Development/Plasticity/Repair

Abnormalities of Neurogenesis in the R6/2 Mouse Model of Huntington's Disease Are Attributable to the *In Vivo* Microenvironment

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Huntington's disease (HD) is an autosomal dominant neurodegenerative condition characterized by movement disorders, psychiatric disturbance, and cognitive decline. There are no treatments to halt or reverse the disease. Mammalian neurogenesis persists into adulthood in the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus. In 2001, our laboratory published the hypothesis that neurogenesis is impaired in neurodegenerative diseases and that this may contribute to disease progression. Since then, it has been shown that neurogenesis is reduced in the DG of transgenic HD mice but increased in the SVZ of HD patients. We sought to characterize neurogenesis further. We found that, in the DG of the transgenic R6/2 mouse model of HD, newborn cell proliferation and morphology, but not differentiation or survival, was compromised. In R6/2 mice, neurogenesis failed to upregulate in the DG in response to seizures. Basal SVZ neurogenesis was similar between R6/2 mice and their wild-type littermates. There was no difference in the *in vitro* growth of adult neural precursor cells (NPCs) between genotypes. These results suggest that abnormal neurogenesis in the R6/2 mouse is not attributable to an intrinsic impairment of the NPC itself but is attributable to the environment in which the cell is located.

Key words: Huntington's disease; R6/2; neurogenesis; seizures; quinolinic acid; doublecortin

Introduction

Huntington's disease (HD) is an inherited neurodegenerative condition characterized by movement disorders, psychiatric disturbance, and cognitive decline. The striatum is particularly vulnerable to degeneration, although other brain areas also dysfunction and degenerate early in the disease (Vonsattel et al., 1985; Lawrence et al., 1998; Rosas et al., 2003).

Mammalian neurogenesis persists into adulthood in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) (Altman and Das, 1965; Kaplan and Hinds, 1977) in many species, including humans (Eriksson et al., 1998; Roy et al., 2000; Palmer et al., 2001) (for review, see Gage, 2000). Adult neurogenesis is relevant because newborn neurons integrate into existing circuitry, are functional, and contribute to olfaction (Gheusi et al., 2000; Carleton et al., 2003) and hippocampal-dependent learning and memory (Shors et al., 2001, 2002; van Praag et al., 2002).

Neurogenesis can be upregulated in response to a variety of stimuli such as ischemia, seizures, and acute lesions, even in non-neurogenic areas (Kempermann et al., 1997; Fallon et al., 2000;

Magavi et al., 2000; Arvidsson et al., 2002; Nakatomi et al., 2002; Parent et al., 2002). We wanted to know whether neurogenesis could be upregulated in response to a chronic lesion (as might be found in the environment of a brain with a neurodegenerative disease such as HD) or, conversely, whether this environment might be nonpermissive for neurogenesis (Armstrong and Barker, 2001). Neurogenesis is reduced in the DG of transgenic HD mice (Gil et al., 2004, 2005; Lazic et al., 2004) but increased in the SVZ of HD patients (Curtis et al., 2003, 2005). In light of this, we sought to characterize neurogenesis further using the R6/2 mouse model of HD (Mangiarini et al., 1996). In particular, we wanted to address whether the abnormalities in HD neurogenesis were attributable to a deficit of the neural precursor cells (NPCs) or a nonpermissive NPC environment. Because neurogenesis is a complex process involving proliferation, migration, differentiation, and survival, we measured all of these parameters in both the DG and SVZ/striatum. It is important to know whether neurogenesis can be upregulated in response to cell dysfunction and death in R6/2 mice, because upregulation of neurogenesis would be required in the disease state and in therapies aimed to enhance neurogenesis. To examine stimulated (upregulated) neurogenesis, we induced seizures by injecting kainic acid (KA) systemically into mice and measured neurogenesis in the DG. We chose to study seizures to stimulate neurogenesis, because this is a well characterized paradigm known to upregulate neurogenesis through a mechanism dependent on altered environmental signals. We also produced a neurotoxic lesion by injecting quinolinic acid (QA) directly into the striatum and measured neuro-

genesis in the SVZ/striatum. Basal neurogenesis was measured using vehicle-treated animals. We compared proliferation, longevity, differentiation fate, and survival of NPCs *in vitro* to establish whether changes in neurogenesis were attributable to the intrinsic properties of the NPC or the microenvironment in which it is located.

Materials and Methods

Animals. A colony of R6/2 transgenic mice was established in the Cambridge Centre for Brain Repair (University of Cambridge, Cambridge, UK). The line was maintained by backcrossing to CBA × C57BL/6 F1 animals. Wild-type (WT) littermates were used as control animals. The mice were housed in mixed genotype single-sex cages in a temperature- and humidity-controlled room on a 12 h light/dark cycle with availability to food and water *ad libitum*. Genotyping was confirmed by PCR, using a method described previously (Hickey and Morton, 2000). Experiments were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses.

KA treatments. A total of 67 mice were used: 34 WT and 33 R6/2; 17 male and 50 female; 35 8-week-old and 32 13-week-old mice. Three mice died during these experiments. KA (20 mg/kg dissolved in sterile water, pH 7.4; Sigma, Gillingham, UK) or vehicle was administered intraperitoneally on the morning of the experiment start date. Thirty-three mice received KA. Seizures were rated according to a modified Racine scale, which assesses the severity of seizure (Racine et al., 1972; Morton and Leavens, 2000). Briefly, seizures were rated as follows; grade 1, mouth movements; grade 2, head nodding; grade 3, forelimb clonus; grade 4, rearing with clonus; grade 5, tonic-clonic seizures.

Bromodeoxyuridine labeling and tissue processing. Because the peak of cell proliferation occurs ~7 d after seizures (Mohapel et al., 2004), mice were given an injection of the thymidine analog bromodeoxyuridine (BrdU) (50 mg/kg) on days 7 and 8 after KA treatment, to label dividing cells. BrdU injections were administered intraperitoneally 12 h apart, for 2 d (i.e., a total of four injections). Mice were killed on days 9, 16, and 32 after KA treatment. Mice were anesthetized using intraperitoneal sodium pentobarbital (Merial, Oxford, UK) and then perfused through the heart with a prewash solution (18 g/liter disodium hydrogen orthophosphate and 9 g/liter sodium chloride, in distilled water, pH 7.3) and then 4% paraformaldehyde (in distilled water, pH 7.4). Brains were postfixed with 4% paraformaldehyde for 24 h and then cryoprotected in 30% sucrose/ 0.1 M PBS. Brains were sectioned coronally at 40 μm and stored in PBS/ azide at 4°C.

Immunohistochemistry. One in every 12 sections was processed immunohistochemically either free-floating or, for fluorojade-B and acetylcholinesterase, mounted on Superfrost-plus glass slides (VWR Scientific, Poole, UK). For antigens labeled with biotinylated antibodies, sections were quenched (10% methanol, 10% hydrogen peroxide, and distilled water) for 5 min, washed, and incubated in a blocking buffer [3% serum in 0.1 M Tris-buffered saline (TBS) with 0.2% Triton X-100] for 60 min at room temperature. Sections were incubated in primary antibody with 1% serum in TBS with 0.2% Triton X-100 overnight at 4°C. Biotinylated secondary antibodies (1:200 in 1% serum in TBS with 0.2% Triton X-100) were applied for 2 h, washed, and visualized using an avidin– biotin–peroxidase system (Vectastain ABC kit; Vector Laboratories, Peterborough, UK) and diaminobenzidine (Sigma). For BrdU immunolabeling, sections were incubated in 2 M HCl at 37°C for 30 min and then washed for 30 min in 0.1 M borate buffer, pH 8.5, before block. For sections in which primary antibodies were visualized using fluorescence-conjugated secondary antibodies, sections were not quenched, and secondary antibody (1:1000, Alexa-488 or Alexa-568; Invitrogen, Paisley, UK) was added for 2 h at room temperature. The sections were washed and then mounted using Vectashield (Vector Laboratories) or Fluorosave (Calbiochem, Beeston, UK) mounting media. The following primary antibodies were used: mouse anti-neuronal nuclear antigen (NeuN) (1:50; Chemicon, Southampton, UK), rat anti-BrdU (1:50; Accurate Chemicals, Westbury, NY), goat anti-doublecortin (DCX) (1:400; Santa Cruz Biotechnology, Heidelberg, Germany), and rat anti-CD11b

(5C6 clone) (1:1000; Serotec, Oxford, UK). The following secondary antibodies were used: horse anti-mouse (1:200; Vector Laboratories), goat anti-rat (1:200; Serotec), donkey anti-goat (1:200; Santa Cruz Biotechnology), and the appropriate Alexa-488 or Alexa-568. Fluorojade-B staining (Chemicon) was performed according to Schmued and Hopkins (2000).

Quantification of numbers of DCX-positive cells. All slides were blind coded for assessment. DCX is a microtubule-associated phosphoprotein (Gleeson et al., 1999) expressed in NPCs and immature hippocampal granule cells for ~3 weeks after birth (Brown et al., 2003b; Kempermann et al., 2004; Rao and Shetty, 2004) and has been validated as a marker for neurogenesis in the DG (Rao and Shetty, 2004; Couillard-Despres et al., 2005). DCX-positive cells were counted using the optical fractionator method (for review, see Gundersen et al., 1999) and CAST-grid package (version 1.09; Olympus Optical, Glostrup, Denmark). The DG was visualized using the 4× objective lens of a light microscope (BX50; Olympus Optical), and the area of interest was highlighted manually. Cells were counted using the 20× objective lens, within a specified field/counting grid ("dissector") of area 6044 μm^2, which was moved across the section systematically on a motorized stage. Cells that overlapped two of the sides of the grid were counted, and cells on the other two sides were not. One in 12 sections were analyzed, and, if any sections were damaged or missing, a correction factor was applied, i.e., the cell count was multiplied by the ratio of sections that should have been counted to the number of sections present. Total cell counts were multiplied by the ratio of reference volume to sampling volume to obtain an estimate of the total number of DCX-positive cells in the DG of each brain.

Quantification of morphological features of DCX-positive cells in the DG. Morphological measurements of DCX-positive cells were performed using a light microscope under a 20× objective lens (Leica/Leitz DMRB; Leica, Nussloch, Germany) and the LUCIA package. Measurements were taken from five areas of each DG, and three DG fields from each animal were examined. The longest measurement from each area was chosen and averaged. The degree of dendritic branching of DCX-positive cells was estimated using an arbitrary visual scale from 0 to 7. Immature DCX-positive cells, with no dendritic branching, were not included in the grading assessment. Sections were assigned a grade according to the maximum grade of branching exhibited. In other words, if some cells exhibited branching grade 5 but some cells exhibited branching grade 2, the section would be assigned an overall grade of 5. The grades of branching were assessed as follows: grade 0, no dendritic branching; grade 1, one or two first-order dendritic branches (in which "first order" refers to a dendritic branch emanating from the apical dendrite, "second order" refers to a branch emanating from a first-order branch, and "third order" refers to a branch emanating from a second-order branch); grade 2, more than two first-order dendritic branches; grade 3, more than two first-order dendritic branches and one or two second-order dendritic branches; grade 4, more than two first-order and second-order dendritic branches; grade 5, more than two first-order and second-order dendritic branches and one or two third-order dendritic branches; grade 6, more than two third-order dendritic branches; grade 7, many third-order dendritic branches.

Quantification of numbers of BrdU-positive cells in the DG. BrdU-positive cells at day 9 were counted using the 40× objective lens of a florescence microscope (Leitz, Wetzlar, Germany) and the Open Lab package (Improvision, Lexington, MA). Every cell was counted throughout the thickness of the section, and total counts were multiplied by the frequency of sections, i.e., 12, to obtain an estimate of the total number of BrdU-positive cells in the DG of each brain.

Quantification of density of CD11b immunoreactivity (microglia). CD11b was semiquantified using the intensity of immunoreactivity because individual cells were not easily identified and not amenable to counting. This was performed by selecting an area of DG using the 20× objective lens of a light microscope (Leica/Leitz DMRB) and measuring the mean intensity of immunoreactivity using the LUCIA package. Sections were kept in chromogen for 3 min per mouse, and illumination, contrast, and brightness were kept consistent for each image in parallel.

Quantification of numbers of BrdU/NeuN-positive cells. It was necessary to count cells that colocalized for BrdU and NeuN using a confocal

microscope to avoid spurious counting of "satellite cells" (Kuhn et al., 1997; Kornack and Rakic, 2001). BrdU and NeuN were considered to be colocalized if nuclear colocalization was seen over the extent of the nucleus in consecutive 1 μm z-stacks, profiles of green and red fluorescence coincided, and when colocalization was confirmed in x–y, x–z, and y–z cross-sections produced by orthogonal reconstructions from z-series using Leica software (version 2.61). Confocal images were taken using a 40× oil objective lens from a Leica microscope. Fluorophores were excited at 488 nm (argon laser) and 568 nm (helium/neon laser). Counting was performed using 1 μm z-stacks, three DG fields were taken for analysis, and five areas were selected (while visualizing the NeuN immunoreactivity), one from the genu of the DG, two from the upper blade and two from the lower blade, ensuring that the areas did not overlap so that cells were not counted twice. In effect, most of the BrdU-positive cells in the DG were counted using this method. This was done for all brains from days 16 and 32 after KA treatment.

SVZ neurogenesis in response to a QA lesion: surgical procedures. Twenty-eight mice, 11 weeks old, were used in this experiment; 16 WT and 12 R6/2. Half of each genotype received a QA injection, and half received vehicle (0.1 M PBS). Weights ranged from 16 to 25 g. Each animal received a subcutaneous injection of analgesic (Rimadyl, 5 mg/kg; Pfizer, Kent, UK) and antibiotic (Terramycin, 60 mg/kg; Pfizer). QA (Sigma) was dissolved in 0.1 M PBS. Under halothane anesthesia, QA (0.66 μl, 40 nmol) or PBS (0.66 μl) was injected using a 1 μl Hamilton syringe, into the striatum using the following coordinates: 0.7 mm rostral to bregma, 1.9 mm lateral to the midline, and 2.5 mm ventral to the dura, with the tooth bar set at zero (Hansson et al., 1999). The injection was given over 2 min, and the syringe was left in place for an additional 3 min and then slowly removed. Body temperature was controlled using a heat pad maintained at 37°C.

BrdU labeling and tissue processing. The BrdU labeling protocol was similar to that described above for the KA experiment. The time points for BrdU administration were chosen on the basis of previous studies showing maximal proliferation at days 6 and 7 (Jin et al., 2001; Arvidsson et al., 2002; Parent et al., 2002). Tissue processing and immunohistochemistry was performed as in the KA experiment, but animals were killed on day 8 and day 31. Acetylcholinesterase staining was performed according to the modified Koelle method (Koelle, 1955).

Quantification of lesion area. Lesion areas, identified by fluorojade-B, were measured using a fluorescence microscope (Leitz); the lesion area on each section was delineated manually using the Open Lab package (Improvision). The lesion area in each section was multiplied by 12 (frequency of section) and 40 μm (section thickness) to calculate lesion volume (in cubic millimeters).

Quantification of numbers of BrdU-positive cells in the SVZ and striatum. Using a light microscope (Leica) under a 25× objective lens, all BrdU-positive cells in the lateral and medial wall of the SVZ from anterior to the septodiencephalic level were counted, because they tended to cluster. Cells at the dorsolateral corner of the SVZ, extending laterally, were not counted. Sections were counterstained with fluorojade-B to identify the lesion. BrdU-positive cells in the striatum were counted using the optical fractionator method described above for the KA experiment.

Quantification of numbers and migration of DCX-positive cells in the striatum. Neuroblast migration was measured as the distance of the furthest lateral DCX-positive neuroblast from the dorsolateral corner of the SVZ. Measurements from each section were taken and averaged using a light microscope (10× objective lens; Leica/Leitz DMRB) and the LUCIA package. The total number of DCX-positive cells in the striatum, including the peri-SVZ area, were counted using a 20× objective lens of a light microscope (BX50; Olympus Optical) and multiplied by 12 (the frequency of sections) to obtain an estimate of the total.

Quantification of numbers of BrdU/NeuN-positive cells in the striatum. For BrdU/NeuN quantification, sections were viewed for NeuN, and six representative samples were taken for counting using confocal images. Three sections were randomly chosen from within the core of the lesion and three sections around the edge of the lesion. Numbers of BrdU/NeuN-positive cells are thus arbitrary and not intended as an estimate of the total number but sufficient for comparison between genotypes and

QA-treated versus vehicle-treated groups. z-Series were performed at 1 μm intervals, and images were viewed using a 40× oil objective lens. Cells were considered double labeled according to the criteria described above for the KA experiment. All of the genotype comparisons are made using cell counts from the ipsilateral side of the brain.

Neurosphere assay. Female R6/2 mice and their WT littermates were killed by CO_2 asphyxiation, and pups (postnatal day 3) were killed by decapitation. Dissections were conducted in PBS with 0.6% glucose, pH 7.4, at 4°C under sterile conditions. Meninges and blood vessels were removed. For the SVZ and DG dissections, each brain was sliced into 2 mm coronal sections. Tissue was washed in PBS/glucose, digested in papain–dispase–DNase solution [0.01% papain, Lorne Laboratories (Twyford, UK); 0.1% dispase, Roche Diagnostics (Hertfordshire, UK); 0.01% DNase, Lorne Laboratories] for 4 min, filtered through 40 μm nylon mesh cell filters (Falcon, Suffolk, UK), and then washed three times. Cell viability was calculated using the trypan blue dye (Sigma) exclusion method. Cells were seeded at a density of 1×10^5 cells/ml in 25 and 75 cm^2 flasks in Neurobasal medium (Invitrogen) with 2% B27 (Invitrogen), 1% penicillin/streptomycin/fungizone (PSF) (0.1 g/liter; Invitrogen), 1% L-glutamine (Sigma), 20 ng/ml basic fibroblast growth factor-heparin (R & D Systems, Oxford, UK), and 20 ng/ml epidermal growth factor (Sigma) (Wachs et al., 2003).

Flasks were incubated at 37°C, 95% air/5% CO_2, in a humidified atmosphere. Media was replaced on day 4 and added on day 7, and neurospheres were dissociated ("passaged") on day 10. To dissociate the neurospheres, calcium-free HBSS (Invitrogen) at pH 7.5 was added to centrifuged neurospheres, and the mixture was gently aspirated. A total of 200 μl of media, pH 11, was then added for 7 min, and finally 200 μl of media, pH 1.8, was added (Sen et al., 2004). Cells were washed, counted using the trypan blue dye exclusion method, and reseeded.

For differentiation, dissociated cells were plated on glass coverslips in 1 ml wells precoated with 0.01 μl/ml poly-D-lysine (Sigma) and, for adult cultures, 1 μg/ml laminin (Sigma). Cells were seeded at 5×10^4 cells per 500 μl in Neurobasal medium with 2% B27, 1% PSF, 1% L-glutamine, and 1% fetal calf serum (FCS) (Invitrogen). An additional 500 μl of media was added on day 4, and cells were fixed in 4% paraformaldehyde after 7 d. Cells were incubated in blocking solution (5% serum in 0.1 M PBS with 0.2% Triton X-100) for 30 min at room temperature and then incubated in primary antibody in 1% serum (in PBS with 0.2% Triton X-100) overnight at 4°C. After washing in PBS, secondary fluorescence-conjugated antibodies were diluted in the fluorescent nuclear stain, *bis*benzimine/Hoechst-33258 (1:5000 in PBS; Sigma), and applied for 2 h at room temperature. After washing, coverslips were mounted on glass slides with 1:1 PBS/glycerol. Galactocerebroside (Gal C) staining was performed on live cells: cells were incubated with primary antibody and 10% FCS for 60 min, washed, and fixed, and the secondary antibody was then applied (1:500, Alexa-488- and Alexa-568-conjugated secondary antibodies; Invitrogen). For BrdU staining, ice-cold methanol was applied to fixed cells for 20 min, and the cells were washed, exposed to 2 M HCl at 37°C, washed in 0.1 M borate buffer at pH 8.5, and then stained in the usual way. The following primary antibodies were used: anti-β-tubulin (1:500; Sigma), anti-glial fibrillary acid protein (GFAP) (1:500; DakoCytomation, Ely, UK), anti-Gal C (gift from P. Gaughwin, Cambridge Centre for Brain Repair), anti-nestin (1:500; Chemicon), anti-BrdU (1:50; Accurate Chemicals), and anti-DCX (1:400; Santa Cruz Biotechnology). Mutant huntingtin (htt) protein was detected using an antibody raised against a human GST-exon-1 fusion protein carrying 53 glutamines (gift from Dr. G. Bates, Guy's Hospital, London, UK) and used at a dilution of 1:1000. Primary antibodies were visualized using Alexa-488- and Alexa-568-conjugated secondary antibodies (1:1000; Invitrogen). Two to four coverslips were stained with each antibody. Slides were examined using a counting grid (0.0625 mm^2) under the 40× objective lens of a fluorescence microscope (Leitz), five random fields were counted from each coverslip, cell counts were expressed as a percentage of Hoechst-positive cells, and images were taken and stored using the OpenLab package (Improvision).

Statistical analysis. Some data were normalized by log transformation or square root (NPC proliferation *in vitro*) to allow parametric statistical analysis, and any transformation is indicated on the appropriate figure.
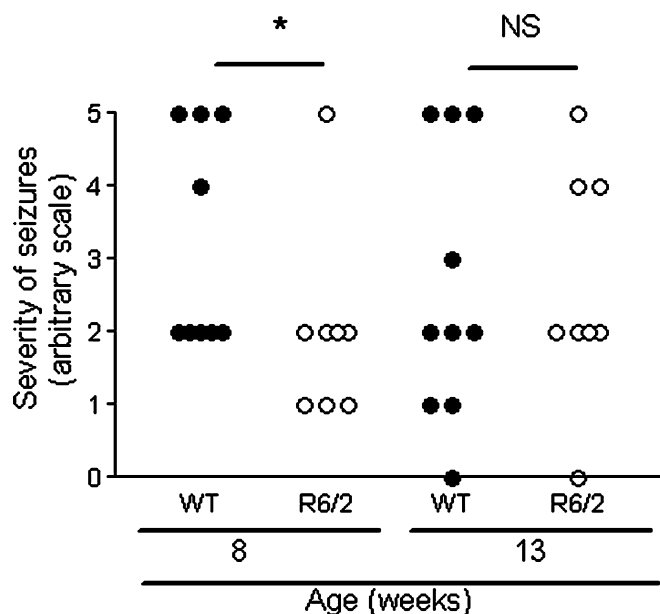
Figure 1. Comparison of seizure severity between genotypes and age. R6/2 and WT mice were given an injection of KA, and seizures were rated on the modified Racine scale. Mice that died (1 8-week-old WT mouse and 2 R6/2 mice aged 8 weeks and 13 weeks) are included in the figure. There was no difference in scoring on the modified Racine scale by genotype ($p = 0.11$), although when each age group is considered separately, younger R6/2 mice had less severe seizures ($p < 0.05$) than older mice ($p = 0.829$). NS, Not statistically significant ($p > 0.05$); the asterisk denotes statistical significance ($p < 0.05$).

Normalization was confirmed by appearance on a quantile–quantile plot and using the Kolmogorov–Smirnov Z test. ANOVA was used to test for differences in BrdU, DCX, and BrdU/NeuN (using age, day, genotypes, treatment, and gender as independent variables), using a full factorial analysis (although it was not possible to model all four- and five-way interactions). *Post hoc* tests were done using an ANOVA with a Bonferroni's correction for multiple comparisons. When data could not be normalized, or ordinal scales were used, nonparametric tests were used (two-tailed Mann–Whitney U test), as indicated in the text. Correlations were made using Pearson's correlation coefficient (parametric data) or Spearman's rank correlation coefficient (nonparametric data). α was set at 0.05, and analysis was performed using SPSS for Windows (version 11.5; SPSS, Chicago, IL).

Results

Seizure severity is similar between genotypes, but younger R6/2 mice have less severe seizures than younger WT mice

KA-induced seizures lasted for up to 3 h, and their severity was graded according to a modified Racine scale (Racine et al., 1972; Morton and Leavens, 2000). All but two mice had seizures that could be graded. Two mice (both 13 weeks old, one WT and one R6/2) did not display gradable seizures but became immobile and hunched, although rousable. Such behavior has been associated with EEG-confirmed seizure activity (Mohapel et al., 2004). One 8-week-old WT mouse and two R6/2 mice (aged 8 and 13 weeks) died during these experiments. The WT mouse died after a series of tonic-clonic seizures; the R6/2 mice died after one prolonged tonic-clonic seizure.

There was no difference in scoring on the modified Racine scale between genotypes ($p = 0.11$) (Fig. 1). However, when each age group was considered separately, younger R6/2 mice had less severe seizures than younger WT mice ($p < 0.05$), but older R6/2 mice had similar scoring on the modified Racine scale to older WT mice ($p = 0.829$). This is consistent with the findings of Morton and Leavens (2000). There was no correlation between
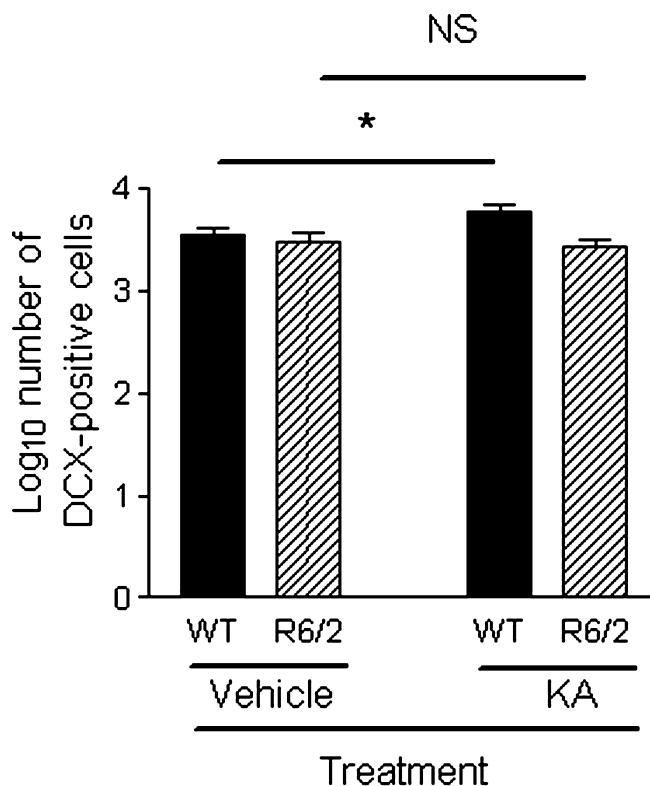

Figure 2. Comparison of neurogenesis as measured by DCX-positive cells in the DG between genotypes in vehicle-treated and KA-treated groups. R6/2 mice had fewer DCX-positive cells in the DG than WT mice ($F_{(1,33)} = 14.422; p = 0.001$). There was a genotype × treatment interaction effect ($F_{(1,33)} = 5.408; p < 0.05$), suggesting that neurogenesis can be upregulated in WT mice but not in R6/2 mice. Data have been log transformed, bars show means, and error bars show means ± 1.0 SEM. NS, Not statistically significant ($p > 0.05$); the asterisk denotes statistical significance ($p < 0.05$).

the scoring on the modified Racine scale and neurogenesis, in both genotypes, as measured by the number of DCX-positive cells in the DG ($p = 0.388; r = 0.155$).

A previous study showed that intraperitoneal KA at a dose of 20 mg/kg causes seizures but no cell loss (Morton and Leavens, 2000). This was confirmed in the present study using fluorojade-B. This dose of KA was chosen to allow a comparison of neurogenesis in response to seizures without the complicating factor of differences in cell loss between genotypes (Morton and Leavens, 2000).

Neurogenesis is lower in R6/2 mice and in older mice

The use of DCX to study neurogenesis has many advantages (Rao and Shetty, 2004; Couillard-Despres et al., 2005), although transcription or handling of DCX in R6/2 mice may be altered and be erroneously interpreted as changes in neurogenesis. Intensity of immunolabeling in individual cells, however, was the same between genotypes, and DCX and BrdU cell counts in the DG correlated closely with one another ($p < 0.001; r = 0.776$). Importantly, the duration of DCX expression remains the same in aging, environmental enrichment and seizure-induced hippocampal neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005).

Mice were killed on days 9, 16, and 32 after KA treatment, and there was no difference in the number of DCX-positive cells in the DG between these time points ($F_{(2,33)} = 0.601; p = 0.554$). There were fewer DCX-positive cells in the DG of R6/2 mice compared with WT mice ($F_{(1,33)} = 14.422; p = 0.001$) (Fig. 2)

(supplemental Table 1, available at www.jneurosci.org as supplemental material). There was a genotype × treatment interaction effect ($F_{(1,33)}$ = 5.408; p < 0.05), suggesting that neurogenesis was greater in the KA-treated group compared with the vehicle-treated group in WT mice, consistent with previous studies (Parent et al., 1997; Scott et al., 1998) but that neurogenesis was the same in the KA-treated group compared with the vehicle-treated group in R6/2 mice. Thus, neurogenesis can be upregulated in WT mice but not R6/2 mice.

There was no significant difference in DCX-positive cells between genotypes in vehicle-treated mice (p = 1.000). That is, basal neurogenesis was similar between WT and R6/2 mice.

There were also fewer DCX-positive cells in the DG of older mice compared with younger mice ($F_{(1,33)}$ = 15.407; p < 0.001). The decline in neurogenesis with age is well recognized (Kuhn et al., 1996) and has been demonstrated for transgenic HD mice (Lazic et al., 2004; Gil et al., 2005).

It is important to consider sex differences in studies of neurogenesis because, for example, NPC proliferation is higher in female rodents compared with males (Tanapat et al., 1999) (for review, see Abrous et al., 2005). We included mice of both sexes for logistical reasons. Seventeen male mice were included in the study, but the numbers of male and female mice were spread evenly across all groups, sex of the mice did not affect neurogenesis ($F_{(1,33)}$ = 2.872; p = 0.100), and there was no interaction between sex and the other variables.

The morphology of DCX-positive cells is altered in R6/2 mice

DCX-positive cells in the DG possessed the morphology of stage 5 immature postmitotic granule cells and had a globular cell body with many fine dendritic processes extending into the molecular layer (Kempermann et al., 2004) (Fig. 3A). Dendritic growth into the molecular layer was greater for the upper blade of the DG than for the lower blade (observation only, not quantified). There were also DCX-positive cells with short bipolar processes arranged tangentially in the SGZ. These cells may correspond to type 2b cells (Kempermann et al., 2004). These short tangential cells could not be discerned individually and thus were not counted.

R6/2 mice had shorter newborn granule neurons than WT mice (as measured from mid cell body to the tip of the apical dendrite), in both vehicle-treated and KA-treated groups ($F_{(1,33)}$ = 31.225; p < 0.001) (Fig. 4A). Older mice had shorter cells than younger mice ($F_{(1,33)}$ = 4.391; p < 0.05). Mice from the KA-treated group compared with the vehicle-treated group had cells of the same length ($F_{(1,33)}$ = 3.837; p = 0.059).

Newborn granule cells in R6/2 mice had a lesser degree of dendritic branching than those from WT mice, as assessed on an eight-point visual scale (p < 0.001) (Fig. 4B). There was a trend for branching to be greater in the KA-treated group in WT mice, but this did not reach statistical significance (WT vehicle treated vs KA treated, p = 0.069; R6/2 vehicle treated vs KA treated, p = 0.760).

DCX-positive cells from R6/2 mice migrated shorter distances from the SGZ into the granule cell layer (GCL) than those in WT mice in both vehicle-treated and KA-treated groups ($F_{(1,33)}$ = 15.128; p < 0.001) (Fig. 4C). Migration of newborn neurons was
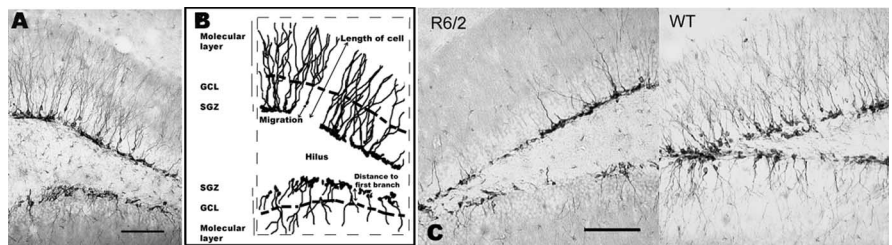
Figure 3. Morphological characteristics of DCX-positive cells in the DG. *A*, Photomicrograph of the DG. *B*, Line drawing of photomicrograph in *A*, illustrating morphological measurements. *C*, Photomicrographs illustrating the morphology of DCX-positive cells in R6/2 and WT mice. Scale bars, 100 μm.
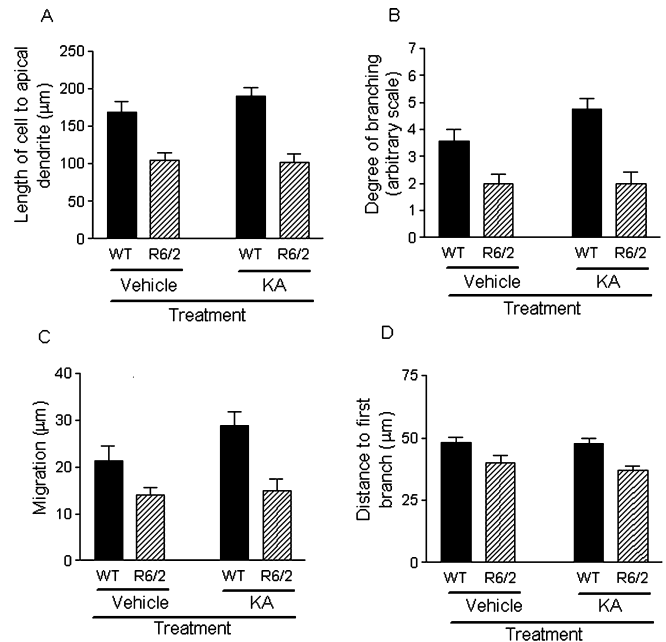
Figure 4. Comparison of the morphological characteristics of DCX-positive cells in the DG. *A*, R6/2 mice have shorter newborn cells, from mid cell body to the tip of the apical dendrites, than those from WT mice ($F_{(1,33)}$ = 31.225; p < 0.001). *B*, The degree of branching of dendritic arbor from three DG sections was assessed on an arbitrary visual eight-point scale (for details, see Material and Methods). R6/2 mice had a lower degree of branching than WT mice (p < 0.001). *C*, Newborn cells from R6/2 mice migrated shorter distances into the GCL from the SGZ than those from WT mice ($F_{(1,33)}$ = 15.128; p < 0.001). *D*, Newborn cells in R6/2 mice had a shorter distance to the first branch of the apical dendrite than those from WT mice ($F_{(1,33)}$ = 13.397; p < 0.001). Bars show means, and error bars show means ± 1.0 SEM.

not greater in WT mice of the KA-treated group compared with the vehicle-treated group (p = 0.174).

In DCX-positive cells from both genotypes, the first branch emanated from a relatively thick apical dendrite, and branching occurred in the GCL, as described previously (Rao and Shetty, 2004). However, newborn cells in R6/2 mice had a shorter apical dendrite from the cell body to the first dendritic branch in both vehicle-treated and KA-treated groups when compared with WT mice ($F_{(1,33)}$ = 13.397; p < 0.001) (Fig. 4D).

Regarding vehicle-treated mice only compared with WT mice, R6/2 mice had shorter DCX-positive cells (p = 0.003), less dendritic branching (p < 0.01), and a trend for shorter initial apical dendrites (p = 0.060).

In summary, newborn granule cells of R6/2 mice had shorter apical dendrites and more sparse dendritic arbor, which emanated close from the cell body, and cells migrated shorter distances when compared with newborn granule cells of WT mice (Fig. 3C).
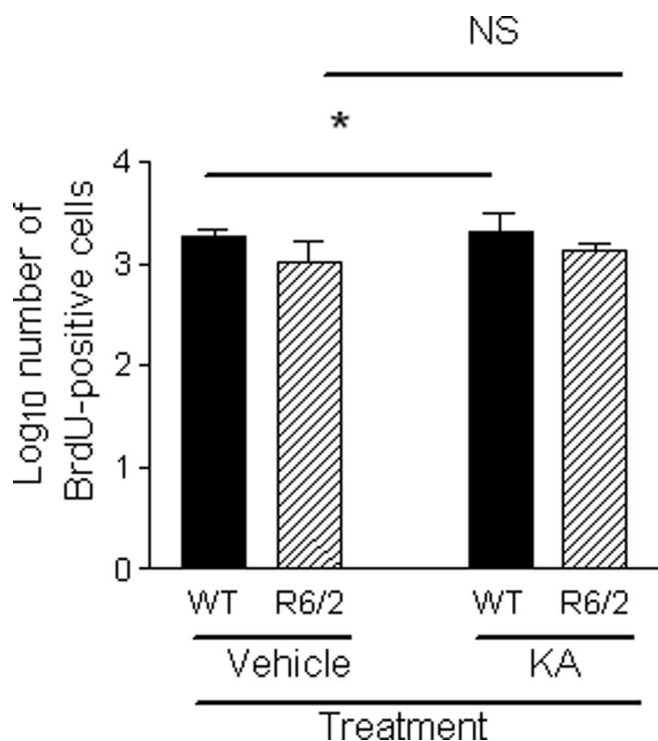
Figure 5. Comparison of cell proliferation in the DG between genotypes. BrdU-positive cells in the DG at day 9 after KA-treatment (1 d after the last BrdU injection) were fewer in number in R6/2 mice compared with WT mice ($F_{(1,10)} = 8.886; p < 0.02$). There was a genotype × treatment interaction effect ($F_{(1,10)} = 7.573; p = 0.02$), suggesting that proliferation was higher in KA-treated WT versus vehicle-treated WT mice but the same in KA-treated R6/2 versus vehicle-treated R6/2 mice. Data have been log transformed, bars show means, and error bars show means ± 1.0 SEM. The asterisk denotes statistical significance ($p < 0.05$). NS, Not statistically significant.

Cell proliferation 1 d after BrdU injection is lower in R6/2 mice compared with WT mice

BrdU-positive cells in the DG at day 9 after KA treatment (i.e., 1 d after BrdU injection) were fewer in number in R6/2 mice compared with WT mice ($F_{(1,10)} = 8.886; p < 0.02; n = 19$) (Fig. 5) (supplemental Table 1, available at www.jneurosci.org as supplemental material), consistent with previous findings (Gil et al., 2004, 2005; Lazic et al., 2004). Proliferation was higher in mice aged 8 weeks than those aged 13 weeks ($F_{(1,10)} = 38.076; p < 0.001$). DCX and BrdU cell counts in the DG correlated closely with one another ($p < 0.001; r = 0.776$). There was no correlation between BrdU-positive cell number in the DG and scores on the modified Racine scale ($p = 0.476; r = 0.274$).

There was no significant difference in BrdU-positive cells between genotypes in vehicle-treated mice ($p = 1.000$). That is, basal NPC proliferation was similar between WT and R6/2 mice.

When both vehicle-treated and KA-treated mice were analyzed, there was a genotype × treatment interaction effect ($F_{(1,10)} = 7.573; p = 0.02$), suggesting that proliferation was greater in KA-treated WT mice compared with vehicle-treated WT mice and that proliferation was the same in KA-treated R6/2 mice compared with vehicle-treated R6/2 mice. This suggests that NPC proliferation is upregulated only in WT mice, not in R6/2 mice, consistent with the DCX data. R6/2 mice are reported to have handling-induced seizures later in life (Mangiarini et al., 1996), so the fact that neurogenesis was the same in R6/2 mice after seizure is important to know when interpreting results from neurogenesis experiments in R6/2 mice. However, handling-

induced seizures in our colony are extremely low (fewer than 1 in 100 mice exhibit such seizures in their lifetime).

Neuronal fate and survival of newborn cells are the same in WT and R6/2 mice

To measure cell fate and survival of newborn cells, we counted the number of BrdU/NeuN-positive cells and the percentage of BrdU-positive cells that expressed NeuN (supplemental Table 1, available at www.jneurosci.org as supplemental material). The number of BrdU-positive cells that coexpressed NeuN (presumptive newborn neurons) at day 16 and day 32 after KA treatment was similar in R6/2 and WT mice (Mann–Whitney U test, $p = 0.394; n = 45$). The percentage of BrdU-positive cells coexpressing NeuN was also similar (Mann–Whitney U test, $p = 0.170$). Survival of BrdU/NeuN-positive cells was no different in the KA-treated group compared with the vehicle-treated group (Mann–Whitney U test, $p = 0.230$).

Comparison of genotypes with the same seizure grade

Most mice (55%) displayed seizures scoring 2 on the modified Racine scale. These mice were analyzed separately ($n = 18$) along with vehicle-injected mice ($n = 49$ in total). There were not enough mice displaying the other grades to analyze separately and produce valid analyses. In this group, there were significant genotype effects. That is, when compared with WT mice, R6/2 mice had fewer DCX-positive cells in the DG ($F_{(1,20)} = 9.269; p < 0.01$), shorter apical dendrites ($F_{(1,20)} = 21.974; p < 0.001$), shorter distance of the apical dendrite from cell body to first branch ($F_{(1,20)} = 9.272; p < 0.01$), shorter migration distances into the GCL ($F_{(1,20)} = 5.500; p < 0.05$), less arbor ramification (Mann–Whitney U test, $p < 0.01$), and no difference in the number of BrdU/NeuN-positive cells ($p = 0.138$). There were fewer BrdU-positive cells in the DG of R6/2 mice ($F_{(1,8)} = 6.441; p < 0.05$), and there was a genotype × treatment interaction effect ($F_{(1,8)} = 7.411; p < 0.05$), again suggesting that NPC proliferation can be upregulated in WT but not R6/2 mice.

In summary, seizure severity does not influence the differences in neurogenesis and newborn neuron morphology between genotypes. This is consistent with Mohapel et al. (2004), who showed that seizure severity does not influence the rate of cell proliferation but reduces newborn cell survival, possibly attributable to cell death. No cell death was seen in our study, as measured using fluorojade-B.

R6/2 mice are resistant to QA-induced striatal lesions

We next examined neurogenesis in the SVZ, the other area of constitutive neurogenesis in the brain, as well as the DG. We also examined the striatum, because it the brain region most vulnerable in HD and a possible target area of newborn neurons from the SVZ (Arvidsson et al., 2002; Parent et al., 2002).

It has been shown previously that transgenic HD mice are resistant to excitotoxic lesions (Hansson et al., 1999, 2001a,b; Hickey and Morton, 2000; Petersen et al., 2001; MacGibbon et al., 2002), although this is age, strain, and excitotoxin dependent (Bogdanov et al., 1998; Hansson et al., 2001a; Petersen et al., 2002; Schauwecker, 2002; Zeron et al., 2002). QA has been used in various doses to induce lesions in mice [1 μl of 8, 20, and 30 nmol (Zeron et al., 2002; Hansson et al., 2001b; Petersen et al., 2002, respectively)], so, in the present study, a larger dose (40 nmol) was given to all animals in an attempt to induce a lesion in R6/2 mice. Fluorojade-B was used to label lesioned tissue, and the area of immunofluorescence was measured (Duckworth et al., 2005). As
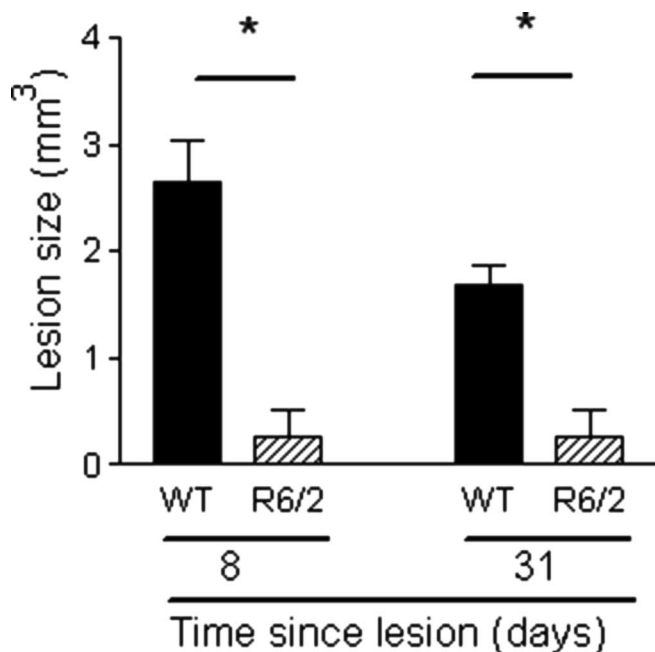
Figure 6. R6/2 mice are resistant to QA-induced striatal toxicity. R6/2 mice had a smaller lesion area than WT mice (Mann–Whitney U test, $p < 0.02$). Lesion size is measured using fluorojade-B and expressed as cubic millimeters. Bars show means, and error bars show means $\pm$ 1.0 SEM. The asterisks denote statistical significance ($p < 0.05$).

Figure 7. Comparison of SVZ proliferation in QA-treated and vehicle-treated animals. There was no significant difference in numbers of BrdU-positive cells in the SVZ in WT mice compared with R6/2 mice in either the QA-treated or vehicle-treated group ($F_{(1,20)} = 3.232; p = 0.087$). The number of BrdU-positive cells was greater in the QA-treated group versus the vehicle-treated group only in WT mice ($F_{(1,20)} = 5.211; p < 0.05$), suggesting that NPC proliferation was upregulated in response to QA lesions only in WT mice. Data have been log transformed, bars show means, and error bars show means $\pm$ 1.0 SEM. NS, Not statistically significant ($p > 0.05$); the asterisk denotes statistical significance ($p < 0.05$).

well as fluorojade-B, brains were stained for acetylcholinesterase, which confirmed the presence of lesions. All WT mice had extensive striatal lesions that also involved the adjacent cortex. However, lesions in the R6/2 mice were smaller at both time points (Mann–Whitney U test, $p < 0.02$) (Fig. 6). Furthermore, whereas all WT mice had distinct lesions, one R6/2 mouse had no lesion (at day 8), two R6/2 mice (one at day 8, one at day 31) had distinct lesions but not as large as the smallest WT mouse lesion, and the other three R6/2 mice had diffuse lesions (i.e., individual fluorojade-B-positive cells were present, but a lesion area could not be measured).

There is no difference in basal SVZ proliferation between genotypes, but proliferation can be upregulated in WT but not R6/2 mice

There was no significant difference in the number of BrdU-positive cells in the SVZ in WT mice compared with R6/2 mice when analyzing both vehicle-treated and QA-treated mice ($F_{(1,20)} = 3.232; p = 0.087$) (Fig. 7) (supplemental Table 2, available at www.jneurosci.org as supplemental material). Similarly, SVZ proliferation was the same between genotypes in vehicle-treated mice ($p = 1.000$). The number of BrdU-positive cells in the SVZ was significantly lower in the day 31 group ($F_{(1,20)} = 63.815; p < 0.001$), suggesting that the NPCs labeled on days 6 and 7 have died, migrated to the olfactory bulb or striatum, or proliferated such that the marker has become diluted. There was a genotype × treatment interaction effect ($F_{(1,20)} = 5.211; p < 0.05$), suggesting that NPC proliferation was upregulated in response to QA lesions only in WT mice. That cell proliferation in the SVZ was higher after striatal lesions in WT mice is consistent with previous studies (Jin et al., 2001; Arvidsson et al., 2002; Parent et al., 2002).
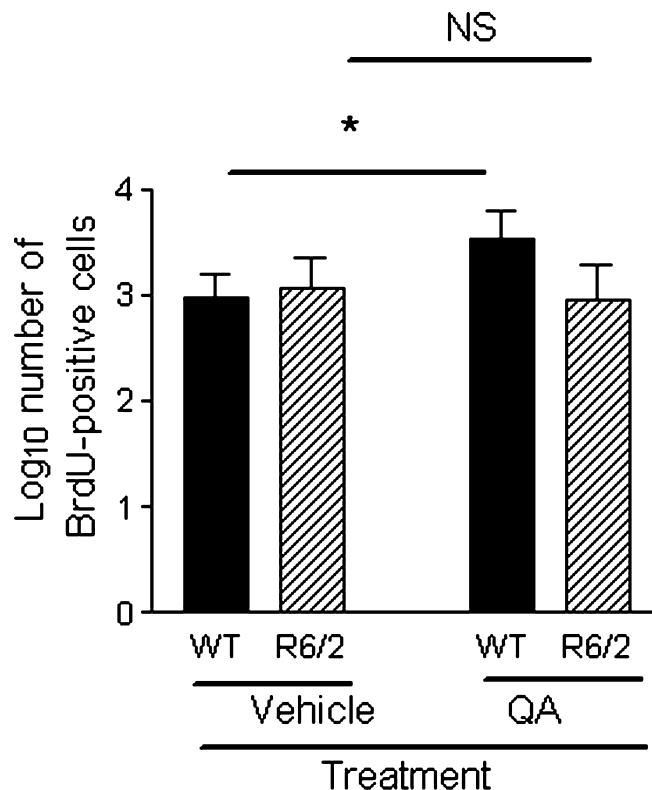
There is no difference in basal neuroblast numbers and migration between genotypes, but neuroblast numbers can be upregulated in WT but not R6/2 mice

There was no difference in lateral migration of neuroblasts overall between genotypes ($F_{(1,20)} = 0.111; p = 0.742$) (Fig. 8 A). Similarly, lateral migration was the same between genotypes in vehicle-treated mice ($p = 1.000$). There was no genotype × treatment interaction effect ($F_{(1,20)} = 2.249; p = 0.149$), suggesting that there was no difference between genotypes in upregulation of neuroblast migration with QA treatment. There was no significant effect of QA treatment on lateral migration of neuroblasts ($F_{(1,20)} = 1.662; p = 0.212$), and, as lesion size increased, lateral migration did not increase (Pearson's correlation coefficient, $p = 0.06; r = 0.514$), but striatal collapse must be considered when interpreting this result. Ventral and lateral migration from the wall of the SVZ was not quantified because it was difficult to ascertain whether a neuroblast had migrated or whether it was an *in situ* progenitor.

Numbers of DCX-positive cells in the striatum were greater in QA-treated mice compared with vehicle-treated mice ($F_{(1,20)} = 22.898; p < 0.001$) (Fig. 8 B) (supplemental Table 2, available at www.jneurosci.org as supplemental material) but the same between genotypes ($F_{(1, 20)} = 0.003, p = 0.956$; genotype × treatment interaction effect, $F_{(1,20)} = 2.164, p = 0.157$). Similarly, numbers of striatal neuroblasts were the same between WT and R6/2 mice in vehicle-treated mice ($p = 1.000$). DCX-positive
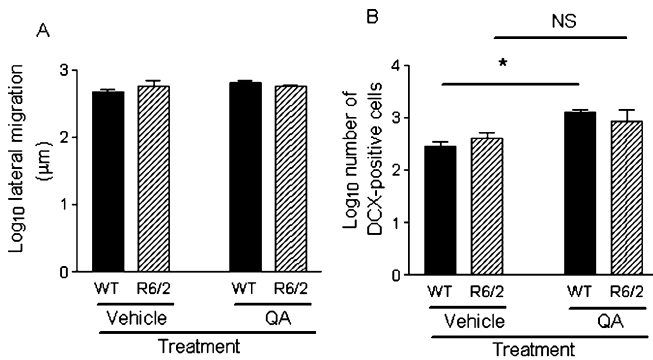
Figure 8. Comparison of neuroblasts between genotypes and treatment (i.e., intrastriatal injection of vehicle QA). ***A***, The distance (in micrometers) of the farthest lateral DCX-positive cell, with the morphology of a migrating neuroblast, from the anterior SVZ was measured from three to four sections and averaged. There was no difference in lateral migration between genotypes ($F_{(1,20)} = 0.111$; $p = 0.742$). There was no genotype × treatment interaction effect ($F_{(1,20)} = 2.249$; $p = 0.149$), suggesting that there was no difference between genotypes in upregulation of neuroblast migration with QA treatment. ***B***, Numbers of DCX-positive cells in the striatum are greater in QA-treated mice compared with the vehicle-treated mice ($F_{(1,20)} = 22.898$; $p < 0.001$) but the same between genotypes ($F_{(1,20)} = 0.003$; $p = 0.956$). DCX-positive cells in the striatum were greater in QA-treated versus vehicle-treated animals only in WT mice ($p < 0.005$) but not in R6/2 mice ($p = 0.441$), suggesting that neurogenesis was upregulated only in WT mice. Data have been log transformed, bars show means, and error bars show means ± 1.0 SEM. NS, Not statistically significant ($p > 0.05$); the asterisk denotes statistical significance ($p < 0.05$).

cells in the striatum were greater in QA-treated versus vehicle-treated animals only in WT mice ($p < 0.005$) but not in R6/2 mice ($p = 0.441$), suggesting that neurogenesis is upregulated only in WT mice. The fact that the interaction effect was not significant, whereas the *post hoc* comparison was, is likely to be explained by the variability of the R6/2 QA-treated group.

There were no obvious morphological differences in SVZ and striatal DCX-positive cells between WT and R6/2 mice (observation only, not quantified). In both genotypes, fusiform-shaped DCX-positive cells were seen in the SVZ, particularly in the dorsolateral corner of the SVZ. DCX-positive cells migrated laterally and ventrally from the dorsolateral SVZ and laterally from the lateral wall of the SVZ. In QA-treated mice of both genotypes, a "ridge" of neuroblasts could be seen migrating from the SVZ (Fig. 9*A*, *A'*), reminiscent of the study by Fallon et al. (2000). In some QA-treated animals, a large number of putative proliferating neuroblasts could be seen in the contralateral side of the corpus callosum and appeared to be migrating toward the lesion (Fig. 9*B*, *B'*). Some cells, in QA-treated animals, had the morphology of migrating neuroblasts, with a single leading process (Fig. 9*A'*) (O'Rourke et al., 1995), whereas others were clustered in "nests" near the wall of the lateral ventricle (Fig. 9*C*). Cells with a more mature morphology were observed adjacent to the lateral wall of the SVZ (Fig. 9*D*) and rostral to the tip of the third ventricle. DCX-positive cells in the striatal parenchyma (distant from the SVZ) were usually isolated, although some clusters were observed, particularly around lesions. DCX-positive cells were seen occasionally in the cortex of both genotypes, and the cells had a more mature morphology, reminiscent of the semilunar-pyramidal neurons described by Nacher et al. (2001). Many isolated cells, lightly immunoreactive for DCX and with possible astrocytic morphology, were also observed throughout the brain (these were counted but not included in the main analysis, and there was no difference between genotypes; data not shown).

Striatal neurogenesis was observed in QA-treated WT but not R6/2 mice

After QA treatment, a large increase in total number of BrdU-positive cells was observed in both R6/2 and WT mice (data not shown). We did not correlate DCX and BrdU counts in the striatum, because many BrdU-positive cells were likely to be proliferating glia (Stoll et al., 1998) and because DCX has not been validated as a marker of neurogenesis in the striatum. We counted BrdU-positive cells that coexpressed NeuN to quantify neurogenesis. BrdU/NeuN colocalization was found only in QA-treated WT mice, consistent with previous studies of striatal lesions (Arvidsson et al., 2002; Parent et al., 2002). Numbers and proportions of BrdU/NeuN-positive cells in QA-treated WT mice were greater in animals with larger lesions (Spearman's rank correlation coefficient, $p < 0.01$, $r = 0.898$; $p = 0.002$, $r = 0.935$, respectively). However, no surviving newborn neurons were found in R6/2 mice. This could be a result of the smaller lesions sustained (Jin et al., 2001), although surviving BrdU/NeuN-positive cells were not seen in the R6/2 mice that sustained relatively large lesions.

In WT and R6/2 mice, NPCs proliferate at the same rate *in vitro*

Proliferation, morphology, migration, and overall neurogenesis in the DG were reduced in the R6/2 mouse, and SVZ/striatal neurogenesis was not upregulated in response to a striatal lesion. This could be attributable to a defect in the NPC itself, its interaction with the environment, or a nonpermissive environment in the R6/2 mouse. To address the first question, we examined NPC *in vitro* from R6/2 and WT mice.

Neurospheres from the SVZ of four R6/2 and five WT mice aged 8 weeks were established as free floating in culture. Neurospheres developed within 4–5 d from seeding and did not differ in gross appearance, size, or number between WT and R6/2 mice. Although the absolute number of NPCs increased from initial seeding after passage, the rate of increase was greatest at early passages and then the rate reached a plateau (Fig. 10*A*). Neurospheres derived from both genotypes could be maintained in culture for up to 70 d (up to nine passages), with no difference in proliferation rate between genotypes (*t* test, $p = 0.206$) (Fig. 10*A*, data to passage 4 shown). This result is consistent with Chu-LaGraff et al. (2001), who established neurospheres from the striatum and cortex of neonatal R6/2 mice. This finding from SVZ-derived neurospheres was confirmed using a range of methods and using different ages of mouse and different regions of the brain, including adult striatum, neonatal cerebellum, hippocampus, and cortex (data not shown). Non-SVZ tissue could not be maintained in large numbers beyond secondary neurospheres, similar to the findings by Seaberg et al. (2005).

Differentiation fate of NPCs is the same in WT and R6/2 mice

There was no difference in differentiation fate between genotypes at passage 1 from the SVZ cultures (Mann–Whitney *U* test, β-tubulin III-positive neurons, $p = 0.96$; GFAP-positive astrocytes, $p = 0.363$) (Fig. 10*B*). There were no obvious morphological differences in differentiated cells between genotypes. There were very few oligodendrocytes/Gal C-positive cells seen in WT cells but none in R6/2 cells. Previous quantitative analyses have shown that Gal C-positive cells are present in very low numbers in dissociated adult NPCs at passage 1 (Wachs et al., 2003). These results were confirmed using adult hippocampal tissue, i.e., there

was no difference in the differentiation fate between genotypes (Mann–Whitney U test; $n = 5$; neurons, $p = 0.23$; astrocytes, $p = 0.56$).

There was no difference in differentiation fate between genotypes at passage 4 from SVZ cultures (Mann–Whitney U test, $p = 0.241$), by which time only GFAP-positive cells were seen. This is consistent with observations from previous studies (Imura et al., 2003; Jain et al., 2003), suggesting that either glial precursors are selected for passage 4 or neuronal precursors are more vulnerable to the dissociation or differentiation procedure. No culture modifications to increase neuronal yield were used in this study, such as addition of retinoic acid (Palmer et al., 1999; Takahashi et al., 1999; Monje et al., 2002). It has been suggested that the tendency to form predominantly glia with time in culture is intrinsic to the precursor cell program (Seaberg et al., 2005).

The majority of cells fixed 1 d after first passage, from both genotypes, expressed nestin, a marker for neuroepithelial stem cells (data not shown) (Frederiksen and McKay, 1988; Lendahl et al., 1990).

Cell survival, measured indirectly by counting the number of Hoechst-positive cells, after 7 d of differentiation was no different between genotypes (t test, $p = 0.429$).

Cells dissociated from neurosphere cultures in R6/2 mice express mutant huntingtin

R6/2 cells expressed mutant htt in a perinuclear location, whereas cells from WT neurosphere cultures did not. This is consistent with data from Chu-LaGraff et al. (2001) who showed, from Western blotting, that exon 1 is expressed in R6/2 NPCs. These results suggest that, despite expression of mutant htt by R6/2 NPCs, the intrinsic properties of proliferation, longevity in culture, differentiation, and survival is similar to those derived from WT mice.

The density of microglia in the DG is the same in WT and R6/2 mice

Neurogenesis was impaired *in vivo* in the R6/2 mouse but intact *in vitro*. This suggests that the *in vivo* environment of the R6/2 mouse may be nonpermissive for neurogenesis. One aspect of the local environment of R6/2 mice, which may be nonpermissive for neurogenesis, is differences in microglia. We chose to study this, following reports that activated microglia reduce neurogenesis (Monje et al., 2002; Ekdahl et al., 2003) and because numbers and morphology of microglia are decreased in R6/2 mice (Ma et al., 2003) but increased in HD patients (Sapp et al., 2001).
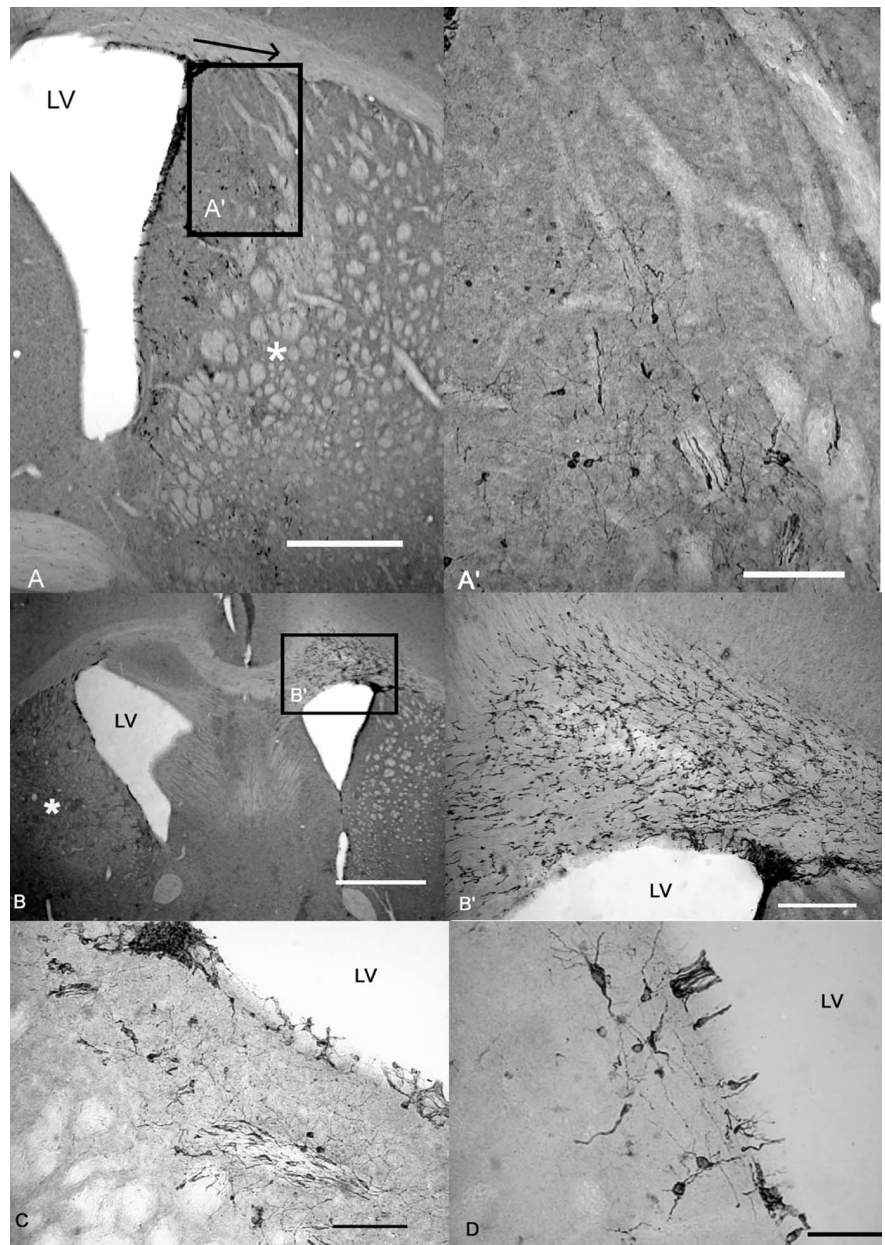

Figure 9. The morphology of DCX-positive cells in the SVZ and striatum. ***A***, Photomicrograph showing the SVZ and striatum from an R6/2 mouse 31 d after lesion. DCX-positive cells migrated laterally from the SVZ toward the lesion (denoted by *). The arrow shows the extent of lateral migration, as used for quantification, from the dorsolateral corner of the SVZ to the furthest neuroblast. ***A′***, The same area highlighted in ***A***; ***A′*** is shown at a higher magnification, illustrating the migratory morphology of DCX-positive cells. ***B***, Photomicrograph showing the SVZ and striatum from an R6/2 mouse 31 d after lesion. The QA injection in this mouse produced a relatively large lesion (denoted by *). A large number of DCX-positive cells were seen in the corpus callosum, with migratory processes directed toward the contralateral lesioned striatum. ***B′***, The same area highlighted in ***B***; ***B′*** is shown at a higher magnification, illustrating migration of DCX-positive cells toward the contralateral side. ***C***, Photomicrograph showing the ipsilateral SVZ from a WT mouse 31 d after lesion, showing a nest of neuroblasts. These were found in mice of both genotypes. ***D***, DCX-positive cells adjacent to the contralateral SVZ in a vehicle-treated WT mouse. Scale bars: ***A***, 500 μm; ***A′***, ***C***, 100 μm; ***B***, 1 mm; ***B′***, 200 μm; ***D***, 50 μm. LV, Lateral ventricle.

Microglia were identified by immunoreactivity for CD11b and mice from the 9 and 16 d time points after KA treatment, from the KA experiment, were examined. Microglia were seen in large numbers in the hilus and molecular layer but were sparse in the GCL. There was no relationship between CD11b immunoreactivity and neurogenesis in the DG, as measured by numbers of DCX-positive cells (Pearson's correlation coefficient, $p = 0.229$; $r = 0.223$). There was no significant difference in microglial den-
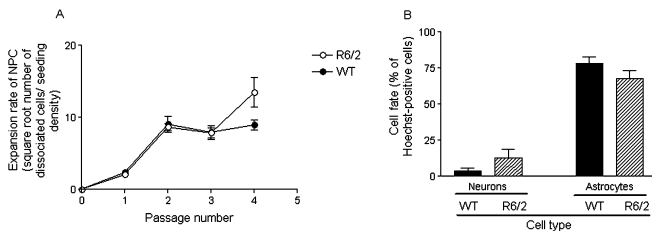
Figure 10. Proliferation and differentiation fate of NPC *in vitro* from adult SVZ. *A*, Comparison of the expansion rate of adult SVZ NPC from WT and R6/2 mice. The SVZ from five WT and four R6/2 mice, aged 9 weeks, were grown as free-floating neurospheres in 10 tissue culture flasks each for the first three passages and two to four each for subsequent passages. The expansion rate represents the number of dissociated cells at each passage divided by the seeding density. The expansion rate of WT and R6/2 mice was the same (*t* test, $p = 0.446$). Data have been transformed by square root, and each point represents means ± SEM of each culture. *B*, Comparison of the differentiation fate of dissociated adult SVZ NPC from WT and R6/2 mice at passage 1. Numbers of neurons or astrocytes are expressed as the percentage of Hoechst-positive cells. Each bar represents means ± SEM for each culture, and statistical comparisons are made using a two-tailed Mann–Whitney *U* test. There was no difference in differentiation fate between genotypes at passage 1 (for β-tubulin III-positive neurons, $p = 0.960$; for GFAP-positive astrocytes, $p = 0.363$).

sity between genotypes ($F_{(1,18)} = 0.326$; $p = 0.575$) or KA-treated mice versus vehicle-treated mice ($F_{(1,18)} = 0.446$; $p = 0.513$). Of note, neurogenesis as measured by DCX was still greater in WT mice compared with R6/2 mice at the 9 and 16 d time points ($F_{(1,24)} = 22.094$; $p < 0.001$). Neuronal death produces microglial activation, and it is possible that there was no significant microglial activation after seizures in our study because no cell loss was seen (Ekdahl et al., 2001).

Discussion

Cell proliferation, migration, and morphology, but not differentiation or survival, was impaired in the DG of R6/2 mice. Neurogenesis failed to upregulate after KA-induced seizures in R6/2 mice. Furthermore, although basal neurogenesis was intact in the SVZ, neurogenesis could not be upregulated in the SVZ/striatum of R6/2 mice. The differences in DG neurogenesis are unlikely to be attributable to an impairment of the NPC per se, because R6/2 NPCs were indistinguishable from WT NPCs *in vitro*. This suggests that the *in vivo* microenvironment is "hostile" to neurogenesis in the R6/2 mouse.

DG neurogenesis is reduced in the R6/1 (Lazic et al., 2004) and R6/2 mouse (Gil et al., 2004, 2005), and this study confirms and extends these findings, lending support to the theory that neurodegenerative diseases are associated with impaired neuroregeneration (Armstrong and Barker, 2001). Our study does not address whether impairments in neurogenesis in R6/2 mice cause (or contribute to) neurodegeneration, nor does it address the question of whether an increase in neurogenesis would be beneficial to an animal with a neurodegenerative disease. To answer such questions, other strategies will be required. For example, models in which neurogenesis can be blocked in adulthood would be invaluable for determining the functional importance of NPCs.

The finding of abnormal morphology of newborn neurons in R6/2 mice is consistent with the reduced sprouting seen in the sciatic nerve of R6/2 mice (Ribchester et al., 2004) and abnormal dendritic branching seen in medium spiny striatal neurons of R6/2 mice (Spires et al., 2004a) and HD patients (Ferrante et al., 1991). Iannicola et al. (2000) reported that hippocampal neurites in a transgenic HD mouse are shortened, and this study supports and extends these findings. We demonstrate for the first time that

newborn (as opposed to mature) neurons are abnormal in the R6/2 mouse. BDNF is upregulated after seizures and causes morphological changes in granule cells (Vaidya et al., 1999; Sathanoori et al., 2004). However, BDNF levels are low in the R6/2 hippocampus (Zuccato et al., 2001; Spires et al., 2004b), and, in R6/2 mice, the increase in BDNF after seizures may not be of sufficient magnitude to increase neurogenesis and produce morphological changes in newborn granule neurons. The altered morphology of newborn granule neurons in the R6/2 mouse may also be attributable to abnormal cytoskeletal reorganization and dendritic growth (for review, see Harjes and Wanker, 2003; Jarabek et al., 2004). These morphological changes may contribute to the cognitive impairment of R6/2 mice. Because newborn neurons show enhanced long-term potentiation (LTP) (Schmidt-Hieber et al., 2004), it is tempting to speculate that fewer or abnormal newborn neurons may contribute to the impaired LTP seen in R6/2 mice (Murphy et al., 2000; Gibson et al., 2005).

The differentiation fate and survival of NPCs were intact in R6/2 mice. Lazic et al. (2004) could not demonstrate BrdU/NeuN colocalization, but Gil et al. (2005) found that, in contrast to our study, the 2 week survival of BrdU-positive cells was lower in R6/2 compared with WT mice. In agreement with our study, percentage colocalization of BrdU with NeuN was no different between genotypes. The reasons for the difference in survival rates between studies are unclear but may involve subtle differences in mouse colonies, such as the frequency of handling-induced seizures and CAG repeat length, and differences in BrdU dosing regimens and time the animals were killed (Prickaerts et al., 2004). Also, the greatest difference in survival rates between genotypes was found for 3.5-week-old mice (Gil et al., 2005), whereas the youngest mice used in our study were 9 weeks old. From our results, it is likely that the reduced neurogenesis seen in R6/2 mice was attributable to reduced cell proliferation. R6/2 mice are resistant to neurotoxic injury in an age-dependent manner (Hansson et al., 1999; Hickey and Morton, 2000), possibly attributable to differences in calcium handling (Hansson et al., 2001b). A similar mechanism may be operating to preserve survival of newborn neurons in the R6/2 mouse.

Basal SVZ proliferation was the same between genotypes, consistent with previous reports (Gil et al., 2004, 2005; Lazic et al., 2004). We show for the first time that neuroblast migration and numbers of striatal neuroblasts are also the same between genotypes.

That neurogenesis in R6/2 mice was impaired in the DG but intact in the SVZ is interesting. Environmental enrichment increases neurogenesis in the DG but not SVZ. This is partly mediated by BDNF (Brown et al., 2003a; Spires et al., 2004b), and BDNF levels are higher in the hippocampus compared with striatum (Katoh-Semba et al., 1997). Thus, the DG may be more vulnerable to deficits of BDNF, and so neurogenesis is impaired in this region rather than the SVZ. Furthermore, there are clear anatomical differences between the DG and SVZ, and there may be intrinsic differences in the potency of stem-like cells (Seaberg and van der Kooy, 2002).

Neurogenesis in the DG of HD patients has not yet been studied, but SVZ proliferation is increased in HD patients (Curtis et al., 2003, 2005). This is in contrast with data presented here. The reason for the discrepancy between humans and mouse models is unknown. One possibility is that the stimulus for SVZ proliferation in humans may be neurodegeneration in the adjacent caudate nucleus, which is minimal in the R6/2 mouse (Mangiarini et al., 1996). It may also be that intrinsic species differences, such as anatomy (Sanai et al., 2004) and neurotransmitter and growth

factor milieu (Kish et al., 1987; Reynolds and Pearson, 1987; Ferrer et al., 2000; Spires et al., 2004), account for differences in neurogenesis. Finally, it is possible that SVZ proliferation may actually increase in mouse models, but their lifespan is so short that they die before this proliferation can be detected.

Neurogenesis in WT mice was upregulated in response to stimulation. It is likely that seizures (rather than a direct pharmacological action of KA itself) are responsible for increasing neurogenesis in our study, because KA does not increase NPC proliferation in the DG *in vitro* (C. Watts, personal communication). Neurogenesis was not upregulated in R6/2 mice, although interpretation of the QA-induced lesion data are complex given that R6/2 mice had smaller lesions. This suggests that these mice have a lower capacity to mobilize the neurogenic process in response to injury, potentially reducing their capacity for endogenous brain repair.

There was no difference *in vitro* between NPC from adult R6/2 and WT mice. Any differences in neurogenesis between R6/2 and WT mice are therefore likely to be attributable to intrinsic differences undetectable by the neurosphere assay, differences in the response of the NPC to the local environment, or differences in the *in vivo* environment. We found no difference in microglial density between genotypes, suggesting that microglial density does not account for the abnormalities in the *in vivo* environment in R6/2 mice that might be responsible for impaired neurogenesis.

There are many possible mechanisms for the reduction in DG neurogenesis in transgenic HD mice, including a reduction in BDNF (Zuccato et al., 2001; Spires et al., 2004b), alterations in neurotransmitter levels such as serotonin (Gould, 1999; Reynolds et al., 1999), and possibly increased glucocorticoids (Cameron and Gould, 1994). Abnormalities of glia may also account for changes in neurogenesis (Song et al., 2002), and we are currently investigating how the function of microglia and astrocytes might impact neurogenesis in R6/2 mice.

Whether or not reduced DG neurogenesis in R6/2 mice is a primary or secondary phenomenon, it is likely that changes in DG neurogenesis will impact cognition and mood (Kempermann, 2002) in HD. Furthermore, the possible lack of upregulation of striatal neurogenesis in R6/2 mice may contribute to the slow neurodegeneration in the striatum, which is characteristic of HD.

We show here that, in a model of chronic neuronal dysfunction, there are abnormalities of neurogenesis that may relate to the microenvironment rather than to the NPC itself. Furthermore, the observation that newborn neurons in R6/2 mice are morphologically abnormal raises the possibility that they are functionally abnormal, and simply increasing endogenous NPC proliferation may not be sufficient to provide therapeutic benefit. This has implications not only for pathogenesis and therapy of human neurodegenerative disease but also for all cell-based therapies used in repair of the adult brain. Thus, for example, in the case of neural transplantation, it may be necessary to alter the microenvironment into which cells are being transplanted; manipulating the microenvironmental milieu (Morton et al., 2005) may be a useful adjuvant therapy.

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