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Adult Neural Stem Cell Proliferation is Not Altered in Transgenic Mice Over-expressing BDNF or Mutant Huntingtin in Forebrain

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Abstract

Stem cells in the adult brain subventricular zone (SVZ) generate new neurons that migrate to the olfactory bulb. About half of the new neurons survive and become functional interneurons. SVZ stem cells are being studied to discover if there are ways to enhance the survival of adult-born neurons, and if they can be used to replace neurons in damaged brain areas. In transgenic mouse models of Huntington's disease (HD), survival of new olfactory neurons is reduced. Crossing this group with mice over-expressing the growth factor brain-derived neurotrophic factor (BDNF) may increase neuron survival. Before testing for neuron survival effects, we quantified SVZ cell proliferation to determine if transgene expression affected SVZ stem cell proliferation. Four groups of mice (BDNF over-expressers, HD mice, two control strains) were analyzed. Mice were given the mitotic cell marker bromodeoxyuridine (BrdU), euthanized 4 hours later, and labeled SVZ cells were counted. The results indicate that neither increased BDNF expression or expression of a human HD mutation have any significant effect on endogenous SVZ cell proliferation in adult mice.

Introduction

Neurogenesis is the process by which new neurons are generated through division, migration, and differentiation (Bath, et al., 2008). For many years, it was believed that vertebrate neurogenesis was observed strictly during embryonic and perinatal development (Ramon y Cajal, 1913). Findings of new neurons generated after maturity by other neuroscientists changed the commonly held view of neurogenesis. In 1965, Altman reported neurogenesis in the dentate granule cells of the postnatal rat hippocampus (Altman & Das, 1965). In later studies, Nottebohm and colleagues demonstrated adult neurogenesis in studies of male songbirds (Paton & Nottebohm, 1980). More recently, neurogenesis has been shown to occur throughout adulthood in mammals in the dentate gyrus of the hippocampal formation, and in the subventricular zone (SVZ) along the lateral ventricles of the forebrain (Abrous et al., 2005; Alvarez-Buylla & Garcia-Verduga, 2002; Ming & Song, 2011;

Whitman & Greer, 2009). The SVZ serves as a source of neuronal progenitors (Zigova, Pencea, Wiegand, & Luskin, 1998). The SVZ stem cells are slowly dividing mitotic cells that have the ability to proliferate and produce cells that can later differentiate into neurons in the adult brain (Abrous, Koehl, & Le Moal, 2005). As illustrated in Figure 1, in the mammalian olfactory system, new, immature neurons are produced in the SVZ lining the lateral ventricles, and these cells migrate in the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into mature interneurons; most become granule cells in the deepest bulb layer (Abrous et al., 2005; Ming & Song, 2011). Only about half of the immature neuroblasts that migrate into the olfactory bulb survive to become interneurons, while the other half undergo programmed cell death within a few weeks of their birth (Whitman & Greer, 2009; Sui, Horne, & Stanic, 2012). The SVZ is believed to be an important reservoir of neuronal progenitors in the adult brain and may be a useful target for cell replacement therapies aimed at treating neurodegenerative diseases (Alvarez-Buylla and Garcia-Verdugo, 2002; Ming & Song, 2011). Understanding the endogenous factors that affect the production and survival of new neurons in adult brain is of interest for this reason.

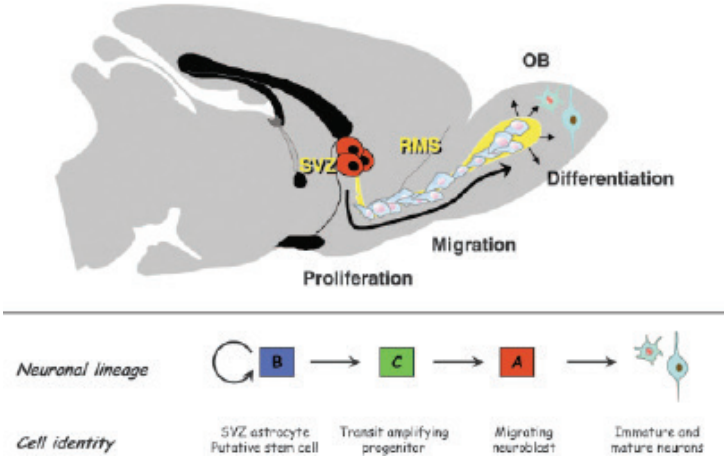

Figure 1. Diagram of a sagittal section through the adult mouse brain illustrating the migration of new neuroblasts from the subventricular zone (SVZ), where proliferating stem cells are located, to the olfactory bulb (OB). Illustration from Abrous et al. (2005) Physiol. Rev. (85): 523-569. (RMS, rostral migratory stream).

Huntington's disease (HD) is an autosomal dominant, chronic neurodegenerative disorder characterized by impairments in motor movements, psychological disturbances, and cognitive decline, which affects about 1 in 10,000 Americans (Phillips, Morton, & Barker, 2005). The motor impairments are caused by the death of neurons in the striatum, an area of the brain that helps to control body movement. This disease is caused by mutations in the huntingtin gene (htt) that result in an abnormally high number of CAG-triplet nucleotide

repeats (encoding polyglutamine), impairing the protein's functions (Kohl, et al., 2010). The normal huntingtin protein (htt) is involved in vesicle trafficking in both the secretory and endocytic pathways in neurons and stimulates transcription of the gene encoding the trophic factor brain-derived neurotrophic factor (BDNF) (Zuccato et al., 2001). The number of new olfactory bulb neurons is reduced in adult HD mouse models that express mutant htt protein (Kohl, et al., 2010). Striatal neurons also degenerate in these mice, and these neurons are known to require BDNF for their maintenance (Rauskolb et al., 2010). When HD mice are crossed with transgenic mice chronically over-expressing BDNF in the brain, striatal neurons survive longer (Gharami et al., 2008). Increased BDNF might also improve the survival of new olfactory bulb neurons in these mice, however this hypothesis has not been examined.

BDNF is a member of the neurotrophin family of peptides. These peptides are growth factors with shared homology that are vital to neuronal survival and maturation during development. The family consists of nerve growth factor, neurotrophin-3, neurotrophin-4/5 and BDNF (Cohen-Cory, Kidane, Shirkey, & Marshak, 2009; Huang & Reichardt, 2001). These factors promote the survival and development of neurons by binding to receptor tyrosine kinases of the Trk receptor family, which stimulates intracellular biochemical pathways that inhibit programmed cell death (Huang & Reichardt, 2001). It has been reported that injecting BDNF into the lateral ventricles or injecting viral vectors that infect SVZ cells which leads to the expression of the BDNF gene, increases survival of new olfactory bulb neurons (Bath, et al., 2008). Delivering BDNF via viral vectors also improved the survival of cells born in the adult SVZ that instead of migrating to the olfactory bulb, migrated to brain areas damaged by quinolinic acid lesions (Henry, Hughes, & Connor, 2007). However, other reports using similar techniques to change BDNF levels, including lateral ventricle injections of BDNF, challenged these findings, and showed that SVZ cell proliferation and olfactory neurogenesis were not stimulated by BDNF in adult mice (Galvao, Garcia-Verdugo, & Alvarez-Buylla, 2008). Therefore, the effects of BDNF are still controversial.

Since increased expression of BDNF in HD transgenic mice helps promote the survival of striatal neurons, it may also help protect adult-born olfactory bulb neurons formed in the adult SVZ of HD mice (Gharami et al., 2008; Kohl et al., 2010). This hypothesis can be tested by crossing HD mice with mice over-expressing BDNF in the olfactory bulb. In order to measure survival rates of new olfactory neurons, the effects of mutant htt expression and BDNF over-expression on stem cell proliferation in the SVZ were first examined. Increases or decreases in proliferation would have an effect on numbers of neurons seen in the olfactory bulb later. If proliferation is not changed, but more adult-born neurons are present in BDNF over-expressers, then this would indicate a survival effect. In this study, SVZ cell proliferation was quantified in four groups of mice: transgenic mice that over-express BDNF (TgBDNF) throughout the forebrain, their wild-type 1 littermates (C571B16 background), transgenic mice expressing mutant htt protein (HD strain R6/2), and their wild-type 2 littermates (B6CBA background strain). The four groups of mice were injected with a mitotic cell marker, bromodeoxyuridine (BrdU), in order to label the actively dividing stem cells in the SVZ (Kee, Sivalingam, Boonstra, and Wojtowicz, 2002). The mice were euthanized four hours post-injection and the density of labeled cells in the SVZ was measured. We found that there was no significant difference in the number of dividing SVZ cells in the four groups of mice, indicating no significant proliferation effect caused by expression of mutant htt or increased BDNF expression. Using this information, we can further evaluate the ability of BDNF to protect new neurons in the adult olfactory bulb of HD mice by crossing the TgBDNF mice with the HD mice.

If more new neurons survive in the crossed mice, in comparison to numbers in HD mice, it will not be because more new cells were born in the SVZ, but instead may show that BDNF can protect new neurons in the adult olfactory bulb from death due to htt after they migrate and differentiate.

Materials and Methods

Animals

The four groups of mice used in this experiment were: (1) transgenic mice over-expressing brain derived-neurotrophic factor in the forebrain under control of the calcium-calmodulin-dependent kinase II alpha promoter (TgBDNF, n=4, Jackson Laboratories, Bar Harbor, ME), (2) their wild-type 1 littermates (C57Bl6 background strain, n=4, Jackson Laboratories), (3) transgenic mice expressing mutant htt protein (R6/2 strain with ~120 CAG repeats in the huntingtin gene, n=2, Jackson Laboratories), and (4) their wild-type 2 littermates (B6CBA background, n=2, Jackson Laboratories). All work was performed according to protocols approved by the FAU Institutional Animal Care and Use Committee. Strains were bred and maintained in the College of Medicine vivarium facilities, and genotyping was performed using the polymerase chain reaction to detect the transgenes in DNA isolated from tail samples. The TgBDNF mice over-express BDNF mRNA and protein in the olfactory bulb granule cell layer, based on earlier in situ cRNA hybridization and Western blot studies in our laboratory (not shown).

Young adult 8-week old male mice were given 100µg/g weight of bromodeoxyuridine (BrdU; Roche Applied Science) in a 5mg/mL solution by intraperitoneal (i.p.) injections over a 1.5 hr period. Mice were treated in matched pairs of transgenic and wild-type littermates. The mice were euthanized with 150mg/kg sodium pentobarbital (i.p.) at four hours after the first injection to allow time for the BrdU to be absorbed and incorporate into actively dividing cells. Mitotic cells begin to show detectable incorporation of the label as early as two hours post-injection, and circulating BrdU is cleared during the next two hours (Abrous, Koehl, & Le Moal, 2005). The mice were perfused transcardially with buffered saline, followed by 4% paraformaldehyde to fix the brain tissue.

Histology

Fixed, dissected mouse brains were frozen after cryoprotection in buffered 25% sucrose for two days to prevent ice crystal formation. The brains were sectioned at 30µm in a cryostat and serial coronal sections (every third) were placed free-floating into 0.1M Trizma-buffered saline (TBS) solution (pH 7.4) in a 6-well tissue culture plate. Sections from transgenic mice were processed in matched batches with sections from their wild-type littermates. Sections were washed in TBS three times at room temperature (RT, defined at 25°C) for 5 minutes each. The tissue was then placed in a 0.6% hydrogen peroxide (H_2O_2) solution to inactivate endogenous peroxidases in the tissue. Three washes in TBS solution were then performed for 5 minutes each at RT. Sections were treated with a 50% formamide/2x saline-sodium citrate (2XSSC) solution for 30 minutes at 65°C. This process helps to denature DNA and permeabilize the nucleus in order for the antibody to BrdU to reach the target sites in the DNA where BrdU is incorporated. The tissue was then washed in 2XSSC twice at RT for 5 minutes each and incubated in 2N HCl for 30 minutes at 37°C for further denaturing. The tissue was transferred to 0.1M sodium borate (pH 8.5) for 10 minutes at RT, and rinsed in TBS buffer. The tissue was then placed in blocking solution (5% normal rabbit serum (NRS) and 0.3% Triton X-100 in TBS) for 60 minutes at RT to prevent the BrdU antibody from binding non-specifically the sections, and to permeabilize the tissue for antibody penetration. After the blocking step, the sections were incubated for 48 hours at 4°C

in a 1:600 dilution of rat anti-BrdU primary antibody (Accurate Scientific Inc.) in 5% normal rabbit serum (NRS) in TBS. The sections were then washed three times in TBS before incubating in the secondary antibody. The secondary antibody was a biotinylated rabbit anti-rat IgG diluted in 3% NRS in TBS. Sections were incubated in the secondary antibody for two hours at RT and were then washed three times in TBS. The tissue was placed in avidin-biotin horseradish peroxidase (HRP) complex (ABC) that was made using the VectaStain Elite kit (Vector Laboratories, Burlingame, CA), made according to the kit instructions. After incubating for 1.5 hours at RT, the reaction product was visualized using freshly made diaminobenzidine (DAB) and hydrogen peroxide from the Impact-DAB kit (Vector Laboratories). Once the tissue was placed in this solution, a brown stain developed within a few minutes wherever the bound BrdU antibody was located. Sections were then rinsed in buffer, and mounted on gelatin-coated glass slides. After dehydration and coverslipping, sections were examined under a microscope (40-100X magnification) to observe the staining.

Cell counts and quantitative analysis

Quantification was carried out by counting the numbers of stained, BrdU-labeled cells in the SVZ using a light microscope. Images were collected using an Olympus AX70 microscope with a digital camera. The number of proliferating cells was recorded as the number of cells per given section through the SVZ. The distance along the SVZ where the cell counts were made was measured from digitized images of each section using NIH Image Software (1.62 Software Program). A minimum of six regularly spaced sections (90μm apart) through the SVZ was used to collect cell counts in the left and right SVZ. Cell counts were made in both the left and right hemispheres (minimum of 12 samples total, per mouse SVZ). The density of brown, BrdU-positive (+) cells was calculated from the number of cells contained within the sample area. The mean density values per animal were used to calculate group mean values, +/- the standard error of the mean (SEM), and the mean density of proliferating cells per group were compared using paired t-tests to test for statistical differences across genotypes. Significance was defined as $p<0.05$.

Results

The density of proliferating SVZ stem cells in adult TgBDNF mice was not significantly different from the density measured in their normal littermates (n=4, Student's paired t-test, p=0.76). Preliminary results of BrdU+ cell counts in HD mice (n=2) also suggest no major difference compared to their wild-type littermates mice (n=2) as reported by Kohl et al. (2010). Even though Kohl et al. (2010) already reported this, we repeated this experiment to be sure we could get the same results using the mice in our laboratory. Female HD mice are infertile and only one in three males is fertile from only 6-9 weeks of age, so they are difficult to breed. After 9 weeks, they develop motor impairments as their striatal neurons degenerate and they die by 13-14 weeks. We continue to breed the strain in order to obtain two more sets of mutant HD and paired WT-2 mice, so that statistical comparisons can be completed using at least four mice per genotype. Figure 2 shows the BrdU-labeled stem cells in the forebrain SVZ in each of the four types of mice. Quantitative comparisons of the densities of BrdU-labeled SVZ cells show no significant differences across the groups, as shown in the bar graph in Figure 3. The mean density of BrdU+ calls in the SVZ of WT-1 mice was at 85.335 cells/mm while the average density of proliferating SVZ cells in TgBDNF was at 82.88 cells/mm, as shown in Table 1. Increased expression of BDNF throughout the forebrain had no effect of SVZ cell proliferation, since the density of proliferating stem cells in the SVZ of mice

that have extra copies of the BDNF gene (the expressed transgene) is not significantly different from the density measured in normal littermates (p=0.76, paired t-test).

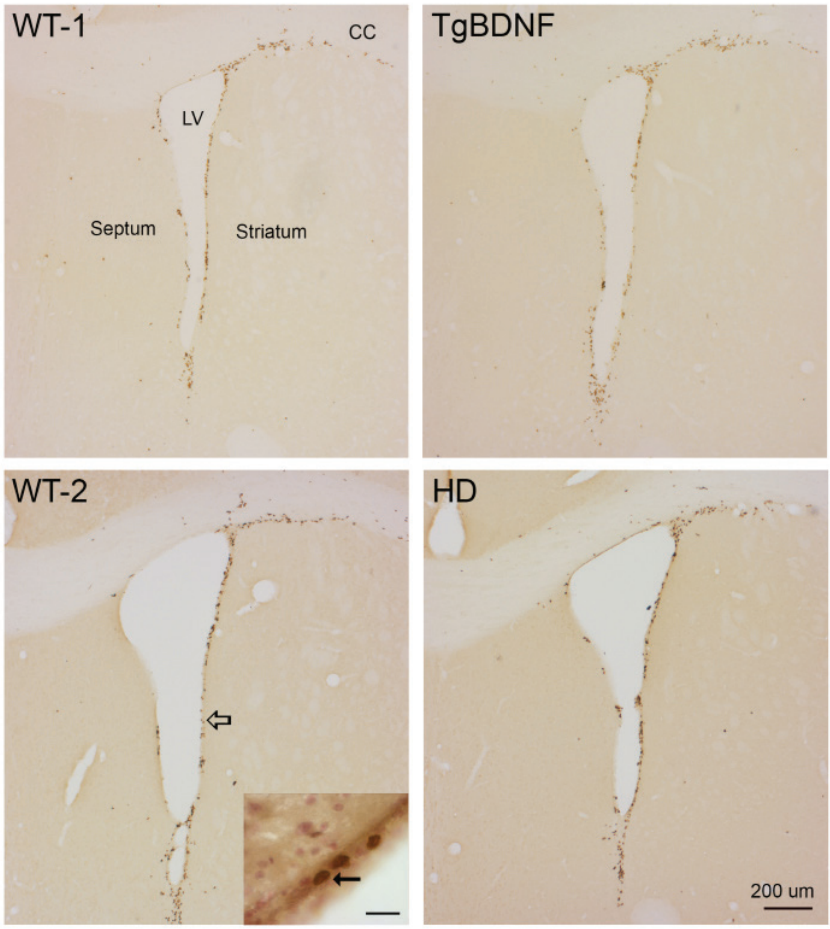

Figure 2. Distribution of proliferating neural stem cells in the forebrain SVZ lining the lateral ventricles (LV) of the four groups of mice. BrdU+ cells appear dark brown. The arrow indicates labeled cells in the SVZ. The inset in the bottom left shows a higher magnification image of BrdU+ cell nuclei. (Cc, corpus callosum). Scale bar in lower right = 200 m. Bar in inset in lower left = 10 m.

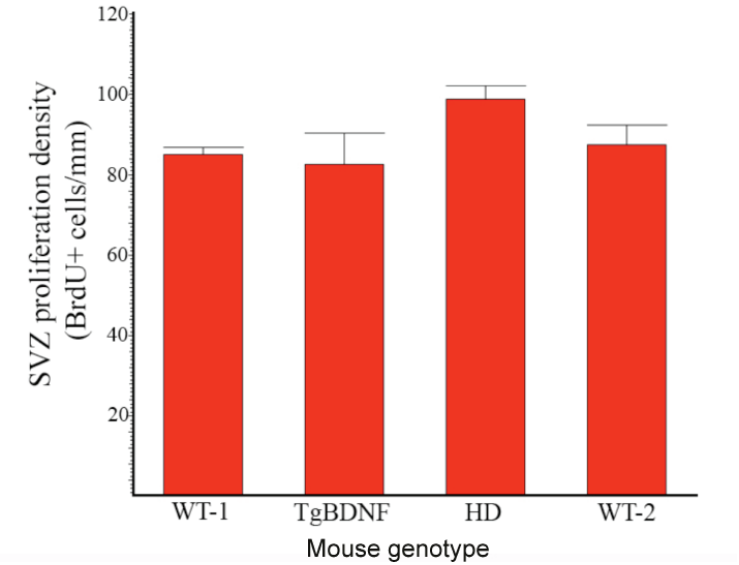

Figure 3. Bar graph comparing the density of proliferating (BrdU+) SVZ cells across groups of mice (mean group values +/- SEM).

Table 1: Numbers of BrdU+ cells/mm of SVZ in each of the groups of mice.

Group	SVZ Proliferation-BrdU+ cells/ mm+/-SEM
WT-1	85.3 +/- 1.8 (n=4)
TgBDNF	82.9 +/- 7.6 (n=4)
HD	99.1 +/- 3.4 (n=2)
WT-2	87.7 +/- 4.8 (n=2)

Table 1. The density of BrdU+ stem cells in the adult SVZ does not show significant differences across genotype, indicating that the expressed transgenes do not alter proliferation rates in vivo. $p>0.5$ for all paired comparisons (paired t-tests).

Discussion

The results of this study indicate the TgBDNF mice do not show a significant change in SVZ cell proliferation compared to their normal littermates. Therefore, increased forebrain BDNF expression does not affect the proliferation of SVZ stem cells in these transgenic mice. This finding is consistent with other BDNF studies that used viral gene transfer or BDNF injections into the lateral ventricles (Galvao et al., 2008). Although only two mice were analyzed in the HD and WT-2 group, preliminary findings show no major differences between these groups and no large reductions in cell proliferation in HD mice. The small sample size was due to the difficulty in breeding the HD mice, because more than half are infertile and die by 14 weeks. However, the results were similar to those previously reported with same HD-R6/2 mice (Kohl et al., 2010, Philips et al., 2005).

Our experiment was the first to analyze the SVZ cell proliferation effects in this transgenic BDNF mouse strain, in which neurons express higher than normal levels of BDNF all over the adult forebrain (Huang et al., 1999). This chronic transgene expression over the animal's lifetime increases endogenous BDNF levels, without using invasive procedures. Previous experiments used direct injections of BDNF or BDNF-encoding viruses into the rodent SVZ to analyze the effects (Bath et al., 2008; Galvao et al., 2008). In studies by Galvao et al. (2008) the lack of cell proliferation effects was shown to be due to lack of full-length TrkB, the receptor for BDNF, in either the SVZ or the RMS. No effects were seen since the functional receptor for BDNF was lacking in the SVZ. Also, truncated TrkB receptors (no tyrosine kinase domain in the receptor) are found throughout the brain on glial cells. These bind BDNF and make it less available to other cells, even when it is directly injected. Our results for the TgBDNF mice provide additional evidence that increasing BDNF in vivo does not affect SVZ cell proliferation, a result that we expected but wanted to confirm. This project does not show whether or not increased BDNF in the olfactory bulb affects long-term survival or maturation of new, adult-born olfactory bulb neurons that migrate from the SVZ to the bulb where they integrate and become functional. This would require counting BrdU+ cells in the bulbs at later time points (2-20 weeks) after BrdU treatment in these mice. Therefore, any changes seen in neuron survival rate will not be due to prior changes in SVZ cell proliferation in these mice.

SVZ cell proliferation effects on later neuronal survival rates in the olfactory bulb have been demonstrated using chemical treatments that reduce stem cell proliferation. Sui et al. (2012) evaluated the effects of reduced progenitor proliferation on neuronal survival at later time points. They concluded that reducing cell proliferation in the SVZ increased (rather then decreased) the number of adult-born, surviving neurons in the adult olfactory bulb compared to normal. They proposed this as a possible mechanism to maintain neuron numbers in the bulb, even with less stem cell proliferation. The granule cell interneurons in the adult olfactory bulb normally die gradually by programmed cell death, which leads to their later replacement from neurons generated by SVZ stem cells. This turnover of granule cell neurons occurs throughout life, and if SVZ stem cell proliferation is permanently blocked, the olfactory bulb actually gets smaller as dying granule cells failed to be replaced (Imayoshi et al., 2008). These findings indicate a correlation between the proliferation rates of SVZ neuronal stem cells and survival rates of the neurons they give rise to.

The results reported from this experiment and others will further our understanding of how to promote survival of new neurons derived from the adult brain SVZ. Further evaluation of survival rates in the olfactory bulbs of TgBDNF mice crossed with the HD mice are planned to see if BDNF can rescue new neurons from death caused by mutant htt. The crossed TgBDNF x HD mice will be examined for changes in SVZ cell proliferation,

but from this experiment we do not expect the crossed mice to show proliferation changes. Any changes in long-term neuron survival in the crossed mice will probably be due to the effects of extra BDNF in the olfactory bulb, as the new neurons reach the adult olfactory bulb and differentiate in their target environment. If there are more maturing neurons that survive, this would show that BDNF is protective for new neurons born in the adult SVZ in the HD mouse model, and may be protective for neurons born from human SVZ neural stem cells as well.

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