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Neurogenesis and Neuroprotection in Huntington Disease

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This thesis, written under the direction of the candidate's thesis advisor and approved by the program chair, has been presented to and accepted by the Biological Sciences Program, at Dominican University of California, in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences.

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“Neurogenesis and Neuroprotection in Huntington Disease”

A thesis submitted to the faculty of

Dominican University of California

&

Buck Institute for Research on Aging

In partial fulfillment of the requirements

For the degree

Master of Science

In

Biology

By

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San Rafael, California

May 2013

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CERTIFICATION OF APPROVAL

I certify that I have read *Neurogenesis and Neuroprotection in Huntington Disease* by Bachir Hadid, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and *The Buck Institute for Research on Aging*.

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ABSTRACT

Adult neurogenesis is attributed to the activity of progenitor cells whose progeny progressively mature to functional neurons under genetic and epigenetic influence.

Due to the significant role of FGF6 (fibroblast growth factor 6), in the skeletal growth, and maintenance of progenitor cells in skeletal muscle, and the role of HDAC inhibitors (histone deacetylase inhibitors) in promoting neurogenesis, and increasing of neuronal proliferation, a combination of FGF6 with HDAC inhibitor CHDI-3 is investigated as a potential treatment to lessen neuronal loss in HD, by enhancing neurogenesis.

Fascinatingly, FGF6 and HDAC inhibitor CHDI-3 treatment was found to be significantly neuroprotective in the expended huntingtin cell lines *Hdh*^{111Q/111Q} model 24hours after treatment during serum withdrawal. Results also show that FGF6 combined with HDAC inhibitor CHDI-3 was found to activate both pERK1/2 and BDNF pathways in the expended huntingtin cell lines *Hdh*^{111Q/111Q} model 1 hour after treatment.

In addition, results from Morris water maze indicate that neurogenesis inhibition negatively regulates the learning and spatial memory, and that neurogenesis occurs more in the young age, and decreases in the old age.

BecausepERK1/2, and BDNF pathways activation have been implicated in neurogenesis, synaptic plasticity, learning, and memory and cell survival, we can conclude that FGF6 and HDAC inhibitors combined can be utilized for future therapeutic treatment to improve learning and memory for HD patients due to its enhancement of neurogenesis.

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ABBREVIATIONS

ANOVA, analysis of variance
BrdU, Bromodeoxyuridine
BDNF, brain-derived neurotrophic factor
CREB, Ca⁺/cAMP-response element binding protein
DCX, doublecortin lissencephalin-X
ECL, electrochemiluminescence
ERK, extracellular signal-regulated protein kinase
FGF6, fibroblast growth factor 6
GCV, gancyclovir
HD, Huntington's Disease
HDAC, histone deacetylase;
Htt, huntingtin
MAPK, mitogen-activated protein kinase
MWM, Morris water maze;
Tg, Transgenic
Sox2, (sex determining region Y)-box 2
SGZ, subgranular Zone
SVZ, subventricular Zone

INTRODUCTION

Huntington's disease

Huntington's disease (HD) is an inherited neurodegenerative disorder with autosomal dominant inheritance. HD is known for characteristic choreiform movements. Cognitive deficits and psychiatric symptoms are frequently observed prior to the onset of the motor symptoms in middle age (Ranen, 1993).

HD affects approximately 1 in 10,000 people in the U.S. (Bates, 2002). HD patients show atrophy of the caudate nucleus, putamen and cerebral cortex. The medium-sized spiny neurons in the caudate and putamen of the basal ganglia, a brain area responsible for coordinating movement, and the cortical layer IV and V neurons in a brain region, which controls thought, perception and memory, are most often affected (Cudkowicz and Kowall, 1990; Hedreen et al., 1991; Albin, 1995). In all cases of HD, the disease results from a mutation in huntingtin (Htt), a ubiquitously expressed protein found at highest levels in neurons. The expanded CAG repeat in the Htt leads to a polyglutamine strand of variable length at the N-terminus, which confers a toxic gain of protein function. The threshold for the repeat length in the disease is 36 repeats or above.

The mechanism by which mutant Htt causes neuronal dysfunction, is still not fully understood. We do know that the mutant forms of Htt are subjected to abnormal folding and aggregation, and may be found in intracytoplasmic or intranuclear inclusions (Schilling et al., 2006).

In addition to the abnormal folding and aggregation of mutant Htt (mHtt), an increase activity of caspases, a family of proteases that are involved in apoptotic cell death, can also generate N-terminal fragments of Htt that can enter the nucleus and form nuclear inclusions in HD (Gafni et al., 2004; Hodgson JG, Agopyan N 1999). It has been shown that mHtt directly binds the acetyltransferase domain of CREB-binding protein (CBP) (Steffan et al., 2001).

There is no cure for HD, and the only available therapy is symptomatic treatment to help the person function for as long and as comfortable as possible (Bonelli and Hofman, 2007). Prior studies have shown that neurogenesis continues in subventricular zone (SVZ) of the lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) in adult mouse, rat, non-human primate, and human brain (Chiasson et al., 1999). Newly generated cells in the SGZ can differentiate into mature, functional neurons and integrate into the DG as granule cells, which are involved in memory formation (Kunlin Jin et al., 2005). Furthermore, an experimental stroke stimulates the proliferation of neuronal stem/progenitor cells located in the SVZ and SGZ of the adult rodent brain (Kunlin Jin et al., 2005), confirming that the resulting newborn cells migrate into ischemic damaged brain regions, where they express mature neuronal markers.

BACKGROUND

HDAC inhibitors in Huntington disease

A previous research confirms that Htt is subjective to the epigenetic mechanism of lysine acetylation and deacetylation via histone acetyltransferases (HAT) and histone deacetylases (HDACs) respectively (Yang and Seto, 2007). The inhibition of HDACs prevents the removal of acetyl groups, and induces transcriptional active gene expression. An experiment was performed with a newly developed HDAC4 inhibitor. The results confirmed a low toxicity level in HD cells, and showed a therapeutic efficacy in preventing motor deficits and neurodegenerative processes in HD model mice (Elizabeth A. Thomas et al., 2008). A recent study indicated that an inhibition of HDAC synergistically promotes neurogenesis induced by a pyridoxine and increase of neuronal proliferation (Dae Young Yoo, et al., 2011). In addition, another current study suggested that HDAC inhibitors in combination with basic FGF could actively drive the Embryonic Stem cells differentiate into neurons (Yao Xing, et al., 2010). Overall, these results demonstrate that HDAC4 inhibitor can be of therapeutic benefit in HD.

NEUROGENESIS IN ADULT BRAIN

Adult neurogenesis is attributed to the activity of progenitor cells whose progeny progressively mature to functional neurons under genetic and epigenetic influence, which occurs in the subventricular-zone, and the subgranular zone (SGZ) –

olfactory-bulb (SVZ, SGZ-OB) system, and dentate gyrus of the hippocampus (Federico Luzzati et al., 2007).

A number of studies have recently demonstrated that neurogenesis is local to dentate gyrus. As a result, the new granular neurons differentiate where they are generated, whereas neuroblast born in the rodent SVZ, undergo a long distance migration to olfactory bulb (Chunmei Zahao et al., 2007).

Ways to utilize the naturally accruing neurogenesis would involve finding pathways that promote cell growth, cell migration, and manipulating multiple signals in internal transduction pathways (Van Praag et al., 2005; Kuhn et al., 1997). The findings suggest that a potential treatment strategy for HD could involve the replacement of dead neurons by stimulating the proliferation of endogenous neuronal precursors, and their migration into damaged regions of the brain. This sparked a research on HD transgenic R6/2 mice treated by subcutaneous administration of fibroblast growth factor FGF-2. This research confirmed that FGF-2 improves neurological deficits and longevity in transgenic mouse model of HD, due to its neuroprotective and neuroproliferative effects (Kunlin Jin et al., 2005).

The Ellerby lab chose to investigate FGF6 because previous research has revealed the significance of FGF6 in skeletal growth in maintenance of progenitor cells in skeletal muscle (Armand AS et al., 2006), and in sustaining ERK phosphorylation (Chen, et al., 2012). Furthermore, the potency of different FGF family members had never been investigated. Comparison of all known FGF family members revealed the family member, FGF6, was a potent neuroprotective protein in

HD cellular models. This evidence directed us to test if FGF6 treatment can lessen neuronal loss, and enhance neurogenesis in HD transgenic zQ175 mice (Armand AS et al., 2006).

Therefore our lab has formulated the following hypotheses for the use of neuroprotection, and neurogenesis as potential treatment to HD pathology.

Hypothesis 1: Activation of two different neurogenesis signaling pathways, using a combination of FGF6, and HDAC inhibitors will increase neuronal plasticity, and eventually will increase learning, memory, and motor function in HD.

Hypothesis 2: Neurogenesis depletion may have a great effect on learning, spatial memory, and aging.

Hypothesis 3: Overexpression of FGF6 or with a combination of HDAC inhibitors, might improve the functional outcomes in zQ175 homozygous full-length knocked HD mice model.

RESULTS

1. FGF6 and HDAC Inhibitor CHDI-3 Displayed Neuroprotection in Huntington's disease Cells' Models.

To confirm if FGF6 and the new novel HDAC inhibitor CHDI-3 that we received from Cure Huntington's Disease Initiative (CHDI) were neuroprotective in HD, we used immortalized mouse striatal cell *Hdh*^{7Q/7Q} and *Hdh*^{111Q/111Q} lines to measure cellular toxicity. We cultured *Hdh*^{7Q/7Q} and *Hdh*^{111Q/111Q} cells, and measured the enzymes activity of caspase-3, -7, and 24 hours after these cells were plated and treated with FGF6, and HDAC inhibitor CHDI-3. The caspase enzyme activity was measured following the manufacturer's protocol (Apo3/7 HTS, Cell Technology Inc. Mountain View, CA). Cell media was removed, and replaced with 25µl of 1X Apo 3 HTS lysis buffer, and with 25µl of DMEM to each well. After gentle shaking for 5 minutes, we removed (20µl) of the samples from the wells to measure the protein concentration in triplicate (BCA Protein Assay Kit, Pierce, Rockford, IL). Then we added 70µl of caspase 3/7 detection reagent with DTT to the remaining of 30µl of sample in each well (the final concentration was 1X caspase reagent and 15 mM DTT). A Fusion Universal Micro plate Analyzer (Perkin Elmer, Waltham, MA) was used to measure the fluorescence signal.

Qualitatively, the measurement of cellular toxicity for FGF6 treatment at 5ng/ml concentration was found to be significantly neuroprotective in the expanded Huntington disease cell model 24 h after treatment (Figure 1A), as well as HDAC inhibitor CHDI-3 at the concentration of 0.5 uM and 1 uM was found to be

neuroprotective in the mouse striatal Huntington's disease cell model after 24 hours of treatment, following the above protocol for the caspase assay (Figure 1B).

Fig: 1A. Caspase activity in *STHdh*Q111/Q111 cells after 24 h serum withdrawal, show a significant reduction in caspase activity after treatment with FGF6.

Fig: 1B. Caspase activity in *STHdh*Q111/Q111 cells after 24 h serum withdrawal; show a significant reduction in caspase activity after treatment with HDAC inhibitor CHDI-3

Neurogenesis signaling pathways

2. Sox2, and BDNF Activation in HD 111Q cell model by FGF6

To determine which neurogenesis signaling pathway FGF6 and HDAC inhibitor CHDI-3 are activating (Figure 2A), the western blot for FGF6, HDAC inhibitor CHDI-3, and the combination with FGF6 and HDAC inhibitor CHDI-3, were done according to the following protocol, and conditions; We incubated the mutant huntingtin striatal cells in serum withdrawal media for 3 hours, next we treated the cells with 5ng/ml of FGF6 for one hour, then we lysed the cells using lysis buffer M-PER, protease inhibitor, and phosphatase inhibitor (Roche), after that we

measured protein concentrations using BCA Protein Assay Kit. We loaded 20µg of each sample per well on a NuPage 4–12% Bis-Tris gel (Invitrogen Corporation, Carlsbad, CA), next we ran all the samples on the same gel at the same time. We transfer over night at 4°C, blocked with milk for one hour, and then we probed the blot with the following antibodies Sox2, BDNF, and beta tubulin for overnight. After that we washed the blot, and we probed the blots with the secondary for one hour, and then put the blot on the same film to avoid the exposure time differences.

Finally to quantify the level of Sox2 and BDNF, we used Image-Quant software to measure the volumetric of the Sox2 and BDNF bands.

Fig: 2A. Neurogenesis signaling pathways

Our quantitative analysis software demonstrated that 5ng/ml concentration of FGF6 increased significantly the expression level of BDNF in the mutant *Hdh^{Q111}* striatal cells, and showed a trend of increase for the level of Sox2. This suggests that FGF6 enhances significantly neurogenesis and plasticity signaling pathway, through the activation of BDNF (Figure 2B).

3.FGF6 mediated pERK1/2 in the mutant *Hdh*^{Q111} striatal cells

To demonstrate if FGF6 activates pERK /ERK the upstream of Sox2, we incubated mutant *Hdh*^{Q111} striatal cells in serum withdrawal for 3 hours, then treated with 5ng/ml FGF6 for one hour, after that we followed the above lab protocol for the western blot, but using this time the primary antibody for pERK, primary antibody for ERK, and b-tubulin, to measure the levels of pERK /ERK using Image-Quant software.

The quantitative analysis result shows that the mutant Huntington's disease striatal cells treated with 5ng/ml of FGF6 exhibited an increase for pERK44/ERK comparing to untreated *Hdh*^{111Q/111Q} cells, and wild type striatal cells, suggesting that FGF6 enhances neurogenesis-signaling pathway through the activation of pERK/ERK pathway (Figure 3A).

Fig: 2B. Immunoblot and its quantification show an increase in Sox2, and BDNF in STHdhQ111/Q111 cells after 1hour treatment with FGF6 in SFM.

Fig: 3A. Immunoblot show increase in ERK phosphorylation in STHdhQ111/Q111 cells after one-hour treatment with FGF6 in SFM, followed by Quantification of the phospho-ERK/ β -tubulin ratio for p44 (ERK1) and p42 (ERK2) bands.

4. FGF6 with HDAC inhibitor CHDI-3 mediated BDNF activation in HD 111Q cell model

We examined the effect of the combination of HDAC inhibitor CHDI-3 with FGF6 on BDNF expression level in mutant *Hdh*^{111Q/111Q} striatal cells, by incubating the mutant *Hdh*^{111Q/111Q} striatal cells in serum withdrawal for 3 hours, then treated the cells with 0.5uM HDAC inhibitor CHDI-3 combined with 2.5ng/ml FGF6 for 1 hour, using the same western blot lab protocol, and the quantitative analysis mentioned above. The results confirmed that mutant huntingtin striatal cells treated with 2.5ng/ml of FGF6 combined with 0.5 uM of HDAC inhibitor CHDI-3, exhibited a significant increase for BDNF in comparison with the serum withdrawal (Figure 4A),

5. FGF6 with HDAC inhibitor CHDI-3 mediated pERK1/2 activation in *Hdh*^{111Q/111Q} cell model

In addition to BDNF, we measure the effect of HDAC inhibitor CHDI-3 and FGF6 on pERK1/2 by incubating mutant Huntington striatal cells in serum withdrawal for 3 hours, then treated them with 2.5ng/ml of FGF6 combined with 0.5 uM of HDAC inhibitor CHDI-3, using the same condition used above. The result showed a significant increase in ERK1/2 phosphorylation (figure 5A), comparing to mutant Huntington's disease striatal cells in the serum withdrawal.

6. pCREB/CREB increase in *Hdh*^{111Q/111Q} cell model by FGF6 and HDAC inhibitor CHDI-3

To confirm if pCREB/ CREB is effected by HDAC inhibitor CHDI-3 combined with FGF6, the mutant huntingtin striatal cells were incubated in serum withdrawal for 3 hours, then treated with 2.5ng/ml of FGF6 combined with 0.5 uM of HDAC inhibitor CHDI-3, for one hour, and using the above western blot protocol, and the quantitative analysis software. The quantification of pCREB/CREB level in the mutant Huntington striatal cells treated with HDAC inhibitor CHDI-3 with FGF6 showed a trend of increase compared to mutant Huntington striatal cells in the serum withdrawal (Figure 6A).

Fig: 4A. Immunoblot and its quantification show an increase of BDNF in STHdhQ111/Q111 cells after 1hour treatment with FGF6 combined with HDAC inhibitor CHDI-3 in SFM

Fig: 5A. Immunoblot show increase in ERK phosphorylation in STHdhQ111/Q111 cells after one-hour treatment with FGF6 combined with HDAC inhibitor CHDI-3 in SFM, followed by Quantification of the phospho-ERK/ β -tubulin ratio for p44 (ERK1) and p42 (ERK2) bands.

Fig: 6A. Immunoblot and its quantification, show an increase in CREB/ β -tubulin, and phospho-CREB/ β -tubulin ration in STHdhQ111/Q111 cells, after one-hour treatment with FGF6 combined with HDAC inhibitor CHDI-3 in SFM.

7. Morris Water Maze (MWM) Test

Conditional inhibition of Neurogenesis/ Immunohistochemistry:

To ensure that the inhibition of neurogenesis occurred for our transgenic TK-DCX mice, treated with anti-viral gancyclovir drug (GCV) for 14 days, the sections of the brains presenting Subventricular zone SVZ of Tg/saline, Tg/GCV, WT/Saline, and WT/ GCV groups were embedded in PFA %4, 7.4 pH, and cut to 7um slides, so we can be able to use immunohistochemistry on the slides. We used immunofluorescent staining of double cortin (DCX) with BrdU. The confocal imaging taken by our lab, clearly showed the ablation of adult stem cells population in SVZ of the mice brain

transgenic TK-DCX, treated with GCV drug, comparing to the wild type mice brain treated with GCV, and Saline (Figure 7A).

Fig: 7.A. Confocal images of ablation of adult cells population in mouse Tg-TK-DCX, GCV, and Wild type saline (K Zafar, 2013).

Neurogenesis inhibition negatively effect learning, spatial memory, and aging

To determine whether learning, and spatial memory requires neurogenesis in young and old mice, we tested learning and spatial memory on five months old, and one year old wild type, and Tg-TK-DCX mice treated with GCV, by an intra-IP injection of Gancyclovir GCV (2.5mg/ml), and saline for 14 days. Saline was used as a vehicle, prior to injection. (N=12 mice used for each group).

Latency to reach the platform (sec) for 5 months–old DCX mice (learning test)

Morris Water Maze data Analysis demonstrated that the control mice; GCV treated wild type mice group, Saline treated wild type mice group, and Saline treated transgenic mice group, performed better than treated transgenic mice with GCV for the latency to reach the platform. The quantitative result using ANOVA confirmed

that treated transgenic mice showed a significant decline in latency time to find the platform comparing to control mice, concluding that there is a decline of learning in treated transgenic mice comparing to the controls (Figure 7B).

Frequency to platform zone for five months old DCX mice (memory test)

Morris Water Maze memory test showed that treated transgenic mice spent significantly less time in the target quadrant for the probe (hidden platform), comparing to the control mice, which spent significantly more time in the target quadrant (Figure 7C).

Fig: 7. B. Latency to reach the platform (sec) for 5 months–old DCX mice (learning test), **C.** Frequency to platform zone for five months old DCX mice (memory test).

B.

C.

Latency to reach platform (sec) for one-year-old DCX mice (learning test) The learning test data analysis for one-year-old mice, showed no significant difference in

latency time to find the platform, between the controls and the treated transgenic mice with GCV, concluding that neurogenesis inhibition had no major effect on learning in the aging mice, like it did for the younger transgenic DCX-TK mice (Figure 7D).

Frequency to platform zone for one-year-old DCX mice (memory test)

The spatial memory test data analysis, showed no significant difference for the time spent in the target quadrant between the control mice and the treated transgenic mice with GCV, concluding that neurogenesis inhibition had no major effect on memory in the aging mice like it did for the younger transgenic DCX-TK mice. (Figure 7E).

Fig: 7. D. Latency to reach platform (sec) for one-year-old DCX mice (learning test),
E. Frequency to platform zone for one-year-old DCX mice (memory test)

D.

E.

DISCUSSION

The caspase-3/7 assay confirmed that the growth factor FGF6 and HDAC inhibitor CHDI-3 are significantly neuroprotective in the Huntington's disease cell model 24 hours after treatment during serum withdrawal

In addition, the western blot results revealed that FGF6 alone, or with the combination of HDAC inhibitor CHDI-3 could enhance two different neurogenesis-signaling pathways, through the activation of pERK1/2 pathway, and the activation of BDNF pathway. The ability to activate two different neurogenesis-signaling pathways, will eventually lead to a significant increase in learning and memory.

Furthermore, the Morris water maze results indicates that the inhibition of neurogenesis contribute to a deficits in the learning and spatial memory in five months old DCX –TK mice treated with GCV, comparing to the control mice.

On the contrary, the inhibition of neurogenesis in the one-year-old mice showed no significant difference in learning and spatial memory between the control mice, and the transgenic DCX –TK mice treated with GCV. These results clearly confirm to us the involvement and the importance of neurogenesis in influencing learning and spatial memory, and revealed to us that neurogenesis occurs more in the younger mice, and less in the aging mice.

CONCLUSION

In conclusion, our study confirmed the involvement and the importance of neurogenesis in influencing learning and spatial memory, and demonstrated that a combination of FGF6 with HDAC inhibitor CHDI-3, have the ability to enhance two different neurogenesis signaling pathways, through the activation of pERK1/2 and the activation of BDNF, leading to an increase in learning and memory. These results suggest that FGF6 combined with HDAC inhibitor CHDI-3, can be utilized for future therapeutic treatment to enhance learning and memory for HD patient.

RESEARCH DESIGN AND METHODS

Induce Neurogenesis in Huntington's disease

Cell culture

The striatal cell lines (Hdh^{7Q/7Q} and Hdh^{111Q/111Q}) were used in this study. Hdh^{7Q/7Q} and Hdh^{111Q/111Q} cells were generously provided by Dr. Marcy MacDonald. The striatal cell lines were maintained at 33°C in a humidified atmosphere of 95% air and 5% CO₂, in Dulbecco's modified eagle medium (DMEM; Cellgro) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were fed with fresh medium every 2–3 days. The PC12 cell lines (23Q and 103Q), kindly supplied by Dr E. S. Schweitzer, were maintained at 37°C in a humidified atmosphere of 90.5% air and 9.5% CO₂, in Dulbecco's modified eagle medium (DMEM; Cellgro) supplemented with 10% heat inactivated horse serum, 5% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin on collagen coated plates and fed every 2-3 days. PC12 cells are inducible cell lines in which mhtt were induced by tebufenozide (1µM).

Caspase 3/7 Assay

Caspase 3/7 activities was measured by using kit as per manufacturer's direction (Mountain View, CA) and normalized by protein concentration expressed as caspase 3/7 activity in RFU/min/mg protein. Briefly, cells were lysed in working cell lysis buffer (50uL, 1:1 lysis buffer: serum free DMEM), cell were lysed by shaking at 700rpm for 5 minutes and a small aliquot (20uL) is taken out to

measure protein. Afterwards 70 uL of working lysis buffer with DTT (20mM), and 2% caspase substrate Rhodamine-DEVD conjugate (zDEVD)₂-Rhodamine 110) added to remaining lysed cells, shake briefly and then read at 37C for 50 reads on Fusion-Alpha FP HT flourometer (Perkin Elmer) at Ex485nm/Em530nm.

Western blot analysis

Cells were harvested in M-PER buffer (Thermo Scientigic) containing a cocktail of protease (Roche) and phosphatase inhibitors (Calbiochem). Cells were lysed by sonication at 30mA for 3x3 sec. Protein concentration was determined using the BCA kit

(Thermo Scientific) following the manufacturer's instructions of protein containing 1x LDS buffer and 100mM DTT is incubated at 95⁰C for 10 min to induce protein denaturation. Following incubation on ice for 2 min, 20µg protein samples were resolved under reducing conditions in MES Buffer (Invitrogen) on precast 4-12% NuPage gels at 200V for 1hr. Proteins are transferred to 40µm nitrocellulose membranes in transfer buffer at a constant 20V for 14hrs at 4⁰C. Membranes were blocked in 3% non-fat milk in TBST for 1hr at room temperature. For phospho-specific antibodies, 3% BSA in TBST was used to block the membranes. Western blots were probed overnight at 4⁰C with primary antibody anti-rabbit phospho ERK (1:1000, Cell Signaling, #4370), anti-rabbit ERK (1:1000, Cell Signaling, #4695), anti-rabbit phospho CREB (1:1000, Cell Signaling, #9191), anti-mouse CREB (1:1000, Cell Signaling, #9104), anti-rabbit BDNF (1:1000, Santa Cruz, sc-546), anti-mouse β Tubulin (1:1000, Sigma #T8328). After 3x10 min wash, blots were

incubated with HRP coupled secondary antibodies (1:3000) for 1 hr and immunoreactivity bands were visualized using the Pierce ECL kit (Thermo Scientific) and Hyperfilm (Kodak).

Adenovirus construction

Standard cloning techniques were used to construct all recombinant AAV-based plasmids. Recombinant expression plasmids containing, the human FGF6 cDNA clone that's been bought through ABGENT, using Invitrogen pcDNA, 4.0 to myc his A, Vector.

The Shuttle vector G0345/ pFBAAVCAGmcsBgHpA, and the control AAV with a GFP, were bought from the Gene Transfer Vector Core of University of IOWA, Carver College of Medicine.

The pFBAAVCAGFGF6 plasmid was constructed by a subcloning protocol. The CAG promoter region from pAAVCAG amplified by polymerase chain reaction (PCR), using the primers FGF6

–SalI_Fwd-5'-(GCATGTCGACATGGCCCTGGGACAGAACT)-3', FGF6_MluI_Rev-5'-(GATCACGCGTTAGAAGGCACAGTCGGAGG)-3', and BGH_Rev-5'-(TAGAAGGCACAGTCGAGG)-3', which contain *SalI*, *MluI* and *BamHI* restriction sites, respectively (underlined). This FGF6 insert in the pFBAAVCAG was then sent to the Gene Transfer Vector Core at University of IOWA, Carven College of Medicine to be complete.

Experimental design and stereotaxic injection

Q175z Homo mice, were injected with AAVCAGFGF6 Adeno Associate virus with a CAG promoter with an insert of FGF6, and injected with the control vector AAVGFP, using stereotaxic and a Hamilton syringe into the both striatum area of the Q175z mouse brain. Each side was injected by 2 μ l of the virus (−2.5 mm anterior to bregma, 3 mm lateral to midline, and 2 mm beneath the dura). Experimental protocols were in accordance with the National Institutes of Health Guidelines for Use of Live Animals and were approved by the Animal Care and Use Committee at the Buck Institute. All possible efforts were made to minimize the number of animals used and their suffering.

Immunohistochemistry

Mice used in our Morris water maze were transcardially perfused with 4% paraformaldehyde and brains were paraffin-embedded, sectioned and deparaffinized with xylene. After antigen retrieval, sections were processed for immunohistochemical localization of DCX double cortin using anti-rabbit DCX (1:50, Santa Cruz, #8607), anti mouse BrdU (1:100, Roche, #1-299-964).

The primary antibodies diluted in 1% BSA overnight. Prior to primary antibody incubation samples were blocked in 10% Donkey serum in TBS. To remove non-specific binding, samples were washed 3 $\times$ in TBS (10min). Antigens were visualized with Alexa Fluor secondary antibodies (1:500).

Tissues were labeled for Brdu and DCX as described previously (Jin et al., 2005).

The stained cells were visualized on a Nikon fluorescence microscope.

(Benraiss et al., 2001; Nunes et al., 2003); (Ivkovic and Ehrlich, 1999)

Animals.

We produced transgenic mice that express herpes simplex virus thymidine kinase (TK) under control of the promoter for doublecortin (Dcx), a microtubule-associated protein expressed in newborn and migrating neurons. Treatment for 14 days with the antiviral drug gancyclovir (GCV) depleted Dcx-expressing and BrdU-labeled cells from the rostral subventricular zone, and abolished neurogenesis. Identification and characterization of transgenic mouse lines was performed by PCR using HSV-TK-specific primers (HSVTK-1: 5'-CCACCACG CAACTGCTGGTG-3'; and HSVTK-2: 5'-CGAGG CGGTGTTGTGTGGTGT-3'). Mice were used in experiments at age 5 to 12 months. Mice were group-housed in an enriched environment with igloo tunnels and unlimited access to food and water. Cages were also equipped with wood shavings, cotton nestlets and a filter top. Temperature, humidity and a 12 h light/dark cycle were tightly controlled. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals to minimize animal suffering and number for the present study.

Morris water Maze

Many neurodegenerative disease mouse models (including polyQ expansion) display learning deficits (Chen et al., *Nature* (2000) 408, 975-9). Learning and memory were assessed in heterozygote Tg polyQ mice and non-Tg experimental animals at 5

and 12 months of age with a Morris water maze in experiments similar to those described (Palop et al., *Proc Natl Acad Sci U S A* (2003) 100:9572). The ability of experimental animals to locate a visible platform in a pool filled with opaque water was tested to exclude differences in vision and swim speed. Animals were trained for 5 days, in 2 sessions, 2.5 hours apart. Each session consisted of 3 consecutive trials. The platform location stayed constant for each mouse, while the starting point at which the mouse will be placed into the water was changed for each trial. Spatial learning was measured as decreases in the time elapsed until each animal reached the hidden platform and as decreases in path length. On days 5 and 7, probe trials in which the platform has been removed was performed, and the time each animal spends in the quadrant where the platform was previously located or frequency of crossing platform zone was recorded as a measure of memory retention. In either case, mice were carefully observed for signs of distress during the test. Any mouse in distress was immediately removed from the water maze.

Rotarod

Animals were tested on a Rotamex Rotarod (Columbus Instruments) on three consecutive days during the dark phase of the 12 h dark cycle. The tests were performed three trials each day, with breaks of at least 1 h between tests. In each trial, mice were placed in separate chambers on the resting rod before rotation was initiated. After 5 seconds of constant slow rotation the speed was increased gradually over the course of 5 minutes from 4 to 40 r.p.m. The timer was stopped either automatically if the mouse falls from the rod as detected by sensor, or manually in

cases when the mouse gripped the rod and started rotating with it. This landing area and this process did not injure the mice.

All experiments and animal care were performed in accordance with the Buck Institute for Age Research Institutional Animal Care and Use Committee guidelines.

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REFERENCES

1. Albin RL (1995) Selective neurodegeneration in Huntington's disease. *Ann Neurol***38**: 835–836.
2. Bates, G., Harper, P., Jones, L., ed. (2002). Huntington's Disease, 3rd edn (New York, Oxford university press).
3. Bonelli R. M., Hofmann P. A Systematic Review of the Treatment Studies in Huntington's Disease since 1990. *Expert OpinPharmacother.* 2007;**8**(2): 141–53
4. Chen, G., Gulbranson, D. R., Yu, P., Hou, Z. and Thomson, J. A. (2012), Thermal Stability of Fibroblast Growth Factor Protein Is a Determinant Factor in Regulating Self-Renewal, Differentiation, and Reprogramming in Human Pluri-potent Stem Cells. *STEM CELLS*, 30: 623–630. doi: 10.1002/stem.1021
5. Chiasson B, Tropepe V, Morshead C, van der Kooy D (1999) Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. *J Neurosci* 19:4462–4471.
6. Chmielnicki E, Benraiss A, Economides AN, Goldman SA.(2004) Adenovirally expressed noggin and brain-derived neurotrophic factor cooperate to induce new medium spiny neurons from resident progenitor cells in the adult striatal ventricular zone.*JNeurosci.*24 (9): 2133-42.
7. Cudkowicz M, Kowall NW (1990) Degeneration of pyramidal projection neurons in Huntington's disease cortex. *Ann Neurol***27**: 200–204
8. Dae Young Yoo, et al.,(2011)Synergistic Effects of Sodium Butyrate, a Histone Deacetylase Inhibitor, on Increase of Neurogenesis Induced by Pyridoxine and Increase of Neural Proliferation in the Mouse Dentate Gyrus. *Neurochem Res* 36:1850–1857
10. Elizabeth A Thomas, et al., (2008) Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037; and [‡]Program in Neurogenetics, Department of Neurology and Semel Institute, University of California, Los Angeles, CA 90095

11. Gafni, J., Hemel, E., Young, J.E Wellington, C.L Hayden, M.R and Ellerby, L.M. (2004). Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/Caspase fragment in the nucleus. *Biol Chem* 279,20211-20220.
12. Gafni and Ellerby (2002). Calpain Activation in Huntington's Disease (Buck Institute for age Research, Novato California). *J. Neurosci* 22 (12): 4842–4849
13. Hao Y, Creson T, Zhang L, et al. Mood stabilizer valproate promotes ERK pathway-dependent cortical neuronal growth and neurogenesis. *J Neurosci*. 2004; 24: 6590–6599
14. Hedreen JC, Peyser CE, Folstein SE, Ross CA (1991) Neuronal loss in layers V and VI of cerebral cortex in Huntington's disease. *Neurosci Lett* **133**: 257–261
15. Hodgson JG, Agopyan N, Gutekunst CA, Leavitt BR, LePiane F, Singaraja R, Smith DJ, Bissada N, McCutcheon K, Nasir J, Jamot L, Li XJ, Stevens ME, Rosemond E, Roder JC, Phillips AG, Rubin EM, Hersch SM and Hayden MR (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* **23**: 181–192
16. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH.(1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci*. 17(15): 5820-9.
17. Kunlin Jin et al., (2005). FGF-2 Promotes Neurogenesis and Neuroprotection and Prolongs Survival in a Transgenic Mouse Model of Huntington's Disease (Buck Institute for age Research, Novato California). *PNAS* 50,18189-18194
18. Langley B, Gensert JM, Beal MF, Ratan RR. Remodeling chromatin and stress resistance in the central nervous system: histone deacetylase inhibitors as novel and broadly effective neuroprotective agents. *Drug Targets CNS Neural Disorder*. 2005; 4: 41–50.
19. Luzzati F, De Marchis S, Fasolo A, Peretto P(2007) Adult neurogenesis and local neuronal progenitors in the striatum. *Neurodegener Dis*.4(4): 322-7
20. Ranen, N., Peyser, CE ., Fostein, SE (1993). A Physician's Guide to the management of Huntington's Disease: Pharmacologic and Non-Pharmacologic Interventions (New York, Huntington's Disease Society of America).

21. Schilling, B., Gafni, J., Torcassi, C., Cong, X., Row, R.H., Lafevre-Bernt, M.A., MA Cusack, M.P., Ratovitski, T., Hirschorn, R., Ross, C.A., et al. (2006). Huntingtin phosphorylation sites mapped by mass spectrometry. Modulation of cleavage and toxicity. *J Biol Chem* 281,23686-23697.

22. Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazanietsev, A., Schmidt, E., Zhu, Y.Z., Greenward, M., et al (2001). Histone deacetylases inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature* 413,739-743.

23. Trettel, F., Rigamonti, D., Hilditch-Maguire, P., Wheeler, V. C., Sharp, A. H., Persichetti, F., Cattaneo, E. & MacDonald, M. E. (2000) *Hum. Mol. Genet.* 9, 2799-2809. .

24. Van Praag H, Shubert T, Zhao C, Gage FH. (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci.*25(38): 8680-5.

25. Yao Xing, et al., (2010) Histone deacetylase inhibitor promotes differentiation of embryonic stem cells into neural cells in adherent monoculture. *Chinese Medical Journal*.

26. Yang XJ, Seto E. (2007) HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene.*26(37)5310-8.

27. Zhao C, Deng W, Gage FH(2008) Mechanisms and functional implications of adult neurogenesis. *Cell.*132(4): 645-60.