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DISRUPTION OF PURKINJE CELL FUNCTION PRIOR TO HUNTINGTIN ACCUMULATION AND CELL LOSS IN AN ANIMAL MODEL OF HUNTINGTON DISEASE

S.E. Dougherty^{a,d}, J. L. Reeves^b, E. K. Lucas^c, K. L. Gamble^d, M. Lesort^d, and R. M. Cowell^{d,*}

^aNeuroscience Graduate Program, University of Alabama at Birmingham

^bNeuroscience Undergraduate Program, University of Alabama at Birmingham

^cDepartment of Psychiatry, Weill Cornell Medical College

^dDepartment of Psychiatry & Behavioral Neurobiology, University of Alabama at Birmingham

Abstract

Huntington Disease (HD) is a devastating neurological disorder characterized by progressive deterioration of psychiatric, motor, and cognitive function. Purkinje cells (PCs), the output neurons of the cerebellar cortex, have been found to be vulnerable in multiple CAG repeat disorders, but little is known about the involvement of PC dysfunction in HD. To investigate possible PC abnormalities, we performed quantitative real time PCR, western blot analysis, and immunohistochemistry experiments to explore the changes in PC markers in the R6/2 mouse model of severe HD. Interestingly, there were reductions in the transcript and protein levels of the calcium-binding proteins parvalbumin and calbindin, as well as the enzyme glutamic acid decarboxylase 67. Immunohistochemistry supported these results, with the most substantial changes occurring in the PC layer. To determine whether the reductions in PC marker expression were due to cell loss, we performed stereology on both presymptomatic and end-stage R6/2 mice. Stereological counts indicated a significant reduction in PC number by end-stage but no change in presymptomatic animals (4 weeks of age). To assess cellular function prior to cell loss and symptom onset, we measured spontaneous firing in PCs from 4-week old animals and found a striking deficit in PC firing as indicated by a 57% decrease in spike rate. Interestingly, huntingtin inclusions were not widely observed in PCs until 12 weeks of age, indicating that soluble huntingtin and/or abnormalities in other cell types may contribute to PC dysfunction. Considering the roles for PCs in motor control, these data suggest that early PC dysfunction potentially contributes to motor impairment in this model of HD.

Introduction

Huntington Disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive deterioration of cognitive, motor, and psychiatric function. HD is caused by an elongation of the CAG repeat in exon 1 of the huntingtin gene, with an

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*To whom correspondence should be addressed., Department of Psychiatry & Behavioral Neurobiology, 728 Sparks Building, 1720 7th Avenue South, Birmingham, AL 35294, Phone: (205) 975-7466, Fax: (205) 975-4879, rcowell@uab.edu.

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inverse correlation between age of onset and repeat length (Group 1993). Expression of mutant huntingtin (mhtt) is ubiquitous throughout the brain and is thought to be involved in a number of pathological interactions. These functions include transcriptional dysregulation (Cha, Kosinski et al. 1998; Luthi-Carter, Hanson et al. 2002; Strand, Aragaki et al. 2005; Hodges, Strand et al. 2006; Imarisio, Carmichael et al. 2008; Bithell, Johnson et al. 2009), interruption of calcium signaling (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011), and disruption of normal synaptic physiology (Klapstein, Fisher et al. 2001; Morton, Faull et al. 2001; Milnerwood and Raymond 2007; Cummings, Andre et al. 2009).

While the striatum is the primarily affected brain region in HD, it is possible that other components of the motor circuit, such as the cerebellum, are involved in motor symptom development. Conflicting reports have been made on the pathology of the cerebellum in HD which range from the region being considered unaffected (Van Raamsdonk, Warby et al. 2007; Carroll, Lerch et al. 2011) to the cerebellum being an important part of the disease progression (Rodda 1981; Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006). The cerebellum appears to be more commonly affected in juvenile HD as exhibited by a loss in overall cerebellar volume (Kageyama, Yamamoto et al. 2003; Fennema-Notestine, Archibald et al. 2004; Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). It is imperative to explore cerebellar pathology in animal models of HD to determine the involvement of this brain region in the pathogenesis of HD.

The output neurons of the cerebellar cortex are the GABAergic Purkinje cells (PCs). Intranuclear inclusions have been found in PCs in postmortem tissue from HD patients and in knock-in animal models of HD (Adachi, Kume et al. 2001). Additionally PCs are vulnerable in both patients and animals models of other CAG repeat disorders (Koeppen 1991; Vig, Fratkin et al. 1996; Vig, Subramony et al. 1998; Garden, Libby et al. 2002; Koeppen 2005; Schulz, Borkert et al. 2010). Investigators have often considered the cerebellum to be unaffected in HD and used the cerebellum as a control region. Nonetheless, there is evidence to suggest a reduced density of PCs in HD patients (Jeste, Barban et al. 1984; Rosas, Koroshetz et al. 2003; Fennema-Notestine, Archibald et al. 2004) raising the possibility that PCs play a meaningful role in HD.

Little is known about PC function or survival in animal models of HD. To investigate the involvement of PC dysfunction in HD, we utilized the R6/2 model. The R6/2 mouse model of HD carries transgenic expression of exon 1 of the human huntingtin gene with an expanded CAG repeat (Mangiarini, Sathasivam et al. 1996). This model has been shown to be a severe model of HD with an abbreviated lifespan, early symptom onset and enhanced disease severity. This increased severity might suggest a more global neurological assault (Ruocco, Bonilha et al. 2008); as such, it follows that investigation of cerebellar changes in the R6/2 mouse could provide insights into the involvement of this brain region in early onset HD.

In these experiments we explored the changes that occur in PCs of R6/2 mice using qRT-PCR, immunohistochemical staining, and electrophysiology. Here we report decreases in GABAergic cell markers and calcium binding proteins in the cerebellum, specifically the PC layer. These changes were accompanied by huntingtin accumulation and PC loss in symptomatic mice but preceded by alterations in cellular physiology. These studies suggest that PCs are vulnerable in R6/2 mice and may contribute to the development of motor symptoms in HD.

Methods

Animals

The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all experimental protocols. The R6/2 mouse line was obtained from Jackson Laboratories and maintained through breeding male F1 generation offspring to WT hybrid (B6CBA) females (B6CBA-Tg (HDexon1)62Gpb/3J). The mice were obtained from Jackson Laboratories by way of an ovarian transplant female with ovaries from a sexually unviable R6/2 +/- female. All experiments were conducted with both male and female animals at ages 2, 4, 7, and 12 weeks. R6/2 mice carried a repeat length of 167 as genotyped by Laragen, Inc (Culver City, CA). These animals were housed in groups of up to 5 animals per cage with food and water *ad libitum*.

Behavioral Assessment

Behavioral analyses were conducted on littermates at four, six or twelve weeks of age during the lights on period (6am - 6pm). All experiments were conducted blind to the genotype of the animals.

Rotarod

The rotarod apparatus (MedAssociates, St. Albans, VT) consisted of a 5-station treadmill with a computer-controlled stepper motor-driven rod with constant speed or accelerating speed modes of operation. Animals were trained on the rotarod for four days. On the first day of the training period, animals were placed on the treadmill at an accelerating speed of 2-24 rotations per minute (rpm) for a maximum of 60 seconds for a total of 5 trials. On the second through fourth days, the animals were placed on the treadmill at a constant speed of 16 rpm for a maximum of 60 seconds for a total of 5 trials per day. On the fifth day, animals underwent two trials each at rotating speeds 16, 20, 24, 28, and 32 fixed rpm. Each trial lasted for a maximum of 60 seconds, during which latencies to fall were recorded. Mice were allowed to rest for at least five minutes between each trial.

Open field

Animals were placed in a square apparatus (27.9 cm²) consisting of 48 infrared beams (MedAssociates) for 30 minutes. Data were collected with Open Field Activity Software (MedAssociates) in one minute intervals over the test period, and total ambulatory time and distance were determined.

Gene Expression Analysis

Protocols were performed as described (Lucas, Markwardt et al. 2010). Briefly, mice were anesthetized with isoflurane and sacrificed by decapitation. Brains were removed, and dissected by anatomical region. Tissues were flash frozen on dry ice and kept in the -80°C freezer until use. At least 12 hours prior to processing, tissues were placed in RNA^{later}-ICE (Ambion Austin, TX) cooled to -80°C and then kept at -20°C until homogenization. Homogenization was performed with a Tissue-Tearor (Biospec Bartlesville, OK) homogenizer in Trizol following the manufacturer's instructions (Invitrogen Grand Island, NY). Taqman PCR was conducted with JumpStart Taq Readymix (Sigma St. Louis, MO) and Applied Biosystems (Carlsbad, California) primers for GAD67 (Mm00725661_s1), calbindin (Mm00486645_m1), parvalbumin (Mm00443100_m1), and β -actin (Mm00607939_s1).

Immunofluorescence

Animals were anesthetized with isoflurane and perfused intracardially with room temperature 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed and postfixed for an additional 24-72 hours. The samples were cryoprotected in graded sucrose over a 5 day period and then embedded and frozen in a mixture of 20% sucrose and Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc. Torrance, CA) and stored at -80°C . Tissue blocks were sectioned at $30\text{ }\mu\text{m}$, mounted onto charged slides (Fisher) and allowed to dry overnight before storage at -80°C . Slides were then thawed and washed with PBS followed by a one hr incubation in 10% serum (from the host of the 2^o antibody) and PBS with 3% bovine serum albumin (BSA). The slides were then incubated overnight with a predetermined concentration of 1^o antibody and 5% serum in 0.3% Triton $\times$ PBS with 3% BSA. This is followed by washing and then a 2 hr incubation with the corresponding fluorescence-conjugated 2^o antibody (Jackson ImmunoResearch West Grove, PA) and 5% serum in 0.3% TritonX PBS with 3% BSA. The slides are then immediately mounted using an antifade media containing DAPI, coverslipped and left to dry at room temperature overnight. When necessary, a Vector (Burlingame, CA) Mouse on Mouse Kit (Fluorescein Cat No. FMK-2201) was used to minimize background staining for mouse-made antibodies.

Western blot analysis

Cerebella were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% sodium dodecyl sulfate, 0.5% deoxycholic acid; pH 8.0) containing a protease inhibitor tablet (Complete Mini, Roche Diagnostics Indianapolis, IN). Total protein concentration was determined with a bicinchoninic acid protein assay kit (Thermo Scientific Waltham, MA), and absorbance was measured at 540 nm. Protein was denatured in sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate, 5% β -mercaptoethanol; pH 6.8) at 95°C . Equivalent amounts of protein were loaded into precast polyacrylamide NuPage gels (Invitrogen). One interblot control sample was loaded onto every gel to permit comparison among gels. Protein was transferred onto nitrocellulose membranes. Blots were blocked with 5% milk in tris buffered saline (TBS; pH 7.6) with 1% Tween (TBS-T) and probed with primary antibodies in 5% IgG-free BSA (Jackson ImmunoResearch) TBS-T overnight at 4°C and peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) in 5% milk TBS-T for one hour at room temperature. Blots were incubated in chemiluminescent substrate (Thermo Scientific Pittsburgh, PA) and exposed to film. The optical density of bands was calculated after background subtraction using UN-SCAN-IT gel analysis software (Silk Scientific Inc. Orem, UT). All bands were normalized to the interblot control band, then to actin and expressed as mean optical density mean $\pm$ SEM.

Antibody Information

Immunostaining was performed with antibodies specific to the following proteins: mouse monoclonal GAD67 (MAB5406, Millipore Billerica, MA) at a dilution of 1:500 for fluorescence, 1:1000 for western; mouse monoclonal Calb (C9848, Sigma) at a dilution of 1:2000 for fluorescence, 1:5000 for western; rabbit polyclonal PV (PV25, Swant) at a dilution of 1:500 for fluorescence; mouse monoclonal PV (Millipore) at a dilution of 1:500 for western; and goat polyclonal Huntingtin (SC-8767, Santa Cruz) at a dilution of 1:100 for fluorescence.

Stereology

Animals were anesthetized with isoflurane and perfused intracardially with room temperature 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed and postfixed for an additional 24-72 hours. The samples were cryoprotected in graded sucrose over a 5 day period and then embedded and frozen in a mixture of 20% sucrose and

Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc. Torrance, CA) and stored at -80°C . Tissue blocks were sectioned at $30\text{ }\mu\text{m}$, mounted onto charged slides (Fisher) and allowed to dry overnight before storage at -80°C . Slides were then thawed and washed with distilled H_2O for 5 minutes followed by a 10 minute incubation in hematoxylin (Sigma). Slides were incubated in running H_2O for 15 minutes followed by a 1 minute incubation in eosin (Sigma). Slides were then dehydrated and cover-slipped using Permount (Fisher).

Cells were counted by an unbiased investigator blinded to animal genotype using StereoInvestigator (MicroBrightField, Inc Williston, VT). Pilot animals were used to determine parameters needed to give optimal counting precision. The optical fractionator method was used to generate an estimate of PCs counted in an unbiased selection of serial sections in a defined volume of the cerebellum. Serially cut $30\text{ }\mu\text{m}$ thick sagittal tissue sections (every twentieth section) were analyzed throughout the entire cerebellum of animals in each cohort ($n=3/\text{genotype}$). The number of PCs within the entire cerebellum was quantified using the optical fractionator method with dissectors placed randomly according to a $263.68 \times 212.13\text{ }\mu\text{m}$ grid. The counting frame was $60 \times 60\text{ }\mu\text{m}$ and the disector height was $26\text{ }\mu\text{m}$. On average, 150 sites were counted per animal. An estimation of cell population number was provided using overall raw counts and mean section thickness.

Cell Attached Loose Patch Recording

Mice (4 week old) were anesthetized with isoflourane and sacrificed by decapitation. Brains were placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 CaCl_2 , 1 MgCl_2 , 25 NaHCO_3 1.25 Na_2HPO_4 and 25 D-glucose with a pH of 7.4 and osmolality of $295 \pm 5\text{ mOsm}$. The ringer solution was bubbled with 95% O_2 /5% CO_2 . Sagittal cerebellar slices ($300\text{ }\mu\text{m}$ thick) were cut using a Vibratome (7000 smz, Campden Instruments: Sarasota, FL). The slices were allowed to rest for 60 min at room temperature ($22\text{-}23^{\circ}\text{C}$); all experiments were then performed at room temperature. Slices were superfused continuously with oxygenated recording ACSF at room temperature. Slices were viewed with an upright microscope (Zeiss Axio Examiner A1) using infrared-differential interference contrast optics. Loose patch recordings were acquired from visually identified PC using Axio Vision 4.8 software. Cellular activity was recorded using internal solution containing the following (in mM) 140 K-gluconate, 1 EGTA, 10 HEPES and 5 KCL, pH 7.3. Pipette tip resistance was 2-5 M Ω . The extracellular recording pipettes, containing the internal solution, were placed under visual control directly adjacent to the soma of the cell of interest. Positive pressure was applied throughout this process followed by a brief release of pressure to form a seal averaging approximately 45 M Ω . Cell-attached, loose patch-clamp recordings were obtained using an Axon CNS Molecular Devices amplifier (Multiclamp 700B), filtered at 10 kHz and digitized at 20 kHz (Digidata 1440A). A 2 min gap-free protocol on Clampex 10.2 software was used for data collection. Detection and analysis of event frequency and inter-event interval were performed semi-automatically using the program Clampfit 10.2. The detection threshold was set for analysis based on the event amplitude from a given cell.

Data Analyses

Data analyses for qRT-PCR, Western Blot assays and loose patch electrophysiology were performed using Microsoft Excel. A two-tailed student t-test assuming unequal variance was utilized to assess statistical significance. Values were considered statistically significant when the p value was less than 0.05. For the measurements of transcripts at different stages of the disease, R6/2 values were normalized to WT values at each independent age, so t-tests were used; thus, the asterisks ($p<0.05$) indicate when R6/2 values were significantly different from values for WT of the same age.

For the open field behavior data, a two-way ANOVA (Age $\times$ Genotype) was performed using PASW 18.0. For post hoc analysis Fisher's least significant difference (LSD) procedure was utilized. Values were considered statistically significant when the p value was less than 0.05.

For the rotarod behavior data, analyses were performed using PASW 18.0. The raw time values measuring latency to fall were binned according to 33rd percentiles (i.e., 0-12.99, 13.00-59.00, and 59.01-60 seconds) for the maximum speed trial (32 rpm). A G-likelihood test was performed on the binned data (binned Proportions were considered statistically significant when the p values were less than 0.05).

For the stereological data, a two-way ANOVA (Age $\times$ Genotype) was performed using PASW 18.0. For post hoc analysis Duncan's multiple comparison procedure was utilized. Values were considered statistically significant when the p value was less than 0.05.

Results

R6/2 mice exhibit an overt motor phenotype at midlife

In order to validate previous reports of the onset and progression of motor symptoms in the R6/2 mouse, we utilized the open field and rotarod behavior paradigms. The open field analysis revealed a hypoactive phenotype which continues throughout the lifespan of these animals ($n = 8/\text{group}$) (fig 1A). A two-way ANOVA revealed significant main effects of genotype and age ($F(1,36) = 16.1$ and $F(2,36) = 11.2$, respectively; $p < 0.01$ for both) but no interaction ($F(2,36) = 2.1$, $p = 0.14$). The rotarod paradigm, which more directly tests balance and motor coordination, revealed coordination dysfunction at 32 rpm at twelve weeks of age ($n = 8/\text{group}$; $\chi^2(2) = 15.02$ $p < 0.01$) (fig 1B). Additionally, there significant differences between WT and R6/2 in the proportion of animals that fell at speeds of 28, 24, and 20 rpm, $\chi^2(2) = 22.18$, 22.18, and 8.00, respectively ($p < 0.05$ for all). There was no difference between WT and R6/2 at 16 rpm at 12 weeks of age ($\chi^2(2) = 2.86$, $p = 0.24$). There were no differences between the two groups at six weeks of age (data not shown) in concordance with previously published data (Mangiarini, Sathasivam et al. 1996).

GABAergic cell markers are decreased in the cerebellum of R6/2 mice around the time of motor onset

To initially determine whether alterations in PCs occur in the R6/2 model, we performed qRT-PCR to evaluate the levels of transcripts that are highly concentrated in PCs. Transcript levels were normalized to the internal control gene, actin, and then reported as fold WT (WT = 1.0). There were no changes in actin between R6/2 and WT mice at any age. There was a decrease in the level of GAD67 ($p < 0.05$) and Calb ($p < 0.05$) transcript at seven and twelve weeks of age ($n = 9/\text{group}$, compared to age-matched WT littermates) and a reduction in PV transcript ($p < 0.05$) by twelve weeks of age (Fig 1C; $n = 9/\text{group}$). Student's t tests were utilized to assess alterations at individual ages since R6/2 values were only compared to values from WT mice of the same age. We then evaluated these changes in expression at the protein level in cerebellum homogenates; protein analysis revealed significant decreases in GAD67, PV, and Calb by twelve weeks of age in the R6/2 mice as compared to WT littermates ($n = 7/\text{group}$, two-tailed student's t test, $p < 0.05$; fig 1D & E). To localize the protein changes to specific cell types, immunohistochemistry was performed; immunoreactivity for GAD67 (fig 2 C&D), Calb (fig 2 G&H) and PV (fig 2 K&L) was reduced markedly in the PC layer of R6/2 cerebellum. Strikingly, the interneurons of the molecular layer retained their basal levels of PV (fig 2L, arrows). These staining patterns suggest alterations specifically in the PCs within the cerebellum of the R6/2 mice. At four

weeks of age, there is no reduction in GAD67, Calb, or PV immunostaining in the R6/2 as compared to WT (fig 2 A&B, E&F and I&J, respectively).

R6/2 mice exhibit a loss of Purkinje cells by end stage

One explanation for the loss of PC markers could be a reduction in the total cell number in the R6/2 mice. In order to investigate whether decreases in protein and transcript levels stem from localized molecular loss within intact cells or from a loss of PC bodies, we performed stereological investigation on R6/2 mice and WT littermates. Stereological counts revealed no significant decrease in PC number at four weeks of age in the R6/2 mice (fig 3A&B). A population estimate using mean section thickness gave an estimate of an average of 84,886 cells in 4 week WT and 107,961 cells in 4 week R6/2 animals. There is a reduction in PC number at twelve weeks of age, decreased 55% as compared to WT littermates (fig 3 C&D). A population estimate using mean section thickness gave an estimate of an average of 97,208 cells in 12-week WT and 43,667 cells in 12-week R6/2 animals. A two-way ANOVA revealed no significant main effects of age or genotype, $F(1,8) = 1.57$ ($p = 0.25$) and $F(1,8) = 4.57$ ($p = 0.07$), respectively. However, there was a significant Age $\times$ Genotype interaction, $F(1,8) = 9.92$, $p < 0.05$ ($n = 3$ animals/group). Duncan's multiple comparison procedure showed that the R6/2 12-week old animals had a mean cell count that was significantly reduced from the other three group's mean cell counts. The lack of a difference in cell number between WT and R6/2 mice at 4 weeks of age suggests that the production and migration of these cells is not affected during development.

Purkinje cells exhibit a reduction in firing rate prior to motor symptom onset and transcriptional changes in R6/2 mice

The motor abnormalities observed in the R6/2 mouse model of HD arise prior to an overt loss of PCs in the cerebellum. In order to elucidate the possible role of early alterations in cellular function in this cell population, cell-attached loose-patch recordings were performed in presymptomatic animals (4 weeks of age). Using an acute slice preparation, overall spike rate was recorded from 4-week-old R6/2 mice and WT littermates. Slices were incubated in ACSF and, once patched, allowed to acclimate to the pipette tip for three minutes in order to eliminate any events in response to mechanical stimulation. Spontaneous events were then recorded for three minutes with no stimulation. Cells with fewer than five spikes within the recording interval were excluded. Loose patch recordings revealed a significant reduction in the spike frequency in the R6/2 mice as compared to WT littermates at four weeks of age, prior to the onset of motor symptoms ($n = 4$ for R6/2 and $n = 5$ for WT with 15 and 17 cells, respectively; two-tailed student t test, $p < 0.05$; fig 4 A&B).

Intranuclear inclusions are not evident in Purkinje cells at the time of electrophysiological deficits

To investigate the pathogenic mechanisms behind the change in firing rate, we questioned whether huntingtin inclusions were present within PCs at the time of electrophysiological deficits. Immunofluorescence using a mhtt-specific antibody revealed the presence of mhtt within the molecular and granule cell layers of the R6/2 cerebellum (fig 5B arrows) while staining was entirely absent in 12-week-old WT (fig 5A). Colocalization of mhtt-positive inclusions with PV (green, Fig. 5B, inset) in the molecular layer of 4-week-old R6/2 mice indicated that molecular layer inclusions were concentrated in molecule layer interneurons. Intriguingly, similar staining patterns were observed at 7 weeks of age, though there were more pronounced inclusions in the interneurons (enlarged in fig 5E) with few inclusions noted in PCs (fig 5C arrows). By 12 weeks of age the R6/2 mice show inclusions in most cells throughout the layers of the cerebellum including pronounced inclusions within the PC layer (fig 5D, enlarged in fig 5F).

Discussion

Huntington Disease is a debilitating neurological disorder. The R6/2 mouse is a severe HD model with an early onset of symptoms and an abbreviated lifespan (Mangiarini, Sathasivam et al. 1996). We show that decreases in PC-specific transcript and protein levels occur as part of the disease etiology in the R6/2 mouse model of HD. Furthermore, we demonstrate that PCs are lost during the course of the disease in R6/2 mice and that prior to degeneration- and overt motor symptom onset, PCs are dysfunctional at a physiological level, with a dramatic reduction in firing rate. This demonstration of presymptomatic functional deficits in PCs of R6/2 mice, prior to the appearance of intranuclear inclusions, is novel and suggests that alterations in cerebellar function may contribute to the onset of motor symptoms in this model.

Initial investigations of transcript levels in the cerebellum indicate a loss of the enzyme GAD67 and the calcium-binding proteins PV and Calb by end-stage in the disease time course. Immunohistological staining revealed that these proteins were lost specifically from the PC layer. While a previous report argued for a role in transcriptional downregulations of PV in the cerebellum (Luthi-Carter, Hanson et al. 2002), the observed loss of PC number associated with transcriptional and protein level changes suggest that these changes are due to a loss of PCs, not transcriptional dysregulation. However, while we did not directly investigate this possibility, reductions in transcript/protein levels in the remaining cells could contribute to alterations in calcium homeostasis and signaling (Llano, DiPollo et al. 1994; Schwaller, Meyer et al. 2002), which has already been noted in other brain regions in HD (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011).

That such cell loss is occurring in the cerebellum is striking as ataxia is not a common finding in this model (Mangiarini, Sathasivam et al. 1996), and PC dysfunction has been shown to cause ataxia-like motor deficits (Walter, Alvina et al. 2006). While ataxia may not be a key symptom of HD, alterations in gait and balance abnormalities have been observed in both young (5 weeks of age) and end stage mice (Carter, Lione et al. 1999; Luesse, Schiefer et al. 2001; Chiang, Chen et al. 2010), in addition to symptoms of dystonia (Mangiarini, Sathasivam et al. 1996; Stack, Kubilus et al. 2005; Stack, Dedeoglu et al. 2007). There are also some reports of ataxia and gait disturbances in HD patients (Koller and Trimble 1985; Grimbergen, Knol et al. 2008), although it is possible that more overt manifestations of ataxia are masked by the traditional motor symptoms. Recent work has suggested that disruption of Purkinje cell function by mutations in the $\alpha 3$ isoform of the sodium-potassium ATP-pump cause the dystonia seen in a Dystonia-Parkinsonism mouse model through aberrant interactions with the basal ganglia (Calderon, Fremont et al. 2011). It is intriguing to postulate that the cerebellum is playing a similar role in the R6/2 model to influence basal ganglia function.

Previously it has been shown that cells of the deep cerebellar nuclei precisely encode spike timing of synchronous PC firing (Person and Raman 2011). Reductions in GABAergic output from the inhibitory PC could lead to over-excitation in these deep cerebellar nuclei and propagation of misfired cellular signals. Additionally, alterations in spiking patterns could contribute to dysfunctional motor output as the cerebellum forms disynaptic connections with the basal ganglia through the thalamus (Bostan, Dum et al. 2010; Bostan and Strick 2010). Interestingly, reports have shown that PC spiking activity is associated with altered levels of GAD67 mRNA (Litwak, Mercugliano et al. 1990; Drengler and Oltmans 1993); it follows that reductions in firing rate could lead to reductions in the mRNA and protein levels that we observed. Further, as PC have been shown to be particularly dependent on activity for survival (Morrison and Mason 1998), it is possible that a reduction in overall PC activity could contribute to cell death.

To determine what mechanisms could underlie PC loss, we investigated alterations in cellular function and cell-specific accumulation of huntingtin prior to cell loss and the onset of overt motor symptoms. At 4 weeks of age, there was a marked reduction in firing rate in R6/2 mice as compared to the WT. Intriguingly, these firing abnormalities occurred without evidence for huntingtin positive inclusions in PCs. Therefore, the observed reductions in firing rate could reflect cell-autonomous effects of soluble mhtt on membrane properties or, since mhtt staining was readily observed in molecular layer interneurons, non-cell autonomous effects of interneuron dysfunction (Garden, Libby et al. 2002). Notably, we observed relative preservation of the molecular layer interneurons, so it is possible that alterations in interneuron function could influence PC firing (increased inhibition). A similar pattern of cell loss (loss of PCs, preservation of interneurons) has been shown previously in a model of spinocerebellar ataxia-1 (Vig, Subramony et al. 1998). Regarding the toxicity of huntingtin, both soluble and aggregated huntingtin have the potential to be toxic (Lajoie and Snapp 2010; Weiss, Kimura et al. 2011); the preservations of the interneurons through the disease time course supports previous reports suggesting the intranuclear inclusions may be protective and not causative in cell death (Kuemmerle, Gutekunst et al. 1999; Arrasate, Mitra et al. 2004).

As previously stated, the R6/2 mouse model is a severe HD mouse model that may more closely replicate a juvenile-onset (JOHD) disease progression (Sawiak, Wood et al. 2009). The most severe cases of HD are JOHD and have the longest recorded repeat length. Intriguingly, case studies have shown that JOHD presents not only with the cardinal features of adult onset HD (rigidity, chorea, dementia) but also additional symptoms including a higher occurrence of seizure activity (Hattori, Takao et al. 1984; Ruocco, Lopes-Cendes et al. 2006). In JOHD, there are multiple reports of cerebellar involvement, atrophy and dysfunction, including fMRI studies showing an incidence of cerebellar atrophy in these most severe patients (Ruocco, Lopes-Cendes et al. 2006; Sakazume, Yoshinari et al. 2009; Nicolas, Devys et al. 2011). Therefore, it is possible that these results may not extend to adult-onset HD mouse models; experiments with HD adult-onset mouse models are required to address this issue.

Our study indicates that the PC population is particularly vulnerable in the R6/2 mouse model. Further experiments are required to determine the extent to which different cell types contribute to PC dysfunction and cell loss, with the goal of designing approaches to rescue this cell population and improve motor function in HD.

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HIGHLIGHTS

- Little information is available regarding the involvement of PCs in the etiology of HD.
- PCs are lost in the R6/2 model of HD evidenced by lost transcripts, protein, and cell number.
- Electrophysiological abnormalities are present in PCs before overt motor symptom onset.
- Firing abnormalities are present before the appearance of intranuclear inclusions within PCs.
- This early PC dysfunction potentially contributes to motor impairment in HD.

Figure 1. R6/2 mice exhibit reductions in Purkinje cell (PC) marker expression around the age of motor symptom onset

Motor impairments were evident by reduced ambulatory distance in the open field paradigm (**A**) and a deficit in rotarod performance at 32 rpm at 12 weeks of age. Rotarod data is shown as percentage of animals per bin with the highest bin (59.1-60) indicative of the most successful run of a full 60 seconds (**B**). Significant deficits were also seen at 20, 24, and 28 rpm speeds while no difference was observed between groups at 6 weeks of age (data not shown). **C**. Using q-RT-PCR to measure transcript levels on cerebellar homogenates, expression of Purkinje markers was reduced in R6/2 mice, with GAD67 and Calb reduced by 7 weeks and PV reduced by 12 weeks ($n=9/\text{group}$ at 7 and 12 weeks, $n=5/\text{group}$ at 2 and 4 weeks). Values for mRNA levels were normalized to actin and then compared to WT (fold = 1). **D**. Western blot analysis revealed a decrease in protein level of GAD67, Calb and PV at 12 weeks of age. ($n=7$ per/group). **E**. Quantification of Western blot. Two-way ANOVA tests were performed on the behavioral data. Two-tailed student's *t*-tests were performed on transcription and Western blot data with significance established as compared to age-matched WT. Error bars: SEM. (* $p < 0.05$).

Figure 2. Immunoreactivity for GAD67, calbindin (Calb) and parvalbumin (PV) is reduced in the Purkinje cell (PC) layer of the cerebellum at 12 weeks of age but unaltered at 4 weeks of age **A-D.** Immunofluorescence with an antibody specific for GAD67 reveals a loss of staining in the PC layer of 12 week old R6/2 animals (**D**) as compared to 12 week old WT (**C**). There is no difference between groups at 4 weeks of age (**A&B**). **E-H.** An antibody specific for Calb shows a decrease in protein levels throughout the molecular and PC layers of 12-week-old R6/2 animals as compared to WT (**H**). There is no difference between groups at 4 weeks of age (**E&F**). **I-L.** Changes, similar to those seen in GAD67, are seen with an antibody specific to PV. Staining shows a decrease in protein levels in the PC layer of 12-week-old R6/2 animals as compared to WT (**L**). Staining is clearly retained in the interneurons of the molecular layer (arrows in **L**). ML: Molecular Layer. PCL: PC Layer. GCL: Granule Cell Layer. Scale bar, 25 μ m.

Figure 3. Cell counts reveal neuronal loss in the Purkinje cell (PC) layer of 12-week-old R6/2 mice

A. Hematoxylin and eosin staining was performed on 30 micron thick cerebellar sections from WT and R6/2 mice. Representative pictures are shown. Staining reveals uniform cell distribution in the cerebellum with no significant differences in stereological counts of PC between WT and R6/2 animals at 4 weeks of age, quantification in **B**. **C.** At 12 weeks there is a significant reduction in the number of PCs by 55%, quantification in **D**. In **C**, representative pictures are shown of 12-week-old animals. Arrows indicate PCs. $n = 3$ /group. Two-way ANOVA, $*p < 0.05$. Error bars represent SEM. Scale bar, 20 μ m. $n = 3$ /group at each age tested.

Figure 4. Purkinje cell spike rate is reduced in presymptomatic R6/2 mice

A, Using presymptomatic R6/2 and age-matched WT mice, loose patch recordings were performed on acute cerebellar slices (300 micron thick); sample traces shown. Recordings lasted for 3 minutes and were then analyzed using clampex software; quantification in **B**. There was a significant difference between event number (spike rate) and interevent interval (data not shown) between the WT and HD mice ($n = 5$ and 4 animals for WT and R6/2 respectively; $*p < 0.05$). Two-tailed student's t -tests were performed with error bars representing SEM.

Figure 5. Huntingtin inclusions are present in the Purkinje cells (PCs) of 12 week R6/2 mice but found in interneurons at a younger age

In order to localize mthtt inclusions to specific cell types in these animals, immunohistochemistry was performed with an antibody specific to mthtt (in red) and calbindin for visualization of the cerebellar layers (in green). In 4-week-old R6/2 animals, mthtt staining is seen in the molecular (arrowheads) and granule cell layers of the cerebellum but not in the PC layer (**B**). (**INSET**) Colocalization of the inclusion pathology in 4-week-old R6/2 with parvalbumin (in green) reveals that these aggregates are found within the molecule layer interneurons. Inclusions are absent in 12-week-old WT animals (**A**). **C**. 7 week old R6/2 mice exhibit staining throughout the cerebellum, including in a few PC (arrows). Inclusions are seen in the interneurons of the molecular layer, enlarged in **E**. In 12 week old R6/2 animals, intranuclear inclusions are seen as punctuate staining throughout the PC layer (arrows) and cerebellum (**D**); representative PCs are highlighted in **F** ML: Molecular Layer. PCL: PC Layer. GCL: Granule Cell Layer. Scale bar (**D**), 25 μ m. Scale bar (**F**), 5 μ m.