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HYPERACTIVITY AND CORTICAL DISINHIBITION IN MICE WITH RESTRICTED EXPRESSION OF MUTANT HUNTINGTIN TO PARVALBUMIN-POSITIVE CELLS

S.E. Dougherty^{a,d}, J.J. Hollimon^d, L.J. McMeekin^d, A.S. Bohannon^d, A.B. West^b, M. Lesort^d, J.J. Hablitz^c, and R. M. Cowell^{d,*}

^aNeuroscience Graduate Program, University of Alabama at Birmingham, 35294

^bDepartment of Neurology, University of Alabama at Birmingham, 35294

^cDepartment of Neurobiology, University of Alabama at Birmingham, 35294

^dDepartment of Psychiatry & Behavioral Neurobiology, University of Alabama at Birmingham, 35294

Abstract

Recent evidence suggests that interneurons are involved in the pathophysiology of Huntington Disease (HD). Abnormalities in the function of interneurons expressing the calcium buffer parvalbumin (PV) have been observed in multiple mouse models of HD, although it is not clear how PV-positive interneuron dysfunction contributes to behavioral and synaptic deficits. Here, we use the cre-lox system to drive expression of mutant huntingtin (mhtt) in parvalbumin (PV)-positive neurons and find that mutant mice exhibit diffuse mhtt immunoreactivity in PV-rich areas at 10 months of age and mhtt aggregates in PV-positive processes at 24 months of age. At midlife, mutant mice are hyperactive and display impaired GABA release in the motor cortex, characterized by reduced miniature inhibitory events and severely blunted responses to gamma frequency stimulation, without a loss of PV-positive interneurons. In contrast, 24 month-old mutant mice show normalized behavior and responses to gamma frequency stimulation, possibly due to compensatory changes in pyramidal neurons or the formation of inclusions with age. These data indicate that mhtt expression in PV-positive neurons is sufficient to drive a hyperactive phenotype and suggest that mhtt-mediated dysfunction in PV-positive neuronal populations could be a key factor in the hyperkinetic behavior observed in HD. Further clarification of the roles for specific PV-positive populations in this phenotype is warranted to definitively identify cellular targets for intervention.

Introduction

Huntington Disease (HD) is a devastating neurological disorder characterized by motor, psychiatric, and cognitive disturbances. HD is caused by an aberrant expansion of the CAG repeat domain within exon one of the huntingtin (htt) gene (Group 1993). At the cellular

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*To whom correspondence should be addressed. Department of Psychiatry & Behavioral Neurobiology, 1720 7th Avenue South SC 729, Birmingham, AL 35294, Phone: (205) 975-7466, Fax: (205) 975-4879, rcowell@uab.edu.

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level mutant htt (mhtt) interferes with various functions including transcriptional regulation (Luthi-Carter, Hanson et al. 2002; Hodges, Strand et al. 2006; Bithell, Johnson et al. 2009), the maintenance of calcium homeostasis (Perry, Tallaksen-Greene et al. 2010; Giacomello, Hudec et al. 2011), and synaptic physiology (Klapstein, Fisher et al. 2001; Milnerwood and Raymond 2007; Cummings, Andre et al. 2009).

Though the mutant protein is ubiquitously expressed, specific neuronal populations are especially vulnerable to the toxic effects of mhtt. Within the striatum, projection neurons undergo neurodegeneration while some regional interneurons are spared (Hodgson, Agopyan et al. 1999; Shelbourne, Keller-McGandy et al. 2007). Studies have indicated that the cerebral cortex is affected as well (Gu, Li et al. 2005; Spampanato, Gu et al. 2008; Gray, Egan et al. 2013) and that reduced trophic support from the cortex may contribute to striatal neuron vulnerability (Zuccato, Ciammola et al. 2001). Interestingly, pan-neuronal expression of mhtt is sufficient to cause hypoactivity and cortical alterations, while pyramidal neuron-specific expression has no impact on behavior or cortical dysfunction, leading investigators to hypothesize that cortical pathology requires the involvement of interneurons (Gu, Li et al. 2005). In support of this hypothesis, early alterations in responses of parvalbumin (PV)-positive interneurons to excitatory neurotransmission are observed in the BACHD mouse model, implicating involvement of this subpopulation in the development of symptoms (Spampanato, Gu et al. 2008).

PV+ interneurons are critical in synchronizing the output of pyramidal neurons (Perney, Marshall et al. 1992; Du, Zhang et al. 1996), with the activation of PV+ interneurons alone being sufficient to drive cortical oscillations (Sohal, Zhang et al. 2009). Synchronization is disrupted in a number of neurological disorders (Gonzalez-Burgos and Lewis 2008; Lodge, Behrens et al. 2009), including HD (Thiruvady, Georgiou-Karistianis et al. 2007; Walker, Miller et al. 2008), making it critical to elucidate the contribution of PV+ interneuron dysfunction to the pathogenesis of HD.

To investigate the role of the PV+ subclass of GABAergic neurons in HD-associated motor and synaptic dysfunction, we utilized a cre-lox system of conditional gene expression (Gu, Li et al. 2005). We bred mice with expression of a floxed stop codon preceding the mhtt gene to mice with a PV promoter-driven cre recombinase enzyme (Hippenmeyer et al. 2005). The resultant mice had expression of mhtt only in PV+ cells and exhibited hyperactivity and reduced GABA release in response to gamma frequency stimulation at midlife. However, 24 month-old mutant mice no longer exhibited behavioral differences or impairments in gamma frequency-stimulated GABA release, potentially due to postsynaptic compensatory changes. These results suggest that mhtt can drive hyperactivity by influencing PV+ neuron function.

Methods

Animals

The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all experimental protocols. The “PVcre” and “mhtt” mouse lines were obtained from Jackson Laboratories and maintained through breeding to WT hybrid (B6CBA) animals. The “mhtt” strain was B6.129-*Gt(ROSA)26Sor^{tm1}(HD*103Q)*Xwy/J (JAX# 007708); these mice harbor a transgene at the *Rosa26* locus composed of a stop codon bounded by lox-p sites, followed by exon 1 of human huntingtin with 103 CAG repeats. When crossed with a mouse expressing cre recombinase driven by a cell-specific promoter, mhtt is expressed in the specific cell of interest. The “PVcre” mouse line used was B6; 129P2-*Pvalb^{tm1}(cre)*Arbr/J (JAX #008069); these mice express cre recombinase in almost all PV-positive cells in the body (Hippenmeyer, Vrieseling et al. 2005) and exhibit maximum

recombination by 3 months of age (Carlen, Meletis et al. 2012). To confirm the neuroanatomical location of recombination with this mouse line, we crossed PVcre mice with the mTmG reporter mouse (JAX#007676; (Muzumdar, Tasic et al. 2007))). All experiments were conducted with both male and female animals at all ages. For all experiments, PVcre littermate mice were used as controls.

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. These mice were used at 12 weeks of age, exclusively as positive controls for immunodetection experiments. R6/2 mice carried a repeat length of 167 as genotyped by Laragen, Inc (Culver City, CA). All 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 six (n=9–14/group), eight (n=6–8/group), ten (n=9–11/group), twelve (n=11–17/group), and twenty-four months of age (n=7–11/group) 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 and 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 and 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 (27.3 cm × 27.3 cm × 20.3 cm) 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.

Chromogenic immunodetection

Animals were anesthetized with isoflurane and perfused intracardially with ice cold 4% paraformaldehyde in phosphate buffered saline (PBS). Animal numbers for all immunostaining experiments were 3–4/group at 6 months, 3/group at 10 months, 6/group at 12 months, and 8/group at 24 months of age. Brains were removed, postfixed for an additional 24–72 hours, cryoprotected in graded sucrose concentrations, 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 µm, mounted onto charged slides (Fisher Scientific; Pittsburg, PA) and allowed to dry overnight before storage at –80°C. Chromogenic immunodetection was performed as in (Lucas, Markwardt et al. 2010). Briefly, slides were thawed and washed with PBS followed by an incubation with 10% serum (1h; from the host of the 2° antibody), the primary antibody (overnight), a

biotinylated secondary antibody (1h), 0.3% hydrogen peroxide in methanol (10 min; to quench endogenous peroxidases), the avidin-biotin-HRP conjugate (30 min; Vector Labs), and diaminobenzamine (~5 min; DAB).

Immunofluorescence

Mice were perfused and brains were removed, post-fixed, and cryoprotected as above, before freezing in isopentane. Brains were sectioned at 40 μ m on a sliding microtome (Leica Microsystems; Buffalo Grove, IL), and sections were stored in 1:1 glycerol/PBS mixture at -20° C until use. For use, sections were blocked with 3% bovine serum albumin (BSA) and 10% serum (from the host of the secondary antibody), and incubated for 30 minutes in 0.3% H_2O_2 in methanol followed by a 30-minute incubation in citrate buffer (for antigen retrieval). Slides were then incubated with primary antibodies (48 h in 5% serum in 0.3% Triton X PBS with 3% BSA), secondary antibodies (24 h in 5% serum in 0.3% TritonX PBS with 3% BSA), and TOPRO3 for visualization of nuclei (Invitrogen). Sections were mounted onto slides and coverslipped using ProLong Gold anti-fade mounting media (Invitrogen). Negative controls included either absence of primary antibody or species-matched IgG in the place of the primary antibody. Representative images were captured on a Leica confocal microscope and processed with Adobe Photoshop CS3; adjustments in contrast and brightness were applied to images where appropriate. In many cases, fluorophores were chosen based on maximal separation (FITC and CY5), but when rhodamine and CY5 channels were used, emission filters were set to <615 nm for rhodamine to limit potential leak through of strong CY5 signals into the rhodamine channel; likewise, with that combination of secondary antibodies, the CY5 emission range was restricted to 640–700 nm to limit any contribution from strong rhodamine intensity. For the display of the recombination images (fig. 1), images of green fluorescence were captured, converted to grayscale, and inverted for comparison to chromagenic staining. For the comparison of inclusion density between 12 and 24 month-old PVcre-mhtt mice, individual immunofluorescence images were captured every 0.5 μ m through a depth of 15 μ m to create a Z-stack, and data are presented as maximum projection images (created using the Leica confocal microscope software).

Antibody Information

Immunostaining was performed with these antibodies: mouse anti-GAD67 (MAB-5406, Millipore Billerica, MA; 1:500 for fluorescence); rabbit polyclonal anti-PV (PV25, Swant; 1:500 for fluorescence); mouse anti-huntingtin (MAB-5492, Millipore; 1:1000 for fluorescence, 1:250–1:500 for DAB); rabbit anti-huntingtin (Chun, Lesort et al. 2001; Lin, Tallaksen-Greene et al. 2001; 1:500 for fluorescence), goat polyclonal huntingtin (SC-8767, Santa Cruz; 1:200 for DAB and 1:100 for fluorescence), mouse anti-synaptotagmin 2 (SYT2; ZNP-1; Zebrafish International Resource Center; 1:50 for fluorescence), and MAP2-FITC (Millipore, 1:500 for fluorescence). Secondary antibodies included biotinylated horse anti-mouse IgG, biotinylated horse anti-goat IgG, biotinylated goat anti-rabbit IgG (Vector Labs), Alexa fluor 488, 594, and 647 donkey antimouse, donkey anti-goat, and donkey anti-rabbit IgG (Jackson ImmunoResearch).

Whole Cell Recordings

Mice were anesthetized with isoflurane and then killed by decapitation (n=3–5 animals and 10–12 cells per condition). Brains were placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 3.5 KCl, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 26 $NaHCO_3$ and 10 D-glucose. The saline was bubbled with 95% O_2 /5% CO_2 . Coronal brain slices (300 μ m thick) were cut using a Vibratome (Ted Pella, Inc., Riverside, CA). The slices were kept for 30 minutes at $37 \pm 1^{\circ}$ C and then stored at room temperature ($22 \pm 1^{\circ}$ C). Slices were superfused continuously with oxygenated recording ACSF at room temperature. Whole-cell patch

clamp recordings were acquired from visually identified pyramidal neurons in layer five of the motor cortex. Position in cortex was verified with streptavidin staining for biocytin filled cells. Recordings were conducted on a Zeiss upright microscope (Carl Zeiss, Thornwood, NY). Cells were voltage clamped at -70 mV, using internal solution containing the following (in mM) 129 CsCl, 2 MgATP, 10 EGTA, 10 HEPES, 0.2 GTP and 2 QX-314, pH 7.2. Pipette tip resistance was 2–5 M Ω . Voltage clamp recordings were obtained using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA) controlled by Clampex 8.0 software via a Digidata 1322A interface (Molecular Devices), filtered at 5 kHz and digitized at 10 kHz. Input resistance and series resistance were monitored by applying a 10 mV voltage step. Spontaneous and evoked IPSCs were pharmacologically isolated with CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 10 μ M) and D-APV (DL-2-amino-5-phosphonovaleric acid, 50 μ M). Spontaneous effects were then further isolated with TTX (tetrodotoxin, 1 μ M). Analysis of spontaneous IPSCs (sIPSCs) was performed using Clampfit 8.0 software, which focused on amplitude, interevent interval and decay time of events. All sIPSCs that fit the template and passed visual inspection were inspected in the analysis. To calculate the paired-pulse ratio, the amplitude of the second IPSC was measured relative to the baseline set immediately before the second stimulus, and normalized to the amplitude of the first IPSC relative to the baseline set immediately before the first stimulus. CNQX, APV and TTX were purchased from Sigma-Aldrich and diluted from stock solutions to final concentrations in ACSF.

Stimulation

Synaptic responses were evoked with a bipolar stimulating electrode consisting of a twisted pair of 25 μ m Formvar insulated nichrome wires. The electrode was positioned in layer V of the motor cortex. A series of paired stimulations at 20, 30 and 100 ms intervals were applied to elucidate pair-pulse ratios. For 66 Hz train recordings, 34 stimulations were applied over 500 ms to evoke a response in patched pyramidal cells. Recordings were taken at $32 \pm 1^\circ\text{C}$.

Quantitative RT-PCR

12 month-old PVcre and PVcre-mthtt mice ($n = 3/\text{group}$) were anesthetized with isoflurane and decapitated for removal of the brain and dissection of the anterior cortex. Total RNA (1 μ g) was isolated, DNase treated, and reverse transcribed as in (Cowell et al. 2007), and actin, PV, GAD67, and peroxisome proliferator activated receptor γ coactivator 1 α (PGC-1 α) cDNA was amplified with inventoried Taqman primer probe sets (Mm00607939_s1, Mm00443100_m1, Mm00725661_s1, and Mm00447183_m1, respectively) and quantified using the calibrator method. Values for PV, GAD67, and PGC-1 α were normalized to actin values, and data are expressed as mean $\pm$ standard error of the mean.

Data Analyses

Data analyses were performed using GraphPad Prism software. A two-tailed student t-test was utilized to assess statistical significance when two groups were compared. For the measurements of multiple groups, one-way ANOVAs were performed followed by post hoc Tukey's Multiple Comparisons test. For longitudinal behavioral analysis two-way ANOVA tests were performed. Values were considered statistically significant when the p value was less than 0.05.

Results

Concentration of Mutant Huntingtin Immunoreactivity in PV-rich regions and processes in Mutant Mice

To determine how PV+ neuron dysfunction contributes to HD-related behavioral and synaptic changes, we generated mice with mhtt expression restricted to PV+ cell populations. In order to confirm the expression of the mhtt protein in the expected cell populations, we first performed immunohistochemical and immunofluorescence studies using antibodies specific for htt (fig. 1–3). While little staining was observed in mutant mice at 6 months of age (not shown), diffuse immunoreactivity was observed throughout the brain in mutant mice at 10 months of age (fig. 1A) in regions of high PV immunoreactivity, including the striatum (fig. 1B), cortex (fig. 1C), hippocampus (fig. 1D), and reticular nucleus of the thalamus (fig. 1E). This result is in line with the documented recombination patterns in these PVcre mice (see methods). To directly compare the neuroanatomical patterns of huntingtin expression to the patterns of recombination, we crossed the PVcre mouse line with the mTmG reporter mouse line, which expresses membrane-bound green fluorescence in cells that undergo recombination. Accordingly, patterns of recombination in the PVcre-mTmG mouse were similar to patterns of htt immunoreactivity in these regions (fig. 1, right column). Higher magnification images of mhtt staining in these regions revealed fibrous, diffuse staining as well as cell-specific patterns of immunoreactivity, especially in the cortex (fig. 1C).

To confirm this staining pattern and determine whether htt expression is expressed specifically in PV+ neurons, we used double-labeling immunofluorescence in floating sections from 10, 12 and 24 month-old mice (figs 2–3). With immunofluorescence, diffuse and cytosolic labeling was not detectable (possibly masked by cytosolic autofluorescence at these ages), while mhtt-positive aggregates were readily detectable. Maximum projection images of Z-stack confocal scans from control and mutant mouse cortex revealed that inclusions were rare in mutant mice at 12 months of age (fig. 2A) but extremely abundant at 24 months of age (fig. 2B, C). Images captured with a single plane confocal microscopy (not a Z-stack) show htt-positive, PV+ inclusions in the thalamus (fig. 2D), hippocampus (fig. 2E), striatum (fig. 2F), and cerebellum (fig. 2G), all areas of the brain with high levels of PV expression. Inclusions always colocalized with PV, most often in processes and rarely cell bodies.

To characterize the subcellular location of these htt-positive inclusions, we used markers for dendrites (microtubule-associated protein; MAP2; fig. 3A), inhibitory axonal terminals (glutamic acid decarboxylase 67; GAD67; fig. 3B), and PV+ axonal terminals (synaptotagmin 2; SYT2; (Sommeijer and Levelt 2012); fig. 3C). We observed that the htt-positive inclusions colocalized in some cases with MAP2 (fig 3A). More often, htt-positive inclusions overlapped with GAD67 in axonal, perisomatic terminals (fig. 3B) and with SYT2, a marker for PV+ axonal terminals in the cortex (fig. 3C). Aggregates were rarely found in the nuclei of PV+ cells, as visualized with the nuclear counterstain topro (fig. 3D).

To determine whether the localization of mhtt to PV+ processes is observed in other HD models, we investigated whether the same pattern exists in the R6/2 model of HD (Mangiarini, Sathasivam et al. 1996). We found that htt-positive inclusions were abundant in all cellular nuclei, including PV+ neuronal nuclei, and that htt-positive inclusions were also found in PV+ processes (fig. 3E, arrowhead). Nuclear inclusions are also observed in the R6/2 mice, in both cortical pyramidal neurons (E, top arrows) as well as PV+ cell bodies (E, bottom arrow). In addition to demonstrating that non-nuclear PV+ inclusions occur in the R6/2 mice, the lack of PV-immunoreactivity overlap with htt in numerous other cells (fig. 3E) demonstrates that leak-through from the htt fluorescence channel was not contributing

to the overlap observed in our colocalization experiments. The patterns of htt immunoreactivity described above were confirmed with three different mthtt antibodies from three different host species; all showed the same staining pattern (data not shown), and no staining was observed when species-matched IgG was used in place of the primary antibody.

PVcre-mthtt Mice Exhibit Hyperactivity

To determine whether the expression of mthtt in PV+ cell populations was sufficient to cause motor dysfunction, we evaluated locomotor activity and coordination using open field analysis and rotarod testing, respectively. Behavior was evaluated at 6, 8, 10, and 12 months of age. PVcre-mthtt showed increased ambulatory distance between 8 and 12 months of age (fig 4A). A two-way ANOVA revealed significant main effects of genotype and age ($F(3,75) = 2.877$ and $F(1,75) = 12.84$, respectively; $p < 0.05$ for both) but no interaction. Binned data revealed that PVcre-mthtt move more than PVcre littermates through the entire recorded period (fig 4B; $F(1,5) = 25.41$, $p = 0.001$). Further investigation of motor performance revealed an increase in total time spent walking (Genotype: $F(1,77) = 13.138$; $p = 0.001$; Age: $F(3,77) = 4.486$; $p = 0.006$; no interaction) as well as a reduction in time spent at rest (Genotype: $F(1,77) = 14.755$; $p < 0.05$; Age: $F(3,77) = 13.294$; $p < 0.05$; no interaction; fig 4C–D), indicating a generalized increase in activity. However, rotarod analysis at 12 months of age revealed no significant difference in the latency to fall between genotypes at all speeds tested (fig 4E), demonstrating that gross motor coordination was not affected. Ambulatory counts for other control groups (PVcre-negative ROSA-positive and complete wild type mice) were not different from PVcre ROSA-negative mice (not shown).

PV+ Cell-specific Expression of mthtt alters GABA Release in the Motor Cortex

To begin to investigate the impact of mthtt expression on the function of PV+ neurons and downstream projection neurons, we used whole cell patch clamp electrophysiology to evaluate inhibitory postsynaptic potentials on layer V pyramidal neurons in the motor cortex. PV+ interneurons densely innervate the perisomatic regions of cortical pyramidal neurons and are critical for the generation of feed-forward inhibition in the cortex (Markram, Toledo-Rodriguez et al. 2004; Freund and Katona 2007). While it is possible that mthtt has effects in all PV+ cellular populations in this mouse model, we chose to investigate inhibitory neurotransmission in the cortex based on the high abundance of mthtt-positive inclusions observed in this region and the dysfunction of these PV+ interneuron populations reported in HD mouse models (see introduction). Initial investigations revealed increases in both amplitude ($p = 0.0004$, $t_{(17)} = 4.408$) and frequency of spontaneous inhibitory postsynaptic potentials (sIPSCs) ($p = 0.0154$, $t_{(18)} = 2.342$; fig 5A–C), indicating an increase in spontaneous inhibitory neurotransmitter release in mutant mice. However, isolation of miniature IPSCs (mIPSCs) with bath application of TTX revealed a reduction in frequency of events, without a change in amplitude ($p = 0.0227$, $t_{(7)} = 2.429$; fig 5D–F), suggesting presynaptic deficits in GABA release. To further investigate presynaptic involvement, we performed evoked recordings in the motor cortex using the paired-pulse paradigm. We observed an increase in the paired-pulse ratio (PPR) in HD mice as compared to their PVcre littermates (fig 5G&H, $p = 0.04$, $t_{(18)} = 2.213$). As PPR is inversely related to presynaptic release, this suggests a reduction in GABA release probability in the cortex of mutant mice, consistent with a reduction in mIPSC frequency.

Reductions in Evoked GABA Release in the Motor Cortex of mutant mice

While the evaluation of spontaneous and miniature IPSCs allows us to investigate synaptic deficits, these approaches do not necessarily identify abnormalities in neurotransmission that occur with physiological levels of network activity. To determine whether abnormalities in

GABA release are present with physiological patterns of activity, we performed repetitive stimulation in the motor cortex at the gamma frequency (66 Hz), the frequency at which PV + interneurons in the cortex entrain regional pyramidal neurons (40–100 Hz; (Ainsworth, Lee et al. 2012)). IPSCs were recorded over a one second period (500 msec of repetitive stimulation and 500 msec following stimulation); measurement of the area under the curve revealed a statistically significant reduction in evoked GABA release (fig 5I&J, $p = 0.0042$, $t_{(17)} = 3.306$) with this stimulation protocol. Overall area in the PVcre mice was 203931 ± 49755 pA·ms while in the PVcre-mthtt mice, the average area was 29578 ± 4180 pA·ms. The difference observed with this stimulation paradigm suggests physiologically relevant reductions in GABA release in the cortex of these mice.

Mutant Mice Show no Alteration in PV Transcript or Protein Levels at 12 months of age

Considering the reduction in inhibitory neurotransmission in these mice, we determined whether there was a loss of PV mRNA and protein in 12 month-old PVcre-mthtt mice. Also, we investigated the expression of the interneuron marker GAD67 and the transcriptional coactivator peroxisome proliferator activated receptor γ coactivator 1 α (PGC-1 α), which has been shown to be highly concentrated in GABAergic cell populations (Cowell, Blake et al. 2007). Additionally, studies have shown that mthtt can interact with and inhibit PGC-1 α transcription (Cui, Jeong et al. 2006). No alterations in these transcripts were observed in 12 month-old PVcre-mthtt mice (fig. 6A), and no overt differences were noted in immunoreactivity for PV in the motor cortex of these mice (fig. 6B, C). These data suggest that mthtt expression in PV-positive neurons is not sufficient to cause a loss of this cell type, at least in the motor cortex.

Phenotypical Alterations at 12 months of age are Absent in Mutant Mice at 24 Months of Age

Considering the neurodegenerative nature of HD, we postulated that the alterations in motor performance and synaptic physiology would continue throughout life and worsen over time, especially considering the increased accumulation of mthtt inclusions with age. In order to explore this possibility, we aged a cohort of animals to 24 months of age. Alterations in basal GABA release were evident and included an increase in sIPSC amplitude ($p = 0.0122$, $t_{(18)} = 2.786$) with no change in sIPSC frequency, as well as no change in mIPSC amplitude or frequency (fig 7A–F). These alterations differ from those observed at 12 months of age and indicate that GABAergic dysfunction is still present at the synaptic level; importantly, the unchanged sIPSC frequency suggests that PV+ neurons have not been lost over time in this model (as suggested by the lack of change in PV transcript expression), as a loss of this interneuron subtype would result in decreases in both mIPSC amplitude and frequency. However, surprisingly, upon paired stimulation, the PPR of the evoked IPSC was normal (fig 8A&B), suggesting that there is no longer any difference in release probability of GABA upon stimulation. Remarkably, this same normalization was seen upon repetitive train stimulation at the gamma frequency (fig 8C&D), indicating that differences were no longer present with a physiological pattern of stimulation. Overall area in the PVcre mice was 162626 ± 65867 pA·ms while in the PVcreRosa mice the average area was 189314 ± 62287 pA·ms. At this same age, these mice no longer demonstrated a hyperactive phenotype; PVcre and PVcre-mthtt mice moved similar distances and spent comparable amounts of time ambulating and resting, as evaluated using the open field paradigm (fig 8E).

Discussion

Huntington Disease is a devastating neurological disorder, with no disease-altering treatments at present. In order to investigate the role of PV+ neurons in the disease etiology of HD we utilized a novel mouse model with PV+ cell-specific expression of exon 1 of the

human *htt* gene with 103 CAG repeats. This restricted expression of *mhtt* to PV+ cell populations results in a unique HD-like phenotype characterized by hyperactivity at midlife and alterations in spontaneous and evoked GABA release in the motor cortex at 12 months of age. Taken together, these data suggest a cellular mechanism by which *mhtt* causes hyperactivity and cortical dysfunction.

HD patients commonly present with chorea, or erratic, involuntary movements. To determine whether mice expressing *mhtt* in PV+ neurons exhibit increased movement, we evaluated their ambulatory activity in the open field paradigm. Interestingly, this is one of the few murine models with *mhtt* overexpression to exhibit overt hyperactivity, as most mouse models of HD typically demonstrate reduced locomotion and impaired coordination (Carter, Lione et al. 1999; Gu, Li et al. 2005; Hickey, Gallant et al. 2005; Albertson, Yang et al. 2011). Electrophysiological characterizations revealed a reduced probability of GABA release in the motor cortex as well as an overall reduction in gamma frequency stimulation-evoked GABA release, and we propose that these alterations could enhance cortical output to the spinal cord and/or the striatum. In fact, imaging studies in patients have shown that there are strong correlations between alterations in excitatory/inhibitory balance in the cortex and motor abnormalities (Ridding, Sheean et al. 1995; Brown, Ridding et al. 1996). As such, this model could be useful for understanding the neurobiological mechanisms underlying the uncontrollable movements associated with HD.

It is important to note, however, that alterations in the function of other PV+ neuron populations could contribute to the observed behavioral phenotype, especially considering that Purkinje cells of the cerebellum and interneurons of the striatum and hippocampus displayed *htt*-positive inclusions. A recent article demonstrates PV+ cell specific dysfunction in the striatum of HD mouse models resulting in altered inhibitory transmission, suggesting that PV+ interneurons could still be playing a role in that region (Cepeda, Galvan et al. 2013). There the authors observe enhanced sIPSC frequency and larger average amplitude of events in R6/2 medium spiny neurons of the striatum. This increase in basal inhibition is similar to the alterations described here. Through paired recordings, they go on to implicate feed forward inhibition from fast spiking PV+ interneurons. However, the coincidence of the “normalization” of motor behavior and the electrophysiological responses to electrical stimulation make it attractive to speculate that cortical dysfunction at least partially contributes to the observed behavioral phenotype. Along those lines, it is important to note that alterations in synaptic activity in the motor cortex can be sufficient to produce changes in motor behavior in the absence of striatal dysfunction (Ridding, Sheean et al. 1995; Brown, Ridding et al. 1996).

It is unclear as to the precise mechanisms that underlie the normalization of GABA release and behavior at 24 months of age. Abundant *mhtt*-positive inclusions were observed in the cortex, and an increase in sIPSC amplitude was evident, suggesting that pyramidal neurons may be compensating to some extent to influence local inhibition. However, an increase in sIPSC amplitude was evident in 12 month-old PVcre-*mhtt* mice as well, meaning that that change, alone, could not be responsible for recovery of function. Related to the progressive nature of HD in other mouse models and in people, pyramidal neurons may be incapable of engaging compensatory responses due to the toxic effects of *mhtt* on pyramidal cell function, leading to progressive neurodegeneration. Regarding the pronounced accumulation of *htt*-positive inclusions, there are conflicting theories on the role of inclusion bodies in the pathology of HD; some studies have shown links between cellular toxicity and inclusion formation, including a direct correlation between inclusion density and CAG repeat length (Becher, Kotzlik et al. 1998), as well as an increased propensity towards cell death (Cooper, Schilling et al. 1998; Martindale, Hackam et al. 1998). Other reports have suggested that the formation of inclusions is neuroprotective (Saudou, Finkbeiner et al. 1998; Arrasate, Mitra

et al. 2004; Bowman, Yoo et al. 2005). In this model, it is possible that the non-aggregated form of mhtt causes the observed dysfunction at the earlier time points while sequestration of mhtt in inclusions normalizes local inhibition, potentially by preventing soluble mhtt's effects on synaptic function (Li, Wyman et al. 2003; Milnerwood and Raymond 2007).

Based on the evidence that action potential-dependent and evoked mechanisms of GABA release are affected at 12 months of age but normalize at 24 months of age in this model, it is intriguing to postulate that soluble htt differentially affects the protein machinery responsible for the exocytosis of synaptic vesicles for spontaneous versus evoked release. A multitude of proteins involved in the control of neurotransmitter release can interact with mhtt, including (but not limited to) VAMPS, SNAREs, complexins, synaptotagmins, myosin, and pacsin (Deitcher, Ueda et al. 1998; Modregger, DiProspero et al. 2002; Young and Neher 2009; Jorquera, Huntwork-Rodriguez et al. 2012; Peng, Rotman et al. 2012). In fact, we found that mhtt localizes with synaptotagmin 2, a presynaptic molecule involved in synchronous neurotransmitter release in PV+ interneurons (McMahon, Missler et al. 1995; Chicka and Chapman 2009; Kochubey, Lou et al. 2011; Kochubey and Schneggenburger 2011). It is possible that soluble mhtt interacts with synaptic release proteins in PV+ interneurons to impair vesicle release as has been demonstrated in excitatory neurons (Li, Wyman et al. 2003), but that the sequestration of the soluble protein into insoluble aggregates could reduce the interaction of mhtt with synaptic vesicles, thereby normalizing neurotransmission at 24 months of age.

One unexpected result was the finding that the mhtt inclusions were rarely nuclear in our model. Previous reports, using similar cre-driven models of conditional expression, showed that immunoreactivity was not only present in the nucleus, but was high there relative to the rest of the neuron (Gu, Li et al. 2005). The lack of intranuclear inclusions in the PV+ neurons could suggest that these neurons may handle the mutant protein differently than excitatory neurons; in fact, even in the R6/2 model, mhtt+ inclusions appear later in PV+ interneurons than in other cell types (data not shown; Meade CA, Reiner et al. 2002). Considering the lower frequency of inclusions at younger ages, it is possible that PV+ neurons are exposed to the soluble form of mhtt for a longer period of time than other neurons, before sequestration into inclusions. Also, a lack of intranuclear inclusions would suggest that the effects of mhtt in these mice are unlikely due to interference of mhtt with transcriptional processes; in fact, a lack of transcriptional alterations in levels of PGC-1 α , PV, and GAD67 in our model supports this theory. Alternatively, it is possible that dysfunction of glutamatergic neuronal populations influences the processing of mhtt in interneurons, causing it to translocate to the nucleus, accounting for the nuclear localization of mhtt in PV+ neurons in other HD models.

In summary, we have found that restricted expression of exon one of mutant huntingtin to PV+ cell populations is sufficient to cause hyperactivity at midlife. This conditional expression also results in reduced inhibitory transmission in the motor cortex. Taken together these data suggest not only a role for PV+ interneurons in the etiology of HD but also a specific niche for these GABAergic cell populations in overall motor control. Furthermore, with the unanticipated longitudinal results in this model, additional experiments are warranted to determine the contribution of pyramidal neuron compensatory effects and/or mhtt sequestration to the normalization of physiology and behavior. Future studies with this model have the potential to shed light on the molecular mechanisms by which mhtt interferes with interneuron function, with the goal of improving motor function in patients with HD.

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Highlights

- Mutant huntingtin expression in parvalbumin cells is sufficient for hyperactivity.
- Cortical pyramidal neurons of these mice exhibit abnormalities in inhibition.
- Gamma frequency stimulation reveals reductions in GABA release in these mice.
- Behavioral & physiological alterations normalize when most inclusions are observed.

Figure 1. Diffuse immunoreactivity for huntingtin is detected throughout parvalbumin-rich regions in PVcre-mthtt mice at 10 months of age

Brains from 10 month-old PVcre-mthtt mice exhibit diffuse huntingtin immunoreactivity throughout the brain as compared to PVcre (littermate control) mice, as revealed by chromogenic immunohistochemistry (A). Immunoreactivity is detectable in the striatum (B), cortex (C), hippocampus (D), and reticular nucleus of the thalamus (E). Similar patterns of labeling are noted in a PVcre mouse crossed with the mTmG reporter mouse (right column in B-E); green fluorescence images were collected on a confocal microscope, converted to grayscale, and inverted to allow for comparison to chromogenic staining. Scale bars: A: 250 μ m. B-E: 10 μ m.

Figure 2. Huntingtin inclusions colocalize with parvalbumin throughout the brain of PVcre-mthtt mice at 24 months of age

Z-stack maximum projection images of huntingtin (htt) immunoreactivity in the cortex of PVcre and PVcre-mthtt reveal little staining in 12 month-old PVcre and PVcre-mthtt mice and 24 month-old PVcre mice (**A1**, **A2**, and **B1**), but large, abundant inclusions are observed in the PVcre-mthtt mice at 24 months of age (**B2**). Single plane confocal images show that htt-positive inclusions consistently colocalize with immunoreactivity for parvalbumin (PV) in the cortex (**C**), thalamus (**D**), hippocampus (**E**), striatum (**F**), and cerebellum (**G**). An example of a “doughnut”-shaped inclusion, common for aggregates containing

polyglutamine-repeat proteins, is displayed in C (top arrowhead). ML: molecular layer. PC: purkinje cell layer. IGL: internal granule cell layer. Scale bars: 10 μ m for all.

Figure 3. Huntingtin inclusions are localized to the dendritic and synaptic compartments of parvalbumin-positive neurons in the cortex

A. Huntingtin (htt) immunoreactivity overlaps with immunoreactivity for microtubule associated protein (MAP2) and parvalbumin (PV; arrowheads). **B.** Htt-positive inclusions overlap with immunoreactivity for glutamic acid decarboxylase 67 (GAD67), a protein concentrated in axonal terminals of inhibitory interneurons which contact the soma of pyramidal neurons (asterisks). Pyramidal neurons lack GAD67, while interneurons (arrow) show immunoreactivity for GAD67 in the cytoplasm. **C.** Htt immunoreactivity also overlaps with immunoreactivity for synaptotagmin 2 (SYT2), a protein concentrated in axonal terminals of PV-positive interneurons. **D.** A small htt-positive inclusion is found in the

nucleus (topro) of a PV-positive interneuron in the cerebellum. PC: purkinje cell. Higher magnification image from figure 2G. **E**. Small htt-positive inclusions overlap with PV immunoreactivity in the cortex from the R6/2 mouse (arrowheads), but many PV-negative htt inclusions are observed, indicating the lack of cross-reactivity between antibodies. Nuclear inclusions are also observed in the R6/2 mice, in both cortical pyramidal neurons (**E**, top arrow) as well as PV+ cell bodies (**E**, bottom arrow). Scale bars: A, B: 25 μ m. C–F: 10 μ m.

Figure 4. PVcre-mthtt Mice Exhibit Hyperactivity

Mice with conditional expression of mthtt in PV+ neurons showed hyperactivity in the open field paradigm at midlife as exhibited by an increase in ambulatory distance (A). Increased activity is observed throughout the time spent in the apparatus with the PVcre-mthtt mice displaying increased ambulatory distance as compared to PVcre littermates at each of the 5 minute intervals (B). Additionally, PVcre-mthtt mice revealed increased ambulatory time (C) and reduced resting time (D) as compared to the littermate PVcre mice. Additional time spent in the arena was accounted for by time spent jumping and rearing which was not significantly different between the groups. Motor coordination is unaffected in the HD

animals at 12 months of age as tested by the rotarod paradigm (**E**). Two-way ANOVA tests were performed on the behavioral data. Error bars: SEM. (n=6–17 animals/group; *p < 0.05).

Figure 5. PV+ Cell-specific Expression of mthtt is Sufficient to Cause Alterations in GABA Release in the Motor Cortex

In acute slices, sIPSCs were recorded from layer V pyramidal neurons in the motor cortex (sample traces in **A**). At 12 months of age there is an increase in average event amplitude and an increase in event frequency (**B&C**). Addition of TTX to the bath allowed for isolation of mIPSCs (sample traces in **D**). Under these conditions there is no change in average event amplitude and a reduction in frequency (**E&F**). **G**. Sample traces of evoked IPSCs in layer V pyramidal neurons in the motor cortex from 12 month old animals show an increase in the ratio of the second response to the first upon paired stimulation. **H**. Upon paired-pulse stimulation at multiple intervals, there is an increase in paired pulse ratio at the

20ms interval in the PVcre-mthtt mice, suggesting a reduction in GABA release probability from the presynaptic interneuron. **I.** Sample traces of evoked IPSCs in layer V pyramidal neurons in the motor cortex from 12 month old animals show a reduction in the overall area under the curve upon repetitive stimulation at the gamma frequency. **J.** Upon repetitive stimulation at gamma frequency (66 Hz), there is a reduction in GABA release as measured by area under the curve in 12 month old PVcre-mthtt mice. Error bars: SEM. (n=3–5 animals and 10–12 cells per condition; *p<0.05, Student's t-test).

Figure 6. The expression of parvalbumin and other transcripts expressed by parvalbuminpositive interneurons is not altered in PVcre-mthtt at 12 months of age
 The expression of parvalbumin (PV), glutamic acid decarboxylase 67 (GAD67), and peroxisome proliferator activated receptor γ coactivator 1 α (PGC-1 α) is not changed in the anterior cortex of PVcre-mthtt mice, as assessed with quantitative RT-PCR (**A**). Representative confocal images of PV immunoreactivity from the motor cortex show similar levels of immunoreactivity between PVcre and PVcre-mthtt mice. (**B** and **C**, respectively). Scale bar: 50 μ m.

Figure 7. Amplitude of spontaneous inhibitory postsynaptic potentials remains elevated in PVcre-mthtt mice at 24 Months of Age

Spontaneous recordings were repeated at 24 months of age; the sIPSCs exhibited an increase in average amplitude (**B**) with no change in frequency (**C**), while mIPSCs displayed no change in average amplitude (**E**) or frequency (**F**). Sample traces shown in **A&D**. Error bars: SEM. (n=3–5 animals and 10–12 cells per condition; *p<0.05, Student's t-test).

Figure 8. Phenotypical Alterations Shown at Midlife are Absent in PVcre-mthtt mice at 24 Months of Age

Application of the paired-pulse stimulation paradigm in 24 month old animals revealed no significant alteration in the release probability of GABA in the PVcre-mthtt mice (**A&B**). **C&D**. Application of the repetitive stimulation paradigm in 24 month old animals revealed no significant alteration in evoked GABA release in the PVcre-mthtt mice. (n=3–5 animals and 10–12 cells per condition; *p<0.05, Student's t-test). Hyperactivity was not evident at this age as measured by ambulatory distance, and time spent ambulatory and at rest, in the open field paradigm (n= 7–11/group; **E**). Error bars: SEM.