Bidirectional control of postsynaptic density-95 (PSD-95) clustering by huntingtin*

Matthew P. Parsons¹, Rujun Kang¹, Caodu Buren¹, Alejandro Dau¹, Amber L. Southwell², Crystal N. Doty², Shaun S. Sanders², Michael R. Hayden² and Lynn A. Raymond¹

¹From the Department of Psychiatry and Brain Research Centre, 2255 Wesbrook Mall
²Centre for Molecular Medicine and Therapeutics, Child and Family Research Institute, 950 W 28th Ave
University of British Columbia, Vancouver, Canada.

Running title: Huntingtin influences PSD-95 clustering

To whom correspondence should be addressed: Lynn A. Raymond, Department of Psychiatry and Brain Research Centre, 2255 Wesbrook Mall, V6T1Z3. University of British Columbia, Vancouver, Canada. Email: lynn.raymond@ubc.ca

Keywords: Huntingtin, PSD-95, palmitoylation, striatum, Huntington disease, synapses, cell culture, patch clamp, post-translational modification

Background: Wild-type huntingtin interacts with synaptic proteins yet its role in synaptic function is unclear.

Results: Huntingtin bi-directionally influences PSD-95 clustering in striatal spiny projection neurons.

Conclusion: Huntingtin plays a role in synaptic protein organization.

Significance: Huntingtin-reducing strategies are being tested as a treatment for Huntington disease. It is therefore critical to understand the role of huntingtin in cellular and synaptic function.

ABSTRACT

Huntington Disease (HD) is associated with early alterations in cortical-striatal synaptic function that precede cell death, and it is postulated that ameliorating such changes may delay clinical onset and/or prevent neurodegeneration. While many of these synaptic alterations are thought to be attributable to a toxic gain-of-function of the mutant huntingtin (mHTT) protein, the role that non-pathogenic huntingtin (HTT) plays in synaptic function is relatively unexplored. Here, we compare the immunocytochemical localization of a major postsynaptic scaffolding protein, PSD-95, in striatal neurons from wild-type (WT) mice and mice overexpressing HTT with 18 glutamine repeats (YAC18; non-pathogenic). We find that HTT overexpression results in a palmitoylation- and BDNF-dependent increase in PSD-95 clustering at synaptic sites in striatal spiny projection neurons (SPNs) co-cultured with cortical neurons. Surprisingly, the latter effect was mediated presynaptically, as HTT overexpression in cortical neurons alone was sufficient to increase PSD-95 clustering in the postsynaptic SPNs. In contrast, antisense oligonucleotide knockdown of HTT in WT co-cultures resulted in a significant reduction of PSD-95 clustering in SPNs. Notably, despite these bidirectional changes in PSD-95 clustering, we did not observe an alteration in basal electrophysiological measures of AMPA and NMDA receptors. Thus, unlike previous studies in the hippocampus, enhanced or decreased PSD-95 clustering alone is insufficient to drive AMPA or NMDA receptors into or out of SPN synapses. In all, our results demonstrate that non-pathogenic HTT can indeed influence synaptic protein localization and uncovers a novel role of Htt in PSD-95 distribution.

INTRODUCTION

Huntington disease (HD) is caused by a CAG triplet repeat expansion in the gene encoding the huntingtin (HTT) protein. This mutation generates an N-terminal polyglutamine (polyQ) expansion within the HTT protein and results in a broad array of deficits in cellular and synaptic function, particularly within the GABAergic spiny projection neurons (SPNs) of the striatum (1). Many of these deficits precede cell death and overt motor...
Huntingtin influences PSD-95 clustering

symptoms, suggesting that the prevention of these early changes may ameliorate disease progression. While much of the cellular toxicity in HD has been generally attributed to a gain-of-function of the polyQ-expanded (mutant) HTT (mHTT), little is known regarding the role of non-expanded (wild-type) HTT (wtHTT) in synaptic function.

Over the past 15-20 years, it has become clear that wtHTT exerts a pro-survival function. Homozygous knockout of wtHTT is embryonic lethal (2-4) and overexpression of wtHTT can protect neurons from excitotoxic insult and other apoptotic stimuli (5,6) as well as reduce the toxicity associated with mHTT (7). However, the precise biological role of wtHTT has been difficult to establish due to its ubiquitous expression both within and outside the central nervous system, its diffuse intracellular localization and its wide variety of interacting partners.

wtHTT interacts strongly with microtubules and vesicular membrane proteins (8,9), leading to the suggestion that wtHTT can facilitate global intracellular vesicular trafficking via these interactions (10). A recent study identified over 700 putative proteins that form complexes with HTT. This interactome revealed networks of proteins involved in diverse biological functions, providing further support for a wide-ranging functional role of wtHTT. Of interest to the present study is the finding that some of the key HTT interactors include proteins involved in neurotransmitter transport as well as pre- and postsynaptic organization and function, thereby suggesting a role for wtHTT in synaptic signaling (11).

The membrane-associate guanylate kinases (MAGUKs) are critical postsynaptic scaffolding proteins. PSD-95, the prototypical MAGUK, is highly enriched at excitatory synapse postsynaptic densities and functions to anchor and organize glutamate receptors and various signaling molecules at the postsynapse (12,13). Interestingly, this scaffold represents a key “hub” in the HTT interactome (11) and the presence of a pathological polyQ expansion in HTT weakens this interaction (14) and mislocalizes PSD-95 to extrasynaptic sites (15). As PSD-95 binds directly to NMDA receptors (NMDARs) (16) and indirectly to AMPA receptors (AMPARs) via transmembrane AMPAR regulatory proteins (TARPs) (17), HTT effects on PSD-95 localization may critically influence AMPAR and/or NMDAR signalling. Indeed, our lab has recently demonstrated an early increase in cell-death-associated (18) extrasynaptic NMDARs in the YAC128 mouse model of HD (19). In the present study, we investigate the role of wtHTT in the subcellular distribution of PSD-95.

EXPERIMENTAL PROCEDURES

Animals were housed and maintained according to the Canadian Council on Animal Care and all procedures were approved by the University of British Columbia Committee on Animal Care. All data were obtained from WT FVB/N controls and YAC18 mice (line 212) that overexpress full-length wtHTT (human) with a polyQ length of 18 on an FVB/N background (20). All data are presented as mean ± S.E.M.

Cortico-striatal co-cultures and transfections - Neuronal culture preparation and maintenance were similar as described previously (21,22). Briefly, striatal and cortical tissue was dissected from E17-18 WT FVB/N or YAC18 embryos in ice-cold Hank’s Balanced Salt Solution (Invitrogen), digested in trypsin and further dissociated in trypsin inhibitor solution. Cells were spun down and transferred to DMEM (Invitrogen) with 10% fetal bovine serum (DMEM+). Striatal cells were immediately transfected with either yellow fluorescent protein (YFP) on a β-actin promoter (a gift from A.M. Craig, University of British Columbia), PSD-95-GFP or a palmitoylation-resistant PSD-95 in which the cysteines are mutated to serines (PSD-95C3,5S-GFP) (23). For all transfections, 2 million cells were suspended in 100 µl of electroporation buffer (Mirus Pro) with 2 µg of DNA, placed in a cuvette (Biorad) and electroporated (AMAXA nucleofector I, program 05). Striatal cells were immediately transfected with either yellow fluorescent protein (YFP) on a β-actin promoter (a gift from A.M. Craig, University of British Columbia), PSD-95-GFP or a palmitoylation-resistant PSD-95 in which the cysteines are mutated to serines (PSD-95C3,5S-GFP) (23). For all transfections, 2 million cells were suspended in 100 µl of electroporation buffer (Mirus Pro) with 2 µg of DNA, placed in a cuvette (Biorad) and electroporated (AMAXA nucleofector I, program 05). Striatal neurons were then plated with non-transfected cortical neurons at a 1:1 ratio on 12mm poly-D-lysine-coated coverslips (Marlenfeld, Germany). Cells were maintained at 37 °C, 5% CO₂, 2-4 hours after plating. DMEM+ was replaced with 500 µl plating medium (PM; neurobasal medium, B27, glutamine and penicillin/streptomycin; Gibco). An additional 500 µl PM was added on DIV3-4 and subsequent half media changes took place every 3-7 days thereafter. For certain experiments, cultures were treated on DIV13-15 prior to fixation. For the BDNF experiments, 5nM BDNF (Tocris) was added directly to the PM for 2 hours. TrkB-Fc (0.7 µg/ml; R&D systems) was added to the PM for 4 hours.
For picrotoxin (PTX)/4-aminopyridine (4AP) experiments, 50 µM PTX and 10 µM 4AP were directly added to the PM for 4 hours.

**Immunocytochemistry and cluster analysis** -
On DIV13-15, coverslips were fixed with 4% paraformaldehyde + 4% sucrose for 15-20 minutes and washed 3-5 times with phosphate buffered saline (PBS). Cells were immediately permeabilized with methanol (5 minutes at -20°C), then subjected to an additional PBS washing and a 5 minute incubation in PBS with 0.03% Triton X-100 (PBST). Coverslips were blocked in PBS with 10% normal goat serum (NGS; 30 minutes at room temperature) and then incubated in primary antibodies overnight. The primary antibody cocktail (diluted in PBST with 2% NGS) consisted of chicken anti-green fluorescent protein (GFP; 1:1000; AbCam ab13970), mouse anti-PSD-95 (1:1000; Thermo Scientific MA 1-045) and guinea pig anti-vesicular glutamate transporter 1 (VGLUT1; 1:4,000; Chemicon AB 5905). Coverslips were washed 4-6 times in PBST and then incubated in secondary antibodies for 1.5 hours at room temperature. The secondary antibody cocktail consisted of Alexa Fluor 488 goat anti-chicken (1:1,000; Invitrogen A11039), Alexa Fluor 568 goat anti-mouse (1:1,000; Invitrogen A11031) and AMCA-conjugated goat anti-guinea pig (1:100; Jackson ImmunoResearch 706-155-148). Coverslips were then washed a final 3-5 times in PBST prior to being slide mounted with fluoromount (SouthernBiotech).

Images were acquired with a Zeiss Axiovert 200M fluorescence microscope using ZEN 2012 software. Primary and secondary dendrites were imaged at 63x magnification (oil-immersion; 1.4 numerical aperture) by taking 12 optical sections in 0.4 µm steps in the z-plane. Of these, 3-5 images centered on the dendrites were flattened using the extended focus function and exported as raw, unprocessed TIFF files. Exposure times were kept constant throughout all experiments. TIFF files were imported into ImageJ and three regions of interest per cell were drawn around primary or secondary dendrites according to the YFP signal. The regions of interest were then superimposed onto the PSD-95 and VGLUT1 images and these images were manually thresholded within the regions of interest to eliminate background and preserve punctate staining. All thresholding was done with treatments/genotypes interleaved and the experimenter blinded to condition. The binary, thresholded images were then analyzed for puncta density, size and co-localization using the analyze particles tool and the colocalization plugin for ImageJ. Values from all three regions of interest were averaged to generate a mean value for each cell. Multiple conditions (genotype and/or treatment) were simultaneously processed (blind/interleaved) and the resultant data were normalized to the control condition by dividing each value by the mean value of the control group for a given parameter. Reported n indicates the number of cells analyzed from a minimum of 3 independent cultures.

**Subcellular Fractionation** - Subcellular fractionation was conducted on tissue from 1-2 month old mice, essentially as previously described (19). FVB/N and YAC18 tissue was paired on the day of dissection. Striatum was dissected on ice in 0.32M sucrose buffer (0.32M sucrose, 10mM HEPES, pH 7.4) containing phosphatase and protease inhibitor cocktails (Roche diagnostics, Mannheim, Germany) and homogenized in 200µl 0.32M sucrose buffer. Fractionation was performed as before (19) to separate the non-PSD and PSD compartments from the P2 crude synaptosomal membranes. Samples were stored at -80°C until use.

The BCA protein assay was used as before (19) but 12.5 µg of sample was heated to 95 °C for 5 minutes and separated (10% SDS-PAGE). Blocking was done at room temperature in 3% BSA/0.5% Tween-20 in TBS for 120 minutes and then membranes were incubated in primary antibody diluted in 3% BSA in TBST (overnight at 4 °C). Secondary antibody incubation and data quantification were as in (19). Primary antibodies used included mouse anti-PSD-95 (UC Davis/NIH NeuroMab, 75-028, 1:1000) and goat anti-beta-actin (Santa Cruz Biotechnology, sc-1616, 1:4500). Secondary antibodies used included donkey anti-goat HRP (Santa Cruz Biotechnology, sc-2020, 1:5000) and sheep anti-mouse HRP (GE Healthcare, NA931V, 1:5000).

**Acyl-biotinyl exchange (ABE) assay** - Striatal tissue was dissected from FVB and YAC18 mice and homogenized in lysis buffer (150mM NaCl, 50mM Tris (pH 7.4), 5mM EGTA containing 1M N- ethylmaleimide (NEM), 0.5mM PMSF and 1 tablet (Roche) protease inhibitor cocktail (epeptin, chymostatin, pepstatin, aprotinin/10 ml buffer)). Triton X-100 was added at 1.7% and cells
were resuspended in Laemmli buffer (4% SDS, 20% glycerol, 10 mM DTT, 0.004% bromphenol blue, 0.125 M Tris HCl, pH 6.8) and samples were analysed by SDS-PAGE. SDS-PAGE gels were transferred onto nitrocellulose membrane (Amersham, 0.5A for 90 min at 4°C) to probe with specific antibodies. Anti-PSD95 (1:1,000) and anti-actin (Sigma, 1:500) primary antibodies were applied overnight in Odyssey blocking buffer (Li-COR Bioscience). The secondary antibodies used were anti-rabbit IRD800 (1:10,000; Rockland) and anti-mouse Alexa Fluor 680 (1:10,000; Invitrogen). The immunoblots were scanned by Odyssey Infrared Imaging System (Li-COR Bioscience), and quantified with ImageJ.

Whole-cell Electrophysiology - On DIV13 to 15, coverslips were transferred to a recording chamber perfused with ACSF (25 °C): 125 NaCl, 2.5 KCl, 1 NaHCO₃, 1.25 NaH₂PO₄, 2.5 MgCl₂, 0.5 CaCl₂, 10 glucose. Coronal sections (400 μm) containing the striatum were obtained using a Leica VT1200S vibratome and immediately transferred to artificial cerebrospinal fluid (ACSF) warmed to 32-35 °C for 30-45 minutes. The composition of ACSF was the same as the slicing solution except the concentrations of MgCl₂ and CaCl₂ were 1 mM and 2 mM, respectively. For recordings, hemisections were transferred to a recording chamber perfused with ACSF that also contained PTX (50 μM), glycine (10 μM) and strychnine (2 μM). Patch pipettes were 3-6 MΩ when filled with the Cs-based internal solution described above and cells (approximately 15 μm soma diameter) in the dorsomedial striatum, visualized with a differential interference contrast microscope, were targeted. Glutamate afferents were stimulated by positioning an ACSF-filled glass pipette (approximately 1 MΩ) 200-300 μm dorsal to the target cell. EPSCs were evoked (evoked EPSC; eEPSC) by 0.04 ms pulses at a stimulation intensity that generated a half-maximal response (typically 150 μA). NMDAR
responses were quantified by subtracting the AMPAR eEPSC at +40 mV (in the presence of 50 μM D-APV) from the dual (AMPA + NMDA) eEPSC at +40 mV prior to D-APV application. AMPAR responses were recorded at -70 mV in the presence of D-APV. Weighted tau ($\tau_w$) was calculated as in (19). All electrophysiological data were analyzed off-line using Clampfit 10.2 software.

RESULTS

Increased levels of wild-type HTT enhance striatal neuronal synaptic clustering of PSD-95 via a palmitoylation-dependent mechanism - The presence of mHTT can influence the localization of PSD-95, with higher expression being observed in non-synaptic fractions (15). Here, we sought to determine whether expression levels of wtHTT can influence PSD-95 localization. We performed triple label immunocytochemistry on cortico-striatal co-cultures obtained from FVB/N wild-type (WT) and wtHTT-overexpressing YAC18 mice (Fig. 1). Striatal neurons were transfected with YFP on the day of plating for identification, and all analyses were obtained from primary and secondary dendrites of SPNs between DIVs 13 and 15. We found a significant enhancement of PSD-95 cluster size (Fig. 1A, B), but not density (Fig. 1E), in cultured YAC18 SPNs compared to WT SPNs. This was associated with an increase in the co-localization of PSD-95 with the presynaptic marker VGLUT1 (Fig. 1C), suggesting enhanced PSD-95 clustering at synaptic sites as a result of wtHTT overexpression. In contrast, we saw no evidence for an increase in the size or density of VGLUT1 puncta (Fig. 1D, F).

PSD-95 is palmitoylated at two N-terminal cysteine residues, C3 and C5 (25). The palmitoylation of PSD-95 is a dynamic process controlling its targeting to synaptic sites (23,26). To determine whether palmitoylation is involved in the enhanced PSD-95 clustering in YAC18 SPNs, we first quantified PSD-95 palmitoylation in striatal tissue from 1 month-old WT and YAC18 mice. In agreement with the increased synaptic clustering observed in culture, we found that PSD-95 palmitoylation was significantly enriched in YAC18 striatum compared to that of WT (Fig. 2A, B). Consistent with the palmitoylation-dependent synaptic targeting of PSD-95 as well as our data in cortico-striatal co-cultures (Fig. 1), we also observed a significant increase in the amount of PSD-95 protein in synaptic fractions (Fig. 2D). This likely represented a re-distribution of PSD-95 as total levels were unaffected (Fig. 2C). In contrast to the striatum, PSD-95 palmitoylation was not different between WT and YAC18 cortical tissue (Fig. 2E) and we saw no evidence for a change in PSD-95 clustering in YFP-transfected cortical neurons in co-culture with SPNs (Fig. 2F). Thus, HTT increases both PSD-95 palmitoylation and clustering selectively in striatal neurons.

To determine whether the enhanced clustering in YAC18 SPNs was palmitoylation-dependent, we transfected SPNs with GFP-tagged PSD-95 and co-cultured them with non-transfected cortical neurons. As we saw with endogenous PSD-95, exogenous PSD-95-GFP clustered more efficiently in YAC18 SPNs as evidenced by the larger cluster size (Fig. 3A, B). When SPNs were transfected with a palmitoylation-resistant PSD-95 construct (PSD-95C3,5S-GFP), clustering was significantly reduced and the GFP signal was much more diffuse (Fig. 3A, B), consistent with previous results in hippocampal neurons (23). Importantly, the ability of wtHTT overexpression to increase PSD-95 clustering was lost in the presence of PSD-95 C3,5S (Fig. 3A, B), suggesting that wtHTT-induced PSD-95 clustering is palmitoylation-dependent. Next, we treated cultures with 4-AP (10 μM; to block voltage-gated potassium currents and prolong action potentials) and PTX (50 μM; to block inhibition thereby increasing glutamate release) for 4 hours, as it has been shown that prolonged enhancement of hippocampal neuronal activity decreases PSD-95 palmitoylation and clustering (26). Interestingly, we found that this treatment had no significant effect on PSD-95 palmitoylation in WT co-cultures (Fig. 3C, D) demonstrating that activity-induced reductions in PSD-95 palmitoylation may be region-specific and is not a global phenomenon. Consistent with this, enhancing excitatory synaptic activity in WT co-cultures for 4 hours did not influence PSD-95 clustering in SPNs (Fig. 3E). On the other hand, the same treatment decreased PSD-95 palmitoylation in YAC18 co-cultures (Fig. 3C, D) and significantly reduced PSD-95 cluster size measured in YAC18 SPN dendrites back to WT levels (Fig. 3E). Together, these results suggest that wtHTT promotes PSD-95 palmitoylation and clustering in SPNs.
Enhanced synaptic PSD-95 clustering in striatal neurons is induced by wtHTT overexpression in cortical neurons and is mimicked and occluded by exogenous BDNF - Next, we turned to chimeric cultures to determine whether pre- and/or postsynaptic wtHTT overexpression is required for the observed effect. In these cultures, YAC18 SPNs were co-plated with WT cortical neurons (and vice versa) and immunocytochemical staining and analyses were carried out on SPN dendrites as before. Interestingly, we found that postsynaptic (striatal) overexpression of wtHTT alone was not sufficient to significantly increase PSD-95 cluster size (Fig. 4). Rather, the presence of YAC18 cortical neurons was sufficient to significantly increase PSD-95 cluster size in WT SPNs by more than 50%, a value almost identical to that observed in Fig. 1 when wtHTT was overexpressed in both the pre- and postsynaptic cells.

Previous work in the cortex has demonstrated that BDNF can act postsynaptically on TrkB receptors to increase the palmitoylation and clustering of PSD-95 (27). Much of the BDNF supply to the striatum originates from the cortex (28) and wtHTT has been shown to facilitate the transcription and trafficking of BDNF (29,30). Thus, we hypothesized that increased BDNF release from YAC18 cortex may account for the enhanced PSD-95 clustering in SPNs. To test this possibility, we applied BDNF (5nM) to the culture medium for 2 hours prior to fixation. As shown previously in the cortex (27), BDNF significantly increased the size of PSD-95 clusters in SPNs from WT co-cultures (Fig. 5A, C) without a significant effect on cluster density (data not shown). In contrast, BDNF treatment did not induce further enhancement of PSD-95 cluster size in SPNs from YAC18 co-cultures, suggesting occlusion of BDNF’s effect (Fig. 5A, C). To our surprise, and in contrast to previous reports in the hippocampus, we saw no evidence for an increase in mEPSC frequency or amplitude in YAC18 SPNs despite the enhanced PSD-95 clustering (Fig. 6A-F). Similarly, when we recorded EPSC bursts in the absence of TTX at -70 mV and then at +40 mV, WT and YAC18 SPNs displayed near-identical rectification indices (Fig. 6G, H) suggesting a lack of change in the GluA2 subunit composition of AMPA receptors.

As PSD-95 directly binds NMDARs, we then asked whether PSD-95 is drawing more NMDARs into the synapse in YAC18 SPNs. Pilot experiments established that isolated NMDAR miniature events are difficult to detect in our co-culture system, both at +40mV in the presence of magnesium and at -70mV in the absence of magnesium. Therefore, in an attempt to quantify synaptic NMDAR current, we calculated the area of EPSC bursts in the absence of TTX before and after MK-801 application. The % reduction in burst area induced by MK-801 should reflect the relative amounts of NMDARs at the synapse. We found that MK-801 reduced a comparable proportion of EPSC burst area in WT and YAC18 SPNs (Fig. 7 A, B), suggestive of similar synaptic NMDAR content. To further evaluate synaptic NMDAR properties, we also evoked NMDAR responses in WT and YAC18 striatal acute slices via stimulation of intrastriatal glutamatergic afferents. In agreement with the results in culture, we found no difference in the
Huntingtin influences PSD-95 clustering

AMPA:NMDA ratio (Fig. 7C, D). Furthermore, NMDAR current decay kinetics were comparable between genotypes (Fig. 7E). Together, these results demonstrate that despite an enhanced clustering of PSD-95 in YAC18 SPNs in co-culture, there is no detectable change in the amount or subunit composition of either AMPA or NMDA receptors under basal conditions. This is in stark contrast to previous reports in the hippocampus (17,31,33,34).

**Knock-down of endogenous wtHTT levels reduces synaptic PSD-95 clustering in striatal neurons** - Numerous HTT-silencing strategies are currently being investigated for their potential use in the clinic for the treatment of HD. At least some of these approaches lack specificity for mHTT over wtHTT, thereby resulting in lower levels of the wtHTT protein as well as the mHTT protein. Moreover, even if specific mHTT knockdown is achieved, the patient will be left with 50% of total HTT levels. This is a major concern, and little is known about how synaptic function is affected by HTT knockdown. Our above results indicate that overexpression of wtHTT increases PSD-95 palmitoylation and clustering at SPN synapses; therefore, it is of interest to determine whether the opposite is true of wtHTT knockdown. To decrease wtHTT levels, cultures were treated with a pan-specific HTT antisense oligonucleotide (ASO; ISIS pharmaceuticals 444664; control ASO 456838, 500 nM) starting on DIV3-4. HTT knockdown was confirmed by western Blot analysis as in (35) (Fig. 8A). We found that wtHTT knockdown in cortico-striatal co-cultures from WT FVB/N mice resulted in an approximately 25% decrease in PSD-95 cluster size (Fig. 8B), suggesting a bi-directional effect of wtHTT levels on PSD-95 clustering. There were no effects of wtHTT knockdown on PSD-95 cluster density (one-way ANOVA, p=0.29) or VGLUT1 cluster size (one-way ANOVA, p=0.43) or density (one-way ANOVA, p=0.60). We also did not see a reduction in the PSD-95 and VGLUT1 co-localization (one-way ANOVA, p=0.75) suggesting that synapses are not lost and rather that the PSD-95 clustering at individual synapses is reduced.

As we were surprised to see a lack of basal alterations in electrophysiological measures of AMPAR content at YAC18 SPN synapses following wtHTT overexpression, we asked whether the decreased PSD-95 clustering results in a decrease in synaptic AMPAR content. Again, we found no change in the frequency (interevent interval) or amplitude of mEPSCs recorded from WT SPNs in co-culture following ASO treatments (Fig. 8C-D), further demonstrating the lack of correlation between PSD-95 and AMPAR content at SPN synapses.

**DISCUSSION**

Due to the increased implementation of research strategies knocking down HTT for the treatment of HD, there exists a major requirement for the proper assessment of wtHTT function. While it is accepted that this protein is absolutely essential for development (2-4), recent work suggests that partial HTT knockdown in adulthood is well-tolerated for up to 6 months (36,37). While lacking a complete, long-term investigation of the effects of HTT knockdown, those studies do suggest that the treatment of HD may not necessarily require specific knockdown of mHTT. On the other hand, as we have seen with HD, what begins as small alterations in synaptic function (1) can give rise to subsequent cell death and a devastating behavioral, cognitive and psychiatric phenotype in a relatively short period of time. Here, we began an investigation into the effects of wtHTT on synaptic function by focusing on the most abundant component of the excitatory synapse postsynaptic density, PSD-95. We show that wtHTT exerts bidirectional control over the amount of PSD-95 clustering at postsynaptic sites in the striatum. The enhanced clustering was due to PSD-95 palmitoylation and was consistent with wtHTT's facilitatory role in BDNF transcription and transport from cortical to striatal neurons (29,30). We also show that knocking down wtHTT is sufficient to decrease PSD-95 clustering.

The effect of wtHTT on PSD-95 was indirect in that wtHTT overexpression was required in the presynaptic cortical rather than the postsynaptic striatal neurons. This came as a surprise as wtHTT interacts directly with PSD-95 and also increases the enzymatic activity of huntingtin interacting protein 14 (HIP14) (38), an important palmitoyl acyltransferase that can palmitoylate PSD-95 (39,40). However, the presently observed effect was clearly driven by wtHTT overexpression in cortical neurons, suggesting that wtHTT overexpression induces the release of a signal that acts postsynaptically to enhance PSD-95 clustering. Our data suggest that...
Huntingtin influences PSD-95 clustering

this factor is BDNF, as the ability for BDNF treatment to increase PSD-95 cluster size was occluded in YAC18 cultures and the enhanced cluster size in YAC18 cultures was reduced to WT levels by the BDNF scavenger TrkB-Fc. It is known that wtHTT enhances the transcription and trafficking of BDNF from cortical to striatal neurons (29,30). Moreover, previous studies indicate that BDNF increases the forward trafficking, palmitoylation and synaptic incorporation of PSD-95 via postsynaptic TrkB receptor activation (27,41): The authors found that BDNF-induced PSD-95 palmitoylation required the activation of signalling pathways downstream of TrkB activation, culminating in a PKMzeta-dependent phosphorylation of the PAT DHHC8. While we did not investigate the contribution of PKMzeta or DHHC8 in the presently observed effect, our data are consistent with a role for presynaptic BDNF release in the postsynaptic clustering of PSD-95.

Despite the reliance of wtHTT overexpression in the cortex, the enhanced PSD-95 palmitoylation and clustering was only observed in the striatum and not in the cortex itself. This suggests that SPNs and cortical neurons may differ in their response to the BDNF and/or additional factors released from the wtHTT-overexpressing cortical neurons. If BDNF is the sole transmitter involved in this effect, then it is surprising that we did not see enhanced PSD-95 palmitoylation and clustering in cortical neurons as BDNF is known to exert this effect on cultured cortical neurons (27,41). While it is possible that additional factors are released from cortical neurons that act specifically on SPNs to induce the effect, we were able to completely restore clustering to WT levels with a BDNF scavenger, suggesting a major role of BDNF itself. Another possibility is that the amount of BDNF released under basal conditions is greater at cortico-striatal synapses compared to cortico-cortical synapses. BDNF is released in an activity-dependent manner and in our co-culture system, we observe much greater basal EPSC frequencies when recording from striatal neurons than from cortical neurons (unpublished observations) suggesting more activity at the cortico-striatal synapse than at the cortico-cortico-synapse in co-cultures. Thus, while exogenous BDNF application to WT cortical neurons may be sufficient to enhance PSD-95 clustering, the basal amount of BDNF released in YAC18 cultures at cortico-cortico synapses appears insufficient to mimic this effect. A third possibility is that SPNs are highly sensitive to the effects of BDNF. The striatum is reliant upon cortical BDNF release for proper development (28) and is also the area most vulnerable to the effects of brain-wide BDNF deprivation (42). Furthermore, exogenous BDNF accelerates the growth of cultured striatal but not hippocampal neurons (42). While the precise reason for the presently observed specificity remains unclear, our data show that Htt overexpression in cortical neurons results in a specific BDNF-dependent increase in PSD-95 clustering in postsynaptic SPNs and are consistent with a prominent role of BDNF signaling in the striatum.

Perhaps most surprising was the observation that altered PSD-95 clustering did not appear to basally alter the quantity or apparent subunit composition of synaptic AMPARs and NMDARs. This is in stark contrast to previous reports in the hippocampus (17,31,33,34). Our results suggest that in the striatum, PSD-95 is not a critical determinant of synaptic AMPAR content. Indeed, work in the cortex also suggests that PSD-95 overexpression does not pull AMPARs into the synapse as is seen in the hippocampus (47,48), suggesting regional differences are at play. Nevertheless, cortical PSD-95 was found to be essential to activity-dependent homeostatic synaptic scaling (47), suggesting that enhanced PSD-95 clustering may influence activity-dependent plasticity in YAC18 SPNs. Even in the hippocampus, the enhanced synaptic AMPAR content following PSD-95 overexpression appears to be activity-dependent (49) and PSD-95 interactions with A kinase anchoring protein 150 are essential to NMDAR-dependent long-term depression (50). Our data further support the idea that PSD-95 alone cannot account for alterations in basal synaptic AMPAR or NMDAR content. The consequences of PSD-95 clustering on synaptic transmission and activity-dependent plasticity in the striatum remain to be fully elucidated and are of interest for future studies.

Notably, we show that wtHTT knockdown results in a decrease in PSD-95 cluster size. While we did not further examine the mechanism underlying the reduction, it is tempting to suggest that this may be due to PSD-95 depalmitoylation. However, it should be pointed out that scavenging

Downloaded from http://www.jbc.org/ by guest on November 4, 2016
Huntingtin influences PSD-95 clustering

BDNF with TrkB-Fc in WT cultures did not mimic the effect of wtHtt knockdown. While this may be due to the relative short treatment of TrkB-Fc (4 hours) compared to ASO treatment (~10 days), it is possible that the declustering effect is dependent upon an entirely separate mechanism. Our ASO treatments were applied directly to the culture medium and thus would be expected to decrease both pre- and postsynaptic levels of wtHtt. As stated, wtHtt interacts directly with PSD-95 (Shirasaki, 2012) and enhances the enzymatic activity of a palmitoylation enzyme that palmitoylates PSD-95 (38), suggesting that the declustering effect may be postsynaptically-driven. Importantly, our data demonstrate that decreasing wtHTT levels can influence neuronal architecture at the synaptic level. This has important therapeutic implications for non-specific Htt-reducing strategies for the treatment of HD and highlights the necessity for additional studies on the precise cellular/synaptic effects of wtHtt knockdown.

REFERENCES

Huntingtin influences PSD-95 clustering


Huntingtin influences PSD-95 clustering


Acknowledgements - This work was supported by grants from the Canadian Institutes of Health Research (Emerging Team grant GPG-102165 and MOP-102517) and the Cure Huntington’s Disease Initiative. MPP holds a Michael Smith Foundation for Health Research Postdoctoral Fellowship. The authors thank Lily Zhang and Liang Wang for their technical assistance as well as Frank Bennett and ISIS Pharmaceuticals for providing pan-specific HTT and control ASOs.

FIGURE LEGENDS

**FIGURE 1.** wtHTT increases PSD-95 cluster size in striatal neurons. A, Images of WT (left) and YAC18 (right) SPN dendrites (YFP) stained for PSD-95 (red) and VGLUT1 (blue). B, Puncta analysis of thresholded images reveal that PSD-95 cluster size is increased in YAC18 SPNs relative to WT (WT n=20, YAC18 n=23, unpaired t-test ***p<0.001). Examples of enlarged puncta are denoted with arrows in A. C, Co-localization of PSD-95 with the presynaptic marker VGLUT1 is also enhanced in YAC18 SPNs, suggesting that the enhanced PSD-95 clustering occurs at synaptic sites (WT n=20, YAC18 n=23, unpaired t-test ***p<0.001). D-F, VGLUT1 cluster size (D) was unaffected as was PSD-95 (E) and VLGUT1 (F) cluster density (unpaired t-test p>0.05). Scale Bar = 5 μm.

**FIGURE 2.** wtHTT increases PSD-95 palmitoylation in the striatum but not cortex. A, Representative ABE assay showing an increase in PSD-95 palmitoylation from YAC18 striatal tissue relative to WT. B, Quantification of the grouped data from the experiments shown in A (n=5 independent experiments in which striatal tissue from WT and YAC18 mice was processed in parallel; paired t-test *p<0.05). C, Western blot analysis of total PSD-95 protein levels in striatal tissue (n=8 independent experiments in parallel; paired t-test, p>0.05). D, Analysis of PSD-95 expression in synaptic fractions reveals a significant increase in synaptic PSD-95 levels in YAC18 striatum (paired t-test *p<0.05). E, ABE quantification of PSD-95 palmitoylation from cortical tissue shows no difference between genotypes (n=7 independent experiments in parallel, paired t-test, p>0.05). F, PSD-95 cluster size in YFP-transfected cortical neurons in co-culture with SPNs is also similar between genotypes (WT n=20, YAC18 n=20, unpaired t-test p>0.05).

**FIGURE 3.** wt-HTT-induced PSD-95 clustering is palmitoylation-dependent. A, Overexpression of PSD-95-GFP results in clear clustering in both WT and YAC18 MSNs whereas clustering is visibly reduced following transfection of the palmitoylation-resistant PSD-95-C3,5S-GFP mutant. B, Grouped data
showing that PSD-95-GFP cluster size is greater in YAC18 SPNs relative to WT (WT n=19, YAC18 n=31, unpaired t-test ***p<0.001). This effect is not seen with the C3,5S construct (WT n=17, YAC18 n=29, unpaired t-test p>0.05), suggesting a palmitoylation-dependent mechanism. C, Representative western blot from ABE assay showing PSD-95 palmitoylation following culture treatment with 4AP (10 µM) and PTX (50 µM, 4 hours) to elevate synaptic activity. D, 4AP/PTX treatment significantly reduces PSD-95 palmitoylation in YAC18 cultures (n=5, one-sample t-test *p<0.05) but not in WT cultures (n=4, one-sample t-test p>0.05). E, Similarly, 4AP/PTX treatment reduced PSD-95 cluster size in YAC18 SPNs (CTL n=25, PTX/4AP n=20, unpaired t-test **p<0.01) but not WT SPNs (CTL n=15, PTX/4AP n=17, unpaired t-test p>0.05). Scale Bar = 5 µm.

Figure 4. Presynaptic mechanisms mediate wtHTT’s effect on PSD-95 clustering. A, Representative pictures of YFP-filled SPN dendrites from WT co-cultures or chimeric cultures consisting of YAC18 SPNs co-cultured with WT cortex and vice versa. PSD-95 staining is shown on the right in red. B, PSD-95 cluster size is significantly enhanced in SPNs only when wtHTT is overexpressed in the presynaptic, cortical cells (WT/WT n=19, 18/WT n=28, WT/18 n=20, one-way ANOVA with Dunnett’s multiple comparison test **p<0.01). Scale Bar = 5 µm.

Figure 5. Effect of wtHTT overexpression on PSD-95 clustering is mimicked by BDNF in WT SPN, and BDNF effect is occluded in YAC18 SPN. A-B, Representative images of YFP-filled SPN dendrites (green) and PSD-95 staining (red) from WT and YAC18 co-cultures treated with BDNF (A; 5nM for 2 hours directly in the culture medium) or TrkB-Fc (B; 0.7 µg/ml for 4 hours directly in the culture medium). C, BDNF treatment significantly increased PSD-95 cluster size in WT SPNs (CTL n=25, BDNF n=24, unpaired t-test, *p<0.05) but not in YAC18 SPNs (CTL n=21, BDNF n=22, unpaired t-test, p>0.05). D, TrkB-Fc treatment significantly decreased PSD-95 cluster size in YAC18 SPNs (CTL n=21, TrkB-Fc n=22, unpaired t-test, **p<0.01) but not in WT SPNs (CTL n=22, TrkB-Fc n=22, unpaired t-test, p>0.05). Scale Bar = 5 µm.

Figure 6. wtHTT overexpression has no effect on mEPSC interevent interval, amplitude or AMPAR rectification index. A-F, mEPSC recordings from WT and YAC18 SPNs co-cultured with cortical neurons. Representative traces are shown in A and D. There was no effect of genotype on the cumulative distribution of mEPSC interevent interval (B, WT n=16, YAC18 n=17, RM two-way ANOVA interaction and genotype p>0.05) or amplitude (E, WT n=16, YAC18 n=17, RM two-way ANOVA interaction and genotype p>0.05). Mean IIEIs (C, WT n=16, YAC18 n=17 unpaired t-test p>0.05) and amplitudes (F, WT n=16, YAC18 n=17, unpaired t-test p>0.05) were also similar between genotypes. G, Isolated AMPAR-mediated responses in the absence of TTX and the presence of PTX (50 µM) and 4AP (10 µM) result in frequently-observed burst EPSCs >100 pA. Average burst EPSC size was determined at a holding potential of -70 mV and then again at +40 mV to quantify an AMPAR rectification index. H, There was no difference in the AMPAR rectification index between WT and YAC18 SPNs (WT n=7, YAC18 n=7, unpaired t-test p>0.05).

Figure 7. wtHTT overexpression does not alter measurements of synaptic NMDARs. A, Burst EPSCs before (top) and after (bottom) rapid application of MK-801 (10 µM) to block synaptic NMDARs in WT (left) and YAC18 (right) SPNs. B, Synaptic NMDAR content was quantified by analyzing the % reduction in the total area of burst EPSCs induced by MK-801. There was no difference between genotypes (WT n=4, YAC18 n=5, unpaired t-test p>0.05). C,D, We also found no difference in the AMPA:NMDA ratio using acute coronal slices from WT and YAC18 mice (WT n=5, YAC18 n=7, unpaired t-test p>0.05). E, NMDAR decay kinetics were also unaltered by wtHTT overexpression (WT n=5, YAC18 n=7, unpaired t-test p>0.05).

Figure 8. Antisense oligonucleotide knockdown of wtHTT decreases PSD-95 cluster size. A, Representative western blot and quantification showing a clear reduction of wtHTT expression following 10-11 day treatment with pan-specific HTT ASO (n=2 independent culture batches). B, Treatment with
pan-specific HTT ASO (500 nM) significantly reduced PSD-95 cluster size in WT SPNs co-cultured with cortical neurons (Conditioned media; CM n=18, CTL ASO n=21, HTT ASO n=22, one-way ANOVA *p<0.05). C-D, despite the deceased PSD-95 clustering, both mEPSC interevent interval (C) and amplitude (D) recorded from SPNs in co-culture with cortical neurons were unaffected (WT n=15, YAC18 n=14, genotype p>0.05, two-way ANOVA).
FIGURE 1.

A

WT YFP PSD-95 YAC18 VGLUT1 MERGE

B

C

D

E

F

Huntingtin influences PSD-95 clustering
Huntingtin influences PSD-95 clustering

FIGURE 2.

A

<table>
<thead>
<tr>
<th>WT</th>
<th>YAC18</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAM</td>
<td>-</td>
</tr>
<tr>
<td>Palmitoylation</td>
<td></td>
</tr>
</tbody>
</table>

WB: PSD-95

B

PSD-95 Palmitoylation (norm)

C

Total PSD-95/actin (norm)

D

Synaptic PSD-95/actin

E

PSD-95 Palmitoylation (norm)

F

PSD-95 Cluster Size (norm)

* n.s.
FIGURE 3.

A

WT  

YAC18  

PSD95-GFP  

PSD95-C3,5S-GFP  

B

PSD-95-GFP Cluster Size (norm)  

WT  

YAC18  

C

WT  

YAC18  

PSD95-palmitoylation (% Control)  

D

WT  

18-PTX  

E

PSD-95 Cluster Size (norm)  

WT  

YAC18  

PTX/4AP  

n.s.  

***  

n.s.  

**  

n.s.
FIGURE 4.
FIGURE 5.
FIGURE 6.
Huntingtin influences PSD-95 clustering

FIGURE 7.

A

\[
\begin{align*}
\text{WT: 4AP} & \quad \text{YAC18: 4AP} \\
\text{WT: 4AP/MK-801} & \quad \text{YAC18: 4AP/MK-801}
\end{align*}
\]

B

\[
\begin{align*}
\% \text{ Area reduction by MK-801} \\
\text{WT} & \quad \text{YAC18}
\end{align*}
\]

C

\[
\begin{align*}
\text{WT} & \quad \text{YAC18}
\end{align*}
\]

D

\[
\begin{align*}
\text{AMPA/NMDA} \\
\text{WT} & \quad \text{YAC18}
\end{align*}
\]

E

\[
\begin{align*}
e\text{EPSC}_{\text{NMDA}}/\text{Ta}_{\text{w}} (\text{ms}) \\
\text{WT} & \quad \text{YAC18}
\end{align*}
\]
Figure 8.
Bidirectional control of postsynaptic density-95 (PSD-95) clustering by huntingtin
Matthew P. Parsons, Rujun Kang, Caodu Buren, Alejandro Dau, Amber L. Southwell, Crystal N. Doty, Shaun S. Sanders, Michael R. Hayden and Lynn A. Raymond

J. Biol. Chem. published online December 17, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.513945

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2013/12/17/jbc.M113.513945.full.html#ref-list-1