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Review

Is Huntingtin Dispensable in the Adult Brain?

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Abstract. Huntingtin (HTT) is an essential protein during early embryogenesis and the development of the central nervous system (CNS). Conditional knock-out of mouse *Huntingtin* (*Htt*) expression in the CNS beginning during neural development, as well as reducing *Htt* expression only during embryonic and early postnatal stages, results in neurodegeneration in the adult brain. These findings suggest that HTT is important for the development and/or maintenance of the CNS, but they do not address the question of whether HTT is required specifically in the adult CNS for its normal functions and/or homeostasis. Recently, it was reported that although removing *Htt* expression in young adult mice causes lethality due to acute pancreatitis, loss of *Htt* expression in the adult brain is well tolerated and does not result in either motor deficits or neurodegeneration for up to 7 months after *Htt* inactivation. However, recent studies have also demonstrated that HTT participates in several cellular functions that are important for neuronal homeostasis and survival including sensing reactive oxygen species (ROS), DNA damage repair, and stress responses, in addition to its role in selective macroautophagy. In this review, HTT's functions in development and in the adult CNS will be discussed in the context of these recent discoveries, together with a discussion of their potential impact on the design of therapeutic strategies for Huntington's disease (HD) aimed at lowering total *HTT* expression.

Keywords: Huntingtin, HTT, Htt, Huntington's disease, HD, cell stress, ROS, DNA damage

INTRODUCTION

Since the discovery of the *Huntingtin* gene (*HTT*) in 1993 [1], Huntingtin's (HTT's) normal functions have been the subject of intense investigation using both cellular and animal models. HTT is one of the larger proteins (3144 amino acids) in the vertebrate and invertebrate proteomes, and it is hypothesized to function as a scaffold in many cellular processes [2]. HTT contains 36 HEAT and HEAT-like repeats (composed of multiple 30–50 amino acid amphiphilic paired alpha helical HEAT motifs in each repeat) that are distributed throughout the protein and contribute to its flexible, superhelical, and solenoid-like structure [3]. HEAT repeats were named after the group of proteins in which they were initially described; Huntingtin, Elongation Factor 3, PR65A scaffolding subunit of protein phosphatase 2A, and Target of rapamycin (TOR) kinase [4]. The HEAT repeat structure of HTT contributes to dynamic interactions with its protein partners, may facilitate its functions in crowded cellular microdomains, and could potentially allow HTT to store and manipulate mechanical energy [5, 6].

The C-terminal portion of HTT is the most evolutionarily conserved, while the N-terminus of vertebrate HTT encoded by the first exon, consisting of an N-terminal 17 amino acid amphipathic helix (N17), the polyglutamine stretch (polyQ), and in mammals, a proline-rich region (PRR), has evolved more recently [7]. The N17 domain is conserved in

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vertebrates and is also present with 80% homology in the marine gastropod mollusk *Aplysia* [8], while the polyQ stretch first arose in deuterostomes [7]. The PRR first appeared in mammals and is hypothesized to contribute to HTT's functions as a protein-protein interaction domain and to modulate the toxicity of the polyQ stretch when it is expanded beyond the pathological threshold in HD [9–12].

Mouse HTT (Htt) is essential in embryonic development and is also required for the formation of the CNS [13–19]. Constitutive knock-out of *Htt* expression in the mouse results in lethality between embryonic day (E) 7.5 to 8.5, a stage when gastrulation has occurred and the development of the nervous system has just started [13–15]. There is increased cell death in the embryonic ectoderm, the germ layer that is the origin of the nervous system, due to the loss of *Htt* expression in the extraembryonic tissues (visceral endoderm and trophoblast) [20]. In *Htt* conditional knock-out mice that have lost *Htt* expression in the developing CNS beginning either during embryogenesis or soon after birth, progressive neurodegenerative phenotypes are present in adult mice, suggesting that *Htt* expression is required in the nervous system for its normal function [21, 22]. However, these experiments did not distinguish between the possibilities that Htt is required only during CNS development (and the neurodegeneration observed in the adult brain is a consequence of developmental defects), or that Htt has essential functions in the adult brain in addition to its roles in development.

Distinguishing between these possibilities and determining if Htt is required in the adult brain became important areas of investigation following the discovery that anti-sense oligonucleotides (ASOs) targeting both normal and mutant *HTT* mRNA can ameliorate HD mouse model phenotypes for up to 3 months after a single dose of the ASOs without any obvious phenotypes due to HTT loss of function [23, 24]. Recently, Wang et al. generated inducible conditional knock-out of *Htt* expression in the adult mouse brain and observed no apparent motor and neuropathological deficits in these mice 6-7 months after the induction of Cre-recombinase expression [25], suggesting that HTT is not required in the adult CNS. However, other recent studies have shown that HTT participates in several cellular functions that are important for neuronal homeostasis and survival, in addition to its critical role in development (summarized in Table 1).

In this review, we will discuss what is currently known about HTT's functions that are important in the developing nervous system and in the adult brain, as well as how new information about normal HTT functions could impact the design of therapeutic strategies for HD.

HTT IN NEURAL DEVELOPMENT

Using an *in vitro* mouse embryonic stem cell (ESC) model, loss of *Htt* expression was shown to disrupt the specification of both primitive and definitive neural stem cells during neural induction [19]. To study Htt's function in later stages of neural development and adulthood *in vivo*, a conditional *Htt* allele with *loxP* sites flanking its promoter and exon 1 was generated (Htt^{flox}) to bypass the requirement of Htt during early embryonic development [21]. Inactivation of *Htt* expression by Cre/*loxP*-mediated recombination is dependent on the expression of *Cre* under the control of tissue- or developmental stage-specific promoters. To confer additional temporal control of recombination, inducible regulation of Cre recombinase activity can be accomplished by fusion of Cre with the ligand-binding domain of the estrogen receptor (ER) [26]. Since many Cre-expressing lines of mice are generated by pronuclear injection, Cre expression levels can vary depending on the transgene insertion site, even when the same promoter is used. If Cre levels are suboptimal, recombination may not occur in every cell and this can result in tissue mosaicism (cells lacking *Htt* expression are interspersed with cells in which the Htt^{flox} allele has not recombined). Some investigations have used hemizygous $Htt^{flox/-}$ mice instead of homozygous $Htt^{flox/flox}$ mice in an attempt to mitigate this issue. Thus, it is important to keep these caveats in mind when comparing *Htt* conditional knock-out mice generated using different Cre lines. A list of the Cre-expressing mouse lines that were used in the studies discussed in this review to inactivate Htt^{flox} expression are presented in Table 2.

When *Htt* expression was inactivated in the developing nervous system using *Camk2a-Cre* transgenic lines (expressing Cre recombinase in forebrain neurons: *R1ag5 Camk2a-Cre* recombining at ~E15 and *L7ag13 Camk2a-Cre* recombining at ~P5) to drive Cre-mediated recombination in mice hemizygous for *Htt* expression ($Htt^{flox/-}$), the conditional knock-out mice exhibited progressive neurodegeneration and gliosis [21]. At 3 months of age, the morphology of the brains of the *Htt* conditional knock-outs appeared relatively normal, except slightly smaller in size and with enlarged ventricles. By 4–6 months of age, how-

Table 1
Proposed normal HTT functions

Proposed Function	Mechanism	References
Microtubule-based transport	HTT, together with HAP1 and the p150Glued subunit of dynactin, associate with the dynein motor complex involved in retrograde transport in neurons [81–85]. HTT can also interact directly with the dynein intermediate chain [85]. In addition, HAP1 interacts with kinesin light chain-1, a subunit of the kinesin anterograde motor complex [86]. Phosphorylation of the HAP1A isoform reduces its association with both p150Glued and kinesin light chain-1 [87], while phosphorylation of HTT at S421 favors recruitment of the kinesin-1 heavy chain to microtubules and vesicles or organelles for anterograde transport in a motor complex containing HTT, dynein, dynactin, and kinesin [88]. Upon de-phosphorylation of HTT S421, the kinesin-1 motor is released and the remaining complex with HTT favors retrograde transport [88].	Li, S.-H. et al., 1998 [81] Li, X.-J. et al., 1995 [82] Engelender, S. et al., 1997 [83] Block-Galarza et al., 1997 [84] Caviston, J.P. et al., 2007 [85] McGuire, J.R. et al., 2006 [86] Rong, J. et al., 2006 [87] Colin, E. et al., 2008 [88]
F-actin-based trafficking	HTT regulates clathrin-mediated endocytosis via several HTT-interacting proteins and their interactions with the actin cytoskeleton [89–95]. HTT forms a complex with HAP40 and Rab5 that can regulate both the long-range movement of early endosomes on microtubules and their localized movements on actin filaments [96, 97]. The mechanism facilitating early endosome movement along F-actin likely involves HTT's interactions with OPTN (FIP2) [98, 99]. OPTN is a coiled-coil vesicle cargo adapter protein that could link the HTT-HAP40-Rab5 complex on early endosomes with the actin motor, myosin VI [99]. OPTN co-localizes with HTT and Rab8 within the Golgi complex, where it recruits myosin VI to the Golgi [100]. This interaction is hypothesized to contribute to post-Golgi trafficking to either the cell surface or the lysosome. The HTT-OPTN-Rab8 complex can also modulate cell polarization and the cell stress response [98].	Rao, D.S. et al., 2001 [89] Waelter, S. et al., 2001 [90] Singaraja, R.R. et al., 2002 [91] Yanai, A. et al., 2006 [92] Kaltenbach, L.S. et al., 2007 [93] Moreira Sousa, C. et al., 2013 [94] El-Daher, M.T. et al., 2015 [95] Pal, A. et al., 2008 [96] Pal, A. et al., 2006 [97] Hattula, K. and Peranen, J., 2000 [98] Caviston, J.P. and Holzbaur, E.L., 2009 [99] Sahlender, D.A. et al., 2005 [100]
Rab11 function and the trafficking of other Rab proteins	HTT can be found in a complex that activates Rab11, and is important for the trafficking of Rab11 vesicles [101, 102]. HTT's activation of Rab11 contributes to the trafficking of recycling endosomes that help to establish apical polarity during epithelial morphogenesis in the mammary gland [103]. HTT, via Rab11 activation, regulates N-cadherin trafficking that is involved in the transition of newly born neurons from a multipolar to bipolar morphology during their migration [31]. HTT has also been proposed to act as a scaffold for Rab11's regulation of GLUT3 expression on the neuronal cell surface [104]. Knock-down of *Drosophila* HTT expression affects the transport of Rab3, Rab19, Rab7, Rab2, and Rab8 vesicles [105].	Li, X. et al., 2008 [101] Power, D. et al., 2012 [102] Elias, S. et al., 2015 [103] Barnat, M. et al., 2017 [31] McClory, H. et al., 2014 [104] White, J.A. et al., 2015 [105]
BDNF transport	HTT enhances the efficiency of both anterograde and retrograde microtubule-based vesicular transport of BDNF via its association with HAP1 and $p150^{Glued}$ [106]. In the brain, BDNF is anterogradely transported in cortical projection neurons to the striatum where HTT also facilitates vesicular transport of TrkB in striatal neurons [107].	Gauthier, L.R. et al., 2004 [106] Liot, G. et al., 2013 [107]

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Proposed Function	Mechanism	References
Ciliogenesis	The HTT-HAP1 interaction contributes to primary cilia formation and is also required for the formation of the motile cilia found on ependymal cells lining the brain ventricles. Formation of cilia requires trafficking of protein components to the pericentriolar material (PCM) that surrounds that centrioles that both anchor and nucleate the microtubules in cilia. In the absence of HTT expression, PCM1, a major component of the PCM, together with pericentrin and ninein, are dispersed from the PCM, and cilia do not form properly [27]. Morpholino knock-down of *Xenopus HTT* in embryos results in reduced numbers and lengths of cilia on the cells covering the embryo's epidermis [29].	Keryer et al., 2011 [27] Haremaki, T. et al., 2015 [29]
Transcription and chromatin modification	In early embryogenesis, Htt regulates polycomb repressor complex 2 function as a chromatin repressor that is important for normal *Hox* gene expression, extraembryonic trophoblast differentiation, and normal histone methylation [108]. HTT interacts with REST/NRSF in the cytosol to help maintain neuronal gene expression, including *BDNF* expression [109]. HTT also interacts with the cAMP Response Element Binding Protein (CBP), a protein involved in the regulation of histone acetylation and deacetylation [110], and regulates the transcription of nuclear receptors [111]. Loss of *Htt* expression in mouse embryonic fibroblasts affects the expression of genes involved in multiple pathways, including protein degradation, lipid metabolism, cell division, development, and extracellular matrix composition [112].	Seong, I.S. et al., 2010 [108] Zuccato, C. et al., 2003 [109] Steffan, J.S. et al., 2000 [110] Futter, M. et al., 2009 [111] Zhang, H. et al., 2008 [112]
Post-transcriptional gene expression regulation	HTT participates in Processing (P)-body formation, RNA transport, and RNA translation; Htt interacts with Argonaut 2 (Ago2) in P-bodies that are involved in post-transcriptional gene silencing [113]. When *Htt* expression is reduced, mRNA transport is also inhibited, and Htt co-localizes with *β-actin*, *BDNF*, the microtubule-dependent anterograde motor protein *Kif5a*, dynein heavy chain (*Dhc*), and its own mRNA [114, 115]. Htt interacts with a variety of RNA binding proteins [44].	Savas, J.N. et al., 2008 [113] Ma, B. et al., 2011 [114] Culver, B.P. et al., 2016 [115] Culver, B.P. et al., 2012 [44]
Neurogenesis	HTT associates with centrosomes in neuronal progenitors undergoing cell division where it regulates mitotic spindle orientation [28, 116]. Htt is also required for the morphogenesis and migration of newly born cortical neurons [31]. In *Xenopus*, loss of *htt* expression disrupts development of the mandibular branch of the trigeminal nerve [28]. In zebrafish, htt contributes to the formation of the anterior neural plate that will eventually become the telencephalon [29]. In both mice and zebrafish, Htt is required for homotypic interactions between neuroepithelial cells during neurulation [117].	Godin, J.D. et al., 2010 [28] Godin, J.D. and Humbert, S., 2011 [116] Barnat, M. et al., 2017 [31] Haremaki, T. et al., 2015 [29] Henshall, T.L. et al., 2009 [30] Lo Sardo, V. et al., 2012 [117]

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Proposed Function	Mechanism	References
Synaptogenesis and synaptic plasticity	In the absence of Htt expression, excess excitatory corticostriatal and thalamostriatal synapses are generated in mice [32]. In *Aplysia*, ApHTT is located in both pre- and post-synaptic sensory neurons where it participates in long-term facilitation [8].	McKinstry, S.U. et al., 2014 [32] Choi, Y.B. et al., 2014 [8]
Signaling pathways	HTT interacts with epidermal growth factor (EGF) receptor signaling complexes containing Grb2 and RasGAP [118]. Screens for HTT-interacting proteins have identified proteins enriched in mTOR and Rho GTPase signaling. In addition, HTT-interacting proteins also participate in the oxidative stress response [93].	Liu, Y.F. et al., 1997 [118] Kaltenbach, L.S. et al., 2007 [93]
Cell stress responses and cell survival	HTT can influence cell death pathways through the regulation of Bcl-2-mediated Caspase3/9 activity [119, 120], and through the modulation of the interaction between the anti-apoptotic Huntingtin-interacting protein-1 (HIP-1) and its pro-apoptotic partner, Hippi [121, 122]. HTT is also required for both the rapid cell stress response and the canonical cell stress response pathways [51, 123]. The HTT N-terminal N17 domain plays a critical role in these processes, along with acting as an ROS sensor [56].	Rigamonti, D. et al., 2000 [119] Rigamonti, D. et al., 2001 [120] Cheng, C.M. et al., 2003 [121] Gervais, F.G. et al., 2002 [122] Nath, S. et al., 2015 [51] Munsie, L.N. and Truant, R., 2012 [123] DiGiovanni, L.F. et al., 2016 [56]
Selective macroautophagy	HTT participates in stress-activated selective macroautophagy [58, 59, 124] and is required for the efficient transport of autophagosomes on microtubules [68].	Ochaba, J. et al., 2014 [59] Rui, Y.N. et al., 2015 [60] Rui, Y.N. et al., 2015 [124] Wong, Y.C. and Holzbaur, E.L., 2014 [68]
DNA damage repair	In response to DNA damage, HTT is phosphorylated at S1181 and S1201 by Cdk5 kinase [45]. Inhibiting this phosphorylation accelerates p53-dependent neuronal cell death when DNA damage is present. HTT also participates in base excision repair as part of a complex with the ataxia-talengiectasia mutated protein (ATM) [50].	Anne, S.L. et al., 2007 [45] Maiuri, T. et al., 2017 [50]

Table 2
Cre lines used to inactivate the Htt^{flox} allele

Studies cited	Cre mouse line	Commercial Source	Reference
Dragatsis et al., [21]	*Camk2a-Cre/R1ag5*	The Jackson Laboratory B6.Cg-Tg(Camk2a-cre)2Szi/J Stock # 027310 | R1ag#5	Dragatsis, I. and Zeitlin, S., 2000 [125]
	Camk2a-Cre/L7ag13		Dragatsis, I. and Zeitlin, S., 2000 [125]
Dietrich et al., [22]	*Wnt1-Cre*	The Jackson Laboratory Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4)11Rth/J Stock # 003829	Danielian, P.S. et al., 1998 [126]
Wang et al., [25]	*CAG-cre/Esr1*	The Jackson Laboratory B6.Cg-Tg(CAG-cre/Esr1)5Amc/J Stock # 004682	Hayashi, S. and McMahon, A.P. 2002 [127]
	Nestin-cre/Esr1	The Jackson Laboratory C57BL/6-Tg(Nes-cre/Esr1*)1Kuan/J Stock # 012906	Burns, K.A. et al., 2007 [128]
	Camk2a-Cre/ERT2	The Jackson Laboratory B6;129S6-Tg(Camk2a-cre/ERT2)1Aibs/J Stock # 012362	Madisen, L. et al., 2010 [129]
Godin et al., [27]	*Nestin-Cre*	The Jackson Laboratory B6.Cg(SJL)-TgN(NesCre)1Kln (current strain name: B6.Cg-Tg(Nes-cre)1Kln/J) Stock # 003771	Tronche, F. et al., 1999 [130]
Barnat et al., [31]	*Nex-Cre*		Goebbels, S. et al., 2006 [131]
McKinstry et al., [32]	*Emx1-Cre*	The Jackson Laboratory B6.129S2-*Emx1*$^{tm1(cre)Krj}$/J Stock # 005628	Gorski, J.A. et al., 2002 [132]
Arteaga-Bracho et al., [33]	*CAG-cre/Esr1* (same mice used in Wang et al., [25])	The Jackson Laboratory B6.Cg-Tg(CAG-cre/Esr1)5Amc/J Stock # 004682	Hayashi, S. and McMahon, A.P. 2002 [127]
Pla et al., [39]	*CaMKCreER*^{T2}	European Mouse Mutant Archive *CaMKCreER*^{T2}	Erdmann, G. et al., 2007 [133]

ever, degeneration in the caudal/lateral region of the cortex was detected with degenerating axon fiber tracts, and by 8 months of age, eosinophilic neurons were visible in the hippocampus along with cortical disorganization and a reduction in myelin staining. At 10 months of age, DNA breaks in the cortex and striatum were detected by *in situ* end-labeling of DNA nicks together with the loss of MAP2-positive dendrites in the forebrain. These results suggest that Htt is essential for the development and/or the maintenance of the CNS.

Subsequently, it was demonstrated that inactivation of *Htt* expression in Wnt1-expressing cell lineages caused congenital hydrocephalus in $Htt^{flox/-}$; *Wnt1-Cre* mice [22]. The hydrocephalus was due to increased cerebral spinal fluid (CSF) production by the choroid plexus, stenosis of the sylvian aqueduct, and a 40% reduction in the size of the subcommissural organ. The Wnt1 cell lineage also gives rise to the ciliated ependymal cells lining the walls of the ventricles [22]. Loss of *Htt* expression in these cells perturbs the cytoplasmic apical distribution of pericentriolar material-1 protein (PCM1), a major component of the PCM located around the centrioles anchoring the motile cilia on the ependymal cells, leading to the absence or shortening of their cilia [27]. Thus, the hydrocephalus observed in the $Htt^{flox/-}$; *Wnt1-Cre* mice is likely due to both abnormal CSF production and defects in cilia function. These results suggested for the first time that Htt contributes to the regulation of CSF homeostasis in the developing CNS.

Htt's role in neurogenesis was revealed by studying $Htt^{flox/-}$; *Nestin-Cre* embryos (the *Nestin* promoter drives Cre expression in neural progenitors and results in wide-spread recombination-sensitive reporter gene expression in the CNS by E10.5). At E14.5, a larger proportion of the $Htt^{flox/-}$; *Nestin-Cre* neuronal progenitors divide with smaller angles in their mitotic cleavage plane compared to controls [28]. This phenotype is recapitulated in embryos

that have their *Htt* expression knocked-down by *in utero* electroporation of anti-*Htt* siRNA constructs, and suggests that loss of *Htt* expression affects neurogenesis by decreasing the numbers of cycling progenitors, and by increasing neuronal differentiation [28]. Htt regulates mitotic cleavage plane orientation by associating with the centrosomes in cells undergoing division. Knock-down of *Htt* expression results in the aberrant localization of $p150^{Glued}$, dynein, and the nuclear mitotic apparatus protein (NuMA, required for mitotic spindle pole assembly and maintenance). This Htt function is highly conserved, as loss of HTT expression in *Drosophila* neuroblasts also perturbs mitotic spindle orientation, and the expression of *Drosophila* HTT in immortalized mouse striatal progenitors lacking *Htt* expression rescues mitotic spindle orientation [28]. In *Xenopus*, loss of *htt* expression results in the failure of the mandibular branch of the trigeminal nerve to develop, a phenotype that may be related to htt's functions in establishing cell polarity [29]. In zebrafish, Htt contributes to both the formation of the most anterior region of the neural plate that will eventually become the telencephalon and pre-placodal tissue, and to the subsequent formation of the peripheral sensory nervous system [30].

To understand Htt's functions in newly born postmitotic neurons, Barnat et al. crossed $Htt^{flox/flox}$ mice with a *NEX-Cre* line (recombining Htt^{flox} at ~E11.5 in postmitotic neurons) and observed a cortical lamination defect in the upper layer of the developing cortex containing the late-born neurons. To study this phenotype further, they electroporated E14.5 $Htt^{flox/flox}$ embryos *in utero* with either a *NeuroD-Cre* construct expressed only in newly born post-mitotic neurons or with a tamoxifen-inducible CRE-ERT2 construct activated at E18.5, a time when the newly born cortical projection neurons have finished their migration [31]. Barnat et al. found that Htt is required for cortical neuron migration, as the laminar organization defect was only observed when *Htt* expression was lost before but not after the cortical neurons finished their migration. This phenotype is caused by the dysregulation of N-cadherin trafficking due to the loss of HTT-dependent activation of Rab11, affecting both neuronal polarization and migration in the developing cortex. Barnat et al. also observed a decrease in dendritic length and branching in those neurons lacking *Htt* expression in comparison to controls at P21 with all three *Cre* lines/constructs, indicating that Htt may have additional functions in neurons after their migration.

Htt^{flox} mice have also been used to investigate the role of Htt in synaptogenesis. Conditional knockout of *Htt* expression in the developing cortex using *Emx1-Cre* (expressing Cre in cortical progenitors and post-mitotic neurons) leads to the excess formation of intracortical and corticostriatal excitatory synapses, but without changes in the number of thalamostriatal excitatory synapses at P21, a time when synapse formation is over in the cortex but before synapse maturation and pruning occurs [32]. By 5 weeks of age, the increase in intracortical synapses disappears, but the synapses appeared to be immature compared to the controls. However, in the 5-week-old conditional knock-out striatum, the number of both corticostriatal and thalamostriatal synapses are significantly increased in comparison to the controls. The medium spiny neurons in the striatum exhibited accelerated maturation of their dendritic spines and enhanced synaptic activity [32]. Thus, in addition to its effect on intracortical synapse formation, loss of *Htt* expression in the cortex appears to have an indirect effect on corticostriatal and thalamostriatal synapse formation, with the caveat that the $Gt(ROSA)26Sor^{tms(CAG-tdTomato)Fawa}$ reporter line used to monitor Cre-mediated recombination recapitulated the specificity of recombination in the Htt^{flox} allele. Nevertheless, these data suggest that Htt is an important regulator of excitatory synapse development in the CNS, in addition to its functions at earlier stages of CNS development.

REDUCTION OF HTT EXPRESSION ONLY DURING CNS DEVELOPMENT AFFECTS THE ADULT BRAIN

The data obtained from *Htt* conditional knockout mice generated with developmentally expressed *Cre* lines, however, cannot be used to distinguish between models requiring continuous *Htt* expression throughout life to support neuronal survival and maintain neuronal homeostasis, and models in which *Htt* expression is required only during important periods in neurodevelopment. In these latter models, the appearance of neurodegenerative phenotypes in the adult conditional knock-out mice are potentially the consequence of the increased susceptibility of a brain developed in the absence of *Htt* expression to age-related stress.

To test these models, Arteaga-Bracho et al. generated compound heterozygous $Htt^{neoQ20/-}$ mice carrying a *CAG-Cre/Esr1* transgene [33]. The

$Htt^{neoQ20/-}$ mice express ∼15% the normal levels of *Htt* due to the insertion of a floxed neomycin phosphotransferase gene (*neo*) within the *Htt* promoter [34]. By administering tamoxifen to the $Htt^{neoQ20/-}$; *CAG-Cre/Esr1* mice at postnatal days 21–26, Cre-mediated excision of the floxed *neo* cassette in the Htt^{neoQ20} hypomorphic allele restores *Htt* expression levels to 50% of wild type levels in the $Htt^{\Delta neoQ20/-}$; *CAG-Cre/Esr1* ($Htt^{d.\ hyp}$) mice (mice with 50% of the normal levels of *Htt* expression do not exhibit obvious developmental phenotypes [13, 15]) [33]. At 6 months of age, the $Htt^{d.\ hyp}$ mice exhibited handling-induced seizures, an abnormal clasping reflex, and motor coordination deficits. By 12 months of age, cortical and striatal neuronal degeneration and white matter tract abnormalities were observed in these mice.

The phenotypes exhibited by the $Htt^{d.\ hyp}$ mice are consistent with the hypothesis that Htt is required during the development of the CNS, and that restoration of Htt levels after this period cannot compensate for its earlier loss. However, whether Htt also has important functions in the adult CNS was not addressed.

ELIMINATING HTT EXPRESSION IN THE ADULT MOUSE

To investigate the consequences of eliminating *Htt* expression in the adult, Wang et al. used tamoxifen-inducible Cre drivers to conditionally knock-out *Htt* expression ubiquitously (using *CAG-Cre/Esr1*), and in the CNS (using *Nestin-Cre/Esr1*) at 2, 4, and 8 months of age in $Htt^{flox/flox}$ mice [25]. Ubiquitous knock-out of *Htt* had an age-dependent effect on lifespan: >95% of the mice with conditional knock-out of *Htt* at 2 months of age died within 10 days following loss of *Htt* expression compared to ∼30% when loss of *Htt* expression occurred at 4 months of age, and ∼5% when *Htt* expression was lost at 8 months of age [25]. For the surviving mice with *Htt* expression lost at 4 or 8 months of age, there were no significant differences between the knock-outs and controls in their motor coordination (accelerating rotarod performance) and in their body weights. There were also no significant differences in the expression of autophagy markers (LC3-II, p62/SQSTM1), and markers for inflammation or activation of cell death pathways (NFκB, FAT10, and Caspase-3) in the brain or in peripheral tissues. Adult CNS knock-outs, however, did not recapitulate the effect on lifespan that was observed in the ubiquitous conditional *Htt* knock-out mice. Fewer mice with the CNS knock-out of *Htt* expression at 2 months of age died (<18%), and all of the CNS Htt conditional knock-outs beginning at 4 and 8 months of age had normal lifespans. The CNS adult conditional knock-out mice were no different from controls in their body weight, forelimb grip strength, and motor coordination on the rotarod (characterized for up to 7 months following the inducible knock-out of *Htt* expression at each age). There were also no significant differences in NeuN, GFAP, p62/SQSTM1, LC3-II and Caspase-3 levels, and no obvious brain atrophy 90 days after the tamoxifen injections at 2 months of age. Wang et al. further used a *Camk2a-Cre/ERT2* transgenic line to eliminate *Htt* expression in the forebrain neurons of 2-month-old mice. They followed these mice for 4 months after tamoxifen injection, and observed no differences between the conditional knock-outs and controls in survival, body weight, rotarod performance, and grip strength.

The early death observed in the ubiquitous knock-out of *Htt* expression in 2-month-old mice is due to acute pancreatitis caused by the activation of digestive enzymes in pancreatic acinar cells [25]. In young mice, Htt interacts with Spink3 and the Htt-Spink3 complex inhibits trypsin activity. The association of Htt with Spink3 declines from 2 to 8 months of age, and thus, loss of *Htt* expression at 2 months of age has a more pronounced effect on aberrant trypsin activation in pancreatic acinar cells. Transgenic expression of a truncated HTT construct (*tHTT*) lacking N-terminal amino acids 1-208 is able to compensate functionally for lack of full-length HTT in both a cell model and in the *Htt* conditional knock-out mice, a result suggesting that the N-terminus is not required for Htt's association with Spink3 [25].

These results indicate that eliminating *Htt* expression in the adult brain is well-tolerated, at least for a period of time, and supports therapeutic strategies aimed at reducing HTT levels. However, further analyses are warranted, as the presence of mutant HTT could potentially affect the consequences of eliminating or reducing either normal or total HTT levels.

ELIMINATING OR REDUCING WILD TYPE OR TOTAL HTT EXPRESSION IN HD MOUSE MODELS

The requirement for normal levels of Htt expression during development was also evident in experiments characterizing hypomorphic $Htt^{neoQ20/neoQ111}$ mice expressing significantly reduced levels of both

wild type and mutant Htt [34]. Approximately 50% of the $Htt^{neoQ20/neoQ111}$ mice exhibited enlarged ventricles, a phenotype that is also observed in $Htt^{neoQ20/-}$ mice [34] and in *Htt* conditional knockout mice. The $Htt^{neoQ20/neoQ111}$ mice exhibited an additional phenotype consisting of an early progressive movement disorder that eventually led to their death with no detectable striatal neuropathology [34]. This phenotype was not observed in the $Htt^{neoQ20/-}$ mice, suggesting that low levels of mutant Htt in the $Htt^{neoQ20/neoQ111}$ mice could potentially exacerbate the effects of reduced total Htt levels.

To investigate the consequences of eliminating wild type *Htt* expression in an HD mouse model, Van Raamsdonk et al. generated YAC128 HD model mice in the $Htt^{-/-}$ background (YAC128–/– mice, [35]). In comparison to YAC128+/+ controls, YAC128–/– mice exhibited an earlier onset of rotarod deficits and a modest decrease in their striatal neuronal cross-sectional area at 12 months of age. In addition, absence of wild type Htt expression exacerbated testicular degeneration and a male-specific survival deficit in the YAC128 HD mouse model, with the caveat that the YAC128+/+ controls have an extra copy of the wild type *Htt* allele [35]. Nevertheless, these observations suggest that the absence or reduction of wild type *Htt* expression starting from conception can exacerbate the toxicity of mutant Htt. However, additional experiments are needed to determine if reducing wild type Htt levels only in the adult will affect HD mouse model phenotypes, as several recent studies suggest that wild type HTT may continue to have important functions in the adult brain.

HTT'S FUNCTIONS IN ADULT NEUROGENESIS

Neurogenesis in the adult rodent brain continues at a low level in the subgranular zone of the dentate gyrus, in the subventricular zone adjacent to the lateral ventricles, and in the hypothalamus (for a recent review, see [36]). In humans, it is debated whether adult neurogenesis also occurs in the striatum [37, 38]. To study HTT's non-cell autonomous functions in adult neurogenesis, Pla et al. injected $Htt^{flox/flox}$; $CaMKCreER^{T2}$ mice with tamoxifen to induce recombination of the Htt^{flox} alleles at 2 months of age, resulting in loss of *Htt* expression in mature neurons throughout the cortex and hippocampus but not in the hippocampal stem cells giving rise to the adult neuronal progenitors and newly born neurons [39]. At 6 months of age (4 months after tamoxifen injection), the $Htt^{\Delta flox/\Delta flox}$; $CaMKCreER^{T2}$ mice exhibited no obvious morphological phenotypes in the cortex and no apoptotic cell death. Two months later (6 months after the tamoxifen injections), a non-cell autonomous long-term survival deficit in newly born neurons was observed in the dentate gyrus 42 days after bromodeoxyuridine (BrdU) labeling. A defect in the dendritic arborization of these newly born neurons was also observed, suggesting that reduced neuronal survival may be related to this phenotype. The non-cell autonomous defects observed in the newly born neurons correlated with deficits in BDNF transport and release from the surrounding mature neurons that lacked *Htt* expression, and with subsequent deficits in TrkB signaling within the newly born neurons [39].

Although $Htt^{\Delta flox/\Delta flox}$; $CaMKCreER^{T2}$ mice did not have any deficits in motor coordination on the accelerating rotarod, they did exhibit increased anxiety in a novelty-suppressed feeding test, in an elevated plus maze, and in an open field test 6 months after tamoxifen injections [39].

In complementary experiments, $Htt^{S1181A/S1201A}$ and $Htt^{S1181D/S1201D}$ knock-in mice were generated to mimic either the absence or constitutive phosphorylation of two cyclin-dependent kinase 5 (Cdk5) phosphorylation sites in HTT that are important for regulating oxidative stress and mutant HTT-induced toxicity in neuronal cell cultures [40]. Blocking phosphorylation at HTT S1181 and S1201 reduced anxiety/depressive-like phenotypes in the mice and increased adult hippocampal neurogenesis by enhancing BDNF transport and delivery of hippocampal BDNF [40]. An issue to be aware of when interpreting these results is the possibility that the S1181A/S1201A and S1181D/S1201D mutations may not completely mimic the effect of the absence or presence, respectively, of phosphorylation at these sites on Htt function. Nevertheless, loss of HTT expression in adult cortical/hippocampal neurons or blocking phosphorylation at specific HTT amino acid residues has the potential to indirectly affect the proliferation and survival of newly born neurons in the hippocampus and cause anxiety-like symptoms.

HTT IN SYNAPTIC FUNCTION

Htt is found at the presynaptic terminal of excitatory synapses where it is proposed to regulate exo-/endocytosis of synaptic vesicles [41], and in

the postsynaptic terminals where it associates with the PSD95 scaffolding protein [42]. In a screen for Htt-interacting proteins at the presynaptic terminal, synaptosomes were purified from the cortex and striatum of adult mice and subjected to chemical cross-linking to stabilize potentially weak protein-protein interactions [41]. Both the Ahnak and Bassoon large scaffolding presynaptic cytomatrix proteins (components of the cytomatrix proteins at active zone, CAZ complex) were identified as Htt interactors by mass-spectrometry, and their interactions with Htt were subsequently validated by co-immunoprecipitation. Bassoon was also identified in another unbiased proteomic analysis of Htt interactions in the brain [43], and Piccolo was identified as a Htt partner in an additional proteomic study [44]. Although no functional validation assays for the interaction of Htt with these presynaptic proteins were performed, these results suggest that Htt may be involved in regulating neurotransmitter release in the adult brain.

The functions of HTT at the synapse are likely conserved. In *Aplysia*, its HTT ortholog (ApHTT) is expressed in both presynaptic and postsynaptic sensory neurons [8]. Serotonin is released during learning in *Aplysia*, and ApHTT mRNA levels are increased by repetitive serotonin application. Knockdown of ApHTT expression in either the presynaptic or postsynaptic neurons eliminates serotonin-induced long-term facilitation, but does not affect short-term facilitation [8]. These data suggest that ApHTT is required for long-term synaptic plasticity that is involved in the formation of long-term memory.

HTT IN STRESS RESPONSES

The results obtained from the characterization of the mouse and *Aplysia* models suggest that loss of *Htt* expression in the adult CNS, with the possible exceptions of adult neurogenesis and long-term synaptic plasticity, is either tolerated or can be functionally compensated for. However, there is an extensive body of work suggesting that HTT participates in many important cellular functions, including sensing and responding to stress that could contribute to maintaining CNS homeostasis.

HTT Participates In DNA damage repair

DNA damage promotes the Cdk5-dependent phosphorylation of HTT S1181 and S1201 both *in vitro* and *in vivo* [45]. In the presence of DNA damage, blocking HTT's phosphorylation at these two sites accelerates p53-dependent neuronal cell death [45]. Based on these observations, it was proposed that HTT participates in the DNA damage response pathway in neurons. A subsequent study demonstrated that blocking HTT phosphorylation at S1181 increases the amount of HTT associated with the microtubule fraction isolated from HeLa cells [40], and thus, it would be interesting in future work to determine if phosphorylation of HTT S1181 and S1201 also promotes HTT's entry into the nucleus. Interestingly, the *Htt* promoter contains multiple p53-responsive binding sites, and expression of *Htt* is regulated by p53 levels [46, 47]. γ-irradiation activates p53 and also increases *Htt* expression in the striatum and cortex [46]. In addition, the brain morphology of conditional knock-out mice lacking *Htt* expression in the developing brain and p53-deficient mice share common features, including enlargement of the lateral ventricles with cortical thinning and increased labeling of DNA nicks [48].

A connection between Htt and another DNA damage repair pathway component, the ataxia-telangiectasia mutated (ATM) protein was first suggested when the reduction of ATM expression was shown to rescue BACHD mouse model phenotypes [49]. Recently, it was demonstrated in a cell culture model that N17-phosphorylated HTT is found at sites of DNA damage in the nucleus, where it co-localizes with ATM to participate in the base excision repair pathway, and this co-localization is dependent on ATM kinase activity [50]. Furthermore, HTT also exhibits an oxidative stress-dependent interaction with other DNA repair proteins such as XRCC1, Flap structure-specific endonuclease 1 (FEN1), APE1, and high mobility group box 1 (HMGB1) [50].

HTT's function in cell stress responses

In addition to the DNA damage response, HTT was shown to participate in both the rapid cell stress and canonical cell stress responses [51–53]. By visualizing HTT in live cells undergoing heat shock, HTT was found to rapidly and reversibly accumulate on early endosomes, forming puncta in the cytoplasm called Huntingtin Stress Bodies (HSBs) [51]. The formation of HSBs correlates with the arrest of both early-to-late and early-to-recycling endosome fusion events. Endosomal trafficking is energy intensive, and the rapid halting of this process provides additional ATP for the cell. In the canonical stress response, HTT translocates from the ER to the nucleus, where it co-localizes with nuclear cofilin-actin rods [52].

The cofilin-actin rods function to transiently halt actin remodeling which, like HSB formation and the arrest of endosomal trafficking, can increase the available ATP pool in the stressed cell.

The HTT N17 domain is proposed to play a critical role in HTT's functions in the cell stress response. It was shown *in vitro* that stress-dependent phosphorylation of HTT's S13 and S16 amino acid residues within the N17 domain promotes its translocation into the nucleus, and its retention in the nucleus by preventing the N17 domain's interaction with the chromosome region maintenance protein 1 (CRM1) [54, 55]. Recently, it was demonstrated that HTT's N17 domain can also act as a sensor for reactive oxygen species (ROS) via sulphoxidation of HTT's methionine 8 (M8) amino acid residue [56]. These observations led to the development of a model in which oxidation of M8 alters the conformation of the N17 domain, triggering both release of HTT from the ER membrane, and enhancing the accessibility of HTT S13 and S16 to phosphorylation. Once phosphorylated, HTT translocates into the nucleus where it is retained in nuclear puncta associated with chromatin. When ROS stress is relieved, de-phosphorylation of S13 and S16 occurs, and HTT translocates back into the cytoplasm by a CRM1-dependent nuclear export mechanism [56]. Oxidative stress and ROS cause DNA damage, and the nuclear entry of N17-phosphorylated HTT allows it to participate with ATM in DNA base excision repair [50].

HTT's functions in selective macroautophagy

Under normal conditions, basal macroautophagy is efficient in the mouse brain, and steady-state levels of LC3-II (an autophagosomal marker) can be difficult to detect [57]. HTT is proposed to function in selective macroautophagy, a protective response to cellular stress [58–60], and thus, chronic or acute stress may be required to reveal HTT's functions in this process [53].

Selective macroautophagy involves the recognition of damaged cellular components (e.g. in the autophagy of protein aggregates, aggrephagy [61]; damaged membranes, lipophagy [62]; excess or damaged peroxisomes, pexophagy [63, 64]; and damaged or old mitochondria, mitophagy [65]) for degradation in the lysosome. Aggregated mutant HTT represents one such cargo that can be eliminated via selective macroautophagy by association of the mutant HTT aggregates with the autophagy adapter proteins ALFY, p62/SQSTM1 and potentially, TOLLIP [66, 67]. In the brains of constitutive conditional *Htt* knock-out mice, LC3-II and p62/SQSTM1 accumulate in the striatal 800xg Triton X100-insoluble pellet fraction beginning at 6 months of age, and both ubiquitin$^+$ and p62$^+$ puncta can be detected in the striatum of 24-month-old conditional knock-out mice [59]. HTT's functions in selective macroautophagy are conserved, as loss of *Drosophila* HTT expression impairs the ability of the mutant flies to mount a stress-activated selective macroautophagy response [59, 60]. Moreover, HTT's function in microtubule-based transport is also required for autophagy, as loss of either *Htt* or *Hap1* expression in primary mouse DRG neurons disrupts transport of autophagosomes to lysosomes leading to an accumulation of autophagosomes and defective cargo degradation [68].

However, the conditional knock-out of *Htt* expression in the adult brain at 2, 4, or 8 months of age did not result in the elevation of LC3-II and p62/SQSTM1 steady-state levels by western blotting or the accumulation of autophagosomes in the striatum 6-7 months following the loss of *Htt* expression. Potential explanations for the difference in autophagy phenotypes exhibited by the conditional knock-out of *Htt* expression during CNS development and conditional knock-out in the mature brain include the possibility that additional aging may be needed (>7 months) following loss of *Htt* expression before alterations in selective macroautophagy can be detected. Alternatively, loss of *Htt* expression during CNS development could sensitize neurons to age-related stress, which can then result in more robust selective macroautophagy phenotypes in older brains.

There is also accumulating evidence suggesting that there may be cross-talk between autophagy and DNA damage repair. Following DNA damage, p53 activation by ATM upregulates the expression of several autophagy genes and *HTT* [46, 47, 69]. Both ATM and p53 are required for DNA damage-induced autophagy [70], and ATM kinase activity can be activated by both mitochondrial and peroxisomal stress that, in turn, can induce pexophagy and, potentially, mitophagy [71, 72]. In addition, knock-out of *Beclin-1* expression (Beclin-1 is involved in both the initiation of autophagosome formation and the formation of autolysosomes [73]) and knock-out of the 200-kDa FAK family-interacting protein (FIP200) (also involved in autophagosome formation [74]), impairs DNA damage repair and decreases cell survival following γ-irradiation [75, 76]. Furthermore, p62/SQSTM1 contains both nuclear localization and nuclear export sequences and, like HTT, can shuttle in

and out of the nucleus [77]. Increased nuclear levels of p62/SQSTM1 resulting from autophagy inhibition can inhibit DNA damage-induced chromatin ubiquitination which, in turn, suppresses DNA damage repair activity [78]. Increased nuclear p62/SQSTM1 can also stimulate the proteosomal degradation of filamin A (FLNA) and RAD51, two proteins that function in the homologous recombination double strand break DNA repair pathway [79].

CONCLUSIONS

The discovery that ASOs targeting *HTT* expression can provide a safe and therapeutically effective "Huntingtin Holiday" for up to 3 months after a single dose of the ASOs was administered to HD model mice [23, 24], contributed to the development of a Phase I clinical trial sponsored by Ionis Pharmaceuticals and Roche using the IONIS-HTT_{Rx} ASO. The trial began in 2015, and is on track to finish by the end of 2017. Currently past the half-way point, the trial is going well with no issues reported so far (http://curehd.blogspot.com/2016/10/ionis-phase-i-huntingtons-disease-trial.html). If the Phase I trial is successful, a phase II trial testing efficacy is scheduled to begin in 2018. The recent observation that the conditional inactivation of *Htt* expression in the adult mouse brain has no obvious motor and neuropathological effects for up to 7 months after the induction of Cre recombinase expression provides additional confidence that HTT knock-down in the CNS can be safe.

Nevertheless, the need for additional preclinical experiments is justified based on the proposed functions for HTT in adult neurogenesis, long-term facilitation, cellular stress responses, selective macroautophagy, ROS sensing, and DNA damage repair. The consequences of disrupting some of these pathways may not be apparent until the adult conditional knock-out mice have survived for over 7 months with reduced *Htt* expression. In addition, although there are no apparent motor deficits in the adult conditional Htt knock-out mice at the ages examined, testing for other behavioral phenotypes (e.g. deficits in learning and memory, increased anxiety, etc.) has yet to be performed. Moreover, mouse models used in these studies were housed in reduced-stress conditions at constant temperature and humidity with unlimited food and water. Future experiments incorporating the application of acute and chronic stress to animal models with reduced levels or complete loss of *Htt* expression should be performed to study the effects of different stressors on the functions and homeostasis of the adult CNS when HTT is low or absent.

Additional experiments are also required to examine the effect of reducing normal Htt levels in adult HD mouse models, as mutant Htt can potentially act as a chronic cellular stressor, and lowering or eliminating normal *Htt* expression in HD models may have different outcomes compared to lowering normal Htt in the absence of mutant Htt.

Although mice have proven to be very useful in HD research, their lifespan, size, brain structure, and living environment differ enough from humans to warrant the use of larger animal models, including non-human primates, in adult loss of HTT function studies (for a review of large HD animal models see [80]). The longer lifespans exhibited by many of the larger HD animal models may be important, for example, in determining if HTT can protect the adult brain from environmental and cellular stress, a function that might not be apparent until enough damage has accumulated with age.

If HTT indeed has functions in the adult brain that are independent from its functions during neural development, it will also be important to determine the minimal amount of HTT that is required to perform its normal functions in adults, as ASO administration usually results in a 60% to 75% reduction in total HTT levels. The results of such studies will help to inform the field about the impact of stress and reduced HTT levels on the effects of HTT knock-down therapeutic strategies, and determine if there is any advantage in promoting reduced stress during a "Huntingtin Holiday".

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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