DNAJB6, a Key Factor in Neuronal Sensitivity to Amyloidogenesis

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DNAJB6, a key factor in neuronal sensitivity to amyloidogenesis

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Abstract:
CAG-repeat expansions in at least 8 different genes cause neurodegeneration. The length of the extended polyglutamine stretches in the corresponding proteins is proportionally related to their aggregation propensity. Although these proteins are ubiquitously expressed, they predominantly cause toxicity to neurons. To understand this neuronal hypersensitivity, we generated iPSC-lines of Spinocerebellar Ataxia-3 and Huntington disease patients. iPSC generation and neuronal differentiation is unaffected by the polyglutamine proteins and show no spontaneous aggregate formation. However, upon glutamate treatment, aggregates form in neurons but not in patient-derived neural progenitors. During differentiation, the chaperone network is drastically rewired, including loss of expression of the anti-amyloidogenic chaperone DNAJB6. Upregulation of DNAJB6 in neurons antagonizes glutamate-induced aggregation, whilst knockdown of DNAJB6 in progenitors results in spontaneous polyglutamine aggregation. Loss of DNAJB6 expression upon differentiation is confirmed in vivo, explaining why stem cells are intrinsically protected against amyloidogenesis, and why protein aggregates are dominantly present in neurons.

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DNAJB6, a key factor in neuronal sensitivity to amyloidogenesis.

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HIGHLIGHTS

Chaperone networks rewires during differentiation
Endogenous expanded Ataxin-3 aggregates in patient-derived neurons but not progenitors
DNAJB6 is critical for polyglutamine protein aggregation in patient-derived cells

SUMMARY

CAG-repeat expansions in at least 8 different genes cause neurodegeneration. The length of the extended polyglutamine stretches in the corresponding proteins is proportionally related to their aggregation propensity. Although these proteins are ubiquitously expressed, they predominantly cause toxicity to neurons. To understand this neuronal hypersensitivity, we generated iPSC-lines of Spinocerebellar Ataxia-3 and Huntington disease patients. iPSC generation and neuronal differentiation is unaffected by the polyglutamine proteins and show no spontaneous aggregate formation. However, upon glutamate treatment, aggregates form in neurons but not in patient-derived neural progenitors. During differentiation, the chaperone network is drastically rewired, including loss of expression of the anti-amyloidogenic chaperone DNAJB6. Upregulation of DNAJB6 in neurons antagonizes glutamate-induced aggregation, whilst knockdown of DNAJB6 in progenitors results in spontaneous polyglutamine aggregation. Loss of DNAJB6 expression upon differentiation is confirmed in vivo, explaining why stem cells are intrinsically protected against amyloidogenesis, and why protein aggregates are dominantly present in neurons.
Differentiation modulates protein homeostasis network (Chaperone Rewiring)
INTRODUCTION

CAG-repeat expansions in at least 8 different genes, including the huntington gene and the ataxin-3 gene, cause neurodegenerative disorders, the onset depending on the length of the repeat expansion (Di Prospero and Fischbeck, 2005). The corresponding proteins have extended polyglutamine stretches, the length of which is also proportionally related to their aggregation propensity (Morley et al., 2002). Whereas the toxicity of the resulting protein aggregates or inclusions is heavily debated (Kampinga and Bergink, 2016), several lines of evidence have revealed that the aggregation process is the driving force initiating disease (Hipp et al., 2019, Kampinga and Bergink, 2016, Labbadia and Morimoto, 2013). Yet, much of the arguments of aggregate-related toxicity is based on model systems in which fragments of the polyQ proteins with large expansions or polyQ-fusion proteins without any of the endogenously flanking sequences were ectopically expressed to high levels in non-neuronal cells. Moreover, although most polyQ proteins are ubiquitously expressed, neurons seem to be selectively sensitive to polyQ aggregation and degeneration, for which no clear explanation has been provided yet. This is remarkable as also most non-polyQ related, amyloid diseases generally affects tissues with low (stem cell related) regenerative potential, including-besides neurons- skeletal muscle, the heart, and the kidney (Chiti and Dobson, 2006).

Stem cell resistance to protein aggregation has been suggested to be associated with extremely efficient protein degradation capacity including highly active proteasomes (Leeman et al., 2018, Vilchez et al., 2012) and elevated lysosomal activity (Leeman et al., 2018). In addition, in proliferating tissues, protein damage that escaped these efficient PQC systems in stem cells, can be disposed through asymmetric segregation leading to rejuvenation of the stem cells (Aguilaniu et al., 2003, Bufalino et al., 2013, Rujano et al., 2006). Whilst these features may explain the resistance of regenerative tissues to protein aggregation diseases, it remains unclear why differentiated cells, in particular neurons, are so hypersensitive to these aggregation processes.

Besides proteasomal and lysosomal activity, molecular chaperones have been long known for their ability to protect cells from toxic protein aggregation (Kampinga and Bergink., 2016, Sakahira et al., 2002, Voisine et al., 2010). For polyQ proteins, in particular the Hsp70 co-chaperones of the DNAJ family have been shown to protect aggregation of ectopically expressed polyQ proteins in a multitude of model systems (Kakkar et al., 2013, Zarouchlioti et al., 2018). This includes overexpression of DNAJB1 in cells (Bailey et al., 2002, Kobayashi et al., 2000, Kuo et al., 2013, Rujano et al., 2007), DNAJB2 in cells (Howarth et al., 2007) and in mice (Labbadia et al., 2012), and DNAJB6 in cells (Hageman et al., 2010, Kakkar et al., 2016), Xenopus (Hageman et al., 2010), Drosophila (Bason et al., 2019) and mouse (Kakkar et al., 2016) models. In addition, the type II chaperonines (CCT) have been shown to reduce polyQ aggregation in cells (Behrends et al., 2006, Shahmoradian et al., 2013, Tam et al., 2006) and in C. elegans (Nollen et al., 2004). If and how expression of any of these chaperones may be related to intrinsic neuronal hypersensitivity, and vice versa to intrinsic stem cells resistance to polyQ aggregation is unknown. Also, whether these chaperones actually are relevant to the aggregation propensity of full length, endogenously expressed polyQ proteins has remained elusive so far.
Here, we utilized patient-derived iPSC-lines to compare polyQ aggregate formation upon neuronal differentiation. Whilst no spontaneous aggregate formation of the endogenous full-length polyQ proteins is observed, we could induce aggregates upon glutamate treatment in neurons, but not in the neural progenitors derived from the same patients. We show that a drastic reorganization of the chaperone network occurs during differentiation, including an almost complete loss of expression of the anti-amyloidogenic chaperone DNAJB6 in neurons. Re-expression of DNAJB6 in neurons antagonizes glutamate-induced aggregation. Inversely, knockdown of DNAJB6 in neural progenitors resulted in spontaneous aggregation of the endogenously expressed polyQ proteins. Our data demonstrate that DNAJB6 levels are a crucial factor in determining sensitivity to poly-Q-related amyloidosis.

**RESULTS**

**Differentiation of human-derived iPSC to neurons is unaffected by endogenous expression of ataxin-3 with poly-Q expansions**

To study aggregation of endogenous, full-length proteins with expanded polyglutamine stretches (ataxin-3polyQ), we generated induced pluripotent stem cell (iPSC) lines from healthy controls and three patients with CAG expansions in the ataxin-3 gene, causing the autosomal dominant Spinocerebellar Ataxia-type 3 (SCA3) (Figure 1A; Figure S1A). Fibroblasts (Zijlstra et al., 2010) as well as their derived iPSCs (Figure 1B) express both wildtype and mutant alleles at equal protein levels, confirming the notion that ataxin-3 is ubiquitously expressed (Ichikawa et al., 2001). In neither the fibroblasts nor in the iPSCs, protein aggregates of ataxin-3 could be detected (not shown). All iPSC lines, irrespective of mutant ataxin-3 expression, exhibited a morphology indistinguishable from human embryonic stem cells (Figure S1B,C) and all could be maintained indefinitely, as shown before (Okita et al., 2011). Pluripotency markers such as OCT-4, SOX-2, SSEA-4, TRA-1-60, and TRA-2-54 were similarly expressed in control and SCA3 patient-derived lines (Figure S1C). Control and SCA3 patient-derived lines were also equally able to differentiate into various germ layers in-vitro (Figure S1D). Since reprogramming somatic cells to iPSCs may induce genomic alterations (Mattis et al., 2012), we generated three clones from each control and SCA3 patient fibroblasts and performed whole-genome SNP sequencing to investigate possible copy number variation (CNV). We observed CNVs in one control and one SCA3 patient line (Figure S1E); these lines were discarded. Diploid control and SCA3 iPSC lines were next differentiated into columnar epithelial cells expressing PAX6 (neural rosettes) (Figure 1C), representing neural tube cells (Figure S2A). Neural rosettes were handpicked and cultured in the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) as spheres and maintained as neural stem cells (NSCs) (Figure 1C). The iPSC-derived NSCs express various multipotency markers, such as the SOX-2 transcription factor, Nestin, and Vimentin (Figure S2B,C). For NSC lines derived from iPSCs of patients with Huntington disease, the CAG-repeat length was found to be increased by up to 10% after multiple passages (Mattis et al., 2012), but under the culture conditions used here, no somatic instability was found in the SCA3-derived lines (Figure S1A).

The expression of ataxin-3polyQ had no effect on the differentiation from iPSCs towards NSCs and neurons, consistent with earlier findings for SCA3-derived iPSCs (Koch et al., 2011) or iPSCs...
derived from patients with Huntington’s disease (HD) (Conforti et al., 2018, Mattis et al., 2012). The iPSC-derived SCA3 neurons show a morphology that is indistinguishable from those derived from controls (Figure 1D) and showed no altered expression of various neuronal markers such as MAP-2 and βIII-tubulin (Figure 1D, Figure S2C). Whole-cell patch-clamp recordings of the control and SCA3 patient-derived neurons after 120 days of differentiation all showed a repetitive firing pattern upon application of depolarizing current (Figure 1E), indicating that the ataxin-3polyQ expression as such has no effect on the basic electrophysiological properties of the neurons.

Directed differentiation of control and SCA3 patient-derived NSCs towards neurons was found to result in a mixed population of glial restricted progenitors and different neuronal subtypes including glutamatergic, GABA-ergic, cholinergic motor neuronal populations with a variable amount of astrocytic contamination (data not shown). To be able to study intrinsic aggregation sensitivity of a controlled, pure neuronal population, we therefore next purified the neurons by a multistep fluorescence-activated cell sorting procedure (Yuan et al., 2011) using the cell-surface markers CD184+ and CD44+ as a signature for NSCs, CD184+/CD44+/CD24- for glia cells, and CD184+/CD44+/CD24- for neurons (Figure 1F and Figure S3). Post-sorting analysis of this neuronal population with cell surface markers showed that 60-80% of the cells expressed CD184+/CD44+/CD24- and this percentage was the same in all lines used (Figure 1F). After sorting, neurons (CD184+/CD44+/CD24-) were plated and cultured for an additional 30 days to allow axonal growth and maturation in the presence of the growth factors BDNF and GDNF (Figure 1A,G) and these were used in the experiments described below.

**Glutamate induces ataxin-3polyQ aggregation in neurons but not stem cells**

To study ataxin-3polyQ aggregation, we fractionated iPSCs, NSCs and the purified neurons into Triton-soluble (TX-100), SDS-soluble (SDS) and SDS-insoluble (formic acid (FA) solubilized) fractions (Figure 2A,B). In none of the different cell populations SDS-insoluble material was detected (Figure 2C), implying that, under the culture conditions used, no spontaneous aggregation occurs. This is consistent with literature data suggesting that the full-length ataxin-3polyQ proteins are not or only moderately aggregation-prone and that secondary events including alterations in protein homeostasis (Balch et al., 2008) or (external) protease-activating triggering events may be required (Kuiper et al., 2017, Kampinga and Bergink, 2016). Based on protocols previously described (Koch et al., 2011), we therefore exposed the iPSCs, NSCs and neurons to the excitatory neurotransmitter L-glutamate. When treating neurons with 0.1mM glutamate, a fraction of the expanded ataxin-3, but not the normal ataxin-3, was found in the SDS-insoluble (FA fraction) of all 3 SCA3-derived neuronal populations (Figure 2C). Immunostaining of L-glutamate-stimulated SCA3-derived neurons with ataxin-3 and 1C2 antibodies (that recognizes polyQ aggregates) confirms the presence of aggregates in SCA3-derived neurons (Figure 2D, Figure S4A). Immunostaining of neurons from controls never showed aggregation (Figure S4A). Interestingly, however, the glutamate treatment did not result in aggregation of ataxin-3polyQ in the iPSCs (data not shown) or in NSCs (Figure 2C) even though the NSCs express functional glutamate receptors as revealed by calcium imaging (Figure S4B). Prolonged glutamate treatment can induce neural progenitor
proliferation (Brazel et al., 2005) which might have confounded our aggregation determination by a dilution effect. However, the short treatment used here had no significant effect of cell proliferation as detected by flow cytometric analyses (Figure S4C). Together, these data suggest that, beyond using asymmetric segregation to deposit polyQ protein aggregates to differentiated daughter cells for rejuvenation (Rujano et al., 2006), stem cells are also intrinsically resistant to formation of polyQ protein aggregates.

Rewiring of the chaperone network during differentiation

Over the last decade, several potential modifiers for SCA3 aggregation have been identified using cell- and animal models and polyQ-containing polypeptide fragments. Many of these modifiers are components of the cellular protein quality control (PQC) system such as heat shock protein (HSP) expression, proteasomal activity or autophagosomal activity (Jimenez-Sanchez et al., 2012, Kakkar et al., 2014, Sakahira et al., 2002, Soares et al., 2019) Here, we addressed whether differential PQC could be a factor related to the neuronal hypersensitivity to polyQ aggregation. Disturbances in protein homeostasis (e.g. as induced upon heat shock) are known to activate a transcriptional cascade known as the heat shock response (HSR), which is under control of a conserved transcription factor (Heat Shock Factor-1) (Åkerfelt et al., 2010). This response increases the levels of various members within different classes of HSP families, of which several members have also been found be present in polyQ inclusions in post-mortem brain tissues (Kim et al., 2002). To test whether differential ability to activate the HSR upon the expression of the mutant ataxin-3polyQ could underlay neuronal hypersensitivity, we measured different HSP protein family members in the various cell populations before and after glutamate treatment. Strikingly, the strictly HSF-1-regulated HSPs, including HSPA1A or HSPA6 (Hageman and Kampinga, 2009), that are not expressed in non-stressed cells, are also not expressed in the different SCA3-derived cell populations, not even when aggregation is induced in SCA3-derived neurons upon treatment with glutamate (Figure 3A). Also, the HSF-1-regulated DNAJB1 (Hsp40) was not upregulated by glutamate in any of the SCA3-derived cell populations, whilst the levels HSPB1 (Hsp27) showed up- as well as down fluctuations depending on the line investigated (Figure 3B). This implies that neither the expression nor the aggregation of endogenously expressed polyQ proteins is sensed as a disturbance in the intracellular protein homeostasis large enough to activate the HSR. This is consistent with earlier suggestions from experimental models with polyQ fragments (Hageman et al., 2010, Hipp et al., 2014) or from analyses in post-mortem brain samples of SCA3 (Seidel et al., 2010) and HD (Seidel et al., 2016) patients.

However, we noticed a number of striking changes in chaperone expression upon differentiation of IPSCs to NCSs and NCSs to neurons, irrespective of ataxin-3polyQ expression. Remarkably, the expression of HSR-regulated DNAJB1 declines upon differentiation from NSCs to neurons, whereas HSPB1 shows an increased expression in neurons (Figure 3B,E). Particularly, expression of two known strong suppressors of polyQ aggregation, DNAJB6 (Kakkar et al., 2016, Månsson et al., 2014a) (Figure 3A,E) and TCP (Figure 3B,E) (Behrends et al., 2006, Shahmoradian et al., 2013, Tam et al., 2006, Vonk
et al. (co-submitted) decline upon differentiation towards neurons. To substantiate the generality of these observations, we also analyzed the expression of these chaperones in a series of iPSC lines derived from controls and HD-patients (Figure S5A,B), from which neurons were generated by two different differentiation protocols (Camnasio et al., 2012, Hu and Zhang, 2009) and that were different from the protocol that we used for SCA3-derived iPSCs. Irrespective of these different differentiation methods, we noted similar changes in chaperone expression (Figure 3C-E) in both control and HD material, the most prominent and consistent ones being the DNAJB6 and TCP-1 down-regulation during differentiation towards neurons (Figure 3C-E, Figure S5C,D). Finally, and consistent with all of the above, database analysis of ribosome profiles of differentiating human ES towards neural crest cells (Werner et al., 2015) revealed a decline in DNAJB6 and all CCT subunits upon differentiation (Figure S6).

**DNAJB6 expression in situ: high in progenitors, low in differentiated cells**

To determine whether DNAJB6 expression levels are generally high in progenitor/stem cells and downregulated during differentiation under more physiologically relevant conditions, we first generated three-dimensional organoids from iPSCs (Lancaster et al., 2013). Immunological staining with the neuronal progenitor marker Sox-2 during organoid growth showed a clear overlap with DNAJB6 expression (Figure 4A: top panel), whereas NeuN-positive neurons were indeed negative for DNAJB6 (Figure 4A: lower panel).

Subsequently, we analyzed DNAJB6 expression in the subventricular zone (SVZ) of adult mice in situ. In contrast to most adult brain areas, the SVZ contains a relatively large population of neural stem/precursor cells (NSCs) located in the walls of the lateral brain ventricles, from which numbers of neuroblasts are produced that migrate into the olfactory bulbs where they differentiate into local circuit interneurons (Alvarez-Buylla and Lim, 2004) (Figure 4B). In the Sox-2 positive progenitor cells within this SZV, DNAJB6 expression is indeed high; inversely, in the NeuN-positive neurons in the cortex of the brain, DNAJB6 expression is nearly absent (Figure 4B), confirming that also in vivo DNAJB6 levels are high in neuronal progenitors and low in differentiated neurons. This is consistent with RNA sequencing data from the BrainSpan consortium (www.brainspan.org) obtained from over 250 samples of prenatal (high percentage of stem cells) and postnatal brains (low percentage of stem cells). Here also, a re-wiring of the chaperone network can be seen with both DNAJB6 and various TCP components of CCT being expressed at higher levels in prenatal than postnatal brain tissue (Figure S7).

To established whether this decline in DNAJB6 expression is a more general feature of cellular differentiation, we turned to intestinal tissue where the stem cell compartment and differentiated cells can be easily distinguished on the basis of their position within the crypts (Clevers, 2013) (Figure S8: left panel). Immunohistochemical analyses clearly showed the highest level of DNAJB6 expression at the basis of the crypts, where the stem cells reside, with differentiated cells showing much lower levels of expression (Figure S8). Interestingly, this corroborates that differentiated cells, but not stem cells,
within the crypts of SCA3 patients were previously found to be positive for ataxin-3polyQ aggregates (Rujano et al., 2006). So, under all these conditions, DNAJB6 expression declines upon stem cell differentiation.

**DNAJB6 expression levels are crucial for sensitivity to amyloid formation**

We have previously identified DNAJB6 as a highly potent anti-amyloidogenic protein in *vitro* (Månsson et al., 2014, Månsson et al., 2014b) and showed that DNAJB6 overexpression in cells, neurons and animal models reduces aggregation of polyQ-containing polypeptide fragment and delayed disease onset (Bason et al., 2019, Gillis et al., 2013, Hageman et al., 2010, Kakkar et al., 2016). To further investigate whether DNAJB6 is indeed a key factor in sensitivity to polyQ aggregation sensitivity, we generated DNAJB6-knockout HEK293 cells using CRISPR/Cas9 technology (HEK293DNAJB6 k/o: Figure 5A). Expression of a fragment of the huntingtin protein with 71 glutamines (GFP-HttQ71) (Figure 5B) in these cells results in low levels of aggregation in HEK293wt as detected by the presence of high molecular weight material (Figure 5B), a filter trap assay (Figure 5C) and by the appearance of GFP-puncta in immunofluorescence (Figure 5D,E). Strikingly, the amount of aggregates increases by a factor of 3 in the HEK293DNAJB6 k/o cells (Figure 5B-E) showing that endogenous levels of DNAJB6 are crucial for the ability of cells to suppress GFP-HttQ71 aggregation. Importantly, re-expression of DNAJB6b in the HEK293DNAJB6 k/o cells fully antagonizes polyQ aggregation (Figure 5B-E). Qualitatively similar data have been observed in DNAJB6-knockout U2OS cells (Figure S9A-C).

Recently, several mutations were identified in the gene that encodes DNAJB6 as the cause of limb-girdle muscular dystrophy type 1D (LGMD1D), a dominant late-onset muscle disease (Couthouis et al., 2014, Harms et al., 2012, Sarparanta et al., 2012, Sato et al., 2013). The disease pathology is characterized by large rimmed vacuoles and cytoplasmic protein aggregates in muscle cells, including DNAJB6 itself (Harms et al., 2012, Sandell et al., 2016). All of the LGMD1D-related mutations reside in the G/F-rich region of DNAJB6. Most of these are point mutations that lead to a substitution of one of the (usually Phe) residues (F89I, F91I/L, F93I/L, P96R/L, F100V). This G/F-rich region, which is found in all DNAJAs and DNAJBs (Kampinga and Craig, 2010), is a structurally disordered flexible region (Pellecchia et al., 1996). Although the function of the G/F-rich region has not been clearly determined yet, it has been suggested to be critical for activity of certain DNAJs in yeast (Yan and Craig, 2015), tentatively by playing a role in substrate recruitment or transfer to HSP70s (Perales-Calvo et al., 2010, Stein et al., 2014, Wall et al., 1995). Expression of three of these mutants together in HEK293 cells revealed that these are not instable (Figure 5F) and revealed that they only had a minor loss of function when analyzed for their ability to suppress the aggregation of a fragment of the huntingtin protein with 119 glutamines (GFP-HttQ119) (Figure 5G) consistent with earlier findings (Sarparanta et al., 2012). Given that the LGMD1D related DNAJB6 mutants are dominant, this implies that a minor drop in the total cellular amount of functional DNAJB6 alone suffices to cause a protein aggregation disease. This further accentuates the importance of functional DNAJB6 levels for the ability of cells to cope with amyloidogenic proteins.
To more directly test whether the drastic drop in DNAJB6 expression in neurons is indeed related to their hypersensitivity towards aggregation of full-length endogenous ataxin-3polyQ aggregation, we first downregulated its expression in NSCs that initially showed high DNAJB6 expression and resistance to ataxin-3polyQ aggregation (Figure 2). Remarkably, siRNA-mediated knockdown of DNAJB6 in NSCs derived from a SCA3 patient (Figure 6A) was found to result in the spontaneous formation of SCA3 aggregates (even without glutamate treatment) as detected by biochemical cell fractionations and microscopic analyses (Figure 6B). As cleavage of ataxin-3 has been suggested as a prerequisite for the initiation of aggregation (Koch et al., 2011, Weber et al., 2017), we wondered whether cleavage of the full-length ataxin-3 protein also played a role in its aggregation under DNAJB6 knockdown conditions. Indeed, upon DNAJB6 knockdown in NSCs cells, cleaved products by two independent ataxin-3 antibodies were detected (Figure S9D) similar to those seen after glutamate or calpain treatment (Koch et al., 2011, Weber et al., 2017). This suggests that cleavage is a key step in the initiation of ataxin-3polyQ aggregation. In addition, these data imply that there are constitutively active proteases, also under non-glutamate activated conditions, that can generate ataxin-3 derived polyQ peptide fragments. Normally these fragments can be chaperoned by DNAJB6, but they accumulate when DNAJB6 is absent and lead to aggregation. We also depleted DNAJB6 in iPSCs and NSCs derived from HD patients (Figure 6C, Figure S9E). Also, here this also resulted in aggregation of the full length polyQ huntingtin protein without the requirement of an external trigger (Figure 6D-F). Both data sets reveal that the relatively high expression levels of DNAJB6 in these NSCs normally suffices to prevent the initiation of aggregation of polyQ proteins, which is consistent with its key role in preventing primary nucleation in the formation of amyloids (Kakkar et al., 2016, Månsson et al., 2014b).

We next wondered whether such polyQ aggregation would affect the fitness of NSCs with polyQ aggregates. Since DNAJB6 levels interfere with neuronal development (Watson et al., 2009), we therefore instead infected control NSCs with either GFP-Q23, GFP-Q43 or GFP-Q71 constructs under a tet-inducible promoter (Figure S9G). It was found that after polyQ expression, NSCs proliferate less rapidly in a Q length-dependent manner (Figure 6G, Figure S9F). However, the NSCs were still able to differentiate (as evidenced by beta-tubulin III expression: Figure 6H) and some of the neurons derived from the GFP-Q43 or GFP-Q71 expressing NSCs (but not GFP-Q23 expressing NSCs) displayed visible inclusions (Figure S9H).

Finally, we addressed whether re-introduction of DNAJB6 would also be able to effectively protects neurons from glutamate-induced aggregation of the endogenously expressed full length ataxin-3 polyQ protein. Hereto, we virally transduced neurons from SCA3 patients with a tetracycline-inducible GFP-tagged DNAJB6 construct (Figure 6I). Using either the long and nuclear isoform DNAJB6a, that shows the most dramatic change upon differentiation (Figure 3A) or the short isoform DNAJB6b that is present in both the cytosol and nucleus (Hageman et al., 2010). Next, we induced DNAJB6 expression 24 or 48 hours prior to glutamate treatment (Figure 6I,J, Figure S9I) and fractionated the extracts. Strikingly, both DNAJB6a and DNAJB6b reduced ataxin-3polyQ aggregation in a concentration-dependent manner (Figure 6J, Figure S9I), consistent with the findings that ataxin-3polyQ aggregates in both the nucleus and cytosol of neurons (Hayashi et al., 2003, Paulson et al., 1997, Seidel et al., 2012).
Since aggregation occurs spontaneously in KO and knock down backgrounds, we argue that the low amount of DNAJB6b in neurons are sufficient to prevent aggregation under normal conditions but not after stimulation.

**DISCUSSION**

Our data as well as those reported by Vonk et al.\textsuperscript{co-submitted} reveal a striking re-wiring of the PQC system upon differentiation of iPSC cells to neurons. They illustrate the versatility of the protein quality control system to adapt to altered proteomes and underscore the importance of adjusting it to protein homeostasis. The strongly reduced expression of anti-amyloidogenic proteins in neurons, in particular of DNAJB6, is directly related to neuronal hypersensitivity to aggregation of polyQ proteins. As the re-wiring of the chaperone network occurs in a more general fashion during differentiation, it does not explain regional hypersensitivities of specific brain areas and specific neuronal subtypes to degeneration (Purkinje cells in cerebellum in SCA3, striatal neurons in HD). In fact, the loss of the anti-amyloidogenic DNAJB6 co-chaperone upon differentiation is not restricted to brain only, but also is seen in the gut. Yet, like in the brain, also in the gut low DNAJB6 levels associated with the presence of polyQ amyloids (Rujano et al., 2006).

**Protein quality control is high in stem cells**

Dividing (neuronal) stem- and progenitor cells are equipped with an extremely efficient chaperone network system (this report, Noormohammadi et al., 2016, Vonk et al.\textsuperscript{co-submitted}). Together with efficient protein degradation capacities (Koyuncu et al., 2018, Leeman et al., 2018, Vilchez et al., 2012) this provides these cells with an intrinsic resistance to imbalances in protein homeostasis that would otherwise endanger their ability to generate progeny. Consistently, it has been demonstrated that stemness in the SVZ is compromised in conditional DNAJB6 knockout mice (Watson et al., 2009). This intrinsic resistance, combined with the ability of stem cells to rejuvenate through asymmetric segregation of protein damage that escaped these efficient PQC systems (Aguilaniu et al., 2003, Bufalino et al., 2013, Ogrodnik et al., 2014, Rujano et al., 2006) may explain why tissues with high regenerative potential are mostly not affected by protein aggregation diseases. The relatively high expression of DNAJB6 in diverse stem/progenitor cell lineages, furthermore points to a central role of DNAJB6 for stem cell fitness. In line with this, DNAJB8, a functional homolog of DNAJB6, is also expressed in cancer stem cells and required for cancer stem cell survival and tumorigenicity (Nishizawa et al., 2012).

**DNAJB6 and hypersensitivity to amyloidogenesis**

Our data show that DNAJB6 expression is a key factor in the sensitivity to polyQ-mediated neurodegeneration. Remarkably, Poly-Q aggregation is easily triggered in neuronal cells with low...
DNAJB6 expression. In line, depletion of DNAJB6 in stem cells, leads to spontaneous polyQ aggregation. We previously demonstrated that DNAJB6 is able to efficiently inhibit the primary nucleation of fragments of polyQ polypeptides, thereby eliminating the formation of polyQ seeds that can initiate an amyloidiogenic cascade (Kakkar et al., 2016). Our data, for the first time, show that such aggregation-inducing polypeptides are being generated spontaneously from full length, endogenously expressed polyQ proteins and that these are eliminated when DNAJB6 levels are sufficiently high. Even in neurons, where DNAJB6 levels are low, these low levels can prevent such spontaneous aggregation. However, upon glutamate treatment, triggering fragmentation of the full-length protein (Koch et al., 2011), these low levels no longer suffice and aggregation is initiated. Re-expression of DNAJB6 into neurons prevents glutamine-triggered aggregation, which is the first demonstration of elevated chaperone expression to protect against aggregation of endogenously expressed, full-length polyQ protein. This is directly consistent with the data obtained with fragments of these proteins containing the polyQ stretch, where we found that DNAJB6 delays onset of aggregation in vitro (Månsson et al., 2014a, Kakkar et al., 2016) and in cellular (Gillis et al., 2013) and organismal models (Hageman et al., 2010, Bason et al., 2019, Kakkar et al., 2016). For DNAJB6 this can be explained because it directly interacts with the polyQ core (Månsson et al., 2014a) and next requires interaction with Hsp70 for the further processing of its bound substrates (Kakkar et al., 2016). In case of other polyQ suppressing chaperones, like CCTs (Tam et al., 2006), DNAJB1 (Kuo et al., 2013) or HSPB7 (Vos et al., 2010), regions flanking the polyQ regions have been shown to be relevant for their action and effects may differ depending on the use of different fragments or full-length proteins.

Our data raise the question why DNAJB6 levels are tuned down in neurons. First, they imply that there is no evolutionary selection against (late onset) amyloid diseases. Second, high levels of DNAJB6 may have antagonizing effect on normal neuronal functioning. One could speculate that neurons may strongly depend on the formation of so-called functional amyloids (including certain prions) required for, for example, transport of RNA-containing granules from the soma of neurons to axonal synapses (Fowler et al., 2007, Shorter and Lindquist, 2005). Indeed, DNAJB6 was recently found to also suppress the formation of prions by sup35NM (Reidy et al., 2016), which is crucial for the formation of liquid/gel droplets to promote survival of yeast during stress (Franzmann et al., 2018). High expression levels of potent anti-amyloidiogenic proteins such as DNAJB6 would negatively impede on these processes.

It is also interesting to note that, whilst the expression of most chaperones is unaffected or declines during differentiation, expression of several members of the group of small HSPs increases (Figure 3 and S8). Small HSPs are known to be very promiscuous “holdases” of many different mis- or unfolded clients (Haslbeck et al., 2005) and may as such act as reservoirs compensating for accumulated damage in cells with lower or altered PQC capacity without interfering with specific functions. In fact, small HSP function as such in the eye-lens, where they maintain protein solubility and hence eye-lens transparency (Slingsby et al., 2013). In addition, small HSPs are upregulated with aging (Walther et al., 2015) and inversely the upregulation of small HSP can increase organismal life span (Morrow et al., 2004, Vos et al., 2010).
In summary, differentiation is associated with a drastic re-wiring of the chaperonome with stem and progenitor cells showing resistance to and differentiated cells hypersensitivity to polyQ aggregation. In particular, DNAJB6 levels appear as a key factor in polyQ aggregation susceptibility. As DNAJB6 not only prevents the initiation of polyQ aggregation, but also was shown to prevent amyloidogenesis triggered by Aβ (Månsson et al., 2018) and α-synuclein (Aprile et al., 2017), our data imply that it likely is a more general and crucial determinant of neuronal hypersensitivity to amyloidosis. If so, this urges for strategies to (re)activate DNAJB6 in neurons as potential treatment of patients with amyloid diseases.
STAR METHODS

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Human subjects
  - Generation of iPSCs of SCA-3 patients with episomal vectors
  - HD-iPS cell lines
- METHOD DETAILS
  - Pluripotency assays for hiPSCs
  - Generation of iPSC-derived neural stem cells and neurons
  - Striatal differentiation
  - Pan-neuronal differentiation
  - Genome-wide SNP genotyping and Genomic CAG repeat length analysis
  - Lentiviral infection of iPSCs
  - Neural differentiation
  - Generation of iPSC-derived cerebral organoids
  - FACS analysis
  - Excitatory stimulation of neurons
  - Immunohistochemistry in brain and intestinal tissue
  - Generation of DNAJB6 knockout (KO) cells
  - DNAJB6 knockdown in NSCs
  - Lentivirus expression in NSCs and neurons
  - Polyglutamine aggregation assays
  - Filter trap
  - Western blotting
  - Immunocytochemistry
  - RT-PCR and qRT-PCR
  - Calcium imaging
  - Electrophysiology

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AUTHOR CONTRIBUTIONS

data and materials. A.T, S.B, E.H.W.G.B, S.C, and H.H.K. analysed the data and wrote the paper, with the other authors provided feedback and editorial comments on the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.


**LEGENDS TO THE FIGURES**

**Figure 1. Directed differentiation and functionality of the control and SCA3 iPSC-derived neurons in-vitro** (A) Schematic representation of the study set-up. (B) Western blot of ataxin-3 expression in SCA3 patient fibroblasts shows normal (bottom) and mutant (top) ataxin-3. (C) Bright field images of iPSC differentiation into embryoid bodies (8 days), neural rosettes (16 days with retinoic acid), and neurospheres cultured in proliferation medium with bFGF/EGF (24 days). Differentiated NSCs (30 days post differentiation) and neurons (90 days post differentiation). Scale bars: 50µm. (D) Differentiated control- and SCA3 iPSC-derived neurons and glial cells at 90 days post differentiation characterized for various markers: βIII-tubulin and MAP-2 (both for neurons) and GFAP (for astrocytes). Scale bars: 50 & 100µm. (E) Electrophysiological activity of control and SCA3 patient iPSC-derived neurons post 90 days. Data on the top show fast inward currents activated by depolarizing voltage steps (voltage clamp). Data on the bottom show repetitive action potentials following activation by 50ms depolarizing current pulses (n=4). (F) Percentage of enriched neuronal population obtained post-sorting based on expression of CD24+, CD44+ and CD184+ (Bars represent SEM, n=3). (G) Purified control and SCA3 patient iPSC-derived neurons based on cell surface marker (CD24+, CD44+ and CD184+) and cultured for 30 days after sorting for axonal regeneration. Scale bars: 50µm.

**Figure 2. Ataxin-3 aggregation in control and SCA3 patient iPSC-derived cells** (A,B) Schematic representation of the protocol for aggregation induction in NSCs (A) and neurons (B); NSCs or neurons were treated with 0.1 mM L-glutamate for 1 hour in total with 30 min interval or left untreated. (C) Subsequently whole cell lysates were fractionated by treatment with TX-100 to yield a TX-soluble fraction (TX-100) and an TX-insoluble pellet. This pellet was treated with SDS to yield an SDS-soluble fraction (SDS) and SDS-insoluble fraction that was dissolved in formic acid (FA). The fractions were run on SDS-PAGE and Western blots were probed with anti-ataxin antibody (*-expanded allele) (n=2). (D) 1C2/MAP2 double immunostaining on healthy control and SCA3 iPSC-derived neurons treated with L-glutamate (150 days post differentiation). Glutamate-induced ataxin-3 inclusions are indicated by arrows. Scale bars: 25µm.

**Figure 3. Heat shock protein expression levels in control and SCA3 patient-derived cells** (A) Representative Western blots of GAPDH and the indicated HSP family members (HSPA6, HSPA1A & DNAJB6) in control and SCA3 patient-derived iPSCs, NSCs and neurons on total fraction treated with L-glutamate and untreated (n=2, technical replicates from same iPSC clone). Heat Shock (30 min 45°C = HS) was used as positive control for stress induced Hsp activation. (B) Representative Western blots of GAPDH and the indicated HSP family members (HSPA8, DNAJB1, HSPB1 & TCP1α) in control and SCA3 patient-derived iPSCs, NSCs and neurons with total fraction treated with L-glutamate and untreated (n=2, technical replicates from same iPSC clone). (C) Representative Western blots of GAPDH and the indicated HSP family members (HSPA6, HSPA8, DNAJB1, & DNAJB6) in control and Huntington patient-derived striatal neurons during various stages of differentiation time points (day0, 7, 13 & 31) on total fraction (n=2, technical replicates from same iPSC clone). (D) Representative Western blots of GAPDH and the indicated HSP family members (HSPB1, HSPA1A & TCP1α) in control and Huntington patient-derived striatal neurons during various stages of differentiation time points (day0, 7,
13 & 31) on total fraction (n=2, technical replicates from same iPSC clone). (E) Quantification of indicated chaperone protein levels in control (circles), HD (triangles) and SCA3 (squares) donor-derived iPSCs, NSCs and neurons. For each cell line, protein levels in NSCs and neurons are plotted relative to those in iPSCs. Note that the DNAJB6 antibody used recognizes two bands, corresponding to the long DNAJB6a (40 kDa) and short DNAJB6b (26 kDa) isoforms.

**Figure 4. DNAJB6 expression levels in cerebral organoids and in the subventricular zone of adult mice**  (A) Derivation of cerebral organoids from control iPS cells (120 days post differentiation), DNAJB6/SOX2 and DNAJB6/NeuN double immunostaining on healthy control cerebral organoids after 120 days of differentiation. Scale bars: 200 and 25µm. (B) Schematic representation of adult mouse subventricular zone for neural stem cell niche. DNAJB6/ SOX2 double immunostaining in the subventricular zone (SVZ) for neural stem cell niche and DNAJB6/NeuN immunostaining in cortex for matured neuron. Scale bars: 25µm.

**Figure 5. DNAJB6-knockout leads to hypersensitivity to polyglutamine aggregation**  (A) Validation of DNAJB6-knockout cell line. Western blot of HEK293T wild-type (WT) and DNAJB6-knockout (KO) cells, transfected with either empty vector (FRT-TO) or with V5-tagged DNAJB6b. (B) Representative image of a polyglutamine aggregation time-course in HEK293T WT and DNAJB6-KO cells. Cells from both genotypes were transfected with GFP-Htt-Q71 alone or in combination with V5-DNAJB6b and collected after 36, 48 or 60 hours. The GFP material accumulated in the stacking portion of the gel corresponds to the amount of Htt-Q71 aggregation. Western blots for the indicated antibodies are shown. (C) Representative image of a filter trap assay in HEK293T WT and DNAJB6-KO cells. Both cell lines were transfected with a GFP-tagged exon 1 fragment of huntingtin with 71 glutamines (GFP-Htt-Q71) with or without co-overexpression of V5-DNAJB6b. PolyQ aggregates were trapped in an acetate nitrocellulose membrane and visualized by immunoblotting for GFP. Dark triangles indicate serial dilutions (1x, 0.2x and 0.04x). Quantification of the percentage of aggregation normalized to wildtype (WT) presented in (b), shown as means ± standard error of the mean of 4 independent biological replicates. (D) Immunofluorescence of HEK293T WT and DNAJB6-KO cells transfected with GFP-Htt-Q71 alone or in combination with V5-DNAJB6b. Soluble polyQ corresponds to the diffuse green staining, while aggregates form puncta. Nuclei were stained with DAPI. Scale bar: 20µm. (E) Quantification of the results shown in (D) represented as the percentage of transfected cells with GFP-positive puncta. Approximately 500 cells per condition were counted. Data are expressed as means ± standard error of the mean. (F) Representative Western blot images of HEK293T cells co-transfected with GFP-Htt-Q71 and either empty vector or one of the indicated V5-DNAJB6b constructs. Soluble levels of GFP and V5-tagged constructs are shown. GAPDH was used as loading control. (G) Representative filter trap assay image of HEK293T cells co-transfected with Htt-Q119-YFP and either empty vector or one of the indicated V5-DNAJB6b constructs. For each condition, a 5-fold serial dilution is shown (1x, 0.2x, 0.04x). Quantifications of GFP band intensities of 3 independent biological replicates are relative to the polyQ-only condition and are expressed as means ± standard error of the mean. *: p< 0.0217, as determined by one-sample t-test (two-tailed).
Figure 6. DNAJB6-knockdown lead to hypersensitivity to polyglutamine aggregation in SCA3 and HD patient-derived cells and overexpression rescue aggregation in SCA3 patient-derived neurons

(A) Schematic representation of siRNA or shRNA mediated knockdown of DNAJB6 in SCA3 and HD patient-derived cells. Insolubilization of Ataxin3 in NSCs SCA3 patients’ cells in mock and DNAJB6 siRNA treated cells. Cells were fractionated resulting in triton soluble (TX100), SDS soluble (SDS) and SDS insoluble (FA) fractions. Western blot for ataxin3 antibody is shown (*-Expanded allele) (n=3, technical replicates from same iPSC clone). (B) Double immunostaining on MOCK and DNAJB6 siRNA treated SCA3 patient-derived NSCs for 1C2/MAP2, ataxin-3 inclusions are indicated by arrows. Scale bars: 25µm. Quantification shows percentage of cells with aggregates (Bars represent SEM, n=3, technical replicates from same iPSC clone). (C) qPCR analysis of DNAJB6 mRNA levels in Control iPSCs (Q$^{33}$) and HD iPSCs (Q$^{71}$). Graphs (relative expression to corresponding non-targeting (MOCK) shRNA control represent the mean ± s.e.m. (n=3). (D) Filter trap analysis of control cells (Q$^{33}$: IPSCs and NSCs) and HD cells (Q$^{71}$: IPSCs and NSCs). Knockdown of DNAJB6 triggers aggregation of polyQ$^{71}$ expanded HTT. (n=3). (E) Immunocytochemistry of Control iPSC (Q$^{33}$) and HD iPSC (Q$^{71}$) upon DNAJB6 knockdown. PolyQ-expanded and Hoechst staining were used as markers of aggregates and nuclei, respectively. Scale bars: 25µm. (n=3, technical replicates from same iPSC clone). Quantification represents the percentage of polyQ aggregate-positive cells/total nuclei in HD-iPSC (Q$^{71}$) (mean ± s.e.m., 800–1000 total cells per condition). We were not able to detect aggregate-containing cells in the control iPSC line (Q$^{33}$) upon DNAJB6 shRNA. Statistical comparisons were made by Student’s t-test for unpaired samples. ***P < 0.001, ****P < 0.0001. (F) Immunocytochemistry of Control-NSCs (Q$^{33}$) and HD-NSCs (Q$^{71}$). Upon neural induction of HD-iPSCs with downregulated levels of DNAJB6, NSCs accumulate mutant polyQ-expanded HTT aggregates. PolyQ-expanded and Hoechst staining were used as markers of aggregates and nuclei, respectively. Scale bar: 25µm. (n=2, technical replicates from same iPSC clone). Graph represents the percentage of polyQ aggregate-positive cells/total nuclei in HD-NSCs (Q$^{71}$) (mean ± s.e.m., 2 independent experiments, 250–300 total cells per condition). We were not able to detect aggregate-containing cells in the control NPC line (Q$^{23}$) on DNAJB6 shRNA. Statistical comparisons were made by Student’s t-test for unpaired samples. Statistical comparisons were made by Student’s t-test for unpaired samples. **P < 0.01, ***P < 0.001, ****P < 0.0001. (G) Quantification of FACS based cell counting on HUES9-derived NSCs as Control, HUES9-derived NSCs transduced with inducible lentiviral GFP-Htt-Q$^{23}$, GFP-Htt-Q$^{43}$ and GFP-Htt-Q$^{71}$ (cells were maintained in tetracycline for 4 days before analysis) (Bars represent SEM, n=3). (H) Schematic representation of the inducible lentiviral GFP-Htt-Q$^{23}$, Q$^{43}$ & Q$^{71}$ in HUES9-derived NSCs for PolyQ aggregation and differentiation assay. Representative Western blots of βIII-tubulin, GFAP and GAPDH on control NSCs, control neurons (120 days differentiated), NSCs overexpressed with GFP-Htt-Q$^{23}$ or Q$^{43}$ or Q$^{71}$ and cultured in neural stem cell medium (4 days with tetracycline) and neuron differentiated from NSCs overexpressed with GFP-Htt-Q$^{23}$ or Q$^{43}$ or Q$^{71}$ maintained in differentiation medium (18 days with tetracycline). (n=2) (I) Schematic representation of the inducible lentiviral GFP-DNAJB6a (nuclear) and b (nuclear and cytoplasmic) overexpression system, GFP-DNAJB6a and b positive SCA3 patient-derived neurons upon addition of tetracycline (48hrs). Scale bar: 50µm. (J) Insolubilization of Ataxin3 in SCA3-2 patient-derived neurons treated with 0.1 mM L-glutamate for 1 hour in total with 30
min interval cells without (0 hrs) or with induced GFP-DNAJB6a or b expression (24 & 48hrs). Subsequently, whole cell lysates were fractionated by treatment with TX-100 to yield a TX-soluble fraction (TX-100) and an TX-insoluble pellet. This pellet was treated with SDS to yield an SDS-soluble fraction (SDS) and SDS-insoluble fraction that was dissolved in formic acid (FA). Total and FA fractions were run on SDS-PAGE and Western blots were probed with anti-ataxin antibody (* refers to the expanded allele), with an anti-GAPDH antibody (for loading) and anti-DNAJB6 antibody (revealing the graded DNAJB6a or b overexpression: top lane).
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be addressed to and will be fulfilled by the Lead Contact, Harm H. Kampinga (h.h.kampinga@umcg.nl).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

The experiments were undertaken with the understanding and written consent of each subject and were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Fibroblast samples were obtained from one healthy individual and three clinically affected and genetically confirmed Dutch patients with SCA3. Subjects were randomly approached for participation in this research. A single experienced neurologist determined the AO in all patients as the age at which the first clinical manifestations of unsteadiness of gait and stance were unmistakably present. The patient group contained heterozygotes only.

Generation of iPSCs of SCA-3 patients with episomal vectors

Human dermal fibroblasts (HDFs) were cultured in Dulbecco's modified Eagle media (DMEM, Gibco) containing 10% fetal bovine serum (FBS), 1 mM non-essential amino acids (NEAAs), 1× GlutaMAX, and 100 units/ml penicillin with 100 µg/ml streptomycin. The episomal iPS reprogramming plasmids, pCXLE-hOCT3/4, pCXLE-hSK and pCXLE-hMLN were purchased from Addgene. The plasmids used in our experiments were mixed in a ratio of 1:1:1 for efficient reprogramming. Three micrograms of expression plasmid mixtures were electroporated into 5× 10^5 HDFs with Amaxa® Nucleofector Kit according to the manufacturer’s instructions. After nucleofection, cells were plated in DMEM containing 10%FCS and 1% penicillin/streptomycin until it reaches 70-80% confluence. The culture medium was replaced the next day by human embryonic stem cell medium (HESM) containing knock-out (KO) DMEM, 20% KO serum replacement (SR), 1 mM NEAAs, 1× GlutaMAX, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin, and 10ng/ml bFGF (Invitrogen). Between 26-32 days after plating colonies developed and colonies with a phenotype similar to human ESCs were selected for further cultivation and evaluation. Selected iPSC colonies were mechanically passaged on matrigel (BD, hES qualified matrigel) coated plates containing mTeSR™1 (defined, feeder-free maintenance medium for human ESCs and iPSCs).

HD-iPS cell lines

Non-integrating HD and control iPS cell lines were generated from control (CTRL-28#6 and CTRL-33#1) and HD (HD-60#5, HD109#1 and HD-180#1) fibroblasts carrying a different number of CAG repeats as described in (Mattis et al., 2015). The lines/clones used in this study were regularly tested and maintained mycoplasma free. Cells were maintained in mTeSR1 medium (Voden) and plated on Matrigel (BD, Becton Dickinson). At 80% of confluence iPSC colonies were mechanically isolated and transferred onto new plates.

METHOD DETAILS

Pluripotency assays for hiPSCs

Subconfluent undifferentiated hiPSCs were harvested by cutting the colonies into small pieces and scraping them off the cell culture dish. Colony fragments were transferred into non-adherent cell culture plates and cultured in hEB medium (DMEM/F12, 20% KSR, 1% NEAA, 1:1000 MycoZap+) for 8 days (medium was changed every other day). At day 9, developing embryoid bodies (EBs) were plated onto gelatin (0.1%) or Matrigel-coated coverslips and cultured for another 2 - 4 weeks. At the end of the differentiation period cells were fixed with 4% PFA and examined for the presence of cells of all three germ layers with immunocytochemistry.
**Generation of iPSC-derived neural stem cells and neurons**

IPSCs and HUES9 embryonic stem cells were dissociated manually and plated on a non-coated dish in human embryonic stem cell medium (HESM). After 4 days, embryoid bodies (EBs) were formed and transferred to neural differentiation medium containing DMEM/F12, 1 mM NEAAs, 1× GlutaMAX, 1% penicillin/streptomycin, and 1× N1 supplement (100X) for another 4 days. EBs were plated on matrigel-coated plates for neural rosette formation for 8-10 days with 0.01mM retinoic acid. Neural rosettes were handpicked and cultured in neural stem cell medium containing DMEM/F12, 1 mM NEAAs, 1×GlutaMAX, 1% penicillin/streptomycin, 1×N1 supplement (100X), 20 ng/mL FGF2 (peprotech), 20 ng/mL EGF (peprotech), and 2µl/ml B27 supplement (Invitrogen). Terminal neural differentiation was induced by dissociating the neural stem cells (NSCs) using accutase (Sigma) for 20 min at 37°C and plating them on a matrigel-coated plated for attachment. The next day, the medium of these cell cultures were changed to neuronal differentiation medium containing DMEM/F12, 1 mM NEAAs, 1× GlutaMAX, 1% penicillin/streptomycin, 1× N1 supplement (100X), 20 ng/mL BDNF (Peprotech), 20 ng/mL GDNF (Peprotech), 1mM dibutyryl-cAMP (Sigma) and 2µl/ml B27 supplement (Invitrogen) for 80-90days.

**Striatal differentiation**

Human HD and control iPS cell lines were exposed to striatal differentiation according to (Delli Carri et al., 2013). Briefly, for neuronal induction cells were plated at a density of 0.6X10⁵ cells per cm² on Matrigel-coated dishes in Matrigel with 10μM ROCK inhibitor (Y-27632, Sigma). The starting differentiation medium included DMEM/F12 (Life Technologies) supplemented with N2 and B27 (Life Technologies), 10uM SB431542 (Evotec) and 500nM of LDN (Evotec). Medium was replaced every day. At day 5 of differentiation 200ng/mL SHHC-25II (Tocris) and 100ng/mL DKK1 (Peprotech), were added and maintained for 3 weeks. At day 15, the cell population was detached by Accutase (Millipore) and replated on Matrigel at density of 2.5 X10⁴ cells per cm². Finally, the cells were terminally differentiated by adding 30ng/mL BDNF.

**Pan-neuronal differentiation**

Neural differentiation of induced pluripotent stem cells (iPSCs) was performed using the monolayer culture protocol following the STEMdiff Neural Induction Medium (Stem Cell Technologies) method based on (Chambers et al., 2009). Briefly undifferentiated pluripotent stem cells were rinsed were treated with 1 ml of Gentle Dissociation Reagent (Stem Cell Technologies) for 10 min after rinsing once with PBS. After the incubation period, pluripotent cells were gently dislodged by adding 2 ml of Dulbecco’s Modified Eagle Medium (DMEM)-F12+10 μM ROCK inhibitor (Abcam). Cells were then centrifuged at 300g for 10 min. Cells were resuspended in STEMdiff Neural Induction Medium+10 μM ROCK inhibitor and plated on polyornithine (15 μg ml⁻¹)Laminin (10 μg ml⁻¹)-coated plates (200,000 cells cm⁻²). For neuronal differentiation, NPCs were dissociated with Accutase (Stem Cell Technologies) and plated into neuronal differentiation medium (DMEM/F12, N2, B27 (ThermoFisher Scientific), 1 μg ml⁻¹ Laminin (ThermoFisher Scientific), 20 ng ml⁻¹ brain-derived neurotrophic factor (BDNF) (Peprotech), 20 ng ml⁻¹ glial cell-derived neurotrophic factor (GDNF) (Peprotech), 1 mM dibutyryl-cyclic AMP (Sigma) and 200 mM ascorbic acid (Sigma) onto polyornithine/laminin-coated plates. Cells were differentiated for 1–2 months, with weekly feeding of neuronal differentiation medium.

**Genome-wide SNP genotyping and Genomic CAG repeat length analysis**

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). The Genomic CAG repeat length analysis of fibroblast, iPSC and NSC samples was performed as previously described in (Verbeek et al., 2004). Genome wide SNP genotyping was performed using 320k cyto Illumina arrays as per the manufacturer’s protocol (Illumina). Data were collected using the Illumina Bead Station scanner and data software. Genotypes were produced using the genotyping module of Genome Studio and copy number variation (CNV) analysis was performed. In addition, the B-allele frequencies and log R ratios were visualized using the genome viewer tool within this package.
Lentiviral infection of iPSCs

Lentivirus (LV)-non-targeting small hairpin RNA (shRNA) control, LV-DNAJB6 shRNA #1 (TRCN0000008781) and LV-DNAJB6 shRNA #2 (TRCN0000008770), in pLKO.1-puro vector were obtained from Mission shRNA (Sigma). Transient infection experiments were performed as follows (Koyuncu et al., 2018). iPSCs colonies growing on Geltrex were individualized using Accutase. Hundred thousand cells were plated on Geltrex plates and incubated with mTesR1 medium containing 10 µM ROCK inhibitor for 1 day. Then, cells were infected with 5 µl of concentrated lentivirus. Plates were centrifuged at 800 × g for 1 h at 30 °C. Cells were fed with fresh media the day after to remove the virus. After 1 day, cells were selected for lentiviral integration using 2 µg ml⁻¹ puromycin (ThermoFisher Scientific). Cells were split for further experiments and collected after 5–7 days of infection.

Neural differentiation

To obtain NPC cultures from Control (Q³³) and HD-iPSC lines (Q⁷¹), we induced neural differentiation using STEMdif Neural Induction Medium (Stem Cell Technologies) following a monolayer culture method as described in Ref (Ju Lee et al., 2017). Undifferentiated iPSCs were rinsed once with phosphate-buffered saline (PBS) and then we added 1 ml of Gentle Dissociation Reagent (Stem Cell Technologies) for 10 min. After the incubation period, we gently dislodged iPSCs and added 2 ml of Dulbecco’s Modified Eagle Medium (DMEM)/F12 (ThermoFisher Scientific) +10 µM ROCK inhibitor (Abcam). Then, we centrifuged cells at 300 × g for 10 min. Cells were resuspended on STEMdiff Neural Induction Medium (Stem Cell Technologies) +10 µM ROCK inhibitor and plated on poly-ornithine (15 µg ml⁻¹)/Laminin (10 µg ml⁻¹)-coated plates at a density of 200,000 cells cm⁻².

Generation of iPSC-derived cerebral organoids

Control iPSCs were dissociated manually and plated on a non-coated dish in human embryonic stem cell medium (HESM). After 4 days, embryoid bodies (EBs) were formed and transferred to neural differentiation medium containing DMEM/ F12, 1 mM NEAAs, 1×GlutaMAX, 1% penicillin/streptomycin, and 1× N1 supplement (100X) for another 4 days. Embryoid bodies were further maintained in differentiation media containing a 1:1 mixture of DMEM/F12 and Neurobasal containing N1 supplement (100X), 2µl/ml B27 supplement without vitamin A (Invitrogen), 3.5 µl l⁻¹ 2-mercaptoethanol, 1:4,000 insulin (Sigma), 1×GlutaMAX and 1 mM NEAAs. Organoids were maintained until further analysis.

FACS analysis

Differentiated NSCs or neurons were dissociated using accutase, neurons stained with antibody and collected in colorless DMEM. Samples were probed with antibodies: Anti-Human CD24-PE (eBioscience; 12-0247-42), Anti-Human CD184 (CXCR4)-APC (eBioscience; 17-9999-42) and Anti-Human/Mouse CD44-FITC (eBioscience; 11-0441-85). The live or stained cells was sorted on a MoFlow-XDP with a 100 nozzle at a pressure of 15-20psi and replated onto matrigel coated coverslips, dishes for maturation or analyzed. For FACS based cell counting, SCA3 iPSC-derived NSCs were plated in a 12 well plate (0.5-1x10⁶ cells per well) and treated with glutamate or left untreated. Next, cells were collected in the same volume and analysed with gating strategies as visualized. Similar conditions mentioned above apply for HUES9-derived NSCs infected with GFP-Q²⁵, GFP-Q⁴³ or GFP-Q⁷¹.

Excitatory stimulation of neurons

SCA3 neurons or control neurons cultured in 6well plates were washed three times with 2 ml HBSS (balanced salt solution) containing 25 mM Tris, 120mM NaCl, 15mM glucose, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, pH7.4. After treatment with L-glutamate 0.1 mM (Sigma no. G8415) in HBSS for 30 min cells were washed again three times and left them to recover for 30min in differentiation media followed by a second 30min L-glutamate treatment in HBSS, and subsequently cultured in differentiation media for 24hr until analysed. For analysis of fragmentation and aggregation of ATXN3 by western blotting, extracts were analysed either immediately after lysis or after fractionation.
Immunohistochemistry in brain and intestinal tissue

For mouse tissue (brain and intestine), we used C57BL/6 mice (Envigo, The Netherlands). Intestinal tissue was fixed with 4% formaldehyde overnight at room temperature, and subsequently dehydrated with a Leica TP 1020 automatic tissue processor before paraffin embedding. Paraffin blocks were sectioned at a thickness of 4 µm using a microtome (Thermo Fisher HM 340E). Brain tissue was fixed with 4% paraformaldehyde for 48 hours at +4°C, and subsequently cryoprotected in 25% sucrose and frozen after OCT embedding. Brains were sectioned using a Leica cryostat at a thickness of 7 µm. Intestinal tissue sections were deparaffinized and re-hydrated before antigen retrieval using a citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6). Frozen brain sections were re-equilibrated at room temperature for 30 minutes before antigen retrieval with the antigen retrieval solution Histo-VT-One (Fine Chemicals Products Ltd, cat#06380-05). Tissue sections were washed in PBS and incubated in 5% serum (donkey, goat or rabbit), 1% bovine serum albumin (BSA) and 0.4% Triton X-100 or 0.1% Tween 20 in PBS for 1 hour at room temperature. After blocking, tissue sections were incubated with the primary antibodies diluted in blocking solution overnight at +4°C. Afterwards tissue sections were washed in PBS and incubated with the relative secondary antibodies diluted in blocking buffer at 1:500 for 1 hour at room temperature. Tissue sections were counterstained with DAPI and mounted with 80% glycerol or Dako mounting medium (Agilent Dako, cat#S3025). Images were obtained using a Leica TCS SP8 confocal microscope (Leica Microsystems) and analysed with Fiji (Image analysis software).

Generation of DNAJB6 knockout (KO) cells

HEK293T and U2OS cells were cultured in DMEM (Gibco) with 10% fetal calf serum, 1% penicillin/streptomycin (Gibco) and 1% GlutaMAX (Gibco) at 37°C with 5% CO2. Cells were subsequently co-transfected with DNAJB6 CRISPR/Cas9 KO(h) and HDR(h) plasmids (1 µg/each; sc-404227 and sc-404227-HDR, respectively, Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen) and selected 24 hours later with puromycin (2 µg/ml; sc-108071, Santa Cruz Biotechnology). The resistant pool of cells was then seeded as single cells in 96-well plates and expanded for approximately 3 weeks under puromycin selection. Selected clones were screened for absence of DNAJB6 expression at the protein level with western blotting, using a home-made rabbit polyclonal anti-DNAJB6 antibody.

DNAJB6 knockdown in NSCs

SCA3 neural stem cells were grown to 70-80% confluence in a twelve well plates and transiently transfected with GENIUS DNA Transfection Reagent (Westburg; cat.no 7-1010) according to the manufacturer's instruction. Cells were transfected with MOCK and siRNA for DNAJB6 (Mock RNAi Human, D-001206-13-20, Dharmacon/GE; SMARTpool Acell DNAJB6 siRNA, E-013020-00-0005, Dharmacon/GE) and harvested after 48hrs for polyglutamine aggregation assay.

Lentivirus expression in NSCs or neurons

Lentivirus was produced in HEK293T cells in 6well plates following transfection with plasmids containing FUW-tet0-GFP-DNAJB6a or b, GFP-Q23, GFP-Q43 or GFP-Q71 together with pMD2-VSV-G and pCMV-D8.91 plasmids. Lentiviral particle containing medium was collected 48hrs post-transfection, filtered through a 0.45µm filter and concentrated with Amicon Ultra 100,000 MWCO centrifugal filters (Millipore). The concentrated supernatant was diluted with 1ml of fresh neural differentiation (ND) medium, containing 8µg/ml polybrene (Sigma-Aldrich) and used to transduce NSCs or neurons. NSCs or neurons were co-infected with a ubiquitin promoter driving M2rtTA, enabling conditional and controlled expression of the GFP-DNAJB6a or b, GFP-Q23, GFP-Q43 or GFP-Q71 i.e. only in the presence of doxycycline. Lentivirus-containing medium was replaced the following day and cells were cultured in ND medium for another 3 days. After 4 days, viral media was removed and replaced with ND medium containing 2µg/ml doxycycline to induce the expression of GFP-DNAJB6a or b, GFP-Q23, GFP-Q43 or GFP-Q71.
**Polyglutamine aggregation assays**

HEK293T or U2OS wild type (WT) and respective DNAJB6-knockout (KO) cells were cultured in 6-wells plates treated with 0.0001% poly-L-lysine at a density of 3x10⁵ cells/well and, 24 hours later, subjected to transient overexpression of exon 1 fragments of huntingtin with either 25 or 71 glutamines (pEGFP-C1 GFP-Htt-Q²⁵ and GFP-Htt-Q⁷¹, respectively) in combination with either pcDNA5 FRT-TO empty vector or human V5-DNAJB6b. Polyethylenimine (6 µg/well) was used as the transfection reagent. Forty-eight hours later (or at other indicated time-points), cells were washed twice with PBS, harvested in FTA lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2% SDS) and sonicated. Protein concentration was measured with the DC protein assay (Bio-Rad). Samples of equal concentration were used for conventional western blotting (5 µg/lane) or the filter trap assay (FTA; 120 µg/lane), as previously described (Hageman et al., 2010, Kakkar et al., 2016). Briefly, the FTA consists on the selective retention of very high molecular weight species, such as polyQ aggregates, on 0.22 µm cellulose acetate membranes with the aid of a Bio-Dot microfiltration apparatus (Bio-Rad). The DNAJB6 disease-associated mutants (F89I, F93L, and P96R) were generated by site-directed mutagenesis from pcDNA5 FRT-TO V5-DNAJB6bWT using the QuickChange II Site-Directed Mutagenesis kit (Agilent, Santa Clara, United States) according to the manufacturer’s instructions. For the analysis of suppression of polyQ aggregation, 0.1 µg of each construct was co-transfected with 0.9 µg of Htt-Q¹¹⁹-YFP plasmid using 6 µg polyethylenimine (PEI; 1 µg/µl) into HEK293T cells cultured in 6-wells plates. After 24 hours, cells were harvested in 200 µl 2% SDS filter trap assay (FTA) buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 2% SDS), sonicated, and stored at -80°C until further use. Protein concentration was estimated with the DC Protein Assay kit (Bio-Rad, Hercules, United States), and FTA samples were prepared as serial dilutions for each sample (0.6, 0.12, and 0.024 µg/µl, respectively). The filter trap assay was performed.

**Filter trap**

IPSCs or NPCs were collected in non-denaturing lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with EDTA-free protease inhibitor cocktail (Roche) and lysed by passing 10 times through a 27 G needle attached to a 1 ml syringe. Protein concentrations of cell lysates were determined with a standard BCA protein assay (ThermoFisher Scientific). Protein concentration is equalized in all the lysates and equal amounts of protein extract are centrifuged at 8000 × g for 5 min at 4 °C. Cell pellet lysates were solubilized with 2% SDS and loaded onto a cellulose acetate membrane assembled in a slot blot apparatus (Bio-Rad). The membrane was washed with 0.2% SDS and retained polyQ proteins were assessed by immunoblotting for anti-polyQ-expansion diseases marker antibody (Millipore, MAB1574, 1:5000).

**Western blotting**

Neuronal cells were washed in PBS and scraped them. Cells were immediately frozen in liquid N2 followed by lysis in RIPA buffer (50mM Hepes pH 7.4, 150mM NaCl, 0.2% Triton X-100) containing 25mM EDTA. For fractionation, lysates containing 1–2 µg/µl total protein dissolved in 50 mM Tris, 150mM NaCl, 0.2% Triton X-100, 25mM EDTA (RIPA buffer) were centrifuged at 22,000g for 30min at 4 °C. The pellet fractions were separated from supernatants (Triton X-100-soluble fraction) and homogenized by sonication in RIPA buffer containing 2% SDS (SDS fraction), β-mercaptoethanol (5%) was added in all the samples and subsequently incubated at 99 °C for 5 min. Gels were loaded with 10-20µg of the Triton X-100 fraction and 40 µl of the SDS fraction. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and then processed for western blotting. Membranes were subsequently incubated with HRP-conjugated secondary antibodies (Amersham) at 1:7000 dilution. Visualization was performed with enhanced chemiluminescence. Samples were probed with primary antibodies : HSPA1A (Stressgen; SPA-810), HSPB1 (Stressgen; SPA-800), DNAJB1 (Stressgen; SPA-400), HSPB8 (Stressgen ;SPA-815F), HSPB6 (Stressgen; SPA-754), GAPDH (Fitzgerald; 10R-G109A), CCT2 (Abcam; ab92746), HSP90B antibody (Abcam; ab3674), HSP70 (SPA815; Amersham), β-actin (Abcam; ab8226). Soluble and aggregated polyQ were detected by western blotting and FTA, respectively, with mouse monoclonal anti-GFP antibody (Clontech; JL-8). DNAJB6 overexpression was detected with
mouse monoclonal anti-V5 antibody (Invitrogen; R960-25) and endogenous GAPDH was used as loading control. Quantification: chaperone protein levels detected by Western blotting were quantified using the Image Lab software (v. 6.0.1, Bio-Rad, Hercules, United States), and normalized by GAPDH levels. For each independent cell line, protein levels in iPSCs were set to 100% and levels in NSCs and neurons were expressed as relative to iPSCs. Dot- and boxplots were generated in R (v. 3.5.0, The R Foundation for Statistical Computing) using the ggplot2 and ggsci packages.

Immunocytochemistry

IPSCs derived from SCA-3 patients, control iPSCs, in vitro differentiated neural rosette, neural stem cells, astrocytes and neurons were fixated with 4% paraformaldehyde for 20 min. Cells were blocked in 5% normal goat serum and 2% Fetal calf serum; subsequently, samples were probed with primary antibodies: SSEA-4 (Hybridoma Bank; MC-813-70), TRA-4 (SantaCruz; sc-5279), Sox-2 (CellSignaling; #4900S), NANOG (AbCam, ab80892), MAP-2 (Millipore; AB5622), GFAP (Dako; Z0334), βIII-tubulin (AbCam; ab7751), GATA4 (SantaCruz; sc-25310), Pax6 (Millipore; AB2237), Vimentin (SantaCruz; sc-7557), Nestin (R&D; MAB1259), Ataxin-3 (Acris Antibodies GmbH; AM21054PU-N), DNAJB6 (made by group Ineke Braakman lab, Utrecht), Musashi (R&D systems; AF2628), polyQ-expansion diseases marker antibody (Millipore; MAB1574). Alexa 488, Alexa 594 and Cy3-conjugated secondary antibodies were used in combination with Hoechst nuclear staining. Confocal imaging was performed with Zeiss LSM 780 confocal laser scanning microscope. Immunofluorescence for Polyglutamine aggregation experiments, cells were grown on glass coverslips and transfected as described above. Forty-eight hours later, cells were washed twice with PBS, fixed in 3.7% formaldehyde for 15 minutes, washed twice with PBS for 5 minutes each, incubated in PBS with 0.2% Triton X-100 for 5 minutes and washed once more for 5 minutes in PBS. Slides were incubated with DAPI (4’,6-diamidino-2-phenylindole; 0.2 µg/ml) for 10 minutes to stain nuclei. Images were obtained using a Leica TCS SP8 confocal microscope (Leica Microsystems). Live cell imaging were obtained using a zeiss cell discoverer 7.

RT-PCR and qRT-PCR

RNA was isolated using the standard Trizol-based procedure. Following cDNA synthesis and PCR reaction, DNA was visualized in an 1% agarose gel (RT-PCR). For qRT-PCR iTaq Supermix with ROX (Biorad, 172-5855) was used. Primer sequences used in this study are listed in key resource table. Another method: Total RNA was extracted using RNAbee (Tel-Test Inc.). Complementary DNA (cDNA) was generated using qScript Flex cDNA synthesis kit (Quantabio). SybrGreen real-time quantitative PCR (qPCR) experiments were performed with a 1:20 dilution of cDNA using a CFC384 Real-Time System (Bio-Rad) following the manufacturer’s instructions. Data were analyzed with the comparative 2ΔΔCt method using the geometric mean of ACTB and GAPDH as housekeeping genes.

Calcium imaging

Cells were washed with 1X HBSS and loaded with 3uM Fluo4AM (ThermoFisher, The Netherlands) by 15 minutes incubation at 37°C after cells were washed again with 1X HBSS and placed in the microscope. Images were acquired using the 40x NA=1.3 oil-objective(Olympus) of a DeltaVision Elite fluorescence microscope (Applied Precision, Issaquah, WA) equipped with a CoolSNAP HQ Camera and 37°C incubation chamber. Lamp intensity was 2% and the FITC filter was used for excitation/emission, the acquisition rate was 1 frame per second for a duration of 120 seconds per well. Glutamate (100µM) was added after 20 seconds. Image sequences were analysed using Fiji.

Electrophysiology

The cells on matrigel coated coverslips were placed in a measuring chamber attached to a microscope (Axioskop 2 FS, Zeiss, Oberkochen, Germany). Membrane currents and voltages were measured using an Axopatch 200 B amplifier (Molecular devices, Sunnyvale, CA, USA) using the whole-cell patch clamp
technique. Pipettes were pulled from 1.2 mm O.D. borosilicate glass (Harvard Apparatus, Edenbridge, UK) and were filled with a solution containing: K-gluconate 140 mM, KCl 10 mM, Hepes 10 mM, MgCl2 4, 1,2-bis (2-aminophenoxy)-ethane-N,N',N,N'-tetraacetic acid (BAPTA) 0.1 mM, Na2ATP 2 mM (280–290 mOsm). The pH was adjusted to 7.40. The bathing solution contained NaCl 130 mM, KCl 3 mM, MgCl2 2 mM, CaCl2 2 mM, NaH2PO4 1.25 mM, NaHCO3 26 mM and glucose 10 mM (mOsm 330). The pH was adjusted to 7.40. When used with these solutions, the pipettes had initial resistances of 5–8 MΩ. Membrane currents were recorded at room temperature (20–22°C) with the amplifier in voltage clamp mode. Currents were low-pass filtered at 2 kHz and sampled at 50 kHz using a Digidata 1320 AD converter (Axon Instruments). The junction potential was corrected with the pipette in the bath solution. After measuring the membrane currents in response to depolarizing voltage steps, the amplifier was switched to current clamp mode. Following measurement of the resting membrane potential, the membrane potential was set to −60 to 70 mV using steady injected current through the patch pipette. Next, the membrane was briefly depolarized by injecting depolarizing current pulses through the pipette (duration 50 ms and 500 ms) in order to evoke action potentials. Voltage clamp step protocols were generated and data analyzed using Pclamp v10 software (Molecular devices).
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**Chemicals, Peptides, and Recombinant Proteins**

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Figure 1

A

Control and SCA3 dermal Fibroblasts → iPSCs → Neural stem cells → Neurons → FACS 90 days → Neurons with aggregates

B

Western blot analysis:
- 70 KDa: α-Ataxin-3
- 55 KDa: α-GAPDH
- 37 KDa: α-GAPDH

C

Control, SCA3-1, SCA3-2, SCA3-3

- Embryoid Bodies
- Neural Rosette
- Neural stem cells
- Neurons

D

Control, SCA3-1, SCA3-2, SCA3-3

Immunofluorescence:
- MAP2/βIII tubulin
- GFAP

E

Control, SCA3-1

- Graphs showing cell proliferation and differentiation

F

Graphs showing CD44/CD184/CD24 and CD44/CD184/CD24

G

Control, SCA3-1, SCA3-2, SCA3-3

30 Day After Scoring
Figure 2

A. Control & SCA3
Neural stem cells

B. Control & SCA3
Neurons

C. Neural stem cells
Neurons

D. SCA3-2
SCA3-3
Supplemental Information

DNAJB6, a key factor in neuronal sensitivity to amyloidogenesis.

Arun Thiruvaluvar¹, Eduardo P. de Mattos¹, Jeanette F. Brunsting¹, Rob Bakels¹, Despina Serlidaki¹, Lara Barazzuol¹, Paola Conforti²,³, Azra Fatima⁴, Seda Koyuncu⁴, Elena Cattaneo²,³, David Vilchez⁴, Steven Bergink¹, Erik HWG Boddeke¹, Sjef Copray¹, Harm H. Kampinga¹

¹Department of Biomedical Sciences of Cells & Systems, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, ²Department of Biosciences, University of Milan, Milan, Italy, ³Istituto Nazionale di Genetica Molecolare, Romeo ed Enrica Invernizzi, Milan, Italy, ⁴Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany.

*shared last authors
Table S1. Generation and characterization of control and SCA3 patient-derived iPSCs

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Figure S1. Generation and characterization of control and SCA3 patient-derived iPSCs

(A) Information on CAG-repeat length and age at onset (AO) of the SCA3 patients from which the iPSCs were generated and CAG-repeat sizes in the SCA3 iPSCs and NSCs after 30 passages. (B) Phase-contrast images of control and SCA3 patient iPSCs and clones derived from patient fibroblasts by non-integrative episomal reprogramming. Scale bars: 50µm. (C) Immunocytochemical detection of pluripotency-associated transcription factors (OCT4, SOX2) and membrane markers (SSEA4, TRA-1-60, TRA-2-54) in control and SCA3 iPSCs. Scale bars: 50µm. (D) In vitro spontaneous differentiation of control and SCA3 patient iPSCs. In vitro, iPSCs differentiated via embryoid bodies (EB) into ectoderm (βIII-tubulin), endoderm (GATA4) and mesoderm (Desmin) on serum-free medium without growth factors (30 days post differentiation). Scale bars: 50µm. (E) SNP analysis on various clones of control and SCA3 patient-derived iPSCs.
Figure S2. Characterization of control and SCA3 patient-derived iPSCs and neural stem cells (A) Immunocytochemical detection of neuroepithelial-associated transcription factor (PAX6; White arrow) in control and SCA3 iPSC-derived cells. The arrows indicate neuroepithelial cells positive for the transcription factor PAX6 in a cell cluster during directed differentiation of pluripotent stem cells to neural stem cells, which were further isolated manually and differentiated into neural stem cells under the presence of mitogens such as EGF and FGF2 (Post 20 days differentiation). Scale bars: 50µm. (B) Immunocytochemical detection of multipotent-associated transcription factor (SOX2) and NSC-markers (nestin, Vimentin) in control and SCA3 iPSC-derived neural stem cells (Post 45 days differentiation). Scale bars: 50µm. (C) Quantitative-PCR analysis of control and SCA3 iPSCs for transcription factors (OCT-4, KLF-4, NANOG, SOX-2 and cMYC), for neural stem cell markers (SOX-2, Nestin and Vimentin), for neuron markers (βIII-tubulin or Tubb-3 and MAP2) and astrocyte markers (GFAP and S100β). (Bars represent SEM, n=3, technical replicates from same iPSC clone).
Figure S3. Purification of control and SCA3 patient iPSC-derived neurons (A) Cell surface marker FAC-sorting of control and SCA3 patient iPSC-derived neurons (CD24^+ , CD44^− and CD184^−) from differentiated mixed culture of glial cells (CD24^− , CD44^+ and CD184^+ ) post 90 days. (n=3, technical replicates from same iPSC clone).
Figure S4. Glutamate-induced Ataxin-3 aggregation in SCA3 patient iPSC-derived neurons  

(A) 1C2/MAP-2 double immunostaining in differentiated control and SCA3 iPSC-derived neurons treated with glutamate or untreated in the presence and absence of BDNF (150 days post differentiation) Scale bars: 100µm and 25µm.  

(B) Ca2+ imaging with Fluo-4 dye in control and SCA3 patient-derived cells reveals a clear increase of intracellular Ca2+ upon exposure to L-glutamate (t: 20sec addition of L-glutamate, measured till t: 120sec) in both NSCs and neurons. Quantification of calcium influx upon L-glutamate treatment (n=99 cells) in control and SCA3 patient-derived cells, plots indicate normalized intensity values and AUC (area under the curve intensity for each cell), Scale bars: 20µm.  

(C) FACS based cell counting for SCA3 iPSC-derived NSCs treated with glutamate or untreated. (Bars represent SEM, n=3, technical replicates from same SCA-3 iPSCs clone).
**Figure S5. Analysis of DNAJB6 levels in HD patient-derived cells** (A) Phase contrast images of HD and control lines at day 30 of differentiation. Immunocytochemistry for MAP2a/b (red) and Hoechst (blue) at day 30 of differentiation in HD and control line. (B) Representative Western blots of β-actin and selective members of the HSP family (HSPA6, HSP90B1, & DNAJB6) in Huntington patient-derived iPS, neural progenitor cells (NPC) and PAN-NEURONAL neurons (NPC and Neurons were derived from the iPS cells in a monolayer differentiation protocol (Mattis et al., 2012). (C) Representative Western blots of β-actin and selective members of the HSP family (HSPA6, HSP90B1, & DNAJB6) in Huntington patient-derived iPS (iPSCs) and striatal neurons (Neu), neurons are derived from iPS cells using an embryoid body method directed toward striatal neuron differentiation (Koyuncu et al., 2018). (D) Quantification of HSPA1A protein levels in control (circles) and HD (triangles) donor-derived iPSCs, NSCs and neurons (left panel). Quantification of DNAJB6a, DNAJB6b and TCP1 in control (circles) and SCA3 (squares) donor-derived iPSCs, NSCs and neurons treated with glutamate (right panels). For each cell line, protein levels in NSCs and neurons are plotted relative to those in iPSCs.
**Figure S6. Analysis of CCT and HSPs expression levels upon differentiation of human ES cells** (A) Ribosome profiling data from human embryonic (hES) stem H1 cells before and after 1 (nd1), 3 (nd3) and 6 (nd6) days of neural induction were generated by (Werner et al., 2015) and retrieved from Gene Expression Omnibus accession GSE62247. For each chaperone, expression patterns of the probe set with the highest average present signal were compared using the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) and are presented as log2 ratios. As an illustration of the successful neural differentiation protocol, expression levels of the stem cell marker OCT4 and of the neural lineage marker PAX6 are shown. P-values refer to the significance of correlation between the hES, nd1, nd3 and nd6 groups, as assessed by the Pearson product-moment correlation coefficient (R).
Figure S7. Comparison of brain-specific messenger RNA levels of several molecular chaperones before and after birth (A) Publicly available RNA sequencing data were extracted from the BrainSpan consortium (www.brainspan.org) existing of 524 samples from different brain areas of human fetuses (ranging from 8 to 37 postconceptional weeks) and of individuals from 4 months to 40 years of age. Selected brain areas include the amygdaloid cortex, anterior cingulate cortex, caudal ganglionic eminence, cerebellar cortex, cerebellum, dorsal thalamus, dorsolateral prefrontal cortex, hippocampus, inferolateral temporal cortex, lateral and medial ganglionic eminences, mediodorsal nucleus of the thalamus, occipital cortex, orbital frontal cortex, parietal neocortex, posterior superior temporal cortex, posteroverentral parietal cortex, primary auditory, visual and somatosensory cortices, striatum, temporal neocortex, upper rhombic lip and ventrolateral prefrontal cortex. Data were analyzed in the R2 Genomics Analysis and Visualization Platform (www.hgserver1.amc.nl). Gene expression values for individual genes are represented as box plots (2.5% to 97.5% confidence interval) of transformed log2 values from reads per kilobase per million. Black dots represent outlier values. For each gene, the mean expression values in the prenatal versus postnatal groups and p-value for the Mann-Whitney test are given.
Figure S8. DNAJB6 expression levels in mouse intestinal crypts. (A) Schematic representation of mouse intestinal crypts, Musashi/DNAJB6 double immunostaining in the mouse intestinal crypts (n=3), Scale bars: 10 & 25µm.
Figure S9. Effect of DNAJB6-knockout (KO) in U2OS cells and DNAJB6-knockdown in HD-derived iPSCs on polyglutamine aggregation. (A) Generation of a DNAJB6-knockout U2OS cell line. Western blot of wild-type (WT) and DNAJB6-knockout (KO) cells. (B) DNAJB6 increases the amount of soluble polyQ. WT U2OS cells or DNAJB6 KO UOS cells were transfected with either GFP-Htt-Q73 or GFP-Htt-Q43 and fractionated. Soluble fractions were loaded and stained with the indicated antibodies. Western blots for the indicated antibodies are shown. (C) Representative image of a filter trap assay in WT and DNAJB6-KO U2OS cells. Both lines were transfected with a GFP-tagged exon 1 fragment of huntingtin with 25 glutamines (GFP-Htt-Q25) and 71 glutamines (GFP-Htt-Q71) with or without co-overexpression of V5-DNAJB6b. PolyQ aggregates were trapped in an acetic nitrocellulose membrane and visualized by immunoblotting for GFP. Dark triangles indicate serial dilutions (1x, 0.2x and 0.04x). (D) siRNA mediated DNAJB6 knockdown in SCA3-derived NSCs lead to cleavage of ataxin-3, cleaved products detected by two independent ataxin-3 antibodies (ataxin-3(ab1) and ataxin-3(ab2)) (E) Western blot of control iPS (Q71) and Huntington patient-derived IPS (Q71) showing the knockdown of DNAJB6 with two shRNA and control with non-targeting shRNA. (F) FACS based cell counting on HUES9-derived NSCs as Control, HUES9-derived NSCs transduced with inducible lentiviral GFP-Htt-Q25, GFP-Htt-Q43 and GFP-Htt-Q71 (cells were maintained in tetracycline for 4 days before analysis) (n=3). (G) Immunostaining on NSCs overexpressed (4 days with tetracycline) with GFP-Htt-Q25, 43 & 71 (n=2). Scale bar: 50μm. (H) Live cell imaging shows positive polyQ aggregates (indicated in white arrow) in NSC-derived neurons overexpressed (18 days with tetracycline) with GFP-Htt-Q43 and GFP-Htt-Q71. Scale bar: 100μm. (I) GFP-DNAJB6b positive SCA3-1 patient-derived neurons upon addition of tetracycline (48hrs) Scale bar: 50μm. Insolubilization of Ataxin3 in SCA3-1 patient-derived neurons treated with 0.1 mM L-glutamate for 1 hour in total with 30 min interval cells without (0 hrs) or with induced GFP-DNAJB6b expression (24 & 48hrs). Subsequently, whole cell lysates were fractionated by treatment with TX-100 to yield a TX-soluble fraction (TX-100) and an TX-insoluble pellet. This pellet was treated with SDS to yield an SDS-soluble fraction (SDS) and SDS-insoluble fraction that was dissolved in formic acid (FA). Total and FA fractions were run on SDS-PAGE and Western blots were probed with anti-ataxin antibody (*-Expanded allele), with an anti-GAPDH antibody (for loading) and anti-DNAJB6 antibody (revealing the graded DNAJB6b overexpression: top lane).