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Interrelationship between the levels of C9orf72 and amyloid-β precursor protein and β-amyloid in human cells and brain samples

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Running title: Interrelationship between the levels of C9orf72, AβPP, and Aβ
Abstract

A subset of C9orf72 repeat expansion-carrying frontotemporal dementia patients display an Alzheimer-like decrease in cerebrospinal fluid amyloid-β (Aβ) biomarker levels. We report that downregulation of C9orf72 in non-neuronal human cells overexpressing amyloid-β precursor protein (AβPP) resulted in increased levels of secreted AβPP fragments and Aβ, while levels of AβPP or its C-terminal fragments (CTFs) remained unchanged. In neuronal cells, AβPP and C83 CTF levels were decreased upon C9orf72 knockdown, but those of secreted AβPP fragments or Aβ remained unchanged. C9orf72 protein levels significantly increased in human brain with advancing neurofibrillary pathology and positively correlated with brain Aβ42 levels. Our data suggest that altered C9orf72 levels may lead to cell-type specific alterations in AβPP processing, but warrant further studies to clarify the underlying mechanisms.

Key words: Alzheimer’s disease; amyloid-β; amyloid-β precursor protein; C9orf72; frontotemporal dementia
Introduction

The GGGGCC hexanucleotide repeat expansion in C9orf72 gene is a major genetic cause of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) [1,2]. The frequency of the C9orf72 repeat expansion varies in different countries. For example, 2-19% of sporadic and 14-48% familial FTD cases, with Finland showing the highest frequency, are expansion carriers [3]. The suggested pathogenic mechanisms in the carriers include haploinsufficiency and formation of RNA foci and dipeptide-repeat (DPR)-containing protein inclusions in cells [1,4-8]. In humans, C9orf72 encodes three mRNA variants, which yield two protein isoforms, the long isoform a (~50 Da) and the short isoform b (~25 kDa). Isoform a is implicated in vesicular trafficking in the endosomal and autophagosomal/lysosomal pathways [9-11] and isoform b in nucleocytoplasmic trafficking [12].

In addition to FTD or ALS, C9orf72 repeat expansion has been identified in some patients with other neurodegenerative disorders, including Alzheimer’s disease (AD). However, the significance of the repeat expansion beyond disorders of the FTD-ALS spectrum is not clear [13-15]. AD is the most common cause of dementia and its central neuropathological hallmarks include intraneuronal neurofibrillary tangles, composed of hyperphosphorylated tau protein, and amyloid-β (Aβ) plaques in the brain parenchyma [16]. Increased levels of total and phosphorylated tau and decreased levels of Aβ42 peptides in the cerebrospinal fluid (CSF) are used as diagnostic biomarkers for AD [17,18]. We have previously reported that 25% of Finnish C9orf72 repeat expansion-carrying FTD patients show decreased CSF Aβ1-42 levels [19]. The patients with such altered biomarker status fulfilled the clinical criteria of the behavioral variant FTD with no clinical signs of AD. In this study, neuropathological confirmation was available only for one patient, showing TAR DNA-binding protein 43 (TDP-43) pathology, but no Aβ or tau pathology [19]. Albeit the reason for the decrease in the CSF Aβ1-42 levels remains thus far unknown, these results suggest that decreased CSF Aβ1-42
levels may not exclude \textit{C9orf72} repeat expansion-carrying FTD patients in clinical diagnostics [19]. Given this AD-like CSF Aβ finding in a subset of \textit{C9orf72} expansion-carrying FTD patients, we asked here if modifying the levels of \textit{C9orf72} affects AβPP processing and generation of Aβ peptides.

\textbf{Materials and methods}

\textbf{siRNAs and cDNA constructs}

Dharmacon (ON-TARGETplus, L-013341-01-005; siRNA 1) or Santa Cruz (sc-92761; siRNA 2) \textit{C9orf72} siRNA pools were used for \textit{C9orf72} knockdown. Silencer Negative control siRNA (Ambion, AM4611) was used as a control. Expression cDNA constructs encoding \textit{C9orf72} isoform a containing a C-terminal green fluorescent protein (GFP) tag (isoform a-GFP) [11] and \textit{C9orf72} isoform b containing a C-terminal myc-DDK tag (isoform b-myc-DDK) were purchased from Origene. Empty plasmid (pcDNA3.1, Invitrogen) was used as a control in the cDNA transfections.

\textbf{Cell culture, transfection, and treatments}

Human embryonic kidney HEK293-AP-AβPP cells overexpressing AβPP751 with a thermostable N-terminal alkaline phosphatase (AP) tag were cultured as previously [20,21]. Human H4 neuroglioma cells overexpressing AβPP751 (H4-AβPP751) were cultured as in [22]. Twenty-five nM of each siRNA or 4 µg of plasmid DNA and 10 µl Lipofectamine 2000 reagent (Invitrogen) were used per transfection according to manufacturer’s instructions to knockdown \textit{C9orf72} or overexpress \textit{C9orf72} protein isoforms. Fresh media was added 6 hours post transfection. A subset of HEK293-AP-AβPP or H4-AβPP751 cells were treated with 100 ng/ml phorbol myristate acetate (PMA) and 250 nM γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) for 3 hours to enhance the
generation of AβPP C-terminal fragments (CTFs) C83 and C99. These samples were used as positive controls for validation of the correct C83 and C99 bands in the Western blot analyses. Cells and conditioned media were collected 48 (overexpression) or 96 hours (knockdown) post transfection for analyses.

**Protein extraction from cells and Western blotting**

Protein extraction and Western blotting were performed as in [21]. Following primary antibodies were used: anti-C9orf72 (1:1000; Santa Cruz, sc-13876); anti-AβPP C-terminus (1:2000; Sigma, A8717); anti-AβPP N-terminus [1:1000; Millipore, MAB348; used to detect total amount of sAβPP fragments (sAβPPTot = sAβPPα + sAβPPβ)]; anti-β-amyloid (1:1000; BioSite, 6E10; used to specifically detect sAβPPα forms); anti-sAβPPβ (1:500, Covance; to specifically detect AβPPβ forms); and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000, Abcam, ab8245). Levels of the investigated proteins were quantified using Quantity One or Image Lab (BioRad) and normalized to those of GAPDH in the same samples. Protein levels in conditioned media were normalized to total protein lysate concentrations in each sample. Data are shown as % of the level compared to that with control siRNA or plasmid-transfected cells (set to 100%).

**Soluble AβPP measurement using alkaline phosphatase assay**

Soluble AβPP fragments containing the N-terminal AP tag (AP-sAβPP) and released into the conditioned media were measured as previously by alkaline phosphatase assay [20].

**Aβ ELISA**

Aβ x-40 and Aβ x-42 levels in conditioned media were measured using Human/Rat β Amyloid 40 (Cat no 294-64701) and Human/Rat β Amyloid 42 (Cat no 292-64501) ELISA Kits (Wako)
according to kit instructions as previously [20]. Aβ concentrations were normalized to total protein lysate concentrations in each sample.

**Human brain sample cohort**

The human brain samples from the inferior temporal cortex used in the analyses have been previously described [23]. The samples were classified into groups according to Braak staging (0-VI) depending on the extent of neurofibrillary pathology [24]. The Ethics Committee of the Kuopio University Hospital has approved the study.

**Extraction and analysis of C9orf72 RNA and protein from the frozen brain tissue samples**

RNA and protein from 71 human brain tissue samples were extracted as described previously [25,26]. Global expression of C9orf72 from 60 RNA samples from the inferior temporal cortex was investigated by using exon array probe FILL_23_P405873 (NM_145005). Assessment was implemented using Agilent One-Color Microarray-Based Exon Analysis as described in [23]. Global expression of C9orf72 was investigated from 36 protein samples from the inferior temporal cortex using SysQuant™ global proteomics (Proteome Sciences) as described in [26]. In total, 25 human brain tissue samples underwent both exon array and proteomic analysis.

**Statistical analyses**

Cell-based data are shown as mean % ± standard error of mean (SEM) of control plasmid- or siRNA-transfected cells. Statistical analyses were performed using GraphPad Prism5 or R. One-way analysis of variance (ANOVA), followed by Newman-Keuls or Fisher’s least significant difference post-hoc test or Mann-Whitney U test, was used to test statistical significance between sample groups. Correlation between variables was assessed using Spearman’s rank-order correlation. The level of statistical significance was defined as p < 0.05.
**Results**

To find out if decreased levels of \textit{C9orf72} result in altered A\textbeta PP processing and A\textbeta generation, we first used siRNA-mediated knockdown of \textit{C9orf72} in non-neuronal HEK293-AP-A\textbeta PP cells that have been widely used to screen for novel substrates for the AD-associated secretases and factors influencing A\textbeta PP processing \cite{20, 27}. Knockdown of \textit{C9orf72} with two different siRNA pools (\textit{C9orf72} siRNA 1 and 2) led to significantly decreased levels of the \textit{C9orf72} protein isoform a by 78.4\% and 73.4\%, respectively, as assessed by Western blotting (Figure 1A and C). Endogenous expression of isoform b was not detectable. We observed a moderate, but statistically not significant, increase in the levels of full-length AP-A\textbeta PP and C83 A\textbeta PP C-terminal fragment (CTF) in \textit{C9orf72} siRNA-transfected cells as compared to control cells (Figure 1A and B). The \textbeta-secretase-cleaved C99 CTFs were not detectable. As a positive control to verify the correct sizes of the observed A\textbeta PP CTFs, a subset of cells was treated with PMA and the \textgamma-secretase inhibitor DAPT. Under these conditions, increase in both C83 and C99 levels could be observed (Figure 1A). Western blotting of conditioned media from the same cells showed a significant increase in the levels of soluble AP-A\textbeta PP\alpha (AP-sA\textbeta PP\alpha) and total soluble AP-A\textbeta PP (AP-sA\textbeta PP\text{tot} = AP-sA\textbeta PP\alpha + AP-sA\textbeta PP\beta) fragments upon \textit{C9orf72} knockdown. The levels of sA\textbeta PP\beta, as assessed by an antibody specifically recognizing this soluble fragment in the media, were not detectable (Figure 1D and E). An alkaline phosphatase-based assay \cite{20}, which detects the levels of AP-sA\textbeta PP\text{tot}, also indicated a significant increase in the conditioned media of \textit{C9orf72} siRNA-transfected cells as compared to control cells (Figure 1 F), confirming the result from Western blot analysis. There were no statistically significant alterations in the levels of endogenous sA\textbeta PP\alpha or sA\textbeta PP\text{tot}, even though there was a small trend towards increased sA\textbeta PP\text{tot} levels (Figure 1D and E). A significant increase in soluble A\textbeta 40 and A\textbeta 42 levels in the conditioned media of \textit{C9orf72} siRNA-transfected cells
was evident compared to control cells. There were no differences in the Aβ42/Aβ40 ratio (Figure 1G).

To study the effects of C9orf72 knockdown in neuronal cells, H4-AβPP751 cells were transfected with C9orf72 siRNA1. Similar to HEK293-AP-AβPP cells, a significant decrease in C9orf72 levels by 73.2% was observed (Figure 2C). Opposite to HEK293-AP-AβPP cells, the levels of mature and immature forms of AβPP and C83 CTF were observed to decrease upon C9orf72 knockdown in H4-AβPP751 cells. The decrease in the levels of immature AβPP was statistically significant. C99 levels were not detectable in the H4-AβPP751 cells even after PMA and DAPT treatment (Figure 2A and B). Also in contrast to the HEK293-AP-AβPP cells, the levels of the soluble sAβPP (sAβPPtot or sAβPPα) fragments remained unchanged in C9orf72 siRNA-transfected H4-AβPP751 cells as compared to control cells. sAβPPβ levels were undetectable in H4-AβPP751 cells (Figure 2D and E). There were no differences in the Aβ40 and Aβ42 levels or Aβ42/Aβ40 ratio in the conditioned media between C9orf72 siRNA-transfected and control cells (Figure 2F). Similar results related to AβPP processing upon C9orf72 knockdown were also obtained in H4 cells without AβPP overexpression (data not shown).

To test whether overexpression of the individual C9orf72 protein isoforms affected AβPP processing, transfection of HEK293-AP-AβPP cells with C9orf72 isoform a-GFP [11] or isoform b-myc-DDK was performed. Western blot analysis of cell lysates from isoform a-GFP- or isoform b-myc-DDK-transfected cells did not indicate differences in full-length AP-AβPP or C83AβPP CTF levels between cells overexpressing the two isoforms or the control cells (Supplementary Figure 1A and B). Western blot analysis of conditioned media from the same cells did not reveal statistically significant differences in AP-sAβPPtot or AP-sAβPPα levels (Supplementary Figure 1C and D). Levels of the endogenously expressed sAβPPα or sAβPPtot did not show alterations by Western blotting (Supplementary Figure 1C and D) or AP-based
assay (Supplementary Figure 1E). The levels of secreted Aβ40 or Aβ42 or Aβ40/Aβ42 ratio remained unchanged in the conditioned media of cells transfected with either of the C9orf72 isoforms compared to control cells (Supplementary Figure 1F).

Finally, the levels of C9orf72 mRNA and protein in an existing human brain sample cohort from temporal cortex subcategorized according to advancing neurofibrillary pathology (Braak staging 0-VI) were examined. We have previously used this cohort to assess AD-related changes in gene expression [23,27]. We observed a small decrease in C9orf72 mRNA levels at Braak stage IV as compared to Braak stage 0, but overall the mRNA levels of C9orf72 did not show major alterations at different Braak stages (Figure 3A). However, the C9orf72 protein levels showed a clear increase along with increasing neurofibrillary pathology and these increases were statistically significant at Braak stages II-VI as compared to Braak stage 0 (Figure 3B). Correlation analyses indicated a highly significant positive correlation between C9orf72 and AβPP mRNA levels (Figure 3C). A similar mild, but not statistically significant, trend in the correlation between C9orf72 and AβPP protein levels could also be observed (Figure 3D). The C9orf72 protein, but not mRNA, levels showed a statistically significant positive correlation with brain Aβ42 levels (Figure 3E and F).

Discussion
The C9orf72 hexanucleotide repeat expansion is suggested to lead to FTD or ALS pathogenesis through haploinsufficiency, resulting in decreased levels of C9orf72 mRNA and protein [1,28], and formation of nuclear RNA foci and cytoplasmic inclusions containing dipeptide repeat (DPR)-containing proteins [1,4-8]. Also, some patients with other neurodegenerative disorders, including AD, have been reported to carry C9orf72 repeat expansion, suggesting its potential involvement in neurodegeneration beyond the FTD/ALS spectrum [13-15]. On the other hand,
in our previous study, we found that 25% of Finnish C9orf72 repeat expansion-carrying FTD patients display decreased CSF Aβ1-42 biomarker levels similarly to AD patients [19].

To assess the potential link between C9orf72 and altered AβPP processing and Aβ production, we modulated expression of C9orf72 by RNA interference-mediated knockdown or overexpression of the individual C9orf72 protein isoforms a and b in non-neuronal HEK293-AP-AβPP or neuronal H4-AβPP751 cells. The knockdown approach was used to model the suggested haploinsufficiency and the overexpression to allow investigation of the effects of individual C9orf72 protein isoforms. Overexpression of either C9orf72 protein isoform did not significantly affect AβPP processing in HEK293-AP-AβPP cells. However, a moderate increase in the levels of full-length AβPP or its C83 CTF upon C9orf72 knockdown was observed. In addition, the levels of secreted sAβPP fragments and Aβ40 and Aβ42 were significantly increased in HEK293-AP-AβPP cells with decreased C9orf72 expression. In contrast to these non-neuronal cells, knockdown of C9orf72 in the neuronal H4-AβPP751 cells overexpressing AβPP, or H4 cells expressing endogenous AβPP, led to decreased levels of full-length AβPP and C83 CTF, but did not influence the levels of the secreted sAβPP fragments or Aβ peptides. These results altogether indicate that alterations in C9orf72 expression may have an impact on AβPP levels and processing, but such effects may be cell type-specific in non-neuronal and neuronal cells.

C9orf72 proteins, especially the isoform a, are implicated in protein trafficking in the endosomal-lysosomal and autophagosomal pathways and suggested to function as GDP-GTP exchange factors for Rab proteins, key regulators of vesicular trafficking [9,11]. It is well established that AβPP trafficking and subcellular localization determine whether AβPP goes through amyloidogenic or non-amyloidogenic processing. AβPP undergoes maturation by glycosylation when trafficking on the secretory pathway from ER to Golgi [29]. The mature, N- and O-glycosylated AβPP then traffics to plasma membrane and is cleaved by α-secretases.
This cleavage prevents Aβ generation and yields sAβPPα potentially having neuroprotective properties [30,31]. Alternatively, AβPP is internalized from the plasma membrane and either recycled via the early endosomal compartment or targeted to later endosomal compartments and lysosomes. Endosomes are the major sites of AβPP amyloidogenic processing by β- and γ-secretase and Aβ production [32,33]. Our results suggest that decreased or increased levels of C9orf72 might not directly affect AβPP cleavage by the secretases, because we did not observe major differences in the levels of α-secretase- or β-secretase-cleaved AβPP CTFs. Moreover, the levels of C83 AβPP CTF normalized to those of full-length AβPP were not altered, also further inferring that the α-secretase-mediated cleavage of AβPP was not affected. The fact that C9orf72 knockdown did not result in increased levels of sAβPPβ or C99 AβPP CTF argues against an enhanced β-secretase-mediated AβPP cleavage. On the other hand, investigations in both non-neuronal and neuronal cells suggested that C9orf72 knockdown may lead to altered levels of full-length AβPP, but whether the AβPP levels increase or decrease, appears to depend on the cell type. Increased levels of both secreted sAβPP and Aβ in HEK293-AP-AβPP cells upon C9orf72 knockdown imply that decreased levels of C9orf72 may lead to alterations in the secretion of these proteins at least in these non-neuronal cells. Alternatively, these increases might result from the initially slightly increased full-length AβPP levels.

Our findings in human brain samples suggest that the expression levels of C9orf72 and AβPP positively correlate with each other. This correlation was statistically significant at the mRNA level and there was a trend towards a positive correlation also at the protein level. These data are in line with the observed decrease of AβPP levels in the neuronal H4 cells either overexpressing or endogenously expressing AβPP upon C9orf72 downregulation. Also, the levels of C9orf72 protein with the brain Aβ42 levels displayed a statistically significant positive correlation. These results together point out that the hexanucleotide repeat expansion-associated
haploinsufficiency leading to decreased levels of $C9orf72$ may have an impact on AβPP expression or the levels of Aβ in human brain.

There are several possible mechanisms, which might underlie the previously observed decrease in CSF Aβ1-42 levels in repeat expansion-carrying FTD patients, including changes in Aβ secretion, clearance, synaptic pools, transport through the blood-brain barrier, or degradation by Aβ-degrading enzymes. Our cell-based findings suggesting potential alterations in the expression of AβPP or secretion of sAβPP and Aβ agree well with these patient data, even though uncovering the specific molecular mechanisms in different cell types still requires further investigations. Therefore, it would be important to investigate the levels of AβPP, its cleavage products, or Aβ in the cells or brain samples derived from the hexanucleotide repeat carriers. To our knowledge, we provide here for the first time information on the relationship between $C9orf72$ and AβPP levels and processing. However, further investigations are warranted in different model systems and patient-derived cells or tissue samples with decreased $C9orf72$ expression to reveal the underlying molecular mechanisms leading to potentially altered AβPP levels and/or processing.

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Conflict of Interest/Disclosure Statement

The authors have no conflict of interest to report.
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Figure legends

**Figure 1.** Knockdown of C9orf72 significantly increases the levels of soluble AβPP fragments and Aβ40 and Aβ42 and mildly the levels of full-length AβPP or C83 C-terminal fragment in non-neuronal HEK293-AP-AβPP cells. A) Western blot of total protein lysates of HEK293-AP-AβPP cells transfected with C9orf72 siRNA 1 or 2 or control siRNA showing the levels of full-length AP-AβPP (AP-AβPP), C83 AβPP C-terminal fragment, endogenous C9orf72 isoform a, or GAPDH (loading control). Treatment with 100 ng/ml PMA and 250 nM DAPT for 3 hours (positive control for the CTFs on the blot) leads to increased levels of both C83 and C99 AβPP CTFs. Endogenous C9orf72 isoform b levels were not detectable. All samples were run on the same gel. Molecular weight markers are indicated on the left as kDa. An unspecific band, detected by the C9orf72 antibody and not affected by the siRNA treatment, is indicated by an asterisk (*). B) Quantification of full-length AP-AβPP and C83 levels normalized to those of GAPDH from A. C83 levels were also normalized to those of AP-AβPP. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. AP-AβPP n = 9; C83/GAPDH n = 6; C83/AP-AβPP n = 6; One-way ANOVA, Newman-Keuls, not significant. C) Quantification of C9orf72 levels normalized to those of GAPDH from A. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. n = 9 for all transfections, One-way ANOVA, Newman-Keuls, ***p ≤ 0.001. D) Western blot of conditioned media of HEK293-AP-AβPP cells transfected with C9orf72 siRNA 1 or 2 or control siRNA showing the levels of the endogenous soluble AβPPα (sAβPPα) and soluble AβPPtot (sAβPPtot = sAβPPα + sAβPPβ) and soluble AP-AβPPα (AP-sAβPPα) and AP-AβPPtot (AP-sAβPPtot) derived from overexpressed AP-AβPP. Soluble sAβPPβ levels remained undetectable. Molecular weight markers are indicated on the left as kDa. E) Quantification of sAβPPα, sAβPPtot, AP-sAβPPα and AP-sAβPPtot from D. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. n = 6 for
all transfections, One-way ANOVA, Newman-Keuls, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. F) Alkaline phosphatase assay-based determination of the soluble AP-sAβPPtot levels in the conditioned media of HEK293-AP-AβPP cells transfected with C9orf72 siRNA 1 or 2 or control siRNA. The assay detects both AP-sAβPPα and AP-sAβPPβ forms (= AP-sAβPPtot). Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. n = 6 for all transfections, One-way ANOVA, Newman-Keuls, ***p ≤ 0.001. G) The levels of Aβ40 or Aβ42, and Aβ40/Aβ42 ratio in the conditioned media from HEK293-AP-AβPP cells transfected with C9orf72 siRNA 1 or 2 or control siRNA. Data are shown as mean % ± SEM of the Aβ levels compared to those in control cells. n = 6 for all transfections, One-way ANOVA, Newman-Keuls, **p ≤ 0.01, ***p ≤ 0.001.

Figure 2. Knockdown of C9orf72 decreases the levels of mature and immature forms of full-length AβPP and C83 C-terminal fragment, but does not lead to changes in the levels of soluble sAβPP fragments or Aβ in neuronal H4-AβPP751 cells. A) Western blot of total protein lysates of H4-AβPP751 cells transfected with C9orf72 siRNA 1 or control siRNA showing the levels of mature and immature forms of AβPP, C83 AβPP C-terminal fragment, endogenous C9orf72 isoform a, or GAPDH (loading control). Treatment with 100 ng/ml PMA and 250 nM DAPT for 3 hours (positive control for the CTFs on the blot) leads to increased levels of C83 AβPP CTFs. Endogenous C9orf72 isoform b levels were not detectable. All samples were run on the same gel. Molecular weight markers are indicated on the left as kDa. An unspecific band, detected by the C9orf72 antibody and not affected by the siRNA treatment, is indicated by an asterisk (*). B) Quantification of mature and immature AβPP and C83 levels normalized to those of GAPDH from A. C83 levels were also normalized to those of total AβPP (mature + immature AβPP). Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. AP-AβPP n = 6; C83/GAPDH n = 6; C83/AP-AβPP n = 6; One-
way ANOVA, Newman-Keuls, **p ≤ 0.01. C) Quantification of C9orf72 levels normalized to those of GAPDH from A. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. n = 6 for all transfections, Mann-Whitney U, **p ≤ 0.01. D) Western blot of conditioned media of H4-\(A\beta\)PP751 cells transfected with C9orf72 siRNA 1 or control siRNA showing the levels of the soluble \(A\beta\)PP\(\alpha\) (\(sA\beta\)PP\(\alpha\)) and soluble \(A\beta\)PP\(\text{tot}\) (\(sA\beta\)PP\(\text{tot}\) = \(sA\beta\)PP\(\alpha\) + \(sA\beta\)PP\(\beta\)). Soluble \(sA\beta\)PP\(\beta\) levels remained undetectable. Molecular weight markers are indicated on the left as kDa. E) Quantification of \(sA\beta\)PP and \(sA\beta\)PP\(\text{tot}\) from D. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. n = 6 for all transfections, One-way ANOVA, Newman-Keuls, not significant. F) The levels of \(A\beta\)40 or \(A\beta\)42, and \(A\beta\)40/\(A\beta\)42 ratio in the conditioned media from H4-\(A\beta\)PP751 cells transfected with C9orf72 siRNA 1 or control siRNA. Data are shown as mean % ± SEM of the \(A\beta\) levels compared to those in control cells. n = 6 for all transfections, One-way ANOVA, Newman-Keuls, not significant.

**Figure 3.** The **C9orf72** mRNA levels slightly decrease and protein levels show an increase in relation to increased neurofibrillary pathology in human temporal cortex. A) **C9orf72** mRNA expression in the human temporal cortex samples was assessed using a FILL_23_P405873 probe, detecting global expression of all **C9orf72** mRNAs. The samples were subcategorized according to the extent of neurofibrillary pathology as assessed by Braak staging. Braak 0 stage indicates no detectable neurofibrillary pathology and Braak I-VI stages indicate increasing neurofibrillary pathology (I = mildest, VI = most severe). **C9orf72** mRNA levels show a slight decrease according to increasing neurofibrillary pathology and the decrease at Braak stage IV is statistically significant (*p ≤ 0.05) as compared to stage 0. Box plots show the median, 25th and 75th percentiles, error bars show 1.5 interquartile ranges. ANOVA, LSD, *p < 0.05, n = 60. B) **C9orf72** protein levels were assessed by LC-MS/MS from a subset of
patients, identifying a C9orf72-specific peptide (DVLMTF). C9orf72 protein levels are significantly increased at Braak stages II-VI according to increasing severity of the neurofibrillary pathology. Box plots show the median, 25th and 75th percentiles, error bars show 1.5 interquartile ranges. ANOVA, LSD, *p < 0.05, n = 36. C) C9orf72 mRNA levels show a significant positive correlation with AβPP mRNA levels in human brain. D) C9orf72 and AβPP protein levels show a trend towards positive correlation in human brain. E) C9orf72 mRNA levels and Aβ42 levels do not correlate with each other in human brain. F) C9orf72 protein and brain Aβ42 levels indicate a significant positive correlation in human brain. Spearman’s rank-order correlation coefficient, C-D; n = 55, E-F; n = 36.

Supplementary Figure 1. Overexpression of C9orf72 protein isoform a or b does not affect AβPP processing in HEK293-AP-AβPP cells. A) Western blot showing the levels of full-length AP-AβPP (AP-AβPP), C83 AβPP C-terminal fragment, and GAPDH (loading control) in HEK293-AP-AβPP cells overexpressing C9orf72 isoform a-GFP, isoform b-myc-DDK, or empty plasmid (control). Overexpressed isoform a-GFP and isoform b-myc-DDK and endogenously expressed isoform a are indicated. Endogenous isoform b levels were not detectable. Asterisks (*) denote unspecific bands detected by the C9orf72 antibody. Molecular weight markers are indicated on the left as kDa. B) Quantification of AP-AβPP and C83 levels normalized to those of GAPDH from A. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. Control n = 7; isoform a-GFP n = 9; isoform b-myc-DDK n = 9. One-way ANOVA, Newman-Keuls, not significant. C) Western blot showing the levels of overexpressed AP-sAβPPα and total AP-sAβPP (AP-sAβPPtot) as well as endogenously expressed sAβPPα and total sAβPP (sAβPPtot) in the conditioned media of HEK293-AP-AβPP cells overexpressing C9orf72 isoform a-GFP, isoform b-myc-DDK, or empty plasmid (control). Molecular weight markers are indicated on the left as kDa. D) Quantification of the AP-
sAβPPtot, AP-sAβPPα, and endogenously expressed sAβPPtot, and sAβPPα levels from C. Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. For AP-sAβPPtot: Control n = 7; isoform a-GFP n = 8; isoform b-myc-DDK n = 9. For AP-sAβPPα: Control n = 7; isoform a-GFP n = 9; isoform b-myc-DDK n = 9. For endogenous sAβPPtot: Control n = 5; isoform a-GFP n = 7; isoform b-myc-DDK n = 7. For endogenous sAβPPα: Control n = 7; isoform a-GFP n = 9; isoform b-myc-DDK n = 9. One-way ANOVA, Newman-Keuls, not significant. E) Alkaline phosphatase assay-based determination of the soluble AP-AβPP (AP-sAβPP) levels in the conditioned media from cells overexpressing C9orf72 isoform a-GFP, isoform b-myc-DDK, or empty plasmid (control). The assay detects both AP-sAβPPα and AP-sAβPPβ forms (= AP-sAβPPtot). Data are shown as mean % ± SEM of the protein levels compared to those in the control cells. Control n = 7; isoform a-GFP n = 9; isoform b-myc-DDK n = 9. One-way ANOVA, Newman-Keuls, not significant. F) The levels of Aβ40 or Aβ42, or Aβ40/Aβ42 ratio in the conditioned media from cells overexpressing C9orf72 isoform a-GFP, isoform b-myc-DDK, or empty plasmid (control). Data are shown as mean % ± SEM of the Aβ levels compared to those in control cells. Control n = 5; isoform a-GFP n = 6; isoform b-myc-DDK n = 6. One-way ANOVA, Newman-Keuls, not significant.
Figure 2

A

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B

Protein levels (% of control)

C

Control siRNA  C9orf72 siRNA 1

Protein levels (% of control)

C9orf72/GAPDH

D

Control siRNA  C9orf72 siRNA 1

kDa

E

Control siRNA  C9orf72 siRNA 1

Medium sAβPP levels (% of control)

F

Control siRNA  C9orf72 siRNA 1

Aβ levels (% of control)

Aβ40  Aβ42  Aβ40/Aβ42