Hallmarks of Alzheimer's disease in stem cell-derived human neurons transplanted into mouse brain

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SUMMARY

Human pluripotent stem cells (PSC) provide a unique entry to study species-specific aspects of human disorders such as Alzheimer's disease (AD). However *in vitro* culture of neurons deprives them of their natural environment. Here we transplanted human PSC-derived cortical neuronal precursors into the brain of a murine AD model. Human neurons differentiate and integrate into the brain, express 3R/4R Tau splice forms, show abnormal phosphorylation and conformational Tau changes and undergo neurodegeneration. Remarkably, cell death was dissociated from tangle formation in this natural 3D model of AD. Using genome wide expression analysis, we observed up-regulation of genes involved in myelination and down-regulation of genes related to memory and cognition, synaptic transmission and neuron projection. This novel chimeric model for AD displays human-specific pathological features and allows the analysis of different genetic backgrounds and mutations during the course of the disease.

INTRODUCTION

Aloïs Alzheimer described in 1906 the main pathological hallmarks of Alzheimer's disease (AD): amyloid-β (Aβ) plaques, neurofibrillary pathology and tangles, astrogliosis and neuronal loss (Alzheimer, 1906). Cerebrovascular amyloid angiopathy, microgliosis, inflammation and major synaptic alteration are other pathological features of AD (Katzman, 1986; McGeer et al., 1988; Crews and Masliah, 2010; Spillantini and Goedert, 2013)**.**

The amyloid hypothesis links abnormally folded Aβ peptides in a linear and causal cascade to the other disease hallmarks, including neuronal tangle formation and

neuronal cell death (Hardy and Selkoe, 2002; critique in De Strooper and Karran, 2016). This hypothesis has provided the theoretical basis for the generation of numerous animal models, diagnostics and therapeutics for AD. However, evolving insights have made increasingly clear that a more complex theory and more elaborate *in vivo* models are needed to understand the long prodromal phase of the disease (De Strooper and Karran, 2016). Rodent AD models (Ashe and Zahs, 2010; LaFerla and Green, 2012) usually over-express mutated forms of the familial AD (FAD) causing genes *APP* and/or *PSEN,* leading to extensive amyloid plaque deposition, Aβ-associated neuroinflammation, and some synaptic dysfunction. However, crucial aspects of the disease process, like neuronal tangle formation and severe neuronal cell loss, have never been convincingly demonstrated in rodents (Kokjohn and Roher, 2009; Morrissette et al., 2009; Crews and Masliah, 2010). The relevance of these genetic models to study sporadic AD, where patients do not carry mutations in *APP* or *PSEN* is debated.

Human embryonic stem cells (ESC) and patient-derived induced pluripotent stem cells (iPS) allow modeling of human neurological disease in a human genetic context (Brennand et al., 2015; Paşca et al., 2014; Studer et al., 2015; van den Ameele et al., 2014)**.** Neurons derived from iPS cells of FAD patients showed altered $A\beta_{42}/A\beta_{40}$ ratio and abnormal Tau phosphorylation (Yagi et al., 2011; Israel et al., 2012; Shi et al., 2012; Kondo et al., 2013; Choi et al., 2014; Muratore et al., 2014; Hu et al., 2015). Recently, a three-dimensional, human neural stem-cell derived culture (Choi et al., 2014) displayed Aβ plaque-like structures and Tau silver positive aggregates (Choi et al., 2014). The cells heavily overexpress exogenous *APP* and *PSEN* FAD genes. No cell loss was detected and neuroinflammatory and vascular components of the disease are lacking in this *in vitro* model. It remains indeed crucial to

complement *in vitro* approaches with *in vivo* experiments to study human neurons in the context of the diseased brain.

Neural precursors derived from PSCs (reviewed in (Suzuki and Vanderhaeghen, 2015)) can be transplanted into the rodent brain, resulting in specific patterns of cortical neuronal maturation, connectivity and synaptic activity, well beyond what can be achieved in a purely *in vitro* condition (Gaspard et al., 2008; Espuny-Camacho et al., 2013). We use here this approach to investigate whether Aβ species generated in an AD mouse model (Radde et al., 2006) are sufficient to induce full AD pathology in non-affected, genetically non-manipulated human neurons. This chimeric model presents numerous Aβ plaques and Aβ-associated neuroinflammation in the human transplant and, importantly, the transplanted neurons show remarkable signs of neurodegeneration. These pathological features are not detected or are far less important in the mouse host brain and in transplanted PSC-derived mouse neurons. Thus human neurons respond to Aβ pathology differently from their murine counterparts *in vivo*.

RESULTS

Human grafted neurons integrate into the mouse brain and are exposed to β-amyloid

We differentiated GFP-expressing human PSCs into cortical precursor cells *in vitro* to implant them as xenografts into the brains of newborn mice (Figure 1 A, Figure S1 E-F) (Espuny-Camacho et al., 2013). We used transgenic Tg (Thy1-APPSw,Thy1-PSEN1*L166P) 21Jckr, further called APP/PS1-21, mice (Radde et al., 2006) and

crossed them with immunodeficient NOD.CB17-Prkdc^{scid}/J, further called NOD-SCID mice (Shultz et al., 1995), to generate AD mice or WT littermates suitable for grafting experiments (Espuny-Camacho et al., 2013). We additionally used GFP-expressing mouse neurons derived from murine PSC as controls (Figure S2 H-I) (Gaspard et al., 2008).

The cells present a normal karyotype (Figure S1 D) or CGH array (not shown) and undergo neuronal and telencephalic/cortical specification *in vitro* as attested by beta III tubulin, Tau, Tbr1 and Ctip2 staining (Figure S1 A-C). The transplanted human neurons express GFP and human-specific markers like Hu Nuclei (Figure S1 G-H) and present cortical (Tbr1$^+$, CTIP2$^+$, Satb2$^+$, Brn2$^+$); telencephalic (Foxg1$^+$), and mature neuronal (NeuN$^+$, MAP2$^+$) identities (Figure 1, B-G, Figure S1 I-J). Electron microscopy combined with GFP-immunogold labeling shows numerous synapses between the human graft and the mouse host (Figure 1 H-I). RNA-seq analysis confirms broad expression of telencephalic, cortical, glutamatergic and some expression of gabaergic, astrocytic and oligodendroglia genes (Figure S1 K). Cholinergic, dopaminergic or neural crest derived cell identities are less abundant or absent (Figure S1 K).

Aβ-plaques are present in the AD mouse host brain (GFP negative), as assessed by Thioflavin, 6E10 and Congo Red staining (arrows; Figure 1 L-N, Figure S2 A-E). Congo Red$^+$ plaques show birefringence when using plane-polarized light (Figure S2 E). Importantly, Aβ plaques are detected within GFP$^+$ human neuronal clusters (arrowheads; Figure 1 L- M', Figure S2 A-B', D-E) and near single human neuronal cells integrated into the mouse host tissue (arrows; Figure 1 N, Figure S2 C). Aβ plaques within human clusters are more diffuse than mouse host dense-cored plaques, and are significantly smaller (Figure 1 O; 120±6 μm^2 in mouse host; 88±8

μm^2 in human grafts). No significant differences in number of Aβ plaques per unit surface are detected (Figure 1 P; 40±3 plaques/mm^2 in mouse host; 71±16 plaques/mm^2 in human grafts). Aβ reactivity is, as expected, not observed in WT mice (Figure 1 J-K).

Aβ plaque ultrastructure is very similar in the human transplants and in the mouse host: one or more cores of extracellular Aβ filaments (asterisks; Figure 1 Q-T) surrounded by dystrophic neurites (arrowheads). Immunogold labeling against human cell-specific markers, i.e. the GFP transgene or endogenous STEM121 marker, confirms that Aβ deposits (asterisk) and dystrophic neurites (DN) are within grafted human clusters (arrowheads point to immunogold particles; Figure S2 F-G). PSC-derived mouse neurons transplanted under similar conditions also present Aβ plaques and DNs inside murine clusters (Figure S2 H-K).

Thus, the transplanted neuronal clusters integrate into the mouse host tissue and are exposed to Aβ deposits produced by the AD mice.

Amyloid-β associated neuroinflammation in the human neuronal transplants

We detect $GFAP^+$ astrocytes (Figure 2 A-B,E-F) and $Iba\text{-}1^+$ microglia cells (Figure 2 C-D,G-H) clustered around $Thioflavin^+$ Aβ deposits in mouse host tissue (GFP^-; arrows) and human grafts in AD mice (GFP^+; arrowheads). Significant increases in the number of $GFAP^+$ astrocytes and $Iba1^+$ microglial cells are seen in AD vs WT mice (Figure 2 M-N), but mouse host and human transplants do not differ significantly. Microglia cells performing phagocytosis are detected (red arrowhead; Figure 2 K). Astrocytes and microglial cells within human clusters are mostly of host origin as attested by the lack of GFP immunoreactivity (arrowheads; Figure 2 I-J,K-L). Ultrastructural analysis of astrocytes and microglia cells in AD mice show an

activated hypertrophic morphology with 76% and 74% of cells showing enlarged and/or phagocytic phenotypes, respectively (Figure 2 P-T and R-V). These phenotypes are rarely found in WT mice (Figure 2 O,Q,T,V), indicating that the elicited immune response is induced by the Aβ-associated pathology and not a consequence of unexpected host-graft reactivity.

Thus, both grafted human neuronal clusters and mouse host tissue are similarly exposed to Aβ-associated neuroinflammatory responses characterized by astrocytic and microglial cell reactivity and recruitment to the Aβ plaque sites.

Extensive neuritic dystrophy and alterations of synaptic markers surrounding Aβ plaques in human neuronal transplants

We examined the grafted human neuronal clusters exposed to Aβ for signs of cellular or neuritic abnormalities. Dystrophic neurite structures (DNs) are found associated with Aβ plaques in both mouse host and human grafts in AD mice 4MPT (Figure 3 A-B,D,E), but not in WT mice (Figure 3 C,F). DNs present a globular structure (Figure 3 B,E) and a heterogeneous content of vesicles (red boxes), dense bodies (red arrowheads), mitochondria (blue boxes), and neurofilaments (green boxes) (Figure 3 G-N). Myelin alterations are also detected (arrowheads; Figure S4 K-L).

Ubiquitin staining confirms the presence of DNs around Thioflavin+ Aβ plaques in mouse host tissue and human clusters 4MPT (Figure 3 O-R) with 85% of Aβ plaques surrounded by DNs in human grafts vs 58% in mouse host (Figure 3 S). DNs are also detected in human clusters in AD mice by GFP or human-specific STEM121 staining (arrowheads; Figure 3 O, Figure 4 C, Figure S2 L-M) but are absent in human grafts in WT mice (Figure 4 A). We observe abnormal accumulations of the presynaptic markers synaptophysin (SYP) and vesicular glutamate transporter 1 (VGlut1) around

Aβ plaques in human clusters in AD mice (Figure 4 C-D,J-K), similar to the accumulations found in human AD brains (Brion et al., 1991) (Figure S3 A-D). These accumulations are absent in human clusters in WT mice (Figure 4 A-B,H-I) and are less important in mouse host or in mouse neurons grafted into AD mice (Figure 4 E-F,L-M, Figure S3 E-H), with 74% and 70% of Aβ plaques in human grafts surrounded by SYP and VGlut1+ structures, respectively, *vs* 23% and 39% in mouse host (Figure 4 G,N). The dendritic marker MAP2 reveals an area devoid of staining around Aβ plaques in human clusters in AD mice (Figure 4 O-P) that is minor in mouse host (Figure 4 Q-R). The absence of MAP2 is mirrored by the accumulation of SYP^+ or Tau^+ structures around Aβ plaques (Figure S3 I-P). The postsynaptic marker Homer1 confirms the reduction of dendritic staining around Aβ plaques in human neuronal clusters (Figure S3 Q-T).

Thus DNs surrounding Aβ plaques display abnormal accumulations of presynaptic and axonal proteins, while human dendritic and post-synaptic proteins become reduced. These neuritic changes are much more subtle in mouse host tissue or mouse neuronal clusters grafted in the AD mice.

Major degeneration and loss of human neurons *in vivo*

Robust neuronal loss is a crucial AD hallmark lacking from existing animal models (Kokjohn and Roher, 2009; Morrissette et al., 2009; Crews and Masliah, 2010). Remarkably, the density of human neurons at 6MPT, evaluated using GFP together with the pan nuclear marker TOPRO3 (Figure 5 A-F) or HuNuclei (Figure S4 A-F), is much lower in transplants in AD mice (Figure 5 D-F) than in WT mice (Figure 5 A-C), although similar amounts of cells were injected. In contrast, nuclei density is similar in mouse host tissue in AD (Figure 5 J-L) vs WT mice (Figure 5 G-I).

Quantification shows that the density of mouse host tissue neurons is not significantly changed (98% in AD vs 100% in WT) while the density of human neurons in AD mice is reduced to 54% compared to WT animals (Figure 5 M). The density of human neurons at 2MPT, before Aβ plaques are detected, is not different between AD and WT animals (Figure 5 M). Thus the human cells integrate normally and similarly in WT and AD mouse brain following transplantation and cell loss occurs specifically in AD animals at later stages when Aβ pathology is present *in vivo.*

Semithin sections of human transplants stained with Toluidine blue also show dense Aβ plaques, disorganized neuropil texture and reduced neuronal density in AD mice (Figure 5 N-O), while no such morphological alterations are seen in mouse host tissue and PSC-derived mouse grafts in AD mice. On the contrary, many healthy-appearing neurons are detected near Aβ plaques (Figure 5 Q, Figure S4 J). Ultrastructural analysis and quantification of neuron density confirm a significant decrease in the number of neurons in human grafts in AD at 6MPT (Figure 5 W; 1463 ± 64 neurons/mm^2 in grafts in WT vs 344 ± 31 neurons/mm^2 in grafts in AD), and the absence of significant differences in mouse host tissue at this age (Figure 5 W; 925 ± 47 neurons/mm^2 in host in WT vs 817 ± 37 neurons/mm^2 in host in AD).

Importantly, up to 33% of human neurons in AD mice show a necrotic phenotype (Figure 5 X) characterized by electron lucent nuclei with highly dispersed chromatin, swelling of cytoplasm, and swelling and disintegration of cytoplasmic organelles (Figure 5 S-S',T-T') (Naganska and Matyja, 2001; Ueda et al., 2007; Burattini and Falcieri, 2013). Enlarged mitochondria with disrupted cristae (red arrowheads), large vacuoles (blue arrowheads), and even rupture of the nuclear membrane (green arrowheads) are also seen (Figure 5 S'-T'). However, such phenotypes are completely absent in human neurons in WT mice (Figure 5 R-R',X). The effects

appear human-specific, as no signs of degeneration are observed in mouse host tissue or in PSC-derived mouse neurons in AD mice (Figure 5 V,X, Figure S4 N). Apoptotic cell death does not contribute strongly to the cell loss at this stage as no increased caspase-3 activated staining (not shown), or TUNEL labeling is observed (Figure S4 O-Z).

Overall, the human grafts exposed to Aβ undergo major neuronal loss and neurodegeneration *in vivo via* a necrosis-mediated mechanism. Moreover, this effect is species-specific as mouse host neurons or grafted mouse neurons exposed to the same Aβ load do not undergo degeneration.

Gradual appearance of the 4R Tau splicing form in human neurons *in vivo*

Adult murine brain mainly expresses the 4R Tau isoform containing four microtubule-binding repeats. During human brain embryonic development only the 3R splice form of Tau is detected, while the adult human brain expresses 3R and 4R Tau splice forms in a 1:1 ratio (Goedert and Jakes, 1990). We thus studied the expression pattern of Tau using specific antibodies raised against 3R or 4R Tau splice forms (Figure S5 M-N). After 2 and 4MPT, 3R Tau is expressed while 4R Tau is detected only in 0.7% of the human neurons (GFP^+ or $STEM121^+$). 4R Tau is widely expressed in mouse host tissue (Figure 6 A-B,E-F,A'-B',E'-F',M, Figure S5 A-C). However, at 6MPT, 89% of the human neurons express high levels of 4R together with 3R Tau (arrows; Figure 6 I-J,I'-J',M, Figure S5 G-I). In addition, RNA-seq analysis of human grafts in old animals (8 MPT) shows a 3R/4R Tau expression close to 1, similar to the ratio in adult human brain (Figure 6 N).

We wondered whether the appearance of 4R Tau could be accelerated in our model using two human iPS lines derived from a patient carrying the pathogenic Tau

mutation Ex10+16, (further indicated as Tau*) (Sposito et al., 2015). This mutation is considered to favor 4R Tau expression and causes Fronto-temporal dementia (FTD) (Hutton et al., 1998). Following *in vivo* transplantation, human Tau* neurons ($HuNCAM^+$) showed no 4R Tau expression at 2MPT (Figure 6 C-D,C'-D'). However, at 4MPT 37% of the human Tau* neurons expressed 4R together with 3R Tau isoforms (arrows; Figure 6 G-H,G'-H',M, Figure S5 D-F) in agreement with previous findings in vitro (Sposito et al., 2015). At 6MPT 84% of Tau* neurons highly expressed 4R together with 3R Tau (arrows; Figure 6 K-L,K'-L',M, Figure S5 J-L).

Human Tau* neurons transplanted into the AD mouse brain show similar phenotypes as described above. We detect numerous Aβ plaques within the grafts (Figure S6 A-H') and DNs (Figure S6 I-J). SYP^+ and $VGlut^+$ accumulations are surrounding Aβ plaques (Figure S6K-L'). Importantly, the density of grafted human Tau* neurons near Aβ plaques is reduced to 28% compared to WT animals (Figure 5 M, Figure S6 M-R). Toluidine blue stained semithin sections (Figure S6 S-T) and EM analysis (Figure S6 U-V) confirm neuronal loss and necrosis that are not seen in human Tau* neurons in WT mice.

Thus, major AD human-specific pathological hallmarks are reproduced with iPS-derived human neurons from a FTD patient transplanted into AD mice.

Presence of pathological Tau species in human neurons *in vivo*

We analyzed Tau pathology in the grafted human neurons. $AT8^+$ hyper-phosphorylated forms of Tau are found in the cell bodies of human neurons and human Tau* neurons (arrowheads; Figure 7 B,E) and in DNs adjacent to Aβ plaques (arrowheads; Figure 7 C,F) at 8 MPT in AD mice. Staining is similar to that found in AD human brain (arrows; Figure S7 J-O) and in AD mouse tissue (arrowheads;

Figure 7 H-I). In fact, more than 95% of Aβ plaques show AT8^{+} DNs in mouse host, human grafts and human Tau* grafts (Figure 7 S).

Importantly, MC1^{+} staining, which reveals a pathological Tau conformation present in human AD brain (Weaver et al., 2000), is also detected in few cell bodies of human neurons and human Tau* neurons (arrowheads; Figure 7 K,N and Figure S7 A-C') and in more than 96% of DNs surrounding Aβ deposits at 8 MPT in AD mice (arrowheads; Figure 7 L,O,T and Figure S7 D-F'), which is similar to human AD brain (arrows; Figure S7 P-U). However, no MC1 staining is found in mouse host tissue in AD mice (Figure 7 Q,R,T and Figure S7 G-I'), as previously described (Kokjohn and Roher, 2009; Morrissette et al., 2009). The lack of MC1 reactivity in AD mouse host is probably not explained by human-specificity of the antibody as it labels also mouse Tau in a pericyte deficient *APP* transgenic mouse model (Sagare et al., 2013). No AT8 nor MC1^{+} staining is detected in WT mice (Figure 7 A,D,G,J,M,P).

Cytoskeletal alterations with abnormal accumulation of straight 10 nm-wide neurofilaments are detected in DN of human neurons in AD mice 6-8 MPT (Figure S7 V-V'). In addition, Hirano bodies, paracrystalline inclusions with an array of symmetrically organized 8-10 nm filaments, are also present (Figure S7 W-W'). However, abnormal filaments in the form of paired helical filaments or twisted ribbons have not been detected.

These results show that even after 6-8 MPT the human neurons do not present definite tangle pathology although clear hyperphosphorylation and pathological conformational alterations of Tau are observed. Thus, there is major neurodegeneration even in the absence of full spread tangle pathology in the human neurons.

Transcriptome analyses reveal human specific signatures of AD

To assess global transcriptional changes in the human grafts in AD vs WT mice, we performed RNA-seq analysis at two time-points: 4-5 MPT (young) and 8 MPT (old mice). Global analyses of gene expression patterns reveal interesting alterations in several gene ontology categories (Ashburner et al., 2000). Genes involved in synaptic transmission, gated channel activity, and neuron projection (GO:007268, GO:0022836, GO:0043005) are significantly down-regulated at 8MPT in human neurons in AD vs WT mice (Figure 8 A, Figure S8, Table S5). Additionally, genes involved in cognition and learning and memory (GO:0050890, GO:0007611) also show a trend towards down-regulation. GO categories related to immune or inflammatory response (GO:0002376, GO:0006954) show a non-significant up-regulation. It should be noted that our analysis includes only the human gene expression programs in the transplanted neurons and not the murine derived immune cells of the brain (Figure 8 A, Figure S8). Genes from the GO category regulation of cell death (GO:0010941) are up-regulated at 8MPT. Representative genes among these categories are shown in Figure 8 B. Significant differential expression of mRNAs and non-coding RNAs is detected already at early but more at late stages in human transplants in AD *vs* WT mice (Tables S2-S5). Interestingly, among the most strongly up-regulated genes in the human neurons in AD mice at 8MPT (Table S4) are a couple of non-coding RNA genes: *LINC01007* and *RP11-89N17.4*. Genes involved in myelination (*MOBP, MAG*, *UGT8, MOG, MBP*) are also up-regulated in the transplanted human cells. Alterations in myelination are known to occur in AD (Ettle et al., 2015; Bartzokis, 2011), but few studies have investigated the role of oligodendrocytes and myelin in AD (De Strooper and Karran, 2016; Bartzokis et al., 2011). In the list of down-regulated genes we find *OTOF, GABRE, TAC1*, *PTK2B*,

and *CACNA1H* involved in neurotransmission and *RHGAP36* involved in vesicle transport and regulation (Table S5). Interestingly *COL25A1* also known as *CLAC* is down-regulated (Table S5). *CLAC* specifically binds to fibrillized Aβ and is present in Aβ plaques in AD patients (Hashimoto et al., 2002; Tong et al., 2010).

To further evaluate the relevance of our model for human AD, we compared the gene expression data from human neurons with a published dataset of human AD samples (Zhang et al., 2013). We performed co-expression clustering analysis using WCGNA (Langfelder et al., 2008) yielding modules of genes showing a similar expression pattern across samples (Figure 8 C). This analysis generated 37 modules from human transplants. The overlap of genes of these modules (left column) with the modules identified by Zhang et al. (right column) is determined using a Fisher exact test. These data demonstrate that the transplanted human neurons show transcriptional changes in gene modules that are also affected in the human AD brain (Zhang et al., 2013).

DISCUSSION

The generation of better *in vivo* models that closely resemble the pathological features present in the human AD brain is instrumental to test new hypotheses with regard to AD etiology and for the validation of new therapeutic approaches. Ideally, such a model would incorporate a human genetic background, be versatile and robust and include aspects such as innate inflammation, cellular heterogeneity and minimal use of transgenes to drive the pathogenesis (De Strooper and Karran, 2016).

In the current study, we transplanted cortical neuronal precursors differentiated from one normal ESC line (H9), and from two FTD iPSC lines derived from the same

patient, into the brain of a well-characterized AD mouse model. Although three cell lines is a limited number, all displayed similar profound degeneration and necrosis when exposed to Aβ in a large number of independent transplantation experiments. In addition, the human specific pathology was compared with host mouse neurons and transplanted PSC-derived murine neurons, which show much more resistance to Aβ pathology than the human neuronal counterparts.

At 4MPT already the human neurons show many signs of neurodegeneration. Dystrophic structures are found and presynaptic components like synaptophysin and vesicular glutamate transporter 1 accumulate, while the dendritic marker MAP2 and the postsynaptic marker Homer1 disappear around Aβ plaques.

At 6MPT a significant loss of human neurons is found to an extent that has never been reported for mouse neurons in the many existing models for AD. Neuronal density is strongly reduced (down to 54%) in human grafts in AD *vs* WT mice whereas no significant differences were observed in mouse host tissue. Importantly, at 2MPT similar densities of human neurons are observed in AD and WT mice, indicating that implantation is normal, and cell loss occurs only later over the course of the disease. EM analysis revealed that 33% of human neurons in AD mice show signs of degeneration at 6MPT. The cell loss appears to be mostly necrotic based on morphological criteria. Genome wide expression analysis of human transplants (8MPT) shows however up-regulation of a broader category of genes related to regulation of cell death. This GO category contains many genes that are only indirectly involved in the regulation of cell death processes. Further work will be needed to clarify whether some of the altered genes play causal roles in the cell death process we observed here.

Strikingly, this major neurodegeneration and human neuronal loss occur in the absence of detectable tangle pathology. It was recently suggested that the induction of neuronal tangles critically depends on the expression of 4R Tau as seen in the human adult brain (Choi et al., 2014). At 2 and 4MPT the human neurons express mainly 3R isoforms, but after 6MPT 4R Tau isoforms are expressed in 89% of the human neurons. In addition, the 3R/4R Tau ratio in human neurons comes close to 1 at 8MPT, as seen in the adult human brain. We detect Tau AT8, and MC1 hyperphosphorylation. MC1 detects definitely pathological conformational changes in Tau (Jicha et al., 1997), and MC1 staining is not readily detected in murine AD models. iPS cells expressing the Tau mutation Ex10+16 linked to FTD (Sposito et al., 2015) show an earlier appearance of 4R Tau, and a higher tendency to cell death when exposed to Aβ. The data are in line with the idea that Tau expression is crucial for Aβ induced toxicity in AD transgenic animals (Roberson et al., 2007; Leroy et al., 2012). However, we did not detect Tau tangles after 8MPT in human neurons nor in human Tau* neurons. Likely even longer periods *in vivo* are required for detectable tangle pathology, or additional seeding is needed to induce the tangle conformation. The NOD-SCID background needed for the experiments does unfortunately not allow analysis beyond 8MPT due to increased incidence of graft-unrelated tumor formation.

Recently, a three-dimensional *in vitro* AD model with human neuronal stem cells that overexpress mutated FAD genes was reported (Choi et al., 2014). This model uses matrigel as a 3D support matrix for the cells, and recapitulates Aβ pathology. The model also presents pathological Tau phosphorylation and Tau filaments but fails to show neurodegeneration or cell death. We have not modified our neurons with APP or PSEN mutations and neurons were grown within the brain, which is arguably a more natural situation than the matrigel used in the cell culture system. It is intriguing

that Tau aggregation was detected in the cell culture system without neurodegeneration, while in vivo neurodegeneration is seen without tangle formation. Further investigations are needed to understand better the relationship, if any, between abnormal Tau conformation and cell death.

A particular important aspect of the current approach is that innate immunity, believed to play an important role in AD (Heneka et al., 2015), remains apparently intact in the model. As shown, host-activated astrocytes and microglia cells are recruited to the human transplants, and similar neuroinflammatory responses are observed in human clusters and mouse host. Activated microglia cells have been proposed as essential for the cell loss in AD (Fuhrmann et al., 2010) and this might explain the lack of cell loss in the 3D cell culture system discussed above.

Lastly, our model allows a genome-wide transcriptome analysis of the human neurons exposed to Aβ at early and late stages of the disease *in vivo*. Intriguingly, we observe two non-coding-RNA sequences among the most up-regulated genes. *RP11-89N17.4* expression might be altered in AD patients (Gui et al., 2015), and data from our lab show dysregulation of *LINC01007* in AD patients (unpublished results). While these findings are still premature, it should motivate additional work exploring the role of non-coding RNAs in neurodegeneration (Salta and De Strooper, 2012). We also notice that *COL25A1* is down-regulated at late stages. Interestingly, rare variants in *COL25A1* have been associated with healthy aging and possible protection against AD (Erikson et al., 2016). Other up-regulated genes are involved in cell death and myelination while many down-regulated genes are implied in memory and cognition, synaptic transmission, gated channel activity, neurotransmitter levels and neuron projection. We validated our model comparing our results with the results of a previous genome-wide gene expression study of AD patient brains (Zhang et al.,

2013). The good concordance, taking into account that Zhang et al. provided data on total brain blocks from patients, while we analyzed gene expression changes in human neurons specifically, is encouraging.

In conclusion, we present here a novel *in vivo* approach to investigate AD mechanisms in human neurons by generating a mouse/human brain chimera. This approach opens the door to explore how different human genetic backgrounds modulate AD related features *in vivo*. This initial work demonstrates clearly the importance of a human molecular and cellular background to observe full neurodegeneration in the presence of Aβ plaques.

Author contributions:

IEC, AMA, IT, PV, and BDS conceived the study and planned experiments. IEC and AMA performed the experiments with the help of MF, AS, KA, SM, JB, LL, NC, LO, EV, ER, SW, AE, JH, KL, JPB. All authors interpreted data. IEC, AMA, PV and BDS wrote the first version of the manuscript. All authors contributed to and approved the final version. RNAseq data analysis was performed by MF.

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FIGURE LEGENDS

Figure 1. Human grafted neurons integrate into the mouse brain and are exposed to Aβ

(A) Schematic representation of the experimental outlay. (B-G) GFP^+ grafted human neurons stained with the GFP antibody (green, B-G) and with the cortical deep layer markers Tbr1 (B) and CTIP2 (C), the upper layer markers Satb2 (D) and Brn2 (E), the telencephalic marker FoxG1 (F) or the mature neuronal marker MAP2 (G). (H-I) Electron microscopy combined with GFP immunogold (arrowheads) shows synaptic contacts from the human transplant to the mouse host tissue (H), and reverse (I) 4MPT. (J-N) GFP (green) and Thioflavin-S (red) stainings show Aβ plaques within human transplants (arrowheads) and in mouse host tissue (arrows) in AD mice (L-N) but not in WT mice (J-K) 4MPT. Panel M' relates to panel M. Panel N shows an isolated GFP^+ neuron close to Aβ plaques (arrows). (O-P) Quantification of Aβ plaque mean area (O) and of the number of Aβ plaques per area (P) in mouse host (n=5) and human grafts (n=5) in AD mice 4MPT. Data are represented as mean +/- SEM, Student's t-test:**$p<0.01$, ns= non-significant. (Q-T) EM images show Aβ deposits (asterisks) within human transplants or mouse host tissue in AD mice 4MPT. Scale bars: 25 µm (B-G, J-N); 500nm (H-I); 2 µm (Q); 1 µm (R); 10 µm (S); and 5 µm (T).

Figure 2. Aβ associated neuroinflammation in the human neuronal grafts

(A-H) Representative micrographs of mouse host tissue (A-D) and GFP^+ human grafts (green, E-H) in AD mice. $GFAP^+$ astrocytes or $Iba1^+$ microglia cells (red) are associated with $Thioflavin^+$ Aβ plaques (blue) 4MPT and 6MPT. Arrows and arrowheads show Aβ plaques in mouse host tissue and within human grafts, respectively. (I-L) GFP^- astrocytes and microglial cells (arrowheads; I-L) are present inside human grafts. Notice host, GFP^-, reactive microglia in phagocytic state (red arrowhead; K). (M-N) Quantification of the number of $GFAP^+$ astrocytes or $Iba1^+$

microglia cells per area in mouse host (WT: n=3 animals 4MPT, n=4 6MPT; AD: n=3 animals 4MPT, n=4 6MPT) and human grafts (WT: n=3 animals 4MPT, n=4 6MPT; AD: n=3 animals 4MPT, n=4 6MPT). Data are represented as mean +/- SEM, two way-ANOVA with Bonferroni posttests: *p<0.05; **p<0.01; ***p<0.001. (O-V) Ultrastructure of hypertrophic reactive astrocytes (P,S) and reactive microglia (R,U) in human grafts 4MPT and 6MPT in AD mice. Notice non-reactive astrocytes and microglia in WT mice (O,Q) and phagocytosing microglia adjacent to dystrophic neurites (R) or degenerating material (U) in human grafts in AD mice. The cytoplasm of these cells has been outlined (O-U). DN: dystrophic neurite; m: microglia. (T,V) Quantification of the percentage of glial cells showing hypertrophic and/or phagocytic morphology in human grafts in WT (n=3) and AD (n=3) mice 6MPT. Data are represented as mean +/- SEM, Student's t-test: ****p<0.0001. Scale bars: 25 μm (A-L); 2 μm (P-S) and 5 μm (O,U).

Figure 3. Neuritic dystrophy surrounding Aβ plaques in human neuronal transplants in AD mice

(A-B,D-E) Ultrastructure of dystrophic neurites (DNs, arrowheads) around Aβ plaques (asterisks) in human grafts and mouse host tissue in AD mice. (C,F) Ultrastructure of axons (red arrowheads) and dendrites (blue arrowheads) in human grafts and mouse host tissue in WT mice. (G-N) Heterogeneous content of DNs in human grafts and mouse host: accumulations of vesicles (red boxes; G,H), dense bodies (arrowheads; I,J); mitochondria (blue boxes; K,L) and neurofilaments (green boxes; M,N). (O,R) Ubiquitin$^+$ DNs (red) close to Thioflavin$^+$ Aβ plaques (blue) in GFP$^+$ human grafts (green) and mouse host tissue in AD mice 4MPT. (S) Quantification of the percentage of Aβ plaques surrounded by ubiquitin$^+$ DNs in mouse host (n=5) and

human grafts (n=5) in AD animals 4MPT. Data are represented as mean +/- SEM, Student's t-test: ****p<0.0001. Scale bars: 25 µm (O-R), 5µm (A), 2 µm (B, D-F, J-L, N); 1 µm (C, G, I, M) and 500 nm (H).

Figure 4. Specific accumulation of neuritic/synaptic markers around Aβ plaques in human neuronal grafts in AD mice

(A-M) Representative images of human clusters in WT and AD mice and AD mouse host tissue stained with GFP (green), Thioflavin (blue) and synaptophysin (SYP, red; B,D,F) or VGlut1 (red; I,K,M) 4MPT. SYP^+ and $VGlut1^+$ accumulations around Aβ plaques in GFP^+ human clusters in AD mice (arrowhead; D,K). Minor accumulations in mouse host tissue in AD mice (arrow; F,M). (G,N) Quantification of the percentage of Aβ plaques surrounded by SYP^+ or $VGlut^+$ accumulations in mouse host (n=5) and human grafts (SYP: n=5, VGlut1: n=4) in AD mice. Data are represented as mean +/- SEM, Student's t-test: **p<0.01; ****p<0.0001. (O-R) Representative images of human clusters and mouse host tissue in AD mice stained with GFP (green), Thioflavin (blue) and MAP2 (red) 4MPT. GFP^+ human clusters show lack of MAP2 around Aβ plaques (arrowheads), while minor effects were detected in the mouse host tissue (arrows). Scale bars: 25 µm (A-R).

Figure 5. Neurodegeneration and neuronal loss in human but not in murine transplants in AD mice

(A-L) Representative images of human grafts (A-F) and mouse host tissue (G-L) in WT and AD mice stained with GFP (green), Thioflavin (blue) and the nuclear marker TOPRO3 (red) 6MPT. (A-F) Areas devoid of $TOPRO3^+$ cells are evident within the human grafts in AD mice (F) together with abundant Aβ plaques within human clusters and in surrounding host tissue (arrowheads and arrows, E). In contrast,

homogeneous staining of mouse nuclei is seen in AD mice (L) even in the presence of Aβ plaques (arrows, K). Human grafts and mouse host tissue in WT mice show homogeneous staining of nuclei (C,I) and no Aβ plaques (B,H). (M) Percentage of relative cell density (number of nuclei per area) of mouse host tissue neurons (AD n=4 animals; WT n=4 animals), human neurons 2MPT (AD n=4 animals; WT n= 3 animals), human neurons 6MPT (AD n= 4 animals; WT n= 4 animals) and human Tau* neurons 6MPT (AD n= 3 animals; WT n= 3 animals) in AD animals compared to the density in WT animals expressed as 100%. Data are represented as mean +/- SEM, one way-ANOVA with Bonferroni posttests: **p<0.01, ***p<0.001, ns= non-significant. (N-Q) Toluidine blue stained semithin sections of human clusters and mouse host tissue in WT and AD mice 6MPT. Human clusters in AD mice (O) show accumulation of dense Aβ plaques (asterisks), disorganized neuropil texture and reduced neuronal density. AD mouse host tissue (Q) show accumulation of dense Aβ plaques (asterisks) but no gross changes in neuropil texture or reduced cell density compared to WT (P). Arrowheads point to neurons. (R-V) Ultrastructure of grafted human and mouse host neurons in WT and AD mice 6MPT. R', S' and T' relate to R, S and T. DN: dystrophic neurites. Signs of neurodegeneration consistent with necrosis are specifically observed in human neurons grafted in AD mice (S, S', T, T'). Red, blue and green arrowheads point to degenerating mitochondria, vacuolar structures and disrupted nuclear membranes, respectively. (W) Quantification of neuronal density expressed as the number of neurons per area in mouse host (WT n=3, AD n=3) and human grafts (WT n=3, AD n=3) 6MPT. Data are represented as mean +/- SEM, two way-ANOVA with Bonferroni posttests: ****p<0.0001; n.s = non-significant. (X) Quantification of the percentage of dying neurons in mouse host (WT n=3, AD n=3) and human grafts (WT n=3, AD n=3) 6MPT. Data are shown as mean

+/- SEM, two way-ANOVA with Bonferroni posttests: ****p<0.0001; n.s = non-significant. Scale bars: 25 μm (A-L); 50μm (N-Q); 5μm (R,T,V); 2μm (S,U,R'); 1 μm (S',T').

Figure 6. Temporal pattern of 3R and 4R Tau expression in human neurons grafted *in vivo*

(A-L') Grafted human neurons (A-B,E-F,I-J) detected with GFP (green) or grafted human Tau* neurons (C-D,G-H,K-L) detected with HuNCAM (green), and stained for 4R Tau (red) after 2,4,and 6MPT. Higher magnification images are shown in A'-L'. Arrows show 4R Tau$^+$ human neurons. (M) Quantification of the percentage of 4R Tau$^+$ cells in human grafts (n=3 animals 4MPT; n=6 6-8MPT) and human Tau* grafts (n=4 animals 4MPT; n=2 6-8MPT). Data are represented as mean +/- SEM, two way-ANOVA with Bonferroni posttests: **p<0.01, ***p<0.001. (N) Quantification of the RNAseq data for 3R/4R Tau ratio in human grafts in young (4-5 MPT) (n=5 WT, 7 AD) and old (8 MPT) (n=2 WT, 5 AD) animals. Results are shown as mean +/- SEM, Student's t-test: ***p<0.0001. Scale bars: 25 μm (A-L').

Figure 7. Tau hyperphosphorylation and abnormal Tau conformational changes in grafted human neurons

(A-R) Immunohistochemistry with AT8 (brown; A-I), or MC1 (brown; J-R) of human grafts, human Tau* grafts, mouse host tissue and non-grafted mouse brain tissue in WT and AD mice. AT8$^+$ neuronal cell bodies (arrowheads; B,E) and DNs around Congo Red$^+$ Aβ deposits (pink, asterisks) (arrowheads; C,F,H,I) in human grafts (B-C), human Tau* grafts (E-F) and in mouse host tissue (H-I) in AD animals. MC1$^+$ neuronal cell bodies (arrowheads; K,N) and DNs around Congo Red$^+$ Aβ deposits (pink, asterisks) (arrowheads; L,O) are found in human grafts and human Tau* grafts

in AD mice but not in mouse host tissue (Q,R). Arrowheads point to soma and DNs$^+$ structures. Asterisks mark Congo Red$^+$ Aβ plaques. Counterstaining with hematoxylin (blue). (S-T) Percentage of Aβ plaques surrounded by AT8$^+$ and MC1$^+$ DNs in human grafts (n=4), human Tau* grafts (n=3) and mouse host tissue (n=3) in AD mice. Results are shown as mean +/- SEM, one-way ANOVA with Kruskal-Wallis test, ****p<0.0001; ns = non-significant. Scale bars: 50µm (A-R).

Figure 8. Transcriptional changes in human neurons grafted *in vivo*

(A) Genes ranked according to the significance of the differential expression of up- (purple) and down-regulated (blue) genes for young (4-5MPT) and old (8MPT) human grafts. The x-axis shows the enrichment score according to the Gene Set Enrichment Analysis (GSEA). Significance of enrichment: ***p<0.001; **p<0.01; *p<0.05. (B) Log fold change of differential expression (brown: down- and green: up-regulation) of a selected set of genes in young and old samples. P-values are corrected for multiple testing: ***p<0.001; **p<0.01; *p<0.05. (C) WCGNA co-expression modules compared to the modules from Zhang et al., 2013. Four extra gene sets (prefix: DE) from the most differentially expressed genes (up & down in old & young mice) are included. Significance of overlap was determined by a Fisher exact test and Benjamini Hochberg multiple testing correction. The plot depicts the 22 modules (out of a total of 37) that show a significant overlap with the Zhang modules, indicating the best matching Zhang module on the right. The bars indicate the adjusted p-value.