Alzheimer's disease-related amyloid-β induces synaptotoxicity in human iPS cell-derived neurons

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Human induced pluripotent stem cell (iPSC)-derived neurons have been proposed to be a highly valuable cellular model for studying the pathomechanisms of Alzheimer's disease (AD). Studies employing patient-specific human iPSCs as models of familial and sporadic forms of AD described elevated levels of AD-related amyloid-β (Aβ). However, none of the present AD iPSC studies could recapitulate the synaptotoxic actions of Aβ, which are crucial early events in a cascade that eventually leads to vast brain degeneration. Here we established highly reproducible, human iPSC-derived cortical cultures as a cellular model to study the synaptotoxic effects of Aβ. We developed a highly efficient immunopurification procedure yielding immature neurons that express markers of deep layer cortical pyramid neurons and GABAergic interneurons. Upon long-term cultivation, purified cells differentiated into mature neurons exhibiting the generation of action potentials and excitatory glutamatergic and inhibitory GABAergic synapses. Most interestingly, these iPSC-derived human neurons were strongly susceptible to the synaptotoxic actions of Aβ. Application of Aβ for 8 days led to a reduction in the overall FM4-64 and vGlut1 staining of vesicles in neurites, indicating a loss of vesicle clusters. A selective analysis of presynaptic vesicle clusters on dendrites did not reveal a significant change, thus suggesting that Aβ impaired axonal vesicle clusters. In addition, electrophysiological patch-clamp recordings of AMPA receptor-mediated miniature EPSCs revealed an Aβ-induced reduction in amplitudes, indicating an impairment of postsynaptic AMPA receptors. A loss of postsynaptic AMPA receptor clusters was confirmed by immunocytochemical stainings for GluA1. Incubation with Aβ for 8 days did not result in a significant loss of neurites or cell death. In summary, we describe a highly reproducible cellular AD model based on human iPSC-derived cortical neurons that enables the mechanistic analysis of Aβ-induced synaptotoxic pathomechanisms and the development of novel therapeutic approaches.

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In Alzheimer's disease (AD), synapse loss and synapse loss are thought to underlie cognitive deficits. 1 Oligomers of the amyloid-β (Aβ) peptide appear to induce synaptic failure as an early event in the etiology of AD. 5–7 However, despite its well-established synapse-impairing effects in rodent models, 5–7 the synaptotoxic actions of Aβ most relevant for the human disease have not been identified in a human model system. Several studies have investigated the synaptotoxic effects of Aβ in cultured rodent neurons and in transgenic mouse models revealing a multitude of potential mechanisms affecting synapses. Postsynaptic Aβ actions result in the loss of functional (α-amino-3-hydroxy-5-methyl-4-isoxazo-l-propionic acid (AMPA)-type) glutamate receptors, 8–10 involve long-term depression-like mechanisms, 9,11,12 and lead to the degradation of the entire postsynapse (dendritic spines). 9,11,13 In addition, several distinct presynaptic Aβ actions on the synaptic vesicle cycle have been described. 10,14 Furthermore, Aβ-induced impairments of axonal transport regulation and Aβ-induced axon degeneration have been found in rodent neurons. 15,16,17 This puzzling diversity of Aβ-induced synapse-related defects raises the question whether all of them are involved in the early pathomechanisms of human AD.

In addition to well-established animal systems, the modeling of human neurological disease pathologies by human induced pluripotent stem cell (hiPSC) technology 18 has been proposed as an innovative approach. 19–21 The in vitro differentiation of hiPSCs to excitable neurons has been reported using a variety of protocols. 22–24 However, quantitative analysis of both functional glutamatergic and GABAergic synapses has been difficult to achieve. 19,25,26 In addition to studying the functional properties of iPSC-derived human neurons from healthy individuals, the in vitro differentiation of

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid; APP, amyloid precursor protein; BP, binding immunoglobulin protein; CHO, Chinese hamster ovary; CHOP, CCAAT-enhancer-binding protein homologous protein; Ctbp2, chicken ovalbumin upstream promoter transcription factor-interacting protein 2; DNOX, 6,7-dinitroquinoxaline-2,3-dione; EB, embryoid body; EPSC, excitatory postsynaptic current; ER, endoplasmic reticulum; FM4-64, 6-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide; GABA, γ-aminobutyric acid; GAD67, glutamic acid decarboxylase 67; iPS, induced pluripotent stem cell; LC3, microtubule-associated protein 1A/1B-light chain 3; MAP2, microtubule-associated protein 2; mIPSC, miniature postsynaptic current; NCAM, neural cell adhesion molecule; PaB, paired box protein 6; PSD95, postsynaptic density protein 95; S Altz, special ARF sequence-binding protein 2; Tbr1, T-box, brain; 1, TTX, tetrodotoxin; UPR, unfolded protein response; WAMP1, vesicle-associated protein 2; vGAT, vesicular GABA transporter; vGlut1, vesicular glutamate transporter 1

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patient-derived iPSCs has been used to model complex neurodevelopmental and neurodegenerative diseases.\textsuperscript{19,27,28} Recently, iPSCs derived from AD patients have been reported to exhibit increased secretion of A\textbeta\ upon in vitro neuronal differentiation; however, neither a loss of synapses nor an impairment of synapse function was detected.\textsuperscript{21,29–33} Here we describe a hiPSC-based, carefully optimized in vitro differentiation protocol, including a novel immunopanning step, which enabled us to study the deleterious effects of application of A\textbeta\ on human cortical neurons and on human synapses.

**Results**

Neural differentiation of hiPSCs and immunopurification of hiPSC-derived immature neurons. hiPSCs were cultured (Supplementary Figure S1) and in vitro differentiated using an embryoid body (EB) system similar to published protocols.\textsuperscript{22} After initial differentiation, EBs were plated on a matrigel substrate leading to the formation of paired box protein 6 (Pax6)-expressing neuroepithelial rosettes (Supplementary Figure S2) that further differentiated to heterogeneous cultures also containing non-neuronal cells (Figures 1a and b). After 6–8 weeks of in vitro differentiation, heterogeneous cultures were dissociated to single cells, which were subjected to immunopurification. Classical immunopanning\textsuperscript{34} with specific modifications was performed using the neural cell adhesion molecule (NCAM) antibody VIN-1S-53 to isolate immature neurons expressing NCAM at a high level. To quantify immunopurification efficiency, dissociated cells without immunopurification (control), dissociated cells isolated by NCAM immunopurification, and dissociated cells non-adherent to the panning plates, respectively (Figure 1c), were immunocytochemically stained for NCAM and the neuronal marker microtubule-associated protein 2 (MAP2) 1 day after immunopurification (Figures 1d and e). The fraction of MAP2-positive cells was strongly increased in cells isolated by NCAM immunopurification (91.2 ± 4.3%) as compared with control cells (28.1 ± 20.6%) and to cells non-adherent to the panning plates (12.2 ± 7.4%) (Figure 1g). The fraction of NCAM-positive cells was also increased by immunopanning (Figure 1f); however, as expected from the low level NCAM expression in neural precursor cells, the increase was less pronounced as compared with MAP2. We next characterized the immunopurified immature neurons using immunocytochemistry. Staining for cortical marker proteins revealed that the vast majority of MAP2-positive cells expressed markers of deep layer cortical neurons (Cltp2 (chicken ovalbumin upstream promoter transcription factor-interacting protein 2), Tbr1 (T-box, brain, 1)), while only 5.0 ± 1.4% of the MAP2-positive neurons expressed the upper layer marker special AT-rich sequence-binding protein 2 (Satb2; Figures 1h and i). Similar to the composition of neuronal cell types in the in vivo cortex, 15.7 ± 1.7% of the MAP2-positive neurons were GABAergic (glutamic acid decarboxylase 67 (GAD67) positive) (Figures 1h and i). Survival of immature neurons was not affected by the immunopurification procedure (Figure 1c). In summary, NCAM immunopurification of hiPSC-derived heterogeneous cultures resulted in highly purified MAP2-positive immature deep-layer cortical neurons.

**Morphological maturation and action potential generation in hiPSC-derived neurons purified by immunopanning.** Further cultivation of the MAP2-positive immature human neurons led to the formation of extended neurites after 1 week and to the formation of a dense neuritic network of mature neurons exhibiting enlarged somata at 8 weeks after immunopurification (Figure 2a). These cultures contained only very few glial cells due to the inhibition of proliferation of non-neuronal cells (see Materials and Methods). To study whether morphologically matured iPS-derived human neurons exhibit essential functional properties typical of cultured cortical neurons, we performed a basic electrophysiological characterization. Whole-cell patch-clamp recordings (at 8 weeks after immunopurification) of the membrane potential (57.8 ± 0.7 mV resting potential) in current-clamp mode revealed the generation of action potentials upon injection of depolarizing current in all neurons tested (Figure 2b). To demonstrate the expression of voltage-dependent Na\textsuperscript{+} currents, we did whole-cell recordings in voltage-clamp mode at a holding potential of −60 mV. Step depolarizations of the membrane potential elicited typical inward Na\textsuperscript{+} currents that were blocked by addition of tetrodotoxin (TTX; 1 μM; Figure 2c). Thus, electrophysiological analysis revealed essential functional properties such as electrical excitability and TTX-sensitive voltage-dependent Na\textsuperscript{+} currents in hiPSC-derived neurons at 8 weeks after immunopurification.

**Characterization of functional synapses in mature hiPSC-derived neurons purified by immunopanning.** At this stage of maturation, the formation of synaptic structures was indicated by vesicle clusters immunocytochemically stained for the synaptic vesicle-associated proteins VAMP2 (vesicle-associated membrane protein 2)/synaptobrevin and...
synapsin I on dendrites (Figures 3a and b). The presence of both glutamate and γ-aminobutyric acid (GABA) containing presynaptic vesicle clusters was confirmed by a punctate immunostaining on dendrites for the vesicular glutamate transporter 1 (vGlut1, colocalized with postsynaptic postsynaptic density protein 95 (PSD95) puncta) and for the vesicular GABA transporter (vGAT), respectively (Figures 3c and d). Moreover, the formation of functional synapses was indicated by spontaneous miniature postsynaptic currents (mPSCs) that were observed by whole-cell patch-clamp.
recording at −60 mV holding potential (Figures 3e and f).

We further characterized spontaneous mPSCs pharmacologically by using specific antagonists. AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were isolated by addition of gabazine (10 μM) and TTX (1 μM), and were blocked by the AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM; Figure 3e). GABA receptor-mediated mPSCs were isolated by addition of DNQX and TTX and were blocked by addition of gabazine (Figure 3f). This demonstrates the presence of both functional glutamatergic and GABAergic synapses. Taken together, our findings demonstrate that at 8 weeks after immunopurification our iPSC-derived neurons exhibit essential functional properties, such as excitability, synaptic activity, and functional glutamatergic and GABAergic synapses.

Aβ induced loss of cycling vesicle clusters in iPSC-derived human neurons. To investigate whether functional synapses in iPSC-derived human neurons are susceptible to the deleterious effects of Aβ, we added Aβ contained in the supernatant from cultures of 7PA2 Chinese hamster ovary (CHO) cells (expressing human APP751 carrying the familial amyloid precursor protein (APP) V717F mutation)35,36 to hiPSC-derived neurons at 8 weeks after immunopurification. Conditioned medium from 7PA2 cells was diluted 1:1 with standard culture medium.37 To test for potential unspecific effects of the 7PA2 supernatant, we immunodepleted Aβ from the 7PA2 conditioned medium by using the anti-Aβ monoclonal antibody IC16.37,38 Aβ-induced defects were analysed 8 days after Aβ application.

First, we studied the effects of Aβ on cycling synaptic vesicle clusters in the processes of iPSC-derived human neurons. Cycling vesicle clusters were stained by the fluorescent dye FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide).39 Uptake of extracellularly added FM4–64 (10 μM) in recycling vesicles
fluorescence signal demonstrating a specific effect of Aβ contained in the 7PA2-conditioned medium. The Aβ-induced reduction of the overall FM4–64 fluorescence signal was largely due to a reduction in the number of FM4–64-stained puncta (vesicle clusters) per area of neuropil as indicated by plotting FM4–64 puncta density versus the mean total fluorescence intensity per punctum (Figure 4b). We also studied the destaining of FM4–64-stained vesicle clusters by triggering vesicle re-exocytosis with extracellular electrical stimulation (1200 stimuli at 20 Hz (for 60 s); Figure 4c). The mean destaining kinetics of FM4–64 puncta were not significantly affected by addition of Aβ, thus further supporting that a reduction in the number of vesicle clusters was the major Aβ-induced alteration.

To further confirm an Aβ-induced impairment of vesicle clusters, we immunocytochemically stained iPSC-derived human neurons for the synaptic vesicle marker vGlut1 after incubation with Aβ. Again, we observed a significant reduction in the density of vGlut1 immunopositive puncta (vesicle clusters) in neurites of Aβ-treated neurons as compared with vehicle-treated control cultures (Figures 4d and e). Addition of immunodepleted 7PA2 supernatant did not affect the number of vGlut1 puncta, again demonstrating a specific effect of Aβ contained in the 7PA2-conditioned medium. To check for Aβ-induced changes in the density of processes and in cell density, we fluorescently stained iPSC-derived human neurons by addition of the permeable dye calcein-AM directly following the FM4–64 staining experiment, the number of neuronal cell bodies and the density of processes (determined by counting processes crossing a randomly chosen line) were not significantly affected (Figures 4g and h). In summary, our results indicate that addition of Aβ results in a strong reduction in the density of vesicle clusters in the neurites of iPSC-derived human neurons.

**Aβ induced functional impairment of glutamatergic synapses in iPSC-derived human neurons.** We next studied whether the addition of Aβ induced deleterious changes at *bona fide* synaptic sites contacting postsynaptic dendrites. To focus on presynaptic vesicle clusters, we reanalysed the FM4–64 puncta (from the FM4–64 staining/destaining experiments described above) that contacted the proximal dendrites of iPSC-derived human neurons. Proximal dendrites arising from neuron somata were identified by calcein-AM staining of whole neurons after FM4–64 staining/destaining experiments, and FM4–64 puncta were analysed after thresholding the FM4–64 fluorescence image (Figure 5a). Eight days after addition of Aβ, we did not observe a significant change in the dendritic density of FM4–64 puncta or a significant change in total FM4–64 fluorescence intensity per punctum or a significant change in the overall presynaptic FM4–64 fluorescence signal per dendrite (Figure 5b). Furthermore, the quantitative analysis of the stimulation-induced destaining of FM4–64 puncta did not reveal any significant changes in exocytosis (Figure 5c).

We also reanalysed the presynaptic vGlut1 immunopositive puncta (described above) located on dendrites, which were identified by MAP2 co-immunostaining of dendrites (Figure 5d). Again, 8 days after addition of Aβ, we observed...
no significant changes in the density of vGlut1 puncta on dendrites (Figure 5e). Taken together, the analysis of FM4–64 fluorescent and of vGlut1 immunopositive puncta on dendrites thus revealed no significant presynaptic alterations at a relatively early phase of incubation with Aβ, suggesting that bona fide presynaptic vesicle clusters and functional release properties were not affected by short-term application of Aβ.

We next addressed whether the deleterious effects of Aβ on functional properties of glutamatergic synapses, which are well described in rodent neurons, are also observed in iPSC-derived human neurons. We recorded AMPA receptor-mediated mEPSCs (AMPA mEPSCs) using the whole-cell patch-clamp technique (1 μM TTX, 10 μM gabazine added, holding potential: −60 mV). We again applied Aβ containing supernatant of the 7PA2 CHO cell line at 8 weeks after immunopurification and studied its effects on AMPA mEPSCs 8 days later. Addition of Aβ resulted in a strong, significant reduction of the mean amplitude of AMPA mEPSCs.
indicating a loss of postsynaptic AMPA receptor function. This amplitude reduction was not observed upon addition of 7PA2 supernatant immunodepleted of Aβ, demonstrating a specific Aβ action. Addition of Aβ did not lead to a significant reduction of the mean frequency of AMPA mEPSCs (Figures 6a and b). The observed trend to a reduced AMPA mEPSC frequency is likely to be caused by limitations in the detection of small amplitude minis. To further confirm an Aβ-induced impairment of postsynaptic AMPA receptors, we performed immunocytochemical stainings for GluA1 (AMPA receptor subunit 1), PSD95, and MAP2 (to visualize dendrites) in iPSC-derived human neurons. Eight days incubation with Aβ-containing 7PA2 supernatant (7PA2) or with immunodepleted 7PA2 supernatant (ID) or with vehicle (control). Means ± S.E.M.; one-way analysis of variance

Figure 5: Presynaptic vesicle clusters are not affected by Aβ application. (a-c) Analysis of FM4–64-stained vesicle clusters on postsynaptic proximal dendrites (from experiments in Figure 4). (a) Thresholded FM4–64-stained puncta (vesicle clusters) on calcein-stained proximal dendrites are illustrated. Scale bar = 10 μm. (b) Quantification of FM4–64 puncta fluorescence on proximal dendrites. Left: Total FM4–64 signal (number of puncta × mean total fluorescence intensity/punctum) of puncta on a proximal dendrite, n = 32/37/28 dendrites. Right: Density of FM4–64 puncta on a proximal dendrite versus mean total intensity (area of punctum × average fluorescence intensity). (c) Activity-induced destaining of FM4–64 puncta on proximal dendrites. Left: Destaining kinetics (for stimulation, see Figure 4). Right: Quantification of decay time constants (τ), n = 32/37/32 dendrites. (d and e) Analysis of vGlut1 immunopositive vesicle clusters on MAP2 immunopositive postsynaptic dendrites (from experiments in Figures 4d and e). (d) vGlut1-positive puncta (vesicle clusters, upper panel) on dendrites immunostained for MAP2 are shown (lower panel). Scale bar: 10 μm. (e) Quantification of the density of vGlut1 positive puncta on dendrites. n = 24/23/24 dendrites. Eight days incubation with Aβ-containing 7PA2 supernatant (7PA2) or with immunodepleted 7PA2 supernatant (ID) or with vehicle (control). Means ± S.E.M.; one-way analysis of variance

(FIGURES 6a and b), indicating a loss of postsynaptic AMPA receptor function. This amplitude reduction was not observed upon addition of 7PA2 supernatant immunodepleted of Aβ, demonstrating a specific Aβ action. Addition of Aβ did not lead to a significant reduction of the mean frequency of AMPA mEPSCs (Figures 6a and b). The observed trend to a reduced AMPA mEPSC frequency is likely to be caused by limitations in the detection of small amplitude minis. To further confirm an Aβ-induced impairment of postsynaptic AMPA receptors, we performed immunocytochemical stainings for GluA1 (AMPA receptor subunit 1), PSD95, and MAP2 (to visualize dendrites) in iPSC-derived human neurons. Eight days incubation with Aβ-containing 7PA2 supernatant (7PA2) or with immunodepleted 7PA2 supernatant (ID) or with vehicle (control). Means ± S.E.M.; one-way analysis of variance

Aβ-induced effects on cell survival, cellular stress and tau protein in hiPSC-derived neurons. To study whether our short-term application of Aβ compromises cell survival, we stained healthy neurons and nuclei of disrupted cells, respectively by performing a live/dead assay (see Materials and Methods). Healthy neurons were fluorescently stained by addition of membrane permeable calcein-AM, which is intracellularly converted to fluorescent calcein. Nuclei without an intact plasma membrane were stained by Ethidium Homodimer 1 (Figure 7a). At 8 days of incubation, we did not observe significant effects of the addition of Aβ on cell survival (Figure 7b). Because Aβ-induced cellular stress might be detectable prior to cell death, we studied the expression of marker proteins for endoplasmic reticulum (ER) stress and autophagy by western blotting analysis. Interestingly, we found a slight Aβ-induced (8 days incubation) increase in the autophagy marker microtubule-associated protein 1A/1B-light chain II (LC3-II) and in the ER stress markers binding immunoglobulin protein (BiP) and CCAAT-enhancer-binding protein homologous protein (CHOP) (Figures 7c and d). This is in line with the recent description

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of increased ER stress in AD patient-derived iPSC lines upon neuronal differentiation. We further analysed Aβ-induced changes in tau protein expression and phosphorylation state by western blotting. Although the expression level of tau protein appeared not to be affected by short-term application of Aβ (8 days incubation), an increased phosphorylation of tau was clearly detectable (Figures 7e and f). This finding in human neurons is in line with the Aβ-induced phosphorylation of tau protein, which has been described in mouse models previously.

In summary, our results indicate that at an early phase of Aβ actions defects in synapse function are clearly revealed in iPSC-derived human neurons by changes in postsynaptic AMPA receptors. In addition, Aβ-induced alterations in neuritic vesicle clusters, in ER stress/autophagy marker proteins, and in tau protein phosphorylation were found. Our work thus establishes that iPSC-derived human neurons represent an innovative model system to study the molecular mechanisms of Aβ-induced synapse damage in AD.
In this study, we used hiPSCs to establish an innovative cell culture model of cortical neurons for specific aspects of AD. We analysed the effects of Aβ on human neurons, human synapses, and synaptic vesicle clusters. We observed a loss of axonal vesicle clusters, a loss of postsynaptic AMPA receptors, and increased tau protein phosphorylation, thus revealing complex deleterious actions of Aβ.

**Discussion**

In this study, we used hiPSCs to establish an innovative cell culture model of cortical neurons for specific aspects of AD. We analysed the effects of Aβ on human neurons, human synapses, and synaptic vesicle clusters. We observed a loss of axonal vesicle clusters, a loss of postsynaptic AMPA receptors, and increased tau protein phosphorylation, thus revealing complex deleterious actions of Aβ.

Without addition of specific morphogens/growth factors,43 iPSCs differentiate to dorsal, cortical-like neuronal progenitor cells characterized by Pax6 expression;20,22,44 however, other contaminating cell types are also generated. The invention of immunopanning protocols34 enables the highly efficient purification of specific types of neuronal precursors, neurons, or astrocytes.45–47 Similarly, the presence of NCAM has been used for selecting neuronally differentiated cells.48,49 Here we have developed an immunopanning procedure based on NCAM antibodies, which yielded > 90% MAP2-positive immature neurons. Immunocytochemical characterization of the purified neurons revealed typical cortical-like cultures consisting of mainly glutamatergic and about 20% GABAergic neurons.

**In vitro differentiation of human iPSCs and immunopurification of iPSC-derived immature human neurons.**

*In vitro* differentiation of human pluripotent stem cells to neurons has been established by several groups.20,22,42 Without addition of specific morphogens/growth factors,43 iPSCs differentiate to dorsal, cortical-like neuronal progenitor cells characterized by Pax6 expression;20,22,44 however, other contaminating cell types are also generated. The invention of immunopanning protocols34 enables the highly efficient purification of specific types of neuronal precursors, neurons, or astrocytes.45–47 Similarly, the presence of NCAM has been used for selecting neuronally differentiated cells.48,49 Here we have developed an immunopanning procedure based on NCAM antibodies, which yielded > 90% MAP2-positive immature neurons. Immunocytochemical characterization of the purified neurons revealed typical cortical-like cultures consisting of mainly glutamatergic and about 20% GABAergic neurons.
Functional maturation of human iPSC-derived immature neurons, including synapse formation. In line with our results, it has been shown that neurons derived from human iPSCs generate action potentials and exhibit voltage-activated Na⁺ and K⁺ currents. In addition, our iPSC-derived human neurons fired repetitive action potentials reminiscent of the firing pattern of regular spiking cortical pyramidal neurons. Similar to primary cultured rodent neurons, the formation of functional synapses has also been observed in human iPSC-derived neurons; however, reliable synapse formation and function appears to be difficult to achieve. Importantly, our iPSC in vitro differentiation protocol resulted reliably in the formation of both functional glutamatergic and GABAergic synapses, thus leading to synaptically active networks.

Synaptotoxic effects of Aβ in human iPSC-derived neurons. We used our innovative model system for studying synaptic aspects of AD. Oligomeric Aβ peptides are thought to be crucial molecular entities in AD and exhibit—in rodent neurons—toxic effects at glutamatergic synapses leading ultimately to synapse loss. The initial deleterious actions of Aβ on the function of glutamatergic synapses might be specifically localized either presynaptically or postsynaptically. In rodent neurons, an initial Aβ-induced removal of postsynaptic AMPA receptors by facilitating AMPA receptor endocytosis has been described. Furthermore, Aβ disrupts membrane trafficking and synaptic recruitment of AMPA receptors by reducing surface expression. In line with an initially postsynaptic mechanism, we found a pronounced Aβ-induced reduction in both the amplitudes of AMPA-mediated mEPSCs and in postsynaptic AMPA receptor clusters in hiPSC-derived neurons. In addition, also presynaptic mechanisms of action of Aβ affecting both exocytosis and endocytosis have been proposed in rodent neurons. However, our analysis of both FM-stained and immunocytochemically stained vesicle clusters asp normal in hiPSC-derived neurons did not reveal any short-term presynaptic effects of Aβ, thus indicating that Aβ-induced alterations in postsynaptic AMPA receptors might be of crucial importance in the human disease.

In addition to Aβ-induced synaptotoxicity, AD is characterized by the formation of neurofibrillary tangles and tau hyperphosphorylation. Aβ-induced tau phosphorylation has been proposed to be an early event leading to the formation of neurofibrillary tangles typical of tauopathy. In line with familial AD patient-derived iPSC-derived neurons, our data demonstrate that Aβ-induced tau phosphorylation occurs in hiPSC-derived neurons after short-term application of Aβ. Furthermore, we observed a slight Aβ-induced increase in proteins related to the ER stress-induced unfolded protein response (UPR). UPR includes enhanced expression of chaperones such as BiP, increased degradation of misfolded proteins, and activates autophagy. Failure of this system to restore ER homeostasis initiates apoptotic mechanisms. In line with our results, Aβ oligomers have been described to induce the UPR.

Effects of Aβ on vesicle clusters in hiPSC-derived neurons. Intriguingly, upon Aβ application we observed a reduced density of non-synaptic axonal vesicle clusters, which are well known to undergo exocytosis and endocytosis. Because we did not observe any Aβ-induced changes in the FM destaining kinetics (reflecting exocytosis) and in the FM fluorescence intensity (reflecting endocytosis), an Aβ-induced impairment of the axonal transport of vesicles without axon degeneration appeared to underlie the reduction in the density of axonal vesicle clusters. In line with this idea, in rodent neurons an impairment of the axonal transport by Aβ has been observed. Aβ-induced defects in the transport of organelles, for example, mitochondria, and in the transport of dense core and synaptic vesicles without axon degeneration have been described previously. Thus our results in iPSC-derived human neurons strongly support an important role of an Aβ-induced impairment in axonal transport of synaptic vesicles at early stages of the human disease.

Conclusion: human iPSC derived neurons as an innovative model system to study toxic effects of Aβ. Here we have established hiPSC-derived neurons as an innovative AD model system to study the effects of Aβ. Toxic effects of Aβ are complex, including both non-synaptic effects on axonal vesicles and bona fide postsynaptic impairment of AMPA receptors. In addition, an Aβ-induced increase in tau protein phosphorylation was found. Most importantly, our iPSC-derived human model system will aid the development of novel AD therapeutics based on the investigation of the molecular mechanisms of deleterious Aβ actions in human neurons.

Materials and Methods

hiPSC culture and neural differentiation. Experiments were approved by the local ethics committee of the University of Düsseldorf. Human iPSC line 8/25 (ref. 65) and in some experiments human iPSC line D6F–9–9T8 (WiCell, provided by Dr. J. Thomson, University of Wisconsin) were cultured according to standard procedures in mTeSR medium (STEMCELL Technologies, Köln, Germany) or in E8 medium on hESC qualified matrigel (Corning, Corning, NY, USA) coated cell culture six-well plates (Sarstedt, Nürnbrecht, Germany). Neural differentiation was performed according to a modified protocol of Li et al. EBs were allowed to form in T25 cell culture flasks (Sarstedt) in NB2B7 medium containing 50% DMEM/F12 (Biochrom, Berlin, Germany), 50% Neurobasal, 2 mM Glutamax, 100 units/ml penicillin and 100 μg/ml streptomycin, 0.1 mM β-mercaptoethanol, 1% B27 without vitamin A (all Gibco, Darmstadt, Germany), 3 μg/ml heparin (Sigma Aldrich, St. Louis, MO, USA), and a modified N2 supplement with final concentrations of 7.5 μg/ml human insulin, 50 μg/ml BSA, 8 μg/ml putrescine, 3.1 ng/ml progesterone, 2.6 ng/ml sodium selenite (all Sigma Aldrich), and 25 μg/ml human holo-transferrin (Calbiochem, Darmstadt, Germany). After 14 days of suspension culture, EBs were plated on 100-mm cell culture dishes coated with growth factor reduced matrigel (Corning) in NB2B7 medium. Six-to-8 weeks after starting differentiation, heterogeneous cultures were harvested for immunopanning by scraping in DPBS without calcium and magnesium (PanBiotech, Aidenbach, Germany).

Immunopanning of hiPSC-derived immature neurons and neuronal maturation. Panning plates (100 mm, bacterial petri dish quality; Falcon/Corning, Darmstadt, Germany) were prepared by incubating goat anti mouse IgG-IgM antibody (Jackson Immunoresearch, Suffolk, UK) at 10 μg/ml in 9 ml of 50 mM Tris HCl (pH 9.5 overnight at 4 °C. After three washing steps with DPBS, the panning plates were incubated overnight at 4 °C with the primary mouse anti-NCAM antibody (VIN-IS-53, kindly provided by Dr. Peter Andrews, University of Sheffield, UK) (0.55 μg/cm² in 0.2% BSA Fraction V (Gibco)/DPBS, 7 ml per plate). Plates were washed twice with DPBS and once with 0.02% BSA. Cells harvested from one 100-mm differentiation plate were enzymatically digested in accutase (Sigma Aldrich) for 30 min. After enzyme removal, cells were dissociated in 0.2% cell culture medium.
Followed by centrifugation. Depletion of Aβ to NHS-Sepharose (GE Healthcare, Chalfont St Giles, UK) by overnight incubation at 4 °C was carried out according to standard protocols, and quantification of images.

Immunocytochemistry and immunoblotting. Immunocytochemistry expresses mutant human APP751 (V717F).35,36 As determined by ELISA (Aβ-immunodepletion. We further thank M Bohndorf for excellent technical assistance. This work was supported by the Forschungskommission (No. 52/2011), Medical Faculty, Heinrich-Heine-University Düsseldorf and the Strategischer Forschungshügel, Heinrich-Heine-University Düsseldorf.

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Fluorescence imaging of cycling vesicle clusters. Cycling vesicle clusters were fluorescently stained by FM–64 uptake.38 FM–64 (10 μM) was added to the extracellular solution (containing in mM: 119 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 25 HEPES, 30 glucose, pH = 7.4), and extracellular stimulation (400 stimuli at 20 Hz) was used to elicit exocytic/endocytic cycling of vesicles and FM–64 uptake. Sixty seconds after stimulation, extracellular FM–64 was removed by washing with extracellular solution (0 mM CaCl2 with addition of DNOX (10 μM) and D-APS (50 μM); 2 min wash; then 2 min wash in the presence of ADNAP-7 (1 mM); and then 10 min wash), and fluorescent FM–64 puncta were imaged.

For studying exocytosis of FM–64 stained vesicles, destaining of FM–64 puncta was elicited by extracellular electrical stimulation (1200 stimuli at 20 Hz for 60 s). After 100 s of destaining, a second stronger stimulation (2000 stimuli at 20 Hz without imaging (for 100 s) was carried out to reach background fluorescence levels. Quantitative image analysis was carried out by defining regions of interest (ROI) around FM–64 puncta. Total fluorescence intensity was determined for each FM–64 punctum using the Metamorph software (Molecular Devices). Total background intensity was determined after the second stimulation within the same ROI and was subtracted from the total fluorescence intensity within the ROI to obtain the total FM–64 fluorescence signal per punctum. Decay time constants of FM–64 destaining were determined by monoeXponentially fitting (SigmaPlot11 software, Systat Software Inc, San Jose, CA, USA) the mean fluorescence decay after averaging the normalized (intensity at the start of stimulation was set to 100%) destaining curves of FM–64 puncta obtained from a field of view (Figure 4) or a proximal dendrite (Figure 5).

Statistics. All data are given as means ± S.E.M. Results were statistically analysed using one-way ANOVA with Holm–Sidak post hoc test (SigmaPlot11 software).

Conflict of Interest. The authors declare no conflict of interest.

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