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Letter to the Editor

Soluble Aβ oligomer-induced synaptopathy: c-Jun N-terminal kinase's role

Dear Editor,

Among the neurodegenerative diseases, Alzheimer disease (AD) is the most common and severe age-related dementia for which there is currently no available treatment. Many studies support the assumption that AD is a spine pathology (Selkoe, 2002; Sivanesan et al., 2013) and that soluble amyloid-β (Aβ) oligomers are causative of AD synaptopathy. Diverse lines of evidence indicate that Aβ oligomers induce formation of pore-like structures on the membrane (Arispe et al., 1993; Lashuel et al., 2002) and interfere with glutamatergic transmission. The Aβ oligomers result in a decreased number of AMPA receptors (AMPA-r) and NMDA receptors (NMDA-r), as well as PSD-95 at the postsynaptic membrane, and thus reduce the strength and plasticity of excitatory synapses (Chapman et al., 1999; Walsh et al., 2002). However, the underlying intracellular mechanisms regulating synaptic changes are only partially known. By understanding the pathophysiological mechanisms leading to synaptic dysfunction and the progression of this dysfunction, better interference in the pathogenesis of AD can be achieved.

We present an *in vitro* model to study the temporal sequence of dendritic spine modifications induced by soluble Aβ oligomers, and to analyse the intracellular signalling pathways leading to AD synaptopathy. This model allows synaptic alterations to be followed in living neurons before and after treatment and reduces bias due to cell variability. This model also permits testing of pharmaceuticals that are designed to reverse the biochemical and structural alterations of synapses induced by Aβ oligomers.

Brainbow hippocampal neurons, which express fluorescent proteins, were used to visualize dendritic spines and study synaptic plasticity (Figure 1A and B). To obtain isolated cells, fluorescent neurons were seeded on a layer of non-fluorescent neurons (ratio: 1/16) (Figure 1A and B). In this way we avoided the need for transfection, infection protocols, and low density cultures that are not well tolerated by neurons. Neurons were treated with a subtoxic dose (Figure 1E) of soluble Aβ1–42 oligomers in order to induce synaptic changes without any signs of neuronal death (Figure 1E). The preparation of synthetic Aβ1–42 that were used to induce *in vitro* synaptic dysfunction had been previously characterized. Oligomeric assemblies were only observed in peptide preparations after a 24-h incubation at 4°C (referred to as oligomers). Immediately after dissolution, the majority of Aβ1–42 remained as unassembled monomeric structures (Figure 1C and D and Supplementary Figure S1).

Subtoxic concentrations of soluble Aβ oligomers induced alterations in the postsynaptic density (PSD) composition of dendritic spines, while monomers had no effect on synaptic plasticity (Supplementary Figure S1F). Exposing the neurons for 3 h to 1 μM Aβ oligomers induced changes in the PSD region, leading to a 68% and 61% drop of GluN2A and GluN2B subunits of NMDA-r, respectively; a decrease of 70% and 65% of GluA1 and GluA2 subunits of AMPA-r, respectively; a 53% loss of PSD-95; and a 76% loss of drebrin (Figure 1F and Supplementary Figure S2A). To assess dendritic spine modifications *in vitro*, we analysed changes in spine density and morphology in neurons exposed to soluble Aβ oligomers. Application of soluble Aβ oligomers (1 μM) for 3 h caused a 25% decrease in total spine number compared with that before Aβ application (Figure 1J and K). The decrease involved all types of spines in a proportional manner. The number of mushroom, stubby, and thin spines decreased by 32%, 25%, and 22%, respectively (Figure 1J and L). Moreover, Aβ oligomer treatment induced a 67% reduction in new spine formation compared with control conditions, and led to spine shrinkage (Supplementary Figure S2E). The number of mushroom spines that became stubby or thin was significantly increased by Aβ oligomer treatment, while the number of spines that became mushroom was decreased (Supplementary Figure S2E). The morphological changes were consistent with the observed biochemical alterations since thin and stubby spines have a less extended PSD region and lower contents of glutamate receptors as well as postsynaptic markers, in comparison with mushroom spines (Tackenberg et al., 2009).

To analyse the pathways involved in Aβ oligomer-induced synaptopathy, we evaluated synaptic changes induced after 30 min and 3 h of Aβ oligomer exposure (1 μM) and correlated them to the activation of two stress signalling pathways, c-Jun N-terminal kinase (JNK) and caspase-3. After 30 min there was no sign of molecular changes: NMDA-r and AMPA-r subunits, PSD-95, and drebrin levels were unaffected by the Aβ oligomer treatment (Figure 1F). However, JNK was already activated at this stage, as indicated by a 2.36-fold increase of the P-JNK/JNK ratio compared with control conditions (Figure 1G and Supplementary Figure S2B). There was no indication of caspase-3 cleavage after 30 min of Aβ oligomer exposure (Figure 1H and Supplementary Figure S2C). Exposing neurons to soluble Aβ oligomers for 3 h induced a biochemical perturbation of PSD (Figure 1F) with a 4.33-fold increase of caspase-3 cleavage (Figure 1H) (Li et al., 2010; D'Amelio et al., 2011), while JNK activity remained elevated by 2.26 folds (Figure 1G). Our results showed that JNK activation was triggered by Aβ oligomers before PSD alterations were induced and JNK activation persisted up to 3 h, at which

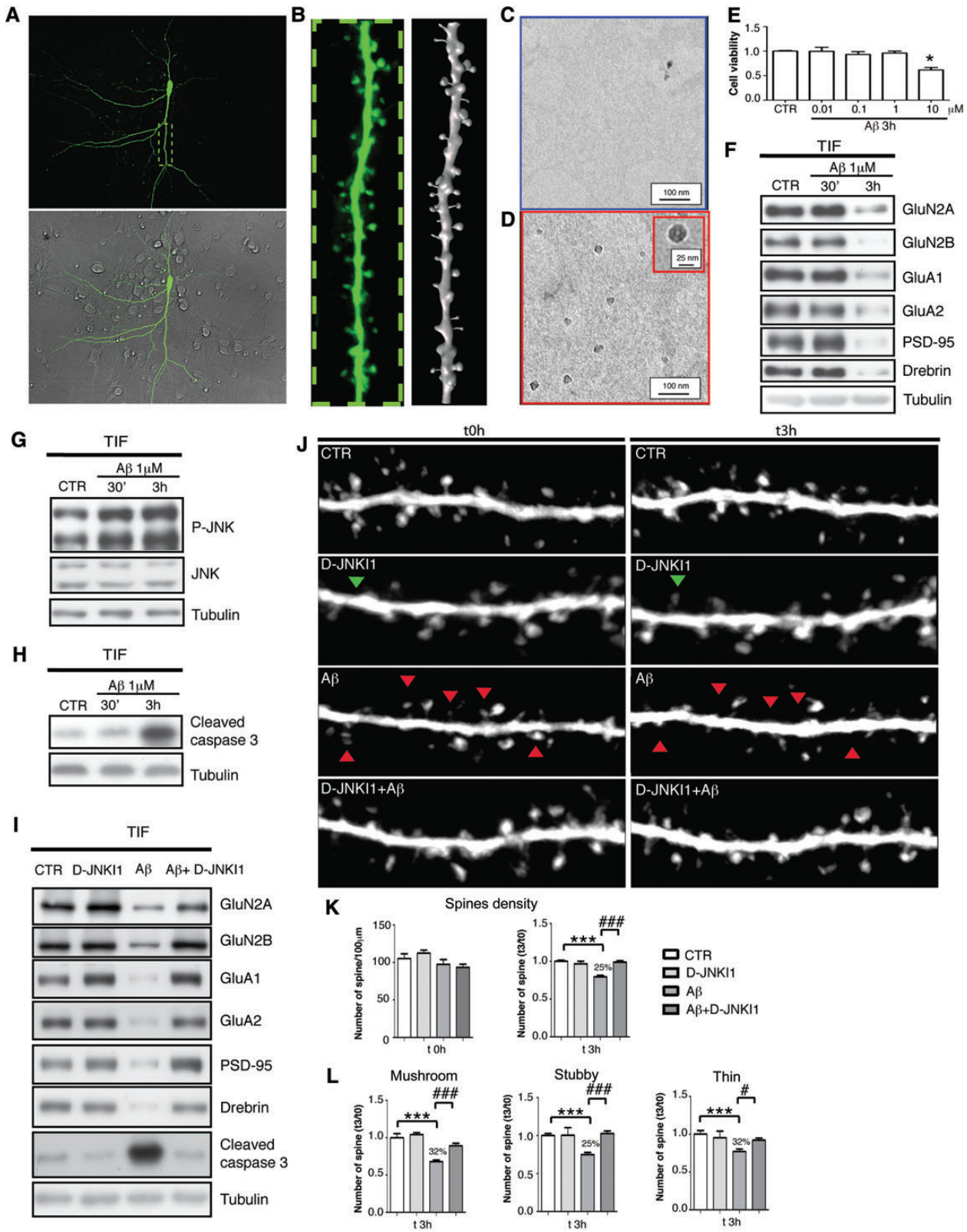

Figure 1 JNK inhibition prevents Aβ oligomer-induced synaptopathy. (**A** and **B**) *In vitro* model to study synaptic plasticity. (**A**) A Brainbow mouse fluorescent hippocampal neuron (green, 20×) seeded on a layer of non-fluorescent neurons in a ratio 1:16 (merge with bright field). (**B**) Higher magnification of a dendrite portion and 3D reconstruction showing that dendritic spines are easily visualized and classified in mushroom, stubby, and thin subtypes. (**C** and **D**) Transmission electron micrographs of monomeric (**C**) and oligomeric (**D**) assemblies. (**E**) The effect of crescent doses of synthetic soluble Aβ oligomers (0.01, 0.1, 1, 10 μM) on neuron viability was assessed with the MTT assay. Soluble Aβ oligomers at 0.01, 0.1, and 1 μM did not affect cell viability after 3 h of treatment. However, Aβ oligomers led to neuronal death at 10 μM (one-way ANOVA, Dunnett's *post hoc*

stage PSD changes and caspase-3 cleavage appeared. These results suggest that JNK activation likely occurs at the onset of AD synaptopathy.

In order to define JNK's role in Aβ oligomer-induced synaptopathy *in vitro*, neurons were pre-treated with the specific cell permeable JNK inhibitor peptide D-JNKI1 and subsequently exposed to Aβ oligomer stimulation. D-JNKI1 completely protected neurons against Aβ oligomer-induced synaptic changes, abolishing modifications of the composition of PSD (GluN2A, GluN2B, GluA1, GluA2, and PSD-95 returned to control levels) (Figure 1I and Supplementary Figure S2D), as well as spine alterations (Supplementary Figure S2E) and loss (Figure 1J–L). Importantly, D-JNKI1 also prevented caspase-3 cleavage (Figure 1I and Supplementary Figure S2D). These results suggest that the JNK pathway triggers synaptic dysfunction *in vitro* following Aβ oligomer exposure.

To better understand the relevance of JNK and caspase-3 pathways in synaptopathy, we compared the effect of D-JNKI1 with z-DEVD-FMK, a caspase-3 inhibitor that partially prevents dendritic spine dysfunction in Tg2576 mice (D'Amelio et al., 2011). In our experimental conditions, z-DEVD-FMK completely inhibited Aβ oligomer-induced loss of AMPA-r, but only partially (about 50%) restored NMDA-r subunits and PSD-95 to control levels (Supplementary Figure S3). Caspase-3 inhibition by z-DEVD-FMK was less efficient in protecting neurons against synaptic changes than JNK inhibition that led to a complete recovery of GluN2A, GluN2B, GluA1, GluA2, PSD-95, and drebrin levels. Our results demonstrate that JNK plays a pivotal role in Aβ oligomer-induced synaptopathy and acts upstream of caspase-3 (D'Amelio et al., 2011).

To our knowledge this is the first demonstration identifying JNK as a key modulator in the degeneration of excitatory synapses. JNK, therefore, represents an innovative target as its inhibition in neurons *in vitro* completely protects synapse degeneration, without interfering with Aβ oligomerization.

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References

Arispe, N., Rojas, E., and Pollard, H.B. (1993). Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. Proc. Natl Acad. Sci. USA *90*, 567–571.

Chapman, P.F., White, G.L., Jones, M.W., et al. (1999). Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. Nat. Neurosci. *2*, 271–276.

D'Amelio, M., Cavallucci, V., Middei, S., et al. (2011). Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease. Nat. Neurosci. *14*, 69–76.

Lashuel, H.A., Hartley, D., Petre, B.M., et al. (2002). Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature *418*, 291.

Li, Z., Jo, J., Jia, J.M., et al. (2010). Caspase-3 activation via mitochondria is required for long-term depression and AMPA receptor internalization. Cell *141*, 859–871.

Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. Science *298*, 789–791.

Sivanesan, S., Tan, A., and Rajadas, J. (2013). Pathogenesis of abeta oligomers in synaptic failure. Curr. Alzheimer Res. *10*, 316–323.

Tackenberg, C., Ghori, A., and Brandt, R. (2009). Thin, stubby or mushroom: spine pathology in Alzheimer's disease. Curr. Alzheimer Res. *6*, 261–268.

Walsh, D.M., Klyubin, I., Fadeeva, J.V., et al. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature *416*, 535–539.

test, $^{*}P < 0.05$, $n = 6$). (**F**–**H**) Synthetic soluble Aβ oligomers induced synaptic activation of JNK before synaptopathy. (**F**) Western blot performed on the triton insoluble fraction (TIF) of neurons treated with Aβ oligomers (1 μM) for 30 min and for 3 h. See for more detail in Supplementary Figure S2A. (**G**) Western blot showing P-JNK and JNK levels in the TIF of neurons exposed to Aβ oligomers (1 μM) for 30 min and 3 h. See for more detail in Supplementary Figure S2B. (**H**) Western blot of caspase-3 activation in the TIF of neurons exposed to Aβ oligomers (1 μM) for 30 min and 3 h. See for more detail in Supplementary Figure S2C. (**I**–**L**) D-JNKI1 prevented Aβ-induced synaptopathy *in vitro*. (**I**) Western blot and relative quantification were performed on the TIF of neurons treated with D-JNKI1 (2 μM, 30 min before Aβ oligomer application) and Aβ oligomers (1 μM, 3 h), either alone or combined. D-JNKI1 prevented Aβ oligomer-induced protein loss from the PSD and caspase-3 activation in the postsynaptic compartment. See also Supplementary Figure S2D. (**J**) Time-lapse photographs showing neurons treated with D-JNKI1 (2 μM, 30 min before Aβ oligomer treatment) alone, Aβ oligomers (1 μM, 3 h) alone, or combined, recorded at time 0 (t0h) and 3 h after the treatment (t3h). Red arrows represent spines that disappeared; green arrows represent newly formed spines. (**K** and **L**) Quantification of dendritic spine density. (**K**) At the starting point (t0h), the total spine density was not significantly different between groups. Graphs show that D-JNKI1 (2 μM, 30 min before Aβ oligomer application) was able to prevent synthetic soluble Aβ oligomer (1 μM, 3 h)-induced reduction in spine density (**K**) and restore the number of mushroom, stubby, and thin spines to control levels (**L**) (two-way ANOVA, Bonferroni *post hoc* test, $^{***}P < 0.001$ CTR vs. Aβ oligomers, $^{\#\#\#}P < 0.001$ Aβ oligomers vs. Aβ oligomers + D-JNKI1, CTR $n = 20$ cells, D-JNKI1 $n = 14$ cells, Aβ oligomers $n = 19$ cells, Aβ oligomers + D-JNKI1 $n = 14$ cells). The number of spines recorded after 3 h of treatment (t3h) was normalized on the number of spines recorded at time 0 (t0h).