

## Letter to the Editor

# The TAT-JNK inhibitor peptide interferes with beta amyloid protein stability

Cell Death and Differentiation advance online publication, 20 July 2007; doi:10.1038/sj.cdd.4402202

Dear Editor,

Alzheimer's disease (AD), the most frequent neurodegenerative disorder in the elderly,<sup>1</sup> is a multifactorial syndrome linked to abnormal metabolism of the transmembrane amyloid precursor protein (APP).<sup>2</sup> Extracellular deposits known as neuritic plaques, a typical neuropathological feature of AD brains, contain large amounts of amyloid-beta-peptide (A $\beta$ ), generated from APP by two endoproteolytic cleavages.<sup>3</sup> The biological origin of A $\beta$  plaques and the mechanism whereby A $\beta$  is involved in AD pathogenesis is still elusive and, despite years of research, the normal functions of APP and its catabolites are also not fully understood.

Proteolysis of APP is regulated not only by the secretase enzymes, but also by a cell signal paradigm,<sup>4</sup> although little is known about the intracellular pathways underlying this processing. Disturbance of normal APP processing may contribute to the disease process as well.<sup>5</sup> Again, very little is known about the biological function of the C-terminal intracellular region (AICD) from which the APP domain is cleaved off by  $\gamma$ -secretase.<sup>6</sup>

The importance of this region is now becoming clearer and several studies have examined the signal transduction pathways that interact with this intracellular portion. Thus, independently of secretases, APP is also cleaved by caspase between Asp664 and Ala665 in the AICD intracellular domain.<sup>7,8</sup> The AICD cytosolic domain of APP is the center of a complex network of protein–protein interactions, including c-Jun N-terminal kinase (JNK)-interacting protein-1b (JIP-1b).<sup>9</sup> JIP-1 is a scaffold protein for JNKs and their upstream kinases, MKK7 and MKL3,<sup>10</sup> and may couple APP to the JNK mitogen-activated protein kinase signalling pathway. A recent study using microarray analysis indicated potent APP-dependent expression of c-jun.<sup>11</sup>

We focused on JNK's role in APP phosphorylation in cortical neurons, using the JNK inhibitor D-JNKI1. This cell-permeable, biologically active peptide consists of the JNK-binding domain of JIP-1/IB1 (JBD<sub>20</sub>), and the HIV-TAT 48–57 transporter sequence<sup>12</sup> and can specifically inhibit JNK in cortical neurons, enabling us to study APP phosphorylation in physiological conditions.

To study the effect of D-JNKI1 on APP processing in primary cultures, adult rat cortical neurons were treated for 24 h with D-JNKI1 at doses of 2, 4 and 6  $\mu$ M. We used three different antibodies in WB analysis: 22C11, 6E10 and  $\beta$ APPs. The first recognizes the full length of APP (mature and immature), the  $\beta$ APPs and  $\alpha$ APPs, the 6E10 antibody

identifies only the  $\alpha$  cut (the  $\alpha$ APPs), and the  $\beta$ APPs antibody is specific for  $\beta$  secretase cleavage.

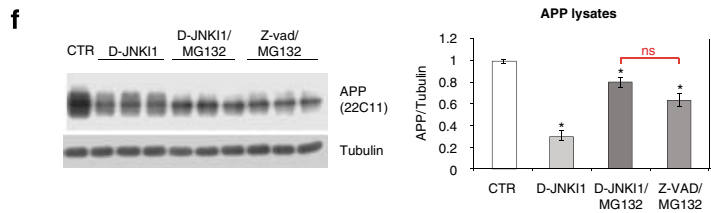
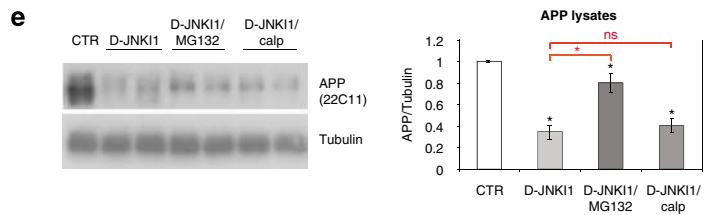
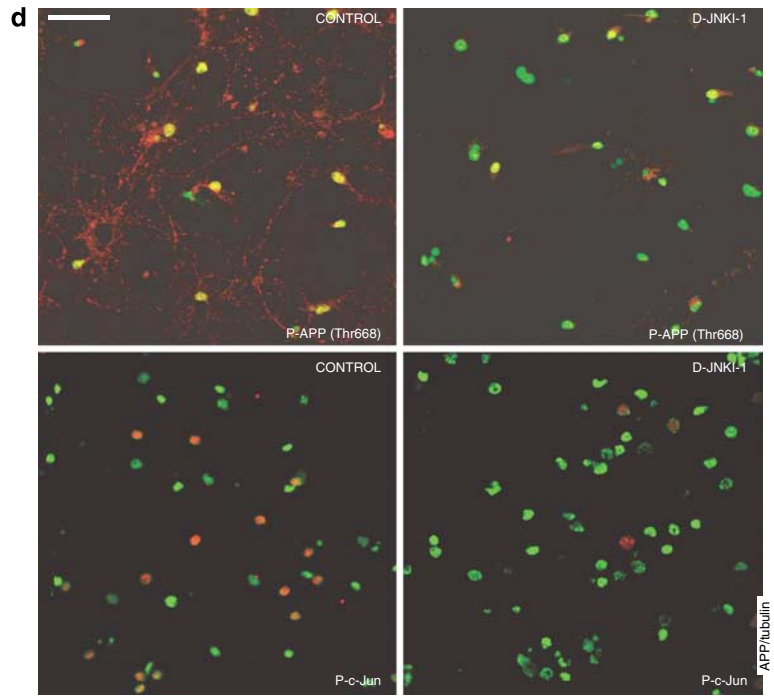
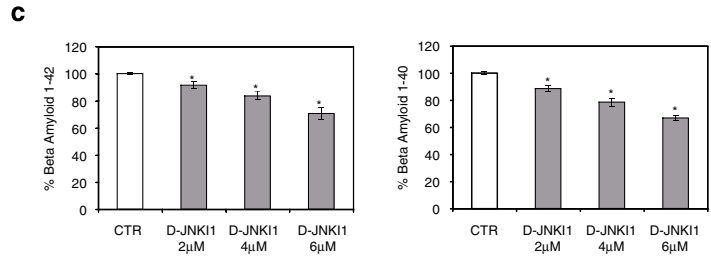
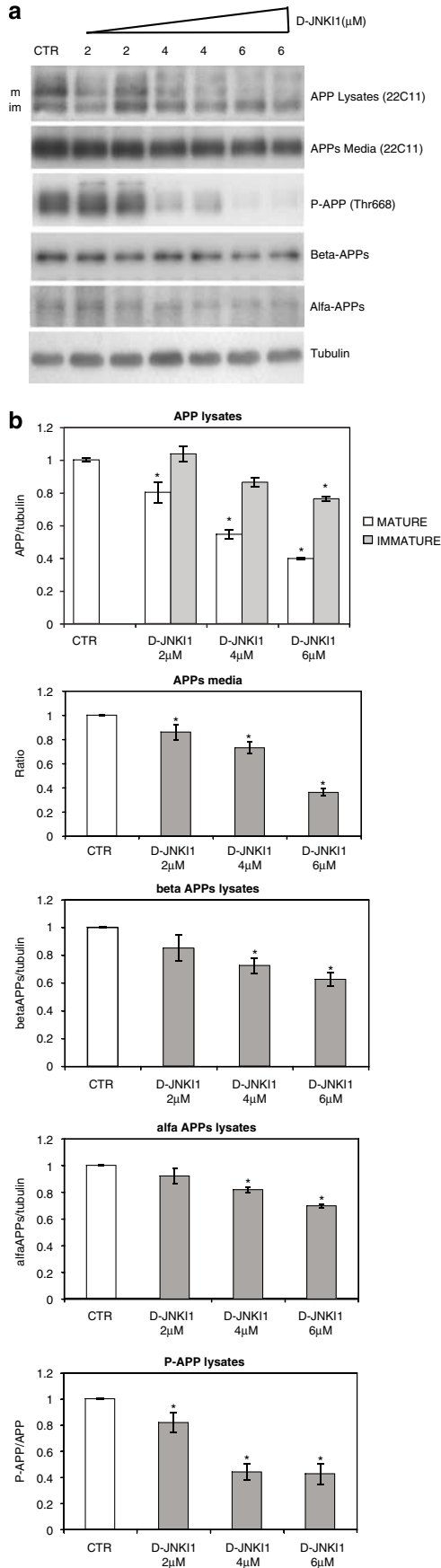
The low concentration, 2  $\mu$ M D-JNKI1, reduced the APP level (mature form) by 20% in cortical neuron lysates (Figure 1a and b). At 4  $\mu$ M, APP was reduced by 45% and at 6  $\mu$ M by 60%. The immature form of APP also declined with 4 and 6  $\mu$ M, but the decrease was significant only with 6  $\mu$ M (24%, Figure 1a and b). Thus, D-JNKI1 reduced the levels of both mature and immature APP in neuron lysates.

There was a clear decrease in the secreted APP (APPs) in the media of treated neurons: 2 and 4  $\mu$ M produced a drop of 30% and 6  $\mu$ M arrived at a 64% reduction (Figure 1a and b). This confirmed the drop in APP with the peptide on the total lysates. The level of  $\beta$ APPs dropped significantly in neurons treated with 4 and 6  $\mu$ M of D-JNKI1, but 2  $\mu$ M had no significant effect. The  $\beta$ APPs level fell 30% with 4  $\mu$ M and 40% with 6  $\mu$ M (Figure 1a and b).

The same quantification was carried out to distinguish the  $\alpha$ APPs;  $\alpha$ APPs did not decrease with 2  $\mu$ M, but it dropped 20% with 4  $\mu$ M and 30% with 6  $\mu$ M (Figure 1a and b). The  $\alpha$ APPs and  $\beta$ APPs presented nearly the same reduction. We also analyzed the media of these neurons with the Beta ELISA kit to measure the production of A $\beta$  fragments. There was a dose-dependent reduction: both A $\beta$ 1-40 and A $\beta$ 1-42 dropped 10% with 2  $\mu$ M of D-JNKI1, 20% with 4  $\mu$ M and 30% with 6  $\mu$ M. This suggests there are no differences between A $\beta$ 1-40 and A $\beta$ 1-42 fragment production (Figure 1c).

Western blot analysis for P-APP in cortical neuron lysates enabled us to correlate APP phosphorylation and its processing and stability. We used the anti-P-APP-Thr<sup>668</sup> antibody, which specifically recognizes only this phosphorylated site of the protein, to evaluate D-JNKI1's inhibitory effect. D-JNKI1 prevented phosphorylation at Thr<sup>668</sup>: 2  $\mu$ M reduced P-APP by 15% and phosphorylation dropped approximately 55% with 4 and 6  $\mu$ M (Figure 1a and b). D-JNKI1 powerfully prevented the phosphorylation of APP at Thr<sup>668</sup> and this strongly correlates with the decline of processing:  $\alpha$ APPs,  $\beta$ APPs and A $\beta$  fragment production were in fact inhibited.

To further analyze the peptide's effect, we investigated P-APP in cortical neurons by immunofluorescence. Staining was sparse and diffusely distributed in the soma, nuclei and dendrites of control neurons, whereas after 24 h exposure to 4  $\mu$ M D-JNKI1, P-APP was markedly reduced and localized in the perinuclear and nuclear regions (Figure 1d, upper panels). This confirmed the strong P-APP reduction seen with Western



blots and showed relocalization inside neurons due to D-JNK11's inhibitory effect on APP.

Immunofluorescence for P-c-jun was also used to verify the real inhibitory effect of D-JNK11. In the control condition, many cortical neurons presented nuclear staining of P-c-jun, because JNK has high basal activity in neurons. After 24 h exposure to the peptide at 4  $\mu$ M, P-c-Jun labelling was markedly reduced into very few neuronal nuclei (Figure 1d, lower panels).

A recent study proposed a connection between APP expression and the JNK pathway.<sup>11</sup> To clarify D-JNK11's effect on APP, we investigated whether it affected APP expression besides APP degradation. We compared APP expression in cortical neurons treated for 24 h with 6  $\mu$ M D-JNK11 and in untreated neurons, using real-time reverse transcription PCR. APP transcript levels were normalized to the tubulin gene unaltered by the peptide. The results clearly showed that APP expression was not changed by D-JNK11 (data not shown). D-JNK11 6  $\mu$ M for 24 h did not change mRNA expression in rat cortical neurons ( $P > 0.05$ , four per group), so we concluded that inhibition of JNK/c-Jun pathway did not influence APP expression.

As both mature and immature full-length APP levels in neuron lysates dropped after D-JNK11 treatment, we investigated whether D-JNK11 caused APP degradation and whether calpains or the ubiquitin–proteolytic pathways were implicated. Cortical neurons were pre-treated with calpastatin (a specific calpain inhibitor) or with MG132 (a proteasome inhibitor against the 26S complex) and D-JNK11, resulting in co-treatment of inhibitors. Western blotting showed that APP degradation after D-JNK11 was strongly inhibited by MG132–D-JNK11, but not by calpastatin–D-JNK11 (Figure 1e). Thus, calpains are not involved in APP degradation, and the proteasome pathway is responsible after D-JNK11 treatment.

Proteasome inhibition had a number of different effects in cells, including caspase 3 activation<sup>13</sup> and APP is a substrate of caspase 3 that cleaves APP in the AICD region.<sup>7</sup> We therefore examined APP levels after co-treatment with MG132 and Z-vad: the effect of Z-vad/MG132 did not differ significantly from D-JNK11/MG132 (Figure 1f). We conclude that caspase-mediated cleavage of APP is independent of proteasomal activity, whereas APP degradation mediated by D-JNK11 is proteasome-dependent.

These results indicate that phosphorylation of AICD at Thr<sup>668</sup> is important for both APP cleavage and degradation in cortical neurons in physiological conditions.

APP is a type I membrane protein of unknown function, whose proteolytic processing, driven by beta- and gamma-secretases, generates the A $\beta$ , one of the pathogenic hallmarks of AD. Its function is unfortunately still poorly understood. Little is known about the AICD/C-terminal cytoplasmic domain that regulates complex protein–protein interactions and intracellular pathways. APP is phosphorylated at multiple sites in the C-terminal cytoplasmic domain<sup>14</sup> and the phosphorylation of Thr<sup>668</sup> is well established, because it induces conformational changes that affect APP function and metabolism.<sup>15</sup>

Lee *et al.*<sup>16</sup> reported that phosphorylated APP, especially phosphorylated at the Thr<sup>668</sup>/C-terminal fragment, accumulated to a high level in human AD brain, raising the logical possibility that this phosphorylation may increase A $\beta$  generation. Activated JNK is significantly increased in AD and is localized in the cytoplasm of neurons, the pattern correlating with the progression of the disease.<sup>17</sup> However, the physiological functions of APP phosphorylation at Thr<sup>668</sup> in neurons remain largely unknown and some possible functions of APP may be clarified by studies of the AICD domain and its partners.

Overall, these results suggest that the cytosolic AICD domain of APP is the center of a complex network that is vital in regulating APP stability. In particular, phosphorylation at Thr<sup>668</sup> in the AICD appears essential for the regulation of APP stability. Inhibition of JNK-mediated phosphorylation of APP causes it to enter the proteasome pathway, supporting the notion that the phosphorylation state of Thr<sup>668</sup> is important in its stability and cleavage. The immunofluorescence approach indicates that P-APP is not only reduced by the peptide, but also re-distributed inside neurons. It is normally localized in soma and dendrites and nuclei of cortical neurons,<sup>18</sup> whereas D-JNK11 treatment limited P-APP staining and distribution to the perinuclear region and nuclei.

We can thus conclude that the D-JNK11 peptide has an important and selective impact on APP stability: by inhibiting APP phosphorylation at Thr<sup>668</sup> it helps reduce the production of  $\beta$ APPs and A $\beta$  fragments and may therefore be important in reducing the neuronal degeneration in AD

**Figure 1** (a) Effects of JNK inhibition on APP processing in cortical neurons. Cortical neurons were pre-treated with 2, 4 and 6  $\mu$ M of D-JNK11 for 24 h and cell lysates were immunoblotted for APP (22C11 antibody),  $\beta$ APPs,  $\alpha$ APPs and P-APP (Thr<sup>668</sup>). Loading control, tubulin. There were dose-dependent reductions of APP (m = mature APP and im = immature APP),  $\beta$ APPs, and  $\alpha$ APPs production and APP phosphorylation. Proteins in the culture media were precipitated and immunoblotted for APPs and the peptide reduced the APPs level. (b) Western blot densitometry clearly showed that the peptide reduced APPs,  $\beta$ APPs,  $\alpha$ APPs and P-APP levels. Quantifications were from eight independent experiments ( $\pm$  S.E.M.);  $*P < 0.05$  versus control. (c) Quantitative determination of beta-amyloid fragments (1–40 and 1–42) in culture media by ELISA assay. The peptide had the same effect on A $\beta$  1–40 and A $\beta$  1–42 (10% decrease with 2 mM D-JNK11, 20% with 4 mM and 30% with 6 mM). Data are mean of eight independent experiments ( $\pm$  S.E.M.);  $*P \leq 0.05$  versus control. (d) P-APP and P-c-jun immunofluorescence. Left-hand panels show control neurons, right-hand panel neurons treated with 4  $\mu$ M D-JNK11 for 24 h, after which the neurons were stained with P-APP (Thr<sup>668</sup>) antibody, red (upper panels) and P-c-Jun antibody, red (lower panels), and nuclei were counterstained with syto13 reagent, green. In the control condition, P-APP labelling was diffusely distributed in soma, dendrites and nuclei of cortical neurons. D-JNK11 strongly reduced the labelling, limiting it to the perinuclear and nuclear regions. P-c-jun staining was strongly reduced by D-JNK11 pre-treatment. Scale bar, 50  $\mu$ m. (e) APP degradation. D-JNK11 treatment (6  $\mu$ M) induced APP degradation (65%), whereas co-treatment with MG132 (5  $\mu$ M) prevented it (reducing APP degradation to 20%). The calpain inhibitor calpastatin had no such effect (2  $\mu$ M). Quantification was carried out on six independent experiments ( $\pm$  S.E.M.);  $*P < 0.05$  versus control. In red, statistical comparison between D-JNK11 and (1) the co-treatment D-JNK11/MG132 and (2) D-JNK11/calpastatin; red asterisk = significant difference determined by Tukey's test. (f) APP degradation. Co-treatment with Z-vad (10  $\mu$ M) and MG132 had minor effect on APP degradation (35%). Red lines indicate the comparison between D-JNK11/MG132 and z-vad/MG132 by Tukey's test: there is no significant difference. Quantification was carried out on three independent experiments ( $\pm$  S.E.M.);  $*P < 0.05$  versus control

pathology. These results clearly suggest the AICD domain is an attractive candidate target for new therapeutic approaches.

**Acknowledgements.** This work was supported by grants from the Botnar Foundation and FNRS (Swiss National Science Foundation) 310000-107888. Dr Repici was supported by a Novartis grant. D-JNK11 peptide was kindly provided by Xigen, SA. We thank Catherine Centeno for assistance, Professor P Davis for kindly providing us the specific anti-P-APP antibody, and JD Baggott for revising the manuscript.

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