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## **Abeta(1-42) induces abnormal alternative splicing of tau exons 2/3 in NGF-induced PC12 cells**

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### **ABSTRACT**

Protein tau plays a pivotal role in the pathophysiology of Alzheimer's disease, where its hyperphosphorylation promotes aggregation and microtubule destabilization. Tau undergoes alternative splicing which generates six isoforms in the human brain, due to inclusion/exclusion of exons 2, 3 and 10. Dysregulation of the splicing process of tau exon 10 is sufficient to cause tauopathy and has shown to be influenced by beta-amyloid peptides, but splicing of other exons is less studied. We studied the effects of beta-amyloid(42) in the alternative splicing of tau exons 2/3 and 6, using untreated and Nerve Growth Factor-induced PC12 cells. Beta-amyloid exposure caused formed cell processes to retract in differentiated cells and altered the expression of exons 2/3 in both undifferentiated and differentiated cells. Expression of exon 6 was repressed in undifferentiated cells only. Our results suggest that beta-amyloid interferes with the splicing process of exons 2/3, favoring their exclusion and thus the expression of immature tau isoforms that are less efficient in stabilizing microtubules and may also be more prone to hyperphosphorylation. The molecular mechanism for this amyloid-tau interaction remains to be determined, but may have potential implications for the understanding of the underlying neuropathological processes in Alzheimer's disease.

**Key words:** Alzheimer's disease, amyloid peptide, splicing regulation, tau isoforms.

### **INTRODUCTION**

Protein tau is involved in microtubule assembly and stabilization; hence the name microtubule-associated protein tau (MAPT) (Weingarten et al. 1975). Nevertheless, tau localization and functions are not limited to microtubules. Microtubule-

binding domains are located at the C-terminus (CT) of the protein while the N-terminus (NT) appears to regulate its localization. Membrane associations of tau have been studied in PC12 cells overexpressing tau and its fragments, where the NTF proved to have a role in neuritic development and microtubule-plasma membrane interactions (Brandt et al. 1995).

Hyperphosphorylation of MAPT in the human brain has for a long time, been known to contribute

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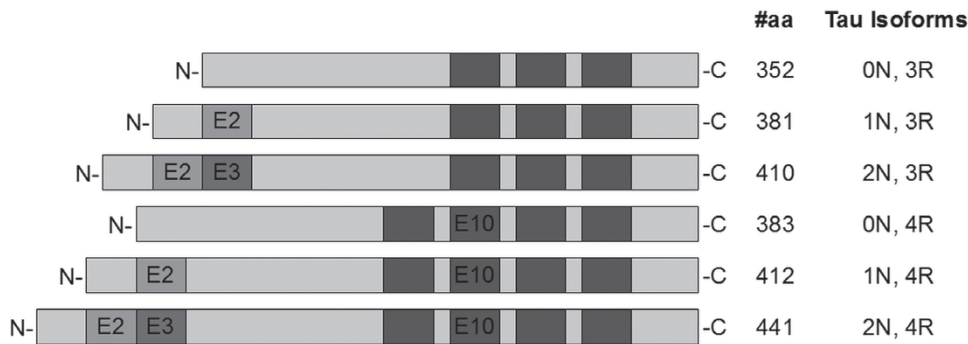
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to Alzheimer’s disease (AD) pathology by tau disassociation from microtubules to aggregate and form neurofibrillary tangles (NFTs), but the phosphorylation state at the serine/threonine residues seems to also be a regulator of tau intracellular trafficking; membrane-associated tau shows dephosphorylation at these sites, which probably increases its association with trafficking proteins that target tau to the plasma membrane. Tau hyperphosphorylation in AD thus, may play yet another role in the pathology by disrupting targeting to the plasma membrane, ultimately inhibiting the protein’s functions there (Pooler and Hanger 2010).

Six tau isoforms ranging from 352 to 441 amino acid residues are found in the human brain (Goedert and Jakes 1990) as a result of the alternative splicing of exons 2, 3 and 10 (Figure 1) (Andreadis et al.

1992). The inclusion of exons 2 and 3 produces isoforms with 0, 1 or 2 insertions near the NTF (0N-, 1N-, 2N-tau) of the tau protein (Andreadis et al. 1992, D’Souza and Schellenberg 2005). Exon 3 never appears independent from exon 2 and is regulated by a weak branch point and a combination of exonic enhancers and silencers (Arikan et al. 2002). Exon 6 seems to be regulated by flanking exons that participate in its inclusion and the modulation of variant ratios (Wei and Andreadis 1998). Exon 10 is a repeat region for microtubule binding (Goedert et al. 1989); its alternative splicing produces almost equally expressing isoforms containing either three (3R-tau without exon 10) or four (4R-tau with exon 10) microtubule-binding domains. Dysregulation of exon 10 alternative splicing has shown to be sufficient to cause neurodegeneration (Liu and Gong 2008).



**Figure 1** - Representation of the six tau isoforms generated in the human brain from the alternative splicing of exons 2, 3 and 10.

Tauopathy is present in AD as a hallmark feature, along with beta-amyloid (Aβ) pathology. The discovery of the interactions between Aβ and tau has implications for the development of strategies that could possibly help prevent neurodegeneration (Chen et al. 2010). We hypothesized that Aβ<sub>42</sub> exposure can induce alterations in the alternative splicing of tau exons 2, 3 and/or 6; to address this, we studied the expression of regions comprising exons 1 to 9 and their possible Aβ-induced alterations, using undifferentiated and differentiated neuron-like PC12 cells induced by nerve-growth factor (NGF) (Greene and Tischler 1976).

**MATERIALS AND METHODS**

**PC12 CELL CULTURES AND AB<sub>42</sub> PEPTIDE EXPOSURE**

PC12 cells were placed in 85% RPMI 1640 culture medium (GIBCO) supplemented with 5% fetal bovine serum, 10% horse serum and 50μl/ml liquid penicillin-streptomycin (GIBCO, SKU# 15140-122). 2,500 cells/ml per well were seeded in Poly-D-Lysine coated 96-well plates and incubated at 37°C with 5% CO<sub>2</sub> atmosphere. A) Undifferentiated PC12 cells were either incubated with 2μM Aβ<sub>42</sub> or without Aβ as controls for a 24 h period. B)

Differentiation was induced by the addition of 50ng/ml NGF-7S (Sigma, SKU# N0513) to the culture medium. Cells were initially incubated for a period of 24 h, after which medium was changed for 1% RPMI 1640 supplemented with horse serum. 10µl of 50µM NGF-7s were added to the medium every 24 h for up to 96 h and then, either 2µM Aβ<sub>42</sub> or no Aβ (for controls) was added for a final 24 h incubation period.

#### CELL MORPHOLOGY

Morphological changes in PC12 cells due to NGF-induced differentiation and Aβ<sub>42</sub> treatment were visualized using a professional binocular inverted microscope (Zeigen, Mexico) and photographed with the complementary digital camera and software provided by the microscope's manufacturer. Images were obtained with the 40x objective, condenser ELWD N.A. 0.35, plan-achromatic objective PL20/0.35.

#### EXPRESSION ANALYSIS OF TAU EXONS BY RT-PCR

Total RNA from PC12 cells was isolated by the Trizol (Invitrogen) method, following manufacturer's instructions; after quantification and integrity assessment, the RNA was stored at -80°C until further utilization. Expression of tau exons 1 to 9 was analyzed by RT-PCR. Retro-transcription was performed with 1µg total RNA using moloney murine leukemia virus reverse transcriptase, M-MLV RT

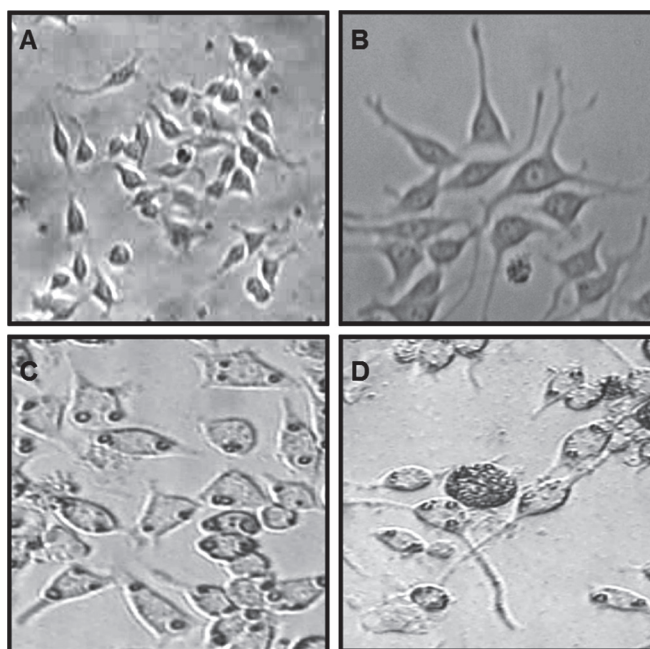
(Invitrogen, SKU# 28025-021), by addition of 1µl oligo(dT)<sub>12-18</sub> (500µg/ml), 1µl dNTPs mix (10mM) and sterile distilled water to a volume of 12µl to the RNA, to heat at 70°C for 3 minutes and quickly chill on ice, followed by addition of 5µl 5X first-strand buffer, 2µl DTT (0.1M) and 1µl M-MLV to incubate at 37°C for 1 h and inactivate the reaction at 94°C for 5 minutes. Amplification of cDNA fragments of regions comprising exons 1 to 5, 5 to 9, 7 to 9 and 6, as well as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene, was performed with 2µl of the synthesized cDNA plus 1µl of each (forward/reverse) primer (50µM), 5µl 10X PCR buffer, 1µl dNTPs mix, 5µl MgCl<sub>2</sub> (50mM), 0.5µl Taq DNA polymerase (5U/µl) and sterile distilled water to a final volume of 50µl, under the following PCR conditions: hot start at 94°C for 2 minutes, 35 cycles of denaturalization at 94°C for 1 minute, annealing at 63°C for 1 minute and elongation at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. Primer sequences and expected PCR products are found in Table I (Vanier et al. 1998). All reagents used for the RT-PCR reactions were from Invitrogen.

#### RESULTS

Morphological changes in NGF-induced PC12 cells were observed as a result of Aβ<sub>42</sub> peptide exposure (Figure 2): 30 min after peptide addition

**TABLE I**  
**PCR pairs of primer sequences and expected product sizes (Vanier et al. 1998).**

<i>Name</i>	<i>Sense</i>	<i>Sequence</i>	<i>Fragment (bp)</i>	<i>Exons</i>
Ex1	Forward	5'-CGCCAGGAGTTTGACACAATGG-3'	Fetal: 189	1+4+5
EX5A	Reverse	5'-TCCTGTCCTGTCTTTGCTTACG-3'	Adult: 280 363	1+2+4+5 1+2+3+4+5
EX5	Forward	5'-CGAGTGGCCGGCGTAAGCAAA-3'	393	5+7+9
EX9	Reverse	5'-CTTTAGGTCTGGCATGGGCAC-3'	591	5+6+7+9
EX7	Forward	5'-GCCAGAAAGGCACATCCAATGC-3'	281	7+9
EX9	Reverse	5'-CTTTAGGTCTGGCATGGGCAC-3'		
EX6S	Forward	5'-AGAAAGCCAAGACATCCACAC-3'	214	6
EX6AS	Reverse	5'-GCGCCCTTGAGTTTCATCTG-3'		
GAPDH	Forward	5'-GCCATCAACGACCCCTTCAT-3'	314	
	Reverse	5'-TTCACACCCATCACAAACAT-3'		



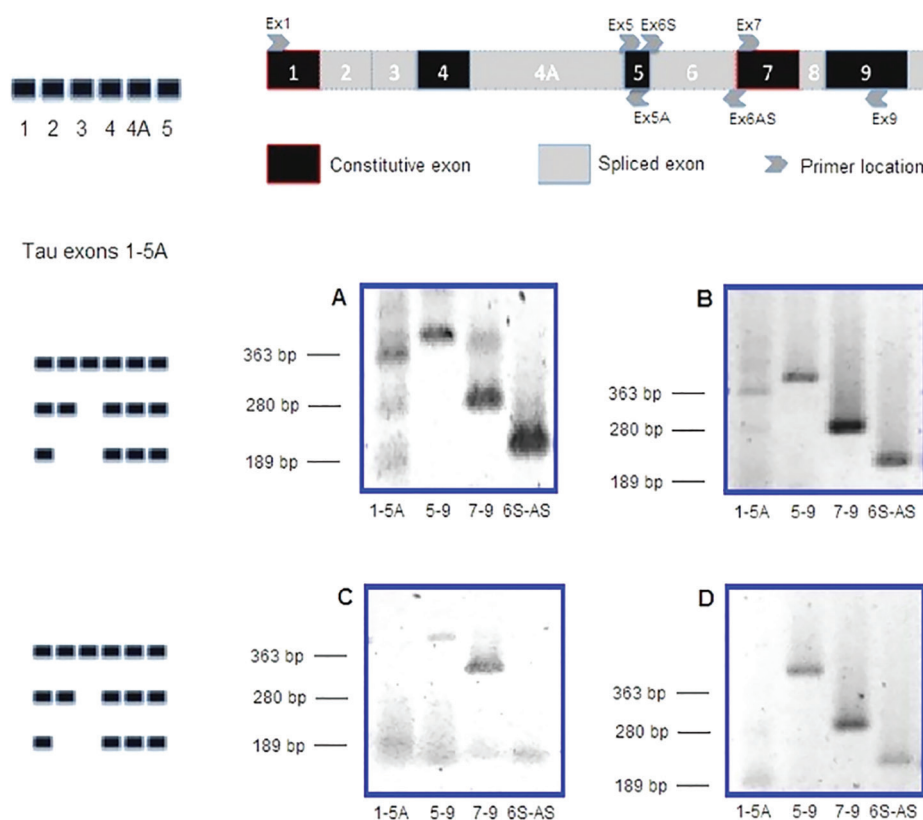
**Figure 2** - A $\beta$ -induced morphological changes in PC12 cells. Untreated **A)** undifferentiated and **B)** NGF-induced cells; differentiated cells after **C)** 30 min and **D)** 24 h of A $\beta$  exposure. A $\beta$ -treatment causes the appearance of inclusions and neurite retraction. Inverted microscope. Magnification: 40X.

to the culture medium, the neuron-like cells started showing inclusions that were followed by retraction of their processes after 24 h.

To investigate the effects of A $\beta_{42}$  in the alternative splicing of tau exons 2, 3 and 6, we analyzed their expression by RT-PCR in untreated undifferentiated and differentiated PC12 cells, in order to establish a control, and in A $\beta$ -treated undifferentiated and differentiated cells. As shown in Figure 3, we first observed a visible decrease in the expression levels of all fragments amplified in cells that were treated with A $\beta_{42}$  peptide, compared to controls. Undifferentiated and NGF-induced cells showed a very similar expression pattern of all amplified fragments when cells were not treated with the A $\beta_{42}$  peptide; nevertheless, when A $\beta_{42}$  was added to cell cultures, we observed a couple of differences between undifferentiated and differentiated cells.

Expression of exons 2 and 3 was investigated using the primer pair Ex1/Ex5A. All three bands

found in the adult brain by Vanier were found in untreated undifferentiated cells, with fragment 189bp corresponding to exons 1+4+5, fragment 280bp to exons 1+2+4+5 and fragment 363bp to exons 1+2+3+4+5, from which fragment 363bp appears to express in a slightly higher level than the others (Figure 3A); fragment 189bp, which corresponds to fetal tau, was not however, detected in differentiated PC12 cells (Figure 3B). When cells were exposed to the A $\beta_{42}$  peptide, exons 2 and 3 were not expressed in either undifferentiated (Figure 3C) or differentiated (Figure 3D) cells, as observed by the amplification of only one band corresponding to fetal tau (189bp fragment). Inclusion of exon 6 was investigated by the use of two primer pairs: Ex5/Ex9 and the exon specific Ex6S/Ex6AS. In undifferentiated A $\beta$ -treated cells, exon 6 amplification with the primer pair Ex6S/Ex6AS showed no expression of this region, and the region amplified with the primer pair Ex5/Ex9



**Figure 3** - Representations of protein tau, exons 1 to 9 (modified from Vanier et al. 1998) and the exons 1 to 5 comprised in the region amplified by primer pair Ex1/Ex5A. Expression of tau exons 2/3 (Ex1/Ex5A), 6 (Ex5/Ex9, Ex6S/Ex6AS) and 8 (Ex7/Ex9) from: untreated **A**) undifferentiated and **B**) NGF-induced differentiated PC12 cells; and from PC12 cells exposed to  $A\beta_{(1-42)}$  peptide in **C**) undifferentiated and **D**) NGF-induced differentiated cells. Differentiation inhibits fetal tau expression.  $A\beta$  exposure promotes exclusion of exons 2/3 in undifferentiated and differentiated cells, and exclusion of exon 6 in undifferentiated cells.

appeared to express in a lower level, compared to differentiated cells. Untreated and  $A\beta$ -treated differentiated cells showed expression using the exon 6 specific primer pair and only one band of 393bp using the non-specific primers. Regarding the expression of exons 7-9, they remain unchanged in their expression, during treatment.

### DISCUSSION

In this study, we demonstrated that  $A\beta_{42}$  peptide exposure causes morphological changes and alters the splicing process of tau exons 2 and 3 in PC12 cells, favoring non-inclusion of both exons (i.e. expression of fetal tau). The study of tau

alternative splicing and the effects  $A\beta_{42}$  peptide can exert on this process, may help to better understand the origin of tau pathology in AD. The splicing of exon 10, which generates tau spliced variants with 3 or 4 microtubule-binding domains, has been widely studied and it has already been demonstrated that alterations in this process can cause neurodegeneration. Dyrk1A-SC35 (Qian et al. 2011), Dyrk1A-9G8 (Ding et al. 2012) and (PKA-C $\alpha$ )-SRSF1 (Shi et al. 2011) interactions contribute to neurodegeneration in the AD brain by altering regulation of tau exon 10 splicing through phosphorylation. In addition, a fairly recent study has provided some insights into the



A $\beta$ -tau relationship demonstrating that A $\beta$  can alter the splicing of tau exon 10 and cause imbalanced expression of 3R/4R-tau through A $\beta$ -(GSK-3 $\beta$ )-SC35 (Chen et al. 2010) interactions.

Similar abnormalities in the alternative splicing of exons 2, 3 and 6 may also induce tau pathology in AD as a response to A $\beta$  aggregation. Mature tau isoforms have demonstrated to participate in the stabilization of neurites when inducing PC12 cell differentiation using NGF (Hanemaaijer and Ginzburg 1991). This suggests that inclusion of MAPT exons 2 and 3 promotes switching from immature to mature tau isoforms that stabilize microtubules. Here, we observed these mature isoforms by analyzing inclusion of exons 2 and 3, and showed 1N- and 2N-tau were expressed in undifferentiated as well as differentiated PC12 cells but, when we exposed these cells to A $\beta_{42}$  peptide, only the immature 0N-tau isoform (fetal tau) was expressed, even in differentiated cells. These results suggest that A $\beta$  peptides may be altering the alternative splicing of tau in the AD brain, promoting the exclusion of exons 2 and 3 and generating immature tau isoforms that make microtubules unstable. Furthermore, fetal tau, similar to the tau found in the NFTs in AD, has shown to be hyperphosphorylated (Smith et al. 1995). Thus, an A $\beta$ -induced exclusion of exons 2 and 3 in AD may destabilize neurites by altering both regulatory mechanisms involving tau: isoforms and phosphorylation.

Exon 6 is contained in high molecular weight tau, but not in the low molecular weight tau found in the human and rat brains; however, exon 6 inclusion has been reported in AR42J tumoral pancreatic acinar cells, using the primer pair Ex5/Ex9, as a second amplified fragment of 591bp to the expected 393bp fragment lacking of exon 6 (Vanier et al. 1998). In our study, we did not find a 591bp fragment, even when exon 6 expression was observed by amplification with the exon specific primer pair Ex6S/Ex6AS. Although we are unaware unclear for this finding, amplification with

exon 6 specific primers showing a high level of expression of the corresponding 214bp fragment in untreated cells is undeniable. We believe inclusion of this exon may be due to the cell line used in this study, as it could be a feature shared by several (or all) tumoral cells; in addition, the inclusion of exon 6 in the longest adult tau isoform has been reported to inhibit neurite elongation in neuroblastoma cells (Luo et al. 2004). These observations open the possibility for this particular long tau species to be constitutively expressed in PC12 cells and decrease its expression upon NGF treatment, when the formation and elongation of cellular processes is induced. Nevertheless, A $\beta_{42}$  exposure affects exon 6 splicing regulation, promoting exclusion in undifferentiated cells and inclusion in differentiated cells, but without promoting expression of the long adult tau isoform also including exons 2 and 3.

It has been shown that the timing and expression pattern of tau alternative splicing is independent of neuronal connectivity and/or environmental signaling (Collet et al. 1997); however, mRNA expression and splicing have recently proven to vary in a region-dependent manner in the human brain (Trabzuni et al. 2012), for which we do not wish to draw any strong conclusions from our results. Nonetheless, even though further study of these observations is required, especially using an AD model, and the specific molecular interactions between A $\beta_{42}$  and the splicing regulation of tau exons 2, 3 and 6 remain to be determined, our results contribute to increasing the evidence to establish interactions between amyloid and tau pathologies in AD involving A $\beta$ -induced alterations in the alternative splicing of protein tau.

Morphologically, reports of A $\beta$  effects on neuronal cells have included the formation of autophagic vacuoles, intracellular aggregates of A $\beta$  (Pajak et al. 2009), and cytoskeleton alterations by accumulation of soluble amyloid- $\beta$  precursor protein (sA $\beta$ PP) (Henriques et al. 2010). Neurite retraction in PC12 cells has been associated with

the inhibition of tau expression (Hanemaaijer and Ginzburg 1991) and phosphorylation during programmed cell death (Nuydens et al. 1997). Although the purpose of this study was not to quantify expression levels of the amplified fragments, we show the relative gene expression, we observed that A $\beta_{42}$  exposure appears to decrease expression and mostly promotes exclusion of tau exons 2 and 3 in undifferentiated as well as NGF-induced cells, while exon 6 expression may be affected only in undifferentiated cells. The study about splicing of exons 2 and 3 is involved, when exposed to amyloid peptide has been little studied in relation to Alzheimer's disease. Most studies are directed at exon 10, and it would be interesting to further investigate these exons and how they might contribute to the generation of Alzheimer's disease.

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**ABBREVIATIONS** - AD: Alzheimer's disease; MAPT: microtubule-associated protein tau; CT: carboxy-terminus; NT: amino-terminus; NFTs: neurofibrillary tangles; 3/4R-tau: 3/4 microtubule-binding repeats tau isoform; A $\beta$ : beta-amyloid peptide; NGF: nerve-growth factor; RT-PCR: retro-

transcription polymerase-chain-reaction; cDNA: complementary deoxyribonucleic acid; mRNA: messenger ribonucleic acid; GSK-3 $\beta$ : glycogen synthase kinase 3-beta; PKA-C $\alpha$ : cyclic adenosine-monophosphate (AMP)-dependent protein kinase catalytic subunit alpha; SC35: serine/arginine rich splicing factor 2, splicing component 35kDa; Dyrk1A: dual-specificity tyrosine phosphorylation-regulated kinase 1A; 9G8: serine/arginine rich factor 9G8; SRSF1: serine/arginine rich splicing factor 1; sAPP: soluble amyloid precursor protein. No conflict of interests exists.

#### RESUMO

Proteína Tau desempenha um papel crucial na fisiopatologia da doença de Alzheimer, onde sua hiper-fosforilação promove agregação e desestabilização de microtúbulo. Tau submete-se a splicing alternativo que gera seis isoformas no cérebro humano, devido a inclusão/exclusão dos exons 2, 3 e 10. A desregulação do processo de splicing do exon tau 10 é suficiente para causar Taupatia e mostrou-se ser influenciado por peptídeos beta-amilóides, entretanto o splicing de outros exons é menos estudado. Nós estudamos os efeitos da beta-amiloide(42) no splicing alternativo de exons tau 2/3 e 6, usando células PC12 não tratadas ou estimuladas pelo fator de crescimento do nervo. A exposição de beta-amiloide levou processos celulares formados a retrair em células diferenciadas e alterou a expressão de exons 2/3 tanto em células indiferenciadas como diferenciadas. A expressão do exon 6 foi reprimida apenas em células indiferenciadas. Nossos resultados sugerem que beta-amiloide interfere com o processo de splicing de exons 2/3, favorecendo a sua exclusão e, portanto, a expressão de isoformas imaturas de tau que são menos eficientes em estabilizar microtúbulos e também podem ser mais propensas a hiper-fosforilação. O mecanismo molecular para essa interação amiloide-tau permanece a ser determinado, mas pode ter implicações potenciais para a compreensão dos processos neuropatológicos fundamentais na doença de Alzheimer.

**Palavras-chave:** Doença de Alzheimer, peptídeo amiloide, regulação de splicing, isoformas tau.



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