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Extracellular tau promotes intracellular calcium increase through M1 and M3 muscarinic receptors in neuronal cells

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Extracellular tau promotes an increase in the level of intracellular calcium in cultured neuronal cells. We have found that such increase is impaired in the presence of antagonists of muscarinic receptors. In order to identify the nature of those receptors, we have tested the effect of different specific muscarinic receptor antagonists on tau promoted calcium increase. Our results indicate that the increase does not take place in the presence of antagonists of muscarinic (mainly M1 and M3) receptors. A similar increase in intracellular calcium was found in non-neuronal cells transfected with cDNA of M1 and M3 muscarinic receptors when tau was added. These results suggest that observed effect of tau protein on neuronal (neuroblastoma and primary cultures of hippocampal and cortical neurons) cells is through M1 and M3 muscarinic receptors. Therefore blocking M1 and for M3 receptors, by using specific receptor antagonists, can prevent that tau toxic effect that could take place in tauopathies.

Keywords: Tau protein; Muscarinic receptors; Alzheimer disease

Introduction

Alzheimer disease (AD) is characterized by the presence in the brain of the patients of two structures, the senile plaques and neurofibrillary tangles (NFT). The main component of NFTs is the microtubule associated protein tau in hyperphosphorylated form. Tau pathology in AD follows a reproducible pattern, whereby hyperphosphorylated (and aggregated) tau first appears in the entorhinal cortex and hippocampus, and from there, the pathology spreads to the surrounding areas (Braak and Braak, 1991). In this pathological process there is neuron death and as a consequence of this, intracellular components like tau in soluble form or forming NFT could be found at the extracellular space, becoming ghost tangles.

It has been proposed that extracellular tau could be toxic for neurons (Gomez-Ramos et al., 2006), playing a role in the spreading pathology of AD. The possibility exists that tau toxicity could be dependent on the interaction of this protein with cellular receptors able to promote an increase in intracellular calcium levels. It has been previously indicated that not only tau protein but others related with neurodegenerative disorders such as α-synuclein (Danzer et al., 2007), β-amyloid peptide or prion protein (Demuro et al., 2005) may induce a disruption of calcium homeostasis when added to cultured neuronal cells. However, the mechanisms for that disruption of calcium homeostasis, differ with the type of assayed protein (Adamczyk and Strosznajder, 2006; Danzer et al., 2007; Gomez-Ramos et al., 2006). In the case of tau protein it has been suggested that changes in the level of intracellular calcium could be mediated by the interaction of tau with muscarinic receptors, that induce calcium release from intracellular stores (Gomez-Ramos et al., 2006).

There are five cholinergic muscarinic receptor subtypes (M1 to M5), all of them being expressed in the Central Nervous System (CNS), but at different levels and in different locations, for example, M1 and M3 appear to be the most abundant muscarinic receptors expressed in hippocampus and entorhinal cortex in adult mouse, whereas M5 is poorly expressed (data from Allen Brain Atlas; http://www.brain-map.org/). The M1–M5 receptors can be subdivided into two major functional classes according to their G-protein coupling preference. The M1, M3 and M5 receptors, selectively couple to G-proteins of the G_q/G_{11} family, whereas the M2 and M4 receptors preferentially activate G_i/G_o-type G-proteins (Wess et al., 2007). Coupling through the first group (M1, M3 and M5), but not through the second group, results in an increase in intracellular calcium (Lanzafame et al., 2003).

In this work, we tried to identify the muscarinic receptor or receptors involved in the response of neuronal cells to extracellular

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tau. Using pharmacological tools and transfected cells with cDNA of muscarinic receptors, we have determined that M1 and M3 muscarinic receptors are involved in this cellular response to tau, whereas M2 did not play any role in tau-induced increase in intracellular calcium. In addition, we have also found that tau induced a sustained intracellular calcium increase in primary cultures of hippocampal and cortical neurons.

Results

Exposure to tau deregulates calcium homeostasis in SH-SY5Y neuroblastoma cells, but not in the presence of atropine

The SH-SY5Y human neuroblastoma cell line is a well characterized model to study muscarinic cholinergic function (Lambert and Nahorski, 1990; Murphy et al., 1991) and we decided to use this cellular model to establish the effect that extracellular tau induces on cholinergic neurotransmission system.

In order to check the functional status of these cells, FURA-2 AM loaded SH-SY5Y cells were exposed to 50 μM ACh (Fig. 1A a). The calcium increase induced by ACh was totally abolished when SH-SY5Y cells were pretreated with 100 μM atropine, a broad muscarinic receptor antagonist (Fig. 1A b). These data suggest that muscarinic are the main cholinergic receptors in this cell line.

Fig. 1B a shows the effect of adding recombinant tau to FURA-2 loaded SH-SY5Y, with a low number of passages (minor than 10). The addition of tau in calcium dependent fluorescence was similar to that found for ACh in these cells; this effect was totally abolished when SH-SY5Y cells were pre-incubated with atropine (Fig. 1B b). This result suggests that the increase in calcium provoked by tau, may be mediated by muscarinic acetylcholine receptors. Nevertheless, when the same experiment was repeated with SH-SY5Y cells with a higher number of passages (>10) (Fig. 1C a), the calcium signaling provoked by tau was more prolonged in time than in the case of cells with a lower number of passages. Also, in this case, the increase in intracellular calcium signaling due to tau addition was abolished when the cells were pre-incubated with 100 μM atropine (Fig. 1C b). This difference in calcium signaling provoked by tau addition can be due to a differential expression of acetylcholine muscarinic receptors in these cells that could depend on the number of cell passages (see below). Also, Fig. 1 indicates a different calcium fluorescence response for ACh and for tau, being more prolonged in time the tau effect than ACh effect.

Characterization of the muscarinic receptors present in SH-SY5Y neuroblastoma cells

As a first step to determine the type(s) of muscarinic receptor involved in tau effect, we have analyzed the expression levels of these receptors in SH-SY5Y neuroblastoma cells by using quantitative RT-PCR in different cell passages. In Fig. 2 it is shown that M1, M2, M3 and M4 receptors; and, in a much lower proportion, M5 are expressed in those neuroblastoma cells. However, we have also found that such expression could slightly change depending on the time of culture of these cells, since after more than 10 culture passages, a decrease in the expression of M2 and an increase in that of M3 and M4 was found. These results strongly suggest that the differences in the intracellular calcium induced by tau on SH-SY5Y depends on the number of days in culture and on the type and levels of muscarinic receptors expressed in these cells.

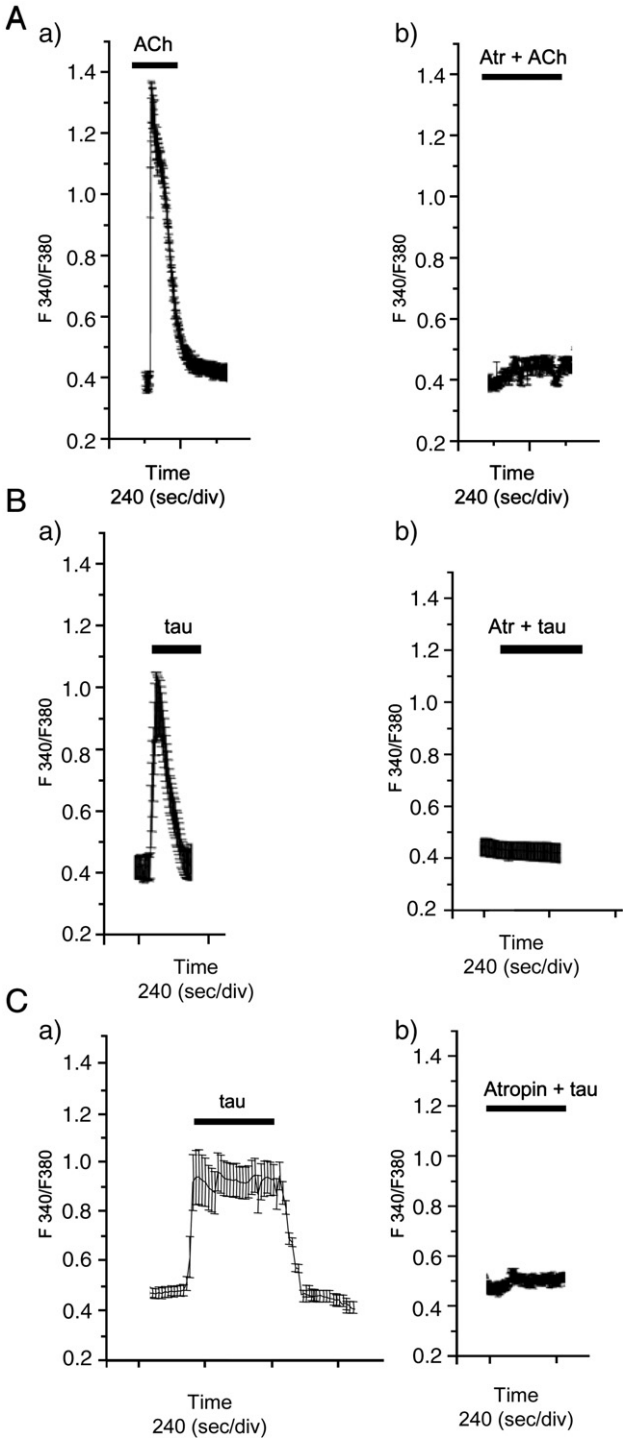

Fig. 1. Muscarinic receptor antagonist atropine, inhibits the intracellular calcium increase induced by tau protein addition. (A) FURA-2 fluorescence time course changes recorded for cultured human neuroblastoma SH-SY5Y cells, which were stimulated with 50 μM acetylcholine (ACh) in the absence (a) or in the presence (b) of 100 μM atropine. Upper solid bars indicate the stimulation periods. (B) FURA-2 fluorescence time course changes recorded for cultured SH-SY5Y cells with a number of passages lower than 10, stimulated with 1 μM tau protein in the absence (a) or in the presence (b) of 100 μM atropine. (C) As in panel B, but in this case, the SH-SY5Y cells assayed have more than 10 passages when they were used. The trace shows the average response of ≥20 cells. Bars indicate standard deviation. In all cases the upper solid bars indicate the stimulation periods.

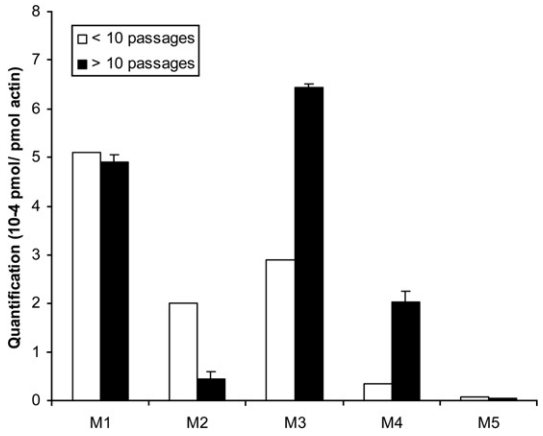

Fig. 2. Changes in the levels of expression of muscarinic receptors in SH-SY5Y human neuroblastoma cells during the culture days. Muscarinic receptor expression in SH-SY5Y neuroblastoma cells with less (□) or more (■) than 10 passages, was analyzed by Real-time PCR, and the obtained results are shown.

Pharmacological analysis to determine the muscarinic receptors involved in tau effect

Since the calcium deregulation induced by tau was prevented by the presence of atropine, a broad antagonist of all muscarinic receptor subtypes (Fig. 1), we decided to perform a pharmacological characterization of muscarinic receptor subtype involved in this phenomenon. For this, we used a battery of selective muscarinic receptor subtype antagonists. Thus, we used Pirenzepine to block the M1 muscarinic receptor (Rosati et al., 1999), Gallamine as M2 receptor antagonist (Gnagey et al., 1999) and 4-DAMP as M3 muscarinic receptor antagonist (Fritz et al., 2005). The results obtained in these studies showed that muscarinic receptors M1 and M3 appear to be the ones involved in the calcium deregulation induced by tau, since both Pirenzepine and 4-DAMP, but not Gallamine, inhibited the calcium increase evoked by tau stimulation (Fig. 3).

Transfection of non-neuronal cells with cDNA expressing M1 and M3 receptors

To confirm the previous pharmacological data, we decided to perform a new set of functional experiments using a cell heterologous expression system for M1 and M3 muscarinic receptors in COS-7 cells. Thus we first measured the expression of muscarinic receptors in these cells by Real-time PCR, and essentially, no expression, compared to that found in neuroblastoma cells was observed (data not shown). To analyze if COS-7 is a good model to test muscarinic function, transient transfection experiments were done. First, we transfected COS-7 cells with pcDNA3.1 empty plasmid (without any cDNA insert), and we found that there was no response to ACh or tau, in those transfected cells (see below, Fig. 5A).

Then, we did a transient transfection using cDNA expressing M1 and/or M3 receptors. Fig. 4 shows the expression of these receptors by western blot (Fig. 4A). Moreover immunological studies using an antibody raised against the tag (HA) of these proteins; or by confocal

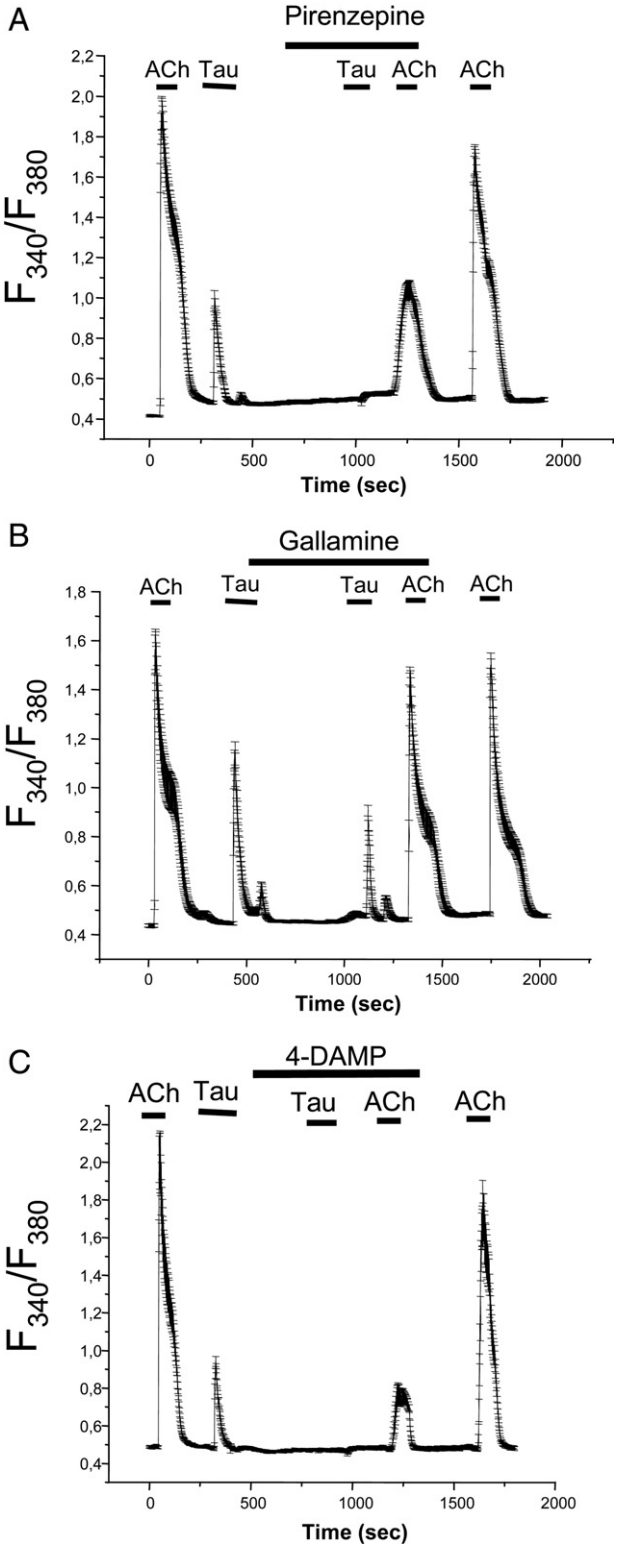

Fig. 3. Pharmacological identification of muscarinic receptor subtypes present in SH-SY5Y cells sensitive to tau stimulation. FURA-2 loaded SH-SY5Y cells were stimulated to 1 μM tau protein in the presence of several muscarinic receptor antagonists: (A) FURA-2 fluorescence time course changes recorded for SH-SY5Y after they were stimulated with ACh and tau in the absence or presence of muscarinic receptor antagonist Pirenzepine (that inhibits M1 receptor). Finally, after washing the cells, 50 μM ACh was applied to confirm the viability of the cells. (B) The same sequential application as in (A), but in this case tau and ACh were applied in the presence of the M2 muscarinic receptor antagonist Gallamine. (C) As in panel B, but using muscarinic receptor antagonist M3 4-DAMP. The trace shows the average response of ≥20 cells. Bars indicate standard deviation. In all cases the upper solid bars indicate the stimulation periods.

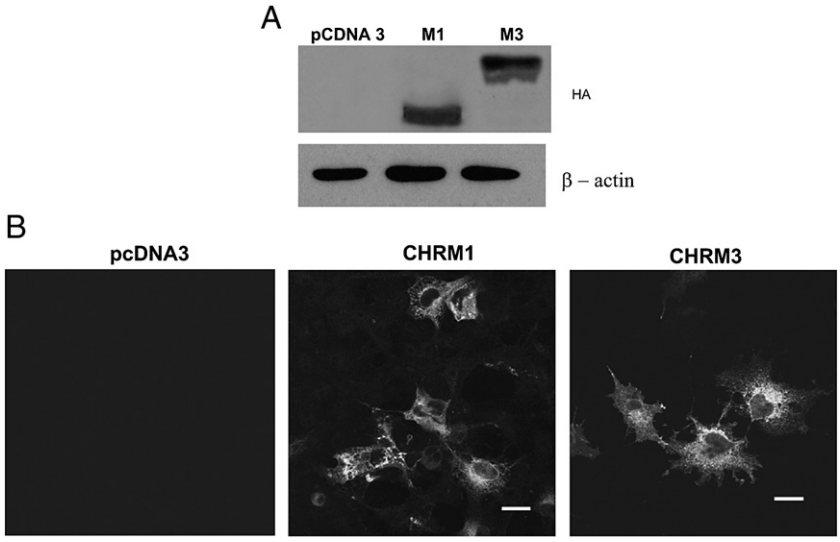

Fig. 4. Transient transfection of COS-7 cells with plasmids containing cDNAs of muscarinic receptors M1 or M3. African green monkey kidney cells COS-7 were transfected with pcDNA3 (empty vector, used as control. CHRM1 (containing full-length human muscarinic acetylcholine receptor 1 cDNA under CMV promoter and 3x HA tag in its N-terminus) and CHRM3 (similar to CHRM1 but, in this case, containing full-length human muscarinic acetylcholine receptor 3 cDNA). (A) Western blot for anti-HA and anti-b-actin from extracts of COS-7 transient by transfected cells with pcDNA, CHRM1 and CHRM3 was done. (B) Confocal image from COS-7 transient by transfected cells with pcDNA3, CHRM1 and CHRM3.

immunofluorescence analysis (Fig. 4B) were also performed, and the expression of these receptors in transfected cells was also observed. No expression was found in the negative control of cells transfected with pcDNA3.1, empty plasmid without any cDNA insert.

When tau protein was added to M1 or M3 expressing cells (Figs. 5B, C) a clear increase in intracellular calcium concentration was found in both cases. Similar results were observed in double M1/M3 transfected cells (Fig. 5D).

After confirming that activation of M3 muscarinic receptor mediates the intracellular calcium increase evoked by tau, we wanted to know which region of tau protein was involved in the muscarinic receptor-dependent intracellular calcium increase. Thus different tau fragments (from N- to C-terminal regions) were assayed in the COS cell model expressing M3 receptor (Fig. 6A). Fig. 6B shows that mainly the tau fragment 3RC is involved in this effect. To have a further analysis of that observation we have tested if a tau peptide, located at the C-terminal region, and comprising residues 391 to 407 may have any effect on calcium response induced by tau in neuroblastoma cells. Fig. 6C shows, indeed, that such tau peptide may be involved in the effect found for the whole tau molecule.

Exposure to tau deregulates calcium homeostasis in primary cultured hippocampal neurons

Tau protein was added to hippocampal or cortical neurons in culture, and intracellular calcium increase was measured in the presence or absence of atropine. Neurons from hippocampus (Fig. 7) were cultured for different days (1, 3 and 7). After one day of culture, day in vitro 1 (DIV 1), less than 20% of the cells respond to either ACh or tau protein. However, after DIV3, and mainly after DIV7, the proportion of cells responding to both ACh and tau protein was clearly increased (Fig. 7B). It is worth highlighting that a higher sensitivity to tau (Fig. 7A) correlates with the increase in the number of neurons expressing M1 receptors (Fig. 7D). As expected, the calcium increase evoked by tau was, in all cases, prevented by atropine pretreatment (Fig. 7C). A potassium dependent depolarization was performed in all studied neurons to confirm that the cells remain functional after the different treatments (Fig. 7C). Similar results were observed on cortical neurons in culture (data not shown).

A difference between ACh and tau responses is that tau response is more prolonged in time than ACh effect, and it could be more toxic for the cell.

Discussion

Thirty years ago, it was described that Alzheimer disease was associated with a severe loss of cholinergic markers in the brain (Bowen et al., 1976; Davies and Maloney, 1976). This observation raised the hypothesis suggesting that cholinergic loss could be related to the severity of the dementia (Perry et al., 1981) and settled the basis for the cholinergic deficit hypothesis in Alzheimer disease (Coyle et al., 1983). However, studies of other pathological features of the disease, such as the presence of senile plaques and the neurofibrillary tangles, have resulted in the formulation of new hypothesis, mainly related with the main component of senile plaques, the amyloid peptide (Hardy and Selkoe, 2002). In comparison with the very high number of publications on amyloid pathology little was done concerning a possible relationship between cholinergic deficits and the pathological features, (for a review see (Mesulam, 2004).

The main component of neurofibrillary tangles is tau protein and, in AD, tau pathology starts at the entorhinal cortex (EC) (Braak and Braak, 1991). In the EC up to 80% of cholinergic axons can be depleted in AD (Geula and Mesulam, 1996) and that depletion has been assigned to neurofibrillary degeneration (Geula and Mesulam, 1999). In the nearby areas, the presence of some muscarinic receptors, such as M2 receptors, was reduced in AD (Mash et al., 1985).

The results of this work are compatible with our previous observation (Gomez-Ramos et al., 2006) suggesting that tau found at the extracellular space, after cell death, could be toxic for neuronal cells and, upon death of the tau-responding cells, the toxicity could propagate to other neighboring cells, and this could be one of the mechanisms to explain the propagation pattern observed for tau

pathology in AD, if the process starts at EC. Indeed, working on SH-SY5Y human neuroblastoma cells, we have found that tau-induced pathology involves a deregulation of calcium homeostasis, that takes place in those cells bearing muscarinic receptors (Gomez-Ramos et al., 2006). The existence of proteins that could activate cholinergic receptors is not a new idea since it has been reported that a rabies virus glycoprotein specifically binds to acetylcholine muscarinic receptors (Kumar et al., 2007).

As a first step to identify the muscarinic receptor able to bind tau, we have identified the muscarinic receptors expressed in SH-SY5Y neuroblastoma cells, and we found that mainly M1, M2 and M3 were expressed. Thus, we propose that SH-SY5Y cells can be a good model for studying tau effect, because M1 receptor is heavily expressed in whole brain (http://www.brain-map.org/) including those regions mainly affected in AD. Besides, a reduction of M2 receptor expression in AD has been described (Mash et al., 1985), and finally, M3 receptors are mainly located on cortex, hippocampus, and retrohippocampal formation (http://www.brain-map.org/). Thus, we have focused our studies in these receptors.

After performing pharmacological studies and cDNA transfection analyses, we found a possible role for M1 and for M3, but not for M2, in tau toxicity in neuroblastoma or non-neuronal transfected cells.

Additionally, it has been shown that M1 and M3 mediated muscarinic stimulation promotes the processing of amyloid precursor protein (APP) by the α-secretase pathway (Nitsch et al., 1992). However, M1 stimulation may also lead to an increase in BACE (APP β-secretase) expression, whereas M2 stimulation decreases BACE expression (Zuchner et al., 2004; Zuchner et al., 2005). In AD, an increase of M1 and M3 receptors with respect to that of M2 has been found (Mash et al., 1985). Thus, tau protein acting through M1 and M3 but not through M2 receptor could act on those sick neurons. In our studies we do not know, at the present, the possible difference for tau or ACh activation of muscarinic receptors and if they could be related, or not, to any of the previous consequences that have been described for muscarinic receptor activation. Nevertheless, since changes in α or β APP secretase activities could take place upon M1/M3 receptor activation that could result in the depletion of Aβ production, we are currently testing if a possible overexpression of Aβ due to tau toxicity may take place and comparing it to the effect of M1/M3 activation by ACh. However, our on-going research on M1/M3 receptor and APP transfected cells, does not suggest any difference between the addition of ACh and that of tau to the cells.

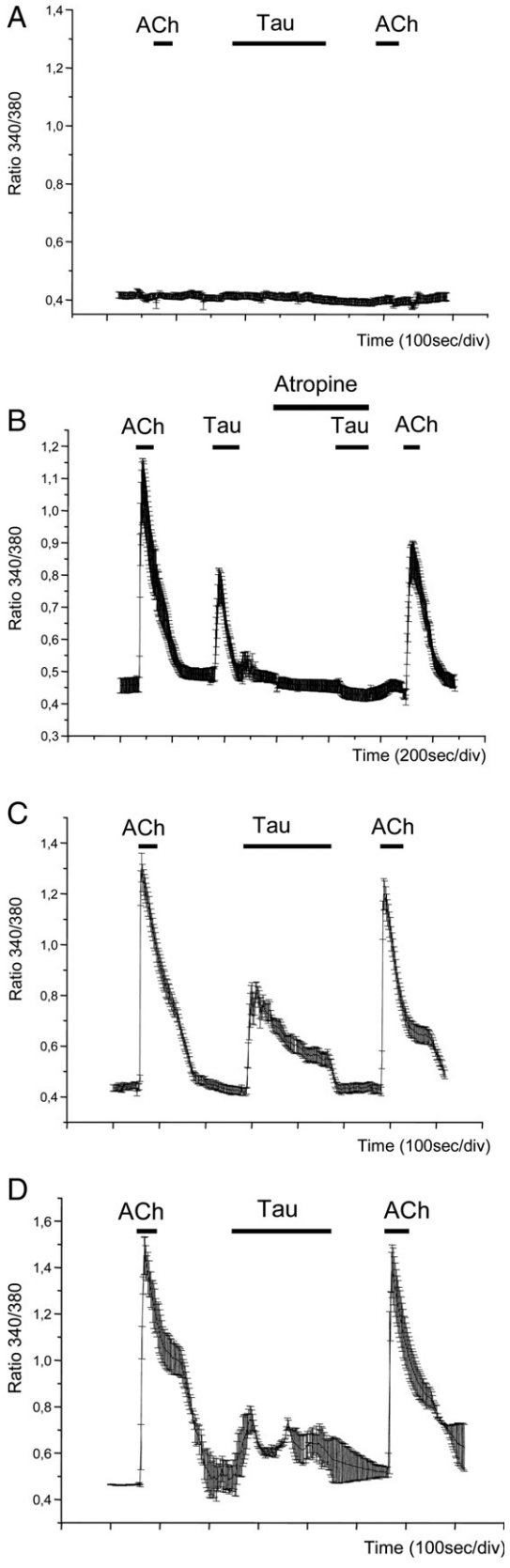

Fig. 5. Tau protein induces intracellular calcium increase in COS-7 cells transfected with CHRM1 and CHRM3 constructs. FURA-2 loaded COS-7 cells transfected with CHRM1, CHRM3 constructs or with empty vector pcDNA3.1 (Ctrl), were stimulated with 1 mM tau protein, and fluorescence time course changes were measured. (A) Control COS-7 cells transfected with pcDNA3.1 were stimulated with a pulse of 50 μM ACh (in order to establish functional cell status) followed by 1 μM tau. The cells do not respond to acetylcholine neither to tau. (B) COS-7 cells transfected with CHRM1 construct were stimulated to ACh, followed by tau protein. The cells respond to tau protein in a similar magnification that occurs in SH-SY5Y human neuroblastoma cells. There was no response to tau when cells were pre-incubated with 100 μM atropine. Finally, after washing the cells with perfusion buffer (see Experimental methods) a pulse of 50 μM ACh was applied in order to confirm cell viability. C) COS-7 cells transfected with CHRM3 were stimulated with 50 μM ACh, 1 μM tau protein and again with 50 μM ACh (after rinsing with perfusion buffer). (D) As in panel C, but in this case the experiment was done with COS-7 cells transfected with both CHRM1 and CHRM3 plasmids. The trace shows the average response of ≥20 cells. Bars indicated standard deviation. In all cases the upper solid bars indicate the stimulation periods.

Finally, we like emphasize that our results on tau action, observed in non-neuronal transfected cells or in neuroblastoma cells, were confirmed in primary cultures of hippocampal and cortex neurons.

Experimental methods

Materials

The muscarinic receptor antagonists: atropine, Pirenzepine, Gallamine and 4-DAMP were purchased from Sigma (St. Louis, MO, USA). Monoclonal mouse anti-HA antibody (clone 12CA5) was obtained from the "Optic and Confocal Microscopy Service" (S.M.O.C., Centro de Biología Molecular "Severo Ochoa", Madrid, Spain). Monoclonal mouse anti-β-actin antibody was obtained from Sigma (St. Louis, MO, USA). Polyclonal rabbit anti-muscarinic acetylcholine receptor M1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mammalian expression plasmids containing CMV promoter and the full-length cDNAs from human muscarinic receptors M1 (CHRM1) and M3 (CHRM3), tagged in its N-terminus with 3x HA epitope, were obtained from UMR cDNA Resource Center (Missouri, MO). pcDNA3.1 plasmid used as control and Lipofectamine 2000 transfection reagent, were obtained from Invitrogen (Carlsbad, Ca). Mammalian expression plasmid containing CMV promoter and cDNA from APP695 was a gift from Dr. Maria Recuero.

Tau peptide comprising the following sequence EIVYKSPVVSGDT-SPRH, residues 391 to 407, (nomenclature of the largest tau isoform), present at the C-terminal region of the protein, was synthesized and purified, as previously described (Perez et al., 2001).

SH-SY5Y Cell culture

Human neuroblastoma SH-SY5Y (Biedler et al., 1978) cells obtained from ATTC (Rockville, Md) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) plus 2 mM glutamine in a humidified atmosphere with 5% CO_2. The day before performing the experiment, the cells were seeded on polylysine-coated glass coverslips at a density of 10^5 cells/coverslip to assay calcium dependent fluorescence analysis.

COS-7 cell culture and transfection

African green monkey kidney fibroblast (COS-7) cells (Gluzman, 1981) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FBS. The day before the transfection, the cells were seeded on polylysine-coated glass coverslips at a density of 5×10^4 cells/coverslip. Cells were transiently transfected with CHRM1, CHRM3 containing a HA tag, or with both plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Control cells were transfected with the empty vector (pcDNA3.1, Invitrogen) at the same conditions. After 24 h of expression, transfected cells were fixed for immunofluorescence analysis or processed for calcium dependent fluorescence signal analysis.

Primary cultures of hippocampal neurons

Hippocampal neurons were cultured as described by Banker and Cowan (1977). Newborn wild-type mice (P0), were sacrificed, and the embryos were removed under sterile conditions. Dissociated hippocampal neurons from the embryos were plated on glass coverslips coated with 1 mg/mL poly (L-lysine)

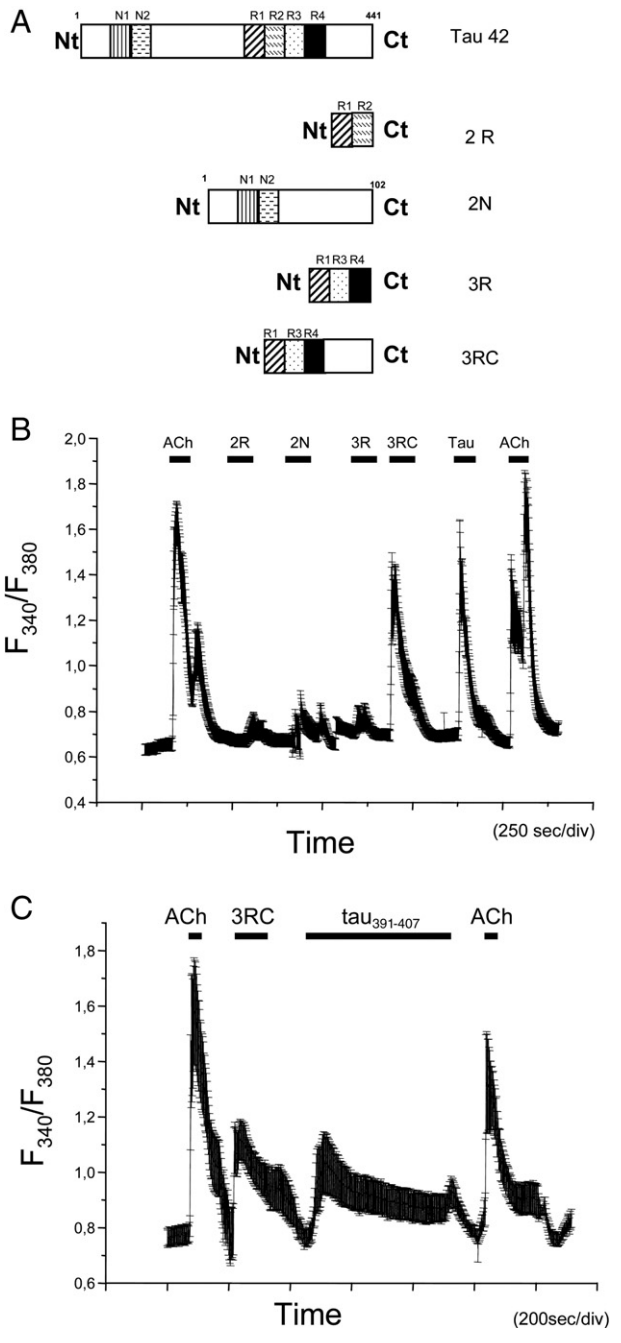

Fig. 6. Effect of tau peptides in COS-7 cells transfected with CHRM3 construct. (A) The whole tau protein and different tau peptides were assayed to determinate the putative region of tau protein involved in deregulation of calcium homeostasis: Tau 42, indicates the longest human tau isoform expressed in the SNC containing 2 inserts in its N-terminal region and 4 repeats in its microtubule binding region (Goedert and Jakes, 1990); 2R, referred to as tau peptide containing first and third repeats of Tau 42; 2N as tau peptide containing the N-terminal region of Tau 42 including its two N-terminal inserts, but no the microtubule binding regions (aminoacids 1–243); 3R referred to as tau peptide containing first, third and fourth repeats of its microtubule binding region and 3RC referred to as the C-terminal region of tau 42, including first, third and fourth microtubule binding repeats. (B) COS-7 cells transiently transfected with CHRM3 plasmid, were stimulated with a sequential sequence, thus first it was assayed at 50 μM ACh (to check cell functionality), and after then were assayed differences in tau peptides at 1 μM: 2N, 2R, 3R, 3RC and finally the full-length tau protein. After tau treatment, the cells were again stimulated with a pulse of 50 M ACh, The cells were washed with perfusion buffer after every application. The trace shows the average response of ≥ 20 cells. Bars indicated standard deviation. In all cases the upper solid bars indicate the stimulation periods. (C) FURA-2 fluorescence time course changes recorded for Cos-7 cells transiently transfected with CHRM3 plasmid, and sequentially stimulated with 50 μM ACh, 3RC peptide and C-terminal tau peptide comprising residues 391 to 407 ($tau_{391-407}$). Again, the cell viability, after tau peptide treatment was tested by a pulse of ACh.

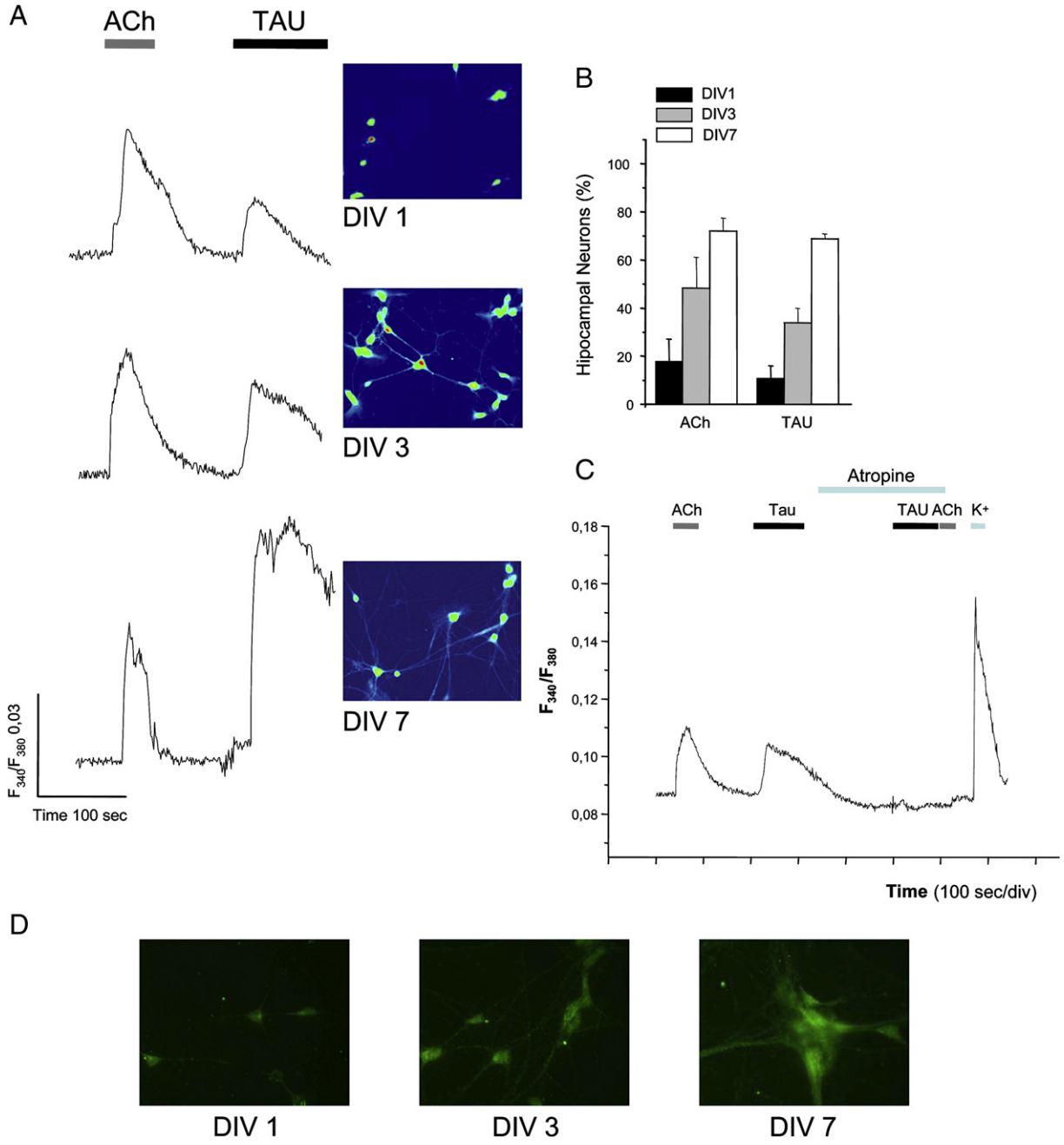

Fig. 7. Tau deregulates calcium homeostasis in primary cultured of hippocampal neurons. (A) Mouse hippocampal neurons cultured for 1, 3 and 7 days, were FURA-2 loaded and the intracellular calcium levels were measured after a sequential application of 50 μM ACh and 1 μM tau. Insets show images showing color representations of the FURA-2 loaded hippocampal neurons responding to tau. (B) Graphic shows the percentage of mouse hippocampal neuronal cells that respond to ACh or tau at 1 (■), 3 (▒) or 7(□) days in vitro. (C) Newborn mouse hippocampal neurons cultured for 3 days, were sequentially stimulated with 50 μM ACh and 1 μM tau in the presence or absence of 100 mM atropine. Finally, the neurons, were depolarizated with 60 μM KCl to confirm their viability after the treatments. (D) Mouse hippocampal neurons were fixed at 1, 3 or 7 days in vitro and processed for immunofluorescence analysis with an antibody that recognizes muscarinic acetylcholine receptor 1.

and 20 μg/mL laminin. After incubation for 3 h in medium containing 10% (v/v) horse serum (Gibco), the cells were transferred to N2- and B27-supplemented medium (Gibco) and incubated for 1, 3 or 7 days.

Immunofluorescence analysis

COS-7 transiently transfected cells and primary cultures of neurons were fixed with 4% paraformaldehyde for 20 min. After the fixation, the coverslips were incubated with phosphate buffered saline (PBS) supplemented with 0.1% Triton X-100 for 10 min, then were incubated with 1% bovine serum albumin (BSA) in PBS/TX-100 for additional 10 min. Primary antibody anti-HA was diluted 1:2000 (vol/vol) in PBS/BSA and incubated in coverslips for 45 min at room temperature. After primary antibody incubation, coverslips were rinsed with PBS and incubated with secondary antibody (Alexa 488, Invitrogen) diluted 1:500 (vol/vol) in PBS/BSA. Finally, the coverslips were rinsed again with PBS and mounted in FluorSave (Calbiochem). Coverslips were analyzed by confocal microscopy in a Confocal MicroRadiance (BioRad) coupled with a vertical microscopy Axioskop 2 (Zeiss).

Calcium dependent fluorescence signal analysis—image acquisition and analysis of the Ca^{2+} response in different cell types

Primary cultures of hippocampal and cortical neurons, human neuroblastoma SH-SY5Y and COS-7 transiently transfected cells, were washed with perfusion buffer (122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose and 20 mM *N*–Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.4), and they were loaded with FURA-2 AM solution (7.5 μM) for 30 min at 37 °C. This period facilitated the intracellular hydrolysis of the FURA-2 AM. Subsequently, the coverslips were washed with fresh medium and mounted in a superfusion chamber in a NIKON Eclipse TE-2000 microscope. In all experiments the cells were superfused first at 1.2 mL/min with perfusion media and then, with 50 μM ACh in order to establish the functional cell status. When tau protein was assayed, the perfusion system was stopped. When the experiments were performed with muscarinic receptor antagonists: atropine (100 μM), Pirenzepine (10 μM), 4-DAMP (2 μM) and Gallamine (2 μM), the cells were incubated for 10 min before adding ACh or tau.

At the end of each experiment, 50 μM Ach pulses were applied to confirm the viability of the cells under study. Cells were visualized using a Nikon microscope containing a ×40 S Fluor 0.5–1.3 oil lens. The wavelength of the incoming light was filtered to 340 nm and 380 nm with the aid of a monochromator (10 nm bandwidth, Optoscan monocromator, Cairin). These wavelengths corresponded to the fluorescence peaks of Ca^{2+}-saturated and Ca^{2+}-free FURA-2 solutions. The 12-bit images were acquired with an ORCA-ER C 47 42–98 CCD camera from Hamamatsu (Hamamatsu City, Japan) controlled by Metafluor 6.3r6 PC software (Universal Imaging Corp., Cambridge, UK). The exposure time was 150 ms for each wavelength and the changing time <5 ms. The images were acquired continuously and buffered in a fast SCSI disk. The time course data represent the average light intensity in a small elliptical region inside each cell. The background and autofluorescence components were subtracted at each wavelength, and the 340 over 380 nm ratio was calibrated into $[Ca^{2+}]_i$ values using Grynkiewicz's equation (Grynkiewicz et al., 1985). The R_{max}, R_{min} and β parameters were calculated from the spectra of small droplets of FURA-2 in Ca^{2+}-saturated and Ca^{2+}-free solutions (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris, 10 mM MOPS and 100 μM FURA-2. Ca^{2+}-free: plus 2 mM EGTA. Ca^{2+}-saturated: plus 2 mM CaCl2, pH 7.4) (Diaz-Hernandez et al., 2001) and the in vitro R_{max} and R_{min} were corrected for the differences with the cytosolic environment using the procedure described by Poenie (1990) and Baitinger et al. (1990).

Protein purification

The recombinant tau isoform (tau42) containing 2 N-terminal inserts and 4 microtubule binding repeats (Goedert and Jakes, 1990), and tau fragments: 2N, containing the amino-terminal half of tau protein, (residues 1–251), tau 2R, containing residues 250–335 (deleted 274–304), tau 3R, containing residues 250–367 (deleted 274–304), and tau 3RC, containing residues 250–441 (deleted 274–304), was isolated as previously described (Perez et al., 1996). The isolated proteins were characterized by gel electrophoresis followed by staining with Coomassie blue.

Gel electrophoresis and western blot

Cells were homogenized at 4 °C in a buffer containing 50 mM HEPES, pH 7.4, 10 mM EDTA, 0.1% Triton X-100, and including the phosphatase inhibitors 20 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM phenylmethanesulfonyl fluoride. The cell lysates were centrifuged at 10,000 g for 5 min at 4 °C and then heated at 100 °C for 5 min in electrophoresis sample buffer. The protein concentration was determined by using the BCA protein assay, at 37 °C (Pierce, Rockford, IL). Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Dassel, Germany). After blocking of non-specific protein binding to the membranes with 0.05% Tween 20 and 5% non-fat dry milk in PBS, the membranes were incubated overnight with the primary antibodies anti-HA (1:2000, vol/vol) or anti-β-actin (1:5000, vol/vol) in blocking buffer at 4 °C. The proteins recognized by the antibodies were visualized by using enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA) after incubation with horseradish peroxidase (HRP)-linked secondary antibodies (Dako A/S, Glostrup, Denmark).

Quantitative RT-PCR

Total RNA was purified from 35 mm dishes of confluent SH-SY5Y cells or COS-7 cells using an RNA Miniprep Kit (Stratagene) according to the manufacturer's recommended protocol. RNA extraction was followed by DNAse treatment to avoid genomic DNA contamination. Then, reverse transcription was performed with both 0.08 μg/μL oligo (dT) primers and 0.2 μg/μL random primers using the first-strand cDNA synthesis kit for RT-PCR (AMV) (Roche Applied Science) protocol.

Genomic DNA was purified from COS-7 cells using the Wizard Plus SV Genomic DNA purification system (Promega). And genomic DNA from SH-SY5Y cells was isolated using a high salt method as follows. First, nuclei lysis buffer (1% Tris EDTA pH 8.0, 0.4 M NaCl and 2 mM EDTA) was added to the cell pellet and cells were centrifuged for 10 min at 4 °C (1200 rpm). The pellet was resuspended in the lysis buffer with 0.3 mg/mL proteinase K (Merck) and 1% SDS and, then, it was incubated at 45 °C overnight. After, 6 M NaCl was added and centrifugation for 15 min (3000 rpm) was performed. The supernatant was centrifuged repeatedly until it was clear of salt. To precipitate DNA, 3 M sodium acetate pH 5.2 and cold 100% isopropanol were added to the final supernatant. DNA was transferred into a new tube containing 70% Ethanol and it was centrifuged for 30 min (14,000 rpm). Finally, the pellet was resuspended in H_2O. Each amplification was performed using genomic DNA from SH-SY5Y as template. The concentrations of PCR products were measured using Nanodrop ND-1000 (Nanodrop Technologies). Micrograms of double stranded DNA (ds DNA) were converted in picomoles: $pmol = \mu g \times 10^6\ pg/1\ \mu g \times 1\ pmol/660\ pg \times 1/N$ (length of the amplicon in bp). Ten-fold serial dilutions in brewer-yeast tRNA (110 ng/μL, Roche Applied Science) of the PCR products starting from 10^{-2} pmol were used as standards for the calibration curves.

Real-time PCR analysis was performed in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a 96-well optical plate. The PCR master mix contained 5 μL of the SYBR Green PCR Master Mix (Applied Biosystems), 1 μL of the template and 150 nM of each forward and reverse primers in a total volume of 10 μL. Table 1 shows primer pairs designed among human sequences for each muscarinic receptor subtype and β-actin used as an endogenous control. Each primer pair reaction for each template was assayed in triplicates.

Table 1
Primers designed for each muscarinic receptor subtype and endogenous β-actin (used as control)

Gene name	Orientation	Sequence	Product length (bp)
Muscarinic receptor 1	Forward	ACCTCTATACCACGTACCTG	122
	Reverse	TGAGCAGCAGATTCATGACG	
Muscarinic receptor 2	Forward	ACCTGTGGTGTGTGACCTTT	128
	Reverse	GCTTGACTGGGTAGGTCAGA	
Muscarinic receptor 3	Forward	CATCATGAATCGATGGGCCT	128
	Reverse	GGCCTCGTGATGGAAAAGTA	
Muscarinic receptor 4	Forward	CAGTTTGTGGTGGGTAAGCG	132
	Reverse	GTACAGCACCGTCATGATGA	
Muscarinic receptor 5	Forward	GATGAGTGCCAGATCCAGTT	103
	Reverse	GACAGTAGAGGATGGTCATG	
β-actin	Forward	CACACTGTGCCCATCTACGA	162
	Reverse	CTCCTTAATGTCACGCACGA	

Thermal cycling conditions were as follows: 10 min at 95 °C, then, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After the amplification, a melting curve was obtained increasing the temperature from 60 °C to 95 °C.

Different controls were carried out for each amplification: RT-PCR-template with no reverse transcriptase as a control of the genomic DNA contamination, no-template reaction as negative control and genomic DNA-template as positive control.

Mean threshold cycle (C_T) values for each amplification performed in triplicates were normalized and analyzed using ABI prism 7000 Sequence Detection System and SDS Software (Applied Biosystems). Melting curves were studied to determine whether the primer pairs used were specific (see Table 1).

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