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Characteristics and consequences of muscarinic receptor activation by tau protein

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Abstract

It was recently suggested that tau protein released as a result of neuronal death is toxic to neighbouring cells, an effect that is mediated through the activation of muscarinic M1 and/or M3 receptors. Nevertheless, why tau protein and not other native muscarinic agonists, like ACh, can induce this neurotoxicity remains unknown. To clarify this issue, we analysed the different responses and properties of muscarinic receptors in response to stimulation by tau or ACh. The results revealed that the tau protein has an affinity for muscarinic receptors of around one order of magnitude higher than that of ACh. Furthermore, while the repeated stimulation with ACh induces desensitization of the muscarinic receptors, reiterate stimulation with tau failed to produce this phenomenon. Finally, we found the tau protein to be very stable in the extracellular milieu. These studies provide valuable information to help understand tau toxicity on neural cells bearing M1 or M3 muscarinic receptors and its contribution to neurodegenerative progression in tauopathies.

1. Introduction

Alzheimer's disease (AD) is characterized by both the occurrence of neuronal death in the brain of patients together with the appearance of two aberrant structures: senile plaques (SP) and intracellular neurofibrillary tangles (NFT). There is a good correlation between neurofibrillary pathology and nerve cell degeneration (Arriagada et al., 1992) and as a consequence of neuron death, intracellular neurofibrillary lesions can reach the extracellular space as ghost tangles. Indeed, in damaged regions like the hippocampus, an inverse correlation between the number of ghost tangles and the number of surviving nerve cells is evident (Bondareff et al., 1989; Cras et al., 1995; Fukutani et al., 1995). The development of neurofibrillary lesions correlates with the progress of the disease from the entorhinal cortex to the hippocampal region, and from there to the cortex (Braak and Braak, 1991). The main component of the NFT is the hyperphosphorylated form of the microtubule associated

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protein tau, and the noxious pathway can be followed by tracking the tau pathology (Braak and Braak, 1991) or tau hyperphosphorylation (Delacourte et al., 1999). The onset of the tau pathology in the entorhinal cortex (Braak and Braak, 1991) is associated with a severe loss of cholinergic markers (Geula and Mesulam, 1996). This loss has been attributed to neurofibrillary neurodegeneration (Geula and Mesulam, 1996), while a reduction in some muscarinic receptors at the nearby areas has also being reported in AD (Mash et al., 1985).

It was recently postulated that when tau accumulates in the extracellular space after cell death it exerts a toxic effect on neuronal cells (Gomez-Ramos et al., 2006). Indeed, the pattern of cell death observed in AD can be explained by the propagation of this toxic effect on neighbouring cells. Moreover, it was proposed that extracellular tau protein induces its toxic effect through activation of the muscarinic M1 and M3 receptors (Gomez-Ramos et al., 2008), identifying the region of the tau protein involved in binding to muscarinic receptors (Gomez-Ramos et al., 2008). However, it remains unclear why tau protein and not ACh, induces a toxic effect through muscarinic receptor activation.

To address this issue, we have analysed different parameters of muscarinic receptor activation by ACh or tau in two different cellular models, as well as in cultured hippocampal neurons. The results obtained showed clear differences in the affinity and desensitization of the muscarinic receptors when activated by either agonist.

2. Experimental procedures

2.1. Materials

A monoclonal mouse antibody against HA clone 12CA5 (Abcam, UK) was obtained from the "Optic and Confocal Microscopy Service" (S. M.O.C., Centro de Biología Molecular "Severo Ochoa," Madrid, Spain) and another against tau, tau5, was purchased from Calbiochem (La Jolla, CA). A polyclonal rabbit antisera against the M1 muscarinic acetylcholine receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mammalian expression plasmids containing the CMV promoter and the full-length cDNAs encoding the human muscarinic M1 (CHRM1) and M3 (CHRM3) receptors, tagged at the N-terminus with 3×HA epitope, were obtained from the UMR cDNA Resource Center (Missouri, MO). The pcDNA3.1 plasmid used as a control and the Lipofectamine 2000 transfection reagent were obtained from Invitrogen (Carlsbad, CA).

2.2. Protein purification

The recombinant tau isoform containing 2 N-terminal inserts and 4 microtubule binding repeats (tau42: Goedert and Jakes, 1990), was isolated as described previously (Perez et al., 1996). The proteins isolated were characterized by gel electrophoresis followed by staining with Coomassie blue. For some experiments, purified tau was labelled with sulfoindocyanine Cy5 (Amersham Biosciences), as described previously (Liu et al., 2004) following the manufacturer's recommendations. Briefly, 1 ml of recombinant tau protein (1,6 mg/ml) was mixed with a sample (see manufacturer) of sulfoindocyanine Cy5 at room temperature for 1 h for coupling to fluorophore. To eliminate the excess of free-dye, the mixture was dyalized overnight at 4 °C against PBS and filtrated by a Sephadex G-50 column. Cy5-coupled tau protein was characterized by Western blotting with an antibody that recognizes Cy5/Cy3 dyes (Sigma).

2.3. SH-SY5Y cell culture

Human neuroblastoma SH-SY5Y (Biedler et al., 1978) cells were obtained from the ATTC (Rockville, MD) and they were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) foetal bovine serum (FBS) and 2 mM glutamine in a humidified atmosphere of 5% CO_2. The day before performing the experiment, the cells were seeded on poly-L Lysine-coated glass coverslips at a density of 1×10^5 cells/coverslip for calcium experiments.

2.4. COS-7 cell culture and transfection

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

In some cases, transfected cells were treated previously with Cy5-labelled tau for 5 min, before fixation and confocal immunofluorescence analysis.

2.5. Primary cultures of hippocampal neurons

Hippocampal neurons were cultured as described previously (Banker et al., 1977). Newborn wild-type mice (P0) were sacrificed and their brains removed under sterile conditions. Dissociated hippocampal neurons were plated on glass coverslips coated with 1 mg/mL poly-L Lysine and 20 μg/mL laminin. After incubating for 3 h in medium containing 10% (v/v) horse serum (Gibco; Carslbad, CA), the cells were transferred to N2- and B27-supplemented medium (Gibco, Carlsbad, CA) and they were incubated for 1, 3 or 7 days.

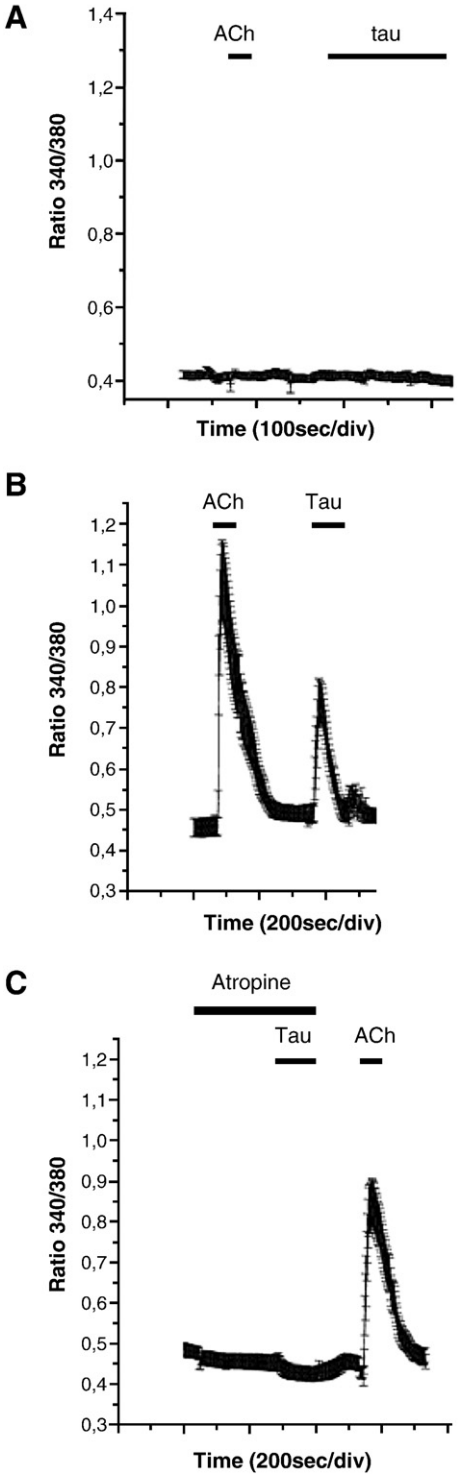
2.6. Immunofluorescence analysis

Transiently transfected COS-7 cells and primary neuron cultures were fixed with 4% paraformaldehyde for 20 min. After fixation, the coverslips were incubated with phosphate buffered saline (PBS) supplemented with 0.1% Triton X-100 for 10 min and they were then incubated with 1% bovine serum albumin (BSA) in PBS/TX-100 for an additional 10 min. The primary anti-HA antibody was diluted 1:1000 (V/V) in PBS/BSA and incubated with the cells on the coverslips for 45 min at room temperature. Subsequently, the coverslips were rinsed with PBS and incubated with the Alexa 488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) diluted 1:500 (vol/vol) in PBS/BSA. Finally, the coverslips were extensively rinsed again with PBS and mounted in FluorSave (Calbiochem, San Diego, CA), and they were analysed by confocal microscopy on a Confocal MicroRadiance system (BioRad, Hercules, CA) coupled to a vertical microscopy Axioskop 2 (Zeiss, Thornwood, NY).

2.7. Microfluorimetric calcium assays

Primary cultures of hippocampal neurons and transiently transfected COS-7 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 then loaded with FURA-2AM solution (7.5 μM) for 30 min at 37 °C. This incubation facilitated the intracellular hydrolysis of the FURA-2AM.

Subsequently, the coverslips were washed with fresh medium and mounted in a superfusion chamber on a NIKON Eclipse TE-2000 microscope. In all experiments the cells were first superfused at 1.2 mL/min with perfusion media before ACh or tau were assayed, and when the tau protein was assayed, the perfusion system was stopped. When the experiments were performed with the muscarinic receptor antagonist, atropine, the cells were exposed to the antagonist for 10 min before adding ACh or tau (Gomez-Ramos et al., 2008).



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 using 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 monochromator, Cairin), wavelengths that corresponded to the fluorescence peaks of the 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 250 ms at each wavelength and the changing time was <5 ms. The images were acquired continuously and buffered in a fast SCSI disk. The time course data represents 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 calculated (Diaz-Hernandez et al., 2001).

2.8. Gel electrophoresis and Western blotting

Equal volumes of culture medium from tau-treated cells were collected at 0, 24 and 48 h and heated at 100 °C for 5 min in electrophoresis sample buffer. The proteins were fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% gels and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Dassel, Germany). After blocking 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 tau-5 antibody (1:1000, vol/vol) in blocking buffer at 4 °C. The proteins recognized by the antibodies were visualized by enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA) after incubation with horseradish peroxidase (HRP)-linked secondary antibodies (Dako A/S, Glostrup, Denmark).

3. Results

3.1. Extracellular tau directly binds to and activates muscarinic M1 and M3 receptors

Neuronal cells express different acetylcholine receptors (different types of nicotinic and muscarinic receptors) and in previous studies (Gomez-Ramos et al., 2008), we found that the tau protein can interact with the M1 and M3 muscarinic receptors. To analyse the differences between the activation of these M1 and M3 muscarinic receptors by tau or by ACh, we used non-neuronal COS-7 cells

Figure 1 Tau protein induces an increase in intracellular calcium in COS-7 cells transfected with M1 receptor cDNA. (A) FURA-2 loaded control COS-7 cells transfected with pcDNA3.1 were stimulated with a pulse of 50 μM ACh (to establish the functional status of the cells), followed by 1 μM tau. The cells did not respond to either ACh or to tau. (B) COS-7 cells transfected with the CHRM1 construct (containing the M1 receptor cDNA) were stimulated with ACh followed by the tau protein, and the cells responded to both ACh and to the tau protein by increasing their intracellular calcium concentration. (C) There was no response to ACh or to tau when cells were pre-incubated with atropine. Finally, after washing the cells with buffer, a pulse of ACh was applied to confirm cell viability. The trace shows the average response of ≥20 cells and the bars indicate the standard deviation. In all cases the upper solid bars indicate the stimulation periods.

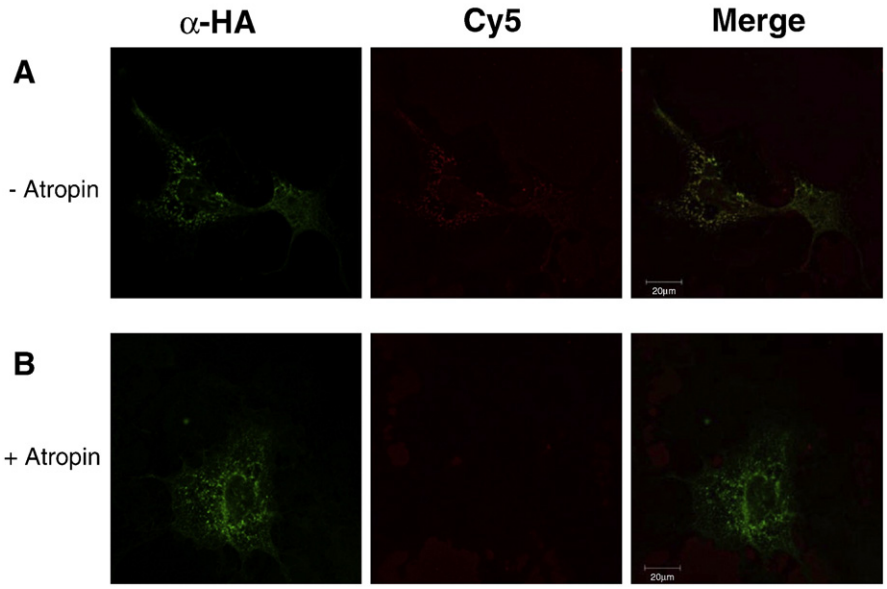


Figure 2 Tau-Cy5 binds to the M1 receptor expressed in COS-7 cells. Recombinant tau protein coupled to the Cy5 fluorescent dye (see Materials and methods) was added to COS-7 cells expressing a M1 receptor tagged at the N-terminus with 3xHA epitope, both in the absence (A) or presence (B) of the muscarinic antagonist, atropine. After 5 min, the cells were fixed with 4% paraformaldehyde, extensively washed with PBS to avoid the excess of signal from tau coupled to Cy5 and processed to be analysed by confocal microscopy. Cy5-tau does not colocalize in atropine pre-treated cells.

(Gluzman, 1981) transiently transfected with cDNA expressing the M1 or M3 receptor. In both cases, we analysed the increase in intracellular calcium induced by muscarinic receptor activation in individual cells by calcium microfluorimetry.

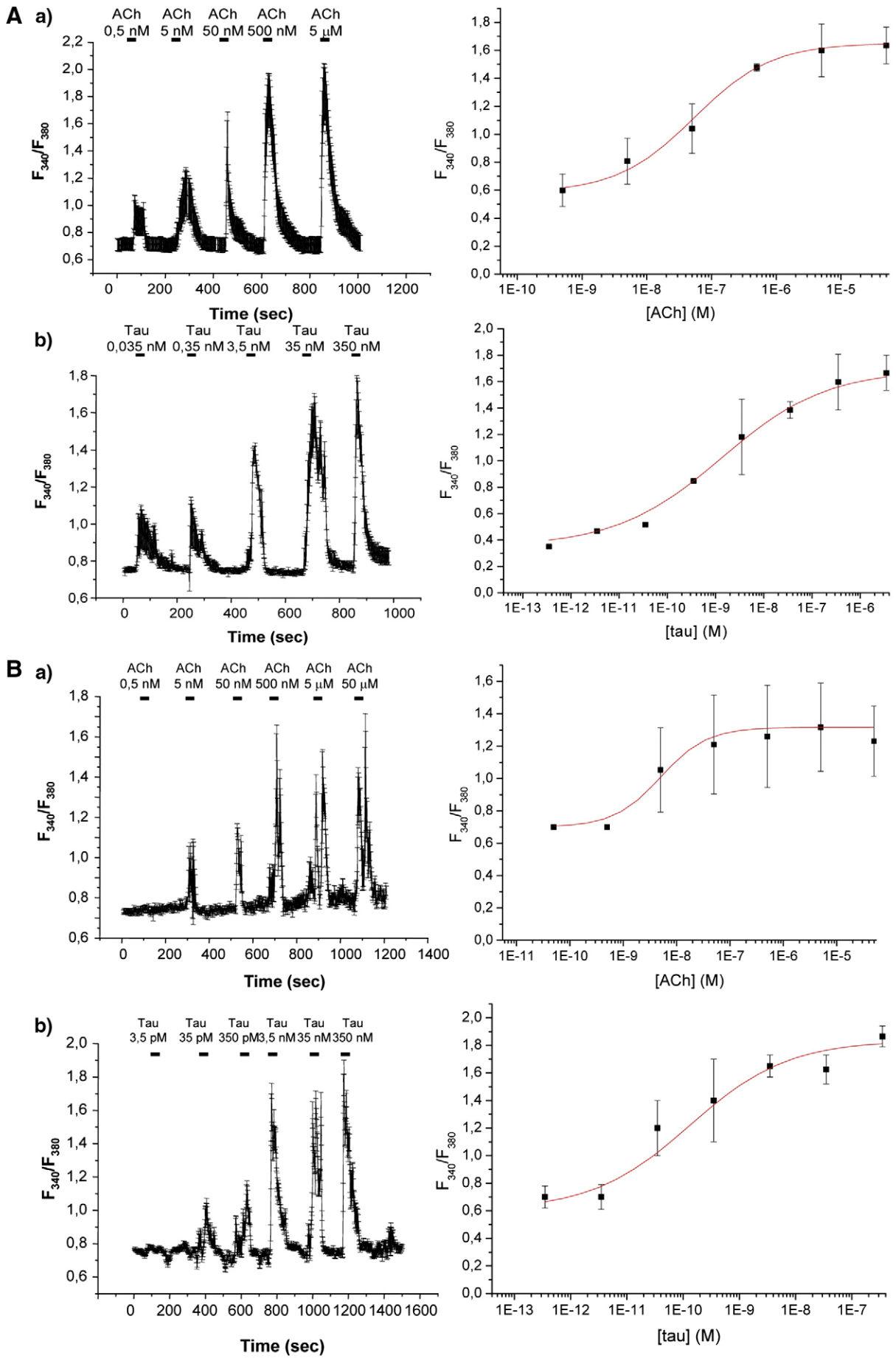
We first confirmed that no intracellular calcium transients were induced when ACh or tau was applied to COS-7 cells transfected with the empty plasmid (pcDNA 3.1 without a cDNA muscarinic receptor insert: Fig. 1A). Accordingly, application of ACh or tau did not induce calcium transients in COS-7 cells transfected with pcDNA 3.1. By contrast, an increase in the intracellular calcium concentration was evident when COS-7 cells were transfected with a plasmid containing the full-length cDNA encoding the human M1 muscarinic receptor (tagged at its N-terminus with the 3xHA epitope: pCHRM1) were exposed to ACh or tau (Fig. 1B). These responses were abolished when cells were pre-treated with atropine, a specific antagonist for muscarinic receptors (Fig. 1C).

To determine whether the effect of tau on the intracellular calcium concentration in COS-7 cells expressing muscarinic receptors was due to a direct interaction between the tau protein and the muscarinic receptors, we performed immunocolocalization studies. We generated a tau protein labelled with the Cy5 (red) fluorescent tag and we assessed whether it co-localized with the muscarinic M1 receptor with the 3xHA epitope at its N-terminus. Muscarinic receptors were visualized by indirect immunofluorescence using an antibody raised against the HA epitope (green). The tau protein completely co-localized with the muscarinic receptor soon after its application to the cultured cells expressing the M1 receptor (Fig. 2). Moreover, the specificity of this interaction between tau and the muscarinic receptors was confirmed by preincubating the cells with the antagonist atropine (Fig. 2 lower panel), which prevented the colocalization of tau with the receptor. Significantly, tau-M1 receptor colocalization was still evident in the presence of an excess of ACh (data not shown), indicating that ACh cannot displace tau binding and that these 2 proteins bound to different sites on these muscarinic receptors.

3.2. Extracellular tau protein exhibits a slightly higher affinity for muscarinic M1 and M3 receptors than ACh

To characterize the agonistic role of tau on the muscarinic receptors, we performed pharmacological studies on COS-7 cells expressing muscarinic M1 or M3 receptors. The intracellular calcium transients in COS-7 cells transfected with pCHRM1 induced by the discrete addition of increasing amounts of either ACh or tau were recorded and analysed (Fig. 3A). The dose-response curves obtained from ACh and tau protein presented monophasic profiles with EC_{50} values of 61.47 (±26.2) nM and 1.29 (±0.45) nM respectively. These data indicate that tau had a higher affinity for the M1 muscarinic receptor than ACh. Similar results were obtained from COS-7 cells transfected with pCHRM3 (Fig. 3B), although in these cells the EC_{50} values obtained were 3.5 (±1.2) nM for ACh and 130.5 (±66.8) pM for tau. Again, tau displayed a higher affinity for M3 muscarinic receptors than ACh.

While the Hill coefficient was similar for the M1 receptor when stimulated by ACh or tau (0.65 ± 0.18 for ACh and 0.41 ± 0.06 for the tau protein), receptor cooperativity for the M3 receptor was significantly different. Thus, while the Hill coefficient of 1.53 (±0.98) reflected a cooperative effect when the M3 receptor was activated by Ach, stimulation of the M3 receptor with tau protein produced a non-cooperative effect with a Hill coefficient of 0.46 (±0.26).


A
a)
ACh 0,5 nM
ACh 5 nM
ACh 50 nM
ACh 500 nM
ACh 5 μM
F340/F380
Time (sec)
[ACh] (M)
b)
Tau 0,035 nM
Tau 0,35 nM
Tau 3,5 nM
Tau 35 nM
Tau 350 nM
F340/F380
Time (sec)
[tau] (M)
B
a)
ACh 0,5 nM
ACh 5 nM
ACh 50 nM
ACh 500 nM
ACh 5 μM
ACh 50 μM
F340/F380
Time (sec)
[ACh] (M)
b)
Tau 3,5 pM
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Tau 350 pM
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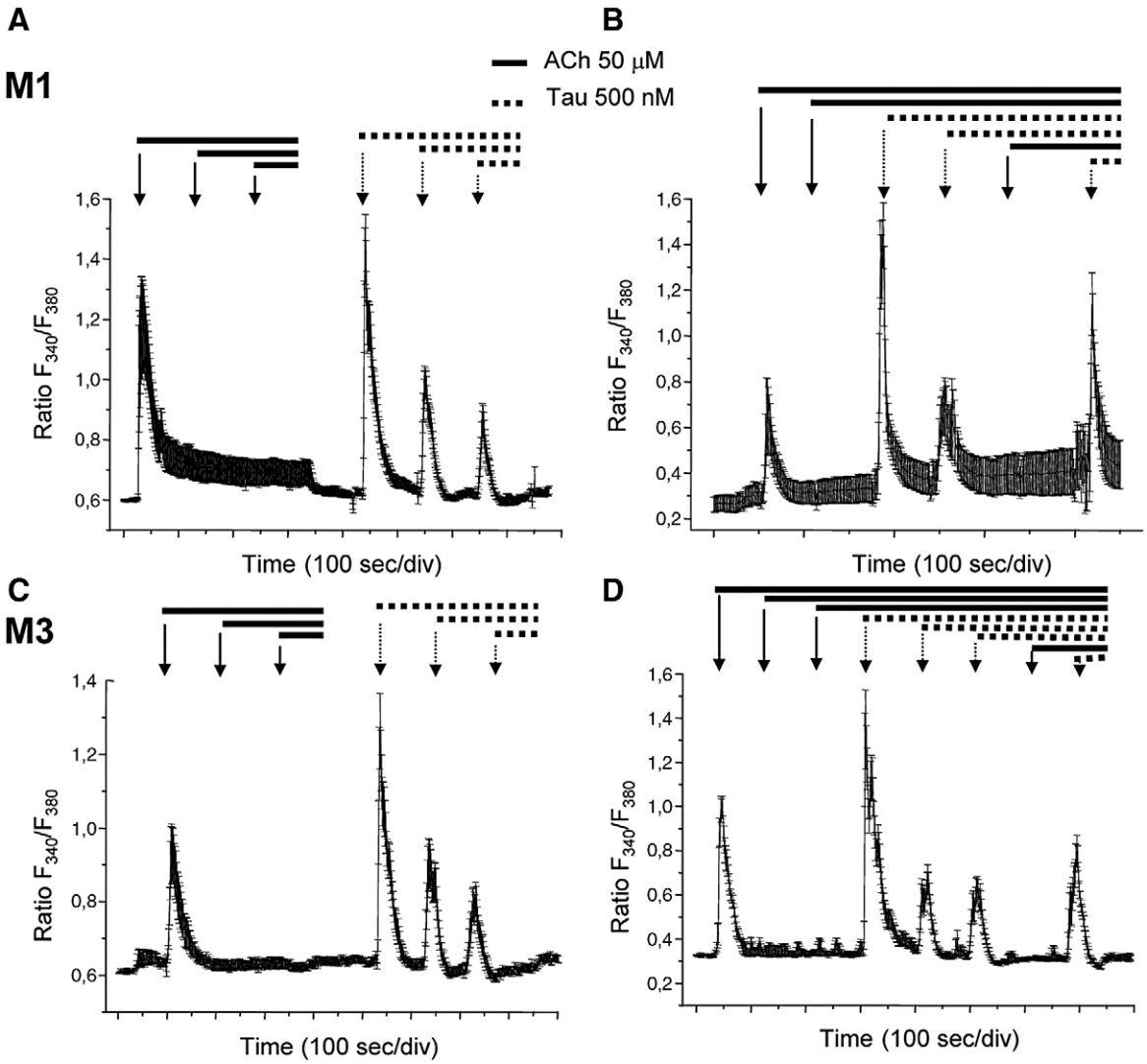

Figure 4 ACh and tau protein exert different effects on M1 and M3 receptors desensitization. In A and B, 50 μM of ACh was added to COS-7 cells transfected with M1 receptor at the times indicated (see continuous lines). The same cells were then exposed to the tau protein (500 nM), without washing, at the times indicated (discontinuous line). The effect of each addition on the increase in intracellular calcium is indicated. C and D, a similar experiment was carried out on COS-7 cells transfected with the M3 receptor. Arrows indicate the time of application.

3.3. Activation of muscarinic receptors by ACh or tau induces different desensitization profiles

As the tau protein can activate muscarinic receptors with a higher affinity than Ach and given that this agonist can desensitize the receptor after repeated stimulation (Waugh et al., 1999), we assessed whether the tau protein might also produce desensitization. This was particularly important since it appeared that these two compounds bind to different sites on the muscarinic receptors.

As expected, when both M1 and M3 receptor were repeatedly stimulated with high ACh concentration, only the first pulse was able to elicit an increase in intracellular calcium the transiently transfected COS-7 cells (Fig. 4A and C). When the same cells that responded to first ACh stimulation alone were washed, they now responded to all the tau stimulations applied (Fig. 4A and C). To confirm that only ACh and not the tau protein could induce muscarinic receptor desensitization, cells expressing M1 or M3 receptors were alternatively stimulated with additive pulses of high concentrations of ACh

Figure 3 Dose response curves for ACh and tau protein on COS-7 cells expressing M1 or M3 receptors. COS-7 cells transfected with CHRM1 (A) or CHRM3 (B) were loaded with Fura-2 and then stimulated with 0.5, 5, 50, 500 nM or 5 μM of ACh (Aa and Ba) and 0.035, 0.35, 3.5, 35 and 350 nM of tau protein (Ba and Bb). The fluorescence ratio is shown with respect to the time of stimulation (left) or with respect to the logarithm of the ACh or tau concentration (right). The trace shows the average response of ≥20 cells and the bars indicate the standard deviation. In all cases the upper solid bars indicate the stimulation periods.

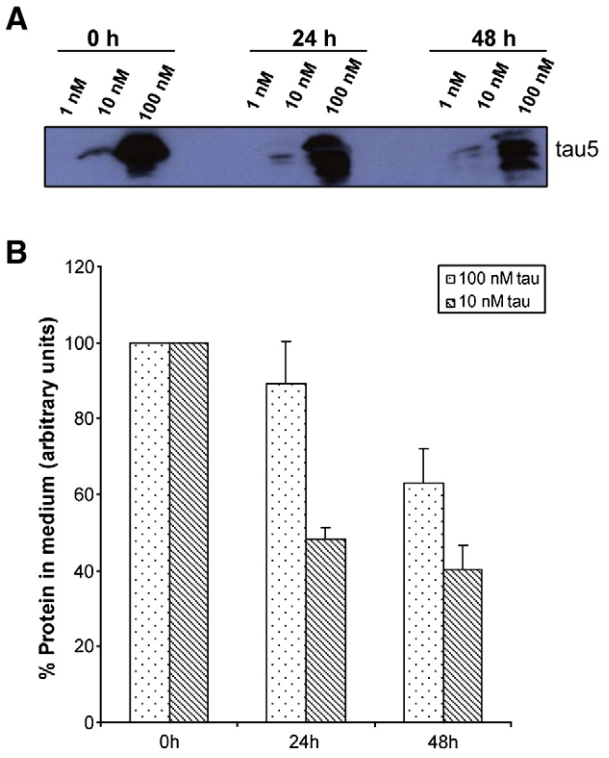

Figure 5 Degradation of the tau protein in the extracellular medium. (A) Tau protein was added to cultures of the SH-SY5Y neuroblastoma cell line at a final concentration of 1, 10 and 100 nM. Equal volume aliquots were recovered from the culture medium at 0, 24 and 48 h and the samples were analysed in Western blots probed with the tau 5 antibody (that recognizes total tau). In B, the quantification of total tau protein with respect to the control (0 h) is obtained by optical densitometry of the results shown in A. The results are the average of three independent experiments and the bars indicate the standard deviation.

and tau. In this experimental paradigm, we again observed that cells responded to all the tau stimuli but only to the first ACh pulse (Fig. 4B and D). Moreover, cells expressing muscarinic receptors respond to a tau stimulus, even when they were unable to respond to a new ACh pulse. These data suggest that although both the ACh and tau protein can activate muscarinic receptors, they do so in a different manner.

3.4. The resistance of tau protein to extracellular degradation

ACh is known to undergo rapid extracellular hydrolysis in neuronal cell cultures due to the action acetylcholinesterases, and the extracellular choline rendered it unable to activate muscarinic receptors (Potter et al., 1984). However, it remains unclear how long tau protein can persist in the extracellular milieu. To answer this question, we measured the tau protein remaining in the media of cultured SH-SY5Y neuroblastoma cells at different times after three distinct concentrations of exogenous tau had been added to the cells (Fig. 5A). Almost 50% of the exogenous tau remained in the medium 48 h after it was added to the culture, although it should be noted that the lower concentration of tau could not be detected in Western blots (Fig. 5A). These results clearly demonstrate that due to its resistance to the extracellular milieu, tau can exert long lasting effects on neural cells.

3.5. Repeated stimulation of hippocampal neurons with tau protein induces additive increases in intracellular calcium

As tau appears to induce a non-desensitizing calcium response in COS-7 cells expressing M1 or M3 muscarinic receptors, similar studies were undertaken in primary neuronal cultures. Accordingly, cultured hippocampal neurons were repeatedly stimulated with pulses of ACh or tau and as expected, only a single and transitory increase in intracellular calcium was detected

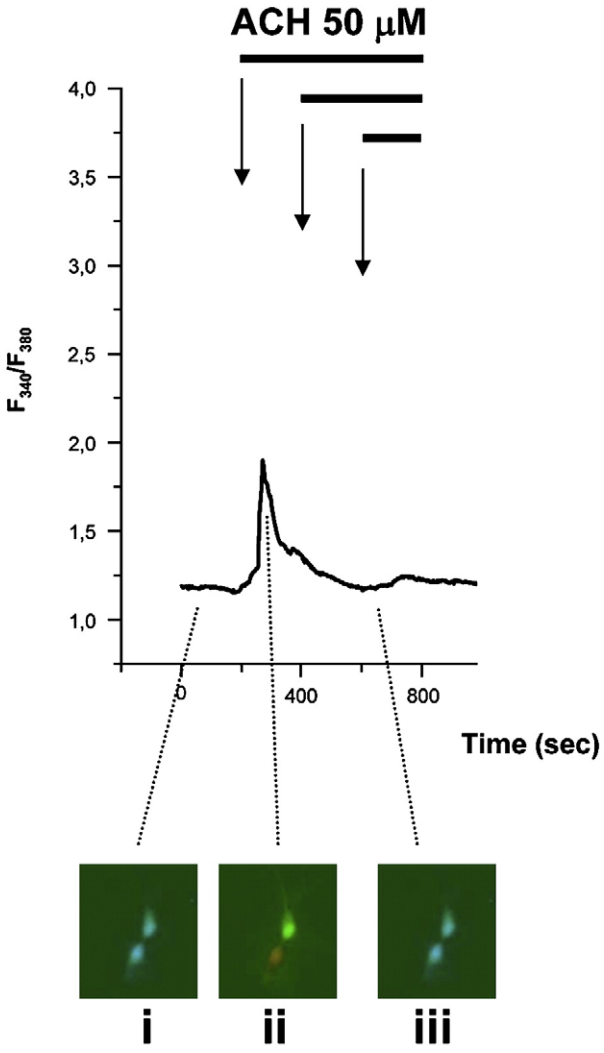

Figure 6 Effect of the successive addition of ACh to hippocampal neurons on the intracellular calcium concentration. ACh (50 μM) was added in consecutive pulses to FURA-2 loaded hippocampal neurons, without washing the cells to remove the ACh and the changes in intracellular calcium were measured respect to time. In the lower panel fluorescent photographs of cells are shown: before the application of ACh (i); a few seconds after the first application of ACh (ii); and before the last application of ACh (iii). Arrows indicate the time of application.

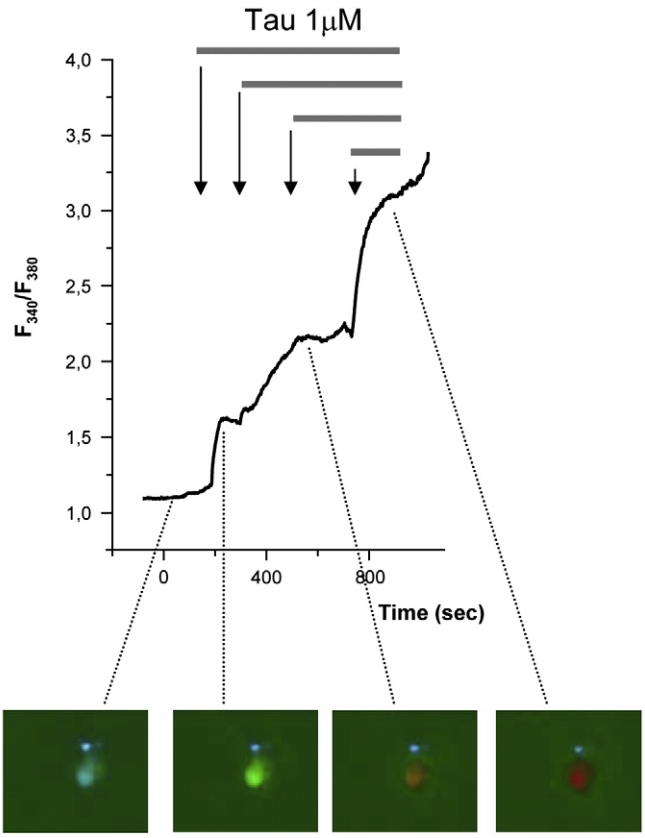

Figure 7 Effect of successive addition of tau protein to hippocampal neurons on the intracellular calcium concentration. Tau (1 μM) was added in consecutive pulses (continuous lines) to FURA-2 loaded hippocampal neurons, without washing the cells after each pulse to remove the tau, and the changes in intracellular calcium were measured. In the lower panel fluorescent photographs of the cells are shown (treated with false colours): before the application of tau (i); a few seconds after the first application of tau (ii); after the second application of tau (iii); and after a fourth application of tau (iv). Arrows indicate the time of application.

when neurons were stimulated with successive pulses of ACh (Fig. 6). However, when neurons were repeatedly exposed to pulses of tau, each stimulus induced an increase in the intracellular calcium (Fig. 7). Interestingly, the calcium increase elicited by tau did not decrease with time, as occurred in the COS-7 cells transfected with muscarinic receptors, rather the calcium waves were additive. Accordingly, hippocampal neurons responding to tau were able to reach very high levels of intracellular calcium and thus, they could trigger calcium-dependent signalling cascades that were inaccessible to ACh.

4. Discussion

In the present work, we have analysed the consequences of M1 and M3 muscarinic receptor activation by ACh or tau protein. After confirming that tau can interact directly with muscarinic receptors, we demonstrated that M1 and M3 receptors possess a nearly 10-fold higher affinity for the tau protein than for ACh. Moreover, we show that unlike Ach, the tau protein does not induce desensitization of native muscarinic receptors expressed by hippocampal neurons. Finally, we showed that tau persists in the extracellular milieu for longer than ACh.

Both tau and ACh were previously shown to specifically activate M1 and M3 receptors (Gomez-Ramos et al., 2006, 2008). However, while both compounds induce an increase in intracellular calcium through M1 and M3 receptor activation, only tau induces neuronal cytotoxicity through this pathway (Gomez-Ramos et al., 2006). This phenomenon may be explained by the kinetic profile of calcium mobilization induced by muscarinic receptor activation. Thus, when muscarinic receptors are stimulated with repetitive ACh stimuli, only the first application can induce an increase in intracellular calcium. However, when exposed to tau, each repeat stimulus induces an increase in intracellular calcium. This behaviour of these muscarinic agonists indicates that ACh but not tau can induce desensitization of muscarinic receptors. In addition, both M1 and M3 receptors possess a higher affinity for tau protein than for ACh. Together, these data might explain why tau produces a neurotoxic effect through the activation of muscarinic receptors that is not observed with classical physiological agonists.

It would be reasonable to think that the progression of neurodegenerative tauopathies depends on neurons expressing muscarinic receptors, as such cells would be highly sensitive to the cytotoxic effects of extracellular tau. However, the capacity of the extracellular tau to diffuse in the extracellular milieu remains unclear. Nevertheless, the slow degradation of extracellular tau in primary cultures of hippocampal neurons contrasts with the short half-life of ACh

(Potter et al., 1983). Thus, the long half-life of tau in the extracellular milieu would support its capacity to activate the muscarinic receptor for longer periods and at sites distant from the primary lesion.

Although the tau protein can activate both M1 and M3 muscarinic receptors, these receptors have a different distribution in the brain. While the M1 receptor is almost ubiquitous in the brain, the M3 receptor is mainly located in the hippocampus (http://mouse.brain-map.org, 2008). Thus, tau acting on the M1 receptor could result in more wide-ranging neuron toxicity than when tau acts on the M3 receptor. However, since M3 receptors are mainly located in the hippocampal area and our studies have been carried out on hippocampal neurons, it should be noted that tau exhibits an affinity of about one order of magnitude higher for M3 than M1 receptors. Due to the relevance of this area in Alzheimer's disease progression, understanding the noxious effect of tau on hippocampal neurons could lead to new hypotheses in order to address neurodegenerative diseases.

Moreover, it is necessary to bear it in mind that from previous studies of our group (Gomez-Ramos et al., 2006) unmodified tau protein seems to be more toxic than phosphotau. Besides, it has been reported that the phosphorylation level between intracellular and extracellular tau can be different (Lai et al., 1995; Wischik et al., 1995). In this way, further analysis about the phosphorylation level of extracellular tau should be carried out to discuss the implication of current data in the pathogenesis of Alzheimer disease.

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Contributors

J.A. and M.T.M-P. designed and supervised the research. A.G-R., M.D-H., A.R., and J.I.D-H. performed the research and contributed to the drafting of the manuscript. M.D-H. and A.G-R planned the experiments and analysed the data. J.A wrote the first draft and the final version of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of the interest

All authors declare that they have no conflicts of interest.

Acknowledgement

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