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Tau Triggers Tear Secretion by Interacting with Muscarinic Acetylcholine Receptors in New Zealand White Rabbits

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Abstract. In recent years, *in vitro* experiments have shown that the spread of Alzheimer's disease is caused by a non-conventional activation of muscarinic receptors by dephosphorylated extracellular tau protein. However, so far, *in vivo* data to support this hypothesis has not been obtained. The eye provides a good model where cholinergic (muscarinic) transmission can be analyzed. The role of muscarinic receptors in the stimulation of lacrimal gland secretion has already been described, and it has been suggested that acetylcholine is the main transmitter controlling tear secretion. In this project, we have studied the interaction between tau and muscarinic receptors by analyzing tear secretion in the eyes of white rabbits. Our results show that tau protein increases tear secretion by 47.2% in a similar way to a muscarinic receptor agonist carbachol (84.3%). The use of muscarinic antagonists indicated that tau interacts with M1 and mainly M3 muscarinic receptors. In summary, tau can bind muscarinic receptors *in vivo* and this may explain the spread of the pathology.

Keywords: Alzheimer's disease, muscarinic receptors, tau, tear secretion

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the presence of senile plaques and neurofibrillary tangles that are aggregates of paired helical filaments, the main component of which is tau protein. One of the main features of this pathology is a huge death of cholinergic (bearing nicotine or muscarinic receptors) neurons [1]. Upon neuronal death, intracellular tau could be released to the extracellular medium and become toxic [2, 3]. Also, the presence of extracellular tau in physiological fluids such as the cerebrospinal fluid of AD patients could be the consequence of previous cell death [4]. Nevertheless, there is evidence to indicate that tau might be released by mechanisms that do not imply cell death [5–13]. Indeed, it has been shown that extracellular tau can induce more tau release by a mechanism that involves the participation of muscarinic acetylcholine receptors.

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This would not only implies that cell death may be the cause of tau spreading from cell to cell, through muscarinic receptors, but also, in the absence of cell death, that the progression of tau pathology could take place by a mechanism involving the binding of tau to muscarinic receptors. Nevertheless, all the studies investigating the possible interactions between tau and muscarinic receptors have been performed *in vitro* with little or no information between the real interactions of both proteins *in vivo*.

The eye, and in particular tear secretion, provides an interesting and elegant model where cholinergic transmission modulation can be studied. The control of tear production is carried out by the autonomic nervous system, and the stimulation of these nerves excites lacrimal glands, promoting secretion of lacrimal electrolytes, water, and proteins.

Although both the parasympathetic and the sympathetic nerves innervate the lacrimal gland, the parasympathetic system predominates, both anatomically and functionally [14–17]. In this sense the most relevant parasympathetic neurotransmitters regulating tear secretion are acetylcholine and VIP. These agonists are all stimulatory and can activate different signaling pathways [18].

In this sense, the use of cholinergic agents can induce or inhibit tear production. As an example, intra-arterial or intraperitoneal systemic administration of acetylcholine, pilocarpine, or other cholinergic agonists stimulates lacrimal gland protein and fluid secretion measured from cannulated lacrimal gland excretory duct of anesthetized rabbits [19–25]. Having said that, the effect of muscarinic antagonists suggests a major role for parasympathetic nerves in stimulation of lacrimal gland secretion since the systemic administration of the muscarinic cholinergic antagonist scopolamine, for instance, produces dry eye in mice [26]. Altogether this indicates that acetylcholine is the main transmitter controlling tear secretion.

Since it has been suggested that there is interaction between tau and muscarinic receptors in some *in vitro* models, but as this has not been fully elucidated *in vivo*, the present experimental work shows how tau can modulate tear secretion *in vivo* and how this process is mediated by its interaction with muscarinic acetylcholine receptors.

MATERIALS AND METHODS

Animals

24 male New Zealand white rabbits (2.5 ± 0.5 kg) were used throughout all the experimentation. The animals were kept in individual cages with free access to food and water. All the protocols described here adhere to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research, and the experiments were carried out in accordance with the principles of the European Communities Council Directive (86/609/EEC).

Compounds and solutions

Carbachol (carbamylcholine), pirenzepine, and galamine were purchased from Sigma (St. Louis, USA). 4-DAMP was purchased from Tocris (Minneapolis, USA). All the other reagents were from Merck (Darmstadt, Germany).

The recombinant unphosphorylated human tau isoform (tau42) containing 2 N-terminal inserts and 4 microtubule binding repeats were isolated as previously described [27].

Hyperphosphorylated tau was purified from an insect cell culture infected with baculovirus-expressing human tau42 protein [28]. Tau peptide comprising the following sequence EIVYKSPVVSGDTSPRH, 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 [29].

Tear volume measurements

Tear secretion was measured by using the Schirmer I test. The tear collection was always performed according to Van Bijsterveld criteria [30]. The Schirmer strip was placed on the temporal tarsal conjunctiva of the lower lid for 5 min.

Control experiments were performed by applying 10 μl of saline solution (NaCl 0.9%) and 5 min after that, the Schirmer strip was applied in the rabbit's lower lid for 5 min. When the experiments were performed, the same volume of the desired compound at the concentration indicated in each case was applied and 5 min after the instillation the Schirmer strip was applied for 5 min as previously indicated. Tear secretion in each case was measured as the length of the wet strip (in mm).

Agonist studies

Single dose experiments designed to study the time-course of the tear secretion process were performed by adding carbamylcholine (carbachol, 100 μM), tau, tau peptide, or phospho tau (all at 1 μM) in a final

volume of 10 μL topically on the rabbit ocular surface. Contralateral eye received the same volume of vehicle (control). In order to see whether carbachol and tau were sharing the same receptors, carbachol (100 μM) was applied and 15 min later 1 μM tau was added.

Carbachol, tau, and the peptide of tau were tried at several concentrations ranging from 10^{-9} M to 10^{-3} M in order to obtain the corresponding concentration-response curves relating the dose of these agonists to the volume of tear production.

Antagonist studies

To study the effect of different antagonists in the inhibition of tear secretion produced by tau, the antagonists were tested alone (in vehicle) or 30 min before the application of tau 1 μM (10 μL). 5 min after that, the Schirmer strip was applied in the rabbit's lower lid for 5 min.

As with the agonists, the ability of the antagonists was checked by assaying them at different concentrations. For these studies, the concentration of tau was fixed at 1 μM (10 μL), and graded doses of pirenzepine, galamine, and 4-DAMP were tested from 10^{-8}M to 10^{-4}M to generate a family of curves to obtain the corresponding pA_2 and IC_{50} values.

Statistical analysis

All data are presented as the mean ± S.E.M. Statistical differences between treatments were calculated using ANOVA test. Plotting and fitting were carried out by GraphPad Prism 5 computer program (GraphPad Software).

RESULTS

The effect of tau and carbachol on tear secretion in New Zealand white rabbits

The protein tau was tested at a single concentration of 1 μM (10 μL). Its application increased tear secretion 47.62 ± 9.12% over the basal tear secretion. Previously, it was indicated that a tau peptide containing the residues 390–423 of the molecule is sufficient to interact with M1/M3 muscarinic receptors present in neuronal cells and that the interaction of that tau peptide with the cell receptors promotes the same effect as the addition of the whole tau molecule [3].

So we tested to see if the selected peptide of the protein tau (1 μM 10 μL) had the same effect, and found that upon addition of tau peptide 390–423, a similar effect to that observed for the whole tau molecule was shown by increasing tear secretion 50.00 ± 7.14% ($n = 8$).

On the contrary, phosphorylated tau did not have any effect in tear secretion (Fig. 1A). Concerning the maximal effects for tau and its peptide, tau peptide presented a peak in tear secretion at 5 min and it continued at 35 min, while the tau protein had no effect at 35 min (Fig. 1B).

Carbachol, the long lasting analogue of the naturally occurring transmitter acetylcholine, was assayed in order to see its effects on tear secretion. As shown in Fig. 1A, a single dose of 10 μL (100 μM) carbachol induced a peak in tear secretion of 84.31 ± 10.38% over basal tear secretion (10 μL vehicle, NaCl 0.9%) whose maximal effect was detected 20 min after the application of the substance (Fig. 1B, $n = 8$).

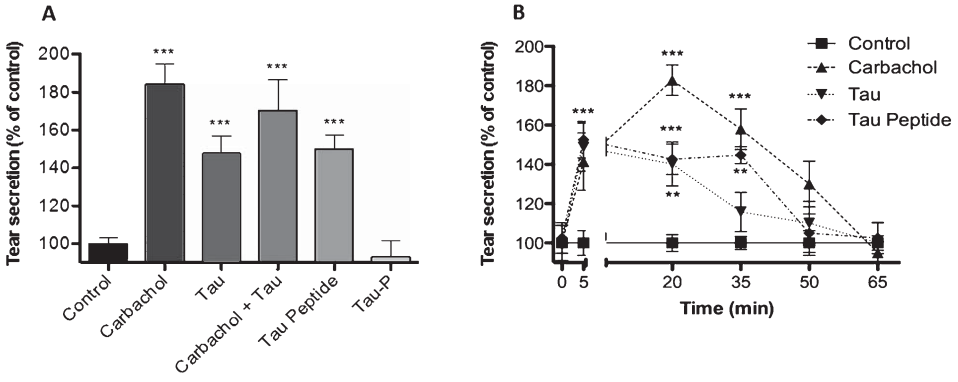

Fig. 1. Effect of carbachol, tau, tau peptide, and phosphorylated tau in rabbit tear secretion. A)Maximal effect of carbachol (100 μM), tau (1 μM), carbachol (100 μM)+tau (1 μM), tau peptide (1 μM), and phosphorylated tau (0.5 μM). 100% represents tear secretion before application of any drug (i.e., at t_0). B) Time-course of carbachol (100 μM), tau (1 μM), and tau peptide (1 μM). Values represent the mean ± s.e.m of eight independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, with respect to control levels (ANOVA tests).

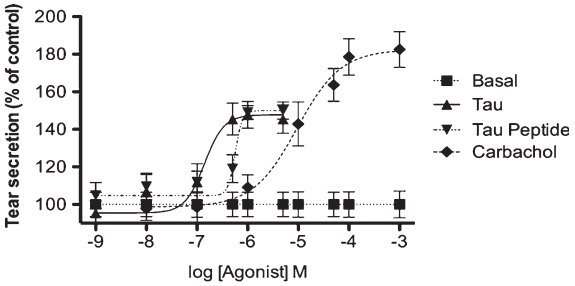

Fig. 2. Concentration-response course for carbachol, tau, and tau peptide. Graded doses of compounds were applied as described in methods. The maximal increased in tear secretion due to carbachol, tau, and tau peptide were $84.3 \pm 10.4\%$, $47.6 \pm 9.1\%$, and $50.0 \pm 7.1\%$, respectively. Values represent the mean $\pm$ s.e.m of eight independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, with respect to control levels (Two-way ANOVA test with Bonferroni posttests).

In order to study if both carbachol and tau were acting via the same receptors, these compounds were applied and its possible additive effect analyzed. As can be seen in Fig. 1A, the addition of both substances did not produce an increase in tear secretion, indicating that they may act via the same receptor.

Dose-response behavior for tau, its peptide, and carbachol on tear secretion

Tau and its peptide were tested in a different range of concentrations (from 10^{-9}M to 10^{-5}M) to fully investigate the effect of those peptides on tear secretion. As a consequence of this concentration-response analysis, it was possible to obtain a sigmoidal curve for each of them with pD_2 values of 6.88 ± 0.12 and 6.27 ± 0.09, for tau and the peptide of tau, which were equivalent to EC_{50}s of 0.13 and 0.54 μM, respectively ($n = 8$) (Fig. 2). Both tau and its peptide were able to produce a maximal tear secretion of $149.45 \pm 9.12\%$ and $152.00 \pm 9.42\%$ at 10^{-6} M, respectively ($n = 8$), with no statistical differences between them.

When carbachol was tested in a broad range of concentrations (from 10^{-8}M to 10^{-3}M) in order to see the concentration response behavior, it was possible to obtain a sigmoid dose-response curve. As can be seen in Fig. 2, carbachol sigmoidal curve obtained presented a pD_2 value of 5.03 ± 0.09 which was equivalent to an EC_{50} of 9.43 μM ($n = 8$). The maximal tear secretion depicted by carbachol was $186.45 \pm 11.77\%$, at 10^{-3}M ($n = 8$), this value being significantly higher than the ones obtained by tau and tau peptide (Fig. 2).

Antagonist studies

We measured the effect of tau in the presence of single doses of muscarinic antagonists in order to see whether or not there was a connection between tau and muscarinic receptors. The antagonists were tested in their ability to modify basal tear secretion by themselves. As can be seen in Fig. 3A, the only one able to produce a significant reduction in the basal tear production was the M3 antagonist 4-DAMP. This compound was able to reduce basal tear secretion by about 11% when compared to control ($n = 4$).

A concentration of 1 μM tau produced an increase in tear secretion of $147.62 \pm 9.12\%$ above basal tear secretion value (basal 100%). Under these stimulated conditions, the effect of tau was challenged by means of several muscarinic receptor antagonists. In this sense, tear secretion was inhibited to $102.66 \pm 4.09\%$ in case of pirenzepine (M1 antagonist), $112.08 \pm 6.36\%$ with galamine (M2 antagonist), and $95.48 \pm 11.46\%$ with 4-DAMP (M3 antagonist) (Fig. 3A).

Since these studies were only performed at single muscarinic antagonist concentrations, which may not reflect the real antagonistic properties of these agents, all the antagonists were tested again in a broad range of concentrations, from 10^{-8}M to 10^{-4}M in order to see the concentration response behavior. As shown in Fig. 3B, pirenzepine, galamine, and 4-DAMP revealed pA_2 values of 6.17 ± 0.09, 5.38 ± 0.18, and 5.83 ± 0.02, respectively, which were equivalent to IC_{50} values of 0.69, 4.18, and 1.48 μM, respectively ($n = 8$).

Concerning the maximal inhibitory effect produced by the antagonists, it is important to emphasize that 4-DAMP was able to block 100% of the tear secretion triggered by tau, while galamine and pirenzepine were unable to completely block the tearing induced by tau (Fig. 3B).

DISCUSSION

In the present experimental work, we have found that human tau protein or its peptide, containing residues 390–423, are acting as agonists of muscarinic receptors M1/M3, and that they can increase tear secretion on rabbits. Since tau peptide with the residues 390–423 of the molecule appears to be sufficient for that increase, we have looked at the sequence of rabbit tau and we found that such sequence was identical in rabbit and human tau. Those tau residues contain some residues that could be modified by phosphorylation and the tau

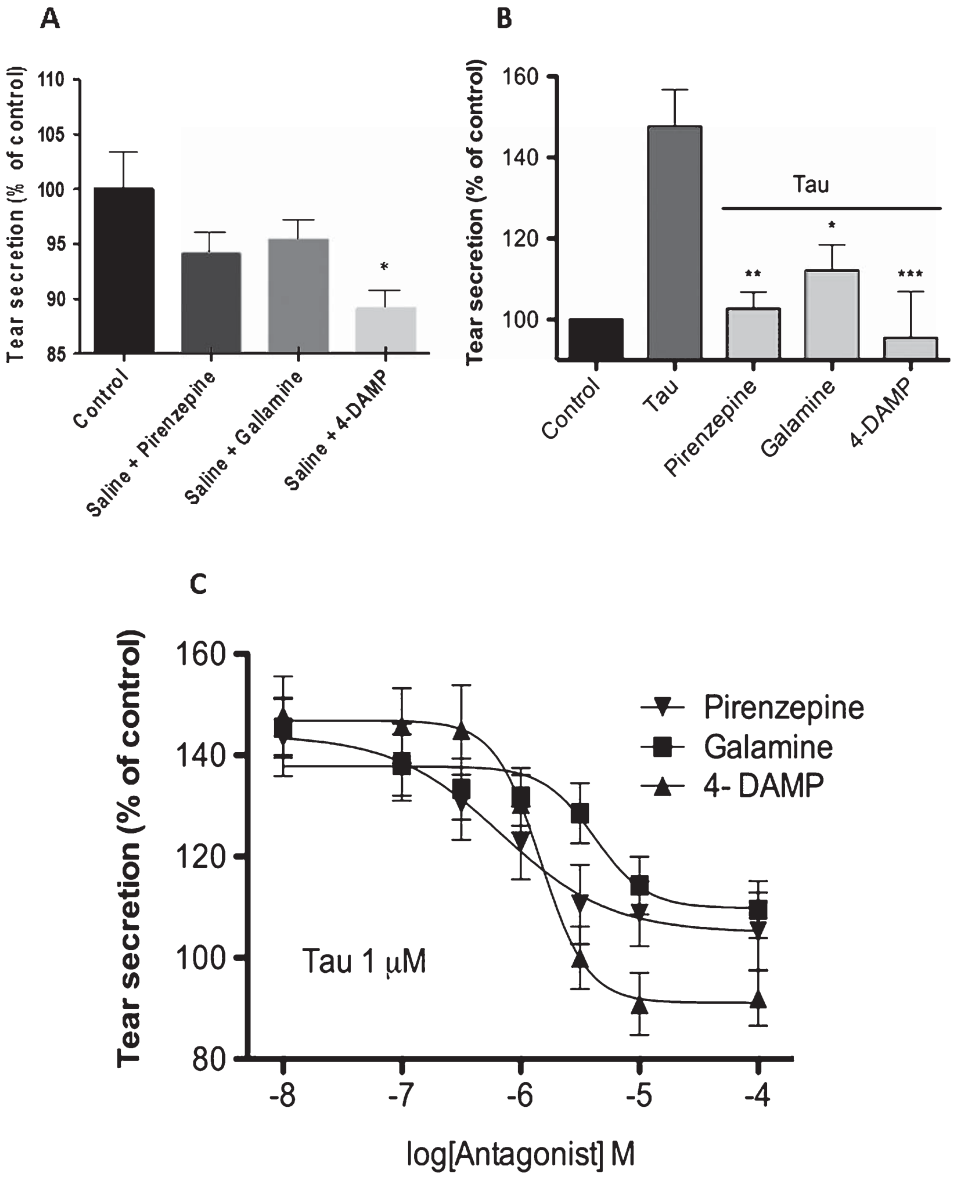

Fig. 3. Antagonism by pirenzepine, galamine, and 4-DAMP (100 μM, 10 μL) of the responses produced by tau. A) Effect of the antagonists applied in the absence of any added compound (prepared in saline solution). B) Maximal inhibition. C) Concentration-response course for the antagonists. Values are the mean ± S.E.M. of eight independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, with respect to control levels (Two-way ANOVA test with Bonferroni posttests).

antibody PHF-1 could recognize such phosphorylation [31]. Our analyses have shown phospho tau is unable to interact with muscarinic receptors. Interestingly, it has been described that tau antibody targeting the 396/404 region reduces tau pathology [32] and it may be due to a possible block of tau-cell receptor interaction.

Rabbit tear secretion has been widely used as a model where physiological and pharmacological research can be performed [33, 34]. Most of the control of tear secretion is carried out by the autonomic nervous system and therefore tearing is influenced by instillation of drugs that modulate this system. Indeed, the application of carbachol or the instillation of muscarinic antagonists clearly demonstrated the importance of the parasympathetic nervous system in the control of tear secretion. Different substances and stimuli have demonstrated their ability to induce tear secretion [35, 36]. Good examples are nucleotides and dinucleotides [37]. They are present in tears and when topically applied they stimulate tearing by act-

ing on purinergic receptors [38]. It is interesting to point out that dinucleotides such as Ap_4A which act as a tear inducer, can increase their effect when the indoleamine melatonin is present in tears [39]. This is another example of cross-talk between different substances and may reflect, together with the interaction between tau and muscarinic receptors here described, that tear secretion can be modulated in different manners. It is noteworthy that in both cases, even being so different, the final effect that is obtained is an increase in tear secretion. This fact raises some new questions to take into consideration. On the one hand, the exogenous application of tau, as demonstrated, induces tear secretion. This might be interesting from a therapeutic point of view since one of the most prevalent ocular diseases is dry eye which very often occurs with a lack of tear secretion [40–42]. Interestingly, tau is stimulating muscarinic receptors (see Fig. 1A), therefore inducing tear secretion without the side effects cholinergic agonists can produce when applied to the eye such as myosis, lens opacity, or a blockade lens accommodation [43–47]. So, this may suggest that either tau or its fragment could be used as a treatment for dry eye if no other side effects are found for this protein. However, caution should be taken with this possibility in view of recent data suggesting that tau could be a prion-like protein involved in the spreading of tau pathology. Also it is interesting to point out that although carbachol induces more tearing at a fixed concentration, the concentration-response analysis indicates about one order of magnitude difference between EC_{50}s, indicating that although carbachol presents a better efficacy (maximal effect), tau is more potent (smaller EC_{50}), which totally concurs with previous reports on neural cells cultured [48]. Altogether, we may consider tau and its peptide as partial agonists of muscarinic receptors.

Another important issue is that tau could be present naturally in tears. In this sense, rabbit's eye has been used as a model to test tau function, but it is not known if tau could be present, in a physiological way, in tears. If tau is present in human tears, and its concentration in patients is concomitantly increased as occurs in the CNS, it would be possible to suggest the quantification of this protein in tears as an easy way to detect the pathology in the very early stages of the disease.

In summary, we have demonstrated that tau is able to stimulate muscarinic receptors, mainly M1/M3, in a model *in vivo*. This interaction may explain in the CNS that the stimulation of these receptors and the concomitant Ca^{2+} increase triggers the release of more tau explaining the spread of the pathology.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2091).

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