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Muscarinic agonists reduce tau phosphorylation in non-neuronal cells via GSK-3β inhibition and in neurons

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Summary. Muscarinic agonists alter the metabolism of amyloid precursor protein, leading to an increase in α-secretase cleavage and a decreased production of amyloidogenic peptides; suggesting that these compounds might modify the Alzheimer's disease process. A second therapeutic target in AD is the accumulation of stably phosphorylated tau into neurofibrillary tangles; an early event correlating with cognitive impairment. Glycogen synthase kinase-3 (GSK-3β) phosphorylates tau and is inhibited via protein kinase C (PKC). As certain muscarinic receptors are linked to PKC, we examined the effect of a range of agonists on GSK-3β phosphorylation of tau. In neurons a non-specific muscarinic agonist, carbachol, reduced tau phosphorylation. In non-neuronal cells expressing the m1 receptor a range of m1 agonists reduced transiently-expressed tau phosphorylation and altered its microtubule-binding properties. These findings link the two pathological process of AD – APP metabolism and tau phosphorylation – and suggest that muscarinic and other cholinergic compounds might have disease-modifying properties.

Keywords: Tau, muscarinic receptor, acetylcholine, Alzheimer's disease, glycogen synthase kinase-3.

Introduction

The first wave of drugs to be made available for the treatment of Alzheimer's disease (AD) all act to reverse the loss of cholinergic neurotransmission

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postulated to be an early part of the pathogenic process (Lawrence et al., 1998). Acetylcholinesterase inhibitors are licensed for use in many countries and cholinergic receptor agonists are undergoing extensive clinical trials. The cholinergic nervous system contains two receptor systems, the nicotinic and the muscarinic. The muscarinic receptors consist of several subtypes (m1, m2, m3, m4, m5), each with individual profiles and functions. Activation of muscarinic m1 receptors is the objective of cholinergic replacement therapy in the treatment of Alzheimer's disease, and clinical trials have been carried out with the direct acting m1 muscarinic agonists, xanomeline, SKB202026, and milameline as well as Lu 25-109 which acts as a partial muscarinic M1 agonist as well as an M2/M3 antagonist (Meier et al., 1995). An antagonistic effect on M2 receptors might reduce the risk of peripheral side-effects seen with some muscarinic compounds due to non-selective M2 receptor stimulation.

The muscarinic agonists were designed to be palliative only; however the observation that modulating cholinergic transmission alters amyloid precursor protein processing [APP; reviewed in Roberson et al. (1997)] suggests that they might have an effect on the disease process itself. More recently it has been reported that in PC12 cells a muscarinic agonist also reduces tau phosphorylation although the mechanism for this effect was not determined (Sadot et al., 1996). As in AD hyperphosphorylated tau aggregates into the paired helical filaments of neurofibrillary tangles, and as these intraneuronal aggregations correlate with cognitive impairment (Lovestone et al., 1997), any effect of muscarinic agonists on tau phosphorylation might also effect pathogenic processes. It is important therefore to determine whether other muscarinic agonists also affect tau phosphorylation and whether this occurs in other cells, most importantly in neurons.

Many kinases phosphorylate tau in vitro but we have previously demonstrated that in intact transfected cells tau is readily phosphorylated by Glycogen Synthase Kinase-3 (GSK-3) (Lovestone et al., 1994) and that this reduces tau binding to and stabilisation of microtubules (Lovestone et al., 1996). GSK-3β is inhibited by lithium (Klein et al., 1996; Stambolic et al., 1996) and as lithium reduces tau phosphorylation in neurons (Lovestone et al., 1999; Muñoz-Montaño et al., 1997) and also alters the microtubule-binding properties of tau (Hong et al., 1997a; Lovestone et al., 1999) this suggests that GSK-3β is a major tau-kinase in neurons. The effect of muscarinic agonists on tau phosphorylation in PC12 cells may be acting via this kinase as some evidence suggests that GSK-3β is regulated through the action of protein kinase C (PKC) (Cook et al., 1996), itself activated following muscarinic signalling.

We therefore examined the phosphorylation of tau induced by GSK-3β in transfected cells in the presence of a number of muscarinic agonists and also examined tau phosphorylation in primary neuronal cells. We have replicated an effect of muscarinic agonists in reducing tau phosphorylation in both neurons and in transfected non-neuronal cells and furthermore show that this alters the biological properties of tau.

Materials and methods

Cell lines

For experiments on neurons, primary cultures were prepared from Sprague-Dawley rat embryos (E17/18). Multiple cortices were aseptically dissected and dissociated by trypsinisation and trituration, resuspended in Neurobasal medium containing B27 supplement, glutamine (2 mM), penicillin (100 I.U.), streptomycin (100 μg/ml), 0.6% D-glucose and 5% (v/v) foetal calf serum (NB27; all Gibco). Cells were plated onto poly-L-lysine and laminin coated culture plates. The media was replaced by serum-free media after 24 hrs and cells cultured for a further 12–14 days prior to experimentation.

In experiments with transfected cells, CHO cells stably expressing the m1 muscarinic receptor were cultured in alpha-MEM with 10% (v/v) foetal calf serum, glutamine (2 mM), penicillin (100 i.u) and streptomycin (100 μg/ml). Transfection with cDNA coding for tau (1N4R) and GSK-3β [constructs previously described (Lovestone et al., 1994)] was done by lipofectamine according to manufacturers instructions. For imaging experiments, cells were transfected with cDNA coding for the largest (2N4R) tau isoform tagged at the N-terminus with green fluorescent protein-tau (EGFP; Clontech). Cells were cultured for 24 hours after transfection prior to experiments.

Treatments

Neurons were treated with the non-specific muscarinic agonist carbachol at 100 μM for 1 to 6 hours. Transfected cells were serum-starved for two hours prior to treatment for 2 hours with carbachol (100 μM) and the m1 agonists xanomeline (50 μM) (Shannon et al., 1994) Lu25-109M (Meier et al., 1995) (100 μM) and SKB202026 (20 μM) and with the GSK-3 inhibitor lithium chloride (10 mM).

Western blotting

Heat stable tau from transfected non-neuronal cells and total cell protein from neuronal cultures was collected as previously described (Lovestone et al., 1994). In brief, non-neuronal cells were scraped into phosphate buffered saline, collected by centrifugation and homogenised in ice-cold MES buffer containing phosphatase and protease inhibitors. Neurons were washed whilst adherent to the culture dish in ice-cold tris-buffered saline before the addition of boiling Laemmli sample buffer (62.5 mM tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue). Protein was separated on SDS-10% (w/v) polyacrylamide gels and electrophoretically transferred onto nitrocellulose. Blots were incubated with primary antibodies (TP70 1/20,000; AT8 1/100, Tau 1 1/2,000) at 4°C overnight and developed either by enhanced chemiluminescence (ECL, Amersham) or by alkaline phosphatase (Bio-Rad) methods. Blots were scanned and analysed using BioRad system one densitometric analysis software. For each individual blot the density of bands in experimental cells were compared to density of control cells (tau alone). Thus tau alone in each case has a value of 1 (SD 0) and all results of experimental pertubations are relative to this. Analysis was by Excel and SPSS for windows software using one-tailed t-test.

Microscopy

For imaging tau in living cells, a tau24-GFP fusion vector was transiently transfected using lipofectamine and after 24 hours cells adherent to coverslips were transferred to a Zeiss axioplan microscope in a chamber maintained at 37°C with a constant flow of 5% (v/v) CO_2. Cells were illuminated at the lowest possible light levels for periods of less than 200–600 ms and imaged using a Pentamax camera (Princeton Instruments) with images collected and analysed using MetaMorh software (Universal imaging) prior to transfer to adobe Photoshop.

Results

Neuronal tau phosphorylation is reduced by carbachol (Fig. 1)

It has previously been demonstrated that in PC12 cells expressing the muscarinic receptor an m1 specific agonist reduces tau phosphorylation (Sadot et al., 1996). To assess the effects of muscarinic agonism on neurons we treated 12–14 day old primary rat cortical cultures with 100 μM carbachol in serum free medium for 1, 3 and 6 hours. Following treatment, total cell protein was subjected to western blotting and analysed using the polyclonal phosphate independent antibody TP70 and the phosphate dependent monoclonal antibodies AT8 recognising tau phosphorylated at serines 202/205 and Tau1 only recognising tau not phosphorylated at an overlapping epitope; serines 199/202 (Goedert et al., 1994; Biernat et al., 1992). Foetal tau, and tau from foetal neuronal cultures, includes a highly phosphorylated species as shown by recognition by the AT8 antibody. Treatment of cultures with carbachol for one hour had little effect on tau phosphorylation – but increasing length of treatment with carbachol resulted in a reduction in tau phosphorylation. The reduced tau phosphorylation was detected as a loss of a slow migrating band on SDS-PAGE using the polyclonal antibody recognising both phosphorylated and unphosphorylated tau (TP70). This reduction in tau phosphorylation in carbachol treated cultures was confirmed by a decrease in recognition by AT8 and an increase with TAU1.

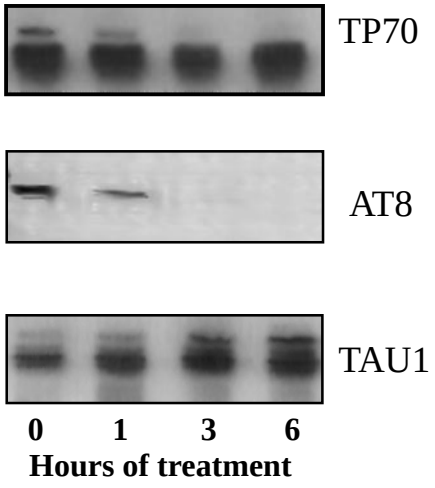

Fig. 1. Carbachol reduces tau phosphorylation in primary neuronal cultures. Primary neuronal cultures maintained for 12–14 days were treated with 100 μM Carbachol and examined by immunoblotting with the phosphate independent antibody TP70 and the phosphate dependent antibodies AT8 and TAU1. A slowly migrating species of tau recognised by TP70 and AT8 was present in untreated control cells and in cells treated for 1 hour but was absent in cultures treated for longer periods

Tau phosphorylation induced by GSK-3β is reduced by muscarinic agonists (Fig. 2 and 3)

We then examined the effect of the relatively non-specific muscarinic agonist carbachol and the relatively specific m1 agonists xanomeline, SKB202026 and Lu-25-109 on tau phosphorylation in non-neuronal cells. CHO cells stably expressing the m1 receptor (CHO-m1) were transiently transfected with either cDNA coding for tau alone or with cDNAs coding for both GSK-3β and tau together. Doubly transfected cells were treated with 10 mM Lithium chloride to inhibit GSK-3 directly and with 2 mM Carbachol, 50 μM xanomeline, 100 μM Lu25-109 and 20 μM SKB202026. Cells were treated for 2 hours in serum-free medium. Results from cells treated with xanomeline are not shown as cell attrition was so extensive following treatment that insufficient protein was obtained for western blot analysis.

Tau expressed in CHO-m1 cells was recognised as three or more bands by the polyclonal antibody TP70 (Fig. 2). These multiple bands have previously been reported in transfected CHO cells (Gallo et al., 1992) and presumably represent different phosphorylation states as alkaline phosphatase treatment reduced tau to a single band. However phosphorylation at the AT8 epitope is minimal in these cells. When cells are transfected with both tau and GSK-3β, however, the mobility of tau on SDS-PAGE is reduced. At the same time, the recognition of tau by the antibody TAU1 is markedly reduced and that by AT8 increased substantially. This pattern of phosphorylation was reversed by treatment with 10 mM Lithium Chloride, a competitive inhibitor of GSK-3 with an EC50 of 2 mM (Klein et al., 1996). These three situations then, tau in

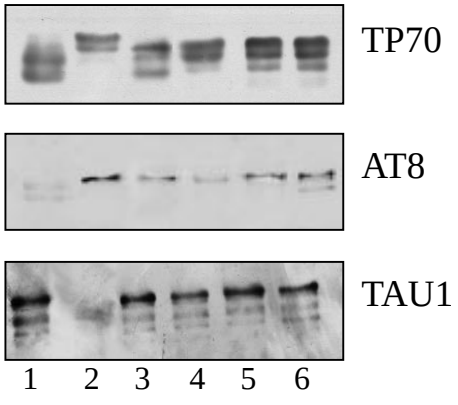

Fig. 2. Muscarinic agonists reduce tau phosphorylation induced by GSK-3β. CHO-m1 cells were transiently transfected with tau (lane 1) or with tau and GSK-3β together (lanes 2–6). When expressed alone tau was present as two main bands (markers on TP70 blot) and a number of ill-defined subsidiary bands with the lower of the principal bands predominating. When expressed together with GSK-3β this was reversed with the upper of the principal bands predominant (lane 2). This change represented phosphorylation as shown by increase in recognition by AT8 and decrease in recognition by TAU1. Treatment with lithium chloride (lane 3), Carbachol (lane 4), Lu25-109 (lane 5) or SKB202026 (lane 6) all reversed the GSK-3β induced pattern of phosphorylation

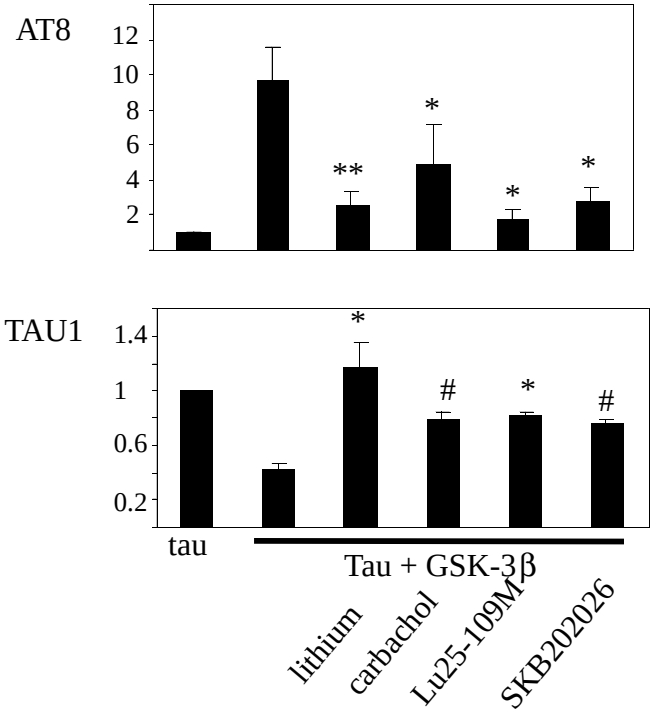

Fig. 3. Effects of lithium chloride and muscarinic agonists on tau phosphorylation – quantitative analysis. Repeated experiments (n = 3) were analysed by densitometry and experimental co-transfected cells normalised to cells expressing tau alone. The AT8 epitope was significantly increased in the presence of GSK-3β co-transfection (tau vs. tau + GSK-3β, $p < 0.005$). Treating these experimental cells with all four compounds reduced AT8 recognition (t-tests shown for tau + GSK-3β vs. tau + GSK-3β+treatment). The reverse was observed for TAU1; the epitope being reduced in the presence of GSK-3β co-transfection (tau vs. tau+GSK-3β, $p < 0.05$) and the compounds attenuating the effects of GSK-3β, approaching significance for carbachol and SKB 202026 and significantly so for the other compounds; error bars represent standard error of the mean

the absence of GSK-3β, tau in the presence of active GSK-3β and tau in the presence of inactive GSK-3β, provided a point of comparison for cells treated with muscarinic agonists.

All three muscarinic agonists analysed resulted in a change in tau phosphorylation. The migration of tau on SDS-PAGE was increased relative to tau from cells expressing tau and GSK-3β in the absence of other treatment. The recognition by the phosphate antibody AT8 was reduced although not abolished and the recognition by the antibody TAU1 was increased although not to the same extent as in lithium chloride treated cells or cells transfected with tau only.

These experiments were repeated and a series of three were analysed by densitometry. For the phosphorylation dependent antibodies, co-transfecting GSK-3β together with tau reduced the recognition by TAU1 by more than

half and increased recognition by AT8 more than 9 fold (Fig. 3). Treatment of cells transfected with tau and GSK-3β with lithium chloride returned the recognition by TAU 1 to the same level as the cells transfected with tau only and substantially reduced recognition by AT8. For both TAU1 and AT8 all three muscarinic compounds reduced the effect of the presence of GSK-3β on the phosphorylation of tau substantially but in each case quantitatively less than the direct inhibitor of GSK-3 lithium chloride. For AT8 the effect of all three compounds was to significantly reduce AT8 recognition in treated co-transfected cells compared to untreated co-transfected cells ($p < 0.05$ in all cases). For TAU1 all three compounds increased TAU1 recognition in treated co-transfected cells compared to untreated co-transfected cells [$p < 0.05$ for Lu25-109M but only approaching significance for SKB202026 and carbachol ($p < 0.06$ for both)]. There were no detectable differences between the three muscarinic agonists using this assay.

The effects of GSK-3β in reducing tau binding to microtubules are attenuated by muscarinic agonists (Fig. 4 and 5)

The use of green fluorescent protein as a tag allowed the properties of tau to be examined in living cells following treatment with muscarinic agonists. When expressed in CHO-m1 cells, tau-EGFP was present in cytoplasm but was also bound to microtubule bundles (Fig. 4A). The microtubule bundles induced by tau-EGFP were similar to those induced in previous experiments using untagged tau (Lovestone et al., 1996). However when tau-EGFP was highly phosphorylated due to co-expression with GSK-3β, the bundles were absent or infrequent and the tau-EGFP was predominantly cytoplasmic (Fig. 4A).

To examine the effects of muscarinic agonists on the properties of tau, CHO-m1 cells transiently transfected with both GSK-3β and tau-EGFP were treated with muscarinic agonists overnight. In each case tau-decorated microtubule bundles were present without any differences between the treatments being apparent (Fig. 4B). The use of EGFP as a tag allowed the effects of removing muscarinic agonism to be examined. CHO-m1 cells transfected with cDNA for both GSK-3β and tau-EGFP were treated overnight with carbachol and subsequently transferred to the microscope stage whilst maintained at 37°C and bathed in 5% CO_2. Cells were allowed to stabilise on the stage for a minimum of 30 min and then imaged for less than 1,000 ms using neutral-density filters to limit the light exposure to the minimum possible. The medium was replaced by serum-free medium without muscarinic agonists and the cells allowed to stabilise at 37°C and 5% CO_2 . After 1 hour bundles were very considerably reduced and the major part of the tau-EGFP signal was diffuse and not associated with microtubules (Fig. 5).

Discussion

In normal neurons tau binds and stabilises microtubules but in AD, hyperphosphorylated tau aggregates into paired helical filaments and microtubules are lost [reviewed in Lovestone et al. (1997)]. GSK-3β induced tau

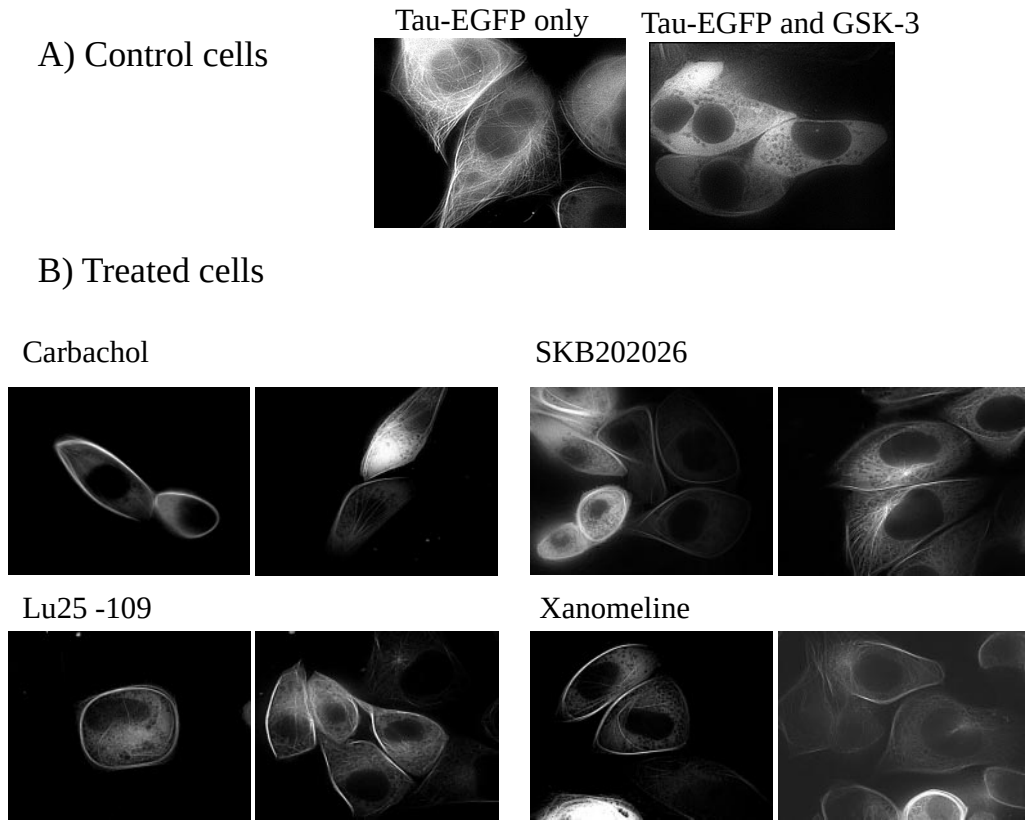

Fig. 4. Muscarinic agonists alter the microtubule binding properties of phosphorylated tau. When expressed in non-neuronal cells, tau, tagged with EGFP was bound to microtubules which were assembled into bundles (**A**). When expressed together with GSK-3β, tau-EGFP was largely cytoplasmic and microtubule bundles were not apparent. All four muscarinic agonists induced a change in the distribution of tau-EGFP such that even in the presence GSK-3β tau was bound to microtubules which were assembled into bundles (magnification ×500)

phosphorylation reduces binding to microtubules (Lovestone et al., 1996) and increasing evidence points to GSK-3β as critical in regulating tau phosphorylation in neurons (Hong et al., 1997a,b; Muñoz-Montaño et al., 1997). Phosphorylation of tau apparently precedes aggregation (Braak et al., 1994), and the cognitive impairment in AD correlates well with tangle formation (Nagy et al., 1995). Slowing the process of tau phosphorylation, possibly via inhibition of GSK-3β, is therefore a potential therapeutic target in AD. Previous observations have demonstrated that muscarinic agonists can reduce tau phosphorylation in PC12 cells expressing the m1 receptor (Sadot et al., 1996). This was intriguing as muscarinic compounds have also been shown to alter the processing of APP (Hung et al., 1993; Nitsch et al., 1992; DeLapp et al., 1998; Haring et al., 1994). Considerable evidence suggested that effects on APP metabolism were acting through PKC (Desdouits-Magnen et al., 1998; Felsenstein et al., 1994; Slack et al., 1997) although the mechanism of action of muscarinic agonists on tau phosphorylation was unclear. However as PKC

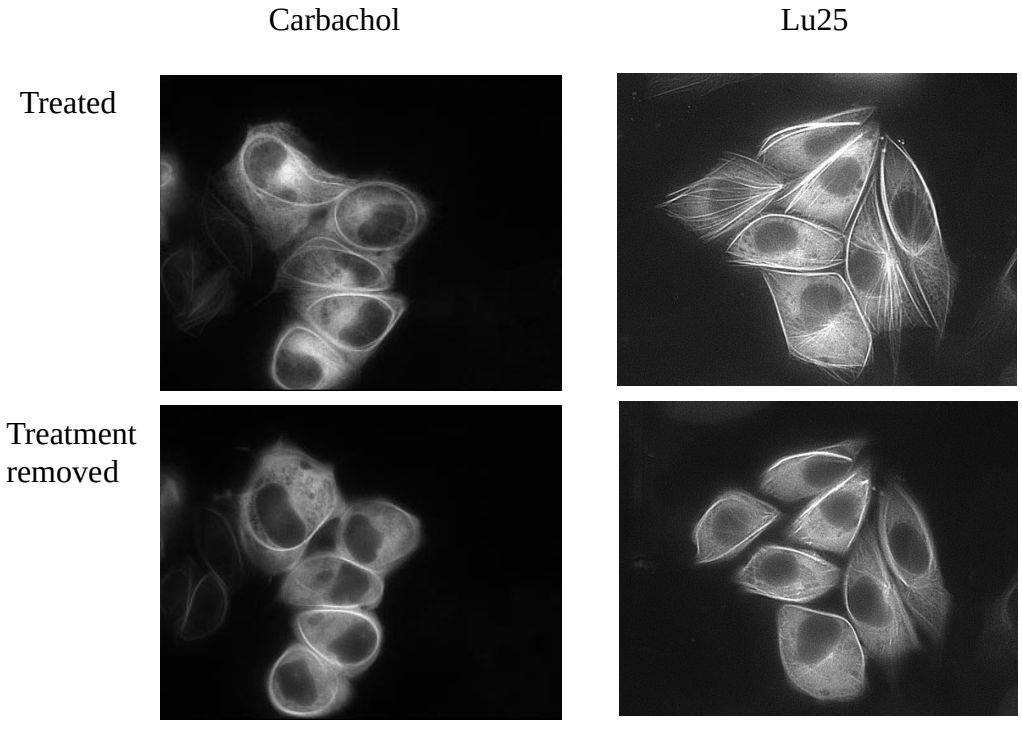

Fig. 5. The muscarinic agonist induced changes in the properties of phosphorylated tau are reversible. Cells transfected with GSK-3β and tau were treated with either carbachol or Lu-25-109 as a muscarinic agonist whereupon microtubule bundles were visualised. The compounds were then removed and cells revisualised whilst maintained at physiological conditions. The tau-EGFP bound microtubule bundles were less prominent one hour after the compounds were removed and most of the tau-EGFP was cytoplasmic (magnification ×750)

also inhibits GSK-3β (Cook et al., 1996), it is possible that the tau phosphorylation changes observed in PC12 cells might result from muscarinic signalling induced inhibition of GSK-3β.

We have examined this hypothesis using three complementary approaches. Firstly we demonstrated that a non-specific muscarinic agonist reduces tau phosphorylation in primary neurons. The developmental expression of the different muscarinic receptors in rat is not fully understood but in slice cultures evidence exists that the m1 receptor is expressed at low levels by E20 and reaches adult levels of expression between postnatal weeks 3–5 (Aubert et al., 1996). As neurons continue to mature in culture with a change in expression in tau isoforms, for example (Ferreira et al., 1997), we expected muscarinic receptors to be expressed in the 12–14 day old cultures we examined.

Having demonstrated that muscarinic agonism alters tau phosphorylation in primary neurons, we examined the effects on GSK-3β induced tau phosphorylation in a non-neuronal cell model. Lithium chloride completely reversed tau phosphorylation in these cells as expected. Both a non-specific

muscarinic agonist, carbachol, and a range of relatively specific m1 agonists reduced tau phosphorylation although to a lesser degree than lithium chloride. In each of these situations some hyperphosphorylated tau was seen as an AT8 reactive band and as a slowly migrating TP70 reactive band. However the proportion of this highly phosphorylated tau was considerably less than in untreated doubly transfected cells. Thus in these cells muscarinic agonists reduce but do not abolish GSK-3β induced tau phosphorylation. Using this model of tau phosphorylation we can be certain that muscarinic agonists reduce tau phosphorylation via GSK-3β. As GSK-3β readily phosphorylates tau in vitro this is most likely to be a direct action of GSK-3β on tau in cells although we cannot exclude the possibility of an indirect effect of GSK-3β via regulation of other kinases or phosphatases.

Finally we demonstrated using living cells that the inhibition of GSK-3β induced tau phosphorylation altered the microtubule binding properties of tau. When cells were treated overnight with muscarinic agonists bundles were present in cells that were doubly transfected with tau-EGFP and GSK-3β. Untreated cells transfected only with tau also showed bundles but doubly transfected untreated cells did not. When the muscarinic agonist was removed bundles were less apparent and tau was more obvious in cytoplasm.

We confirm that muscarinic agonists reduce tau phosphorylation and that this is via a GSK-3β mediated process. The finding that both APP metabolism and tau phosphorylation are altered by muscarinic signalling is interesting and raises the possibility of a pathogenic link, possibly via the muscarinic second messenger, PKC.

Additional evidence needs to be generated to confirm a cholinergic-linked connection between the changes in APP metabolism and tau phosphorylation. However, it is a remarkable finding that cholinergic compounds may indeed affect the pathogenic processes underlying the formation of both plaques and tangles in AD. These findings now need to be extended to animal studies.

Acknowledgements

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