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Research Paper

BAG2 expression dictates a functional intracellular switch between the p38-dependent effects of nicotine on tau phosphorylation levels via the α7 nicotinic receptor

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ABSTRACT

The histopathological hallmarks present in Alzheimer's disease (AD) brain are plaques of Aβ peptide, neurofibrillary tangles of hyperphosphorylated tau protein, and a reduction in nicotinic acetylcholine receptor (nAChR) levels. The role of nAChRs in AD is particularly controversial. Tau protein function is regulated by phosphorylation, and its hyperphosphorylated forms are significantly more abundant in AD brain. Little is known about the relationship between nAChR and phospho-tau degradation machinery. Activation of nAChRs has been reported to increase and decrease tau phosphorylation levels, and the mechanisms responsible for this discrepancy are not presently understood. The co-chaperone BAG2 is capable of regulating phospho-tau levels via protein degradation. In SH-SY5Y cell line and rat primary hippocampal cell culture low endogenous BAG2 levels constitute an intracellular environment conducive to nicotine-induced accumulation of phosphorylated tau protein. Further, nicotine treatment inhibited endogenous expression of BAG2, resulting in increased levels of phosphorylated tau indistinguishable from those induced by BAG2 knockdown. Conversely, overexpression of BAG2 is conducive to a nicotine-induced reduction in cellular levels of phosphorylated tau protein. In both cases the effect of nicotine was p38MAPK-dependent, while the α7 antagonist MLA was synthetic to nicotine treatment, either increasing levels of phospho-Tau in the absence of BAG2, or further decreasing the levels of phospho-Tau in the presence of BAG2. Taken together, these findings reconcile the apparently contradictory effects of nicotine on tau phosphorylation by suggesting a role for BAG2 as an important regulator of p38-dependent tau kinase activity and phospho-tau degradation in response to nicotinic receptor stimulation. Thus, we report that BAG2 expression dictates a functional intracellular switch between the p38-dependent functions of nicotine on tau phosphorylation levels via the α7 nicotinic receptor.

1. Introduction

Alzheimer's disease (AD), the most common form of neurodegenerative dementia, affects more than 35 million patients worldwide (Prince et al., 2013). The disease is characterized by a decline in memory performance followed by a severe global cognitive impairment, and eventual death (Jicha and Carr, 2010). AD involves progressive loss of neurons and synapses, beginning in the entorhinal cortex, the limbic cholinergic system, and later spreading to the neocortex (Braak et al., 2006). The histopathological hallmarks of AD brain are extracellular amyloid plaques of Aβ peptide, intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein, and a reduction in nicotinic acetylcholine receptor (nAChR) levels (Burghaus et al., 2000; Wevers et al., 1999). The regions of the brain most affected by AD also express high levels of α7nAChRs (D'Andrea and Nagele, 2006). Despite this, the relationship between these three features of AD remains poorly characterized.

The microtubule-stabilizing activity of tau protein is regulated as a function of its phosphorylation (Iqbal et al., 1994), which correlates negatively with microtubule binding affinity, and results in microtubule destabilization and impaired intracellular transport (Bendiske et al., 2002). Hyperphosphorylated tau is also toxic to the intracellular environment and leads to neuronal death (Shimura et al., 2004). Levels of tau phosphorylation in AD brain are approximately 4-fold higher than normal (Khatoon et al., 1994). Accumulation of phosphorylated tau (phospho-tau; p-tau) is likely a consequence of imbalance between tau kinases and phosphatases, as well as a failure in protein degradation pathways (Poppek et al., 2006). While the effects of nAChRs activation on tau phosphorylation have been extensively studied, little is known concerning the relationship between nicotinic receptors and phospho-

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tau degradation machinery. In addition, the role of nAChRs on tau phosphorylation is particularly controversial (Oz et al., 2013).

Activation of nAChRs has been variously reported to have both neuroprotective and neurotoxic effects (Berger et al., 1998; Laudenbach et al., 2002; Oz et al., 2013). Several studies indicate that Aβ binds to α7nAChR (Wang et al., 2000), leading to tau phosphorylation (Busciglio et al., 1995; Oz et al., 2013; Wang et al., 2003) and cell death (D'Andrea et al., 2001; Wang et al., 2000, 2003) other studies show contrary results which describe α7nAChR-mediated reduction hyperphosphorylated tau in vivo (Bitner et al., 2009) and in vitro (Alonso et al., 2011; Del Barrio et al., 2011). When activated by endogenous agonist or by nicotine, α7nAChR also protects neurons against Aβ toxicity (Akaike, 2006; Arias et al., 2005; Kihara et al., 1997; Liu et al., 2007) and against toxicity induced by glutamate (Shimohama et al., 1996), oxidative stress (Cormier et al., 2003) and rotenone (Takeuchi and Yamagishi, 2009). These apparently contradictory data suggest a complex role for α7nAChR function in the context of dynamic regulation of tau phosphorylation, and may yield insight into the mechanisms responsible for the loss of function and neuronal death that are characteristic of AD.

The co-chaperone BAG2 inhibits tau ubiquitination by CHIP and delivers phospho-tau to the proteasome for ubiquitin-independent degradation (Carrettiero et al., 2009b). Because nicotine activates tau phosphorylation via p38 (Oddo et al., 2005), and it is the only kinase known to phosphorylate BAG2 (Ueda et al., 2004), which in turn regulates phospho-tau levels via protein degradation, we wished to investigate whether BAG2 influences the effect of nicotine on phospho-tau levels via the α7 nicotinic receptor. Our findings show that the expression of BAG2 dictates a functional intracellular switch between the p38-dependent functions of nicotine on tau phosphorylation levels via the α7 nicotinic receptor in SH-SY5Y neuroblastoma cell line and rat primary hippocampal cell culture. Endogenous levels of BAG2 expression constitute an intracellular environment conducive to nicotine-induced accumulation of phospho-tau protein. Conversely, overexpression of BAG2 is conducive to a nicotine-induced reduction in cellular levels of phospho-tau protein. Taken together, these findings reconcile the apparently contradictory effects of nicotine on tau phosphorylation by suggesting a role for BAG2 as an important regulator of p38-dependent tau kinase activity and phospho-tau degradation in response to nicotinic receptor stimulation.

2. Material and methods

2.1. Antibodies, reagents and plasmids

Mouse Tau-5 monoclonal antibody (1:1000 dilution; Sigma) recognizes phosphorylated and non-phosphorylated forms of tau (total tau); phospho-tau was detected with rabbit anti-phospho-tau (pS199/202) polyclonal antibody (1:1000 dilution; Sigma). Mouse anti-β-actin monoclonal antibody (1:6000 dilution; Sigma) was used to normalize Western blot bands. DAPI (4,6-diamidino-2-phenylindole; Vector) stained mounting medium was used to label cell nuclei. Anti-mouse Tx-RED-conjugate secondary antibody was used for fluorescence microscopy (Sigma). FITC-labeled (5 μM) and unlabeled (10 μM) α-bungarotoxin, (α-Bgt; Molecular probes) was used to detect α7nAChR by incubation for 45 min. Nicotine ((−)-nicotine; Sigma) was dissolved in water and used for 6 h treatment at indicated concentrations (10, 50 and 100 μM). The nicotinic antagonists methyllycaconitine (MLA, 10 nM, Sigma) and mecamylamine (MECA, 2 μM, Sigma) were diluted in H_2O and used at indicated concentrations. The p38MAPK inhibitor SB203580 was dissolved in dimethylsulfoxide and used at a final concentration of 5 μM. BAG2 was overexpressed by transfection with pEGFP-BAG2 and pEGFP control plasmids as previously described (Carrettiero et al., 2009b), and Lipofectamine 2000 Transfection Reagent (Invitrogen) was used according to the manufacturer's instructions. Twenty-four hours after transfection, cell medium was replaced with medium containing MLA, MECA or SB203580 at indicated concentrations for 3 h after the addition of nicotine to final concentrations indicated above. Knockdown of BAG2 expression was achieved using a specific anti-shRNA expressed from commercially-available pre-prepared lentivirus (Dharmacon) and used according to manufacturer instructions, together with non-silencing control lentivirus. Transduction was confirmed via confirmation of expression of GFP marker via fluorescent microscopy.

2.2. Primary hippocampal cell culture

Wistar rats were obtained by the Universidade Federal do ABC (UFABC), and all procedures and protocols were in strict accordance with international and UFABC Institutional Guidelines for Animal Experimentation. Approximately 15 newborn rats, 1–2 days old, per experiment were used for isolation of primary hippocampal neurons (Carrettiero et al., 2009a, b). Brains were rapidly removed and placed in 4 °C HBSS buffer solution (Invitrogen; pH 7.4, $NaHCO_3$ (25 mM), Glucose (13 mM)). Dissected hippocampi were trypsinized (0.05%; Invitrogen) for 15 min at 37 °C in a shaking water bath. Tissue was washed 2× with HBSS and manually dissociated with a fire-bored Pasteur pipette. The solution was centrifuged (300 × *g*) for 5 min, and resuspended in Neurobasal medium (Gibco® BRL, Rockville, MD, USA) supplemented with 2% B27 (Gibco® BRL, Rockville, MD, USA), 0.25 mM L-glutamine (Sigma Chemical Co St. Louis, MO, EUA), 0.25 mM GlutaMAX (Gibco® BRL, Rockville, MD, USA) and gentamicin (Gibco® BRL, Rockville, MD, USA). After counting, the cells were plated on glass coverslips coated overnight with poly-D-lysine (Sigma-Aldrich), at 3.1×10^5 cells per cm^2. Neurons were cultured at 37 °C in a humidified incubator with 95% air/5% CO_2 for 10 days before use.

2.3. SH-SY5Y cell culture

SH-SY5Y human neuroblastoma cells (ATCC) were cultivated according to de Paula et al. (2015). Undifferentiated SH-SY5Y cells, which, like in vivo differentiated neurons, express low levels of endogenous BAG2 compared to differentiated SH-SY5Y cells (Santiago et al., 2015). Briefly, cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and F12 (Gibco® BRL) supplemented with 10% fetal bovine serum (Sigma) and 200 U/ml penicillin and streptomycin (Gibco® BRL) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were subcultured every 2 days.

2.4. Immunocytochemistry

After treatments cell cultures were fixed in a 1:1 methanol-acetone for 10 min at −20 °C, washed (3 × 1 min) with PBS, and permeabilized with 0.1% Triton X-100 for 15 min. Cells were washed 2× in PBS and incubated for 20 min in blocking medium (2% NGS, normal goat serum, 4% BSA, 0.2% Triton X-100). Cells were incubated with primary antibody in blocking medium overnight at 4 °C, washed (3 × 5 min) with PBS and incubated for an additional 1 h with secondary antibodies. After washing (3 × 5 min), the dishes were mounted with mounting media containing DAPI, washed in PBS and visualized by fluorescence microscopy. For each transfection experiment 100 cells were chosen randomly per plate. Optical density (OD) values of cells stained with phospho-tau (S199/202) antibody were quantified by pixel intensity using Image J software and normalized to background.

2.5. Protein extraction and Western blot

After treatments cells were lysed with RIPA buffer (1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM DTT, 1% Protease Inhibitor Cocktail (Sigma Aldrich), 1 mM PMSF). Total protein concentration was estimated by Bradford assay. Total cell lysates (20 μg) were resolved by 12%