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Anticholinergics boost the pathological process of neurodegeneration with increased inflammation in a tauopathy mouse model

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ABSTRACT

Anticholinergics, and drugs with anticholinergic properties, are widely and frequently prescribed, especially to the elderly. It is well known that these drugs decrease cognitive function and increase the risk of dementia. Although the mechanism of anticholinergic drug-induced cognitive impairment has been assumed to be functionally reduced acetylcholine (ACh) neurotransmission, some data have indicated that anticholinergics might enhance the pathology of Alzheimer's disease. In this study, we investigated the pathological effects of anticholinergics on neurodegeneration. We chronically administered two anticholinergics, trihexyphenidyl (TP) and propiverine (PP) (the latter with less central anticholinergic action), to neurodegenerative tauopathy model mice 2 to 10 months old. Furthermore, because the ACh nervous system regulates both central and peripheral inflammation, we administered TP or PP to PS19 mice in which we had artificially induced inflammation by lipopolysaccharide injection. Tau pathology, synaptic loss, and neurodegeneration in the hippocampal region, as well as tau insolubility and phosphorylation, were markedly increased in TP-treated mice and mildly increased in PP-treated mice. Furthermore, immunohistochemical analysis revealed microglial proliferation and activation. Moreover, anticholinergics increased interleukin-1β expression in both the spleen and brain of the tauopathy model mice intraperitoneally injected with lipopolysaccharide to induce systemic inflammation. Interestingly, these alterations were more strongly observed in TP-treated mice than in PP-treated mice, consistent with the level of central anticholinergic action. Anticholinergic drugs not only impair cognitive function by decreased ACh neurotransmission, but also accelerate neurodegeneration by suppressing an ACh-dependent anti-inflammatory system. Anticholinergics should be less readily prescribed to reduce the risk of dementia.

Introduction

Anticholinergics are widely used for treating overactive bladder or Parkinson's symptoms in elderly people. Moreover, many drugs commonly prescribed to elderly people (such as antiemetics, antispasmodics, bronchodilators, anti-arrhythmic drugs, antihistamines) have anticholinergic properties. It is well known that these drugs might induce cognitive decline and increase the severity of dementia symptoms (Ancelin et al., 2006; Moore and O'Keeffe, 1999; Tune et al., 1992). Surprisingly, a large-scale cohort study of elderly individuals identified mild cognitive impairment (MCI) in 80% of patients on anticholinergic therapies, and concluded that this therapy imparts a 5-fold increased risk of MCI (Ancelin et al., 2006). A 6-year observational study in an African-American population indicated that 53% of the population used a possible anticholinergic and 11% used a definite anticholinergic; the number of definite anticholinergics was associated with an increased risk of cognitive impairment (Campbell et al., 2010a). Similarly, a 4-year follow-up study in France indicated that 7.5% of the participants were taking anticholinergics, and that the use of these drugs was associated with an increased risk of cognitive decline and dementia, which remained significant after adjustment for the multiple possible codeterminants of cognitive decline (Carriere et al., 2009). An observational study in Germany showed that 37% of the participants used anticholinergics, and that they had an increased risk of dementia (hazard ratio = 2.081) in an anticholinergic activity-dependent manner (Jessen et al., 2010). Moreover, there are significant associations reported between high serum anticholinergic activity and poor mini-mental state examination (MMSE) scores (Mulsant et al., 2003). One explanation of these observations is functional suppression of the cholinergic system, which is closely related to cognitive function. An autopsy study, however, demonstrated a larger amount of amyloid plaques and neurofibrillary tangles in the brains of Parkinson's disease (PD) patients treated with anticholinergics than in PD patients not treated with anticholinergics, suggesting that anticholinergic therapy might potentially enhance or induce Alzheimer's disease (AD) pathology and neurodegeneration (Perry et al., 2003).

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To assess the effects of anticholinergics on neurodegeneration, trihexyphenidyl (Artane™, TP), which is preferentially used for the treatment of PD in Japan, and propiverine (BUP-4™, PP), which is used for the treatment of overactive bladder and has a lower central anticholinergic action (Kobayashi et al., 2007; Oka et al., 2001; Suzuki et al., 2007), were administered to the P301S tauopathy model mice (PS19 mice). Moreover, to investigate the effects of these anticholinergics on systemic inflammation, we administered TP or PP to PS19 mice in which we had artificially induced systemic inflammation by lipopolysaccharide (LPS) injection.

Methods

Tau transgenic (PS19) mice and drug treatment

The generation of transgenic (Tg) mice carrying the human tau gene with a P301S mutation has been previous described by our group (Yoshiyama et al., 2007). Briefly, a cDNA construct of human T34 isoform tau (1N4R) harboring the P301S mutation was cloned into the MoPrP.Xho expression vector containing a mouse prion (MoPrP) promoter (Borchelt et al., 1996) at the XhoI site. A 15-kb NotI fragment containing T34 and the MoPrP promoter, together with its 39 untranslated sequences, was used as the transgene to create tau Tg mice on a B6C3H/F1 background. A stable Tg line (PS19) and non-Tg offspring were identified by PCR analysis of tail genomic DNA.

TP and PP were mixed with powdered food (TP, 1.5 mg/100 g; PP, 5 mg/100 g) and administered to groups of 15 PS19 mice and six WT mice at 2 months of age. The estimated doses per body weight per day were 1.5 mg/kg TP and 50 mg/kg PP, respectively. Ten PS19 and six WT mice were fed powdered food containing no drug, and served as the non-treated control (nTC) group.

All experiments were approved by the Committee of Animal Care and Control in Chiba East National Hospital.

Immunohistochemistry

The methods of tissue preparation and immunohistochemical analysis have been previously described (Yoshiyama et al., 2000, 2007). Briefly, mice were deeply anesthetized and transcardially perfused with 15 ml of phosphate-buffered saline (PBS). The brains and spinal cord were removed, immersion-fixed for 24 h in 4% paraformaldehyde in PBS, then stored in 15% sucrose–PBS at 4 °C until further use. The tissue was sectioned with a cryostat in 20-μm-thick sections, which were used for immunohistochemical analysis. Streptavidin–biotin-peroxidase (Vector Laboratories, CA), as well as double-labeling immunofluorescence, was used for staining. The antibodies and their dilutions used are listed in Table 1.

Tau insolubility and phosphorylation states

Proteins were extracted from brains using high-salt (0.75 M NaCl) reassembly buffer (RAB) [0.1 M MES, 1 mM EGTA, 0.5 mM MgSO4, 0.02 M NaF, 1 mM PMSF, and protease inhibitor cocktail (protease inhibitor cocktail set I, Calbiochem®, Merck, Germany)] by centrifugation at 50,000×*g* for 40 min at 4 °C in a Beckman TL-100 ultracentrifuge. The RAB-insoluble pellets were re-homogenized with 1 M sucrose in RAB and centrifuged at 40,000×*g* for 20 min at 4 °C to remove myelin and associated lipids. The resulting pellets were dissolved with 1 ml/g tissue in a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 5 mM EDTA; pH 8.0, and protease inhibitor cocktail) and centrifuged at 40,000×*g* for 20 min at 4 °C. The supernatants were used as RIPA-soluble samples, while the RIPA-insoluble pellets were extracted with 70% FA to recover highly insoluble protein.

Imaging and statistical analyses

Images were captured with a Nikon Eclipse 80i microscope and a Nikon DXM 1200C digital camera, and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). The results were expressed as the mean ± SEM. Statistically significant differences were determined by analysis of variance using SPSS statistical software (SPSS, Chicago, IL). Statistical significance was set at $P<0.05$.

Western blot analysis

Brains were homogenized, and diluted to 2 ml/g tissue with ice-cold 1% triton high salt RAB in the presence of protease inhibitor cocktail (protease inhibitor cocktail set I, Calbiochem®, Merck, Germany), followed by centrifugation at 50,000×*g* for 40 min at 4 °C in a Beckman

Table 1
Primary antibodies used in this study.

Antibody	Immunogen	Host	Dilution for IHC	Dilution for WB	Source
17026	Total tau	Rabbit		×1000	Ishihara et al., 1999
AT8	Peptides containing phospho-S202/T205 tau	Mouse	×1000	×500	Inogenetics (Alpharetta, GA)
T1	aa 189–207 of non-phosphorylated tau	Mouse		×500	Binder et al., 1985
AT180	Peptides containing phospho-T231/S235 tau	Mouse		×500	Inogenetics (Alpharetta, GA)
pTau (Ser46)	Peptides containing phospho-S46 tau	Goat		×1000	Santa Cruz Biotechnology (Santa Cruz, CA)
pTau (S422)	Peptides containing phospho-S422 tau	Rabbit		×1000	Abcam (Cambridge, UK)
Syntaxin	Synaptic vesicle containing fractions of immunoprecipitated human brain homogenate	Mouse	×500	×1000	Santa Cruz Biotechnology (Santa Cruz, CA)
Iba1	C terminus of Iba1	Rabbit	×1000		Wako (Osaka, Japan)
IL-1β	aa 117–269 of human IL-1β	Rabbit	×100		Santa Cruz Biotechnology (Santa Cruz, CA)
SAPK/JNK	Human JNK2	Rabbit		×1000	Cell Signaling (Beberly, MA)
Phospho SAPK/JNK	Phosphopeptide surrounding Thr183/Tyr185 of human SAPK/JNK	Rabbit		×1000	Cell Signaling (Beberly, MA)
GSK-3β	aa 345–420 of human GSK-3β	Rabbit		×200	Santa Cruz Biotechnology (Santa Cruz, CA)
Phospho-GSK-3β	aa containing phosphorylated ser9 of human GSK-3β	Goat		×200	Santa Cruz Biotechnology (Santa Cruz, CA)
cdk5	aa 1–291 of human cdk5	Mouse		×200	Santa Cruz Biotechnology (Santa Cruz, CA)
Phospho-cdk5	aa containing phosphorylated Ser159 of human cdk5	Goat		×200	Santa Cruz Biotechnology (Santa Cruz, CA)
p38	Human p38	Rabbit		×1000	Cell Signaling (Beberly, MA)
Phospho-p38MAPK	Phosphopeptides surrounding Thr180/Tyr182 of human p38	Rabbit		×1000	Cell Signaling (Beberly, MA)
Phospho-p38MAPK	Phosphopeptides surrounding Thr180/Tyr182 of human p38	Mouse		×1000	Cell Signaling (Beberly, MA)
CD11b	Mouse CD11b (5C6)	Rat	×100		Serotec (Kidlington, UK)
CD68	Mouse CD68 (FA11)	Rat	×200		Serotec (Kidlington, UK)

IHC, immunohistochemistry; WB, Western blotting.

TLX ultracentrifuge. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL), and proteins were resolved by SDS–PAGE, followed by Western blotting. Signals were detected by enhanced chemiluminescence (GE Healthcare, WI) according to the manufacturer's instructions. The antibodies and their dilutions used are listed in Table 1.

Results

Tau pathology and neuronal degeneration are enhanced by anticholinergics in PS19 mice

The Tg mouse expressing 4R1N human tau with P301S mutation (line PS19) used in this study exhibits synaptic loss and microglial activation beginning at 3 months of age, followed by abnormal tau accumulation starting around 6 months of age. Neuronal loss and brain atrophy can be observed at about 8 months (Yoshiyama et al., 2007). To investigate the effects of anticholinergics on tau pathology, TP or PP was administered to these mice from age 2 to 10 months. Brain sections were histopathologically or immunohistochemically analyzed at 10 months of age. Eight of 15 (53% survival rate) TP-treated PS19 mice, 11 of 15 (73% survival rate) PP-treated PS19 mice, and seven of 10 (70% survival rate) nTC PS19 mice, and all six WT mice survived to 10 months of age.

To compare the tau pathology among the groups, brain sections were immunostained with AT8, an antibody specific for phosphorylated tau. Although the distribution of tau-positive neurons, which were mainly located in the hippocampus, amygdala, and spinal cord, was similar in each group, TP-treated PS19 mice showed much stronger tau pathology in the CA1 (Fig. 1I) and CA3 (Fig. 1J) regions, compared with nTC PS19 mice (Figs. 1A, B). Moreover, PP-treatment caused intermediate tau pathology phenotype (Figs. 1E, F). AT8 immunostaining with Nissl counterstaining (Figs. 1C, G, K) showed a disintegrated laminar structure of the pyramidal neurons in the CA3 region in PP (Fig. 1G) and TP (Fig. 1K) treated PS19 mice, and a high and low frequency, respectively, of morphologically abnormal neurons with pyknotic nuclei in TP-treated and PP-treated mice. Interestingly, most of pyknotic neurons exhibited no intraneuronal tau accumulation (Figs. 1C, G, K). Statistical analysis of the AT8-positive neurons in the CA1 and CA3 regions revealed a significantly increased number of AT8-positive neurons, compared with the number in nTC, in TP-treated mice in CA1 (Fig. 1M) and PP-treated mice in CA3 (Fig. 1N). Although the number of AT8-positive neurons in the CA3 region in TP-treated mice was lower than expected, this might have been because of the decreased numbers of neurons following neuronal cell death upon abnormal tau accumulation. Therefore, Nissl-stained sections were examined to determine the number and shape of neurons remaining in the hippocampus. Low-magnification images of the hippocampal regions (Figs. 1D, H, L) revealed a severe selective neuronal loss in the CA3 region, as well as hippocampal atrophy, in PS19 mice treated with TP (Fig. 1L, arrowheads). Statistical analysis of the remaining neurons in the CA3 regions of each group demonstrated significant neuronal loss with TP treated mice, compared with nTC mice (Fig. 1N), despite a lack of significant neuronal loss in the CA1 region in any group (Fig. 1M). To assess synaptic degeneration, syntaxin immunoreactivity was measured by Western blotting. A significant reduction in syntaxin immunoreactivity was observed in TP-treated PS19 mouse brains (Fig. 1O).

Tau insolubility and phosphorylation are increased by anticholinergics

Since more tau-positive neurons were observed in TP- and PP-treated PS19 mice, the mice were subsequently analyzed for soluble and insoluble tau by sequential extraction of brain samples with three buffers of increasing extraction strength, as previously described (Ishihara et al., 1999). Tau solubility decreased in the order nTCs > PP-treated > TP-treated (Fig. 2A). In particular, a slowly migrating band (Figs. 2A, C, arrow), which was highly phosphorylated and specifically recognized by AT8 (Fig. 2C, arrow), was more intense in TP-treated than in the PP-treated mouse brain samples in the FA fractions. Samples in the RAB fractions were also hyperphosphorylated in TP-treated PS19 mice (AT180 and pS422 in Fig. 3).

Tau kinases are activated in anticholinergics-treated PS19 mice

To assess tau kinase activities causing tau phosphorylation, activated and inactivated forms of GSK3β, CDK5 and MAPKs were measured in TP-treated, PP-treated and nTC PS19 mice, and WT mice by Western blotting (Fig. 3). For all of these enzymes except GSK3β, the phosphorylated (p-) form is the activated form; p-GSK3β is the inactivated form. Activation status in TP-treated, PP-treated, and nTC PS19 mice was compared. TP treatment clearly enhanced the activation of GSK3β, CDK5, and ERK, but not p38 or JNK. PP treatment mildly increased GSK3β and CDK5 activation. These results indicate that anticholinergics enhanced tau phosphorylation by activating tau kinases.

Microglial activation is induced by anticholinergics

Microglial-mediated neuroinflammation plays an important role in neurodegeneration in a number of neurodegenerative diseases including tauopathies, in fact, PS19 mice show microglial activation preceding tau pathology and neuronal loss (Yoshiyama et al., 2007). Moreover, our recent research indicated that enhancing cholinergic activity with donepezil suppressed neuronal inflammation including microglial activation (Yoshiyama et al., 2010). Thus, we speculated that anticholinergics might enhance microglial activation. Microglia in the brains of TP- or PP-treated as well as nTC PS19 mice were analyzed immunohistochemically using microglia-specific antibodies. Double-immunolabeling for Iba1 and tau clearly demonstrated marked microglial activation in the brains of TP-treated PS19 mice (Figs. 4G–I) compared with nTC PS19 mice (Figs. 4A–C). Higher magnification images revealed enlarged, Iba1-positive microglia with thick processes in the CA3 region in TP-treated PS19 mice (Fig. 4K), whereas a few, small microglia with thin processes were observed in the WT mouse brain (Fig. 4J, insert). In the CA1 region of TP-treated PS19 mice, the microglia were elongated parallel to the neuronal processes (Fig. 4L). Immunohistochemistry of activated microglial markers, including CD11b (Figs. 4O–R) and CD68 (cys. 4S–V), demonstrated obvious microglial activation in TP-treated PS19 mice (Figs. 4Q, R, U, V). Interestingly, CD68-positive microglia were prominent in the granular cell layer of the hippocampus, including the CA3 (Fig. 4V) and CA1 (Fig. 4Q) regions. However, Iba1-positive (Figs. 4G–I) and CD11b-positive (Figs. 4Q, R) microglia were almost uniformly distributed throughout the white matter and gray matter in the hippocampus, suggesting that CD68-positive microglia might be pathologically activated and directly involved in the neurodegeneration. Microglial activation was mainly observed in the hippocampus, amygdala, entorhinal cortex, and brain stem, corresponding to regions where the tau pathology was predominantly observed (data not shown). Statistical analysis of the percentage of Iba1-positive areas in the CA1 or CA3 clearly demonstrated that TP treatment enhanced microgliosis in each hippocampal region (Figs. 4M, N).

LPS-induced systemic and neuronal inflammation is accelerated by anticholinergics

Our data suggested that neuroinflammation enhanced by anticholinergics was involved in the acceleration of tau pathology and neurodegeneration in PS19 mice. Next, we assessed the effects of anticholinergics on systemic and neuronal inflammations artificially induced by LPS-injection. Immunohistochemistry for IL-1β in the spleen showed enhanced staining in TP-treated mice (Fig. 5A) compared with PP-treated mice (Fig. 5B) and nTC mice (Fig. 5C). Similarly, neuroinflammation represented by immunohistochemical staining for IL-1β (Figs. 5D–F) and Iba1 (Figs. 5G–I) demonstrated enhanced neuroinflammation in TP-

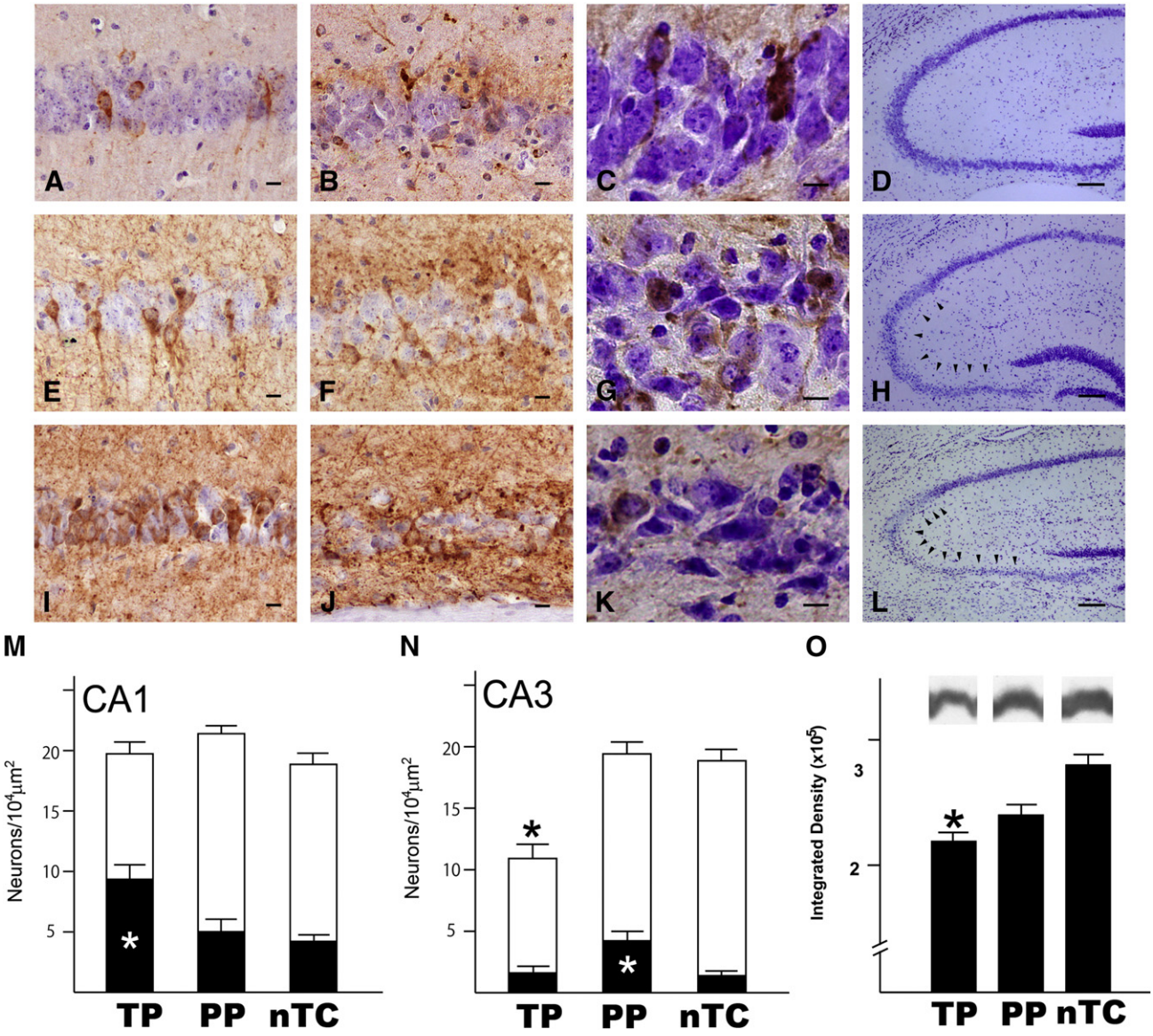

Fig. 1. Anticholinergics trihexyphenidyl and propiverine accelerate neurodegenerative process in a tauopathy mouse model (PS19). Brains from PS19 mice treated with trihexyphenidyl (TP) (I–L) or propiverine (PP) (E–H), and non-treated control (nTC) (A–D) PS19 mice were stained with AT8 (an antibody to phosphorylated tau) (A–C, E–F, I–K). Immunohistochemical staining for AT8 counterstained with hematoxylin in the CA1 (A, E, I) and the CA3 (B, F, J) regions demonstrate that the proportion of AT8-positive pyramidal neurons is slightly increased in PP-treated (E, F) and clearly increased in TP-treated PS19 mice (I, J), compared with nTC PS19 mice (A, B). Interestingly, the pyramidal neurons in TP- or PP-treated PS19 mice are poorly stained with hematoxylin, compared with neurons in nTC. The number of neurons in the CA3 region of TP-treated PS19 mice is apparently decreased (N). In contrast, although there are more AT8-postive neurons in the CA1 region in TP-treated PS19 mice, the number of neurons in is not obviously decreased (M). To confirm the neuronal loss, hippocampal sections are stained with Nissl staining (D, H, L). Low-magnification images indicate decreased affinity for the stain in pyramidal neurons, especially in the CA3 region (arrows); the reduction is moderate in PP-treated (H) and severe in TP-treated (L) PS19 mice. In PP-treated PS19 mice, some atrophic neurons with pyknotic nuclei are scatteringly seen in neurons faintly stained with Nissl (G). In TP-treated PS19 mice, the number of neurons is decreased; they are pyknotic and their shape is indicative of atrophy (K). The alteration in shape and Nissl-stainability of neurons is not related to tau accumulation (C, G, K). Statistical analysis indicated that in the CA1 region, the number of AT8-positive neurons was increased in TP-treated PS19 mice compared to nTC PS mice, although the total neuron number was unchanged (M); however, in the CA3 region, the number of AT8-positive neurons was increased in PP-treated PS19 mice but not in TP-treated PS19 mice, and the ratio of AT-8 positive neurons to the total number of neurons was increased both in TP-treated and PP-treated PS19 mice. The neuronal loss was significant in TP-treated mice. To quantify the synaptic loss, signals of syntaxin in Western blots was measured revealing a significant reduction in TP-treated mice (O). *, $p<0.05$, to nTC. Scale bars, 10 µm (A–C, E–G, I–K); 100 µm (D, H, L).

treated mice (Figs. 5D, G). These results suggest that anticholinergics enhanced not only neuroinflammation but also systemic inflammation induced by LPS injections. Meanwhile, only administration of PP or TP to PS19 mice without LPS-injection did not show obvious enhancement in IL-1β expression or microglial activation (data not shown).

Discussion

Our study provides evidence that anticholinergics enhance tau pathology, neurodegeneration and neuroinflammation as well as systemic inflammation in the neurodegenerative tauopathy mouse model PS19. With increasing interest in the effects of lifestyle on the development dementia, the relationship of dementia to the use of anticholinergics has attracted attention. These drugs have been shown to increase cognitive impairment and dementia (Campbell et al., 2010b; Carriere et al., 2009; Jessen et al., 2010), but they are widely and frequently prescribed to elderly people (7.5–50%) with no particular cautions. The cognitive impairment and dementia caused by anticholinergics was previously suspected to be due to functional disruption of acetylcholine (ACh) neurotransmission; however, our results indicate that these drugs might accelerate neurodegenerative processes other than disrupted neurotransmission. Interestingly, PP, which has less central anticholinergic

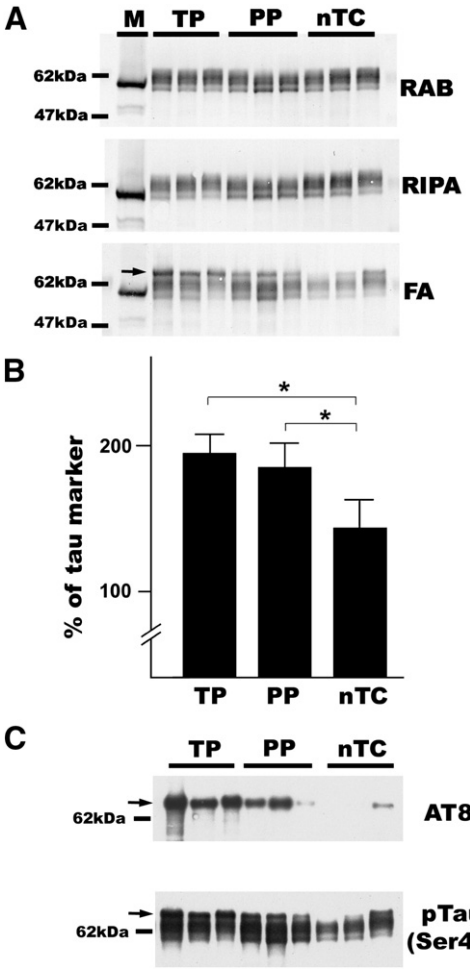

Fig. 2. Increased tau insolubility in the brain of PS19 mice treated by trihexyphenidyl (TP) or propiverine (PP). To examine tau solubility in PS19 mice treated with TP or PP, 2 μg of RAB-HS-extracted protein, 4 μg of RIPA buffer-extracted protein, and 5 μl of FA-extracted samples from brains of 10-month-old TP- or PP-treated PS19 mice, and non-treated control (nTC) mice were immunoblotted with 17026, a tau-specific antibody (A). The tau protein levels are almost identical in the RAB and RIPA fractions. In contrast, the tau protein levels are clearly different in the FA fractions, with stronger signals following TP or PP treatment and weaker signals in the nTC PS19 mice. Moreover, slowly migrating tau bands in the FA fractions (arrow) are clearly observed in TP- and PP-treated PS19 mice. The signal intensities of tau protein in the FA fractions from TP- and PP-treated PS19 brains are significantly stronger than those from nTC PS19 brains (B). Insoluble tau proteins in the FA fractions of TP-treated and PP-treated mice are more phosphorylated (pTau); in particular, slower migrated band (arrow) is strongly positive for AT8 (C). M, 1N4R recombinant tau protein; RAB, reassembly buffer; RIPA, radioimmunoprecipitation assay; FA, formic acid. *, $p<0.05$.

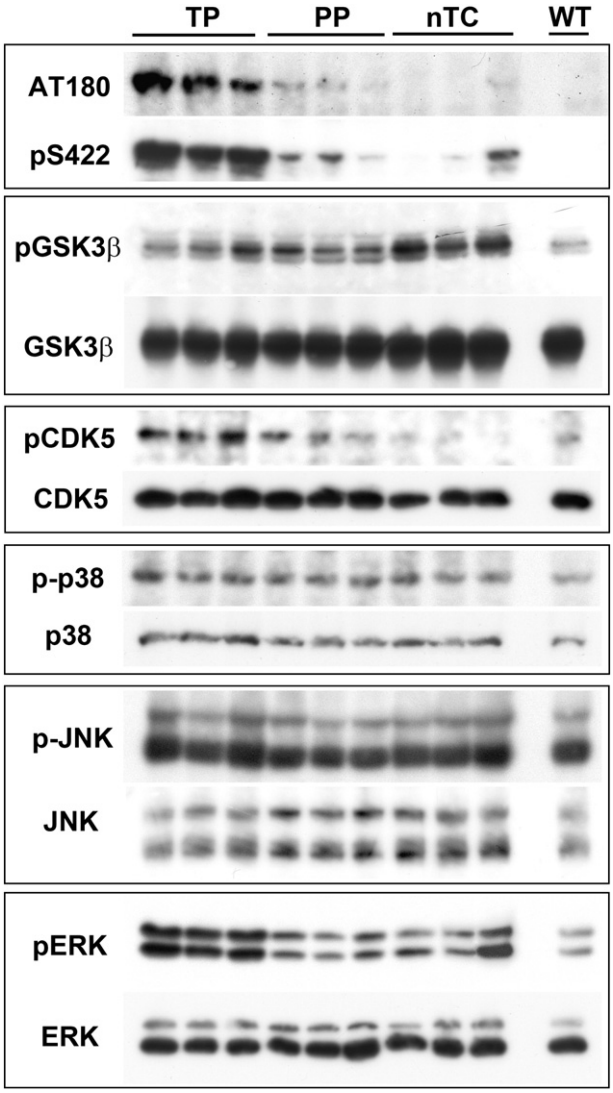

Fig. 3. Tau phosphorylation and Tau kinesis activities. To assess the kinase activities involved in tau phosphorylation, Western blot analysis is performed for active and inactive kinases, including GSK3β, CDK5 and MAPKs (p38, JNK, ERK), in brain samples from trihexyphenidyl (TP)- or propiverine (PP)-treated PS19, non-treated control (nTC) PS19, and non-treated WT mice. Basal kinase expression in the brain is similar among the groups; however, the activated forms of CDK5 and MAPKs (p-) are increased and the inactivated form of GSK3β (pGSK3β) is decreased in TP-treated PS19 mice compared with those in nTC PS19 mice, indicating that TP treatment activates tau kinases in PS19 mice.

activity, showed a milder pathological and biochemical deterioration than TP. This finding agrees with the clinical observation that anticholinergic activity levels are proportional to the risk of dementia (Jessen et al., 2010).

The positive correlation of microgliosis with both tau pathology and neuronal loss, in PS19 mice treated with TP, PP or in nTCs (Figs. 2, 4) provides a mechanism for how ACh could modify neurodegeneration. Because microgliosis precedes tau pathology and neuronal loss, and early administration of FK506, an immunosuppressant, attenuates those pathologies, neuroinflammation and microglial activation might be initiating processes that leads to pathology in PS19 mice (Yoshiyama et al., 2007). Thus, one possible mechanism to explain the worsening of neuropathology, such as tau pathology and neuronal loss, is modulation of inflammatory reactions by chronic administration of anticholinergics. TP aggravated not only microgliosis in the brain, but also IL-1β expression in the spleen of PS19 mice with LPS-induced systemic inflammation, indicating the inflammation-enhancing properties of TP (Fig. 4). Recent evidence supports an anti-inflammatory function of ACh, and there are some reports demonstrating that acetylcholinesterase inhibitors (AChEIs) exert inhibitory influences on neuroinflammation and generalized systemic inflammation (Pollak et al., 2005; Tyagi et al., 2007). Peripheral administration of AChEIs, or an antisense oligonucleotide to AChE, significantly attenuated IL-1β production in the hippocampus and blood of mice, with concomitant reduced AChE activity (Pollak et al., 2005). Furthermore, we recently reported that donepezil, an AChEI, ameliorated pathological changes in PS19 mice (Yoshiyama et al., 2010). Taken together, the cholinergic nervous system might be involved in neurodegeneration and inflammation. It is well-known that neuronal inflammation may play an important role in the pathogenesis of neurodegenerative disorders including AD. Acute and chronic systemic inflammation may be associated with an increased risk of developing AD (Bonotis et al., 2008; Holmes et al., 2009; Tan et al., 2007). Because early depletion of ACh is one of the pathological and biochemical hallmarks of AD, a depleted ACh system in the brain might

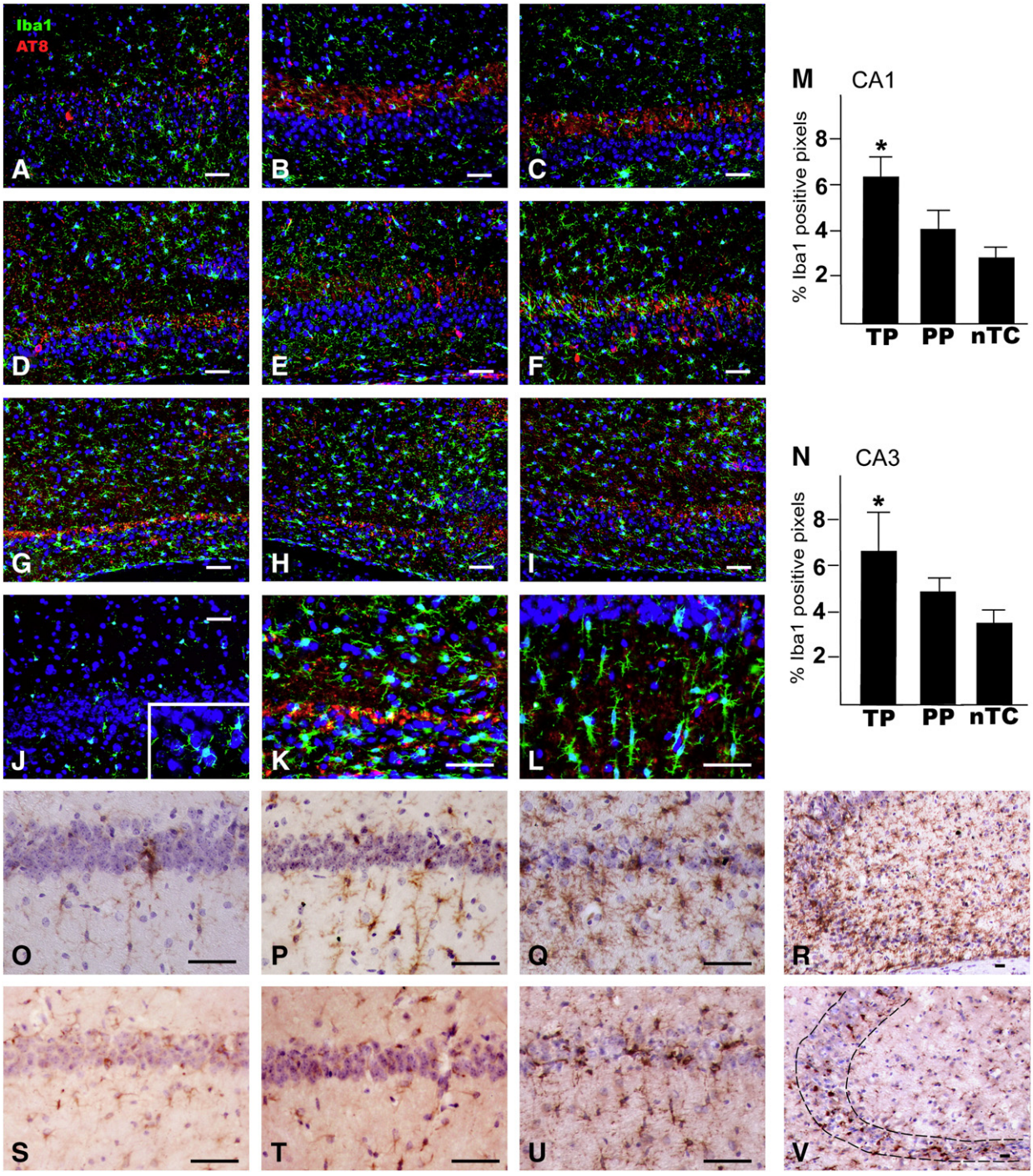

Fig. 4. Anticholinergics enhance microglial activation in PS19 mice. Sections of the hippocampal CA3 (A–K) and CA1 (L) regions were immunofluorescently double-stained with Iba1 antibody (green) and 17026 (tau antibody, red), and sections of the CA1 region were immunohistochemically stained with CD11b (O–R) and CD68 (S–V). Low-magnification images demonstrate more microglial activation in trihexyphenidyl (TP)-treated PS19 mice (G–I) than in propiverine (PP)-treated (D–F) or non-treated control (nTC) PS19 mice (A–C). High-magnification images (K, L) of the hippocampus in TP-treated PS19 mice clearly demonstrate activation of microglia, which show enlarged cytoplasm with thick processes in the CA3 region (K) and elongated rod-like cytoplasm with thick processes parallel to the nerve fibers in CA1 region (L), whereas a few, small microglia with thin processes were observed in the WT mouse brain (J). The activation of microglia was quantified by measurement of Iba1-positive area. The Iba1-positive area in TP-treated mice was significantly increased compared with that in nTC PS19 mice, both in the CA1 (M) and CA3 (N) regions. Although there is no statistical significance of PP-treatment in Iba1-psositive area, other activated microglial markers including CD11b and CD68 also indicate that TP-treatment (Q, U, R, V) markedly and PP-treatment (P, T) mildly activates microglia compared with nTC (O, S). A low-magnification image stained with anti-CD68 clearly demonstrates a concentration of CD68-positive microglia in the granular neuronal cell layer (V, outlining) in the hippocampus (compare V to R). *, $p<0.05$, to nTC. Scale bars, 10 μm (A–L, O–Q, S–U); 100 μm (R, V).

accelerate neurodegeneration by enhancing inflammatory reactions in AD. Some studies have indicated that sustained low-grade systemic inflammation, including elevated inflammation related cytokines and proteins, is observed in AD (Heneka and O'Banion, 2007; Lee et al., 2009; Yaffe et al., 2004). This low-grade systemic inflammation in AD possibly results from neurodegeneration in ACh system.

ACh acts through two different types of receptors: nicotinic and muscarinic. It has been demonstrated that anti-inflammatory effects of ACh

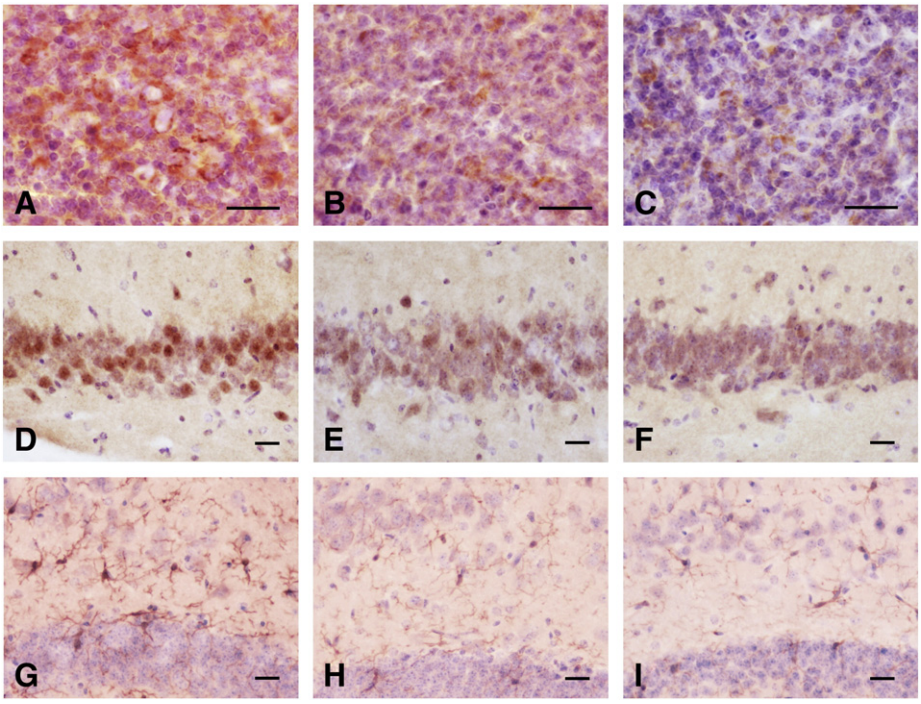

Fig. 5. Anticholinergics accelerate systemic and neuronal inflammation induced by lipopolysaccharide (LPS) injection. Three-month-old PS19 mice fed powdered food mixed with trihexyphenidyl (TP) (A, D, G), propiverine (PP) (B, E, H) or no drug (nTC) (C, F, I) were peritoneally injected with 1 mg/kg LPS twice a week for 1 month, and then sacrificed at 4 months old. IL-1β expression in the spleen (A–C) is increased in the TT-treated mice (A) compared with the nTC mice (C). Similarly, neuronal IL-1β expression in the CA3 region (D–F) in the TT-treated mice is enhanced (D) in accordance with the microglial activation detected with Iba1 antibody (G–I). Scale bars, 20 μm.

are mainly regulated by nicotinic ACh receptors in macrophages (Wang et al., 2003) and microglia (De Simone et al., 2005). Because most clinically used anticholinergics are classified as anti-muscarinic agents, anticholinergics may not exert their inflammation-enhancing effects by directly suppressing nicotinic receptors on immunocompetent cells. Interestingly, AChEIs suppresses TNF production in the blood and spleen following LPS-induced systemic inflammation in mice (Pavlov et al., 2009). Meanwhile, the suppressive effect on blood TNF was reversed by surgical transection of the cervical vagus nerve or pre-treatment with atropine sulfate, a blood–brain barrier-permeable muscarinic receptor antagonist. However, atropine methyl nitrate, a blood–brain barrier-impermeable muscarinic receptor antagonist, produced no effect. A highly selective, centrally acting AChEI, huperzine A, significantly reduced blood TNF levels (Pavlov et al., 2009). Moreover, muscarine injection into the lateral ventricle reduced the levels of inflammatory mediators in both brain and spleen in experimental intracerebral hemorrhage rats (Lee et al., 2010). These data indicate a centrally controlled muscarinic receptor-dependent anti-inflammatory mechanism for both the central nervous system and peripheral systemic organs.

Anticholinergic drugs not only impair cognitive function by decreased ACh neurotransmission, but also accelerate neurodegeneration by suppressing ACh-dependent anti-inflammatory system. Anticholinergics should be less readily prescribed, to reduce the risk of dementia. Moreover drugs targeting the ACh-dependent anti-inflammatory system might be candidates for reducing the risk of dementia.

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