Rho-kinase ROCK inhibitors reduce oligomeric tau protein

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Abstract

Neurofibrillary tangles, one of the pathological hallmarks of Alzheimer's disease, consist of highly phosphorylated tau proteins. Tau protein binds to microtubules and is best known for its role in regulating microtubule dynamics. However, if tau protein is phosphorylated by activated major tau kinases, including glycogen synthase kinase 3β or cyclin-dependent kinase 5, or inactivated tau phosphatase, including protein phosphatase 2A, its affinity for microtubules is reduced, and the free tau is believed to aggregate, thereby forming neurofibrillary tangles. We previously reported that pitavastatin decreases the total and phosphorylated tau protein using a cellular model of tauopathy. The reduction of tau was considered to be due to Rho-associated coiled-coil protein

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kinase (ROCK) inhibition by pitavastatin. ROCK plays important roles to organize the actin cytoskeleton, an expected therapeutic target of human disorders. Several ROCK inhibitors are clinically applied to prevent vasospasm postsubarachnoid hemorrhage (fasudil) and for the treatment of glaucoma (ripasudil). We have examined the effects of ROCK inhibitors (H1152, Y-27632, and fasudil [HA-1077]) on tau protein phosphorylation in detail. A human neuroblastoma cell line (M1C cells) that expresses wild-type tau protein (4R0N) by tetracycline-off (TetOff) induction, primary cultured mouse neurons, and a mouse model of tauopathy (rTG4510 line) were used. The levels of phosphorylated tau and caspase-cleaved tau were reduced by the ROCK inhibitors. Oligomeric tau levels were also reduced by ROCK inhibitors. After ROCK inhibitor treatment, glycogen synthase kinase 3β, cyclin-dependent kinase 5, and caspase were inactivated, protein phosphatase 2A was activated, and the levels of IFN-γ were reduced. ROCK inhibitors activated autophagy and proteasome pathways, which are considered important for the degradation of tau protein. Collectively, these results suggest that ROCK inhibitors represent a viable therapeutic route to reduce the pathogenic forms of tau protein in tauopathies, including Alzheimer's disease.

Keywords

ROCK inhibitor; Alzheimer's disease; Tau protein; Phosphorylation; Tau oligomer

1. Introduction

Neurofibrillary tangles (NFTs), which are one of the pathological hallmarks of Alzheimer's disease (AD), are composed of highly phosphorylated tau proteins. Microtubule-associated protein tau serves several functions, the most well-known of which is the regulation of microtubule dynamics. However, a major hypothesis in the field for the disease process leading to abnormal phosphorylation is that once tau is phosphorylated by activated tau kinases, such as glycogen synthase kinase 3β (GSK3 β) or cyclin-dependent kinase 5 (Cdk5), or inactivated tau phosphatase, including protein phosphatase 2A (PP2A), its affinity for microtubules is reduced, leading to unbound tau that can aggregate into oligomeric species and eventually NFTs. Therefore, the imbalance of protein kinase and phosphatase activities is thought to be a major factor leading to the disease pathogenesis in AD (Gong et al., 1995; Kanaan et al., 2013; Lovestone and Reynolds, 1997).

Small GTPases of the Rho family are important regulators of many types of cellular behavior, including cell motility and proliferation, apoptosis, and cytoskeletal dynamics. These effects of Rho are mediated by Rho effectors, including Rho-associated coiled-coil protein kinase (ROCK) (Riento and Ridley, 2003) and Dia (Sakamoto et al., 2012). ROCK is a serine/threonine kinase that plays essential roles in cellular behavior, from the contraction of smooth muscle to the migration of cells and neurite outgrowth. ROCK also functions in centrosome positioning and the regulation the cell volume, which are affected in many pathophysiological conditions (Riento and Ridley, 2003). Two types of ROCKs exist, ROCK1 and ROCK2, and they both may play the same physiological roles. Expression of ROCK1 is mainly in non-neuronal tissues, whereas the expression of ROCK2 is mainly in the brain and spinal cord, and its expression increases with aging (Koch et al., 2014). ROCK

inhibitors are approved for clinical usage such as for the prevention of vasospasm after subarachnoid hemorrhage (fasudil: HA-1077) (Olson, 2008) and as treatment for glaucoma (Ripasudil) (Yamamoto et al., 2014). The ROCK inhibitor, fasudil, was effective in reducing the behavioral deficits and neuropathology in a transgenic mutant alpha-synuclein mice model of Parkinson's disease (Tatenhorst et al., 2016). It was previously reported that fasudil increases survival, improves motor function, and stimulates regenerative responses in an amyotrophic lateral sclerosis mouse model (Tönges et al., 2014). Some studies suggest that ROCK inhibition mitigates tau pathology in drosophila models of tauopathy (Gentry et al., 2016); however, whether ROCK inhibitors have effects in mammalian models of tauopathy remain unclear. We previously reported that pitavastatin reduced the total tau and phosphorylated tau protein levels, suggesting that pitavastatin reduces the amount of tau by preventing the maturation of small G proteins, especially Rho-ROCK (Hamano et al., 2012).

Here, we examined the effect of ROCK inhibitors on the prevention of tau oligomerization, phosphorylation, and caspase cleavage and the underlying mechanisms using transfectant M1C cells expressing wild-type tau protein (4R0N) (Hamano et al., 2008, 2012, 2016; Shirafuji et al., 2018) and using a mouse model of tauopathy (Shirafuji et al., 2018). ROCK inhibitors significantly reduced the amount of pathological forms of tau while facilitating tau degradation, suggesting that these compounds effectively reduce tau pathology.

2.   Materials and methods

2.1.   Materials

Lab-Tek chambered cover glass was from Nunc (Rochester, NY, USA). Other tissue culture supplies were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Living cell numbers were quantitated by ATP assay using the CellTiter-Glo Luminescent Cell Viability Assay Kit (catalog# G755A, Promega, Madison, WI, USA), as previously reported (Hamano et al., 2012, 2016; Shirafuji et al., 2018). The ROCK inhibitor H1152 was from Calbiochem (catalog #555552–500UG, Darmstadt, Germany) and Y-27632 was from WAKO (catalog #257–00511, Tokyo, Japan). Suc-LLVY-AMC fluorogenic substrate (Biomol International) for 20S chymotrypsin-like activity and Bz-Val-Gly-Arg-AMC (Biomol International) fluorogenic substrate for 26S proteasomal activity assay were from Biomol International (Plymouth Meeting, PA, USA). All other chemicals were from Sigma (Saint Louis, MO, USA) unless stated otherwise.

2.2.   Antibodies

The epitopes identified by the anti-tau antibodies applied in this project are presented in Fig. 1A. All antibodies were examined extensively and applied in many article (DeTure et al., 2002; Gamblin et al., 2003; Hamano et al., 2008, 2009, 2012, 2016; Ko et al., 2004; Shirafuji et al., 2018). The monoclonal antibody Tau5 was purchased from Invitrogen (Carlsbad, CA, USA). TauC3, Bcl-2, Cdk5, and phospho-Cdk5 (Ser 159) (pCdk5), PP2A, and demethylated PP2A (DPP2A) antibodies were from Santa Cruz (catalog #sc-32240, #sc-7382, sc-6247, #sc-271981, #sc-80665, and #sc-13601). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Millipore (catalog #MAB374, Billerica, MA, USA). Monoclonal antibodies against phosphorylated tau protein, PHF-1,

and CP13 were from Dr Peter Davies (The Feinstein Institute for Medical Research, Manhasset, NY, USA). AT180 and AT270 were from Thermo Scientific (catalog #MN1040 and MN1050, Rockford, IL USA), and polyclonal rabbit anti-Tau phosphoserine 199/202 (PS199/202) antibody was from EMD Millipore Corporation (catalog #AB9674, Temecula, CA, USA). Tau Oligomer Complex I antibody (TOC1), which detects tau dimers and high-ordered oligomers, was originally developed in Dr Lester Binder's laboratory (Hamano et al., 2016; Patterson et al., 2011; Shirafuji et al., 2018; Ward et al., 2014). Monoclonal antibodies against microtubule-associated protein 1 light chain 3 (LC3) and ubiquitin (P4D1) was from Cell Signaling (catalog #3868, #3936, Danvers, MA, USA). Polyclonal antibodies against GSK3β, phospho-GSK3β (Ser9) (pGSK3β), Akt, phospho-Akt (pAkt) (Ser 473), and cleaved caspase-3 were from Cell Signaling (catalog #9315, #9336, #9272, #9271, and #9661), interferon g (IFN-γ) was from abcam (catalog #ab9657, Tokyo, Japan), and p62 was from Medical and Biological Laboratories Co Ltd (MBL) (catalog #PM045, Nagoya, Japan). The antibodies were used at the following dilutions: Tau5 (1:1000), PHF-1 (1: 100), CP13 (1:100), AT180 (1:1000), AT270 (1:1000), PS199/202 (1:1000), TauC3 (1:2000), TOC1 (1:1000), anti-cleaved caspase-3 (1:500), anti-GAPDH (1:2000), anti-GSK3β (1:1000), anti-pGSK3β (1:500), anti-pCdk5 (1:1000), anti-Cdk5 (1:1000), anti-Bcl-2 (1:1000), anti-IFN-γ (1:1000), anti-PAkt (1:500), anti-Akt(1:1000), anti-LC3 (1:1000), and anti-p62 (1:1000).

2.3. Cell culture

We seeded M1C cells at $1.5–2 \times 10^6$ cells/plate in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, G418 (400 μg/mL; Life Technologies, Gaithersburg, MD, USA), and Tet (2 μg/mL). After 24 hours from plating, tau induction was performed by replacing the old medium with fresh medium containing 1 ng/mL of Tet (TetOff induction). We treated replica cultures of M1C cells with the ROCK inhibitor after TetOff induction for 4 days, and harvested cells at the end of a 5-day induction period (Fig. 1B). All experiments were performed in triplicate, and the experimenters were blinded to the treatment group when performing the outcome measures.

2.4. Primary neural cell culture

Isolated primary neurons from Slc:ICR mice at embryonic day 16 (E16) were seeded on Lab-Tek chambered cover glasses precoated with polyethyleneimine at a density of 5×10^4 per well, or in 3.5 cm plastic dishes precoated with polyethyleneimine at a density of 4×10^5 per dish for Western blotting. Cultured neurons were maintained in serum-free Neurobasal medium (Invitrogen) with 5 μM Ara C to prevent non-neuronal cell proliferation (Hamano et al., 2012, 2016; Han et al., 2005; Shirafuji et al., 2018). All experiments in the present study were carried out using pure neuronal cells (>95% neuronal). The purity of the cells was estimated by staining with neuron-specific anti-NeuN (Millipore). All experiments were performed in triplicate, and the experimenters were blinded to the treatment group when performing the outcome measures.

2.5. Animals and treatment

Animal procedures were approved by the University of Fukui and the National Center for Geriatrics and Gerontology. The extensively characterized mouse line rTg4510 was used as

a model of tauopathy (Jackson Lab, FL) (Santacruz et al., 2005). rTg4510 mice have the tau responder and activator transgenes that drive the overexpression of the four-repeat human tau, encoding the 4R0N isoform with the P301L mutation, which can cause the inherited form of human tauopathy (Lee et al., 2001; Santacruz et al., 2005). All mice were male progeny of crosses between mice with the activator transgene in a 129 background and those with the tau responder transgene in an FVB background (Charles River Laboratories Japan, Inc, Yokohama, Japan). Food/water were given ad libitum and housed in a light/dark cycle (12 h/12 h) at 23 °C. Male mice were randomized to either the control group (N = 5) supplied standard water or the fasudil group (N = 5) supplied fasudil in the water (12 mg/kg/d). Starting at 6–9 months of age, mice underwent fasudil supplementation for 4 weeks until they were 7–10 months old. During this experiment, mice in all groups regularly increased in weight, and the weight was not significantly different between the 2 groups. After sacrifice, we removed the brains and dissected in 2 sample sets per hemisphere. Sample one contained the hippo-campus and cerebral cortex, and sample 2 contained the hypothalamus, basal ganglia, cerebellum, and brainstem. For the fractionation study, brain samples were processed as described in Section 2.6 (Shirafuji et al., 2018). The experimenters were blinded to the treatment group when performing the outcome measures.

2.6. Fractionation study

Harvested M1C cells and brain samples of mice were homogenized in Tris buffer with protease and phosphatase inhibitors [30 mM sodium fluoride, 30 mM β-glycerophosphate, 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail (Roche, Germany)]. To obtain lysates, homogenates were centrifuged at $180 \times g$ for 15 minutes. Portions of lysates were fractionated further based on their solubility in Tris buffer or 2% sarkosyl to obtain SN1, SN2, and S/P fractions, as reported previously (Hamano et al., 2008, 2009, 2012, 2016; Shirafuji et al., 2018). The supernatant obtained by centrifugation of lysates at $150,000 \times g$ for 15 minutes at 4 °C was the SN1 fraction. The resuspended pellet in buffer containing 10% sucrose, 10 mM Tris/HCl (pH 7.4), 0.8 M NaCl, 1 mM EGTA, protease inhibitor cocktail, and 1% sarkosyl was centrifuged at $150,000 \times g$ for 15 minutes to generate a supernatant (SN2) and sarkosyl-insoluble pellet (S/P). Supernatants (SN1 and SN2) and the sarkosyl-insoluble pellet (S/P) re-suspended in Tris buffer were subjected to Western blot analysis directly or stored at −20 °C or −70 °C.

2.7. Western blotting and dot blot

Cells collected by scraping were homogenized in a lysis buffer [20 mM MES, pH 6.8, 80 mM, NaCl, 1 mM MgCl2, 2 mM EGTA, 10 mM NaH2PO4, 20 mM NaF, and protease inhibitor cocktail (Roche, Germany)] and centrifuged at $180 \times g$ for 15 minutes at 4 °C. The protein concentration of lysates was measured by the bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA). Lysates or fractionated samples were mixed with Laemmli sample buffer containing 1% β-mercaptoethanol. Samples of equal protein amount (10 μg, corresponding to $0.1–0.2 \times 10^5$ cells per lane) were resolved by 10%–20% gradient SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P; Merck Millipore, Darmstadt, Germany) for immunoblotting. Under nonreducing conditions, the lysate or fractionated samples were mixed with Laemmli sample buffer without 1% β-mercaptoethanol. Dot blot samples

(without Laemmli sample buffer) were spotted onto PVDF membranes directly at a concentration of 2 μg of protein (Hamano et al., 2016; Shirafuji et al., 2018; Ward et al., 2014). Both Western and dot blots were incubated in 1% gelatin in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature (RT). After washing with TBS-T, blots were immersed with monoclonal antibodies against Tau5, PHF-1, CP13, AT180, AT270, TauC3, GAPDH, Bcl-2, LC3, PP2A, DPP2A, or P4D1, or polyclonal antibodies against GSK3βb, pGSK3β, cleaved caspase-3, PAkt, Akt, or p62 for 1 hour at RT at the dilutions described previously. After the third wash, blots were immersed in horseradish peroxidase-conjugated sheep anti-mouse IgG or goat anti-rabbit IgG for 30 minutes at RT. After a third rinse, immunoreactive proteins were visualized using the enhanced chemiluminescence prime (ECL Prime) system (Amersham, Buck-inghamshire, UK) (Hamano et al., 2008, 2009, 2012, 2016; Shirafuji et al., 2018). Immunoreactivity was measured on film using Adobe Photoshop Elements Version 16 (Adobe, San Jose, CA, USA) or captured images directly by Image Quant LAS 4000mini (GE Healthcare UK, Amersham Plaec, Backinghamshire, UK), and ImageJ software (version 1.51, NIH, Bethesda, MD, USA) was used for densitometry measurements of immunoreactive bands. Cell lysates were also probed for the level of the inactive form of GSK3β, which can be identified by labeling with antibodies against Ser9-phosphorylated GSK3β (Hamano et al., 2012, 2016; Ma et al., 2009; Shirafuji et al., 2018).

2.8.   mRNA expression

To quantify the expression level of tau mRNA, total RNA was extracted from M1C cells by TRIzol reagent (Invitrogen) and converted to cDNA for PCR amplification. The cycling conditions for PCR were 94 °C, 5 minutes (1 cycle): 94 °C, 30 seconds: 50 °C, 30 seconds: 72 °C, 20 seconds (29 cycles): 72 °C, 15 minutes. Primer sequences for tau were 5′-TGAGCCCCGCCAGGAGTTC-3′ and 5′-TTGGAGGGGGCGGGGTTTTTG-3′, and generated products of 446 or 355 bp for tau isoforms contained or lacked exon 2 (4R0N), respectively (Hamano et al., 2012). Primer sequences for GAPDH were 5′-TTGATTTTCGAGGGATCTCG-3′ and 5′-GAGTCAACGGATTTGGTCGT-3′ (product size: 238 bp) (Hamano et al., 2012). Using the StepOnePlus Real-Time PCR System, we performed quantitative real-time PCR using 20 ng of cDNA in a 20 μL reaction mixture per sample. We calculated the relative gene expression by the δCT method (Bunpo et al., 2009). We ran all samples in triplicate. For the analysis of Tau mRNA levels, we performed the following TaqMan gene expression assays (Applied Biosystems): MAPT (Hs00902188_mL) and GAPDH (Hs99999905_mL) (Hamano et al., 2012, 2016; Shirafuji et al., 2018).

2.9.   Morphological study

We performed morphological studies of M1C cells seeded on 24-well plates at a density of 2.6–3.5 cells $\times 10^3/cm^2$ with or without ROCK inhibitor exposure after TetOff induction. We performed phase-contrast imaging of M1C cells using an inversion microscope (IX-70: Olympus, Tokyo, Japan) with a digital camera (DP-70: Olympus) and processed the images using Adobe Photoshop Elements Version 16 for publication.

2.10. Immunocytochemical study

M1C cells seeded on Lab-Tek chambered cover glasses underwent TetOff induction with or without ROCK inhibitor (H1152, or Y-27632) (1 or 10 μM) for the final 24 or 48 hours of induction. After exposure, cells were fixed in 4% paraformaldehyde/0.1 M phosphate buffer. Fixed samples were rinsed with phosphate-buffered saline and permeabilized with permeabilization buffer (0.25% Triton X-100 in phosphate-buffered saline). They were then blocked with blocking buffer containing 3% goat serum and 1% bovine serum albumin, and incubated with primary antibodies (1:100), including P44, PHF-1, CP13, AT180, TauC3, or TOC1 antibodies, followed by Alexa 488 anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and Alexa 594 anti-rabbit IgG (Molecular Probes). To visualize immunoreactivity, we used a confocal laser microscope (TCS SP II; Leica, Heidelberg, Germany).

2.11. Proteasome activity assay

Lysates containing 50 μg of protein was used to measure proteasome activity. We placed samples in a reaction buffer (50 μL/sample) with 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 20% glycerol, 5 mM sodium pyrophosphate, 30 mM sodium fluoride, 30 mM β-glycerophosphate, and protease inhibitor cocktail (Sigma) in a 96-well plate. Then, we mixed them with 100 μM Suc-LLVY-AMC fluorogenic substrate (Biomol International) for 20S chymotrypsin-like activity (Hamano et al., 2008; Osna et al., 2010; Shirafuji et al., 2018) or 100 μM Bz-Val-Gly-Arg-AMC (Biomol International) fluorogenic substrate for 26S trypsin-like activity. We incubated the reaction mixture at 37 °C in the dark for 60 minutes and measured the fluorescence signals using excitation/emission wavelengths at 360 nm/460 nm assess chymotrypsin-like activity or at 380/460 nm for trypsin-like activity on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.12. Immunoprecipitation of ubiquitinated tau

After normalization of protein levels, tau was immunoprecipitated with the Tau5 antibody that recognizes total tau as described previously (Wood et al., 2005). The resulting immunoprecipitates were transferred to PVDF membranes and probed with anti-ubiquitin antibody (P4D1).

2.13. Statistical analysis

All values are shown as the mean ± SD, in the figures. We assessed the differences among more than 2 groups using one-way analysis of variance followed by Bonferroni post hoc test if the data had a normal distribution and with the Kruskal-Wallis test followed by Dunn's post hoc test if they deviated from the normal distribution. The differences between 2 groups were evaluated by Student's t-test if the data had a normal distribution and by the Mann-Whitney U test if they deviated from the normal distribution (IBM SPSS Statistics, version 22, IBM Corp, Armonk, NY, USA), with $p < 0.05$ considered significant.

3. Results

3.1. Total tau protein level was reduced by ROCK inhibitors

First, the cytotoxicity of the ROCK inhibitors used in this study was examined. The ROCK inhibitor (H1152) (0.1–10 μM) did not cause morphological change (Fig. 2A). Cell death was not observed under these conditions in the ATP assay (0.01 μM-100 μM ROCK inhibitor [H1152] treatment) (Fig. 2B). To assess the effects of the ROCK inhibitors on tau, M1C cells underwent TetOff induction to express wild-type tau (4R0N) for 5 days, and the ROCK inhibitor (H1152) (1 or 10 μM) was added during the last 24 hours of TetOff induction. Cell lysates obtained from cultures were analyzed by Western blotting using the Tau5 antibody (Fig. 2C). These blots were probed with anti-GAPDH to confirm that the loading amount among the lanes was the same (Fig. 2C). The total tau amount was reduced in a dose-dependent manner by ROCK inhibitor (H1152) exposure. When cells were exposed to 1 μM ROCK inhibitor, Tau5 detected 45–60-kDa bands at 76.0% of the levels observed for the vehicle control. Another ROCK inhibitor, Y-27632, also reduced the amount of total tau. Control cells were exposed to dimethyl sulfoxide, the vehicle used to dilute the ROCK inhibitor Y-27632. One to 10 μM Y-27632 reduced the tau amount dose dependently (Supple Fig. 1A). The ROCK inhibitor (H1152) did not alter tau mRNA levels according to RT-PCR and quantitative real-time PCR (Fig. 2D and E).

3.2. The phosphorylated tau protein level was reduced by ROCK inhibitors

On Western blot analysis, phosphorylated tau levels detected by PHF-1, CP13, AT270, and AT180 (Fig. 1) decreased after ROCK inhibitor (H1152) treatment (Fig. 3A–D). Immunocytochemical analyses revealed that the PHF-1, CP13, and AT180-positive phosphorylated tau level was markedly reduced by the ROCK inhibitor (Fig. 3E and F). The phosphorylation ratios (PHF-1/Tau5, CP13/Tau5, AT180/Tau5, and AT270/Tau5) were also decreased by 10 μM ROCK inhibitor (H1152) treatment. Another ROCK inhibitor, Y-27632, also reduced the phosphorylated tau (PHF-1) level, as observed on Western blot analysis (Supple Fig. 1B and C).

3.3. Tau kinases GSK3β and Cdk5 were inactivated, and tau phosphatase PP2A was activated by the ROCK inhibitors

Phosphorylation levels of GSK3β at Ser9, which is a major tau kinase, were upregulated by H1152 (Fig. 4A) and Y-27632 (Supple. Fig. 2A) ROCK inhibitors, whereas the total amount of GSK3β did not change. This suggests that GSK3β was inactivated by the ROCK inhibitors (Fig. 4A, Supple Fig. 2A). Phosphorylated Akt (pAkt) was increased by ROCK inhibitor (H1152) treatment (Fig. 4B). However, the total amount of Akt did not change, suggesting that Akt was activated by the ROCK inhibitor (Fig. 4B). Another tau kinase, Cdk5, was downregulated by H1152 (Fig. 4C) and Y-27632 (Supple. Fig. 2B). ROCK inhibitor (H1152) reduced DPP2A and total PP2A was unchanged, suggesting that PP2A, a major tau phosphatase, was activated by ROCK inhibitor (Fig. 4D).

3.4. Caspase inactivation by ROCK inhibitor was observed

The amount of cleaved caspase-3 was reduced by the ROCK inhibitors H1152 (Fig. 5A) and Y-27632 (Supple Fig. 3) in MC1 cells. The antiapoptotic agent, Bcl-2, was upregulated by ROCK inhibitor (H1152) treatment (Fig. 5B). Immunoblots (Fig. 5C) and immunocytochemistry (Fig. 5D) of MC1 cells treated with the ROCK inhibitor (H1152) also demonstrated a reduction of TauC3-positive tau species, which represent pathological tau that is C-terminally truncated by caspase-3. The proinflammatory cytokine IFNγ level was also reduced by the ROCK inhibitor (H1152) (Fig. 6).

3.5. ROCK inhibitor reduced oligomeric tau

ROCK inhibitors decreased the amount of aggregated tau protein in the sarkosyl-insoluble fraction (H1152; Fig. 7A, Y-27632; Supple Fig. 4A). Of note, the amount of oligomeric tau in the Tris-insoluble, sarkosyl-soluble fraction was also decreased by 1 μM ROCK inhibitor (H1152 and Y27632) under nonreducing conditions (Fig. 7B, Supple Fig. 4B). TOC1, which is a tau oligomer-specific antibody, detected the reduction of 120 kDa oligomeric tau in the Tris-insoluble, sarkosyl-soluble fraction (Supple Fig. 4B) by the ROCK inhibitor (Y-27632). Dot blot analysis also confirmed the reduction of TOC1-positive oligomeric tau after ROCK inhibitor (H1152 and Y27632) treatment (Fig. 7C; Supple Fig. 4C). Immunocytochemical study demonstrated the reduction of TOC1-positive oligomeric tau by 1 μM ROCK inhibitor (H1152) (Fig. 7D).

3.6. ROCK inhibitors upregulated autophagy and activated proteasome

ROCK inhibitors upregulated autophagy in MC1 cells. LC3-II, a marker of autophagy, was upregulated by the ROCK inhibitor (H1152), especially by 10 μM ROCK inhibitor treatment (Fig. 8A). Another marker of autophagy, p62, was reduced after ROCK inhibitor (H1152) treatment (Fig. 8B). These results suggested that autophagy was upregulated. ROCK inhibitor (H1152) treatment decreased the level of ubiquitinated tau, especially in high molecular weight ubiquitinated tau (Fig. 8C). Chymotrypsin-like 20S proteasomal activity was also activated by the ROCK inhibitor (H1152) (Fig. 8D). Furthermore, trypsin-like, 26S proteasomal activity was upregulated by the ROCK inhibitor (Fig. 8E). These results suggest that the ROCK inhibitor activated the proteasomal system.

3.7. Endogenous tau was reduced by the ROCK inhibitor

Endogenous total tau (Tau5) and phosphorylated tau (PHF-1) levels in noninduced M1C cells (Fig. 9A) were reduced by the ROCK inhibitor (H1152). Oligomeric tau detected by dot blot analysis of TOCI was not identified in noninduced cells (Fig. 9B). Moreover, in the cultured mouse neurons, total tau and phosphorylated tau levels were reduced by ROCK inhibitor (H1152) treatment according to immunocytochemical (Fig. 9C) and Western blotting assays (Fig. 9D).

3.8. ROCK inhibitor reduced total tau and phosphorylated tau levels in P301L mice

The ROCK inhibitor fasudil reduced total tau and phosphorylated tau levels in P301L mice (Fig. 10A). It also reduced the level of phosphorylated tau in the Tris-insoluble, sarkosyl-soluble (SN2) fraction and sarkosyl-insoluble fraction (S/P) (Fig. 10B). Under nonreducing

conditions, the ROCK inhibitor fasudil also reduced the amount of high molecular weight tau (Fig. 10C). It also reduced the amount of oligomeric tau detected by TOC1 in the dot blot analysis (Fig. 10D).

4. Discussion

ROCK is associated with the cell-substratum (adhesion) as well as cell-cell adhesion, migration, invasion, apoptosis, and transformation. ROCK is of interest as a potential treatment for several disorders such as cancer, neurodegenerative disorders, renal failure, asthma, glaucoma (Yamamoto et al., 2014), osteoporosis, wound healing of the corneal endothelium (Okumura et al., 2011), erectile dysfunction, insulin resistance (Olson, 2008), pulmonary arterial hypertension (Antoniu, 2012), and coronal vasospasm (Shimokawa and Takahashi, 2015). Furthermore, ROCK inhibitors improved the corneal endothelial cell restoration in bullous keratopathy (Kinoshita et al., 2018). Koch et al. reported that ROCK2 inhibition improved neurite regeneration and suggested prosurvival effects in neurons (2014). The ROCK inhibitor Y-27632 increased neuronal survival after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine lesions (Borrajo et al., 2014) and oxygen glycogen depletion. It was also reported that knockdown of ROCK2 reduced calpain and caspase-3 activity in neurons. On the other hand, pAkt activity, collapsin response-mediated protein-2, and autophagic flux were increased by knockdown of ROCK (Koch et al., 2014). Indeed, under stressed conditions with little caspase activation, the caspase-mediated cleavage of ROCK1 and consequent activation of ROCK1 may enhance or accelerate apoptosis (Chang et al., 2006). One of the most closely studied downstream targets of ROCK is LIM domain kinase (Ohashi et al., 2000), and LIM domain kinase activation results in growth cone collapse. Thus, ROCK signaling pathways are involved in many cellular processes, many of which have implications in pathways that may alter the pathological events involving tau protein.

The relationship between ROCK inhibition and tau metabolism is not well known (Castro-Alvarez et al., 2011; Gentry et al., 2016). Previously, it was reported that tau was phosphorylated by Rho-kinase ROCK at Thr245, Thr377, and Ser409 (Amano et al., 2003). Castro-Alvarez et al. demonstrated that ventricular injection of a ROCK inhibitor (Y-27632) reduced NFT, and learning and special memory was improved in the mouse model of ischemia (2011). Furthermore, in progressive supranuclear palsy and corticobasal degeneration, the ROCK1 or ROCK2 expression level was increased (Gentry et al., 2016). Depletion of ROCK1 or ROCK2 by RNAi decreased both tau mRNA and tau protein in a human neuroblastoma cell line. Pharmacological inhibitors of Rho kinase resulted in the reduction of tau in soluble and insoluble fractions by activating autophagy and reducing tau mRNA levels (Gentry et al., 2016).

In this cell culture study, H1152 and Y-27632 were used as the ROCK inhibitors to examine the influence of ROCK signaling on tau pathology. H1152 is a specific inhibitor of ROCK2 that is membrane permeable with a Ki value of 1.6 nM but poorly inhibits the serine threonine kinase (Ikenoya et al., 2002). Cell-permeable Y-27632 is a highly potent and selective both ROCK1 (Ki = 220 nM) and ROCK2 (Ki = 300 nM) inhibitor that competes

with ATP to bind the catalytic domain (Davies et al., 2000; Ishizaki et al., 2000). Fasudil is also an inhibitor of both ROCK1 (Ki = 82 nM) and ROCK2 (Ki = 91 nM).

We found that ROCK inhibitors decreased phosphorylated tau levels. We found that GSK3β and Cdk5, important tau kinases, were downregulated by the ROCK inhibitor (H1152). In this study, Akt activation was observed with ROCK inhibition (H1152) (Fig. 4B). Akt was previously reported to form complexes with ROCK2, PDK1, and PKCb2 (Lin et al., 2014), suggesting that ROCK inhibition induces Akt activation indirectly. Akt is known to phosphorylate Ser9 in GSK3β, facilitating its inactivation. Thus, Akt-mediated inhibition of GSK3β by phosphorylation of Ser9 may play a role in the observed GSK3β inactivation (Chu et al., 2017; Cross et al., 1994, 1995) after ROCK inhibition, which may in turn reduce the levels of phosphorylated tau. However, other studies suggested that Akt directly phosphorylates tau (Ksiezak-Reding et al., 2003; Dickey et al., 2008), but this was unlikely in this model because the phospho-tau levels decreased after ROCK inhibitor treatment.

We also examined whether ROCK inhibitors reduced total tau protein levels. ROCK inhibitors alter the autophagic-lysosomal system, one cellular mechanism for degrading tau (Hamano et al., 2008, 2018). Indeed, ROCK inhibition reduced total tau levels, which may have occurred via the autophagy system. LC3-II, is an LC3-phosphatidylethanolamine conjugate and marker of autophagosomes in many cell lines (Hamano et al., 2018; Mizushima, and Komatsu, 2011). The level of LC3-II was upregulated by ROCK inhibition (H1152) in MC1 cells. The amount of p62, a well-known autophagy substrate, decreased after ROCK inhibitor treatment. These results suggest that ROCK inhibitors upregulate autophagy, as reported previously (Bauer, and Nukina, 2009; Koch et al., 2014). Krüger et al. (2012) reported that trehalose upregulates autophagy, and tau degradation via autophagy pathways was observed. Activation of autophagy is considered to be a possible therapy for tauopathy, including AD (Hamano et al., 2018; Hochgräfe et al., 2015).

The ubiquitin proteasomal system is another main cellular machinery that controls protein levels and removes impaired, abnormally folded or mutated proteins to control the quality of cytoplasmic and nuclear proteins (David et al., 2002; Oddo et al., 2004). ROCK signaling also regulates the proteasomal degradation systems (Bauer and Nukina, 2009); therefore, we examined whether the effects of ROCK inhibition on tau degradation were associated with alteration of the proteasomal pathway. ROCK inhibitors activated 20S and 26S proteasome. Unmodified tau may be identified by a subunit of 20S proteasomes and subjected to slow degradation (Lee et al., 2013), and the 20S proteasome (core particle) and trypsin-like activity were significantly reduced in postmortem AD brains (López Salon et al., 2000).

ROCK inhibitors (H1152, Y-27632, and fasudil) reduced tau in the sarkosyl insoluble fraction, which contains paired helical filament (PHF) tau aggregates that comprise NFTs. Of note, ROCK inhibitors reduced the amount of high-molecular weight tau in the Tris-insoluble, sarkosyl-soluble fraction under nonreducing conditions, which represents oligomeric species of tau (Hamano et al., 2008, 2016; Shirafuji et al., 2018). The oligomeric nature of these tau species was confirmed using the TOC1 antibody, which specifically detects oligomeric tau. Tau oligomers have cytotoxic effects (Fá et al., 2016; Patterson et al., 2011). Oligomeric tau is considered to be an important factor in AD progression (Lasagna-

Reeves et al., 2011; Maeda et al., 2006; Meraz-Ríos et al., 2010; Sahara et al., 2007), and oligomers are observed in the early phase of AD and closely correlate with hyperphosphorylation. When tau oligomers were injected into the brain, endogenous tau aggregation was facilitated, leading to the propagation of tau pathology comparable to that in studies of prions (Holmes et al., 2014). Oligomeric tau also induces dysfunction of synapses and mitochondria (Meraz-Ríos et al., 2010; Lasagna-Reeves et al., 2011).

Tau cleaved by caspase at the C-terminus enhances tau aggregation and cytotoxicity (Gamblin et al., 2003), and caspase upregulation precedes and leads to PHF formation (de Calignon et al., 2010). The C-terminus of tau inhibits tau aggregation in vitro and cleavage of the C-terminus by caspase can enhance tau polymerization (Berry et al., 2003; Gamblin et al., 2003). In this study, inactivation of caspase-3 was also observed after ROCK inhibition. Caspase inactivation was associated with reduced amounts of caspase-cleaved tau (Fig. 5C). This may be one of the manners by which aggregated tau was reduced after ROCK inhibition. Of note, tau species cleaved by caspase are involved in cell toxicity and increased tau secretion (Chung et al., 2001; Plouffe et al., 2012). The ROCK inhibitor-mediated reduction of caspase-cleaved tau (at D421) was associated with a decrease in sarkosyl-insoluble tau and cell death in M1C cells. Moreover, the level of the proinflammatory cytokine, IFNγ, was also reduced by ROCK inhibitors. The presence of IFNγ was reported to lead to caspase activation (Zhang et al., 2014). Thus, ROCK inhibition may mitigate caspase-mediated tau pathologies through multiple mechanisms in neurons.

In summary, ROCK inhibitors inactivated tau kinases, including GSK3β, associated with Akt activation. ROCK inhibitors also inactivated caspase, associated with decreased tau truncation at the C-terminus and the formation of tau oligomers which are 2 forms of tau related to toxicity. Furthermore, ROCK inhibitors upregulated autophagy and reduced total tau levels (Fig. 11). These findings suggest that ROCK inhibitors could provide beneficial effects against pathogenic forms of tau via multiple mechanisms and may represent a viable therapeutic approach for AD and other tauopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

Amano M, Kaneko T, Maeda A, Nakayama M, Ito M, Yamauchi T, Goto H, Fukata Y, Oshiro N, Shinohara A, Iwamatsu A, Kaibuchi K, 2003 Identification of Tau and MAP2 as novel substrates of Rho-kinase and myosin phosphatase. J. Neurochem 87, 780–790. [PubMed: 14535960]

Antoniu SA, 2012 Targeting RhoA/ROCK pathway in pulmonary arterial hypertension. Expert Opin. Ther. Targets 16, 355–363. [PubMed: 22449260]

Bauer PO, Nukina N, 2009 Enhanced degradation of mutant huntingtin by rho kinase inhibition is mediated through activation of proteasome and macro-autophagy. Autophagy 5, 747–748. [PubMed: 19411823]

Berry RW, Abraha A, Lagalwar S, LaPointe N, Gamblin TC, Cryns VL, Binder LI, 2003 Inhibition of tau polymerization by its carboxy-terminal caspase cleavage fragment. Biochemistry 42, 8325–8331. [PubMed: 12846581]

Borrajo A, Rodriguez-Perez AI, Villar-Cheda B, Guerra MJ, Labandeira-Garcia JL, 2014 Inhibition of the microglial response is essential for the neuroprotective effects of Rho-kinase inhibitors on MPTP-induced dopaminergic cell death. Neuropharmacology 85, 1–8. [PubMed: 24878243]

Castro-Alvarez JF, Gutierrez-Vargas J, Darnaudéry M, Cardona-Gómez GP, 2011 ROCK inhibition prevents tau hyperphosphorylation and p25/CDK5 increase after global cerebral ischemia. Behav. Neurosci 125, 465–472. [PubMed: 21517148]

Chang J, Xie M, Shah VR, Schneider MD, Entman ML, Wei L, Schwartz RJ, 2006 Activation of Rho-associated coiled-coil protein kinase 1 (ROCK-1) by caspase-3 cleavage plays an essential role in cardiac myocyte apoptosis. Proc. Natl. Acad. Sci. U. S. A 103, 14495–14500. [PubMed: 16983089]

Chu J, Lauretti E, Praticò D, 2017 Caspase-3-dependent cleavage of Akt modulates tau phosphorylation via GSK3β kinase: implications for Alzheimer's disease. Mol. Psychiatry 22, 1002–1008. [PubMed: 28138159]

Chung CW, Song YH, Kim IK, Yoon WJ, Ryu BR, Jo DG, Woo HN, Kwon YK, Kim HH, Gwag BJ, Mook-Jung IH, Jung YK, 2001 Proapoptotic effects of tau cleavage Product generated by caspase-3. Neurobiol. Dis 8, 162–172. [PubMed: 11162250]

Cross DA, Alessi DR, Vandenheede JR, McDowell HE, Hundal HS, Cohen P, 1994 The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem. J 303, 21–26. [PubMed: 7945242]

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA, 1995 Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789. [PubMed: 8524413]

David DC, Layfield R, Serpell L, Narain Y, Goedert M, Spillantini MG, 2002 Proteasomal degradation of tau protein. J. Neurochem 83, 176–185. [PubMed: 12358741]

Davies SP, Reddy H, Caivano M, Cohen P, 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J 351, 95–105. [PubMed: 10998351]

de Calignon A, Fox LM, Pitstick R, Carlson GA, Bacskai BJ, Spires-Jones TL, Hyman BT, 2010 Caspase activation precedes and leads to tangles. Nature 464, 1201–1204. [PubMed: 20357768]

DeTure M, Ko LW, Easson C, Yen SH, 2002 Tau assembly in inducible transfectants expressing wild-type or FTDP-17 tau. Am. J. Pathol 161, 1711–1722. [PubMed: 12414518]

Dickey CA, Koren J, Zhang YJ, Xu YF, Jinwal UK, Birnbaum MJ, Monks B, Sun M, Cheng JQ, Patterson C, Bailey RM, Dunmore J, Soresh S, Leon C, Morgan D, Petrucelli L, 2008 Akt and CHIP coregulate tau degradation through coordinated interactions. Proc. Natl. Acad. Sci. U. S. A 105, 3622–3627. [PubMed: 18292230]

Fá M, Puzzo D, Piacentini R, Staniszewski A, Zhang H, Baltrons MA, Li Puma DD, Chatterjee I, Li J, Saeed F, Berman HL, Ripoli C, Gulisano W, Gonzalez J, Tian H, Costa JA, Lopez P, Davidowitz E, Yu WH, Haroutunian V, Brown LM, Palmeri A, Sigurdsson EM, Duff KE, Teich AF, Honig LS, Sierks M, Moe JG, D'Adamio L, Grassi C, Kanaan NM, Fraser PE, Arancio O, 2016 Extracellular tau oligomers Produce an immediate impairment of LTP and memory. Sci. Rep 6, 19393. [PubMed: 26786552]

Gamblin TC, Chen F, Zambrano A, Abraha A, Lagalwar S, Guillozet AL, Lu M, Fu Y, Garcia-Sierra F, LaPointe N, Miller R, Berry RW, Binder LI, Cryns VL, 2003 Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A 100, 10032–10037. [PubMed: 12888622]

Gentry EG, Henderson BW, Arrant AE, Gearing M, Feng Y, Riddle NC, Herskowitz JH, 2016 Rho kinase inhibition as a therapeutic for progressive supranuclear palsy and corticobasal degeneration. J. Neurosci 36, 1316–1323. [PubMed: 26818518]

Gong CX, Shaikh S, Wang JZ, Zaidi T, Grundke-Iqbal I, Iqbal K, 1995 Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. J. Neurochem 65, 732–738. [PubMed: 7616230]

Hamano T, Gendron TF, Causevic E, Yen SH, Lin WL, Isidoro C, Deture M, Ko LW, 2008 Autophagic-lysosomal perturbation enhances tau aggregation in transfectants with induced wild-type tau expression. Eur. J. Neurosci 27, 1119–1130. [PubMed: 18294209]

Hamano T, Gendron TF, Ko LW, Yen SH, 2009 Concentration-dependent effects of proteasomal inhibition on tau processing in a cellular model of tauopathy. Int.J. Clin. Exp. Pathol 2, 561–573. [PubMed: 19636403]

Hamano T, Yen SH, Gendron T, Ko LW, Kuriyama M, 2012 Pitavastatin decreases tau levels via the inactivation of rho/ROCK. Neurobiol. Aging 33, 2306–2320. [PubMed: 22133277]

Hamano T, Shirafuji N, Makino C, Yen SH, Kanaan NM, Ueno A, Suzuki J, Ikawa M, Matsunaga A, Yamamura O, Kuriyama M, Nakamoto Y, 2016 Pioglitazone prevents tau oligomerization. Biochem. Biophys. Res. Commun 478, 1035–1042. [PubMed: 27543203]

Hamano T, Hayashi K, Shirafuji N, Nakamoto Y, 2018 The implications of autophagy in Alzheimer's disease. Curr. Alzheimer Res 15, 1283–1296. [PubMed: 30289076]

Han P, Dou F, Li F, Zhang X, Zhang YW, Zheng H, Lipton SA, Xu H, Liao FF, 2005 Suppression of cyclin-dependent kinase 5 activation by amyloid precursor protein: a novel excitoprotective mechanism involving modulation of tau phosphorylation. J. Neurosci 25, 11542–11552. [PubMed: 16354912]

Hochgräfe K, Sydow A, Matenia D, Cadinu D, Könen S, Petrova O, Pickhardt M, Goll P, Morellini F, Mandelkow E, Mandelkow EM, 2015 Preventive methylene blue treatment preserves cognition in mice expressing full-length pro-aggregant human Tau. Acta Neuropathol. Commun 3, 25. [PubMed: 25958115]

Holmes BB, Furman JL, Mahan TE, Yamasaki TR, Mirbaha H, Eades WC, Belaygorod L, Cairns NJ, Holtzman DM, Diamond MI, 2014 Proteopathic tau seeding predicts tauopathy in vivo. Proc. Natl. Acad. Sci. U. S. A 111, E4376–E4385. [PubMed: 25261551]

Ikenoya M, Hidaka H, Hosoya T, Suzuki M, Yamamoto N, Sasaki Y, 2002 Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. J. Neurochem 81, 9–16. [PubMed: 12067241]

Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S, 2000 Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. Mol. Pharmacol 57, 976–983. [PubMed: 10779382]

Kanaan NM, Pigino GF, Brady ST, Lazarov O, Binder LI, Morfini GA, 2013 Axonal degeneration in Alzheimer's disease: when signaling abnormalities meet the axonal transport system. Exp. Neurol 246, 44–53. [PubMed: 22721767]

Kinoshita S, Koizumi N, Ueno M, Okumura N, Imai K, Tanaka H, Yamamoto Y, Nakamura T, Inatomi T, Bush J, Toda M, Hagiya M, Yokota I, Teramukai S, Sotozono C, Hamuro J, 2018 Injection of cultured cells with a ROCK inhibitor for bullous keratopathy. N. Engl. J. Med 378, 995–1003. [PubMed: 29539291]

Ko LW, Rush T, Sahara N, Kersh JS, Easson C, Deture M, Lin WL, Connor YD, Yen SH, 2004 Assembly of filamentous tau aggregates in human neuronal cells. J. Alzheimers Dis 6, 605–622. [PubMed: 15665401]

Koch JC, Tönges L, Barski E, Michel U, Bähr M, Lingor P, 2014 ROCK2 is a major regulator of axonal degeneration, neuronal death and axonal regeneration in the CNS. Cell Death Dis 5, e1225. [PubMed: 24832597]

Krüger U, Wang Y, Kumar S, Mandelkow EM, 2012 Autophagic degradation of tau in primary neurons and its enhancement by trehalose. Neurobiol. Aging 33, 2291–2305. [PubMed: 22169203]

Ksiezak-Reding H, Pyo HK, Feinstein B, Pasinetti GM, 2003 Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. Biochim.Biophys. Acta 1639, 159–168. [PubMed: 14636947]

Lasagna-Reeves CA, Castillo-Carranza DL, Sengupta U, Clos AL, Jackson GR, Kayed R, 2011 Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice. Mol. Neurodegener 6, 39. [PubMed: 21645391]

Lee VM, Goedert M, Trojanowski JQ, 2001 Neurodegenerative tauopathies. Annu. Rev. Neurosci 24, 1121–1159. [PubMed: 11520930]

Lee MJ, Lee JH, Rubinsztein DC, 2013 Tau degradation: the ubiquitin-proteasome system versus the autophagy-lysosome system. Prog. Neurobiol 105, 49–59. [PubMed: 23528736]

Lin G, Brownsey RW, Macleod KM, 2014 Complex regulation of PKCβ2 and PDK-1/AKT by ROCK2 in diabetic heart. PLoS One 9, e86520. [PubMed: 24466133]

López Salon M, Morelli L, Castaño EM, Soto EF, Pasquini JM, 2000 Defective ubiquitination of cerebral proteins in Alzheimer's disease. J. Neurosci. Res 6, 302–310.

Lovestone S, Reynolds CH, 1997 The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. Neuroscience 78, 309–324. [PubMed: 9145789]

Ma T, Zhao Y, Kwak YD, Yang Z, Thompson R, Luo Z, Xu H, Liao FF, 2009 Statin's excitoprotection is mediated by sAPP and the subsequent attenuation of calpain-induced truncation events, likely via rho-ROCK signaling. J. Neurosci 29, 11226–11236. [PubMed: 19741129]

Maeda S, Sahara N, Saito Y, Murayama S, Ikai A, Takashima A, 2006 Increased levels of granular tau oligomers: an early sign of brain aging and Alzheimer's disease. Neurosci. Res 54, 197–201. [PubMed: 16406150]

Meraz-Ríos MA, Lira-De León KI, Campos-Peña V, De Anda-Hernández MA, Mena-López R, 2010 Tau oligomers and aggregation in Alzheimer's disease. J. Neurochem 112, 1353–1367. [PubMed: 19943854]

Mizushima N, Komatsu M, 2011 Autophagy: renovation of cells and tissues. Cell 147, 728–741. [PubMed: 22078875]

Oddo S, Billings L, Kesslak JP, Cribbs DH, LaFerla FM, 2004 Abeta immuno-therapy leads to clearance of early, but not late, hyperphosphorylated tau aggregates via the proteasome. Neuron 43, 321–332. [PubMed: 15294141]

Ohashi K, Nagata K, Maekawa M, Ishizaki T, Narumiya S, Mizuno K, 2000 Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. J. Biol. Chem 275, 3577–3582. [PubMed: 10652353]

Okumura N, Koizumi N, Ueno M, Sakamoto Y, Takahashi H, Hirata K, Torii R, Hamuro J, Kinoshita S, 2011 Enhancement of corneal endothelium wound healing by Rho-associated kinase (ROCK) inhibitor eye drops. Br. J. Ophthalmol 95, 1006–1009. [PubMed: 21398412]

Olson MF, 2008 Applications for ROCK kinase inhibition. Curr. Opin. Cell Biol 20, 242–248. [PubMed: 18282695]

Osna NA, White RL, Donohue TM Jr., Beard MR, Tuma DJ, Kharbanda KK, 2010 Impaired methylation as a novel mechanism for proteasome suppression in liver cells. Biochem. Biophys. Res. Commun 391, 1291–1296. [PubMed: 20026058]

Patterson KR, Remmers C, Fu Y, Brooker S, Kanaan NM, Vana L, Ward S, Reyes JF, Philibert K, Glucksman MJ, Binder LI, 2011 Characterization of prefibrillar Tau oligomers in vitro and in Alzheimer disease. J. Biol. Chem 286, 23063–23076. [PubMed: 21550980]

Plouffe V, Mohamed NV, Rivest-McGraw J, Bertrand J, Lauzon M, Leclerc N, 2012 Hyperphosphorylation and cleavage at D421 enhance tau secretion. PLoS One 7, e36873. [PubMed: 22615831]

Riento K, Ridley AJ, 2003 Rocks: multifunctional kinases in cell behaviour. Nat. Rev.Mol. Cell. Biol 4, 446–456. [PubMed: 12778124]

Sahara N, Maeda S, Murayama M, Suzuki T, Dohmae N, Yen SH, Takashima A, 2007 Assembly of two distinct dimers and higher-order oligomers from full-length tau. Eur. J. Neurosci 25, 3020–3029. [PubMed: 17561815]

Sakamoto S, Ishizaki T, Okawa K, Watanabe S, Arakawa T, Watanabe N, Narumiya S, 2012 Liprin-a controls stress fiber formation by binding to mDia and regulating its membrane localization. J. Cell Sci 125, 108–120. [PubMed: 22266902]

Santacruz K, Lewis J, Spires T, Paulson J, Kotilinek L, Ingelsson M, Guimaraes A, DeTure M, Ramsden M, McGowan E, Forster C, Yue M, Orne J, Janus C, Mariash A, Kuskowski M, Hyman B, Hutton M, Ashe KH, 2005 Tau suppression in a neurodegenerative mouse model improves memory function. Science 309, 476–481. [PubMed: 16020737]

Shimokawa H, Takeshita A, 2005 Rho-kinase is an important therapeutic target in cardiovascular medicine. Arterioscler. Thromb. Vasc. Biol 25, 1767–1775. [PubMed: 16002741]

Shirafuji N, Hamano T, Yen SH, Kanaan NM, Yoshida H, Hayashi K, Ikawa M, Yamamura O, Kuriyama M, Nakamoto Y, 2018 Homocysteine increases tau phosphorylation, truncation and oligomerization. Int. J. Mol. Sci 19, 891.

Tatenhorst L, Eckermann K, Dambeck V, Fonseca-Ornelas L, Walle H, Lopes da Fonseca T, Koch JC, Becker S, Tönges L, Bähr M, Outeiro TF, Zweckstetter M, Lingor P, 2016 Fasudil attenuates aggregation of α-synuclein in models of Parkinson's disease. Acta Neuropathol. Commun 4, 39. [PubMed: 27101974]

Tönges L, Günther R, Suhr M, Jansen J, Balck A, Saal KA, Barski E, Nientied T, Götz AA, Koch JC, Mueller BK, Weishaupt JH, Sereda MW, Hanisch UK, Bähr M, Lingor P, 2014 Rho kinase inhibition modulates microglia activation and improves survival in a model of amyotrophic lateral sclerosis. Glia 62, 217–232. [PubMed: 24311453]

Ward SM, Himmelstein DS, Ren Y, Fu Y, Yu XW, Roberts K, Binder LI, Sahara N, 2014 TOC1: a valuable tool in assessing disease progression in the rTg4510 mouse model of tauopathy. Neurobiol. Dis 67, 37–48. [PubMed: 24631720]

Wood DW, Nye JS, Lamb NJC, Fernanez A, Kitzmann M, 2005 Intracellular retention of caveolin 1 in presenilin-deficient cells. J. Biol. Chem 280, 6663–6668. [PubMed: 15613480]

Yamamoto K, Maruyama K, Himori N, Omodaka K, Yokoyama Y, Shiga Y, Morin R, Nakazawa T, 2014 The novel Rho kinase (ROCK) inhibitor K-115: a new candidate drug for neuroprotective treatment in glaucoma. Invest. Ophthalmol. Vis. Sci 55, 7126–7136. [PubMed: 25277230]

Zhang MC, Liu HP, Demchik LL, Zhai YF, Yang DJ, 2014 LIGHT sensitizes IFN-gamma-mediated apoptosis of HT-29 human carcinoma cells through both death receptor and mitochondria pathways. Cell Res 14, 117–124.

A
Phospho-tau antibodies

## B
TetOff induction of Tau
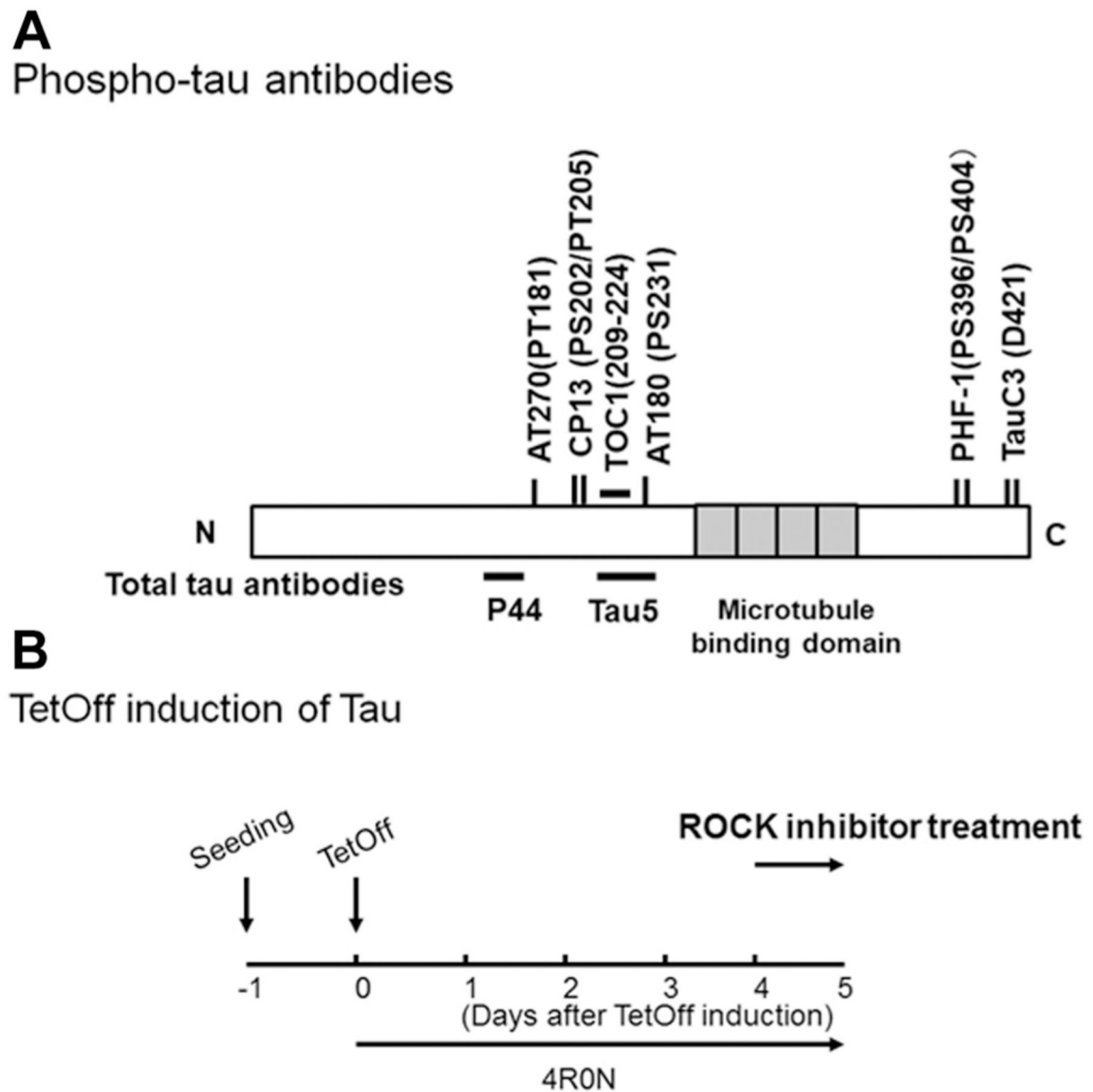
Fig. 1.
Tau antibodies used in this study and the experimental timeline. (A) Schematic representation of 4R0N tau and distinct epitopes recognized by different tau antibodies. (B) Treatment schedule of tetracycline off (TetOff) induction. On day 0, TetOff induction with 1 ng/mL of tetracycline was initiated. On day 4, ROCK inhibitor (H1152, or Y-27632) treatment was initiated. On day 5, cells were harvested. Abbreviation: ROCK, Rho-associated coiled-coil protein kinase.
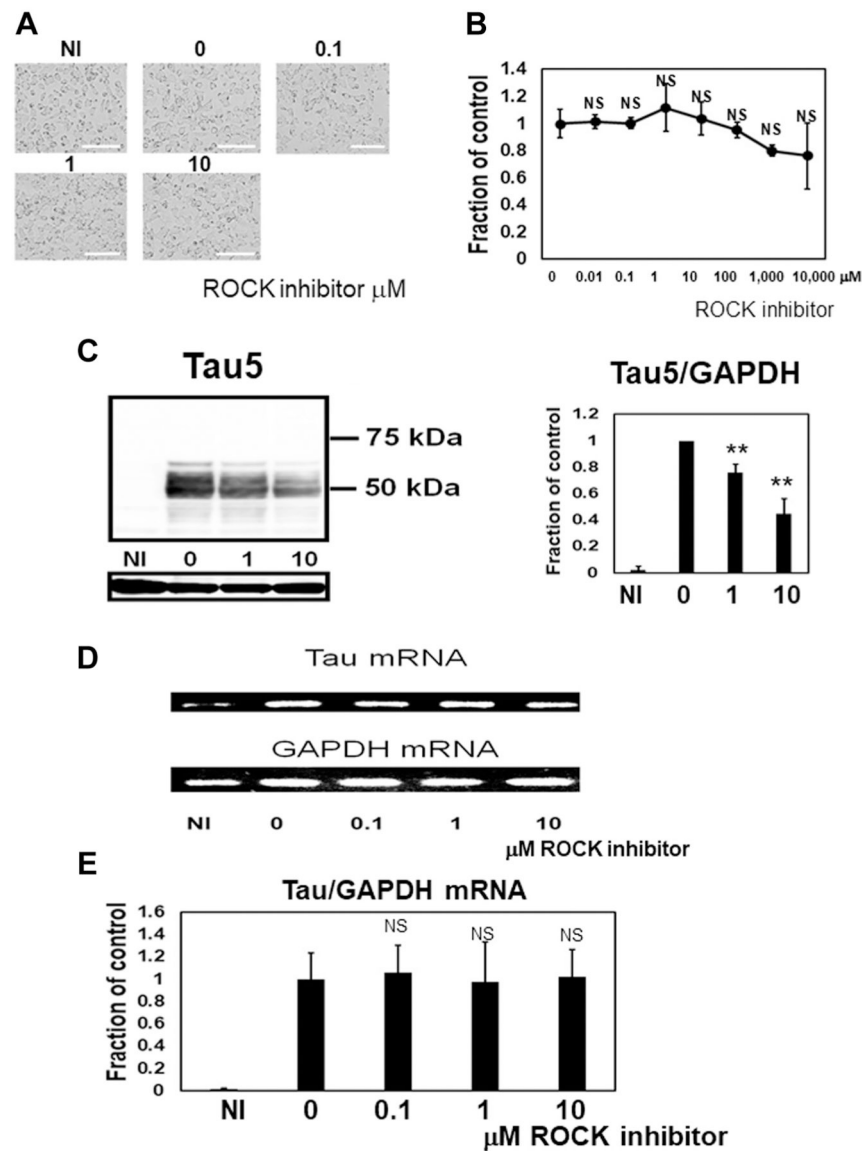
Fig. 2.
Western blot analysis showed reduced total tau levels by the ROCK inhibitor (H1152). The ROCK inhibitor (H1152) does not have cytotoxic effects. (A) ROCK inhibitor did not cause morphological change. Scale 25 μm. (B) No cell death under these conditions was observed in the ATP assay (0.01 μM-100 μM). N = 4, Data: ± SD. (C) To examine the effect of ROCK inhibitor on tau, M1C cells were induced to express tau for 5 d and exposed to ROCK inhibitor (1 or 10 μM) during the final day of the induction period. Lysates from cultures were analyzed by Western blot using the Tau5 antibody. These samples were probed with anti-GAPDH to confirm that loading among lanes was equal. Total tau levels decreased dose dependently after ROCK inhibitor treatment. In cultures treated with 1 μM ROCK inhibitor, the antibody Tau5 detected 45–60 kDa bands at 76.0% of the levels exhibited by the vehicle control. NI: noninduced cells, 0: untreated cells, 1: 1 μM ROCK inhibitor (H1152)-treated cells, 10: 10 μM ROCK inhibitor-treated cells. N = 6, **$p < 0.01$, Bar: ± SD. Tau mRNA

was not affected by ROCK inhibitor treatment according to real-time PCR (D) and quantitative PCR (qPCR). N = 4, Bar: ± SD (E). Data from Tau5/GAPDH and Tau QPCR/ GAPDH qPCR followed a normal distribution and were analyzed with one-way ANOVA followed by Bonferroni post hoc test, whereas the Kruskal-Wallis test followed by Dunn's post hoc test was used for ATP assay because the data deviated from a normal distribution. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NS, not significant; ROCK, Rho-associated coiled-coil protein kinase.
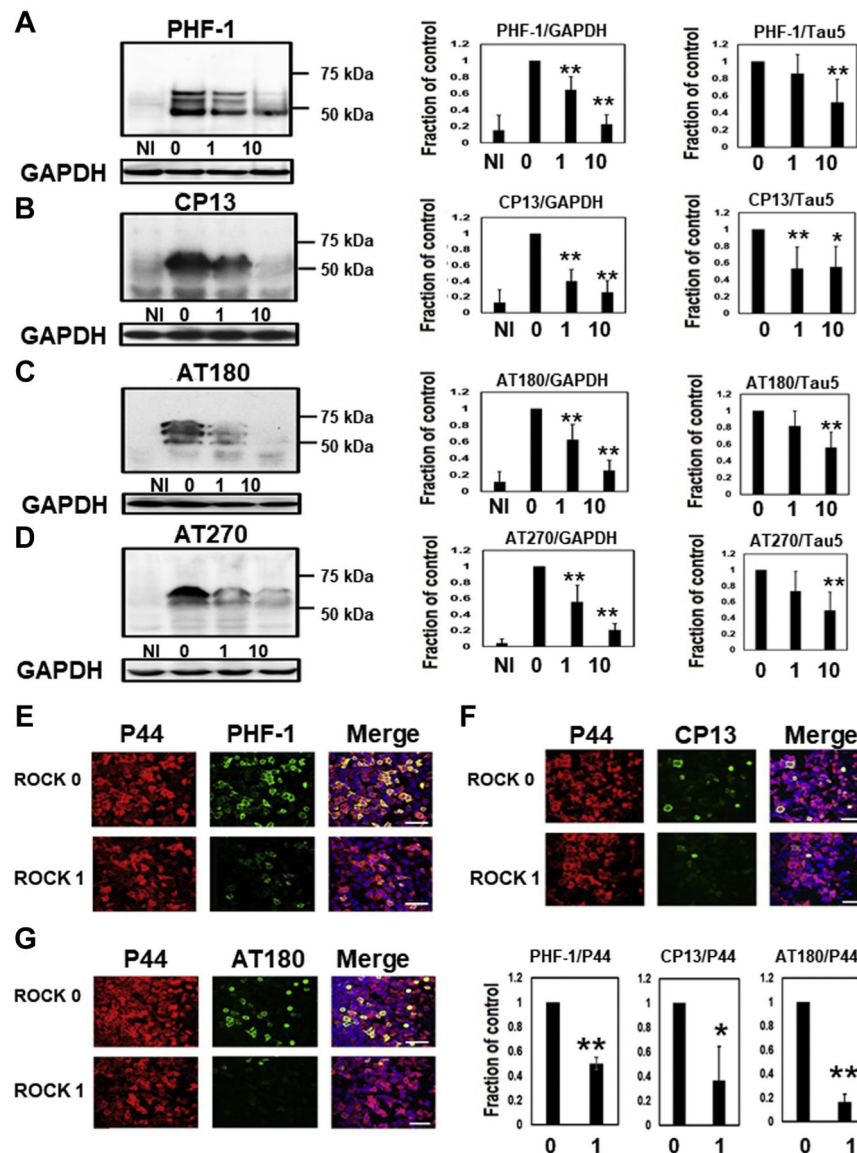
Fig. 3.
Phosphorylated tau levels were reduced in M1C cells by the ROCK inhibitor. Western blot analysis revealed reduced phosphorylated tau levels based on PHF-1 (A), CP13 (B), AT180 (C), and AT270 (D) by ROCK inhibitor (H1152). N = 5, **p < 0.01, *<0.05, Bar: ± SD. NI: noninduced cells, 0: untreated cells, 1: 1 μM ROCK inhibitor-treated cells, 10: 10 μM ROCK inhibitor treated cells. According to the immunocytochemical study, PHF-1(E), CP13 (F), and AT180 (G)-positive phosphorylated tau was markedly reduced by the ROCK inhibitor (H1152) (0): 0 μM ROCK inhibitor, (1): 1 μM ROCK inhibitor. N = 5, **p < 0.01, *<0.05, Bar: ± SD, Scale 75 μM. Data from PHF-1/GAPDH, CP13/GAPDH, AT180/GAPDH, AT270/GAPDH, PHF-1/Tau5, AT180/Tau5, and AT270/Tau5 followed a normal distribution and were analyzed with one-way ANOVA followed by Bonferroni post hoc test, whereas the Kruskal-Wallis test followed by Dunn's post hoc test was used for CP13/Tau5 because the data deviated from a normal distribution. For the immunocytochemical study of

PHF-1/P44, CP13/P44, and AT180/P44, the data followed a normal distribution and were analyzed with Student's *t*-test. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ROCK, Rho-associated coiled-coil protein kinase.
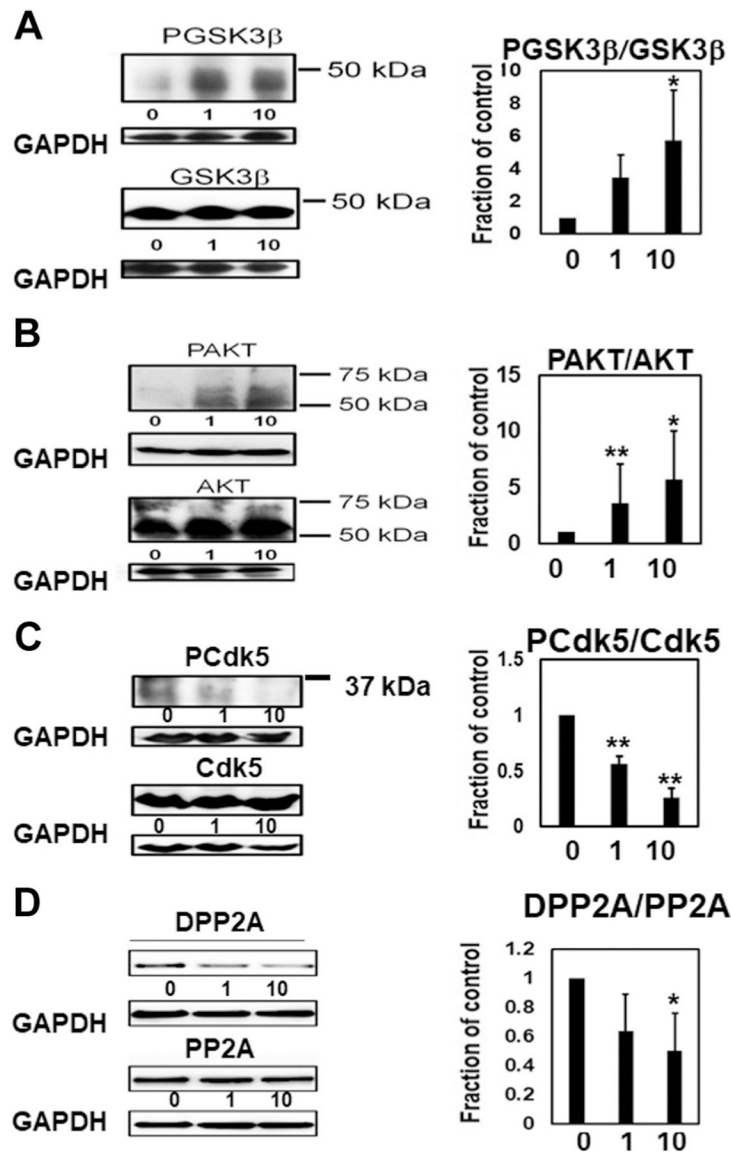
Fig. 4.

Tau kinase inactivation by the ROCK inhibitor (H1152). GSK3β was inactivated by the ROCK inhibitor. 0: 0 μM ROCK inhibitor, 1: 1 μM ROCK inhibitor, 10: 10 μM ROCK inhibitor. N = 4, *$p < 0.05$, Bar: ± SD (A). Akt activation was also observed with the ROCK inhibitor treatment. 0: 0 μM ROCK inhibitor, 1: 1 μM ROCK inhibitor, 10: 10 μM ROCK inhibitor. N = 4, **$p < 0.01$, *<0.05, Bar: ± SD (B). Cdk5, another tau kinase, was inactivated by ROCK inhibitor treatment. N = 3, **$p < 0.01$, Bar: ± SD (C). Inactivated PP2A (DPP2A) was decreased, whereas total PP2A was unchanged by ROCK inhibitor treatment. N = 3, *$p < 0.05$, Bar ± SD (D). Data from GSK3β/GSK3β, PCDK5/CDK5, and DPP2A/PP2A followed a normal distribution and were analyzed with one-way ANOVA followed by Bonferroni post hoc test, whereas the Kruskal-Wallis test followed by Dunn's post hoc test was used for PAKT/AKT because the data deviated from a normal distribution. Abbreviations: Cdk5, cyclin-dependent kinase 5; DPP2A, demethylated PP2A; GSK3β,

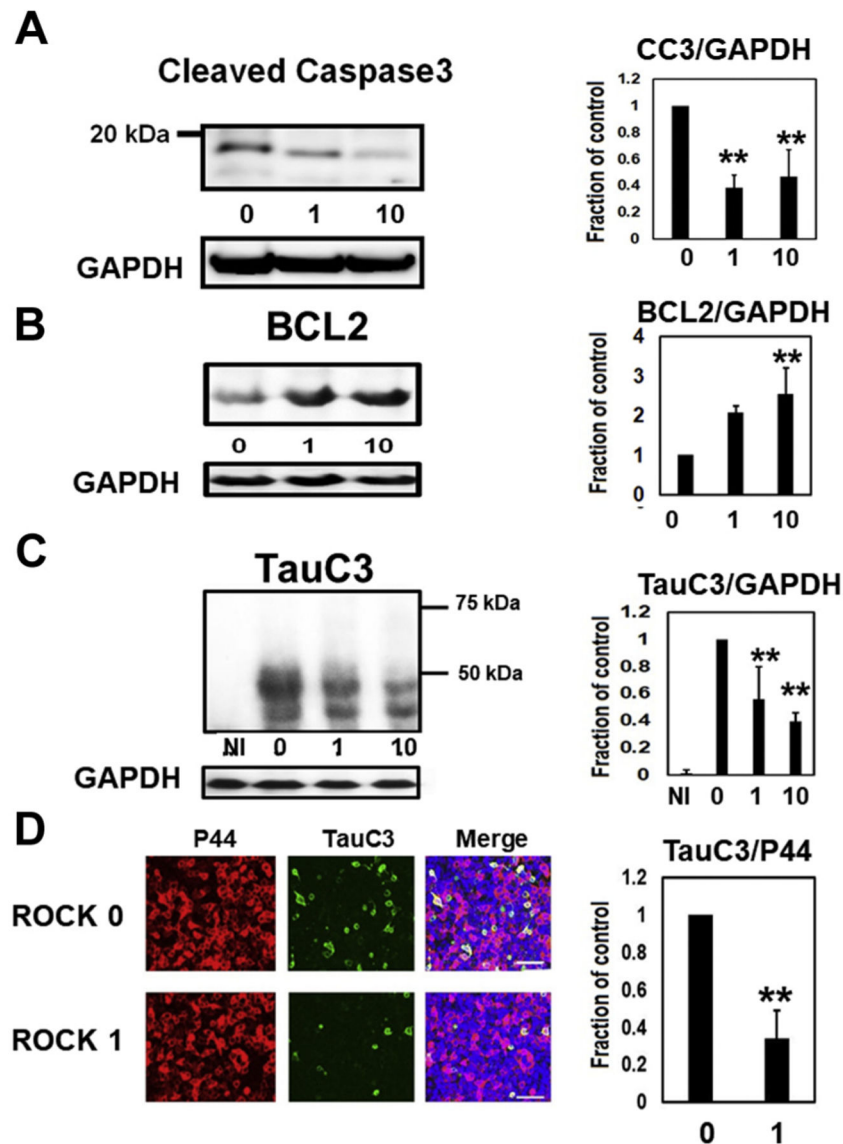glycogen synthase kinase 3β; pCdk5, phosho-Cdk5; PP2A, protein phosphatase 2A; ROCK, Rho-associated coiled-coil protein kinase.

Fig. 5.
The ROCK inhibitor (H1152) inactivated caspase-3 and activated BCL-2 in M1C cells. (A) The amount of cleaved caspase-3 was reduced by the ROCK inhibitor. 0: 0 μM ROCK inhibitor,1: 1 μM ROCK inhibitor, 10: 10 μM ROCK Inhibitor. N = 4, $**p < 0.01$, Bar: ± SD. (B) Bcl-2 activation was also observed after ROCK inhibitor treatment, N = 3, $**p < 0.01$, Bar: ± SD. Caspase-cleaved tau was reduced by ROCK inhibitor treatment, as shown by Western blotting (N = 4), $**p < 0.01$, Bar: ± SD (C), and immnocytochemical study. N = 4, $**p < 0.01$, Bar: ± SD, Scale 75 μM (D). Data from cleaved caspase3/GAPDHP, TauC3/GAPDH, and BCL2/GAPDH followed a normal distribution and were analyzed with one-way ANOVA followed by Bonferroni post hoc test. The immunocytochemical data from TauC3 followed a normal distribution and were analyzed with Student's t-test. Abbreviations: CC3, cleaved caspase-3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ROCK, Rho-associated coiled-coil protein kinase.

IFNγ

GAPDH

IFN γ/GAPDH
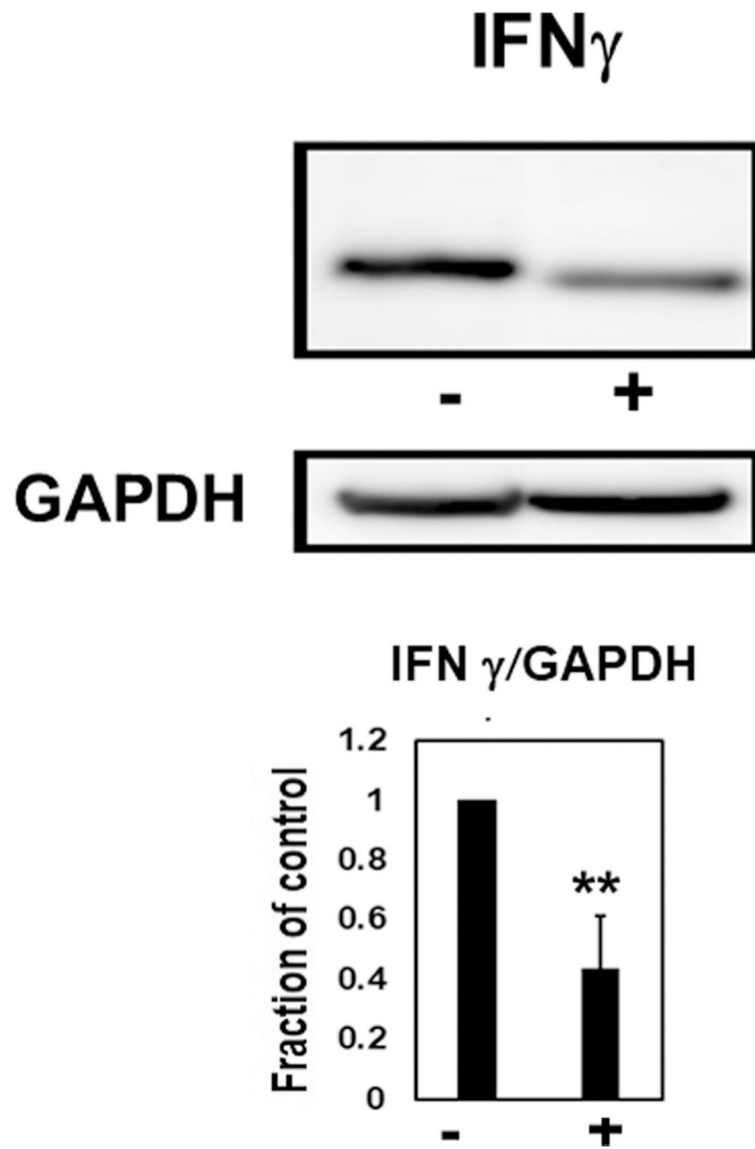
Fig. 6.
The ROCK inhibitor (H1152) reduced proinflammatory cytokine levels. IFNγ was reduced by the ROCK inhibitor (H1152). −: 0 μM ROCK inhibitor, +: 1 mM ROCK in hibitor. N = 3, **$p < 0.01$, Bar ± SD. Data from IFNγ after normal distribution were analyzed with Student's *t*-test. Abbreviation: ROCK, Rho-associated coiled-coil protein kinase; IFNγ, interferon γ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
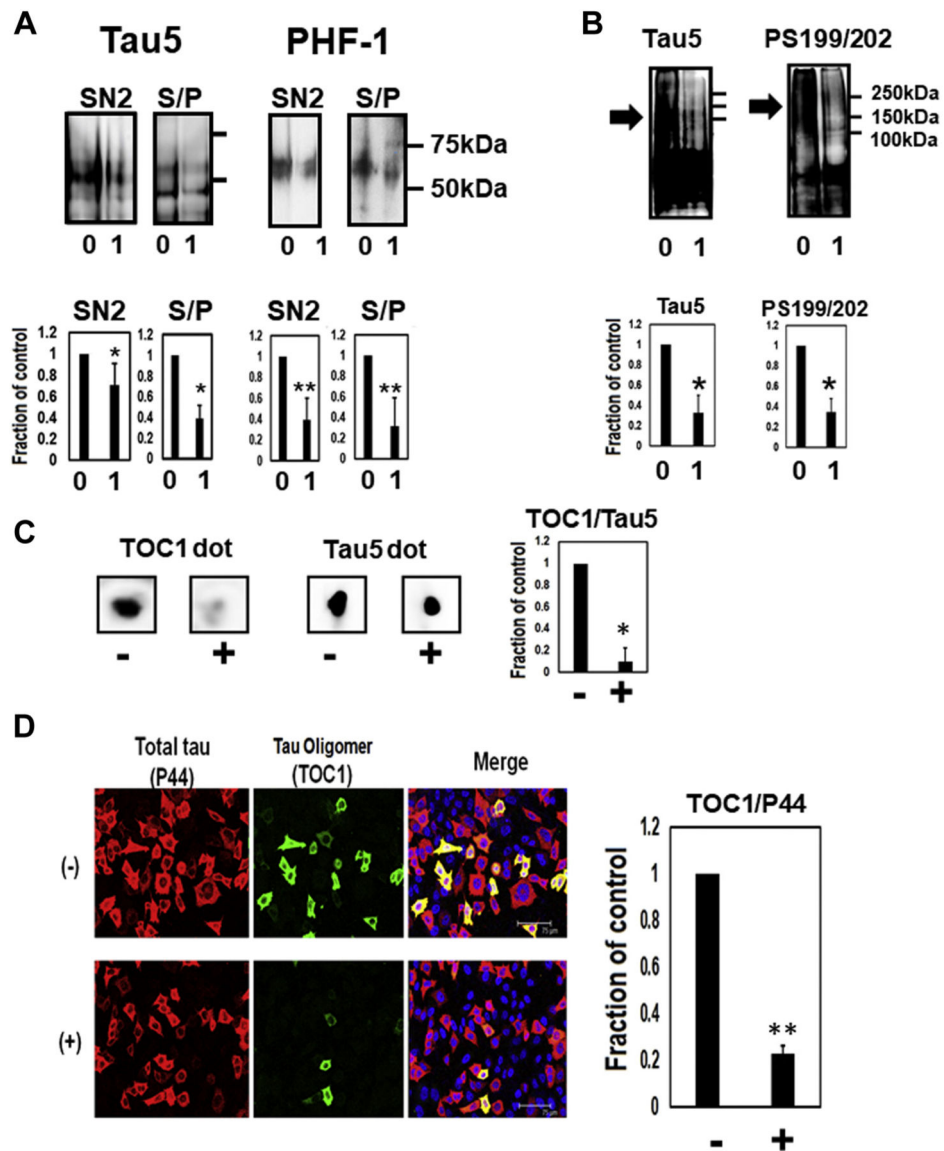
Fig. 7.
The ROCK inhibitor (H1152) reduced tau in the sarkosyl-insoluble fraction and oligomeric tau. The ROCK inhibitor (H1152) reduced total tau (Tau5) and phosphorylated tau (PHF-1) in the sarkosyl insoluble fraction (S/P) and Tris-insoluble, sarkosyl-soluble fraction (SN2). (N = 4) **$p < 0.01$, *$p < 0.05$, Bar ± SD (A). In the Tris-insoluble, sarkosyl-soluble fraction, the levels of high molecular weight smear total tau (Tau5) and phosphorylated tau (PS199/202) were decreased by the ROCK inhibitor (H1152) under nonreducing conditions. N = 3, *$p < 0.05$ (B). (C) Dot blot analysis of lysate demonstrated the reduction of oligomeric tau by ROCK inhibitor (H1152). −: ROCK inhibitor (−), +: 1 μM ROCK inhibitor-treated cells. N = 4, *$p<0.05$, Bar ± SD. (D) Immunocytochemical study also showed that ROCK inhibitor treatment decreased oligomeric tau as detected by TOC1. −: DMSO, +: 1 μM ROCK inhibitor (H1152), Scale: 75 μM, **$p < 0.01$, Bar ± SD. For the total tau (Tau5) and phosphorylated tau (PHF-1) in the sarkosyl insoluble fraction (S/P) and

Tris-insoluble, sarkosyl-soluble fraction (SN2), high molecular weight smear total tau and phosphorylated tau, and immunocytochemical study of TOC1, the data followed a normal distribution and were analyzed with Student's *t*-test, whereas dot blot data were analyzed with the Mann-Whitney *U*-test because the data deviated from a normal distribution. Abbreviation: ROCK, Rho-associated coiled-coil protein kinase; TOC1, Tau Oligomer Complex I antibody.
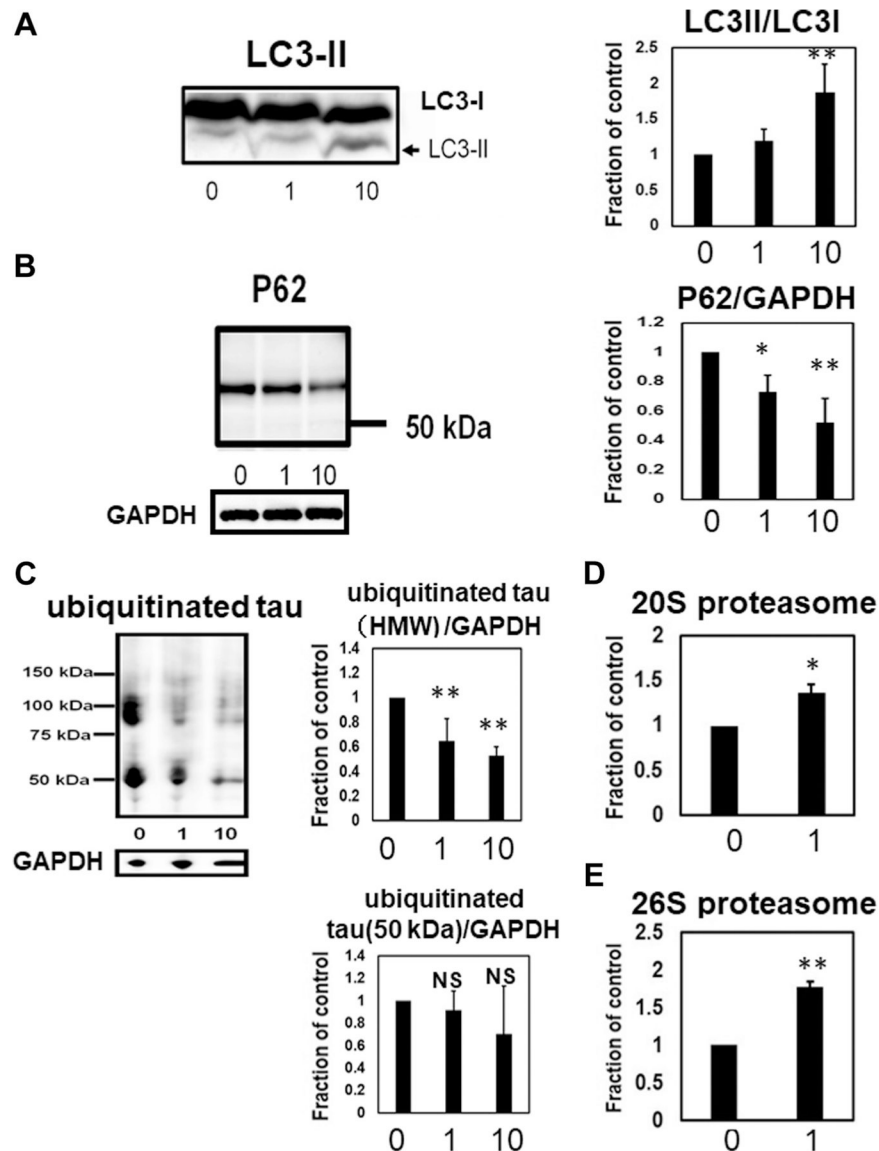
Fig. 8.

The ROCK inhibitor (H1152) activated autophagy and proteasome (20S and 26S). LC3-II, which is the marker of autophagy, was upregulated by ROCK inhibitor (10 μM) treatment. N = 4, **$p < 0.01$, Bar: ± SD (A). P62, which is one of the substrates of autophagy, was decreased by ROCK inhibitor treatment. N = 4, **$p < 0.01$, *$p < 0.05$, Bar: ± SD (B). ROCK inhibitor treatment reduced the amount of ubiquitinated tau protein, suggesting that the proteasomal system was upregulated by the ROCK inhibitor. N = 3, **$p < 0.01$, Bar: ± SD. (C). Chymotrypsin-like 20S proteasomal activity (N = 3) *$p < 0.05$, Bar: ± SD (D), and trypsin-like 26S proteasomal activity (N = 3), **$p < 0.01$, Bar: ± SD (E) were also upregulated by the ROCK inhibitor. Data from LC3-II/LC3-I, P62/GAPDH, and ubiquitinated high molecular weight tau followed a normal distribution and were analyzed with one-way ANOVA followed by Bonferroni post hoc test, whereas the Kruskal-Wallis test followed by Dunn's post hoc test was used for 50 kDa ubiquitinated tau because the data

deviated from a normal distribution. The Chymotrypsin-like 20S proteasomal activity and trypsin like 26S proteasomal activity data followed a normal distribution and were analyzed with Student's *t*-test. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NS, not significant; ROCK, Rho-associated coiled-coil protein kinase, LC3, microtubule-associated protein 1 light chain 3.
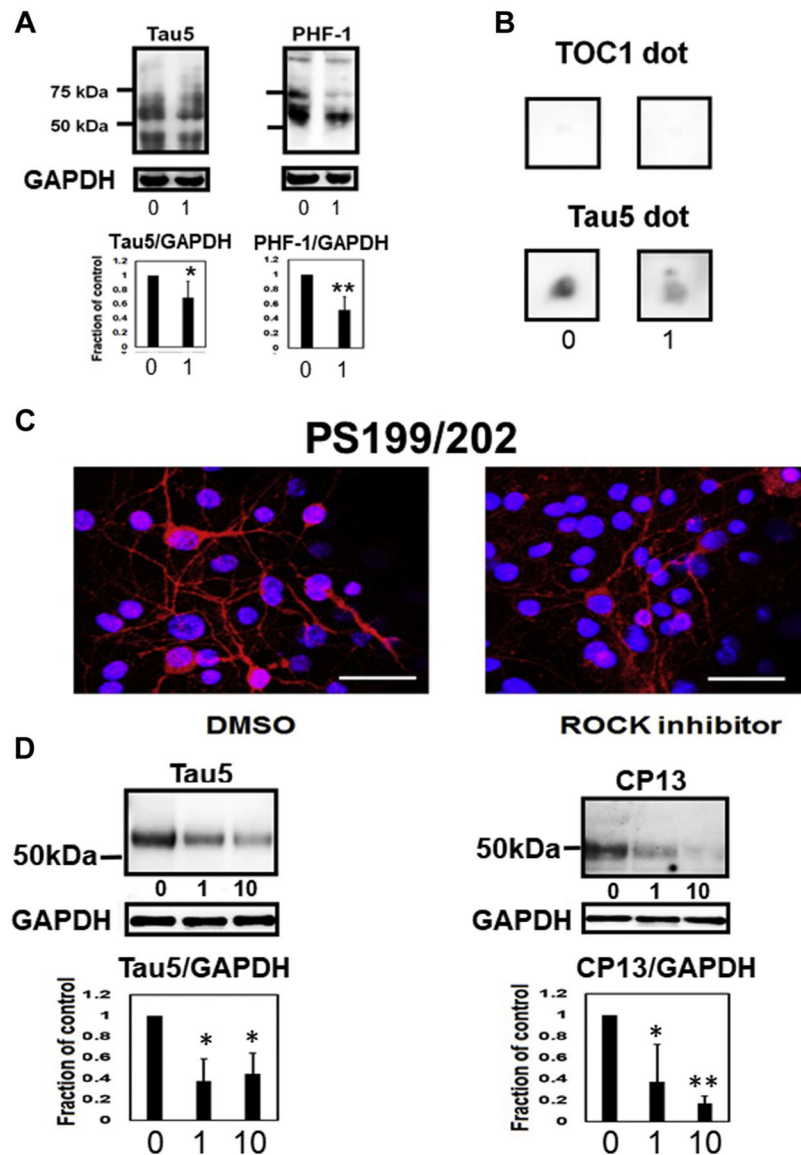
Fig. 9.
Endogenous tau reduction by the ROCK inhibitor (H1152). Endogenous tau in noninduced M1C cells was reduced by ROCK inhibitor (H1152) treatment. Total tau (Tau5) and phosphorylated tau (PHF-1) levels were reduced by 1 μM ROCK inhibitor treatment. N = 4, $**p < 0.01$, $*p < 0.05$, Bar: ± SD (A). Oligomeric tau detected by TOC1 was not identified in noninduced cells. N = 3 (B). Endogenous tau was reduced in mouse primary neurons by ROCK inhibitor treatment as compared with untreated neurons, which was confirmed by immunocytochemistry (PS199/202), scale 37.5 μm (C), and Western blot analysis detected the reduction of total (Tau5) and phosphorylated (CP13) tau. 0: 0 μM ROCK inhibitor, 1: 1 μM ROCK inhibitor, 10: 10 μM ROCK inhibitor. N = 3, $**p < 0.01$, $* < 0.05$, Bar: ± SD (D). Data from noninduced M1C cells Tau5 and PHF-1 followed a normal distribution and were analyzed with Student's *t*-test. Data from mouse neuron Tau5 and CP13 followed a normal distribution and were analyzed with one-way ANOVA followed by Bonferroni post hoc test.
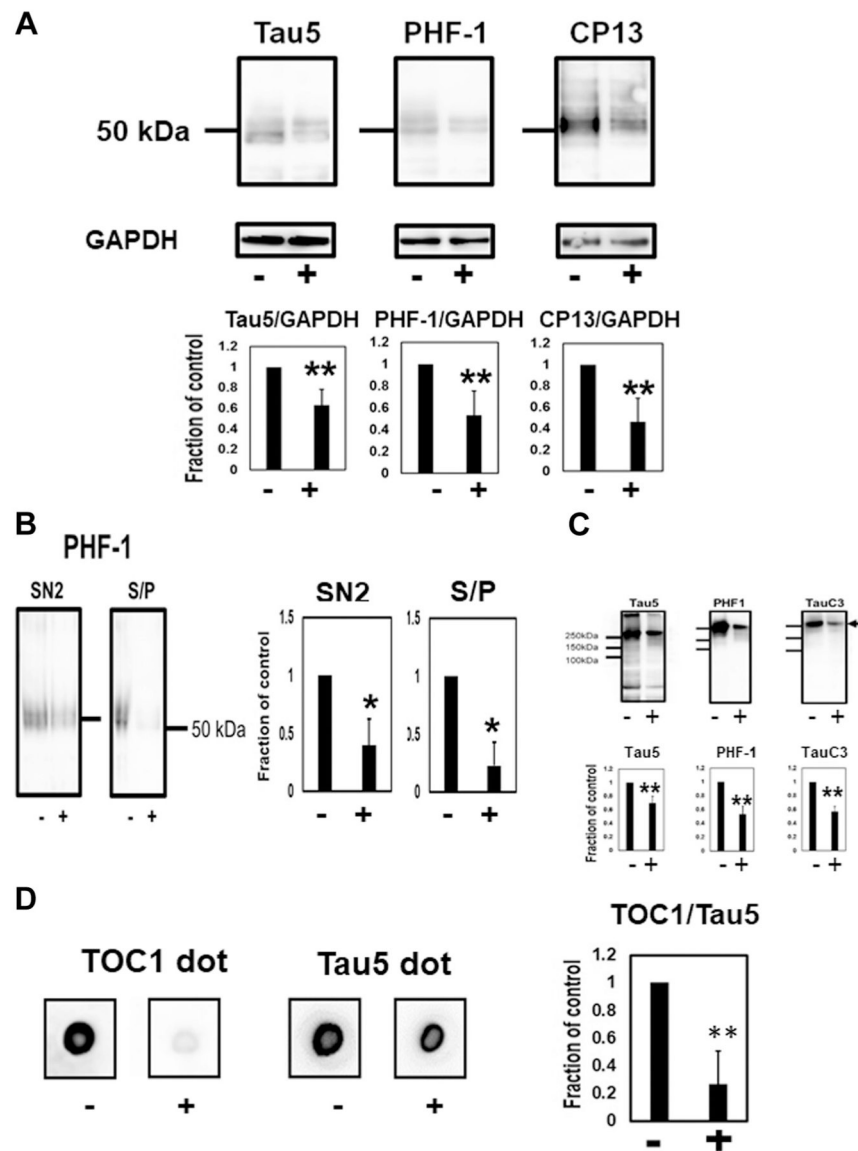
Abbreviation: ROCK, Rho-associated coiled-coil protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TOC1, Tau Oligomer Complex I antibody.

Fig. 10.
The tau protein level was reduced by ROCK inhibitor (fasudil) treatment in the mouse model of tauopathy. The ROCK inhibitor (fasudil) reduced the amount of total tau (Tau5) and phosphorylated tau (PHF-1, CP13) in the mouse model of tauopathy. N = 3, $**p < 0.01$, Bar: ± SD (A). ROCK inhibitor (fasudil) also reduced phosphorylated tau (PHF-1) in Tris-insoluble sarkosyl soluble (SN2) and sarkosyl-insoluble fraction (S/P). N = 3, $*p < 0.05$, Bar: ± SD (B). ROCK inhibitor reduced the amount of oligomeric high molecular weight (>250 kDa) total tau (Tau5), phosphorylated tau (PHF-1), and C-terminal-truncated tau (TauC3) under nonreducing conditions of lysate. N = 3, $**p < 0.01$, Bar: ± SD (C). The ROCK inhibitor (fasudil) reduced the amount of oligomeric tau detected by TOC1. N = 4, $**p < 0.01$, Bar: ± SD (D). Data from PHF-1, and CP13 in lysate, PHF-1 in SN2 and S/P fraction, Tau5, PHF-1, and TauC3 in high molecular weight tau in nonreducing condition, and TOC1 dot blot followed a normal distribution and were analyzed with Student's *t*-test,
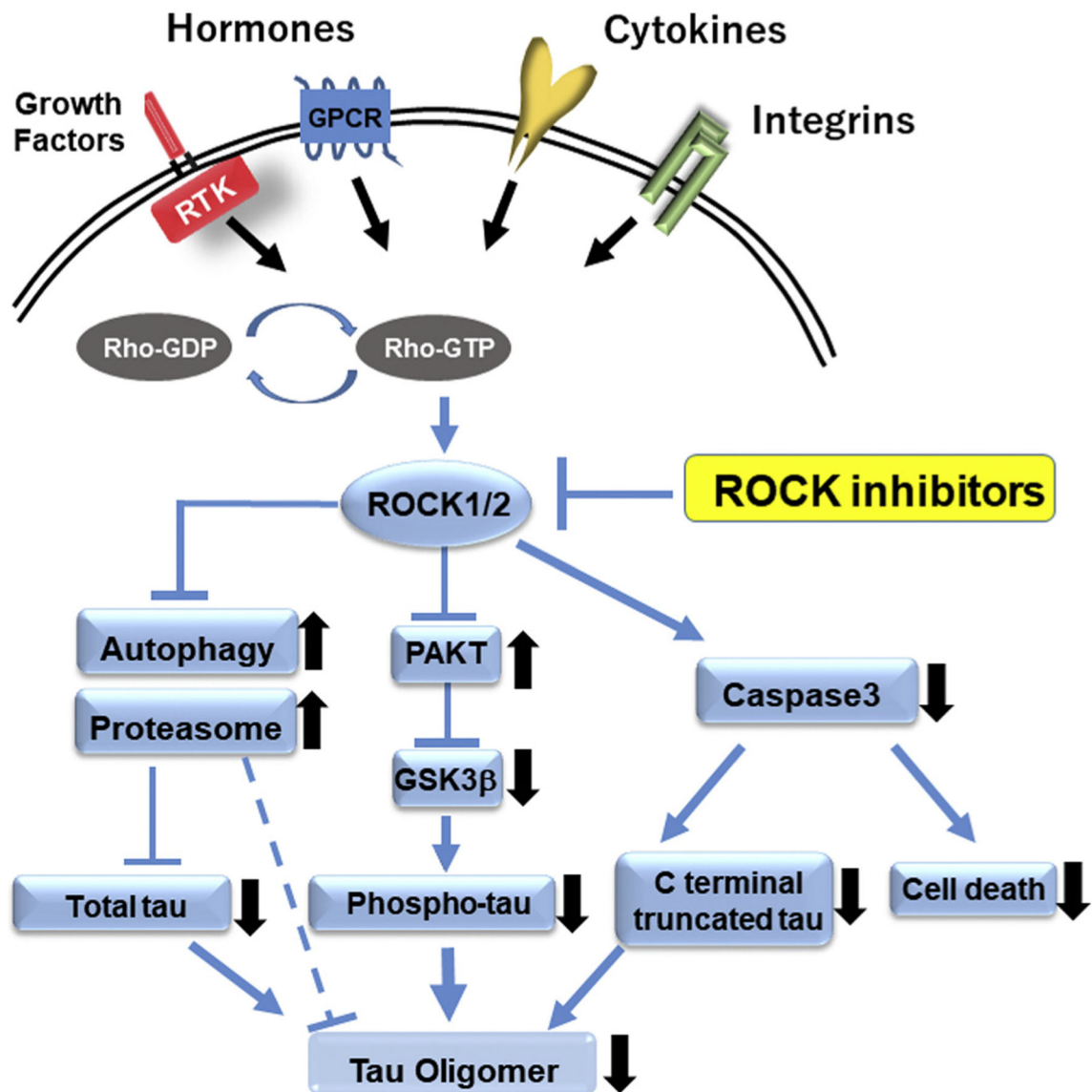
whereas Tau5 in lysate was analyzed with the Mann-Whitney *U*-test because the data deviated from a normal distribution. Abbreviation: ROCK, Rho-associated coiled-coil protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TOC1, Tau Oligomer Complex I antibody.

Fig. 11.
Proposed mechanisms by which ROCK inhibitors reduce the amount of oligomeric tau. ROCK inhibitors inactivated tau kinases, including GSK3β, via AKT activation. ROCK inhibitors also inactivated caspase, and decreased the C-terminal truncation of tau and formation of tau oligomers, which are 2 forms of tau linked to toxicity. ROCK inhibitors also upregulated autophagy and proteasome and reduced total tau levels. Abbreviations: GSK3β, glycogen synthase kinase 3β; ROCK, Rho-associated coiled-coil protein kinase.