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Inhibition of Death-associated Protein Kinase 1 Attenuates *Cis* P-tau and Neurodegeneration in Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is the leading cause of mortality and disability in young people and may lead to the development of progressive neurodegeneration, such as that observed in chronic traumatic encephalopathy. We have recently found that the conformation-specific *cis* phosphorylated form of tau (*cis* P-tau) is a major early driver of neurodegeneration after TBI. However, not much is known about how *cis* P-tau is regulated in TBI. In this study, we demonstrated a novel critical role of death-associated protein kinase 1 (DAPK1) in regulating *cis* P-tau induction after TBI. We found that DAPK1 is significantly upregulated in mouse brains after TBI and subsequently promotes *cis* P-tau induction. Genetic deletion of DAPK1 in mice not only significantly decreases *cis* P-tau expression, but also effectively attenuates neuropathology development and rescues behavioral impairments after TBI. Mechanistically, DAPK1-mediated *cis* P-tau induction is regulated by the phosphorylation of Pin1 at Ser71, a unique prolyl isomerase known to control the conformational status of P-tau. Furthermore, pharmacological suppression of

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Author Contributions

N.K. designed the studies, performed the experiments, analyzed the data, and wrote the manuscript. B.W., K.K., Y.N., and C.Q. helped with TBI experiments and provided technical assistance. T.H.L. and X.Z.Z. conceived and supervised the project, designed the studies, analyzed the data, and wrote the manuscript. All authors reviewed the manuscript.

†These authors jointly supervised this work.

Conflict of Interest

X.Z.Z. and Kun Ping Lu are inventors of *cis* P-tau antibody technology, which was licensed by BIDMC to Pinteon Therapeutics. Both X.Z.Z. and K.P.L. are founders of and own equity in Pinteon. Their interests were reviewed and are managed by BIDMC in accordance with its conflict of interest policy. Other authors do not have any competing interests.

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DAPK1 kinase activity dramatically decreases the levels of Pin1 phosphorylated at Ser71 as well as *cis* P-tau after neuronal stress. Thus, DAPK1 is a novel regulator of TBI that, in combination with its downstream targets, has a major impact on the development and/or outcome of TBI, and targeting DAPK1 might offer a potential therapeutic impact on TBI-related neurodegenerative diseases.

Keywords

cis phosphorylated tau (*cis* P-tau); cistauosis; death-associated protein kinase 1 (DAPK1); Pin1; traumatic brain injury (TBI)

1. Introduction

Traumatic brain injury (TBI) is a major cause of death and disability in young people under the age of 45 (Ghajar, 2000; Werner and Engelhard, 2007). More than 50 million people worldwide suffer TBI each year, and approximately half of the world's population is estimated to experience at least one TBI in their lifetimes (Maas et al., 2017). In the United States, TBI is one of the most important public health issues, and approximately 5.3 million people have TBI-related disabilities and sequelae (Langlois et al., 2006). TBI patients are typically classified into the broad categories of mild, moderate or severe, and the severity is determined by the evaluation of coma and loss of consciousness (Saatman et al., 2008). The most common type of head injury is a concussion or mild TBI (mTBI) that occurs during contact sports, such as American football and boxing, or recreation (Mez et al., 2017; Selassie et al., 2013). Repeated mild traumatic brain injury (rmTBI) is a major risk factor for chronic traumatic encephalopathy (CTE), which is associated with psychiatric alterations, such as memory and mood disorders (Aungst et al., 2014; Hay et al., 2016; Mez et al., 2017; Washington et al., 2016). Similarly, single moderate to severe TBIs (ssTBIs), which are seen in motor vehicle accidents or military explosions, is also associated with neuropsychiatric symptoms and cognitive disability, which may increase the risk of chronic neurological dysfunction (Albayram et al., 2017; Fleminger et al., 2003; Kondo et al., 2015; Rosenfeld et al., 2012; Stocchetti et al., 2017).

In most cases, the chronic sequelae of TBI include the general neuropathological features and clinical symptoms of classically defined neurodegenerative diseases (Crane et al., 2016; Washington et al., 2016; Wilson et al., 2017). A neuropathological signature in patients with CTE is extensive neurofibrillary tangles, which is also a hallmark of Alzheimer's disease (AD) and other related neurodegenerative disorders, commonly known as tauopathies (Blennow et al., 2012; DeKosky et al., 2013; Goldstein et al., 2012; McKee et al., 2013; Omalu et al., 2005; Smith et al., 2013). Tauopathies are characterized by abnormally hyperphosphorylated tau that aggregates into bundles of filaments (Grundke-Iqbal et al., 1986; Iqbal et al., 2010). In the brains of AD patients, tau is 3 to 4 times more hyperphosphorylated than in the brains of normal subjects, and it is polymerized into paired helical filaments (PHFs) with straight filaments, forming neurofibrillary tangles (NFTs) (Arriagada et al., 1992; Iqbal et al., 2010). The pathology associated with neurodegenerative diseases occurs years or decades after TBI, but the underlying mechanisms leading to

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chronic neurodegeneration are unclear. In addition, there are currently no treatments to prevent neurodegenerative diseases, such as CTE or AD.

The unique prolyl isomerase Pin1 catalyzes pathogenic *cis* to physiological *trans* conversions, especially at the phosphorylated Thr231-Pro motif in tau (Lu et al., 2016). As a precursor of tau-induced pathological changes, *cis* phosphorylated tau (*cis* P-tau) is an early driver of the neurodegeneration associated with AD, CTE and TBI (Kondo et al., 2015; Lu et al., 2016; Albayram et al., 2017). We have previously shown that *cis* P-tau was prominently induced by TBI within a few hours in mice or in response to neurological stress *in vitro*, which led to the pathological spread of *cis* P-tau, which is termed cistauosis (Kondo et al., 2015; Lu et al., 2016). Cistauosis mediates apoptotic neuronal cell death through the disruption of the axonal microtubule network and mitochondria transport system and the spread of pathological tau to other neurons (Kondo et al., 2015; Lu et al., 2016). Eventually, cistauosis leads to extensive tau-mediated neurodegeneration and atrophy in the brain (Kondo et al., 2015; Lu et al., 2016). Cistauosis is effectively improved by treatment with a monoclonal *cis* P-tau antibody (Kondo et al., 2015; Albayram et al., 2017). In a previous study, a *cis* P-tau antibody prevents neuronal cell death and progressive neurodegeneration after TBI by targeting intracellular *cis* P-tau for proteasomal degradation and preventing extracellular *cis* P-tau from spreading to other neurons (Kondo et al., 2015; Lu et al., 2016; Albayram et al., 2017). Although this *cis* P-tau antibody is being evaluated in clinical trial for the treatment, diagnosis, and prevention of neurodegenerative disease (clinical trial NCT04096287), the upstream mechanisms underlying *cis* P-tau induction are still unknown.

Death-associated protein kinase 1 (DAPK1), as a calcium/calmodulin (Ca^{2+}/CaM)-dependent serine/threonine (Ser/Thr) kinase, has a critical function in the regulation of neuronal cell death (Bialik and Kimchi, 2006; Chen et al., 2019; Kim et al., 2019; Singh et al., 2016). Overexpression of DAPK1 results in neuronal cell death, whereas lack of DAPK1 expression is resistant to apoptotic stimulation in neuron (Bialik and Kimchi, 2006; Fujita and Yamashita, 2014; You et al., 2017). Moreover, DAPK1 induces neuronal cell death in response to pathological conditions through the N-methyl-D-aspartate (NMDA) receptor (Fujita and Yamashita, 2014; Martin and Wang, 2010; Tu et al., 2010). A genome-wide association study demonstrated that genetic modification of DAPK1 is significantly associated with late-onset AD (Li et al., 2008; Li et al., 2006). Interestingly, the DAPK1 levels are highly increased in the brains of AD patients compared with the brains of age-matched normal subjects (Chen et al., 2020; Kim et al., 2014; Kim et al., 2016a). Moreover, DAPK1 promotes microtubule destabilization and neuron differentiation, which lead to tau toxicity and neurodegeneration (Wu et al., 2011). DAPK1 overexpression increases tau stability and induces various phosphorylation sites of tau associated with AD, particularly Thr231, Ser262, and Ser396, in neurons (Duan et al., 2013; Kim et al., 2014; Kim et al., 2019). Although DAPK1 is a potential molecular target for neuronal cell death and AD, the role of DAPK1 in TBI has not yet been determined.

Here, we comprehensively assessed the impact of DAPK1 on the pathogenesis of TBI caused by closed-head injury. In this study, we found that DAPK1 expression is significantly enhanced by TBI via axonal injury in the brain. Mechanistically, we demonstrated that the DAPK1-dependent regulation of *cis* P-tau and hyperphosphorylated tau is mediated by its

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phosphorylation on Pin1 at Ser71, leading to Pin1 inactivation. Furthermore, DAPK1 downregulation prevents tau-related pathologies in neurons and improves functional brain damage following TBI. Consequently, DAPK1 may represent a potential novel therapeutic target for the treatment of acute and chronic neurodegenerative diseases, including TBI and AD.

2. Materials and methods

2.1. Reagents and antibodies

The DAPK1 inhibitor (4Z)-4-(3-pyridylmethylene)-2-styryl-oxazol-5-one was purchased from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS), penicillin-streptomycin, neurobasal-A medium B27 supplement and GlutaMAX supplement were acquired from Thermo Fisher Scientific (Waltham, MA, USA). The antibodies used in the current study are listed in Supplemental Table S1.

2.2. Traumatic brain injury model

Male C57BL/6 mice were acquired from Jackson Laboratories (Bar Harbor, ME, USA). DAPK1 KO mice were described previously (Gozuacik et al., 2008; Kim et al., 2014; You et al., 2017). The mice were housed (12:12-h light-dark cycle) for at least 1 week before the TBI experiments. The mouse model of closed-head TBI was established by weight drop as previously described (Flierl et al., 2009). Briefly, the mice were anaesthetized for 25-50 s using 4% isoflurane in a 70:30 mixture of air:oxygen. The anaesthetized mice were placed on a Kimwipe (Kimberly-Clark, Irving, TX, USA) and positioned so that the center of the head was placed directly below a hollow glass tube. A metal weight of 54 grams was used to impact on the dorsal aspect of the skull, causing rotational acceleration of the head. The sham mice were anesthetized but were not subjected to impact. All the mice were allowed to recover in room air. All these and the subsequent animal experiments were approved by Beth Israel Deaconess Medical Center Institutional Animal Care & Use Committee (IACUC) and complied with the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Immunostaining analysis

For the immunohistochemical analysis of the mouse brain tissues, the mice were anesthetized and intracardially perfused with cold PBS. The mouse brains were fixed with 4% paraformaldehyde/PBS at 4 °C for 24 h. The fixed tissues were embedded in paraffin. The paraffin sections (5 μm thick) of the brain were deparaffinized with histoclear (National Diagnostics, Atlanta, GA, USA) and rehydrated with descending grades of ethanol. The slides were briefly boiled in antigen unmasking solution (Vector, Burlingame, CA, USA), for antigen enhancement. After antigen retrieval, the sections were incubated in a blocking buffer containing PBS, 10% FBS or 1% gelatin and 0.1% v/v Tween 20. For the immunocytochemistry, cells were washed twice with ice-cold PBS and fixed with 4% formaldehyde/PBS. The cells were permeabilized with 0.1% Triton X-100 and PBS for 10 min at room temperature. The primary antibodies were added to the blocking buffer and incubated with the samples overnight at 4 °C. For double immunofluorescence staining, the samples were incubated with Alexa Fluor 488- or 568-conjugated isotype-specific secondary antibodies (Thermo Fisher Scientific) and Hoechst (MilliporeSigma, Burlington, MA, USA)

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for 1 h at room temperature. The samples were washed four times with PBST or PBS after each step. The stained sections were visualized with a Keyence microscope (Osaka, Japan) or Zeiss confocal microscope (Ostalbkreis, Baden-Württemberg, Germany). The immunostaining images were analyzed and colocalization was quantified using Fiji/ImageJ Coloc 2.

2.4. Cell culture

The human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (Manassas, VA, USA). The SH-SY5Y cells were cultured in Gibco Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific). The cultures were maintained at 37 °C under 5% CO_2 in a humidified atmosphere. Primary neurons were prepared from the cerebral cortex of early postnatal (P0-P1) mouse brains. The neurons were cultured on culture dishes precoated with poly-D-lysine (MilliporeSigma) in DMEM (high glucose) supplemented with 10% FBS, 1% GlutaMAX supplement, 2.5% HEPES, 10% Nutrient Mixture F12 HAM (Millipore Sigma), and 0.25% penicillin/streptomycin at 37 °C under 5% CO_2. After adaptation for 2 h, the culture medium was replaced with neurobasal-A medium containing 2% B27 supplement, 1% GlutaMAX supplement and 1% penicillin/streptomycin. The SH-SY5Y cells and primary cortical neurons were pretreated with a DAPK1 inhibitor (0.1-0.5 μM) for 48 h, and cell stress was induced under normoxic or 0.5% oxygen conditions for 48 h at DIV 10-12.

2.5. Immunoblotting analysis

Brain tissues or cultured cells were lysed in RIPA buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl) containing protease and phosphatase inhibitor cocktails (MilliporeSigma). The soluble protein concentration was determined by the Bradford (Bio-Rad, Hercules, CA, USA). After separation with SDS-polyacrylamide gels, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (PerkinElmer, Waltham, MA, USA). The membranes were incubated with various primary antibodies at 4 °C overnight, after which they were washed three times with Tris-buffered saline (TBS) containing 1% Tween-20. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad). The blots were visualized using the Western Lightning Plus-ECL reagent (PerkinElmer). Quantification was performed by densitometry using Fiji/ImageJ Coloc 2.

2.6. Elevated plus maze

The elevated plus maze was used to assess anxiety/risk-taking behavior after TBI. The elevated plus maze device (Lafayette Instruments, Lafayette, IN, USA) consists of two open and two closed arms (30 × 5 cm) extending out from a center point (decision zone) to form a plus shape. The entire device is raised 58 cm above the floor. A mouse was placed at the center of the plus maze, facing an open arm, and allowed it to explore the device for 5 min. The maze was washed with 70% ethanol and dried between each mouse experiment. A computer-assisted video-tracking system (Noldus Ethovision XT) was used to record the total time spending in two closed arms as ‘safe’ decision, the two open arms as risk-taking

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and the center as decision making. The percent time spent in the open arms was used anxiety/risk-taking behavior.

2.7. T maze

The T maze was used to test spatial memory after mouse brain injury. The T maze apparatus is composed of a T shape with three closed arms, including the start, left and right arms, separated by 90-degree angles. The stem of the maze is 38 cm long, the arms are 27.5 cm long each, and the walls are 16.5 cm high. The width of the alleys is 8 cm. The T maze test included training and testing sessions. During the training session, a mouse was placed in the start arm and allowed it to randomly explore all three arms for 5 min. After a day, the test session was performed. First, when a mouse entered the right arm, the right door was closed, and the mouse was left for 10 s. After a 3-5 min delay, the mouse was placed in the start arm again and allowed it to choose either the left or the right goal arm. The T maze test is an experiment based on the tendency of a mouse to explore novel places. Therefore, in general, entering the left arm was considered as the right choice. This test was performed up to three times a day, and the testing results were analyzed as previously described (Rodriguez et al., 1992; Szelest and Cohen, 2006).

2.8. Y maze spontaneous alternation test

The Y maze was used to test hippocampal-dependent spatial leaning and working memory (short-term) for the mice subjected to TBI as previously described (Holcomb et al., 1998; Tchantchou and Zhang, 2013). The Y maze device has a symmetrical Y shape with three arms that are 38 cm long, 8 cm wide, and 16.5 cm high separated by 120-degree angles. The mouse was placed at the end of an arm and allowed it to freely explore all three arms of the maze for 5 min. The sequence and number of entries into each arm were tracked and recorded with a video camera (Noldus Ethovision). The test session takes advantage of the innate tendency of the mouse to explore novel, unexplored areas, and each alternation was defined as consecutive entry into three different arms (Canas et al., 2009; Dauge et al., 2001; Tchantchou et al., 2004). The percentage of alternations was calculated based on the previously described formula: total number of alternations/(total number of arm entries - 2) × 100 (Maurice et al., 1996).

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism v.7 software. All the experiments were repeated with at least three independent replicates, and the number of repeats was increased according to effect size or sample variation. In the case of ssTBI, approximately 20% of the mice died immediately after brain damage. All the surviving mice were analyzed except the dead mice. The data are presented as the mean ± SD or SEM of individual experiments. Statistical significance was determined by two-tailed Student’s t-test for quantitative variables, one-way ANOVA for continuous or multiple independent variables or one-way ANOVA with Tukey’s multiple comparisons test. The difference between mean values was considered statistically significant at $p < 0.05$.

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3. Results

3.1. TBI induces cis P-tau and DAPK1 in mouse brains

A previous study has shown that *cis* P-tau is robustly induced in neurons under stress *in vitro* and after TBI in mice (Kondo et al., 2015). After injury, apoptosis and the spread of tau in neurons as well as the destruction of the axonal microtubule network are driven by *cis* P-tau (Kondo et al., 2015). However, the upstream mechanisms underlying *cis* P-tau induction are still unknown. Since DAPK1 expression is increased by neuronal injury and promotes tau phosphorylation, including at Thr231, we examined the potential role of DAPK1 in *cis* P-tau induction in TBI. (Kim et al., 2019; Pei et al., 2015).

For this purpose, we conducted closed-head TBI experiments on 10- to 11-week-old C57BL/6 mice, where ssTBI was induced by a single drop of a weight of 54 g from a height of 60 inches, while rmTBI was induced by five daily drops of a weight of 54 g from a height of 32 inches on a point near the center of the mouse head (Fig. 1A). After ssTBI, the mice were stunned and took a longer time to wake up than the mice subjected to rmTBI. ssTBI almost caused convulsive symptoms and decreased movement, but the skull was not fractured. First, we validated the total Thr231 phosphorylation of tau using a non-conformation-specific monoclonal antibody. Interestingly, total Thr231 phosphorylation of tau was increased in the cerebral cortex (Fig. 1B, C, Supplemental Fig. S1A). To test the effect of TBI on tau Thr231 phosphorylation in the *cis* conformation, we used *cis* P-tau specific mouse monoclonal antibody. The *cis* P-tau levels were robustly increased 48 h after ssTBI ($p = 1.517 \times 10^{-16}$) and rmTBI ($p = 3.458 \times 10^{-5}$) in the cortical regions (Fig. 1D, E, Supplemental Fig. S1B, 2A, B). Consisting with the immunostaining results, the *cis* P-tau and P-tau (Thr231) levels were increased after TBI damage as shown by the immunoblotting assay in a time-dependent manner (Fig. 1G, H, Supplemental Fig. S2C, D).

To examine whether TBI affects DAPK1 expression, we first detected DAPK1 expression after TBI using immunostaining and found that DAPK1 was significantly increased in the cerebral cortex after ssTBI or rmTBI (Fig. 1D, F, Supplemental Fig. S1C, 3A, B). In addition, we investigated the time-dependent protein expression of DAPK1 after ssTBI. In the cerebral cortex, DAPK1 showed a trend of increasing expression beginning at 24 h ($p = 0.485$), and its expression was robustly increased at 48 h ($p = 5.999 \times 10^{-5}$) (Fig. 1G, H). The DAPK1 expression showed the similar pattern to the *cis* P-tau expression that both were induced and able to colocalized in neurons by ssTBI (Fig. 1D). Moreover, rmTBI induced a marked increase in DAPK1 protein expression in the cerebral cortex immediately after the fifth injury (Supplemental Fig. S3C, D). Thus, these results suggest that TBI induces DAPK1 protein accumulation along with increased *cis* P-tau levels in the cerebral cortex.

3.2. DAPK1 promotes the phosphorylation of cis tau after TBI

To evaluate whether DAPK1 has any effects on neuronal injury after TBI in mouse brains. We first examined the expression level of microtubule-associated protein 2 (MAP2), a sensitive neuronal damage maker, in wild type (WT) and DAPK1 knockout (KO) mice 48 h after TBI. We found that MAP2 was decreased significantly in WT mice, but not in DAPK1 KO mice (Supplemental Fig. S4A, B), indicating that DAPK1 KO may prevent neurons from

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damage in mice after TBI. Moreover, the above results show that TBI induces simultaneously the pathological *cis* tau and the expression of DAPK1 in the mouse brains (Fig 1), we investigated whether DAPK1 regulates *cis* P-tau expression after TBI. Interestingly, the phosphorylation of tau at Thr231 (P-tau (Thr231)), representing a total phosphorylated tau without conformation specificity, was prominently increased 48 h after TBI in WT mice, but not in DAPK1 KO mice (Fig. 2A, B, I, J). After TBI at 2 months, the level of P-tau (Thr231) in the WT mice was increased more significantly compared to that at 48 h time point (Fig. 2C, D), while DAPK1 deletion mice displayed no P-tau (Thr231) induction with its level similar to WT or DAPK KO sham mice (Fig. 2C, D). More importantly, the conformation specific phosphorylation of tau at Thr231, a pathological tau epitope, *cis* P-tau was unable to induce in the DAPK1 KO mice after TBI at 48 h and 2 months (Fig. 2E-J). In contrary, the pathological *cis* P-tau was dramatically increased at 48 h, and further accumulated at 2 months after TBI in the WT mice (Fig. 2E-J), consistent with our previous findings (Kondo et al., 2015). These results indicate that TBI induces neuronal damage, the phosphorylation of tau, particularly the pathological *cis* P-tau through DAPK1 activation and that the downregulation of DAPK1 may have a protective effect against the axonal damage induced by TBI.

3.3. TBI induces the phosphorylation of Pin1 at Ser71 through DAPK1

Given that DAPK1 KO abolishes *cis* P-tau induction in TBI mice, a critical question is how DAPK1 regulates *cis* P-tau induction after TBI. Notably, we have previously shown that DAPK1 plays an important role in negatively regulating the activity of Pin1, which in turn controls conformational changes of phosphorylated Ser/Thr-Pro motifs, including pT231-Pro motif in tau (Albayram et al., 2017; Lu and Zhou, 2007; Zhou and Lu, 2016) . Pin1, a unique peptidyl-prolyl *cis-trans* isomerase (PPIase), is associated with various cellular processes, including the progression of the cell cycle, inflammation, neuron function and apoptosis (Lee et al., 2011b; Lu and Zhou, 2007). Notably, deregulation of Pin1 affects a variety of diseases, such as age-related diseases, especially AD (Lee et al., 2011b; Lu and Zhou, 2007). DAPK1 catalytic activity has been shown to be responsible for the phosphorylation of Pin1 at Ser71, which negatively regulates the activity of Pin1 (Kim et al., 2019; Lee el al., 2011a). Therefore, we speculated that DAPK1 may regulate *cis* P-tau via Pin1 phosphorylation in response to the axonal injury caused by TBI. To study the molecular mechanisms by which DAPK1 regulates *cis* P-tau, we investigated the phosphorylation of Pin1, specifically at Ser71 using a pSer71-specific Pin1 antibody that we developed and characterized previously (Lee et al., 2011a). Although Pin1 expression didn't change in both WT and DAPK1 KO mice after TBI (Fig. 2E-H, Supplemental Fig. S5A-D), the phosphorylation of Pin1 at Ser71 (pSer71-Pin1), like the pathological *cis* P-tau, was significantly induced at 48 h, and accumulated at 2 months in WT mice after TBI (Fig. 2K-N). In contrast, such induction was abolished in the DAPK1 KO mice at 48 h and 2 months after TBI (Fig. 2K-N), indicating that the *cis* P-tau induction linked the phosphorylation of Pin1. Thus, these results demonstrate that DAPK1 mediates Pin1 phosphorylation to inhibit its isomerization activity, thereby increasing *cis* P-tau levels after TBI.

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3.4. DAPK1 deletion prevents neuropathological features after TBI

We investigated whether TBI-mediated secondary pathologies are associated with DAPK1 in neurons. TBI initially causes mechanical damage to the brain, including neurons, axons, glia, and blood vessels (Kabadi and Faden, 2014; Schimmel et al., 2017). Primary damage mediates multiple biochemical cascades that, in turn, cause secondary cell damage, such as neuroinflammation and neurodegeneration, in the brain (Schimmel et al., 2017; Sullivan et al., 1999). Glial fibrillary acidic protein (GFAP), an astroglial activation and gliosis marker, was gradually increased from 12 h to 48 h in the cerebral cortex of the WT mice after ssTBI (Supplemental Fig. S6). However, GFAP expression was significantly suppressed in the DAPK1 KO mice subjected to TBI (Fig. 3A, B). We also examined microglial activation, which plays a critical role in neuroinflammation during brain injury with diffuse axonal damage (Xiong et al., 2018). Inhibited activation of the TBI-induced ionized calcium-binding adaptor molecule 1 (Iba-1), a common marker of microglial activation, was observed to be also suppressed in the DAPK1 KO mice subjected to TBI (Fig. 3E, F). We next asked whether DAPK1 affects TBI-induced secondary neuronal damage in deeper regions of the brain following cerebral cortex damage. In the hippocampus, GFAP and Iba-1 expression was markedly decreased in the DAPK1 KO mice compared to the WT mice at 2 months after TBI (Fig. 3C, D, G, H), indicating that DAPK1 not only prevents neuroinflammation in the cortex during acute TBI, but also inhibits neuroinflammation in other brain regions later after TBI.

To examine the effects of DAPK1 on the development of tauopathy, we investigated the consequences of the chronic stage of TBI in DAPK1 KO mice. To detect tauopathy, we immunostained mouse brains with a tau oligomer-specific antibody (T22). In contrast to sham-treated WT mice, T22 staining was highly elevated in the cortical regions of WT mice subjected to ssTBI after 2 months (Fig. 4A, B). In the chronic stage at 6 months after ssTBI, WT mice displayed not only prominent tau oligomerization in deeper brain regions (Fig. 4C, D), but also significant neuronal loss detected by immunofluorescence staining with neuronal nuclear marker (NeuN) (Supplemental Fig. S4C, D). Remarkably, the DAPK1 KO mice exhibited little tau oligomerization at both 2 and 6 months after TBI (Fig. 4A-D). More importantly, DAPK1 KO prevented TBI mice even at chronic stage from neuronal loss (Supplemental Fig. S4C, D). In addition, tau phosphorylation at specific epitopes related to neurodegenerative disease was also examined to evaluate the chronic stage of TBI mice. Antibodies detecting early tau tangles (AT8 and AT100) were used to show that the DAPK1 KO mice exhibited remarkable diminished tau pretangle formation that were comparable to the sham mice, while the WT mice showed high levels of AT8 and AT100 staining 6 months after ssTBI (Fig. 4E-H). Thus, DAPK1 KO prevents TBI mice from tauopathy development and pretangle tau formation.

3.5. DAPK1 deletion rescues TBI-related behavioral and memory impairments

Next, we evaluated the effects of DAPK1 on the behavioral or functional outcomes associated with neurodegeneration after TBI. To examine the behavioral change in TBI mice, we used an elevated plus maze apparatus to evaluate anxiety/risk-taking behaviors in mice (Adhikari et al., 2011; Kondo et al., 2015). All the groups spent similar amounts of time in the decision arm or center point either at 2 months after ssTBI or 10 days after

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rmTBI (Fig. 5A, B, Supplemental Fig. S7A, B). Among WT mice, the sham mice stayed mainly in the two closed arms, which are considered as safe areas, but the mice subjected to TBI spent 3 times longer in both open arms, indicating increase risk-taking behavior (Fig. 5A, B, Supplemental Fig. S7A, B), as shown previously (Kondo et al., 2015). In contrast, both the sham-treated DAPK1 KO mice and those DAPK1 KO mice subjected to TBI showed minimal risk-taking behavior patterns (Fig 5A, B, Supplemental Fig. S7A, B), indicating that DAPK1 KO averts risk-taking behavior in TBI mice.

In general, memory impairment is one of the most common symptoms of patients in the early stages of neurodegenerative diseases (Morris et al., 2013; Panegyres, 2004; Sandry, 2015). To test spatial memory, we used the T maze for TBI mice at the intermediate and chronic stages. In both stages, WT mice subjected to TBI exhibited spatial memory deficits compared to the sham-treated WT mice (Fig. 5C, D, Supplemental Fig. S7C). Moreover, the mice subjected to TBI showed impaired spontaneous alteration both at 3.5 and 5 months after TBI, examined by Y maze apparatus to test hippocampal-dependent spatial leaning and working memory (Fig. 5E, F, Supplemental Fig. S7D). Strikingly, the DAPK1 KO mice rescued behavioral changes associated with spatial learning and memory defect examined by the T and Y mazes (Fig. 5C-F, Supplemental Fig. S7C, D). These results demonstrate the potential beneficial effect of DAPK1 inhibition in preventing the development of pathological and functional outcomes after TBI.

3.6. Pharmacological inhibition of DAPK1 suppresses hypoxic stress-induced cis P-tau in neuronal cells

Given the dramatic beneficial effects of DAPK1 KO on the induction of *cis* P-tau and the development of pathological and functional outcomes after TBI, we next aimed to examine whether the pharmacological inhibition of DAPK1 kinase activity attenuates the expression of *cis* P-tau in neuronal cell models. In this context, cellular stress was induced with two conditions, namely, serum starvation and 0.5% hypoxia, in the SH-SY5Y neuroblastoma cell line and primary neurons, as we described previously (Kondo et al., 2015). We chose (4Z)-4-(3-pyridylmethylene)-2-styryl-oxazol-5-one because it has been shown to efficiently and selectively inhibits the DAPK1 enzymatic activity (Okamoto et al., 2009). We previous showed this chemical specifically inhibits DAPK1 activity and suppresses DAPK1-mediated neuronal death (You et al., 2017). Under serum starvation and 0.5% hypoxic conditions, DAPK1 expression in SH-SY5Y neuroblastoma cells was significantly increased compared to that observed under normoxic conditions at 48 h (Fig. 6A, B). Furthermore, we confirmed that DAPK1 was highly expressed in the primary mouse cortical neurons exposed to hypoxia at 48 h (Fig. 6C, D).

Moreover, the levels of *cis* P-tau and phosphorylated Pin1 at Ser71 (pSer71-Pin1) were also increased under cell stress conditions in both the SH-SY5Y cells and primary neurons (Fig. 6E-L). In contrast to untreated cell, the DAPK1 inhibitor reduced the levels of *cis* P-tau and phosphorylated Pin1 at Ser71 in a dose-dependent manner in SH-SY5Y cells (Fig. 6E-H). Interestingly, the phosphorylation of Pin1 at Ser71 was effectively reduced even at a low concentration (0.1 μM) of the DAPK1 inhibitor (Fig. 6G, H). In the primary neurons, the induction of *cis* P-tau and phosphorylated Pin1 at Ser71 by hypoxia was also blocked by the

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DAPK1 inhibitor (Fig. 6I-L). These results suggest that inhibition of DAPK1 may have potential as a therapeutic strategy for treating TBI.

4. Discussion

Although cumulative evidence suggests that DAPK1 may play key roles in neuronal function under stress, the underlying mechanisms are unknown, and studies related to the overall impact of TBI are rare. In this study, we discovered that DAPK1 critically regulates TBI-related pathology by increasing *cis* P-tau levels, thereby increasing tau hyperphosphorylation and tauopathy in TBI. First, DAPK1 protein is highly expressed in the cerebral cortex in a time-dependent manner in mice subjected to either ssTBI or rmTBI. Second, DAPK1 deletion dramatically reduces the P-tau (T231) and *cis* P-tau levels in the brain after TBI. Third, the DAPK1-mediated induction of *cis* P-tau is regulated by the inhibition of Pin1 activity by its phosphorylation at Ser71, which is unable to regulate the conformational status of P-tau. Fourth, DAPK1-KO attenuates TBI-related pathologies, including neuroinflammation, tau oligomerization, and pretangle formation. Fifth, DAPK1 deletion prevents TBI-mediated anxiety and memory impairments. Finally, inhibiting DAPK1 with a pharmacological DAPK1 inhibitor significantly decreases the *cis* P-tau and phosphorylated Pin1 levels. Thus, these results provide the most direct evidence for an essential role of DAPK1 in regulating the levels and cellular functions of *cis* P-tau in TBI and suggest that DAPK1 might be a novel therapeutic target for treating TBI and preventing TBI-related neurodegeneration.

Compelling studies suggest that *cis* P-tau is an early driver of neurodegeneration after closed-head injury and that there is an important link between TBI and neurodegenerative diseases (Albayram et al., 2017; Kondo et al., 2015; Lu et al., 2016; Nakamura et al., 2012). Pin1, a phospho-specific *cis/trans* prolyl isomerase, has been shown to protect against tauopathies by restoring the conformation and function of phosphorylated tau (Liou et al., 2003; Lu et al., 1999). Pin1 is colocalized with hyperphosphorylated tau in AD and other tauopathies, and this colocalization exhibits an inverse relationship with tau accumulation (Holzer et al., 2002; Liou et al., 2003; Ramakrishnan et al., 2003). DAPK1 is responsible for the phosphorylation of Pin1 at Ser71, thereby inhibiting its cellular function and catalytic activity (Lee et al., 2011a). However, the molecular mechanisms associated with neurodegeneration caused by TBI remain unclear. Herein, we found that DAPK1 is a key modulator of the phosphorylation of Pin1 at Ser71 and continuously induces the phosphorylation of *cis* tau in the brain after brain injury. After neuronal damage, Pin1 phosphorylation is increased, accompanied with increased *cis* P-tau, particularly at the Thr231-Pro motif, which is regulated by Pin1; however, these findings are not observed in DAPK1 KO mice. These findings may indicate that DAPK1 is a critical regulator of Pin1 and *cis* P-tau in brain neurodegenerative diseases caused by TBI.

Primary damage after TBI is caused by physical and mechanical impacts associated with brain damage, including contusion, hemorrhage and axonal damage (Kokiko-Cochran and Godbout, 2018; Maas et al., 2008). Secondary damage, such as excitotoxicity injury, oxidative stress, and widespread neuroinflammation, occurs within hours to days after primary injury, and this cascade simultaneously and synergistically affects the outcome

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(Dorsett et al., 2017; Hall et al., 2010; Kokiko-Cochran and Godbout, 2018; Maas et al., 2008; Simon et al., 2017). Neuroinflammation from TBI is a response of the central nervous system that can be affected by age, gender, genetic factor, location and severity of damage (Simon et al., 2017). Chronic inflammation in neurons is one of the strong risk factors for neurodegenerative diseases associated with progressive pathologies, including atrophy, neuronal loss, and axonal degeneration (Kokiko-Cochran and Godbout, 2018; Soares et al., 1995). Astrocytes are known to be critical markers of multicellular responses to central nervous system trauma and disease. Mechanical forces from TBI initiate astrocyte responsiveness and astrocyte-microglial interactions (Burda et al., 2016). Our results showed that the representative markers of neuroinflammation, namely, GFAP for astrocyte activation and Iba-1 for microglial activation, were acutely increased in the cerebral cortex after TBI. Both markers were observed in a deep brain region, such as the hippocampus, 2 months after injury. However, in the DAPK1 deletion mice, inflammatory responses to the neuronal damage were not observed. Intrinsic tau oligomers induce neurodegeneration by mediating the accumulation of pathogenic tau in human neurons (Usenovic et al., 2015). The accumulation of tau oligomers is associated with the development of cognitive and motor deficiencies during the progression of tauopathy in animal models (Berger et al., 2007). Moreover, the AT8 monoclonal antibody is used to identify tau phosphorylation at Ser202 and Thr205 and detects various tau aggregates, including pretangles, in the brain (Castellani and Perry, 2019). In addition, phosphorylation at Thr212 and Ser214 is detected by the monoclonal antibody AT100, which is a better indicator of more developed tau pathology (Allen et al., 2002). Our results demonstrate that the deletion of DAPK1 effectively prevented the development of tauopathy, as shown by abolishing tau oligomers, and pretangle epitopes 2 to 6 months after TBI. Further studies are required to determine whether long-term inhibition of DAPK1 expression attenuates chronic tauopathies caused by TBIs, including PHF1 and NFTs.

Signs of TBI-mediated neurodegenerative disease include dysarthria, gait disturbance, tremor, cognitive impairment, and, in some cases, dementia (Castellani and Perry, 2019). Among these signs, memory deficits are most common in the early stages of neurodegenerative diseases (Panegyres, 2004; Sandry, 2015). It has been reported that FK506 may inhibit DAPK1 and reduce neuronal apoptosis in diffuse axonal injury (Huang et al., 2017). However, since FK506 also regulates several other downstream targets, including cytokines, BAD, and cytochrome c, the role of DAPK1 in axonal injury was still not known (Dumont, 2000; Huang et al., 2017). We found that the DAPK1 KO mice subjected to TBI exhibited minimal risk-taking behavior and improved short-term memory compared to the WT mice subjected to TBI. Furthermore, a DAPK1 inhibitor that pharmacologically suppresses its kinase activity significantly decreased the levels of *cis* P-tau and phosphorylated Pin1 at Ser71 under cellular stress, such as hypoxia and serum depletion. Since *cis* P-tau induction and tauopathy are important characteristics in TBI, our finding of the effect of DAPK1 deletion in reducing TBI-mediated *cis* P-tau induction and the neuronal loss needs further studies on the roles on DAPK1 inhibition in ameliorating tauopathy via *cis* P-tau downregulation in TBI animal models. Furthermore, it might be interesting to examine whether treatment with a DAPK1 inhibitor reverses the TBI-mediated severe cognitive impairment observed in animal models and whether there is a possible

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association between DAPK1 and *cis* P-tau in human brains following TBI. Furthermore, the intensive cross-talking between TBI and the signals upstream of DAPK1 needs to be studied to determine the underlying mechanisms. Excitatory toxicity caused by NMDA receptors affects acute neurological diseases, such as ischemic stroke, as well as chronic neurodegenerative disease including AD (Chen et al., 2020; Choi, 1988; Parsons and Raymond, 2014). DAPK1 directly binds to NR2B subunit of NMDA receptor and mediates brain injury following ischemic stroke (Tu et al., 2010). In addition, DAPK1 interacts with tau to phosphorylate it, specifically at Ser262, and is involved in neuronal cell loss in a stroke mouse model (Pei et al., 2015). Moreover, DAPK1 mediates phosphorylation of amyloid precursor protein (APP) at Thr668 through c-Jun N-terminal kinase 3 (JNK3) and glycogen synthase kinase-3 beta (GSK-3β) activation (Kim et al., 2016b). Therefore, these mechanisms may have the potential as strong upstream signaling candidates for DAPK1 to induce neuronal damage in TBI.

Taken together, our results support a model in which TBI directly upregulates DAPK1 expression and increases *cis* P-tau levels via increased DAPK1-mediated phosphorylation of Pin1 at Ser71. Inhibition of DAPK1 expression or activity reduces *cis* P-tau accumulation and pretangle formation and rescues TBI-mediated behavioral impairments (Fig. 7). Our work has thus demonstrated a novel role of DAPK1 in the regulation of *cis* P-tau, tau phosphorylation and TBI and may offer a potential novel therapeutic approach for the treatment of human TBI and tauopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Death-associated protein kinase 1 (DAPK1) has a novel critical role in traumatic brain injury (TBI).
- DAPK1 expression is highly increased after TBI and subsequently promotes *cis* P-tau induction via increased DAPK1-mediated phosphorylation of Pin1.
- DAPK1 inhibition prevents TBI-related neuropathological features.
- DAPK1 deletion rescues risk-taking behavior and memory impairments after TBI.
- DAPK1 may provide a potential therapeutic target for TBI-related neurodegenerative disease.

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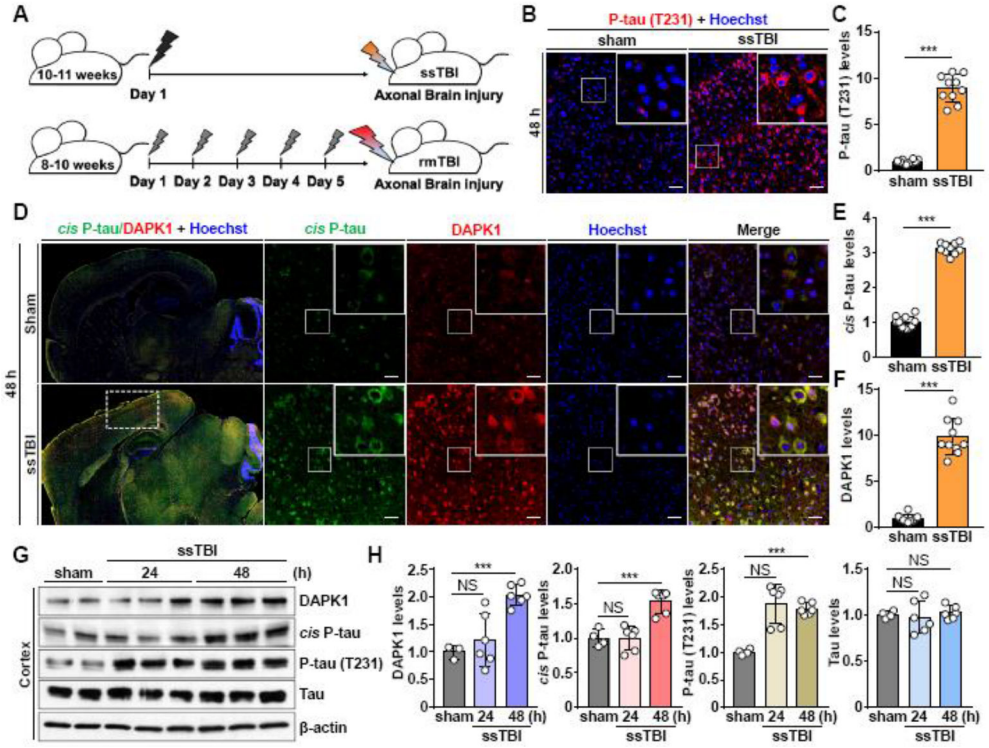

Fig. 1. Severe TBI leads to robust induction of *cis* P-tau, particularly at the Thr231-pro motif, and DAPK1 in the brain.

A. Schematic representation of the experimental setup. Mice were subjected to TBI by weight drop with a weight of 54 grams dropped from a height of 60 inches for ssTBI. B-F. Forty-eight hours after ssTBI, the mouse brains were collected for immunofluorescence staining to detect P-tau (T231) (B), *cis* P-tau (D) and DAPK1 (D). P-tau (T231) and DAPK1, red; *cis* P-tau, green; DNA, blue. Quantification of P-tau (T231) (C), *cis* P-tau (E) and DAPK1 (F) immunostaining; p-values for P-tau (T231) (C), *cis* P-tau (E) and DAPK1 (F) comparisons were calculated using two-tailed Student's t-tests. G. The mouse brains were subjected to immunoblotting 24 h and 48 h after ssTBI. H. The immunoblotting assays to detect DAPK1, *cis* P-tau, P-tau (T231) and tau were quantified and analyzed respectively. The levels of tau and β-actin are presented as the loading controls. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. All the values were combined and are expressed as the mean ± SD (***$p<0.001$). Experiments were performed in triplicate with at least three mice per group per experiment. Scale bar= 50 μm. NS, no significance.

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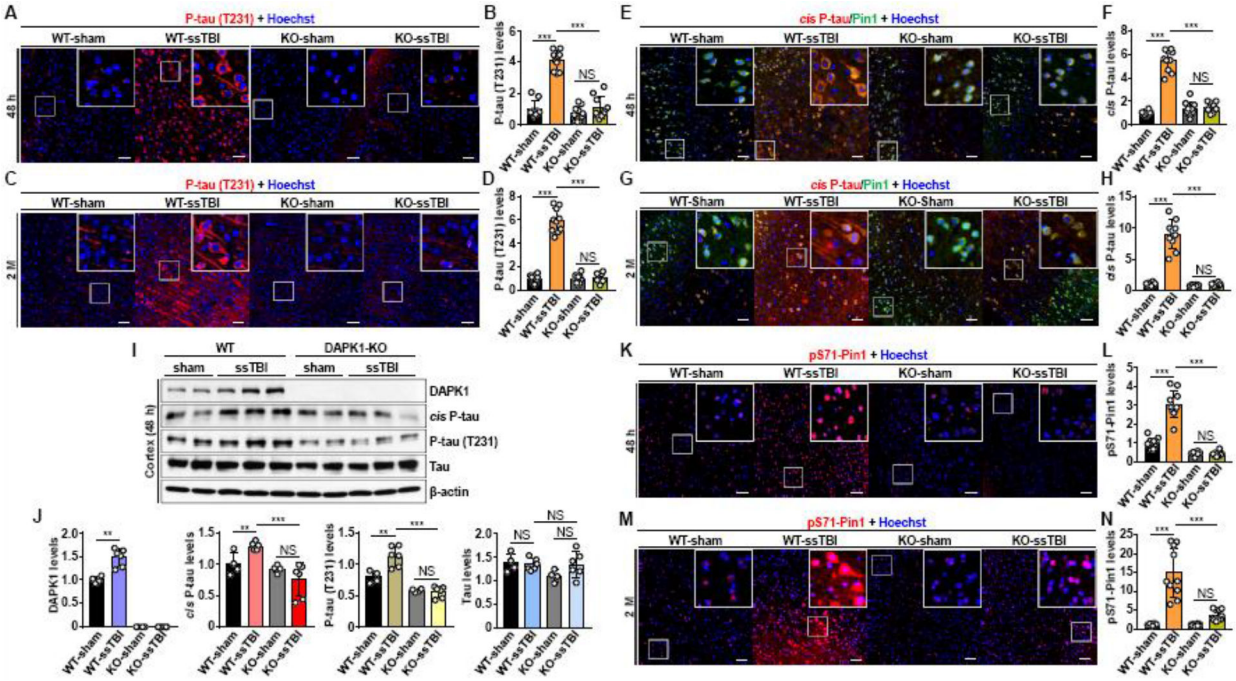

Fig. 2. Severe TBI induces prominent induction of *cis* P-tau and phosphorylated Pin1 through DAPK1.

A-D. Forty-eight hours (A) and 2 months (C) after TBI, WT and DAPK1 KO mouse brains were collected, and P-tau (T231) was detected by immunofluorescence. P-tau (T231), red; DNA, blue. The images of P-tau (T231) immunostaining were quantified and analyzed using one-way ANOVA with Tukey's multiple comparisons test (B, D). E-H. WT and DAPK1 KO mouse brains were collected 48 h (E) and 2 months (G) after injury, and *cis* P-tau and Pin1 were detected by immunofluorescence. *cis* P-tau, red; Pin1, green; DNA, blue. The images of *cis* P-tau immunostaining were quantified and analyzed using one-way ANOVA with Tukey's multiple comparisons test (F, H). I. The mouse cerebral cortex lysates were analyzed by immunoblotting with the indicated antibodies. J. The immunoblotting results were quantified as a ratio of the β-actin housekeeping gene. The p-values of all the representative data were calculated using one-way ANOVA with Tukey's multiple comparisons test. K-N. TBI-injured WT and DAPK1 KO mouse brains were harvested 48 h (K) and 2 months (M) and were analyzed by immunofluorescence with a pS71-Pin1 antibody. pS71-Pin1, red; DNA, blue. Quantification of pS71-Pin1 48 h (L) and 2 months (N) immunostaining; p-values for pS71-Pin1 were calculated using one-way ANOVA with Tukey's multiple comparisons test. The results shown are the mean ± SD (***$p<0.001$, **$p<0.01$). Experiments were performed in triplicate with at least three mice per group per experiment. Scale bar= 50 μm. NS, no significance.

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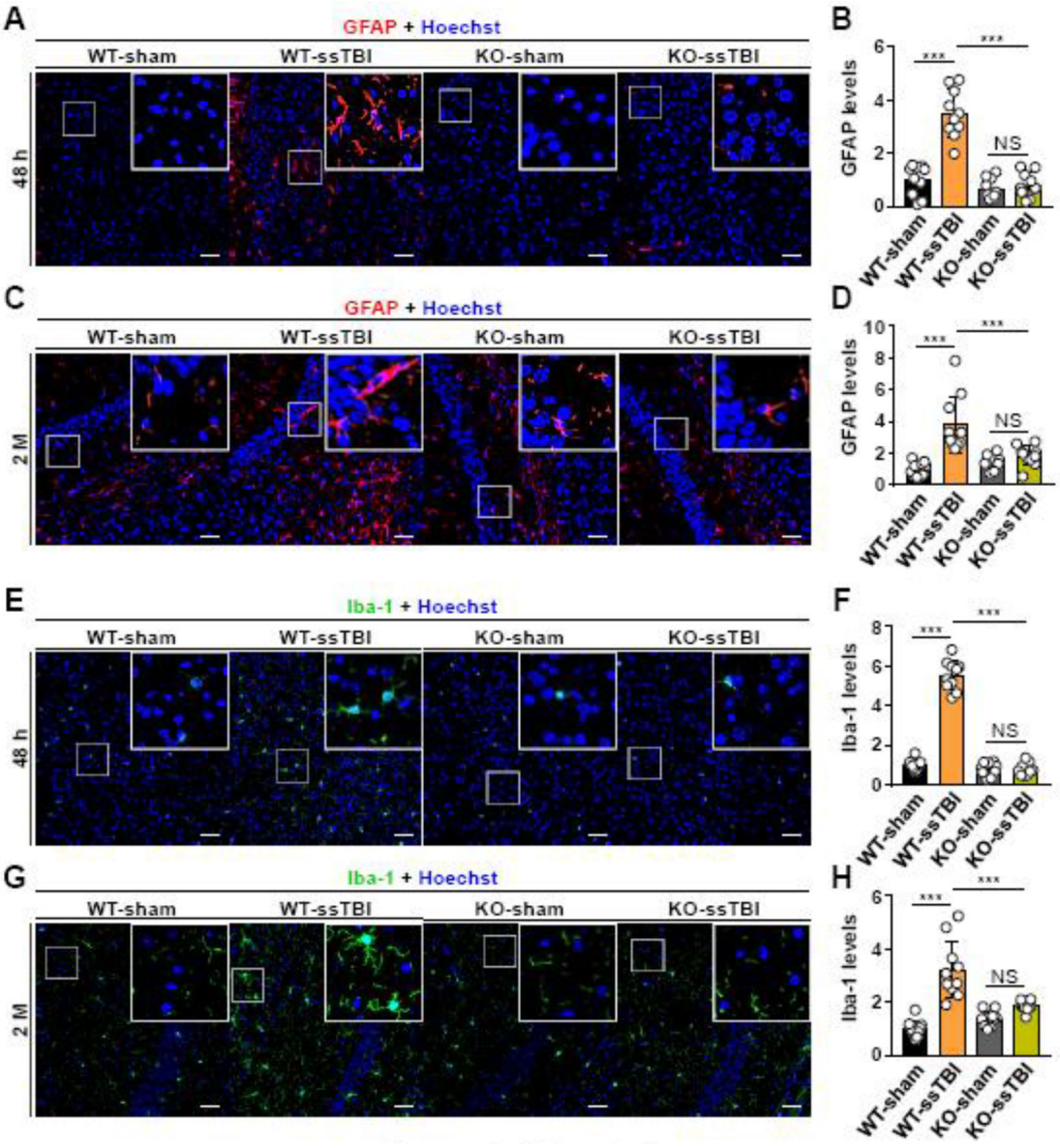

Fig. 3. Eliminating DAPK1 prevents neuroinflammation features after severe TBI.

WT and DAPK1 KO mouse brains were injured by a single weight drop. Forty-eight hours after TBI, the mouse brains were subjected to immunofluorescence to detect GFAP (A), an astrocyte activation marker, and Iba-1 (E), a microglial activation marker, in the cerebral cortex. Two months after injury, the mouse brains were subjected to immunofluorescence staining (C. G). In the hippocampus, a deep brain region, immunostaining for GFAP (C) and Iba-1 (G) was confirmed using a microscope. GFAP, red; Iba-1, green; DNA, blue. The immunostaining images were quantified and analyzed using one-way ANOVA with Tukey's multiple comparisons test (B, D, F, H). The results shown are the mean ± SD (***$p<0.001$). Experiments were performed in triplicate with at least three mice per group per experiment. Scale bar= 50 μm. NS, no significance.

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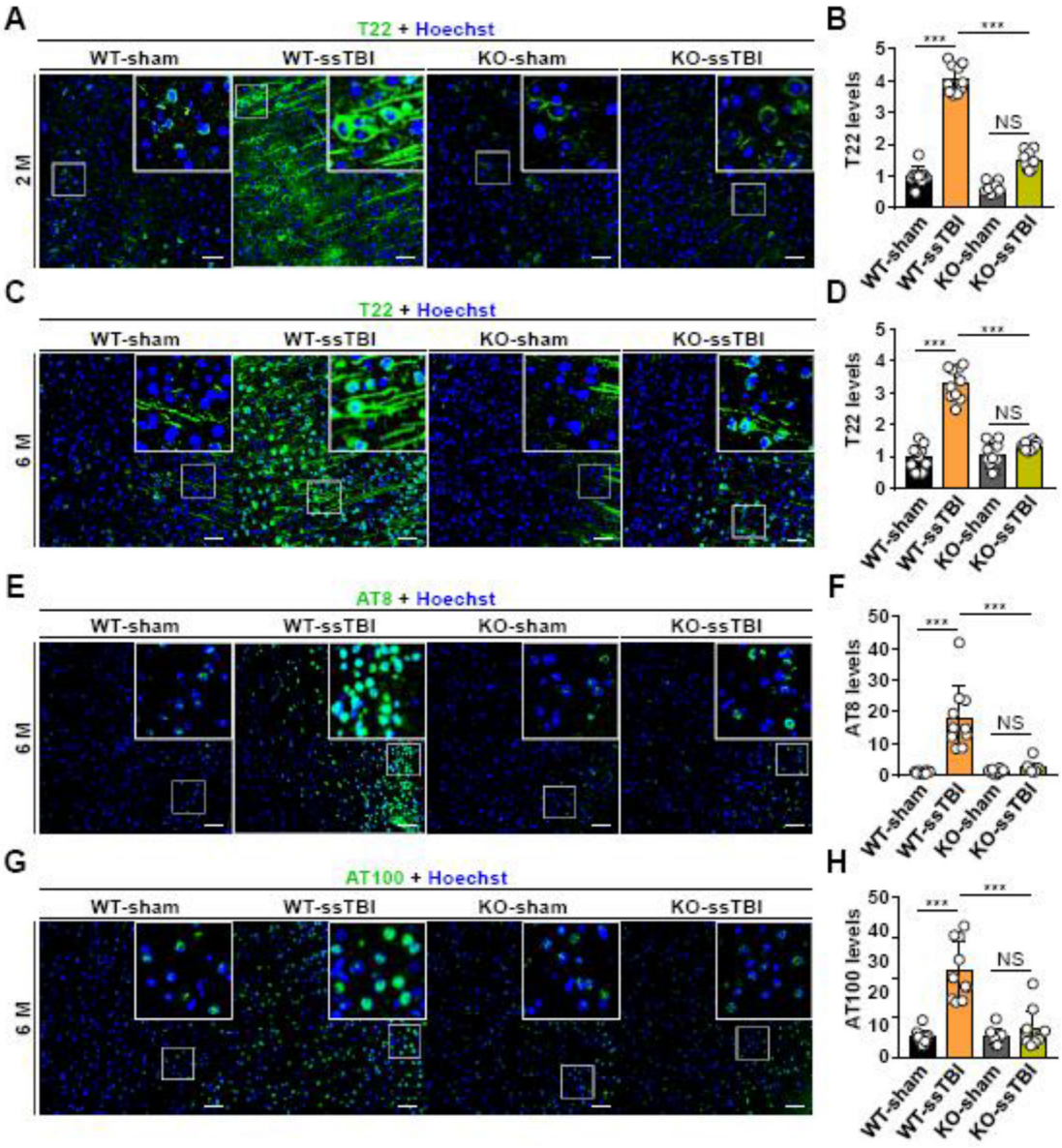

Fig. 4. DAPK1 deletion prevents neuropathological features following axonal injury.

A-D. Two months (A) and 6 months (C) after axonal injury, the brains from the WT and DAPK1 KO mice were analyzed by immunofluorescence to detect oligomeric tau (T22). T22, green; DNA, blue. The images of T22 immunostaining at two months (B) and at 6 months (D) were quantified and analyzed using one-way ANOVA with Tukey's multiple comparisons test (Dorsett *et al.*). E-H. Early pretangle pathologies were detected in the cerebral cortex of mouse brains by immunofluorescence with the AT8 (E) and AT100 (G) antibodies 6 months after axonal injury. AT8 and AT100, green; DNA, blue. The images of AT8 (F) and AT100 (H) immunostaining were quantified and analyzed using one-way ANOVA with Tukey's multiple comparisons test; the results shown are the mean ± SD (***$p<0.001$). Experiments were performed in triplicate with at least three mice per group per experiment. Scale bar= 50 μm. NS, no significance.

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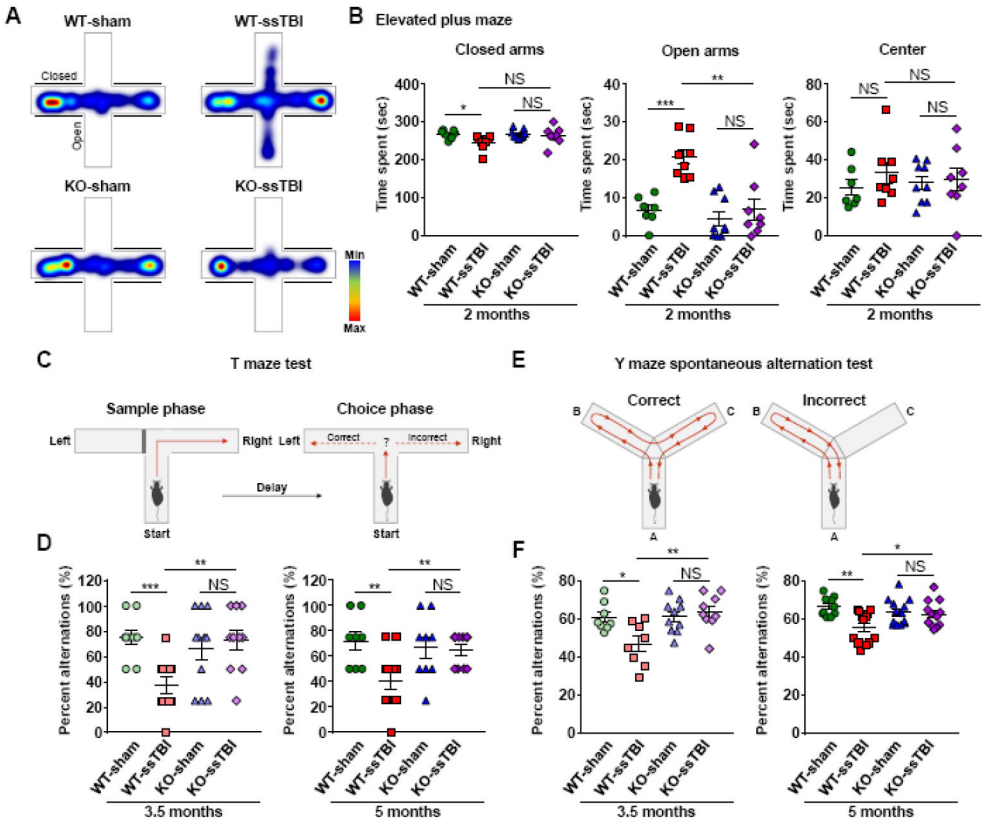

Fig. 5. DAPK1 KO reverses TBI-induced behavioral impairments in mice.

A, B. Mice were subjected to the elevated plus maze test (n= 7-9), and the time spent in the four arms was measured and quantified. C, D. T maze tests were performed to measure spatial working memory at 3.5 months (n= 10-11) and 5 months after TBI (n= 8-11). The behavior results regarding spatial working memory were quantified as a ratio of percentage alternations. E, F. Y maze spontaneous alternation tests were performed to measure spatial leaning and working memory at 3.5 months (n= 8-10) and 5 months (n= 10-15) after TBI. The Y maze behavior results were quantified as a ratio of percentage alternations. The p-values of all the representative data were calculated using one-way ANOVA with Tukey's multiple comparisons test. All the results shown are the mean ± SEM (***p<0.001, **p<0.01, *p<0.05). NS, no significance.

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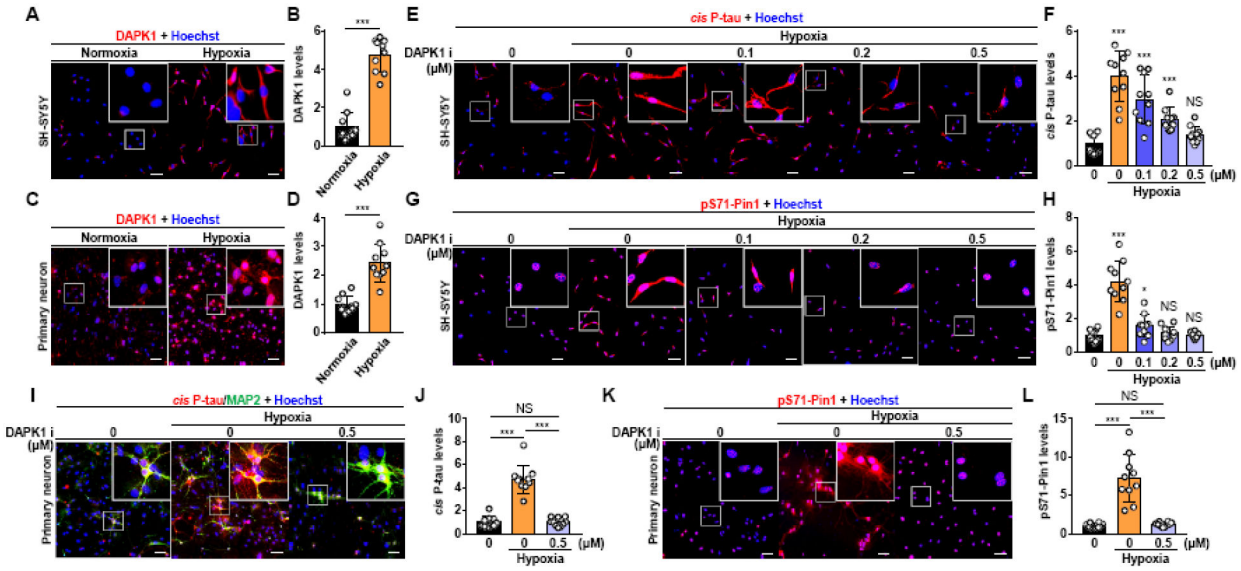

Fig. 6. *Cis* P-tau is induced in stressed neurons and blocked by a DAPK1 inhibitor.

A-D. SH-SY5Y cells were cultured without serum and placed in a 0.5% hypoxia chamber for 48 h (A). Primary neurons derived from the cerebral cortex of early postnatal (P0-P1) mouse brains at DIV 10-12 were treated with 0.5% hypoxia (C). DAPK1 expression was detected by immunofluorescence (A, C), and DAPK1 expression was quantified (B, D). DAPK1, red; DNA, blue; p-values were calculated using two-tailed Student's t-test. E-H. SH-SY5Y cells were pretreated with several doses of a DAPK1 inhibitor in the absence of serum for 48 h, and the cells were then treated with hypoxia for 48 h in the absence and presence of the DAPK1 inhibitor at various doses. The expression of *cis* P-tau (E) and pS71-Pin1 (G) in the cells was examined by immunofluorescence staining and quantified (F, H). *cis* P-tau and pS71-Pin1, red; DNA, blue. I-L. Primary neurons were pretreated with a DAPK1 inhibitor at the indicated dose for 48 h, and then, the cells were treated with 0.5% hypoxia for 48 h. Finally, the cells were collected for immunofluorescence (I, K). *cis* P-tau and pS71-Pin1, red; MAP2, green; DNA, blue. The images of *cis* P-tau (J) and pS71-Pin1 (L) immunostaining were quantified and analyzed using one-way ANOVA with Tukey's multiple comparisons test; the results shown are the mean ± SD (***p<0.001, *p<0.05). All experiments are representative of three independent experiments. Scale bar= 50 μm. NS, no significance.

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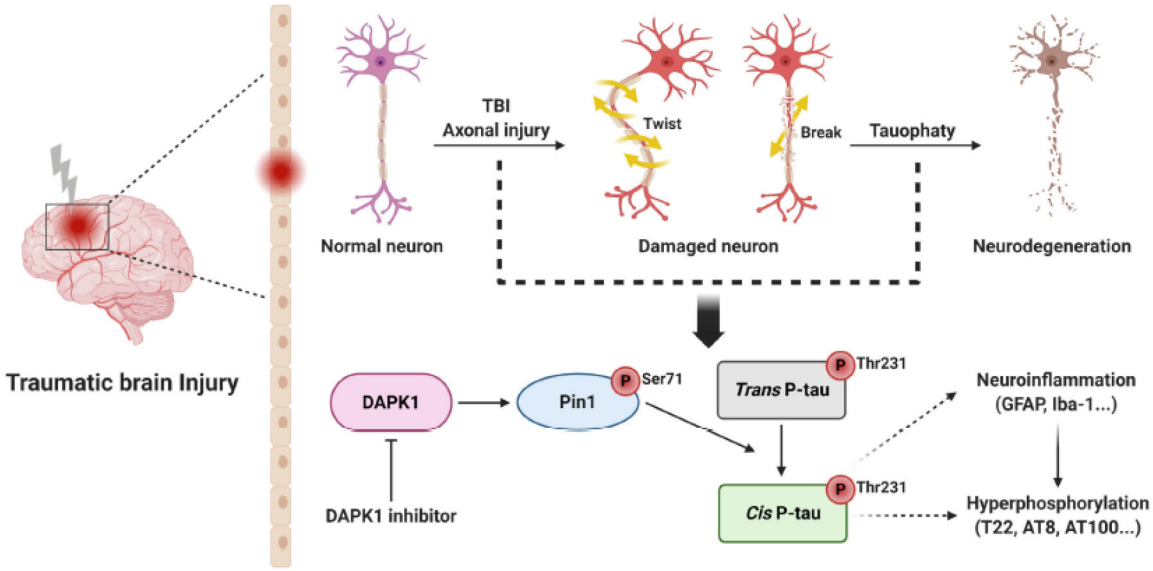

Fig. 7. Schematic diagram summarizing the proposed role of the regulation of DAPK1 after axonal injury.

TBI induces the expression of DAPK1, which causes Ser71 phosphorylation of Pin1. DAPK1-mediated Pin1 phosphorylation robustly promotes *cis* P-tau accumulation, especially at the phosphorylated Thr231-Pro motif in tau. Under these pathological mechanisms due to axonal injury contribute neuroinflammation and hyperphosphorylation of tau. However, the inhibition of DAPK1 expression not only prevents the conformational *cis* P-tau accumulation, but also prevents neuropathological and functional consequences after injury. Figure was created using Biorender.