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ARTICLE

GSK3β negatively regulates TRAX, a scaffold protein implicated in mental disorders, for NHEJ-mediated DNA repair in neurons

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Abstract

Translin-associated protein X (TRAX) is a scaffold protein with various functions and has been associated with mental illnesses, including schizophrenia. We have previously demonstrated that TRAX interacts with a Gsα protein-coupled receptor, the A_{2A} adenosine receptor ($A_{2A}R$), and mediates the function of this receptor in neuritogenesis. In addition, stimulation of the $A_{2A}R$ markedly ameliorates DNA damage evoked by elevated oxidative stress in neurons derived from induced pluripotent stem cells (iPSCs). Here, we report that glycogen synthase kinase 3 beta (GSK3β) and disrupted-in-schizophrenia 1 (DISC1) are two novel interacting proteins of TRAX. We present evidence to suggest that the stimulation of $A_{2A}R$ markedly facilitated DNA repair through the TRAX/DISC1/GSK3β complex in a rat neuronal cell line (PC12), primary mouse neurons, and human medium spiny neurons derived from iPSCs. $A_{2A}R$ stimulation led to the inhibition of GSK3β, thus dissociating the TRAX/DISC1/GSK3β complex and facilitating the non-homologous end-joining pathway (NHEJ) by enhancing the activation of a DNA-dependent protein kinase via phosphorylation at Thr^{2609}. Similarly, pharmacological inhibition of GSK3β by SB216763 also facilitated the TRAX-mediated repair of oxidative DNA damage. Collectively, GSK3β binds with TRAX and negatively affects its ability to facilitate NHEJ repair. The suppression of GSK3β by $A_{2A}R$ activation or a GSK3β inhibitor releases TRAX for the repair of oxidative DNA damage. Our findings shed new light on the molecular mechanisms underlying diseases associated with DNA damage and provides a novel target (i.e., the TRAX/DISC1/GSK3β complex) for future therapeutic development for mental disorders.

Introduction

Many genes involved in DNA repair or associated with the sensitivity to DNA damage have been implicated in mental disorders (such as bipolar disorder, depression, and schizophrenia) [1–3]. Among various forms of DNA damage, DNA double-strand breaks (DSBs) are the most menacing lesions [4]. Elevated oxidative stress, which may cause oxidative DNA damage, was found in the brains of patients with bipolar disorder or schizophrenia [5, 6]. Most importantly, incomplete repair of oxidative DNA damage may aggravate the development of psychotic disorders [3, 5, 7]. DNA DSBs can be repaired via at least two principal pathways: the homologous recombination pathway and the non-homologous end-joining (NHEJ) pathway [8]. HR requires sister chromatin as the template and is error-free, while NHEJ is highly efficient but intrinsically error-prone [4]. In neurons, the NHEJ pathway is initiated by a DNA-dependent protein kinase (DNA-PK) that consists of a catalytic subunit (DNA-PKcs) and a Ku protein subunit [9]. In

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response to DNA DSBs, DNA-PKcs is autophosphorylated at T2609, a step required for the recruitment of DNA repair proteins into the breakage site and the initiation of DNA repair [8].

Translin-associated protein X (TRAX) was initially recognized as an interacting protein of Translin, a DNA/RNA-binding protein that controls mRNA transport, translation, DNA recombination, and RNAi production [10–13]. The exact function of TRAX remains elusive and is of great interest because the human *TRAX* gene is clustered with the *Disrupted-in-Schizophrenia 1 (DISC1)* gene at 1q42 and has been implicated in schizophrenia, autism, depression, and bipolar disorder [14–17]. The *DISC1* gene is a well-recognized candidate gene for schizophrenia [18] and has been shown to inhibit glycogen synthase kinase-3 (GSK3β) through a direct physical interaction [19, 20]. The functional interaction between TRAX and DISC1 has also been implicated in the control of the volume of several brain regions during neurodevelopment [21].

Accumulated evidence suggests that TRAX regulates multiple functions by interacting with various interaction partners. In response to stresses (e.g., γ-irradiation) that cause DSBs, TRAX interacts with an activator of DNA-PK (i.e., C1D), suggesting that TRAX may facilitate DNA repair [22]. We previously demonstrated that, in TRAX-null mouse embryo fibroblasts, the lack of TRAX causes reduced phosphorylation of ATM and H2AX, and subsequently impairs DNA repair in response to stresses. By interacting with ATM and stabilizing the MRN complex at DSBs, TRAX plays an important role in the ATM/H2AX-mediated DNA repair machinery [23]. In addition to Translin and C1D and ATM, TRAX has several other interacting proteins, including the A_{2A} adenosine receptor ($A_{2A}R$) [24, 25]. Adenosine is a modulator that is important for controlling the homeostatic bioenergetic network that is composed of receptor-mediated signaling pathways, cellular bioenergetics, and epigenetics [26]. Because adenosine has been functionally associated with dopamine and glutamate, the major neurotransmitter systems involved in schizophrenia pathophysiology, the modulation of the adenosinergic system might, therefore, be an important therapeutic approach for both the genesis and treatment of schizophrenia [26, 27]. In line with this hypothesis, adenosine augmentation is believed to be beneficial for schizophrenia [28, 29]. There are four types of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3). The $A_{2A}R$ is a G-protein-coupled receptor and is located in many brain areas, with the highest level of expression in the striatum [30]. Stimulation of the $A_{2A}R$ activates cAMP/PKA signaling and evokes a TRAX-dependent pathway required for neuronal differentiation [24, 25]. Interestingly, the $A_{2A}R$ level is differentially altered in different brain regions of patients with schizophrenia [31, 32], suggesting that the function of the $A_{2A}R$ might be critical for the development of schizophrenia. In addition, the activation of the $A_{2A}R$ reduces oxidative stress, which is believed to contribute to the pathogenesis of schizophrenia [5], in a rat neuron-like cell line [33] and in human medium spiny neurons (MSNs) derived from induced pluripotent stem cells (iPSCs) [34].

In the present study, we investigated the molecular mechanism underlying the TRAX-dependent protection of rodent neurons and human MSN-like neurons from oxidative stress-evoked DNA damage. Our findings showed that TRAX forms a complex with DISC1 and GSK3β at resting conditions. GSK3β is a serine/threonine kinase that controls multiple functions via phosphorylation-mediated signal cascades. The inhibition of GSK3β by $A_{2A}R$ activation or a GSK3β inhibitor (SB216763) released TRAX from the complex to mediate the DNA-PK-dependent NHEJ repair. The results of the present study shed new light on the mechanistic role of DISC1/GSK3β in regulating DNA damage caused by elevated oxidative stress in neurons. Drugs that target the TRAX/DISC1/GSK3β complex provide novel possibilities for the development of therapeutic treatments for mental disorders (such as schizophrenia and bipolar disorder).

Materials and methods

Cell cultures and transfection

PC12 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 10% heat-inactivated horse serum [34]. Cells were incubated in a humidified incubator at 37 °C with 10% CO_2. The permanent PC12-shTRAX cell line was established via the transfection of PC12 cells with the linearized pSuper-shTRAX-F vector, as previously described [24], which contains a small hairpin RNA (5'-GCTGTACACCTTGAAACAG-3') for the knockdown of TRAX in PC12 cells and a neomycin resistance gene as a selective marker. The colonies were selected by G418 (500 μg/ml; Biochrom, Holliston, MA, USA) for 2 weeks. Down-regulation of TRAX was confirmed by Western blotting to assess the amount of TRAX in PC12-shTRAX cells. PC12-shTRAX cells were cultured in DMEM supplemented with 5% heat-inactivated FBS, 10% heat-inactivated HS and G418 (250 μg/ml). Cells were incubated in a humidified 37 °C incubator with 10% CO_2.

Primary hippocampal neurons were prepared as described previously [35]. Cells were maintained under 5% CO_2 for 14 days and subjected to the indicated treatments.

Human iPSCs were derived from normal subjects and differentiated into MSN-like neurons as described previously [34].

Lentivirus preparation and infection

Lentiviruses were prepared using standard procedures by the National RNAi Core Facility (NRC), Academia Sinica. PC12 cells were infected with lentiviruses at the multiplicity of infection (MOI) of 50 in the presence of polybrene (8 μg/ml) (Sigma-Aldrich) for 3 days and subjected to the subsequent treatments.

iPSC-derived neurons were infected with lentiviruses as previously described [34]. Briefly, neurons were infected with the desired lentiviruses at the MOI of 10 for 3 days and subjected to the indicated treatment(s).

Constructs and site-directed point mutation

The pcDNA3.1-rTRAX-V5, pcDNA3.1-ΔNLS-rTRAX-V5 and pSuper-shTRAX-F constructs were developed as previously described [24]. The DNA fragments of rat GSK3β and human GSK3β were amplified from cDNA of PC12 cells and HEK293T cells, respectively, and cloned into pcDNA3.1-Myc (Invitrogen) using polymerase chain reaction (PCR) and the primers described in Table S1. The DNA fragment of rat DISC1 was amplified from the cDNA of PC12 cells by PCR and cloned into pCMV-Tag2B (Stratagene, La Jolla, CA, USA) using the primers listed in Table S1.

The point mutation of GSK3β-S9A was created by a method described earlier [36] using standard protocols and site-specific primers (5'-GACCGAGAACCACCG- CCTTTG CGGAGAGC-3' and 5'- GCTCTCCGCAAAGGCGGTGG TTCTCGGTC-3'). pLKO1-h-shTRAX and pLKO1-r-shC1D were purchased from the NRC, Academia Sinica (Taipei, Taiwan).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

SDS–PAGE and Western blot analysis were performed as described previously [24]. Briefly, cell lysates were prepared by vibrating cells in a RIPA buffer (150 mM Sodium Chloride, Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl; pH 8.0). The protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). The immunoreactive bands were visualized via a light emitting non-radioactive method (ECL; Millipore). The sources of the antibodies used in western blot analyses are listed below: anti-γH2AX (Millipore; 05–636), anti-α-tubulin (Genetex, Irvine, CA, USA; GTX76511), anti-TRAX [25], anti-β-actin (Genetex; GTX11003), anti-DNA-PK_{CS} Thr2609 (Abnova, Cambridge, United Kingdom; PAB10324), anti-DNA-PK_{CS} (Abcam, Cambridge, United Kingdom; ab1832), anti-GSK3β Ser9 (Cell Signaling, Danvers, MA, USA; 9336), anti-GSK3β (Genetex; GTX83315), anti-β-catenin (Genetex; GTX61089), anti-DISC1 (Thermo; 710203), anti-$A_{2A}R$ (Santa cruz, Dallas, Texas, USA; SC-32261), and anti-PARP (Genetex; GTX112864).

Immunofluorescence staining and quantitation

Immunofluorescence staining was performed as described previously [37]. The images were examined with a laser-scanning confocal microscope (LSM 700, Carl Zeiss; Oberkochen, Germany). Quantitative analysis was performed by determining the immunofluorescence intensity of the target protein(s) using ImageJ [38] and normalized with the cell number on each slide. At least 100 cells were scored in each condition. The sources of the antibodies used in immunofluorescence staining are listed below: anti-γH2AX (Millipore; 05–636), anti-TUJ1 (Biolegend; MRB435P), and anti-TRAX [25].

Immunoprecipitation

Cell lysates were prepared by vibrating cells in a non-denaturing lysis buffer (137 mM NaCl, 1% NP-40, 20 mM Tris-Cl pH 8.0, 2 mM EDTA) for 1 h at 4 °C. The Protein A or G Dynabeads® (Novex™, Thermo Fisher Scientific, Waltham, MA, USA) were incubated with a proper primary antibody (2 μg/per sample) at RT for 10 min. The cell lysates (1–3 mg) were added to the Dynabead-antibody (Ab) complex at RT and gentlely rotated for 1 h. Immunoprecipitates were collected by placing the tube containing the reaction mixture onto a Magnetic Separation Rack for 1 min to collect the Dynabead–Ab complexes, which was subsequently washed with a washing buffer (0.02% Tween-20 in PBS) three times. The immunoprecipitated proteins were released from the immunocomplexes by adding 30 μl of the SDS–PAGE reducing sample buffer (50 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 1% β-mercaptoethanol) to the collected Dynabead–Ab complexes and boiled at 95 °C for 10 min.

Neutral comet assay

The Neutral Comet assay was performed as described previously [39]. Briefly, DNA damage (DNA breaks) was evaluated with a comet assay kit (TREVIGEN, Gaithersburg, MD, USA) following the manufacturer's protocol. Cells at a density of 1×10^5/ml were combined with molten LMAgarose (at 37 °C) at a ratio of 1:10 (v/v), pipetted onto

a CometSlide, lysed for 1 h, and subjected to electrophoresis. At least 100 cells were scored in each sample and were analyzed with the comet 5.0 image system (Kinetic Imagine, Liverpool, UK).

Annexin V apoptosis detection assay

The Annexin V apoptosis detection assay was performed as described previously [40]. Briefly, cells were resuspended in a binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) at a density of 10^6/ml. The cell suspension (100 µl) was incubated with Annexin V-FITC (10 µl) and propidium iodide (20 µg/ml, 5 µl) (Enzo, Farmingdale, NY, USA) for 10 min and analyzed via flow cytometry (LSR ll, Biosciences, San Jose, CA, USA).

Proximity ligation assay (PLA)

The PLA analysis was performed as described previously [41]. Cells were fixed with 4% paraformaldehyde for 20 min and then treated with 0.05% NP-40 in PBS for 20 min. PLA was carried out following the manufacturer's protocol. The mages were examined with a laser-scanning confocal microscope (LSM700-meta). Quantitative analysis was performed by measuring the number of PLA signals, which were normalized to the cell number. At least 100 cells were evaluated for each condition. The sources of the antibodies used in PLA are listed below: anti-TRAX [25], anti-DISC1 (Thermo; 710203), and anti-GSK3β (Genetex; GTX83315).

NHEJ assay, DNA extraction and quantitative PCR (qPCR)

The NHEJ assay was performed as described previously with slight modifications [42]. To generate DSBs, pEGFP-C3 was cleaved by NheI at a site between the promoter and the GFP reporter gene. The linearized DNA was resolved and isolated from electrophoresis gels with a Gel Extraction kit (Geneaid, New Taipei City, Taiwan) and transfected into cells with Lipofectamine 2000 as described above. Cells were co-transfected with the linearized pEGFP-C3 and a control circular DNA (pDsRed-monomer-C1) that assessed transfection efficiency at a ratio of 9:1 (w/w). 2 days post-transfection, transfected plasmids inside the cells were purified with the UltraClean™ Tissue & Cells DNA Isolation Kit (MO BIO, Carlsbad, CA, USA), and subjected to qPCR via an ABI PRISM 7700 Sequence Detection System (Life Technologies, Carlsbad, CA, USA), the SYBR Green PCR Master Mix (Life Technologies), and the primers (pEGFP-C3-NHEJ-F and pEGFP-C3-NHEJ-R for the linearized pEGFP-C3; pDsRed-C1-F and pDsRed-C1-R for pDsRed-monomer-C1) listed in Table S1. The amount of DNA amplified using pEGFP-C3-NHEJ-F and pEGFP-C3-NHEJ-R reflected the amount of repaired DNA, and the values were normalized to those obtained from the amplification of pDsRed-C1-F and pDsRed-C1-R to control for transfection efficiency.

Statistical analysis

All experiments were reliably and independently conducted at least three times. The data are shown as the mean ± SEM. Our data meet the assumption of normal distribution. No data was excluded. Imaging quantitation was performed blinded. SigmaPlot (Version 10; Systat Software Inc.; San Jose, CA, USA) was used for data analysis. Statistical significance was determined by one or two-way analysis of variance, or Student's *t* test as indicated. P values < 0.05 are considered statistically significant.

Results

TRAX mediates the protective effect of the $A_{2A}R$ on DNA damage in rodent and human neurons

We previously demonstrated that the stimulation of the $A_{2A}R$ rescues oxidative stress-induced DNA damage and apoptosis in human iPSC-derived MSNs via a cAMP/PKA-dependent pathway [34]. Because TRAX is an $A_{2A}R$ interacting protein [25] and plays a central role in the ATM-mediated DNA repair [23], TRAX might contribute to the $A_{2A}R$-induced repair of DNA damage evoked by elevated oxidative stress. We first assessed whether the activation of the $A_{2A}R$ also rescued oxidative DNA damage in rodent neurons as in human neurons. PC12 cells were treated with H_2O_2 (100 µM) for 4 h to induce oxidative DNA damage. As shown in Fig. 1a, treatment with H_2O_2 evoked significant DNA DSBs as detected with the neutral comet assay. Pretreatment with an $A_{2A}R$-specific agonist, CGS21680 (CGS) [24] effectively reduced the DNA DSBs (Fig. 1a). We next evaluated DNA damage in PC12 cells by H_2O_2 (100 µM) from 1 to 4 h by measuring the level of a DNA DSBs marker (γH2AX) [43] via Western blot analysis. Pretreatment of PC12 cells with CGS consistently reduced the amount of γH2AX up to 4 h (Fig. 1b). Similarly, exposure to H_2O_2 increased the number of γH2AX foci and the level of γH2AX, which can be ameliorated by pretreatment with CGS in mouse hippocampal neurons (Fig. 1c). Such oxidative stress-induced DNA damage, if unrepaired, would lead to apoptosis, as determined by the level of Annexin V (Fig. 1d). Pretreatment with CGS reduced not only the amount of γH2AX but also the level of Annexin V, evoked by H_2O_2 (Fig. 1d). Since an $A_{2A}R$-selective inhibitor (SCH58261, SCH) effectively eliminated

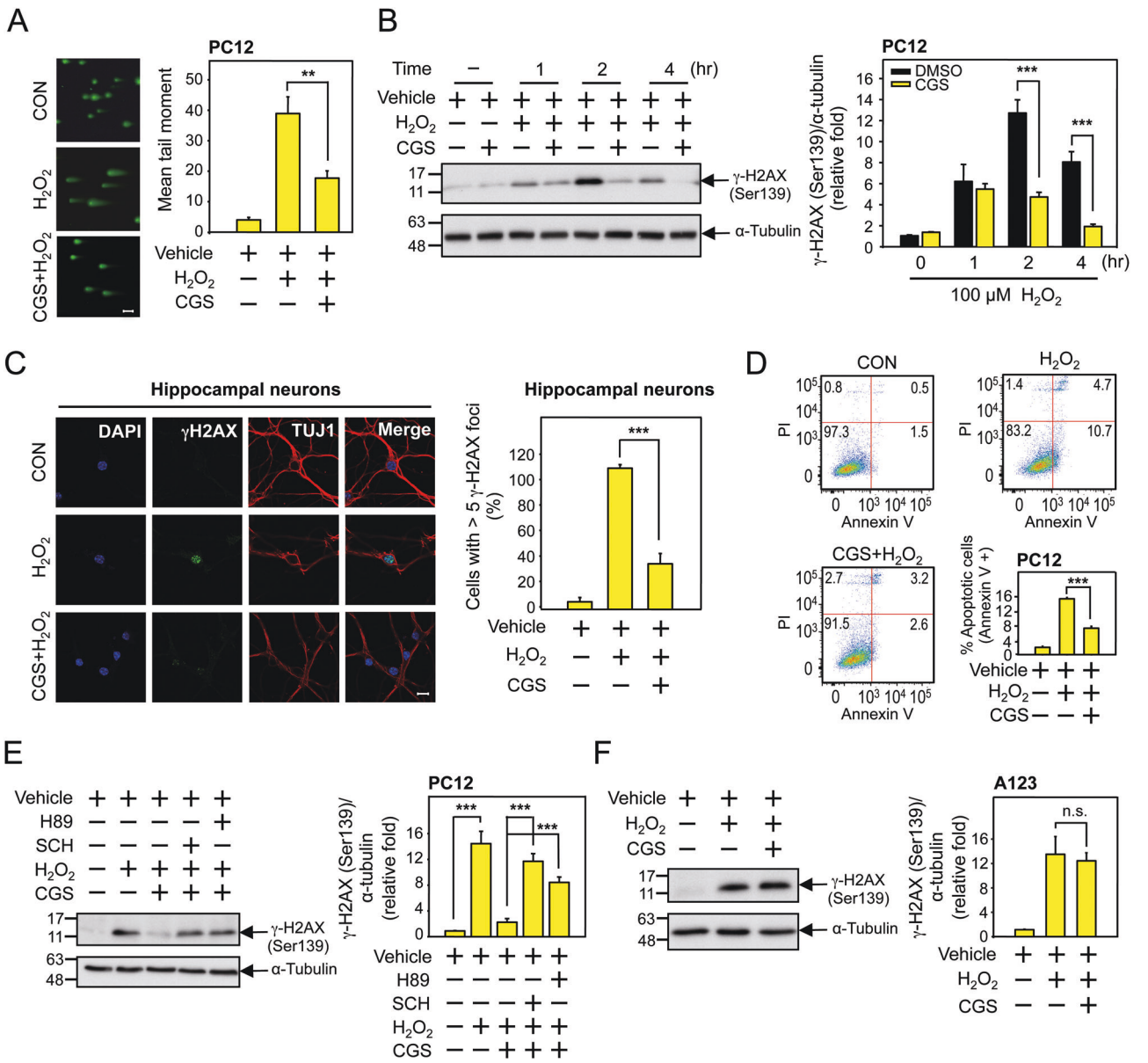
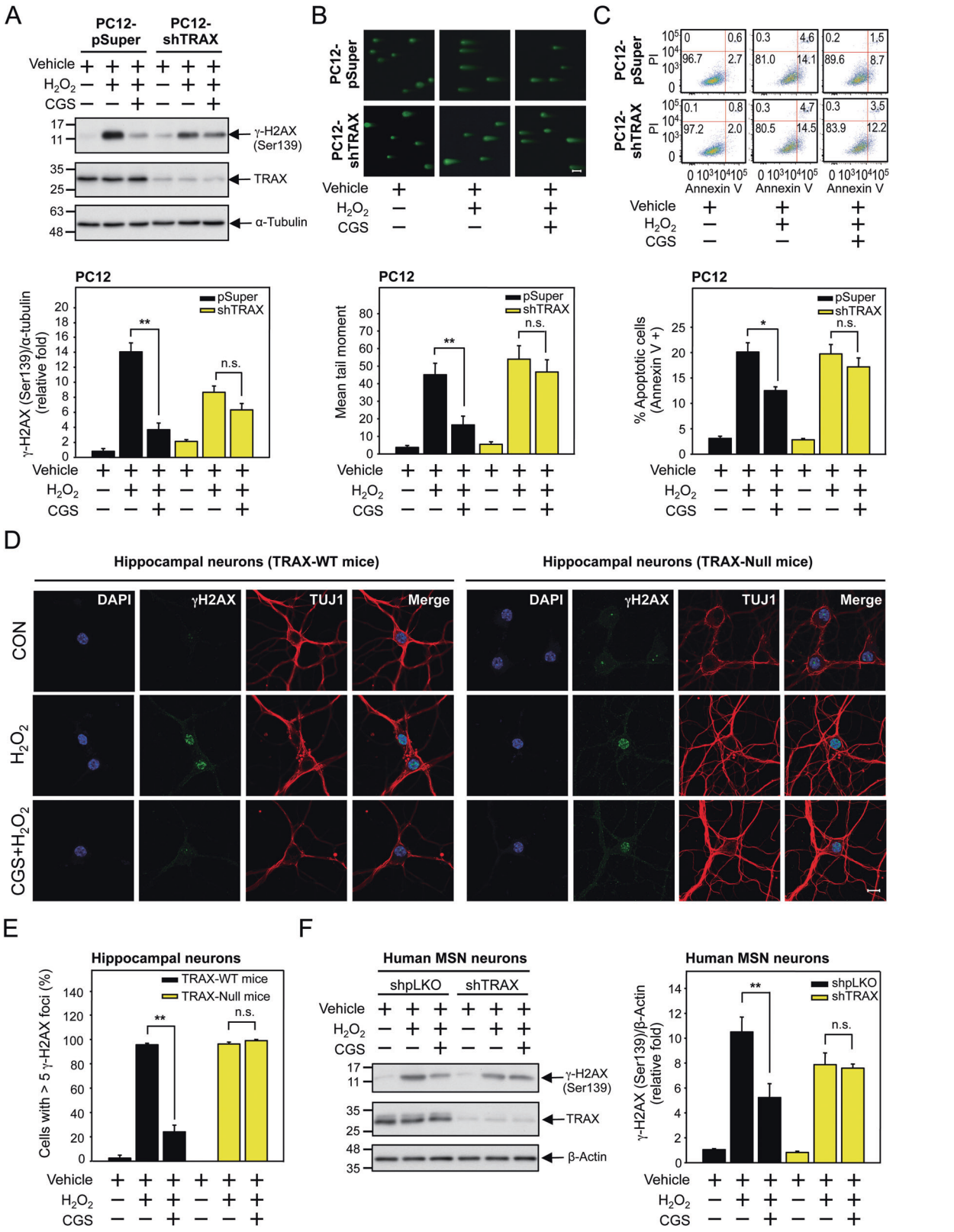


Fig. 1 Activation of the A_{2A}R ameliorates oxidative stress-induced DNA damage and toxicity. **a** PC12 cells were treated with an agonist of the A_{2A}R (CGS21680, CGS; 10 μM) or vehicle for 1 h to activate the A_{2A}R, followed by the addition of H_2O_2 (100 μM) for 4 h. The extent of DNA damage was analyzed via the neutral comet assay. The mean tail moment was quantified by using COMETscore. v1.5 software. Scale bar, 50 μm. **b** PC12 cells were pretreated with CGS (10 μM) or vehicle for 1 h and then treated with H_2O_2 (100 μM) for the indicated time. Cells were lysed and subjected to SDS–PAGE and Western blot analysis using the anti-γH2AX and anti-α-Tubulin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **c** Primary hippocampal neurons (DIV14) harvested from wild-type B6/C57 mice were treated with CGS (10 μM) or vehicle for 1 h and then treated with H_2O_2 (100 μM) for 2 h. DNA damage was assessed by determining the number of DNA foci per cell by immunofluorescence staining using the anti-γH2AX antibody (green) in neurons identified by a neuronal marker (TUJ1, red). The percentage of cells with > 5 γH2AX foci per cell in at least 100 cells were determined in each condition. Scale bar, 10 μm. **d** PC12 cells were treated with CGS (10 μM) or vehicle for 1 h, followed by treatment with H_2O_2 (100 μM) for 4 h. The survival of these treated cells was measured using the Annexin V apoptosis detection assay kit. Cells were co-stained with Annexin V-FITC and propidium iodide (PI) for 10 min followed by flow cytometric analysis. These experiments were repeated three times. **e**, **f** PC12 cells were treated with an inhibitor of protein kinase A (H89; 10 μM), an A_{2A}R-selective inhibitor (SCH58261, SCH; 1 μM) or vehicle for 30 min and then treated with CGS (10 μM) or vehicle for 1 h to activate the A_{2A}R, followed by the addition of H_2O_2 (100 μM) for 4 h (**e**). A123, a cAMP-dependent protein kinase (PKA)-deficient PC12 cell line, was treated with CGS (10 μM) or vehicle for 1 h to activate the A_{2A}R, followed by treatment with H_2O_2 (100 μM) for 4 h (**f**). Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-γH2AX and anti-α-Tubulin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. Data are presented as the mean ± SEM from at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control


A
PC12-pSuper
PC12-shTRAX
Vehicle
H2O2
CGS
γ-H2AX (Ser139)
TRAX
α-Tubulin
B
PC12-pSuper
PC12-shTRAX
C
PC12-pSuper
PC12-shTRAX
PI
Annexin V
PC12
pSuper
shTRAX
γ-H2AX (Ser139)/α-tubulin (relative fold)
Mean tail moment
% Apoptotic cells (Annexin V +)
n.s.
D
Hippocampal neurons (TRAX-WT mice)
Hippocampal neurons (TRAX-Null mice)
DAPI
γH2AX
TUJ1
Merge
CON
H2O2
CGS+H2O2
E
Hippocampal neurons
TRAX-WT mice
TRAX-Null mice
Cells with > 5 γ-H2AX foci (%)
F
Human MSN neurons
shpLKO
shTRAX
γ-H2AX (Ser139)
TRAX
β-Actin
γ-H2AX (Ser139)/β-Actin (relative fold)

◀ **Fig. 2** The protective effect of an A_{2A}R agonist is mediated by TRAX. **a-c** PC12-pSuper and PC12-shTRAX cells were treated with an A_{2A}R agonist (CGS21680, CGS; 10 μM) or vehicle for 1 h to activate the A_{2A}R and then treated with H_2O_2 (100 μM) for 4 h. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-γH2AX, anti-TRAX and anti-α-Tubulin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times (**a**). The extent of DNA damage was analyzed via the neutral comet assay. The mean tail moment was quantified with COMETscore.v1.5 software, scale bar, 50 μm (**b**). Apoptosis was assessed with the Annexin V apoptosis detection kit. Cells were co-stained with Annexin V-FITC and PI for 10 min followed by flow cytometric analysis. These experiments were repeated three times (**c**). **d**, **e** Primary hippocampal neurons (DIV14) from TRAX-WT and TRAX-null mice were treated with CGS (10 μM) or vehicle for 1 h and then treated with H_2O_2 (100 μM) for 2 h. DNA damage was assessed by determining the number of DNA foci per cell by immunofluorescence staining using the anti-γH2AX antibody (green) in neurons identified by a neuronal marker (TUJ1, red). The percentage of cells with >5 γH2AX foci per cell in at least 100 cells were determined in each condition. Scale bar, 10 μm. **f** Human MSN neurons were infected with lentivirus expressing TRAX shRNA or control shRNA for 3 days. Human MSN neurons were treated with an A_{2A}R agonist (CGS21680, CGS; 10 μM) or vehicle for 1 h to activate the A_{2A}R and then treated with H_2O_2 (100 μM) for 4 h. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-γH2AX, anti-TRAX and anti-β-Actin antibodies as indicated. The amount of target protein was quantified and normalized to that of β-Actin, the loading control. These experiments were repeated three times. Data are presented as the mean ± SEM from at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared to control

the protective effect of CGS on oxidative DNA damage (Fig. 1e), the action of CGS was mediated by the A_{2A}R.

Because stimulation of the A_{2A}R leads to activation of the cAMP/PKA pathway, we next performed experiments to evaluate the involvement of PKA. As shown in Fig. 1e, pretreatment with a PKA inhibitor (H89) effectively reversed the protective effect of CGS. In addition, no protective effect of CGS on oxidative DNA damage was observed in a PKA-deficient PC12 cell line (A123) [44] (Fig. 1f). These findings confirmed that the protective effect of CGS on oxidative DNA damage was mediated by the A_{2A}R and required PKA in rodent neurons.

To investigate the role of TRAX in the protective effect of the A_{2A}R on oxidative DNA damage, we generated a stable cell line (termed PC12-shTRAX) that permanently expressed an shRNA against the rat TRAX (shTRAX-F, [24]) and reduced the expression of endogenous TRAX in PC12 cells (Fig. 2a). We previously showed that TRAX plays a critical role in the detection of DNA damage by directly interacting with ATM and stabilizing the Mre11-Rad50-Nbs1 complex at double-strand breaks in mouse embryo fibroblasts [23]. Similarly, the lack of TRAX in the striatum of TRAX-null mice markedly hindered the ability of striatal neurons to detect the X-irradiation-induced DNA damage as monitored by the amount of γH2AX (Fig. S1). In line with the above findings, we found that reduction of TRAX jeopardized the ability to repair DNA damage evoked by oxidative stress and led to an impairment in detecting γH2AX in PC12-shTRAX cells compared to control cells (PC12-pSuper, Fig. 2a). Reduction of TRAX alone (PC12-shTRAX) did not affect the number of DSBs-containing cells, detected via the Comet assay, at the basal level and the H_2O_2-treated cells ($p = 0.83$ and 0.50, verses PC12-pSuper cells, respectively; Fig. 2b). Most importantly, down-regulation of TRAX in PC12 cells prevented the A_{2A}R-evoked protective effect on oxidative DNA damage and apoptosis (Fig. 2a–c). Similarly, no effect of CGS on oxidative stress induced-γH2AX foci formation at DSBs was found in primary neurons prepared from TRAX-null mice (Fig. 2d, e). To validate this role of TRAX in human neurons, human iPSC-derived MSNs were infected with lentiviruses harboring an shRNA against human TRAX for 3 days. The endogenous level of TRAX in human neurons infected with shTRAX was significantly reduced when compared with neurons infected with a control virus (sh-pLKO, Fig. 2f). The suppression of TRAX in human MSNs also markedly reduced the CGS-evoked protective effect on oxidative damage (Fig. 2f). Taken together, these findings suggest that TRAX is required for the protective effect of the A_{2A}R on oxidative DNA damage in both human and rodent neurons.

TRAX contributes to the ATM/DNA-PK-mediated NHEJ pathway evoked by A_{2A}R activation

TRAX has been implicated in DNA repair because it interacts with an activator (i.e., C1D) of DNA-PK, a major enzyme responsible for NHEJ [8], in response to stresses that cause DSBs [22]. To evaluate whether the stimulation of the A_{2A}R activated DNA-PK_{CS}, PC12 cells were treated with H_2O_2 to elevate oxidative stress for 1–4 h in the absence or presence of pretreatment with CGS. The activation of DNA-PK_{CS} was evaluated by determining the phosphorylation level of DNA-PK_{CS} at Thr^{2609} [8] by Western blot analysis. Our results showed that CGS pretreatment enhanced DNA-PK_{CS} activation in PC12 cells under the conditions tested (Fig. 3a). Down-regulation of TRAX markedly removed the activation/phosphorylation of DNA-PK_{CS} when compared to PC12-pSuper cells (Fig. 3b), suggesting that stimulation of the A_{2A}R led to the activation of DNA-PK_{CS} in a TRAX-dependent manner.

Surprisingly, the down-regulation of C1D in PC12 cells did not affect the protective effect of the A_{2A}R on oxidative DNA damage (Fig. S2), suggesting that C1D was not involved. Because we previously demonstrated that TRAX directly interacts with ATM [23] and an earlier study showed that ATM is required for the activation/phosphorylation of DNA-PKcs at Thr^{2609} [45], we went on to

determine whether ATM is involved in the activation of DNA-PK$_{CS}$ during A_{2A}R activation. Treatment with the ATM inhibitor KU55933 effectively abolished the CGS-mediated enhancement of DNA-PK$_{CS}$ activity (Fig. 3c), suggesting that ATM functions upstream of DNA-PK$_{CS}$ during the activation of the A_{2A}R to facilitate DNA repair.

Because DNA-PK$_{CS}$ is the major enzyme that mediates NHEJ [8], we next conducted experiments to determine

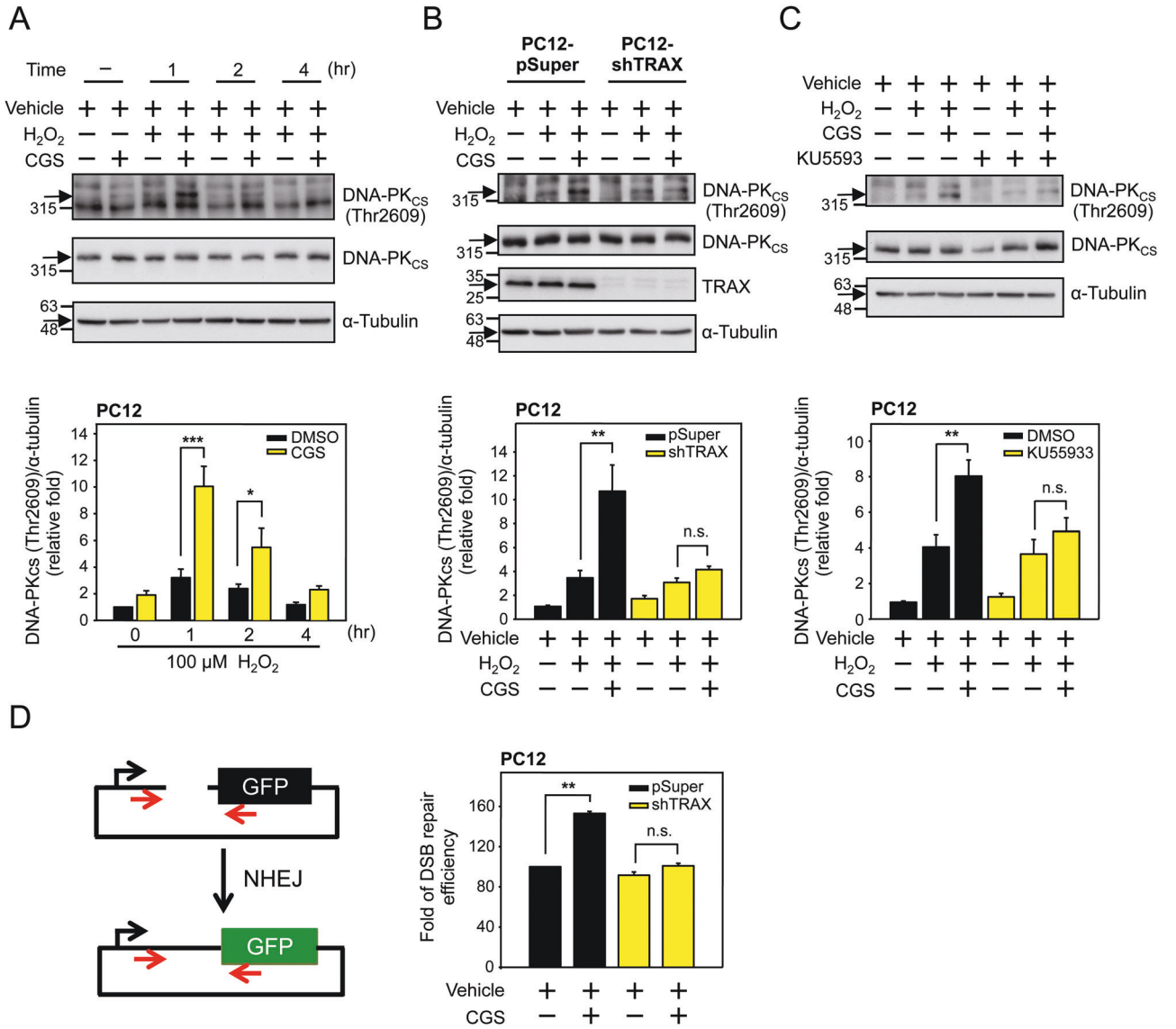
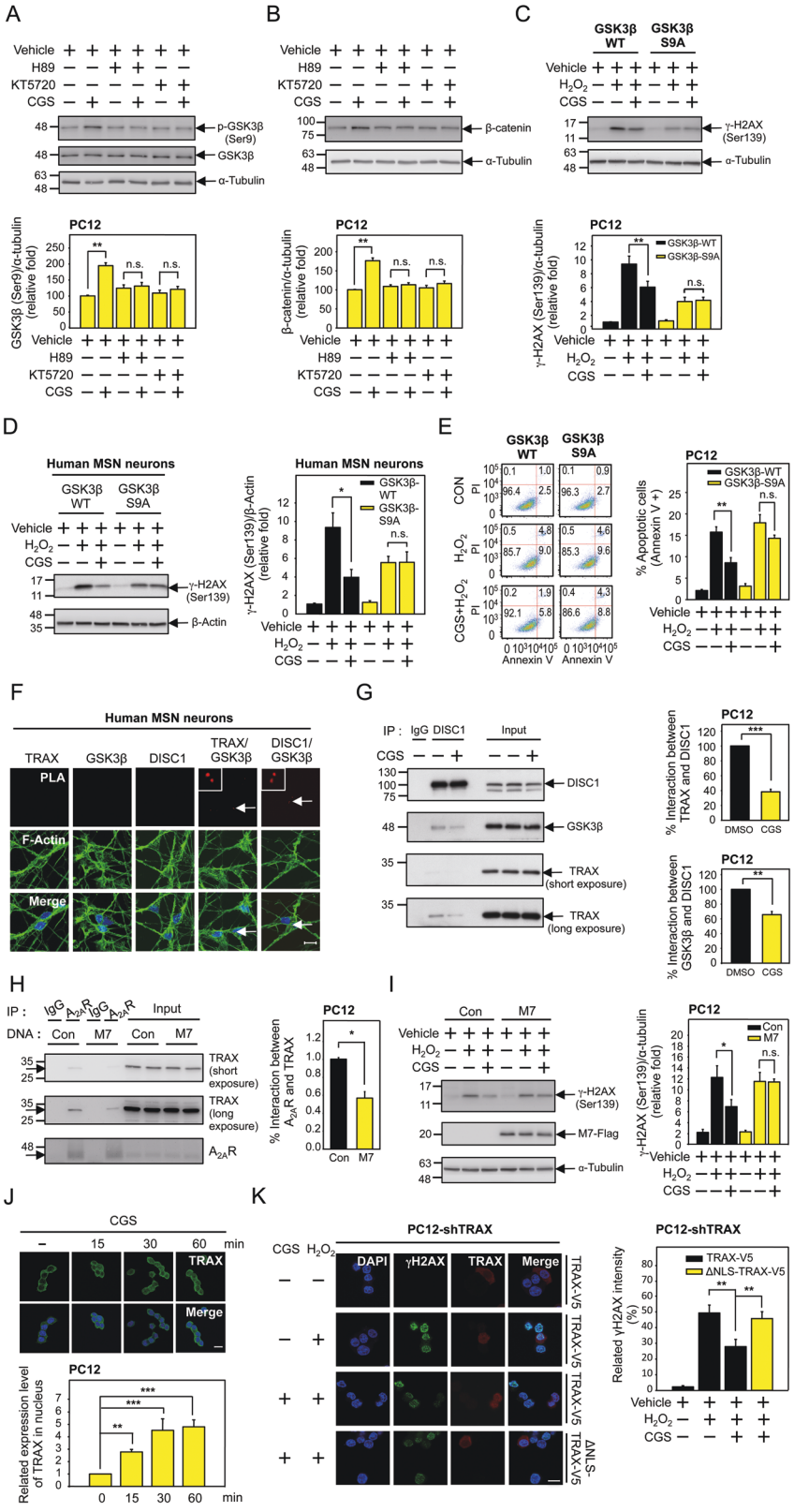


Fig. 3 TRAX and ATM contribute to the DNA-PK-mediated NHEJ pathway evoked by A_{2A}R activation. **a** PC12 cells were treated with an A_{2A}R agonist (CGS21680, CGS; 10 μM) or vehicle for 1 h. Following the treatment, cells were treated with H_2O_2 (100 μM) for the indicated time periods. Cells were lysed and analyzed by immunoblotting with anti-DNA-PKcs T2609, anti-DNA-PKcs and anti-α-Tubulin antibodies. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **b** PC12-pSuper and PC12-shTRAX cells were treated with CGS (10 μM) for 1 h and treated with H_2O_2 (100 μM) for 1 h. Cells were lysed and analyzed by immunoblotting with anti-DNA-PKcs T2609, anti-DNA-PKcs, anti-TRAX and anti-α-Tubulin antibodies. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **c** PC12 cells were treated with an inhibitor of ATM (KU55933; 10 μM) or vehicle for 30 min and then treated with CGS (10 μM) or vehicle for 1 h to activate the A_{2A}R, followed by the addition of H_2O_2 (100 μM) for 1 h. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-DNA-PKcs T2609, anti-DNA-PKcs and anti-α-Tubulin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **d** Schematic illustration of the NHEJ assay in vivo. A single DSB was generated in this plasmid substrate pEGFP-C3 with NheI to cut between the promoter and the GFP gene. Linearized plasmids and the circular pDsRed-monomer-C1 as the internal control for transfection efficiency were cotransfected into the PC12-pSuper cells and PC12-shTRAX cells. The region of the forward primer binding site is in the promoter and the reverse primer binding site is in the GFP gene. After transfection for 2 days, cells were collected for qPCR. Data are shown as the mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control


A
Vehicle
H89
KT5720
CGS
p-GSK3β (Ser9)
GSK3β
α-Tubulin
PC12
GSK3β (Ser9)/α-tubulin (relative fold)
B
β-catenin
β-catenin/α-tubulin (relative fold)
C
GSK3β WT
GSK3β S9A
H2O2
γ-H2AX (Ser139)
γ-H2AX (Ser139)/α-tubulin (relative fold)
GSK3β-WT
GSK3β-S9A
D
Human MSN neurons
β-Actin
γ-H2AX (Ser139)/β-Actin (relative fold)
E
CON
CGS+H2O2
PI
Annexin V
% Apoptotic cells (Annexin V +)
F
TRAX
GSK3β
DISC1
TRAX/ GSK3β
DISC1/ GSK3β
PLA
F-Actin
Merge
G
IP :
IgG
DISC1
Input
DISC1
TRAX (short exposure)
TRAX (long exposure)
% Interaction between TRAX and DISC1
% Interaction between GSK3β and DISC1
DMSO
CGS
H
DNA :
Con
M7
$A_{2A}R$
% Interaction between $A_{2A}R$ and TRAX
I
M7-Flag
J
CGS
TRAX
Merge
Related expression level of TRAX in nucleus
min
K
PC12-shTRAX
DAPI
γH2AX
TRAX
Merge
TRAX-V5
ΔNLS-TRAX-V5
Related γH2AX intensity (%)

◀ **Fig. 4** The TRAX/DISC1/GSK3β (TDG) complex is involved in the protective effect of an $A_{2A}R$ agonist. **a**, **b** PC12 cells were treated with an inhibitor of protein kinase A (H89; 10 μM and KT5720; 10 μM) or vehicle for 30 min and then treated with CGS (10 μM) or vehicle for 1 h (**a**) and 8 h (**b**). Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-GSK3β Ser9, anti-GSK3β, anti-β-catenin and anti-α-Tubulin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **c–e** In PC12 cells and human MSN neurons, cells were infected with lentivirus expressing GSK3β or a constitutively active GSK3β mutant (GSK3β-S9A) for 3 days. Cells were treated with an $A_{2A}R$ agonist (CGS21680, CGS; 10 μM) or vehicle for 1 h to activate the $A_{2A}R$ and then treated with H_2O_2 (100 μM) for 4 h. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-γH2AX, anti-α-Tubulin and anti-β-Actin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin or β-Actin, the loading controls. These experiments were repeated three times (**c**, **d**). Apoptosis was assessed by co-staining with Annexin V-Cy5 and PI for 10 min followed by flow cytometric analysis (**e**). **f** Protein–protein interactions were monitored by the proximity ligation assay [31] using the corresponding antibodies as described in the Methods section [31]. Each red dot represents the detection of protein–protein interaction. Scale bar, 10 μm. **g** PC12 cells were treated with an $A_{2A}R$ agonist (CGS21680, CGS; 10 μM) or vehicle for 1 h. Cells were lysed, subjected to immunoprecipitation with anti-DISC1 and immunoblotted with anti-DISC1, anti-TRAX and anti-GSK3β antibodies. The amount of target protein was quantified and normalized to that of the input. These experiments were repeated three times. **h** PC12 cells were infected with the lentivirus expressing $A_{2A}R_{253–410}$-Flag (M7) for 3 days. Cells were lysed, subjected to immunoprecipitation with an anti-$A_{2A}R$ antibody or a control IgG, followed by Western blot analysis using the indicated antibody. The amount of TRAX that interacted with $A_{2A}R$ was quantified and normalized to that of the input. These experiments were repeated three times. **i** PC12 cells were infected with lentivirus expressing $A_{2A}R_{253–410}$-Flag for 3 days. Cells were treated with CGS (10 μM) or vehicle for 1 h to activate the $A_{2A}R$ and then treated with H_2O_2 (100 μM) for 4 h. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-γH2AX, anti-Flag and anti-α-Tubulin antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading controls. These experiments were repeated three times. **j** PC12 cells were treated with CGS (10 μM) during the indicated time period. Cells were subjected to immunostaining with an anti-TRAX antibody (Green). Scale bar, 10 μm. **k** PC12-shTRAX cells were transfected with TRAX-V5 and ΔNLS TRAX-V5 plasmids. Cells were treated with CGS (10 μM) or vehicle for 1 h to activate the $A_{2A}R$ and then treated with H_2O_2 (100 μM) for 4 h. Cells were subjected to immunostaining with anti-γH2AX (Green) and anti-V5 (Red) antibodies. Scale bar, 10 μm. Data are presented as the mean ± SEM from at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control

whether the activation of the $A_{2A}R$ enhanced the activity of the NHEJ pathway. PC12-shTRAX and PC12-pSuper cells were transfected with a linearized plasmid (pEGFP-C3) that is to be repaired through NHEJ. The amount of the repaired, circular form of pEGFP-C3 was quantified by qPCR. As shown in Fig. 3d, CGS treatment significantly enhanced the activity of the NHEJ pathway in PC12-pSuper cells, but not PC12-shTRAX cells. Collectively, these findings suggest the activation of the $A_{2A}R$ using CGSfacilitated DNA repair upon oxidative stress via a TRAX/ATM/DNA-PK-mediated NHEJ pathway.

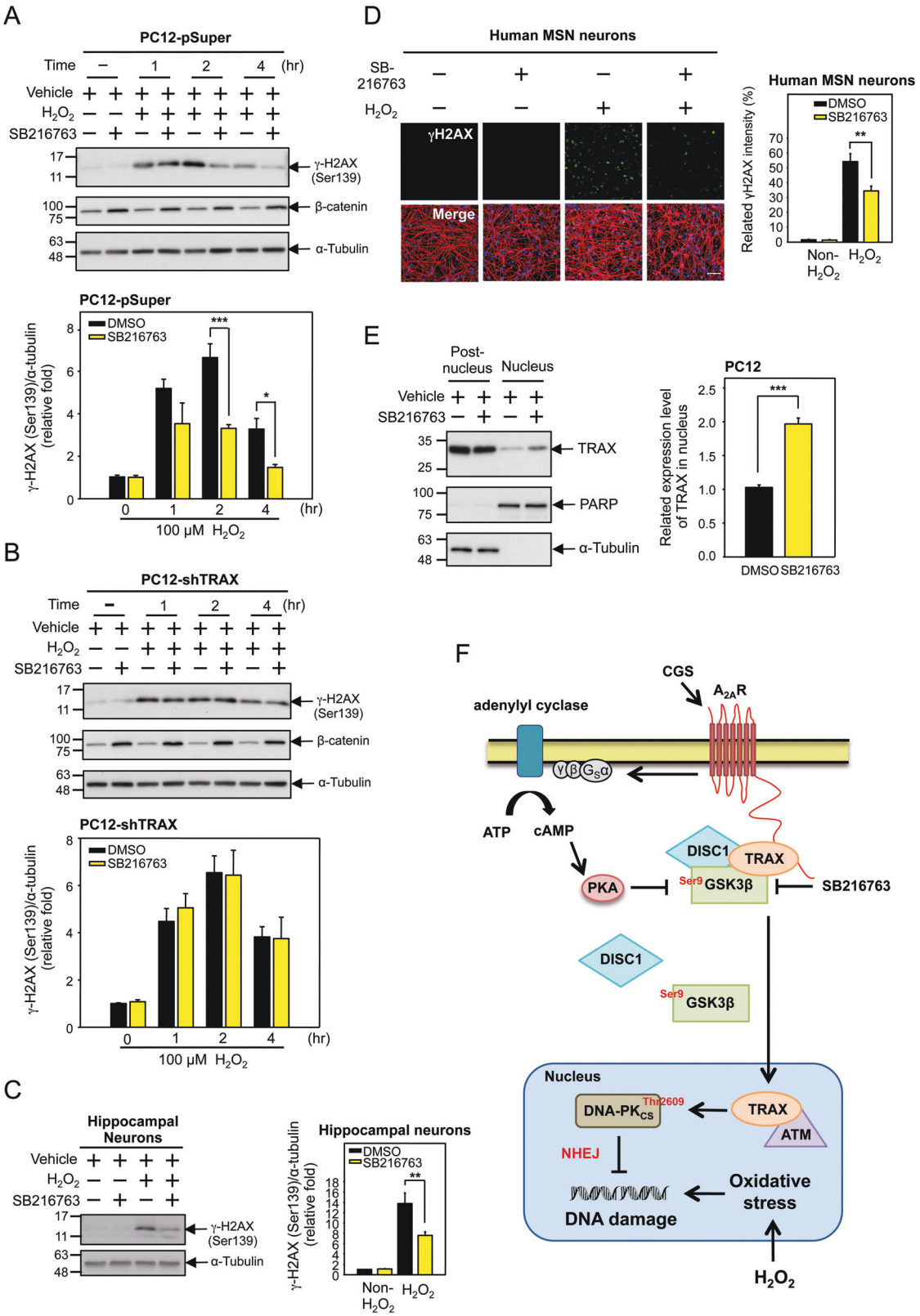
GSK3β binds with DISC1 and TRAX and negatively regulates the TRAX-mediated DNA repair during oxidative DNA damage

Because GSK3β has been negatively implicated in neuroprotection and DNA-PK-mediated repair [42, 46] and $A_{2A}R$ activation leads to the serine phosphorylation and inhibition of GSK3 activity [47, 48], we next evaluated whether GSK3β is involved in DNA-PK-mediated DNA repair during $A_{2A}R$ stimulation. As shown in Fig. 4a, stimulation of the $A_{2A}R$ by CGS enhanced the phosphorylation of GSK3β at Ser^9, which suppressed GSK3β activity. No inhibition/phosphorylation of GSK3β was observed in the presence of PKA inhibitors (H89 or KT5720, Fig. 4a). Because previous studies have shown that mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) may function upstream of GSK3β [49], we next tested whether these two kinases are involved in the regulation of GSK3β in PC12 cells. As shown in Figure S3A, pretreatment of PC12 cells with a MAPK inhibitor (UO126, 10 μM) or a PI3K inhibitor (LY294002, 20 μM) did not affect the $A_{2A}R$-mediated inhibition of GSK3β. Collectively, these results demonstrated that $A_{2A}R$ stimulation reduced GSK3β activity via a pathway that requires activation of PKA, but not MAPK or PI3K.

In addition to assessing the GSK3β activity by determining the level of GSK3β-Ser^9 phosphorylation, we also evaluated a downstream target of GSK3β to validate the activation of GSK3β. Specifically, expression of β-catenin is negatively regulated by GSK3β [20]. Consistent with the inhibition of GSK3β by $A_{2A}R$, stimulation of $A_{2A}R$ increased the level of β-catenin, and this effect was reversed by two PKA inhibitors (H89 and KT5760; Fig. 4b). PKA assay revealed that stimulation of $A_{2A}R$ enhanced PKA activity, which was blocked by PKA inhibitors (Fig. S3B). Taken together, these results confirmed that activation of $A_{2A}R$ inhibited GSK3β through a PKA-dependent pathway.

To assess whether GSK3β plays an important role in DNA repair, we created a constitutively active GSK3β mutant (GSK3β-S9A) [50]. The overexpression of a GSK3β-S9A in PC12 cells (Fig. 4c) and in human MSNs derived from iPSCs (Fig. 4d) abolished the protective effect of CGS on oxidative DNA damage assessed by the level of γH2AX. In addition, the overexpression of GSK3β-S9A eliminated the protective effect of CGS on apoptosis evoked by oxidative DNA damage (Fig. 4e), confirming the importance of GSK3β inhibition in the protective effect of the $A_{2A}R$ in PC12 cells.

Previous studies suggest that GSK3β physically interacts with DISC1 [19, 51, 52], an important scaffold protein that


A
PC12-pSuper
Time – 1 2 4 (hr)
Vehicle + + + + + + + +
H2O2 – – + + + + + +
SB216763 – + – + – + – +
17
11
γ-H2AX (Ser139)
100
75
β-catenin
63
48
α-Tubulin
PC12-pSuper
DMSO
SB216763
γ-H2AX (Ser139)/α-tubulin (relative fold)
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0 1 2 4 (hr)
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PC12-shTRAX
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Vehicle + + + + + + + +
H2O2 – – + + + + + +
SB216763 – + – + – + – +
γ-H2AX (Ser139)
β-catenin
α-Tubulin
PC12-shTRAX
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C
Hippocampal Neurons
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γ-H2AX (Ser139)/α-tubulin (relative fold)
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Related γH2AX intensity (%)
Non- H2O2
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E
Post-nucleus
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Vehicle + + + +
SB216763 – + – +
TRAX
PARP
α-Tubulin
PC12
***
Related expression level of TRAX in nucleus
DMSO SB216763
F
CGS
A2AR
adenylyl cyclase
γβGsα
ATP
cAMP
PKA
DISC1
TRAX
Ser9
GSK3β
SB216763
DISC1
Ser9
GSK3β
Nucleus
DNA-PKcs
Thr2609
TRAX
ATM
NHEJ
Oxidative stress
DNA damage
H2O2

◀ **Fig. 5** Inhibition of GSK3β enables TRAX-dependent DNA repair. **a**, **b** PC12-pSuper and PC12-shTRAX cells were treated with a GSK3β inhibitor (SB216763, 10 μM) or vehicle for 2 days. After the above-mentioned treatment, cells were subjected to H_2O_2 (100 μM) for 1–4 h as indicated. The levels of γH2AX, β-catenin, and α-Tubulin (a loading control) were evaluated by Western blot analysis using the indicated antibodies. The relative amounts of target proteins were quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **c** Primary hippocampal neurons (DIV14) harvested from wild-type B6/C57 mice were treated with SB216763 (10 μM) or vehicle for 1 day and then treated with H_2O_2 (100 μM) for 2 h. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-γH2AX and anti-α-Tubulin antibodies, as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin, the loading control. These experiments were repeated three times. **d** Human iPSCs-derived MSN neurons were treated with SB216763 (10 μM) or vehicle for 1 day and then treated with H_2O_2 (100 μM) for 4 h. DNA damage was assessed by determining the intensity of the DNA damage marker γH2AX by immunofluorescence staining using the anti-γH2AX antibody (green) in neurons identified by a neuronal marker (TUJ1, red). Scale bar, 50 μm. **e** PC12 cells were treated with a GSK3β inhibitor (SB216763, 10 μM) or vehicle for 2 days and nuclear and cytosolic fractions were isolated. Cells were lysed and subjected to SDS–PAGE, followed by Western blot analysis using the anti-TRAX, anti-α-Tubulin and anti-PARP antibodies as indicated. The amount of target protein was quantified and normalized to that of α-Tubulin or PARP, the loading controls for post-nucleus and nucleus fractions, respectively. These experiments were repeated three times. The data are presented as the mean ± SEM from at least three independent experiments0. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to control. **f** A schematic representation showing that TRAX interacts with the $A_{2A}R$ and forms complexes with GSK3β and DISC1 at the resting stage. Inhibition of GSK3β by either the activation of an $A_{2A}R$/PKA-dependent pathway or a GSK3β-specific inhibitor (SB216763) would release TRAX from the complex to facilitate the ATM/DNA-PK- dependent NHEJ in the nuclei

functionally interacts with TRAX [21]; we therefore evaluated whether GSK3β forms a complex with DISC1 and TRAX. HEK293T cells were transfected with expression constructs of V5-tagged TRAX (TRAX-V5), Myc-tagged GSK3β (GSK3β-Myc) and Flag-tagged DISC1 (DISC1-Flag) for 48 h, and subjected to immunoprecipitation. Our results showed that both DISC1-Flag and GSK3β-Myc existed in the immunocomplex with TRAX-V5 (Fig. S4A). Similarly, IP of TRAX successfully pulled down endogenous GSK3β and DISC1 from rat brains (Fig. S4B) and primary neurons (Fig. S4C). By the in situ proximity ligation assay, we also observed the interaction of GSK3β/TRAX and GSK3β/DISC1 in human MSN cells derived from iPSCs (Fig. 4f), supporting the idea that the TRAX/DISC1/GSK3β complex might exist in these cells.

Given that TRAX is an interacting protein of the $A_{2A}R$ [25], we further examined whether the interaction among TRAX, DISC1 and GSK3β was regulated by $A_{2A}R$ activation. The stimulation of the $A_{2A}R$ using CGS significantly reduced the complex formation of TRAX, DISC1 and GSK3β in PC12 cells (Fig. 4g and S4D). The importance of the $A_{2A}R$ was further evaluated by the overexpression of $A_{2A}R_{253–410}$ (designated M7) [25], a DNA fragment encoding the 7th transmembrane domain plus the C-terminus of the $A_{2A}R$. Of note, the C-terminus of the $A_{2A}R$ is the binding domain for TRAX [25]. Overexpression of M7 in PC12 cells reduced the interaction of $A_{2A}R$ and TRAX (Fig. 4h). As shown in Fig. 4i, the expression of M7 in PC12 cells reversed the CGS-mediated rescue of oxidative DNA damage as assessed by the levels of γH2AX. Immunofluorescence analysis further demonstrated that stimulation of the $A_{2A}R$ by CGS enhanced the levels of TRAX (Fig. 4j), but not those of GSK3β or DISC1 (Fig. S5), in the nuclei of PC12 cells. While the overexpression of wild-type (WT) TRAX-V5 in PC12 cells with low TRAX (i.e., PC12-shTRAX) rescued the $A_{2A}R$-mediated DNA repair during oxidative stress, a TRAX variant (designated ΔNLS-TRAX-V5) that lacked the nuclear localization signal (NLS) produced no effect (Fig. 4k). TRAX thus appeared to execute its effect on DNA repair in nuclei.

Our findings collectively suggest that TRAX, DISC1 and GSK3β form complexes that play a critical role in the regulation of repair of oxidative DNA damage. Stimulation of the $A_{2A}R$ disrupted the TRAX/DISC1/GSK3β complex and allowed TRAX to enter nuclei for DNA repair.

GSK3β blockade enables TRAX to mediate DNA repair during oxidative DNA damage

Our findings led us to hypothesize that inhibition of GSK3β might result in the disassembly of its interaction with DISC1 and TRAX and subsequently release TRAX to facilitate DNA repair. To test this hypothesis, PC12 cells were treated with the indicated GSK3β inhibitor (SB216763, 10 μM; Fig. 5a) to suppress GSK3β activity before the exposure to oxidative stress (H_2O_2) for 1–4 h as indicated. Treatment with a GSK3β inhibitor significantly enhanced the expression level of a downstream target (i.e., β-catenin, [20]) of GSK3β, demonstrating the effective blockade of GSK3β. Most intriguingly, although the initial oxidative DNA damage assessed by the amounts of γH2AX in the first hour of H_2O_2 treatment was similar, the levels of γH2AX in PC12-pSuper cells treated with GSK3β inhibitors were much lower than those of the vehicle-treated cells in the later time course (2 and 4 h; Fig. 5a).

Similarly, inhibition of GSK3β by SB216763 protects cells from oxidative stress-induced DNA damage in primary neurons and iPSCs-derived human MSN cells (Fig. 5c, d). Consistent with our hypothesis that TRAX plays a critical role in this DNA repair process, a GSK3β inhibitor SB216763 exerted no effect on the repair of DNA damage in PC12-shTRAX cells that had low levels of TRAX (Fig. 5b). Similar to the effect of CGS (Fig. 4j), inhibition of GSK3β by SB216763 also increased the level of TRAX in

the nucleus (Fig. 5e), supporting that TRAX may function in the nuclei. These results collectively suggested that blockade of GSK3β facilitates the TRAX-mediated DNA repair process.

Discussion

We have previously reported that TRAX plays a critical role in the ATM/γH2AX-mediated DNA repair pathway by directly interacting with ATM at DSBs in the nuclei [23]. In the present study, we provide evidence to demonstrate that TRAX formed complexes with GSK3β and DISC1 at the resting stage (Fig. 4 and S4). Inhibition of GSK3β by either the activation of an A_{2A}R/PKA-dependent pathway (Figs. 4a, b and S3B) or a GSK3β-specific inhibitor (SB216763; Fig. 5a) would release TRAX from the complex to facilitate the ATM/DNA-PK- dependent NHEJ in the nuclei (Figs. 4j and 5e). The function of TRAX in DNA repair was confirmed in a rat neuron-like cell line (PC12 cells), primary mouse hippocampal neurons, and human MSN cells derived from iPSCs (Fig. 2), suggesting that facilitation of the repair for oxidative DNA damage is a conserved function of TRAX. Because a deficiency in DNA repair has been implicated in many mental disorders (including bipolar disorder and schizophrenia) [2, 3], our findings provide an intriguing explanation for the association between the human TRAX gene and these mental illnesses [15, 16, 53].

TRAX is a multi-functional scaffold protein that regulates various cellular activities by interacting with many proteins for a growing list of different functions, including proliferation [54], trafficking of brain-derived neurotrophic factor (BDNF) mRNA [55], production of RNAi [10], neuritogenesis [25], and DNA repair [23]. In the present study, we report that DISC1 and GSK3β are two additional interacting proteins of TRAX. In addition to functionally associating with DISC1 [21], we found that TRAX also physically interacts with DISC1 (Fig. 4 and S4), a risk gene for schizophrenia. In fact, the human DISC1 and TRAX genes are located nearby at 1q42. This DISC1/TRAX locus is associated with mental illnesses, including schizophrenia [15, 16]. Mice lacking TRAX (TRAX-null) showed abnormal behaviors as evaluated by the open-field and the elevated plus-maze tests (data not shown). In addition, *DISC1* mutant mice that lack exons 2 and 3 of the *DISC1* gene also showed similar behaviors to those of TRAX-null mice [56]. Importantly, the association of TRAX with DISC1 and GSK3β is regulated by the activity of GSK3β. The inhibition of GSK3β via various approaches allowed TRAX to mediate the repair process of oxidative DNA damage (Figs. 4, 5). We hypothesized that the TRAX/DISC1/GSK3β complex might serve as a tightly controlled unit and could be regulated by stimuli that inhibit GSK3β and subsequently release TRAX for the subsequent action (s). It is of interest to evaluate whether TRAX contributes to other known actions of GSK3β or/and DISC1 in the future.

Lithium, an inhibitor of GSK3β, is a widely used mood stabilizer in the treatment of many mental diseases, notably bipolar disorder [57]. Although the molecular mechanism underlying its clinical effectiveness remains largely elusive, the inhibition of GSK3β has been implicated in multiple actions of lithium [58, 59]. Most interestingly, lithium can also be used to treat ethanol toxicity and ischemia [60, 61], in which high oxidative stresses occur. The observations in the present study are consistent with those studies and suggest that lithium is neuroprotective against stresses that cause DNA damage. For example, lithium enhances DNA–PK-mediated DNA repair in irradiated hippocampal neurons [42]. More recently, lithium has been shown to protect retinal neurocytes from ischemia-induced damage by the up-regulation of DNA ligase IV, a key NHEJ ligase, via a pathway that requires nuclear respiratory factor 1 (Nrf-1) and the activation of cyclic AMP-response element binding protein-1 (p-CREB1) [62]. The results of our study revealed a new component, TRAX, which mediates the protective effect of a GSK3β inhibitor on oxidative DNA damage in neurons (Fig. 5). In addition to mental disorders and neuronal traumas, oxidative DNA damage or inferior DNA repair systems have also been reported in neurodegenerative diseases [9, 63–66]. For example, multiple reports suggest that DISC1 is a candidate gene for Alzheimer's disease [67]. Dysregulations of DISC1 and GSK3β are part of the Huntington's disease (HD) pathogenesis [68, 69]. Lithium, which blocks GSK3β and regulates other signaling pathways, has also been considered one of the beneficial reagents for the amelioration of degenerative diseases [57, 70]. Given the clinical importance of lithium, further investigations are warranted to assess the potential involvement of TRAX/DISC1/GSK3β in various mental diseases, brain traumas, and neurodegenerative diseases.

Beyond the blockade of GSK3β directly using SB216763, our study demonstrated that stimulation of the A_{2A}R, an interacting protein of TRAX, activated PKA that inhibited GSK3β and subsequently freed TRAX to facilitate the ATM/DNA-PK- mediated NHEJ pathway in the nuclei (Figs. 4, 5f). Stimulation of the A_{2A}R might promote DNA repair in neurons and restore neuronal functions. Consistent with this hypothesis, several approaches have been explored for adenosine augmentation, which may result in the activation of adenosine receptors and ameliorate the symptoms of schizophrenia [28, 29]. Accumulated evidence suggests that adenosine contributes to neurodevelopmental processes, which have been implicated in schizophrenia [26]. Thus, it is possible that adenosine augmentation not only can be used as a treatment for schizophrenia but also prevent the genesis of schizophrenia [26, 27]. Our results

provide potential mechanistic insights into the role of adenosine and $A_{2A}R$ in schizophrenia and other mental disorders associated with inferior DNA repair [1–3].

Another interesting aspect is the regulation of the TRAX/$A_{2A}R$ complex. Our results suggest that inhibition of GSK3β may lead to dissociation of TRAX from $A_{2A}R$. Immunoprecipitation of TRAX effectively pulled down $A_{2A}R$ from detergent-soluble fractions of PC12 cells. Treatment with SB216762 markedly reduced the amount of $A_{2A}R$ existing in the TRAX-immunocomplex (Fig. S6A–E), indicating that the interaction between $A_{2A}R$ and TRAX was also regulated by GSK3β. Because stimulation of $A_{2A}R$ also suppresses GSK3β (Fig. 4), TRAX can be released not just from DISC1 and GSK3β but also from $A_{2A}R$ during $A_{2A}R$ stimulation. This would explain why stimulation of $A_{2A}R$ by CGS enhanced the level of TRAX in the nuclei of PC12 cells (Fig. 4j) and support our hypothesis that TRAX mediates its function in DNA repair in nuclei. Of note, although the level of TRAX and $A_{2A}R$ were not altered by treatment with SB216762, the amount of TRAX immunoprecipitated by the anti-TRAX antibody was slightly higher than that from control cells (Fig. S6F). This finding is interesting because the anti-TRAX antibody was raised against the last 18 amino acids located in the C-terminus of TRAX [25]. It is possible that dissociation from its binding partners may expose the C-terminus of TRAX, and result in a more efficient immunoprecipitation of TRAX. It is currently unknown whether $A_{2A}R$ and TRAX are substrates of GSK3β. Analyses using the NetPhos 3.1 Server [71] suggest that TRAX has one potential GSK3 phosphorylation site and that $A_{2A}R$ has seven potential GSK3 phosphorylation sites located at its C-terminus where TRAX binds [25]. Further investigations are required to evaluate whether GSK3β regulates the formation of the $A_{2A}R$/TRAX complex by direct phosphorylation of TRAX or $A_{2A}R$ or through an indirect manner.

Stimulation of the $A_{2A}R$ also effectively protects human MSN cells derived from iPSCs of HD patients from oxidative stress [34] and delays the symptoms of HD mice that suffer from high oxidative stress [30, 72, 73]. The binding of TRAX to the C-terminus of the $A_{2A}R$ provides an efficient means by which to control the release of TRAX from the TRAX/DISC1/GSK3β complex and from $A_{2A}R$, and may also facilitate other TRAX-dependent functions, including the transport of BDNF mRNA [55]. This is of great interest since the chronic blockade of the $A_{2A}R$ in vivo markedly suppressed the neuronal plasticity mediated by BDNF in the hippocampus [74]. Because $A_{2A}R$ agonists have been considered antipsychotic drugs [75], and the DISC1/TRAX locus is associated with schizophrenia [14–17], the role of TRAX in mediating the actions following the activation of the $A_{2A}R$ is of great importance and requires further investigation.

TRAX was identified 15 years ago and has been implicated in a wide variety of cellular functions ranging from nuclear machinery to events occurring in the cytoplasm. Studies from different laboratories suggest that TRAX exerts its functions via a direct interaction with different binding partners. Our study revealed at least two pathways (i.e., the stimulation of the $A_{2A}R$ and the blockade of GSK3β) to regulate the complex that TRAX binds to and shed new light on the role of GSK3β/DISC1 in the NHEJ of oxidative DNA damage. Future investigation on the regulation of the TRAX/DISC1/GSK3β complex may pave the way for the development of novel therapeutic treatments for mental illness and neurodegenerative diseases with high oxidative DNA damage or/and poor DNA repair.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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