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Article

JNK Inhibitor Suppresses CASK Deficiency-Induced Cerebellar Granular Cell Death in MICPCH Syndrome Model Mice

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Abstract: Microcephaly with pontine and cerebellar hypoplasia (MICPCH) syndrome is a severe neurodevelopmental disorder caused by a deficiency in the X-linked gene calcium/calmodulin-dependent serine protein kinase (CASK). A better understanding of the role of CASK in the pathophysiology of neurodevelopmental disorders may provide insights into novel therapeutic and diagnostic strategies for MICPCH syndrome and other neurodegenerative diseases. To investigate this, we generated CASK knockout (KO) cerebellar granule (CG) cell culture from CASK floxed (CASK^{flox/flox}) mice by infecting lentiviruses expressing iCre. We performed RNA-sequencing (RNA-seq) on these cells and found that CASK-KO CG cells underwent apoptosis by activating intracellular Jun N-terminal kinase (JNK) signaling and upregulating reactive oxygen species (ROS) -related gene expression. We also performed mouse gait analysis and limb clasping behavior experiments on trans-heterozygous CASK-KO and Hprt-eGFP (CASK^{+/-}Hprt^{eGFP/+}) mice. The CASK^{+/-}Hprt^{eGFP/+} mice exhibited cerebellar ataxic phenotypes as judged by the scores of these experiments compared to the CASK wild-type control (CASK^{+/+}Hprt^{eGFP/+}) mice. Interestingly, the administration of the JNK inhibitor, JNK-IN-8, in CASK-KO CG cell cultures increased CG cell survival by reducing ROS generation. Moreover, injection of JNK-IN-8 into the cerebellum of CASK^{+/-}Hprt^{eGFP/+} mice suppressed CG cell death and alleviated cerebellar ataxic phenotypes *in vivo*. In conclusion, JNK-IN-8 suppresses the cell death and activation of ROS pathway in CASK-KO CG cells in both *in vitro* and *in vivo* models, suggesting its potential as a therapeutic strategy for cerebellar neurodegeneration in MICPCH syndrome.

Keywords: CASK; cerebellum granule cell; Reactive oxygen species; MAPK; JNK inhibitors; MICPCH syndrome

1. Introduction

Microcephaly with pontine and cerebellar hypoplasia (MICPCH) syndrome is a severe neurodevelopmental disorder with numerous symptoms associated with microcephaly and pontocerebellar hypoplasia, usually found in girls, and lethality in boys [1–3]. MICPCH syndrome is caused by the deficiency of the X-linked gene calcium/calmodulin-dependent serine protein kinase (CASK) that encodes a membrane associated guanylate kinase (MAGUK) family protein, containing a CaMK domain, two L27 domains, a PDZ domain, an SH3 domain, and a C-terminal guanylate kinase domain [4]. CASK has been originally identified as an intracellular binding partner for Neurexins [5] and considered to function for regulating formation and maintenance of synapse and neurotransmitter release in the brain [6]. CASK knockout (KO) mice showed significant developmental abnormalities and lethality [7]. The excitatory/inhibitory (E/I) balance is disrupted in cultured neurons derived from CASK-KO mice. Female CASK heterozygote KO (CASK-hKO) mice

may be the models that mimic the genetic background of most MICPCH syndrome. These mice are subjected to the effects of X-chromosome inactivation, resulting in a mosaic distribution of CASK-expressing and CASK-KO cells in the cerebral cortex [8]. Patch-clamp electrophysiology in acute brain slices has shown that excitatory synaptic input to CASK-KO pyramidal neurons is increased, while inhibitory synaptic input is reduced in CASK-hKO mice [8]. This is attributed to insufficient transcription of *Grin2B*, which encodes the GluN2B subunit of the N-methyl-D-aspartate (NMDA) receptor, due to a deficiency in the complex formed between guanylate kinase domain of CASK and the T-box transcription regulatory factor TBR1 [8].

Mutations in CASK identified in the human patients have been shown to affect brain development and function. *In vitro* experiments demonstrated these mutations cause neurodegeneration and apoptosis in cerebellar granule cells (CG cells) [9]. However, the detailed interaction between the CASK deficiency and the degeneration of CG cells has not been completely investigated. A better understanding of the role of CASK in maintenance of the cerebellar structure may provide insights into the prevention of the cerebellar hypoplasia in MICPCH syndrome.

Ding et.al., reported that the knockdown of CASK in hepatocellular carcinoma (HCC) activates the Jun N-terminal kinase (JNK), a kinase that belongs to the family of stress-activated serine/threonine protein kinases of the mitogen-activated protein kinase (MAPK) pathway [10]. JNK signaling plays a key role in cell death by regulating reactive oxygen species (ROS) [11,12], and the administration of the JNK inhibitor significantly attenuates CASK deficiency-mediated autophagic cell death [10]. This finding suggests that CG cell death in CASK-KO mice may be also involved in the JNK and MAPK pathways, and inhibiting such pathways may rescue CG cell survival.

To investigate the molecular mechanism underlying cerebellar neurodegeneration caused by CASK deficiency, we performed RNA-sequencing (RNA-seq) analysis on CASK-KO CG cell culture. We identified that the JNK pathway-related genes were upregulated in these cells. The administration of JNK inhibitor rescued the CASK-KO CG cells in both *in vitro* and *in vivo*. These results suggest that JNK inhibitors have the potential to serve as a therapeutic strategy for cerebellar neurodegeneration in MICPCH syndrome.

2. Materials/Subjects and Methods

2.1. Animals and Housing Conditions

Floxed CASK (CASK^{flox/flox}) mice and wild type mice (CASK^{+/+})(B6;129-Casktm1Sud/J, JAX Stock #006382) were as described previously[9], Hprt-eGFP mice was obtained by other members in our laboratory. Male homozygous CASK KO (CASK^{-/-}) mice and female heterozygous CASK KO mice (CASK^{+/-} mice) were obtained by crossing CASK^{flox/flox} mice and ZP3-Cre mice [13] (C57BL/6-Tg (Zp3-cre)93Kw/J, JAX Stock #003651). Heterozygous CASK KO with Hprt-eGFP (CASK^{+/-}Hprt^{eGFP/+}) mice was obtained by crossing female CASK^{+/-} mice and Hprt-eGFP mice.

All animals were maintained with a 12:12 light-dark cycle with food and water ad libitum. The room temperature was maintained at 23 ± 2°C. Animal experiments were reviewed by the Committee for Animal Experiments and were approved by the president of Shinshu University No.021045. The methods were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University and the ARRIVE guidelines 2.0.

2.2. Plasmid and Lentivirus Preparation

Lentivirus-CTL: EGFP was removed by digestion with BamHI and EcoRI from pFSy(1.1)GW (Addgene #27232) and self-ligated. pFSyGW-iCre (lentivirus-iCre): BamHI-iCre-EcoRI was cloned into the BamHI/EcoRI site of pFSy (1.1)GW. Empty lentivirus (pFSy-control, lentivirus-CTL) was used as control. Construction of all other plasmid used in this study was described previously[9].

2.3. Cell Culture

Primary CG cells were prepared from wild-type C57BL/6, CASK^{flox/flox}, Hprt^{eGFP/eGFP}, or CASK^{+/-flox}Hprt^{eGFP/+} mice at P6, as described previously[9,14]. In short, isolated cerebella were incubated with phosphate-buffered saline (PBS) containing 1% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% DNase I (Sigma-Aldrich) for 3 min and dissociated by passing through a fire-polished Pasteur pipette in PBS containing 0.05% DNase I, 0.03% trypsin inhibitor (Sigma-Aldrich), and 2 mM MgCl₂. Cells were plated on coverslips coated with mouse laminin (ThermoFisher Scientific, Waltham, MA, USA) and poly-L-lysine (Sigma-Aldrich) at a density of 2×10^6 cells/well, cultured in Neurobasal-A (ThermoFisher Scientific) supplemented with 2% B-27 supplement (ThermoFisher Scientific), 5% fetal bovine serum (FBS) (Biowest, Nuaille, France), 100 U/mL of Antibiotic-Antimycotic Mixed (Nacalai tesque, Kyoto, Japan), and 2 mM GlutaMAX I (ThermoFisher Scientific) for 24 h at 37 °C in a 5% CO₂ atmosphere, then cultured in the same medium without FBS.

The cultured cells were infected with either an empty lentivirus (pFSy(1.1)GW) or the lentivirus-iCre (pFSyGW iCre) at DIV1. After the first infections, the culture medium was replaced with the fresh medium, followed by infection with an empty lentivirus or a lentivirus expressing CASK. For drug effect testing, after the initial infections, the culture medium was replaced with the fresh medium, followed by a 0.1 μ M, 1 μ M, 10 μ M, 20 μ M, 50 μ M or 100 μ M of inhibitors (Table S2) or DMSO (0.1%) as a control. The cells were harvested in DIV4 and prepared for RNA extraction. The cells were fixed in DIV7 by 4%PFA+4% sucrose for further experiments.

2.4. RNA Extraction and Purification

Total RNA was extracted using TRIzol (Ambion, Carlsbad, CA, USA) followed by the manufacturer's instructions, purified with the PureLink™ RNA Mini Kit (ThermoFisher Scientific). After purification, the total RNA was stored at -80°C for further study.

2.5. RNA-Sequencing Analysis

RNA-seq was mainly completed by Takara Bio (TAKARA BIO INC., Shiga, Japan). Briefly, the reverse transcription was performed with the SMART (Switching Mechanism at 5' End of RNA Template) method with the SMART-Seq Stranded Kit. Then the PCR product purification was conducted using AMPure XP (Beckman Coulter, California, USA). The RNA-seq library was prepared by Nextera XT DNA Library Prep Kit. Sequence was performed with NovaSeq 6000 (Illumina, San Diego, USA). The results normalized by using the TPM (Transcripts Per Million), data visualization was performed using Rstudio[15] (R 4.3.0; Posit Software; BOSTON, USA), gene ontology analysis was performed with the Metascape[16] and gene set enrichment analysis (GSEA)[17,18].

2.6. Stereotaxic Surgery

P6 CASK^{+/-flox}Hprt^{eGFP/+}, CASK^{+/-}Hprt^{eGFP/+}, or CASK^{+/-} mice were anesthetized on ice. Using Neuros syringes (65460-02, Hamilton, USA) and approximately 10 mg/ml (2% DMSO, 30% PEG 300, 5% Tween 80, 63% ddH₂O) JNK-IN-8 or DMSO, 6.6 mg/kg was injected bilaterally in the following stereotactic coordinates of the cerebellum-anteroposterior (AP) - 1.2 mm, mediolateral (ML) \pm 1.0 mm, and dorsoventral (DV) - 1.3 mm, taking lambda as the reference point using a stereotaxic device (Narishige, Tokyo, Japan). Each mouse injected 2ul drugs and 1ul 0.4%trypan blue. After the surgery, keep the heater on for 30 min before moving back to the mother. The mice were kept for 2 weeks after injection, before further experiments.

2.7. Limb-Clasping Scoring

One week before scoring, mice were allowed to acclimate to the vivarium. Mice were held by the tail for 60 s and recorded by the GoPro 9 (GoPRO, San Mateo, USA). During tail suspension, limb-clasping behavior was evaluated with the following standards: 0 - No limb clasping. Normal escape

extension: 1 - 1 hind limb exhibits incomplete splay and loss of mobility. Toes exhibit normal splay; 2 - Both hind limbs exhibit incomplete splay and loss of mobility. Toes exhibit normal splay; 3 - Both hind limbs exhibit clasping with curled toes and immobility; 4 - Forelimbs and hind limbs exhibit clasping and are crossed, curled toes and immobility [19].

2.8. Mouse Foot Gait Analysis by Ink

Habituate mice to the testing room and gait tunnel with the room light. Place the food at the surface level at the end of the gait tunnel to serve as a goal box. Mice's forelimbs are coated with red ink, and the hindlimbs are coated with blue ink before they walk across a white paper. The papers with paw prints were used to measure the toe spread, stride length, and stride width [20].

2.9. Data Visualization and Statistical Analyses

GraphPad (version 10.2.3(403), GraphPad Software; San Diego, CA, USA) and RStudio (R 4.3.0) were used for data visualization, and the quantitative variables are expressed as the mean \pm standard error of the mean (SEM). Data statistical analyses were performed using GraphPad (version 10.2.3(403) with two-way analysis of variance (2-way ANOVA) followed by Tukey's correction, $p < 0.05$ viewed as statistical significance between each group.

2.10. Additional Materials and Methods

Additional methods are described in Supplementary Methods. Sequences of primers are listed in Table S1. Reagents and antibodies cat numbers are listed in Table S2. Original blot images are shown in Original Data 1.

3. Results

3.1. CASK Deletion Causes Activation of JNK Signaling and Apoptosis Pathway in CG Cells

To investigate the molecular mechanism underlying cerebellar hypoplasia in MICPCH syndrome, we first studied postnatal day 0 (P0) CASK^{-/-} mice. We found the body size of CASK^{-/-} mice was significantly smaller than that of CASK^{+/+} mice (Fig. S1A). Morphological abnormality was also observed in the H&E stained cerebellar slices in CASK^{-/-} mice (Fig. S1B). Since CASK^{-/-} mice die at P0, the capability of *in vivo* analysis in CASK^{-/-} mice was limited.

We then decided to study gene expression profiling in CASK deficient cells in comparison with that in wild-type cells. For this, we performed RNA-seq analysis in cultured CG cells. We prepared dissociated CG cell cultures from CASK^{flox/flox} and CASK^{+/+} mice and infected them with lentiviruses expressing iCre at day *in vitro* (DIV) 1 to generate CASK-KO and wild-type control CG cell cultures (Fig. 1A). Since CASK-KO GC cells started dying at DIV4, we collected mRNA from CASK-KO and control CG cell cultures at DIV4. The expression of CASK in CASK^{flox/flox} iCre CG cells was significantly ($p < 0.001$) decreased compared to that in CASK^{+/+} iCre CG cell in the RNA-seq results, as expected (Fig. 1B). We found the expression levels of JNK signaling and apoptosis-related genes such as c-Jun ($p < 0.01$), Fos12 ($p < 0.01$), Gem ($p < 0.01$) and Cxcl2 ($p < 0.05$) were significantly increased in CASK^{flox/flox} iCre CG cells compared to those in CASK^{+/+} iCre CG cells. We selected genes of which expression were significantly changed in CASK-KO CG cells compared to that in \wild-type cells and performed Gene Ontology analysis (Fig. S2A, 430 in total). The most significantly changed ones were related to the regulation of the neurotransmitter transport pathway (Fig. 1C) and the localization process (Fig. 1D). This suggests that the deletion of CASK may cause abnormality in neurotransmission, highlight the significance of CASK in the synaptic function also in CG cells. GSEA indicated that cell death related signaling such as apoptosis and Reactive Oxygen Species (ROS) pathway were enhanced in CASK^{flox/flox} iCre CG cells (Fig. 1E). To further investigate the molecular interaction, we conducted IPA signaling analysis for the representative genes and found that the CASK deletion caused upregulation of the apoptosis and inflammation signaling, such as IL-1 β and

Tnf, (Fig. 1F). These results suggest that the CASK deletion causes the activation of JNK signaling and apoptosis pathway in CG cells.

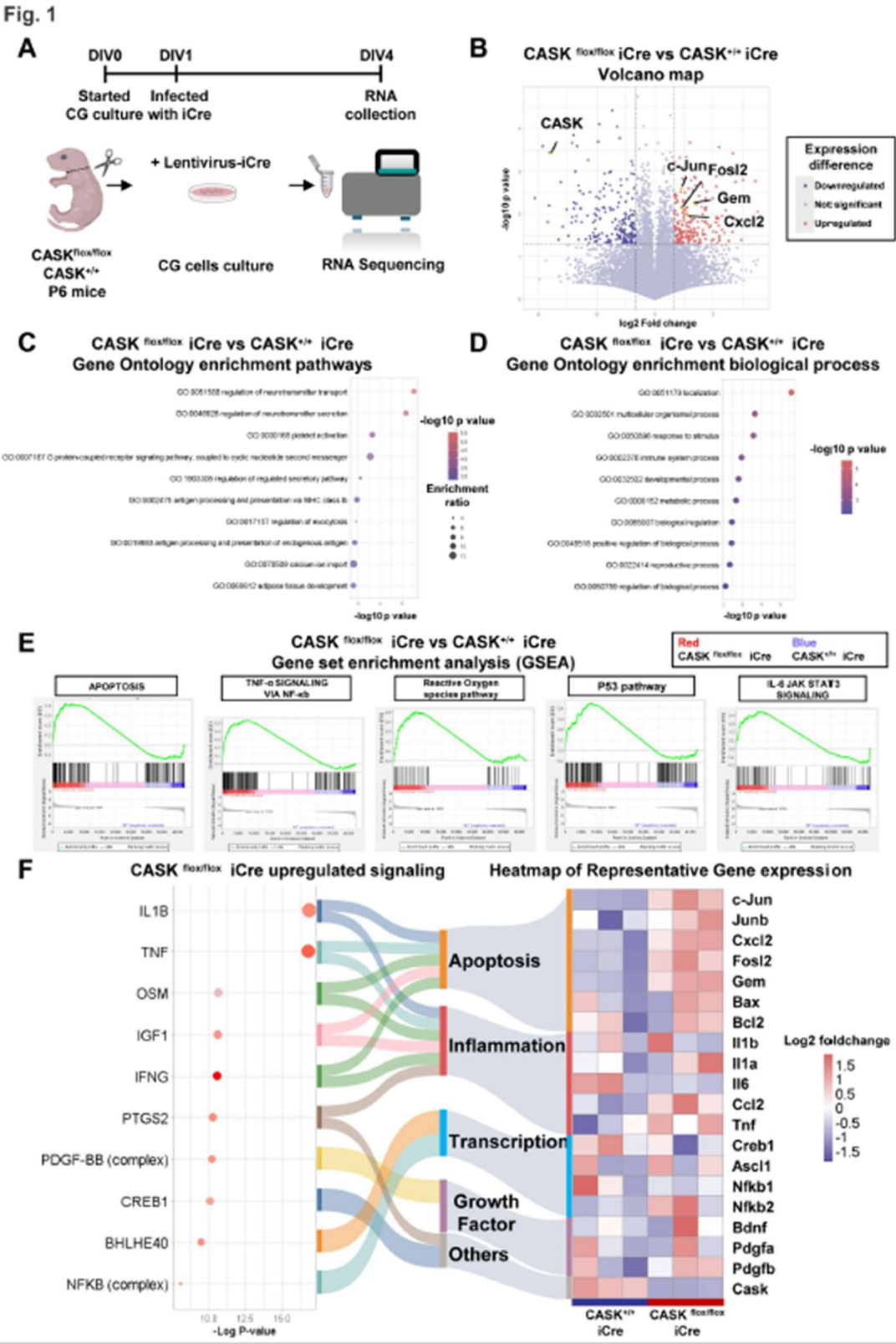


Figure 1. RNA-seq analysis showed activation of JNK signaling and apoptosis pathway in CASK^{flx/flx} iCre CG cells. **A.** Experimental design of the RNA-seq analysis. P6 CASK^{flx/flx} and CASK^{+/-} mice were used for CG cell culture. Cells were infected with iCre in DIV1, RNA was collected in DIV4 and Protein was collected in DIV5. (n=3 in each group) **B.** Volcano map of gene expression difference between CASK^{flx/flx} iCre CG cells and CASK^{+/-} iCre CG cells. Red indicated genes upregulated in CASK^{flx/flx} iCre CG cells, blue indicated genes

downregulated in CASK^{flox/flox} iCre CG cells. (Threshold: $p < 0.05$, Fold change over 2) C. The top 10 Gene Ontology enrichment pathways in CASK^{flox/flox} iCre CG cells analyzed by Metascape. D. The top 10 Gene Ontology enrichment process in CASK^{flox/flox} iCre CG cells analyzed by Metascape. E. Representative Gene Set Enrichment Analysis (GSEA) results of apoptosis related pathways. F. Upregulated signaling in CASK^{flox/flox} iCre CG cells was analyzed using IPA analysis, and the heatmap of the related gene expression detected by RNA-seq.

3.2. The Levels of p-c-Jun and p-JNK in CASK^{flox/flox} iCre CG Cells Are Significantly Increased

To confirm the RNA-seq results, we performed realtime-qPCR and western blotting analysis in the extracts from CG cell cultures infected with lentivirus expressing iCre (Fig. 2A). The expression of CASK in CASK^{flox/flox} iCre was almost undetectable in these experiments indicating gene removal by Cre recombination was successful (Fig. 2B). The expression of genes related to JNK signaling such as c-Jun ($p < 0.001$) and c-Fos ($p < 0.001$) in CASK^{flox/flox} iCre CG cells were significantly upregulated compared to the CASK^{+/+} iCre CG cells. The expression of ROS related genes such as Cyba, Cybb, Ncf1 and Ncf2 was also significantly upregulated in CASK^{flox/flox} iCre infected CG cells. On the other hand, gene expressions such as IL-1 β , NF- κ B1, NF- κ B2, and Bax/Bcl-2 were not significantly changed between each group (Fig. 2B). Therefore, we decided to focus on the protein phosphorylation changes in JNK signaling and ROS pathway.

In western blotting analysis on these cells, we found the phosphorylated c-Jun ($p < 0.05$) and JNK ($p = 0.1725$) increased in CASK^{flox/flox} iCre CG cells compared to the CASK^{+/+} CTL CG cells (Fig. 2C). This suggests the knockout of CASK promotes phosphorylation signaling of JNK, that will cause cell apoptosis in CG cells. These results indicate that the inhibition of JNK signaling may be effective for preventing cell death in CASK deficient CG cells.

Fig. 2

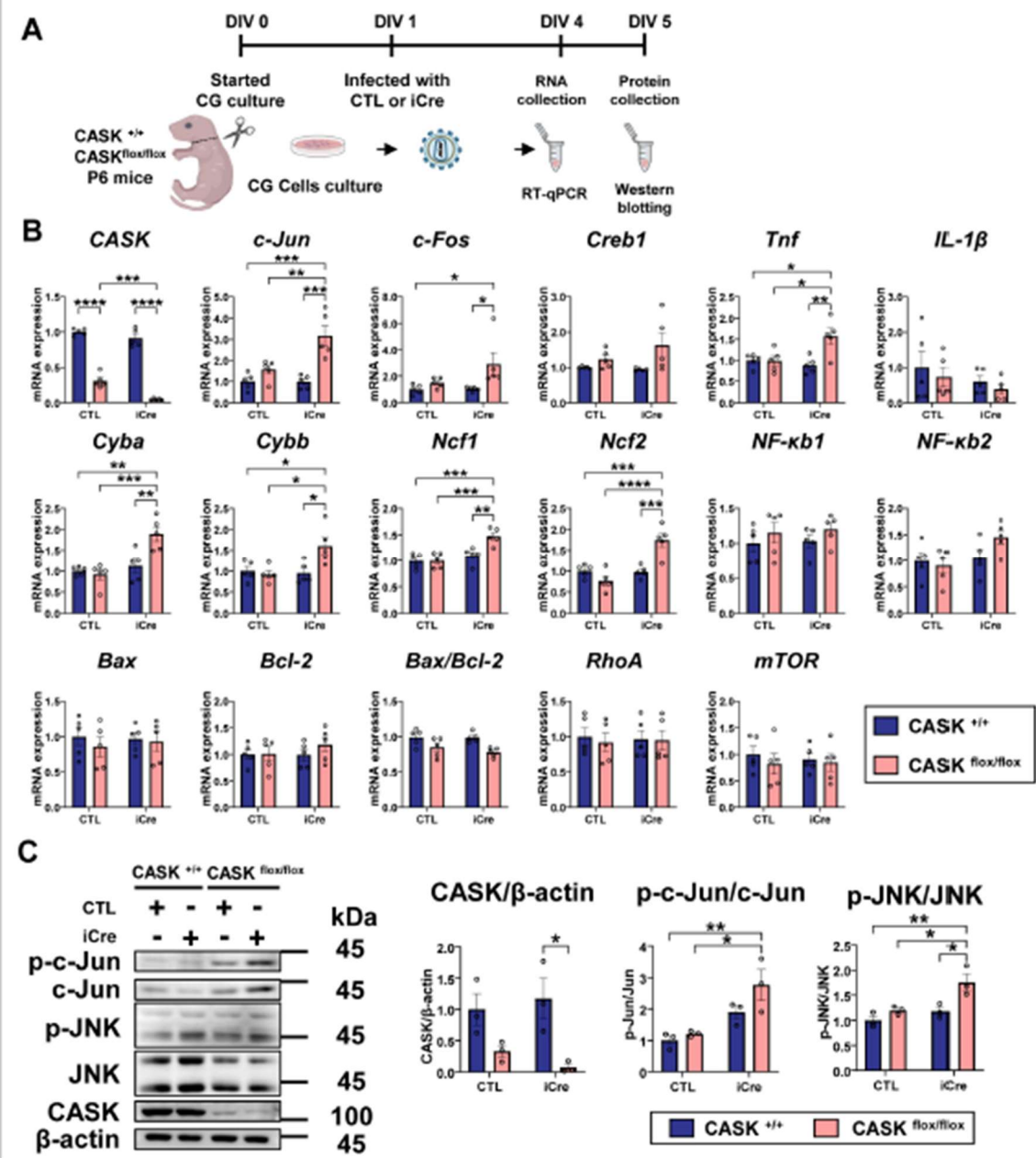


Figure 2. Realtime-qPCR analysis and western blotting confirmed the activation of JNK signaling in CASK^{flx/flx} iCre CG cells A. Experimental design of realtime-qPCR and western blotting. CG cells were infected with lentivirus-iCre and lentivirus-CTL in DIV1, RNA and protein samples were collected in DIV4. B. Realtime-qPCR analysis on CG cells. (n=5 in each group) C. Western blotting analysis on CG cells. (n=3 in each group) * indicates difference between the groups. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$ and **** indicates $p<0.0001$.

3.3. Administration of JNK Inhibitors Promotes the Survival Rate in CASK^{flx/flx} iCre CG Cells In Vitro

To investigate the effects of JNK inhibition on CG cells, we administrated JNK inhibitors on CG cells with different concentrations and stained the cells with NeuN and DAPI to evaluate the survival rate changes (Fig. 3A). Without administration, the survival rate of CASK^{flx/flx} iCre CG cells was significantly lower than the CASK^{+/+} CTL CG cells (Fig. 3B). We found the administration of 1 μ M of JNK-IN-8 and JNK-IN-7 significantly ($p<0.0001$) increased the survival rate of CASK^{flx/flx} iCre CG cells compared to those untreated. However, administration of 10 μ M of these drugs showed cytotoxicity. The administration of Tantisertib, DB07268, and SP600125, another JNK inhibitors,

increased the survival rate of CASK KO CG cells as the concentration increased (Fig. 3C, S3A, B). These results indicate that the inhibition of JNK signaling promotes the survival rate in CASK^{flx/flx} iCre CG Cells.

Fig. 3

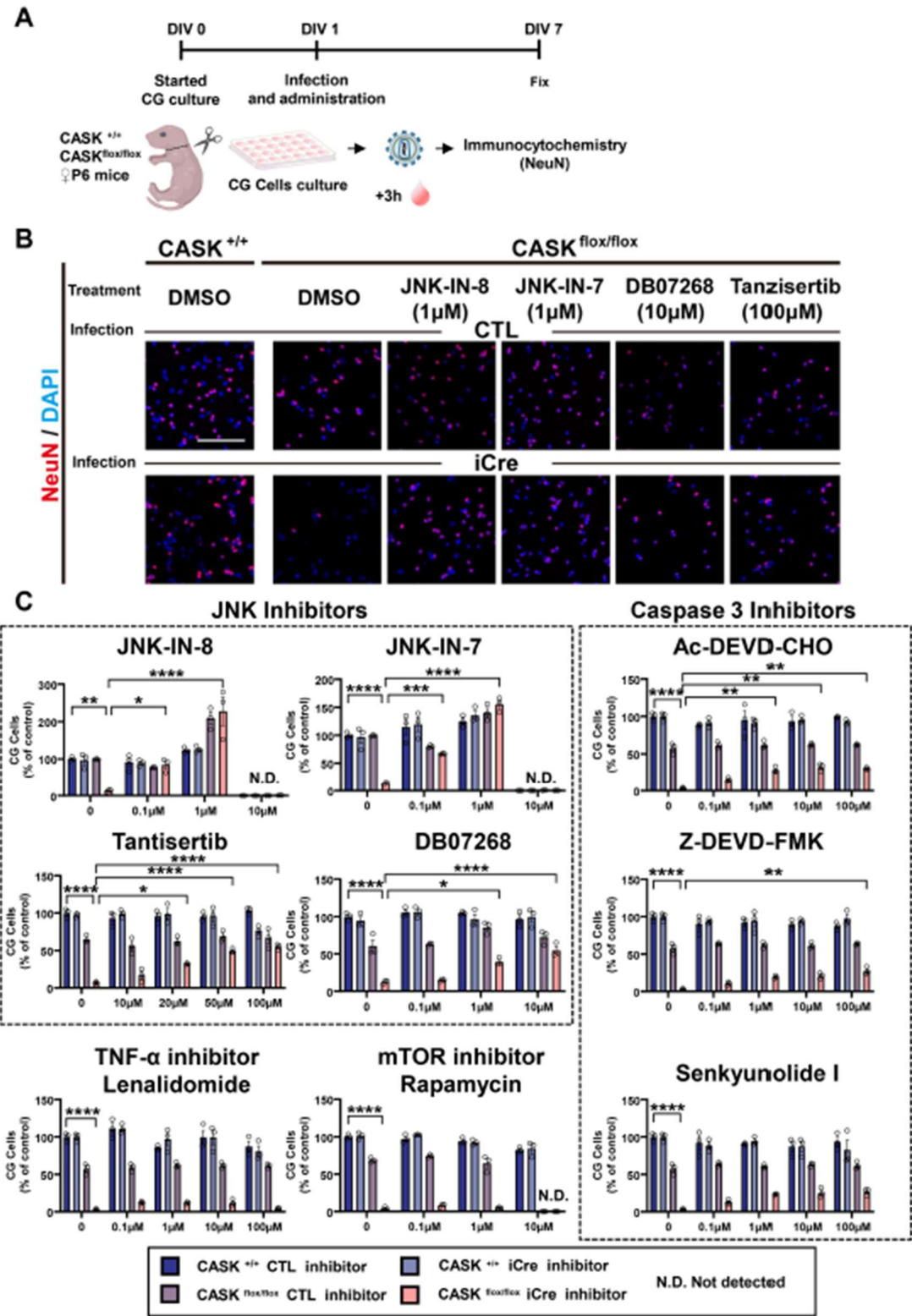


Figure 3. Administration of JNK inhibitors promoted the *in vitro* survival rate in CG cells A. Experimental design of the intracellular signaling inhibitors administration on CG cells. CG cells were infected with lentivirus-iCre and lentivirus-CTL in DIV1, 3 hours later the inhibitors were administrated. In DIV 7, the CG cells were fixed and stained with NeuN and DAPI. B. Images of CG cells stained with NeuN and DAPI, scale bar = 100 μ m.

C. Changes in cell survival rate calculated by the NeuN and DAPI double positive cells. And the group of CASK^{+/+} CTL CG cells with DMSO was used as the control. (n=3) * indicates difference between the groups. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$ and **** indicates $p<0.0001$.

To verify the result, we also administrated JNK signaling related inhibitors, such as the inhibitors of Caspase 3, TNF, mTOR, and p38 signaling (Fig. 3C, S3A, B). Those inhibitors showed a minor effect on the survival rate of CASK-KO CG cells, but not comparative to the effect of JNK inhibitors. These indicate that the JNK inhibition has the strongest relationship with CG cell death caused by CASK deletion. JNK-IN-8 showed strongest rescue effects on CASK deficient CG cell survival among JNK inhibitors we tested. To investigate the effect on apoptosis, we administrated the JNK-IN-8 on CG cells and performed TUNEL staining (Fig. 4A). We found the number of TUNEL and NeuN double positive cells was significantly ($p<0.0001$) increased in CASK^{flox/flox} iCre CG cells compared to CASK^{+/+} CTL CG cells (Fig. 4B, C). After the administration of JNK-IN-8, the number of double positive cells was significant ($p<0.0001$) reduced in CASK^{flox/flox} iCre CG cells. As expected, the number of TUNEL and NeuN double positive cells were decreased in CASK^{flox/flox} iCre CG cells after the infection with Lenti-CASK ($p<0.0001$). The result of TUNEL indicated that the administration of JNK-IN-8 promoted the survival rate in CASK^{flox/flox} iCre CG cells, by preventing the apoptosis caused by the deletion of CASK.

We further verified the inhibition effect of JNK-IN-8 on JNK signaling in CG cells (Fig. 4D). The p-c-Jun/c-Jun and p-JNK/JNK were suppressed by the JNK-IN-8 administration. We also tested the effect of Lenti-CASK infection on CASK^{+/+} CG cells. Although Lenti-CASK infection reduced p-JNK/JNK compared to the uninfected control, the effect was smaller than that of the JNK-IN-8 administration (Fig. S3C).

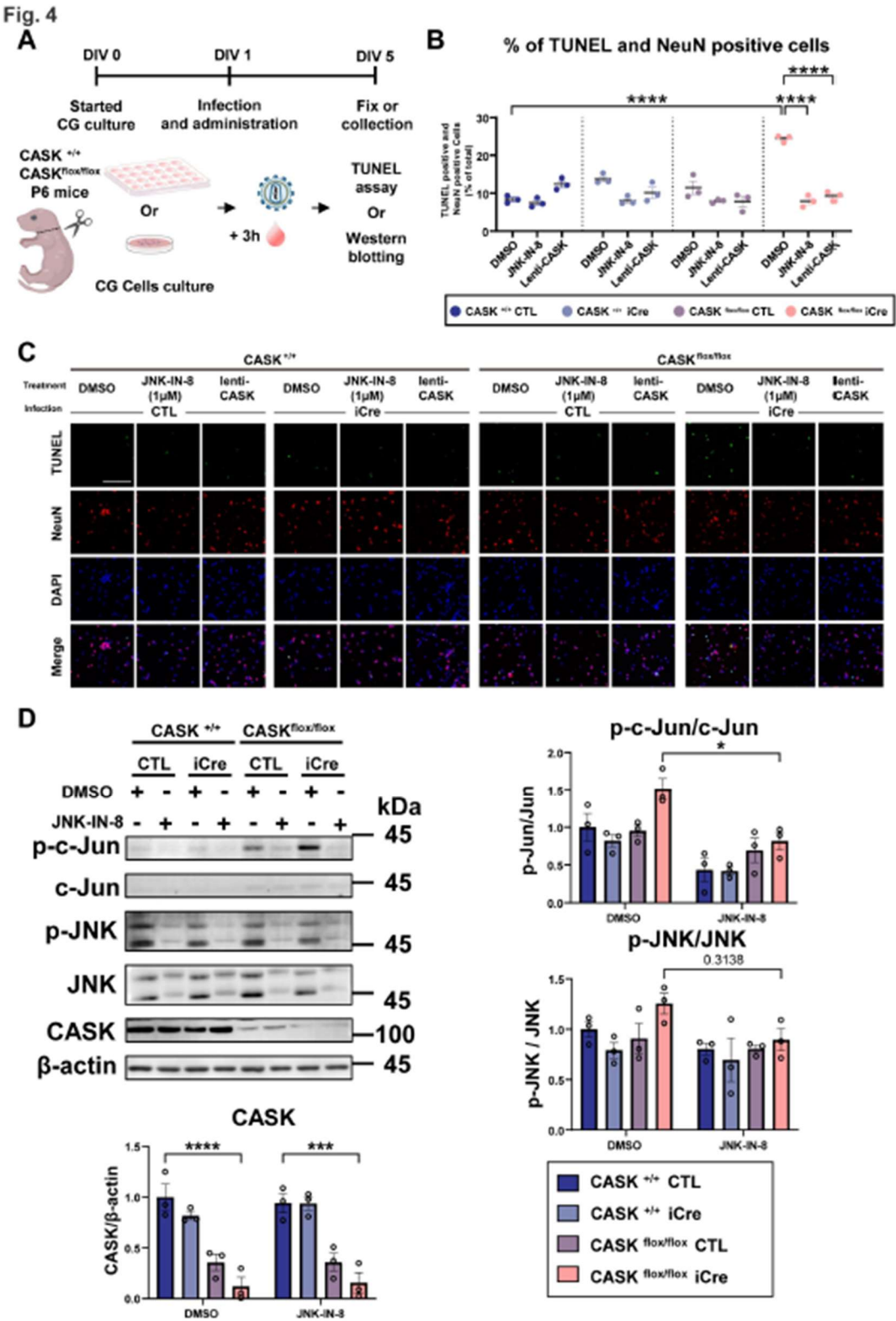


Figure 4. JNK inhibitor reduced apoptosis and JNK signaling in CASK^{flx/flx} iCre CG cells A. Experimental design for TUNEL assay and western blotting. CG cells was infected with lentivirus-iCre and lentivirus-CTL in DIV1, 3 hours later the inhibitors were administrated. In DIV5, the CG cells were fixed for TUNEL assay staining or collected for western blotting. B. Percentage of TUNEL and NeuN double positive cells. (n=3) C. Images of CG cells with TUNEL, NeuN, and DAPI triple staining, scale bar =100 μm. D. Western blotting analysis on CG cells with or without JNK-IN-8 administration. (n=3 in each group) * indicates difference between the groups. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$ and **** indicates $p<0.0001$.

3.4. Gene Expression Analyses Indicates that JNK Inhibition Specifically Reduced Apoptosis in CASK^{flx/flx} iCre CG Cells

To further confirm the JNK inhibitor's effect on the alteration of the gene expression in CG cells, we examined the expression levels of those genes in JNK-IN-8- or DMSO-treated CG cells by realtime-qPCR (Fig. 5A, S4). We found that the expression levels of c-Jun ($p<0.0001$), c-Fos ($p<0.01$), and Tnf ($p<0.001$) were significantly reduced after the administration of JNK-IN-8 in CASK^{flx/flx} iCre CG cells. In addition, the levels of ROS and ROS related genes were also reduced by the administration of JNK-IN-8 in CASK^{flx/flx} iCre CG cells (Fig. 5A, B). By RNA-seq analysis, we found apoptosis genes such as Gem and Cxcl2 were significantly reduced in JNK-IN-8 treated CASK^{flx/flx} iCre CG cells compared to those in DMSO treated CASK^{flx/flx} iCre CG cells (Fig. 5C, D). From GESA analysis (Fig. 5E), cell death related signaling such as apoptosis and ROS pathway was suppressed by the administration of JNK-IN-8. However, the other apoptosis signaling related genes such as Bax and Bcl2 were not significantly affected by the administration of JNK-IN-8 (Fig. 5D), which was also verified by realtime-qPCR (Fig. S4), indicating that JNK signaling plays a specific role in suppressing apoptosis and ROS caused by CASK deletion. By inhibiting JNK signaling, CG cell survival was improved in CASK deficient CG cells by suppressing the expression of apoptotic genes *in vitro*. This indicates application of JNK inhibitors may be the method for preventing cerebellar hypoplasia in MICPCH syndrome.

Fig. 5

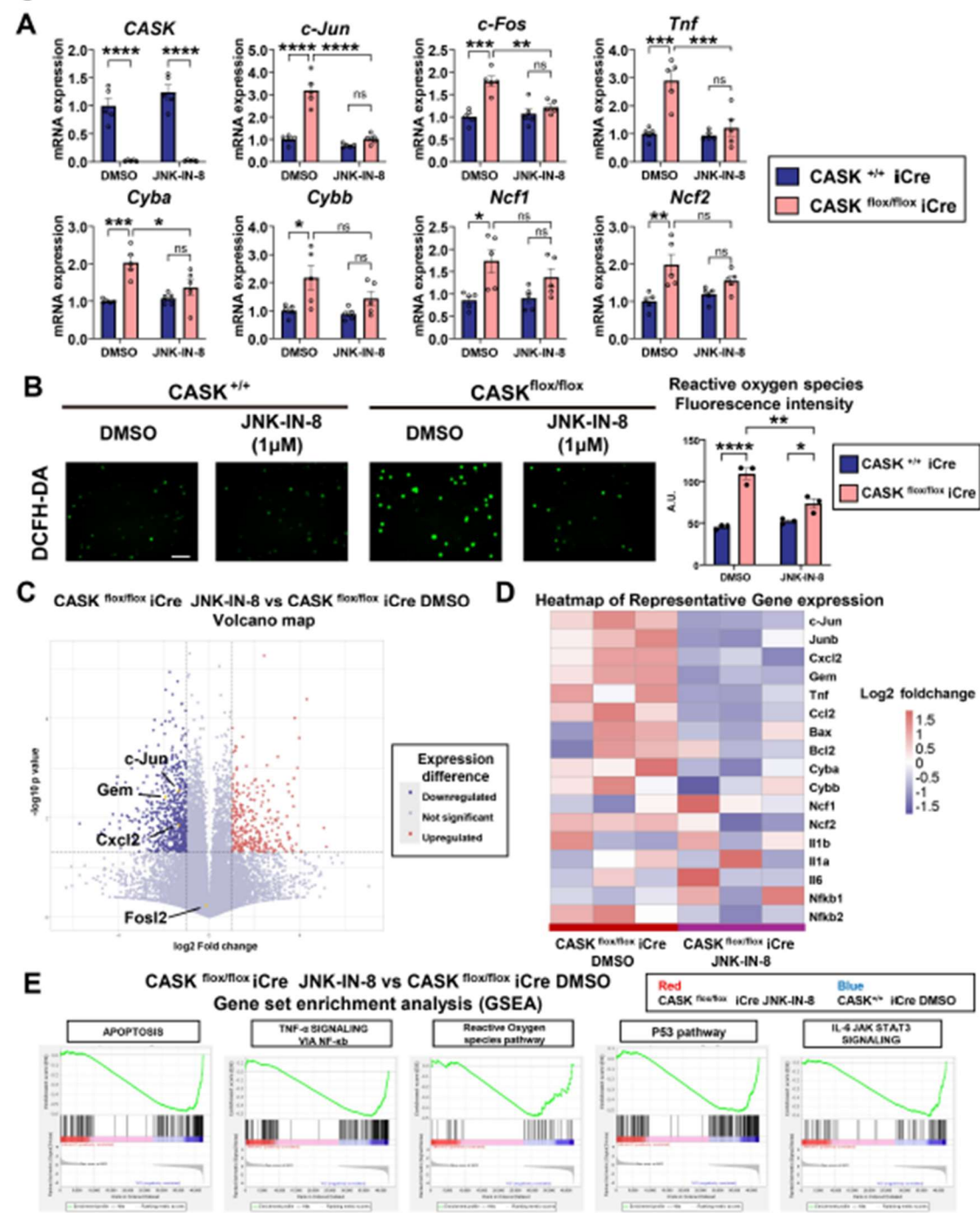


Figure 5. JNK inhibitor reduced apoptosis and reactive oxygen species pathway. A. Realtime-qPCR analysis on JNK-IN-8 or DMSO treated CG cells. (n=5 in each group) B. Reactive oxygen species visualization and quantification of fluorescence intensity, scale bar =100 μ m. (n=3) C. Volcano map of gene expression difference between JNK-IN-8 treated CASK *flox/flox* iCre CG cells and DMSO treated CASK *flox/flox* iCre CG cells. Red indicated genes upregulated in JNK-IN-8 treated CG cells, blue indicated genes downregulated in JNK-IN-8 treated CG cells. Detected by RNA-seq. (Threshold: $p < 0.05$, Fold change over 2) D. Heatmap of the representative gene expression detected by RNA-seq. E. Representative Gene Set Enrichment Analysis (GSEA) results of apoptosis related pathways. * indicates difference between the groups. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

3.5. The Administration of the JNK Inhibitor *In Vivo* Shows Preventative Effect Against Neurodegeneration

Due to the perinatal lethality of CASK knockout, we evaluated the effect of JNK inhibition on the cerebellar hypoplasia *in vivo* in female CASK heterozygote knockout mice, of which genetic background is representative to the majority of MICPCH syndrome. Our previous study demonstrated that X-chromosome inactivation produced mosaic distribution of the CASK expressing and CASK non-expressing neurons in the brain of female CASK heterozygote knockout mice [8]. To investigate whether the CG cell death in female CASK heterozygote knockout mice is specific to CASK knockout cells or not, we utilized Hprt-eGFP mice to visualize the pattern of X-chromosome inactivation. In Hprt-eGFP mice, the eGFP expression cassette under the control of CAG promoter was knocked in the hprt locus on the X-chromosome [21]. We generated CASK flox and Hprt-eGFP trans-heterozygote mice (CASK^{flox}; Hprt^{eGFP}) by crossing CASK flox and Hprt-eGFP mice. In these mice, the cells expressing eGFP indicate that the X-chromosome inherited from Hprt-eGFP mice is intact and express wild-type CASK. In contrast, the cells that are not expressing eGFP indicate of which X-chromosome inherited from Hprt-eGFP is inactivated and have active X-chromosome with CASK flox allele. We prepared CG cell culture from CASK^{flox}; Hprt^{eGFP} mice and infected with lentivirus expressing iCre to create CASK KO and CASK intact culture (Fig. S5). We examined the survival rate by NeuN and DAPI staining (Fig.6 A, B). By analyzing immunocytochemistry-stained images, we found the percentage of the CG cells without eGFP expression is gradually reduced in these cultures, indicating CASK knockout cells die selectively without affecting CASK intact cells (Fig.6 C). We administered JNK-IN-8 or DMSO in the co-cultured CG cells 3 hours after the iCre infection. We found the DMSO administration did not increase the percentage of CASK knockout CG cells (Fig.6 D). In contrast, JNK-IN-8 administration rescued the reduction of CASK knockout cells and the ratio between eGFP positive and negative cells was almost 1:1 (Fig.6 D). These results suggest that apoptosis occurs selectively in CASK knockout CG cells and it can be prevented by the administration of JNK-IN-8.

Fig. 6

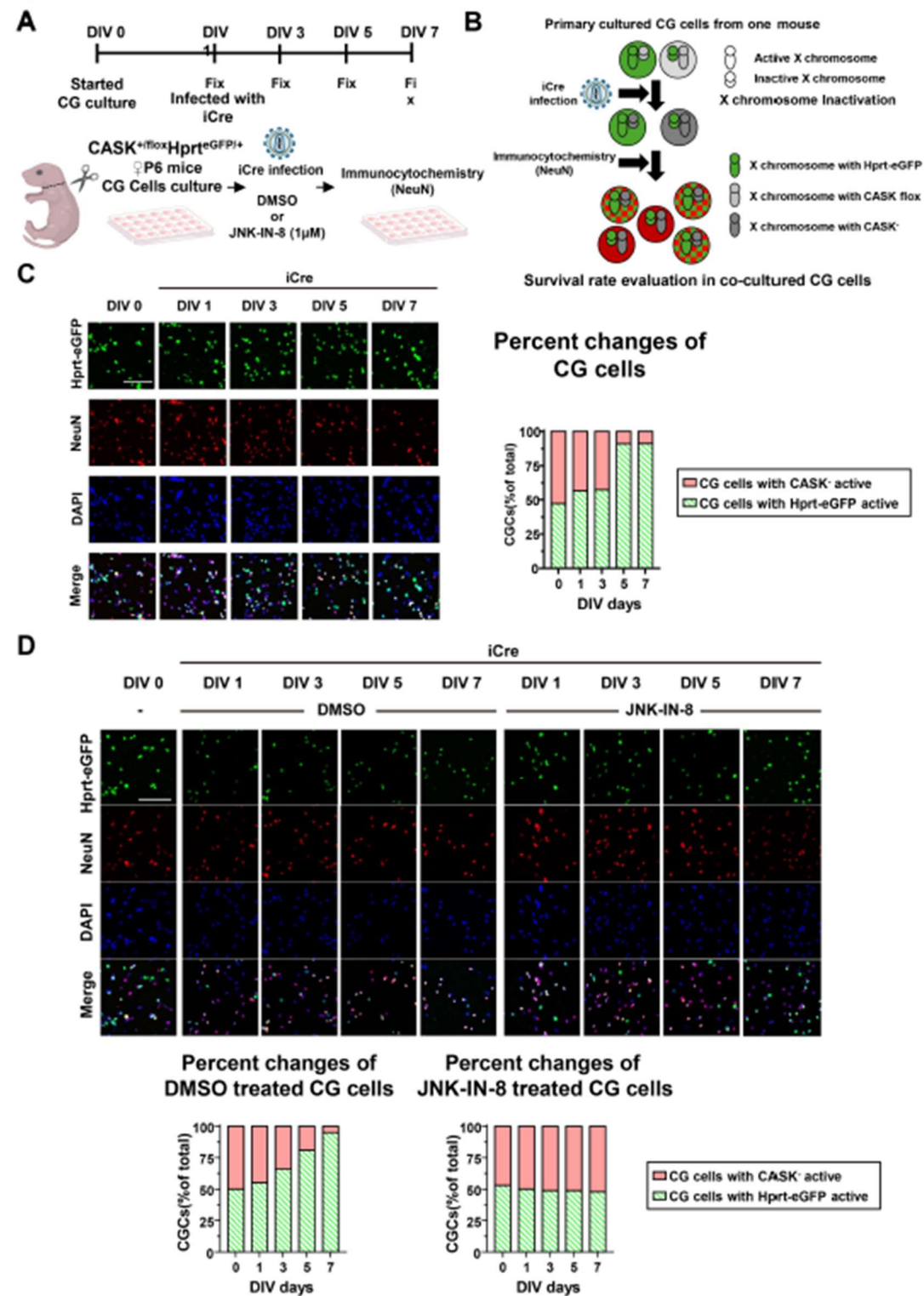


Figure 6. Survival rate evaluation on heterozygous CASK knockout CG cells A. Experimental design on CASK^{+/-}Hprt^{eGFP/+} CG cells. B. Schematic diagram showing how survival rate evaluation was conducted on different X chromosome activated CG cells in co-cultivation. And the CG cells with double positive of eGFP and NeuN were viewed as the CG cells with Hprt-eGFP X chromosome active. The NeuN positive CG cells without eGFP florescent light were viewed as the CG cells with CASK knockout (CASK⁻) X chromosome active. C. Immunocytochemistry stained Images of eGFP, NeuN, and DAPI in CG cells. And the percent changes of CG cells were evaluated by those images, scale bar =100 μm. (n=3) D. Immunocytochemistry stained Images of eGFP,

NeuN, and DAPI in JNK-IN-8 or DMSO administrated CG cells. And the percent changes of CG cells were evaluated, scale bar =100 μ m. (n=3).

Next, to evaluate the effect of JNK-IN-8 on heterozygous CASK knockout mice *in vivo*, we administrated JNK-IN-8 in the cerebellum of P6 CASK^{+/-}Hprt^{eGFP/+} and CASK^{+/+}Hprt^{eGFP/+} mice and analyzed 2 weeks after the injection (Fig.7 A). Before injection, the body weight of CASK^{+/-}Hprt^{eGFP/+} mice was significantly (P6: $p<0.05$; P13: $p<0.05$; P20: $p<0.001$) lower than CASK^{+/+}Hprt^{eGFP/+} mice (Fig.7 B, S6A). The JNK-IN-8 administration significantly ($p<0.05$) increased the body weight of CASK^{+/-}Hprt^{eGFP/+} mice, 2 weeks after the injection. In H&E stained cerebellum slices, we found the size of the cerebellum in CASK^{+/-}Hprt^{eGFP/+} mice was smaller than that of CASK^{+/+}Hprt^{eGFP/+} mice at P20. The administration of JNK-IN-8 increased the size in CASK^{+/-}Hprt^{eGFP/+} mice (Fig.7 C). Moreover, by immunohistochemistry staining or H&E stained on slices from Lobules IX and Lobules IV/V, we found granular cell (GC) layer of the CASK^{+/-}Hprt^{eGFP/+} was thinner and the cell density was lower than those of CASK^{+/+}Hprt^{eGFP/+} (Fig.7 D, S6B). Almost 100% of cells in CASK^{+/-}Hprt^{eGFP/+} GC layer are NeuN and eGFP double positive, indicating that only those CASK expressing CG cells were survival. After the JNK-IN-8 administration, the ratio of such double positive cells significantly ($p<0.05$) reduced in both Lobules IX and Lobules IV/V. Indicating that some CASK knockout CG cells were recovered in those layers. We found similar results in Purkinje cell (PC) layer (Fig. S7). These morphological results indicated that JNK-IN-8 administration *in vivo* prevents cerebellar cell loss in of CASK^{+/-}Hprt^{eGFP/+} mice.

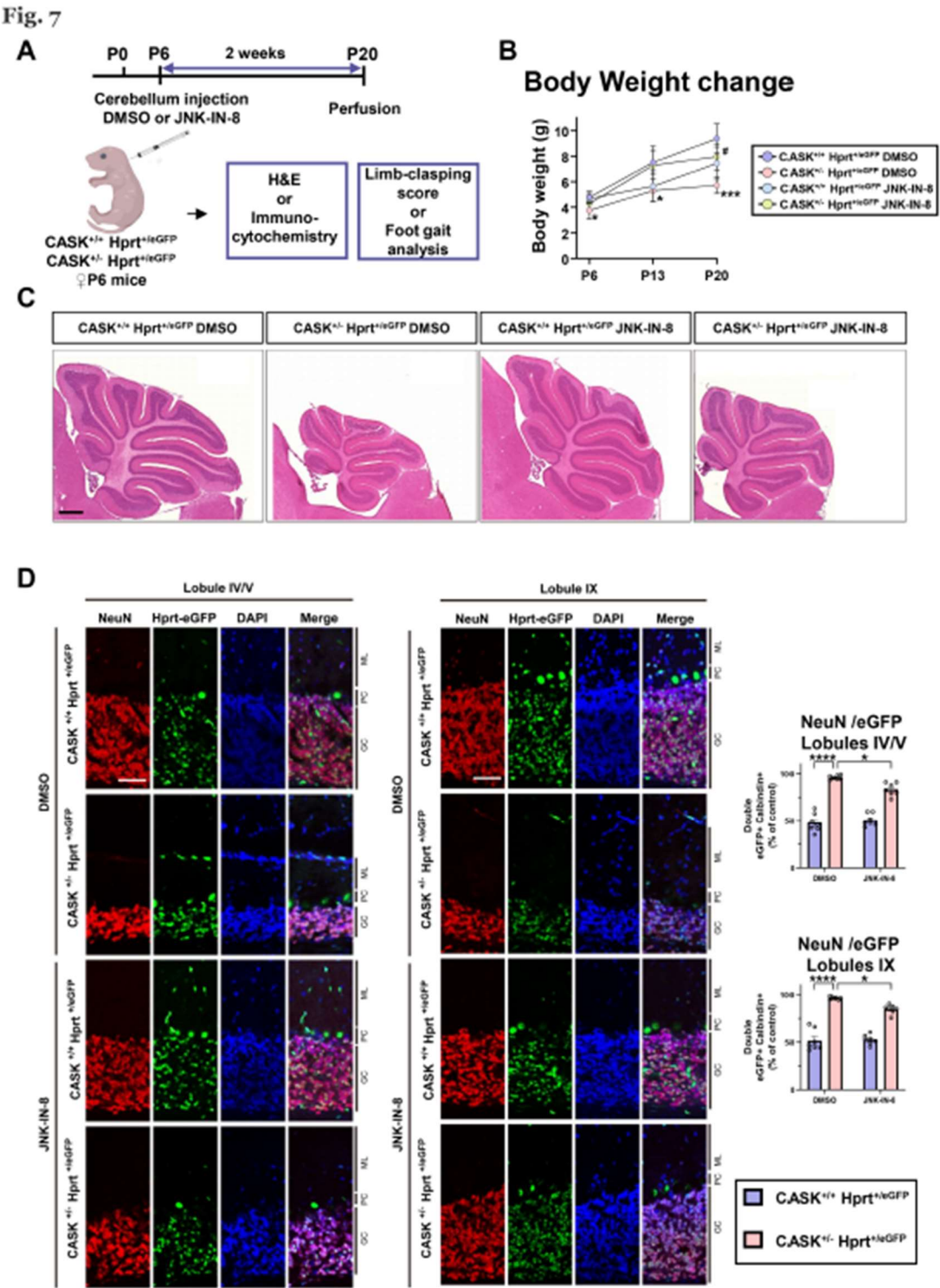


Figure 7. JNK-IN-8 administration *in vivo* cerebellum development of CASK^{+/+}-Hprt^{eGFP/+} mice **A.** Experimental design on CASK^{+/+}-Hprt^{eGFP/+} and CASK^{+/+}-Hprt^{eGFP/+} mice. **B.** Body Weight changes in CASK^{+/+}-Hprt^{eGFP/+} and CASK^{+/+}-Hprt^{eGFP/+} mice. (n=5)(* indicates difference between CASK^{+/+}-Hprt^{+/eGFP} DMSO and CASK^{+/+}-Hprt^{+/eGFP} DMSO, # indicates difference between CASK^{+/+}-Hprt^{+/eGFP} JNK-IN-8 and CASK^{+/+}-Hprt^{+/eGFP} DMSO) **C.** Photography of H&E stained cerebellum slices, scale bar= 500µm. **D.** Immunohistochemistry stained Lobules IV/V slices and Lobules IX. ML, molecular layer; PC, Purkinje cell layer; GC, granular cell layer. The ratio of double positive cells of NeuN and eGFP was calculated. (n=7) * indicates difference between the groups. *, # indicate $p<0.05$; *** indicates $p<0.001$ and **** indicates $p<0.0001$.

Lastly, we examined the effect of the administration of the JNK inhibitor on the behavior of CASK^{+/-}-Hprt^{eGFP/+} mice. We performed limb clasp test on these mice that can monitor cerebellar ataxia. CASK^{+/-}-Hprt^{eGFP/+} mice exhibited clasp and crossing of their limbs and immobilized indicating they have cerebellar ataxia (Fig. 8A). However, after the JNK-IN-8 injection into cerebellum at P6, the mobility was improved, and the limb-clasping score was significantly decreased ($p<0.0001$) compared to untreated mice. We also performed gait analysis on these mice. We found the stride length and toe spread of CASK^{+/-}-Hprt^{eGFP/+} mice were significantly shorter than CASK^{+/-}-Hprt^{eGFP/+} (Fig. 8B). These behavioral analyses indicated that the cerebellar function of CASK^{+/-}-Hprt^{eGFP/+} mice was improved by the administration of JNK-IN-8.

Fig. 8

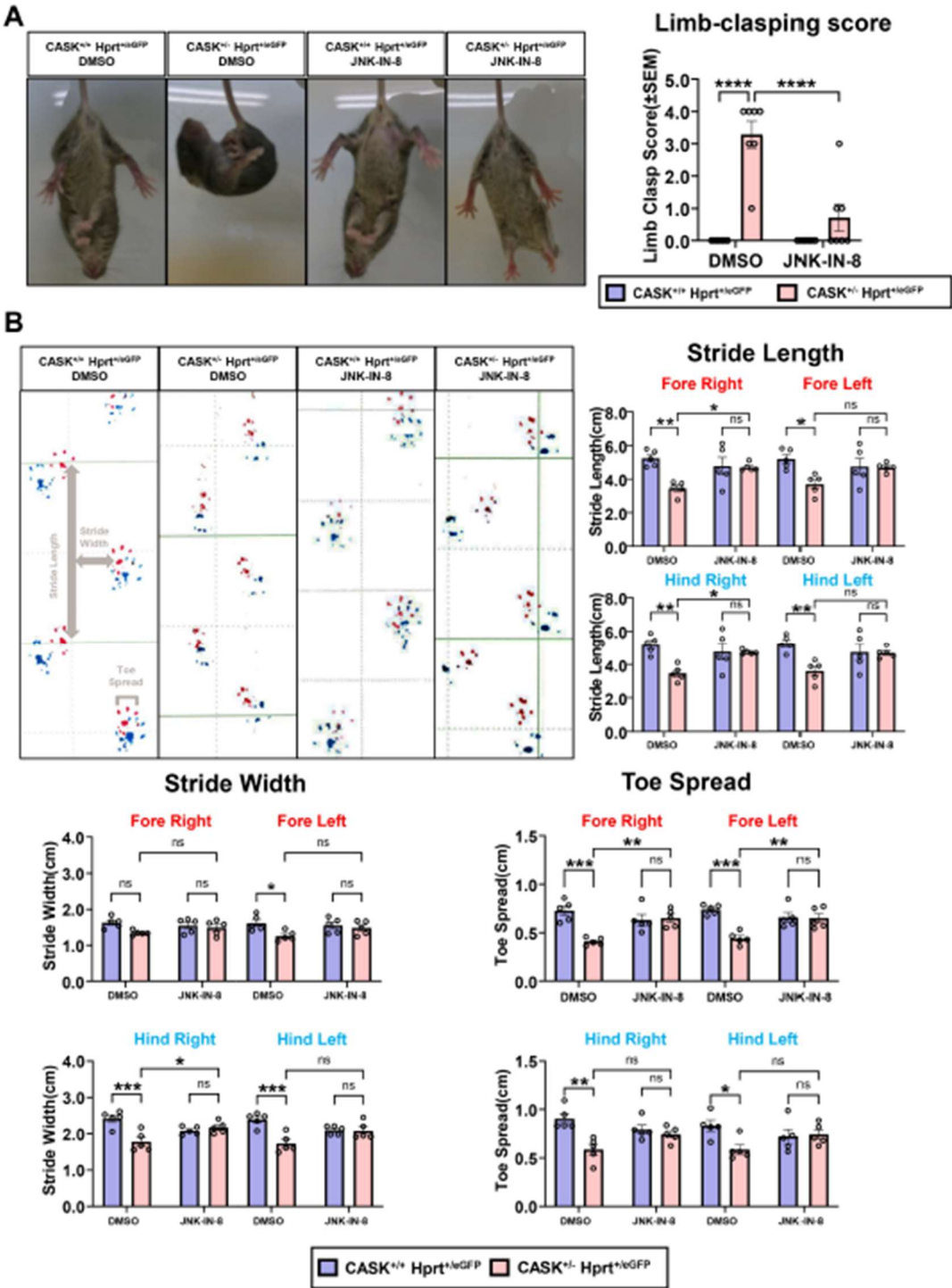


Figure 8. behavioral analyses indicated that JNK-IN-8's administration improved cerebellum function. A. Photography on P20 CASK^{-/-}Hprt^{eGFP/+} and CASK^{-/-}Hprt^{eGFP/+} mice, treated with or without JNK-IN-8. Limb-clasping scores were calculated by their behavioral performance. (n=7) B. Gait analysis record and the stride length, stride width, and toe spread were measured separately from each limb. (n=5) * indicates difference between the groups. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$ and **** indicates $p<0.0001$.

4. Discussion

Although apoptosis caused by CASK deletion has been well documented in previous neurological studies [2,22,23], the specific molecular pathways mediating CG cell death have remained largely unexplored. Our study is the first to perform bulk RNA-seq and pathway-level analysis on cerebellar granule (CG) cells from CASK^{flox/flox} iCre mice, revealing that CASK deficiency induces significant activation of the JNK signaling and reactive oxygen species (ROS) pathways. This result was further confirmed by qPCR, western blotting, and TUNEL assays, supporting the hypothesis that CASK plays a crucial role in maintaining cellular homeostasis in the cerebellum.

Our findings are consistent with previous reports linking JNK activation and ROS accumulation to neurodegenerative processes [25,26]. The upregulation of apoptosis-related genes such as c-Jun, Fosl2, Gem, and Cxcl2, alongside the increased phosphorylation of JNK and c-Jun, strongly suggest that the JNK pathway is a key effector of CG cell death in the absence of CASK. Importantly, JNK-IN-8 treatment effectively suppressed both the JNK phosphorylation and the transcriptional activation of ROS-related genes, thereby rescuing cell survival. Among the JNK inhibitors tested, JNK-IN-8 showed the most robust and selective effect, highlighting its potential as a therapeutic compound for CASK-related neurodegeneration.

Moreover, our *in vivo* experiments in heterozygous CASK knockout mice (CASK^{+/-}Hprt^{eGFP/+}) provided compelling evidence that JNK inhibition alleviates cerebellar hypoplasia and motor deficits. Notably, eGFP-negative cells—representing the CASK-deficient population due to X-inactivation—were selectively lost in untreated mice, but their survival was preserved following JNK-IN-8 administration. Behavioral improvements in limb-clasping and gait analysis further confirmed the functional recovery of cerebellar circuits, particularly in the hemisphere receiving JNK-IN-8 injections.

Nevertheless, our study has several limitations. First, while CG cells were the primary focus, the potential involvement of other cerebellar cell types, such as Purkinje cells and interneurons, was not fully explored. Second, our omics analysis focused primarily on apoptosis and oxidative stress pathways; other altered processes, including metabolism-related changes [31], may also play a role and warrant future investigation. In addition, while bulk RNA-seq provided valuable global insight, single-cell resolution would help distinguish cell-type-specific responses and mosaic expression patterns due to X-inactivation. Further studies utilizing single-cell RNA-seq or spatial transcriptomics would be particularly informative.

Lastly, while our results strongly support the use of JNK inhibitors to rescue CASK-dependent phenotypes, the long-term safety and efficacy of such compounds *in vivo* remain to be established. The possibility of off-target effects or compensation by parallel pathways cannot be excluded. Nonetheless, our work provides a molecular framework that bridges CASK deficiency and cell death signaling in the cerebellum and opens the door for targeted therapeutic strategies in MICPCH syndrome.

5. Conclusions

Our study demonstrates that CASK deletion in cerebellar granule cells leads to apoptosis and neurodegeneration through activation of the JNK signaling and ROS pathways. Using RNA-seq, molecular assays, and behavioral analysis *in vitro* and *in vivo*, we showed that pharmacological inhibition of JNK signaling—particularly via JNK-IN-8—effectively suppresses CG cell death and

alleviates cerebellar ataxia in a mouse model of MICPCH syndrome. These findings identify the JNK pathway as a critical mediator of CASK-related cerebellar pathology and support the potential utility of JNK inhibitors as therapeutic agents for neurodevelopmental disorders involving CASK dysfunction.

Declaration of competing interest: We have no conflicts to declare.

Availability of Data and Materials The RNA-seq data and other raw datasets are available at [https://] (link to be inserted), or from Q.G. upon request (gq_2222@126.com). Materials used in the study are available from K.T. upon request (ktabuchi@shinshu-u.ac.jp).

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