Apolipoprotein E Affects In Vitro Axonal Growth and Regeneration via the MAPK Signaling Pathway

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Abstract

Following central nervous system injury in mammals, failed axonal regeneration is closely related to dysneuria. Previous studies have shown that the obvious effects of apolipoprotein E (ApoE) on traumatic brain injury (TBI) were associated with an axonal mechanism. However, little information on the actions of ApoE and its isoforms on axonal regeneration following TBI was provided. In our study, the cerebral cortices of ApoE-deficient (ApoE$^{-/-}$) and wild-type (ApoE$^{+/+}$) mice were cultured in vitro, and an axonal transection model was established. Interventions included the conditioned medium of astrocytes, human recombinant ApoE2/3/4 isoforms and inhibitors of the JNK/ERK/p38 pathway. Axonal growth and regeneration were evaluated by measuring the maximum distance and area of the axons. The expression levels of β-tubulin III, MAP2, ApoE, p-JNK, p-ERK and p-p38 were detected by immunofluorescence and western blotting. The results showed that ApoE mRNA and protein were expressed in intact axons and regenerated axons. Axonal growth and regeneration were attenuated in ApoE$^{-/-}$ mice but recovered by exogenous ApoE. Human recombinant ApoE3 positively influenced axonal growth and regeneration; these effects were mediated by the JNK/ERK/p38 pathway. These results suggest ApoE and its isoforms may have influenced axonal growth and regeneration via the MAPK signaling pathway in vitro.

Keywords

apolipoprotein E, axon, transection, regeneration, MAPK

Introduction

After a mammalian central nervous system (CNS) injury, such as stroke, neurodegenerative disease and traumatic brain injury (TBI), the damage usually contributes to irreversible neurological impairment, which can involve coma, sensory disorder, paralysis, etc. Axonal regeneration failure is regarded as the main reason for persistent neurological dysfunction following a CNS injury[1]. Promoting axonal regeneration might be a potential treatment strategy for CNS injury. In contrast to the initial research on axonal regeneration, which supposed that injured axons failed to regenerate in the mammalian CNS, recent studies support that injured axons possess an intrinsic regeneration capability, which can be stimulated by certain regulating molecules[2,3].

As a major apolipoprotein in the brain, apolipoprotein E (ApoE) mainly transports cholesterol and other lipids via ApoE membrane receptors[4]. Accumulating evidence shows an association between ApoE and TBI[5,6]. In animal experiments, ApoE considerably relieved impaired neurological function compared with conditions without it[7,8]. The three isoforms in humans, in contrast to those in animals, are ApoE2, ApoE3 and ApoE4[9]. Some clinical studies found that ApoE also significantly affected patients with TBI, and the prognosis of patients carrying the ApoE3 isoform was better than that of patients carrying the ApoE2/4 isoforms[10,11]. However, the protective mechanism of ApoE on TBI has not been fully revealed.

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Accumulating studies indicate that the effect of ApoE on TBI is associated with the axons. In the peripheral nervous system (PNS), several studies have demonstrated that ApoE and its isoforms promote axonal extension of the dorsal root ganglia and axonal regeneration of sciatic and olfactory nerves[12–14]. In the CNS, ApoE and its isoforms can also promote axonal extension of retinal ganglion cells and cortical neurons[15–17]. However, the function of ApoE and its isoforms in axonal regeneration has not been fully elucidated. Just as axons are almost unavoidably damaged or are unable to cross an injury in TBI, here we speculate that ApoE and its isoforms can improve TBI prognosis by promoting axonal regeneration.

To simulate an environment similar to that of living neurons and better observe the axonal growth and regeneration, explants from cortical brain tissue were cultured, and an axonal transection model was established to imitate axonal injury in TBI. We attempted to explore the potential role of ApoE and its isoforms in axonal growth and regeneration.

Materials and Methods

Experimental Design and Cortical Explant Culture

All procedures were evaluated and approved by the Animal Care and Use Committee. A total of 61 neonatal mice ($n^{ApoE+/+} = 22$, $n^{ApoE-/-} = 39$) were randomly assigned to the following experimental parts: Part 1: Model establishment: $n^{ApoE+/+} = 2$; Part 2: Descriptive study: $n^{ApoE+/+} = 12$, $n^{ApoE-/-} = 15$; Part 3: Mechanism study: $n^{ApoE+/+} = 8$, $n^{ApoE-/-} = 24$. Usually, more than 10 cortical explants could be obtained for one brain cortex, and the best six mature explants would be adopted in our experiments. Based on this proportion, excluding the four neonatal mice used for astrocytes conditioned medium, a total 342 of cortical explants (57 pups) were used in our experiments.

A previously reported procedure of culturing cortical explants was applied to this study[18,19], but the planting method was improved for obtaining an intact axon sample in this study. Briefly, cortical brain tissue was obtained from postnatal day 1[20] ApoE-deficient (ApoE-/-) mice and wild-type littermate (ApoE+/+) mice and chopped into approximately 0.3 mm pieces by a scalpel. The tissue pieces were transferred into a centrifuge tube containing 8 ml of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Invitrogen, Waltham, MA, USA), allowed to subside naturally, and then gently transferred into another centrifuge tube that also contained 8 ml of DMEM/F12; this process was repeated 5–8 times until the liquid was clear. The brain tissue pieces were moved into a 10 cm diameter dish that was filled with DMEM/F12. A 1 mm diameter glass micropipette with a fire-polished tip was used to pick up the cortical explant tissue pieces and gently place them onto a coverslip one by one. After 4 h of incubation at 37°C in a humidified 95% air/5% CO_2 incubator, the entire medium was changed to Neurobasal medium with 10%
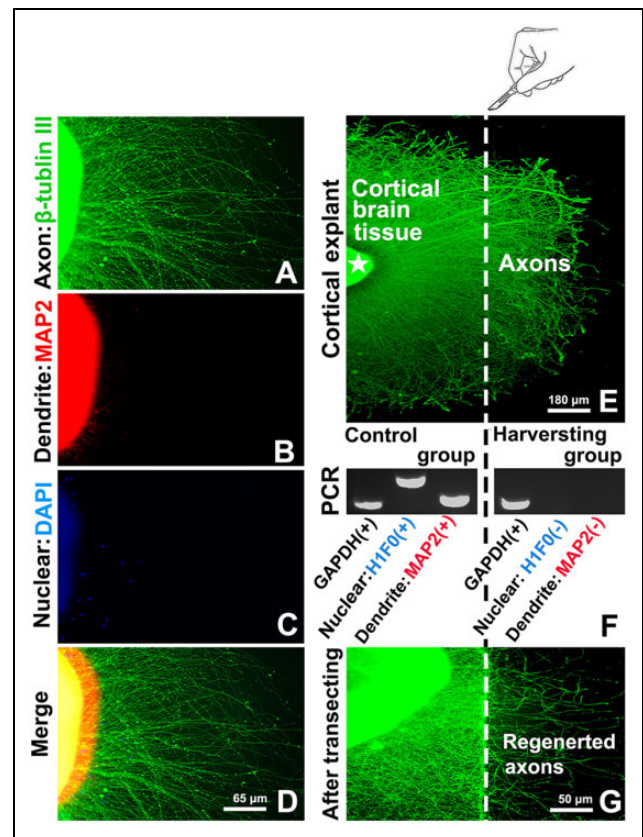

Fig 1. Culturing cortical explants and establishing an axonal transection model. **A, B, C, D.** In mature cortical explants, the axons (marked by β-tubulin III, green), dendrites (marked by MAP2, red) and nuclei (marked by DAPI, blue) were respectively colored by immunofluorescence staining. The image shows that the axons are longer than the dendrites and that the dendrites and nuclei are only located around the edge of the cortical brain tissue. **E.** After transecting along the white dotted line, the distal axons on the right side of the dotted line were taken as the harvested group. The remaining part of the cortical explant was taken as the control group. **F.** The control and harvested group were analyzed by RT-PCR, and the results showed that H1f0 (existing only in nuclei) and MAP2 (only existing in dendrites) were positive in the control group but negative in harvested group. **G.** After transecting the axons and harvesting the distal axons, the rest of cortical explant was continually incubated for 2 days. Then, the sparsely regenerated axons were observed as they crossed the transection line and extended.

fetal bovine serum (FBS, Invitrogen) and supplement with Neurobasal medium containing 2% B27 (Neurobasal/B27, Invitrogen).

Axonal Transection and Sample Collection

After culture for 7 days in vitro, the distal axons in the cortical explants were transected by a scalpel under a microscope (40×; Fig. 1E). The distally transected axons were separately moved from the cortical explant into medium by a 0.22 mm diameter needle. All medium was transferred into a centrifuge tube and centrifuged (10,000 g) for 10 min

at 4°C. The precipitate was taken as the RT-PCR/Western blot sample. For observing and detecting the regenerated axons, the distal axons were removed, and the rest of the cortical explant was placed into an incubator for next 48 h. Then, the regenerated axons that crossed the transecting line were collected by a second transection just as the sample that was collected for RT-PCR/Western blot.

Conditioned Medium (CM) of Astrocytes

Primary culture of astrocytes. Astrocytes were obtained from the ApoE$^{-/-}$ and ApoE$^{+/+}$ mice (postnatal day 1). Briefly, the brain cortex was collected and incubated with 0.25% trypsin (SigmaAldrich, St. Louis, MO, USA) for 20 min at 37°C before adding 10% FBS to terminate the chemical digestion. After gentle blowing-suction, filtering and centrifugation, the cells were resuspended in DMEM/F12 with 10% FBS and seeded into dishes.

Preparation of the astrocyte CM. The ApoE$^{-/-}$ and ApoE$^{+/+}$ astrocytes were washed three times in serum-free DMEM/F12 and incubated with 2 ml of serum-free DMEM/F12 for 24 h. Then, serum-free DMEM/F12 without cellular debris was collected as the astrocyte-ApoE$^{-/-}$-CM (CM$^{ApoE-/-}$) and the astrocyte-ApoE$^{+/+}$-CM (CM$^{ApoE+/+}$).

Incubation of explants with the astrocyte CM. After planting the explants, the astrocyte-ApoE$^{+/+}$-CM or astrocyte-ApoE$^{-/-}$-CM was separately added into culture media[21].

Human Recombinant ApoE Protein and JNK/ERK/p38 Inhibitors

Three human recombinant ApoE proteins, isoforms 2, 3 and 4 (PeproTech, Rocky Hill, NJ, USA), were separately added into Neurobasal/B27 to culture the explants of ApoE$^{-/-}$ mice after planting. The ApoE final concentration was 10 μg/ml in medium, which is similar to the concentration of ApoE in human cerebrospinal fluid (CSF)[22–24]. In addition, after axonal transection, the explants of the ApoE$^{-/-}$ mice were continuously cultured in Neurobasal/B27 with human recombinant ApoE2/3/4 proteins for 24 h.

JNK/ERK/p38 inhibitors, which included SP600125, U0126, and SB203580 (Beyotime, Shanghai, China), were also used as interventional means. The final concentration of the three inhibitors was 10 μM, and they were dissolved in dimethyl sulfoxide (DMSO, Beyotime)[25,26]. In the control group, only DMSO was added without the inhibitors. After explant planting, the explants were separately treated by medium with the JNK, ERK and p38 inhibitors for 24 h. Subsequently, the media were replaced with fresh media without the inhibitors[27].

Immunofluorescence

Coverslips that contained the explants were rinsed three times with phosphate-buffered saline (PBS). The coverslips were fixed with 4% paraformaldehyde at room temperature for 20 min, and 0.1% Triton X-100 was added to them for 1 h. Normal goat serum was utilized to dilute primary antibodies at the following dilutions: β-tubulin III antibody (1:1000, Sigma-Aldrich), MAP2 antibody (1:200, Boster, Wuhan, China), and ApoE antibody (1:100, Santa Cruz, Dallas, TX, USA). Coverslips were incubated with primary antibodies overnight at 4°C. FITC (green) and Alexa 555 (red) were diluted with PBS and used for secondary antibodies. Coverslips with secondary antibodies were incubated for 30 min at 37°C in a water bath. The nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI, Beyotime). The excess DAPI was washed off, and the coverslips were mounted with 50% glycerol. In addition, phalloidin (1:100, FITC labeled, SigmaAldrich) was used to dye F-actin in growth cones. A fluorescence microscope (NIKON TE2000-U, Tokyo, Japan) was used to capture all images.

RT-PCR

The samples for RT-PCR came from intact axons, from the axons that regenerated after the transection and from the residual explants. Total RNA was extracted by a MicroElute Total RNA Kit (Omega, Norcross, GA, USA) following the manufacturer's protocol[28]. A PrimeScript RT reagent Kit (Takara, Tokyo, Japan) was used to reverse transcribe RNA to cDNA, and Premix Taq Version 2.0 (Takara) was used to amplify genes by PCR. We examined typical mRNA involved in axon regeneration, including H1f0 (GenBank ID: 14958), MAP2 (GenBank ID: 17756), GAPDH (GenBank ID: 14433) and ApoE (GenBank ID: 11816). The primer sequences (forward, reverse) and product lengths were as follows: H1f0, 5'-ggaccacccccaagtattcagac-3', 5'-actaggcgcttgatggacaact-3', 159 bp; MAP2, 5'-atcaggaga-cagggaggagaact-3', 5'-aggggtagtaggtgtggaggtg-3', 124 bp; GAPDH, 5'-gacatcaagaaggtggtgaagc-3', 5'-gaaggtgaa-gagtgggagtt-3', 117 bp; ApoE, 5'-aaccgcttctgggattacctg-3', 5'-cgtcatagtgtcctccatcagtg-3', 117 bp. The PCR conditions were 94°C for 30 s, 55°C for 30 s and 72°C for 1 min for a total of 35 cycles. The PCR products were separated in 4% agarose gels and scanned by a Gel Imager system (Bio-Rad, Hercules, CA, USA). ImageJ was used for image processing and analysis.

Western Blotting

After collection of the intact axons and the regenerated axons, Western blotting was performed as previously described[29,30]. Primary antibodies used in the western blot assay were ApoE (1:500, Santa Cruz), p-JNK (1:1000, Santa Cruz), p-ERK (1:1000, Santa Cruz), p-p38 (1:1000, Santa Cruz), and β-actin (1:2000, Santa Cruz). Then, they were

incubated with the corresponding secondary antibodies at room temperature. The Western blot results were analyzed via ImageJ software.

Assessment of Axonal Growth and Regeneration

Axonal growth. In mature cortical explants, the maximum distance of axonal growth and the area of the axons were used to assess the axonal growth ability. The maximum distance of axons was determined by the maximum straight-line distance from the edge of the cortical brain tissue to the axonal terminal. The area of axons was determined by the total axonal coverage area in a cortical explant (see Fig. 4A, C).

Axonal regeneration. At 48 h after transection of the cortical explants, the maximum distance of regenerated axons was determined by the maximum straight-line distance from the transection line to the axonal terminal. The area of regenerated axons was determined by the total regenerated axonal coverage area that existed outside the transection line (see Fig. 4E, G). In every experimental group, 10 cortical explants were examined. The maximum distance and the area of axons/regenerated axons were measured via ImageJ software.

Statistical Analysis

Data are expressed as the mean $\pm$ SD. The Statistical Package for the Social Sciences (SPSS, Armonk, NY, USA) software was used for data analysis. The data were evaluated by *t*-tests or analysis of variance (ANOVA), followed by S-N-K test for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Establishing the Axonal Transection Model

After the cortical explants grew to maturity, the cortical brain tissue was located in the center and contained numerous neurons, and the tissue was surrounded by radial axons (Fig. 1E). In addition, outgrowth of dendrites from neurons mixed with axons was observed. To obtain intact axon samples without dendrites or cell bodies for the subsequent experiments, the axons, dendrites and nuclei were separately stained by immunofluorescence (Fig. 1A, B, C). The merged results show the dendrites and nuclei existed only around the edge of the cortical brain tissue in mature explants (Fig. 1D). Therefore, to obtain intact axon samples, only the distal axons were transected and harvested (Fig. 1E). In addition, RT-PCR was further used to detect the purity of the axonal samples. The results showed that no dendrites or nuclei were mixed into the harvested axons (Fig. 1F). After harvesting the axons, the rest of cortical explant was placed into a cell incubator for further culture. Two days later, a limited number of regenerated axons was observed and used for the follow-up experiments (Fig. 1G).

ApoE mRNA and Protein Expression in Intact Axons and Regenerated Axons

Immunofluorescence staining, RT-PCR and western blotting were used to analyze the expression and localization of ApoE mRNA and protein in the ApoE$^{+/+}$ group. In axons, the RT-PCR results showed that ApoE mRNA was present in the intact axons, and the immunofluorescence and western blot results showed that ApoE protein was also expressed in axons (Fig. 2A, B). In addition, the RT-PCR results showed that ApoE mRNA was present in the regenerated axons. The ApoE protein was also found in the regenerated axons via immunofluorescence and western blotting (Fig. 2C, D). On the other hand, in the axons and regenerated axons of the ApoE$^{-/-}$ group, no ApoE mRNA or ApoE protein existed (Fig. 2).

ApoE Protein Expression in the Growth Cones of Intact Axons and Regenerated Axons

Growth cones exist at the axonal tip and are regarded as the leader of axonal growth and regeneration. To investigate the role of ApoE in axonal regeneration, it was essential to determine whether ApoE is expressed in the growth cone. In cortical explants, the growth cones of intact axons and regenerated axons were both stained by F-actin and ApoE. The merged results showed that ApoE was present in the growth cones of intact axons and regenerated axons (Fig. 3A, B).

Axonal Growth and Regeneration were Attenuated in ApoE$^{-/-}$ mice and Improved by Exogenous ApoE

As mentioned in the experimental procedures, axonal growth and regeneration were assessed by the maximum distance and area of axons (Fig. 4A, C, E, G). The maximum distance and area of the axons in the ApoE$^{-/-}$ group and the ApoE$^{-/-}$+CM$^{ApoE-/-}$ group were significantly decreased compared with those in the ApoE$^{+/+}$ group, but the CM$^{ApoE+/+}$ obviously relieved the ApoE$^{-/-}$-attenuated effect (Fig. 4B, D). In the regenerated axons, the maximum distance and area were significantly attenuated in the ApoE$^{-/-}$ group compared with those in the ApoE$^{+/+}$ group. The CM$^{ApoE-/-}$ mildly improved the maximum distance and significantly improved the area compared with the ApoE$^{-/-}$ group, but the CM$^{ApoE+/+}$ significantly improved both the maximum distance and area compared with the condition without CM in the ApoE$^{-/-}$ group (Fig. 4F, H).
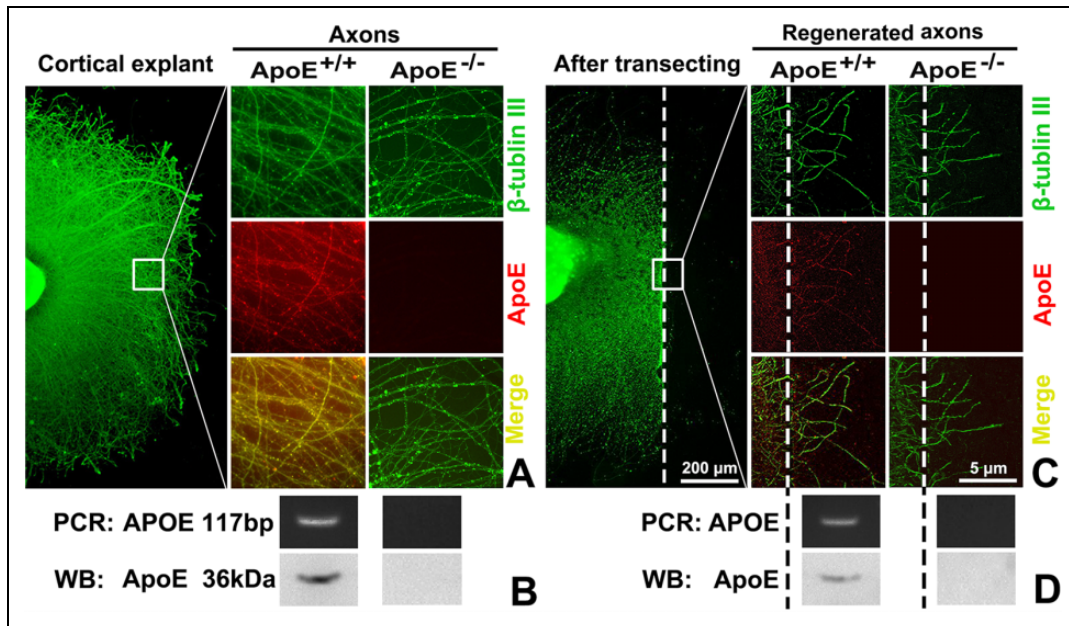
Fig 2. ApoE mRNA and protein expression in intact axons and regenerated axons of ApoE$^{+/+}$ mice. **A, B.** The immunofluorescence shows axons (green) and ApoE (red), and the merged result shows ApoE overlapped with axons (yellow). Further analysis of the distal axons by RT-PCR and Western blotting showed that ApoE mRNA and protein were expressed in the intact axons of ApoE$^{+/+}$ mice. Besides, in the axons of ApoE$^{-/-}$ mice, no ApoE mRNA or ApoE protein existed. **C, D.** In the regenerated axon samples, the axons and ApoE were colored by immunofluorescence staining, and the merged results also showed that ApoE was present in the regenerated axons. RT-PCR and Western blotting further showed that ApoE mRNA and protein were present in the regenerated axons. However, there was no ApoE mRNA or ApoE protein expressed in the regenerated axons of ApoE$^{-/-}$ mice.
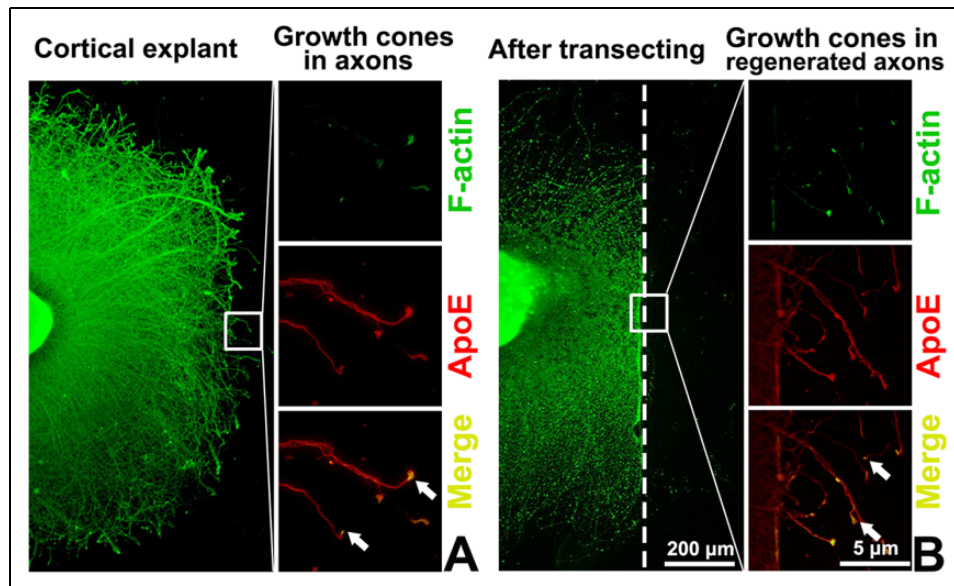

Fig 3. ApoE protein expression in intact axonal growth cones and regenerated axonal growth cones in ApoE$^{+/+}$ mice. **A.** In the intact axons, the growth cones are marked by F-actin (green) and ApoE (red). The image shows only the axonal tip, which is the position of the growth cone and is displayed as green. In the merged image, the area of the growth cones is displayed as yellow. **B.** In the regenerated axons, the growth cones were also distinctly stained by F-actin (green), and the regenerated axons were stained by ApoE (red). In the merged image, ApoE is present in the growth cones of the regenerated axons.
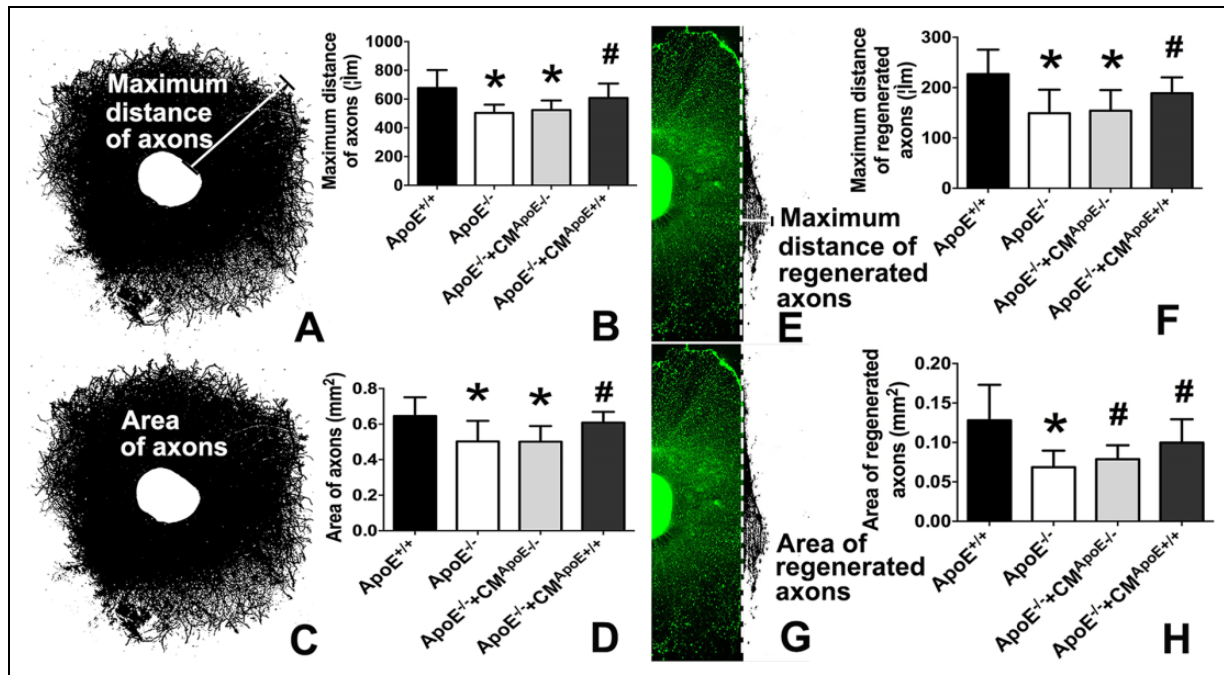
Fig 4. The effects of ApoE on axonal growth and regeneration. In intact axons: **A, C.** The schematic diagram illustrates the "maximum distance" and "area" in axons. **B, D.** In the ApoE$^{-/-}$ group and the ApoE$^{-/-}$+CM$^{ApoE-/-}$ group, the maximum distance and area were significantly decreased compared with those in the ApoE$^{+/+}$ group, but the maximum distance and area of the ApoE$^{-/-}$+CM$^{ApoE+/+}$ group were increased compared with those in both the ApoE$^{-/-}$ group and ApoE$^{-/-}$+CM$^{ApoE-/-}$ group. In regenerated axons: **E, G.** The schematic diagram illustrates the "maximum distance" and "area" in regenerated axons. **F, H.** The maximum distance and area were significantly attenuated in the ApoE$^{-/-}$ group compared with those in the ApoE$^{+/+}$ group. The CM$^{ApoE-/-}$ mildly improved the maximum distance and significantly improved the area, but the CM$^{ApoE+/+}$ significantly improved both the maximum distance and area compared with the condition without CM in the ApoE$^{-/-}$ group (*$p < 0.05$ vs. ApoE$^{+/+}$, #$p < 0.05$ vs. the ApoE$^{-/-}$ group and the ApoE$^{-/-}$+CMApoE$^{-/-}$ group).

ApoE Improved Axonal Growth and Regeneration via the JNK/ERK/p38 Pathway

In consideration of the ApoE effect on axonal growth and regeneration, the possible molecular mechanisms were explored. In intact axons and regenerated axons, the expression levels of p-JNK, p-ERK and p-p38 were obviously decreased in the ApoE$^{-/-}$ group compared with those in the ApoE$^{+/+}$ group (Fig. 5).

Considering that the axonal growth and regeneration were attenuated and the level of p-JNK/ERK/p38 was decreased in the ApoE$^{-/-}$ group, the JNK/ERK/p38 inhibitors were used to further explore whether ApoE influenced axonal growth and regeneration by a p-JNK/ERK/p38-mediated mechanism. The results showed that the JNK/ERK/p38 inhibitors significantly suppressed the axonal growth and regeneration (Fig. 6).

Human Recombinant ApoE4 Negatively and ApoE3 Positively Influenced Axonal Growth and Regeneration

In contrast to those in mice, three isoforms of ApoE exist in humans. Explants from ApoE$^{-/-}$ mice were cultured, and human recombinant ApoE2, ApoE3 or ApoE4 was separately added into the culture medium to achieve the same concentration as ApoE in human CSF. The "maximum distance" and "area" were still used to quantify axonal growth and regeneration. In intact axons, the maximum distance was improved by ApoE2 and significantly improved by ApoE3, but only ApoE3 significantly improved the area (Fig. 7A). In regenerated axons, the maximum distance and area were significantly improved by ApoE3 (Fig. 7B). In contrast, ApoE4 did not improve the maximum distance or area in intact axons or in regenerated axons (Fig. 7A, B).

ApoE3 Improved Axonal Growth and Regeneration via the JNK/ERK/p38 Pathway

In view of the distinctive effects of ApoE3 and ApoE4 on axonal growth and regeneration in the cortical explants, the relationship between ApoE3/ApoE4 and the p-JNK/ERK/p38 pathway was further investigated. Compared with ApoE3, p-JNK and p-ERK were obviously reduced by ApoE4 in intact axons. However, in the regenerated axons, only p-JNK was significantly decreased by ApoE4 (Fig. 8).

In addition, of the three human ApoE isoforms, only ApoE4 obviously inhibited axonal growth and regeneration. The level of p-JNK/ERK/p38 was also downregulated by
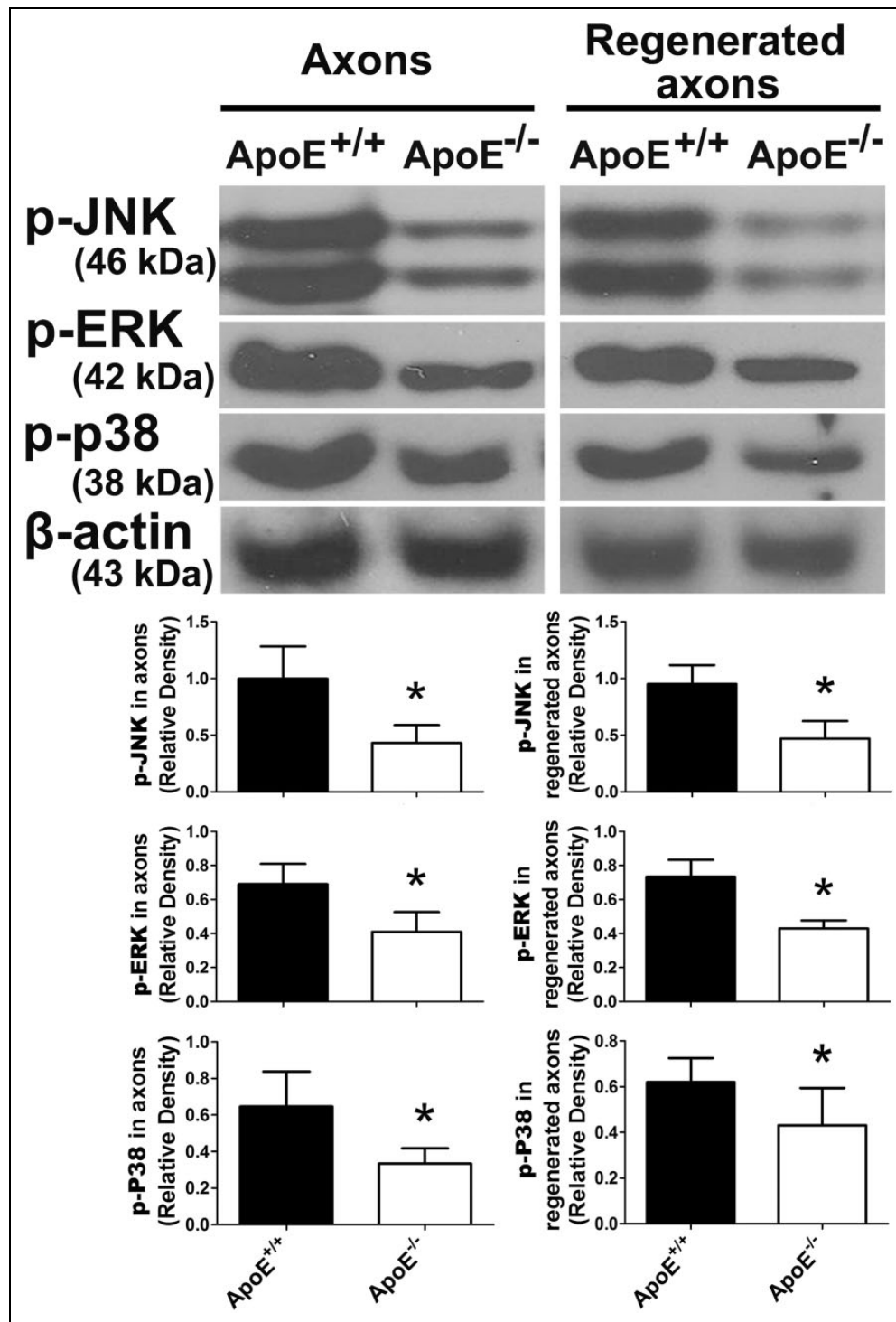
Fig 5. The expression levels of p-JNK, p-ERK and p-p38 were decreased in the ApoE^{-/-} group. In intact axons and regenerated axons, the expression levels of p-JNK, p-ERK and p-p38 were significantly decreased in the ApoE^{-/-} group compared with those in the ApoE^{+/+} group (*$p < 0.05$ vs. ApoE^{+/+}).

ApoE4, but ApoE3 showed an opposite effect, more so than that of ApoE2. Therefore, whether ApoE3 assisted axonal growth and regeneration via p-JNK/ERK/p38 was investigated by adding JNK/ERK/p38 inhibitors. The inhibitors markedly attenuated the positive effects of ApoE3 on axonal growth and regeneration (Fig. 9A, B).
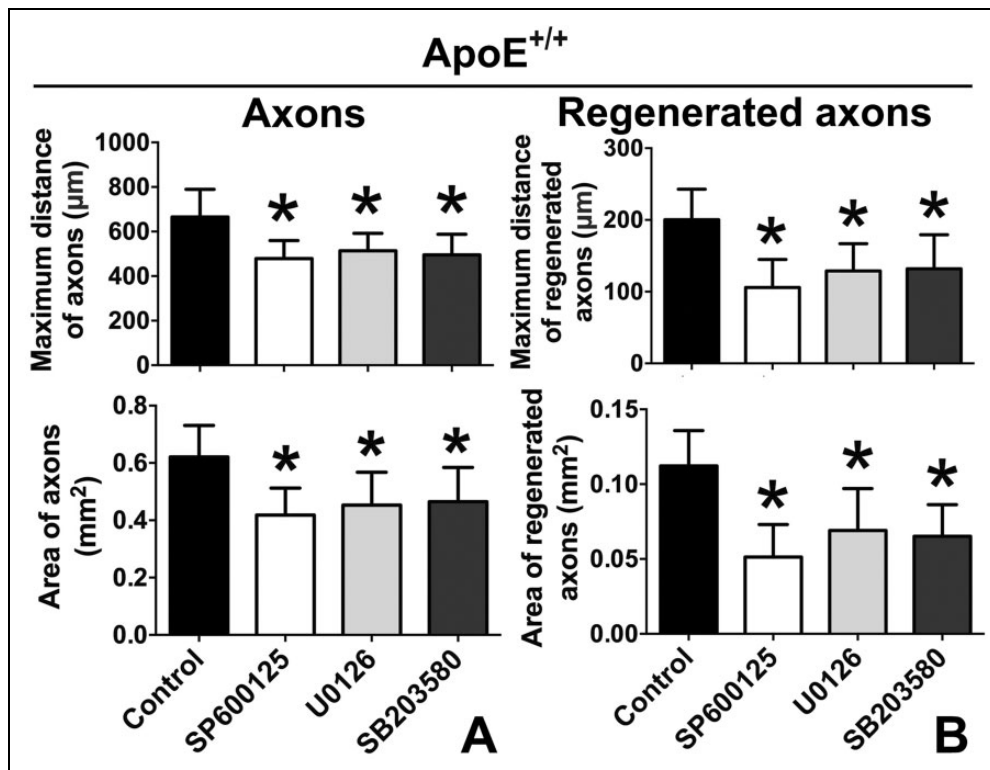
Fig 6. The JNK/ERK/p38 inhibitors attenuated the axonal growth and regeneration in the ApoE^{+/+} axons. **A**. In the intact axons, the maximum distance and area were significantly attenuated by the JNK/ERK/p38 inhibitors (*$p < 0.05$ vs. control). **B.** In the regenerated axons, the JNK/ERK/p38 inhibitors also obviously decreased the maximum distance and area (*$p < 0.05$ vs. control).

Discussion

In this study, we discovered that ApoE not only exists in regenerated axons, but also plays a role in promoting axonal regeneration in the mammalian CNS. In addition, human recombinant ApoE4 negatively influenced axonal regeneration and ApoE3 did so positively, and these effects were mediated by the JNK/ERK/p38 pathway.

The cortical explant culture was chosen as our experimental model based on the following considerations. First, the in vitro cortical explant culture allowed axonal growth and regeneration without obstructions, which are usually composed of glial scars in vivo[2,3]. This study examined the intrinsic capacity of axonal regeneration, and the cortical explant model allowed axons to freely grow and regenerate without barriers, which was suitable for our research goals. Second, the morphological structure of axons could be effectively exhibited in this model. As mentioned in Fig. 1, entire axons were radially distributed around the cortical brain tissue in the mature explants, making it convenient for us to observe the axonal growth and regeneration. In addition, because the axons gathered around the cortical brain tissue, the axonal transection and recovery were easy; for example, precisely transecting the axons to observe the regeneration and collecting enough axonal samples for RT-PCR and western blotting, etc., were easy using this model. Finally, the explant culture provided a similar environment to that in vivo for neurons. In primary neuron cultures, the neurons are isolated and cultured away from glial cells. However, in explant cultures, a piece of cortical brain tissue that contains neurons is cultured, so the surrounding environment of the neurons better simulates that of the brain in vivo.

Previous studies have suggested a correlation between ApoE and TBI[5,6]. One study has also shown that ApoE exists in the cell bodies and axons of mouse primary cortical neurons in vitro[31], and the subsequent research also showed that ApoE was increased in injured neurons in vivo[21,32]. These previous results suggested that ApoE might participate in axonal growth, but whether it was involved in axonal regeneration was unknown. Our research provides supporting evidence for this possibility. After the regenerated axons grew from the site of axonal transection, ApoE was observed in the regenerated axons by immunofluorescence. To further validate the role of ApoE in axonal regeneration, the growth cone, located at the tip of axon, was also analyzed in this study. Growth cones play a vital role in guiding axonal growth and regeneration[33,34], and there are a large number of mRNA and proteins in a tiny growth cone that actively take part in many pathological and physiological processes in the CNS[35,36]. Our results showed that ApoE exists not only in the growth cones of intact axons but also in the growth cones of regenerated axons, which indicates that
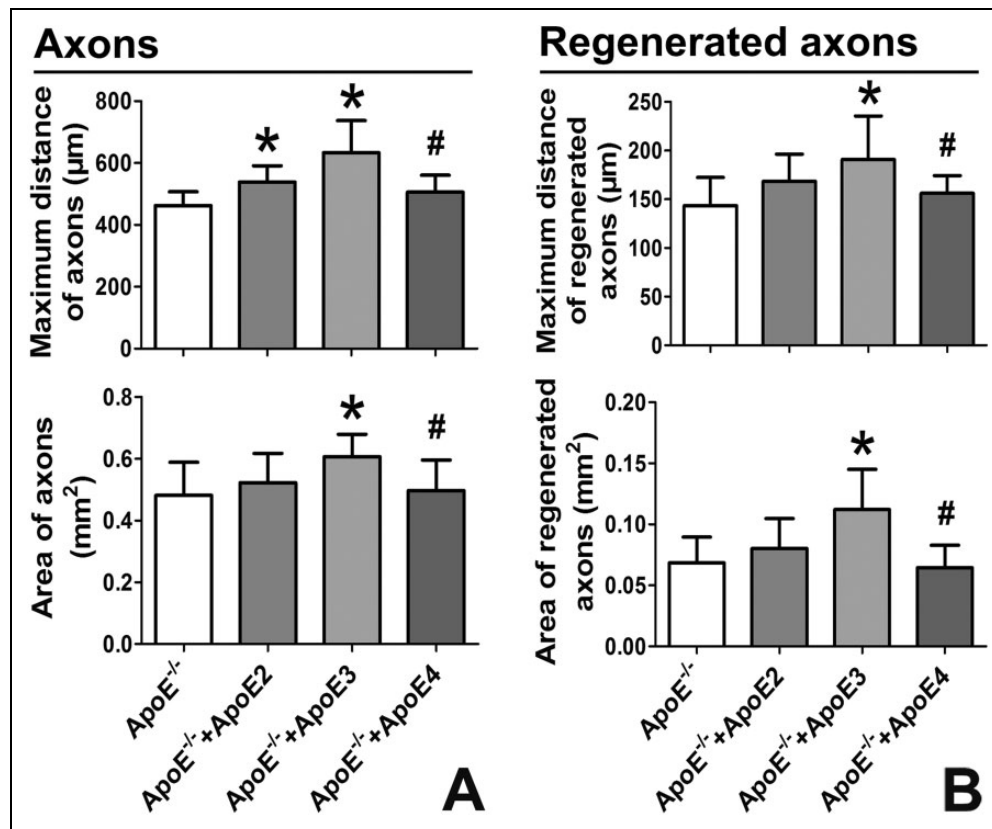
Fig 7. ApoE3 rather than ApoE4 improved the axonal growth and regeneration. **A.** In the intact axons, ApoE2 improved and ApoE3 significantly improved the maximum distance, and only ApoE3 significantly improved the area, but ApoE4 did not improve the maximum distance or area. **B.** In the regenerated axons, ApoE3 significantly improved the maximum distance and area, but ApoE4 did not improve the maximum distance or area. (*$p < 0.05$ vs. ApoE$^{-/-}$, #$p < 0.05$ vs. ApoE$^{-/-}$+ApoE3)

ApoE might participate in axonal regeneration as well as the corresponding new growth cone formation.

Although axons are an important component of neurons, most of the proteins are synthesized in the neuronal soma then slowly transported to distal axons through the transport system within the axons. In contrast, a small amount of proteins are rapidly transcribed and translated in the axon itself[37–39]. After axonal injury, proteins that can be timely and locally synthesized may be more essential for axonal repair or regeneration[39,40]. Therefore, whether ApoE mRNA was present in axons was further analyzed in our research. The results showed that ApoE mRNA exists in regenerated axons, which implied that injured axons could synthesize ApoE protein, and that it may even be used for axonal regeneration. However, it was completely possible that the intact axons and regenerated axons had taken up exogenous ApoE that was produced by glial cells. Indeed, considering the nature of the cortical explant culture, our study cannot differentiate these two possibilities, but our data demonstrate the importance of ApoE to axonal growth and regeneration.

Further, of the three human ApoE isoforms, ApoE3 and ApoE4 distinctively affected axonal growth. In the mouse

PNS, Comley et al. found that ApoE4 obviously negatively affected peripheral nerve regeneration and neuromuscular junction re-innervation[41]. However, the specific influence of the ApoE isoforms on axonal regeneration in the CNS has not been well studied. In our axonal transection model of cortical explants, the results supported that ApoE3 significantly improved axonal growth and regeneration, but the effect of ApoE4 was opposite. The above results might partly reveal an unfavorable prognosis for TBI patients with the ApoE4 genotype with regard to injured axons. To address this phenomenon, we examined the possibly related signaling molecules. As an essential signaling pathway protein, the MAPK protein family has demonstrated involvement in axonal regeneration by a series of experiments[42]. In a study by Korwek et al., the expression of JNK and ERK was not significantly different between ApoE-deficient and wild-type mice. However, the phosphorylated ratio of JNK to ERK was significantly attenuated[43]. This result demonstrated an association between ApoE and MAPK. Therefore, in our study, p-JNK, p-ERK and p-p38, which belong to the MAPK family, were detected in the regenerated axons of ApoE$^{-/-}$ and ApoE$^{+/+}$ mice. Our results were consistent with those of Korwek and colleagues. In addition, considering
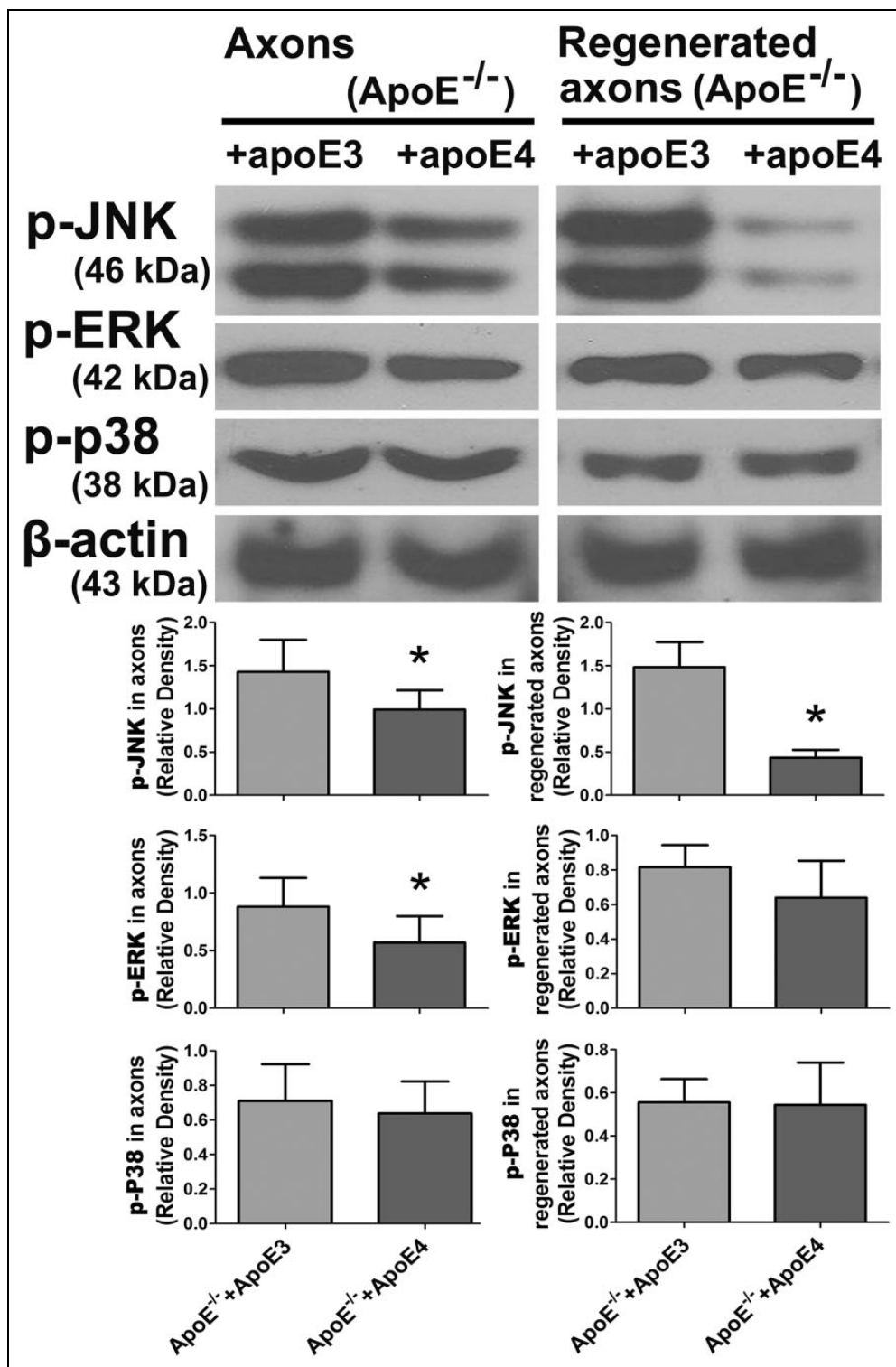
Fig 8. ApoE4 significantly decreased the expression of p-JNK in regenerated axons. In intact axons, the expression levels of p-JNK and p-ERK were obviously decreased in the ApoE$^{-/-}$+ApoE4 group compared with the ApoE$^{-/-}$+ApoE3 group. However, in the regenerated axons, only the expression of p-JNK was significantly decreased in the ApoE$^{-/-}$+ApoE4 group compared with that in the ApoE$^{-/-}$+ApoE3 group (*$p < 0.05$ vs. ApoE$^{-/-}$+ApoE3).
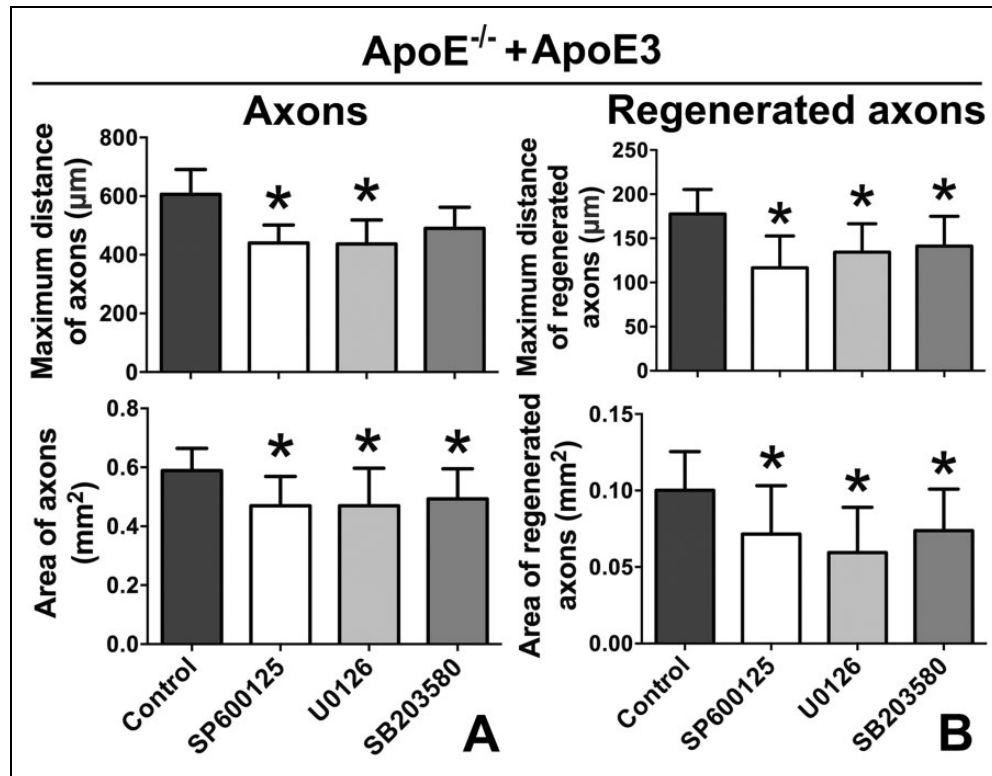
Fig 9. Inhibiting the JNK/ERK/p38 pathway attenuated the positive effects of ApoE3 on axonal growth and regeneration. **A.** In the intact axons, the area was significantly attenuated by the JNK/ERK/p38 inhibitors, but the maximum distance was only attenuated by the JNK and ERK inhibitors (*$p < 0.05$ vs control). **B.** In the regenerated axons, the JNK/ERK/p38 inhibitors also obviously decreased the maximum distance and area (*$p < 0.05$ vs. control).

that ApoE3 and ApoE4 play distinctive roles in axonal regeneration, p-JNK, p-ERK and p-p38 expression levels were also detected after ApoE3/4 intervention in ApoE$^{-/-}$ mice. The measurements showed that ApoE4 significantly attenuated p-JNK compared with ApoE3 in regenerated axons. In addition, the JNK/ERK/p38 inhibitor interventions further demonstrated the above results.

There were two obvious limitations in our study. First, the previous studies did not pay enough attention to the relationship between ApoE and axonal regeneration, so our research was mainly concentrated on descriptive studies, as the mechanistic research on ApoE was relatively insufficient. Furthermore, we focused only on the MAPK7 family, but we cannot rule out the possibility that ApoE influences axonal growth and regeneration via other signaling proteins. Second, axonal growth and regeneration are extremely complicated pathophysiological processes, not only because of the axon itself but also its surrounding environment. In our research, only the intrinsic capacity of growth and regeneration with ApoE were assessed by the axonal transection model in vitro. The effects of external factors on axonal growth and regeneration were not explored in this study. Overall, we merely discovered a partial role of ApoE, and the relationship of ApoE to axonal growth and regeneration warrants further investigation.

Conclusions

ApoE$^{-/-}$ and ApoE$^{+/+}$ mice were used in this study, explants from the cerebral cortex were cultured, and an axonal transection model was established in vitro. ApoE mRNA and protein were expressed not only in intact axons but also in regenerated axons. The capabilities of axonal growth and regeneration were significantly improved by ApoE. Further, ApoE3 positively but ApoE4 negatively influenced axonal growth and regeneration, and these effects were mediated by the JNK/ERK/p38 pathway. Our results revealed that ApoE and its isoforms influenced axonal growth and regeneration via the MAPK signaling pathway in mice, which perhaps partially explains why ApoE isoforms can influence the prognosis of TBI patients.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Statement of Human and Animal Rights

All procedures performed in studies involving animals (No human in this study) were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Provincial People's Hospital.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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