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Targeting IGF1/IGF1r signaling relieve pain and autophagic dysfunction in NTG-induced chronic migraine model of mice

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Abstract

Background Chronic migraine is a severe and common neurological disorder, yet its precise physiological mechanisms remain unclear. The IGF1/IGF1r signaling pathway plays a crucial role in pain modulation. Studies have shown that IGF1, by binding to its receptor IGF1r, activates a series of downstream signaling cascades involved in neuronal survival, proliferation, autophagy and functional regulation. The activation of these pathways can influence nociceptive transmission. Furthermore, alterations in IGF1/IGF1r signaling are closely associated with the development of various chronic pain conditions. Therefore, understanding the specific mechanisms by which this pathway contributes to pain is of significant importance for the development of novel pain treatment strategies. In this study, we investigated the role of IGF1/IGF1r and its potential mechanisms in a mouse model of chronic migraine.

Methods Chronic migraine was induced in mice by repeated intraperitoneal injections of nitroglycerin. Mechanical and thermal hypersensitivity responses were assessed using Von Frey filaments and radiant heat, respectively. To determine the role of IGF1/IGF1r in chronic migraine (CM), we examined the effects of the IGF1 receptor antagonist ppp (Picropodophyllin) on pain behaviors and the expression of calcitonin gene-related peptide (CGRP) and c-Fos.

Result In the nitroglycerin-induced chronic migraine model in mice, neuronal secretion of IGF1 is elevated within the trigeminal nucleus caudalis (TNC). Increased phosphorylation of the IGF1 receptor occurs, predominantly co-localizing with neurons. Treatment with ppp alleviated basal mechanical hypersensitivity and acute mechanical allodynia. Furthermore, ppp ameliorated autophagic dysfunction and reduced the expression of CGRP and c-Fos.

Conclusion Our findings demonstrate that in the chronic migraine (CM) model in mice, there is a significant increase in IGF1 expression in the TNC region. This upregulation of IGF1 leads to enhanced phosphorylation of IGF1 receptors on neurons. Targeting and inhibiting this signaling pathway may offer potential preventive strategies for mitigating the progression of chronic migraine.

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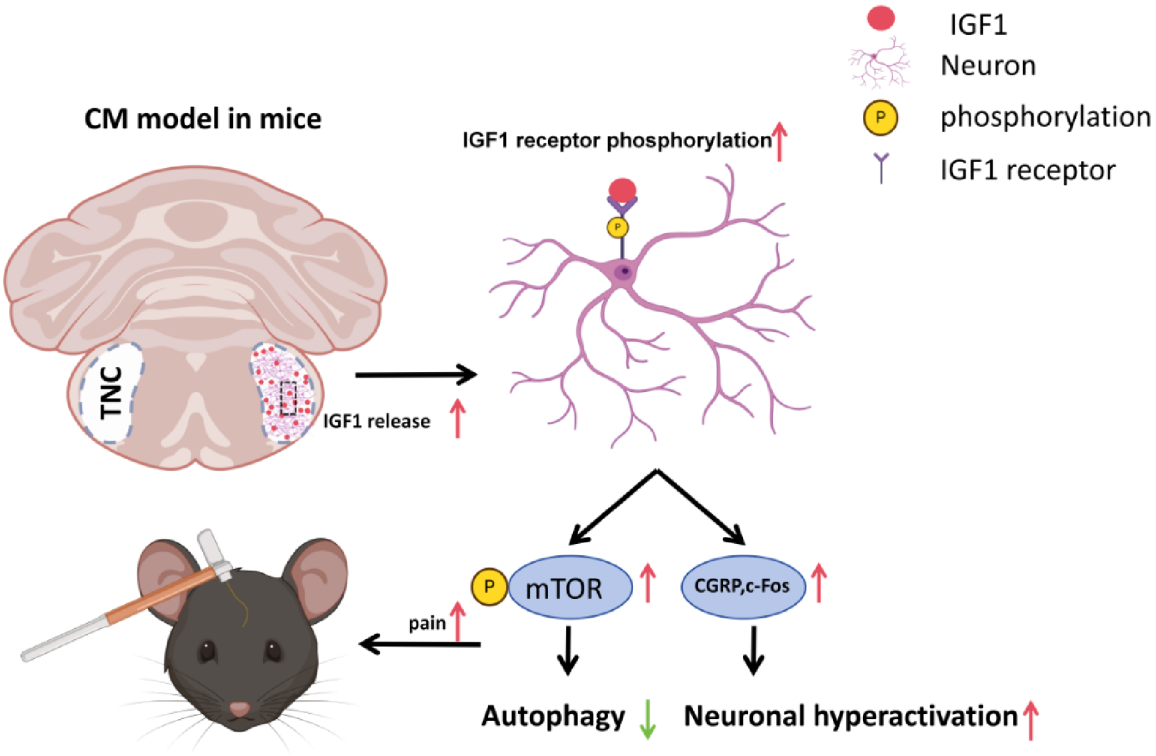
Keywords Chronic migraine, IGF1, IGF1r, mTOR, Autophagy

Graphical abstract



Background

Migraine is a serious neurological disorder characterized by moderate to severe headache and accompanied by symptoms such as photophobia and phonophobia. It ranks among the most common primary headaches [1, 2]. Epidemiological findings indicate that approximately 3% of episodic migraine sufferers progress to CM each year [3]. Chronic migraine, as a highly disabling primary headache disorder, often causes patients unable to live a normal life or work, and severely impact the quality of life. However, the mehcanism of CM is not clear [4]. Central sensitization is considered one of the potential mechanisms underlying the chronification of migraine [3].

Central sensitization refers to the enhanced response to normal or sub-threshold sensory stimuli in the central nervous system (CNS). Under normal circumstances, the CNS regulates pain transmission and perception through a balance of inhibitory and excitatory signals. However, in cases of central sensitization, this balance is disrupted, leading to amplification of pain signals, also known as hyperalgesia [5]. Previous reports indicate that central sensitization may be induced by pro-inflammatory cytokines such as IL-6, TNFα, CGRP and so on, and autophagy is involved in the neuroinflammatory processes associated with central neurodegenerative diseases and stroke [6, 7]. TNC is a crucial brain region involved in processing thermal sensation related to pain in the head and face [8].

Insulin-like growth factor 1 (IGF1) is a small peptide molecule expressed abundantly throughout the central nervous system and plays a significant role in growth and development processes [9, 10]. Moreover, previous studies have indicated its association with various neurological disorders such as Parkinson's disease, Alzheimer's disease, and others [11–13]. IGF1 in the brain has two primary sources: endocrine and autocrine. Autocrine IGF1 is mainly produced in response to pulses of growth hormone in the liver, constituting a classic negative feedback regulatory mechanism. After synthesis in the liver, IGF1 reaches the central nervous system through the blood-brain barrier via the choroid plexus [14]. On the other hand, IGF1 is also produced autonomously within the nervous system [15].

IGF1 primarily exerts its effects through binding to the IGF1 receptor (IGF1r) [16]. Upon binding, IGF1 initiates intracellular signaling cascades such as the phosphatidylinositol 3-kinase-protein kinase

B (PI3K-Akt) and mitogen-activated protein kinase (MAPK) pathways, while mTOR serves as a downstream effector [17, 18]. Activation of these pathways influences diverse physiological processes such as autophagy, neuronal growth, and apoptosis [19, 20]. Increasing evidence suggests that IGF1 is closely associated with pain perception, and the abnormal activation of its signaling pathway may contribute to the development of chronic pain conditions. In patients with chronic migraine (CM), studies have shown a correlation between changes in IGF1 levels and the severity of pain; however, the precise mechanisms underlying this relationship remain unclear [21, 22].

Therefore, in this study, we investigated the sources of IGF1 and its impact on pain in a chronic migraine mouse model. We explored the effects of IGF1/IGF1r signaling pathway activation and its downstream effects on pain, discussing potential physiological mechanisms. Our experimental results indicate that in the nitroglycerin-induced chronic migraine model in mice, there is an increase in IGF1 expression and activation of its downstream pathways, leading to impairment of autophagy.

Methods

Animal

The male C57BL/6 mice used in this experiment were purchased from the Model Animal Research Center of ShanghaiTech University, A total of 60 male mice, weighing between 20 and 30 g, were used in this experiment. 8–10 weeks. License number 20,240,819,002. All animal experiments were approved by the Animal Ethics Committee of ShanghaiTech University and conducted in accordance with the standards outlined in the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences, USA, and guidelines from the National Institutes of Health.

All animals were housed under standard conditions with controlled temperature (22–24 °C) and humidity (40–60%), and subjected to a 12-hour light-dark cycle. They were provided with ample food and water ad libitum (unrestricted) throughout the housing period. Mice were given one week to acclimatize to the experimental environment before the commencement of experiments. Random allocation of mice into different experimental groups was performed prior to the start of the study [23].

Establishment of the chronic migraine model

CM model referenced in this study was based on previous research methodologies [24]. The nitroglycerin (NTG) (Baiyunshan, China) stock solution was prepared at a concentration of 5.0 mg/ml dissolved in a mixture of 30% ethanol, 30% propylene glycol, and water. Before each experiment, it was diluted to 1 mg/ml using 0.9% sterile saline solution.

Mice were injected intraperitoneally with either 10 mg/kg of NTG solution or sterile saline solution every other day for a total of nine days (five injections in total). Behavioral tests were conducted on all experimental animals 2 h before and after NTG injection.

Drug administration

To investigate the effects of the IGF1/IGF1r signaling pathway on pain behavior in the chronic migraine model in mice induced by NTG, mice were injected with an IGF1r antagonist (ppp, Selleck, USA) at a dose of 40 mg/kg/day. This injection was administered before NTG intraperitoneal injections and after baseline threshold measurements of the mice. The ppp (IGF1r antagonist) was dissolved in a solution containing 8% DMSO and 92% corn oil. To investigate the effects of IGF1 on CM mouse model in autophagic flux, mice were intravenously injected with 100 μg/kg of IGF1 (IGF1 Protein, MCE, USA) via the tail vein after baseline threshold measurements. IGF1 was diluted in 0.9% sterile saline solution. To study the role of autophagy in headache induced by intravenous injection of IGF1, the autophagy inducer rapamycin (RAPA, Aladin, China) was administered intraperitoneally at a dose of 1 mg/kg in a similar manner. Rapamycin was dissolved in a solution containing 10% DMSO and 90% corn oil. All drug solutions were freshly prepared on the day of use.

Behavioral tests

All behavioral tests were conducted in a dimly lit, quiet environment between 9:00 AM and 3:00 PM. Mice were acclimated to the behavior room for 3 days prior to the behavioral testing. Some migraine patients may experience allodynia in the skin of the head and face [25]. Therefore, in animal models, we measured the mechanical withdrawal thresholds of mice following both paw and periorbital mechanical stimulation. The experiment employed a double-blind design, and all data were analyzed by another blinded observer. Measurement was conducted using the up-down method during the assessment [26]. In brief, applying von Frey filaments vertically to the tested area (ranging from 0.008 to 2 g), a positive response indicates decreased filament strength, while a negative response indicates increased filament strength [27]. The initial strength is 0.16 g. Each filament is applied for 3 s with a 3-minute interval between applications. Finally, calculate the 50% mechanical threshold using an online tool. https://bioapps.shinyapps.io/von-Frey-app/ [28]. For periorbital mechanical withdrawal threshold testing,

place the mouse in a 4-ounce paper cup, allowing free movement of the head and front paws while preventing body rotation within the cup. The periorbital region includes from the mouse's eye corner to near the midline position. In the periorbital mechanical threshold withdrawal experiment, a positive response is defined as the mouse quickly withdrawing its head from the stimulus or scratching the facial area with the ipsilateral front paw [29]. For hind paw mechanical threshold testing, the mouse is placed in a bottomless box made of transparent acrylic, positioned on top of a wire mesh. During measurement, von Frey filaments are applied vertically to the exact center of the mouse's hind paw, avoiding the fat pad. A positive response in the hind paw experiment is defined as withdrawal, shaking, or licking of the paw [24].

Measurement of thermal withdrawal latency

To measure thermal threshold, mice were placed in a testing apparatus surrounded by a transparent acrylic board, with the bottom heated to 55 degrees Celsius (15 cm radius), using a YLS-6B Intelligent Hot Plate (Jinan, China). A positive response was defined as the mouse licking its hind paw or jumping. A maximum cutoff time of 30 s was set to prevent thermal injury to the mouse paws [30].

Immunofluorescence staining

Twelve hours following the final behavioral test, we conducted immunostaining analysis on each group of mice. The mice were deeply anesthetized with 1% pentobarbital and transcardially perfused with pre-chilled 1X phosphate-buffered saline (PBS, pH = 7.4), followed by perfusion with 4% paraformaldehyde (PFA). Immediately after perfusion, the mice were dissected to extract the brain, which was then fixed in 4 °C PFA for 48 h. Then, the brain tissue was sequentially dehydrated in 20% and 30% sucrose solutions until the tissue sank. Using a vibrating microtome (Leica, Wetzlar, Germany, Cat# VT1000S), 40 μm thick coronal sections were obtained from the fixed tissue. Before staining, the tissue sections were washed three times for 3 min each in clean PBS. Subsequently, the primary antibody was diluted in 1× PBS buffer containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA), and the sections were incubated overnight at 4 °C with the free-floating tissue slices. The next day, brain sections were washed three times for 5 min each with 1× PBS. Next, the tissue sections were incubated at room temperature for 2 h with the secondary antibody diluted in a buffer containing 0.3% Triton X-100 and 1% BSA in 1× PBS. Throughout the entire process, all experimental procedures were conducted in a dark environment. Finally, the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Yeasen Biotech, China, Cat# 40728ES03) for 20 min. These antibodies were used for immunofluorescence staining: NeuN(1:500, Abcam, Cat# ab177487), IGF1(1:500, Abcam, Cat# ab9572), IBA1 (1:500, Abcam, Cat# ab5076), c-Fos(1:500, CST, Cat#2250), CGRP(1:1000, SCBT, Cat#sc-57053), p-IGF1r(1:100, Abclonal, Cat#AP0367). The secondary antibody used was goat anti-mouse Alexa Fluor 647(1:1000, Yeasen, Cat#33213ES60), goat anti-rabbit Alexa Fluor 555(1:1000, Yeasen, Cat#33213ES60), donkey anti-goat Alexa Fluor 555(1:1000, Thermo Fisher Scientific, Cat# A-21432)donkey anti-mouse Alexa Fluor 647(1:1000, Thermo Fisher Scientific, Cat# A-31571).

Immunofluorescence imaging data analysis

To ensure accurate and unbiased image analysis, confocal images were randomly captured from each fluorescence channel using a Zeiss LSM980 inverted microscope (Carl Zeiss, Oberkochen, Germany). Fixed gains and exposure times were used for all channels during image acquisition. Each mouse was randomly selected for analysis of 2–3 brain sections, obtained at magnifications of 10×, 40×, and 63×.According to the mouse brain atlas [31], To delineate the TNC (trigeminal nucleus caudalis) region based on morphological observations under an optical microscope, we utilized ImageJ software (version 1.8.0_112) to analyze the average optical density of CGRP. For quantification of c-Fos-positive cells, we manually traced the TNC area in ImageJ according to the mouse brain atlas and quantified the number of c-Fos-positive cells.

Quantitative reverse transcription-PCR

According to the manufacturer's protocol, RNA extraction from mouse brain tissue was performed using TRIzol (Invitrogen, Carlsbad, Cat# 15596018). Subsequently, cDNA synthesis was carried out using a cDNA synthesis kit (Transgene Biotech, Cat# AT311-03). Quantitative reverse transcription PCR (qRT-PCR) was performed using 2× SYBR Green qPCR Master Mix (Selleck, Cat# B21202). The reverse transcription PCR (RT-PCR) was conducted using the Quant Studio 7 Flex PCR System (Thermo Fisher Scientific, Cat# 4485700).The reaction conditions were as follows: initial denaturation at 95 °C for 20 s, followed by 40 cycles at 95 °C for 15 s denaturation, 60 °C for 30 s annealing, and 72 °C for 30 s extension. The relative expression of mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression using the 2−ΔΔCT method.

Primer Sequence: IGF1F GTGGATGCTCTTCAGTT CGTGTG, IGF1rTCCAGTCTCCTCAGATCACAGC, IGF1rF CGGGATCTCATCAGCTTCACAG, IGF1rR T

CCTTGTTCGGAGGCAGGTCTA, GAPDHF AGGTC GGTGTGAACGGATTTG, GAPDHR TGTAGACCAT GTAGTTGAGGTCA.

Western blot

Using 1% pentobarbital to deeply anesthetize mice, the mouse brain tissue was extracted. The TNC was immediately collected and stored at -80 °C. The tissue was homogenized in cold RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Beyotime, Shanghai, China) for 30 min. The tissue lysates were centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was collected, and the protein

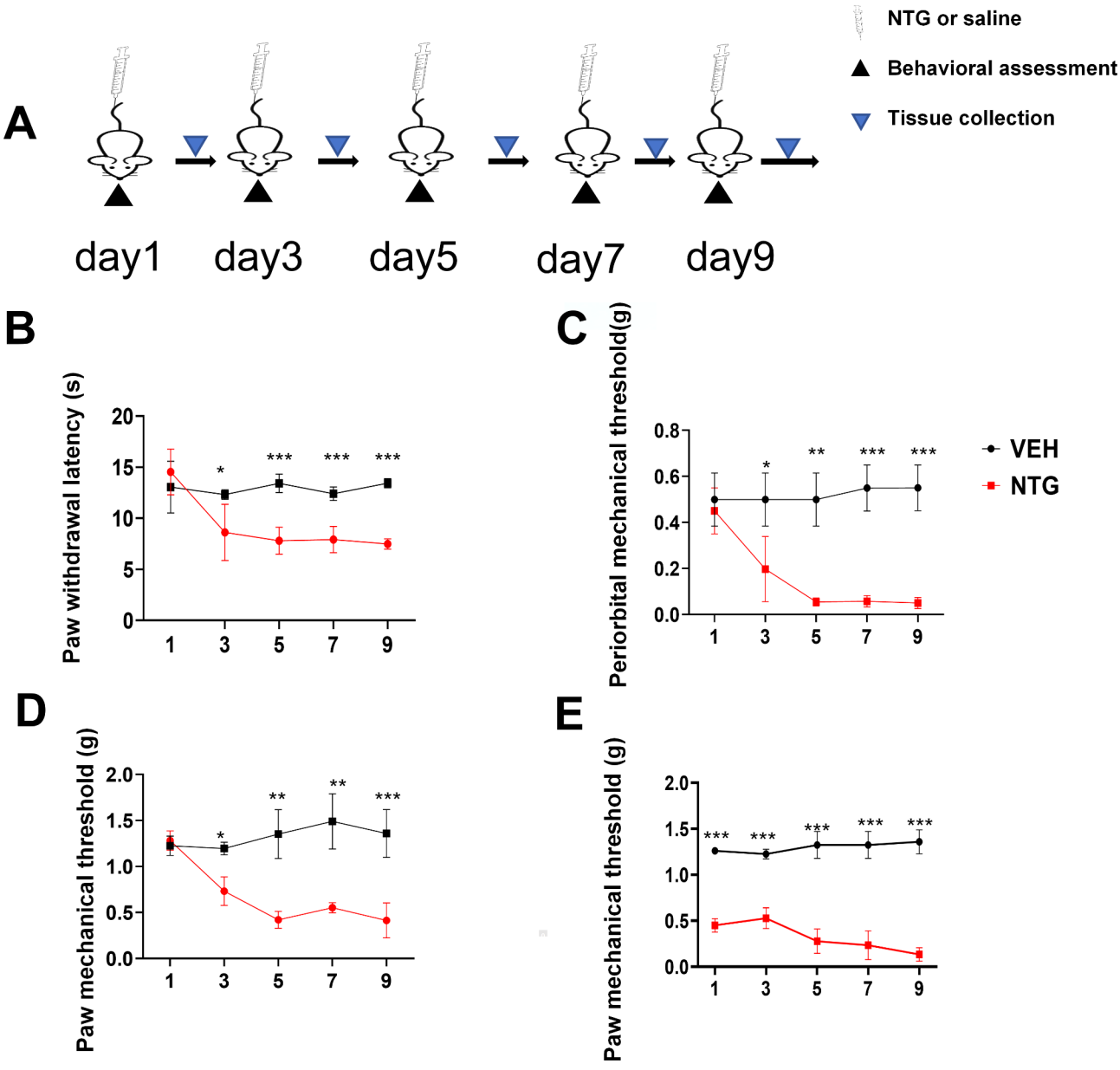


Fig. 1 Repeated NTG administration induced mechanical allodynia and thermal hyperalgesia in mice. (**A**) Experimental paradigm illustrating repeated intraperitoneal administration of nitroglycerin (NTG; 10 mg/kg) every 2 days for 9 days in C57Bl/6J mice, with physiological saline as the control group. Additionally, a group of mice was euthanized 24 h after a single injection to collect trigeminal nucleus caudalis (TNC) tissue for subsequent experiments ($n = 5$ mice/group; two-tailed independent sample t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). **B**, **C**, **and D** illustrate behavioral tests performed before saline or nitroglycerin injections. Following repeated administration, mice exhibited mechanical allodynia and thermal hyperalgesia as evidenced by the open field and paw withdrawal tests. (**B**) Thermal withdrawal latency. (**C**) Periorbital mechanical withdrawal threshold. (**D**) Hind paw non-acute phase mechanical withdrawal threshold. (**E**) Measurement of hind paw acute phase mechanical withdrawal threshold 2 h after single injection. ($n = 5$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$)

concentration was determined using the bicinchoninic acid (BCA) method with a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China, Cat# P0010). Equal amounts of tissue protein (40 μg) were separated on 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Beyotime, Shanghai, China) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Subsequently, the membranes were blocked in 5% non-fat milk at room temperature for 2 h. Subsequently, the membranes were incubated overnight with primary antibodies. IGF1(1: 1000, Abcam, Cat# ab9572), c-Fos(1:2000, CST, Cat#2250), CGRP(1:2000, SCBT, Cat#sc-57053), p-IGF1r (1:1000, Abclonal, Cat#AP0367), GAPDH(1:2000,abways, AB0037), p62 (1:1000, Abclonal, Cat#A19700), Phospho-mTOR (1:2000, CST, Cat#5536), mTOR(1:2000, CST, Cat#2972). After overnight incubation, the membranes were washed in TBS-Tween and then incubated with the following secondary antibodies for 1 h at room temperature: Goat Anti-Rabbit IgG(H+L) HRP(H+L;1:5000, sparkjed, Cat#EF0002). The immunoreactive bands were visualized using the Amersham Imager 600 chemiluminescence imaging system (GE Healthcare, Beijing, China). Finally, band densities were evaluated using ImageJ software version 1.8.0. Protein band densities were normalized to GAPDH as an internal reference.

Statistical analysis

Data analysis was conducted using PRISM 9.3 software (GraphPad, San Diego, CA). For behavioral test data, including drug administration and different time points, We assessed the normality of the data using the Shapiro-Wilk test. Data that met the criteria for normality are reported as mean ± standard error of the mean (SEM), while data that did not follow a normal distribution are reported as mean ± standard deviation (SD). A two-tailed independent samples t-test was used to evaluate differences between two groups. For non-normally distributed data, we employed non-parametric tests, such as the M-U test.

Result

Repeated intraperitoneal injections of nitroglycerin induced migraine-like behavior in mice

Based on previous reports, we established a chronic migraine-like model in male mice by administering intraperitoneal injections of nitroglycerin at a dose of 10 mg/kg every other day(Fig. 1A-D, $^{*}p<0.05$, $^{**}p<0.01$). Furthermore, mechanical allodynia and thermal hyperalgesia were assessed in mice using the von Frey and hot plate tests, respectively, 30 min before and 2 h after nitroglycerin injection. Experimental results indicated that mice in the nitroglycerin group exhibited a persistent decrease in baseline thresholds for mechanical allodynia, thermal hyperalgesia, and spontaneous nociceptive behavior, reaching their lowest levels on the ninth day. In addition, compared to the vehicle (VEH) group, the nitroglycerin group exhibited acute mechanical allodynia and thermal hyperalgesia 2 h after injection (Fig. 1E, $^{*}p<0.05$, $^{**}p<0.01$).

In the nitroglycerin-induced chronic migraine mouse model, CGRP and c-Fos expression increased TNC, along with a gradual rise in IGF1 expression

TNC (trigeminal nucleus caudalis) refers to a crucial component of the trigeminal sensory pathway responsible for processing pain and sensory information from the head and face. We focused on changes in the trigeminal nucleus caudalis (TNC) brain region related to activation of neurons and headache related substances, including CGRP and c-Fos expression.The immunofluorescence results indicated a significant increase in the number of c-Fos-positive cells in the NTG group mice (Fig. 2A, $p<0.01$). The fluorescence intensity of CGRP was significantly increased (Fig. 2B, $p<0.01$). These data indicate that the chronic migraine (CM) model we established is reliable and suitable for further research. Simultaneously, we monitored the expression levels of IGF1 during the modeling process. Twenty-four hours after nitroglycerin injection, TNC samples were collected for qPCR. The experimental results demonstrated a gradual increase in IGF1 expression in the mouse chronic migraine model, particularly on the seventh and ninth days (Fig. 2C, $p<0.01$). On the eleventh day, TNC samples were subjected to Western blot, revealing a significant increase in IGF1 expression in the mouse trigeminal nucleus caudalis (TNC) brain region (Fig. 2E, $p<0.01$).

Elevated IGF1 in the TNC is primarily mediated through autocrine signaling within the nervous system and predominantly co-localizes with neuronal cells

IGF1 in the central nervous system originates primarily from two sources. One of these is autocrine secretion within the nervous system. The other is the growth hormone (GH) secreted by the hypothalamic-pituitary axis, which acts on the liver to produce IGF1 that enters the circulation, crosses the blood-brain barrier, and reaches the central nervous system. After confirming the increased expression of IGF1 in the mouse chronic migraine model, our first step was to investigate the sources of this IGF1. We collected liver samples from mice on the eleventh day post-behavioral testing for qPCR validation and found no significant change in IGF1 expression (Fig. 3B, $p>0.05$).

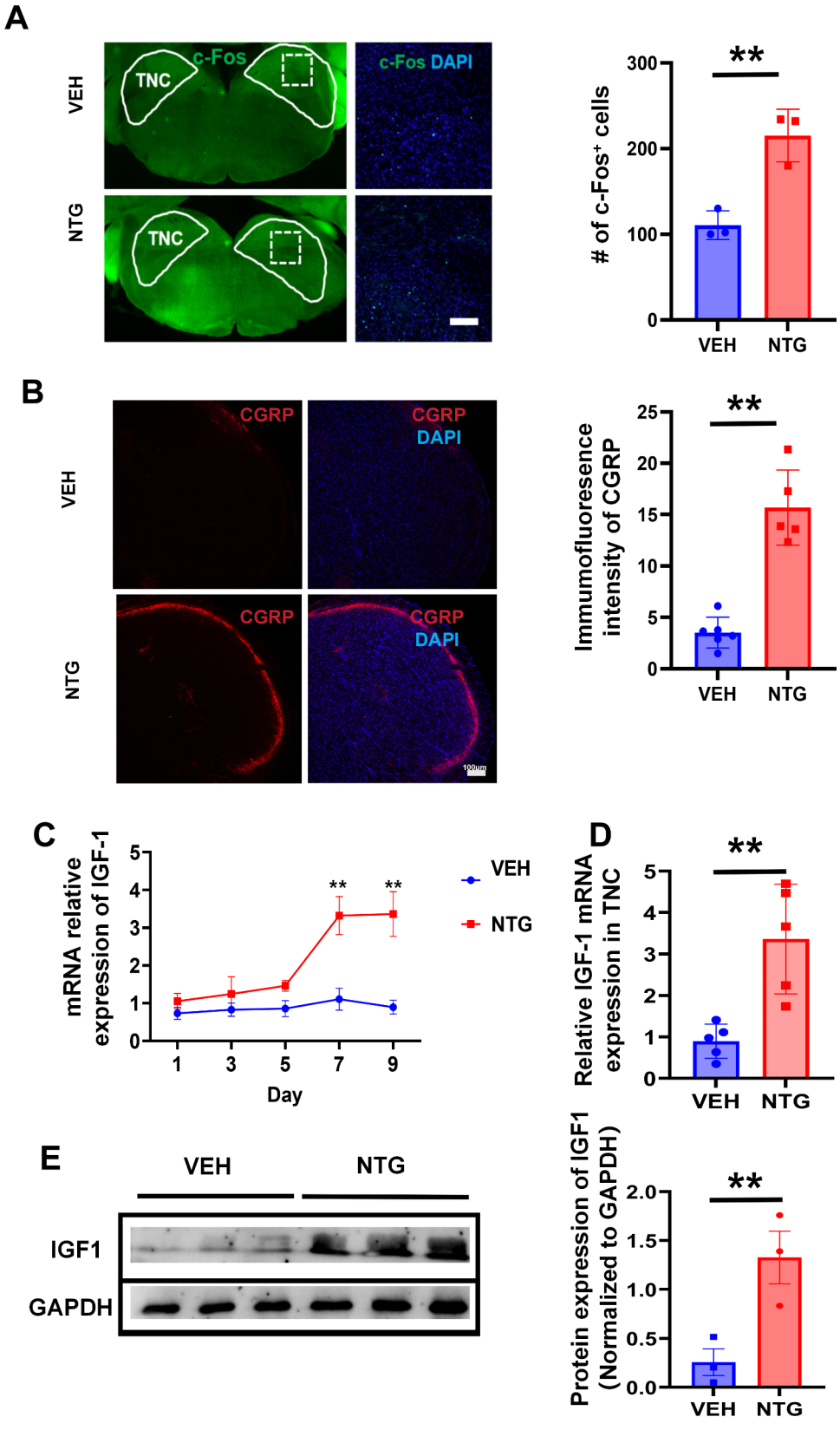


Fig. 2 (See legend on next page.)

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Fig. 2 Gradual increase in IGF1 expression in the trigeminal nucleus caudalis (TNC) brain region following repeated intraperitoneal injections of NTG in mice. (**A**) In the mouse chronic migraine model, there is a significant increase in the number of c-Fos + cells in the trigeminal nucleus caudalis (TNC) brain region. ($n = 3$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**B**) In the mouse chronic migraine model, there is a significant increase in CGRP expression in the trigeminal nucleus caudalis (TNC) brain region ($n = 5$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**C**) Changes in IGF1 mRNA expression during the induction of chronic migraine in mice ($n = 4$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**D**) On the 11th day of the mouse chronic migraine model, there is a significant increase in IGF1 expression in the trigeminal nucleus caudalis (TNC) brain region ($n = 5$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**E**) Western blot representative image showing changes in IGF1 expression in the trigeminal nucleus caudalis (TNC) brain region during the mouse chronic migraine model ($n = 3$ mice/group; two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$)

We performed Western blot validation on circulating blood samples and found no significant difference in IGF1 expression (Fig. 3c, $p > 0.05$) (Fig. 3D). Additionally, immunofluorescence demonstrated that IGF1 in the TNC brain region predominantly co-localized with neurons, with minimal co-localization observed with microglia. Therefore, in the mouse chronic migraine model, the elevated IGF1 in the trigeminal nucleus caudalis (TNC) brain region primarily originates from autocrine secretion within the nervous system and predominantly co-localizes with neurons.

In the mouse model of chronic migraine, there is a significant increase in the phosphorylation of IGF1 receptors in TNC

After confirming the increased expression of IGF1 in the TNC brain region, we monitored its downstream effects. Considering that IGF1r is a classical tyrosine kinase receptor whose activity is primarily regulated by phosphorylation, we assessed the phosphorylation level of IGF1r at the Tyr-1161 site (p-IGF1r). We observed that in NTG-treated mice, there was an increase in IGF1 receptor phosphorylation in TNC (Fig. 4, A-B, $^{*}p < 0.05$, $^{**}P < 0.01$). Moreover, this phosphorylated IGF1 receptor predominantly co-localized with neurons (Fig. 4, D, $^{*}p < 0.05$, $^{**}P < 0.01$). In the mouse model of chronic migraine, there was a significant increase in the number of P-IGF1r in the TNC brain region (Fig. 4, C, $^{*}p < 0.05$, $^{**}P < 0.01$).

Blocking the IGF1/IGF1r pathway alleviates mechanical and thermal hyperalgesia in a mouse model of chronic migraine and reduces CGRP expression in the TNC

Picropodophyllin (PPP, AXL1717) is a selective inhibitor of IGF1r with high specificity. To investigate whether activation of the IGF1/IGF1r axis in a mouse model of chronic migraine affects pain behavior, mice were intraperitoneally injected with the IGF1r antagonist (40 mg/kg/day) two hours prior to intraperitoneal injection of nitroglycerin (NTG) (Fig. 5A). Behavioral assessments were conducted half an hour before and two hours after nitroglycerin injection. Experimental results demonstrate that PPP significantly attenuated hyperalgesia in NTG-treated mice (Fig. 5B-E). Concurrently, there was a reduction in CGRP expression in the TNC brain region of mice (Fig. 5F).

PPP inhibited the excessive activation of the mTOR pathway in a mouse model of chronic migraine and alleviated neuronal autophagy dysfunction

Numerous studies indicate that activation of IGF1r involves the initiation of phosphoinositide 3-kinase (PI3K)-Akt signaling, which regulates various critical cellular functions such as protein synthesis and autophagy. One of the downstream effectors of Akt signaling is the mTOR pathway. Therefore, to investigate its downstream mechanisms, we assessed the impact of the IGF1r antagonist on the mTOR pathway in a mouse model of chronic migraine using Western blot. Our data indicate overactivation of the mTOR pathway in a chronic migraine mouse model (Fig. 6A, D). Inhibition of IGF1r reduces the activation of the mTOR pathway. Concurrently, we analyzed the expression levels of the autophagy-related protein p62, revealing increased expression in the chronic migraine mouse model. IGF1r antagonism decreases the expression of p62 (Fig. 6E). c-Fos was employed as a marker of activation of neurons, and our results demonstrate that in the mouse model of chronic migraine, the administration of IGF1 receptor antagonist reduced c-Fos expression in TNC region (Fig. 6C).

Intravenous IGF1 injection in mice decrease the facial mechanical withdrawal threshold, while mTOR inhibitor treatment alleviates pain behaviors

To validate the effect of IGF1 on facial pain behaviors in mice, we administered IGF1 via tail vein injection (100 μg/kg). Experimental results show that intravenous IGF1 injection causes a decrease in the facial mechanical pain threshold in mice (Fig. 7A). Treatment with the mTOR pathway antagonist rapamycin alleviates headache behaviors in mice (Fig. 7A). Subsequently, we conducted Western blot analysis on mice, which demonstrated IGF1-induced activation of IGF1r in the TNC brain region (Fig. 7D), along with an increase in phosphorylated mTOR activation (Fig. 7C). Additionally, expression levels of c-Fos (Fig. 7E) and

p62 (Fig. 7F) were elevated in the TNC brain region of mice.

Discussion

Our study has revealed several novel findings. Firstly, we observed elevated expression of IGF1 in the TNC in NTG-induced chronic migraine mouse model. And the increased IGF1 expression was observed to enhance phosphorylation of IGF1r. Subsequently, we identified the source of this IGF1, primarily from autocrine mechanisms within the nervous system. We then intervened in the IGF/IGF1r signaling pathway in the NTG-induced chronic migraine model and found that inhibiting IGF1 receptor phosphorylation alleviated pain behaviors in mice and ameliorated mTOR-related autophagic dysfunction. Furthermore, we discovered that intravenous injection of IGF1 induced headache-like behaviors in mice, accompanied by excessive activation of autophagy inhibition-related signaling pathways. Inhibiting this pathway alleviated autophagy suppression and reduced headache-like behaviors in mice.

CM is a severe neurological disorder characterized by recurrent episodes of intense headaches accompanied by sensory disturbances. Cutaneous allodynia is a common clinical feature, with approximately 80% of migraine patients experiencing increased sensitivity to touch on the side of the head where pain occurs [32]. Cutaneous allodynia is a common clinical symptom of migraine. Approximately 80% of migraine patients experience increased sensitivity to touch on the side of the head where pain occurs. This abnormal sensory hypersensitivity in the head and face is believed to be associated with central sensitization [5]. Previous studies have indicated that CGRP plays a pivotal role in the chronicization of migraine, and its antagonists are extensively utilized in clinical practice for treating migraines [8, 33, 34]. c-Fos is a protein encoded by an immediate early gene. Its expression level reflects neuronal activity and is considered a reliable marker related to response to pain and other noxious stimuli [5, 35, 36].

In this study, we choose a chronic migraine mouse model by repeated intraperitoneal injections of nitroglycerin every other day. Nitroglycerin can trigger a migraine-like attack in patients that is indistinguishable from spontaneous migraine [37]. This model is widely accepted as a reliable CM model [24]. NTG is a well-established trigger for migraine attacks [38, 39]. Previous studies have demonstrated that a single intraperitoneal injection of NTG reduces acute mechanical pain thresholds in mice [25]. Repeated injections of NTG can further reduce baseline mechanical withdrawal thresholds over time [39]. These behavioral manifestations resemble the abnormal facial and head skin pain experienced by chronic migraine patients during both attack periods and interictal phases [25, 40]. In addition, administration of NTG induces migraine-like symptoms in mice, including reduced activity, and facial grimacing behaviors. These symptoms resemble the features of light sensitivity and exacerbated headache upon movement observed in patients with CM [41]. Because both male and female mice can successfully establish CM models, we chose male mice to establish the experimental model in order to avoid the potential influence of hormonal fluctuations on experimental outcomes [24]. Due to the gender differences in the prevalence of migraine, the sexual dimorphism of IGF1 involvement in the pathophysiological mechanisms of CM warrants further exploration.

IGF1 is a small peptide molecule that plays crucial roles both centrally and peripherally, such as in neuronal growth, as previously reported [42, 43]. Furthermore, IGF1 is closely associated with multiple neurological disorders [11]. For brain-derived IGF1, it can be categorized into two sources: endocrine (systemic) secretion and autocrine secretion within the brain. That is to say, on one hand, the nervous system can autocrinally secrete IGF1 and act locally within the nervous system [44]. On the other hand, IGF1 is the principal effector of the growth hormone (GH) axis. It is produced by the liver in response to growth hormone secretion, released into the bloodstream, and crosses the blood-brain barrier to reach the brain [14].

The relationship between IGF1 and pain is close, yet several questions remain unanswered. For example, Miura et al.'s study demonstrated that local administration of IGF1 induces dose-dependent thermal and mechanical hypersensitivity in pain responses [45]. Kohno et al. found that upregulation of IGF1 in CD11c microglia in the spinal dorsal horn can alleviate neuropathic pain 21 days after peripheral nerve injury (PNI) [46]. In a rat model of chemotherapy-induced peripheral neuropathy (CIPN), the expression of IGF1 protein in the spinal cord is significantly reduced. Intravenous or intraperitoneal injection of IGF1 (1 μg) alleviates chemotherapy-induced pain-like behaviors [47]. We hypothesize that these seemingly conflicting conclusions arise from differences in animal models, central-peripheral differences, and the source of IGF1. Therefore, in our study, we focused on these aspects. Our experimental results indicate that in the NTG-induced mouse model of chronic migraine, the increased expression of IGF1 primarily originates from neurogenic autocrine mechanisms and predominantly co-localizes with neurons. In patients with migraine, studies have found an association between changes in

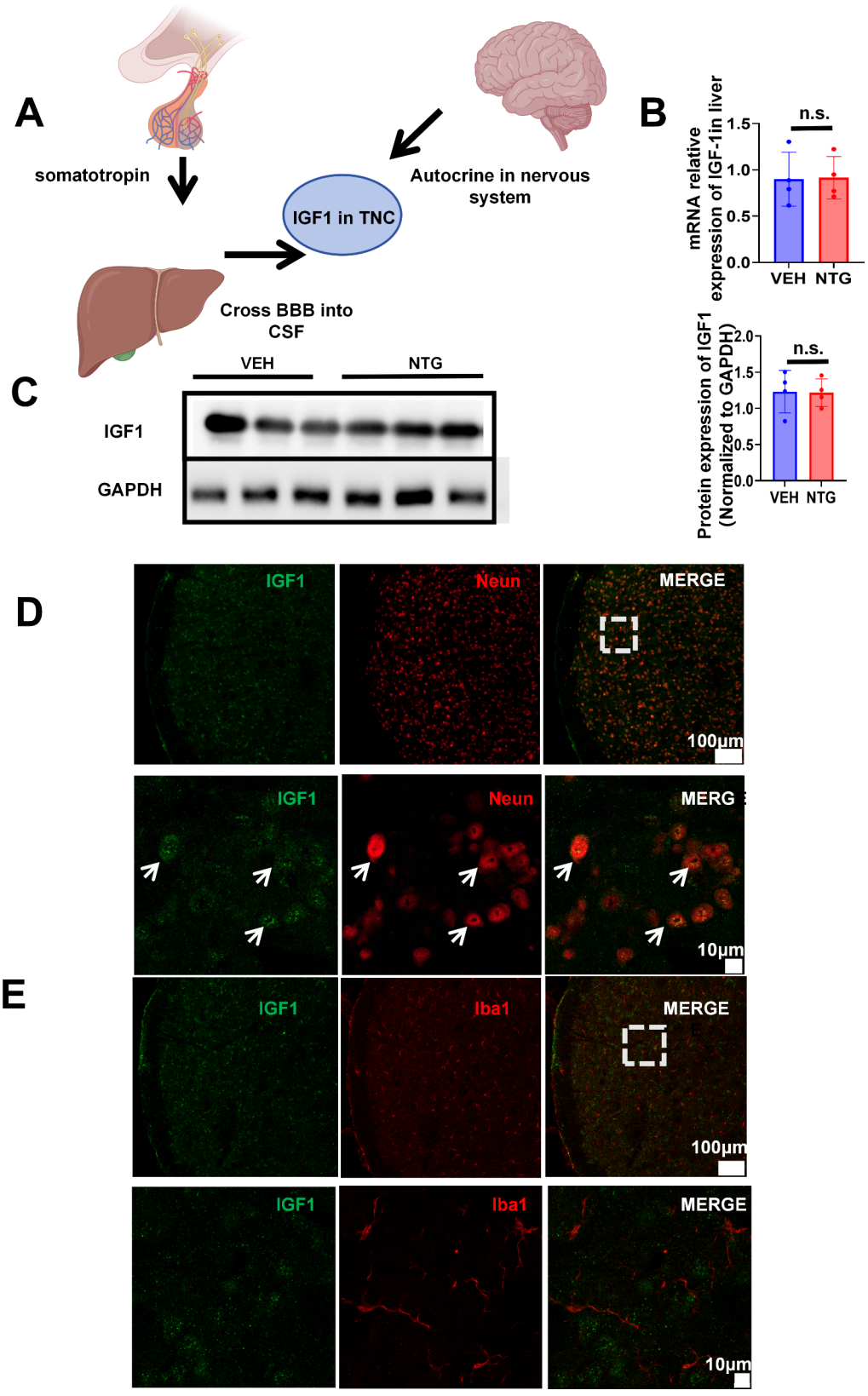

Fig. 3 (See legend on next page.)

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Fig. 3 In the mouse model of chronic migraine, the increase in IGF1 in the trigeminal nucleus caudalis (TNC) brain region primarily originates from autocrine secretion within neuronal cells, with no significant changes observed in IGF1 levels in the liver and blood. (**A**) Schematic representation of potential sources of IGF1 in the TNC brain region. (**B**) In the mouse model of chronic migraine, there is no significant change in IGF1 expression in the liver ($n = 4$ mice/group; compared to saline group, two-tailed independent samples t-test, n.s. $p > 0.05$). (**C**) Western blot representative image showing no significant difference in IGF1 expression in circulating blood of mice in the chronic migraine model ($n = 3$ mice/group; compared to saline group, two-tailed independent samples t-test, n.s. $p > 0.05$). (**D**) In the trigeminal nucleus caudalis (TNC) brain region of the mouse chronic migraine model, IGF1 predominantly co-localizes with neurons. Green represents IGF1, and red represents Neun (neuronal marker). (**E**) In the trigeminal nucleus caudalis (TNC) brain region of the mouse chronic migraine model, IGF1 shows no significant co-localization with microglia. Green represents IGF1, and red represents Iba1 (microglial marker)

IGF1 levels and the severity of pain; however, the specific mechanisms underlying this relationship remain unclear. IGF1 primarily exerts its effects through binding to the IGF1r [16]. Furthermore, IGF1r is a classical tyrosine kinase receptor whose activity is primarily regulated by phosphorylation [48]. Therefore, we measured the phosphorylation levels of IGF1r to quantify the activation status of the IGF1/IGF1r signaling pathway. Our results indicate that in the mouse CM model, there is an increase in IGF1r phosphorylation, suggesting overactivation of the IGF1/IGF1r signaling pathway. Upon activation of IGF1r, intracellular signaling cascades are initiated, including mTOR, which acts as one of its downstream effectors [17]. The mTOR signaling pathway plays a crucial role in regulating autophagy and serves as a major negative regulator of this process [49]. P62 is an endogenous autophagic substrate that recruits other proteins for autophagic degradation [50, 51]. Both overexpression of these proteins indicate exacerbation of autophagy inhibition. Our data demonstrate aggravated autophagic dysfunction in the CM model, consistent with previous reports [52]. Furthermore, the IGF1r inhibitor (ppp) can alleviate autophagic dysfunction in mice. Additionally, administration of ppp alleviated pain behaviors in mice. Our immunofluorescence results also demonstrate that phosphorylated IGF1r predominantly co-localizes with neurons.

Due to the administration of ppp primarily via intraperitoneal injection, although ppp can penetrate the blood-brain barrier [53]. However, we cannot exclude the possibility of effects on other pain-related regions. To investigate the relationship between elevated IGF1 levels and headache behaviors associated with autophagic dysfunction, we intravenously injected IGF1 into mice. Our results demonstrate that intravenous injection of IGF1 induced headache-like behaviors in mice, accompanied by increased phosphorylation of IGF in the TNC brain region and overactivation of autophagy-related pathways. RAPA is an autophagy inducer that can alleviate autophagic dysfunction [54]. Experimental results demonstrate that RAPA mitigated headache-like behaviors induced by IGF1. Because IGF1/IGF1r is closely linked to the mTOR pathway, and mTOR is a crucial negative regulator of autophagy, We hypothesize that IGF1 exacerbates autophagic dysfunction through the activation of IGF1r in the TNC brain region, leading to neuronal hyperactivation and the manifestation of headache behaviors. However, we cannot rule out the involvement of the peripheral nervous system in this process. Further exploration is required to elucidate the specific mechanisms involved.

Conclusions

Our study in a NTG-induced chronic migraine mouse model revealed several significant findings. Elevated expression of IGF1 in the TNC led to enhanced phosphorylation of IGF1r, which was primarily sourced from autocrine mechanisms within the nervous system. Intervention in the IGF/IGF1r signaling pathway through inhibition of IGF1 receptor phosphorylation alleviated pain behaviors and improved mTOR-related autophagic dysfunction. Additionally, intravenous injection of IGF1 induced headache-like behaviors in mice, associated with heightened activation of autophagy inhibition-related signaling pathways. These findings suggest that targeting the IGF/IGF1r signaling pathway could potentially alleviate autophagy suppression and reduce headache-like behaviors, highlighting its therapeutic relevance in chronic migraine management.

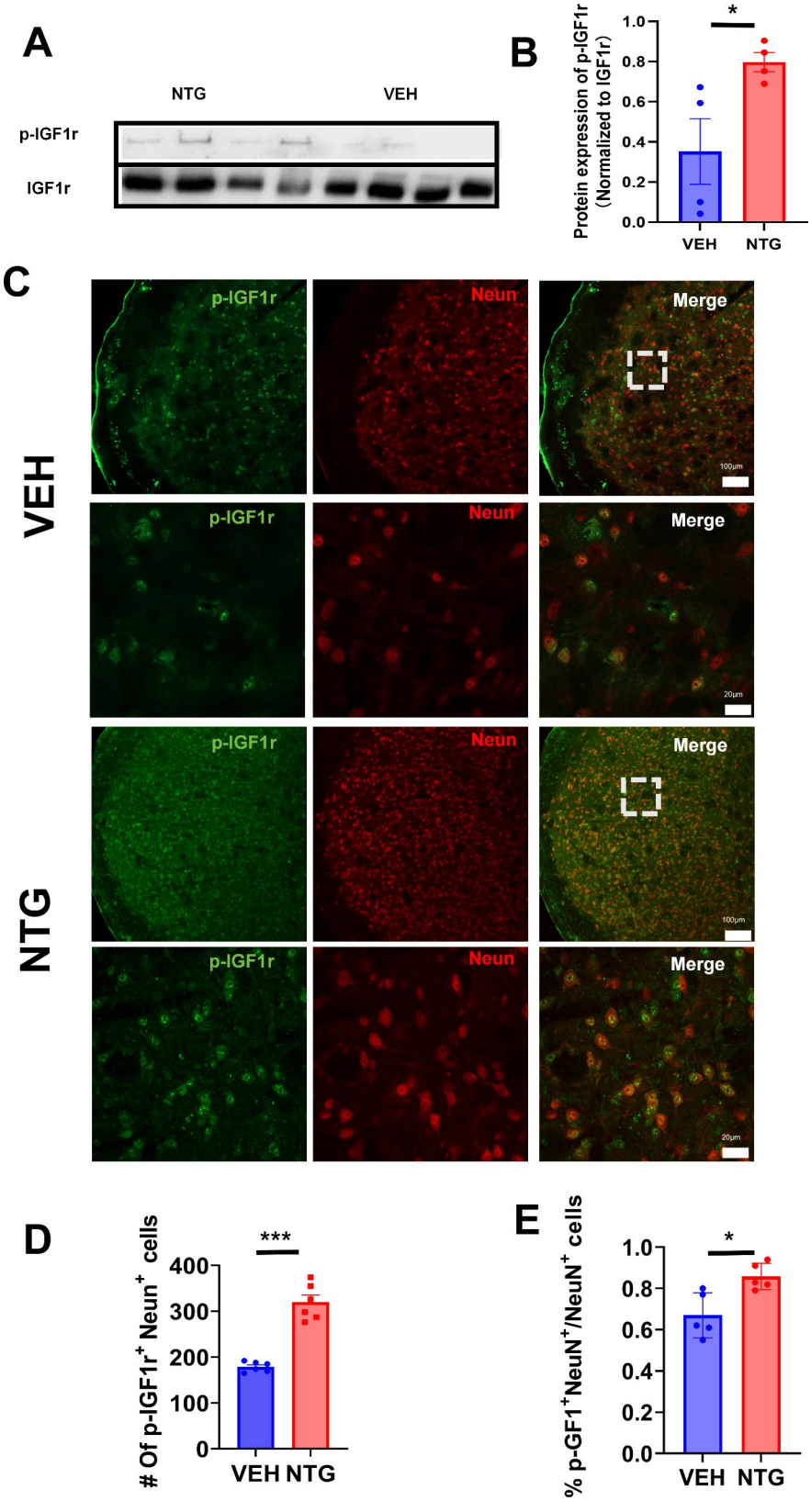

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Fig. 4 In the mouse model of chronic migraine, there is an increase in IGF1 receptor phosphorylation in the trigeminal nucleus caudalis (TNC) brain region. Moreover, phosphorylated IGF1r predominantly co-localizes with neurons. (**A**) Western blot representative image showing the phosphorylation levels of IGF1r in the TNC brain region of the mouse chronic migraine model. (**B**) Increased phosphorylation of IGF1r in the TNC brain region of mice induced by NTG in the chronic migraine model ($n=4$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p<0.05$, $^{**}P<0.01$). (**C**) Phosphorylated IGF1r primarily co-localizes with neurons in the mouse chronic migraine model. Green represents phosphorylated IGF1r, and red represents Neun (neuronal marker). (**D**) Increased number of neurons with phosphorylated IGF1r in the TNC brain region compared to the control group ($n=6$ mice/group; compared to saline group, two-tailed independent samples t-test, $^{*}p<0.05$, $^{**}P<0.01$). (**E**) In a chronic migraine model of mice, the proportion of neurons with phosphorylated IGF1 receptors in the TNC brain region significantly increased compared to the saline group ($n=5$ mice/group; two-tailed independent samples t-test, $^{*}P<0.05$, $^{**}P<0.01$)

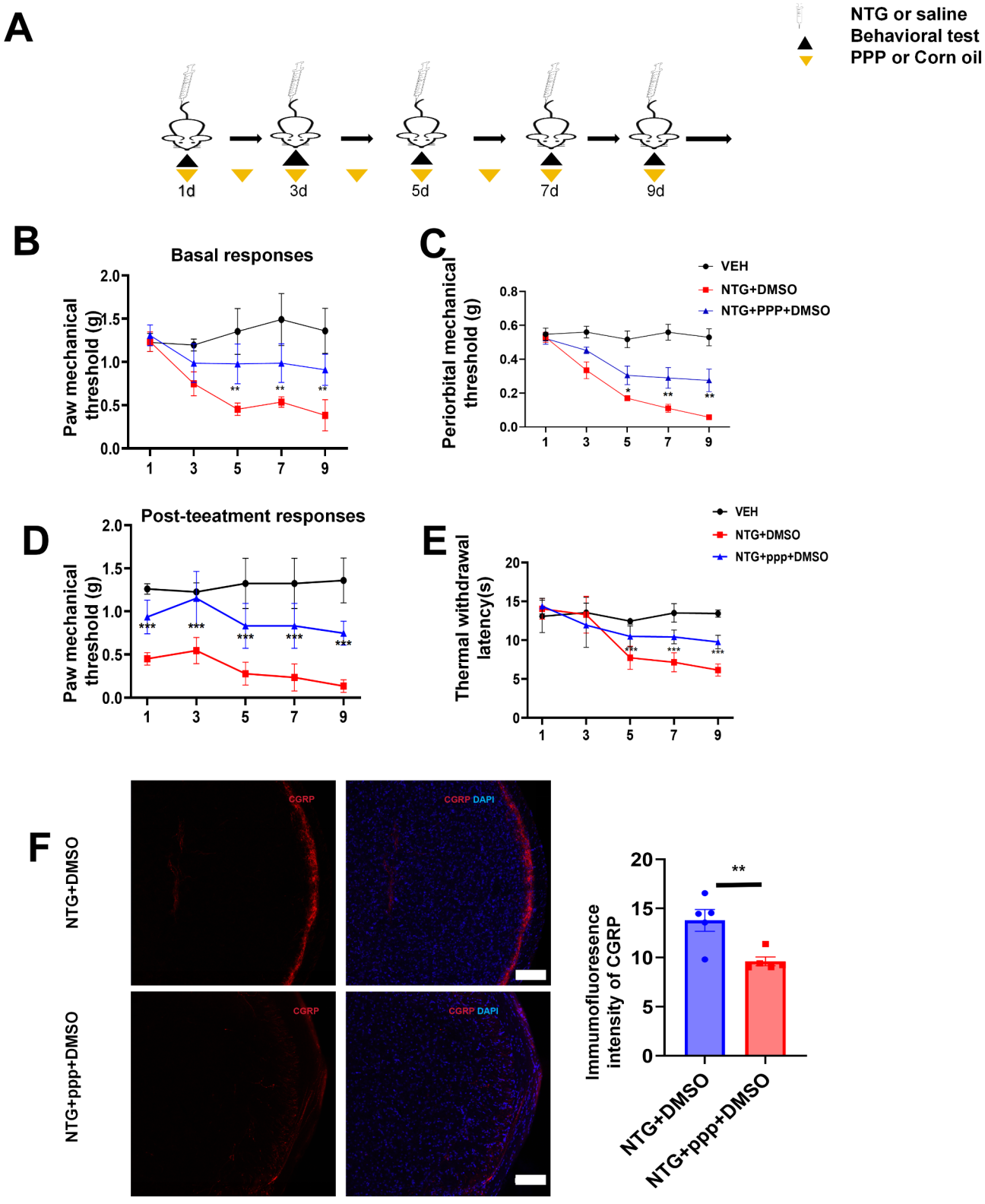

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Fig. 5 Blocking the IGF1/IGF1r pathway alleviates mechanical and thermal hyperalgesia and reduces CGRP expression in the TNC brain region in a mouse model of chronic migraine. (**A**) Experimental schematic diagram. In the NTG-induced chronic migraine mouse model, mice were intraperitoneally injected with the IGF1 receptor antagonist Picropodophyllin (ppp) at 40 mg/kg two hours before baseline behavioral tests. (**B**) The IGF1 receptor antagonist attenuated the decreased mechanical withdrawal threshold in the mouse plantar test ($n = 5$ mice/group; compared to the NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**C**) The IGF1 receptor antagonist attenuated the decreased mechanical withdrawal threshold around the orbit induced by nitroglycerin in mice ($n = 5$ mice/group; compared to the NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**D**) The IGF1 receptor antagonist attenuated the acute mechanical withdrawal threshold decrease in the mouse plantar test induced by nitroglycerin ($n = 5$ mice/group; compared to the NTG vehicle group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**E**) The IGF1 receptor antagonist attenuated the decreased thermal withdrawal latency induced by nitroglycerin in mice ($n = 5$ mice/group; compared to the NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**F**) The IGF1 receptor antagonist reduced CGRP expression in the TNC brain region induced by nitroglycerin in mice ($n = 5$ mice/group; compared to the NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$)

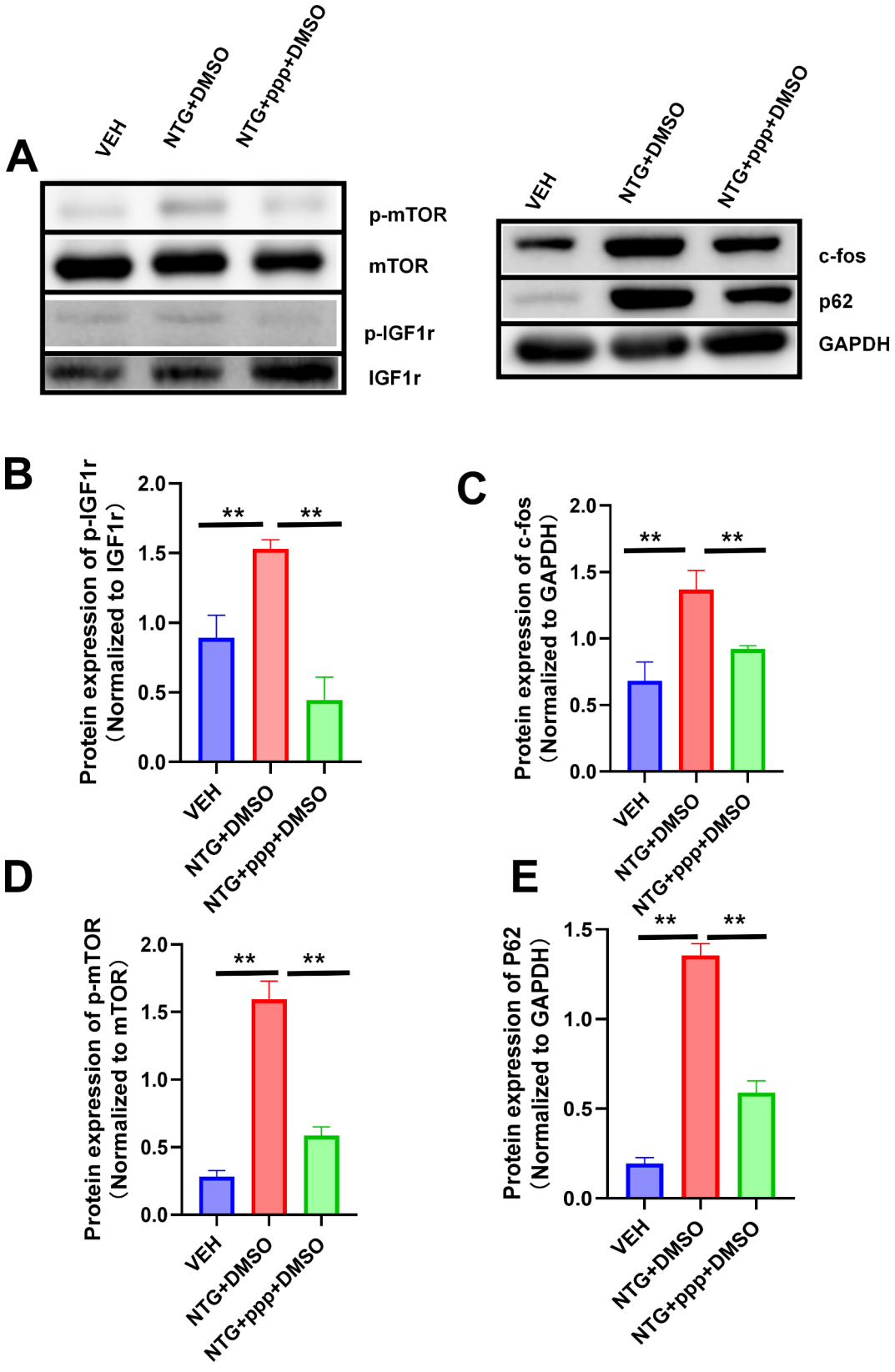

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Fig. 6 PPP inhibited the overactivation of the mTOR pathway in a chronic migraine mouse model and alleviated neuronal autophagy dysfunction. (**A**) Representative Western blots. (**B**) In the chronic migraine mouse model, phosphorylation of IGF1 receptor (IGF1r) is increased in the NTG group. PPP can inhibit this NTG-induced increase in IGF1r phosphorylation ($n = 3$ mice/group; compared to NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**C**) In the chronic migraine mouse model, expression of c-Fos is increased in the NTG group. Administration of PPP reduces c-Fos expression in the TNC ($n = 3$ mice/group; compared to NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**D**) In the chronic migraine mouse model, phosphorylation of mTOR is increased in the NTG group. PPP can inhibit this NTG-induced increase in mTOR phosphorylation ($n = 3$ mice/group; compared to NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (**E**) In the chronic migraine mouse model, expression of p62 is increased in the NTG group. PPP can inhibit p62 expression in the NTG model ($n = 3$ mice/group; compared to NTG group, two-tailed independent samples t-test, $^{*}p < 0.05$, $^{**}p < 0.01$)

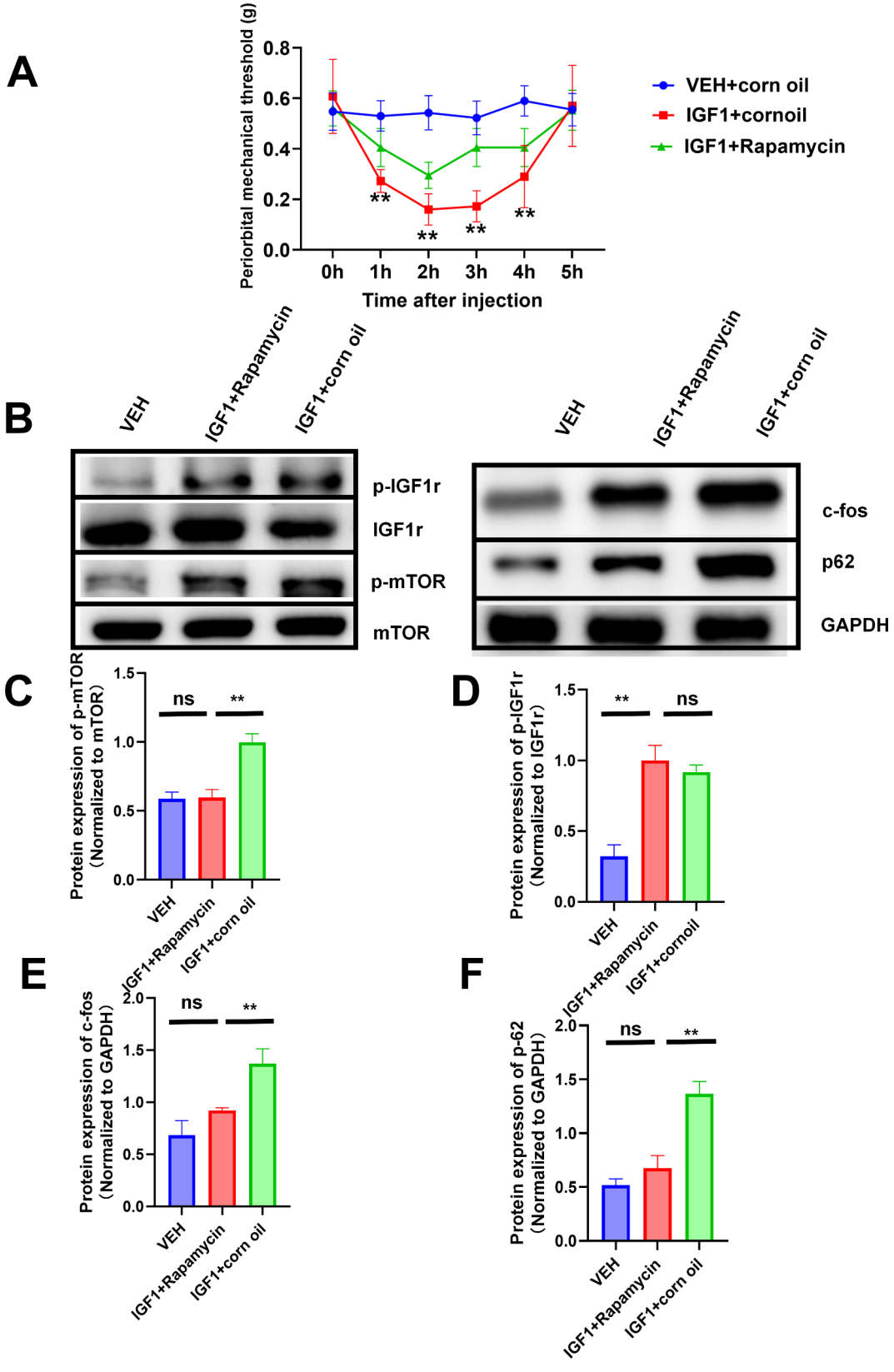

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Fig. 7 Tail vein injection of IGF1 in mice lowers the facial mechanical withdrawal threshold. An mTOR inhibitor alleviates pain behaviors and ameliorates neuronal autophagy dysfunction. (**A**) Tail vein injection of IGF1 causes a decrease in the facial mechanical withdrawal threshold in mice, and the autophagy inducer rapamycin partially restores the facial mechanical withdrawal threshold in mice. (**B**) Representative Western blots. (**C**) Administration of IGF1 increases phosphorylation of mTOR in the TNC of mice ($n=3$ mice/group; compared to NTG group, compared to saline group, two-tailed independent samples t-test, $^{*}p<0.05$, $^{**}p<0.01$). (**D**) Administration of IGF1 increases phosphorylation of IGF1 receptor (IGF1r) in the TNC of mice, and rapamycin administration does not affect IGF1 receptor phosphorylation ($n=3$ mice/group; compared to IGF1 group, compared to saline group, two-tailed independent samples t-test, $^{*}p<0.05$, $^{**}p<0.01$). (**E**) Administration of IGF1 increases c-Fos expression in the TNC of mice, and rapamycin reduces the increase in c-Fos induced by IGF1 ($n=3$ mice/group; compared to NTG group, compared to IGF1 group, two-tailed independent samples t-test, $^{*}p<0.05$, $^{**}p<0.01$). (**F**) Administration of IGF1 increases p62 expression in the TNC of mice, and rapamycin reduces the increase in p62 induced by IGF1 ($n=3$ mice/group; compared to NTG group, compared to IGF1 group, two-tailed independent samples t-test, $^{*}p<0.05$, $^{**}p<0.01$)

Abbreviations

CM	Chronic migraine
ppp	Picropodophyllin
CGRP	Calcitonin gene-related peptide
TNC	Trigeminal nucleus caudalis
CNS	Central nervous system
IGF1	Insulin-like growth factor 1
IGF1r	IGF1 receptor
PI3K-Akt	Phosphatidylinositol 3-kinase-protein kinase B
MAPK	Mitogen-activated protein kinase
RAPA	Rapamycin
GH	Growth hormone
PNI	Peripheral nerve injury
CIPN	Chemotherapy-induced peripheral neuropathy

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Author contributions
This study was designed by YGW, TXW. TXW and ZCL participated in behavioral testing and immunofluorescence staining. KBZ and JGG performed the statistical analysis. TXW, ZCL, and YHX write the manuscript. JSG, KBZ, revised the manuscript. WTX and YGW provided supervision and a final check. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval
All animal experiments performed in this study were approved by the Animal Ethics Committee of ShanghaiTech University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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