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Research article

Spinal RAMP1-mediated neuropathic pain sensitisation in the aged mice through the modulation of CGRP-CRLR pain signalling

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ABSTRACT

Pain sensitivity varies depending on both the state and age of an individual. For example, chronic pain is more common in older individuals, but the underlying mechanisms remain unknown. This study revealed that 18-month-old mice (aged) experienced more severe and long-lasting allodynia and hyperalgesia in the chronic constriction injury (CCI)-induced pain state compared to 2-month-old mice. Interestingly, the aged mice had a higher baseline mechanical pain threshold than the adult mice. The expression of spinal receptor-active modification protein 1 (RAMP1), as a key component and regulator of the calcitonin gene-related peptide (CGRP) receptor for nociceptive transmission from the periphery to the spinal cord, was reduced in the physiological state but significantly increased after CCI in the aged mice compared to the adult mice. Moreover, when RAMP1 was knocked down using shRNA, the pain sensitivity of adult mice decreased significantly, and CCI-induced allodynia in aged mice was reduced. These findings suggest that spinal RAMP1 is involved in regulating pain sensitivity in a state- and age-dependent manner. Additionally, interfering with RAMP1 could be a promising strategy for alleviating chronic pain in older individuals.

1. Introduction

Chronic pain affects 20%–45 % of the population [1] and significantly impacts quality of life, affects medical resource

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consumption, and leads to various secondary social problems [2–6]. The incidence of chronic pain increases with age, and the high prevalence of chronic pain among older individuals is recognised as an epidemiological feature [7–10]. Recent studies have shown that older people have a higher physiological pain threshold compared to young people [11–14], suggesting that pain characteristics may differ between age groups in both physiological and neuropathic conditions. However, there is limited research on the different pain characteristics of older and younger people. Therefore, there is an urgent need to conduct in-depth research to explore the underlying mechanism of age-related changes in pain threshold.

The spinal dorsal horn (SDH) is the first site where pain information is transferred from the periphery to the brain and is considered a key site for the modulation of chronic pain [15–19]. Sensory neurons in the SDH of older individuals exhibit extensive degenerative changes [20], particularly in lamina I [21,22]. However, pain-related changes in the SDH have been rarely studied in the context of ageing.

Calcitonin gene-related peptide (CGRP) is a widely recognised neuroactive substance that is closely associated with the transmission of nociceptive information. Its primary afferent fibres and terminals mainly terminate in laminae I–II of the SDH [23–25]. The CGRP receptors include the calcitonin receptor-like receptor (CRLR), G Protein-Coupled Receptors (GPCR), and receptor-active modification protein 1 (RAMP1). RAMP1 is a single transmembrane protein that determines ligand specificity and ensures that CRLR targets the cell surface. Anxiety triggers the release of CGRP from the ends of primary sensory neurons in the dorsal horn and peripheral tissues of the spinal cord. Subsequently, CGRP activates CRLR/RAMP1 in spinal cord neurons to induce nociception [26]. However, there is a lack of research on changes in the CGRP and CRLR/RAMP1 nociception signalling systems in the context of ageing.

This study aimed to investigate the physiological and pathological pain mechanisms in ageing animals using various methods of pain measurement, with a specific focus on changes in the pain transmission signal system involving CGRP and CRLR/RAMP1 in aged mice.

2. Methods

2.1. Animals

This study included adult (8-week-old; 20–25 g) and aged (18-month-old; 30–35 g) male C57/BL6 mice. The mice were purchased from the Shanghai Laboratory Animal Research Center and were housed in an animal facility. They were given ad libitum access to water and standard laboratory food pellets. Prior to the experiments, the mice (n = 186) were habituated to their environment (maintained at 22–24 °C, with 50%–60 % relative humidity, and a 12-h light/dark cycle) for 7 days.

All protocols were approved by the Animal Care and Use Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). The procedures were conducted in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, NIH Publication No. 86–23, revised 1985) and the policies of the International Association for the Study of Pain regarding the use of laboratory animals. Efforts were made to minimise suffering due to surgery and to reduce the overall number of animals.

2.2. Chronic pain model (chronic constriction injury of the sciatic nerve)

The mice were anaesthetised with pentobarbital sodium (50 mg/kg body weight) administered intraperitoneally. An incision was made parallel to the sciatic nerve, and the left sciatic nerve was exposed. Four 5-0° loose intestinal ligations were then performed around the nerves, with a 1-mm interval between each ligation. The muscles and skin were sutured after the procedure. For the sham surgery, the sciatic nerve was exposed without any ligation. Local analgesia was provided using lidocaine gel, and a bacitracin ointment was applied to prevent postoperative infection.

2.3. Preparation of the shRNA-(RAMP1) adeno-associated virus (AAV)

The viral solution used in this study was rAAV-U6-shRNA-(RAMP1)-CMV-EGFP-SV40 pA (AAV2/9, 5E+12 vector genomes/mL) from BrainVTA, Wuhan, China. The viral vector was rAAV-U6-shRNA-CMV-EGFP [27]. The shRNA was driven by the U6 promoter, and the EGFP was driven by the CMV promoter. AAV-U6-shRNA(scramble)-CMV-EGFP-SV40 pA was used as a negative control to eliminate the influence of other interfering factors.

2.4. SDH microinjection

To knockdown RAMP1 expression in the L4/5 SDH, we performed direct SDH microinjection using recombinant adeno-associated virus 2/9 (AAV2/9) and RAMP1 shRNA. The mice were anaesthetised with isoflurane, and oxygen was delivered through a nasal catheter at a rate of 0.8–1.0L/min. After anaesthesia, the mice were shaved with an animal razor for skin preparation. The surface of the mouse's back skin was disinfected with iodophor, and a 2–3 cm incision in the middle of the lower back was made to expose the lumbar spine. The spine was fixed to the spinal cord adapter to prevent its movement while the mouse was breathing. The surrounding muscles were separated and removed to expose the L4 and L5 SDH as needed. After SDH exposure, a Hamilton syringe was used to inject the viral solution (5e+12 v.g./mL, 200 nL at each injection point) into the left L4 and L5 SDH. After the injection, the syringe was maintained for another 10 min and the skin incision was sutured.

The mice injected with the virus showed no signs of paralysis or other abnormalities, suggesting a minimal immune response to

virus injection. To confirm the effectiveness of the positive and control AAVs in infecting the SDH, we examined frozen sections of the left L4–6 SDH to detect the abundance of green fluorescence (EGFP) after behavioural testing. We used the SDH on the contralateral side to exclude nonspecific emissions and interpret the background fluorescence (Fig. 5C). Each group included 5–10 mice.

2.5. Western blot

Western blot analysis was performed as previously described, with slight modifications [28,29]. The left L4–6 SDH was collected, flash-frozen, and homogenized in cooled sodium dodecyl sulphate lysis buffer (P0013G, Beyotime). The crude homogenate was centrifuged at 4 °C for 15 min at 12,000×*g*, and the supernatant was collected. The protein concentration was measured, and the samples were heated to 100 °C for 15 min and electrophoresed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were then transferred onto polyvinylidene difluoride membranes (IPVH00010, Immobilon-P). The membranes were blocked with 1 % bovine serum albumin at 4 °C overnight and then incubated with anti-RAMP1 antibody (1:2000, ab156575, Abcam, Cambridge, UK), rabbit polyclonal CRLR antibody (1:2000, NLS6731, Novus Biologicals, Cambridge, UK), HRP anti-beta actin antibody (1:2000, ab49900, Abcam), and anti-beta tubulin antibody (1:2000, ab6046, Abcam) at 4 °C overnight under gentle agitation. The membranes were washed and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000, ab7090, Abcam) for 1 h at room temperature. The blots were detected using the ChemiDoc XRS + System (Bio-Rad, Hercules, CA, USA), and the band density was measured using Image J software. The experiment was repeated more than twice with a sample size greater than five each time.

2.6. Immunofluorescence

For immunofluorescence, samples were perfused with phosphate-buffered saline (PBS) and 4 % paraformaldehyde (PFA), and then fixed in 4 % PFA overnight. The spinal cord was stored in a 20 % sucrose solution overnight at 4 °C and then transferred to a 30 % sucrose solution until it sank to the bottom. The dehydrated tissues were embedded in an optimal cutting temperature compound, and 20-μm-thick frozen sections were washed with PBS. Subsequently, the sections were treated with immunofluorescence staining blocking buffer (P0102, Beyotime) for 1 h at room temperature. The primary antibodies used were as follows: rabbit anti-RAMP1 antibody (1:2000, ab156575, Abcam); mouse anti-CGRP antibody (1:1000, ab81887, Abcam); mouse anti-Gfap antibody (1:1000, ab81887, Abcam); mouse anti-Iba1 antibody (1:1000, ab283319, Abcam); rabbit polyclonal CRLR antibody (1:1000, NBP1-59073, Novus Biologicals); FITC-conjugated isolectin B4 antibody (1:500, L2140-1 MG, Sigma-Aldrich, St Louis, MO, USA); and rabbit anti-c-Fos antibody (1:500, ab222699, Abcam). Most primary antibodies were stained at 4 °C overnight except for c-Fos, which required longer staining time of 72 h in a 4 °C. To prevent antibody volatilisation, another c-Fos primary antibody was added at the 36th h of incubation. All sections were incubated with anti-mouse immunoglobulin G (IgG) conjugated to Alexa Fluor® 488 (1:1000, 4408, Cell Signaling Technology) or anti-rabbit IgG conjugated to Alexa Fluor® 594 (1:1000, 8889, Cell Signaling Technology). The conjugated antibodies were also used for nuclear staining with DAPI. Images were captured with a confocal microscope (Olympus, FV1200) and processed using Image J software. Each treatment group included 3–4 mice. The experiment was repeated more than three times, with a sample size of more than three for each group.

2.7. Von frey test

The paw withdrawal thresholds were evaluated using the von Frey test using a series of calibrated hairs ranging from 0.02 g to 2.0 g (Touch Test® Sensory Evaluators, North Coast). Before the experimental operation, the mice were allowed to adapt to the housing facilities and were treated daily for at least 3 days. The formal test was performed on the central area of the plantar surface of the left hind paw (0.02, 0, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g). Each hair sample was combed five times at intervals of 5 s, and three of the five consecutive hair plucks were considered responsive.

2.8. Laser heat pain (thermal stimulation)

Each mouse was habituated in a small plastic cage (7.5 × 15 × 15 cm) with a vent on a glass plate at the top for 30 min. Laser heating (Hargreaves Model 390, IITC Life Science) was used to heat the middle of the plantar surface of each hind paw by passing a beam of light through a hole in the light box and glass plate. The light beam was turned off when the mice lifted their feet, and the time (latency) from stimulation to foot withdrawal was measured. This experiment was repeated every 10 min, and the average value from three experiments was obtained. A cutoff time of 20 s was applied to avoid tissue damage.

2.9. Capsaicin pain model

Each mouse was habituated for 3 days, spending 30 min per day in a 50 mL centrifuge tube and a small plastic cage (7.5 × 15 × 15 cm) with air vents on the top of the glass plate and centrifuge tube. The mice were considered well adapted when they exhibited a quiet state. Each mouse was then placed head-first into the centrifuge tube, and the left hind foot was gently pulled out. Subcutaneous injection of 20 μL of 0.1 % capsaicin was administered to the foot sole. The mice were subsequently placed in a transparent plastic cage, and their behaviour was video-recorded 30 min after the injection using a P50 Pocket camera (HAIWEI). The duration of palm-licking after the injection was recorded. Each behavioural experiment was repeated at least twice, with a sample size of more than five mice

per group.

For c-Fos immunohistochemistry experiments, mice were placed in mouse cages after the injection of 20 μL of 0.1 % capsaicin on the soles of their feet. After 30 min, the mice were injected again following the same protocol. A total of four injections were administered, each lasting for 2 h. Thereafter, the spinal cords were collected as described in section 2.6.

2.10. Intrathecal injection

Mice were anaesthetised with sevoflurane until the righting reflex disappeared. The microsyringe needle was carefully inserted between the grooves of the L4 and L5 vertebral bodies, and the presence of the tail bullet indicated successful entry into the intradural space. Once a tail flick was observed, 0.1 mg/kg of α-CGRP (C0292, Sigma Aldrich) was injected.

2.11. Statistical analysis

Data are presented as mean ± standard error of the mean. Comparisons between two groups and among three or more groups were performed using the Student's t-test and one-way analysis of variance, respectively. All statistical analyses were conducted using GraphPad Prism 8.0. The test level was set at $\alpha = 0.05$, and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Differences in pain response between the aged and adult mice

We used various pain models and measures to differentiate between aged and adult mice and to understand the impact of aging on pain. The results showed that aged mice had a higher mechanical pain threshold and longer thermal pain latency (Fig. 1A–B). In contrast, adult mice exhibited more sensitive nociception after a subcutaneous injection of 0.1 % capsaicin (Fig. 1C).

The thermal paw withdrawal latency test revealed significant differences in the effects of capsaicin injection between the capsaicin-injected and non-injected paws of adult mice at both 20 min and 40 min (Fig. 1D). While the mechanical pain levels of adult mice returned to baseline at 10 weeks after CCI, allodynia persisted much longer in aged mice, and their mechanical pain threshold did not fully recover to baseline within our observation period (Fig. 1E). Additionally, latency values at 6 weeks after CCI were significantly shorter in aged mice compared to their preoperative values, whereas no significant difference was observed between preoperative and postoperative values in adult mice (Fig. 1F). Immunofluorescence analysis using c-Fos showed greater activation in adult mice experiencing capsaicin induced acute pain (Fig. 1G–H), whereas in aged mice, greater activation was observed in the chronic pain state, specifically at 6 weeks after CCI (Fig. 1I–J). These findings support the idea that aged mice exhibit insensitivity to acute pain stimuli and experience persistent allodynia in chronic pain.

3.2. CGRP-induced allodynia in adult mice

CGRP participates in nociceptive information transmission from the periphery to the spinal cord. Under physiological conditions, intrathecal injection of 0.1 mg/kg α-CGRP reduced the mechanical pain threshold in adult mice but had no effect on aged mice (Fig. 2A). In the chronic pain state, both adult and aged mice showed exacerbated mechanical allodynia, with a further decrease in threshold (Fig. 2B). Immunofluorescence analysis revealed that nerve fibre terminals containing CGRP primarily terminated in laminae I, II, and V of the SDH, and no significant difference in CGRP expression was observed between aged and adult mice (Fig. 2C). Analysis of CGRP levels in plasma and the SDH preoperatively and postoperatively in mice with CCI showed that CGRP increased only at 2 weeks postoperatively in adult mice, while in aged mice, CGRP increased in the SDH but not in plasma at 6 weeks postoperatively (Fig. 2D–E). Therefore, we hypothesised that CGRP contributes to allodynia in the aged group.

3.3. Age-dependent changes of RAMP1 expression in chronic pain models

As previously mentioned, CGRP receptors consist of CRLR and RAMP1. Immunofluorescence analysis revealed that neuronal cells in the SDH highly expressed CRLR and RAMP1. Interestingly, the expression of RAMP1 was significantly reduced in aged mice, while CRLR expression did not show significant changes (Fig. 3A–B). To analyse these findings further, four mice were selected from each group, and ten 20-μm-thick slices were collected from each mouse. The fluorescence intensities of CRLR and RAMP1 were measured, averaged, and compared between the two groups in the left SDH. Two sets of immunofluorescence images were obtained under the same conditions for comparison, including the material, staining, and confocal development. The voltage, laser intensity, and offset used for confocal imaging were consistent between the groups. Additionally, it was found through immunofluorescence staining and subsequent microscopic analysis that RAMP1 did not co-localize with markers for microglia (IBA-1) and astrocytes (GFAP) (Fig. 3C). Moreover, western blotting results showed a gradual decrease in RAMP1 expression in the SDH with age (Fig. 3D–E)(Supplementary Figs. 1–3).

Moving on to the expression in the dorsal horn, RAMP1 expression was significantly lower in 18-month-old mice compared to 2-month-old mice, while CRLR expression did not show significant differences (Fig. 4A–C)(Supplementary Figs. 4–5). In adult mice, RAMP1 expression in the SDH significantly increased at 2 weeks after CCI, returning to normal levels 6 weeks after CCI. Conversely, CRLR expression did not show significant changes (Fig. 4D–F)(Supplementary Figs. 6–7). In aged mice, RAMP1 expression in the SDH

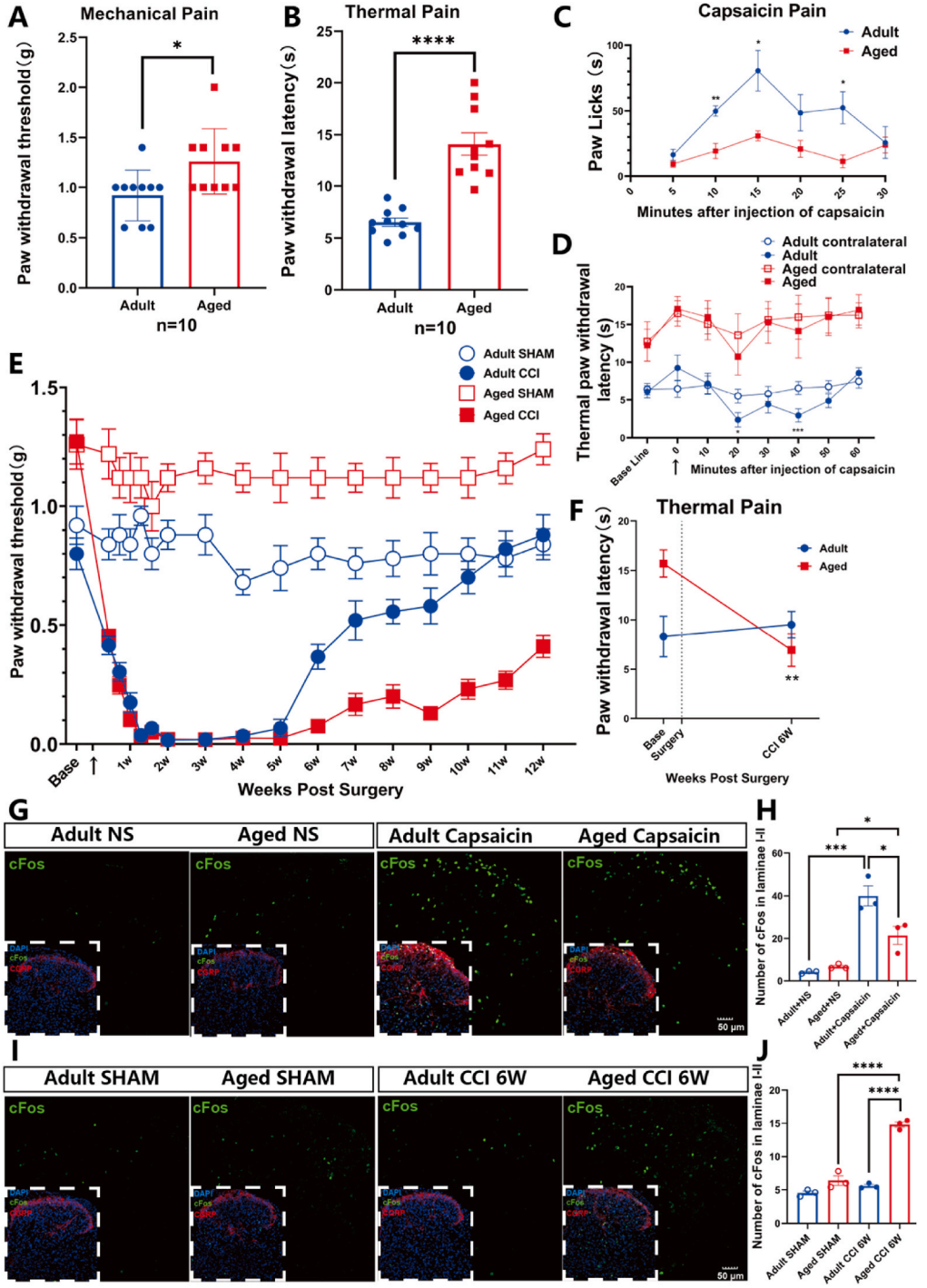

Fig. 1. Aged mice responded to pain stimuli differently from adult mice. A. The mechanical pain threshold was higher in aged mice than in adult mice ($p = 0.0181$, $n = 10$). B. Thermal pain incubation period in aged mice was longer than that in adult mice ($p < 0.0001$, $n = 10$). C. Adult mice were more sensitive to lower-dose capsaicin than aged mice in physiological conditions (10 min: $p = 0.0026$,15 min: $p = 0.0144$,25 min: $p = 0.0142$, $n = 5$). D. Dynamics of thermal paw withdrawal latency after injection of capsaicin. Significant differences were observed between capsaicin and contralateral (non-injected) paws in adult mice at both 20 min and 40 min (20 min: $p = 0.0313$, 40 min: $p = 0.0005$, $n = 5$). The bold arrow indicates the time of injection of capsaicin. E. The trend of mechanical pain threshold in the CCI model. Aged mice showed difficult abnormal noalgesia and noalgesia after surgery. Adult mice recovered at 4 weeks and 10 weeks after surgery ($n = 10$). The bold arrow indicates the time of CCI surgery. F. Aged mice showed a shorter latency of heat pain at 6 weeks after CCI ($p = 0.0076$), whereas adult mice showed no difference ($n = 10$). G–H. Adult mice were more sensitive to acute pain stimuli and had a higher cFos activation in the spinal cord dorsal horn after plantar injection of capsaicin compared to aged mice (Adult NS vs. Capsaicin: $p = 0.0002$, Aged NS vs. Capsaicin: $p = 0.0449$, Aged Capsaicin vs. Adult Capsaicin: $p =$

0.0142, n = 3). I–J. Aged mice were more sensitive to chronic pain stimuli and had higher cFos activation in the SDH than adult mice at 6 weeks after CCI (Aged SHAM vs. CCI 6W: $p < 0.0001$, Adult CCI 6W vs. Aged CCI 6W: $p < 0.0001$, n = 3).
CCI, chronic constriction injury; SDH, spinal dorsal horn.

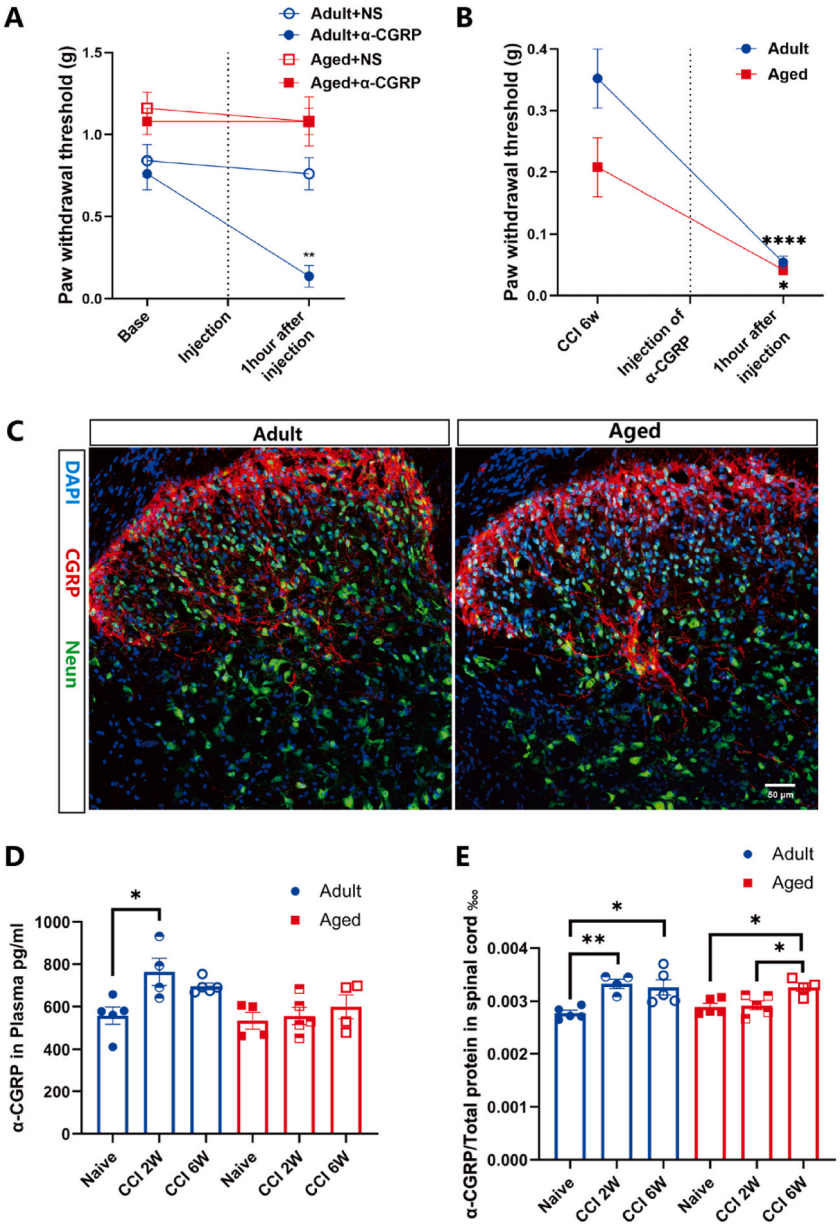

Fig. 2. Responses of aged and adult mice to CGRP in physiological and chronic pain states. A. In the physiological state, 18-month-old aged mice responded differently to intrathecal α-CGRP compared to 2-month-old adult mice that showed abnormal nociception after intrathecal α-CGRP and showed no difference between intrathecal α-CGRP and saline ($p = 0.0021$, n = 5). B. Both aged and adult mice treated at 6 weeks in the CCI model showed abnormal nociception after intrathecal injection of α-CGRP in a chronic pain state (Adult: $p < 0.0001$; Aged: $p = 0.0174$, n = 5) C. The distribution of CGRP in SDH, mainly in lamina I-II, showed no difference between adult and aged mice D. The amount of α-CGRP in the blood, measured by ELISA, for adult and aged mice in the physiological state and after CCI at 2 weeks and 6 weeks after CCI ($p = 0.03$, n = 4–5). E. The amount of α-CGRP in the SDH, measured by ELISA, of adult and aged mice in the physiological state and after CCI at 2 weeks and 6 weeks after CCI (Adult Naive vs CCI 2W: $p = 0.0083$; Adult Naive vs. CCI 6W: $p = 0.0129$; Aged Naive vs. CCI 6W: $p = 0.0209$; Aged CCI 2W vs. CCI 6W: $p = 0.0301$, n = 4–5).
CCI, chronic constriction injury; CGRP, calcitonin gene-related peptide; ELISA, enzyme-linked immunosorbent assay; SDH, spinal dorsal horn.

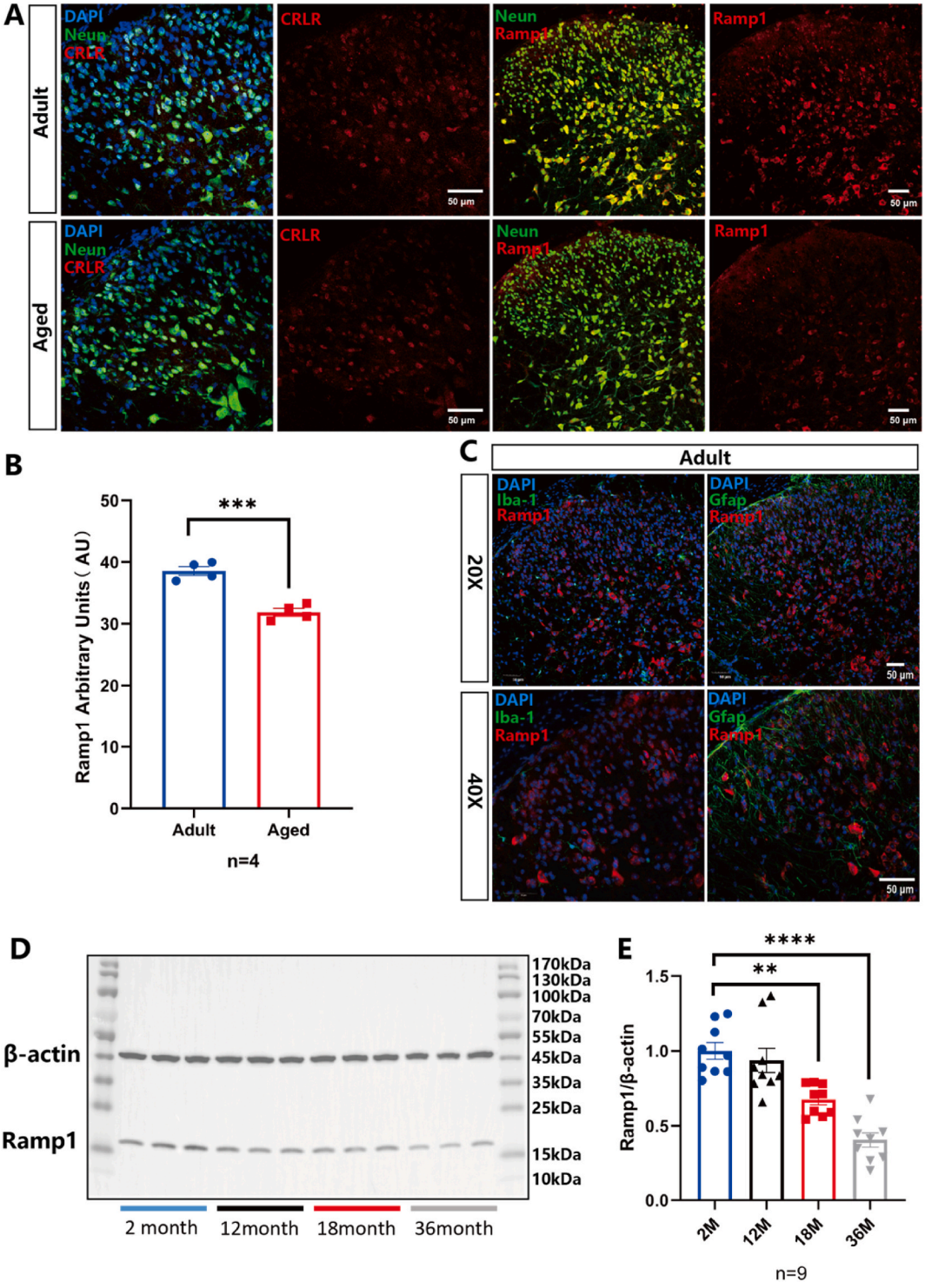

Fig. 3. RAMP1 expression in aged mice is different from that in adult mice in the physiological state. A. The expression of CRLR and RAMP1 in the SDH was co-labelled with the Marker (Neun) of neuronal cells. Aged mice expressed significantly lower RAMP1 than adult mice. B. Immunofluorescence intensity statistics of CRLR and RAMP1 in Fig. 3A. RAMP1 expression in the SDH of aged mice was significantly lower than that of adult mice ($p = 0.0004$, $n = 4$). C. The expression of RAMP1 in the SDH is rarely co-labelled with the astrocyte (Gfap) and microglial (Iba-1) markers. D–E. The protein expression levels of RAMP1 in the SDH decreased with age (2M vs. 18M: $p = 0.002$; 2M vs. 36M: $p < 0.001$, $n = 9$).
CRLR, calcitonin receptor-like receptor; RAMP1, receptor-active modification protein 1; SDH, spinal dorsal horn.

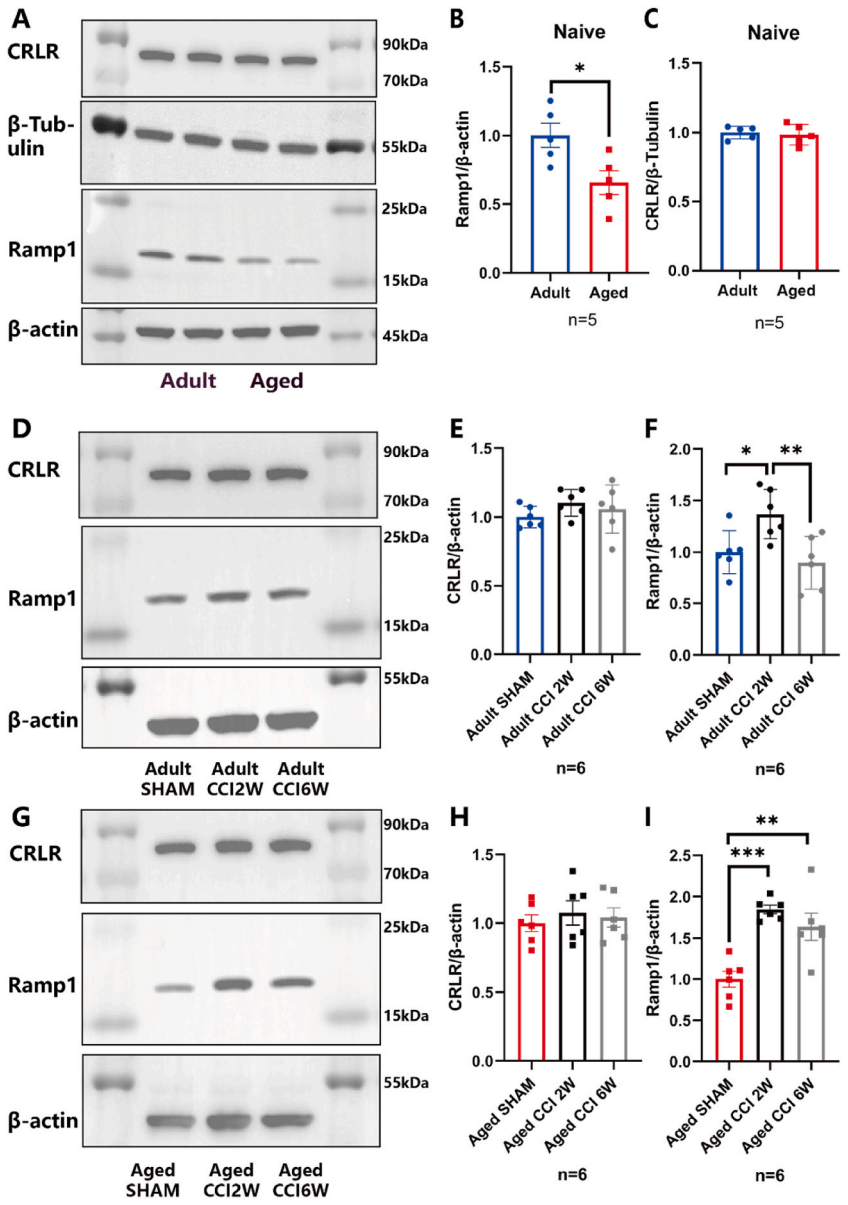

Fig. 4. Changes in RAMP1 expression in aged and adult mice under chronic pain conditions. A–C. RAMP1 expression was lower in aged than in adult mice, and no difference was observed in CRLR expression ($p = 0.0244$, $n = 5$). D–F. RAMP1 expression was upregulated at 2 weeks (Adult SHAM vs. Adult CCI 2W: $p = 0.0403$; Adult CCI 2W vs. Adult CCI 6W: $p = 0.0091$, $n = 6$) and was not different from the physiological state at 6 weeks after surgery G–I. RAMP1 expression was upregulated at both 2 and 6 weeks after CCI (Aged SHAM vs. Aged CCI 2W: $p = 0.0003$; Aged SHAM vs. Aged CCI 6W: $p = 0.0036$, $n = 6$)
CCI, chronic constriction injury; CRLR, calcitonin receptor-like receptor; RAMP1, receptor-active modification protein 1.

consistently increased after CCI, remaining significantly higher at 6 weeks postoperatively compared to the preoperative physiological state. However, CRLR expression did not show significant changes (Fig. 4G–I)(Supplementary Figs. 8–9). Based on these results, the age-related decrease in RAMP1 expression in the SDH seems to be an important factor contributing to the insensitivity to acute pain in aged mice. Furthermore, the persistent increase in RAMP1 expression in the SDH may explain the persistence of allodynia after CCI.

3.4. AAV-induced reduction of RAMP1 expression and corresponding pain behavioural changes

AAV application successfully downregulated RAMP1 expression in the SDH, as shown in Fig. 5A and B (Supplementary Figs. 10–11). However, CRLR expression was not affected. On the AAV-injected side of the SDH, the intensity of the green fluorescence was enhanced, indicating a significant knockdown of RAMP1 expression. In contrast, no green or red fluorescence indicating RAMP1

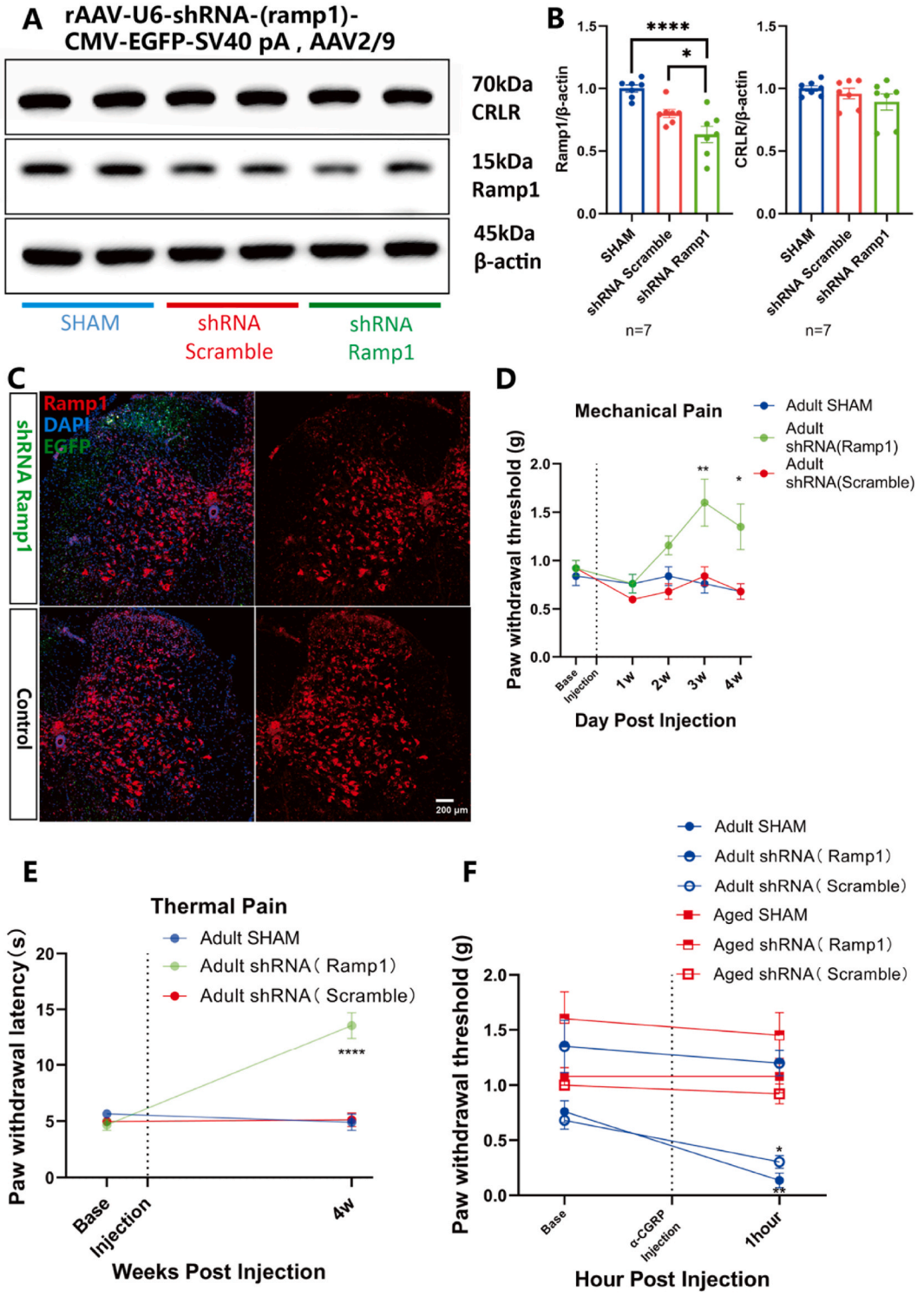

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Fig. 5. Changes in pain threshold after RAMP1 knockdown in mice under a physiological state. A–B. Four weeks after injecting AAV into the L4–6 SDH of adult mice, RAMP1 was downregulated in the SDH (SHAM vs. shRNA RAMP1: $p < 0.0001$; shRNA Scramble vs. shRNA RAMP1: $p = 0.0458$, and there was no difference in CRLR expression, $n = 7$) C. Immunofluorescent expression of RAMP1 was reduced in the L4–6 SDH of adult mice after a 4-week injection of AAV. Viral expression was concentrated in the dorsal angle range of the spinal cord. D. Mechanical pain thresholds increased in the RAMP1 knockdown group than in the negative control and sham groups at 3 weeks (8w RAMP1 vs. 8w SHAM: $p = 0.0095$) and 4 weeks (8w RAMP1 vs. 8w SHAM: $p = 0.0163$; 8w RAMP1 vs. 8w scramble: $p = 0.0072$) ($n = 5$). E. Thermal pain thresholds increased in the RAMP1 knockdown group than in the negative control and sham groups at 4 weeks ($p < 0.0001$, $n = 5$). F. No nociception occurred after 4 weeks of intrathecal injection of α-CGRP in the RAMP1 knockdown group adult mice (Adult SHAM: $p = 0.0013$; Adult shRNA-scramble: $p = 0.0396$, $n = 5$)
AAV, adeno-associated virus; CGRP, calcitonin gene-related peptide; RAMP1, receptor-active modification protein 1; SDH, spinal dorsal horn; shRNA, short hairpin RNA.

expression was observed on the contralateral side (Fig. 5C). At 4 weeks after viral interference with RAMP1 expression in the SDH of adult mice, the mechanical (Fig. 5D) and thermal pain thresholds (Fig. 5E) were significantly higher in the RAMP1 knockdown group compared to the negative control and sham groups. This suggests that the knockdown of RAMP1 may mimic the physiological insensitivity to acute pain observed in aged mice. Meanwhile, the CGRP response after intrathecal injection indicated that adult mice

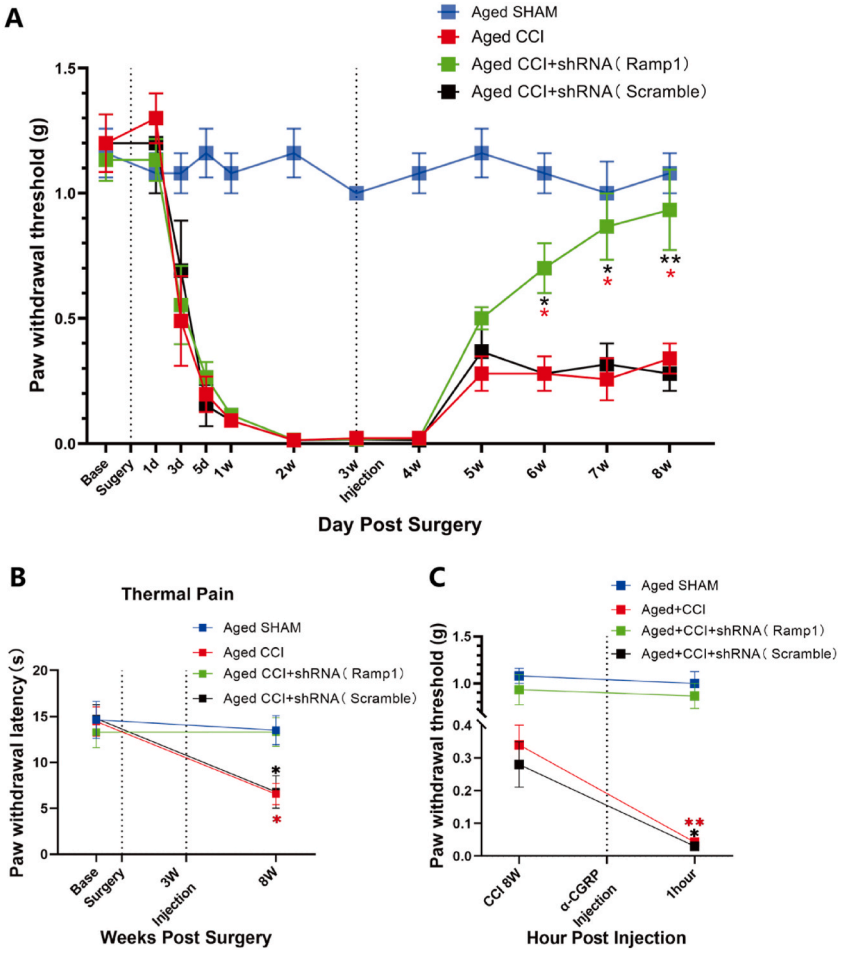

Fig. 6. Changes in pain threshold after RAMP1 knockdown in aged mice under a chronic pain state. A. At 3 weeks after CCI, the 3-week injection of sh RNA-RAMP1 in the L4–6 SDH was significantly higher than that in the CCI and control virus groups (6 weeks: 18m RAMP1 vs. 18m CCI: $p = 0.0164$; 18m RAMP1 vs. 18m scramble: $p = 0.0164$; 7 weeks: 18m RAMP1 vs. 18m CCI: $p = 0.0121$; 18m RAMP1 vs. 18m scramble: $p = 0.0242$; 8 weeks: 18m RAMP1 vs. 18m CCI: $p = 0.0136$; 18m RAMP1 vs. 18m scramble: $p = 0.0067$) and returned to the mechanical pain threshold to physiological state level at 4 weeks of virus expression ($n = 6$). B. The thermal pain threshold of aged CCI mice treated with sh RNA-RAMP1 at 5 weeks was significantly higher than that in the CCI and control virus groups, and there was not statistical difference with the SHAM group (Aged CCI: $p = 0.0124$; Aged CCI + shRNA-Scramble: $p = 0.0163$, $n = 6$). C. α-CGRP after intrathecal injection of sh RNA-RAMP1 in the L4–6 SDH for 5 weeks was significantly higher than that in the CCI and control virus groups (Aged CCI: $p = 0.0032$; Aged CCI + shRNA-Scramble: $p = 0.0112$, $n = 6$).
CCI, chronic constriction injury; CGRP, calcitonin gene-related peptide; RAMP1, receptor-active modification protein 1; SDH, spinal dorsal horn; shRNA, short hairpin RNA.

with RAMP1 knockdown exhibited similar responses to aged mice, with decreased sensitivity to CGRP and no change in mechanical pain threshold (Fig. 5F).

To investigate the impact of RAMP1 expression on chronic pain in aged mice, we injected the same AAV in aged mice 3 weeks after CCI, which is the most painful period. The aged mice with CCI experienced significant pain relief 3 weeks after viral injection (Fig. 6A), and both their mechanical pain thresholds and thermal pain latencies returned to baseline levels 5 weeks after injection (Fig. 6A–B). Moreover, no difference in response sensitivity to CGRP was observed between aged mice undergoing the sham operation and those with RAMP1 knockdown in the CCI group (Fig. 6C). This suggests that RAMP1 knockout reversed the effect of CCI on aged mice, not only in terms of mechanical pain threshold but also their insensitivity to CGRP.

4. Discussion

This study focused on investigating the phenomenon and mechanism of pain sensitivity in ageing. It was found that aged mice were not sensitive to acute pain stimulation but experienced persistent allodynia after CCI. This may be attributed to a decrease in the expression of RAMP1 with ageing and an increase in RAMP1 expression after CCI. The decreased RAMP1 expression resulted in impaired CGRP signalling, while continuous elevation of RAMP1 led to excessive CGRP signalling. Consequently, aged mice exhibited bipolar characteristics in terms of acute and chronic pain states.

In general, the level of pain sensitivity in older individuals compared to young people depends on various factors, including the type of pain stimulation, the site of stimulation, and the pathological state. While some clinicians believe that older people experience less pain than younger people, current evidence suggests that the older population has specific pain thresholds compared to other age groups [30–33]. A meta-analysis of over 50 studies found that pain thresholds increase with age [34]. Moreover, the incidence of asymptomatic myocardial ischemia is clinically associated with age due to the higher pain thresholds observed in older patients [12]. In terms of specific pain sites, the prevalence of headache, abdominal pain, back pain, and chest pain peaks in individuals around the age of 55 and decreases afterwards [35,36]. Additionally, joint, foot, and leg pain increases with age until 90 years [37,38]. Age-related changes in peripheral nerves mainly affect A-δ fibres, leading to functional, structural, and biochemical modifications. Overall, pain thresholds tend to increase with age, while pain tolerance thresholds remain constant or decrease. Additional noteworthy changes in the pain perception system include a significant decrease in descending inhibitory capacity and an associated increase in central sensitisation [39].

Pharmacokinetics and pharmacodynamics are crucial factors to consider when prescribing analgesics to older patients. Pharmacokinetic changes in older patients include reduced absorption, alterations in drug distribution influenced by lipophilicity, enhanced therapeutic responses to protein-bound drugs due to hypoalbuminemia, as well as diminished hepatic metabolism and renal excretion [40,41]. Both peripheral and central nervous systems undergo pharmacodynamic changes in ageing individuals. Pre-existing cognitive deficits, reduced neural myelination, and decreased receptor density may make older patients more susceptible to increased side effects of commonly prescribed drugs [42]. Furthermore, older patients are more prone to long-term pain, and drugs that target peripheral sensitisation are less effective [20]. Considering these factors, it is important to fully understand how the nervous system functions during clinical treatment, including the interactions between pharmacokinetics and pharmacodynamics in older individuals. In order to investigate the underlying mechanisms and identify potential targets for future therapies, we developed animal models to simulate these conditions. Our results demonstrated that adult mice were sensitive to painful stimuli in their physiological state, exhibiting a lower mechanical pain threshold and heat pain latency compared to aged mice. Furthermore, they were susceptible to pain perception abnormalities even at low concentrations of capsaicin. However, in the context of chronic pain, adult mice recovered from mechanical hyperalgesia and allodynia sooner than aged mice, as evidenced by the restoration of the mechanical pain threshold after CCI surgery. These fundamental observations provide a foundation for a comprehensive study on the impact of ageing on pain.

The spinal cord is the first stop in the transfer of peripheral pain information to central receptive regulation and plays a critical role in the transition from acute to chronic pain [19]. CGRP serves as the primary neurotransmitter that transmits nociceptive information from peripheral nerves to the spinal cord. Peripheral CGRP-positive nerve endings terminate in the nociceptive areas of the superficial layer of the spinal cord. CGRP, released from these peripheral nerve endings transmits nociceptive information from the spinal cord through CRLR receptors on nociceptive sensory-related neurons of the spinal cord [43]. Within this pathway, CGRP interacts with CRLR receptors on nociceptive sensory neurons. CRLR, a non-canonical G protein-coupled receptor, forms through the hetero-dimerisation of two different peptides [44]. It demonstrates the highest affinity for CGRP when complexed with RAMP1 [45]. RAMP1 is essential for transporting CRLR to the plasma membrane, where it forms heterodimers to create an intact receptor complex that can bind agonists [46,47]. Previous bioluminescence resonance energy transfer experiments have shown that CRLR can interact with signalling partners only in the presence of RAMP1 after CGRP stimulation [48]. Moreover, our results indicate that RAMP1 expression in mouse SDH neurons is age-dependent. RAMP1 expression decreases in the SDH of aged mice in the physiological state and increases in the chronic pain state. Therefore, the downregulation of RAMP1 in aged mice leads to insensitivity to pain stimuli, whereas the upregulation of RAMP1 in the CCI model leads to neuropathic pain.

Small interfering RNAs (siRNAs) are accurate and effective for gene modulation. The discovery of siRNAs was first documented in *Caenorhabditis elegans* in 1998, and subsequent studies have shown the clinical potential of synthetic siRNA or shRNA in various diseases, including neurodegenerative diseases [49,50]. siRNA-based therapies have now entered the pharmaceutical market, and siRNA drugs are becoming the standard model of drug therapy with three drugs already approved and many others in advanced stages of development [51]. In this study, we used an AAV to target RAMP1 and treat chronic pain in older individuals. Specifically, we microinjected the rAAV 2/9-U6-shRNA-(RAMP1)-CMV-EGFP virus into the superficial layer at the L4–6 SDH of the mice. This approach aimed to inhibit RAMP1 expression in neuronal cells within this region. Control mice were microinjected with rAAV

2/9-U6-shRNA-(Scramble)-CMV-EGFP into the superficial layer of the L4–6 SDH. The behavioural results obtained 3 weeks after the injection revealed an elevated mechanical pain threshold in both adult and aged mice, and the mechanical pain threshold in aged mice with chronic pain started to recover. Overall, our results support the idea that RAMP1 is a potential target for chronic pain treatment in older populations. Therefore, downregulating RAMP1 expression in the dorsal horn may help alleviate chronic pain in older individuals.

Allodynia, which is the experience of pain triggered by light touch or warmth, is a prominent feature of peripheral neuropathic pain [52]. This is why we chose to test mechanical pain as a way to better assess the extent of chronic pain in mice after CCI. Initially, we also considered experimental methods involving non-noxious stimulation, such as the minefield, hanging tail, sugar water preference, and sticky paper experiments. However, due to the declining exercise ability of mice with age, no experimental method can completely eliminate all confounding factors. In addition to mechanical and heat pain, we conducted a capsaicin injection experiment to observe the spontaneous foot-licking behaviours of the mice. This helped to partly compensate for the deficiency in mechanical pain. Capsaicin injection is a relatively strong inflammatory stimulus that elicits a response in both adult and aged mice. Nevertheless, our study has some limitations. First, due to limited experimental technology, we were unable to conduct electrophysiological examinations to determine if SDH neurons respond to α-CGRP after RAMP1 knockdown. Additionally, we did not investigate the sensitivity of aged mice to acute painful stimuli after RAMP1 overexpression in the SDH. Furthermore, while our study would benefit from more accurate control groups, such as the inclusion of sham controls in Fig. 2D and E, our primary focus was to compare CGRP changes over time between the 2-week and 6-week periods under chronic neuropathic pain and to assess differences between adult and aged mice as per our initial design. In future studies, we plan to add a naive group as a control and improve our experimental design, incorporating additional control groups, to meet higher standards.

In conclusion, our findings revealed the bipolarized characteristics of aged mice in response to acute and chronic pain using the acute pain stimulation and a chronic pain model. Insensitivity to pain stimulation in aged mice is associated with age-related downregulation of the CGRP receptor modification protein RAMP1 in the SDH. Reducing RAMP1 expression in the SDH of adult mice can decrease their sensitivity to pain stimuli, whereas reducing RAMP1 in the chronic pain state can improve pain abnormalities and expedite the recovery from chronic pain in aged mice. These results collectively indicate that increased RAMP1 expression and activity are key factors contributing to hyperalgesia in the context of chronic pain and ageing.

Ethics statement

This research is approved by the Ethnic Committee for Experimental Use of Animals of Shanghai Jiao Tong University School of Medicine (Document #SYXK-2013-0050).

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CRediT authorship contribution statement

Yi Qin: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Xuemei Chen:** Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zhangjie Yu:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Xiaoxin Zhou:** Validation, Investigation. **Yihao Wang:** Validation, Methodology. **Qi Li:** Methodology. **Wanbing Dai:** Methodology. **Yizhe Zhang:** Methodology. **Sa Wang:** Writing – review & editing, Writing – original draft. **Yinghui Fan:** Methodology, Funding acquisition. **Jie Xiao:** Writing – review & editing, Investigation, Funding acquisition. **Diansan Su:** Funding acquisition, Formal analysis, Conceptualization. **Yingfu Jiao:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Weifeng Yu:** Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35862.

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