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Spinal cord protein interacting with C kinase 1 is required for the maintenance of complete Freund's adjuvant-induced inflammatory pain but not for incision-induced post-operative pain

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Abstract

Protein interacting with C kinase 1 (PICK1) is a PDZ-containing protein that binds to AMPA receptor (AMPA) GluR2 subunit and protein kinase C α (PKC α) in the central neurons. It functions as a targeting and transport protein, presents the activated form of PKC α to synaptic GluR2, and participates in synaptic AMPAR trafficking in the nervous system. Thus, PICK1 might be involved in many physiological and pathological processes triggered via the activation of AMPARs. We report here that PICK1 knockout mice display impaired mechanical and thermal pain hypersensitivities during complete Freund's adjuvant (CFA)-induced inflammatory pain maintenance. Acute transient knockdown of spinal cord PICK1 through intrathecal injection of PICK1 antisense oligodeoxynucleotide had a similar effect. In contrast, knockout and knockdown of spinal cord PICK1 did not affect incision-induced guarding pain behaviors or mechanical or thermal pain hypersensitivities. We also found that PICK1 is highly expressed in dorsal horn, where it interacts with GluR2 and PKC α . Injection of CFA into a hind paw, but not a hind paw incision, increased PKC α -mediated GluR2 phosphorylation at Ser880 and GluR2 internalization in dorsal horn. These increases were absent when spinal cord PICK1 was deficient. Given that dorsal horn PKC α -mediated GluR2 phosphorylation at Ser880 and GluR2 internalization contribute to the maintenance of CFA-induced inflammatory pain, our findings suggest that spinal PICK1 may participate in the maintenance of persistent inflammatory pain, but not in incision-induced post-operative pain, through promoting PKC α -mediated GluR2 phosphorylation and internalization in dorsal horn neurons.

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Keywords

Protein interacting with C kinase 1; AMPA receptors; Protein kinase C α ; Spinal cord; Inflammatory pain; Incisional pain

1. Introduction

The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) are heterotetrameric cation channels composed of receptor subunits GluR1-4 [2,13]. They mediate the majority of fast excitatory postsynaptic transmissions. Therefore, changes in postsynaptic membrane trafficking or in synaptic targeting of AMPAR subunits alter excitatory synaptic strength. These changes are recognized as a key mechanism that underlies various forms of synaptic plasticity in the central nervous system [6,11].

Recent evidence indicates that peripheral noxious insults drive changes in synaptic AMPAR subunit trafficking in dorsal horn neurons and that such changes may contribute to dorsal horn central sensitization (a specific form of synaptic plasticity) in persistent pain [28].

Subcutaneous injection of complete Freund's adjuvant (CFA) into a rat hind paw, which produces long-lasting peripheral inflammation and persistent inflammatory pain, leads to GluR2 internalization at synapses in dorsal horn neurons during the maintenance period [16, 21,22]. This internalization requires NMDA receptor (NMDAR)-triggered spinal cord protein kinase C α (PKC α) activation and PKC α -mediated phosphorylation of GluR2 at Ser880 [21]. Furthermore, preventing CFA-induced dorsal horn GluR2 internalization through targeted mutation of the GluR2 PKC α phosphorylation site impairs CFA-evoked nociceptive hypersensitivity during the maintenance period [21]. These findings suggest that dorsal horn PKC α -mediated GluR2 phosphorylation and subsequent GluR2 internalization might participate in the maintenance of pain hypersensitivity in persistent inflammatory pain.

Protein interacting with C kinase 1 (PICK1), a scaffolding protein that is enriched at mammalian synapses, was initially reported to interact with PKC α [26], but it has several other binding partners that are involved in synaptic function [7-9,14,33,34,36]. PICK1 has a single PDZ (PSD-95/Discs large/ZO-1 homologous) domain. Via this domain, PICK1 interacts with GluR2 and PKC α , recruits intracellular PKC α to synaptic GluR2, leads to GluR2 phosphorylation at Ser880, and promotes GluR2 internalization in brain neurons [19,23,31]. Given that PICK1 binds to GluR2 as well as PKC α in spinal cord and that dorsal horn PKC-mediated GluR2 phosphorylation and internalization contribute to the maintenance of inflammation-induced pain hypersensitivity [21], it is very likely that spinal cord PICK1 participates in the maintenance of inflammatory pain.

In the present study, by combining a PICK1 genetic knockout strategy with a PICK1 antisense (AS) oligodeoxynucleotide (ODN) knockdown approach, we characterized the functional role of PICK1 in inflammatory pain induced by injection of CFA into a hind paw. Because spinal AMPAR activation participates in dorsal horn sensitization that underlies postoperative pain [25,38], we also examined the involvement of spinal cord PICK1 in a model of post-operative pain induced by a hind paw incision.

2. Materials and Methods

2.1. Animal preparation

Male mice (10–12 weeks old) and male Sprague-Dawley rats (250–300 g) were housed on a standard 12-h light/dark cycle, with water and food pellets available *ad libitum*. PICK1 knockout (KO) mice (C57BL/6J genetic background) were generated as described previously

[27]. PICK1 KO male and female mice are viable with normal appearance [27]. Male PICK1 KO mice and wild-type (WT) littermates were obtained by interbreeding PICK1 heterozygous mice. To minimize intra- and inter-individual variability of behavioral outcome measures, animals were trained for 1–2 days before behavioral testing was performed. Animal experiments were conducted with the approval of the Animal Care and Use Committee at Johns Hopkins University and were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experimenters were blind to mouse genotype and drug treatment condition in the behavioral testing.

2.2. Persistent inflammatory pain model

The CFA-induced inflammatory pain model was carried out as described previously [5,39]. Briefly, mice and rats were injected subcutaneously with CFA solution (1 mg/ml *Mycobacterium tuberculosis*; 20 µl in mice and 100 µl in rats) into the plantar side of one hind paw. Control animals received identical volumes of saline. Behavioral tests were performed 1 d prior to injection and at 2 h, 1 d, 3 d, 5 d, 7 d, 9 d, and 12 d after injection.

2.3. Incisional pain model

The incisional pain model in mice and rats was performed as described previously [1,25]. Briefly, the animal was placed under anesthesia with isoflurane, and a longitudinal incision (8 mm in mice and 10 mm in rats) was made with a number 11 blade through the skin, fascia, and muscle of the right hind paw, starting 0.3 cm in mice and 0.5 cm in rats from the proximal edge of the heel and extending toward the toes. After hemostasis with slight pressure, the skin was closed with two 6-0 silk sutures and the wound covered with triple antibiotic ointment. After surgery, animals were allowed to recover in cages with sterile bedding. On the second postoperative day, sutures were removed during brief anesthesia. Control animals underwent a sham procedure (sham incision) that consisted of the administration of isoflurane for 30 min, without incision. Pain behaviors were measured 1 d before incision and 4 h, 1 d, 2 d, 3 d, 5 d, and 7 d after incision.

2.4. Intrathecal administration of PICK1 AS ODN

The nucleotide sequence of the PICK1 cDNA was obtained from the GenBank database (GenBank accession number NM_053460). The AS ODN corresponding to the PDZ domain nucleotides 109–126 (5'-CTGGGCCCCCTCCTCCGAT-3') of PICK1 mRNA, as well as the missense (MS) ODN (5'-GCCCCGCTTCTCCCAGCTG-3'), was designed. The ODNs were searched to exclude nonspecificity of the AS ODN and to show that the MS ODN did not match any confounding sequences in the GenBank database. The ODNs were made and purified by high-performance liquid chromatography (Integrated DNA Technologies, Inc., Coralville, IA), dissolved in saline, and stored at –70°C.

For repeated injection of ODNs, an intrathecal polyethylene (PE-10) catheter was inserted into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement segments through an incision at the atlanto-occipital membrane according to the method previously described [21,22,29]. The rats were allowed to recover for 5 to 10 days before being used experimentally. Rats showing any neurological deficits postoperatively were discarded. Every 24 h for 4 days, the rats were injected intrathecally with saline (10 µl; control), AS ODN (10 µg/10 µl), or MS ODN (10 µg/10 µl), followed by an injection of 10 µl of saline to flush the catheter. On the fifth day, some rats were euthanized for examination of PICK1 expression in the spinal cord. The rest were entered into the CFA model group or the incisional pain model group, as described above.

2.5. Behavioral testing

Guarding behaviors were assessed as described previously [1,25,37]. Briefly, unrestrained animals were placed on a stainless steel mesh floor. The incised and intact paws were closely observed during a 1-min period repeated every 5 min for 1 h. Depending on the position of each paw during the majority of the 1-min scoring period, a score of 0, 1, or 2 was given. The paw was considered to be fully weight-bearing (score = 0) if the paw was blanched or distorted by the mesh. If the paw was completely off the mesh, a score of 2 was recorded. If the area of the wound touched the mesh without blanching or distorting, a 1 was given. At the end of the 1-h session, the 12 scores were summed to give a total score of 0–24. The difference between the score from the incised paw and the intact paw was the cumulative pain score for that 1-h period.

To measure paw withdrawal responses to noxious thermal stimuli, the animal was placed in a Plexiglas chamber on a glass plate above a light box. Radiant heat from Model 336 Analgesia Meter (IITC Inc./Life Science Instruments, Woodland Hills, CA, USA) was applied by aiming a beam of light through a hole in the light box through the glass plate to the middle of the plantar surface of each hind paw. When the animal lifted its foot, the light beam automatically shut off. The length of time between the start of the light beam and the foot lift was defined as the paw withdrawal latency. Each trial was repeated five times at 5-min intervals for each side. A cut-off time of 20 s was used to avoid tissue damage to the hind paw.

To measure paw withdrawal responses to repeated mechanical stimuli, the animal was placed in a Plexiglas chamber on an elevated mesh screen. In mice, two calibrated von Frey monofilaments (0.24 and 4.43 mN; Stoelting Co., Wood Dale, IL, USA) were employed. Each von Frey filament was applied to the hind paw for approximately 1 s, and each trial was repeated 10 times to both hind paws. In rats, a single trial of mechanical stimuli consisted of eight applications of a calibrated von Frey filament (8.01 mN, Stoelting Co.) within a 2–3 s period. Each trial was repeated 10 times at 3-min intervals on each hind paw. The occurrence of paw withdrawal in each of these 10 trials was expressed as a percent response frequency [(number of paw withdrawals/10 trials) $\times$ 100 = % response frequency], and this percentage was used as an indication of the amount of paw withdrawal.

Locomotor function was examined in mice and rats according to methods described previously [29,30]. The following tests were performed with the experimenter blind to the mouse genotype and rat treatment: (1) Placing reflex: the experimenter held the animal's hind limbs slightly lower than the forelimbs and brought the dorsal surfaces of the hind paws into contact with the edge of a table. The experimenter recorded whether the hind paws were placed on the table surface reflexively. (2) Grasping reflex: the experimenter placed the animal on a wire grid and recorded whether the hind paws grasped the wire on contact. (3) Righting reflex: the experimenter placed the animal's back on a flat surface and noted whether it immediately assumed the normal upright position. Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex exhibited in five trials. In addition, the animal's general behaviors, including spontaneous activity, were observed.

2.6. Fractionation of 150,000 spin gravity (150 k-g) proteins

Subcellular fractionation was carried out as described previously with minor modification [12,21]. Briefly, the fourth and fifth lumbar segments of the spinal cord were homogenized in homogenization buffer [250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine] and centrifuged at $1,000 \times g$ for 10 min at 4°C. The supernatant was collected and centrifuged at $20,000 \times g$ for 20 min at 4°C. Again the supernatant was collected and subsequently centrifuged at $150,000 \times g$ for 1 h at 4°C; the final pellet was used as the 150 k-g fraction.

2.7. Immunoprecipitation and Western blot analysis

Co-immunoprecipitation and Western blotting were carried out as described previously [21, 30]. Briefly, the affinity-purified polyclonal rabbit anti-PICK1 or anti-GluR2 antibody was incubated with 100 μ l of a 1:1 slurry of protein A-sepharose for 1 h, and the protein-antibody complex was spun down at 2,000 rpm for 4 min. The solubilized fractions from the tissues were then added to the sepharose beads and the mixture incubated for 2–3 h at 4°C. The mixture was washed with modified RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM NaPO₄, 150 mM NaCl, 2 mM NaF, 1 mM PMSF, 1 mg/ml leupeptin, 10 mM sodium pyrophosphate). The proteins were separated by SDS-PAGE on 4% stacking/7.5% separating gels and then were electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked with 3% non-fat dry milk and then incubated with primary rabbit anti-GluR2 (1:500; Chemicon, Temecula, CA), rabbit anti-PICK1 (1:200; provided by Dr. R.L. Haganir), rabbit anti-PKC α (1:1,000; Santa Cruz, CA), rabbit anti- α -adaptin (1:500; Chemicon), or mouse anti- β -actin (1:4,000; Sigma, St. Louis, MO) overnight. β -actin was used as a loading control. The proteins were detected by using horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence reagents provided with the ECL kit (Amersham, Piscataway, NJ) and exposure to film. The intensity of blots was quantified with densitometry and normalized to the corresponding β -actin.

The blot density from naïve WT mice was set as 100%. The relative density values from naïve KO mice and in the treated groups from both WT and KO mice were determined by dividing the optical density values from these groups by the value from the naïve WT mice.

2.8. Statistical analysis

Data from behavioral tests and Western blots were analyzed with a one-way or two-way analysis of variance (ANOVA) and are shown as mean $\pm$ SEM. When ANOVA showed significant differences, pair-wise comparisons between means were tested by the post hoc Tukey method. Significance was set at $P < 0.05$. All statistical analyses were carried out with the statistical software package of SigmaStat.

3. Results

3.1. Effect of PICK1 knockout and knockdown on CFA-induced inflammatory pain

PICK1 is a scaffolding protein without any receptor-like or enzyme-like activities. We used a genomic strategy to examine whether targeted disruption of the PICK1 gene affects CFA-induced inflammatory pain. Consistent with previous studies [5,30], subcutaneous injection of CFA into a hind paw led to long-term mechanical pain hypersensitivity and thermal pain hypersensitivity on the ipsilateral side in WT mice ($n = 14$). The paw withdrawal frequencies in response to 0.24 mN (low intensity) and 4.43 mN (moderate intensity) von Frey filaments applied to the injected hind paw were significantly greater than those at baseline, a behavioral indication of mechanical pain hypersensitivity (Fig. 1A and B). Similarly, the paw withdrawal latency of the injected hind paw in response to heat was significantly less than at baseline, an indication of thermal pain hypersensitivity (Fig. 1C). Pain hypersensitivity appeared at 2 h, reached a peak level on day 1, and persisted for 5 days or longer after CFA injection (Fig. 1). PICK1 KO mice ($n = 12$) displayed basal paw withdrawal responses to mechanical and thermal stimuli that were similar to those of WT mice (Fig. 1). However, their nocifensive responses after CFA injection differed. Although mechanical and thermal pain hypersensitivities were present at 2 h post-CFA, they were significantly reduced during the remainder of the observation period. The magnitude of response frequencies to low- and moderate-intensity mechanical stimuli in the KO mice was significantly lower than that observed in the WT mice from 1 to 7 days post-CFA injection ($P < 0.05$; Fig. 1A and B), and the magnitude of paw withdrawal latencies was significantly higher than that observed in the WT mice from 1 to 5

days post-CFA injection ($P < 0.05$; Fig. 1C). As expected, no significant changes were seen in the contralateral hind paw in either WT or PICK1 KO mice after CFA injection (Fig. 1).

To further confirm our observations from PICK1 KO mice, we used a PICK1 AS ODN approach to knock down the spinal cord PICK1 in rats. MS ODN was designed as a control. On day 1 after four days of intrathecal injection of 10 μ g AS ODN (but not 10 μ g MS ODN), PICK1 protein expression was specifically and selectively reduced in the spinal cord lumbar enlargement segment (Fig 2A). The levels of PICK1 in the saline-treated ($n = 4$), AS ODN-treated ($n = 4$), and MS ODN-treated ($n = 4$) groups were reduced by 5% ($P > 0.05$), 35% ($P < 0.05$), and 7% ($P > 0.05$), respectively, compared to the value of the naïve group ($n = 4$). PICK1 expression remained depressed for at least 1 day (25% less than the naïve group value on day 2 after the final ODN injection; $n = 4$; $P < 0.05$) and recovered at day 4 after the final AS ODN injection (10% less than the naïve group; $n = 4$; $P > 0.05$). No significant differences were observed in the expression of PICK1-interacting proteins GluR2 or PKC α , or in the GluR2-binding protein GRIP1 in the lumbar enlargement segment of naïve, saline-treated, AS ODN-treated, or MS ODN-treated groups (Fig. 2A).

The basal paw withdrawal responses to mechanical and thermal stimuli were similar among saline-treated (control), AS ODN-treated, and MS ODN-treated groups. Furthermore, the CFA-injected control rats developed long-term mechanical and thermal pain hypersensitivities on the ipsilateral side, but not on the contralateral side ($n = 6$; Fig. 2B and C), consistent with the results in mice and previous reports [21,22,39]. However, we found that spinal cord PICK1 knockdown significantly attenuated CFA-induced mechanical and thermal pain hypersensitivities on the ipsilateral side 1 d post-CFA (that is, day 2 after the final ODN injection), but not 2 h, 3 d, or 5 d post-CFA (Fig. 2B and C). Compared to values of the saline-treated group, intrathecal injection of 10 μ g AS ODN ($n = 5$) inhibited the CFA-induced increase in paw withdrawal frequency by 34% ($P < 0.05$) and blocked the CFA-induced reduction in paw withdrawal latency by 62% ($P < 0.05$) 1 d post-CFA. MS ODN ($n = 5$) did not affect the CFA-induced pain hypersensitivity during the observation period (Fig. 2B and C), and there was no significant difference in paw withdrawal frequency or latency between the saline-treated and MS ODN-treated groups.

3.2. Effect of spinal PICK1 knockout and knockdown on incision-induced post-operative pain

Next, we examined the role of spinal cord PICK1 in post-operative pain. Consistent with previous studies [1,37], a hind paw incision led to substantial guarding behaviors and mechanical and thermal pain hypersensitivities on the ipsilateral side of WT mice ($n = 10$ /test; Fig. 3). These pain behaviors were significantly greater than those at baseline at 4 h, lasted for 3–5 days, and disappeared 7 days post-incision. Like the WT mice, the PICK1 KO mice did not show any pre-incision guarding behaviors. Furthermore, the deficiency of PICK1 did not alter incision-induced guarding behaviors, mechanical hypersensitivity, or thermal pain hypersensitivity on the ipsilateral or contralateral side ($n = 12$ /test; Fig. 3). The guarding scores, paw withdrawal latencies, and paw withdrawal frequencies in the WT and PICK1 KO mice were not statistically different at any single time point.

A KO strategy may be of limited value by itself because of potential compensatory mechanisms during development. To exclude the possibility that intact incisional pain behaviors in PICK1 KO mice might be attributable to such compensation, we used the ODN strategy to examine the effect of acute transient knockdown of spinal PICK1 on incisional pain. Similar to the mice, a hind paw incision in the rats produced marked guarding behaviors and mechanical and thermal pain hypersensitivities on the ipsilateral side (but not on the contralateral side) in the control group ($n = 5$; Fig. 4). Intrathecal injection of neither 10 μ g PICK1 AS ODN ($n = 6$) nor 10 μ g MS ODN ($n = 5$) affected incision-induced changes in the guarding scores, paw

withdrawal latencies, or paw withdrawal frequencies on the ipsilateral or contralateral side compared to the saline-treated group during the observation period.

3.3. Effect of PICK1 knockout or knockdown on locomotor functions in mice and rats

To exclude the possibility that the effect of spinal PICK1 knockout or knockdown on CFA-induced pain behaviors was caused by impaired locomotor functions (or reflexes), we examined locomotor functions of experimental animals. As shown in Tables 1 and 2, both PICK1 knockout mice and the ODN-treated rats exhibited normal locomotor functions, including placing, grasping, and righting reflexes. Convulsions and hypermobility were not observed in any of the PICK1 KO mice or treated rats. In addition, we did not observe any significant difference in general behaviors, including spontaneous activity, between WT and PICK1 KO mice or between the saline-treated and the ODN-treated rats.

3.4. Effect of PICK1 deletion on GluR2 phosphorylation and internalization in spinal cord after CFA injection or incision

So far, our behavioral results suggest that spinal cord PICK1 may be required for the maintenance of CFA-induced inflammatory pain, but not for incisional pain. We further examined the potential mechanisms that underlie these events. Using Western blot analysis, we detected abundant PICK1 protein in the dorsal horn but little in the ventral horn and dorsal root ganglion of mice (Fig. 5A) and rats (data not shown). We were unable to obtain a detailed cellular distribution of PICK1 in these regions because the PICK1 antibody is inadequate for immunohistochemistry. Two PICK1-binding proteins, GluR2 and PKC α , were also expressed highly in the dorsal horn (Fig. 5A). Co-immunoprecipitation revealed that PICK1 interacted with both GluR2 and PKC α in the spinal cord of WT mice. PICK1 antibody immunoprecipitated not only PICK1 but also GluR2 and PKC α , and GluR2 antibody immunoprecipitated GluR2 as well as PICK1 and PKC α (Fig. 5A). These findings are in agreement with those of our previous study in the rat spinal cord [21] and indicate the existence of a complex formed by PICK1, GluR2, and PKC α in the spinal cord of WT mice and rats. However, in the spinal cord of PICK1 KO mice, GluR2 antibody immunoprecipitated only itself (not PICK1 or PKC α), and PICK1 antibody did not immunoprecipitate any of the three proteins (Fig. 5B). The data suggest that PICK1, as a transport protein, is required for the coupling of PKC α to GluR2 in mouse spinal cord.

We have previously shown that in dorsal horn, PKC α phosphorylates Ser880 of GluR2, which is then internalized into the cell [21]. These events are considered to underlie the maintenance of CFA-induced inflammatory pain [21]. Thus, we finally examined whether spinal cord PICK1 deficiency affected PKC-mediated GluR2 phosphorylation at Ser880 and its internalization in spinal cord during the maintenance of CFA-induced inflammation. Consistent with our work in rats [21], subcutaneous injection of CFA into a WT mouse hind paw induced a significant increase in the level of GluR2 phosphorylation at Ser880 in the ipsilateral (but not contralateral) L₄₋₅ dorsal horn at 1 day (n = 5). This phosphorylation was increased by 1.8-fold of the value in the naïve group (n = 5; $P < 0.05$; Fig. 6A). CFA injection did not alter the expression of total GluR2 or PICK1 in dorsal horn (Fig. 6A). PICK1 deficiency did not affect the basal level of GluR2 phosphorylation at Ser880 (n = 5), but it abolished the CFA-induced increase in GluR2 phosphorylation at Ser880 (n = 5; Fig. 6A). The phosphorylated level was 0.96-fold of the value in naïve WT mice ($P > 0.05$; Fig. 6A). As expected, saline injection did not change the basal level of GluR2 PKC phosphorylation at Ser880 in WT (n = 5) or PICK1 KO (n = 5) mice (Fig. 6A).

GluR2 is transported away from the synaptic membrane mainly via clathrin-coated pit-dependent endocytosis. We used differential centrifugation to collect a 150-k-g spin fraction (which contains abundant endocytosed, clathrin-coated vesicles [20,21]) from the ipsilateral

L₄₋₅ dorsal horn tissues of WT and PICK1 KO mice after CFA or saline injection. Consistent with the findings in rats [21], subcutaneous injection of CFA into a WT mouse hind paw produced GluR2 internalization in the ipsilateral dorsal horn 1 day after CFA injection (n = 5). The amount of GluR2 in the 150-k-g fraction was increased by 1.65-fold of the value in the naïve group (n = 5; $P < 0.05$; Fig. 6B). PICK1 knockout significantly attenuated this CFA-induced GluR2 internalization (n = 5). The amount of GluR2 in the 150-k-g fraction was 1.15-fold of the value in the naïve WT group ($P > 0.05$; Fig. 6B). No significant differences were observed in the amount of GluR2 in the 150 k-g fraction from the dorsal horns of WT and PICK1 KO mice between the saline-treated and naïve groups (n = 5/genotype; Fig. 6B).

Interestingly, hind paw incision did not alter the level of GluR2 phosphorylation at Ser880 or the amount of total GluR2 protein in the ipsilateral and contralateral L₄₋₅ dorsal horns from either WT (n = 4/time point) or PICK1 KO (n = 4/time point) mice (Fig. 7A). The incision also did not change the level of GluR2 in the 150-k-g fraction from the ipsilateral or contralateral L₄₋₅ dorsal horns of WT (n = 4/time point) and PICK1 KO (n = 4/time point) mice during the observation period (Fig. 7B). These findings indicate that incisional noxious input, unlike CFA-induced inflammatory input, does not induce PKC α -mediated phosphorylation of GluR2 Ser880 or GluR2 internalization in dorsal horn neurons, although spinal PICK1 binds to PKC α and GluR2 and presents PKC α to synaptic GluR2 (Fig. 5B).

4. Discussion

Peripheral tissue incision and inflammation produce persistent pain hypersensitivities in rodent animal models that mimic clinical post-operative pain and chronic inflammatory pain, respectively. Uncovering the mechanisms that lead to the hypersensitivity may lead to novel therapeutic strategies for its prevention and/or treatment. Although evidence documented over the last three decades has implicated AMPAR involvement in persistent post-operative pain and inflammatory pain, how AMPARs participate in these two disorders is not completely understood. Here, we report that spinal PICK1, an AMPAR GluR2 subunit-binding protein, might be required for the maintenance of CFA-induced inflammatory pain, but not for incision-induced post-operative pain. PICK1 may be a new target for the treatment of persistent inflammatory pain.

Our results showed that spinal PICK1 deficiency caused by the congenital knockout of PICK1 gene or acute transient knockdown of PICK1 significantly reduces mechanical and thermal pain hypersensitivities during the maintenance of CFA-induced inflammatory pain. This anti-hyperalgesic effect might be related to the functional role of spinal PICK1 in CFA-induced AMPAR GluR2 subunit internalization during inflammatory pain maintenance. PICK1 binds to both PKC α and GluR2 and can present the activated form of PKC α to synaptic GluR2 in dorsal horn [21]. It has been shown previously that during the maintenance period of CFA-evoked pain hypersensitivities, spinal NMDAR-triggered PKC α activation leads to phosphorylation of GluR2 at Ser880, disruption of GluR2 binding to its synaptic anchoring protein ABP/GRIP, and promotion of GluR2 internalization in dorsal horn neurons [16,21, 22]. GluR2 internalization results in an increase in AMPAR Ca²⁺ permeability. The increase in intracellular Ca²⁺ in dorsal horn neurons might initiate or potentiate a variety of Ca²⁺-dependent intracellular cascades that are associated with the maintenance of inflammatory pain [28]. The present study demonstrated that PICK1 is required for CFA-induced GluR2 phosphorylation and GluR2 internalization in dorsal horn, as they were abolished by PICK1 knockout. Given that the presence or absence of synaptic GluR2 in the AMPAR complex greatly influences AMPAR Ca²⁺ permeability, it is very likely that the anti-hyperalgesic effect caused by PICK1 deficiency might result from the failure of activated PKC α to be recruited to GluR2. Without the resulting association, no changes would occur in GluR2 phosphorylation or trafficking in the dorsal horn.

It is noteworthy that mice and rats deficient in PICK1 still displayed some level of pain hypersensitivity during the maintenance phase of CFA-induced inflammatory pain. Preventing CFA-induced spinal GluR2 internalization through targeted mutation of the GluR2 PKC phosphorylation site also did not completely abolish CFA-induced pain hypersensitivity during the maintenance period [21]. These findings suggest that PICK1-mediated GluR2 internalization is just one of the key factors involved in CFA-induced inflammatory pain maintenance. Moreover, PICK1 interacts with several proteins in addition to GluR2, including ASIC1, ASIC2, ephrin-B ligands, Eph receptor tyrosine kinase, metabotropic glutamate receptor subunit 7, dopamine transporter, and class I ADP-ribosylation factors [9,14,33,34]. These PICK1-mediated interactions might also be involved in inflammatory pain maintenance. In addition, PICK1 is expressed in other pain-related regions of the nervous system, such as the dorsal root ganglion. Thus, the detailed mechanisms by which the deficiency of PICK1 affects CFA-induced inflammatory pain maintenance remain to be explored.

Spinal AMPARs also participate in dorsal horn sensitization that underlies incision-induced post-operative pain. Intrathecal or epidural administration of AMPA/kainate receptor antagonists reduces pain behaviors caused by incision [15,18,35]. Intrathecal application of these antagonists also attenuates incision-induced increases in background activity of dorsal horn neurons and in the responses of dorsal horn wide dynamic range neurons to mechanical stimuli [38]. We expected that a hind paw incision, like CFA-induced peripheral inflammation, would lead to PICK1-dependent dorsal horn GluR2 internalization and that blocking this internalization through targeted disruption of the PICK1 gene would contribute to dorsal horn sensitization after incision. However, our results showed no significant change in either PKC α -mediated GluR2 phosphorylation at Ser880 or GluR2 internalization in dorsal horn from WT and PICK1 KO mice after a hind paw incision. Neither knockdown nor knockout of spinal PICK1 affected incision-induced pain hypersensitivities during the observation period. The mechanisms that underlie these unexpected results are unclear, but they might be related to NMDAR-independent dorsal horn sensitization under incisional pain conditions. It has been demonstrated that intrathecal application of NMDAR antagonists does not affect pain behaviors or the enhanced responsiveness of dorsal horn neurons that occurs after incision [4,38]. These findings indicate that spinal cord NMDARs and their downstream signal cascades (such as PKC α) might be inactivated after incision. Given that spinal PKC α activation is triggered by NMDARs in dorsal horn [21], this might be one reason that incision does not alter the level of spinal cord GluR2 phosphorylation or internalization.

Our findings indicate that different peripheral nociceptive insults may produce distinct changes in spinal cord AMPAR subunit trafficking. This view is further supported by recent studies on different pain models. Capsaicin-induced acute inflammation increased the amount of membrane GluR1 protein and correspondingly decreased the level of cytosolic GluR1, without affecting GluR2 trafficking in dorsal horn neurons [10,17]. The injection of carrageenan or formalin into a hind paw led to an increase only in the level of GluR1 in the plasma membrane of dorsal horn neurons [3,24]. In addition, CFA-induced persistent inflammation was shown to produce GluR1 membrane insertion and GluR2 internalization in dorsal horn [16,21,22]. We also found that a hind paw incision does not alter the levels of membrane or cytosolic GluR1 proteins in the dorsal horn (data not shown). It will be very interesting to further characterize spinal cord AMPAR subunit trafficking under other persistent pain conditions (such as nerve injury-induced neuropathic pain).

In conclusion, we demonstrated that knockout and knockdown of spinal cord PICK1 significantly attenuates pain hypersensitivities during CFA-induced inflammatory pain maintenance, without affecting incision-induced post-operative pain behaviors. Furthermore, we showed that spinal PICK1 deficiency abolishes CFA-induced GluR2 phosphorylation at Ser880 and GluR2 internalization in dorsal horn. Given that the latter two events contribute to

CFA-induced inflammatory pain maintenance [21,28], spinal cord PICK1 might participate in the maintenance of persistent inflammatory pain through promoting GluR2 phosphorylation at Ser880 and GluR2 internalization in dorsal horn neurons. It should be noted that genetic strategies, including knockout and knockdown approaches, might be impractical in the clinical setting. A recent study identified a small-molecule inhibitor (FSC231) that specifically bound to the PICK1 PDZ domain, inhibited PICK1 interaction with GluR2, and accelerated recycling of GluR2 after internalization in response to NMDAR activation [32]. Thus, FSC231 may represent a promising novel strategy for treating persistent inflammatory pain.

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Fig. 1.

Effect of targeted disruption of PICK1 gene on CFA-induced inflammatory pain. CFA injection into a hind paw produced significant increases in paw withdrawal frequencies in response to 0.24 mN (low intensity) (A) and 4.43 mN (moderate intensity) (B) mechanical stimuli and marked decreases in paw withdrawal latencies in response to thermal stimuli (C) on the ipsilateral (ipsi) side in wild-type (WT) mice. The PICK1 knockout (KO) mice exhibited intact mechanical and thermal pain hypersensitivities at 2 h after CFA injection and significantly reduced mechanical and thermal pain hypersensitivities during the remainder of the observation period (A, B, and C). No significant changes in paw withdrawal frequencies or latencies were seen on the contralateral (con) side after CFA injection in either WT or KO mice. * $P < 0.05$, ** $P < 0.01$ vs corresponding time points in PICK1 KO mice.

Fig. 2.

Effect of acute transient knockdown of spinal PICK1 on CFA-induced inflammatory pain. (A) Intrathecal injection of 10 μ g PICK1 AS ODN, but not saline or 10 μ g MS ODN, significantly reduced expression of PICK1, but not GluR2, PKC α , or GRIP1, in the lumbar enlargement segments compared to that in naïve rats. Top: Representative Western blots. Bottom: Statistical summary of the densitometric analysis expressed relative to naïve rats after normalization to corresponding β -actin. (B and C) Intrathecal injection of 10 μ g PICK1 AS ODN, but not saline or 10 μ g MS ODN, significantly attenuated the CFA-induced increase in paw withdrawal frequency in response to mechanical stimulation (B) and the CFA-induced decrease in paw withdrawal latency in response to thermal stimulation (C) on the ipsilateral side at 1 d, but not 2 h, 3 d, or 5 d after CFA injection. * $P < 0.05$ vs the corresponding time point in the saline-treated group. Sal, saline; AS, antisense; MS, missense

Fig. 3.

The targeted disruption of PICK1 gene does not affect incision-induced post-operative pain. (A) Guarding pain scores at different time points after incision. (B) Paw withdrawal latencies in response to thermal stimulation at different time points after incision. (C and D) Paw withdrawal frequencies in response to 0.24 mN (C) and 4.43 mN (D) mechanical stimuli at different time points after incision. Ipsi, ipsilateral; con, contralateral.

Fig. 4.

Spinal PICK1 knockdown does not affect incision-induced post-operative pain. (A) Guarding pain scores at different time points after incision. (B) Paw withdrawal latencies in response to thermal stimulation at different time points after incision. (C) Paw withdrawal frequencies in response to mechanical stimulation at different time points after incision. Sal, saline; AS, antisense; MS, missense.

Fig. 5.

Expression of PICK1 and PICK1 binding to GluR2 and PKC α in dorsal horn. (A) Western blotting showed higher levels of PICK1, GluR2, and PKC α in the dorsal horn (DH) and lower levels in the ventral horn (VH) and dorsal root ganglion (DRG). β -actin was used as a loading control. (B) Left panel: GluR2 antibody immunoprecipitated GluR2, PICK1, and PKC α in spinal cord from WT mice and only GluR2 itself in spinal cord from PICK1 KO mice. Right panel: PICK1 antibody immunoprecipitated GluR2, PICK1, and PKC α in spinal cord from WT mice, but not from PICK1 mice. IB: immunoblotting. IP: immunoprecipitation.

Fig. 6.

Effect of spinal PICK1 deficiency on CFA-induced GluR2 phosphorylation at Ser880 and GluR2 internalization in dorsal horn. (A) CFA (but not saline) injection increased GluR2 phosphorylation at Ser880 (GluR2-p), but not total GluR2 or PICK1, in the ipsilateral dorsal horn of WT mice after 1 day. This increase was not seen in the ipsilateral dorsal horn of PICK1 KO mice. Top: Representative Western blots. Bottom: Statistical summary of the densitometric analysis expressed relative to naïve WT mice after normalization to corresponding β -actin. * $P < 0.05$ vs naïve WT mice. (B) CFA (but not saline) injection increased the level of GluR2 in the 150-k-g fraction from the ipsilateral dorsal horn of WT mice after 1 day. This increase was not detected in the ipsilateral dorsal horn of PICK1 KO mice. Top: Representative Western blots. Bottom: Statistical summary of the densitometric analysis expressed relative to naïve WT mice after normalization to corresponding α -adaptin. * $P < 0.05$ vs naïve WT mice.

Fig. 7.

Paw incision does not induce GluR2 phosphorylation at Ser880 or GluR2 internalization in dorsal horn. (A) The levels of GluR2 phosphorylation at Ser880 (GluR2-p) and total GluR2 in the ipsilateral dorsal horns of WT (left panel) and PICK1 KO (right panel) mice at the different time points after a hind paw incision. Top: Representative Western blots. Bottom: Statistical summary of the densitometric analysis expressed relative to naïve (N) mice after normalization to corresponding β -actin. (B) The levels of GluR2 in the 150-k-g fraction from the ipsilateral dorsal horn of WT (left panel) and PICK1 KO (right panel) mice at the different time points after hind paw incision. Top: Representative Western blots. Bottom: Statistical summary of the densitometric analysis expressed relative to naïve (N) mice after normalization to corresponding α -adaplin.

Table 1

Mean (SEM) changes in locomotor function

Genotype	Functional Test		
	Placing	Grasping	Righting
WT	5 (0)	5 (0)	5 (0)
PICK1 KO	5 (0)	5 (0)	5 (0)

n = 10/genotype, 5 trials

Table 2

Mean (SEM) changes in locomotor function

Group	Functional Test		
	Placing	Grasping	Righting
Saline	5 (0)	5 (0)	5 (0)
AS ODN	5 (0)	5 (0)	5 (0)
MS ODN	5 (0)	5 (0)	5 (0)

n = 5/group, 5 trials; AS, antisense; MS, missense; ODN, oligodeoxynucleotide