Biphasic Activation of Extracellular Signal-regulated Kinase in Anterior Cingulate Cortex Distinctly Regulates the Development of Pain-related Anxiety and Mechanical Hypersensitivity in Rats after Incision

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ABSTRACT

Background: A recent study has demonstrated that surgical incision induces an anxiety-like behavior but its relationship with incision-evoked mechanical hypersensitivity remains elusive. Extracellular signal-regulated kinase (ERK) activity in the anterior cingulate cortex (ACC) is important for the affective pain. The current study aims to explore ERK1/2 activity in the ACC and its role in the development of anxiety and mechanical hypersensitivity after incision.

Methods: Anxiety-like behavior was measured by elevated plus maze experiment and open field test after hind paw incision. ERK1/2 phosphorylation was determined by immunohistochemistry and Western blot. Cannulae were implanted into the bilateral ACC for the intra-ACC injection of ERK inhibitors PD98059 and U0126. Brushing (innocuous stimulus) was used to investigate its effect on ERK activation under the incision-evoked painful condition.

Results: The anxiety-like behavior induced by the hind paw incision persisted longer than mechanical hypersensitivity. One hind paw incision resulted in a biphasic ERK activation in bilateral ACC. Inhibiting ERK activation in the early phase of postoperative pain, pain-related anxiety and mechanical hypersensitivity whereas inhibiting ERK activation in the late phase only reduced the anxiety-like behavior. During the time interval between two phases of ERK activation, brushing the incised skin dramatically increased ERK phosphorylation in the ACC.

Conclusions: These data suggest that in the early phase of postoperative pain, pain-related anxiety and mechanical hypersensitivity are tightly linked and regulated by the ERK activity in the ACC. However, in the late phase of postoperative pain, ERK activation in the ACC is only required for the expression of pain-related anxiety but not mechanical hypersensitivity.

PAIN is a multifaceted and highly personal experience. It is generally viewed according to two dimensions: the sensory and affective dimensions. The sensory-discriminative dimension involves stimulus localization and intensity and is assessed in a number of ways, including the visual analog scale, whereas the affective-motivational dimension involves the affective component of pain and is measured by rating unpleasantness. In a chronic pain state, the negative affect including anxiety and depression is well known to accompany the pain perception during disease progression.
The pain-related negative affect is more disabling than pain itself and has a severe effect on the daily activities in chronic pain patients. Despite extensive studies about the role of negative affect and its relation with the pain perception in the chronic pain, less is known about the role of negative affect especially anxiety in surgical pain and underlying mechanisms.

Compared with chronic pain, postoperative pain is more transitory and a major concern for perioperative management. However, studies of postoperative management have placed emphasis on seeking novel therapies for better pain perception control and fewer side effects even though alleviation of preoperative anxiety was reported to reduce postoperative pain perception and opioid consumption. Our recent study demonstrated that hind paw incision also induced an anxiety-like behavior along with the mechanical hypersensitivity, a hallmark of pain perception. Moreover, the expression of anxiety-like behavior persisted longer than that of mechanical hypersensitivity, suggesting that there may be a dissociation of anxiety-like behavior from mechanical hypersensitivity in response to a surgical incision. Supporting this assumption, our study further showed that both gabapentin and morphine attenuated pain-related anxiety and mechanical hypersensitivity evoked by incision; however, combined use of gabapentin and morphine had an additive analgesic effect but not an additive anxiolytic effect. Previous clinical studies also show that a low dose of morphine (0.04–0.06 mg/kg) significantly reduces the affective but not the sensory aspect of pain. The mechanism underlying the dissociation of sensory and affective aspects in incisional pain remains to be determined.

Accumulating evidence suggests that anterior cingulate cortex (ACC) is implicated in the processing of affective pain. In patients with chronic pain, a surgical lesion of the ACC attenuates the pain-related depression and unpleasantness. In the experimental rats, ablation of ACC abolishes the formalin-induced conditioned place avoidance. More recent studies further demonstrate that the activations of extracellular-signal-regulated kinase (ERK) and N-methyl-D-aspartate receptor in the ACC may contribute to the pain-like aversion in response to formalin injection. On the other hand, ACC is believed to be an important cortical region regulating the sensory nociceptive information in the chronic pain state (pain after amputation) and under nociceptive electric stimuli. Furthermore, blocking protein kinase Mζ in the ACC alleviates the neuropathic pain hypersensitivity. Thus, ACC may act as a substrate to link the affective and sensory aspects of pain.

Extracellular signal-regulated kinase (ERK), a family member of mitogen-activated protein kinase, has been implicated in the pain hypersensitivity in the spinal cord and learning and memory in the hippocampus. Recent studies have shown that the ERK is activated in the ACC after tissue or nerve injury. For example, persistent increased ERK phosphorylation in the ACC is observed in formalin-induced pain, and the up-regulation of phosphorylated (p-) ERK contributes to the induction and expression of pain affect in this chemical inflammatory pain. Moreover, p-ERK expression in the ACC is also highly expressed in the phantom pain model, and the up-regulation of p-ERK is enhanced in the synaptic sites during touch-evoked allodynia under this painful condition. All of these data suggest that ERK activity is important for synaptic plasticity in the ACC during the induction and expression of various types of pain.

Given that ERK activity in the ACC is important for the induction and expression of pain affects, the current study hypothesized that ERK activity in the ACC contributed to the development of pain-related anxiety and mechanical hypersensitivity in incisional pain. In the current study, we demonstrated that hind paw incision induced a biphasic activation of p-ERK in the bilateral ACC. More importantly, the ERK activation in the first phase contributed to the induction of anxiety-like behavior and mechanical hypersensitivity evoked by incision. However, the ERK activation in the second phase only contributed to the expression of pain-related anxiety but not mechanical hypersensitivity.

Materials and Methods

Animals and Reagents

The study was carried out on male Sprague-Dawley rats (150–250 g) obtained from Central South University Animal Services (Changsha, China). The experimental protocol was approved by the Animal Care and Use Committee of Central South University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of rats used and their suffering. PD98059 (10 mM) and U0126 (2 mM) were purchased from Sigma Chemical Company (St. Louis, MO) and were dissolved in 10% and 35% dimethylsulfoxide, respectively, according to the description of the manufacturer for intra-ACC injection.

Surgical Preparation

The right hind paw surgical incision in the rats was established under 1.5% isoflurane anesthesia as described previously. In brief, a 1-cm-long longitudinal skin incision was made, and the plantaris muscle was increased with the blade. The skin was then sutured and antibiotic ointment applied to the plantar hind paw. The sham-operated animals underwent the same procedure except that the incision was not carried out. A thoracic incision model was also performed to measure the behavior study as previously reported by us.

Elevated Plus-maze Test

Anxiety-like behavior was tested by elevated plus-maze (EPM) test as described previously. Briefly, after habituation of approximately 1 day in the experimental room, rats were placed in the middle of an apparatus comprising two
open arms and two closed arms that extended from a common central platform. The behavior was recorded by a video camera for 10 min. The percentage of time spent in open arms was recorded and used for statistical analysis.

Open Field Test
An open field test was undertaken as described by others previously. Briefly, animals were placed directly into one corner of the open field (120 cm × 120 cm), which was divided into a grid of 8 × 8 squares. Movement of the animal in the area was recorded during the 10-min testing session. An observer blind to the experimental conditions recorded the movement through the videotapes. Exploration was defined as the number of squares crossed during the testing session.

Nociceptive Testing
Mechanical allodynia was assayed using nylon von Frey filaments as described by Chaplan et al. The experiment was performed by two authors who were blind to the experimental treatment. Rats were placed on wire mesh platforms in clear cylindrical plastic enclosures, and filaments were applied to the center of the plantar surface of the unincised hind paw or on the wound edge of the incised hind paw. Withdrawal of the hind paw from the floor was scored as a response. When no response was obtained, the next stiffer filament in the series was applied to the same paw; if a response was obtained, a less stiff filament was next applied. Paw withdrawal threshold was estimated based on the testing proceeded in this manner.

Intra-ACC Drug Infusions
Rats were anesthetized with intraperitoneal injection of chloral hydrate (40 mg/kg) and securely placed into a stereotactic device with bregma and lambda at a horizontal level. Two 30-gauge stainless steel cannulae with 33-gauge stainless steel stylet plugs were bilaterally implanted 0.5 mm above the ACC injection site [anteroposterior + 2.6 from bregma, mediolateral ± 0.6, dorsoventral −2.5] according to the atlas of Paxinos and Watson (1998). The cannulae were anchored to the cranium with stainless steel screws and dental acrylic. Animals were allowed to recover for 5 days. For the rats with immunohistochemistry, the cryostat sections were used with cresyl violet staining to verify cannula position and injection site.

Microinjection was performed through a 33-gauge stainless steel injection cannula that was connected to a PE-10 tubing. A total volume of 0.5 µl per hemisphere of either vehicle or drugs was infused more than 5 min. After injection, the cannula was kept in place for an additional 5 min to minimize the drugs leaking out through the injection track.

Nissl (Cresyl Fast Violet) Staining
To identify the injection site into the ACC, a transverse section of the brain was cut for Nissl staining in an independent set of rats with intra-ACC injection of drugs or vehicle. The frozen transverse sections were then immersed in cresyl fast violet staining solution at 60°C for 5–10 min followed by differentiation in 70% ethanol. The sections were dehydrated in an ascending series of ethanol and passed through xylene before a coverslip with Permount as mountant was placed. They were then viewed under a Nikon (Tokyo, Japan) light microscope.

Immunohistochemical Protocol
At indicated time points, one set of rats was sacrificed by overdose of chloral hydrate (80 mg/kg) after behavior studies. The brains from the control and experimental rats were fixed for 4 h with 4% paraformaldehyde after perfusion and cryoprotection by immersion in 20% sucrose in phosphate buffer (pH 7.4) overnight. Transverse sections of the brain were cut at cryostat and mounted on 3-aminopropyl triethoxysilane-coated slides, and mouse anti-p-ERK antibody (dilution 1:200; Cell Signaling Technology, Danvers, MA) was incubated at room temperature overnight. The secondary reagents used for localization were biotinylated goat antimouse immunoglobin and ABC kit (Vector Laboratories, Burlingame, CA). The diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Company) was used as a peroxidase substrate. To define the density of p-ERK immunoreactivity, relative optic density of positive staining was performed using HPIAS-1000 image analysis (Tongji Qingping Company, Wuhan, China) as described in our recent studies. The measurements were performed by an author who was blind with respect to treatments.

Western Blot
After behavioral tests, another set of rats was sacrificed by overdose of chloral hydrate and the brains removed rapidly. The ACC was dissected on ice using a surgical blade according to the atlas of Paxinos and Waston (1998) followed by nitrogen quickly frozen in liquid. Frozen samples were homogenized in a lysis buffer containing protease inhibitors cocktails (Roche Applied Science, Mannheim, Germany) and PMSF (Sigma Chemical Company). Samples were then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatants were used for Western blotting.

Equal amounts of protein (approximately 50 µg) were loaded and separated in 10% Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. The resolved proteins were transferred onto polyvinylidene difluoride membrane (Amersham Biosciences Corporation, Piscataway, NJ). The membrane was blocked in 10% nonfat milk for 2 h at room temperature and incubated overnight at 4°C with mouse anti-phospho-ERK (p-ERK1/2; 1:1000, Cell Signaling Technology), mouse anti-ERK (total ERK1/2; 1:2000, Cell Signaling Technology) and mouse anti-p-ERK antibody (dilution 1:1000). The membrane was incubated with horseradish peroxi-
idase (1:2,000, Pierce, Rockford, IL) for 2 h at room temperature followed by exposure onto x-ray films for 1–10 min.

Quantitation and Statistical Analysis

For the quantification of Western blot signals, x-ray films with blotted bands were scanned. BIORAD Image Analysis System (Bio-Rad Laboratories, Inc., Hercules, CA) was then used to measure the integrated optic density of the bands. For the quantification of immunoreactive signals, six nonadjacent sections (30 μm) through the ACC were randomly selected. The numbers of p-ERK-labeled cells were counted inside the optic field using a computerized image analysis system (Leica Qwin 500, Leica, Wetzlar, Germany). Four to five rats were included in each group for semiquantification of Western blot and immunohistochemistry results. For behavioral data, all animals survived and were subjected to the behavior tests.

Statistical analysis was performed using software SPSS 13.0 (SPSS Inc., Chicago, IL) and Prism (Graphpad Software, San Diego, CA). Data are presented as mean ± SD. Differences between groups were compared using one-way or two-way ANOVA followed by post hoc Dunnett testing or Tukey post hoc multiple comparison test where appropriate. Unpaired two-tailed Student’s t test was used if only two groups were applied. A value of P < 0.05 was considered as significant difference.

Results

Effect of Surgical Incision on Anxiety-like Behavior and Mechanical Pain Hypersensitivity

Our recent study showed that the hind paw incision induced the anxiety-like behavior as determined by the EPM experiment and open field test. The incision-evoked anxiety-like behavior lasted longer than the incision-induced mechanical allodynia, a hallmark of pain hypersensitivity induced by surgery. Consistent with this finding, the hind paw incision rapidly induced mechanical hypersensitivity and anxiety-like behavior. At postoperative hour 1, there was an approximately 80% decrease in paw withdrawal threshold (P < 0.01, fig. 1A) and an approximately 65% decrease in time spent in open arm (P < 0.01, fig. 1B) compared with the sham-operated rats. At 5 days after operation, the paw withdrawal threshold levels in the incised rats were comparable with those in the sham-operated rats (P = 0.52 vs. sham operation, fig. 1C). However, at this time point, there was still an approximately 50% decrease in the time spent in open arm in the incised group (P < 0.05, fig. 1D). The open field test also showed the reduced time spent in the inner area in response to the incision (P < 0.05, fig. 1E). No significant difference was observed in the total travel distance between the incised group and the sham operation (P = 0.26, fig. 1F).

Fig. 1. Effects of the surgical incision on mechanical nociceptive threshold (A and C) and anxiety-like behavior (B, D, E) in rats. A dramatic decrease of PWT is observed in the rats at postoperative hour 1 (Sham, n = 6; Inci, n = 6; A) but not at postoperative day 5 (Sham, n = 6; Inci, n = 7; C). Time spent in open arm is significantly less in the rats at postoperative hour 1 (Sham, n = 6; Inci, n = 6; B) and postoperative day 5 (Sham, n = 6; Inci, n = 7; D) as determined by EPM experiment. (E) The decreased time spent in inner area in the rats at postoperative day 5 (Sham, n = 6; Inci, n = 7) as measured by open field test. Open field test revealed no significant difference in the total travel distance between the incised rats and the sham operation (Sham, n = 6; Inci, n = 7; F). Inci, incised group; PWT = paw withdrawal threshold; EPM = elevated plus maze. * P < 0.05 versus sham operation; ** P < 0.01 versus sham-operation. Unpaired two-tailed Student t test.

Biphasic ERK Activation in the ACC after Surgical Incision

It is well documented that ERK1/2 phosphorylation in the ACC is involved in the affective and sensory dimensions in chronic pain. We examined whether a surgical incision also induced ERK phosphorylation in the ACC. Our preliminary experiment showed no significance in p-ERK expression among the sham-operated control rats with different time points. Thus, the sham-operated control rats at different time points were combined as the sham-operated control rats for the quantitative analysis. As shown in figure 2, mild immunoreactivity for p-ERK was observed in the ACC in the sham operation (fig. 2, A and J). The expressed p-ERK was mainly localized in the pyramidal-shaped neurons. One-way ANOVA analysis revealed that there was a significant difference in the p-ERK positive staining in bilateral ACC at the different time points after the incision and the sham-operated groups (ipsilateral, F7,32 = 35.14, P < 0.01; contralateral, F7,32 = 36.21, P < 0.01). The following Dunnett post hoc test showed that increased number of p-ERK labeled cells was detected at postoperative min 15 (44.2 ± 10.62 vs. 14.2 ± 5.59 for the ipsilateral side and 40.5 ± 8.52 vs. 12.2 ± 6.54 for the contralateral side, fig. 2, B and K-L) and...
Postoperative min 30 (fig. 2C). The increased p-ERK expression was transient and returned to the control level at 1 h after surgical incision (fig. 2D and E). Interestingly, p-ERK expression was increased again at postoperative hour 6 (ipsilateral, 67.8 ± 10.35; contralateral, 56.5 ± 9.54) and sustained up to 3 days after incision (ipsilateral, 84.6 ± 10.62; contralateral, 78.6 ± 15.66, fig. 2, F–I, K, and L). Western blot was conducted to further confirm the upregulation of p-ERK in the ACC after hind paw incision (fig. 3A and B). There was no significant difference in total ERK expression at the different time points after incision (F9,27 = 0.98, P = 0.45, fig. 3C). Analysis of Western blot also showed that the activation of p-ERK presented the biphasic phases, in which the first phase was from postoperative min 15 to min 30, and the second phase of activation was from postoperative hour 6 (0.99 ± 0.17 vs. 0.72 ± 0.12, sham operation) and maintained up to postoperative day 5 (0.99 ± 0.08 vs. 0.72 ± 0.12, sham operation) (fig. 3B).

**ERK Activation in the First Phase Contributed to the Induction of Anxiety-like Behavior and Mechanical Hypersensitivity in Response to Incision**

Given that surgical incision induced a biphasic ERK activation in the ACC, a critical brain region implicated in the affective response to noxious stimuli, we postulated that the biphasic ERK activation may distinctly contribute to the induction and expression of pain-related negative emotion and mechanical hypersensitivity. To investigate the role of ERK activation in the ACC in pain-related anxiety and mechanical hypersensitivity, two mitogen-activated protein kinase inhibitors, U0126 and PD98059, were administered through the cannulae implanted into the bilateral ACC (fig. 4A). As previous studies...

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**Fig. 2.** Effect of the surgical incision on the expression of p-ERK in neurons of the ACC. (A–J) Representative photomicrographs showing the p-ERK immunoreactivity in the ACC at different time points (15 min, 30 min, 1 h, 6 h, 24 h, and 72 h) after hind paw incision or sham operation (15 min and 24 h). Surgical incision biphasically increases the expression of p-ERK in the ACC. (I) Image of higher magnification in the inset of H. (K and L) Time course of the number of p-ERK-labeled cells in the ipsilateral side (K) and contralateral side (L) of ACC after hind paw incision (n = 4–5 for each time point). Bar, 200 μm. ACC = anterior cingulate cortex; p-ERK = phosphorylated extracellular signal-regulated kinase. **P < 0.01 versus sham-operation. One-way ANOVA followed by Dunnett post hoc test.
have reported that 2 nmol U0126 or 10 nmol PD98059 could successfully inhibit ERK phosphorylation,23 the current study thus used the same dose to block ERK activation. Indeed, in the vehicle-treated incised rats, the band of Western blot of p-ERK was increased again and the increase is sustained for 5 days after incision. \( P < 0.05 \) versus sham operation; \( n = 4–5 \) for each group. GAPDH as the loading control.

Effect of the surgical incision on the expression of p-ERK

As shown in figure 5, the pretreatment by U0126 or PD98059 (inhibiting the first phase of p-ERK activation) significantly increased the paw withdrawal threshold induced by incision (17.92 ± 6.99, U0126 and 20.36 ± 6.46, PD98059 vs. 7.94 ± 6.32, vehicle treatment, \( F_{2,16} = 5.97 \), fig. 5A). In the U0126- and PD98059-treated groups, the time spent in the open arm (15.4 ± 9.17, U0126 and 13.47 ± 10.98, PD98059, vs. 3.92 ± 2.11’ vehicle treatment, fig. 5B) and the time spent in the inner area (13.89 ± 0.05, U0126 and 12.90 ± 11.42, PD98058, vs. 2.05 ± 3.81 vehicle, fig. 5C) were significantly longer than those in vehicle-treated groups in response to surgical incision. Notably, the partial reversal of incision-induced anxiety-like behavior was not due to the increased locomotor activity because there was no significant difference of total travel distance among all the groups (\( F_{2,16} = 0.09, P = 0.91 \)). An incision on the thoracic region also triggered a similar anxiety response (data not shown).

**ERK Activation in the Second Phase Contributed to Pain-related Anxiety, But Not Mechanical Hypersensitivity**

To further determine the role of the ERK activation in the second phase, U0126 was administered into ACC through an implanted catheter at postoperative hour 6. After 1 h of inhibitor injection, animal behaviors were examined. Two-way ANOVA analysis followed by the Tukey post hoc multiple comparison test showed that there was no significant difference in the paw withdrawal threshold levels between vehicle-treated and U0126-treated group at the same time points (baseline and postoperative hour 6, fig. 6A). This finding suggests that blocking the ERK activation in the second phase has no effect on mechanical hypersensitivity. In contrast, EPM experiment (11.40 ± 1.98, U0126 vs. 6.13 ± 1.29’ vehicle, \( P = 0.04 \), fig. 6B) and open field test (7.26 ± 0.89, U0126 vs. 2.65 ± 0.48, \( P < 0.01 \), fig. 6C) showed that intra-ACC infusion of U0126 at this time point could partially reverse the reduced time spent in open arm and inner area. The total travel distance in the U0126-treated group was not significant compared with that in the vehicle-treated group (fig. 6D).
subjected to brushing (0.96 p-ERK in the ACC was greatly increased in the incised rats activated. As shown in figure 7A and B, the expression of p-ERK expression in the ACC was significantly increased in the incised rats subjected to brushing (0.96 ± 0.08) compared with the sham-operated groups subjected to brushing (0.63 ± 0.03) or incision only (0.66 ± 0.04) without affecting the total ERK expression when normalized to glyceraldehyde-3-phosphate dehydrogenase (F2,9 = 0.57 ± 0.07, P = 0.57). The immunohistochemical analysis showed that the up-regulated p-ERK expression was mainly localized in pyramidally shaped neurons (fig. 7C–E).

**Effect of Brushing the Incised Skin on the Expression of p-ERK During the Interphase of ERK Biphasic Activation**

The incision rapidly induced the biphasic ERK activation in the ACC (the first phase), which contributed to the induction of both mechanical hypersensitivity and anxiety (fig. 5A). However, the first phase of ERK activation was transient and it returned to the basal level at postoperative hour 1, when the incision evoked intense allodynia. Previous studies have shown that in the chronic pain rat model, p-ERK in the ACC is activated in response to the nonnoxious mechanical stimuli, brushing. Given that allodynia, a painful response to a usually innocuous stimulus, is the hallmark of postoperative pain along with the spontaneous pain and hyperalgesia, we used brushing in the hind paw subjected to the incision and investigated whether p-ERK expression in the ACC was activated. As shown in figure 7A and B, the expression of p-ERK in the ACC was greatly increased in the incised rats subjected to brushing (0.96 ± 0.08) compared with the sham-operated groups subjected to brushing (0.63 ± 0.03)

**Discussion**

There are several important findings in the current study. First, the current study further confirmed our previous finding that the temporal expression of anxiety was not correlated with that of mechanical hypersensitivity after surgical incision. Second, one hind paw incision led to a biphasic activation of ERK in the ACC. Third, blocking ERK activation in the first phase could inhibit the induction of pain-related anxiety and mechanical hypersensitivity, whereas the second phase of ERK activation only contributed to the expression of pain-related anxiety but not mechanical hypersensitivity. Finally, innocuous stimuli (brushing) could induce the p-ERK activation in the resting phase of ERK activation in the ACC after the surgical incision.

**Dissociation of Pain Affect from Pain Perception at the Later Phase after the Surgical Incision**

Extensive studies have demonstrated that in chronic pain, the affective and sensory dimensions are developed together,
Fig. 7. Effect of brushing on the p-ERK expression in the ACC at postoperative hour 1. (A) Representative Western blot of ERK phosphorylation in the sham-operated group subjected to brushing (sh+Brush), group with 1 h after incision (Inci), group with 1 h after incision subjected to brushing (Inci+brush). (B) Semiquantitative analysis of Western blots shows the activation of p-ERK after brushing (n = 4 for each group). (C–E) Representative microphotographs of immunoreactivity for p-ERK in the ACC. Bar, 200 μm. ACC = anterior cingulate cortex; p-ERK = phosphorylated extracellular signal-regulated kinase. **P < 0.01 versus the group with 1 h postincision. One way ANOVA followed by Tukey post hoc test.

interact with each other, and contribute to the morbidity and progression of the pain state. However, few studies were used to investigate the role of the affective dimension in postoperative pain. Our recent study has observed that a surgical incision induced anxiety-like behavior as well as mechanical hypersensitivity, a hallmark of sensory dimension in pain. More interestingly, the expression of anxiety-like behavior induced by the incision lasted longer than that of mechanical hypersensitivity. In line with our recent findings, the rats with hind paw incision spent much less time in the open arm in the EPM experiment and the inner area in the open field respectively test at postoperative day 5 when the mechanical hypersensitivity was diminished. These findings suggest that there is a dissociation between the expression of pain-related anxiety and pain perception in incisional pain. The dissociation of anxiety from pain perception observed in the current study is also observed in chronic back pain patients. In a previous human functional image study, N-acetyl aspartate as a neuronal marker was measured by proton magnetic resonance spectroscopy and correlated with pain perception or anxiety. The authors observed that the mapping between brain regions variation of N-acetyl aspartate and pain perception was different from that between the same brain regions variation of N-acetyl aspartate and anxiety. Thus, it is suggested that there be a dissociation of pain perception and anxiety in patients with chronic back pain. However, recent experimental studies showed that neuropathic pain could induce the development of anxiety-like behavior, which is positively correlated with that of mechanical hypersensitivity. This discrepancy may be due to the different study designs (clinical trial and animal studies).

Notably, the induction of mechanical hypersensitivity and anxiety-like behavior by incision was rapid and simultaneous in the current study. Anatomically, the noxious stimulus from the peripheral tissue is transported into the brain area through two ascending nociceptive pathways, the spinothalamic and nociceptive pathways that feed the area of the brain concerned with affects, and the spinothalamic pathway that probably relays nociceptive information to areas of cortex. In the current study, the fact that both aspects (affective and sensory) of pain were rapidly induced by the incision suggests that in the early phase of incisional pain, the pain-related anxiety and perception may be tightly linked, i.e., the noxious stimulus induced by incision activates the distinct brain regions involving the affective and sensory aspects of pain, respectively. However, once activated, the activity of these brain areas may not be dependent on the noxious stimulus, resulting in the dissociation of anxiety and perception in the late phase of postoperative pain.

The Biphasic ERK Activation in the ACC Distinctly Regulates Affective and Sensory Aspects in Incisional Pain

Several studies have shown that ERK in the ACC is activated and contributes to the affective aspect in the inflammatory and phantom pain. Similarly, the current study also found that the incision resulted in ERK activation in the ACC. However, the incision-evoked ERK activation is bi-phasic and different from those in inflammatory pain or neuropathic pain. The activation pattern of p-ERK in formalin-induced inflammatory pain is still not fully determined. In a recent study by Wei and Zhuo, p-ERK in the ACC was observed to be transiently up-regulated in the formalin test. However, a more recent study reported the persistent up-regulation of p-ERK up to 24 h in the rostral ACC after formalin hind paw injection. The different expression profiles of p-ERK in the ACC in these two studies may be due to the different time courses (90 min vs. 24 h) and subregions of ACC. Notably, rostral ACC is closely associated with pain-related negative affect because destruction of neurons originating from rostral, but not caudal, ACC reduces formalin-induced conditioned place avoidance. In the current study, ERK activation in the first phase returned to the baseline at postoperative hour 2 and is consistent with Wei and Zhuo’s study. The fact that ERK inhibitors attenuated the induction of pain-related anxiety and mechanical hypersensitivity indicates that the first phase of ERK activation in the ACC contributes to the affective and perceptive aspects of pain. The finding that ERK activation in the ACC modulated pain perception after surgical incision is in agreement with the recent studies showing that ACC contributed to the transient nociceptive response in the complete Freund’s adjuvant injection-induced inflammatory pain. In addition, a recent study also reported that ACC plays an
important role in the short-term but not long-term nociceptive information processing. In contrast, other studies reported that ERK activation in the ACC has no effect on biphasic nociceptive response in the formalin test. This discrepancy may be due to different types of injury and/or different time course studied. In this regard, complete Freund’s adjuvant injection into the hind paw may lead to a different noxious stimulus with the formalin injection. Taken together, the findings of the current study suggest that the induction of ERK phosphorylation in the ACC by the incision modulates the two aspects of pain that may be tightly coupled in the early phase of incisional pain.

On the other hand, the reappearance of ERK activation in the late time course may be correlated with those in phantom pain or neuropathic pain. In these two types of pain, ERK activation was observed 14 days after amputation and 3 days after chronic constriction nerve injury, respectively. The persistent activation of p-ERK in the ACC in the later time course may suggest the occurrence of central plasticity in synapses of the ACC because p-ERK activation is critical for the long-term potentiation in the ACC. In the current study, the finding that blocking ERK activation in the second phase only reduced pain-related anxiety but had no effect on mechanical hypersensitivity strongly indicates that ERK activation in the ACC in late phase only contributes to the affective dimension. This finding also supports the dissociation of pain-related anxiety and mechanical hypersensitivity in the later time course of the incisional pain.

**Effect of Brushing on the ERK Activity in the ACC in the Interphase of the Biphasic ERK Activation**

In the current study, there was a time interval when p-ERK expression returned to the basal level of sham operation during the biphasic ERK activation. The existence of the resting phase suggests that the primary afferents alone from injured tissue may not be sufficient to activate the p-ERK expression in the ACC. Interestingly, the innocuous stimulus brushing greatly up-regulated p-ERK expression in the ACC. Consistent with this finding, brushing also increased ERK activation in the ACC in the phantom pain. These results strongly indicate that under a painful condition, an innocuous stimulus can induce strong activity-dependent neural plasticity in the ACC. The enhanced p-ERK expression in neurons and their dendrites may contribute to the local synaptic plasticity or pain modulation during the development of alldynia in postoperative pain.

**Study Limitation and Clinical Implication**

There are several limitations in the current study. First, mechanical hypersensitivity was examined as the indicator of pain perception whereas two other common features of postoperative pain, guarding pain and thermal hyperalgesia, were not tested and correlated with the anxiety-like behavior in the current study. Guarding pain after incision is believed to reflect pain at rest after surgery and correlates well with spontaneous activity in the dorsal horn neurons. However, in the current study, the biphasic activation but not persistent activation of p-ERK in response to the incision suggests that the spontaneous activity alone may not be sufficient to induce ERK activation in the ACC that is required for pain-related emotional response. Second, EPM and open field tested were used to measure the anxiety-like behavior in the current study. The observation that the decreased time in the open arm in response to incision may be argued by the possibility of decreased locomotor activity due to touch-evoked pain (mechanical hypersensitivity). In the current study, the locomotor activity was not examined by the classic experiment, the rotarod test. Instead, the total travel distance was calculated in the open field test, which may also reflect the locomotor activity indirectly. There is no statistical difference in the total travel distance between incised and sham-operated rats, suggesting that pain did not affect overall activity. In addition, intra-ACC injection of the ERK inhibitors during the second phase of ERK activation only increased the time spent in the open field but did not affect mechanical hypersensitivity. All of these findings strongly suggest that the decreased time spent in open arm and inner area is due to pain-related anxiety.

The finding that pain-related anxiety is dissociated from mechanical hypersensitivity at the later time course after an incision suggests that the negative emotional response should be considered in the treatment of postoperative pain. It is well documented that preoperative anxiety contributes to postoperative pain and morphine consumption. Thus, treatment of preoperative anxiety is believed to be important for the management of postoperative pain. However, current postoperative pain management is mainly focused to alleviate the sensory pain. Our study suggests that treatment of anxiety induced by surgical trauma also may be important for the management of postoperative pain. In this regard, consecutive oral administration of gabapentin during the perioperative period of coronary artery bypass graft surgery provided better postoperative pain control up to 1 month after surgery.

In conclusion, the current study showed that the hind paw incision induced a biphasic activation of ERK in the ACC. The ERK activation in the first phase contributed to the induction of pain-related anxiety and mechanical hypersensitivity whereas the ERK activation in the second phase only contributed to the expression of anxiety. The fact that the dissociation of affective and sensory aspects in the late time course of postoperative pain strongly suggests that the treatment of pain-related anxiety also may be important for the management of postoperative pain clinically.

**References**

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