Glutamatergic activation of anterior cingulate cortex mediates the affective component of visceral pain memory in rats

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ABSTRACT

Studies of both humans and animals suggest that anterior cingulate cortex (ACC) is important for processing pain perception. We identified that perigenual ACC (pACC) sensitization and enhanced visceral pain in a visceral hypersensitive rat in previous studies. Pain contains both sensory and affective dimensions. Teasing apart the mechanisms that control the neural pathways mediating pain affect and sensation in nociceptive behavioral response is a challenge. In this study, using a rodent visceral pain assay that combines the colorectal distension (CRD)-induced visceromotor response (VMR) with the conditioning place avoidance (CPA), we measured a learned behavior that directly reflects the affective component of visceral pain. When CRD was paired with a distinct environment context, the rats spent significantly less time in this compartment on the post-conditioning test days as compared with the pre-conditioning day. Effects were lasted for 14 days. Bilateral pACC lesion significantly reduced CPA scores without reducing acute visceral pain behaviors (CRD-induced VMR). Bilateral administration of non-NMDA receptor antagonist CNQX or NMDA receptor antagonist AP5 into the pACC decreased the CPA scores. AP5 or CNQX at dose of 400 mM produced about 70% inhibition of CRD-CPA in the day 1, 4 and 7, and completely abolished the CPA in the day 14 after conditioning. We concluded that neurons in the pACC are necessary for the “aversiveness” of visceral nociceptor stimulation. pACC activation is critical for the memory processing involved in long-term negative affective state and prediction of aversive stimuli by contextual cue.

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1. Introduction

Human brain imaging studies have revealed new roles of cortical neuronal networks in chronic visceral pain (Mayer, Naliboff, & Craig, 2006). Studies of both humans and animals consistently suggest that ACC and its related area are important for processing pain perception (Sikes & Vogt, 1992; Traub, Silva, Gebhart, & Solodkin, 1996). Our series of published observations characterized neural electrophysiological activity of ACC during processing of visceral nociceptive stimulation (Gao, Wu, Owyang, & Li, 2006). We have identified that perigenual ACC (pACC) sensitization (Gao et al., 2006) and enhanced visceral pain in a visceral hypersensitive rat model with colonic anaphylaxis evoked by intraperitoneal injection of chicken egg albumin (EA) (Cao, Wu, Chen, Owyang, & Li, 2008). Allodynia and hyperalgesia in these rats appear to be mediated by enhanced glutamate N-methyl-D-aspartate (NMDA)-receptor 2B (NR2B-receptor) activities (Fan et al., 2009; Wu et al., 2008). Hypersensitivity to colorectal distension (CRD) can be observed up to 7 weeks after the initiation of colonic anaphylaxis and is independent of mucosal inflammation suggesting mediation by a mechanism for learning and triggering of pain memories in the medial thalamus-ACC neuronal circuitry (Fan et al., 2009).

Pain contains both sensory and affective dimensions. Except for human experiments where self report is possible, teasing apart the mechanisms that control the neural pathways mediating pain affect and sensation in an overall animal nociceptive behavioral response is a challenge. It is well documented that the ACC is involved in pain processing and encoding of negative affects in humans, which results in pain-related unpleasantness (Tolle, Kaufmann, Siessmeier, et al., 1999). Research suggests that ACC neuronal activity in rodents is related to stimulus–reward learning (Bussey, Everitt, & Robbins, 1997). However, the role of the ACC in the mediation of sensory and affective components of visceral pain is not easily distinguished in animal experimental study. Recently, Johannsen, Fields, and Manning (2001), Johansen and Fields (2004) introduced a formalin-induced conditioned place avoidance (F-CPA) model to distinguish somatic pain emotion from pain sensation in rats. Until now the visceral pain-related affective processing, and the role of ACC in mediating long-term visceral pain aversion have not been investigated.

Noxious visceral stimuli such as colorectal distension (CRD) produce vigorous cardiovascular and visceromotor responses (VMR) (Ness & Gebhart, 1998). CRD also produces avoidance
behavior in rats (Ness & Gebhart, 1998) and pain in humans (Lipkin & Sleisenger, 1957). Using a rodent visceral pain assay that combines the CRD model with the conditioned place avoidance (CPA) paradigm, we measured a learned behavior that directly reflects the affective component of visceral pain (CRD-induced CPA). We hypothesize that pACC plays a critical role in the mediation of pain-affective processing, learning and memory in rats under physiological state. Findings described herein show that rats subjected to visceral pain insult display a significant place aversion to a pain-associated environment that persistent up to 14 days in the absence of any additional conditioning. Destruction of pACC neurons had no effects on acute visceral pain responses measured by CRD-induced VMR. However, ACC lesion significantly reduced CPA scores suggesting that the ACC are necessary for the “aversiveness” of visceral nociceptor stimulation, which reflects the affective pain component. Moreover, pre-training microinjection of AMPA receptor antagonist CNQX into pACC blocked the acquisition of CPA learning suggesting activation of the glutamate AMPA receptors are required for long-term visceral aversive information processing in the physiological state.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich (St Louis, MO). All protocols were approved by the Institutional Animal Care and Use Committee at the City University of Hong Kong and the Special Health Service Division Department of Health Hong Kong. Subjects were adult male Sprague–Dawley rats (275–300 g). For surgical preparations, rats were anesthetized with a mixture of xylazine and ketamine according to the protocol described in our previous publication (Li, Wu, Zhu, et al., 2003).

2.1. Visceromotor Response (VMR) to colorectal distension (CRD)

Details of this protocol were described in our previous publications (Chen et al., 2008, 2009). Briefly, 32-gauge stainless steel wires were implanted in the external oblique pelvic muscles 4–6 days before the beginning of the experimental procedures. Graded-pressure CRD (40, and 60 mm Hg) was produced by rapidly injecting saline into a colonic balloon over 1 s and maintaining the distension for 30 s. The results of electromyography were quantified by calculating the area under the curve (AUC), which is the sum of all recorded data points multiplied by the sample interval (in seconds) after baseline subtraction.

2.2. Conditioned place avoidance (CPA)

Place conditioning apparatus (Johansen et al., 2001) consisted of three wooden compartments (45 × 45 cm, one neutral compartment and two conditioning compartments with distinctive visual cues with removable doors to allow room isolation when necessary). One conditioning compartment had horizontal stripes on the walls and an odor of 1.0% acetic acid, whereas the other had vertical stripes and standardized cinnamon scent associated with it. Walls of uniform color characterized and no distinctive odor characterized the neutral compartment. The experimental process consists of three distinct sessions: a pre-conditioning session (days 1), conditioning session (day 2–5), and post-conditioning session (e.g., test days, 1, 4, 7, 10 and 14 days after conditioning day 5). Rats were handled by the experimenter for habituation purpose on each of 2 days before behavioral testing. On the first day, rats were individually placed in the neutral compartment and were allowed to explore the two conditioning compartments.

1. Pre-conditioning Day (Day 1). On day 1, the entrance connected to each compartment was opened. Each rat was allowed to move freely throughout the entire apparatus (i.e., all three compartments) for 20 min. The times spent by the rat in each compartment were recorded. Animal spending more than an 80% (time spent > 16 min) or less than 20% (time spent < 4 min) of the total time in a chamber were eliminated from further testing (approximately 15% of total animal).

2. Conditioning Days (Days 2–5). The conditioning phase of all experiments consisted of 4 days. In the morning, rats received nothing, and were randomly confined with one of the compartment for 45 min. In the afternoon, rats received treatment being paired with CRD 40 mm Hg or CRD 0 mm Hg in the other conditioning compartment for 45 min. A polyethylene tube (i.d., 1.67 mm) attached to a balloon (length, 40 cm) lightly coated with a surgical lubricant was placed in the colon and secured to the base of the tail. Colorectal distension (40 mm Hg) was produced by rapidly injecting saline into the colonic balloon over 1 s and maintaining the distension for 30 s with 3-min interval, and repeated five times. The pressure was regulated with the distention control device and monitored using a pressure transducer. 0 mm Hg CRD was served as sham treatment.

In separate groups of rats the effects of s.c. injection of U69,593 (a k-opioid receptor agonist) or s.c. vehicle were paired with a distinct compartment in a place conditioning apparatus.

1. Post-conditioning Days (1, 4, 7, 10 and 14 days after conditioning day (day 5), test days). The same trial was performed as pre-conditioning session (day 1). On day 1, 4, 7, 10, and 14 after the conditioning phase, each rat was allowed to move freely throughout the three compartments for 20 min with no aversive stimulus (CRD) present. The time spent in each compartment was recorded.

2.3. ACC lesions generated with ibotenic acid

Previous studies by other investigators demonstrated that neurons originating from the rostral, but not caudal, ACC are involved in somatic pain-related aversion (Johansen & Fields, 2004; Li, Ren, Xiao, et al., 2009; Ness & Gebhart, 1998). We reported that perigliallular pACC responsible for modulating visceral hyperalgesia in the visceral hypersensitive rats (Cao et al., 2008; Fan et al., 2009). Thus, in this study axon-sparing (excitotoxic) lesions were generated in the pACC as described previously (Cao et al., 2008). Briefly, 0.6 μL of ibotenic acid (0.5 mg/mL) was microinfused over a period of 6 min at each of the following coordinates in the rostral ACC: (1) anterior posterior (AP) + 4.2 mm from bregma, 0.7 mm lateral to midline (L), 2.5 mm ventral to brain surface; (2) AP + 3.0 mm, L + 0.8 mm, 2.5 mm ventral to brain surface. This procedure was repeated in the opposite hemisphere. Pain behavioral testing (VMR) or CPA conditioning was performed after a recovery period of 6 days. The extent of the lesions was determined by histologic studies, and reconstructions were made on charts derived from the atlas of the rat brain by Paxinos and Watson (1998).

2.4. Chronic ACC cannulation

For microinjection studies, chronic guide cannulae (33-gauge, Small Parts) were implanted using stereotaxic procedure described previously (Fan et al., 2009; Li et al., 2003). Double (1.2 mm spacing between barrels) stainless steel guide cannulae were implanted 1 mm above the ACC injection site (coordinates from Bregma: anterior/posterior (AP), +4.0 to +2.0 mm, dorsal/ventral (DV), +2.5 mm, medial/lateral (ML), +0.7 mm on each site. The VMR
experiments were performed 6 days postoperatively. All animals (lesion, microinjection) recovered normally from surgery as evidenced by a weight gain on the test day. The location of the point of termination of the cannula track was determined by histologic studies. Serial coronal sections (50 μm) were cut with a cryostat along the path of the cannula, mounted on gelatin-coated slides, and stained with thionine (Fan et al., 2009).

In separate group of rats the double stainless steel guide cannulae were implanted into the caudal ACC: coordinates from Bregma: anterior/posterior (AP), −0.05 mm to −0.6 mm, dorsal/ventral (DV), +2.5 mm, medial/lateral (ML), +0.7 mm on each site. The caudal ACC includes portions of postgenual Brodmann areas 24a and 24b (Vogt, Vogt, & Farber, 2003).

2.5. Glutamate antagonist studies

Using conditioned place aversion in rats, there were studies showing that glutamatergic activation of pACC is necessary to produce formalin-induced CPA (Johansen & Fields, 2004; Johansen et al., 2001). In this study, we examined the effects of NMDA and non-NMDA antagonists on CRD-induced conditional place avoidance. The rat was placed in an injection chamber. Injectors were inserted into the guide cannula after removal of the dummy cannula. The glutamate N-methyl-D-aspartate (NMDA) receptor blocker aminophosphonopentanoic acid (AP5; 200, and 400 mmol/L), and non-NMDA antagonists on CRD-induced conditional place avoidance. The doses of AP5 and CNQX were chosen in accordance to previous studies. The glutamate antagonist blocker cyanonitroquinoxaline dione (CNQX; 200, and 400 mmol/L) were administrated. A total volume of 60 nL per hemisphere was microinjected 10 min before CPA training. The same volume of vehicle (saline) was administrated into the ACC as control. The doses of AP5 and CNQX were chosen in accordance to previous studies, which showed that similar doses of glutamate receptor antagonists inhibited pACC pain-related aversion (Lei, Sun, Gao, Zhao, et al., 2004), avoidance (Mello e Souza, Roesler, Madruga, et al., 1999) and visceral pain responses in rats (Cao et al., 2008). Each rat served as its own control. Vehicle and a single drug were tested on each rat. Previous studies have shown that after microinjection of [3H][3-methyl-His2]-TRH (60 nL) into the preoptic nucleus, more than 75% of the radioactivity was found within a diameter of 600 μm from the injection site (Siren, Vonhof, & Feuerstein, 1991). Postoperative care was taken as previously described (Li et al., 2003).

2.6. Statistical analyses

Statistical comparisons of the VMR in various groups were made using one-way repeated measures analysis of variance, followed by multiple comparisons adjusted by the Bonferroni test using baseline values as a covariate and 2 main factors (i.e., distention level as the repeated factor and group as the independent factor) (Fan et al., 2009). Results were expressed as means ± SEM. P < 0.05 was considered statistically significant. For the CPA data, the amount of time spent in the conditioning compartment (i.e., compartment paired with CRD) on the post-conditioning days (i.e., test days) was subtracted from the amount of time spent in the same compartment on the pre-conditioning day. These processes produced a magnitude of CPA score for each rat. Magnitude of CPA scores for sham lesion vs. lesion animals were compared by using a Student’s t test. The differences in CPA scores among drug-treated groups were compared using two-way repeated-measures ANOVA followed by multiple comparisons adjusted by the Bonferroni test. In addition, the absolute times spent in the conditioning compartment on the pre-conditioning day vs. the post-conditioning days were presented.

3. Results

3.1. Visceromotor responses to colorectal distension

Under basal conditions (CRD, 0 mm Hg), there was no significant difference between normal control rats and conditioning training rats. Graded CRD pressures of 20, 40, and 60 mm Hg caused an increase in the number of muscle contractions to 1 ± 0.02, 20 ± 4, and 33 ± 7 in normal control and 0.5 ± 0.02, 22 ± 4, and 30 ± 5 contractions per 5 s in the rats after conditioning (Fig. 1). The mean amplitude of the electromyogram (AUC, in microvolts per second) is shown in Fig. 1B. These results provide evidence that the pain sensitivity has not been change in rats following 4 days CRD-conditioning training.

3.2. ACC lesions do not affect the visceromotor responses to distension

Three cytoarchitectural regions of the cingulated cortex have been identified in rats: the pACC with perigenual and subgenual parts, the midcingulate cortex, and the retrosplenial cortex (Vogt et al., 2003). A similar classification has been applied to the rabbit (Sikes, Vogt, & Vogt, 2008). In the present study, perigenual ACC (pACC) neurons were defined as the area corresponding to perigenual Brodmann area 24b, portions of perigenual 24a, and caudodorsal area 32 (Fan et al., 2009; Vogt & Peters, 1981). Areas of the rostral ACC are rich in nociceptive input, as reported in the literature (Gao et al., 2006; Johansen et al., 2001). It should be noted that the criteria used to define rostral and caudal ACC in current studies do not correspond to the terminology of ACC regions in the primates and humans (Mayer et al., 2006). Lesions were generated in the pACC of 6 normal rats and 8 rats before conditioning. One rat in the conditioning group died postoperatively. Histologic evaluation confirmed that lesions were successfully generated in the pACC. Examination showed that neuronal loss extended from 4.2 to 2.7 mm anterior bregma, destroying perigenual Cg1 and Cg2. Damage to the dorsal prelimbic cortex was detected in two rats (Figs. 1, 2 and 4). The infralimbic cortex, the posterior cingulated cortex, and the corpus callosum were not damaged. Lesions in the pACC did not change the VMR in normal control rats and the rats after 4 days conditioning (Fig. 1). These observations are consistent with our previous reports (Cao et al., 2008) suggesting that the pACC region of the forebrain is not involved in the mediation of behavioral visceral pain responses in the rats under physiological condition.

3.3. Conditioned place avoidance (CPA)

No initial preference for any of the 3 components in the place-conditioning apparatus was detected on the pretest days. When CRD (40 mm Hg) was paired with a particular compartment in the place-conditioning apparatus the ACC sham lesion rats spent significantly less time in this compartment on the post-conditioning test days as compared with the pre-conditioning test day. Effects were lasted for 14 days Magnitudes of CPA (pre-condition minus post-condition) are 398 ± 20, 246 ± 16, and 214 ± 23, 87 ± 9, and 34 ± 7 s in 1, 4, 7, 10, and 14 days after training, respectively. We show that pACC lesion did not alter CRD induced visceromotor responses compared with sham lesion rats (Fig. 1). However, pACC lesioned rats displayed significantly lower magnitude of CPA scores (113 ± 24, 85 ± 25, 73 ± 9, 34 ± 11, and 10 ± 4 in 1, 4, 7, 10, and 14 days after conditioning training in the lesion rats vs. 302 ± 18, 252 ± 26, and 226 ± 14, 98 ± 8, and 26 ± 5 in 1, 4, 7, 10, and 14 days after training in the sham lesion rats, respectively (Fig. 2A). Unlike the effects of pACC lesions, CRD did produce CPA scores that were not significantly different in caudal ACC lesion.
rats compared with caudal ACC sham lesion (323 ± 11, 255 ± 22, and 211 ± 8, 101 ± 7, and 28 ± 6 vs. 344 ± 23, 226 ± 21, 198 ± 5, 83 ± 11, and 22 ± 7 in 1, 4, 7, 10, and 14 days after conditioning training (Fig. 3). It appears that pACC lesion reduces the aversive aspect of the visceral pain responses without reducing acute CRD-induced pain behavioral reflex.

In the control group (0 mm Hg CRD, 4 rats), no significant differences of the time spent in the conditional compartment compared between the pre-conditioning (396 ± 24) and post-conditioning (377 ± 19 in day 1 and 407 ± 24 in day 4).

![Fig. 1. Visceromotor response (VMR) to graded intensities of CRD in normal control rats and the rats following conditioning training and brain lesion maps associated with rats with pACC lesions (5 control, and 5 after conditioning training). (A) Representative abdominal muscle electromyograms of the VMR to graded-pressure CRD recorded from the external oblique pelvic muscle in normal control rat and pACC lesion rat after conditioning training. (B) Effects of ablation of the rostral ACC using ibotenic acid on the VMR to graded-pressure CRD in normal control and conditioning rats. Mean amplitude of the abdominal muscle contraction expressed as AUC after baseline subtraction was presented. Data were collected from 4 control rats sham lesion, 5 conditioning sham lesion; 5 ACC lesion control rats, and 5 ACC lesion conditioning training rats. Analysis of variance showed no significant effect for distention level, as well as interaction between distention level and group. Values are presented as means ± SE. These results suggest that ablation of the rostral ACC did not affect CRD-induced acute pain-related behaviors in the rats following 4 days conditioning training. (C) Representations of the examples of the largest (gray) and smallest (black) lesions in the pACC lesion in the groups. Sections are in the coronal plane, numbers in mm to Bregma. (D) Statistical comparisons of the VMR in various groups were made by two-way repeated ANOVA, followed by multiple comparisons adjusted by Bonferroni test. Results were expressed as means ± SE. P < 0.05 was considered statistically significant. No significant was found in this study.

![Fig. 2. Conditioned place aversion produced by colorectal distension (40 mm Hg) (n = 5), the rats with pACC lesions (n = 5) and pACC sham lesions (n = 5). Data are represented as mean ± SEM. (A) For the CPA score the amount of time spent in the conditioning compartment on the post-conditioning days were subtracted from the amount of time spent in the same compartment on the pre-conditioning day. The rats spent less time in this compartment on the post-conditioning test day as compared with the pre-conditioning test day. Compared with sham lesion rats lower CPA scores were observed in the ACC lesion rats because they spent more time in the conditioning compartment after conditioning training. These results provide direct evidence that neurons in the ACC are necessary for visceral pain-related aversion learning. Results were expressed as means ± SE. Statistical significance was determined by using the appropriate Student t-test (paired or unpaired) *P < 0.05, **P < 0.01 Student’s t test, as compared with rats with sham lesion of the pACC. (B) Representations of the examples of the largest (gray) and smallest (black) lesions in the pACC lesion in the groups. Sections are in the coronal plane, numbers in mm to Bregma.]
3.4. U69,593-induced CPA

To clarify whether neurons in the pACC are responsible for specifically with aversiveness of visceral nociceptor-activating stimuli, or pACC is associated with aversive stimuli in general, we examine the effects of lesion of pACC on CPA induced by an aversive, but non-nociceptive-activating stimulus.

Mu-opioid receptor agonists function as rewarding stimuli, whereas agonists at kappa-opioid receptors induce aversive states. These motivational effects have been attributed to interactions of exogenous opioids with endogenous reward pathways in the brain. The \( \kappa \)-opioid receptor agonist U69,593, which is known to be aversive (Shippenberg, Bals-Kubik, & Herz, 1993) when injected systemically was administrated s.c. and paired with a distinct compartment in the apparatus. The conditioning procedure was similar to that used the CRD-induced CPA. The scores of rats in these studies are shown in Fig. 4. Unlike the CRD-CPA (Fig. 2), lesions of pACC did not reduce CPA elicited by systemic U69,593 (120 ± 17, 43 ± 8, and 117 ± 15, 48 ± 7 in the sham lesion and lesion rats, respectively, in 1 and 4 days after conditioning training Fig. 4). Thus, whereas, pACC lesion reduced CRD-induced CPA, a CPA elicited by a nociceptive stimulus (Fig. 2), pACC lesions did not affect CPA induced by non-nociceptive stimulus. Therefore, pACC lesions do not have a general disruptive effect on learning in the place-conditioning paradigm, but the lesions cause a deficit in the acquisition or expression of visceral pain-related CPA.

Fig. 3. Conditioned place aversion produced by colorectal distension (40 mm Hg) \((n = 5)\), the rats with caudalACC lesions \((n = 5)\) and caudalACC sham lesions \((n = 5)\). Data are represented as mean ± SEM. (A) For the CPA score the amount of time spent in the conditioning compartment on the post-conditioning days were subtracted from the amount of time spent in the same compartment on the pre-conditioning day. The rats spent less time in this compartment on the post-conditioning test day as compared with the pre-conditioning test day. Compared with sham lesion rat, CPA scores were not significantly different from the caudal ACC lesion rats. These results provide evidence that neurons in the caudal ACC are not responsible for visceral pain-related aversion learning. Appropriate Student \( t \)-test (paired or unpaired) was used in lesioned rats as compared with rats with sham lesion of the pACC. No significant was found in this study. (B) Representations of the examples of the largest (gray) and smallest (black) lesions in the caudal ACC lesion in the groups. Sections are in the coronal plane, numbers in mm to Bregma.

Fig. 4. Conditioned place aversion produced by the \( \kappa \)-opioid receptor agonist U69,593 (non-nociceptive stimulus) in the rats with pACC lesions \((n = 5)\) and pACC sham lesions \((n = 5)\). Data are represented as mean ± SEM. (A) For the CPA score the amount of time spent in the conditioning compartment on the post-conditioning days were subtracted from the amount of time spent in the same compartment on the pre-conditioning day. The rats spent less time in this compartment on the post-conditioning test day as compared with the pre-conditioning test day. Student \( t \)-test (paired or unpaired) was used. Compared with sham lesion rats, no significant differences of CPA scores were observed in the ACC lesion rats. These results suggest that neurons in the ACC are not necessary for non-nociceptive stimulus learning. (B) Representations of the examples of the largest (gray) and smallest (black) lesions in the pACC lesion in the groups. Sections are in the coronal plane, numbers in mm to Bregma.
3.5. Roles for NMDA- and non-NMDA receptors in CRD-induced CPA

We have shown previously, that microinjection of either non-NMDA receptor antagonist CNQX (400 mmol/L) or NMDA receptor antagonist AP5 (400 mmol/L) into the pACC did not change the VMR to graded pressure CRD in rats (Cao et al., 2008). In this study, microinjection of CNQX or NMDA receptor antagonist AP5 (400 mmol/L) did not change the numbers of spontaneous muscle contractions in 5 normal rats. Graded CRD pressures of 20, 40, and 60 mm Hg caused an increase in the number of muscle contractions to 1 ± 0.03, 19 ± 4, and 30 ± 3 contractions per 5 s, respectively following vehicle treatment. CRD pressures of 40, and 60 mm Hg induced an increase in the number of muscle contractions to 21 ± 6, 32 ± 5; and 17 ± 4, 34 ± 7 in the rats after microinjection of CNQX and AP5, respectively. These results suggest that administration of glutamate receptor antagonists into the pACC had no effect on the VMR to CRD in normal rats.

To determine if glutamatergic transmission in the ACC is responsible for mediating visceral pain-induced CPA glutamate receptor antagonist studies were performed. A total of 38 rats were studied. Histologic studies confirmed the accuracy of the microinjection sites in 34 of total 38 rats. The microinjection sites in these 4 rats were outside the ACC; therefore, the data from these 4 rats were excluded from the statistical analysis. Administration of AP5 at concentration of 200 mM had no effect on the CRD-induced CPA. AP5 400 mM reduced the CPA score from 369 ± 21, 245 ± 17, 204 ± 19, 78 ± 11, 25 ± 13 to 279 ± 21, 190 ± 19, 148 ± 17, 70 ± 2, and 22 ± 11 in the day 1, 4, 7, 10, and 14 after conditional training, in the vehicle treated rats and conditioning rats, respectively (Fig. 5A). Microinjection of CNQX 200 mM into pACC produced mild reduction in CRD-CPA scores to 259 ± 19, 158 ± 21, 105 ± 21, 67 ± 11, and 23 ± 8 in the day 1, 4, 7, 10, and 14. CNQX at dose of 400 mM markedly suppressed CRD-induced CPA to 147 ± 23, 92 ± 21, 78 ± 8 and 46 ± 1.5 in the day 1, 4, 7 and 10 after training, and completely abolished the aversive responses in the day 14 (Fig. 5A). These observations suggest that glutamate NMDA and AMPA receptor activations in ACC neuronal network are critical in the acquisition of visceral pain-induced long-term aversive learning. The locations of microinjection sites in the pACC are shown in Fig. 5B.

4. Discussion

Pain is considered both a sensation and an emotion and shows considerable complexity and subjectivity. In both clinical and laboratory settings the perception of pain bears a poor relationship to the intensity of the noxious stimulus. Mildly noxious stimuli can be perceived as very painful whereas very noxious stimuli can produce no pain whatsoever. Although the sensory and affective dimensions of pain can be readily dissociated in humans, it is a challenge to quantify pain affect in non-human animals. Electrophysiological recordings from ACC neurons showed that ACC cells responded to peripheral noxious stimuli (Cao et al., 2008; Sikes & Vogt, 1992; Wu et al., 2008). In humans neuroimaging studies have further confirmed these observations and showed that the ACC, together with other cortical structures, were activated by acute noxious stimuli, psychological pain, and social pain (Mayer et al., 2006; Rainville, Duncan, Price, et al., 1997). Surgical lesions of this area do not remove the sensation of pain but they remove the associated emotional response and suffering (Davis, Hutchison, Lozano, et al., 1994; Hutchison, Davis, Lozano, Sikes & Vogt, 1992). Our previous studies have shown that lesions in the rostral ACC did not change the VMR in normal rats, which suggests that this region of the forebrain is not involved in the behavioral component of the visceral pain response in rats that are not visceroally hypersensitive. In contrast, using visceral hypersensitive rat model we demonstrated that lesions in the rostral ACC caused a reduction in the number of muscle contractions in the visceral hypersensitive rats, suggesting that the perigenual ACC plays a critical role in the modulation of sensory aspect of visceral pain in viscerally hypersensitive rats (Cao et al., 2008; Fan et al., 2009). However, the role of ACC in modulating visceral pain-related affective memory in the physiological condition has not been investigated. Rodents do not have the forebrain structures...
to generate the cognitive feelings of humans. The use of behavioral paradigms visceromotor responses (VMR) to assess visceral pain in the conscious rat may help to identify the regulatory role of the ACC in visceral pain sensation. The VMR induced by CRD is a brainstem-mediated reflex contraction of the abdominal musculature. In response to stimuli that cause pain, all animals show musculoskeletal and autonomic responses, the so-called pseudo-affective reflex responses (Ness & Gebhart, 1990). In this study, we demonstrated that both pre-conditioning and post-conditioning rats showed pressure-dependent increases in the CRD-induced VMR. These responses were not significantly changed after conditioning training suggesting the place conditioning paradigm did not change the sensitivity of visceral pain. Unlike viscerally hypersensitive rats (Cao et al., 2008; Fan et al., 2009) lesions in the ACC did not change the CRD-induced VMR in both pre-conditioning and post-conditioning rats. These observations suggest that ACC neuronal network is not involved in the mediation of visceral pain sensation under the experimental condition.

Then, we test our hypotheses that ACC play a key role in the mediation of visceral pain-affective processing as well as learning and memory in physiological condition. We showed that CRD-induced CPA when paired with a distinct environment context. After 4 days of conditioning phase training, our findings demonstrated that animals subjected to visceral pain insult display a significant conditioned place aversion to a pain stimuli-paired environment. In conditioned avoidance procedures, animal avoid stimuli based on the formation of a negative association between the given stimuli and the environment. This tendency for animal to avoid environmental cues that have been deemed aversive is believed to be affective important (Bevins & Besheer, 2005). Therefore, conditioned place avoidance (CPA) test reflected the affective component of pain induced by visceral nociceptive stimulus. However, it is never possible to know with certainty how closely an animal “visceral pain” model reflects pain as experienced by humans. The fact that colorectal distension produces CPA in addition to other nociceptive behaviors indicates that colorectal distension is an aversive stimulus to the animal in a manner resembling the response to noxious stimuli in humans.

To investigate whether ACC is involved in the acquisition of learned aversive behaviors, bilateral infusions of the excitotoxin ibotenic acid were made into the ACC to produce neuronal cell loss. In sham lesion rats, CRD-induced a high CPA score that persisted for 2 weeks. In contrast, the group of rats subjected to ACC lesion before conditioning, they spent equal or more time in the conditioning compartment compared with sham lesion rats. Thus, ACC lesion blocked CPA learning. These results suggest that the ACC is causally involved with the perception of visceral pain-related unpleasantness.

Glutamate is the major fast excitatory transmitter in the ACC, and postsynaptic AMPA receptors mediate majority of postsynaptic responses. Johansen and Fields (2004) used a tonic somatic pain model, the formalin test model, combines with the place-conditioning paradigm demonstrated that glutamatergic activation in the ACC is necessary and sufficient for pain-like aversion. The formalin model has been described as a persistent model of tonic pain. To distinguish with a tonic persistent somatic pain, we employed a normal rat model with colorectal distension, and show that no visceral hypersensitivity was detected in this model after 4 days conditioning training in current study. Our published data of ACC neuronal electrophysiological recording have identified colorectal distension-responsive neurons in the pACC, and demonstrated that reverse microdialysis of the AMPA receptor antagonist CNQX reduced basal and abolished CRD-induced ACC neuronal firing in normal rats (Wu et al., 2008). In supporting our previous observations (Cao et al., 2008; Fan et al., 2009), we show that administration of CNQX or N-methyl-D-aspartate (NMDA) receptor antagonist AP5 had no effect on acute visceral pain behaviors (CRD-induced VMR). It appears that glutamate receptors activation in the ACC did not responsible for the modulation of visceral pain sensation under normal physiological condition in rat. Here we demonstrate that administration of CNQX and AP5 at a dose of 400 mM markedly suppressed CRD-induced CPA in the day 1, 4, 7 and 10 after training, and completely abolished the aversive responses in the day 14. Using whole-cell patch clamp recordings, previous study have demonstrated that cingulate long-term synaptic potentiation require the functional recruitment of GluR1 AMPA receptors (Hiroki, Wu, Zhao, et al., 2007). In the ACC, NMDA receptors are highly expressed. Voltage dependence is a major characteristic of NMDA receptors. At resting membrane potentials, NMDA receptors are inactive because of the blocking of pores by extracellular Mg2+ (Mayer, Westbrook, & Guthrie, 1994). However, stimulation of nociceptive afferents by colorectal distension following 4 days conditional phase may results in depolarization beyond a critical threshold with removal of the Mg2+ block and

### Table 1

Raw total time spent during pre and postconditioning for all groups in the CRD paired and unpaired compartment.

<table>
<thead>
<tr>
<th></th>
<th>Preconditioning</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRD-paired compartment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>449 ± 20</td>
<td>80 ± 14</td>
<td>204 ± 21</td>
<td>245 ± 29</td>
<td>371 ± 18</td>
<td>424 ± 20</td>
</tr>
<tr>
<td>AP5 400 mM</td>
<td>491 ± 21</td>
<td>212 ± 30</td>
<td>301 ± 14</td>
<td>343 ± 22</td>
<td>421 ± 28</td>
<td>469 ± 23</td>
</tr>
<tr>
<td>CNQX 200 mM</td>
<td>468 ± 30</td>
<td>209 ± 18</td>
<td>310 ± 22</td>
<td>363 ± 27</td>
<td>401 ± 24</td>
<td>445 ± 23</td>
</tr>
<tr>
<td>CNQX 400 mM</td>
<td>474 ± 30</td>
<td>327 ± 13</td>
<td>382 ± 18</td>
<td>396 ± 28</td>
<td>428 ± 26</td>
<td>467 ± 16</td>
</tr>
<tr>
<td>Sham-caudal ACC lesion</td>
<td>486 ± 12</td>
<td>163 ± 23</td>
<td>231 ± 27</td>
<td>275 ± 34</td>
<td>385 ± 19</td>
<td>458 ± 27</td>
</tr>
<tr>
<td>Caudal ACC lesion</td>
<td>452 ± 26</td>
<td>108 ± 9</td>
<td>226 ± 16</td>
<td>254 ± 29</td>
<td>369 ± 11</td>
<td>430 ± 31</td>
</tr>
<tr>
<td>Sham-pACC lesion</td>
<td>459 ± 27</td>
<td>77 ± 11</td>
<td>207 ± 41</td>
<td>233 ± 20</td>
<td>361 ± 18</td>
<td>433 ± 39</td>
</tr>
<tr>
<td>pACC lesion</td>
<td>465 ± 31</td>
<td>352 ± 17</td>
<td>380 ± 21</td>
<td>392 ± 25</td>
<td>431 ± 29</td>
<td>455 ± 32</td>
</tr>
<tr>
<td><strong>CRD-unpaired compartment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>472 ± 30</td>
<td>676 ± 21</td>
<td>477 ± 14</td>
<td>579 ± 32</td>
<td>374 ± 26</td>
<td>395 ± 12</td>
</tr>
<tr>
<td>AP5 400 mM</td>
<td>476 ± 20</td>
<td>619 ± 23</td>
<td>407 ± 21</td>
<td>457 ± 18</td>
<td>436 ± 36</td>
<td>322 ± 31</td>
</tr>
<tr>
<td>CNQX 200 mM</td>
<td>490 ± 22</td>
<td>512 ± 26</td>
<td>498 ± 32</td>
<td>501 ± 27</td>
<td>568 ± 23</td>
<td>414 ± 14</td>
</tr>
<tr>
<td>CNQX 400 mM</td>
<td>487 ± 34</td>
<td>589 ± 32</td>
<td>556 ± 21</td>
<td>545 ± 31</td>
<td>471 ± 16</td>
<td>355 ± 42</td>
</tr>
<tr>
<td>Sham-caudal ACC lesion</td>
<td>501 ± 29</td>
<td>660 ± 37</td>
<td>490 ± 34</td>
<td>540 ± 12</td>
<td>420 ± 36</td>
<td>396 ± 24</td>
</tr>
<tr>
<td>Caudal ACC lesion</td>
<td>443 ± 13</td>
<td>580 ± 32</td>
<td>521 ± 19</td>
<td>490 ± 12</td>
<td>415 ± 10</td>
<td>402 ± 15</td>
</tr>
<tr>
<td>Sham-pACC lesion</td>
<td>480 ± 21</td>
<td>572 ± 13</td>
<td>509 ± 24</td>
<td>466 ± 17</td>
<td>401 ± 27</td>
<td>383 ± 34</td>
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<tr>
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<td>520 ± 24</td>
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<td>371 ± 26</td>
</tr>
</tbody>
</table>
increases the availability of NMDA receptors. In this study, our data suggest that activation of both glutamate NMDA and glutamate AMPA receptors in the ACC is required for the induction of visceral pain related negative affects in the physiological state.

Moreover, in using the CPA paradigm, we were able to show that a negative affective state was maintained for 14 days following acute visceral pain insult in the absence of further conditioning. It is known that the ACC greatly contributes to the formation of contextual fear memory and inhibitory avoidance memory (Malin, Ibrahim, Tu, et al., 2007; Zhao, Toyoda, Lee, et al., 2005). Memory consolidation is a neural process during which a newly learned knowledge is gradually stabilized into a permanent memory. This process includes both synaptic and systematic reorganizations. The former involves stabilization of local synaptic modifications, whereas the latter involves reorganization of the brain regions that support storage of long-term memory, which is a relatively protracted process (Frankland & Bontempi, 2005). The hippocampus and medial temporal lobe structure are crucial for the processing of recently acquired information. However, with time lapsed, extra-hippocampus regions, especially the neocortical areas, became essential for the storage of long-lasting memory (Frankland, O’Brien, Ohno et al., 2001). It appears that pain is likely to be reflected in a matrix of neuronal structures rather than in a fixed pain center. A ‘neuromatrix’ incorporating, for example, the ACC, the prefrontal, the insula cortices, and the amygdala, hippocampus, may be involved in the learning and retrieval processing of visceral pain. Present study provide causal evidence, which indicates that neurons in the ACC is required for visceral pain-related averse learning, but without distinguishing a role for ACC neurons in its acquisition (i.e., in averse learning) vs. expression (i.e., in retrieval). Further studies are needed to investigate whether ACC is involved in the long- and (or) short-term expression of learned visceral averse behaviors.

The observation that acute visceral pain insult lead to the persistence of negative affective state in rodent is very interesting in light of clinical data, which indicates how the emotional or motivational effects of pain may live much longer than the pain itself. The treatment of painful condition remains a challenge for researchers, partially due to that the pain is complex and difficult to reproduce in animal. The CPA paradigm allowed us to investigate the aspects of visceral pain-induced behavior by challenging an animal’s ability to recollect where the pain was perceived. Our studies support the theory of the ongoing nature visceral pain-induced affective disorder observed in the clinic, such as the irritable bowel syndrome (IBS), and underscores the importance of memory in visceral pain perception (Fan et al., 2009; Mayer et al., 2006).

In conclusion, using a method that assesses visceral pain-induced CPA, the present study indicates that neurons in the ACC not only encodes visceral pain related negative affect, it is also essential for learning that underlies recognition of pain-predictive cues and avoidance.

Acknowledgments

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References


Mayer, E. A., Naliboff, B. D., & Craig, A. D. (2006). Neuroimaging of the brain-gut syndrome (IBS), and underscores the importance of memory in viscer al pain perception (Fan et al., 2009; Mayer et al., 2006).

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