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Research report

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Abstract

The present study investigated the effect of lesions of the anterior cingulate cortex (ACC) on mechanical allodynia/hyperalgesia after L5 ligation or on inflammatory nociceptive responses following formalin injection in the rat. For both the neuropathic and inflammatory pain models, three groups of animals were used. The control groups consisted of a group of sham lesioned animals and a group of animals that had unilateral damage to the ACC or unilateral/bilateral damage to surrounding cortical tissue. The third group consisted of animals that had at least 75% bilateral damage of the ACC. Subjects received L5 ligation or a 0.05-ml injection of 1% formalin into the plantar surface of the hindpaw. In contrast to the control groups, bilateral ACC lesions significantly decreased inflammatory nociceptive responses during the prolonged, tonic portion of the formalin test (20–35 min). The difference between the groups was most prevalent in the amount of time spent licking the paw. However, ACC lesions did not significantly attenuate the enhanced mechanical paw withdrawal threshold in the neuropathic nociceptive model. These results suggest a differential role of the ACC in the modulation of different types of pain conditions. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Sensory systems*Topic:* Pain modulation: anatomy and physiology*Keywords:* L5 ligation; Tonic pain; Limbic system; Mechanical hyperalgesia; Formalin test**1. Introduction**

Several lines of evidence indicate that the anterior cingulate cortex (ACC) is an important brain structure for the processing of noxious information. Electrophysiological recordings have demonstrated nociceptive specific cells with whole body receptive fields within the ACC of the rat [16], human [17], and rabbit [31]. Also, nociceptive neurons in the macaque ACC appear to encode the anticipation of pain that precedes avoidance behavior [20]. Behavioral studies have found that temporary lesions produced by lidocaine microinjection into the ACC and cingulum bundle produce a delay in the onset of self-mutilation [33] and reduced nociceptive responses in the prolonged, tonic portion of the formalin test [34]. Electrical stimulation of the cingulum bundle and the surrounding

ACC cortical tissue reduces nociceptive responses in the formalin test in rats [13]. The finding that both temporary lesions and electrical stimulation of the cingulum bundle and surrounding cortical tissue produce a marked decrease in nociceptive responses points to the complex role of the ACC in pain processing [13].

Human brain imaging studies reveal that the ACC is activated during acute pain conditions [2,4,7,18,21,28] and during deep brain stimulation of the thalamus in patients with pain [6]. Clinically, the surgical destruction of the ACC and main projection pathway significantly reduces malignant and nonmalignant pain [1,14,29,30,36,37], possibly by a selective decrease in the emotional component of pain, although an alteration in the sensory component of pain perception has also been noted [5,32]. One of the more intriguing clinical outcomes is that surgical destruction of the ACC and cingulum bundle provides significant pain relief in only 50–75% of patients [3]. One possible reason for the variability in clinical efficacy is that certain types of pain are resistant to the effect of cingulotomies

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[26], with those pain conditions associated with a high degree of anxiety being more treatable [3].

Animal models of neuropathic and inflammatory pain are extensively used to study the mechanisms of chronic pain [10,38]. One method to model neuropathic pain is by tight ligation of the L5 spinal nerve [19]. The behavioral test paradigms are designed to measure reflexive withdrawal responses to mechanical and thermal stimulation. The leftward shift in the stimulus–response function following nerve injury is thought to reflect certain characteristics of clinical pain conditions, such as allodynia (reduced withdrawal thresholds for normally innocuous stimulation) and hyperalgesia (enhanced withdrawal response to normally nocuous stimulation). One method to model an inflammatory condition is the formalin test, which involves a subcutaneous (s.c.) injection of dilute formaldehyde into the plantar region of the rat hindpaw [11]. Unlike the reflexive behavioral test paradigms for neuropathic pain, behavioral responding (i.e. paw elevation and licking) in the formalin test is continuous and cannot be eliminated with simple reflexive responses. The temporal profile of the formalin test consists of an early acute phase followed after a short delay by a second, longer phase of responding. The second phase of sustained behavioral responding activates brain structures composing the frontolimbic network [27], of which the ACC is a critical component involved in higher order processing of noxious input (i.e. affect and motivation) [8,35]. The purpose of the present experiment was to examine the effect of ACC lesions on neuropathic and inflammatory nociceptive conditions. It was hypothesized that lesions of the ACC would attenuate behavioral responses during the prolonged second phase of the formalin test but have no effect on the early acute phase or on the increased sensitivity to mechanical stimuli following peripheral nerve injury.

2. Materials and methods

2.1. Subjects

Seventy-six male Sprague–Dawley albino rats (University of Texas at Arlington vivarium), weighing 300–400 g at the time of surgery, served as subjects. They were housed in pairs and maintained on a 12-h light, 12-h dark cycle with free access to food and water throughout the study. The animals were maintained and cared for in accordance with the guidelines set forth by the International Association for the Study of Pain [39]. The experimental protocol was approved by the University of Texas at Arlington Animal Care and Use Committee.

2.2. Induction of neuropathic and inflammatory hyperalgesia

Experimental nociceptive conditions were induced by

tight ligation of the L5 spinal nerve ($n=41$) or by a s.c. injection of 1% formalin into the plantar surface of the hindpaw ($n=35$). Animals receiving L5 ligation were anaesthetized with halothane in 100% O₂ (3% induction, 2% maintenance) and then placed in a prone position to access the left L4–L6 spinal nerves. Under magnification, approximately one-third of the L6 transverse process was removed. The L5 spinal nerve was identified and carefully dissected free from the adjacent L4 spinal nerve and then tightly ligated using a 6-0 silk suture [19]. The wound was treated with an antiseptic solution, the muscle layer was sutured and the wound was closed with wound clips. Animals that displayed signs of L4 damage (i.e. impaired motor function of the left hindpaw region) were excluded from further testing. Animals receiving a formalin injection were lightly restrained during the 0.05-ml s.c. injection of formalin solution into the plantar surface of the left hindpaw [11].

2.3. Surgical lesions of the anterior cingulate cortex

All subjects ($n=76$) were anaesthetized with acepromazine (0.5 mg/kg s.c.) followed by a combination of ketamine (50 mg/kg i.m.) and xylazine (5 mg/kg i.m.). Animals were securely placed into a stereotaxic device with the skull level between bregma and lambda. A stainless steel electrode (0.7 mm diameter) insulated except at the tip, was directed towards the region of the ACC. The following stereotaxic coordinates were used: 0.8 mm lateral to bregma; 3.2 mm dorsal; at 15° [25]. Electrolytic lesions were made using a constant current of 1 mA for 20 s. Twenty-two animals received bilateral ACC lesions while 34 received unilateral and/or dorsal lesions. Sham lesioned animals ($n=20$) underwent the same surgery as lesioned animals except no current was passed. The wound was cleaned with an antiseptic solution and closed with a 4-0 silk suture.

Animals were sacrificed with sodium pentobarbital and perfused with normal saline and 10% formaldehyde 3–5 days after behavioral testing was complete. The brains were removed and stored in 10% formaldehyde for several days. Histological analysis was performed by microscopic inspection of 50- μ m coronal sections mounted on glass slides and stained with thionin.

2.4. Measurement of mechanical paw withdrawal threshold following L5 ligation

Behavioral testing was performed using different methods for each experimental pain condition. Three days after L5 ligation, animals were placed within a Plexiglas chamber (20×10.5×40.5 cm) and allowed a 15-min habituation period. The chamber was placed on top of a mesh screen to allow for easy administration of mechanical

stimuli to the plantar surface of both hindpaws. Mechanical threshold measurements for each hindpaw were obtained using eight von Frey monofilaments (5, 7, 13, 27, 43, 64, 106, and 202 mN) using the up/down method [9]. Each trial started with a von Frey force of 13 mN delivered for approximately 1 s to the right and then the left hindpaw. For each paw, if a withdrawal response to the mechanical stimulus was not observed, then the next higher force was delivered. If a withdrawal response was observed, the next lower force was delivered. This procedure was performed until no withdrawal response was observed at the highest force (202 mN) or until four stimuli were administered following the initial response. The 50% mechanical paw withdrawal threshold for each paw was calculated using the following formula: $[X_{th}] \log = [vFr] \log + ky$ where $[vFr]$ is the force of the last von Frey used, $k=0.2268$ which is the average interval (in log units) between the von Frey monofilaments, and y is a value that depends upon the pattern of withdrawal responses [9]. If an animal did not respond to the highest monofilament (202 mN), then $y=1.00$ and the 50% mechanical paw withdrawal response for that paw was calculated to be 340.5 mN. Threshold testing was performed three times and the scores were averaged to determine the mechanical paw withdrawal threshold for the right and left paws for each animal immediately before and 7–10 days after ACC lesions. The difference between the left and right paw withdrawal threshold was calculated and averaged across subjects within each group.

2.5. Measurement of formalin-induced responses

On the day of testing (7–10 days following ACC lesion), animals were placed in a 30×30×30 cm Plexiglas chamber and allowed to habituate for at least 15 min. To allow for easy viewing of behavioral responses, a mirror was placed below the test chamber at a 45° angle. Subjects were administered a 0.05-ml injection of 1% formalin solution into the plantar surface of the left hindpaw. The left hindpaw was chosen to compare results with L5 ligated animals that were ligated on the left side. Behavioral testing began immediately after injection and lasted for 45 min. The amount of time the animal spent with the injected paw down, elevated, or licking the paw was recorded for the nine 5-min time periods. A weighted pain score for each animal was calculated using the following formula: $\text{Pain Score} = (\text{time spent with inflamed paw elevated} + 2(\text{time spent licking inflamed paw}))/300$. A pain score of zero reflects the entire duration of the 5-min period was spent with the paw down and a pain score of two indicates the entire duration of the 5-min period was spent licking the injected paw. In addition to weighted pain scores, the raw scores for each of the behavioral categories (i.e. paw down, paw elevated, and paw licking) were also summed over each 5-min time interval.

2.6. Data analysis

The left and right paw difference in mechanical paw withdrawal thresholds was analyzed using a repeated measures ANOVA with group (three levels) as a between-subjects factor and test time period (two levels) as the within-subjects variable. The formalin pain score and each of the separate behavioral categories were analyzed using a repeated measures ANOVA with group (three levels) as a between-subjects variable and test time period (nine levels) as the within-subjects factor. Additional analysis of group differences at each 5-min test time period was performed using a one-way ANOVA followed by post-hoc comparisons (protected *t*-test).

3. Results

3.1. Histology

Under magnification, coronal sections were examined for signs of tissue damage and the anterior/posterior extent of damage was recorded for each animal. A schematic representation of the greatest (3.4 mm) and least (1.5 mm) amount of tissue damage is illustrated in Fig. 1A. The frequency of animals demonstrating different extents of anterior/posterior tissue damage for subjects that had bilateral lesions of the cingulate cortex is illustrated in Fig. 1B. The extent of tissue damage for the two groups of bilateral cingulate cortex lesioned animals reveals a virtually identical pattern. For the bilateral cingulate lesion groups, the mean anterior/posterior extent of the lesion was 2.14 ± 0.17 and 2.11 ± 0.11 mm for the L5 ligation and formalin models, respectively. Although there was some minor variability among animals with regard to the most anterior and posterior extent of damage, the common damage extended from 1.2 mm (except for one animal in the L5 ligation model) to -0.26 mm [25]. There was at least 75% bilateral damage to the ACC at the maximum medial/lateral extent of tissue damage (Fig. 1A,C). Damage was also localized to the medial/dorsal region of the frontal cortex. In no case was there evidence of damage to the corpus collosum or the cingulum bundle. The control ACC lesioned animals consisted of unilateral, unilateral/dorsal or bilateral/dorsal damage relative to the area of the ACC, with similar anterior/posterior extent of tissue damage in the bilateral ACC lesion groups.

3.2. Mechanical paw withdrawal thresholds following L5 ligation

The average pre- and post-lesion mechanical paw withdrawal threshold (left–right paw difference) for animals that had bilateral, unilateral/dorsal or sham lesions of the ACC is illustrated in Fig. 2. The difference between the ligated left paw and the non-ligated control paw prior to

Fig. 1. (A) Schematic representation of the maximum (dot pattern) and minimum (line pattern) anterior/posterior extent of tissue damage for animals with bilateral lesions of the ACC. (B) Frequency histogram illustrating the extent of tissue damage for animals with bilateral ACC damage and tested in either the neuropathic condition (L5 ligation, $n=12$) or the inflammatory condition (formalin test, $n=10$). The location of damage was virtually identical in both pain conditions, with the anterior extent of damage beginning in a few animals at 2.2 mm from bregma and ending in a few animals at -1.4 mm from bregma. All other animals had tissue damage within this range, as indicated by the values on the horizontal axis corresponding to plates from the atlas of Paxinos and Watson [25]. Animals in both conditions had at least 75% bilateral ACC damage between 1.0 and -0.26 mm from bregma. (C) Photomicrograph of a representative subject with at least 75% bilateral ACC damage. Scale bar, 1 mm. CC, corpus collosum; CB, cingulum bundle.

ACC lesion reflects the common finding that L5 ligation causes an enhanced response to mechanical stimulation of the ligated paw [19]. The overall statistical analysis of mechanical paw withdrawal threshold revealed no significant main effect for group ($P>0.99$) and no significant group $\times$ time period interaction ($P>0.55$). To ensure a lack of difference among groups, additional analyses revealed no difference among the groups prior to ($P>0.55$) or following ($P>0.80$) the stereotaxic procedure for ACC damage.

3.3. Mean pain scores following formalin injection

The overall analysis of mean pain scores revealed a

significant main effect for group ($P<0.05$), but no group $\times$ test period interaction ($P>0.25$). Additional analyses at each 5-min test time period revealed significant differences among the groups, with the bilateral ACC lesion group demonstrating significantly lower mean pain scores from 15 to 35 min following the formalin injection compared to sham lesion control subjects (Fig. 3).

To further explore the effect of ACC lesions on formalin pain responses, the three behavioral categories (paw down, paw elevation, paw lick) were analyzed separately for differences among groups. The mean duration of time that the groups spent with the paw down is illustrated in Fig. 4A. The overall analysis revealed a significant main effect for group ($P<0.05$), but no significant group $\times$ time period

Fig. 2. Mean ($\pm$ S.E.M.) mechanical paw withdrawal thresholds prior to the surgical procedure for ACC lesions (pre-lesion) or following the ACC lesion (post-lesion) for animals that had ligation of the L5 spinal nerve. Represented are L5 ligated animals that had bilateral damage to the ACC (bilateral ACC, $n=12$), incomplete damage to the ACC (unilateral/dorsal, $n=19$) or had the electrode inserted, but no current passed (sham lesion, $n=10$).

interaction ($P>0.40$). Additional analyses (one-way ANOVA followed by LSD post-hoc comparisons) at each 5-min test time period revealed that the bilateral ACC lesion group spent a significantly greater amount of time between 15 and 35 min with the paw down compared to the sham lesion group. It should also be noted that the unilateral/dorsal ACC lesion group had values that were between the bilateral ACC and sham lesion groups. The mean duration of time that the groups spent with the paw elevated is illustrated in Fig. 4B. The overall analysis revealed a lack of a significant main effect for group ($P>0.45$) and a lack of a significant group $\times$ time period interaction ($P>0.10$). Indeed, additional analyses revealed

Fig. 3. Mean ($\pm$ S.E.M.) pain scores for each 5-min interval of a 45-min formalin test period for animals that had bilateral damage to the ACC (bilateral ACC, $n=10$), incomplete damage to the ACC (unilateral/dorsal, $n=15$) or had the electrode inserted, but no current was passed (sham lesion, $n=11$). * $P<0.05$; ** $P<0.01$ versus sham lesion control.

Fig. 4. (A) Mean ($\pm$ S.E.M.) duration of time with the paw down during each 5-min interval of a 45-min formalin test period for animals that had bilateral damage to the ACC (bilateral ACC, $n=10$), incomplete damage to the ACC (unilateral/dorsal, $n=15$) or had the electrode inserted but no current passed (sham lesion, $n=11$). (B) Mean ($\pm$ S.E.M.) duration of time with the paw elevated during each 5-min interval of a 45-min formalin test period for animals that had bilateral damage to the ACC (bilateral ACC, $n=10$), incomplete damage to the ACC (unilateral/dorsal, $n=15$) or had the electrode inserted, but no current passed (sham lesion, $n=11$). (C) Mean ($\pm$ S.E.M.) duration of time licking the injected paw during each 5-min interval of a 45-min formalin test period for animals that had bilateral damage to the ACC (bilateral ACC, $n=10$), incomplete damage to the ACC (unilateral/dorsal, $n=15$) or had the electrode inserted, but no current passed (sham lesion, $n=11$). * $P<0.05$; ** $P<0.01$ versus sham lesion control.

that the bilateral and unilateral/dorsal ACC lesion groups spent significantly less time with the paw elevated during one 5-min test time period (25 min). The mean duration of time that the groups spent licking the injected paw is illustrated in Fig. 4C. The overall analysis revealed a significant main effect for group ($P < 0.01$) but no significant group $\times$ time period interaction ($P > 0.20$). Additional analyses at each 5-min test time period revealed that the bilateral ACC lesion group spent significantly less time between 20 and 35 min licking the paw compared to the sham lesion group. It should also be noted that the unilateral/dorsal ACC lesion group had values that were between the bilateral ACC and sham lesion groups.

4. Discussion

Although human brain imaging studies indicate that the anterior cingulate cortex (ACC) is an important supraspinal structure for the processing of noxious information [2,6,18,28], there are still a number of fundamental questions that remain unanswered. Indeed, we are unaware of any study that has examined the basic issue of the effect of ACC lesions on different types of experimental nociceptive conditions. Consequently, this experiment was designed to investigate if electrolytic lesions of the ACC would have differential effects on neuropathic and inflammatory conditions. The main finding is that ACC lesions had no effect on enhanced mechanical paw withdrawal thresholds following ligation of the L5 spinal nerve (neuropathic condition, Fig. 2) or on behavioral responses during the first phase of the formalin test (Fig. 3). However, ACC lesions did cause a significant decrease of nociceptive responses during the prolonged phase of the formalin test (inflammatory condition) compared to the unilateral/dorsal and sham ACC lesioned control groups.

Previous studies in the rat have found decreased nociceptive responses following either a temporary block of ACC function with lidocaine microinjection [33] or by focal brain stimulation [13]. To more closely resemble the clinical approach of cingulotomy for chronic pain treatment, we examined the effect of an electrolytic lesion of the rat ACC on both neuropathic and inflammatory conditions. The only effect of ACC lesion was on formalin nociceptive responses during the prolonged portion of the formalin test from 20 to 40 min following the injection. Examination of categorical behavioral responses indicates that the nature of the decrease in formalin pain following bilateral ACC lesions is primarily the result of a decrease in the duration of the licking response (Fig. 4C). Licking behavior following formalin injection likely reflects the behavioral outcome of higher order mechanisms partially involving the function of the ACC. It might be argued that the decrease in licking behavior may be due to behavioral modifications that are unrelated to nociceptive processing. However, this is unlikely since licking behavior was not

significantly altered during the first 5 min of the formalin test. Rather, we believe that the present results suggest that the primary consequence of ACC lesions is to interrupt the processing of noxious information related to the negative hedonic value of the stimulus (licking), rather than the intensity of the stimulus (elevation). Indeed, preliminary studies using a recently developed behavioral test paradigm to directly measure the aversive nature of stimulus-evoked nociceptive processing in the rat [22] indicate that AAC lesions selectively interrupt the quality (emotional), but not the quantity (sensory) [23].

It is interesting to note that only bilateral ACC lesions produced a marked decrease of behavioral responses during the prolonged phase of the formalin test. The lack of a unilateral effect in the present study is surprising, given that nociceptive specific cells located within the ACC have whole body receptive fields and that unilateral ACC lidocaine microinjection and electrical stimulation result in antinociception [13,33,34]. It should be noted, however, that incomplete lesions of the ACC (unilateral, unilateral/dorsal and bilateral/dorsal) produced intermediate pain scores relative to the bilateral and sham lesioned groups throughout the duration of the prolonged second phase of the formalin test (Fig. 3, 20–40 min). The small extent of damage by unilateral ACC lesions may be the reason that this finding was not duplicated in the ACC. However, this seems unlikely since our lesions affected at least the same amount of tissue and most likely a greater amount than affected by lidocaine microinjection and focal brain stimulation. It remains possible that more complete unilateral lesions would produce a greater decrease of formalin pain responses. Another possibility is that the lesion control group consisted of animals that also had no ACC damage (unilateral/dorsal, bilateral/dorsal) and the creation of a group consisting entirely of subjects with unilateral ACC damage might provide the power to detect a significant decrease of formalin pain responses. What remains clear, however, is that damage of the ACC can significantly attenuate inflammatory pain responses.

Although the bilateral ACC lesion caused a clear decrease in formalin responding, there was no effect of the lesion on the enhanced mechanical paw withdrawal threshold following L5 ligation. Mechanical paw withdrawal thresholds after bilateral ACC lesions were not different compared to control animals (Fig. 2). The relevance of this finding remains unclear since there are at this time insufficient clinical reports to evaluate if cingulotomies are a useful therapeutic approach for neuropathic pain. However, the symptoms of reflex sympathetic dystrophy, which mimic many of the clinical signs of neuropathic pain, can be treated by cingulotomy [29].

The finding that ACC lesion decreases inflammatory but not neuropathic nociceptive behavior suggests that the ACC plays a differential role in the modulation of different types of pain conditions. One possible reason for the differential effect might be that the enhanced mechanical

paw withdrawal threshold in the neuropathic experimental model is a reflection of allodynia and not susceptible to the effect of ACC lesions. A second reason might be related to the function of the ACC and closely associated prefrontal cortex for the production of behavioral responses that are produced by the different experimental nociceptive conditions. For instance, activation of the prefrontal cortex might reflect cognitive evaluation, attention and movement planning, such that the organism recognizes that a stimulus is painful and then performs a behavior to terminate the stimulus [2,15]. The ACC is thought to be involved in processing the affective aspect of a pain experience, learning associated with prediction/avoidance of a noxious stimulus and the implementation or inhibition of a motor response selection during the presence of a noxious stimulus [8,28,35]. In terms of complexity, paw withdrawal responses can be elicited in spinal animals [12] whereas the paw licking response of the formalin test likely requires supraspinal activity. Therefore, ACC lesions might interrupt more complex behaviors associated with motor response selection for formalin-induced noxious stimulation. Indeed, examination of formalin pain responses indicates that paw licking is decreased, but paw elevation is unaltered, suggesting that paw withdrawal responses (including paw elevation) and paw licking responses are mediated by different neural systems. Since the ACC is thought to process the affective aspect of a pain experience, a decrease in formalin pain responses, primarily by decreasing licking behavior might reflect an interruption in the affective and motivational aspects of nociceptive processing.

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