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GABA_A but not GABA_B Receptors in the Rostral Anterior Cingulate Cortex Selectively Modulate Pain-Induced Escape/Avoidance Behavior

Stacey C. LaGraize^{1,3,*} and Perry N. Fuchs²

¹ Department of Psychology, University of Texas at Arlington, Arlington, Texas 76019

² Departments of Psychology and Biology, Bioscience and Bioengineering Center, University of Texas at Arlington, Arlington, Texas 76019

Abstract

The rostral anterior cingulate cortex (rACC) is involved in supraspinal nociceptive processing. ACC lesions relieve persistent pain, but do not affect the patient's ability to localize a noxious stimulus. Since the rACC has a high density of GABA receptors, it is possible that pain processing is influenced by these receptors in the rACC. The present experiments examined the involvement of rat rACC GABA_A and GABA_B receptors in regard to sensitivity to mechanical stimulation and escape/avoidance behavior in response to a noxious stimulus following L5 spinal nerve ligation. Rats were or were not afflicted with a neuropathic pain condition by an L5 spinal nerve ligation. rACC microinjection of 10 µg/µl GABA, a GABA_A agonist (0.001 µg/µl, 0.1 µg/µl, or 0.5 µg/µl muscimol), a GABA_B agonist (0.1 µg/µl, 1 µg/µl, or 5 µg/µl baclofen), or saline, did not alter mechanical withdrawal thresholds. Importantly, following 10 µg/µl GABA, 0.1 µg/µl, or 0.5 µg/µl muscimol microinjected into the rACC, place escape/avoidance behavior to a noxious mechanical stimulus was attenuated in injured animals. The attenuation was specific to the rACC and was blocked by a preadministered microinjection of the appropriate antagonist(s) into the rACC. In conclusion, microinjection of GABA and higher doses of muscimol did not decrease mechanical hyperalgesia but did attenuate place escape/avoidance behavior that is associated with mechanical stimulation of the ligated paw. These results provide additional support for the role of the rACC in higher order supraspinal processing of noxious events and suggest that rACC GABA_A receptors significantly contribute to this processing.

Keywords

GABA; Limbic System; Nociception; Escape; Avoidance; Cingulate Cortex

Chronic pain conditions affect the lives of millions of individuals every day. Pain is considered to include sensory/discriminative, motivational/affective, and cognitive/evaluative dimensions (Melzack and Casey, 1968). Spinal and supraspinal mechanisms underlying the sensory dimension of pain have received much attention, while the underlying mechanisms of the

*Corresponding Author: Stacey C. LaGraize, Ph.D., Department of Oral and Craniofacial Biomedical Sciences, University of Maryland Dental School, 650 W. Baltimore Street, Baltimore, MD 21201 USA, (T) 410-706-3673, (F) 410-706-0865, SLagraize@umaryland.edu.

³Present Address: Department of Oral and Craniofacial Biomedical Sciences, University of Maryland Dental School, 650 W. Baltimore Street, Baltimore, MD 21201 USA, SLagraize@umaryland.edu

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affective dimension of pain remain unclear. Many structures in the limbic system are involved in affective drives and escape behavior. As a result, these brain regions may be involved in behavior resulting from negative affect that is associated with pain. The anterior cingulate cortex (ACC) is one limbic system structure that may play a role in the affective dimension of pain (Vogt, 1985; Devinsky et al., 1995; Johansen et al., 2001; Gao et al., 2004; LaGraize et al., 2004;). Imaging studies have reported increased ACC activity during noxious stimulation as well as chronic pain conditions (Hsieh et al., 1995a, 1995b, 1999; Porro et al., 1998; Tolle et al., 1999). Positron emission tomography studies have revealed increased activation in the ACC following film- and recall-induced emotion and altered states of pain affect by hypnosis (Rainville et al., 1997, 1999; Lane et al., 1998). Additionally, Fos expression in the ACC is increased following formalin-induced conditioned place avoidance in rodents (Lei et al., 2004). Lesion or stimulation of the ACC or cingulum bundle has been shown to decrease nociceptive responding in the formalin test (Vaccharino and Melzack, 1989; Fuchs et al., 1996; Donahue et al., 2001). More specifically, previous results from our laboratory show that following ACC lesion, paw licking is significantly decreased in the formalin test (Donahue et al., 2001). Paw licking most likely involves supraspinal mechanisms and thus, ACC lesions may disrupt the higher order processing of noxious input. Additionally, activation or deactivation of the ACC in neuropathic rodents appears to selectively reduce pain affect assessed by the place escape/avoidance paradigm without affecting paw withdrawal threshold in response to a mechanical stimulus (LaGraize et al., 2001, 2004). On the other hand, chemical ablation of the rostral ACC (rACC) has been shown to reduce pain affect as measured by a formalin-induced conditioned place preference paradigm (Johansen et al., 2001). Thus, the ACC is clearly involved in pain affect.

Currently, the pharmacological mechanisms underlying pain affect are largely unknown. The ACC has a substantial GABAergic termination (Vogt, 1993). This termination is primarily found in layer Ia where there is a high density of GABA_A receptors. Binding studies have reported GABA_A and GABA_B receptors within the ACC (Chu et al., 1990; Bozkurt et al., 2005), with more binding sites for GABA_A than for GABA_B (Chu et al., 1990). Behaviorally, muscimol microinjection into the ACC attenuates formalin-induced conditioned place preference conditioning, possibly through facilitation of polysynaptic excitatory transmission (Wang et al., 2005). Therefore, the purpose of the following experiments was to examine pharmacological mechanisms in the rACC as related to pain affect with a focus on GABA_A and GABA_B mechanisms. Preliminary findings have been reported in abstract form (LaGraize et al., 2003, 2004).

Materials and Methods

Two hundred and eighty-three male Sprague-Dawley rats (University of Texas at Arlington vivarium), approximately 3–4 months old at the time of surgery, were housed in pairs and maintained on a 12:12 light:dark cycle with free access to food and water throughout the study. The animals were maintained and cared for in accordance to the guidelines outlined by the International Association for the Study of Pain (Zimmerman, 1983). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Arlington. All behavioral testing was conducted during the light cycle. Additionally, behavioral testing was performed ‘blind’ with respect to microinjection substance. Histological analysis was performed ‘blind’ to ACC implant condition, neuropathic condition (sham versus ligation) and behavioral outcome. Table 1 lists the groups and the number of animals used in each group.

Cannula implants

Cannulas were implanted into the rostral ACC (hit) or a surrounding region (miss) one week prior to behavioral testing (rACC hit: n=243; rACC miss: n=40). All animals were administered a s.c. injection of acepromazine (0.65 mg/kg). Five minutes following the acepromazine injection, the animals were deeply anesthetized by an intramuscular injection of ketamine (50 mg/kg) and xylazine (2.61 mg/kg). The animals were then positioned in a stereotaxic frame with blunt-tipped ear bars and a midline incision was made. A burr hole was drilled and a guide cannula (23-gauge) was lowered into the rACC (AP: 1.9; L: 0.6; D: 2.9 mm) or a surrounding region (AP: 1.9; L: 2.6; D: 2.4 mm). The guide cannula was then secured with cranioplastic cement. To prevent clogging of the guide cannula, a stylet (Hypodermic Tube; 0.0120" OD, 0.0055") was inserted and secured with cranioplastic cement until the microinjection was administered.

Induction of neuropathic pain condition

The experimental pain condition was induced using tight ligation of the L5 spinal nerve (n=183; Kim and Chung, 1992). All animals were anesthetized with isoflurane in 100% O₂ (3% induction, 2% maintenance administered through a face mask) and placed in the 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. The wound was treated with an antiseptic solution, the muscle layer sutured, and the wound closed with wound clips. Additional animals served as sham surgery controls where everything mentioned above was performed with the exception of ligation of the L5 spinal nerve (n=100).

Measurement of mechanical hyperalgesia

Behavioral testing was performed using identical methods for both ligated and sham ligated groups. Three days following L5 ligation (and seven days following ACC implant), animals were placed within a Plexiglas chamber (20 x 10.5 x 40.5 cm) and allowed to habituate for 15-min. The chamber was positioned on top of a mesh screen so that mechanical stimuli could be administered to the plantar surface of both hindpaws. Mechanical paw withdrawal threshold measurements for each hindpaw were obtained using the up/down method (Dixon, 1980) with eight von Frey monofilaments (4, 6, 10, 18, 40, 78, 141, and 217 mN). Each trial began with a von Frey force of 10 mN delivered to the right hindpaw (contralateral) for approximately 1-sec, and then the left hindpaw (ipsilateral). If there was no withdrawal response, the next higher force was delivered. If there was a response, the next lower force was delivered. This procedure was performed until no response was made at the highest force (217 mN) or until four stimuli were administered following the initial response. The 50% paw withdrawal threshold for each paw was calculated using the following formula: $[X_{th}]_{log} = [vFr]_{log} + k_y$ where $[vFr]$ is the force of the last von Frey used, $k = 0.2487$ 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. If an animal does not respond to the highest von Frey hair (217 mN), then $y = 1.00$ and the 50% withdrawal threshold response for that paw was calculated to be 383.99 mN. Withdrawal threshold testing was performed three times and the 50% withdrawal values were averaged over the three trials to determine the mean withdrawal threshold for the contralateral and ipsilateral paw for each animal. In L5 ligated animals, the criterion for continuation in the experiment was that the withdrawal threshold value for the ipsilateral paw had to be at least 50% of the withdrawal threshold value for the contralateral paw. In sham ligated animals, the withdrawal threshold value for the ipsilateral paw had to be greater than 50% of the withdrawal threshold value for the contralateral paw. Animals were threshold tested prior to drug administration and immediately following place escape/avoidance testing. All behavioral testing was completed 45 min following microinjection. Figure 1 illustrates the time line for

behavioral testing. Withdrawal threshold difference scores (ipsilateral – contralateral) were used for simplification due to the large number of groups. A large negative difference indicates hypersensitivity of the ipsilateral paw. A small difference indicates that the withdrawal threshold for each paw was similar.

Microinjections

Following withdrawal threshold testing, animals were lightly restrained, the stylet was removed and an injection cannula (Hypodermic Tube; 0.0120" OD, 0.0055") extending 0.5 mm below the guide cannula was inserted. GABA (10 µg/µl), muscimol (0.001, 0.1, or 0.5 µg/µl), baclofen (0.1, 1, or 5 µg/µl), or saline was microinjected using a 1-µl syringe (Hamilton), which was attached to the injection cannula by PE 10 tubing. A volume of 0.5 µl was injected over a 90 s period and the injection cannula remained untouched for an additional 60 s to allow for absorption into the brain region and to minimize injectate along the track of the cannula. Five minutes following the microinjection, animals were tested in the place escape/avoidance paradigm.

Additional L5 ligated animals were used to ensure the effects of GABA and muscimol were specific. Following withdrawal threshold testing, animals were administered a series of two 0.5 µl microinjections (dimethyl sulfoxide (DMSO) followed by 10 µg/µl GABA, 0.5 µg/µl bicuculline + 1 µg/µl phaclofen followed by 10 µg/µl GABA, or 0.1 µg/µl muscimol followed by 0.5 µg/µl bicuculline) separated by five minutes. The procedure for microinjections was identical to that described earlier except that two microinjections were administered separated by a 5-min period. Five minutes following the second microinjection, animals were tested in the place escape/avoidance paradigm.

Place escape/avoidance paradigm testing

Place escape/avoidance testing (LaBuda and Fuchs, 2000a, 2000b, 2001, 2005; LaGraize et al., 2003, 2004, 2006) was performed following pre-microinjection withdrawal threshold testing and drug administration. No training is necessary for this behavioral test. Animals were placed within a 16 x 40.5 x 30.5 cm Plexiglas chamber that was positioned on top of a mesh screen. One half of the chamber is painted white (light area) and the other half of the chamber is painted black (dark area). During behavioral testing, animals were allowed unrestricted movement throughout the test chamber for the duration of a 30-min test period. Testing began immediately with suprathreshold mechanical stimulation (476 mN von Frey monofilament) applied to the plantar surface (L4/L5 dermatomes) of the hindpaws at 15-sec intervals. The mechanical stimulus was applied to the left paw (ipsilateral to ligation) when the animal was within the preferred dark area of the test chamber and the right paw (contralateral to injury) when the animal was within the non-preferred light area of the test chamber. Shamligated animals were mechanically stimulated in an identical manner as the experimental group. The location of the animal at each 15-sec interval when it is stimulated is recorded and converted to a percentage of time spent in the light side of the chamber.

Histology

Following behavioral testing, animals were sacrificed with sodium pentobarbital and perfused with saline and 10% formalin. 80-µm coronal sections of brain tissue stained with thionin were examined under magnification to determine the cannula placement according to the atlas of Paxinos and Watson (1998).

Statistical analyses

Withdrawal threshold difference scores for sham ligated and L5 ligated animals in the various microinjection groups prior to or following microinjection was performed using the Kruskal-

Wallis ANOVA by ranks. The Mann-Whitney U test was used to compare sham and L5 ligated groups within each drug condition at each time point. Additionally, in cases where all animals were ligated, withdrawal threshold difference scores for animals in the various microinjection groups prior to or following microinjection was performed using the Mann-Whitney U Test. The change (pre vs. post microinjection) in withdrawal threshold difference scores for each group was assessed using the Wilcoxin Matched Pairs Test. The overall analysis of place escape/avoidance behavior for sham ligated and L5 ligated animals in the various microinjection conditions was performed using a two-factor neuropathy X microinjection ANOVA. In cases where all animals were ligated, an independent t-test was performed to examine any differences due to microinjection condition. Following overall parametric analyses, differences were further analyzed using Fisher's LSD post hoc comparisons. Alpha level was set at 0.05 for all statistical tests.

Drugs

All drugs used in the present experiments were obtained from Sigma. GABA, muscimol, and baclofen were dissolved in physiological saline. Bicuculline and phaclofen were dissolved in DMSO.

Results

GABA Microinjection and Mechanical Paw Withdrawal Threshold

The mean withdrawal threshold difference scores for sham ligated or L5 ligated animals before and after microinjection (saline or 10 µg/µl GABA) into the rACC are illustrated in Figure 2A. A Kruskal-Wallis ANOVA by ranks examining withdrawal threshold difference scores revealed a significant effect for group prior to microinjection ($H_{3,38} = 29.09$, $p < 0.001$) and following microinjection ($H_{3,38} = 28.67$, $p < 0.001$). Mann-Whitney U tests revealed that both prior to or following microinjection, sham ligated animals showed significantly smaller withdrawal threshold difference scores as compared to ligated animals that received the same drug ($p < 0.001$). Wilcoxin Matched Pairs Tests revealed that within each group, there were no changes in withdrawal threshold difference scores due to microinjection, except for sham ligated animals that received a 10 µg/µl GABA microinjection into the rACC. In this group, the withdrawal threshold difference scores became smaller (although in the opposite direction) following the microinjection indicating that the scores of the ipsilateral and contralateral paws were more similar following microinjection. Of importance is that the animals in this group were not hyperalgesic. Figure 2E illustrates the distribution of injection sites for animals in these experimental groups (left panel – L5 ligation; right panel – sham ligation).

The mean withdrawal threshold difference scores for L5 ligated animals before and after microinjection (saline or 10 µg/µl GABA) into a region adjacent to the rACC are illustrated in Figure 2B. A Mann-Whitney U Test examining withdrawal threshold difference scores prior to or following microinjection did not reveal any significant differences. Additionally, Wilcoxin Matched Pairs Tests revealed no changes in withdrawal threshold difference scores due to microinjection. Thus, there were no differences in withdrawal threshold for both ligated groups prior to or following microinjection into a region adjacent to the rACC. Figure 2F illustrates the distribution of injection sites for animals in these experimental groups.

GABA Microinjection and Place Escape/Avoidance Behavior

The mean percentage of time that animals spent in the light side of the place escape/avoidance chamber for sham ligated and L5 ligated animals following a microinjection (saline or 10 µg/µl GABA) into the rACC is illustrated in Figure 2C. A two-factor ANOVA examining place escape/avoidance behavior following microinjection that included L5 ligated and sham ligated animals that received a microinjection into the rACC revealed a significant interaction ($F_{1,34}$

= 5.34, $p < 0.05$). Post hoc analyses indicated that all groups spent significantly less time in the light side of the chamber as compared to L5 ligated animals that received a saline microinjection in the rACC. Of primary importance is the finding that L5 ligated animals that received a microinjection of 10 $\mu\text{g}/\mu\text{l}$ GABA into the rACC spent significantly less time in the light side of the chamber than L5 ligated animals that received a saline microinjection into the rACC. Additionally, the amount of time spent in the light side of the chamber for L5 ligated animals that received a microinjection of 10 $\mu\text{g}/\mu\text{l}$ GABA into the rACC did not differ from either of the sham ligated groups.

The mean percentage of time that L5 ligated animals spent in the light side of the place escape/avoidance chamber following a microinjection (saline or 10 $\mu\text{g}/\mu\text{l}$ GABA) into a region adjacent to the rACC is illustrated in Figure 2D. An independent t-test did not reveal any significant differences for microinjection condition which indicates that both L5 ligated groups escape/avoided the suprathreshold mechanical stimulation.

GABA Microinjection Blocked by Bicuculline/Phaclofen Microinjection and Mechanical Paw Withdrawal Threshold

The mean withdrawal threshold difference scores for L5 ligated animals before and after microinjections (DMSO followed by 10 $\mu\text{g}/\mu\text{l}$ GABA or 0.5 $\mu\text{g}/\mu\text{l}$ bicuculline + 1 $\mu\text{g}/\mu\text{l}$ phaclofen followed by 10 $\mu\text{g}/\mu\text{l}$ GABA) into the rACC is illustrated in Figure 3A. A Mann-Whitney U Test examining withdrawal threshold difference scores prior to or following microinjection did not reveal any significant differences. Additionally, Wilcoxin Matched Pairs Tests revealed no changes in withdrawal threshold difference scores due to microinjection. Figure 3C illustrates the distribution of injection sites for animals in these experimental groups.

GABA Microinjection Blocked by Bicuculline/Phaclofen Microinjection and Place Escape/Avoidance Behavior

The mean percentage of time spent in the light side of the place escape/avoidance chamber is illustrated in Figure 3B. An independent t-test revealed that L5 ligated animals that received a microinjection of DMSO followed by a microinjection of 10 $\mu\text{g}/\mu\text{l}$ GABA into the rACC spent significantly less time in the light side of the chamber as compared to L5 ligated animals that received a microinjection of 0.5 $\mu\text{g}/\mu\text{l}$ bicuculline + 1 $\mu\text{g}/\mu\text{l}$ phaclofen followed by a microinjection of 10 $\mu\text{g}/\mu\text{l}$ GABA into the rACC ($t_{20} = -2.11$, $p < 0.05$). Thus, microinjection of a GABA_A and a GABA_B antagonist prior to a microinjection of GABA into the rACC blocked the attenuation of place escape/avoidance behavior seen following a microinjection of GABA into the rACC.

Muscimol Microinjection and Mechanical Paw Withdrawal Threshold

The mean withdrawal threshold difference scores for sham ligated or L5 ligated animals before and after microinjection (saline, 0.001 $\mu\text{g}/\mu\text{l}$, 0.1 $\mu\text{g}/\mu\text{l}$, or 0.5 $\mu\text{g}/\mu\text{l}$ muscimol) into the rACC are illustrated in Figure 4A. A Kruskal-Wallis ANOVA by ranks examining withdrawal threshold difference scores revealed a significant main effect for group prior to microinjection ($H_{7,81} = 62.64$, $p < 0.001$) and following microinjection ($H_{7,81} = 62.78$, $p < 0.001$). Mann-Whitney U tests revealed that both prior to or following microinjection, sham ligated animals showed significantly smaller withdrawal threshold difference scores as compared to ligated animals that received the same drug ($p < 0.001$). Wilcoxin Matched Pairs Tests revealed no changes in withdrawal threshold difference scores due to microinjection within each group. Of importance, a microinjection of saline, 0.001 $\mu\text{g}/\mu\text{l}$, 0.1 $\mu\text{g}/\mu\text{l}$, or 0.5 $\mu\text{g}/\mu\text{l}$ muscimol into the rACC did not alter withdrawal threshold for L5 ligated or sham ligated animals. All L5 ligated groups had significantly larger mean withdrawal threshold difference scores as compared to sham ligated groups which indicates increased sensitivity of the injured paw to mechanical

stimulation. Figure 4E illustrates the distribution of injection sites for animals in these experimental groups (left panel – L5 ligation; right panel – sham ligation).

The mean withdrawal threshold difference scores for L5 ligated animals before and after microinjection (saline or 0.1 µg/µl muscimol) into a region adjacent to the rACC are illustrated in Figure 4B. A Mann-Whitney U Test examining withdrawal threshold difference scores prior to or following microinjection did not reveal any significant differences. Additionally, Wilcoxin Matched Pairs Tests revealed no changes in withdrawal threshold difference scores due to microinjection. Thus, there were no differences in withdrawal threshold for both ligated groups prior to or following microinjection into a region adjacent to the rACC. Figure 4F illustrates the distribution of injection sites for animals in these experimental groups.

In summary, all sham ligated groups (saline, 0.001 µg/µl, 0.1 µg/µl, or 0.5 µg/µl muscimol in the rACC; saline or 0.1 µg/µl muscimol in an adjacent region) were less sensitive to mechanical stimulation both prior to and following microinjection as compared to their ligated counterparts that received a saline microinjection.

Muscimol Microinjection and Place Escape/Avoidance Behavior

The mean percentage of time that animals spent in the light side of the place escape/avoidance chamber for sham ligated and L5 ligated animals following a microinjection (saline, 0.001 µg/µl, 0.1 µg/µl, or 0.5 µg/µl muscimol) into the rACC is illustrated in Figure 4C. A two-factor ANOVA examining place escape/avoidance behavior following microinjection that included L5 ligated and sham ligated animals that received a microinjection into the rACC revealed a significant interaction ($F_{3,73} = 10.72$, $p < 0.001$). Post hoc analyses indicated that all sham ligated groups spent significantly less time in the light side of the chamber as compared to L5 ligated animals that received a saline microinjection into the rACC. Of primary importance is the finding that L5 ligated animals that received a microinjection of either 0.1 or 0.5 µg/µl muscimol into the rACC spent significantly less time in the light side of the place escape/avoidance chamber than L5 ligated animals that received a saline microinjection into the rACC. In addition, the amount of time spent in the light side of the chamber for L5 ligated animals that received a microinjection of either 0.1 or 0.5 µg/µl muscimol into the rACC did not differ from any of the sham ligated groups.

The mean percentage of time that animals spent in the light side of the place escape/avoidance chamber for L5 ligated animals following a microinjection (saline or 0.1 µg/µl muscimol) into a region adjacent to the rACC is illustrated in Figure 4D. An independent t-test examining place escape/avoidance behavior following microinjection did not reveal any significant differences. The attenuation of escape/avoidance behavior following activation of GABA_A neurons in the rACC and not following activation of GABA_A neurons in an adjacent region indicates that these results are specific to this supraspinal region.

Muscimol Microinjection Blocked by Bicuculline Microinjection and Mechanical Paw Withdrawal Threshold

The mean withdrawal threshold difference scores for L5 ligated animals before and after microinjections (DMSO followed by 0.1 µg/µl muscimol or 0.5 µg/µl bicuculline followed by 0.1 µg/µl muscimol) into the rACC are illustrated in Figure 5A. A Mann-Whitney U Test examining withdrawal threshold difference scores prior to or following microinjection did not reveal any significant differences. Additionally, Wilcoxin Matched Pairs Tests revealed no changes in withdrawal threshold difference scores due to microinjection. Figure 5C illustrates the distribution of injection sites for animals in these experimental groups.

Muscimol Microinjection Blocked by Bicuculline Microinjection and Place Escape/Avoidance Behavior

The mean percentage of time spent in the light side of the place escape/avoidance chamber is illustrated in Figure 5B. An independent t-test revealed that L5 ligated animals that received a microinjection of DMSO followed by a microinjection of 0.1 µg/µl muscimol spent significantly less time in the light side of the chamber as compared to L5 ligated animals that received a microinjection of 0.5 µg/µl bicuculline followed by a microinjection of 0.1 µg/µl muscimol ($t_{20} = 2.65$, $p < 0.05$). In summary, there were no differences in withdrawal threshold values prior to or following microinjection of any combination of drugs. However, a GABA_A antagonist followed by a GABA_A agonist microinjected into the rACC blocked the attenuation of place escape/avoidance behavior.

Baclofen Microinjection and Mechanical Paw Withdrawal Threshold

The mean withdrawal threshold difference scores for sham ligated or L5 ligated animals before and after microinjection (saline, 0.1 µg/µl, 1 µg/µl, or 5 µg/µl baclofen) into the rACC are illustrated in Figure 6A. A Kruskal-Wallis ANOVA by ranks examining withdrawal threshold difference scores revealed a significant main effect for group prior to microinjection ($H_{7,80} = 63.19$, $p < 0.001$) and following microinjection ($H_{7,80} = 62.81$, $p < 0.001$). Mann-Whitney U tests revealed that both prior to or following microinjection, sham ligated animals showed significantly smaller withdrawal threshold difference scores as compared to ligated animals that received the same drug ($p < 0.001$). Wilcoxin Matched Pairs Tests revealed that within each group, there were no changes in withdrawal threshold difference scores due to microinjection, except for sham ligated animals that received a 5 µg/µl baclofen microinjection into the rACC. In this group, the withdrawal threshold difference scores became smaller (although in the opposite direction) following microinjection indicating that the scores of the ipsilateral and contralateral paws were more similar following microinjection. Again, of importance is that the animals in this group were not hyperalgesic. In essence, a microinjection of a GABA_B agonist or saline into the rACC did not alter withdrawal threshold for L5 ligated or sham ligated animals. Figure 6C illustrates the distribution of injection sites for animals in these experimental groups (left panel – L5 ligation; right panel – sham ligation).

Baclofen Microinjection and Place Escape/Avoidance Behavior

The mean percentage of time that animals spent in the light side of the place escape/avoidance chamber for sham ligated and L5 ligated animals following a microinjection (saline, 0.1 µg/µl, 1 µg/µl, or 5 µg/µl baclofen) into the rACC is illustrated in Figure 6B. A two-factor ANOVA examining place escape/avoidance behavior following microinjection that included animals that received a microinjection into the rACC revealed a significant main effect for neuropathy ($F_{1,72} = 35.92$, $p < 0.001$). Post hoc analyses indicated that all sham ligated groups spent significantly less time in the light side of the chamber as compared to L5 ligated animals that received a saline microinjection into the rACC. The interaction was not significant indicating that the microinjection had no effect on place escape/avoidance behavior for L5 ligated or sham ligated animals.

Histology

Figure 2G illustrates photomicrographs that are representative examples of cannula tip placements within the rACC (top panel) and in a region other than the rACC (bottom panel). Under magnification, histological analysis revealed that the anterior-posterior extent of cannula tip placements was between 4.2 mm and 1.0 mm relative to bregma (Paxinos and Watson, 1998). In no case was there damage to the corpus callosum or the cingulum bundle.

Discussion

The current study examined the role of rACC GABA receptors in neuropathic pain and escape/avoidance behavior. More specifically, we addressed two main issues: 1. the role of rACC GABA_A receptors in neuropathic pain and escape/avoidance behavior and 2. the role of rACC GABA_B receptors in neuropathic pain and escape/avoidance behavior. GABA_A or GABA_B agonist administration in the rACC did not alter withdrawal threshold following L5 ligation. However, administration of a GABA_A agonist, but not a GABA_B agonist, led to an attenuation of place escape/avoidance behavior induced by a noxious stimulus in L5 ligated animals. Following nerve ligation and prior to microinjection, animals displayed decreased threshold to mechanical stimulation compared to sham ligated animals.

Following microinjection of any dose of GABA, muscimol, baclofen, or saline, injured animals remained hypersensitive to mechanical stimuli as measured by withdrawal threshold compared to control animals.

All sham ligated groups spent 16–32% of the test period in the light side of the escape/avoidance chamber, which is typical of control animals (Labuda and Fuchs, 2000a, LaGraize et al., 2004; LaGraize et al., 2006). L5 ligated groups that received a microinjection of saline, 0.001 µg/µl muscimol, 0.1 µg/µl, 1 µg/µl, or 5 µg/µl baclofen into the rACC or a microinjection of saline, 10 µg/µl GABA, or 0.1 µg/µl muscimol into an adjacent region spent 52–76% of the test period in the light side of the chamber, which is typical of injured animals (Labuda and Fuchs, 2000a, LaGraize et al., 2004; LaGraize et al., 2006). These groups escaped/avoided noxious stimulation in the escape/avoidance chamber. Interestingly, L5 ligated animals that received a microinjection of 10 µg/µl GABA, 0.1 µg/µl, or 0.5 µg/µl muscimol into the rACC spent 17–26% of the test period in the light side of the chamber. Thus, the escape/avoidance behavior typically seen in L5 ligated animals was attenuated in these groups to control levels. The effective doses in the rACC did not attenuate escape/avoidance behavior when administered in a region adjacent to the rACC, indicating an effect that is specific to the rACC. Additionally, the effects of the agonist were receptor-mediated as demonstrated by ability of the antagonist to block the effect. The effects of pain processing following GABA, muscimol, or baclofen microinjection into the rACC are interesting in that there is no study to date that has examined GABA_A and GABA_B involvement within this region in regard to both the sensory dimension of pain and the aversiveness that develops alongside a persistent neuropathic pain condition.

Although the sensory and affective dimensions of pain can be readily dissociated in humans (Melzack, 1975; Rainville et al., 1997), it is a challenge to quantify pain affect in non-human animals. Recent reports from our laboratory have used the place escape/avoidance paradigm as an additional measure of nociceptive processing (LaBuda and Fuchs, 2000a, 2000b, 2001, 2005; LaGraize et al., 2001, 2004, 2006). The most parsimonious explanation for the escape/avoidance behavior displayed by rodents following repeated suprathreshold mechanical stimulation of the injured hindpaw when they are located within the preferred side of the chamber is that it is a result of the aversive quality of the stimulation. Without influencing withdrawal threshold, an attenuation of escape/avoidance behavior in L5 ligated animals has been demonstrated following morphine microinjection into the ACC (LaGraize et al., 2006) or following electrolytic lesion of the ACC (LaGraize et al., 2004). These experiments provide additional support for the role of the ACC in one aspect of pain processing while not affecting the sensory processing of nociception. More specifically, if these experiments only examined threshold to mechanical stimulation, the conclusion would be that the ACC is not involved in nociceptive processing. Thus, these experiments provide additional support for the place escape/avoidance paradigm as a tool for examining an additional aspect of pain processing. Additionally, the evidence resulting from the place escape/avoidance paradigm is in agreement

with clinical studies which have reported that the ACC is involved in attenuating negative affect associated with chronic pain conditions (Foltz and White, 1962; White and Sweet, 1969).

Manipulations within the rACC can obviously influence place escape/avoidance behavior, but what the behavior represents may be a controversial issue. Ablation (Gabriel et al., 1991) and excitation (Johansen and Fields, 2004) of the ACC have been reported to disrupt the acquisition of avoidance behavior. Perhaps following microinjection of GABA or muscimol into the rACC, L5 ligated animals are unable to learn the association between the mechanical stimulus and the side of the escape/avoidance chamber. If this were the case, following stimulation to the injured paw, animals would temporarily escape to the other side of the chamber. Animals would then return to the preferred side of the chamber and receive the noxious stimulation to the injured paw again. This behavior would repeat and there would be an increased frequency of crossing the center line of the chamber. In the place escape/avoidance chamber, there is an initial period of exploration (frequent line crossings during the first 5–8 min) followed by the animals ‘choosing’ a side and not moving much for the remainder of the test. Consequently, if acquisition of avoidance behavior was disrupted, animals would have more center line crossings and this was not the case (data not shown). Thus, it is unlikely that the acquisition of escape/avoidance behavior is disrupted in this paradigm.

An additional alternative for the current findings is that following microinjection of a GABA or muscimol into the rACC, there is a disruption of working memory. In this circumstance, animals may not ‘remember’ that the mechanical stimulation in the preferred dark area of the chamber is noxious. Following the noxious stimulation in the dark area of the chamber, they would escape to the light area and promptly return to the dark area because they do not ‘remember’ that the stimulation they received in that area was noxious. Thus, injured animals that received GABA or muscimol microinjected into the rACC should have increased mobility throughout the test period as measured by crossing the center line of the chamber. Again, this was not the case (data not shown) which indicates that working memory was intact following rACC microinjection. The current data are further supported by research reporting that prefrontal cortex lesions in rats did not lead to a disruption of working memory measured by the radial arm maze as opposed to prefrontal cortex lesions that included the prelimbic and infralimbic cortices (Fritts et al., 1998). Additional research indicates that following frontal cortex lesions, there is no impairment of working memory or reference memory (Blokland et al., 2003).

Although evidence suggests that the ACC is involved in various aspects of learning and memory, it is unlikely that these phenomena alter place escape/avoidance behavior. The most parsimonious explanation is that the stimulus that is administered in the dark side of the chamber is more aversive to L5 ligated animals and thus, they escape/avoid that stimulus. Following a microinjection of 10 µg/µl GABA, 0.1 µg/µl or 0.5 µg/µl of muscimol into the rACC, this behavior is attenuated indicating a role for GABA_A receptors within this region in escape/avoidance behavior. However, our findings fail to implicate GABA_B receptors within this region in this behavior. Support for the ACC in emotional processing comes from human studies where the ACC is activated (Bush et al., 1998; Whalen et al., 1998) or deactivated (Drevets and Raichle, 1998) during emotional stimuli. For example, words with emotional valence (e.g., “murder”) resulted in activation of the ACC (Bush et al., 1998; Whalen et al., 1998).

One explanation for the involvement of GABA_A receptors, and not GABA_B receptors, is that within the ACC, there are at least twice as many binding sites for GABA_A as compared to GABA_B (Chu et al., 1990). However, the functional significance of higher receptor densities remains unknown and most likely does not account for the present findings. A more likely

explanation for the involvement of GABA_A and not GABA_B receptors in the supraspinal modulation of pain may be related to the differential sites of action of GABA_A and GABA_B receptors (i.e. pre- or postsynaptic) and the underlying mechanisms associated with the action of GABA_A and GABA_B agonists (Sylantsev et al., 2005). Although the precise mechanism(s) of GABAergic modulation of pain affect remains unknown, the present findings provide additional support for a recent report indicating that muscimol ACC microinjection blocks the acquisition of the formalin induced conditioned place avoidance response (Wang et al., 2005)

The neural representation of the attenuation of place escape/avoidance behavior is unknown. It is likely the effect involves the rACC via reciprocal connections with other limbic system and brainstem structures. It is known that nociceptive information reaches the rACC by projections from the medial thalamus and periaqueductal gray area (Hsu and Shyu, 1997; An et al., 1998; Floyd et al., 2000). Through projections from the rACC to the amygdala and periaqueductal gray area, various other physiological and psychological components may be altered. Information that is transmitted and terminates in the ACC may, in turn, be used in the descending transmission of information regarding supraspinal pain processing. It is proposed that the rACC is involved in the supraspinal processing of nociceptive information from the periphery. Additional experiments are needed in order to fully understand the involvement of the rACC in pain processing.

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Figure 1.
Time line for behavioral testing.

Figure 2.

Withdrawal threshold difference scores, place escape/avoidance behavior, and histological representation for animals that received either a saline or GABA microinjection. A: Mean withdrawal threshold difference scores ($\pm$ SEM) for sham ligated and L5 ligated animals prior to and following microinjection of saline or 10 $\mu\text{g}/\mu\text{l}$ GABA within the rACC. *** $p < 0.001$ versus Ligation/same drug condition at the same time point. B: Mean withdrawal threshold difference scores ($\pm$ SEM) for L5 ligated animals prior to and following microinjection of saline or 10 $\mu\text{g}/\mu\text{l}$ GABA into a region adjacent to the rACC. C: Mean percentage of time spent in the light side of the place escape/avoidance chamber ($\pm$ SEM) during the 30-min test period for sham ligated or L5 ligated animals following microinjection of saline or 10 $\mu\text{g}/\mu\text{l}$ GABA

within the rACC. * $p < 0.05$ versus Ligation/Saline; ** $p < 0.01$ versus Ligation/Saline. D: Mean percentage of time spent in the light side of the place escape/avoidance chamber ($\pm$ SEM) during the 30-min test period for L5 ligated animals following microinjection of saline or 10 $\mu\text{g}/\mu\text{l}$ GABA into a region other than the rACC. E: Histological representation of cannula tip placements within the rACC for animals that received a microinjection of saline (open circles) or 10 $\mu\text{g}/\mu\text{l}$ (closed circles). The anterior-posterior extent of cannula tip placements was between 3.7 mm to 1.7 mm relative to bregma according to the atlas of Paxinos and Watson (1998). Left panel: L5 ligated animals. Right panel: Sham ligated animals. F: Histological representation of cannula tip placements in a region other than the rACC for L5 ligated animals that received a microinjection of saline (open circles) or 10 $\mu\text{g}/\mu\text{l}$ GABA (closed circles). The anterior-posterior extent of most of the cannula tip placements was between 4.2 mm to 1.7 mm relative to bregma (one cannula tip placement was at 1.0 mm relative to bregma and not included in this figure for simplicity) according to the atlas of Paxinos and Watson (1998). G: Photomicrographs of coronal sections depicting representative examples of cannula tip placements. Top panel: Example of a cannula placed within the rACC. Bottom panel: Example of a cannula placed in a region adjacent to the rACC. Ligation/Saline/hit, $n = 10$; Ligation/10 $\mu\text{g}/\mu\text{l}$ GABA/hit, $n = 10$; Sham Ligation/Saline/hit, $n = 8$; Sham Ligation/10 $\mu\text{g}/\mu\text{l}$ GABA/hit, $n = 10$; Ligation/Saline/miss, $n = 10$; Ligation/10 $\mu\text{g}/\mu\text{l}$ GABA/miss, $n = 10$.

Figure 3.

Withdrawal threshold difference scores, place escape/avoidance behavior, and histological representation for animals that received a microinjection of DMSO or bicuculline + baclofen within the rACC followed by a microinjection of 10 µg/µl GABA into the same region. A: Mean withdrawal threshold difference scores ($\pm$ SEM) for L5 ligated animals prior to and following microinjections of DMSO/10 µg/µl GABA or 0.5 µg/µl bicuculline + 1 µg/µl phaclofen/10 µg/µl GABA into the rACC. B: Mean percentage of time spent in the light side of the place escape/avoidance chamber ($\pm$ SEM) during the 30-min test period for L5 ligated animals following microinjections of DMSO followed by 10 µg/µl GABA or 0.5 µg/µl bicuculline + 1 µg/µl phaclofen followed by 10 µg/µl GABA into the rACC. * $p < 0.05$ versus 0.5 µg/µl bicuculline + 1 µg/µl phaclofen/10 µg/µl GABA. C: Histological representation of cannula tip placements within the rACC for L5 ligated animals that received microinjections of DMSO followed by 10 µg/µl GABA (open triangles) or 0.5 µg/µl bicuculline + 1 µg/µl phaclofen followed by 10 µg/µl GABA (closed triangles). The anterior-posterior extent of cannula tip placements was between 3.7 mm to 1.7 mm relative to bregma according to the atlas of Paxinos and Watson (1998). DMSO/10 µg/µl GABA, $n = 11$; 0.5 µg/µl bicuculline + 1 µg/µl phaclofen/10 µg/µl GABA, $n = 11$.

Figure 4.

Withdrawal threshold difference scores, place escape/avoidance behavior, and histological representation for animals that received either a saline or muscimol microinjection. A: Mean withdrawal threshold difference scores ($\pm$ SEM) for sham ligated and L5 ligated animals prior to and following microinjection of saline, 0.001 $\mu\text{g}/\mu\text{l}$ muscimol, 0.1 $\mu\text{g}/\mu\text{l}$ muscimol, or 0.5 $\mu\text{g}/\mu\text{l}$ muscimol within the rACC. *** $p < 0.001$ versus Ligation/same drug condition at the same time point. B: Mean withdrawal threshold difference scores ($\pm$ SEM) for L5 ligated animals prior to and following microinjection of saline or 0.1 $\mu\text{g}/\mu\text{l}$ muscimol into a region adjacent to the rACC. C: Mean percentage of time spent in the light side of the place escape/avoidance chamber ($\pm$ SEM) during the 30-min test period for sham ligated or L5 ligated

animals following microinjection of saline, 0.001 $\mu\text{g}/\mu\text{l}$ muscimol, 0.1 $\mu\text{g}/\mu\text{l}$ muscimol, or 0.5 $\mu\text{g}/\mu\text{l}$ muscimol within the rACC. *** $p < 0.001$ versus Ligation/Saline. D: Mean percentage of time spent in the light side of the place escape/avoidance chamber ($\pm$ SEM) during the 30-min test period for L5 ligated animals following microinjection of saline or 0.1 $\mu\text{g}/\mu\text{l}$ muscimol into a region other than the rACC. E: Histological representation of cannula tip placements within the rACC for animals that received a microinjection of saline (open circles), 0.001 $\mu\text{g}/\mu\text{l}$ muscimol (closed circles), 0.1 $\mu\text{g}/\mu\text{l}$ muscimol (open squares), or 0.5 $\mu\text{g}/\mu\text{l}$ muscimol (closed squares). The anterior-posterior extent of cannula tip placements was between 3.7 mm to 1.7 mm relative to bregma according to the atlas of Paxinos and Watson (1998). Left panel: L5 ligated animals. Right panel: Sham ligated animals. F: Histological representation of cannula tip placements in a region other than the rACC for L5 ligated animals that received a microinjection of saline (open circles) or 0.1 $\mu\text{g}/\mu\text{l}$ muscimol (closed circles). The anterior-posterior extent of cannula tip placements was between 3.2 mm to 2.2 mm relative to bregma according to the atlas of Paxinos and Watson (1998). Ligation/Saline/hit, $n = 10$; Ligation/0.001 $\mu\text{g}/\mu\text{l}$ muscimol/hit, $n = 8$; Ligation/0.1 $\mu\text{g}/\mu\text{l}$ muscimol/hit, $n = 11$; Ligation/0.5 $\mu\text{g}/\mu\text{l}$ muscimol/hit, $n = 11$; Sham Ligation/Saline/hit, $n = 11$; Sham Ligation/0.001 $\mu\text{g}/\mu\text{l}$ muscimol/hit, $n = 11$; Sham Ligation/0.1 $\mu\text{g}/\mu\text{l}$ muscimol/hit, $n = 9$; Sham Ligation/0.5 $\mu\text{g}/\mu\text{l}$ muscimol/hit, $n = 10$; Ligation/Saline/miss, $n = 10$; Ligation/0.1 $\mu\text{g}/\mu\text{l}$ muscimol/miss, $n = 10$.

Figure 5.

Withdrawal threshold difference scores, place escape/avoidance behavior, and histological representation for animals that received a microinjection of DMSO or bicuculline within the rACC followed by a microinjection of 0.1 µg/µl muscimol into the same region. A: Mean withdrawal threshold difference scores ($\pm$ SEM) for L5 ligated animals prior to and following microinjections of DMSO/0.1 µg/µl muscimol or 0.5 µg/µl bicuculline/0.1 µg/µl muscimol into the rACC. B: Mean percentage of time spent in the light side of the place escape/avoidance chamber ($\pm$ SEM) during the 30-min test period for L5 ligated animals following microinjections of DMSO followed by 0.1 µg/µl muscimol or 0.5 µg/µl bicuculline followed by 0.1 µg/µl muscimol into the rACC. * $p < 0.05$ versus 0.5 µg/µl bicuculline/0.1 µg/µl muscimol. C: Histological representation of cannula tip placements within the rACC for L5 ligated animals that received microinjections of DMSO followed by 0.1 µg/µl muscimol (open triangles) or 0.5 µg/µl bicuculline followed by 0.1 µg/µl muscimol (closed triangles). The anterior-posterior extent of cannula tip placements was between 3.2 mm to 1.7 mm relative to bregma according to the atlas of Paxinos and Watson (1998). DMSO/0.1 µg/µl muscimol, $n = 11$; 0.5 µg/µl bicuculline/0.1 µg/µl muscimol, $n = 11$.

Figure 6.

Withdrawal threshold difference scores, place escape/avoidance behavior, and histological representation for animals that received either a saline or baclofen microinjection. A: Mean withdrawal threshold difference scores (± SEM) for sham ligated and L5 ligated animals prior to and following microinjection of saline, 0.1 µg/µl baclofen, 1 µg/µl baclofen, or 5 µg/µl baclofen within the rACC. *** $p < 0.001$ versus Ligation/same drug condition at the same time point. B: Mean percentage of time spent in the light side of the place escape/avoidance chamber (± SEM) during the 30-min test period for sham ligated or L5 ligated animals following microinjection of saline, 0.1 µg/µl baclofen, 1 µg/µl baclofen, or 5 µg/µl baclofen within the rACC. ** $p < 0.01$ versus Ligation/Saline; *** $p < 0.001$ versus Ligation/Saline. C: Histological representation of cannula tip placements within the rACC for animals that received a microinjection of saline (open circles), 0.1 µg/µl baclofen (closed circles), 1 µg/µl baclofen (open squares), or 5 µg/µl baclofen (closed squares). The anterior-posterior extent of cannula tip placements was between 3.7 mm to 1.7 mm relative to bregma according to the atlas of Paxinos and Watson (1998). Left panel: L5 ligated animals. Right panel: Sham ligated animals. Ligation/Saline, $n = 10$; Ligation/0.1 µg/µl baclofen, $n = 9$; Ligation/1 µg/µl baclofen, $n = 10$; Ligation/5 µg/µl baclofen, $n = 10$; Sham Ligation/Saline, $n = 9$; Sham Ligation/0.1 µg/µl baclofen, $n = 11$; Sham Ligation/1 µg/µl baclofen, $n = 11$; Sham Ligation/5 µg/µl baclofen, $n = 10$.

The groups included in the experiments as well as the number of animals within each group.

Table 1

GABA MICROINJECTION:			
rACC Hit		rACC Miss	
Sham Ligation	Ligation	Sham Ligation	Ligation
Saline (n=8) 10 µg/µl GABA (n=10)	Saline (n=10) 10 µg/µl GABA (n=10)		Saline (n=10) 10 µg/µl GABA (n=10)
GABA MICROINJECTION REVERSED BY BICUCULLINE/PHACLOFEN MICROINJECTION:			
rACC Hit		rACC Miss	
Sham Ligation	Ligation	Sham Ligation	Ligation
	DMSO/10 µg/µl GABA (n=11) 0.5 µg/µl Bic + 1 µg/µl Phac/10 µg/µl GABA (n=11)		
MUSCIMOL MICROINJECTION:			
rACC Hit		rACC Miss	
Sham Ligation	Ligation	Sham Ligation	Ligation
Saline (n=11) 0.001 µg/µl Muse (n=11) 0.1 µg/µl Muse (n=9) 0.5 µg/µl Muse (n=10)	Saline (n=10) 0.001 µg/µl Muse (n=8) 0.1 µg/µl Muse (n=11) 0.5 µg/µl Muse (n=11)		Saline (n=10) 0.1 µg/µl Muse (n=10)
MUSCIMOL MICROINJECTION REVERSED BY BICUCULLINE MICROINJECTION:			
rACC Hit		rACC Miss	
Sham Ligation	Ligation	Sham Ligation	Ligation
	DMSO/0.1 µg/µl Muse (n=11) 0.5 µg/µl Bic/0.1 µg/µl Muse (n=11)		
BACLOFEN MICROINJECTION:			
rACC Hit		rACC Miss	
Sham Ligation	Ligation	Sham Ligation	Ligation
Saline (n=9) 0.1 µg/µl Bac (n=11) 1 µg/µl Bac (n=11) 5 µg/µl Bac (n=10)	Saline (n=10) 0.1 µg/µl Bac (n=9) 1 µg/µl Bac (n=10) 5 µg/µl Bac (n=10)		