Role for NMDA receptors in visceral nociceptive transmission in the anterior cingulate cortex of viscerally hypersensitive rats

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Wu X, Gao J, Yan J, Fan J, Owyang C, Li Y. Role for NMDA receptors in visceral nociceptive transmission in the anterior cingulate cortex of viscerally hypersensitive rats. Am J Physiol Gastrointest Liver Physiol 294: G918–G927, 2008. First published February 7, 2008; doi:10.1152/ajpgi.00452.2007.—We have identified colorectal distension (CRD)-responsive neurons in the anterior cingulate cortex (ACC) and demonstrated that persistence of a heightened visceralafferent nociceptive input to the ACC induces ACC sensitization. In the present study, we confirmed that rostral ACC neurons of sensitized rats [induced by chicken egg albumin (EA)] exhibit enhanced spike responses to CRD. Simultaneous in vivo recording and reverse microdialysis of single ACC neurons showed that a low dose of glutamate (50 μM) did not change basal ACC neuronal firing in normal rats but increased ACC neuronal firing in EA rats from 18 ± 2 to 32 ± 3.8 impulses/10 s. A high dose of glutamate (500 μM) produced 1.95-fold and a 4.27-fold increases of ACC neuronal firing in sham-treated rats and in EA rats, respectively, suggesting enhanced glutamatergic transmission in the ACC neurons of EA rats. Reverse microdialysis of the 3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) reduced basal and abolished CRD-induced ACC neuronal firing in normal rats. In contrast, microdialysis of N-methyl-d-aspartate (NMDA) receptor antagonist AP5 had no effect on ACC neuronal firing in normal rats. However, AP5 produced 86% inhibition of ACC neuronal firing evoked by 50 mmHg CRD in the EA rats. In conclusion, ACC nociceptive transmissions are mediated by glutamate AMPA receptors in the control rats. ACC responses to CRD are enhanced in viscerally hypersensitive rats. The enhancement of excitatory glutamatergic transmission in the ACC appears to mediate this response. Furthermore, NMDA receptors mediate ACC synaptic responses after the induction of visceral hypersensitivity.

anterior cingulate cortex; visceral hypersensitivity; NMDA and non-NMDA receptors

HYPERSENSITIVITY TO VISERAL distending stimuli has been shown in patients with irritable bowel syndrome (IBS). Patients with IBS have lower pain thresholds during rectal distension, accompanied by the development of excessive reflex motor activity in the rectum (28). It has been shown that peripheral sensitization results from an increase in the sensitivity and excitability of the afferent nerve itself and/or the dorsal horn of the spinal cord (15, 25). The brain interprets and influences the perception of pain sensation signals transmitted from the gut. The anterior cingulate cortex (ACC) has a functional relationship to emotional and motivational responses and to the brain’s cognitive functions, including attention and memory, as well as sensory perception (7, 18, 33, 40, 45). Experiments in animals have demonstrated that the ACC receives nociceptive inputs (14, 41). Chronic pain is reduced in patients with ACC lesions (8).

Our electrophysiological studies of viscerally hypersensitive rats showed that colorectal anaphylaxis results in enhanced ACC spontaneous activity, decreased colorectal distension (CRD) pressure threshold that stimulate ACC neurons, and increased magnitude of the ACC response (14). Furthermore, a population of rostral ACC neurons is capable of hypersensitive-discriminative coding, specifically for visceral afferent input (13, 14). These findings suggest that the persistence of a heightened tonic visceral afferent nociceptive input to the ACC enhances the neuronal response of the ACC to noxious visceral stimulation and furthermore provides evidence of ACC neuronal plasticity in viscerally hypersensitive rats.

Glutamate is a major mediator of excitatory signals in the central nervous system and is involved in many physiological and pathological processes, such as excitatory synaptic transmission, synaptic plasticity, cell death, stroke, and chronic pain (26). Just as in other regions of the central nervous system, fast excitatory synaptic transmission within the ACC is mediated by the excitatory amino acid glutamate (35, 48). Glutamate exerts its signaling role by acting on glutamate receptors, including N-methyl-d-aspartate (NMDA), 3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate, and metabotropic glutamate receptors. NMDA receptors are ionotropic glutamate receptors that require glycine as a coagonist, display voltage-dependent inhibition by extracellular Mg2+, and have high permeability to Ca2+. These properties contribute to the unique role of NMDA receptors in neuronal plasticity (11, 30). In most cases, synaptic responses are primarily mediated through postsynaptic AMPA/kainate receptors, since NMDA receptors are blocked by Mg2+ at resting membrane potential. However, there are reports that NMDA receptors contribute to synaptic transmission and modulation in the cortex, hippocampus, and spinal cord (34, 16).

In the ACC, NMDA receptors are highly expressed, although their function remains unclear. Genetic enhancement of NMDA receptor function in forebrain structures, including the hippocampus and the ACC, intensifies behavioral responses to tissue inflammation (48). The present study was designed to characterize the electrophysiological properties of ACC neurons that are activated by CRD. Single ACC neuronal discharges were examined in sham-treated rats and in viscerally hypersensitive rats. To study the effects of local pharmacological manipulations of ACC neurons, we performed extracellular single neuronal recording at the same time as reverse microdialysis. The responses of CRD-excited ACC neurons to...
application of glutamate were characterized. Finally, the roles of NMDA and non-NMDA receptors in the mediation of ACC neuronal responses to noxious visceral stimulation were examined in both viscerally hypersensitive and normal states.

**METHODS**

**Ethical Approval**

Experimental procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Materials**

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Experiments were performed on adult male Sprague-Dawley rats (275–300 g). The animals were housed four per plastic cage and maintained on a 12:12-h light-dark cycle (lights on at 7 AM) and given access to food and water ad libitum.

**Rat Model of Visceral Hypersensitivity and Colonic Anaphylaxis**

Previous studies have shown that intestinal anaphylaxis alters intestinal motility (37) and triggers neuronal activations in the nucleus of the solitary tract (NTS) (38). This model has been shown by number of investigators (19) including ourselves (14) to be suitable for the study of visceral hypersensitivity.

The rats were sensitized to chicken egg albumin (EA) with an injection of 1 ml ip normal saline containing EA (10 μg) as the antigen and aluminum hydroxide (10 mg) as the adjuvant. On day 3, colorectal anaphylaxis was induced. Antigen solution was perfused through a Silastic enema tube at 50 μl per min for 30 min (EA, 10 μg/ml in 40 mM d-glucose, made isotonic with NaCl). CRD (30 mmHg for 30 s with a 3-min interval, repeated 5 times) was performed 30 min after EA instillation. This entire procedure was performed once a day for 3 consecutive days. No significant inflammatory changes were observed in the colon 7 days after colonic anaphylaxis. After an interval of 7–10 days, ACC electrophysiological recordings were performed. Successful sensitization was verified by the detection of aluminum hydroxide deposits in the abdominal cavity during laparotomy. No inflammation changes were observed in colon morphology 7–10 days after initiation of visceral hypersensitivity. Rats injected with 1 ml ip normal saline served as the sham-treated controls. Three days after the saline injection, these rats were subjected to an intracolonic infusion of saline and CRD (30 mmHg maintained for 30 s), performed on 3 consecutive days, without EA challenge. Recording studies were conducted 7–10 days after the third day using the same protocol as used with the sensitized rats (14).

**Electrophysiological Recording of ACC Neurons**

Rats were anesthetized with an injection of a mixture of α-chloralose (80 mg/kg ip) and urethane (800 mg/kg ip). Intravenous supplements of anesthetic were given every 3.5 h, with one-fourth of the initial intraperitoneal dose. Body temperature was maintained at 36.5 ± 0.5°C by use of a heating blanket. The electrocardiogram was monitored continuously. A craniotomy was performed. An opening was made 1.0–5.0 mm anterior to bregma and 0.1–2.0 mm lateral to midline to record neurons in the ACC. All wound margins were covered with Nupercainal. Heart rate and respiratory rate were monitored throughout the experiment to ensure a consistent anesthetic response. Signs of arousal, such as increased heart rate and respiratory rate and coordinated movements, were absent, but ACC neuronal activity remained robust. Glass microelectrodes with tip diameters of 0.08 mm and 14 MΩ impedance were filled with neurobiotin (4%, Vector Laboratories, Burlingame, CA) in 1.0 M KCl-Tris buffer (pH 7.6) and lowered into the rostral ACC by with a micromanipulator (coordinates: 1.5–3.8 mm anterior to bregma, 0.3–1.0 mm lateral to midline, 1.5–3.5 mm ventral to brain surface). The rostral ACC, as defined by Vogt and Peters (44), is the area corresponding to perigenual Brodmann area 24b, portions of perigenual 24a, and caudal dorsal area 32. In rats, the rostral cingulate receives most afferents from the mediodorsal thalamic nucleus (21). Areas of the rostral ACC are rich in nociceptive input, as reported in the literature (14, 20). After penetrating the surface of the cortex and avoiding blood vessels near the midline, the recording electrode was advanced until the spontaneous activity of a single unit could be accurately discriminated from the background neuronal noise. The recording had uniform spike amplitude and could be maximized and separated from neighboring neurons. Noise levels were typically 40–50 μV. Only well-isolated neurons that showed a signal-to-noise ratio of at least 4:1 were analyzed.

The signals were amplified by a high-input impedance preamplifier (A-M Systems, Carlsborg, WA), displayed, and stored on a personal computer with a 166-MHz Pentium processor using Axoscope software (Axon Instruments, Union City, CA). Data were recorded digitally (Datawave Systems, Thornton, CO).

**Drug Application**

To study the effects of local pharmacological manipulations on ACC neurons in vivo, we performed extracellular single neuronal recording at the same time as reverse microdialysis of glutamate and various glutamate antagonists. Reverse microdialysis is a useful tool for administering drugs into localized regions of brain tissue (24, 51). This technique provides administration of drugs in vivo with no change in tissue pressure or volume and no current or voltage stimuli (51). Microdialysis probes (Bioanalytical Systems, West Lafayette, IN) with 3–4 mm of exposed membrane (320-μm diameter, ~6,000 Da permeability) were implanted into the ACC (coordinates: 1.5–3.8 mm anterior to bregma, 0.3–1.0 mm lateral to midline, and lowered about 4.2 mm at a 30° angle) with a micromanipulator at 5 μm/s. Once the probe reached the targeted region of the ACC, it was fixed in the stereotactic carrier for the remainder of the experiment. After implantation, the probes were perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 147 NaCl, 3.0 KCl, 0.8 MgCl2, 1.2 CaCl2, 2.0 NaH2PO4, and 2.0 Na2HPO4 at a rate of 2 μl/min by use of a microperfusion pump (World Precision Instruments, Sarasota, FL). Glass microelectrodes were filled with neurobiotin and lowered into the rostral ACC ~1 mm lateral or rostral to the probe and angled at 10° toward the probe. The distance between microdialysis probe and recording electrode was 0.5–1.0 mm. The distance was estimated from the stereotaxic coordinates as well as from the number of histological sections. Electrophysiological recordings were initiated ~2 h after probe implantation. Given that stable neurons recorded in animals undergoing microdialysis in the present study exhibited electrophysiological properties similar to those recorded in intact controls, it is likely that the effects of local microdialysis on ongoing synaptic activity and neuronal excitability were minimal.

**Labeling and Histological Identification of Recording Sites: Juxtacellular Injection**

On completion of the experiment, recorded neurons were labeled by injecting them with neurobiotin by the technique of juxtacellular iontophoresis as we described previously (14, 32, 36). Continuous electrophysiological control ensured that the neurons remained alive. The animals were allowed to survive for 6–8 h after the juxtacellular injection of neurobiotin and then were deeply anesthetized with pentobarbital sodium and perfused transcardially with 50 ml PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and serial coronal sections were cut with a cryostat along the path of electrode penetration. Sections were incubated with peroxidase-conjugated avidin-biotin complex (1:100; ABC; Vector Laboratories) for histological identification of recording sites, and plotted on standard adapted from Paxinos and Watson (31, Fig. 1). Details of the filling
protocol and histological procedures have been described previously (14).

**Experimental Design**

**ACC neuronal activity in response to CRD in control and EA rats.** ACC neuronal spontaneous discharge was monitored for 2 min to confirm the stability of the basal firing frequency. The basal firing rate was assessed over 30 s to quantify the resting discharge in both control and EA rats. Every neuron isolated on the basis of spontaneous activity was studied to determine its response to CRD. The colon was emptied with an enema (1 ml of saline). A polyethylene tube (ID 1.67 mm) attached to a water-filled latex balloon (4 cm length) which was lightly coated with a surgical lubricant was inserted into the anal canal to the rectum, a distance of 9–10 cm, and secured to the tail. Graded-pressure distension was produced by rapidly injecting saline into the balloon over 1 s and maintaining the distension for 30 s. Pressure within the balloon was monitored by connecting the catheter by way of a three-way stopcock to a pressure transducer (World Precision Instruments) (5, 14). Neurons responding to 50 mmHg CRD were tested twice to make sure the responses were consistent and repeatable. A neuron was deemed responsive to CRD if its spike firing rate increased or decreased at least 10% from its predistension baseline activity. Neuronal discharge rates were measured 30 s before, 30 s during, and 120 s after CRD, with 5-min intervals in between, and evaluated on a time histogram (5-s bin width). Consistent monitoring of each neuron was ensured by careful study of the firing pattern, the amplitude, and the waveform. Previously, we have reported that in normal rats ACC neurons activated by 50 mmHg CRD, but not by 30 mmHg CRD. This study, ACC neuronal responses induced by 50 mmHg CRD were examined in normal rats, whereas 30 and 50 mmHg CRD were applied on the visceral hypersensitive rats.

**ACC neuronal activity in response to glutamate.** We hypothesized that enhancement of synaptic excitatory glutamatergic transmission in the ACC plays a key role in the mediation of enhanced postsynaptic ACC neuronal activation in viscerally hypersensitive rats. In a group of rats, 50 mmHg CRD-excited neurons were subjected to application of glutamate by reverse microdialysis. After characterizing the CRD-excited ACC neurons, steady-state basal activity was recorded. Each neuron was further tested in response to glutamate. Before each drug application, ACSF was perfused through the microdialysis probe for 2 min; then, by use of a zero dead-volume liquid switch (CMA Microdialysis, North Chelmsford, MA), ACSF containing glutamate (50, 100, and 500 μM) was perfused. It is estimated that the time elapsed between the switch from ACSF to ACSF with the drug ensured that drug was being delivered into the brain during a given recording period. The dialysis tubing dead space (8 μl) and perfusate flow rate (2 μl/min) were considered, and the syringes containing the drug were switched 4 min before assessment of the effect of glutamate. This infusion system was similar to the reverse dialysis system reported by other laboratories (2, 24, 51). The concentration of drugs in the tissue immediately adjacent to the probe was estimated to be ~10% (for 2-mm probes) or 25% (for 4-mm probes) of the concentration in the perfusion fluid and substantially less at the soma of the neuron being recorded (30). Drugs were dissolved in ACSF at a concentration 100 times that predicted to be needed, according to data from in vitro studies (10, 17). ACC neuronal firings were examined before and after microdialysis of glutamate (50, 100, and 500 μM) in normal and sensitized rats. Effective doses of glutamate were derived from previous studies (24, 50, 51). Each ACC neuron was tested for three doses of glutamate administration with 15-min washout periods. In our preliminary studies, different concentrations of glutamate were administered in a cumulative fashion. When concentrations were tested individually in some experiments, no difference was found compared with the cumulative concentration-response data.

**Roles for NMDA and non-NMDA receptors in visceral nociceptive transmission in the ACC in normal and viscerally hypersensitive rats.** In separate groups of rats, electrophysiological recording of ACC neuronal basal activity and the responses evoked by CRD were conducted. The effects of NMDA and non-NMDA antagonists on 50 mmHg CRD-excited ACC neurons in normal rats and 30 and 50

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**Fig. 1. Location of neurons recorded in the anterior cingulate cortex (ACC).** Coronal sections from the caudal to rostral regions of the ACC (i.e., bregma 1.70, 2.70, and 3.70 mm) show the electrophysiological recording sites (adapted from the atlas of the rat brain by Paxinos and Watson; Ref. 31). Squares and circles indicate CRD-excited ACC neurons in the normal and sensitized rats, respectively. Cl, claustrum; Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2; IL, intralimbic cortex; M2, secondary motor cortex; MO, medial orbital cortex; PrL, prelimbic cortex; VO, ventral orbital cortex.
mmHg CRD-excited ACC neurons in EA rats were examined. The NMDA receptor blocker aminophosphonopentanoic acid (AP5) (100 μM, 500 μM, and 2.0 mM) and the AMPA/kainate receptor blocker 6-cyano-7-nitroquinoline-2,3-dione (CNQX) (100 μM, 500 μM, and 1.0 mM) were administered by reverse microdialysis. One dose of antagonist was applied to each CRD-excited neuron. The doses of AP5 and CNQX were chosen in accordance with previous studies, which showed that similar doses of glutamate receptors antagonists inhibited ACC laminar transmembrane currents (layer II/III and layer V) during noxious electrical stimulation of medial thalamus (53) and suppressed pain-related aversion (23), avoidance (27a), and visceral pain responses in rats (5).

Data analysis and statistics. Single neuronal responses were examined using Datapac 2,000 (RUN Technologies, Mission Viejo, CA). The prestimulus discharge frequency was assessed for 30 s to quantify the resting discharge. The discharge frequency during CRD was also measured for 30 s. The mean and standard deviation of ACC neuronal firing during the 30-s control period was compared with the activity after CRD and after administration of glutamate antagonists. Data of spontaneous firing in various experimental groups were evaluated using the Dunn T3 multiple-comparisons method, after one-way ANOVA. Statistical comparisons of the CRD responses and the glutamate dose responses in various groups were made using one-way repeated-measures ANOVA followed by multiple comparisons adjusted by the Bonferroni test. Results were expressed as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

ACC Neuronal Responses to CRD and Glutamate in Control Rats

A total 37 control rats were examined for their responses to CRD (50 mmHg) and reverse microdialysis of glutamate. One neuron per rat was labeled with neurobiotin. Neurobiotin labeling failed in 6 of 37 rats. Of these six rats, the juxtacellular configurations were lost during the injection in three rats, and histological studies revealed an absence of stained cells in three rats, presumably because neurobiotin was deposited extracellularly. These rats were excluded from further study. Histological localization of the CRD-responsive neurons showed that the recording electrodes were successfully placed in the ACC of 31 rats. All of the recordings had uniform spike amplitude and could be minimized and separated from the recordings of the neighboring neurons. One hundred nine neurons were recorded in the 31 rats. Spontaneous activity was sufficiently stable to permit recording for 45–70 min. Among the 109 neurons, 78 showed no response to CRD, and 26 (24% of total 109 neurons; Fig. 1) exhibited an excitatory response characterized by increased spike firings from baseline 8.2 ± 0.85 to 16 ± 1.5 impulses/10 s and thus were referred to as CRD-excited neurons (Fig. 2). Eight CRD-excited neurons were examined for responses to graded doses of glutamate. The remaining 18 CRD-excited neurons were examined for the effects of various glutamate antagonists on ACC neuronal responses to CRD. Five of 109 neurons were inhibited by 50 mmHg CRD (CRD-inhibited neurons); their spontaneous activity was reduced to 0.28 ± 0.02 impulses/10 s. These CRD-inhibited neurons were not tested further. Microdialysis of vehicle (ACSF, 2 μl/min) had no effect on ACC neuronal firing. Application of glutamate at a dose of 50 μM (concentration in the microdialysis fibers) had no effect on ACC neuronal firing. Glutamate at concentrations of 100 and 500 μM significantly increased ACC neuronal firing from basal 8.2 ± 0.5 to 12 ± 1.0 and 16 ± 1.5 impulses/10 s (1.44- and 1.92-fold increases, respectively) in normal rats (Figs. 3 and 4). The excitatory effect of glutamate was relatively quick, typically occurring within 3–10 s after the start of the glutamate perfusion. The spike responses recovered after 10–30 s, indicating that the effect is reversible.

Effects of Glutamate Receptor Antagonists on ACC Neuronal Activation Evoked by CRD in Normal Rats

As mentioned, glutamate receptors antagonist studies were performed in 18 CRD-excited neurons. One dose of antagonist was applied to each CRD-excited ACC neuron. Application of the AMPA glutamate receptor antagonist CNQX at concentrations of 100 and 500 μM (in the microdialysis fibers) significantly decreased basal ACC neuronal firings from 8.2 ± 0.5 to 5 ± 0.25 and 4 ± 0.02 impulses/10 s, a 40 and 49% inhibition of spontaneous firings, respectively. CNQX at concentrations of 100 and 500 μM decreased ACC neuronal firings in response to 50 mmHg CRD from vehicle 15.5 ± 2 impulses/10 s (a 91% increase over basal) to 9 ± 1 and 7 ± 0.2 impulses/10 s (78 and 70% increases over basal), respectively, suggesting an inhibition of CRD-induced ACC firings compared with vehicle (5 neurons in each group, P < 0.05) (Figs. 5 and 6). In contrast, microdialysis of the NMDA receptor antagonist AP5 at concentrations of 100 and 500 μM did not change ACC neuronal spontaneous firing. Intra-ACC administration of AP5 had no effect of 50 mmHg CRD-evoked ACC neuronal responses (from vehicle 16 ± 2 to 16.5 ± 3.5 impulses/10 s after AP5 infusion; 4 neurons in each group) (Figs. 5 and 6). In another study, 31 neurons from 7 normal rats were examined. Seven of 31 neurons responded to 50 mmHg CRD, and six neurons (~20%) exhibited an excitatory response to 50 mmHg (from basal 8.0 ± 0.5 to 17.5 ± 2 impulses/10 s). Histological studies confirmed that the neurons were located in the cingulate cortex area (not shown in Fig. 1 because the overlaps of some neurons). Administration of AP5 at concentration of 2.0 mM (in the microdialysis fibers) did not change spontaneous firings, and had no effect of 50 mmHg CRD-evoked ACC firings (Fig. 6).

ACC Neuronal Responses to CRD and Graded Doses of Glutamate in Sensitized Rats

A total of 48 EA rats were studied. During the injection of neurobiotin, the juxtacellular configurations were lost in four rats. These rats were excluded. Histology studies revealed an absence of stained cells in two rats. The data from these two
animals were not presented. Recording electrodes were successfully placed in the ACC of 42 EA rats. Eighty-four neurons from the 42 EA rats were characterized and reported. Three types of neurons were classified according to their CRD (50 mmHg) response: 36 of 84 (43%) did not respond to CRD, 48 of 84 (57%) were activated by CRD. Among the latter 48 activated neurons, 35 were excited by 50 mmHg CRD which represented 42% of total 84 neurons examined. Compared with 24% neurons were identified as CRD-excited neurons in normal rats, these observations suggest that significant increases in the numbers of CRD-excited ACC neurons in the visceral hypersensitive rats compared with normal rats (14). In EA rats, the average spontaneous activity recorded in the CRD-excited neurons was significantly higher than that recorded in the control rats (18 ± 2 in the EA rats vs. 8.2 ± 0.7 impulses/10 s in the control rats). ACC spike firings in response to 50 mmHg CRD increased from basal 18 ± 2.5 to 43 ± 5 impulses/10 s, respectively. Original action potential recordings are presented in Fig. 2. These observations suggest that viscerally hypersensitive rats have an increased ACC neuronal excitability. Thirteen of the 84 neurons (16%) were CRD-inhibited neurons; ACC neuronal discharge was reduced from basal 18 ± 2.5 to 3.5 ± 0.25 impulses/10 s at a distension pressure of 50 mmHg. Eight of 30 CRD-excited neurons were examined for responses to glutamate (Fig. 1). ACC administration of glutamate at doses of 50, 100, and 500 μM markedly increased ACC neuronal firings in the EA rats from basal 18 ± 2 impulses/10 s to 32.5 ± 3 and 52 ± 4 and 78 ± 5.5 impulses/10 s, respectively (1.7-, 2.8-, and 4.3-fold increases). Examples of original recordings are shown in Fig. 3. Mean neuronal firing frequencies are shown in Fig. 4.

Effects of Glutamate Receptor Antagonists

Data were collected from five to six CRD-excited ACC neurons in each group. Local application of the AMPA glutamate receptor antagonist CNQX by reverse microdialysis at concentrations of 100 and 500 μM did not significantly change spontaneous ACC neuronal firing or the increase in ACC firing evoked by 50 mmHg CRD in the EA rats. In contrast, microdialysis of the NMDA receptor antagonist AP5 at concentrations of 100 μM and 500 μM suppressed basal ACC neuronal firings from 18 ± 1.5 to 13.5 ± 0.5 and 8.0 ± 0.05 impulses/10 s, respectively. Local administration of CNQX at 1.0 mM reduced spontaneous firings to 12 ± 2 impulses/10 s (see Figs.
Similar to the responses of 50 mmHg CRD-excited ACC neurons, the percentages of increases in neuronal firings over basal did not change (Fig. 10). Microdialysis of AP5 100 and 500 μM reduced the basal firings to 9 ± 1 and 6 ± 0.5 impulses/10 s, respectively. Administration of AP5 (100 and 500 μM) abolished 30 mmHg-CRD-induced ACC responses (Fig. 8B).

### DISCUSSION

Studies of both humans and animals consistently suggest that the ACC and its related areas are important for processing pain perception. Lesions of the medial frontal cortex, including the ACC, significantly inhibit acute nociceptive responses and injury-related aversive memory behaviors (20, 22). Stimulation of the ACC facilitates the spinal nociceptive tail-flick reflex (4) and enhances visceromotor responses to CRD in viscerally hypersensitive rats (5). Patients with IBS show enhanced activation of the dorsal ACC, and a reduction of this pattern is associated with a reduction in IBS symptoms (28). These findings suggest a possible dysfunction of the emotional and sensory components of the brain pain experience system in IBS.

Electrophysiological recordings and anatomical studies demonstrate that neurons within the ACC respond to noxious stimuli (18, 41, 47, 49). We have provided direct electrophysiological evidence of the sensitization of ACC neurons in viscerally hypersensitive rats induced by colorectal anaphylaxis. We have shown that viscerally hypersensitive rats exhibit enhanced ACC spontaneous activity, decreased CRD pressure threshold, and increased the magnitude of ACC neuronal response to visceral stimulation (14). There are significant increases in the numbers of CRD-excited ACC neurons in the visceral hypersensitive rats compared with normal rats (14). It appears that colorectal anaphylaxis resulted in sensitization of high-threshold receptors and brought into play previously un-responsive silent nociceptors. Furthermore, a group of rostral 8A and 9) and produced a mild decrease in ACC firings induced by 50 mmHg CRD from vehicle 43 ± 3.5 to 36.5 ± 4 impulses/10 s. However, the percentages of increases in ACC neuronal firings over basal did not change (Fig. 10). CNQX at the higher concentration 10 mM does not produce further inhibition (data not shown). Local administration of AP5 100 and 500 μM abolished 50 mmHg CRD-evoked ACC responses in the EA rats (from vehicle 42.5 ± 3 to 16.5 ± 2.5 and 10 ± 1 impulses/10 s, respectively, after AP5 infusion) (Fig. 7 and 8A). In a separate 22 EA rats, 57 neurons were characterized in the responses to 30 mmHg CRD. Twenty of the 57 neurons were 30 mmHg CRD-excited neurons (from 19.5 ± 4 spikes/10 s basal to 36 ± 5 spikes/10 s) (Fig. 8A). Microdialysis of CNQX at 500 μM had no effects on basal and CRD-evoked ACC firings. CNQX at 1 mM slightly reduced spontaneous firing to 16 ± 3 impulses/10 s (Fig. 9) and decreased ACC firings induced by 30 mmHg CRD to 30 ± 4 impulses/10 s (Fig. 8A).

Fig. 5. Recording of CRD-excited neurons of the ACC during infusion of 3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) or N-methyl-D-aspartate (NMDA) receptor antagonists in normal rats. In normal rats compared with application of vehicle (A), microdialysis of artificial cerebrospinal fluid containing AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) decreased basal and the 50 mmHg CRD-evoked ACC spike firing (B). C–E: recordings from different ACC neurons in normal rats (application of vehicle are not shown). CNQX at high dose abolished (C) the 50 mmHg CRD-evoked ACC spike firing. Administration of aminophosphonopentanoic acid (AP5) at 100 μM (D) or 500 μM (E) did not change basal firing; and had no effect on ACC neuronal responses to CRD.

Fig. 6. Spike firing rates of 50 mmHg CRD-excited ACC neurons during infusion of glutamate receptor antagonists in normal rats. Results were collected from 4–5 neurons per group. There was a significant increase in response to 50 mmHg CRD (see Fig. 2) during reverse microdialysis of vehicle (artificial cerebrospinal fluid). Application of AMPA receptor antagonist CNQX dose dependently decreased basal ACC neuronal firings and suppressed or completely abolished the evoked ACC spike firings in response to 50 mmHg CRD. *P < 0.05 compared with vehicle 0 mmHg. In contrast, NMDA receptor antagonist AP5 had no effect on either basal or CRD-evoked ACC neuronal activity in normal rat. The percent inhibition of spontaneous firings and percent increase of evoked ACC neuronal firings over basal are shown in Figs. 9 and 10.
ACC neurons in viscerally hypersensitive rats exhibit enhanced activities evoked by CRD. However, neuronal responses evoked by cutaneous noxious heat stimulation did not change significantly, suggesting that a population of rostral ACC neurons is capable of discriminative coding for hypersensitivity, specifically visceral hypersensitivity. Investigation of synaptic mechanisms within the ACC will provide valuable insight into plastic changes in the ACC and increase understanding of the neurophysiological mechanisms that are involved in the generation of visceral hypersensitivity.

Among many neurotransmitters, glutamate is the major neurotransmitter mediating excitatory transmission in synapses throughout the central nervous system related to pain transmission and modulation and neuronal plasticity (3, 17, 39). In this study, we recorded single ACC neuronal discharges in response to CRD in sham-treated rats and in a well-characterized rat model of visceral hypersensitivity induced by intraperitoneal injection of EA followed by repeated colonic EA challenge and CRD to evoke colonic anaphylaxis (14). We showed that reverse microdialysis of glutamate into the CRD-excited ACC neurons increased ACC neuronal spike activities in both normal and viscerally hypersensitive rats.

Importantly, viscerally hypersensitive rats showed a reduction of the glutamate dose threshold that stimulates ACC neurons (100 μM glutamate in normal rats and 50 μM glutamate in EA rats), and they showed an increased magnitude of the ACC response to graded doses of glutamate. These observations suggest that enhancement of synaptic excitatory glutamatergic transmission in the ACC may play a key role in the induction of sensitization of postsynaptic ACC neurons in viscerally hypersensitive rats.

Glutamate mediates synaptic transmission by binding to postsynaptic AMPA, NMDA, and kainate receptors (17). In most cases, synaptic responses are primarily mediated through postsynaptic AMPA/kainate receptors. The AMPA receptor opens in response to glutamate binding and mediates most of the rapid excitatory postsynaptic current. Most ACC pyramidal neurons in layers II/III and V display intense GluR2/3 (AMPA) immunostaining in the perikaryal cytoplasm and proximal dendrites (49). However, in the ACC, NMDA receptors are highly expressed; the contribution of glutamate receptor sub-

![Fig. 7. Recording of CRD-excited neurons of the ACC during infusion of AMPA or NMDA receptors antagonists in viscerally hypersensitive rats. ACC neurons of viscerally hypersensitive rats had high spontaneous activities and enhanced responses to CRD (see Fig. 2). Microdialysis of artificial cerebrospinal fluid containing AMPA receptor antagonist CNQX 500 μM (A) did not change basal or CRD-evoked ACC neuronal firing. B: CNQX at 1.0 mM reduced spontaneous firings to 12 ± 0.2 impulses/10 s and produced a mild decrease in ACC firings induced by 50 mmHg CRD from vehicle 43 ± 3.5 to 36.5 ± 4 impulses/10 s. Application of NMDA receptor antagonist AP5 at 100 μM (C) or 500 μM (D) significantly decreased basal ACC neuronal activities and suppressed or completely eliminated CRD-induced ACC neuronal responses to CRD.

![Fig. 8. Spike firing rates of 50 mmHg (A) and 30 mmHg (B) CRD-excited ACC neurons during infusion of glutamate receptor antagonists in viscerally hypersensitive rats. Results were collected from 4–5 CRD-excited ACC neurons per group. There was a marked increase in response to 50 mmHg CRD in viscerally hypersensitive rats during the infusion of vehicle (see Fig. 2). Application of AMPA receptor antagonist CNQX at 100 or 500 μM had no effect on either basal (CRD 0 mmHg) or evoked ACC neuronal activity (CRD 50 mmHg). AP5 dose dependently decreased basal ACC neuronal activities and suppressed or completely abolished the CRD (50 mmHg)-evoked ACC spike firings. *P < 0.05 vs. vehicle 0 mmHg (1-way repeated measures ANOVA followed by the Bonferroni test). The percent inhibition of spontaneous firings and percent increase of evoked ACC neuronal firings over basal are shown in Figs. 9 and 10. These observations suggest that NMDA glutamate receptors are responsible for the mediation of ACC neuronal activities evoked by noxious visceral stimulation in viscerally hypersensitive rats.

**G924 NMDA RECEPTORS IN THE ANTERIOR CINGULATE CORTEX**

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types to the transmission of visceral noxious afferent input to the ACC has not been characterized. Consistent with these previous reports, our present results show that blockade of ACC non-NMDA receptors in the normal rats reduced the spontaneous activity and eliminated most ACC neuronal responses to 50 mmHg CRD. Thus, under normal conditions, transient pain transmission occurs in the ACC through the release of glutamate acting by way of the AMPA/kainate receptors, which do not have a Mg$^{2+}$ block, resulting in membrane depolarization. Furthermore, NMDA receptors do not significantly contribute to spontaneous ACC neuronal activity, nor to acute noxious visceral stimulation-evoked ACC responses.

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 Mg$^{2+}$ (27) even in the presence of glutamate and do not contribute to transient pain transmission (9, 12) in normal situations. However, stimulation of nociceptive afferents following induction of visceral hypersensitivity causes an increased release of glutamate in the ACC. This results in depolarization beyond a critical threshold with removal of the Mg$^{2+}$ block and increases the availability of NMDA receptors. In this study, we show that in sensitized rats reverse microdialysis of the NMDA receptor antagonist AP5 into the ACC neurons dose dependently reduces the basal activity and eliminates the ACC firings evoked by noxious CRD.

Administration of CNQX at 1.0 mM mildly reduces basal firings, suggesting that the background activities of ACC neurons are mediated by both NMDA and non-NMDA receptors in visceral hypersensitive status. Further studies are needed to investigate the roles of NMDA and non-NMDA receptors for synaptic plasticity in the ACC in visceral hypersensitive rats. However, the evoked activities (percentages of increases in CRD-induced ACC neuronal firings) did not change after administration of CNQX. These observations suggest that a key step in the development of ACC sensitization is the activation and modulation of the NMDA receptors that receive primary afferent input. The increase of NMDA receptor activation may consequently amplify the visceral pain (52). This phenomenon of NMDA receptor-mediated ACC sensitization is consistent with a well-established mechanism in both animal and human models of somatic pain hypersensitivity, a mechanism that is attenuated by NMDA receptor antagonists (54). Our behavioral findings in visceral hypersensitive rats (5) are consistent with the currently electrophysiological observations and suggested that NMDA receptor activation of ACC neurons plays a critical role in the modulation of visceral pain responses (5).

Whereas AMPA and kainate receptors mainly contribute to sensory transmission, NMDA receptors are critical for the induction of long-term plastic changes lasting from hours to days (6, 29). Unlike other ionotropic receptors, NMDA receptors are 5–10 times more permeable to Ca$^{2+}$, a critical intracellular signaling molecule, than to Na$^+$ or K$^+$. Ca$^{2+}$ influx by way of NMDA receptors from the extracellular space into the postsynaptic cells triggers a cascade of signaling molecules, including protein kinases, protein phosphatases, and immediate early genes, as well as enzymes that produce diffusible retrograde messengers (1, 46). The function of NMDA receptors as coincidence detectors and their permeability to Ca$^{2+}$ makes these receptors the best candidates for a central mechanism in memory formation. Recent studies further suggest that the activation of supraspinal structures including ACC neurons can also facilitate spinal responses (4) and pain responses in visceral hypersensitive rats (5). The consequence of this positive feedback control leads central neurons to a much enhanced and overexcited status; a weak input leads to significantly greater neuronal action potentials. Such a mechanism most likely contributes to several chronic pain-related states.

In summary, we demonstrated that blocking non-NMDA receptors with CNQX had inhibitory effects on the background activity and noxious CRD-evoked response in normal rats. However, NMDA antagonist AP5 did not affect the background activity or the CRD-induced response in normal rats. In

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**Fig. 9.** Percent inhibition of spontaneous firings of CRD-excitatory ACC neurons in the normal rats (50 mmHg CRD) and the EA rats (30 and 50 mmHg CRD). In normal rats, AMPA glutamate receptor antagonist CNQX at concentrations of 100 μM and 500 μM produced a 40 49% inhibition of ACC spontaneous firings, respectively. Microdialysis of the NMDA receptor antagonist AP5 at concentrations of 100 μM, 500 μM, and 2.0 mM did not change ACC neuronal spontaneous firing. In the EA rats, CNQX at concentrations of 100 and 500 μM did not change spontaneous ACC neuronal firing, CNQX at 1.0 mM slightly reduced spontaneous firing but did not significantly change the ACC firings induced by 30 and 50 mmHg CRD (Fig. 10). Microdialysis of AP5 markedly reduced the spontaneous firings in the EA rats.

**Fig. 10.** Percent increase in CRD-excitatory ACC neuronal firings over basal in the normal rats and EA rats. In the normal rats, AMPA glutamate receptor antagonist CNQX suppressed ACC neuronal firing evoked by 50 mmHg CRD dose dependently. However, application of AP5 had no effect on these responses. In the EA rats, microdialysis of CNQX had no effect on the increases of ACC neuronal firings in response to 30 and 50 mmHg CRD. In contrast, administration of AP5 completely abolished ACC neuronal responses to 30 mmHg CRD and markedly reduced ACC firings in response to 50 mmHg CRD. *P < 0.05, normal 50 mmHg compared with normal 50 mmHg vehicle; EA 30 mmHg and EA 50 mmHg compared with EA 30 mmHg vehicle and EA 50 mmHg vehicle, respectively.
contrast, in sensitized rats, AP5 significantly inhibited the enhanced background activity and abolished the ACC neuronal responses to CRD. Thus ACC nociceptive transmission is mediated by glutamate AMPA receptors in normal circumstances. In viscerally hypersensitive rats, the synaptic transmission in the ACC neurons is enhanced. This enhancement is mediated mainly by NMDA receptor activation. Understanding of the mechanisms of ACC neuronal plasticity may provide the rationale for novel targets for the treatment of chronic pain in gastrointestinal disorders. Specifically, the results of this study suggest that the enhancement of synaptic excitatory glutamatergic transmission in the ACC may play a role in the mediation of nociceptive sensitization in functional gastrointestinal disorders.

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REFERENCES


