Up-Regulation of Anterior Cingulate Cortex NR2B Receptors Contributes to Visceral Pain Responses in Rats

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Background & Aims: **Electrophysiologic and behavioral studies have shown that increased *N*-methyl-D-aspartate (NMDA)-receptor activation of anterior cingulate cortex (ACC) neurons has a critical role in modulating visceral pain responses in viscerally hypersensitive (VH) rats. This study aimed to identify the NMDA receptor subtypes in perigenual ACC (pACC) neurons involved in the facilitation of visceral nociception.** ***Methods:*** **We performed in vivo electrophysiologic recordings of pACC neurons and examined the visceromotor response (VMR) to colorectal distention (CRD) in normal and VH rats induced by colonic anaphylaxis. The NR2A-subtype–receptor antagonist [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) and the NR2B-receptor–antagonist Ro25-6981 were microinjected into the pACC. To down-regulate NR2B-receptor gene expression, an NR2B-specific small interfering RNA (siRNA) and a plasmid (pEGFP-N1) that expressed the green fluorescent protein were administered into ACC neurons by electroporation.** ***Results:*** **Reverse microdialysis of NVP-AAM077 had no effect on basal and CRD-induced ACC neuronal firing in VH and control groups. In VH rats, Ro25-6981 (500 μmol/L) inhibited ACC neuronal firing, evoked by 30 and 50 mm Hg CRD, by 98% and 52%, respectively. NVP-AAM077 did not affect the VMR in either group. Ro25-6981 significantly suppressed the VMR in VH but not normal rats. Immunoblot analysis showed increased NR2B-receptor expression in the pACC of VH rats. NR2B siRNA-treated VH rats showed a significant reduction in the VMR, compared with controls.** ***Conclusions:*** **The NR2B subunit of the NMDA receptor has a critical role in the modulation of ACC sensitization and visceral pain responses in VH rats.**

Human brain imaging studies have revealed new roles of cortical neuronal networks in chronic visceral pain.[1] Experiments in animals show that the anterior cingulate cortex (ACC) receives nociceptive inputs[2–4] and suggest that ACC neuronal activity is related to stimulus–reward learning.[5] Electrophysiologic studies in our laboratories have shown that ACC sensitization occurs in viscerally hypersensitive (VH) rats.[6] Allodynia and hyperalgesia in these rats appear to be mediated by enhanced glutamate *N*-methyl-D-aspartate (NMDA)-receptor activities in the ACC.[7] Hypersensitivity to colonic distention can be observed up to 7 weeks after the initiation of colonic anaphylaxis and is independent of mucosal inflammation. This suggests mediation by a mechanism for learning and triggering of pain memories in the ACC neuronal circuitry. Nociceptive transmission in the ACC is mediated by glutamate α-amino-3-hydroxy-5-methyl-isoxazole propionic acid receptors in normal circumstances. In VH rats, the synaptic transmission in ACC neurons is enhanced. This enhancement is mediated mainly by NMDA-receptor activation,[8] indicating neuronal plasticity in the ACC circuitry in the VH state.

NMDA receptors contain heteromeric combinations of the NR1 subunit plus one or more of the subunits NR2A–2D. Although NR1 is distributed widely in the brain, NR2 subunits show regional specificities. In human beings and rodents, the subunits NR2A and NR2B predominate in forebrain structures.[9] In the ACC, the NMDA receptor containing NR2A or NR2B subunits contributes to most NMDA-receptor currents.[10] Mice that genetically overexpress the NR2B-receptor subtype in the forebrain show enhanced responsiveness to painful stimuli[11] and superior learning ability and memory of different behavioral tasks.[12] Considering the distinct roles that NMDA receptors may serve, identification of the receptor subtype in the ACC that mediates visceral hypersensitivity will promote our understanding of the molecular mechanisms underlying nociceptive processes in the VH state.

We hypothesize that ACC sensitization and visceral hyperalgesia in our VH rat model may be mediated by changes in the expression and the function of NMDA-receptor subtypes in ACC neurons. To test this hypoth-

Abbreviations used in this paper: ACC, anterior cingulate cortex; AUC, area under the curve; CRD, colorectal distention; EA, egg albumin; GFP, green fluorescent protein; NMDA, *N*-methyl-D-aspartate; NVP-AAM077, [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid; pACC, perigenual anterior cingulate cortex; RNAi, RNA interference; siRNA, small interfering RNA; VH, viscerally hypersensitive; VMR, visceromotor response.

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esis, we used VH rats to characterize the electrophysiologic properties of neurons of the perigenual ACC (pACC; ie, areas 24b, 24a, and 32)[2] that are activated by colorectal distention (CRD). Single neuronal recording of pACC neurons was combined with reverse microdialysis of NR2A and NR2B subtype-selective NMDA-receptor antagonists [(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) and Ro25-6981 to identify which receptor subtypes mediate visceral hypersensitivity. The selectivity of these 2 antagonists has been established in vitro.[13,14] Visceromotor response (VMR) studies were conducted in parallel to examine the antinociceptive effects of subtype-selective NMDA-receptor antagonists. Finally, to down-regulate the gene expression of the NR2B subtype of the NMDA receptor, NR2B-specific small interfering RNA (siRNA) was administered into the pACC neurons of VH rats by electroporation. Our results suggest that activation of ACC NR2B-containing NMDA receptors may be involved in ACC sensitization and hyperalgesia in the VH state.

Materials and Methods

Experimental procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan. Experiments were performed on adult male Sprague–Dawley rats (weight, 275–300 g). The animals were housed 4 per plastic cage and maintained on a 12-hour:12-hour light:dark cycle (lights on at 7 AM) and given access to food and water ad libitum. For surgical preparations, rats were anesthetized with a mixture of xylazine and ketamine.[6]

Viscerally Hypersensitive Rat Model

The rats were sensitized to chicken egg albumin (EA) with an intraperitoneal injection of normal saline (1 mL) containing EA (10 mg) as the antigen and aluminum hydroxide (10 mg) as the adjuvant. Beginning on day 3, the antigen solution was perfused into the colon and CRD was performed 30 minutes after EA instillation. VMR studies were conducted 5–7 days after the induction of colonic anaphylaxis.[7,8]

Electrophysiologic Recording of pACC Neuronal Activity in Response to CRD in Control and VH Rats

Three cytoarchitectural regions of the cingulate cortex have been identified in rats: the ACC with perigenual and subgenual parts, the midcingulate cortex, and the retrosplenial cortex.[2] A similar classification has been applied to the rabbit.[3] In the present study, neurons were recorded in the pACC using glass microelectrodes (tip diameters, 0.08 μm; impedance, 20–40 MΩ) at the following coordinates: 2.0–3.8 mm anterior to bregma, 0.5–1.0 mm lateral to midline, and 2.5 mm ventral to the brain surface. These areas correspond to perigenual 24b and portions of perigenual 24a and area 32. The recording electrode was advanced until the spontaneous activity of a single unit could be discriminated accurately from the background neuronal noise. The recording had uniform spike amplitude and could be maximized and separated from neighboring neurons. Noise levels typically were 40–50 μV. We analyzed only well-isolated neurons that showed a signal-to-noise ratio of at least 4:1.[7,8] A neuron was deemed responsive to CRD if its spike firing rate increased or decreased at least 10% from its predistention baseline activity. Neuronal discharge rates were measured 30 seconds before, 30 seconds during, and 120 seconds after CRD, with 5-minute intervals between each measurement, and evaluated on a time histogram (5-s bin width). On completion of the experiment, recorded neurons were labeled by injecting neurobiotin using the technique of juxtacellular iontophoresis.[7] Brain sections were incubated with peroxidase-conjugated avidin–biotin complex (1:100; Vector Laboratories, Burlingame, CA). Thionine was used as a counterstain.

Data on spontaneous firings in various experimental groups were evaluated using the Dunnett T3 multiple comparisons method after 1-way analysis of variance (ANOVA). Statistical comparisons of the CRD-pressure responses in various groups were made using the 1-way repeated-measures ANOVA, followed by multiple comparisons adjusted by the Bonferroni test. Results were expressed as means ± SEM. A P value of less than .05 was considered statistically significant.

Studies of Antagonists of NMDA-Receptor Subtypes

The NR2A-selective antagonist NVP-AAM077[15] (Novartis, Basel, Switzerland) or the NR2B-selective antagonist Ro25-6981[13] (Tocris Bioscience, Ellisville, MO) were infused into the pACC using reverse microdialysis. One dose of antagonist was applied to each CRD-excited neuron.

Reverse microdialysis was performed as described previously.[8,16] Briefly, microdialysis probes with 3–4 mm of exposed membrane were implanted into the pACC at the following coordinates: 2.0- to 3.8-mm anterior to bregma, 0.5- to 1.0-mm lateral to midline; and lowered about 4.0 mm at a 30° angle. Glass microelectrodes filled with neurobiotin were lowered into the pACC about 1-mm lateral or rostral to the probe and angled at 10° toward the probe.[7] Investigators have reported serial measurements of drug concentrations in tissue after microdialysis.[17] In our study, drugs were dissolved in artificial cerebrospinal fluid at a concentration 100 times that predicted to be necessary according to data from in vitro studies.[18] Artificial cerebrospinal fluid containing NVP-AAM077 or Ro25-6981 (50, 100, and 500 μmol/L) was perfused. The doses were chosen in accordance with the results of previous brain slice recordings in which NVP-AAM077 or Ro25-6981 at a concentration of 0.4–3.0 μmol/L blocked