Intrathecal Coadministration of D-APV and Morphine Is Maximally Effective in a Rat Experimental Pancreatitis Model

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Background: Many studies have demonstrated that either glutamate *N*-methyl-D-aspartate (NMDA) receptor antagonists or opioid receptor agonists provide antinociception. Spinal coadministration of an NMDA receptor antagonist and morphine has an additive action for control of various pain states in animal models. The current study examined spinal coadministration of low doses of NMDA receptor antagonist, D-(-)-2-Amino-5-phosphonovalerate (D-APV), and μ-opioid receptor agonist, morphine sulfate (MS), in reducing visceral nociception using an acute bradykinin induced pancreatitis model in rats.

Methods: An intrathecal catheter was surgically inserted into the subarachnoid space for spinal drug administration in Sprague-Dawley rats. A laparotomy was performed for ligation and cannulation of the bile-pancreatic duct. Rats were pretreated intrathecally with artificial cerebrospinal fluid (aCSF), D-APV, MS, or combined administration of D-APV and MS. These treatments were given 30 min before noxious visceral stimulation with bradykinin injected through the bile-pancreatic catheter. Spontaneous behavioral activity tests, including cage crossing, rearing, and hind limb extension, were conducted before and after bradykinin injection into the bile-pancreatic duct to assess visceral nociception.

Results: Spinal pretreatment of D-APV or low doses of MS partially reduced visceral pain behaviors in this model. Pretreatments with combinations of low doses of MS (0.05–0.5 μg) and D-APV (1 μg) were maximally effective in returning all spontaneous behavioral activities to baseline.

Conclusions: Spinal administration of combined doses of NMDA receptor antagonist, D-APV, and MS reversed three behavioral responses to induction of an acute pancreatitis model. These results suggest that in the clinical management of visceral pain, such as pancreatitis, restricted usage of glutamate antagonists might be useful as adjuvant potentiation at the onset of morphine therapy.

PAIN is the primary cause of suffering in patients with visceral organ disorders. Pain arising from visceral organs has not been as widely investigated as pain arising from somatic tissues. In the past decade, studies of peripheral and central nervous system mechanisms involved in the generation and maintenance of visceral pain have used various animal models.[1–13] Visceral pain is still poorly understood from a pharmacologic point of view as well. The lack of information hinders efforts to develop better treatment strategies for clinical use.

There is growing evidence that *N*-methyl-D-aspartate (NMDA) receptors are important in the transmission of nociceptive signals from the periphery *via* the spinal cord to the brain, and that NMDA receptors are involved in the induction and maintenance of the central sensitization for visceral structures.[14–16] Use of NMDA receptor antagonists as antinociceptive agents has been successfully demonstrated in visceral pain models. Enhanced responses to colorectal distention in the presence of colonic inflammation were attenuated by NMDA receptor blocker MK-801 and non-NMDA receptor antagonist DNQX.[17] The NMDA receptor antagonist (APV) has been used to block the effects of turpentine sensitization[18] and all NMDA-produced nociceptive effects[16] in the visceromotor response to colorectal distension. This evidence provides support for the hypothesis that spinal glutamate receptors, particularly those of the NMDA subtype, play an important role in regulating spinal encoding of afferent information after visceral tissue inflammation or injury.

Opioid receptor agonists can exert their action on both pre- and postsynaptic sites on primary afferent endings and have been widely used for controlling pain, particularly pain arising from viscera. Both μ and δ opioid receptor agonists have been shown to inhibit the visceromotor response to colorectal distension.[19–21] Side effects, however, often limit clinical usefulness of μ-receptor agonists for long-term use in pain treatment. Tolerance-induced decreases in analgesia also limit their therapeutic efficacy. The NMDA receptor antagonists have limited usefulness in the clinic because of their side effects, including dysphoria, nightmares, motor disturbance, and sedation. These side effects can decrease in combination with other drugs.[22,23] Some investigations demonstrate that NMDA receptor antagonists can prevent morphine tolerance.[24,25] Recently, investigators have found that spinal coadministration of glutamate receptor (NMDA or AMPA) antagonists and a low dose of morphine have synergistic antinociceptive effects in various pain states.[26–30]

The experiments described in this article include analysis of the behavioral manifestations of acute experimental pancreatic pain induced by bile-pancreatic duct ligation and infusion of bradykinin in the conscious rat. The hypothesis tested was that glutamate-related mechanisms have a role in visceral nociceptive transmission from the inflamed pancreas and that combined use of an NMDA receptor antagonist and a μ-receptor agonist en-

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hances antinociceptive effects in this animal model. These studies provide insights for improvement of current therapy for intractable pain caused by visceral disease.

Materials and Methods

All experiments were approved by the Animal Care and Use Committee at our institution and are consistent with the guidelines of this committee as well as the policies on the Ethical Treatment of Research Animals published by the International Association for the Study of Pain. Ninety male Sprague-Dawley rats (250–300 g) were used for the study. The animals were housed in a room with a constant ambient temperature of 22°C, a 12 h light–dark cycle, and free access to food and water.

General Procedures

Animals received an intrathecal catheter. After 4 to 5 days of recovery, a laparotomy for the bile-pancreatic duct ligation and cannulation was performed for delivery of bradykinin. Bradykinin served as a noxious visceral stimulus that evoked visceral nociceptive responses and induced severe acute pancreatitis that was evident histologically.

The next day rats received intrathecal aCSF, NMDA receptor antagonist (D-APV), μ-opioid receptor agonist MS, or a combination of both drugs. Thirty minutes later, 0.5 ml of bradykinin (10^{-5} M, Sigma, St. Louis, MO)[10] in lactated Ringer's solution (Baxter Healthcare Corp., Deerfield, IL) was injected into the pancreas. Based on a previous physiologic study, 10^{-10}-10^{-4} M bradykinin is effective in producing nociceptive responses in this model without producing desensitization.[10] Spontaneous behavioral activity of the rats was observed during the first 10 min in a novel cage and quantified (1) before any surgery (naïve), (2) after intrathecal surgery, (3) the next day after laparotomy (baseline), and (4) after noxious activation of pancreatic afferent fibers with bradykinin and intrathecal aCSF or drug treatment. Histologic samples were taken immediately following the last behavioral test.

Intrathecal and Intraductal Catheter Placements

Rats were anesthetized with methohexital sodium (brevital sodium, 40 mg/kg, intraperitoneal) prior to receiving an intrathecal catheter for administration of drugs according to Yaksh and Rudy.[31] A 4.5 cm intrathecal catheter (32-guage, ReCathCo, Allicon Park, PA) was implanted intrathecally through an opening in the atlanto-occipital membrane to the spinal T6–7 level. The catheter was linked to soft polyethylene tubing (PE10) (i.d. 0.28 mm, o.d. 0.61 mm; Clay Adams Brand, Becton Dickinson Primary Care Diagnostics Becton Dickinson and Company) and then connected to PE20 tubing,

which was tunneled under the skin, and remained subcutaneous until use. Rats were allowed to recover for 4 to 5 days. After the recovery period, spontaneous behavioral activities of rats returned to levels observed in naïve rats. Rats with behavioral deficits were excluded from the study (n = 5).
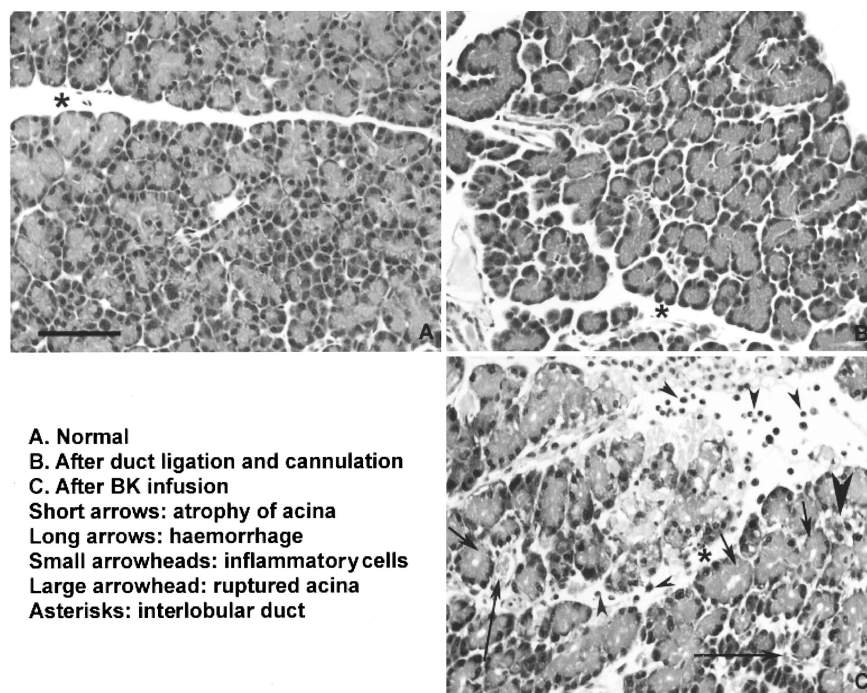
Rats were reanesthetized with methohexital sodium as described previously for a ventral midline laparotomy. The PE10 tubing catheter connecting to PE20 tubing was inserted into the common bile-pancreatic duct.[6] The PE20 tubing was tunneled under the skin to exit at the nape of the neck, and the exposed end of the tubing was sealed. The rats were allowed to recover overnight. Any rat with a swollen abdomen or that became completely inactive compared to before laparotomy was excluded from the study (n = 5).

Behavioral Testing

Behavioral tests were conducted during the first 10 min after rats were introduced to a novel, clear transparent cage. The timing of the behavioral observations was determined through consideration of results of previous studies in our laboratory,[10] including physiology experiments that found that cell firing was sustained for 3–6 min after bradykinin stimulation. Three spontaneous activities observed were (1) cage crossing (forward locomotion across the centerline of the cage), (2) exploratory rearing behavior (standing on the hind limb with or without support of the cage walls) and (3) hind limb extension (stretching or twisting of the hind limbs behind or under the body), which is a specific pain related behavior. The number of times each behavior occurred spontaneously in the novel cage environment was counted and recorded for every 10-s block of time, continuing for 10 min.[10] The mean of the counts for each behavioral measure was used as the animal's behavioral response to induction of pancreatitis and response to pretreatment with D-APV, MS, and combined treatment. The experimenter analyzing animal behavior was blind to whether the animals had received drug treatment or aCSF.[32]

Intrathecal Administration of Drugs

An NMDA receptor antagonist, D-APV (Tocris Cookson Inc., Ellisville, MO) and MS (Paddock Laboratories Inc., Minneapolis, MN), a μ-opioid receptor agonist, were assessed in this study. Drugs were dissolved in saline and diluted in aCSF to their final concentrations (in 10 μl). The drugs were administered intrathecally with a Hamilton syringe (Reno, NV), followed by 10 μl of aCSF to flush the catheter. A dose response curve for each drug was generated by examining the effects of pretreatment with the drug in animals with pancreatitis. In the D-APV pretreatment group, the drug doses injected intrathecally were of 0.5 (n = 6), 1 (n = 7), and 2 μg (n = 6). In the MS pretreatment group, the doses used were 0.05

A. Normal
B. After duct ligation and cannulation
C. After BK infusion
Short arrows: atrophy of acina
Long arrows: haemorrhage
Small arrowheads: inflammatory cells
Large arrowhead: ruptured acina
Asterisks: interlobular duct

Fig. 1. Photographs illustrating hematoxylin/eosin stained histologic sections taken from rat pancreas. The pancreatic tissues were taken from normal rat (*A*), after ligation and cannulation of the bile-pancreatic duct (*B*), and after bradykinin infusion (*C*). The section thickness was 4-μm. Scale bar = 50 μm.

μg (n = 6), 0.1 μg (n = 6), 0.5 μg (n = 6), and 1 μg (n = 7). In the drug control group, 20 μl of aCSF was infused intrathecally (n = 7). After the dose response curve was generated for D-APV, a single dose of D-APV (1 μg) was combined with various doses of MS (0.01 μg, n = 6; 0.05 μg, n = 7; 0.1 μg, n = 6; 0.5 μg, n = 6 and 1 μg, n = 6;) to examine the effects of coadministration of both drugs.

Histopathology of the Pancreas

Rats received an overdose of sodium pentobarbital and the pancreatic tissue was collected from naïve rats (n = 3), from rats 1 day after laparotomy and cannulation (n = 3), and from rats 10 min after bradykinin injection (n = 3). The tissue was cut into small blocks (about 3 × 3 × 2 mm) and washed with phosphate buffer saline (PBS, pH 0.1 M), followed by block fixation in 4% buffered paraformaldehyde for 2 to 3 days. The tissue was embedded in paraffin and cut at 4 μm thickness sections, and stained with hematoxylin/eosin (HE). The pancreatic tissues were examined and photographed with an Olympus microscope (Melville, NY) equipped with an advanced SPOT digital camera system for histopathologic analysis.

Statistical Analysis

Comparisons tested included behavior of rats with bradykinin-induced pancreatitis with and without drug pretreatments. The results of the behavioral testing were not normally distributed and were therefore analyzed using nonparametric statistics. Pairwise comparisons within groups for values before (baseline) and after drug treatment were analyzed using the Wilcoxon test. Comparisons were made between groups using the Mann–Whitney U test. A *P* value of less than 0.05 was considered a significant difference. The data were expressed as average $\pm$ SEM. As a matter of interest, analysis of variance (ANOVA) was also performed using Newman-Keuls *post hoc* comparisons and the results were identical. The Prizm software program was used to determine the ED_{50} from the dose response curve.

Results

Histopathology

In control rats the pancreas showed entirely normal acinar architecture (fig. 1A). In rats with 24 h of bile-pancreatic duct obstruction, the pancreatic interstitial space was edematous and acinar cell vacuolization was evident. There was no evidence of acinar cell destruction or hemorrhage. Infiltration of polymorphic nuclear leukocytes was minimal (fig. 1B). After obstruction of the bile-pancreatic duct and bradykinin infusion, the ducts were ruptured. There was acinar atrophy (loss of zymogen staining and separation of acini), stromal proliferation associated with the presence of inflammatory cells (including lymphocytes and neutrophils) and hemorrhage (fig. 1C).

No Effect of Intrathecal Catheter Placement on Rat Spontaneous Behavioral Activities

After a 4 to 5 day recovery period, intrathecal catheter placement did not have an affect on rat spontaneous behavioral activities. The numbers of cage crossings and exploratory rearing were 23.71 $\pm$ 1.97 and 32.43 $\pm$ 3.37 in naïve rats (n = 7); and 23.67 $\pm$ 0.8 and 31.97 $\pm$ 0.98 in rats with intrathecal surgery (n = 30), respectively.
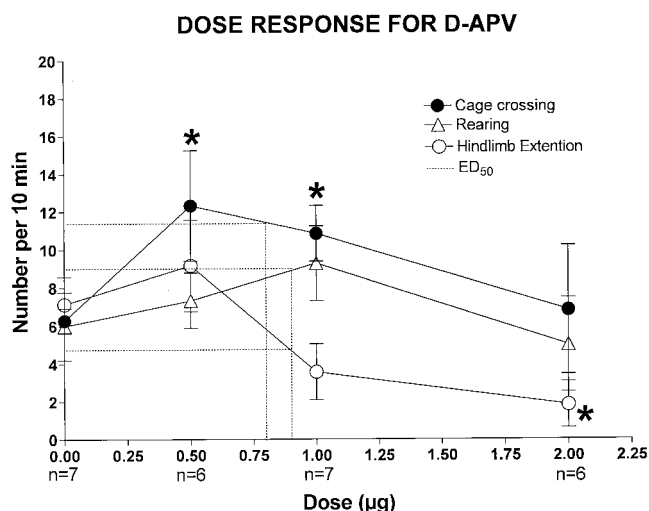
Fig. 2. Dose-response curve illustrating the antinociceptive effect of intrathecal administration of D-APV on spontaneous behavioral activities in rat with acute pancreatitis induced by bradykinin infusion into the pancreas. Values are represented as the mean ± SEM. Comparisons were made within groups using Wilcoxon matched pairs test. The 0.0 μg dose represents aCSF treated rats (drug control group). * = $P < 0.05$ (*vs.* drug control group). The ED_{50} for D-APV is indicated for each behavioral measure.

Hind limb extension was not evident in either naive rats or in rats after intrathecal surgery. Since there were no significant differences in the spontaneous behavioral activities between the two groups of rats ($P > 0.05$), the group with intrathecal surgery was included in the control group for comparisons with other groups in this study.

Spontaneous Behavioral Activity Subsequent to Obstruction of the Bile-Pancreatic Duct

Bile-pancreatic duct ligation and cannulation resulted in significant decreases in crossing and rearing behaviors and development of hind limb extension after 24 h compared with control rats ($P < 0.01$). The numbers of cage crossing and rearing events were 23.67 ± 0.79 (range 13–35) and 31.97 ± 0.98 (range 20–43) in control rats and 12.54 ± 0.56 (range 6–22) and 13.68 ± 0.68 (range 5–27) in rats with duct obstruction, respectively. Hind limb extension was not present in control rats, but was evident in rats with ductal obstruction (5.04 ± 0.51, range 0–16). Spontaneous behavioral activities in rats with ductal ligation prior to chemical stimulation with bradykinin were used as their own baseline control measurements for comparison with drug pretreatments in the following studies.

Effect of Bradykinin Infusion into Pancreas on Rat Spontaneous Behavioral Activities

After bradykinin infusion into the pancreas, a significant further reduction in cage crossing and rearing behaviors occurred compared with their own baseline
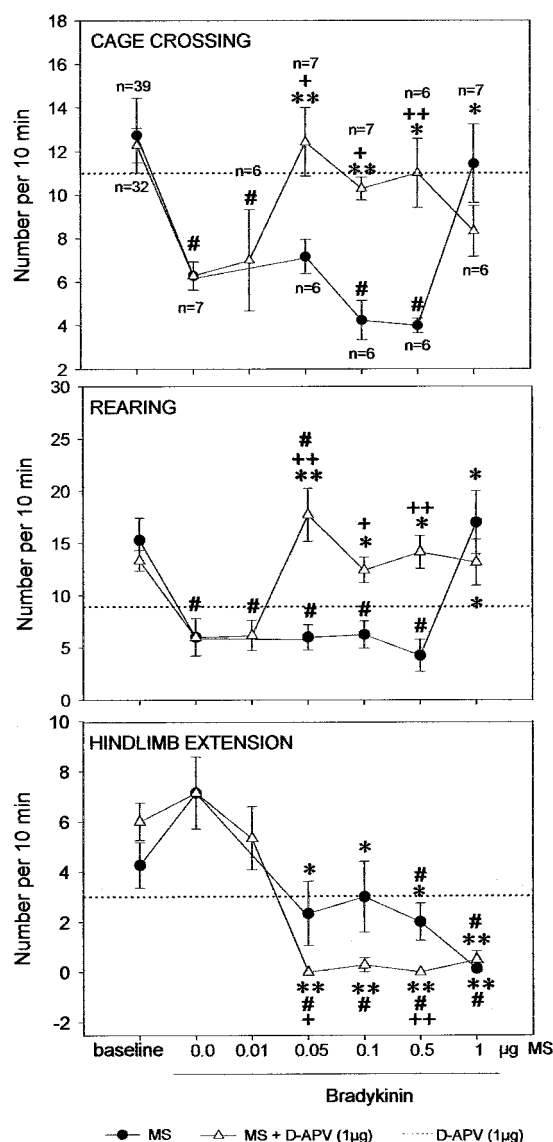

Fig. 3. Dose-response curves illustrating the antinociceptive effects of intrathecal morphine (dash-dot) and coadministration of D-APV (1 μg) with various low doses of MS (0.01, 0.05, 0.1, 0.5, or 1.0 μg; [dash-triangle]) on spontaneous behavioral activities in rats with pancreatitis induced by bradykinin infusion into the pancreas. Values are represented as the mean ± SEM. Comparisons were made within groups using Wilcoxon matched pairs test. Comparisons were made between groups using Mann–Whitney U test. The 0.0 μg dose represents the aCSF treated rats (drug control group). # and ## = $P < 0.05$ and 0.01 (*vs.* baseline). * and ** = $P < 0.05$ and 0.01 (*vs.* drug control group). + and ++ = $P < 0.05$ and 0.01 (*vs.* MS treated group).

($P < 0.05$, figs. 2, 3). While further increases in hind limb extensions following bradykinin were observed, they were not significantly higher than after ductal obstruction by cannulation.

Effect of D-APV Pretreatment on Spontaneous Behavioral Activities

Rats that received intrathecal aCSF pretreatment (0.0 μg drug control group) as well as bradykinin infusion were

used in comparisons with drug-pretreated animals. Compared to the spontaneous activity of the aCSF treated drug control group, intrathecal preadministration of low doses (0.5–1 μg) of D-APV significantly increased the number of cage crossings ($P < 0.05$). Increases in rearing were nonsignificant after D-APV, and hind limb extension was decreased. The ED_{50} was 0.80 μg for cage crossing, 0.96 μg for rearing, and 0.98 μg for hind limb extension (fig. 2). The hind limb extension behavior was attenuated significantly in rats pretreated with the highest dose of D-APV (2 μg), but side effects were evident, suggestive of sedation including flaccid paralysis and lack of activity. Behavioral data reported here for D-APV, as well as that for morphine and combined doses reported below, were similar at later time points (45 and 60 min) extending through the peak effect of these agents in pilot studies (data not shown).

Effect of MS Pretreatment on Spontaneous Behavioral Activities

The MS significantly attenuated numbers of hind limb extensions ($P < 0.05$–0.01), but did not restore cage crossing and rearing, compared to that of the aCSF treated drug control group. Only the maximum dose of MS (1 μg) significantly increased cage crossing and rearing above that of the aCSF treated drug control group ($P < 0.05$–0.01, fig. 3). The ED_{50} for low doses of MS were higher than the dose range used (up to 1 μg), thus MS had no significant effect on crossing and rearing behaviors. There was a significant effect on hind limb extension in this model at all doses. The ED_{50} was 9.84 μg for cage crossing, 9.25 μg for rearing, and 0.67 μg for hind limb extension.

Interaction Between D-APV and MS

Intrathecal coadministration of low doses of MS (0.05, 0.1, and 0.5 μg) and D-APV (1 μg), prevented both the development of significant pain-related behavioral activity observed in rats with pancreatitis and restored spontaneous exploratory behaviors (fig. 3). Thus, the low dose of NMDA receptor antagonist, D-APV, combined with low doses of MS improved all behavioral measures. In the absence of bradykinin, behavior was unaffected by the same doses of coadministered D-APV and MS (n = 3; 1 μg + 0.05 μg).

Discussion

In the past decade, several animal models have been employed to investigate peripheral and central nervous system (CNS) mechanisms underlying pain arising from visceral organs. Nonetheless, the knowledge of neuronal mechanisms as well as clinical management of visceral pain states remains unsatisfactory.

In this study we investigated the role of NMDA and opioid receptors in an acute experimental pancreatitis model in rats. The results indicate that spinal administration of a low dose of NMDA receptor antagonist, D-APV or low doses of MS (0.05–0.5 μg) were partially effective for antinociception in this model. Higher doses resulted in development of motoric deficits and/or sedation. This is despite the intrathecal administration directly onto thoracic spinal levels (T6-T11) innervated by pancreatic afferents. The particular measures of pain related behaviors observed in this model were resistant to D-APV unlike other visceral pain models in which it has been found to be effective.[3,16,18]

Coadministration of low doses of MS with D-APV (1 μg) resulted in elimination of the pain-related behaviors induced in this acute pancreatitis model in rats. Potentiated action of these two agents brought the two spontaneous behavioral activities back to baseline as well as totally eliminating hind limb extension, indicative of successful visceral antinociception.

Histopathological Change Correlated with Behavioral Change

The manipulation of the pancreas, including surgical placement of the catheter into the pancreatic duct in this model, resulted in acute edematous histopathologic changes in the pancreas. This effect of induced acute pancreatitis has been reported by Merriam *et al.*[33] The reduction of normal, spontaneous behavioral activities and onset of hind limb extension, a specific pain-related behavior, in the conscious rat with ligation-induced pancreatitis indicated that ligation of the bile-pancreatic duct itself produced visceral nociception arising from the pancreas.

The secondary bradykinin insult further contributes to a severe acute pancreatitis. Exposure to bradykinin secondary to the catheter-induced changes included ruptured pancreatic ducts, acinar atrophy, infiltration of inflammatory cells, as well as hemorrhage. These histopathologic changes after bradykinin stimulation were paralleled by more severe alterations in nociceptive behaviors, including significant reduction of cage crossing and rearing behaviors compared to the histopathologic and behavioral changes produced by ductal ligation (baseline). A nonsignificant increase in the number of hind limb extensions also occurred. These spontaneous behaviors have been reported previously to be associated with increased visceral nociception.[6,10,34,35]

NMDA Receptor Antagonists

Previous studies demonstrated that NMDA receptor antagonists provide significant antinociceptive effects in visceral pain models involving hollow organs.[17,18,36] The activation of NMDA and non-NMDA receptors in the spinal cord differentially modulates visceral nociceptive input. Spinal NMDA receptor antagonist, APV, blocked

all NMDA-produced facilitation of visceromotor responses to noxious CRD stimulation, while AMPA receptor antagonist, DNQX, blocked quisqualic acid-produced inhibitory effects.[16] Intrathecal administration of APV prevented hyperreflexia after urinary bladder inflammation[3] and blocked the effect of turpentine sensitization on visceromotor response to CRD.[18]However, the effectiveness of the NMDA receptor antagonist for antinociception in the pancreas, a solid endodermally derived organ, was limited. We found that only the reduction in crossing following bradykinin was abrogated by intrathecal NMDA receptor antagonist D-APV. Spinal D-APV did not bring all of spontaneous behavioral activities back to baseline, providing a limited antinociceptive effect in this pain model by this agent alone. Since this is in contrast to the reported effectiveness of D-APV in other visceral pain models, this may denote differences in the model used and/or methodological, species, or organ differences.

Morphine and NMDA Receptor Antagonists

Opioids have been widely and successfully used for management of visceral pain in patients and in animal studies. Many previous studies have shown that μ-opioid receptors can modulate visceral nociceptive transmission in the spinal cord. Intrathecal administration of morphine produces a dose-dependent attenuation of visceromotor responses to noxious colorectal distension.[19,20,37]

It has been shown that NMDA receptor activation increases excitation in neurons leading to an inability of opioids to effectively reduce somatic nociceptive transmission.[38] Thus, it has been proposed that NMDA receptor antagonism enhances the antinociceptive effect of morphine and thus may improve the analgesic efficacy of morphine.[30,39] The current study, using a visceral pain model, supports the proposal that antagonism of NMDA receptor action potentiates the effects of morphine. The results of the current study showed that spinal coadministration of low doses (0.05–0.5 μg) of the opioid receptor agonist morphine sulfate, and a 1 μg dose of NMDA receptor antagonist D-APV, produced antinociception. Coadministration of these two agents maximally increased spontaneous behavioral activities including eliminating hind limb extension in this rat model of acute pancreatitis. These results concur with findings of previous studies in somatic pain models demonstrating that the combined administration of morphine with NMDA receptor antagonists can produce powerful potentiation of morphine's actions on nociceptive events.[27-30,40]

In conclusion, our results imply that spinal administration of the NMDA receptor antagonist, D-APV, can be used as a therapeutic adjuvant producing potent antinociception when combined with low doses of morphine. Coadministration of D-APV (1 μg) potentiated the analgesic effect of low doses of morphine in this model. These findings may be important to developing im-

proved strategies for pain control and adjuvant potentiation of morphine therapy for treatment of developing visceral pain states.

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