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New neostigmine-based behavioral mouse model of abdominal pain

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Abstract:
Background: Animal models of visceral pain have gained much attention as an important tool to elucidate the possible mechanisms underlying functional gastrointestinal (GI) disorders. Here we report the development of a new, minimally invasive behavioral model of abdominal pain induced by *ip* administration of neostigmine in mice.
Methods: Spontaneous behavioral responses evoked by *ip* injection of neostigmine were compared to pain-related behaviors induced by acetic acid solution (*ip*), mustard oil (MO) and capsaicin (both *ic*). Pain behaviors were quantified by assessment of defined postures (licking of the abdomen, stretching, squashing of the abdomen and abdominal contractions). Neuronal activation of spinal cord was measured by determining the number of c-Fos-positive cells.
Results: Neostigmine (2.5 μg/kg, *ip*), acetic acid solution (*ip*), MO and capsaicin (both *ic*) induced spontaneous behavioral responses in mice, which were blocked by morphine (3 mg/kg, *ip*), suggesting the involvement of pain signaling pathways. Injection of neostigmine enhanced c-Fos expression in spinal cord neurons.
Conclusion: The neostigmine model represents a new minimally invasive mouse model to study visceral pain. Based on the neuronal activation pattern in the spinal cord we suggest that this model may be used to study abdominal pain signaling pathways in the GI tract.

Key words:
abdominal pain, analgesia, behavioral pain responses, colorectal distension, gastrointestinal disorders

Introduction

Visceral pain is a frequent symptom in functional gastrointestinal (GI) disorders, like irritable bowel syndrome (IBS) and functional dyspepsia [12]. Visceral pain is diffuse and poorly localized, mainly due to the structure and physiology of visceral nociceptive pathways, e.g., a relatively low number of afferent fibers [2, 6, 7, 10, 12]. Cellular and molecular mechanisms of visceral pain signaling, as well as its perception and physiological processing, are clearly distinct from those involved in somatic pain, and need to be studied separately.

Characterization of the mechanisms underlying visceral nociception is of both, scientific and clinical importance. Studies in humans are mainly based on brain imaging techniques to map the sites activated in clinically evoked or pathophysiological visceral pain [41]. In animals, the most direct approach is the electrophysiological recording of the primary afferent or second-order neurons [12, 18].

Mimicking GI disorders and visceral pain in animals is difficult and therefore only a few experimental models have been validated. The main limitation is the assessment and measurement of pain responses in animals. Electromyography is a common technique used to characterize abdominal muscle contractions in response to colorectal distension (CRD) [34, 36], but the CRD-based model is invasive, technically challenging and time consuming. Recently, models based on the observation of animal behavior and scoring of specific pain-related responses have gained much attention [16, 24]. These models are less invasive, require less animal preparation (e.g., surgical intervention) prior to the assessment of nociception, and allow studies in freely moving animals. However, based on comparative studies, behavioral models seem less sensitive. Both mice and rats are used [18, 19, 37]; however, mouse models of visceral pain have become more popular due to the development of transgenic strains and thus bigger scientific impact.

Several factors need to be considered prior to validation of a behavioral animal model of visceral pain. A test can be considered viscero-specific only when the substances used produce strong smooth muscle contractions sufficient to excite visceral receptors (e.g., acetylcholine; ACh or hypertonic saline) [13, 17, 24, 26] or mild inflammation (e.g., mustard oil; MO) [26]. However, the response should last long enough to be of clinical significance, which is not the case for behaviors evoked by intraperitoneally (*ip*)-injected ACh [26]. Furthermore, high level of invasiveness has to be considered as a major drawback for the MO-based model, where MO is administered intracolonically (*ic*).

In an effort to develop a minimally invasive method of assessment of visceral nociception, we describe here a new model of pain based on the spontaneous behavioral responses following *ip* administration of a reversible acetylcholinesterase inhibitor, neostigmine, in mice. The pain responses to neostigmine were compared to behaviors evoked by acetic acid solution (*ip*), MO and capsaicin (both *ic*), noxious stimuli used in well-established mouse models of visceral pain [16, 23, 24]. Furthermore, the effects of morphine on pain-related behaviors evoked by neostigmine, acetic acid solution, MO and capsaicin were examined. To further evaluate the effects of the applied irritants on the nociceptor activation in the central nervous system, quantification of c-Fos positive neurons in the spinal cord was performed.

Materials and Methods

Animals

Male Swiss albino mice (CD1, Charles River, Canada), weighing 28–30 g were housed at a constant temperature (22°C) and maintained under a 12-h light/dark cycle in sawdust-lined plastic cages with free access to laboratory chow and tap water. The animal use for these studies was approved by the University of Calgary Animal Care Committee and the experiments were performed in accordance with institutional animal care guidelines that follow the guidelines established by the Canadian Council on Animal Care.

All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs

All drugs and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Oakville, ON, Canada). Capsaicin was purchased from Tocris Bioscience (Ellisville, MO, USA).

Measurement of pain-related behavioral responses

Mice were randomly assigned to experimental groups (n = 6–10). The animals were habituated to a raised wire mesh (5 × 5 mm apertures) under a clear plastic box (20 × 20 × 15 cm) for 20 min one day before the assay and again 20 min prior to the experiment.

Neostigmine (1, 2.5 and 10 μg/kg) or vehicle was administered *ip* and the pain-related behaviors were recorded on a videotape for 20 min for later analysis by a separate observer blinded for experimental conditions. Pain-related behaviors: 1) licking of the abdomen in the absence of other grooming behavior, 2) stretching the abdomen, 3) squashing of lower abdomen against the floor, and 4) contracting the abdominal wall and adopting "arched" posture (abdominal retractions) were each counted as 1, as characterized previously [24].

The writhing test was performed as described earlier [23]. Acetic acid solution (0.25, 0.75 and 1.5%, vol/vol in 0.9% NaCl, 10 ml/kg) was administered *ip*. Mice were then placed in individual cages and the total number of writhes was counted 5 min after acetic acid injection during four periods of 5 min each.

Behavioral responses to *ic* mustard oil (MO) and *ic* capsaicin were determined based on the methods described by Laird et al. [24]. For the assessment of the MO-induced pain behaviors, 50 μl of MO (0.25 and 1% v/v in 70% ethanol) or vehicle was administered *ic* under isoflurane anesthesia. Vaseline was applied to the perianal area to avoid stimulation of perianal somatic areas. After 5 min of recovery, spontaneous behaviors were counted for 20 min. Capsaicin-induced pain behaviors were initiated by *ic* administration of 50 μl of capsaicin solution (0.1 and 0.3% w/v in 10% ethanol – 10% Tween 80 – 80% saline) under isoflurane anesthesia and recorded as described above.

Effect of morphine on pain-related behavioral responses

In separate groups the animals were pre-treated with morphine (3 mg/kg in saline, *ip*) 20 min prior to the administration of the irritant. The behavioral responses to neostigmine (*ip*), acetic acid solution (*ip*), MO and capsaicin (both *ic*) were assessed as described above.

c-Fos staining

Immediately after the behavioral tests, mice were anesthetized and transcardially perfused with 10% paraformaldehyde. Spinal cords (lumbar to sacral region) were fixed overnight at 4°C, then placed in 30% sucrose for 24 h at 4°C, and finally embedded in OCT (Sakura Finetek, Torrance, CA, USA), and sectioned at 20 μm. Sections were washed in phosphate-buffered saline (PBS) containing 3% serum and 0.3% Triton X-100, and incubated with a primary c-Fos antibody (Anti-c-Fos (Ab-5) (4-17) Rabbit pAb, 1:10,000 , EMD Millipore, Darmstadt, Germany) for 48 h at 4°C. Sections were washed with PBS and incubated with a secondary antibody, Alexa Fluor 555 (1:1,000; Molecular Probes, Eugene, OR, USA) at room temperature for 2 h. Washed sections were then mounted and imaged using a Zeiss LSM-510 META confocal inverted microscope (Carl Zeiss, Jena, Germany) equipped with 20× objective.

Statistics

Statistical analyses were performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as the means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* test; p values < 0.05 were considered statistically significant.

Results

Spontaneous pain-related behaviors

Administration of neostigmine (1, 2.5 and 10 μg/kg, *ip*) evoked pain-related behaviors, which were characterized by abdominal licking, abdominal retractions, squashing the abdomen against the floor and stretching the whole body (Fig. 1A). The behaviors counted as pain-related were distinct from the grooming behaviors, which were observed in non-treated animals. The total number of spontaneous behaviors in neostigmine-treated mice was dose-dependent and significantly higher than that in vehicle-injected animals (Fig. 1A).

Abdominal licking (approx. 80–90% of all behaviors) and, to a lesser extent, abdominal retractions

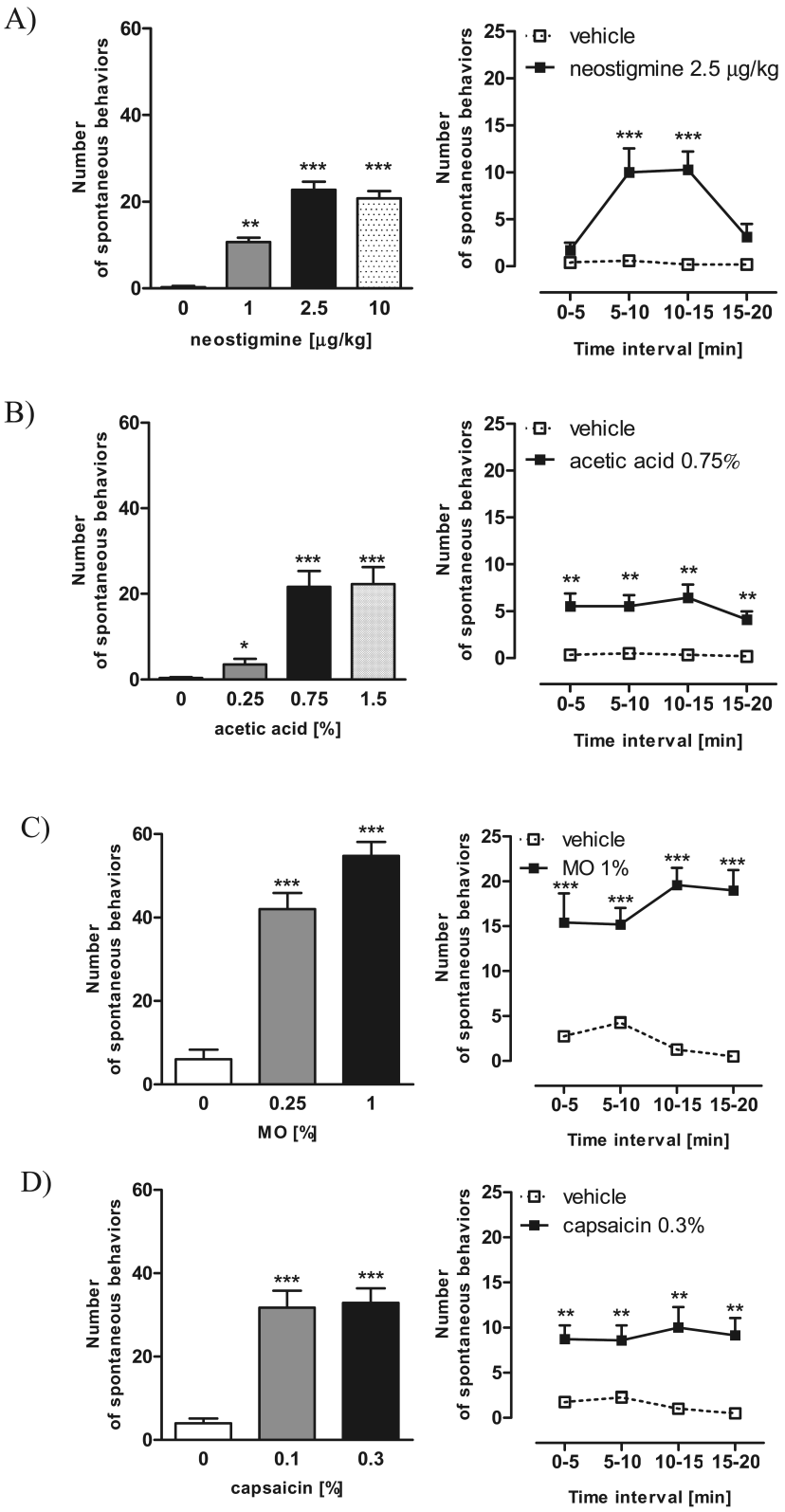

Fig. 1. Total number of behavioral pain responses (stretching, licking and squashing of abdomen, abdominal retractions) evoked by *ip* injection of neostigmine (**A**), *ip* injection of acetic acid solution (**B**), *ic* administration of MO (mustard oil) (**C**) and *ic* administration of capsaicin (**D**). Responses were determined in 5-min episodes for 20 min. Data represent the mean ± SEM for n = 6–10. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as compared to vehicle-treated animals

were the most frequently observed, while stretching or abdominal constrictions were the least observed behaviors. The time course showed that the maximum response was observed 5 to 15 min after injection, with the latency to first behavior greater than 1 min (Fig. 1A). The total number of spontaneous behaviors evoked by the highest dose of neostigmine tested (10 μg/kg, *ip*) was not significantly different from that observed at 2.5 μg/kg, *ip* (20.3 ± 2.3 and 22.7 ± 1.9, respectively). Therefore, a dose of 2.5 μg/kg (*ip*) was used in subsequent experiments.

The *ip* injection of acetic acid solution (0.25, 0.75 and 1.5%, v/v in 0.9% NaCl, 10 ml/kg) elicited pain-related response in mice (Fig. 1B). The total number of pain-evoked behaviors induced by acetic acid solution at a dose of 0.75%, which was selected for further studies, was 21.7 ± 3.7. The time course of the experiments demonstrated a non-significant decrease in the number of behavioral responses after acetic acid solution treatment in the last 5 min-period of the test (Fig. 1B).

MO and capsaicin (both *ic*) produced significant pain-related behavioral response in mice, compared to vehicle-treated animals (Fig. 1C and D). The most predominant behavior (approx. 80–95% of all behaviors) was abdominal licking. The time course of the experiments showed that the total number of responses was similar throughout the test for both MO and capsaicin (Fig. 1C and D) and was less transient than the neostigmine model. For comparison with previously published data, the doses of 1% (*ic*) for MO and 0.3% (*ic*) for capsaicin were used in further assays.

Effect of morphine on spontaneous pain-related behaviors

Pre-treatment with morphine (3 mg/kg, *ip*, 20 min before the assay) significantly decreased the number of spontaneous behaviors evoked by neostigmine (2.5 μg/kg, *ip*) (Fig. 2A), suggesting that they are pain-related [24]. The *ip* administration of morphine also blocked pain-related responses evoked by acetic

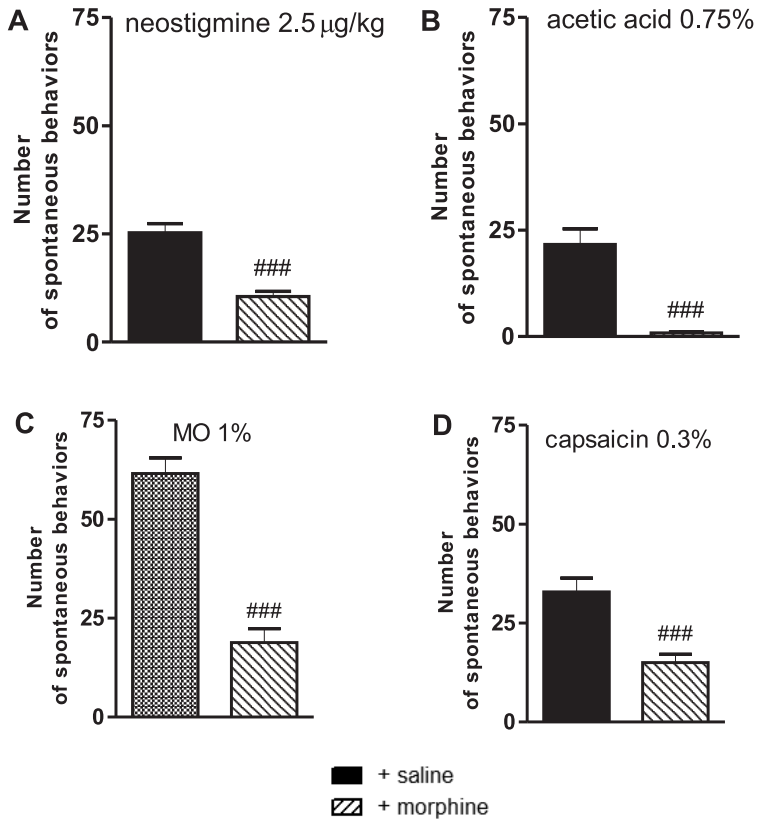

Fig. 2. Effect of morphine (3 mg/kg, *ip*, 20 min pre-treatment) on pain-related behavioral response in mice evoked by (**A**) neostigmine (2.5 μg/kg, *ip*), (**B**) acetic acid solution (0.75%, *ip*), (**C**) MO – mustard oil (1%, *ic*) and (**D**) capsaicin (0.3%, *ic*). Data represent the mean ± SEM for n = 6–10; ### $p < 0.001$, as compared to respective controls (animals injected *ip* with saline)

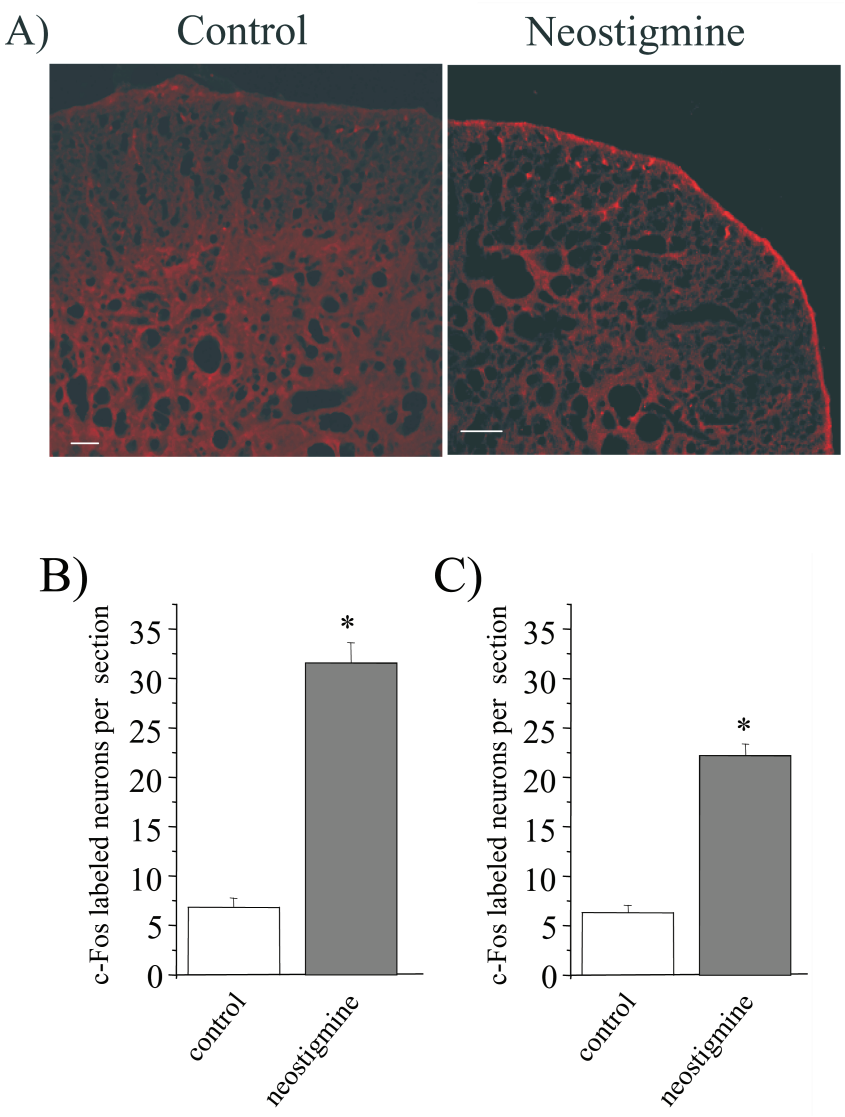

Fig. 3. Intraperitoneal administration of neostigmine enhances c-Fos expression in spinal cord neurons. (**A**) Confocal images of spinal cord sections (L5–S1) in control and neostigmine (2.5 µg/kg, *ip*) treated animals. Scale bar: 50 µm. (**B**) Quantification (the mean ± SEM) of c-Fos positive neurons from seven sections taken in the lumbar spinal cord (L2–L4) following injection of saline and neostigmine (2.5 µg/kg, *ip*) (n = 6 mice per group). (**C**) Quantification (the mean ± SEM) of c-Fos positive neurons from seven sections taken in the lumbosacral spinal cord (L5–S1) following injection of saline and neostigmine (2.5 µg/kg, *ip*) (n = 6 mice per group). Values are mean number of the total section (Laminae I–X) ± SEM from 7 sections per group (* $p < 0.05$, ANOVA)

acid solution (0.75%, *ip*), MO (1%, *ic*) and capsaicin (0.3%, *ic*) (Fig. 2B–D, respectively). When given alone, morphine had no effect on locomotor activity and did not alter the spontaneous behavior of the observed animals.

c-Fos staining and neuronal response

The number of c-Fos positive cells in sections (laminae I–X) from upper (L2–L4) and lower (L5–S1) spinal cord segments was determined to characterize the neuronal activation by nociceptive input received from the colon [14, 16, 32, 44]. As shown in Figure 3, the *ip* injection of neostigmine (2.5 µg/kg) in mice significantly increased the number of c-Fos positive cells in sections from upper (31.5 ± 2.0 neurons per section) and lower (22.2 ± 1.19 neurons per section) spinal cord segments compared with non-treated control animals (6.9 ± 0.9 and 6.3 ± 0.7 neurons per section, respectively).

Discussion

Here we report a new behavioral model of abdominal pain evoked by *ip* injection of a reversible acetylcholinesterase inhibitor, neostigmine, in mice.

Functional GI disorders and many diseases of internal organs induce visceral pain sensitization. Distinguishing visceral from somatic pain is difficult due to structural overlap of afferent fibers and common signaling patterns, including peripheral and central sensitization associated with enhanced neuronal excitability [9, 11, 40]. According to Laird et al. [24] a pain test is considered viscero-specific when substances that produce strong contractions of visceral smooth muscle are used (e.g., ACh or hypertonic saline) [13, 42]. Most of the longer-lasting irritants applied *ic* induce inflammation and – since peritoneum becomes involved – the response is regarded as mixed visceral and somatic [26].

In our newly developed model, we employed neostigmine, a reversible acetylcholinesterase inhibitor, which increases the levels of endogenous ACh, previously shown to excite visceral nociceptors [42], but does not influence macroscopic mucosal damage, adhesions or diarrhoea or induce colitis [33]. The action of the irritant lasts long enough to be assessed in a standardized fashion. In addition, contrary to the methods based on the *ic* instillation, there is no risk of irritation of the perianial area, which also limits overlap with somatic pain pathways. In addition, the relatively short lasting effect of neostigmine, compared to *ic* administered irritants, is more favorable in terms of animal care issues.

Several lines of evidence imply that the newly developed model can be used to study pain signaling pathways in the abdomen. As shown by the c-Fos staining, which is a useful marker of neuronal activity, the *ip* administration of neostigmine activated neurons in the superficial laminae in upper and lower spinal cord. Previous studies demonstrated that the neuronal activation and the increase in c-Fos expression in these segments requires nociceptive input from the colon and rectum [18, 22, 29, 32, 38]. Taken together, our data suggest that the new model can be used to study abdominal pain pathways.

This is further supported by the characteristics of pain behaviors observed after neostigmine injection. Abdominal stretching or writhing, a spontaneous pain reaction typical of the *ip* administration of acetic acid solution, was a marginally observed posture. Instead, abdominal licking and – to a lesser extent – abdominal retractions were predominant. Similar differences in behavioral response profiles between the writhing test and colorectal abdominal pain models in rats and mice were shown earlier ([24] and citations therein: [28, 31]).

The applicable value of our newly developed model has been evidenced in experiments with morphine, a classical opiate analgesic commonly used to relieve acute and chronic pain. At the dose used, morphine decreased the number of neostigmine-evoked behavioral responses, but did not influence locomotor activity or alter spontaneous behavior of the animals, suggesting anesthetization, but not sedation. In 1990, Presley et al. [38] showed that formalin injection increased cFos immunoreactivity in laminae I and II of the dorsal horn. Morphine inhibited the formalin-evoked cFos immunoreactivity and suppressed the pain-induced behavioral response, suggesting an analgesic effect at the spinal level. Numerous other studies have emphasized the role and distribution of opiates and opioid receptors in the superficial dorsal horn of the spinal cord [1, 27] and opioid peptides have been found in high concentration in these regions [15, 20, 21]. It is noteworthy that the superficial dorsal horn is densely innervated by the raphe-spinal axons that are suggested to mediate opiate-activated descending bulbospinal controls [3–5]. Therefore, based on previous findings and our results, we believe that morphine mediated an analgesic effect by dampening the neuronal activity of nociceptive circuit in the dorsal horn of the spinal cord.

The correct assessment of visceral pain in experimental studies is hindered by a more difficult access to visceral tissues, compared to superficial structures, and the requirement of surgical intervention in most of the methods ([24], for review see: [19, 37]). Presently, one of the most common techniques used to assess visceral nociception is based on the electromyographic recording of the abdominal muscle contractions in response to mechanical activation of visceral afferents after graded colorectal distension (CRD) [34, 36]. This method has been successfully used in both rats [35, 36, 39, 43] and mice [25, 31]. In mice, however, it is particularly challenging and only short lasting distensions allow comparable results [30]. Nevertheless, this model is mostly used for its high sensitivity, being able to detect subtle changes in visceral nociception. For instance, this model was used to reproduce in mice visceral hypersensitivity induced

by culture supernatants from IBS patient biopsies [8], while this hypersensitivity was not detectable by behavioral measures. In contrast to the electromyographic recording, the neostigmine-based assay requires no prior surgery, thus eliminating the need for recovery and maximizing the number of animals which can be used for a successful experiment. Furthermore, unlike invasive *ic* administration-based assays, a longer survival time after visceral stimulation decreases dramatically the invasiveness category and eliminates ethical issues.

Another important factor that makes our technique an attractive alternative to currently used mouse models is that the behavioral response can be graded, as it is proportional to the intensity (dose) of the stimuli. There is a similar correlation between the distending pressure and the electromyographic signal [34, 36], which is not the case with the techniques based on the *ic* instillation of irritant solutions to induce visceral pain.

Conclusions

Here we report a new behavioral model to study the abdominal pain response in mice, which is minimally invasive, dose-dependent, and reproducible. Considering all these advantages, it might become an attractive alternative to currently used animal models, especially when testing compounds intended to target and reduce abdominal pain. In our newly described model, the spinal activation suggests that the nociceptive input originates in the colorectum and thus may be helpful to study abdominal signaling pathways, including visceral pain pathways.

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The authors declare no conflicts of interest.

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