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Menthacarin treatment attenuates nociception in models of visceral hypersensitivity

Adesina Omoloye[1] | Sabrina Weisenburger[2] | Martin D. Lehner[2] | Benjamin Gronier[1]

[1]Leicester Institute for Pharmaceutical Innovation, Leicester School of Pharmacy, De Montfort University, Leicester, UK

[2]Preclinical R&D, Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany

Correspondence
Benjamin Gronier, Leicester School of Pharmacy, De Montfort University, Leicester LE1 9BH, UK.
Email: bgronier@dmu.ac.uk

Funding information
Dr. Willmar Schwabe GmbH & Co

Abstract

Background: Chronic visceral hypersensitivity is closely associated with irritable bowel syndrome (IBS), a very common disorder which significantly impairs quality of life, characterized by abdominal pain, and distension. Imaging studies have found that IBS patients show higher metabolic activities and functional differences from normal controls in the anterior cingulate cortex (ACC), in response to visceral pain stimulation. Non-clinical data and clinical data suggest that medicinal products containing essential oils such as peppermint or caraway oil exert beneficial effects on IBS symptoms.

Methods: We assessed acute and long-term treatment effects of a mixture of peppermint and caraway essential oils (Menthacarin) on brain electrophysiological markers of gut pain sensitivity in two rat models of visceral hypersensitivity.

Key Results: Chronic administration of corticosteroids and acute repeated mechanical hyperstimulation under anesthesia induced hyperalgesia and hypersensitivity, characterized by an increase in electrophysiological excitatory responses of ACC neurons to colorectal distension (CRD) and an increase in the proportion of neurons responding to otherwise subthreshold stimulation, respectively.

Long-term, but not acute, oral administration of Menthacarin (60 mg kg^{-1} day^{-1}) significantly reduced the net excitatory response to CRD in normally responsive control animals and counteracted the development of visceral hyperalgesia and hypersensitivity induced by repeated corticosterone administration and acute mechanical stimulation.

Conclusions & Inferences: The present study shows that, using the CRD method, chronic Menthacarin administration at a clinically relevant dose attenuates the neuronal discharge associated with visceral pain stimuli in the rat ACC, particularly in models of hypersensitivity, suggesting a potential for treating exaggerated visceral pain sensitivity.

KEYWORDS
anterior cingulate cortex, colorectal distension, electrophysiology, irritable bowel syndrome, Menthacarin, visceral hypersensitivity

1 | INTRODUCTION

Chronic visceral hypersensitivity is often closely associated with functional gastrointestinal disorders such as irritable bowel syndrome (IBS), a very prevalent and debilitating condition affecting up to 16% of the general population.[1] Patients suffering from visceral hypersensitivity and hyperalgesia not only experience abdominal pain, but tend to have increased in pain sensation, whereby normal visceral physiological stimulations are perceived as discomfort and pain.[2] In most of cases, patients tend to have lower pain thresholds during rectal distension, accompanied by an exaggerated reflex motor activity in the rectum.[3]

In IBS, these symptoms occur without any identifiable organic cause, tissue damage or significant inflammation, though intestinal permeability may be increased in a significant proportion of patients.[4] IBS patients generally suffer persistent abdominal pain or discomfort in combination with aberrant bowel pattern and present as one of the three predominant subtypes: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C) or mixed (IBS-M).

IBS is a major public health concern that imposes a substantial economic burden to the society.[5] As the disease is chronic and treatments are only partially successful, IBS may trigger or worsen significant psychological disturbances, indicating that patients can be both affected mentally and physically. Surveys indicate that on average, middle-age patients would be willing to sacrifice between 10 and 15 years of their life for a permanent and immediate cure for their IBS.[5] Perhaps not surprisingly, a significant proportion of patients with IBS symptoms have associated psychiatric co-morbidity, mainly generalized anxiety disorders, major depression, and suicide attempt/ideation.[6,7] The relation with psychiatric ailment, however, is complex, as psychosocial stress is a well-described contributor to intestinal disturbance and may therefore be involved in the development of IBS symptoms,[7] causing intestinal disruption and/or irritation, with change in intestinal secretion/permeability.

Available treatments for IBS symptoms and visceral hypersensitivity have only limited efficacy depending on the nature of the symptoms and their severity. Some patients may moderately respond to dietary modifications, lifestyle changes, or medications affecting gut motility. Tricyclic antidepressants, serotonin uptake inhibitors, 5-HT3 agonists or opioid agents may be helpful to control bowel movement and severe pain symptoms, but clinical trials often report modest results,[8] sometimes with unpleasant side effects.[1] Given the partial failure of classical pharmacotherapies, it is reasonable to consider alternative types of therapies including herbal remedies, which may be offered as adjunct therapies with conventional treatments or as monotherapies. Herbal remedies usually include a number of potentially active ingredients which provide the advantage of acting on multiple therapeutic targets simultaneously, possibly in a synergetic way. This multimodal action may be more effective for complex disorders like IBS, where different components of the digestive track could collectively contribute to the disease.

Key points

- The anterior cingulate cortex (ACC) plays a crucial role in visceral pain processing.
- Nociceptive signals induced by colorectal distension (CRD) alter neuronal activity of ACC neurons in anaesthetised rats.
- Chronic administration of Menthacarin, a combination of peppermint/caraway oils, attenuated CRD-induced ACC neuronal discharges in controls and visceral hypersensitivity models.

Peppermint and Caraway oils are classical remedies with a very long historical use for relieving gastrointestinal ailments and for their pain-relieving effects.[9] Menthacarin, is a proprietary combination of peppermint oil (90 mg WS® 1340) and caraway oil (50 mg WS® 1520) with specified quality. Clinical data from three randomized controlled trials on patients suffering from functional disorders have established that Menthacarin produces significant relief in IBS associated symptoms.[10]

The anterior cingulate cortex (ACC) is a critical brain region of the frontal cortex involved in pain processing and considered to be particularly crucial in mediating the affective component of pain.[11] Electrophysiological and imaging studies have well established that ACC neuronal activity is increased during a peripheral pain stimulus.[12] Interestingly, imaging studies have shown that metabolic activity is increased in the ACC region in disease associated with visceral hyperalgesia.[3,13] In animal models, visceral pain perception and sensitivity (nociception) can be assessed, even in deeply anesthetized preparations, by measuring individual neuronal activity in the ACC[14] during a pain stimulus administered by colorectal distension (CRD), which was obtained by inflating a balloon in the colorectal area.

Different animal models have been described to mimic hyperalgesia and visceral hypersensitivity, which are major clinical manifestations of IBS.[15,16] Chronic treatment with corticosterone is a pharmacological model of hyperalgesia associated with visceral hypersensitivity, which, to some extent, mimics chronic psychological stress which is known to be closely associated with IBS.[17] Another more acute and nonpharmacological model of visceral hypersensitivity consists of mechanical hyperstimulation,[18–20] where short and repetitive phases of CRD are delivered to the animal before the investigation, causing a long-lasting exaggerated visceral nociceptive response.

The aim of the present study was to assess the therapeutic potential of Menthacarin on visceral pain responses, measured as the electrophysiological response of ACC neurons to CRD in anesthetized rats in normal and in hyperalgesic conditions, respectively. We mainly examine whether Menthacarin treatment can prevent the development of visceral hyperalgesia using the two hypersensitivity models presented above.

2 | MATERIALS AND METHODS

2.1 | Drugs and mixtures

Menthacarin®, a proprietary combination of 90 mg peppermint oil (WS® 1340) and 50 mg caraway oil (WS® 1520) with specified quality was provided by Dr. Willmar Schwabe GmbH & Co. KG, Germany. All other drugs/chemicals were purchased from Sigma (Sigma-Aldrich, UK).

2.2 | Experimental animals

Male Sprague Dawley rats (250–350 g) were used. All animal experiments were conducted in strict accordance with the UK Home Office guidelines and the Animal Scientific Procedures Act (1986). Rats were housed in groups of two to four per cage in wood shaving bedding, in open double-Decker cages (188 cm^2 floor area). Cage were kept in a room maintained at 20–22°C with humidity rates between 45% and 65% under a 12:12 light/dark cycle with lights on at 07:00 in adequate ventilation conditions of air renewal according to Home Office code of practice. Food and water were both provided ad libitum. Animals were allowed a 3-day acclimatization period after delivery. All experiments were performed during the light phase and with permission from the UK Home Office and De Montfort University Ethics Committee.

2.3 | Electrophysiological recordings

Experiments were conducted in the afternoon (typically between 1 and 7 p.m.). Animals were not presented with food from 8 to 2 h before the experiments to prevent fecal pellets to interfere with the experiments. Rats were deeply anesthetized with urethane (1.5–1.7 g kg^{-1}, i.p.) and installed on a stereotaxic equipment (Kopf Instruments) and trepanned (1.0–5.0 mm anterior to bregma and 0.1–2.0 mm lateral to midline; trepanation hole: 2.5 mm diameter) for electrophysiological investigations. Electrodes for recordings were manufactured in-house from borosilicate capillaries (1.5 mm, Harvard Apparatus Ltd., UK), pulled on a PP-830 vertical electrode puller (Narishige, Japan) and filled by hand with an electrolyte solution of NaCl 147 mM. The tip of the electrode was broken down under a microscope to an external diameter of 1–1.5 µm. Typical electrode resistance was in the range 4–8 MΩ. Outputs from the electrode were sent to a Neurolog AC pre-amplifier and amplifier (Digitimer, UK). If necessary, signal amplification was manually adjusted to record whole neuronal action potential amplitudes. Signals were filtered and sent to an audio amplifier, a Tektronix 2201 digital storage oscilloscope and a 1401 interface connected to a computer running Spike 2 v5.21 (CED, UK) for data capture and analysis. Descent of the electrode was carried out using a hydraulic micromanipulator (MO-103, Narishige, Japan). The recording electrode was lowered slowly in the ACC (coordinates: 2–3 mm anterior to bregma, 0.3–1.2 mm lateral to midline, 1.5–4.5 mm ventral to brain surface, Figure S1) until clear single unit neuronal activity was identified. The ACC neuron was then recorded and tested for its electrophysiological response to CRD at various pressures. An average of three to four cells per electrode descent were recorded. Electrode descents were repeated up to four times, with each descent distant from one another of at least 150 µm.

Neurons, presumed to be pyramidal, were identified according to previous electrophysiological criteria established in our and other laboratories[21,22]: a broad action potential (>1 ms), with a biphasic or triphasic, large waveform (>2 mV), starting with a positive inflection, a slow firing rate typically between 1 and 50 spikes/10 s and irregular firing pattern, occasionally with burst activity. For each electrode descent (1.5–4.5 mm ventral to brain surface) we recorded individually all neurons encountered (provided the recording was stable) and tested electrophysiological responses to CRD application.

2.4 | Colorectal distension

A polyethylene tubing attached to a 5 cm long balloon (made from nitrile rubber) was carefully inserted in the terminally anesthetized animal through the anal canal (8–9 cm down) and secured to the base of the tail. CRD pressures were produced by rapidly inflating the balloon with air at pressures varying from 15 to 75 mmHg (typically 10–20 mmHg increment) using a 50-mL plastic syringe. Pressure within the balloon was monitored by connecting the catheter to a digital pressure transducer (Testo).

Graded-pressure CRDs (15, 30, 50, 60, and 75 mmHg) were applied to establish stimulus–response curves. Typically, most cells from naïve rats are unresponsive to pressure below 40 mmHg. Whenever possible, each CRD pressure was tested twice with 3-min intervals to get a stable response at the same intensity (or with higher intensity if no response was observed from the initial application) to make sure the responses were consistent and repeatable. At the end of the experiments the animals were euthanized with an overdose of chloral hydrate administered via an intravenous catheter previously inserted in the lateral tail vein. The intestinal tissue was then visually checked to rule out very rare cases of perforation.

2.5 | Treatment

Corticosterone (from a 15 mg mL^{-1} suspension in ethanol 1% and 12% peanut oil) and Menthacarin, or their combination, were diluted in sucrose solution (10%), administered orally by gavage (3 mL kg^{-1}) once a day for the indicated periods, at the doses of 8 and 60 mg kg^{-1}, respectively. Vehicle (10% sucrose) was also administered in the same conditions. Treatments were done in the morning between 8 and 10 a.m. The last administration was done at least 6 h prior to start of the CRD protocol.

2.6 | Data analysis and statistics

A neuron was considered responsive to CRD if its spike firing rate increased or decreased by at least 15% from its pre-CRD baseline activity and if this was observed on at least two (generally subsequent) CRD applications. Mean neuronal discharge rates are measured 50 s before, 50 s during, and 120 s after CRD, with 3–4 min intervals in between, and evaluated on a time histogram (10-s bin width) (as shown in Figure 1). The net response was expressed as the difference between the mean neuronal excitatory discharge during the CRD and baseline activity recorded immediately before CRD application, in stable recording conditions. Occasionally, in case of protracted response, the supplementary spikes occurring within 30 s after the CRD application were also averaged and included in the response, provided the firing returned to normal levels.

Data were expressed as mean ± SD; *n* represents the number of neurons tested. Unless otherwise stated, all experiments involving acute or chronic treatments were carried out in groups of five to seven animals. At least 15 neurons were recorded per animal. Control animals consisted of naïve (6 rats) or chronically treated rats for 7–14 days (8 animals) with vehicle (sucrose 10%). Both groups displayed similar excitatory responses to CRD (Figure S2). Consequently, all control values were pooled together to make up a unique control group ($n = 49$ neurons). Control animals were tested within the same time-frame and on the same batches of animals as the drug-tested animals. Comparison between groups were made using Student's *t*-test or one-way ANOVA test, followed, if appropriate, by Newman–Keuls test. The proportions of specific types of response in different groups of animals were compared using the chi-squared test. Statistical analyses were performed using PRISM software.

3 | RESULTS

A total of 233 neurons from the ACC were recorded and tested for CRD in control animals (17 naïve or sucrose-treated rats). The basal firing activity of the neurons recorded was variable (0.5–60 spikes/10 s) but typically below 20 spikes/10 s (average 13.1 ± 45.6 spikes/10 s), in agreement with previous electrophysiological data performed in this area.[21] All neurons tested exhibited the electrophysiological characteristics of pyramidal neurons (see Section 2).

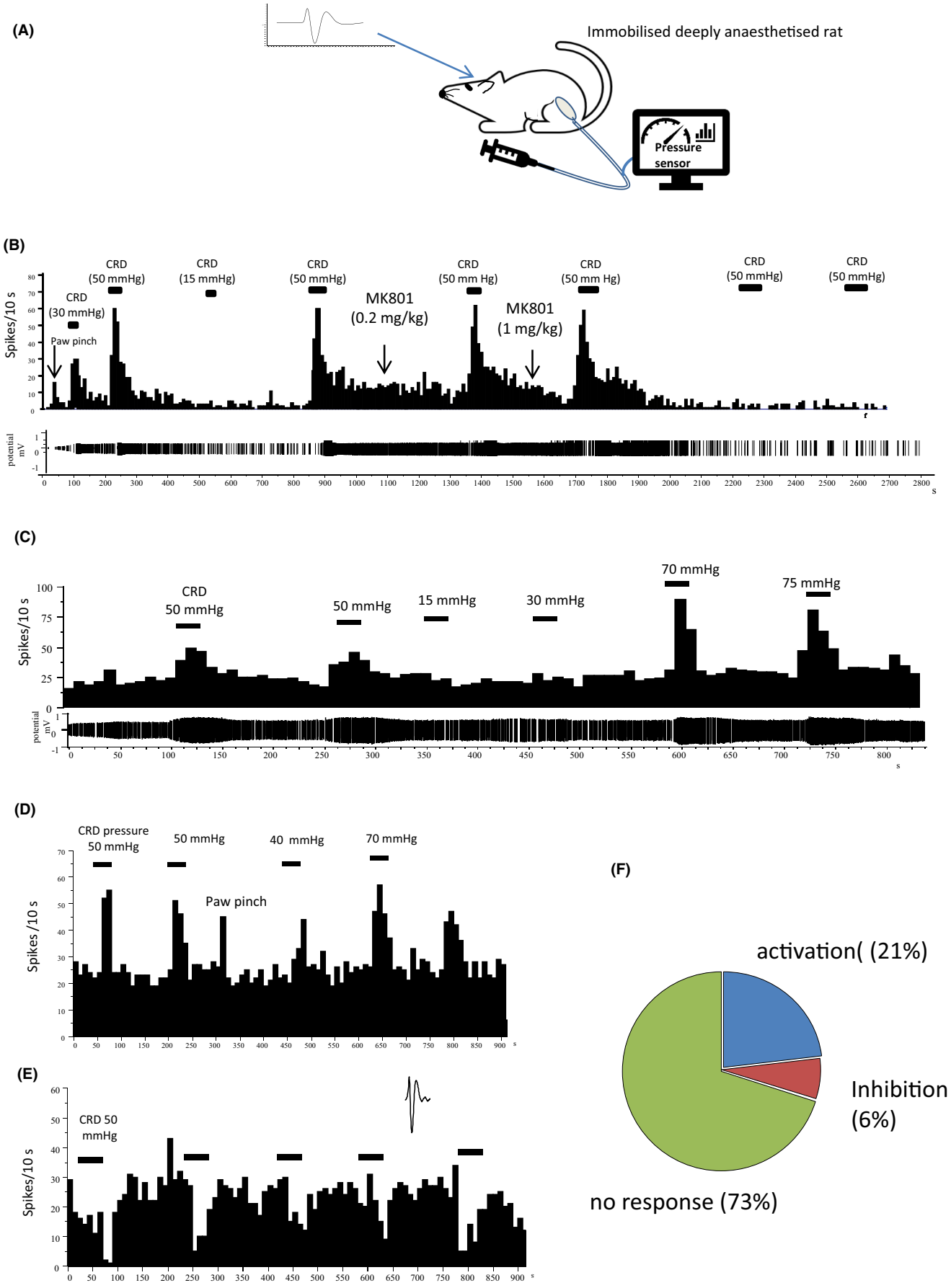
3.1 | Control conditions

Figure 1 shows individual electrophysiological responses of some representative ACC neurons to colorectal distension application at various pressures.

Overall, only a subpopulation of the ACC neurons tested responded to CRD application (mostly at 50 mmHg) by a change in firing rate (estimate 27% of the total number of neurons tested). As indicated in methods, only neurons showing changes in firing activity by more than 15% of their basal firing activity in two separate CRD applications were considered responsive neurons. In the neurons responding to CRD (50 mmHg) by an activation of firing, the average firing rate increased from 15.6 ± 13.2 spikes/10 s to 24.4 ± 18.0 spikes/10 s, and activation frequently persisted after stimulation, but decreasing progressively back to initial baseline values. However, there were variable responses from one neuron to one another, some neurons showing large increases in firing rate (5–10 times the baseline activity), while other neurons showed more modest responses (15%–30% increase obtained consistently for two to three CRD applications). Interestingly, the excitatory component caused by CRD application was inhibited by administration of the glutamate NMDA antagonist dizocilpine (MK-801, 1.2 mg kg^{-1}, cumulative dose, i.p.). This finding suggests that CRD probably activates a neuronal pathway involving the release of glutamate on the ACC neurons that controls the affective component of pain (Figure 1B), in agreement with previous investigation.[23]

The neuronal response to CRD was pressure-dependent (Figure 1B–E). Responses to CRD were often only detectable at pressures ≥40 mmHg, which correspond to the pain threshold in non-anesthetized conditions.[24] Unless otherwise stated, responsive neurons from control animals were all responsive to a CRD pressure of 50 mmHg. CRD activation was first tested at low (15–30 mmHg), then medium (50 mmHg) and, whenever possible, higher pressures (70–75 mmHg). Only a small proportion of neurons tested were responsive to pressure of 30 mmHg or below (2 out of the 49 responsive neurons tested in control conditions). For all responsive neurons we calculated the net excitatory CRD response expressed as the difference between the mean neuronal excitatory discharge during the CRD application and baseline activity recorded immediately before CRD application (typically during the 50 s preceding CRD), in stable recording conditions, as indicated in Figure 2. As expected, electrophysiological responses

FIGURE 1 (A) Schematic illustration of the experimental CRD protocol. Each time the balloon is inflated (50 s duration), the responding recorded neuron responds (or does not respond) by a change in firing activity. (B–E) Representative individual firing rate histograms and spike train of an ACC neuron responding to colorectal distention applied at various pressures. (B) Neuron displays a strong increase in firing activity each time a CRD is applied. Interestingly, this response is inhibited by the administration of the glutamate NMDA receptor antagonist MK801. (C, D) Neurons exhibit sharp activation, which was dependent on the intensity of the pressure applied. Note that, in control condition, pressure below 40 mmHg (pressure threshold to cause noxious stimuli in conscious subjects) rarely generated a response. (E) Neuron responds by inhibition of firing activity to CRD. On top is shown the corresponding ACC neuron action potential waveform. The figures also show that the neurons recorded are also activated by a paw pinch. (F) Pie diagram showing the proportion of neurons responding to CRD (50 mmHg): by an activation of firing, an inhibition, or no effects.



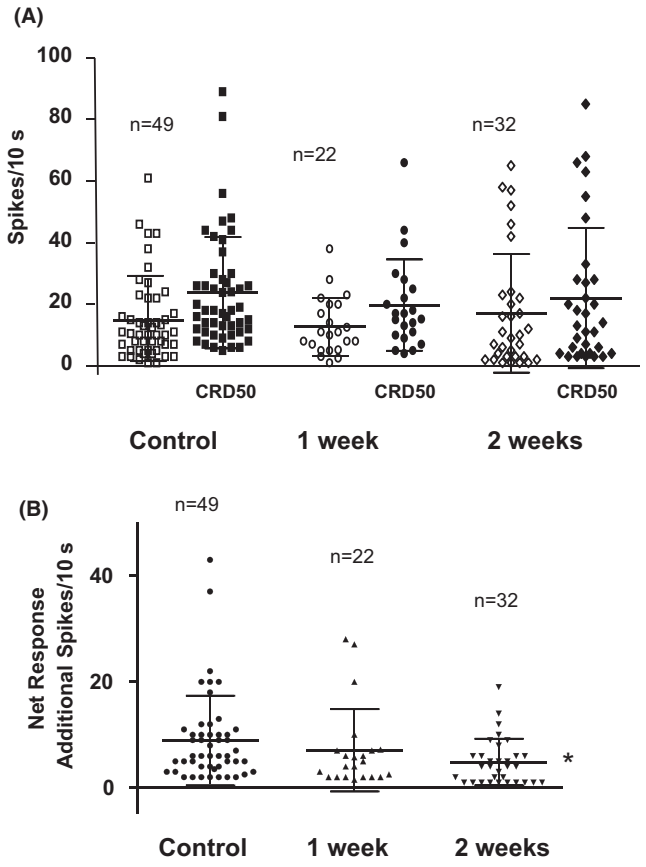


FIGURE 2 Long-term Menthacarin treatment attenuates the neuronal electro-physiological response to CRD. (A) Scatter plots showing CRD-sensitive ACC neurons individual firing activities before and during applications of CRD (50 mmHg, 60 s) in control (14 animals) and in animals treated with Menthacarin (60 mg kg^{-1} day^{-1}, oral) for 1 week (3 animals) and 2 weeks (5 animals). In this and subsequent figures the number of recorded neurons is indicated on the top of each corresponding graph. Bars indicate means ± SD. (B) Scatter plots showing individual excitatory responses to CRD (50 mmHg), of the same ACC neurons as in (A), expressed as the difference between the stimulated and corresponding baseline activity in control conditions and after administration of Menthacarin for 1 week or 2 weeks. A 2-week, but not a 1-week, administration of Menthacarin significantly reduced the responses. The net excitatory response is significantly decreased following an exposure of 2 weeks with Menthacarin. *$p < 0.05$, compared to control, Newman–Keuls test, following significant ANOVA ($F_{2,100} = 3.2$, $p < 0.04$).

of ACC neurons to CRD 75 mmHg were significantly higher than their responses to CRD 50 mmHg (net response increased from 7.0 ± 9.3 to 12.7 ± 12.2 additional spikes/10s, $p < 0.001$, $n = 34$, paired Student's *t*-test, Figure S3). However, maximal CRD responses tended to reach a plateau between 60 and 75 mmHg (Figure 1C,D). Therefore, only responses to pressures lower or equal to 50 mmHg were used for comparison between groups and analysis.

Among the neurons recorded in control conditions, 61 neurons (26%) showed a response to CRD applied at 50 mmHg, of which 49 (21%) showed an excitatory response and 12 neurons (5%) a clear inhibitory response (Figure 1F).

3.2 | Menthacarin treatment

3.2.1 | Single oral administration

After single oral administration of Menthacarin (60 or 100 mg kg^{-1}, 3 animals tested) the net electrophysiological response to CRD application, expressed as the difference between the mean neuronal excitatory discharge during CRD and baseline activity (6.1 ± 5.0 additional spikes/10 s, $n = 15$), did not change significantly from control (9.0 ± 8.4, additional spikes/10 s $n = 49$; $p < 0.67$; Student's *t*-test).

3.2.2 | Chronic treatment

A one-week administration of Menthacarin (60 mg kg^{-1}/day, 3 animals tested) did not significantly change the net electrophysiological response to CRD (7.1 ± 7.5 additional spikes/10 s, $n = 22$; Figure 2). In contrast, long-term Menthacarin (60 mg kg^{-1} day^{-1}) administration for 2 weeks (5 animals tested) resulted in a small but significantly lower net excitatory CRD response (4.8 ± 4.5 additional spikes/10 s, $n = 32$) compared to controls ($p < 0.05$; Newman–Keuls test, following significant one-way ANOVA ($F_{2,100} = 3.2$, $p < 0.04$); Figures 2A,B). This reduction was associated with a decrease in the proportion of cells responding by a neuronal activation in the Menthacarin long-term treatment group (12.5% vs. 21% in control), although the difference was not statistically significant ($p = 0.08$, chi-squared test).

The proportion of cells showing inhibitory responses and the magnitude of these responses were not different between Menthacarin-treated and control animals.

3.3 | Effects of Menthacarin treatment on visceral hypersensitivity

3.3.1 | Corticosterone-induced visceral hypersensitivity

Chronic administration of corticosterone (8 mg kg^{-1} day^{-1}) for 14 days (6 animals tested) resulted in a significant 53% increase in the net CRD response in otherwise untreated animals ($p < 0.001$, Newman–Keuls test after significant ANOVA ($F_{2,115} = 13.4$; $p < 0.0001$); Figure 3), indicating that long-term administration caused visceral hyperalgesia. In contrast, in animals treated with Menthacarin ($n = 5$) concomitantly to corticosterone administration, the neuronal excitatory response to colorectal distension (50 s, 50 mmHg) was not potentiated (Figure 3). This indicates that Menthacarin apparently prevented or counteracted the hyperalgesic effect of corticosterone on CRD.

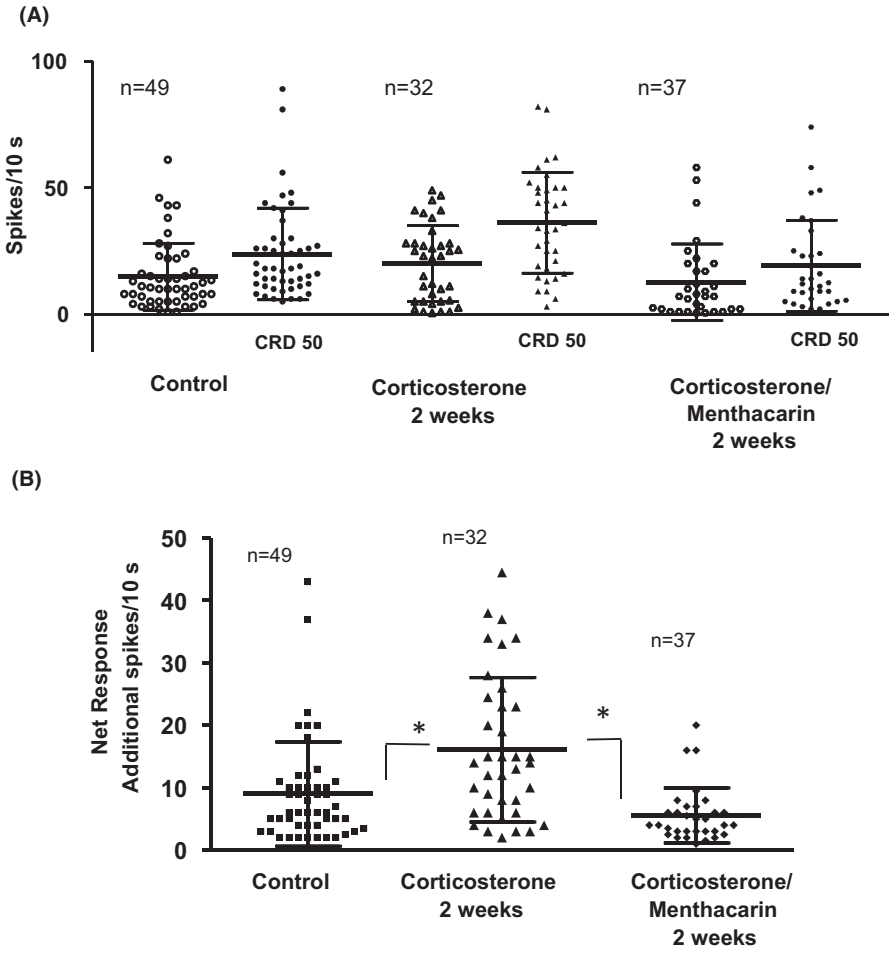


FIGURE 3 Menthacarin attenuates corticosterone-induced visceral hyperalgesia. (A) Scatter plots showing individual firing activity of responsive ACC neurons before and during CRD (50 s, 50 mmHg) in control (14 animals), corticosterone (6 animals) and in Menthacarin-corticosterone combination treatment groups (5 animals). (B) ACC excitatory net responses to colorectal distension (CRD) (60 s, 50 mmHg) in controls, corticosterone and in Menthacarin/corticosterone combination treatment groups, from the same neurons as in (A). Corticosterone treatment significantly increased the net response compared to control animals. Menthacarin counteracted the corticosterone-mediated effects. $^{*}p < 0.001$, compared to control and to corticosterone/Menthacarin combination treatment; Newman–Keuls test after significant ANOVA ($F_{2,115} = 13.3$; $p < 0.0001$).

In addition, in the corticosterone-treated animals, a significant proportion of responsive neurons ($n = 17$, 41% of the 50 mmHg-responsive neurons) were also sensitive to a lower pressure of CRD application (30 mmHg), an effect that was only rarely observed in naive animals, confirming visceral hypersensitivity (Figure 4A,B). Furthermore, this increase in the proportion of responsive neurons at low CRD pressure (30 mmHg) was not seen in the Menthacarin/corticosterone combination treatment group (no responsive neurons found).

Together, these findings suggest that corticosterone chronic administration increases both visceral nociceptive response and threshold sensitivity, but these effects can be prevented by Menthacarin.

3.4 | Mechanically induced hypersensitivity

As an alternative approach to the chronic corticosteroid administration model, an acute model was additionally employed, consisting of a series of colorectal distensions applied at higher strength and at short intervals (8 stimulations at 75 mmHg for 1 min at 1-min intervals) in the deeply anesthetized animals. This procedure was adapted from previous studies carried out in conscious animals.[18,19]

Thirty minutes after applying this protocol, neurons were recorded and we found that most of the responding neurons tested developed (at least for several hours) significantly higher electrophysiological responses to CRD compared to control animals, indicating hypersensitivity. In untreated animals, this mechanical stimulation induced hyperalgesia, as evidenced by the significant increase in CRD response compared to naïve animals (Figure 5). The net increase in neuronal response with mechanical stimulation (14 spikes/10 s) was similar to that observed following chronic corticosterone treatment (16 spikes/10 s).

A 3- to 4-day treatment (net response = 17.1 ± 16.8 additional spikes/10 s, $n = 14$), or an acute administration of 60 mg kg^{-1} of Menthacarin (net response = 13.8 ± 12.8 additional spikes/10 s, $n = 17$), had no effects on the hyperresponsivity of ACC neurons after the multiple CRD application protocol. In contrast, long-term pre-treatment of the animals for 14 days with Menthacarin completely prevented the hyperalgesic effects of the mechanical stimulation (Figure 5).

Interestingly, a large proportion of the responsive neurons to CRD 50 mmHg were also showing sensitivity to lower pressure (30 mmHg) (Figure 6), as it was already observed for the corticosterone treated animals. This hypersensitive response was also normalized by prolonged Menthacarin therapy (only one neuron out of

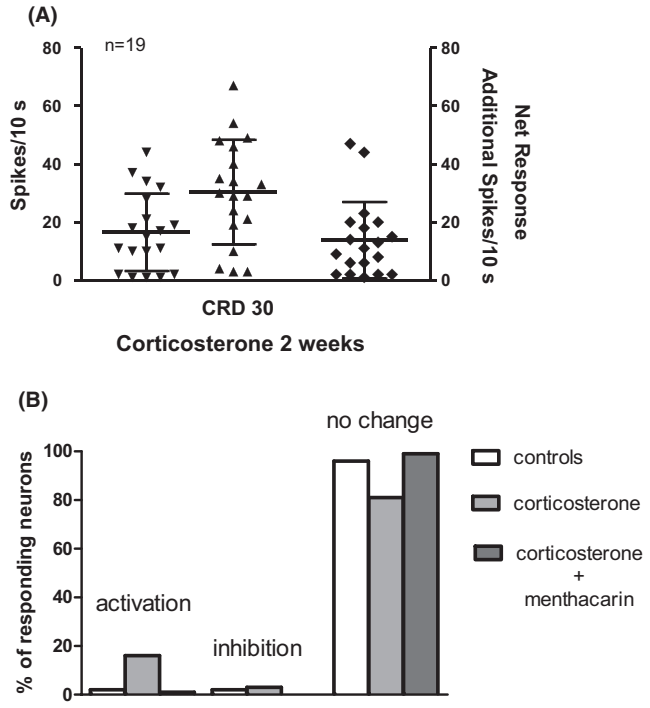


FIGURE 4 Chronic corticosterone induced visceral hypersensitivity is abolished by Menthacarin. (A) Scatter plots showing individual firing activity of responsive ACC neurons before and during CRD at lower pressure (50 s, 30 mmHg) in corticosterone treated animals (left) and the individual net excitatory responses from the same neurons (right).
(B) Proportion of neurons responding to a CRD of 30 mmHg from corticosterone-treated and control rats, and rats treated with the combination Menthacarin/corticosterone. In control animals and in animals chronically treated with the combination corticosterone/Menthacarin virtually no neurons (<3 neurons) were sensitive to the low pressure.

the 69 neurons tested was showing sensitivity to CRD 30 mmHg). Overall, these results suggest that both, corticosterone treatment and mechanical colorectal stimulation induced a visceral hyperalgesic and hypersensitive state that can be successfully prevented by Menthacarin therapy.

4 | DISCUSSION

The present investigation using the colorectal distension (CRD) method shows that a 2-week repeated daily administration of Menthacarin, a proprietary mixture of peppermint oil and caraway oil, reduces the neuronal discharge associated with visceral pain stimuli in the rat anterior cingulate, which is a brain region involved in the emotional control of nociception.

We employed chronic administration of corticosterone and mechanical hyperstimulation as pharmacological and non-pharmacological challenges to induce a state of visceral hyperalgesia and hypersensitivity. Both models have been shown to display features of visceral hypersensitivity, assessed for example by measuring the visceromotor response in conscious animals.[17] In our study, both challenges produced an exaggerated electrophysiological response of ACC neurons to CRD compared to control conditions, causing a robust augmentation of their neuronal discharge during CRD. In addition, neuronal discharges could be observed already at a lower pressure threshold normally not causing neuronal activation, which suggests the induction of visceral hypersensitivity. It has been established that chronic corticosterone administration can decrease epithelial tight junction protein levels and increase colonic permeability to low-molecular weight macromolecules in the colon.[25] This may result in an increased release of soluble mediators such as histamine or tryptase in the intestinal mucosa as a result of increased paracellular permeability, causing a mucosal inflammation process and visceral hyperalgesia.[25] The mechanism causing this down-regulation of gap-junction protein is unknown but may be caused by a down-regulation of glucocorticoid receptors, which may directly or indirectly affect transcription of tight-junction proteins.[26]

Our intervention studies demonstrated that long-term (2 weeks), but not the short-term (<7 days), administration of Menthacarin significantly attenuates the electrophysiological response to visceral stimuli in both chronic and acute models. Hence, long-term Menthacarin treatment seems to exert a certain degree of analgesic effect on visceral nociception per se, and effectively prevents or counteracts development of visceral hyperalgesia and hypersensitivity. The mechanism by which Menthacarin caused this reduction in visceral hypersensitivity has not been addressed in this study and will deserve further investigations.

It will be of particular interest to examine the two essential oil components separately. There is some evidence that small-intestine release peppermint oil, one of the two ingredients of Menthacarin, can have beneficial effects on symptoms of IBS,[27] though overall efficacy in terms of pain response did not reach statistical significance in a recent randomized-controlled trial.[28] In addition, peppermint oil has an interesting antimicrobial effect.[29] Caraway oil, the other Menthacarin active ingredient, is traditionally used for its beneficial effect on functional dyspepsia, and is known to have, like peppermint oil, a strong muscle inhibitory and pro-secretory activity in the human intestine in in vitro conditions.[30]

Menthacarin, when administered chronically, may provide long-term protective effects on the intestinal mucosa, reducing the consequence of corticosterone treatment and of mechanical hyperstimulation on tissue integrity and function (e.g., intestinal permeability, as mentioned above) which may be at the origin of visceral hyperalgesia. Long-term corticosterone treatment, like chronic stress, can enhance visceral and somatosensory pain perception.[17] Functional visceral pain has been associated with activation of the ATP ion-gated channels, voltage-gated sodium and calcium channels, as well as up-regulation of different TRP receptors such as transient receptor potential vanilloid type-1 (TRPV1)[31] and transient receptor potential ankyrin 1 (TRPA1). The receptors TRPV1 and TRPA1 play a particularly important role in visceral pain and hypersensitivity states as well as inflammatory processes. There is

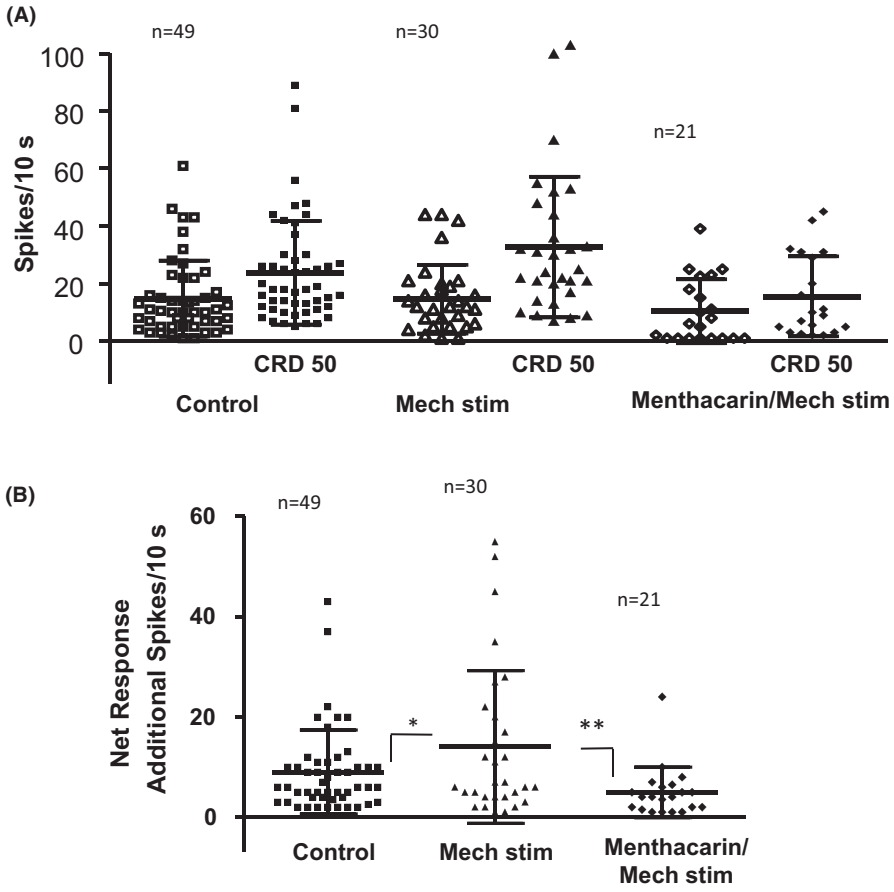
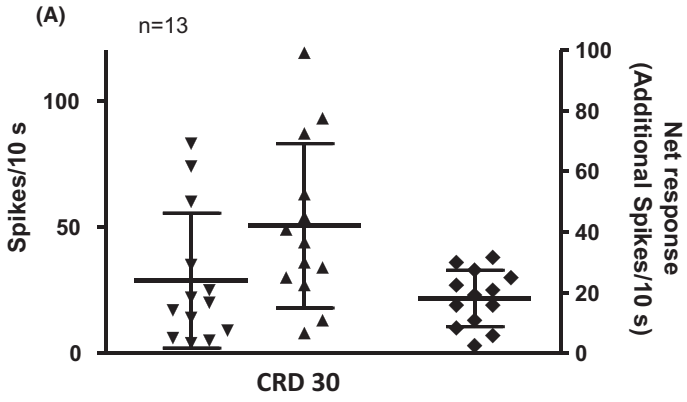


FIGURE 5 Menthacarin attenuates visceral hyperalgesia induced by mechanical stimulation. (A) Scatter plots showing individual firing activities of responsive ACC neurons before and during CRD (60 s, 50 mmHg) in control and in animals previously subjected to multiple mechanical stimulations under no treatment conditions ($n = 5$ animals) and during a 2-week treatment with Menthacarin ($n = 5$ animals). (B) ACC excitatory net responses to colorectal distension (CRD) (60 s, 50 mmHg) from the same neurons as in A. Multiple mechanical stimulations significantly increased the net response compared to control animals. Menthacarin counteracted the corticosterone-mediated effects. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control and to Menthacarin treatment, respectively; Newman–Keuls test after significant ANOVA ($F_{2,97} = 4.8$; $p < 0.01$).

evidence that TRPV1 and TRPA1 nerve fibers are increased in the mucosa of IBS patients and may contribute to the pathophysiology of pain in IBS.[32,33] Repeated administration of corticosterone in healthy control rats that reproduced the corticosterone levels observed during chronic stress exposures (similar doses as in our study) caused upregulation of the TRPV1 receptor.[17,32] Menthol, one of the main components of Menthacarin, is known to elicit activation of transient receptor potential cation channel subfamily M member 8 (TRPM8) which is also involved in diverse aspects of cellular function and sensory perception and is also present on nociceptive neurons.[34] TRPV1 and TRPM interact with each other, often negatively,[35] to modulate pain perception. Though the molecular action of menthol is complex and not totally elucidated, it has the ability to sensitize or desensitize its receptors (TRPM) based on cellular conditions.[36] Interestingly, a chronic treatment with menthol is known to down-regulate TRPV1 receptor in the gastro-esophagus system and help to reduce tissue damage caused by acid reflux in animal and in vitro models.[37] Besides menthol, also other terpene constituents of essential oils such as (+)-carvone from caraway oil act as agonists on different TRP receptors.[38,39] Data from calcium entry studies in cells expressing human TRPs demonstrated TRPM8 activation in cells by Menthacarin, peppermint and caraway oil, as well as early activation and subsequent desensitization of the nociceptor TRPA1 upon repeated exposure.[40] In line with these data, Zhang et al. recently showed that exposure to Menthacarin induced early calcium entry in murine sensory neurons and colonic organoids, followed by tachyphylaxis upon repeated exposure.[41] Although transient in vitro desensitization can be induced quite rapidly, sustained nociceptor desensitization via modulation of TRP function or expression in vivo could require a more prolonged treatment compatible with the relatively long delay of Menthacarin therapy to exert efficacy as observed in our study (2 weeks).

In addition to the reduction in visceral pain responses, as shown in the present study, Menthacarin displays a number of additional pharmacological activities relevant for the treatment of functional gastrointestinal disorders such as IBS. Recent in vitro findings revealed a strong muscle inhibitory and epithelial prosecretory action of peppermint and caraway oils on intestinal mucosa at clinically relevant concentrations.[30] Both actions were nerve-independent and involved contractility of intestinal muscle through an inhibition of L-type (voltage-dependent) calcium channels. Such an effect may occur through the blockade of voltage-gated Na^{+}/Ca^{++} channels[30,42] through carvone derivatives, which are important active ingredients of caraway oil, and menthol, which can also interact negatively with voltage-gated Na^{+}/Ca^{++} channels. This effect on muscle contraction and mucosa secretion may have protective effects and be complementary, helping to keep the bolus moist while having an antispasmodic effect. Interestingly, caraway essential oil has anticonvulsive effect on animal model of epilepsy, possibly via inhibition of L-type (voltage-dependent) calcium channels.[42]



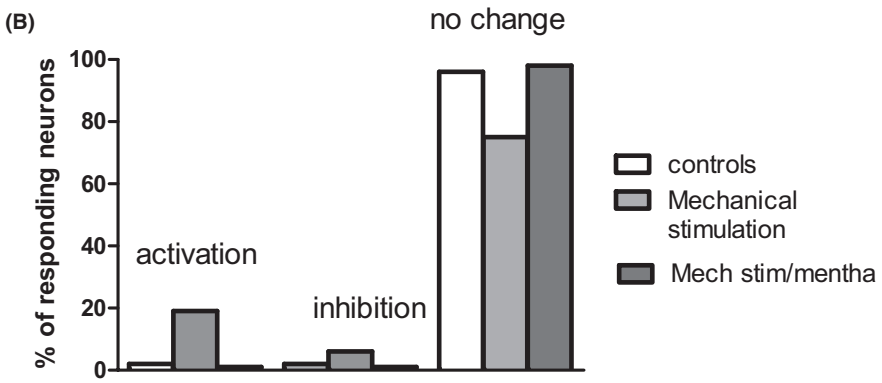


FIGURE 6 Mechanical stimulation-induced visceral hypersensitivity is abolished by Menthacarin. (A) Scatter plot showing the net electrophysiological responses of ACC neurons to low (30 mmHg) CRD pressures in rats previously subjected to multiple mechanical stimulations. (B) Proportion of neurons responding to a CRD of 30 mmHg from rats previously subjected to multiple mechanical stimulations under no treatment conditions, or during a 2-week treatment with Menthacarin, or in control animal. In control animals and in stimulated animals pretreated with Menthacarin, virtually no neurons (2 neurons) were sensitive to the lower pressure.

There is also experimental evidence that menthol or carvone derivatives can act on γ-aminobutyric acid type A receptors, potentially reducing neuronal excitability and spontaneous synaptic transmission in pain neuronal pathways.[43,44]

Alongside these putative pharmacological actions, Menthacarin administration may also cause some long-term functional changes within the gastrointestinal mucosa by altering gut microbiota composition. To note, perturbation of the gut microbiota, though not a common characteristic in all IBS patients, may cause alterations in intestinal function resembling those found in IBS patients.[45] We previously found in our laboratory that Menthacarin had interesting in vitro anti-microbiological effects on pathogenic gut species, while not affecting beneficial species (unpublished data), in agreement with other studies showing that both menthol and (+)-carvone exhibit antibacterial and antifungal activities.[46,47] A recent investigation on visceral hypersensitivity models of IBS, induced by water avoidance stress in maternally separated rats,[48] showed that visceral hypersensitivity was associated with both mycobiome and microbiome dysbiosis. Interestingly, the reversal of visceral hypersensitivity in this animal model by Menthacarin treatment was associated with a shift of the mycobiome composition to similar characteristics as in control animals. Though perhaps less investigated than the gut microbiota, gut mycobiota has likely an important impact on health.[49] In the present study, we did not assess potential effects of Menthacarin treatment on gut microbiome and hence can only speculate that Menthacarin may favor an enrichment of specific beneficial bacterial and fungal strains in the flora, which may reduce inflammatory process and possibly promote the production of substances which can act centrally, directly or through efferent nerves (e.g., vagus or splanchnic nerves), to change visceral pain perception. Though a 7-day treatment with Menthacarin, at similar doses as in our study in normal visceral sensitive rats, appeared to primarily affect mycobiota diversity, nothing is known about a longer exposure to the oil combination on the microbiota.[48] To note, in our study, 2 weeks of treatment were required to reduce hypersensitivity. Interestingly, there is evidence that menthol chronic administration can alter gut microbiota in mice, causing an increase in production of short chain fatty acids (e.g., butyrate), which are known to improve intestinal barrier integrity and have protective effects against inflammation.[50,51]

There are some limitations in the interpretation of our study results. Menthacarin appears to induce persistent changes in visceral pain processing which would be expected to have beneficial effects on IBS patients. However, the experimental procedures used to induce hypersensitivity in our study (corticosterone treatment and previous mechanical stimulation) do not reflect exactly the IBS clinical situation, where the visceral tissues are thought to be in a mild post-inflammatory state.[52] Mechanical stimulation, which could cause hidden mechanical damages, is an acute

manipulation causing hypersensitivity, which is not directly comparable to the pathophysiological processes in IBS. Prolonged corticosterone treatment was found to produce visceral hypersensitivity, but there is no clear evidence that gluco-corticosteroid levels are high in IBS patients[53] and alteration in corticosteroid levels cannot solely explain the functional changes occurring in IBS. In future studies, it will be relevant to study the effects of Menthacarin in other laboratory models that may, in other aspects, mimic other IBS features, such as the low-dose acetic acid (or other irritant substances) pre-exposure and CRH (corticotropin-releasing hormone) pre-treatment,[54] or in stress-related models. However, these models have also their limitation. Though CRH (generally administered intracerebroventricularly) can initiate visceral hyperalgesia and changes in colonic motility, as observed in IBS, CRH antagonists seem to have limited clinical effect in IBS patients (yet some new CRH drugs are still under development).[55] Irritant substances models could mimic some characteristics of IBS but may cause microstructural damages of the intestine tissue (as the mechanical stimulation) which are at variance of the complex pathophysiology of the disease.[15,16] Stress-related models, like neonatal maternal deprivation or repeated water avoidance stress, are well-recognized models of visceral hypersensitivity that can precipitate IBS symptoms, and cause depression-like symptoms in the animals. Psychosocial stress may indeed be an important contributor for the development of IBS, causing intestinal disruption and/or irritation, with change in intestinal secretion/permeability via activation of a number of neural, endocrine, and immune pathways.[16] However, the relation with mood disorders is complex, as symptoms of IBS may also in turn aggravate depressive symptoms or greatly impact on mental health. Though depression and IBS overlap, co-morbidity do not concern all patients (10%–40% of patients, depending on IBS subtype according to some studies[56]).

Another limitation of our study concerns the gender of our subjects which was only male, while most studies carried out in western countries the disease preferentially affects females.[57,58] Nevertheless, IBS is also affecting a very significant proportion of male subjects (nearly 9% of male population), and in many countries of the southern hemisphere (Asia and South America) there is no significant gender prevalence.[59] Interestingly, IBS seems particularly more prevalent in women between 20 and 30 years, an age where sex hormones can peak at their highest levels. Different evidence suggests that sex steroids can influence visceral pain and intestinal motility.[60] Examining variation in electrophysiological pain response at the different stages of the estrus cycle would be a particularly relevant study to carry out in the absence of treatment and in Menthacarin-treated female rats. However, it is important to first establish whether Menthacarin can protect against hypersensitivity and hyperalgesia in the absence of other complicating endocrine factors.

In conclusion, our study demonstrates a protective effect of a long-term administration of Menthacarin at clinically relevant dose against visceral hypersensitivity in two animal models of visceral hyperalgesia and hypersensitivity. Though the mechanisms involved in this analgesic effect need to be elucidated, Menthacarin has a therapeutic potential to attenuate visceral pain perception in disorders involving visceral hypersensitivity.

AUTHOR CONTRIBUTIONS

Study concept: BG, ML, SW; Data collection: AO, BG; Data analysis: BG; Data interpretation: all authors; Drafting manuscript: BG; Reviewing manuscript: BG, ML, SW; All authors have accepted the final draft of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Sabrina Weisenburger and Martin D. Lehner are employees of Dr. Willmar Schwabe GmbH & Co. KG, a manufacturer of medicinal products containing Menthacarin®. Adesina Omoloye and Benjamin Gronier declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Benjamin Gronier https://orcid.org/0000-0001-8107-2070

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