Possible role for TRPV1 in neomycin-induced inhibition of visceral hypersensitivity in rat

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Abstract Transient receptor ion channel 1 (TRPV1) is a nociceptor involved in visceral hypersensitivity. Aminoglycosides like neomycin are not only potent antibiotics but *in vitro* data suggest that neomycin also acts as a TRPV1-antagonist and alleviates somatic pain responses. To what extent neomycin reduces visceral hypersensitivity remains unknown. Therefore, we aimed to investigate whether neomycin can inhibit *in vivo* TRPV1-dependent hypersensitivity responses in two rat models of visceral pain. In the first model rats were pretreated with intraperitoneal (*i.p.*) capsazepine, the selective TRPV1 antagonist SB-705498, neomycin or vehicle alone and 30 min later instilled with intracolonic TRPV1-activating capsaicin. Likewise, rats were pretreated with 10 days oral neomycin and then subjected to intracolonic capsaicin. The visceromotor response (VMR) to distension was measured before and after capsaicin application. In addition, the VMR to distension was measured in adult maternal separated rats before and after acute stress. Before the 2nd distension protocol these rats were treated with *i.p.* neomycin, amoxycillin or vehicle alone. Our results showed that capsaicin administration induced an enhanced VMR to distension that was prevented by *i.p.* capsazepine, SB-705498 and neomycin. Oral neomycin treatment changed bacterial faecal content but could not inhibit capsaicin induced visceral hypersensitivity. In maternal separated rats acute stress induced an enhanced response to distension that was reversed by *i.p.*

neomycin, but not amoxycillin. These data indicate that (*i.p.*) neomycin can inhibit visceral hypersensitivity to distension in a nonbactericidal manner and suggest that TRPV1-modulation may be involved.

Keywords *in vivo*, neomycin, rat, transient receptor ion channel 1, visceral hypersensitivity.

Abbreviations: AUC, area under curve; CFU, colony forming units; IBS, irritable bowel syndrome; *i.p.*, intra peritoneal; MS180, 180 minutes daily maternal separation; NH, nonhandled; SIBO, small intestinal bacterial overgrowth; TRPV1, transient receptor ion channel 1; VMR, visceromotor response; WA, water avoidance.

INTRODUCTION

Aminoglycosides like neomycin and gentamycin are commonly used in the treatment of serious infections caused by aerobic Gram-negative bacilli. Interestingly, data obtained in different rodent models suggested that these antibiotics are also potent analgesics.^{1–3} In mice, intracerebroventricular administration of neomycin produced dose-dependent antinociceptive activity in hot plate and tail flick tests.¹ Similar results were reported for a rat model of incisional pain where intrathecal neomycin administration reduced incisional allodynia.² Blenk *et al.* showed that not only central but also peripheral administration of neomycin can be effective.³ Transection of the left ventral ramus of the L5 spinal nerve led to mechanical allodynia which was prevented by local application of neomycin immediately after cutting the nerve. Although these reports clearly showed the analgesic potential of neomycin, the possible mechanism of action was not addressed. *In vitro* investigations however suggested that neomycin is a TRPV1 antagonist.

Transient receptor ion channel 1 is a sensory-neuron-expressed, nonselective, ligand-gated cation

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channel essential for several modalities of pain sensation. It can be activated by a number of different stimuli including noxious temperature, extracellular low pH and the nonphysiological stimulus capsaicin.⁴ Using whole-cell patch-clamp recordings of acutely isolated rat DRG neurons Zhou *et al.* were the first to show that neomycin inhibits capsaicin-induced currents.⁵ Later results, obtained with cloned TRPV1 heterologously expressed in *Xenopus* oocytes, suggested that neomycin inhibits TRPV1 by allosteric binding and altering channel gating.⁶ Whether TRPV1 is not only an *in vitro* but also an *in vivo* target of neomycin remains to be investigated. Another unanswered question is whether neomycin-induced analgesia is restricted to somatic pain alone or also effective in visceral pain models. We chose to investigate these issues in two TRPV1 dependent rat models of colonic hypersensitivity.

MATERIAL AND METHODS

All experimental protocols were approved by the Ethical Animal Research Committee of the University of Amsterdam.

Animals

Long-Evans rats (Harlan, Horst, The Netherlands) were housed at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands) under conditions of controlled light (06:00–18:00 h), temperature (20–22 °C) and humidity (45%). Water and food (SDS; Technilab BMI, Someren, The Netherlands) were available *ad libitum*. Rats used for the maternal separation protocol (nonhandled (NH) and maternal separated animals) were bred in our own animal facilities. Nonhandled rats used for the intra-colonic capsaicin experiments were directly obtained from Harlan. Vehicle was always investigated in separate groups. All experiments were carried out with male animals only and none of the animals were used in more than one experiment.

Colonic distension protocol

Colonic distensions were performed with a latex balloon (Ultra-cover 8F, International Medical Products BV, Zutphen, The Netherlands) and carried out as described previously.⁷

Distensions in maternal separated rats (see Model 2: neonatal maternal separation and water avoidance (WA) for description of the separation model) were performed at the minimum age of 3 months and achieved by inflation of graded volumes of water (1.0, 1.5 and 2.0 mL) into the balloon. Length and diameter of the balloon during a 2 mL maximum volume distension were 18 and 15 mm respectively. After each 20 s distension period the volume of water was quickly removed and an 80 s resting period was introduced. Animals who received intracolonic capsaicin (see Experimental models) were studied at 2 months of age (to economize costs of purchase and housing) and subjected to lower distension volumes; 0.8, 1.2 and 1.6 mL.

Measurement of the visceromotor response (VMR) in freely moving rats and EMG data analysis

We used a telemetric transmitter (Physiotel Implant TA10AE-F20; Data Sciences International (DSI), St Paul, MN, USA) positioned in the right side of the abdominal cavity to record the EMG signals from two connected electrodes placed in the abdominal muscles. During distension protocols, animals were placed in a standard macrolon cage that was positioned on top of a flat receiver with an equal surface area (DSI). The receiver was linked to a Biopac MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA) and a personal computer via a raw data analog converter (Data Sciences International). Data were acquired with AcqKnowledge software (Biopac Systems Inc., Santa Barbara, CA, USA) and analyzed as described before.⁷ Briefly, each 20-s distension period and its preceding 20-s of baseline recording were extracted from the original raw EMG data file. After correction for movement and breathing, data were rectified and integrated. Absolute data sets were then obtained by subtracting the 20-s baseline recording from the 20-s distension result. Similar to previous studies^{7,8} the final results are given as normalized data sets, which were calculated from the absolute data by setting the highest prestress (or precapsaicin) value at 100%.

Experimental models

Model 1: intracolonic capsaicin in NH rats After positioning of the balloon catheter the precapsaicin VMR to colonic distension was established at $t = 0$ min. At $t = 60$ min, just before re-positioning of the balloon catheter, capsaicin (Sigma-Aldrich, St Louis, MO, USA) was administered under short isoflurane anesthesia. Capsaicin concentration and application methodology were taken from Laird *et al.*;⁹ we first applied Vaseline (Boots Healthcare, Hilversum, The Netherlands) to the perianal area to avoid stimulation of somatic areas. Subsequently, 100 μ L (0.1%) of the TRPV1 agonist capsaicin, or vehicle alone (10% ethanol, 10% Tween-80, 80% saline), was given with a fine cannula with a rounded tip inserted rectally, 2 cm from the anus. Animals were allowed to recover and the second distension protocol was performed at $t = 90$ min.

Model 2: neonatal maternal separation and water avoidance (WA) Primiparous pregnant rats reared NH male pups; second time pregnant dams reared male pups that were subjected to the maternal separation protocol. During maternal separation dams were separated from the nest from postnatal day 2 to 14 for 3 h per day (MS180) as described previously.⁷ After weaning on postnatal day 22, rats were raised in pairs of two. Nonhandled pups were nursed normally.

In our earlier investigations we showed that acute WA-stress induces enhanced sensitivity to colonic distension in adult MS180- and not in NH rats. Stress-induced hypersensitivity was still present at 24 h post-WA and was not induced by sham WA-stress.⁷ Therefore, whenever acute-stress was applied in the present investigations, distensions and concurrent EMG recordings were performed just before and 24 h after 1 h WA during which rats were positioned on a pedestal surrounded by water.

lines, $*P = 0.018$, $n = 7$). Next, i.p. capsazepine (10 mg kg^{-1}) was administered 30 min prior to intracolonic capsaicin. This resulted in a complete inhibition of capsaicin-induced hypersensitivity to distension (continuous lines, Fig. 2B, $n = 7$) whereas precapsaicin administration of vehicle alone could not prevent this enhanced response (dotted lines, $**P = 0.028$, $n = 7$). Similar to capsazepine, the specific TRPV1-antagonist

SB-705498 (30 mg kg^{-1}) prevented capsaicin-induced visceral hypersensitivity (Fig. 2C, continuous lines, $n = 8$), whereas vehicle alone was unable to inhibit the enhanced VMR to distension (dotted lines, $\#P = 0.012$, $n = 8$).

As shown in Fig. 2D, neomycin (100 mg kg^{-1} i.p.) is able to inhibit capsaicin-induced hypersensitivity to colonic distension when administered 30 min prior to

Figure 2 Effects of intracolonic capsaicin on VMR to distension in nonhandled (NH) rats. Values are mean $\pm$ SEM. Differences in area under the curve were evaluated for statistical significance. In contrast to vehicle alone (dotted lines), intracolonic capsaicin application induced an enhanced response to colonic distension (A; continuous lines, pre- vs postcapsaicin, $*P = 0.018$). Pretreatment with nonselective TRPV1 antagonist capsazepine (continuous lines B), selective TRPV1 antagonist SB-705498 (continuous lines C) or neomycin (continuous lines D) inhibited the capsaicin induced hypersensitivity to distension that was still apparent when rats were pretreated with vehicle alone (dotted lines, $**P = 0.028$, $P = 0.012$ and $\#P = 0.028$ respectively). 3E shows a summary of the intracolonic capsaicin data but now depicting AUC (P -values of pre- vs postcapsaicin application as mentioned before). $115 \times 199 \text{ mm}$ ($600 \times 600 \text{ DPI}$).

Figure 3 Effects of 10 days oral neomycin treatment in nonhandled (NH) rats. Values are mean $\pm$ SEM. Treatment resulted in the reduction of aerobic faecal bacteria on day 2 and 10 (A, $*P = 0.028$ and $**P = 0.028$ respectively). Anaerobic bacterial load was increased at day 10 (A, $***P = 0.028$). Visceromotor response (VMR) to distension was unchanged (area under curve) after 10 days of oral neomycin (B, dotted lines) and neomycin treatment was unable to prevent enhanced capsaicin-induced VMR to colonic distension at day 10 (dotted line with triangles vs continuous line, $*P = 0.017$). 254 $\times$ 104 mm (600 $\times$ 600 DPI).

capsaicin (continuous lines, $n = 7$). In contrast, vehicle alone is unable to prevent the enhanced response to distension (dotted lines $^{*}P = 0.028$, $n = 7$). Fig. 2E shows a summary of the intracolonic capsaicin data by depicting AUC: similar inhibitory results are obtained with preadministration of capsazepine, SB-705498 and neomycin.

Ten days oral neomycin treatment resulted in a 1-log reduction of aerobic faecal bacteria on day 2 and a 2-log reduction on day 10 (Fig. 3A, $*P = 0.028$ and $**P = 0.028$ respectively). In contrast, the anaerobic bacterial load showed a 1-log increase at day 10 (Fig. 3A, $***P = 0.028$). Distension results obtained before and after 10 days oral neomycin showed no changes in VMR due to neomycin treatment *per se* (Fig. 3B, dotted lines, $n = 10$). In addition, 10 days oral neomycin could not prevent the enhanced capsaicin-induced VMR to colonic distension at day 10 ($*P = 0.017$).

WA-induced hypersensitivity to distension

Water avoidance was unable to induce an enhanced response to distension in NH rats (Figs 4A,C, dotted lines). Similarly, when NH rats were treated with neomycin (Fig. 4A) or amoxicillin (Fig. 4C, continuous lines) the post-WA response to distension remained unchanged. In contrast, WA induced an enhanced VMR in vehicle treated MS180 rats (Figs 4B,D, dotted lines, $*P = 0.018$ and $^{*}P = 0.018$). Neomycin treatment, 30 min prior to the post-WA distension protocol, inhibited the enhanced response to distension (Fig. 4B, continuous lines), whereas amoxicillin treated rats remained hypersensitive (Fig. 4D, continuous lines, $^{*}P = 0.028$). Fig. 4E shows a summary of the WA results. In this case AUC is depicted instead of relative response (P -values as mentioned above).

Compliance

For each individual distension volume we compared pressures generated before and after application of the different compounds (capsaicin, capsazepine, SB-705498, neomycin or amoxicillin). We did not observe significant differences. This suggests that there are no compound induced changes in compliance (see Table 1).

DISCUSSION

Previous *in vitro* experiments revealed that neomycin acts as a TRPV1-antagonist.^{5,6} In the present study its *in vivo* analgesic properties were assessed in two different TRPV1-dependent rat models of visceral pain. In the first model, colonic instillation of the TRPV1-agonist capsaicin led to an enhanced VMR to colonic distension. This response could be prevented by i.p. administration of capsazepine and SB-705498 (nonselective- and selective TRPV1-antagonists, respectively). Intraperitoneal administration of neomycin inhibited the capsaicin-induced response to the same extent as these compounds, suggesting that neomycin may also act as an *in vivo* TRPV1 antagonist.

The possibility of neomycin alleviating visceral hypersensitivity in a nonbactericidal but TRPV1-dependent manner was further investigated in the rat model of maternal separation. In this model, daily separation of mother and pups during the neonatal period leads to altered behavior of the mother towards these pups and, subsequently, to an enhanced stress response at adult age.¹⁷ In adult MS180 Long-Evans rats, acute stress induces an increased VMR to colonic distension which is not observed in NH animals.^{7,8}

Figure 4 Effects of WA on VMR to distension in NH and MS180 rats. Values are mean $\pm$ SEM. Differences in area under the curve were evaluated for statistical significance. WA did not induce an enhanced VMR to distension in NH rats (A and C, dotted lines) and post-WA response to distension remained unchanged when rats were treated with neomycin (A) or amoxycillin (C, continuous lines). WA induced an enhanced VMR in vehicle treated MS180 rats (B and D, dotted lines, * $P = 0.018$ and $P = 0.018$ respectively) which was prevented by neomycin (B, continuous lines) but not amoxycillin treatment (D, continuous lines, # $P = 0.028$). E shows a summary of the results; AUC is depicted instead of relative response (P -values as mentioned above). 131 $\times$ 198 mm (600 $\times$ 600 DPI).

Table 1 No significant differences (Wilcoxon signed ranks) were observed when comparing volume-induced pressures before and after compound application

Distension volume (mL)	Pressure $\pm$ SEM (mmHg)									
	Capsaicin		Capsazepine		SB-705498		Neomycin		Amoxycillin	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1.0	19.5 $\pm$ 1.0	20.5 $\pm$ 1.0	19.0 $\pm$ 1.5	18.0 $\pm$ 1.7	24.0 $\pm$ 0.8	24.0 $\pm$ 0.8	19.2 $\pm$ 1.0	20.8 $\pm$ 1.6	18.8 $\pm$ 1.8	18.3 $\pm$ 1.3
2.0	35.5 $\pm$ 2.4	33.0 $\pm$ 1.3	41.6 $\pm$ 4.7	42.0 $\pm$ 4.2	37.0 $\pm$ 1.7	35.8 $\pm$ 2.3	34.8 $\pm$ 2.3	33.2 $\pm$ 2.1	33.5 $\pm$ 3.6	34.0 $\pm$ 2.3
3.0	66.0 $\pm$ 3.6	69.5 $\pm$ 5.9	78.8 $\pm$ 4.3	78.0 $\pm$ 5.0	69.5 $\pm$ 2.4	70.5 $\pm$ 2.1	72.8 $\pm$ 2.2	72.4 $\pm$ 2.8	70.8 $\pm$ 4.6	69.0 $\pm$ 5.0

Our recent data indicated that stress-induced hypersensitivity in MS180 rats depends on mast cell degranulation and subsequent TRPV1 modulation.

The latter was shown by capsazepine¹⁶ and SB-705498 (unpublished observation) mediated reversal of enhanced colonic sensitivity. In the present

study neomycin, administered (i.p.) 30 min prior to the post-WA distension protocol, completely inhibited stress-induced hypersensitivity. The short time-interval between the administration of neomycin and the subsequent post-WA distension protocol makes a bactericidal effect rather unlikely. Nevertheless, to rule out this possibility, we investigated the effect of i.p. administration of the nonaminoglycoside-antibiotic amoxycillin in the same experimental set up. In contrast to neomycin, this broad spectrum penicillin was unable to reverse the stress-induced increase in VMR in MS180 rats.

Others showed that neomycin blocks capsaicin evoked membrane currents in cultured dorsal root ganglia^{5,6} and cloned-TRPV1 heterologously expressed in *Xenopus* oocytes.⁶ The results of the oocyte study indicated that neomycin inhibits TRPV1 activity by allosteric binding and altering channel gating. It should however be emphasized that for our *in vivo* studies, interactions with other ion channels or membrane receptors can not be excluded. For example, therapeutically relevant concentrations of neomycin indeed block voltage-activated Ca^{2+} channels.¹⁸ Whether this mechanism plays a role in the present study is unknown: neomycin has a preference for P-type Ca^{2+} channels and to our knowledge their role has not been investigated in any of the colonic hypersensitivity models. Neomycin also inhibits TTX-resistant Na^{+} currents in isolated small dorsal root ganglia neurons¹⁹ and it was shown previously that colitis-induced hyper-excitability of colonic sensory neurons is associated with increased Nav1.8 currents.²⁰ In addition, when intracolonic capsaicin was administered to Nav1.8-null mutants, significantly fewer nociceptive behaviours were observed compared to wild-type mice.²¹ These data suggest that the neomycin-induced reversal of capsaicin-induced hypersensitivity to distension may, in addition to TRPV1, also relate to Nav1.8 modulation. Nevertheless, in our models complete inhibition of visceral hypersensitivity was achieved by the TRPV1-selective antagonist SB-705498, suggesting that enhanced sensitivity to distension mainly depended on TRPV1.

In the present investigations we used the VMR to colorectal distension as an indirect measure for visceral hypersensitivity. Visceral hypersensitivity is thought to be an important pathophysiological mechanism in the irritable bowel syndrome (IBS).²² This functional bowel disorder is characterized by abdominal pain or discomfort associated with defecation or change in bowel habit. One of the new theories about its etiology suggests that small intestinal bacterial overgrowth (SIBO) leads to symptoms of IBS.²³ When neomycin was used to eradicate possible SIBO in a

placebo-controlled patient trial,²⁴ a modest but significant reduction in global symptom or composite score was achieved. Especially because increased numbers of colonic TRPV1⁺ mucosal nerve fibers were reported to correlate with abdominal pain scores²⁵ it is tempting to speculate that neomycin also targeted TRPV1 in the IBS patient study. Our current results can however not justify such a conclusion.

While aminoglycosides are poorly absorbed from the gut, the IBS patients received oral treatment. When neomycin was added to the drinking water of NH rats, a 10-days treatment protocol resulted in a clear shift in the bacterial composition of faecal pellets, but capsaicin-induced hypersensitivity to distension could not be prevented. This negative finding suggests that, at least in rats, absorption of neomycin is not high enough to reach effective systemic concentrations. However, a direct translation to the IBS data is not possible. Firstly, it is known that the permeability of the human gut is considerably higher than that of rats.²⁶ Secondly, gut permeability in IBS patients may be compromised^{27,28} and therefore lead to enhanced neomycin absorption compared with controls (0.58% of an orally administered 2 g dose is absorbed in 48 h in normal human subjects).²⁹ The latter makes it impossible to reliably calculate the absorbance rate of neomycin in these patients (data on serum or urine concentrations are not available). Thus, a definitive conclusion on this intriguing possibility requires further experiments in humans, which is beyond the scope of these investigations.

Earlier results indicated that neomycin is a potent analgesic in somatic pain responses. *In vitro* investigations suggested that this may relate to a TRPV1-antagonistic effect. We report here that neomycin is also capable of reversing established visceral hypersensitivity. Moreover, our data indicate that, regarding its anti-nociceptive properties, TRPV1 is an *in vivo* target of neomycin. Finally, these data again show that TRPV1 is an important candidate target molecule to develop effective treatment strategies in disorders associated with visceral hypersensitivity.

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