Interactive involvement of brain derived neurotrophic factor, nerve growth factor, and calcitonin gene related peptide in colonic hypersensitivity in the rat

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Background and aims: Neurotrophins are involved in somatic and visceral hypersensitivity. The action of nerve growth factor (NGF) on sensory neurones contributes to the development of referred colonic hypersensitivity induced by trinitrobenzene sulfonic acid (TNBS). Based on data on brain derived neurotrophic factor (BDNF) and calcitonin gene related peptide (CGRP) in pain, the aims of the present study were: (1) to investigate the involvement of BDNF and CGRP in this model of referred colonic hypersensitivity, (2) to test the effect of exogenous BDNF and CGRP on the colonic pain threshold, and (3) to investigate the relationship between BDNF, NGF, and CGRP by testing antineurotrophin antibodies or h-CGRP 8-37 (a CGRP antagonist) on bowel hypersensitivity induced by these peptides.

Methods: Colonic sensitivity was assessed using a colonic distension procedure.

Results: Anti-BDNF antibody and h-CGRP 8-37 reversed the induced decrease in colonic threshold (33.4 (2.1) and 40.3 (4.1) mm Hg, respectively, compared with a vehicle score of approximately 18 mm Hg; $p < 0.001$). BDNF (1-100 ng/rat intraperitoneally) induced a significant dose dependent decrease in colonic reaction threshold in healthy rats. This effect was reversed by an anti-BDNF antibody and an anti-NGF antibody (33.4 (0.6) v 18.7 (0.7) mm Hg ($p < 0.001$), anti-NGF v vehicle). NGF induced colonic hypersensitivity was reversed by h-CGRP 8-37 but not by the anti-BDNF antibody. Finally, antineurotrophin antibody could not reverse CGRP induced colonic hypersensitivity (at a dose of 1 µg/kg intraperitoneally).

Conclusion: Systemic BDNF, NGF, and CGRP can induce visceral hypersensitivity alone and interactively. This cascade might be involved in TNBS induced referred colonic hypersensitivity in which each of these peptides is involved.

Functional digestive disorders are often associated with a decrease in visceral pain threshold indicating visceral hypersensitivity.^{1,2} Indeed, patients suffering from irritable bowel syndrome (IBS) demonstrate a lower visceral sensory threshold to colorectal balloon distension.³ It has been suggested in IBS that there is heightened pain sensitivity of the brain-gut axis, with a normal pattern of activation.² We have previously shown that injection of trinitrobenzene sulfonic acid (TNBS) into the proximal colon provoked chronic colonic hypersensitivity, measured in conscious rats by a decreased pain threshold in response to colonic distension. This chronic hypersensitivity was found in the distal non-inflamed colon and persisted for 21 days.⁴ It mimicked certain characteristics of IBS and so it can be used as a model to experimentally explore the pathophysiological aspects of this disorder. We have previously shown that the action of nerve growth factor (NGF) on sensory neurones contributes to the development of visceral hypersensitivity in this model.⁵ NGF induced colonic hypersensitivity, which was prevented by an anti-NGF antibody, was also able to prevent TNBS induced referred non-inflammatory colonic hypersensitivity.

Brain derived neurotrophic factor (BDNF) is a type of neurotrophin which has been studied for its role in neuronal survival and development. Recently, much attention has focused on the role of BDNF as a neuromodulator, especially in inflammatory pain states. In humans, it is now known that BDNF is upregulated and associated with pain in chronic

pancreatitis.^{6,7} In animals, levels of BDNF or TrkB receptor were increased in models of bladder inflammation⁷ and nerve injury.^{8,9} These increases were associated with pain behaviour.¹⁰ Data on the effects of exogenous BDNF on pain remain inconclusive but several studies have shown that BDNF has nociceptive effects.^{11,12} Finally, BDNF has been shown to be involved in the pathophysiology of pain via sequestration. Anti-BDNF antibody relieved mechanical or thermal hyperalgesia in rat models of nerve ligation.^{8,13} The sequestering antibody trkB-IgG significantly reduced behavioural nociceptive responses evoked after subcutaneous injection of dilute formalin or carrageenan into one hind paw.¹⁴⁻¹⁶ Antibodies against different neurotrophins (NGF, BDNF, and neurotrophin 3), delivered directly to the injured dorsal root ganglia, significantly reduced (with different time sequences) the percentage of paw withdrawal responses evoked by von Frey hairs after spinal nerve injury.¹² Taken together, these data suggest that BDNF seems to be involved in the mechanism of hyperalgesia.

It is now well established that calcitonin gene related peptide (CGRP) is implicated in several models of visceral pain.¹⁷⁻²⁰ CGRP is by far the most abundant peptide of

Abbreviations: NGF, nerve growth factor; TNBS, trinitrobenzene sulfonic acid; BDNF, brain derived neurotrophic factor; CGRP, calcitonin gene related peptide; IBS, irritable bowel syndrome; SP, substance P; BSA, bovine serum albumin; h-CGRP 8-37, human calcitonin gene related and peptide fragment 8-37

capsaicin sensitive afferent fibres of gastrointestinal origin, accounting for up to 80% of overall peptide immunoreactivity.^{21–22} Recently, morphological colonic distribution of CGRP and substance P (SP) immunoreactive nerves was investigated in our model of TNBS induced referred non-inflammatory colonic hypersensitivity.²³ This study showed that the inflammatory process in the proximal colon induced, at a distance (that is, in the distal colon), a highly significant increase in SP and CGRP innervation of the myenteric plexus.²³

Based on data on BDNF, CGRP, and NGF on pain and, for NGF, on the model of TNBS induced referred non-inflammatory colonic hypersensitivity, the aims of the present study were to determine the involvement of BDNF and CGRP in the same model, and to test the link between NGF, BDNF, and CGRP to determine their respective involvement in the peripheral pathophysiology of non-inflammatory colonic hypersensitivity.

METHODS

Animals

This study was carried out on adult male Wistar rats (Janvier, Le Genest-St-Isle, France) weighing 320–350 g. Animals were housed three per cage under conditions of controlled temperature ($20 \pm 1^\circ\text{C}$), hygrometry ($50 \pm 5\%$), and lighting (lights on from 7am to 7pm) for at least one week prior to the experiments. They were deprived of food for 18 hours but allowed access to water *ad libitum* up to the start of the experiments. All studies were performed in accordance with the ethical guidelines of the International Association for the Study of Pain.²⁴ Great care was taken, particularly with regard to housing conditions, to avoid or minimise discomfort to the animals.

Assessment of colonic sensitivity

A latex balloon (5 cm length) was inserted through the anus and placed in the distal colon, 5 cm from the anus. To maintain this position, the catheter was taped onto the base of the tail. Animals were individually placed without restraint in polypropylene cages for the distension session. After a 30 minute acclimatisation period, the balloon was progressively inflated from 0 to 75 mm Hg, in 5 mm Hg increments, every 30 seconds. Each cycle of colonic distension was controlled by an electronic barostat (ABS, St-Dié, France). The colonic reaction threshold was determined as the pressure inducing the first abdominal contraction and consequently interruption of the cycle. Abdominal contraction corresponds to waves of contraction of oblique musculature with inward turning of the hind limb, to the hump backed position, or to squashing of the lower abdomen against the floor.²⁵ Similar behaviours have previously been used as colonic reaction thresholds to noxious stimuli.^{26–28} To average the colonic reaction threshold, each rat was subjected to four distension cycles (D1–D4) with a 10 minute interval between each cycle. At the end of the four cycles, animals were sacrificed by cervical dislocation.

Induction of colonic hypersensitivity

Under anaesthesia (acepromazine 12 mg/kg intraperitoneally and ketamine 80 mg/kg intraperitoneally), injection of TNBS, dissolved in 30% ethanol, was given at 50 mg/kg (1.5 ml/kg) into the proximal colon, 1 cm from the caecum. Following administration, rats were individually housed in polypropylene cages and maintained for seven days under the controlled conditions described above. Corresponding controls were healthy animals housed under the same conditions. It has previously been shown that sham operated animals had no pathophysiological signs of visceral inflam-

mation in both proximal- and distal colon-like saline or EtOH 30% injected animals.⁴ Mean weight of animals before injury was of 361 (4) g; three days after injury mean weight was 348 (5) g and seven days after injury mean weight was 349 (7) g.

Experimental protocol

Three series of experiments were conducted. For each treated group, $n = 7$ or 8 animals were used.

To determine the involvement of BDNF and CGRP in the TNBS model of referred non-inflammatory colonic hypersensitivity, the first series of experiments were conducted on seven groups of TNBS treated rats (50 mg/kg into the proximal colon), seven days before distension, and in one group of healthy rats without any intracolonic administration. Three groups of TNBS treated rats received anti-BDNF antibody (36 $\mu\text{g/kg}$) or its vehicle (bovine serum albumin (BSA) 0.1%), or a control isotype antibody (40 $\mu\text{g/kg}$) intraperitoneally, 30 minutes before distension. Four other groups of TNBS treated rats received h-CGRP 8–37, a CGRP receptor antagonist, at a dose of 75, 150, or 225 $\mu\text{g/kg}$, or its vehicle (BSA 0.1%) intravenously, 30 minutes before distension.

In the second series of experiments, the effect of exogenous BDNF was tested to confirm its ability to induce colonic hypersensitivity. Four groups of rats received BDNF in 0.1% BSA at doses of 1, 10, or 100 ng /rat, or its vehicle (0.1% BSA), intraperitoneally (0.5 ml/rat), 30 minutes before distension.

The third series of experiments was performed to test the reciprocal influence of an anti-BDNF or anti-NGF antibody, or of h-CGRP 8–37, on hypersensitivity induced by BDNF, NGF, or CGRP. In the initial experiment, the effect of an anti-BDNF and anti-NGF antibody on BDNF induced hypersensitivity was studied. Administration of the antibody and peptide was quasi-simultaneous, both intraperitoneally, 30 minutes before distension. The control group received 0.1% BSA or anti-BDNF antibody (36 $\mu\text{g/kg}$) while the other groups received 100 ng BDNF/rat with either the anti-BDNF (18 or 36 $\mu\text{g/kg}$ or control isotype antibody 40 $\mu\text{g/kg}$) or anti-NGF antibody (1/2000, 2 ml/kg, a dose which blocks the hyperalgesic effect of NGF²⁹), or vehicle.

The aim of the second experiment was to test the effect of anti-BDNF antibody and h-CGRP 8–37 on NGF induced hypersensitivity. The control group received NGF vehicle (BSA 0.1%) whereas the other three groups were treated with NGF (10 ng/rat intraperitoneally) and received the anti-BDNF antibody (36 $\mu\text{g/kg}$ intraperitoneally), h-CGRP 8–37 (300 $\mu\text{g/kg}$ intravenously, a dose which blocks the hyperalgesic effect of CGRP²³), or vehicle, 30 minutes before distension.

Finally, in a third experiment, we studied the effect of anti-NGF and anti-BDNF antibodies on CGRP induced hypersensitivity. The control group received CGRP vehicle (BSA 0.1%) whereas the other three groups were treated with CGRP (1 $\mu\text{g/kg}$ intraperitoneally) and received the anti-NGF (1/2000, 2 ml/kg intraperitoneally) or anti-BDNF antibody (36 $\mu\text{g/kg}$ intraperitoneally), or vehicle (BSA 0.1%), 30 minutes before distension.

All the experiments were performed blind by the same investigators. Treatments were administered randomly. Different animals were used for each experimental study.

Compounds

BDNF was a human recombinant form (expressed in *Escherichia coli*) and NGF-2.5S was obtained from mouse submaxillary gland. Monoclonal antihuman BDNF (IgG1 isotype) was purified from a mouse hybridoma. The corresponding control isotype antibody was a purified mouse

Figure 1 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (36 µg/kg intraperitoneally 30 minutes before distension), its vehicle (bovine serum albumin (BSA) 0.1%), a control isotype antibody (40 µg/kg) (A), or human calcitonin gene related and peptide fragment 8–37 (h-CGRP 8–37) antagonist (75, 150, and 225 µg/kg, intravenously, 30 minutes before distension) (B) on colonic reaction threshold of trinitrobenzene sulfonic acid (TNBS) treated rats (50 mg/kg, 30% ethanol, into the proximal colon, seven days before distension), in response to colonic distension in the distal colon. Results are expressed as mean (SEM) (n=7–8 per group). * $p<0.05$, *** $p<0.001$ versus control group; ††† $p<0.001$ versus colonic reaction threshold of TNBS treated rats + vehicle.

IgG1. Polyclonal neutralising NGF antibody was developed in rabbit against NGF-2.5S from mice. The corresponding control antibody was a rabbit IgG. Calcitonin gene related peptide (α -CGRP, human) was of synthetic origin, as was the human calcitonin gene related and peptide fragment 8–37 (h-CGRP 8–37), a selective peptidic CGRP1 receptor antagonist.^{29–31} TNBS was purchased from Fluka (Buchs, Switzerland) and was dissolved in 30% EtOH. Other compounds were supplied by Sigma-Aldrich Chemical Co (St Louis, Missouri, USA) and were dissolved in BSA 0.1%.

Expression of results and statistical analysis

Results are expressed as mean (SEM) of balloon pressure in mm Hg that induce the first abdominal contraction. Statistical comparisons between the different groups were made using one way ANOVA followed by a Bonferroni post hoc test to compare several treatments. Differences were considered statistically significant at $p<0.05$.

Figure 2 Effect of exogenous brain derived neurotrophic factor (BDNF) (0–100 ng/rat in 0.1% bovine serum albumin (BSA), intraperitoneally (IP)) on colonic reaction threshold of rats in response to colonic distension. Results are expressed as mean (SEM) (mm Hg) (n=7–8 per group). *** $p<0.001$ versus vehicle treated control group.

RESULTS

Evaluation of the involvement of BDNF and CGRP in TNBS induced referred colonic hypersensitivity

Rats treated with TNBS (50 mg/kg into the proximal colon) seven days before distension had a significant decrease in colonic reaction threshold in response to distal colonic distension (18.6 (1.3) v 41.3 (1.8) mm Hg for controls; $p<0.001$) (fig 1A). Administration of anti-BDNF antibody (36 µg/kg intraperitoneally), which has no effect in healthy rats, produced a colonic reaction threshold of 41.4 (1.8) mm Hg, which was not significantly different from control colonic reaction thresholds of healthy rats (41.2 (1.35) mm Hg). The same dose of anti-BDNF antibody significantly reduced (but not totally reversed) TNBS induced referred non-inflammatory colonic hypersensitivity (33.4 (2.1) v 18.6 (1.3) mm Hg ($p<0.001$), anti-BDNF v vehicle). The isotype control antibody had no effect on this decrease. h-CGRP 8–37 inhibited the decrease in reaction threshold in a dose dependent manner: at a dose of 225 µg/kg, inhibition was maximal with a colonic reaction threshold of 40.3 (4.1) mm Hg compared with that of TNBS treated rats injected with vehicle (17.5 (0.7) mm Hg; $p<0.001$) or healthy control rats (42.4 (3.2) mm Hg) (fig 1B).

Effect of BDNF on pain threshold in response to colonic distension

The colonic pain threshold of control rats, treated with vehicle only (0.1% BSA), was 40.4 (1.2) mm Hg. Intraperitoneal injection of BDNF in 0.1% BSA (1–100 ng/rat), 30 minutes before distension, induced a significant dose dependent decrease in colonic pain threshold (fig 2). With a dose of 1 ng/rat, the decrease was not statistically significant compared with control rats (35.4 (1.8) v 40.4 (1.2) mm Hg, respectively). For BDNF at 10 and 100 ng/rat, the colonic pain threshold was 21.5 (2.7) and 25.5 (2.1) mm Hg ($p<0.001$ v control threshold). The dose of 100 ng BDNF/rat was chosen for the following experiments.

Reciprocal influence of neurotrophin antibodies or CGRP antagonist on hypersensitivity induced by various peptides

Effect of anti-BDNF and anti-NGF antibody on pain threshold in response to colonic distension of BDNF treated rats

Anti-BDNF antibody (36 µg/kg intraperitoneally) reversed the decrease in colonic pain threshold induced by simultaneous intraperitoneal injection of BDNF (100 ng/rat)

Figure 3 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (0, 18, or 36 µg/kg in bovine serum albumin (BSA) 0.1% intraperitoneally) (A) and an anti-nerve growth factor (NGF) antibody (1/2000, 2 ml/kg intraperitoneally) (B) on colonic distension threshold of BDNF treated rats (100 ng/rat in 0.1% BSA intraperitoneally). Results are expressed as mean (SEM) (mm Hg) (n=7–8 per group). ***p<0.001 versus control group; † p<0.05; †††p<0.001 versus colonic reaction threshold of BDNF treated rats + vehicle.

(38.1 (2.6) v 18.6 (0.8) mm Hg; p<0.001) for BDNF treated rats receiving vehicle (fig 3A). The threshold obtained for the anti-BDNF treated group was not significantly different from the control threshold. Half the dose of anti-BDNF antibody had a small effect on this decrease. Colonic pain threshold was similar in isotype control antibody treated rats and vehicle treated rats. Similarly, anti-NGF antibody (1/2000, 2 ml/kg intraperitoneally) inhibited the BDNF induced decrease in colonic pain threshold (33.4 (0.6) v 18.7 (0.7) mm Hg; p<0.001) for BDNF treated rats receiving vehicle (fig 3B). Again, the threshold obtained for the anti-NGF treated group was not significantly different from the control threshold (without BDNF).

Effect of anti-BDNF and h-CGRP8–37 on pain threshold in response to colonic distension of NGF treated rats

Rats treated with NGF (10 ng/rat intraperitoneally) had a significant decrease in pain threshold in response to colonic distension (18.2 (1.1) mm Hg v 40.3 (0.9) mm Hg for the control group; p<0.001) (fig 4). The anti-BDNF antibody (36 µg/kg intraperitoneally) was unable to reverse this decrease. On the other hand, h-CGRP8–37 (300 µg/kg intravenously) reversed NGF induced colonic hypersensitivity (32.1 (3.6) mm Hg v vehicle treated rats 18.2 (1.1) mm Hg;

Figure 4 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (36 µg/kg intraperitoneally in bovine serum albumin (BSA) 0.1%) and human calcitonin gene related and peptide fragment 8–37 (h-CGRP8–37) antagonist (300 µg/kg intravenously) on colonic reaction threshold of nerve growth factor (NGF) treated rats (10 ng/rat, in 0.1% BSA intraperitoneally). Results are expressed as mean (SEM) (mm Hg) (n=7–8 per group). ***p<0.001 versus control group; †††p<0.001 versus colonic reaction threshold of NGF treated rats + vehicle.

p<0.01). Colonic reaction threshold of the h-CGRP8–37 treated group was not statistically different from the control threshold (without NGF).

Effect of anti-NGF and anti-BDNF on pain threshold in response to colonic distension of CGRP treated rats

Rats treated with CGRP (1 µg/kg intraperitoneally) had a significant decrease in pain threshold in response to colonic distension (21.0 (1.5) v 39.7 (2.1) mm Hg for the control group; p<0.001) (fig 5). Neither anti-NGF (1/2000, 2 ml/kg intraperitoneally) nor anti-BDNF antibody (36 µg/kg intraperitoneally) was able to reverse this CGRP induced decrease.

DISCUSSION

We have previously shown that NGF contributes, via capsaicin sensitive fibres, to the development of TNBS induced referred colonic hypersensitivity in response to distal

Figure 5 Effect of an anti-brain derived neurotrophic factor (BDNF) antibody (36 µg/kg intraperitoneally) and an anti-nerve growth factor (NGF) antibody (1/2000, 2 ml/kg intraperitoneally) on colonic reaction threshold of calcitonin gene related peptide (CGRP) treated rats (1 µg/kg in 0.1% bovine serum albumin intraperitoneally). Results are expressed as mean (SEM) (mm Hg) (n=7–8 per group). *p<0.05; ***p<0.001 versus control group; NS, non-significant difference from colonic reaction threshold of CGRP treated rats + vehicle.

colonic distension, a model that can mimic IBS in rats.⁴⁻⁵ In our study, this model was used to test the same hypothesis with other neurotrophins, BDNF and CGRP, and the relationship between BDNF, NGF, and CGRP in colonic hypersensitivity was also investigated.

Our main findings were that: (1) anti-BDNF antibody and CGRP antagonist alleviated TNBS induced referred colonic hypersensitivity, which demonstrates (in common with NGF) involvement of this neurotrophin and this neuropeptide in the pathophysiology of a model which mimics certain aspects of IBS; (2) confirmation that BDNF and CGRP induce colonic hypersensitivity; and (3) BDNF, NGF, and CGRP can induce colonic hypersensitivity in an interactive manner.

Take together, the ability of anti-BDNF antibody to markedly reduce the TNBS induced decrease in distal colonic distension thresholds and its inability to modify this parameter in healthy rats demonstrates that the neurotrophin is involved in colonic hypersensitivity. This conclusion is in agreement with the demonstrated hyperalgesic effect of low doses of exogenous BDNF which, in terms of magnitude and onset, are similar to that induced by exogenous NGF in the colorectal distension test.⁵ It is also in accordance with previous studies demonstrating a nociceptive effect of exogenous BDNF in various models. Exogenous BDNF, like exogenous NGF, triggered a persistent mechanical allodynia when delivered directly to the intact dorsal root ganglia.¹² Injection of BDNF into the rat hind paw has been shown to induce thermal hyperalgesia.¹¹ These data are also in accordance with other studies where BDNF mRNA and protein expression were upregulated in some models of inflammatory and neuropathic pain.^{8-9, 32-34}

BDNF and NGF are thought to be involved in long term plasticity.³⁵⁻³⁶ However, in our studies (the present work and Delafoy and colleagues⁵), seven days after injury (TNBS in the proximal colon), a single injection of antibodies was effective at the same dose as that used to reverse the acute hyperalgesic effect of their respective neurotrophins, suggesting ongoing release of the two neurotrophins in non-inflammatory colonic hypersensitivity. Moreover, it is important to note that anti-BDNF and anti-NGF⁵ antibodies did not completely reverse this hypersensitivity. Accordingly, we hypothesise that two mechanisms may be involved in neurotrophin mediated non-inflammatory colonic hypersensitivity. The first mechanism, involving early released neurotrophins inducing long term plastic changes insensitive to acutely administered antibodies injected several days after injury, and a second one (predominant) involving ongoing release of neurotrophins inducing hyperalgesic effects which can be reversed by antibodies. The suspected ongoing release of neurotrophin is in agreement with some of the data in the literature. In a model of visceral hypersensitivity induced by neonatal maternal deprivation, adult deprived rats treated with anti-NGF antibodies exhibited normal gut permeability and visceral sensitivity to rectal distension.³⁷ Anti-BDNF antibody, injected three days after injury, has been shown to relieve mechanical or thermal hyperalgesia in rat models of nerve ligation.⁸⁻¹³ Antibodies to different neurotrophins (NGF, BDNF, neurotrophin 3) significantly reduced the percentage of foot withdrawal responses evoked by von Frey hairs when injected up to 14 days after spinal nerve injury.¹² Moreover, findings of an increase in expression of neurotrophins, in visceral pain models or in patients, indirectly suggest ongoing involvement of neurotrophins. In a model of colonic hypersensitivity induced by neonatal stress in rats, NGF expression (protein and mRNA) increased for up to 12 weeks of life.³⁷ In *Trichinella spiralis* infected rats, which provide a model of small intestine hypersensitivity, NGF protein and mRNA levels were significantly increased in intestine tissue three days post infection.³⁸ In inflamed gut of

patients with colitis, tissues are rich in mast cells and high levels of NGF were observed.³⁹ BDNF is also upregulated and associated with pain in patients with chronic pancreatitis.⁶

Moreover, the data obtained in this study provide further insight into the "hypersensitising" action of BDNF. The efficacy of low systemic doses of BDNF, its short delay of action in inducing a decrease in distension thresholds, and its inability to cross the blood-brain barrier⁴⁰ suggest a peripheral action of this neurotrophin. However, release of endogenous BDNF and the mechanisms by which it occurs, in our model of colonic hypersensitivity, remains to be determined.

The pronociceptive role of CGRP has been well established.^{17-20, 41} Accordingly, in our study, as with BDNF, we have shown the ability of CGRP to induce colonic hypersensitivity and its involvement in TNBS induced referred colonic hypersensitivity. This latter result is also consistent with the previously described peripheral involvement of CGRP in the mechanism of neurogenic inflammation,^{25, 42} which could be the case here.

Another major finding of this study was that involvement of BDNF, NGF, and CGRP in induction of non-inflammatory colonic hypersensitivity is linked. One hypothesis would be an interactive involvement according to an ordered cascade. Indeed, in our study, the anti-BDNF antibody only reversed BDNF induced hypersensitivity and was inactive on both NGF and CGRP induced hypersensitivity. Secondly, BDNF induced hypersensitivity was reversed by the anti-NGF antibody. Thirdly, NGF induced hypersensitivity was alleviated only by h-CGRP 8-37. Finally, none of the anti-neurotrophin antibodies tested was able to reduce CGRP induced hypersensitivity. Taken together, these results suggest that the cascade is as follows: first BDNF, which needs the involvement of NGF, which in turn needs CGRP to induce colonic hypersensitivity. This cascade is supported by the fact that anti-BDNF, anti-NGF, and CGRP antagonist reversed TNBS induced referred colonic hypersensitivity by 65%, 86%, and 93%, respectively. We may hypothesise that TNBS induces release of neurotrophins from peritoneal mast cells, for example,^{39, 43} in first BDNF and then NGF, which in turn triggers release of CGRP from capsaicin sensitive primary sensory afferents. This is in agreement with: (i) our previous demonstration that neonatal treatment with capsaicin reduces NGF and TNBS induced hypersensitivity, suggesting that capsaicin sensitive primary afferents are required in the development of colonic hypersensitivity⁵; (ii) the fact that CGRP is present in these sensory afferent fibres⁴⁴⁻⁴⁶ and that capsaicin, which induces depletion of CGRP,⁴⁷ also reduces, for example, peritoneal irritation induced visceral pain^{17, 19, 20}; and (iii) the fact that NGF has been shown to be involved in CGRP secretion in sensitive neurones^{28, 48-51} and to regulate expression of neuropeptide genes in adult sensory neurones.⁵² Recent work shows that NGF increases CGRP release due to TRPV1 agonists, such as anandamide.⁵³

Accordingly, we know that in our model of colonic hypersensitivity there is a highly significant increase in SP and CGRP innervation of the myenteric plexus in the distal colon.²³ However, regarding partial inhibition of TNBS induced referred colonic hypersensitivity by neurotrophin antibodies, we may also speculate that other pathways, neurotrophin independent, could be involved in CGRP receptor involvement. Indeed, the existence of the suspected cascade needs to be confirmed (for example, study of the time course involvement of the various mediators) and the link between these three mediators may also involve another mechanism, such as a synergistic interaction.

In conclusion, our results highlight the importance of BDNF and CGRP, as previously shown for NGF, in

non-inflammatory colonic hypersensitivity and demonstrate their interactive involvement. Due to the similar characteristics of the animal model used with IBS, these findings can help in understanding the pathophysiology of IBS and may offer interesting pharmacological perspectives.

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