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Adrenergic Stimulation Mediates Visceral Hypersensitivity to Colorectal Distension following Heterotypic Chronic Stress

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Abstract

Background & Aims—Chronic stress exacerbates or causes relapse of symptoms such as abdominal pain and cramping in patients with irritable bowel syndrome (IBS). We investigated whether chronic stress increases plasma norepinephrine and sensitizes colon-specific dorsal root ganglion (DRG) neurons by increasing the expression of nerve growth factor (NGF) in the colon wall.

Methods—Heterotypic chronic stress (HeCS) was induced in male Wistar rats and neurologic and molecular responses were analyzed. Tissues were analyzed for NGF expression.

Results—HeCS significantly increased the visceromotor response to colorectal distension; expression of NGF increased in colonic muscularis externa and mucosa/submucosa. Rheobase decreased, resting membrane potential was depolarized, and electrogenesis of action potentials increased in colon-specific thoracolumbar DRG neurons. Luminal administration of resiniferatoxin in distal colon, systemic administration of anti-NGF antibody, or inhibition of the NGF receptor TrkA by k252A or antisense oligonucleotides in thoracolumbar DRG blocked the chronic stress-

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Contributions by the authors:

Topic	Winston	Xu	Sarna
Study concept And design			X
Analysis of data	X	X	
Acquisition of data	X	X	
Interpretation of data			X
Drafting manuscript			X
Revision			X
Statistical analysis	X	X	
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induced visceral hypersensitivity to colorectal distension. Blockade of $\alpha 1/\alpha 2$ - and $\beta 1/\beta 2$ -adrenergic receptors prevented the stress-induced visceral hypersensitivity and increased expression of NGF in the colon wall. HeCS did not induce any inflammatory response in the colon wall.

Conclusion—The peripheral stress mediator norepinephrine induces visceral hypersensitivity to colorectal distension in response to HeCS by increasing the expression of NGF in the colon wall, which sensitizes primary afferents in the absence of an inflammatory response.

Keywords

irritable bowel syndrome; nerve growth factor; dorsal root ganglia; norepinephrine; corticotrophin releasing hormone

Abdominal discomfort/cramping and altered bowel habits are the defining symptoms of irritable bowel syndrome (IBS)¹. The etiologies of both symptoms are not fully understood. However, clinical studies show that chronic stress exacerbates or precipitate both symptoms of IBS^{2, 3}. This suggests that chronic stress may impair the cellular functions of some of the same cells that cause motility dysfunction or visceral hypersensitivity in IBS patients in the first place. However, the mechanisms by which chronic stress causes cellular dysfunction in these cells may differ from those that cause the underlying dysfunction in IBS. Animal studies support this hypothesis. A recent study found that alterations in the transcription rates of genes encoding key cell-signaling proteins of the excitation-contraction coupling in colonic circular smooth muscle cells underlie motility dysfunction of faster colonic transit and increase in defecation rate in a model of post-infective IBS⁴. Another study found that chronic stress enhances the transcription rate of some of the same genes encoding key cell-signaling proteins that accelerate colonic transit and defecation rate, but by different mechanisms⁵.

Clinical studies show that the primary spinal afferents mediate visceral hypersensitivity to colorectal distension (CRD) in IBS patients^{6, 7}. However, all psychological stress responses begin in the CNS. The release of corticotrophin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus is an early and essential step in the initiation of all psychological stress responses⁸. The central release of CRH and other mediators, such as arginine vasopressin, stimulate the neuroendocrine system comprised of autonomic neurons and the hypothalamus-pituitary-adrenal (HPA)-axis, which modulates the adaptive and maladaptive responses of peripheral organs in a stress- and cell-type specific manner. Both acute and chronic stressors induce visceral hypersensitivity to colorectal distension in rats by releasing CRH in the hypothalamus^{9, 10}. However, we do not know which pathways or which stress mediators of the neuroendocrine system transmit the central signal to colon-specific primary afferent neurons in the dorsal root ganglia (DRG) to modulate their sensitivity to CRD.

In a recent study⁵, we found that nine-day heterotypic chronic stress (HeCS) significantly increases the plasma concentration of norepinephrine. In the present study, we tested the hypothesis that norepinephrine released by the central component of the stress response acts as a messenger to induce the expression of neurotrophin, nerve growth factor (NGF), in the distal colon, which sensitizes the colon-specific neurons in the thoracolumbar DRG to induce visceral hypersensitivity to CRD. We chose chronic stress because clinical findings indicate that chronic stress, rather than short-term acute stress^{2, 3} exacerbates the symptoms of IBS. In addition, variable stressors are less likely to show adaptation compared to repeated applications of the same stressor.

Methods

Animals

We used six to ten week-old male Wistar rats housed at 22°C with a 12-hour light/dark cycle. The Institutional Animal Care and Use Committee at UTMB approved all procedures performed on animals.

Heterotypic chronic stress protocol

The rats were subjected to nine consecutive days of a heterotypic stress protocol comprised of three randomly arranged stressors, 60-minutes of water avoidance stress, 45-minutes of cold restraint stress at 4°C or 20-minutes of forced swimming stress, as described previously⁵. The control rats were brought to the lab and handled identically without subjecting them to the stress protocol.

Measurement of visceromotor response to graded CRD

Electromyographic activity from the external oblique muscle was recorded in response to colorectal distention (CRD). CRD was performed by rapidly inflating the balloon to constant pressures: 20, 30, 40, 50, 60 & 80 mm Hg, for 20 seconds followed by 2-minute rest. The net value for each distension period was calculated by subtracting the baseline value derived from the average AUC for 20 seconds before and 20 seconds after the distention period. Additional details are provided in the Supplement.

Tissue isolation

About six cm length of the distal colon was opened along the mesentery. Mucosa/submucosa was separated from the muscularis externa. Tissues were either snap frozen in liquid nitrogen or were divided into 8 strips and washed in Hanks balanced salt solution and incubated in Dulbecco's modified eagle medium + 10% fetal calf serum + penicillin/streptomycin containing norepinephrine for 24-hours.

Electrophysiology

Under general 2% isoflurane anesthesia, the lipid soluble fluorescent dye, DiI-I (1,1'-diolel-3,3,3',3'-tetramethylindocarbocyanine methanesulfonate, Invitrogen), 25 mg in 0.5 ml methanol was injected in 2 µl volumes at eight to ten sites in the distal colon wall starting at the pelvic girdle and moving toward the cecum. These sites were within the colon segment used for colorectal distension. Thoracolumbar DRG neurons were isolated from DRG T13, L1 and L2, 16 days later. The methods used for isolation of DRG and current clamp recordings have been described previously¹¹ (see supplement for additional details).

Intrathecal Catheter/osmotic pumps

K252a, a non-specific antagonist of tyrosine kinase receptors including the high affinity trkA receptors¹², or previously validated antisense and mismatched oligonucleotides¹³ were administered by osmotic pump attached to an intrathecal catheter. Please see Supplement for details.

NGF neutralizing antibody (16 µg/kg i.p. in 0.5 ml in phosphate buffer saline (PBS)) was purchased from R&D systems (Minneapolis, MN). Control rats received PBS containing an equal concentration of goat non-immune serum.

Please see supplement for details on mast cell counts, myeloperoxidase and tryptase measurements and pharmacological reagents

Statistics

Data are expressed as mean $\pm$ SE. One-way ANOVA followed by Fisher post-hoc analysis and t-test were used for comparison of means. The effect of HeCS and treatments were analyzed using two-way repeated measures ANOVA.

Results

Chronic Stress-induced Visceral Hypersensitivity is Associated with Increase in Excitability of Colon-specific TL DRG Neurons

We found that 9-day HeCS significantly increases the visceromotor response to graded CRD at pressures of 40, 50, 60 and 80 mm Hg, compared to pre-stressed baseline response (Figure 1A) (n=14 rats). The increase in visceromotor response persists for at least 8-hours after the last stressor, but it returns to basal levels 24-hours post-HeCS. By contrast, 9-day sham stress in age-matched control rats had no significant effect on the visceromotor response to CRD (Figure 1B) at 8 and 24 hours (n=10 rats). However, there was a significant decline in the VMR to CRD in sham stressed rats at 7 days compared to baseline at 40, 50, 60, and 80 mm Hg.

Acutely dissociated thoracolumbar colon-specific afferent neurons, identified by the presence of retrograde label DiI injected into the colon wall, showed a significant decrease in Rheobase (Figures 2A and 2B), depolarization of resting membrane potential (Figure 2C), and a significant increase in the number of action potentials evoked at 2X rheobase (Figures 2D and 2E), compared to neurons from the sham-stressed control rats (n=22 and 31 neurons respectively). HeCS did not alter the other electrophysiological characteristics, such as cell diameter, capacitance, input resistance and action potential amplitude, duration, threshold and overshoot, of DRG neurons (data not shown).

The role of afferent nerve endings in the above remodeling of colon-specific DRG neurons was investigated by desensitizing them with intraluminal infusion of 200 μ g/kg resiniferatoxin¹⁴ in the distal colon, one-day prior to the beginning of HeCS protocol. The treatment of sham-stressed rats with resiniferatoxin had no significant effect on rheobase and resting membrane potential of colon-specific DRG neurons (Figures 2B and 2C) (n=20 neurons). However, in rats subjected to HeCS, resiniferatoxin-treatment significantly blocked the reduction in rheobase and increase in the number of action potentials at 2X (Figures 2A, 2B, 2D and 2E) (n=23 neurons).

HeCS Induces NGF Expression in the Distal Colon

We found that HeCS significantly increases the expression of NGF in the muscularis externa and in mucosa/submucosa of the distal colon, when compared with those in sham-stressed rats (Figure 3A and Supplement Figure 1). Immunohistochemical staining of cross-sections detected NGF immunoreactivity in smooth muscle cells and in the myenteric plexus in muscularis externa and in epithelial cells in mucosa/submucosa. NGF immunoreactivity increased in both tissue-types of the chronically stressed rats, compared with and sham-stressed rats (Figure 3B). By contrast, HeCS had no significant effect on the expression of trkA receptors in the muscularis externa ($100 \pm 11\%$ vs $108 \pm 27\%$) or in mucosa/submucosa ($100 \pm 17\%$ vs. $92 \pm 5\%$).

Effect of Antagonism of Peripheral NGF on HeCS-induced Increase in visceromotor response

The rats treated with 16 μ g/kg i.p. NGF antibody¹⁵, 30 minutes before each daily stress session, showed no significant increase in visceromotor response to CRD following HeCS, compared to their pre-stress baseline responses (Figure 3C). Rats receiving non-immune serum showed significant increase in visceromotor response following HeCS (Figure 3C).

Effects of Antagonism of trkA Receptors in thoracolumbar DRG on HeCS-induced Increase in Excitability of Colon-specific Afferent Neurons

Next, we investigated whether NGF high affinity receptor trkA in DRG mediates the HeCS-induced increase in visceromotor response to CRD. We administered either trkA pharmacological inhibitor k252a, or trkA anti-sense oligonucleotide intrathecally by a surgically implanted osmotic pump for the entire nine-day period of HeCS. The intrathecal infusions started 24-hours prior to the first session of HeCS. The control rats received infusion of mismatch oligonucleotides. The rats treated with intrathecal k252a showed no significant difference between their post-HeCS and baseline responses to CRD (Figure 3D). The control rats that received mismatch oligonucleotide during stress showed significant increase in visceromotor response to CRD following HeCS, compared to baseline responses (Figure 3D). There was no significant difference between the post-HeCS and baseline responses of rats treated intrathecally with the trkA antisense oligonucleotides (Figure 3D).

The expression of trkA in the thoracolumbar DRG of rats treated with antisense oligonucleotides decreased significantly to about 50% of that in rats treated with mismatch oligonucleotides (Figure 3F). No significant difference in trkA levels were observed in the thoracic DRG, several segments rostral to the catheter tip, or in lumbosacral DRG, several segments caudal to the catheter tip, indicating that the knockdown effect was localized to the DRG near the catheter tip (Figure 3F).

Intrathecal application of k252a significantly attenuated the HeCS-induced decrease in rheobase (Figure 4A), depolarization of resting membrane potential (Figure 4B), and increase in the number of action potentials at 2X rheobase (Figure 4C). Taken together, these findings support an essential role of NGF receptor trkA and NGF in colon-specific thoracolumbar DRG neurons in the induction of visceral hypersensitivity by HeCS.

Effect of NGF on colon-specific thoracolumbar DRG neuronal excitability in vitro—Next, we investigated whether exposure to NGF *in vitro* alters the excitability of colon-specific thoracolumbar DRG neurons. We incubated acutely dissociated thoracolumbar DRG neurons from naïve rats with either high NGF (250 ng/ml) or low NGF (2.5 ng/ml) for 24 hours and measured passive and active electrophysiological properties of DiI labeled colonic sympathetic afferents. The resting membrane potential (Figure 5A) and rheobase (Figure 5B) significantly decreased in neurons treated with 250 ng/ml NGF, when compared with those treated with 2.5 ng/ml NGF. The number of action potentials generated at 2X rheobase was greater in neurons treated with high NGF than that with low NGF controls, but it did not reach statistical significance ($p=0.11$, data not shown). These findings demonstrate that exposure to higher concentrations of NGF *in vitro* produces changes in electrophysiological properties of colon-specific thoracolumbar DRG neurons that are similar to those produced by HeCS.

The Role of Norepinephrine in Inducing Visceral Hypersensitivity and NGF Expression in Distal Colon

We reported recently that nine-day HeCS significantly elevates plasma concentration of norepinephrine⁵. To determine whether norepinephrine contributes to the induction of visceral hypersensitivity, rats subjected to HeCS were treated once daily before each stress session with phentolamine (2 mg/kg i.p.) + propranolol (2 mg/kg i.p.). Sham-treated rats served as controls. Visceromotor responses to CRD were compared with their respective pre-stress baselines (Figure 6A). Phentolamine plus propranolol blocked the HeCS-induced increase in visceromotor response to CRD and elevation of NGF in the muscularis externa and mucosa/submucosa (Figure 3A).

We incubated strips of muscularis externa or mucosa/submucosa with norepinephrine for 24 hours *in vitro*. Norepinephrine (Figures. 6B and 6C) concentration-dependently increased the expression of NGF in muscularis externa and in mucosal/submucosa.

The Roles of Mast cells, Inflammatory Mediators and Peripheral CRH₁/CRH₂ Receptors in the Induction of Visceral Hypersensitivity by HeCS

We observed no differences in mast cell numbers in either muscularis externa or in mucosa/submucosa between HeCS-treated and sham-stressed controls (Figure 7A). HeCS reduced the spontaneous release of tryptase from muscularis externa, but not that induced by ionomycin (Figure 7B). HeCS had no effect on the spontaneous or ionomycin-induced release of tryptase in mucosal/submucosal tissues (Figure 7C). Nine-day HeCS had no significant effect on MPO in the muscularis externa or the mucosa/submucosa (Figure 7D). It also did not alter the concentrations of TNF α and IL-1 β in these tissues (data not shown). Finally, the inhibition of CRH₁/CRH₂ receptors by astressin had no significant effect on the generation of NGF by norepinephrine in muscularis externa (Figure 7E), but astressin blocked this effect in the mucosa/submucosa (Figure 7F).

Discussion

Our findings show that HeCS induces visceral hypersensitivity to CRD that lasts for at least 8 hours, but it returns to baseline by 24 hours. Choudhury et al.⁵ found that HeCS induces colonic circular smooth muscle hyperreactivity to ACh that also lasts for about 8 hours and returns to baseline by 24 hours. We found that adrenergic stimulation mediates the induction of visceral hypersensitivity by HeCS. The blockade of α_1/α_2 - and β_1/β_2 -adrenergic receptors before the daily application of stress prevented the induction of visceral hypersensitivity by HeCS. By contrast, Bradesi et al.¹⁰ found that 10-day homotypic water avoidance stress induces visceral hypersensitivity that lasts for about 40 days. However, they did not investigate which peripheral stress hormone/neurotransmitter mediates the prolonged induction of hypersensitivity in their model. Note that plasma/urine levels of norepinephrine are elevated in IBS patients^{16, 17}.

The mechanoreceptors in the gut wall are distributed throughout its thickness^{18–21}. Accumulating evidence from human⁷ and animal studies^{19, 20} shows that splanchnic neurons with mechanoreceptors in the muscularis externa/serosa mediate afferent responses to rapid step distensions that mimic the physiological rapid compression of the gut wall by giant migrating contractions²². *In vitro*, the mucosal afferents respond primarily to stimulation by von Frey hairs that mimics the flow of digesta^{19, 20}; the mucosal afferent nerves do not respond to circumferential stretch¹⁹. *In vivo*, the mucosal afferents respond to slow distension that mimics slow lumen filling by fecal material, but not to rapid step balloon distension⁶. In addition, desensitization of the mucosal afferent mechanoreceptors by lidocaine does not affect pain-perception to rapid balloon distension in IBS patients⁶. However, lidocaine blocks the sensory perception to slow distension. It seems that the mucosal and muscularis externa afferent neurons may respond differentially to slow and rapid distensions of the gut wall. Therefore, we chose to investigate the effects of HeCS on the expression of various potential mediators of DRG-sensitization separately in the muscularis externa and in mucosa/submucosa.

Western blotting and immunohistochemical staining with NGF antibody and ELISA measurements showed that HeCS enhances the expression of NGF in the muscularis externa as well as in mucosa/submucosa. The inhibition of α_1/α_2 - β_1/β_2 adrenergic receptors blocks the increase in the expression of NGF in both tissues, suggesting that they mediate the expression of NGF in the colon wall in response to HeCS. In addition, *in vitro* incubation of both tissue-types with norepinephrine enhances the expression of NGF. Prior reports show that numerous cell-types, including smooth muscle cells²³, glia²⁴, immune cells²⁵ epithelial cells²⁶ and neurons²⁷ are capable of generating NGF. In our study, the smooth muscle cells

and mucosa seemed to show the largest increase in NGF immunoreactivity in the colon wall, but we did not quantitate it. We found that neutralization of peripheral NGF by its antibody blocks the increase of visceromotor response to CRD. Together, the above data suggest that the up regulation of NGF throughout the thickness of the distal colon wall by HeCS-induced release of norepinephrine is an intermediate step in the induction of visceral hypersensitivity to CRD.

NGF in the periphery complexes with trkA receptors and migrates retrograde to the DRG neurons^{28, 29}. The inhibition of retrograde migration of this complex by desensitization of afferent nerve endings with resiniferatoxin blocked the induction of visceral hypersensitivity to CRD. The pharmacological blockade of trkA receptors or their suppression by antisense oligonucleotide in the thoracolumbar DRG also blocked the induction of visceral hypersensitivity to CRD. Taken together, NGF expression in the colon wall is critical for the induction of visceral hypersensitivity to CRD by HeCS.

Patch-clamp recordings from colon-specific thoracolumbar DRG neurons showed that HeCS decreases rheobase, depolarizes resting membrane potential and increases the electrogenesis of action potentials, when compared with those in age-matched sham-stressed controls. Systemic administration of NGF antibody that does not cross the blood-brain barrier blocked these effects. This suggests that the alterations in the electrophysiological characteristics of colon-specific thoracolumbar DRG neurons may primarily be due to increase of NGF in the colon wall, rather than due to a direct effect of plasma norepinephrine on the DRG neurons³⁰ or due to descending inhibition from the CNS³¹.

Voltage-gated sodium channels (Na_v) play a critical role in regulating the sensitivity of DRG neurons to peripheral insult. The C-type DRG neurons express predominantly the slowly inactivating TTX-R Na_v1.8 and TTX-R Na_v1.9 channels^{32, 33}. The Na_v1.8 channels make major contribution to the electrogenesis of action potentials^{32, 34}, whereas the Na_v1.9 channels regulate the resting membrane potential of these neurons³⁵. The ionic mechanisms underlying visceral hypersensitivity are specific to the pathophysiology of pain and its targeted organ³⁶. Visceral hypersensitivity in rat colonic inflammation results mainly from alterations in the electrogenesis of action potentials due to remodeling of the Na_v1.8 channels³⁷. Our data show that visceral hypersensitivity induced by HeCS in rats is due to alterations in the electrogenesis of action potentials and changes in the resting membrane potentials of the colon-specific DRG neurons. According to the above-established roles of Na_v1.8 and Na_v1.9 in regulating the electrophysiological characteristics of the DRG neurons, HeCS may remodel both types of Na_v channels. The persistence of hypersensitivity in isolated DRG neurons for at least 8-hours after the last stressor suggests altered gene expression of the Na_v channels^{38, 39}.

NGF is a key signaling protein in the sensitization of visceral afferent neurons during inflammation^{40–43}. The administration of exogenous NGF mimics inflammatory hyperalgesia⁴³, while systemic administration of NGF antibody blocks the induction of inflammatory hyperalgesia¹⁵. During inflammation, NGF interacts with mast cells in a reciprocal synergistic reaction^{25, 44}. NGF acts on trkA receptors on mast cells to degranulate them, while the degranulation of mast cells releases NGF. It is noteworthy that while both NGF and trkA receptors are up regulated during inflammation⁴³, only NGF expression is enhanced in the muscularis externa by non-inflammatory stress mediator norepinephrine.

Other studies found increase in mast cell numbers or their activation and increase in MPO activity and proinflammatory cytokines in mucosa/submucosa in response to chronic stress^{9, 10} or maternal deprivation⁴⁵. However, the consensus seems to be that the overall intensity of inflammatory responses in these models is several-fold less than that in models of TNBS-induced inflammation. By contrast, we did not find any evidence of increase in MPO,

proinflammatory cytokines or mast cell activation/hyperplasia in the muscularis externa or mucosa/submucosa in response to HeCS, which induces robust visceral hypersensitivity to CRD. In addition, NGF increases in both tissue-types in response to HeCS, which was blocked by inhibiting α_1/α_2 - β_1/β_2 -adrenergic receptors. Norepinephrine increases the expression of NGF *in vitro* in muscularis externa mucosa/submucosa, and this increase is blocked by the inhibition of adrenergic receptors. Taken together, norepinephrine induces the expression of NGF in the colon wall in the absence of any detectable inflammatory response.

CRH receptors have been identified in the mucosa as well as in the myenteric and submucosal plexi^{46, 47}. Our findings show that these receptors in the muscularis externa that includes the myenteric and submucosal plexi do not mediate the generation of NGF by norepinephrine. However, the CRH receptors in the mucosa mediate the expression of NGF in mucosal/submucosal tissues.

In conclusion, 9-day heterotypic chronic stress induces visceral hypersensitivity to CRD that lasts for at least 8-hours after the last stressor. We reported previously that HeCS elevates the plasma level of norepinephrine that alters gene expression of key cell signaling proteins of the excitation-contraction coupling in colonic circular smooth muscle cells to induce hyperreactivity to acetylcholine⁵. Our findings in this study in relation to the well-established elements of the stress response are summarized in a schematic diagram in Figure 8: 1) Chronic stress releases CRH and angiotensin vasopressin from the paraventricular nucleus in the hypothalamus. 2) CRH and arginine vasopressin stimulate the locus ceruleus/norepinephrine system. In parallel, CRH releases adrenocorticotrophic hormone from the pituitary, which releases corticosterone from the adrenal cortex. 3) The activation of the greater splanchnic sympathetic preganglionic neurons releases norepinephrine from the chromaffin cells in the adrenal medulla into the blood stream^{48, 49}. The activation of the sympathetic neurons also releases norepinephrine locally in peripheral organs. 4) Norepinephrine enhances the expression of NGF in the colon wall. 5) NGF complexes with trkA receptors and the complex transports retrograde to the thoracolumbar DRG²⁹. 6) NGF/trkA complex in the DRG sensitizes the ion channels. 7) The hypersensitization of these ion channels amplifies the afferent signals in response to colonic distension to increase in its perception. This sensitization occurs in the absence of a detectable inflammatory response in the muscularis externa or in mucosa/submucosa. Based on the topology and phenotypes of afferent nerve endings in the colon wall^{7, 18–21}, increase of NGF in muscularis externa may mediate the induction of visceral hypersensitivity by HeCS, whereas, increase of NGF in mucosa/submucosa may mediate altered physiological responses to digesta in the lumen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IBS	Irritable bowel syndrome
(HPA)-axis	Hypothalamus-pituitary-adrenal
CRH	Corticotrophin-releasing hormone
DRG	Dorsal root ganglia

HeCS	Heterotypic Chronic Stress
NGF	Nerve Growth Factor
(trkA) [NGF high affinity receptor]	Tropomyosin related kinase A
CRD	Colorectal Distention
DiI	1,1'-dilinoylel-3,3,3',3'-tetramethylindocarbocynine perchlorate

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Figure 1.

(A) HeCS significantly increased visceromotor to graded CRD, $n=14$ rats, $*p<0.05$ compared to pre-stress baseline responses 8-hours following the last stressor. No significant increase was observed at 24-hours and 7-days after HeCS. (B) Sham stressed control rats showed no change in their visceromotor response at 8 and 24 hours but, significant differences were found between baseline and 7 days at 40, 50, 60, 80 mmHg $p<0.05$, $n=10$ rats.

Figure 2. Effects of HeCS and colonic resiniferatoxin (RTX) treatment on electrophysiological properties of DiI labeled colon-specific thoracolumbar DRG neurons. Changes in electrophysiological properties of colon specific DRG neurons from control (Ctr, n=31 neurons), HeCS (n=22 neurons), HeCS rats pretreated with RTX (n=23 neurons) and control rats treated with RTX (n=20 neurons). (A) Current clamp traces showing action potentials induced by current injection at rheobase. (B) Graph showing changes in rheobase. (C) Graph showing changes in resting membrane potential. (D) Current clamp traces showing the number of action potentials at 2X rheobase. (E) Number of action potentials (AP) induced by current injection at 2X rheobase. *p<0.05, control vs HeCS, #p<0.05 HeCS vs HeCS + RTX.

Figure 3.

(A) HeCS significantly increased NGF in muscularis externa and mucosa/submucosa, which was blocked by inhibition of α_1/α_2 and β_1/β_2 -adrenergic receptors prior to each daily stress session. $n=8$ rats in each group, $*p<0.01$, control (Ctr) vs. HeCS and HeCS vs. HeCS + adrenergic receptor antagonists. (B) Immunohistochemical staining for NGF (brown) in distal colon cross-sections from control and HeCS rats. Sections were counterstained with hematoxylin. (C) NGF antagonism by systemic administration of neutralizing antibody significantly reduced HeCS-induced increase in visceromotor response. HeCS, $n=4$ rats, HeCS + NGF Ab, $n=5$ rats, $*p<0.05$ baseline vs HeCS; $+p<0.05$ HeCS vs. HeCS + NGF Ab. (D) Intrathecal administration of trkA antagonist k252A ($n=5$) or antisense oligonucleotide ($n=3$) suppressed HeCS-induced increase in visceromotor response. $*p<0.05$ baseline vs. HeCS, $+p<0.05$, HeCS + mismatch oligonucleotide (MM) vs. HeCS + NGF Ab. (E) Western blots showing the effects of intrathecal treatment with either trkA antisense (AS) or mismatch (MM) oligonucleotide on trkA receptor expression in thoracolumbar (TL), thoracic (Th) or lumbosacral (LS) DRG. $n=4$, $*p<0.05$ mismatch vs antisense oligonucleotides.

Figure 4.

Intrathecal treatment with trkA antagonist k252a blocked the HeCS-induced increase in excitability of colon-specific thoracolumbar DRG neurons (Ctr= control, n=13 neurons from 5 rats; HeCS n=22 neurons from 5 rats; HeCS pre-treated with resiniferatoxin, n=23 neurons from 5 rats). (A) Rheobase. (B) Resting membrane potential. (C) Number of action potentials (AP) at 2X rheobase. *p<0.05, control vs. HeCS, #p<0.05 HeCS vs. HeCS + k252a.

Figure 5.

Electrophysiological properties of colon-specific thoracolumbar DRG neurons that were incubated for 24 hours with either high NGF (250 ng/mL or low NGF (2.5 ng/mL) in vitro. Neurons incubated with high NGF showed a significant decline in resting membrane potential (A) and rheobase (B), * $p < 0.05$, low NGF vs high NGF.

Figure 6.

(A) In vivo intraperitoneal administration of α_1/α_2 - and β_1/β_2 -adrenergic receptor antagonists blocked the HeCS-induced increase in the visceromotor response to graded CRD (n=3). Rats subjected to HeCS were treated once daily before each stress session with a combination of phentolamine (2 mg/kg i.p.) + and propranolol (2 mg/kg i.p.). *In vitro* incubation of muscularis externa/serosa (B) and mucosa/submucosa (C) for 24-hours with norepinephrine concentration-dependently increased the expression of NGF, *p<0.05, n=6 strips (Thirty strips of each tissue type were prepared from distal colon of 4 rats (about 8 strips/rat) and evenly distributed among the experimental groups.

Figure 7.

(A). HeCS did not increase the number of mast cells in the muscularis externa or mucosa/submucosa (n=4 rats). (B) and (C). HeCS did not increase the spontaneous or ionomycin-induced release of tryptase from the muscularis externa/or the mucosa/submucosa. Instead, it decreased the spontaneous release of tryptase in the muscularis externa. n=4 rats, * $p < 0.05$. (D) HeCS did not increase MPO protein in the muscularis externa or mucosa/submucosa. n=4 rats. (E) and (F). CRH1/CRH2 receptor antagonist astressin (AST) had no effect on the norepinephrine-induced expression of NGF in the muscularis externa/serosa, but it blocked this increase in mucosa/submucosa, * $p < 0.05$, n=4 strips. Strips were incubated with 10 μ M astressin for 30 min prior to the addition of norepinephrine (NE).

Figure 8.

A schematic illustrating the proposed mechanism of HeCS-induced visceral hypersensitivity to CRD in relation to the well-established elements of the stress response. **Step 1:** Stress releases of CRH and angiotensin vasopressin from the paraventricular nucleus in the hypothalamus. CRH releases adrenocorticotrophic hormone and other mediators from the pituitary. **Step 2:** CRH and arginine vasopressin stimulate the locus ceruleus/norepinephrine system. **Step 3:** The activation of the sympathetic preganglionic neurons releases norepinephrine/epinephrine in blood stream from the adrenal medulla. **Step 4:** Norepinephrine elevates the expression of NGF in colonic muscularis externa and mucosa/submucosa. **Step 5:** NGF complexes with trkA receptors and the complex transports retrograde to the thoracolumbar DRG. **Step 6:** NGF/trkA complex sensitizes colon-specific neurons by modulating the expression and characteristics of ion channels **Step 7:** The amplification of afferent signal in response to CRD is perceived as abdominal pain/discomfort.