Changes of the neuropeptides content and gene expression in spinal cord and dorsal root ganglion after noxious colorectal distension

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Abstract

Visceral pain/hypersensitivity is a cardinal symptom of functional gastrointestinal disorders. With their peripheral and central (spinal) projections, sensory neurons in the dorsal root ganglia (DRG) are the “gateway” for painful signals emanating from both somatic and visceral structures. In contrast to somatic pain, the neurochemical pathways involved in visceral pain/hypersensitivity have not been well studied. We hypothesized the neuropeptide changes in spinal cord and DRG during visceral pain would mirror similar changes in somatic nociception. Noxious (painful) colorectal distension (CRD) was done by distending a rectal balloon up to 60 mm Hg phasically for 1 h in Sprague–Dawley rats. The spinal content of calcitonin gene-related peptide (CGRP), substance P (SP), galanin and vasoactive intestinal peptide (VIP) as well as their mRNAs in DRG were measured at 0, 4 and 24 h after the CRD. Visceromotor reflex (VMR) was measured by recording the electromyogram at the abdominal muscle in response to CRD. Distal colorectum was removed for evaluating the presence of inflammation. No significant evidence of histological inflammation was seen in the colonic mucosa/submucosa after repeated CRD, which is confirmed by myeloperoxidase assay. The spinal content of CGRP and SP decreased significantly 4 h after CRD, while galanin and VIP levels increased gradually and reached highest level at 24 h (p < 0.05). The mRNAs in DRG were measured at 0, 4 and 24 h after the CRD. Visceromotor reflex (VMR) was measured by recording the electromyogram at the abdominal muscle in response to CRD. Distal colorectum was removed for evaluating the presence of inflammation. No significant evidence of histological inflammation was seen in the colonic mucosa/submucosa after repeated CRD, which is confirmed by myeloperoxidase assay. The spinal content of CGRP and SP decreased significantly 4 h after CRD, while galanin and VIP levels increased gradually and reached highest level at 24 h (p < 0.05). The mRNAs in DRG were measured at 0, 4 and 24 h after the CRD. Visceromotor reflex (VMR) recording showed the rat’s colon became hypersensitive 4 h after CRD, a sequence parallel to the spinal changes of CGRP and SP in timeframe. Noxious mechanical distension of the colorectum causes an acute change in the spinal levels of excitatory neurotransmitters (CGRP and SP), probably reflecting central release of these peptides from sensory neurons and contributing to the hypersensitivity following the noxious CRD. This is followed by a slower change in the levels of the inhibitory neurotransmitter galanin and VIP. Such stimulation results in significant alternation of the gene expression in DRG, reflecting the plasticity of the neuronal response. In the absence of visceral inflammation, the aforementioned neuropeptides are important mediators in the processing of visceral pain/hypersensitivity.

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1. Introduction

A generally accepted characteristic of the patients with irritable bowel syndrome is a lower threshold for reporting
and visceral structures. Calcitonin (CGRP), substance P (SP), galanin and vasoactive intestinal peptide (VIP) are synthesized by nociceptor neurons in the DRG. Many studies support the hypothesis that these excitatory and inhibitory neuropeptides are important biochemical mediators in somatic pain pathways, which are evidenced by a significant alteration of the neuropeptides in spinal cord and DRG after somatic stimulation [5–13].

The above neuropeptides can also be found in the majority of visceral afferents [14], but the role of the aforementioned neuropeptides in mediating spinal mechanism of visceral pain is less well studied. Preliminary evidence has suggested a similar function for these neuropeptides in the processing of visceral pain/hypersensitivity. For example, in the spinal cord dorsal horn, SP contributes to the generation of central sensitization, while NK-1 receptor antagonists attenuate sensitization of dorsal horn neurons following repetitive noxious colorectal distension (CRD) [15]. Likewise intrathecal administration of a CGRP receptor antagonist attenuated the visceromotor reflex (VMR) in rats with colonic inflammation [16]. These observations suggest that CGRP and SP are also the main neuropeptides in the mediating visceral pain/hypersensitivity. Given the similarity of the function of neuropeptides identified in both somatic and visceral pain, we hypothesized that changes of neuropeptides in spinal cord and DRG in response to noxious (painful) colorectal distension (CRD) would mirror similar changes reported in somatic pain. We used a well-characterized and reproducible model of noxious visceral stimulation involving repeated CRD in rat [17–20] to study the pattern of release of key neuropeptides in the spinal cord and changes of the gene expression in primary sensory neurons. Since repeated CRD can induce a visceral hypersensitivity [21], we further evaluate the correlation between the alteration of VMR and changes of spinal neuropeptides following the noxious visceral stimulation.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (300–420 g) were used in this study. They were maintained on a 12 h light–dark cycle with standard laboratory chow and tap water ad libitum.

2.2. Experiment protocol

The study was conducted with the following 3 series of experiments.

In the first experiment, we evaluated the changes of spinal neuropeptides in rats and their gene expression in DRG. Rats were placed in plastic tunnels (6 cm diameter, 25 cm length) and, during the 3 days preceding the experiments, they were trained to stay in the tunnels and to undergo the CRD procedure. This training period minimized stress reactions during the experiments. The balloon used for distention was a latex glove finger (7 cm long) attached to a catheter (Disposable Rectal Catheter for Abdominal Pressure Measurements, 3.5 mm diameter, 20 cm length, Medtronic, Skovlunde, Denmark). The balloon was checked for possible leakage immediately before and after the experiment. The balloon was then inserted through the anal canal down to the rectum in the conscious rat, and the tube was secured to the base of the tail with tape. Then the tube was connected to a barostat (Medtronic, Skovlunde, Denmark). CRD consisted of 1 h of intermittent rapid phasic distentions (30-s duration; 60 mm Hg) with an interpulse (30-s duration; 0 mmHg). In the control rats (sham group), the tube was retained in the colon without distension. DRG and spinal cord were then removed at different time points (0, 4 and 24 h) after CRD (n = 6 in each group and each time point). In brief, each animal was anesthetized with chloral hydrate (0.6 mg/100 g intraperitoneal injection; Ferak, German) and perfused transcardially with 150 ml ice-cold Ca2+-free Tyrode’s solution (100 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, 0.4 mM MgSO4, 1.2 mM NaH2SO4, 5.5 mM glucose, 26 mM NaHCO3, bubbled with 95% O2/5%CO2) at 4 ℃. DRGs and spinal cord (from the level of T13 to S1) were quickly removed from the animals and frozen on dry ice. The DRGs from one animal were pooled to give one sample. Both DRGs and spinal cord were stored at –80 ℃ until further measurement of peptide levels and their gene expression (see below).

In the second experiment, two groups of rats (sham and CRD groups, n = 8 in each group) were implanted with electromyogram (EMG) electrodes made from Teflon-coated gauge stainless steel wire (7 strand, A-M Systems, Inc., Rlsborg, WA, USA) in the external oblique muscle of the abdominal wall at least 7 days before the experiment. On the day of experiment, one-hour CRD or sham was performed with same protocol described in the experiment 1. Thereafter, a 5-minute rapid phasic distensions (30-s duration; 60 mm Hg) with an interpulse (30-s duration; 0 mm Hg) were given to the rats at 0, 4 and 24 h after the one-hour CRD. The CRD procedure with simultaneous electromyogram (EMG) recording was then performed as described above. The EMG was recorded using a CED 1401 instrument and analyzed using Spike 2 for windows software (Cambridge Electronic design, Cambridge, UK). The raw EMG signal was rectified off line. The baseline activity was subtracted, and the area of the rectified response was divided by the duration of CRD.

In the third experiment, we determined the inflammation in colonic mucosa. The distal 7 cm colorectum was separated after CRD or tube insertion in another set of animals (n = 4 at each time point, in either the control or experiment group). It was divided longitudinally into two halves: one half was fixed in 10% formalin, and the other half was used for myeloperoxidase (MPO) analysis. The severity of inflammation in colonic mucosa (none, mild,
moderate, and severe) was graded on a formalin-fixed, paraffin-embedded, H and E-stained section after a blinded review by our gastrointestinal pathologist (Lai CR). MPO activity was determined spectrophotometrically by the Abs260. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the endogenous control, with each sample normalized to GAPDH content.

2.4.2. Primers and DNA probe

Primers and probes were designed using Primer Express software (Perkin-Elmer). The probes were designed specifically to span an intron in order to avoid potential amplification of small amounts of contaminating DNA in the analysed samples. The probes were labelled at the 5' end with 6-carboxy fluorescein (FAM) and at the 3' end with 6-carboxy-tetramethyl rhodamine (TAMRA). Primers and probes were stored at −20 °C until use. Table 1 shows the primers and probe sequences for the following: CGRP (GenBank accession no: L29188), preprotachykinin (PPT, GenBank accession no: M15191), galanin (GenBank accession no: J03624), VIP (GenBank accession no: X02341), and GAPDH (GenBank accession no: AF106860).

2.5. Single-step real-time quantitative RT-PCR

Real-time quantitative RT-PCR analysis was performed using the Taqman One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA, USA). Total RNA (50 ng) was reverse transcribed in a 25 μl reaction mixture at 48 °C for 30 min. After 10 min denaturation at 95 °C for 15 s and extension at 60 °C for 60 s in the presence of the fluorescent oligonucleotides indicated above, RT-PCR analyses were carried out at least twice for each gene transcript to determine consistency of results, and GAPDH mRNA was assessed as an internal control for RNA quality. Reactions were recorded and analysed using the ABI 7700 Prism Sequence Detection System (Perkin-Elmer Applied Biosystems, Warrington, USA). We adopted the comparative Cₜ (threshold cycle) method to get the relative changes in the target gene. Briefly, the Cₜ value is a unitless value defined as the fractional cycle number at which sample the fluorescence signal passes a fixed threshold above baseline. Samples with a high starting copy number show an increase yields for the DRGs of each rat was determined spectrophotometrically by the Abs260. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the endogenous control, with each sample normalized to GAPDH content.

Table 1

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers</th>
<th>Probes (5'FAM-3' TAMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P</td>
<td>Sense 5'-AAGTGTGTTGCACACCTGAGCTGCA-3' Antisense 5'-AACCAAGGGAAGCCGCAAGA-3'</td>
<td>ACAGGAGGTTCCTCCTGGCTCCAGCAGCA</td>
</tr>
<tr>
<td>CGRP</td>
<td>Sense 5'-ATGTTGCTACTGCCCCACAGGAAGGTC-3' Antisense 5'-ACATTGGTGGCGCAAATGGT-3'</td>
<td>TCAGCAGCTCCTGGAC</td>
</tr>
<tr>
<td>VIP</td>
<td>Sense 5'-TTCGAGGTCTCAGGACCAGGA-3' Antisense 5'-TACTGGGAAATCAGACCTCGT-3'</td>
<td>CTAGGCTTACACATCCACAGCAACAGCAGAAGG</td>
</tr>
<tr>
<td>Galanin</td>
<td>Sense 5'-TCTGGGACACATCCACCTGT-3' Antisense 5'-CAGCTACAAAGGAGACAAGAAGT-3'</td>
<td>CACCCTTGCTTCTCAGTATAATTTAAGTCATTCTGCCC</td>
</tr>
<tr>
<td>GADPH</td>
<td>Sense 5'-ATGTATCCGTTGGAATCTGAC-3' Antisense 5'-AGCCCAGGATGCCCTTTATG-3'</td>
<td>TCGGGCCGCTTCCACCAC</td>
</tr>
</tbody>
</table>
in fluorescence early in the PCR process, resulting in a low $C_T$ number, whereas a lower starting copy number results in higher $C_T$ numbers. Then the $\Delta C_T$ is determined by subtracting the average GAPDH $C_T$ value from the average target gene value, in both controls and experiment group. The $\Delta \Delta C_T$ is a subtracted value of $\Delta C_T$ between the controls and experiment group. The folds of increment of target genes were expressed as $2^{-\Delta \Delta C_T}$.

2.6. Statistical analysis

Results were expressed as the mean±S.E.M. Statistical analysis was conducted using a Student’s $t$ test, ordinary or repeated-measures ANOVA test, followed by a Tukey’s test to determine significance.

3. Results

3.1. Changes in the content of CGRP, SP, galanin and VIP in the spinal cord (Fig. 1)

By 4 h following CRD, there was a 20.0% decrease in the tissue content of CGRP and a 24.4% decrease in the SP of the spinal cord when compared with the controls ($p<0.05$) (Fig. 1). These neuropeptides then gradually returned to the control level after 24 h. On the contrary, the spinal galanin and VIP levels progressively increased and reached its peak (98.2% for galanin and 189.3% for VIP, $p<0.05$) at 24 h. No significant difference was seen in spinal cord levels of any of the neuropeptides at any time point in the control rats.

3.2. Quantitative analysis of CGRP, PPT, galanin and VIP expressions in DRG at different time point (Figs. 2 and 3)

After CRD, the $\Delta C_T$ level of CGRP and PPT were persistently decreased and reached its maximum at 24 h (Figs. 2 and 3). This implied a 3- and a 12-fold increase for CGRP mRNA and PPT mRNA 24 h after CRD, respectively. The $\Delta C_T$ level of galanin at 0 h was the lowest among the 3 time points, which implies the galanin mRNA reached its highest (23-fold from controls) immediately after CRD and returned to normal four hours after CRD. The $\Delta C_T$ level of VIP persistently decreased from 0 to 24 h after CRD, which denotes a persistent upregulation of VIP mRNA of about 2-fold throughout the 24 h period after CRD.

3.3. Viscero-motor response after one-hour CRD (Fig. 4A and B)

After the one-hour CRD, the colon become hypersensitive at 4 h (between CRD and control group at 4 h after CRD:

![Fig. 1. Line graphs illustrating the average CGRP, SP, galanin and VIP content in the spinal cord in control and balloon-distended rats. The CGRP and SP were significantly decreased at 4 h after colorectal distension (CRD), while the galanin and VIP progressively increased and reached its peak at 24 h ($n=6$ for each group). Data are expressed as mean±SEM; *: Control vs. CRD, $p<0.05$. (between control and CRD group), #: 0 vs. 24 h in CRD groups, $p<0.05$; and : 4 vs. 24 h in CRD groups, $p<0.05$ (within CRD group).]
sham vs. CRD, \( p < 0.01 \); within the CRD group: 4 vs. 0 h and 24 h, \( p < 0.05 \) (Fig. 4A and B). Within the CRD group, the EMG signal at four hours after CRD increased by 39.7% than that at 0 h. This hypersensitivity returned to baseline at 24 h. In the sham stimulation group, no significant difference in VMR was noted among the 3 time points.

Fig. 2. Real-time PCR analysis of CGRP, prepro-tachykinin (PPT), galanin and VIP mRNA in rat DRG. \( \Delta C_T \) is determined by subtracting the average GAPDH \( C_T \) value from the average target gene value, in both controls and experiment group. Each \( \Delta C_T \) value is a mean of six independent experiments ± S.E.M. *: Control vs. CRD, \( p < 0.05 \). (between control and CRD group), #: (4 or 24 h) vs. 0 h, \( p < 0.05 \); and #: 4 vs. 24 h, \( p < 0.05 \) (within CRD groups).

Fig. 3. Bar graphs illustrating the expression of CGRP, prepro-tachykinin (PPT), galanin and VIP mRNA in rat DRG. The folds of increment of target genes were expressed as \( 2^{-\Delta C_T} \). The \( \Delta C_T \) is the subtracted value of \( C_T \) between the controls and experiment group.
3.4. Colonic inflammation and MPO activity after CRD

To determine whether the CRD procedure induced an inflammatory response in the colonic mucosa, colons were examined histologically and MPO activity were measured. Following CRD, no evidence of acute inflammatory response could be identified in the mucosa or submucosa by histological examination at any time point, a finding confirmed by MPO activity in the colonic wall (Fig. 5). The mean value of MPO was not different at any time point.

4. Discussion

The current study has demonstrated that a noxious mechanical distension of the rectum can cause alterations in the spinal levels of the CGRP, SP, galanin and VIP with significant changes in the expression of their genes in primary sensory neurons. Such changes have been previously reported in the setting of peripheral inflammation during which numerous endogenous substances are released or synthesized (e.g., nerve growth factor, prostaglandins, leukotrienes, bradykinin, histamine, 5-HT), with the potential to alter nociceptive processing [23–27]. In our study, we found no evidence of overt inflammation in the rectal mucosa after CRD. Although a contributory role for subtle tissue injury cannot be completely excluded, our results demonstrate that ‘pure’ mechanical distension in a hollow viscus can by itself result in significant alternations in peripheral neurotransmitters.

CGRP and SP have clearly been shown to be important in the mediation of somatic pain. The central release of SP from DRG neurons in response to peripheral stimulation facilitates glutamate-induced calcium flux with subsequent activation of key enzymes and gene expression responsible for increased excitability [26]. Noxious stimulation also results in the spinal release of CGRP, which is colocalized with SP in DRG neurons [27]. Although the exact role of CGRP has yet to be clarified, it appears to protect SP from degradation and thus may have a facilitatory role in nociception [28]. Recent studies have suggested a role for both CGRP and SP in visceral pain as well. For example, administering CGRP or tachykinins receptor antagonists can reverse the abdominal contraction induced by peritoneal irritation or CRD [16,21,29]. Similarly, Traub et al. found a significant decrease in CGRP and SP labelled DRG cells after experimental colitis was induced by intracolonic zymosan [15]. It has also been suggested that SP may in fact have a more important role in visceral pain as compared with somatic pain, since transgenic mice lacking the SP receptor
NK-1 do not develop hyperalgesia after visceral inflammation but still do so after somatic inflammation [30]. In our study, we found that the spinal content of both CGRP and SP was significantly decreased 4 h after CRD and had returned to control levels by 24 h. Similar findings have been reported in somatic pain models [8,31–35]. According to these reports, the spinal cord content of both peptides decreased within the first 24 h of inflammation and then gradually returned to normal during the following days. Such changes are also observed in rat models of nerve injury [6,9]. The acute decrease in neurotransmitter content in the current study is presumably a result of increased release from the central terminals of DRG neurons and subsequent uptake and/or degradation. Our results therefore provide more evidence to support the concept that CGRP and SP are involved in the acute response to noxious stimulation in the gut.

Galanin is a 29-amino acid peptide, which is present in a small population of primary sensory neurons and is also colocalized with CGRP and SP [36]. Electrophysiological and behavioral studies have suggested that under normal conditions galanin exerts tonic inhibition of nociceptive input to the central nervous system [36]. Galanin agonists have also been reported to be useful in the treatment of inflammatory and neuropathic pain [37]. After peripheral nerve injury or inflammation, galanin levels increased in the DRG or spinal cord [38,39]. Together, these findings suggest that galanin may be part of a physiological “antinociceptive” response to painful stimuli. The role of galanin in processing “visceral pain” has not been studied. Our data suggests that spinal galanin levels progressively increase and reach a significant level 24 h after CRD. Since we did not study the actual release, the implications of this finding are open to interpretation. However, given the increase in galanin mRNA in DRG neurons (see below), one possible explanation is that the increase in galanin is a reflection of the accumulation of this peptide in the central projections of primary sensory neurons. Analogous to somatic models, an increase in galanin could therefore represent a “compensatory” mechanism in response to noxious visceral stimulation.

VIP, a 28 amino-acid polypeptide, is normally co-expressed with galanin in the small-to-medium DRG neurons and is highly concentrated in the superficial laminae of the spinal cord [12]. The locations of VIP in DRG or the spinal cord suggests its association with nociception [12]. In somatic pain, VIP is known to be upregulated in DRG and its central projections in the dorsal horn of the spinal cord [12]. In addition, VIP participates in spinal reflex hyperexcitability induced by conditioning stimulation [40]. This observation suggests that VIP may be excitatory neurotransmitters or the neuromodulator of sensory transmission in neuropathic pain states (somatic pain). VIP has also been suggested as taking over the excitatory role of SP following peripheral nerve injury [13]. The role of VIP in “visceral pain” is still unknown, but it may function in a similar way to somatic pain.

Our data also shows that the DRG mRNA for CGRP, PPT, galanin and VIP were all significantly upregulated after CRD. The increase in mRNA can result in an increase in the production of the CGRP, SP, galanin and VIP by the primary afferent neurons. This contributes to the normalization of the spinal levels of CGRP and SP, as well as the compensatory increase of galanin and VIP after CRD. Although changes in the biosynthesis of sensory neuropeptides have been found in several animal models of tissue inflammation or nerve damage [6,7,9,40,41], induction of plastic changes in the sensory neurons of the DRG in response to CRD has not been previously reported. On the contrary, Reinschagen et al. reported no change in the expression of SP in DRG after colonic inflammation induced by formalin instillation in guinea pigs [42]. The reason for this discrepancy is not clear, but it may be resulted from the differences in experimental techniques, animal species or the nature of the noxious stimulus.

In the current study, we demonstrated that visceral sensitization can occur after short-term mechanical distension, similar to Gschossmann’s observation showing a 50% increment in VMR after phasic CRD [21]. We further showed that the visceral hypersensitivity can return to normal 24 h after the initial one-hour noxious CRD. This timeframe in the development of visceral hypersensitivity paralleled with the changes in spinal CGRP and SP. Several reports have already suggested both CGRP and SP are important mediators in the induction and maintaining of visceral hypersensitivity. For example, intravenous or intrathecal infusion of a CGRP antagonist can inhibit the visceral hypersensitivity induced by intracolonic instillation of acetic acid [16] or phasically colorectal distension [21]. On the other hand, giving NK1 receptor antagonist can also inhibit the visceral hypersensitivity induced by chemical irritation to the rabbit colon [43]. Therefore, we suggest that the CGRP and SP may also be responsible for the observed visceral hypersensitivity in our study.

In conclusion, a short-term noxious mechanical distension of the rectum can induce an acute change in the spinal levels of CGRP and SP and a slower change in the levels of the galanin and VIP. These results support the hypothesis that CGRP, SP, galanin and VIP, in the absence of visceral inflammation, are also important mediators in the visceral pain/hypersensitivity. Repeated mechanical visceral stimulation can induce visceral hypersensitivity, which may be due to the release of CGRP and SP in spinal cord. The changes in the spinal neuropeptides are accompanied by an upregulation of the expressions of their genes in DRG, reflecting the plasticity of the neuronal response.

Acknowledgements

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