Effects of neonatal maternal separation on neurochemical and sensory response to colonic distension in a rat model of irritable bowel syndrome

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Am J Physiol Gastrointest Liver Physiol 292: G849–G856, 2007. First published November 16, 2006; doi:10.1152/ajpgi.00400.2006.—Early life stress has been implicated as a risk factor for irritable bowel syndrome (IBS). We studied the effect of neonatal maternal separation on the visceral motor response and the expression of c-fos, 5-HT, and its receptors/transporters along the brain–gut axis in an animal model of IBS. Male neonatal Sprague-Dawley rats were randomly assigned to a 3-h daily maternal separation (MS) or nonhandling (NH) postnatal days 2–21. Colorectal balloon distention (CRD) was performed for assessment of abdominal withdrawal reflex as a surrogate marker of visceral pain. Tissues from dorsal raphe nucleus in midbrain, lumbar-sacral cord, and distal colon were harvested for semi-quantitative analysis of c-fos and 5-HT. The expression of 5-HT expression, 5-HT3 receptors, and 5-HT transporter were analyzed by RT-PCR. Pain threshold was significantly lower in MS than NH rats. The abdominal withdrawal reflex score in response to CRD in MS rats was significantly higher with distension pressures of 40, 60, and 80 mmHg. In MS rats, the number of c-fos-like immunoreactive nuclei at dorsal horn of lumbar-sacral spinal cord increased significantly after CRD. 5-HT content in the spinal cord of MS rats was significantly higher. In the colon, both 5-HT-positive cell number and 5-HT content were comparable between MS and NH groups before CRD. Post-CRD only MS rats had significant increase in 5-HT content. Protein and mRNA expression levels of 5-HT3 receptors and 5-HT transporter were similar in MS and NH rats. Neonatal maternal separation stress predisposes rats to exaggerated neurochemical responses and visceral hyperalgesia in colon mimicking IBS.

visceral hyperalgesia; neonatal maternal separation; serotonin; 5-HT; c-fos

IRRITABLE BOWEL SYNDROME (IBS) is a common functional disorder characterized by symptoms of abdominal pain and discomfort associated with alterations in bowel habits in the absence of a demonstrable structural abnormality. The symptoms are believed to involve changes in perception to visceral stimuli, leading to visceral hypersensitivity (19, 20). Abnormal central processing of nociceptive signals in the brain–gut axis plays an important role in the pathogenesis of visceral hypersensitivity. The processing of visceral nociceptive signals involves an integrated network of various structures, which include sensory cortex, thalamus, hypothalamus, and midbrain.

These centers modulate processing of the nociceptive signals at the spinal cord level through descending pathways (33).

Pharmacological studies have identified receptors for different neuromediators involved in processing the information transmitted along afferent pathways. Recently, there is growing interest in serotonin (5-HT) for its possible involvement in IBS. 5-HT distributes predominantly in the gastrointestinal tract [90% in enterochromaffin cells (EC) and 10% in enteric neurons] and exists in the brain only in minute amounts. Release of 5-HT from EC cells acts as transducers for activation of afferent projections to the central nervous system (13, 16). 5-HT released by EC cells within the mucosa by intraluminal distension or irritation stimulated 5-HT3 receptors located on the primary afferent neurons of both splanchnic and vagal fibers, thereby modulating sensory response (32). Alteration in 5-HT signaling may contribute to gastrointestinal dysfunction and hypersensitivity in IBS (4, 12). Recent work has demonstrated distinct changes in intestinal EC cells number, 5-HT content, and 5-HT release and reuptake in IBS patients (10, 12, 37). 5-HT3 receptor antagonist blocked the transmission of afferent signals, thus reducing visceral hypersensitivity and relieve pain in IBS patients (3, 15).

On the other hand, the immediate proto-oncogene c-fos, expressed in dorsal horn neurons in the spinal cord following visceral stimulation, has been used to index neuronal activation secondary to visceral nociception (11, 18, 34). Repetitive colorectal balloon distention (CRD) induced c-fos expression in spinal cord and increased in Fos-like immunoreactive neurons. Fos-like immunoreactive nuclei were most commonly found in the lumbar-sacral spinal segments following CRD, which corresponded to afferent projections from the pelvic nerve (34). Morphine has been shown to attenuate c-fos expression in the rat spinal cord following noxious CRD, leading to pain relief (14, 35).

Maternal separation of newborn rats is a well-established model of early life stress that results in permanent changes in central nervous system (6, 9). Coutinho et al. (9) showed that maternal separated rats suffered from stress-induced visceral hyperalgesia and increased colonic motility that mimic IBS in human. To date, the impact of early life stress on neurochemical responsiveness of visceral nociceptive pathway is unclear. We studied the effect of neonatal maternal separation stress on the physiological response and the neuronal reactivity to nociceptive visceral stimulus. The expression of 5-HT and c-fos

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along the brain-gut axis was evaluated in this experiment to delineate the neurological mechanisms of visceral hypersensitivity in IBS.

**METHODS**

**Animals**

Sprague-Dawley neonates were obtained from Laboratory Animal Services Center of The Chinese University of Hong Kong on postnatal day 1. To avoid the influence of estrogen and hormonal cycles on neurochemical and sensory response of bowel to stimulations, we used only male pups in this experiment. The litters were randomly assigned to one of two rearing conditions: 1) maternal separation (MS) group or 2) control, nonhandling (NH) group.

During postnatal days 2–21, inclusive, litters were exposed to a 180-min period of daily maternal separation (9). Manipulation commenced at 0800 with removing the dams and placing them into separation cages, whereas the litters were removed as a group in an isolation cage in an adjacent thermoregulated room maintained at 20°C. Litters were then returned to their maternity cage with the foster dam. Eight pups were fostered as a group by a dam housed in a single isolation cage in an adjacent thermoregulated room maintained at 20°C. Animals were housed on a 12:12-h light-dark cycle with access to food and water ad libitum. Litters were weaned on day 22. Animal care and experimental procedures were conducted following institutional ethics guidelines and conformed to the requirements of the Institutional Animal Care and Use Committee of the University of Maryland-Baltimore and animal ethics committee of Chinese University of Hong Kong.

**CRD and Behavioral Testing in Rats**

On day 60, CRD was given and abdominal withdrawal reflex (AWR) was quantitatively measured in each individuals of both MS and NH groups (1, 22). Rats were first anesthetized with isoflurane. The balloon was constructed from a latex glove finger (6 cm of length) and connected via a Y connector to a syringe pump and a sphygmomanometer, which was used to keep the pressure at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

CRD (at 10, 20, 40, 60, and 80 mmHg, 20-s inflation and followed by a 4 min interval of deflation) and the threshold intensity (increments of 10 mmHg starting at 10 mmHg) were obtained by an observer unaware of the randomization. The AWR scores were as follows: 0, no behavioral response to CRD; 1, brief head movement followed by immobility; 2, contraction of abdominal muscles; 3, lifting of abdomen; 4, body arching and lifting of pelvic structures. The threshold intensity of CRD was defined as the stimulus intensity that evoked a visually identifiable contraction of the abdominal wall (i.e., score 2 or above) (1). All the measurements were performed twice, and the average of the results was obtained for further analysis. Because c-Fos should be evaluated 1 h poststimulus, we added four additional 80 mmHg distensions in the CRD. After the 14 phasic distensions (each lasted for 20 s) and the 4-min intervals between each distension (thus 20 s x 14 + 4 min x 13 = 57 min 40 s), adding the time required for dismantling the balloon, syringe pump, and sphygmomanometer, it was roughly 1 h poststimulation before we evaluated the neurochemical response.

**Tissue Preparation and Immunohistochemistry**

Immediately after CRD, adult rats were injected with 7% chloroform (35 mg/100 g body wt) intraperitoneally and perfused transcardially with 0.85% normal saline solution (~100 ml) followed by 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4, ~300 ml). Tissues including distal colon (6–7 cm from anus), lumbosacral spinal cord (L6–S1), and midbrain were dissected out and postfixed in the same fixative overnight. Afterward tissues were cryoprotected in 25% sucrose in 0.1 mol/l phosphate buffer for ~48 h and were embedded in OCT compound and frozen in isopentane chilled in liquid nitrogen. Every fifth sections of the tissue (15-μm thickness for colon and 40-μm thickness for spinal cord and brain) were obtained (17, 31).

We chose to sample tissue from the colon, lumbosacral cord, and dorsal raphe nucleus (DRN) in the brain-gut axis for the following reasons: 1) 5-HT distributes mainly in the gastrointestinal tract (mostly in the mucosa and some in enteric plexus) and only a minute amount is located in the brain. Although mucosal 5-HT was reported to play an important role in visceral hyperalgesia and the role of enteric plexus is less clear, we examine both the colonic mucosa and the enteric plexus in this study. 2) Most 5-HT in the brain is concentrated in the raphe nucleus. Although the DRN is not the only component of the pain pathway, it has a major role in visceral pain.

### Table 1. Primers and annealing temperatures used for PCR experiments

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence 5’-3’</th>
<th>Temperature Cycle Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT3A (sense)</td>
<td>cat gta tgc cat cct cca cg</td>
<td>50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min</td>
</tr>
<tr>
<td>5-HT3A (antisense)</td>
<td>cca cgt cca caa act cat tg</td>
<td></td>
</tr>
<tr>
<td>5-HT3B (sense)</td>
<td>gtt gaa gac ata gac ctt ggc ttc ctg ag</td>
<td></td>
</tr>
<tr>
<td>5-HT3B (antisense)</td>
<td>acc ctt cgc ttc ttc gca cct cat cag a</td>
<td></td>
</tr>
<tr>
<td>SERT (sense)</td>
<td>acc gat gtg asg aga atg ct</td>
<td></td>
</tr>
<tr>
<td>SERT (antisense)</td>
<td>gga cga cat cct cta tgc agt</td>
<td></td>
</tr>
<tr>
<td>β-actin (sense)</td>
<td>tgt cac cca ctg gga cga ta</td>
<td></td>
</tr>
<tr>
<td>β-actin (antisense)</td>
<td>ggg gtt gtc aag gtc tca aa</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Effect of increasing distension pressure CRD on the AWR score and threshold pressure in MS and NH groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Threshold Pressure, mmHg</th>
<th>AWR at Various Balloon Pressures</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mmHg</td>
<td>20 mmHg</td>
<td>40 mmHg</td>
</tr>
<tr>
<td>MS group (n = 24)</td>
<td>19.37±8.38</td>
<td>1.15±0.85</td>
<td>2.06±0.89</td>
</tr>
<tr>
<td>NH group (n = 24)</td>
<td>24.37±9.24</td>
<td>1.04±0.62</td>
<td>1.94±0.76</td>
</tr>
<tr>
<td>P value</td>
<td>0.036</td>
<td>0.958</td>
<td>0.817</td>
</tr>
</tbody>
</table>

Values are means ± SD. CRD, colorectal balloon distension; AWR, abdominal withdrawal reflex; MS, maternal separation; NH, nonhandling.
null group (post-CRD).

Fig. 1. Fos expression in lumbosacral spinal cord after colorectal balloon distention (CRD) (4). The brownish dots that represent c-fos immunoreactivity (c-fos-like immunoreactive nuclei) are found predominantly in laminae I and II. However, a scanty amount was found also in laminae V, VII, and X. The number of c-fos-like immunoreactive nuclei significantly increased in the maternal separation (MS) group after CRD (P < 0.05). D, dorsal horns; NH, nonhandling group (post-CRD).

Transmission (21, 26). The DRN is important not only in direct or indirect descending pain control but also in ascending pain modulation all the way to the thalamus and the prefrontal cortex (23, 27). Therefore, we chose to study 5-HT expression in the DRN. 3) Both thoracolumbar spine and lumbosacral spinal cord receive afferent input from the descending colon and the rectum. We only evaluated c-fos at the level of the lumbosacral spinal cord because the role of thoracolumbar spine segment in afferent input is less clear. Moreover, visceral afferent stimuli from the distal colon and rectum are predominantly conveyed to the lumbosacral spinal cord via the pelvic nerve (4). Therefore, many investigators chose the lumbosacral spinal cord for c-fos detection (17, 31). Noxious CRD induced expression of c-fos in the lumbosacral spinal cord bilaterally, mainly in laminae I, II, lateral V and VI, VII, and X (17). In the present study, c-fos expression in lumbosacral spinal segments was counted bilaterally in the entire dorsal horn (laminae I–VI) and laminae VII and X.

Immunohistochemical analysis of c-fos expression in spinal cord. Lumbosacral spinal cord (L6–S1) was dissected and processed as mentioned above. Sections were rinsed 3 × 10 min in 0.01 mol/l PBS (pH 7.4), preincubated for 20 min with 0.3% H2O2 in 0.01 mol/l PBS, rinsed 3 × 10 min in 0.01 mol/l PBS, then preincubated for 30 min with 5% normal goat serum (NGS) in 0.01 mol/l PBS and incubated overnight at room temperature with rabbit anti-Fos polyclonal antibody (Oncogene Research Products, San Diego, CA; rabbit anti-rat, 1:16,000) in 0.01 mol/l PBS with 1% NGS and 0.3% Triton X-100. After rinsing, sections were incubated for 90 min with secondary goat anti-rabbit immunoglobulin G (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) 1:200 in 0.01 mol/l PBS with 1% NGS and 0.3% Triton, rinsed 3 × 10 min in 0.01 mol/l PBS, incubated for 90 min with tertiary antibody (avidin DH/biotinylated horseradish peroxidase H complex, Vectastain ABC kit) 1:100 in 0.01 mol/l PBS, and rinsed 2 × 10 min in 0.01 mol/l PBS. A peroxidase reaction was performed to visualize Fos immunolabeling by incubating with 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.01% H2O2 for 5 min before decanting and stopping the reaction with 0.01 mol/l PBS. After rinsing, sections were mounted on gelatin-coated glass slides, air dried, dehydrated in ethanol, cleared in xylol, and coverslipped. To assess antibody specificity, incubation with the primary antibody was omitted for some sections, and no significant staining was observed in this case. Numbers of Fos-like immunoreactive nuclei were counted bilaterally in eight consecutive sections through the L6-S1 segment at ×100 magnification, and the average number of immunoreactive cells was used for subsequent analysis.

Immunohistochemical analysis of 5-HT, 5-HT3 receptors, and 5-HT transporter in colon. Colon tissues were washed in 0.1 mol/l PBS, incubated with a 5-HT polyclonal antibody (Alpha Diagnostic International, San Antonio, TX; rabbit anti-rat, 1:1,000), 5-HT3R affinity-purified polyclonal antibody (Oncogene Research Products, San Diego, CA; rabbit anti-rat, 1:10) or 5-HT transporter polyclonal antibody (Oncogene Research Products; rabbit anti-rat, 1:200) for 12 h, followed by a 2-h incubation of goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:200; Jackson Immuno Research, West Grove, PA) at room temperature. Primary and secondary antibodies were diluted in Triton X-100 (0.3% in 0.1 mol/l PBS). NGS (Vectastain ABC kit) was added to a final concentration of 10% to reduce the nonspecific background staining.

Double immunostaining of c-fos and 5-HT in DRN in the midbrain. To confirm that DRN participated in modulating pain response and projected to extensive regions of the brain including the limbic system and the hypothalamus, the c-fos-positive cells were double stained with 5-HT (2). Brain tissue was placed in a mixture of 0.3% H2O2 until oxygen bubbling ceased and was incubated in 10% NGS in PBS for 1 h to block sites of nonspecific binding of serum products. Fos immunoreactivity was detected by a standard avidin-biotin peroxidase protocol. This method produced a blue-black nuclear reaction product. Sections were then incubated overnight at room temperature in polyclonal rabbit anti-5-HT antibody diluted 1:20,000 in a solution of PBS containing 2% NGS and 0.3% Triton X-100. After incubation, sections were rinsed and incubated in the appropriate biotinylated secondary antisera and avidin-biotin peroxidase complex (Vector, 1:200 dilution in 1% NGS-PBS). Cytoplasmic 5-HT immunoreactivity was detected with 3,3-diaminobenzidine to produce a brown reaction product. Controls for the Fos-immunoreactive and 5-HT-immunoreactive cells were con-
transport protein (5-HTT) was quantified by real-time RT-PCR. Seg-

duction of 5-HT is mainly found in the lamina propria (white arrowhead) or close to the mucosal surface (white arrow) of the distal colon (×20 magnification). Expression and distribution of 5-HT are significant higher in MS than in NH rats after CRD ($P < 0.001$).

**Image Analysis**

All stained sections of harvested tissues (brain, spinal cord, and colon) were examined under Zeiss AxioPhot 2 imaging Universal Fluorescence Photo-Microscope with Axio cam (Universal Microscope), and images were captured by a SPOT cooled color digital camera equipped with Spot32 software (Diagnostic Instrument). All the images were analyzed by Metamorph 4.0 software (Universal Imaging), which performed automatic measurement of areas defined using an interactive threshold editing function. The latter resulted in colored overlay that marked which pixels in the image to be measured. Thresholding an image defined “objects” to be measured and segmented them from background. The process consisted of deciding whether a pixel was part of an object to be measured or was merely part of the background based on the intensity of the pixel. We segmented the image by selecting an upper and lower threshold to define a range of acceptable grayscale levels, and the image processor would group all the contiguous pixels that fall within that range into object.

In this study, areas of c-fos and 5-HT expression were stained in brown. They were quantified in the following areas of the nociceptive surface (white arrow) of the distal colon (19.37 ± 8.38 mmHg) ($P < 0.001$) 384.4 ± 103.4 mmHg) in the animals before and after CRD. The greenish dots by immunofluorescence represent 5-HT activities. Distribution of 5-HT is mainly found in the lamina propria (white arrowhead) or close to the mucosal surface (white arrow) of the distal colon (×20 magnification). Expression and distribution of 5-HT are significant higher in MS than in NH rats after CRD ($P < 0.001$).

**Measurement of the 5-HT Content of the Colon and Spinal Cord**

A segment of distal colon (~6 cm from anus) and lumbosacral spinal cord were removed from the animal, homogenized in 0.2 N HClO$_4$, and centrifuged at 10,000 g for 5 min. The supernatant was neutralized with 0.5 ml of 1.0 M borate buffer (pH 9.25) and centrifuged at 10,000 g for 1 min. The 5-HT content was analyzed by commercially available enzyme immunoassay according to the manufacturer’s instructions (Invitrogen). Real-time quantitative PCR amplification reactions were carried out in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBRgreen I as the detection format in a 25-μl volume. The specific primers used are listed below (Table 1).

**Data Analyses**

All experimental data were presented as means ± SE. Statistical analyses were performed by using SPSS11.0 (SPSS, Chicago, IL). Comparisons between two groups were made by $t$-test or Mann-Whitney $U$-test as deemed appropriate. Comparisons between three or more groups were done with one-way ANOVA. Differences between means at a level of $P < 0.05$ were considered to be significant.

**RESULTS**

Forty-eight rats (24 in the MS group and 24 in the NH group) were used in this study. Histological sections from the colons of 16 rats (8 MS, 8 NH) were examined. The tissues showed no structural abnormality. Mucin depletion or increase in intraepithelial lymphocytes was not seen in any of the tissue examined.

**Colorectal Distention and Pain Response in Rats**

The threshold pressure to elicit abdominal muscle contraction in response to CRD was significantly higher in the animals of the NH group (24.37 ± 9.24 mmHg) than in the MS group (19.37 ± 8.38 mmHg) ($P = 0.036$). A graded response was seen in both groups with a stepwise increase in balloon pres-

| Table 4. 5-HT content in colon and lumbosacral spinal cord in MS and NH animals before and after CRD |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | Colon (ng/g tissue) | Spinal Cord (ng/g tissue) |
|                 | Pre-CRD          | Post-CRD        | Pre-CRD          | Post-CRD        |
| MS group (n = 5) | 384.4 ± 145.4    | 682.4 ± 101.9  | 661.0 ± 97.1     | 639.1 ± 103.4   |
| NH group (n = 5) | 509.5 ± 81.8     | 470.0 ± 151.3  | 474.5 ± 91.0     | 402.9 ± 57.2    |
| $P$ value       | 0.177            | 0.017           | 0.004            | 0.001           |

Values are means ± SD. After CRD, colonic content of 5-HT was significantly higher in MS animals. The 5-HT contents of spinal cord in MS animals were significantly higher than that of NH animals before and after CRD.
sure from 10 to 80 mmHg (Table 2). MS rats showed an exaggerated abdominal response to visceral pain compared with NH rats. The AWR score in response to CRD in the MS group was significantly higher than NH group at distension pressures of 40, 60, and 80 mmHg (Table 2).

c-fos Expression in Spinal Cord

In NH animals, total numbers of Fos-like immunoreactive cells were very low and hence difficult to assess regional distribution. Repetitive CRD caused a significant increase in Fos-positive cells in the lumbosacral spinal in NH rats. The expression sites were located in the dorsal horns of the spinal gray matter, predominantly in laminae I and II, but a scanty amount was also found in laminae V, VII, and X (Fig. 1). The number of c-fos-like immunoreactive nuclei were significantly increased in the MS compared with the NH group (89.4 ± 27.5 vs. 41.8 ± 16.7; P < 0.05) after CRD (Fig. 1).

5-HT, 5-HT3 Receptors, and 5-HT Transporter in Colon and Spinal Cord

The numbers of EC cells expressing 5-HT were comparable between MS and NH groups before CRD. After CRD, there was an increase in 5-HT positive cell in the MS group (from 295.1 ± 21.1 to 383.9 ± 29.6) but not in the NH group (from 288.4 ± 63.3 to 292.9 ± 54.7) (Table 3). The distribution of 5-HT in the colon is shown in Fig. 2. Most of these cells are found in the lamina propria or at the base of the mucosal surface. A scanty amount of 5-HT expression was also found in enteric plexus, but only in sporadic distribution in some sections. The colons and lumbosacral spinal cords were homogenized, and 5-HT was measured by enzyme immunoassay. 5-HT content of the colon was comparable between the MS and NH groups before CRD. After CRD, there was a significant increase in 5-HT content in the colon of the MS group but not the NH group (Table 4). The content of 5-HT of spinal cord was significantly increased in MS compared with the control group both before and after CRD.

5-HT3 receptors and 5-HTT immunoreactivity in both the mucosa and in the neurons of the myenteric plexus are shown in Figs. 3 and 4. Both expression intensity and distribution of 5-HT3 and 5-HTT were similar in the MS and NH groups. To determine whether the expression of 5-HT3A, 5-HT3B subunit, and 5-HTT were altered in CRD, real-time RT-PCR was used to quantify mRNA encoding 5-HT3A, 5-HT3B subunits, and 5-HTT in colons from adult rats treated with MS and control animals. Transcripts encoding 5-HT3A, 5-HT3B subunit, and 5-HTT were normalized to those encoding β-actin. The ratio of mRNA encoding 5-HT3A, 5-HT3B subunit, and 5-HTT to that of β-actin was not significantly different in the MS and NH rats (Table 5).
5-HT and c-fos Double Staining in DRN in Midbrain

Within the DRN, the Fos-immunoreactive cells were mainly located in the ventrolateral and dorsal subdivision after CRD (Fig. 5). The number of Fos-immunoreactive cells was significantly increased in the MS group when compared with the NH group (Table 5). Double-immuno-labeled fos/5-HT neurons in the DRN are shown in Fig. 5. The MS group showed a significant increase in the number of Fos/5-HT-immunoreactive cells compared with the NH groups (Table 6).

**DISCUSSION**

In rats, early life stress in the form of separation of neonates from the mother has been demonstrated to result in permanent changes in the central nervous system. Well characterized changes include unrestrained secretion of corticotrophin-releasing factor and increase expression of its receptors (24), increased regional norepinephrine release (30), downregulation of β2-receptors, decreased benzodiazepine receptor, and γ-aminobutyric acid type A receptor (6). Recently, it has also been demonstrated that neonatal maternal separation leads to altered stress-induced responses to viscerosomatic nociceptive stimuli mimicking features of IBS (9).

Rosztoczy et al. (28) used two protocols of maternal separation and studied the effects of rectal distension in adult male and female rats. From day 1 to day 14 during the postnatal period, maternal separation was introduced for 2 h per day by either removing all pups from their home cage (type M) or separating half of the littermates (type P). The authors reported that the development of long-term visceral hyperalgesia depends on the type of maternal deprivation and the sex of the animals. Although both separation models induced hyperalgesia, type M separation has more profound effects than type P, and females responded more remarkably than males. In our present study, to produce greater effects of visceral hyperalgesia without the influence of hormonal cycles, we used type M (i.e., total separation) and male pups. It is known that rearing conditions also have significant effects on acute and delayed stress-induced visceral hypersensitivity (29). Daily separation of 180 min from the dam leads to visceral hyperalgesia. Yet separation for 15 min per day leads to hypoalgesia. To mimic the clinical conditions of IBS, we chose to use the 180-min separation instead of 15 min. Although Schwetz et al. (29)
immunoreactive cells were more highly expressed in the MS than in NH rats. Double immunoreactivity in DRN (Fig. 5). Double immunostaining of 5-HT and c-fos in dorsal raphe nucleus (DRN). Double immunoreactivity in DRN \( \times 20 \) c-fos immunoreactive nucleus (small black blue particles, solid arrow), 5-HT immunoreactive cells (brown particles, arrowhead) and c-fos/5-HT double immunoreactive cells (brown particles with a dark nucleus, dotted arrow). Both c-fos and c-fos/5-HT double immunoreactive cells were more highly expressed in the MS than in NH rats.

Focused on central mediators of visceral hyperalgesia, namely corticotrophin-releasing factor and its receptor, we studied the two neurotransmitters of peripheral pain perception and modulation, namely 5-HT and c-fos. We believe that our data and those of Schwetz et al. are complementary to each other in the understanding of stress related visceral hypersensitivity.

Assessing painful symptoms in animals has been a major obstacle in the study of IBS. AWR is introduced as a semi-quantitative method gauging involuntary motor reflex in response to visceral pain (1, 22). It involves a supraspinal loop in the brain-gut axis. The AWR is reproducible and graded with intensity of the stimuli. In the present study, we used the model of neonatal maternal separation and demonstrated that threshold of visceral nociceptive response was reduced by using colorectal balloon distention and grading of abdominal reflex (1). Compared with control animals without early life stress, the MS rats demonstrated painful responses to a much lower balloon distension pressure and exaggerated abdominal withdrawal responses to high pressures. The aggravated response to noxious CRD in MS rats compared with NH rats indicates visceral hyperalgesia. Unlike previous studies that showed only changes in bowel motor function by counting fecal pellet output (5), this study demonstrates visceral hyperalgesia in animals reared in early life stress environment.

Noxious CRD also induced expression of the proto-oncogene c-fos in the lumbosacral spinal cord of the animals. Fos-like immunoreactive nuclei were found predominantly in the dorsal horn. Induction of c-fos is thought to be largely indicative of c-fiber afferent activation (8, 25). The majority of afferent nerves innervating the colon are c-fibers, most of which showed a graded response to increasing stimulus intensity. In the present study, the number of c-fos-like immunoreactive nuclei was significantly increased in MS but not in NH animals after CRD. This suggests that maternal separation led to hyperactivation of neurons in the dorsal horn in response to CRD. The hyperactivation of neurons in the dorsal horn in MS rats can develop in response to either peripheral tissue irritation or to descending influences originating in the brain stem (36).

Previous studies have shown that 5-HT is found primarily in mucosal EC, where it appears to serve as a neurotransmitter of the enteric nervous system and a paracrine molecule signaling other neural activity such as bowel contraction, relaxation, intestinal secretion, and pain sensation (13). Postprandial level of 5-HT is significantly higher in patients with IBS (4). In the present study, there was no significant difference in the number of 5-HT-positive cells in the gut between the MS and NH groups at baseline. After CRD stimulation, a significant increase in cells expressing 5-HT in MS animals was documented. Thus whereas the early life stress event has not increased the number of 5-HT-secreting cells, an exaggerated response of 5-HT will occur on encountering stressful events or noxious stimuli. The result suggests that the early life event in maternal separation leads to permanent alterations in the central nervous system that sensitizes certain neurochemical changes along the brain-gut axis, causing an abnormal response of intestinal 5-HT expression to visceral stimuli. The increased responsiveness is not a result of altered expression of 5-HT receptors and 5-HT transporters because we have detected no difference in mRNA of these mediators. Hyperalgesia is therefore unlikely a result of increased affinity of 5-HT to receptors or a result of increased uptake capacity of 5-HT.

Besides its function at the level of the intestinal wall, 5-HT is also known to be an important modulator of nociception in the central nervous system (13, 16). The increase in c-fos expression in the DRN after CRD suggests that some neurons

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**Table 6. 5-HT and c-fos double staining in DRN in MS rats and NH rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Fos-ir Cells</th>
<th>Number of Fos-ir/5-HT Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS group (n = 8)</td>
<td>144.0 ± 34.6</td>
<td>26.3 ± 8.7</td>
</tr>
<tr>
<td>NH group (n = 8)</td>
<td>110.0 ± 12.0</td>
<td>16.9 ± 5.4</td>
</tr>
<tr>
<td>P value</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. DRN, dorsal raphe nucleus. Fos-ir, Fos-immunoreactive cells; Fos-ir cells/5-HT, both 5-HT and fos-immunoreactive cells. Number of c-fos and c-fos/5-HT coexpressed cells is higher in MS compared with NH group.
in this nucleus were activated by visceral stimuli. Animals in the MS group had upregulated expression of c-fos in DRN compared with that in the NH group, indicating enhanced perception of MS rats to visceral pain in the higher center other than the spinal cord. Among the activated neurons, most are serotoninergic (double positive for Fos and 5-HT). These findings suggest that enhanced responsiveness of central serotoninergic pathways also plays an important role in early stress-induced visceral hyperalgesia. The activation of the DRN's and its expression of 5-HT inferred not only its role in pain modulation, but also its control through extensive connections on other impact regions of the nervous system in this IBS rat model.

In conclusion, this study shows that early life stressful event leads to exaggerated neurochemical responsiveness to visceral stimuli. Visceral hyperalgesia in maternal separated rats is related to increased expression of c-fos and 5-HT in response to stress. This model would be useful for future studies on the pathogenesis and treatment of IBS.

GRANTS

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REFERENCES