Microglia-derived TNF-α inhibiting GABAergic neurons in the anterior lateral bed nucleus of the stria terminalis precipitates visceral hypersensitivity induced by colorectal distension in rats

Ning-Ning Ji [a,b,c,1], Qing-Xiang Meng [a,b,c,1], Ying Wang [a,b,c], Zi-Ming Zhou [a,b,c], Yu Song [a,b,c], Rong Hua [d], Yong-Mei Zhang [a,b,c,*]

[a] *Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, Jiangsu, China*
[b] *Jiangsu Province Key Laboratory of Anesthesia and Analgesia Application Technology, Xuzhou Medical University, Xuzhou, Jiangsu, China*
[c] *NMPA Key Laboratory for Research and Evaluation of Narcotic and Psychotropic Drugs, Xuzhou, Jiangsu, China*
[d] *Institute of Emergency Rescue Medicine, Xuzhou Medical University, Xuzhou, Jiangsu, China*

ARTICLE INFO

ABSTRACT

Irritable bowel syndrome (IBS) is a common and debilitating gastrointestinal disorder that is exacerbated by stress and characterized by abdominal pain. Although microglia in the CNS have been implicated as an important mediator of the stress response, the role of microglia and microglia-GABAergic neuron interactions in the limbic area, most notably BNST, in the development of colorectal hypersensitivity has not been determined. We established a neonatal colorectal distension-induced chronic visceral hyperalgesia model in rats. The results showed that the frequency of spontaneous discharges of alBNST GABAergic neurons and the expression of GAD65/67 were significantly decreased in rats with chronic visceral pain. Moreover, ablation of BNST GABAergic neurons significantly reduced the visceral pain threshold in normal rats. Meanwhile, the number of M1 proinflammatory microglia and the expression of the M1 proinflammatory microglia-derived cytokines IL-6 and TNF-α were increased in the alBNST of rats with chronic visceral pain. Furthermore, alBNST infusion of the microglial inhibitor minocycline or IL-6 and TNF-α neutralizing antibodies significantly increased the visceral pain threshold. The decreased frequency of spontaneous discharges of alBNST GABAergic neurons in rats with chronic visceral pain was mimicked by a bath perfusion of TNF-α, but not IL-6, and was abolished by a perfusion of the microglial inhibitor minocycline. In addition, the alBNST infusion of the microglial inhibitor minocycline upregulated the expression of GAD65/67. Moreover, ablation of BNST GABAergic neurons significantly decreased the visceral pain threshold in normal rats, which was not reversed by a subsequent infusion of the microglial inhibitor minocycline. Our findings revealed this microglia-GABAergic neuron circuit in the alBNST, and this microglia-driven disinhibitory mechanism is essential for brain and gut dysfunction in stressful condition, providing a novel potential target for treating patients with IBS presenting visceral pain that is worsened during episodes of stress.

1. Introduction

Irritable bowel syndrome (IBS) is a common and debilitating gastrointestinal disorder that is exacerbated by stress and characterized by abdominal pain and alteration of bowel movements in the absence of colonic mucosal damage (Moloney et al., 2015; Chey et al., 2015). In patients with IBS, several brain regions responsible for the modulation of the hypothalamic-pituitary-adrenal (HPA) axis, such as the central extended amygdala, thalamus, and prefrontal cortex, are associated with emotional arousal, endogenous pain modulation, and gastrointestinal hyperactivity (Berman et al., 2008; Price, 2003; Tillisch et al., 2011; Wilder-Smith, 2011). In support of these clinical observations, our prior research in experimental models has demonstrated that the anterior ventral bed nucleus of the stria terminalis (avBNST), a critical node of the central extended amygdala, is a vital structure for mediating visceral nociception with the activation of HPA axis in the stressful condition

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(Kovner et al., 2019; Yu et al., 2020). Here, we focused on another important node of the central extended amygdala, the anterior lateral bed nucleus of the stria terminalis (alBNST), to fully explore the role of the entire central extended amygdala in chronic visceral pain.

As validated in previous studies, neuroimmune interactions within the central nervous system (CNS) mediate the pathogenesis of chronic pain (Ji et al., 2016; Ren and Dubner, 2010). In particular, microglia, CNS-resident immune cells, play a central role in the development of central sensitization and neuropathic pain (Inoue and Tsuda, 2018; Ji and Suter, 2007). Under this pathological condition, activated microglia release proinflammatory cytokines such as IL-1β and TNF-α, which enhance pain sensation by increasing neuronal excitability to facilitate synaptic transmission (Old and Malcangio, 2012; Burke et al., 2016). Regarding visceral sensitivity, microglia have recently been identified to mediate central sensitization in a rat model of narcotic bowel-like syndrome (Agostini et al., 2010). Albeit microglia in the CNS have been implicated as an important mediator of the activation of HPA axis (Lehnardt, 2010; Wohleb et al., 2016), the role of microglia in the limbic area, most notably BNST, in the development of colorectal hypersensitivity has not been determined. Therefore, the aim of the current study was to investigate whether and how modification of the microglial plasticity in the alBNST modulates the neuronal sensitivity that contributes to the activation of HPA axis and the development of colorectal hypersensitivity.

We established a rodent model of neonatal colorectal distension (CRD), in which we have shown persistent visceral hypersensitivity to mechanical luminal distension, to address our experimental aim (Ji et al., 2018, 2020; Song et al., 2020). Using the CRD model, we investigated the morphological and functional modifications of microglia and GABAergic neurons, the most dominant neuronal subtype in the alBNST, in the stressful condition. In addition, ablation of alBNST GABAergic inhibitory neurons resulted in a decrease in the visceral pain threshold, confirming their essential role in visceral hypersensitivity. Since activation of microglia can modify neuronal activity by releasing inflammatory cytokines, we thus assessed cytokine expression levels in the alBNST. Finally, we hypothesized that pharmacological modulation of microglial activity within the alBNST would alter the functions GABAergic inhibitory neurons and subsequently increase colorectal sensitivity. Therefore, we stereotaxically microinjected either the microglial inhibitor minocycline or neutralizing antibodies against inflammatory cytokines into the alBNST and assessed colorectal sensitivity by measuring the visceral pain threshold in freely moving animals, and we used brain slice electrophysiology to explore the "crosstalk" between microglia and GABAergic inhibitory neurons.

Our findings suggest that alBNST GABAergic inhibitory neurons are inhibited by microglia-derived TNF-α and that this microglia-driven disinhibitory mechanism is essential for visceral hypersensitivity induced by colorectal distension in rats. Pharmacological inhibition of microglia alleviates visceral hypersensitivity, which suggests the potential of microglia inhibitors as a novel intervention agent for visceral pain.

2. Materials and methods

2.1. Animals

Adult male and female Sprague-Dawley (SD) rats (8–12 weeks) were provided by the Lukang Animal Feed Distribution Center in Rencheng District, Jining, Shandong, China. One male rat and two female rats were mated to produce offspring. After separation on postnatal day 21, male juvenile rats were grouped into 4 per cage and were applicable at 8 weeks old in this study. The animals were housed under pathogen-free conditions with soft bedding under a 12-h light-dark cycle, controlled temperature (22 ± 2 °C) and 50 ± 5% relative humidity. Food and water were available ad libitum. This experiment followed the guidelines of the International Association for the Study of Pain (IASP), with the

principle of minimizing the number of experimental animals and avoiding harming. This experiment was approved by the Experimental Animal Ethics Committee of Xuzhou Medical University.

2.2. Chronic visceral pain model

We established a chronic visceral pain model by repeated colorectal distension at 8th, 10th and 12th day after birth. The 3 mm × 12 mm PTA balloon Dilation Catheter (C. R. Bard, USA) was used for neonatal colorectal distension (Neonatal CRD) by insertion via the rectum into the descending colon with 0.3 ml water at a pressure of 60 mmHg for 60 s. Then, the balloon was deflated and withdrawal. Each neonatal rat was treated twice a day with an interval of more than 30 min each time. Newborn rats were weaned at the 21st day after birth, and male rats were reared till adulthood (8th week). Adult colorectal distension (Adult CRD) denotes that the adult rats were treated with colorectal distension with a rubber balloon at a pressure of 60 mmHg for 60 s, totally 10 times at an interval of 15 s. Neonatal + adult CRD means that rats undergoing both neonatal CRD and adult CRD. Control rats were handled similarly to those in neonatal CRD group except that no colonic insertion was made. Rats in this group were gently held and touched on the perineal area at 8th, 10th and 12th day after birth. All groups of rats were tested for the visceral pain threshold. Behavioral test was performed 2 h after adult CRD, as our previous study has shown that after adult CRD procedure, the pain threshold returned to normal in the adult CRD group, whereas the pain threshold remained significantly lower in the neonatal + adult CRD group (Ji et al., 2020).

2.3. Assessment of pain threshold

Distention balloons (4.5 cm in length) were inserted via the rectum into the descending colon of mildly sedated adult rats under light isoflurane anesthesia and secured by taping the attached tubing to the rats tail. The animals were then placed into a transparent plexiglas box (20 × 10 × 18 cm) and allowed to wake up and adapt 30 min prior to measurement. Abdominal Withdrawal Reflex (AWR) was used for the responses of abdominal wall to colorectal stimuli. AWR scoring standard: 0, no evident behavioral changes; 1, immobile or only slight head movement; 2, contraction of abdominal muscles, but without lift off the desktop; 3, contraction of abdominal muscles with lift off the desktop; 4, arching of abdominal wall and back. At the initial stage of the test, the line of sight is flush with the abdomen of the rat while the pressure starts from 0 mmHg with the pressure of the balloon increasing by 10 mmHg per second. When the abdominal wall lifted off the desktop (AWR score ≥3), the reading of the sphygmomanometer was recorded as the visceral pain threshold. Each rat was tested thrice at a 1–2 min interval, with averages of the threshold recorded. The observers were blinded to treatment when recording the AWR.

2.4. Electromyography (EMG) recording

The EMG response to CRD was applied to assess visceral hypersensitivity in rats. When recording the VMR, anesthetic equipment (RWD, Shenzhen, China) maintained the same level of sedation in rats under light isoflurane anesthesia. Two Teflon-coated silver wires separated by 1 cm were inserted into the external oblique muscle. The EMG signal was amplified and filtered using a BL-420N biological signal collection and processing system from Chengdu Taimeng Co., Ltd (Chengdu, China). The high frequency filter was set at 2 kHz, time constant at 0.001 s, sampling frequency at 40 Hz, sensitivity at 500 μV, and scanning speed at 250 ms/div. Visceral hypersensitivity can be quantified by spike-to-spike measures - the voltage changes between the peak (highest amplitude value) and trough (lowest amplitude value, which can be negative).

2.5. Western blotting analysis

To identify the level of protein expression, whole protein samples were analyzed by western blotting. Under isoflurane anesthesia (RWD, China), the anterolateral region of BNST was rapidly isolated from rat brain as protein sample. Then, samples were put into a pre-cooled EP tube with radioimmunoprecipitation assay (RIPA; Beyotime, China) lysis buffer and phenylmethanesulfonyl fluoride (PMSF; Beyotime, China), followed by centrifugation (12,000 rpm, 15 min, 4 °C). The BCA protein measurement kit (Beyotime, China) was used to detect the protein concentration and to adjust to the identical concentration across groups. Then, equal amounts (40 μg) of protein samples were electrophoresed by 8–10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, UK). Membranes were blocked with 5% non-fat milk for 2 h at room temperature (r/t), incubated with the primary antibodies overnight at 4 °C. The mainly primary antibodies were rabbit anti-GAD65/67 antibody (1:1000, ab112497, ab97739, Abcam, USA), mouse anti-GAPDH antibody (1:2000, AC002, ABclonal, USA), rabbit anti-IL-6 antibody (1:600, sc-57315, Santa Cruz, USA), rabbit anti-TNF-α antibody (1:600, 17590-1-AP, Proteintech, China), rabbit Anti-CD86 antibody (1:200, 13395-1-AP, Proteintech, China), rabbit anti-CX3CR1 antibody (1:1000, ab8021, Abcam). Then, HRP-coupled secondary antibodies (Beyotime, China) were applied for 2 h at r/t. The membranes were probed with a BeyoECL Plus kit (Beyotime, China). Data were analyzed with the ImageJ software (NIH USA).

2.6. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The anterolateral region of the bed nucleus of the stria terminalis was isolated from deeply anesthetized rat under isoflurane anesthesia. Total RNA was extracted by TRIzol reagent (Invitrogen, USA) and qualified by a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, USA). RNA extracts (500 ng) were reverse transcribed into complementary DNA (cDNA) using Hiscript II Q RT SuperMix (Vazyme Biotech, China). Following reverse transcription, qRT-PCR was performed with AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, China) on a Light Cycler 480II instrument (Roche Diagnostics, Switzerland). The thermal cycling conditions comprised 30-s polymerase activation at 95 °C, 40 cycles of 10-s denaturation at 95 °C, 30-s annealing at 62 °C and 30-s extension at 72 °C. Primers for GAPDH, GAD65 and GAD67 were designed according to NCBI published sequences, as follows: GAPDH: forward 5′-TGAAG-CAGGCATCTGAGG-3′, reverse 5′-CGAAGGTGGAAGAGTGGGAG-3′, GAD65: forward 5′-TCTCAAAGGTGGCGCCAGTGATTA-3′, reverse 5′-TTGGTGAGTTGCTGCAGGGTTTGA-3'); GAD67: forward 5′-ACAAA TGCCTGGAGCTGGCTGAAT-3′, reverse 5′-TTGTGTGCTCAGGCTCAC-CATTGA-3'. At the end of the PCR, all fluorescence data underwent melting analysis to confirm amplicon specificity. Results were normalized to GAPDH. Changes of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Immunofluorescence (IF) staining

Following behavioral testing, rats were deeply anesthetized with isoflurane and then perfused with 300 ml normal saline (100 ml/100 g), followed by 4% polyformaldehyde (PFA, 100 ml/100 g). Whole brains, including BNST, were immediately dissected and post-fixed in 4% PFA overnight at 4 °C, followed by immersion in 30% sucrose at 4 °C for 3 days. The brain tissues were coronally serial sliced on a cryostat (CM1800, Leica, Germany) at a thickness of 30 μm and stored in PBS at 4 °C prior to immunofluorescence staining. Brain slices were blocked with 1% BSA at r/t for 2 h, followed by incubation with the appropriate primary anti-body overnight at 4 °C. Primary antibodies were used for Immunofluorescence including goat anti-Iba1 antibody (1:400, ab48004, Abcam, USA), mouse anti-NeuN antibody (1:400, ab104225, Abcam, USA) (used to mark microglia and neurons, respectively). The

slices were subsequently incubated with the appropriate secondary antibody for 2 h at r/t. Alexa Fluor 488 or Alexa Fluor 594 donkey anti-goat and Alexa Fluor 488 donkey anti-mouse IgG (1:1000, Life Technologies, USA) were used as secondary antibody. 4′,6-diamidino-2-phenylindole (DAPI, KGI Biological, China) was used for nuclear staining. Images were obtained with 10 × and 20 × lens with the use of a confocal laser microscope (LSM80, Zeiss, Germany). For quantification of cells, three sections around the BNST were counted manually using NIH ImageJ software. The sections used were at the same coordinates for each group.

2.8. Microinjection of adenovirus-associated vector (AAV) and drugs

To label GABAergic neurons in BNST of rats, adeno-associated virus (AAV) vector containing Dlx5/6-EGFP (1.5×10^{12}VG/ml, 0.5 μl, BrainVTA, China) was employed. Similarly, to destroy GABAergic neurons in BNST of rats, adeno-associated virus (AAV) vectors containing flex-taCaspase 3-TEVp (5.2×10^{12}VG/ml, BrainVTA, China) and vgat-Cre (4.8×10^{12}VG/ml, 1:2,0.5 μl, BrainVTA, China) were employed. For the virus infusion, rats were deeply anesthetized with isoflurane and placed in a stereotaxic apparatus (RWD, China). Following skin incision to expose the skull, the coordinates of alBNST region was targeted with reference from the Rat Brain in Stereotaxic Coordinates (third edition) edited by George Paxinos and Charles Watson: AP +0.1 mm, ML ± 1.25 mm, DV -6.4 to 6.5 mm from bregma. Micro syringes (Gaoge, China) were used to microinject viruses, and then sutured surgical incisions. Then, rats were put into their cages after being awake. After 3 weeks period of recovery, animal behavior was tested and fluorescence microscopy and in vitro electrophysiology were used to observe GABAergic neurons. To examine the effect of minocycline (10 μg/μl, 0.5 μl/site, Sigma, USA), anti-TNF-α (1 ng/μl, 0.5 μl/site, Santa Cruz, USA) and anti-IL-6 (65 ng/μl, 0.5 μl/site, Santa Cruz, USA) on the visceral pain threshold, drugs were injected in BNST of rats under isoflurane anesthesia, and the pain threshold was tested after administration.

2.9. Brain slice electrophysiology

Electrophysiological experiments were performed in the cell-attached model at r/t. Rats were deeply anesthetized under isoflurane followed by transcardial perfusion with ice-cold high-sucrose cutting solution. The slices of the BNST (300 μm) were obtained on a vibratome (VT1200S, Leica, Germany) in ice-cold high-sucrose cutting solution: 80 mM NaCl, 3.5 mM KCl, 4.5 mM MgSO4, 0.5 mM CaCl2, 1.25 mM NaH2PO4, 90 mM sucrose, 25 mM NaHCO3, and 10 mM glucose (pH = 7.35). The osmolarity was adjusted to 295–305 mOsm. For the recording of spontaneous firing frequency in GABAergic neurons of BNST-AL region, the slices were incubated in high-sucrose cutting solution at 34 °C for 15–30 min and then transferred into the artificial cerebral spinal fluid (ACSF) solution for recovery of 1 h at r/t. The artificial cerebral spinal fluid (ACSF) consisted of 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.2 mM MgSO$_4$, 26 mM NaHCO$_3$, 10 mM glucose, and 2.4 mM CaCl$_2$ (pH = 7.35). The osmolarity was adjusted to 295–305 mOsm. All solutions were saturated with 95% O2 and 5% CO2. Cell-attached patch clamp recording from EGFP$^+$ neurons was performed with patch pipette filled with internal solutions containing: 10 mM phosphocreatine-Tris, 10 mM HEPES, 10 mM EGTA, 2 mM ATP-Mg, 0.5 mM GTP-Na, 115 mM K gluconate, 20 mM KCl, 1.5 mM MgCl2 (pH = 7.25). The osmolarity was adjusted to 285 mOsm. Spontaneous firing frequency was recorded using a MultiClamp 700B amplifier (Axon Instruments, USA) and analyzed with Clampex and Clampfit 10 (Axon Instruments, USA).

2.10. Statistical analyses

All data are expressed as the mean ± standard error of the mean (SEM). Statistical differences between two groups are analyzed using independent samples Student's *t*-test. For comparison of multiple

groups, one-way or two-way analysis of variance is used. qRT-PCR analysis was performed by Kruskal-Wallis test. Statistical analyses are performed with SPSS 16.0 (IBM, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Adult CRD amplifies visceral hypersensitivity in rats subjected to neonatal CRD

The rat model of visceral hypersensitivity was established by neonatal CRD and subsequent CRD in adulthood. Rats were subjected to neonatal CRD on postnatal days 8, 10, and 12 and adult CRD in week 8, and the behavioral test was performed in weeks 8–10 (Fig. 1a). One-way ANOVA revealed a decrease in the pain threshold and an increase in the spike-to-spike amplitude in the rat groups (neonatal CRD; neonatal + adult CRD) (Fig. 1b, one-way ANVOA, n = 6, F(3, 20) = 15.41, $P < 0.01$; Fig. 1c, one-way ANVOA, n = 8, F(3, 28) = 74.37, $P < 0.01$). Additionally, One-way ANOVA revealed that the neonatal + adult CRD group presented decreased visceral pain threshold and increased spike-to-spike amplitude as compared with neonatal CRD group (Fig. 1b, one-way ANVOA, n = 6, F (3, 20) = 15.41, $P < 0.01$; Fig. 1c, one-way ANVOA, n = 8, F (3, 28) = 74.37, $P < 0.01$), suggesting that rats experiencing neonatal CRD presented with underlying visceral hypersensitivity that was amplified by subsequent CRD in adulthood. Therefore, for the convenience of this study, we treated the rats that have undergone neonatal and adult CRD as visceral hypersensitivity rats in the continued study.

3.2. alBNST GABAergic inhibitory neuronal activity is inhibited in rats with visceral hypersensitivity

As an initial step towards evaluating the role of the alBNST in visceral hypersensitivity, we focused on GABAergic inhibitory neurons – the most dominant neuron subtype in the alBNST. First, we compared the expression of GAD65/67, enzymes that catalyze the decarboxylation of glutamate to GABA. Kruskal-Wallis test and one-way ANOVA revealed decreased expression of GAD65/67 mRNA and protein, respectively, within the alBNST of rats with visceral hypersensitivity compared with control rats, as determined using qRT–PCR and western blotting. (Fig. 2a, Kruskal-Wallis test, $P = 0.0288$, n = 5; Fig. 2b, Kruskal-Wallis test, $P = 0.0316$, n = 4; Fig. 2c, one-way ANOVA, F (3, 28) = 3.437, $P = 0.0303$, n = 8; Fig. 2d, one-way ANOVA, F (3, 20) = 5.104, $P = 0.0087$, n = 6). Next, t-test revealed that the number of alBNST GABAergic inhibitory neurons was decreased in rats with visceral hypersensitivity (Fig. 2g, t-test, n = 3, t = 7.671, df = 4, $P < 0.01$). In addition, we quantified the physiological activity of alBNST GABAergic inhibitory neurons and t-test revealed that rats with visceral hypersensitivity exhibited a decrease in spontaneous neuronal firing rates of GABAergic neurons compared with control rats (Fig. 2j, t-test, t = 5.366, df = 36, $P < 0.01$). Based on these results, alBNST GABAergic inhibitory neurons mostly participate in visceral hypersensitivity.
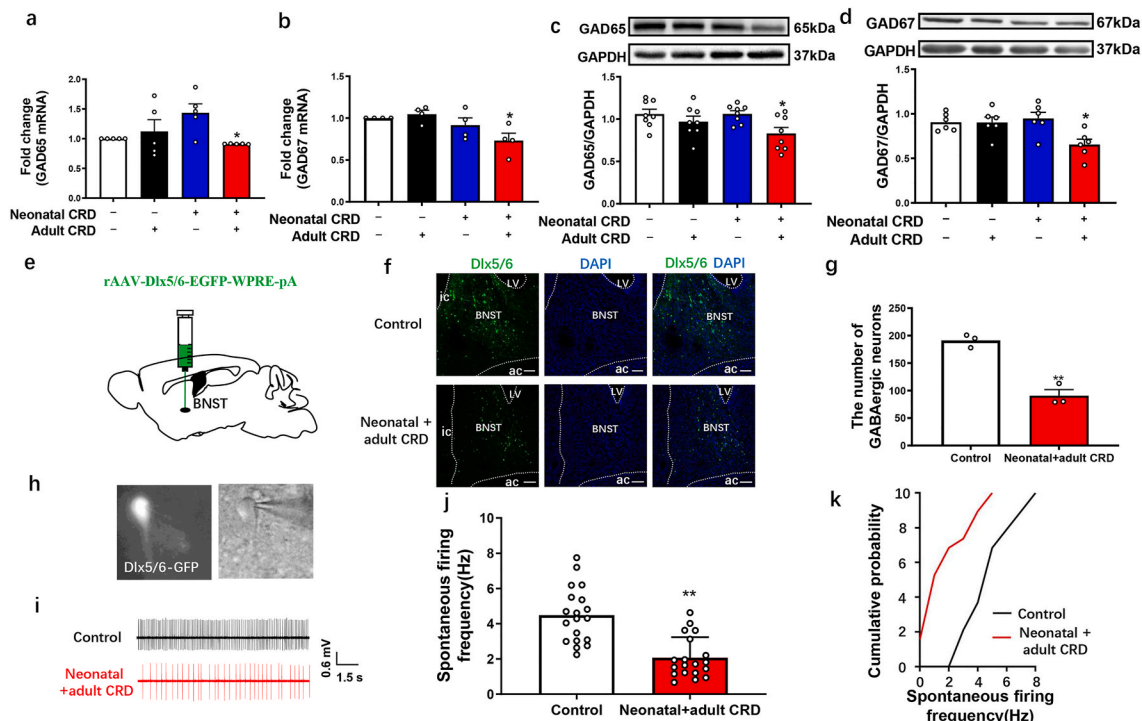
3.3. Ablation of alBNST GABAergic inhibitory neurons induces visceral hypersensitivity

We ablated alBNST GABAergic inhibitory neurons to further characterize their precise role in visceral hypersensitivity. Consistent with the effect of Caspase 3 on inducing cell apoptosis, Caspase 3 gradually eliminated GABAergic inhibitory neurons in the alBNST, resulting in an average loss of 52.72% of GABAergic inhibitory neurons 3 weeks after the virus injection (Fig. 3a and b). t-test revealed that compared with the control rats, Caspase 3-injected rats developed a sharp decrease in the visceral pain threshold and an increase in the spike-to-spike amplitude (Fig. 3c, t-test, t = 2.403, df = 8, n = 5, $P < 0.05$). These data therefore suggest that alBNST GABAergic inhibitory neurons are able to modulate visceral hypersensitivity.
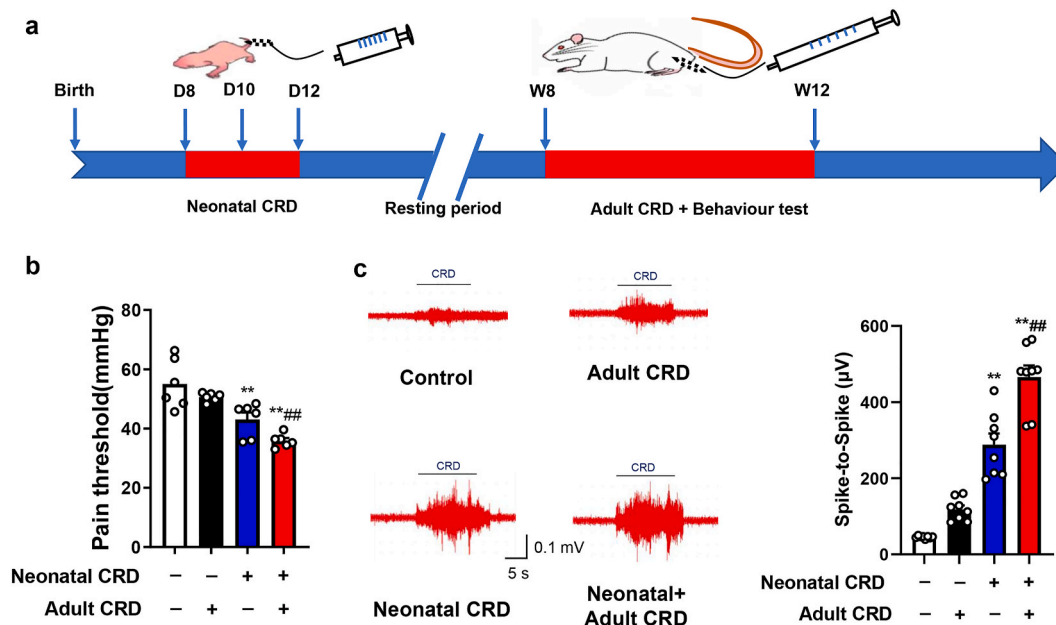

Fig. 1. Adult CRD amplifies visceral hypersensitivity in rats subjected to neonatal CRD. (a) A schematic depiction of the chronic visceral pain protocol. (b) The neonatal CRD induced significant visceral pain hypersensitivity compared with the control group. Rats which underwent both neonatal CRD and adult CRD presented lower pain threshold compared with rats in the neonatal CRD group. (**$P < 0.01$ compared with the control group; ##$P < 0.01$ compared with the neonatal CRD group). (c) Left: Representative EMG recordings at 60 mmHg CRD. Right: The neonatal CRD rats presented an increase in spike-to-spike amplitude compared with that in control or adult CRD groups. Rats which underwent both neonatal CRD and adult CRD presented an increase in spike-to-spike amplitude compared with that in neonatal CRD groups. (**$P < 0.01$ compared with the control group; ##$P < 0.01$ compared with the neonatal CRD group). The data are expressed as the mean ± SEM.

Fig. 2. alBNST GABAergic inhibitory neuronal activity is inhibited in rats with visceral hypersensitivity. (a–b) qRT-PCR analysis of GAD65/67 mRNA in the alBNST region showed that GAD65/67 expression decreased in the neonatal + adult CRD group compared with the control group. Results are normalized to the housekeeping gene GAPDH. (c–d) The protein expression of GAD65/67 downregulated in the neonatal + adult CRD group compared with the control group. (e) Schematic diagram of injecting rAAV-Dlx5/6-EGFP-WPRE-pA which marked alBNST GABAergic neurons. (f) The co-localization of GABAergic neurons (green) and DAPI (blue) was assessed by confocal microscopy in alBNST. (g) Quantification of alBNST GABAergic neurons numbers indicated that the number of GABAergic neurons decreased in the neonatal + adult CRD group compared with the control group. Scale bar = 200 μm. (h) Specific labeling of GABAergic neurons in alBNST region with rAAV-Dlx5/6-EGFP-WPRE-pA. (i–k) Spontaneous firing frequency of alBNST GABAergic neurons was significantly decreased in the neonatal + adult CRD group compared with control group. The data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
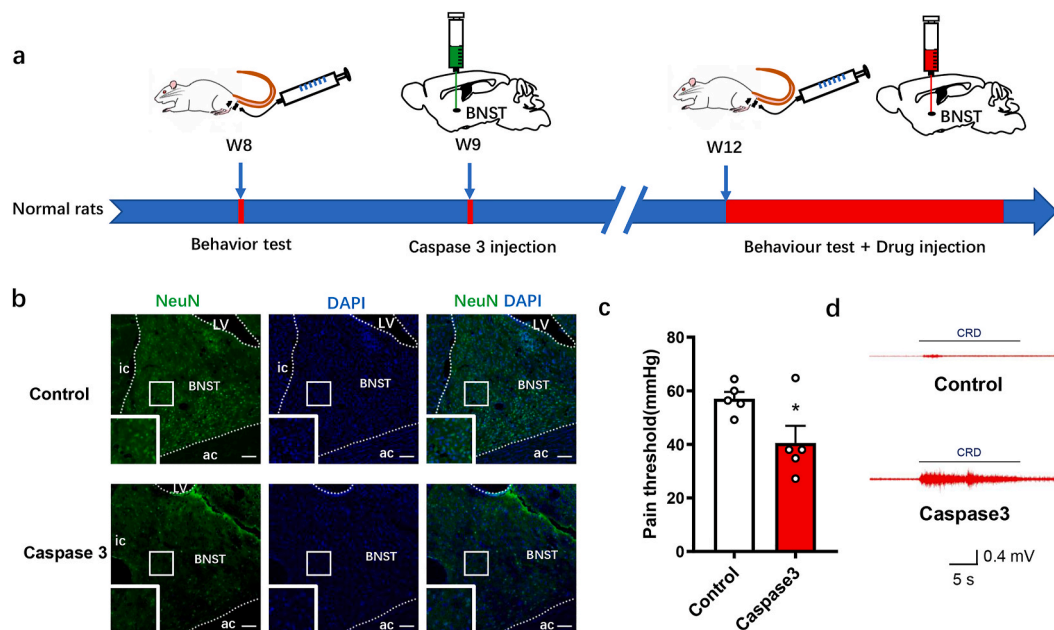
Fig. 3. Ablation of alBNST GABAergic inhibitory neurons induces visceral hypersensitivity. (a) A schematic depiction of mixed virus microinjection and behavioral test. (b) 3 weeks after microinjection of mixed virus (rAAV-flex-taCaspase3-TEVp-WPRE-pA, AAV2/9; rAAV-EF1α-DIO-mCherry-WPREs-pA, AAV2/9; rAAV-vgat-Cre-WPRE-pA, AAV2/9), the count of neurons was significantly decreased in alBNST. (c) Visceral pain threshold was decreased significantly after microinjecting mixed virus. Scale bar = 100 μm. (d) Representative EMG recordings at 60 mmHg CRD. The data are expressed as the mean ± SEM. *$P < 0.05$.

3.4. Microglia polarize to the M1 proinflammatory phenotype within the alBNST of rats with visceral hypersensitivity

Given that the activated microglia can reportedly affect neuronal activity through proinflammatory cytokines, we next focused on the role of microglia to explore whether GABAergic neurons are modulated by microglia in rats with visceral hypersensitivity. Notably, one-way ANOVA revealed that rats with visceral hypersensitivity displayed a substantially increased number of microglia and a remarkably decreased average branch length of microglia in the alBNST compared with control rats (Fig. 4b, one-way ANOVA, F (3, 8) = 5.940, P = 0.0197; Fig. 4d, one-way ANOVA, F (3, 32) = 4.757, P = 0.0075). These findings indicate the activation of microglia within the alBNST of rats with visceral hypersensitivity. We assayed the expression of CD86 (M1 phenotype marker) and CX3CR1 (M2 phenotype marker) using western blotting to further determine the microglial phenotype. One-way ANOVA revealed that CD86 expression was increased in rats with visceral hypersensitivity compared with control rats, whereas the expression of CX3CR1 was not changed (Fig. 4e, one-way ANOVA, F (3, 24) = 4.954, P = 0.0081; Fig. 4f, one-way ANOVA, F (3, 16) = 1.479, P = 0.2579), indicating that microglia polarized to the M1 proinflammatory phenotype. The production of M1 proinflammatory cytokines (TNF-α and IL-6) was measured using western blotting to further confirm this hypothesis. One-way ANOVA revealed that the expression of IL-6 and TNF-α was increased in rats with visceral hypersensitivity compared with control rats (Fig. 4g and h, one-way ANOVA; g: F (3, 28) = 4.016, P = 0.0170; f: F (3, 20) = 5.643, P = 0.0057). Thus, alBNST microglia polarize to the M1 proinflammatory phenotype and generally participate in visceral hypersensitivity.

3.5. Pharmacological inhibition of microglia and microglia-derived proinflammatory cytokines within the alBNST alleviates visceral hypersensitivity

Microglia and microglia-derived proinflammatory cytokines were inhibited by a local microinjection of the microglial inhibitor minocycline or neutralizing antibodies, respectively, to further assess the exact role of microglia in visceral hypersensitivity. t-test revealed an average loss of 47.74% of microglia, as visualized via immunofluorescence staining (Fig. 5c, t-test, t = 5.914, df = 10, n = 6, P = 0.0001), and two-way ANOVA revealed an increased visceral pain threshold and reduced spike-to-spike amplitude after the minocycline infusion (Fig. 5e and f). Similarly, two-way ANOVA revealed that both TNF-α- and IL-6-neutralizing antibodies increased the visceral pain threshold and reduced the spike-to-spike amplitude (Fig. 5d, e, g, two-way ANOVA; d: F (3, 18) = 13.21, P < 0.0001; e: F (3, 30) = 28.35, P < 0.0001; g: F (3, 30) = 62.62, P < 0.0001). Based on these data, microglia modulate visceral hypersensitivity, and this effect may be achieved by microglia-derived proinflammatory cytokines.
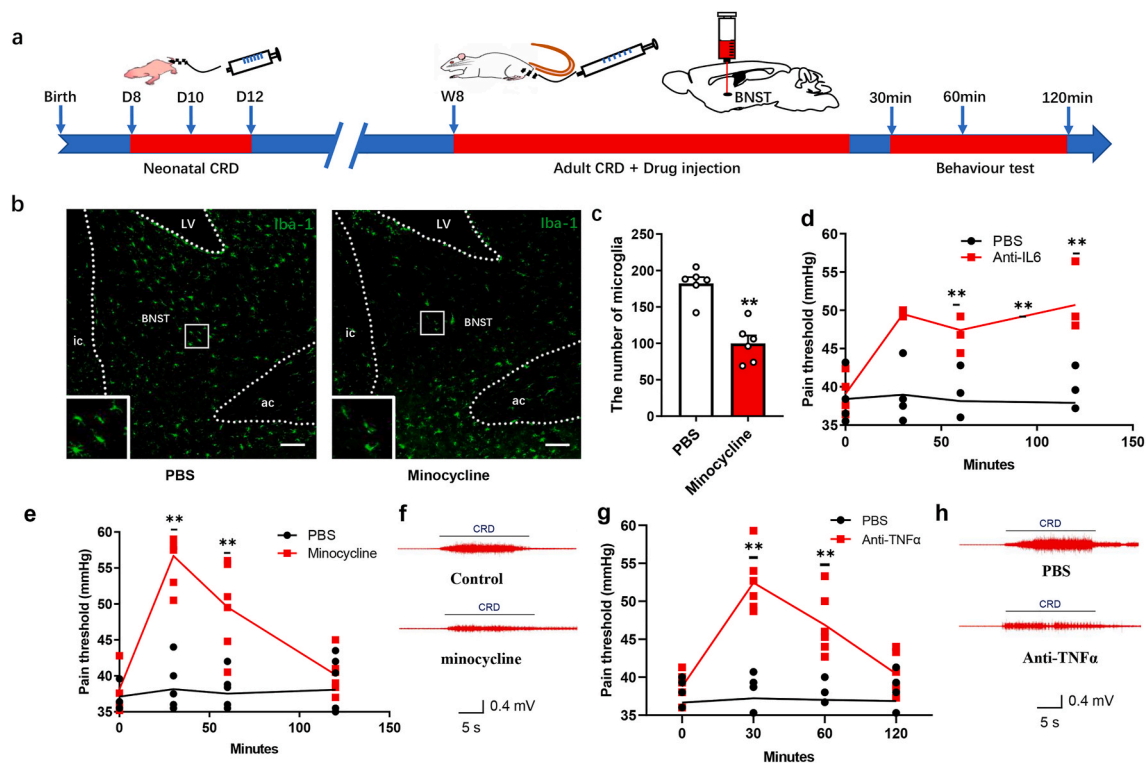
3.6. Microglia-derived TNF-α regulates visceral hypersensitivity by suppressing GABAergic inhibitory neuronal activity

On the grounds that both alBNST GABAergic inhibitory neurons and microglia regulate the pain threshold, we next tested whether microglia regulate visceral hypersensitivity by suppressing GABAergic inhibitory neuronal activity. One-way ANOVA revealed a substantial decrease in GAD65/67 expression in rats with visceral hypersensitivity as compared with control rats, a change that was reversed by an alBNST infusion of the microglial inhibitor minocycline (Fig. 6b and c, one-way ANOVA; b: F (3, 16) = 4.976, P = 0.0126, n = 5; c: F (3, 12) = 3.840, P = 0.0387, n = 4). In addition, t-test revealed that the action potential firing rate of alBNST GABAergic inhibitory neurons was increased by perfusion of the microglial inhibitor minocycline (Fig. 6d, t-test, t = 2.648, P = 0.0244, df = 10, n = 6). These data indicate that microglia decrease GABAergic neuronal activity.

We adopted a strategy that combines Caspase 3 to eliminate GABAergic inhibitory neurons and an infusion of the microglial inhibitor minocycline in the alBNST to further investigate the essential role of GABAergic neurons in activating microglia-induced visceral
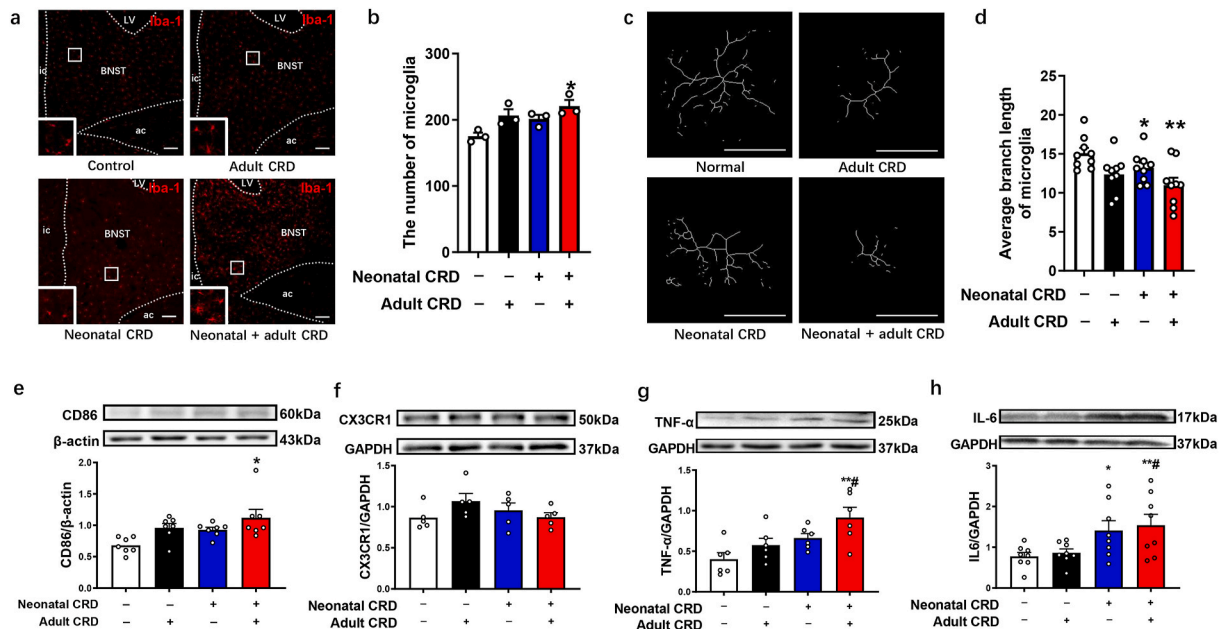
Fig. 4. Microglia polarize to the M1 proinflammatory phenotype within the alBNST of rats with visceral hypersensitivity. (a–b) The number of alBNST microglia was increased in the adult CRD group and neonatal + adult CRD group compared with the control group. (c–d) The average branch length of alBNST microglia was decreased in the adult CRD group and the neonatal + adult CRD group compared with the control group (*P < 0.05, **P < 0.01). (e–f) The expression of alBNST CD86 (M1 phenotype marker) was significantly increased in the neonatal + adult CRD group compared with the control group (*P < 0.05) and the expression of CX3CR1 (M2 phenotype marker) was not changed between four groups. (g–h) The expression of alBNST IL-6 and TNF-α displayed significantly increased in the neonatal + adult CRD group compared with the control group and the adult group. (*P < 0.05, **P < 0.01 compared with the control group; #P < 0.05 compared with the adult group). Scale bar = 100 μm. The data are expressed as the mean ± SEM.

Fig. 5. Pharmacological inhibition of microglia and microglia-derived proinflammatory cytokines within the alBNST alleviates visceral hypersensitivity.
(a) A schematic depiction of drugs administered in neonatal + adult CRD rats. In treatment group, rats were treated with minocycline, anti-IL-6, or anti-TNF-α. (b–c) 30 min after microinjection of minocycline, the number of microglia was decreased compared with PBS group. (**P < 0.01). (d–g) Basal pain threshold were determined, and then rats were microinjected with minocycline, anti-IL-6, or anti-TNF-α. 30, 60, 120 min later, visceral pain threshold was tested. Representative EMG recordings at 60 mmHg CRD were recorded 30 min after drug administration. (**P < 0.01 compared with PBS group). Scale bar = 100 μm.

hypersensitivity. One-way ANOVA revealed that ablation of BNST GABAergic neurons significantly decreased the visceral pain threshold in control rats, which was not reversed by a subsequent infusion of the microglial inhibitor minocycline (Fig. 6e, one-way ANOVA, F (4, 20) = 4.182, P = 0.0127, n = 5). Therefore, GABAergic inhibitory neurons are necessary for microglia to regulate visceral hypersensitivity.

We assessed the effects of TNF-α and IL-6 perfusions on GABAergic neurons by patch clamp recording to further determine whether microglia modulate GABAergic neuronal activity via microglia-derived proinflammatory cytokines. *t*-test revealed that similar to rats with visceral hypersensitivity, alBNST GABAergic neurons displayed a significantly decreased frequency of spontaneous discharges upon perfusion with TNF-α, rather than IL-6 (Fig. 6g–h, *t*-test; g: t = 2.960, df = 18, P = 0.0084, n = 10; h: t = 4.912, df = 14, P = 0.0002; n = 8). These findings suggest that TNF-α mostly modulates GABAergic neuronal activity in visceral hypersensitivity.

Taken together, these findings suggest that microglia-derived TNF-α regulates visceral hypersensitivity by inhibiting GABAergic inhibitory neuronal activity.

4. Discussion

In the current study, we specifically focused on neuroinflammation in the microglia-GABAergic neuron circuit in the alBNST in visceral hypersensitivity. We showed that microglial activation and microglia-driven inhibition of GABAergic neurons in the alBNST were sufficient for visceral hypersensitivity in stressful condition, whereas an infusion of a microglial inhibitor into the alBNST reversed visceral hypersensitivity. Moreover, an infusion of a neutralizing antibody against the microglia-derived cytokines IL-6 and TNF-α exerted a similar effect, suggesting the pivotal involvement of alBNST microglia in visceral pain. However, upon the ablation of BNST GABAergic neurons by Caspase 3,

infusion of the microglial inhibitor minocycline failed to relieve visceral pain, which indicated the essential role for GABAergic neurons in modulating visceral pain induced by microglia. Moreover, similar to rats with visceral hypersensitivity, alBNST GABAergic neurons displayed a significantly decreased frequency of spontaneous discharges after perfusion with TNF-α but not IL-6, suggesting that microglia-derived TNF-α mostly suppresses GABAergic neuronal activity in visceral hypersensitivity. Our findings revealed this microglia-GABAergic neuron circuit in the alBNST, and this microglia-driven disinhibitory mechanism is essential for brain and gut dysfunction in stressful condition, hence providing a novel potential target for treating patients with IBS presenting visceral pain that is worsened during episodes of stress.

We selected this brain region based on previous evidence obtained from our laboratory and from other researchers suggesting that the BNST is a key brain region that regulates both stress and visceral nociceptive responses (Lebow and Chen, 2016; Radley and Johnson, 2018). Our previous research in experimental models has shown that the anterior ventral bed nucleus of the stria terminalis is an important structure for mediating visceral nociception with the activation of HPA axis in stressful condition. Sha et al. described that BNST electrical stimulation inhibited visceral pain (Sha et al., 1993). Moreover, the anterior lateral division of the BNST (alBNST) serves as an extra-hypothalamic conduit between the CeA and the paraventricular nucleus (PVN) of the hypothalamus, a primary brain region in the stress axis (Dent et al., 2007; Paxinos, 2005). Therefore, alBNST is an ideal candidate for the current study.

Since GABAergic neurons are the dominant neuronal subtype (Nguyen et al., 2016), accounting for more than 80% of neurons in the BNST, we initially investigated the morphological and functional changes of GABAergic neurons in stressful condition by colorectal distension. Rats with visceral hypersensitivity exhibited a decrease in both the expression of GAD65/67 and spontaneous neuronal firing rates
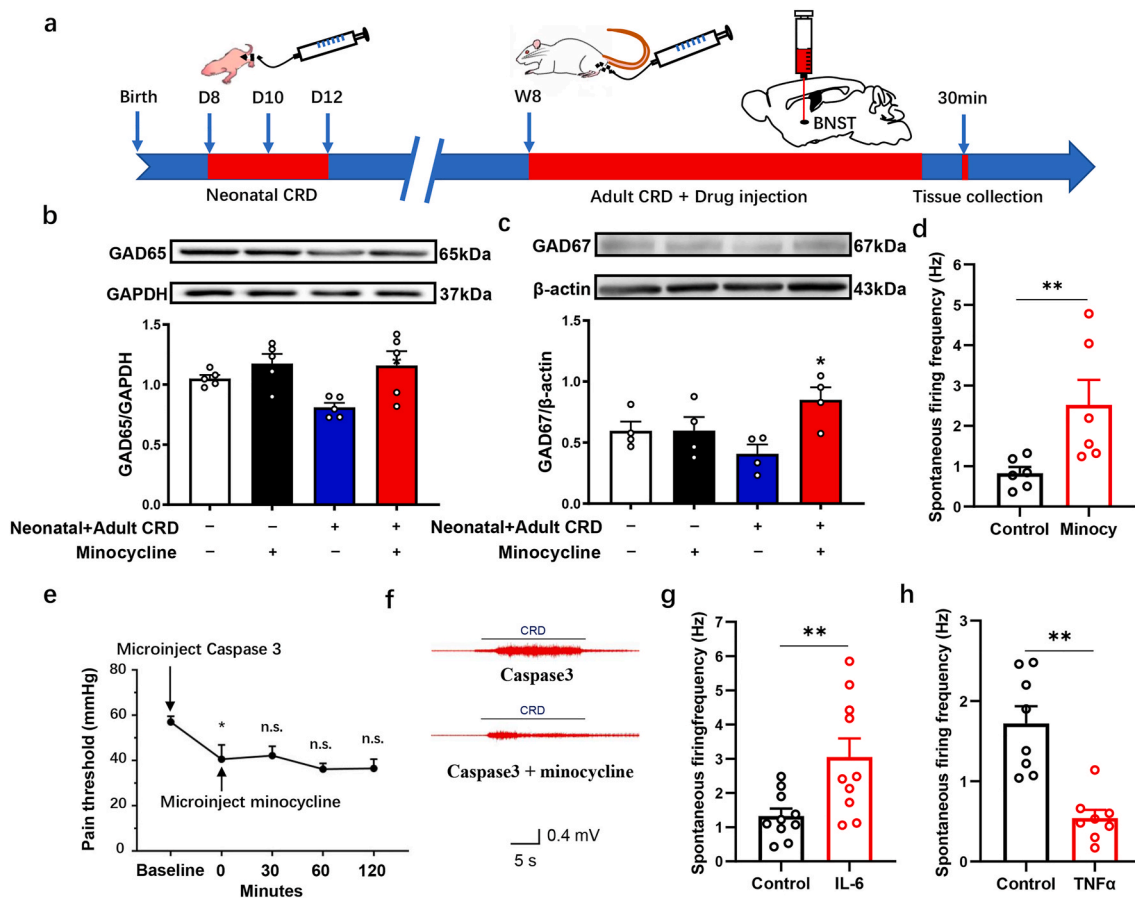
Fig. 6. Microglia-derived TNF-α regulates visceral hypersensitivity by suppressing GABAergic inhibitory neuronal activity. (a) A schematic depiction of drugs administered in the neonatal + adult CRD rats. (b–c) 30 min after microinjection of minocycline, the protein expression of GAD65/67 was increased in the minocycline group compared with the PBS group (*P < 0.05). (d) Effect of minocy (abbreviation for minocycline) perfusion on GABAergic neuronal frequency of spontaneous discharges (**P < 0.01). (e–f) Basal pain threshold was determined, and 3 weeks after microinjection of Caspase 3, rats were microinjected with minocycline. 0, 30, 60, 120 min later, visceral pain threshold was tested (*P < 0.05, [n.s.]P > 0.05 compared with 0 min). (g–h) Effects of TNF-α and IL-6 perfusions on GABAergic neuronal frequency of spontaneous discharges (**P < 0.01).

of GABAergic neurons, and ablation of alBNST GABAergic inhibitory neurons by Caspase 3 produced a marked decrease in the visceral pain threshold. These data support the alBNST as the key brain region regulating both stress and visceral nociceptive responses and indicate that inhibition of GABAergic inhibitory neuronal activity is the cause of visceral hypersensitivity.

Despite our finding that neonatal CRD induces visceral hypersensitivity via stress response, we cannot simply exclude that repeated CRD in this early neonatal period results in sensitization of pain perception and processing. Sensitization of the primary (peripheral afferent), secondary (spinal), or tertiary (brainstem/thalamic) neuron can promote chronic pain signaling. In response to the peripheral stimuli (repeated CRD), the afferent fibers can release neuromodulators to further stimulate nerve activity, leading to remodeling and persistent excitation of the second order neuron, which promotes chronic pain and can invoke similar central remodeling to cause persist pain. We will conduct in-depth research in this direction in the future.

Although the regulation of neuronal activity in the brain has long been viewed as an exclusive prerogative of neurons, recent findings have suggested that immune cells in the brain – the microglia – might be involved in this process (Li et al., 2012; Eyo et al., 2014; Akiyoshi et al., 2018; Kato et al., 2016; Wake et al., 2009; Peng et al., 2019; Cserép et al., 2020). Similar to inhibitory neurons, microglia sense neuronal activation and suppress excessive neuronal activity. However, these reports focused on homeostatic microglia and excitatory neuronal activation, and thus a study aiming to determine whether microglia regulate

inhibitory GABAergic neuronal activity would be intriguing. The previous decade has seen a rapid increase in studies of the role of microglia in pain, with a unique focus on microgliosis and microglial activation in the spinal cord after nerve injury and neuropathic pain. Recent studies reported region-specific brain microglial activation in a neuropathic pain model, including the medial prefrontal cortex, anterior cingulate cortex, nucleus accumbens, amygdala, hippocampus, and ventral tegmental area (Taylor et al., 2017). Microglial activation markers are upregulated in the anterior cingulate cortex after peripheral nerve injury, and microinjection of minocycline, an inhibitor of microglial activation, into the anterior cingulate cortex reduces pain behaviours (Miyamoto et al., 2017). Moreover, our previous research showed that microglial activation and the release of the proinflammatory cytokines TNF-α and IL-1β in the PVN in stressful condition, whereas an intra-PVN infusion of minocycline attenuated microglial activation and visceral hypersensitivity (Zhang et al., 2016). Hence, we hypothesize that microglial activation modulates inhibitory GABAergic neuronal activity and subsequently precipitates visceral hypersensitivity. Consistent with the hypothesis that homeostatic microglia suppress excessive excitatory neuronal activity, activated microglia suppress inhibitory neuronal activity in the alBNST. Here, we report that a microinjection of the microglial inhibitor minocycline reversed the decrease in GAD65/67 expression and spontaneous neuronal firing rates of GABAergic neurons in CRD rats and alleviated visceral pain. Based on these data, activated microglia suppress inhibitory GABAergic neuronal activity in the alBNST and then induce visceral hypersensitivity.

Previously, it is reported that the purinergic receptor P2RY12 played a key role in the ability of homeostatic microglia to sense neuronal activation and suppress excessive neuronal activity (Badimon et al., 2020). However, P2RY12 expression may be downregulated in reactive microglia in response to various inflammatory and neurodegenerative diseases, including Alzheimer's and Huntington's diseases (Haynes et al., 2006; Krasemann et al., 2017; Mildner et al., 2017). Therefore, although activated microglia, which function as homeostatic microglia by inhibiting excessive neuronal activity, suppressed inhibitory GABAergic neuronal activity, the underlying mechanism should be distinctive. Previous studies have revealed that activated microglia release proinflammatory cytokines such as IL-1β and TNF-α, which enhance pain sensation by increasing neuronal excitability to facilitate synaptic transmission (Chen et al., 2018). Consistent with these reports, we observed increased IL-6 and TNF-α levels in the aIBNST and enhanced inhibitory GABAergic neuronal activity following the perfusion of IL-6. Nevertheless, inhibitory GABAergic neuronal activity was suppressed by perfusion with TNF-α, which exerted the opposite effect on excitatory neuronal excitability.

Earlier studies focused on projection neurons due to their critical contribution to hyperalgesia (Mantyh et al., 1997; Todd, 2010), while recent progress suggests the important contribution of interneurons (GABAergic neurons) to pain processing (Duan et al., 2014). Moreover, disinhibition — the reduction or loss of inhibitory synaptic transmission in the spinal dorsal horn pain circuit — has been strongly implicated in central sensitization (Latremoliere and Woolf, 2009). In the current study, similar to projection neurons, GABAergic neurons played a crucial role in visceral hypersensitivity. Indeed, the inhibition of GABAergic neuronal activity naturally leads to an increase in projection neuron excitability, and this disinhibition subsequently produces visceral hypersensitivity. Based on accumulating evidence, microglia drive central sensitization via microglial mediators. Proinflammatory cytokines powerfully modulate inhibitory synaptic transmission at multiple sites in the spinal dorsal horn (Ji et al., 2013), and microglia are a major source of cytokines, including TNF, IL-6 and IL-1β (Hanisch, 2002). Unlike most of proinflammatory cytokines that increase the activity of excitatory neurons, the role of inflammatory cytokines in GABAergic neurons may be more complex. At the presynaptic level, IL-1β and IL-6 inhibit the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in the spinal pain circuit. At postsynaptic sites, IL-1β and IL-6 reduce the sIPSC amplitude. At extrasynaptic sites, GABA receptor activity is suppressed by IL-1β (Kawasaki et al., 2008; Yan et al., 2015). Furthermore, TNF rapidly inhibits spontaneous action potentials in GABAergic neurons (Zhang et al., 2010). Here, we revealed that TNF-α suppressed the activity of GABAergic neurons in the aIBNST.

Previous studies have demonstrated significant changes in nociceptive thresholds across the estrous cycle in female rats: the stages at which threshold was highest were proestrus (Kepler et al., 1989; Molina et al., 1990), diestrus-2/proestrus (Martinez-Gomez et al., 1994), and diestrus-2 (Frye et al., 1993) using tail flick tests, and diestrus-1/diestrus-2 in paw and tail pressure tests (Kayser et al., 1996), and in hotplate and tail withdrawal tests (Craft and Bernal, 2001). Our previous results (Chen et al., 2015) indicated that ovarian hormones are essential to the modulation of visceral hypersensitivity. Compared with male neonatal CRD rats, female neonatal CRD rats exhibited enhanced vulnerability in response to CRD during pro-oestrus/oestrus. In contrast, there were no significant differences during metoestrus/dioestrus. Since male rats were used in this study, the conclusion should strictly be restricted to males and should be extended to females with more caution. Given the evidence for a sex difference in the function and circuitry of the BNST and the fact that visceral pain is more robust in female rodents and humans, more research should be focused on female rats and effects of sex differences on visceral pain in the future. In both preclinical and clinical studies, a comparison of both sexes will further our understanding of individual differences in sensitivity to pain and analgesia, thus improving our ability to treat and prevent pain in all people.

In summary, we assayed the functional importance of the microglia-dependent inhibition of GABAergic neuronal activity in rats with visceral hypersensitivity. Pharmacological inhibition of microglia increased the activity of GABAergic neurons and alleviated visceral hypersensitivity, while the opposing result was obtained when aIBNST GABAergic neurons were ablated. In addition, the administration of TNF-α neutralizing antibody relieved visceral hypersensitivity and the administration of TNF-α exactly mimicked the decrease in GABAergic neuronal activity in rats with visceral hypersensitivity. Collectively, these findings suggest that microglia-derived TNF-α suppresses the activity of aIBNST GABAergic neurons and then participates in visceral hypersensitivity. This novel microglia-regulated disinhibition is essential for brain and gut dysfunction in stressful condition, providing a novel potential target for regimen for patients with IBS presenting with visceral pain that is worsened during episodes of stress.

Funding

CRediT authorship contribution statement

Ning-Ning Ji: Methodology, Formal analysis, Writing – original draft. **Qing-Xiang Meng:** Investigation. **Ying Wang:** Investigation. **Zi-Ming Zhou:** Data curation. **Yu Song:** Validation. **Rong Hua:** Conceptualization. **Yong-Mei Zhang:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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