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Research Article

Effects of NK2 antagonist GR 159897 on the levels of stress hormones and pro- and anti-inflammatory cytokines in the serum of rats subjected to repeated stress

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Summary

GR 159897 is a potent, selective NK2 receptor antagonist with anxiolytic properties that inhibits the contraction of experimental animals' tracheal and intestinal smooth muscle. This study evaluated its effects on stress hormones and cytokines in rats under repeated stress.

Methods: Wistar rats underwent water avoidance stress (WAS) and heterotypic stress (HS). Measurements included fecal pellet output, colorectal transit, and plasma levels of CTRH, ACTH, corticosterone (CORT), and cytokines.

Results: Control rats gained weight, while WAS rats lost weight. Stressed rats treated with GR 159897, increased weight significantly. In HS, there was no significant change in the body weight (BW), and the administration of GR 159897 resulted in a noticeable increase in BW. WAS and HS caused an increase in fecal pellet excretion output in animals, and GR159897 significantly reduced the average number of pellets in WAS animals. CRH levels were significantly different in WAS and HS groups compared to controls. GR 159897 reduced CRH in WAS rats. ACTH levels increased in stress-subjected groups, significantly in HS rats, but GR 159897 decreased its concentration in both stress groups. CORT levels indicated significant differences among the control, WAS, and HS groups. GR 159897 reduced CORT levels in WAS+GR group, but not statistically significant to WAS group. GR 159897 significantly reduced elevated IL-6 levels in stressed rats.

Conclusion: The ability of GR 159897 to reduce colorectal contractions, fecal pellet output, stress hormone levels, and proinflammatory cytokines leads us to conclude that NK2 receptor antagonists could help treat conditions like IBS or inflammatory bowel disease.

Key words: ACTH, corticosterone, heterotypic stress, inflammatory bowel disease, irritable bowel syndrome, water avoidance stress

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Introduction

Psychological stress is believed to play a major role in functional gastrointestinal disorders, especially irritable bowel syndrome (IBD), leading to worsening of symptoms. The available data indicate that delay of gastric emptying and stimulation of the colonic passage is a major response of the SHR to acute or

short-term stress. Thus, it can be assumed that these changes play a pathophysiological role in dyspeptic symptoms and changes in the frequency and consistency of stools in patients with stress-related functional disorders of the gastro-intestinal tract (GIT) (Mönnikesa et al. 2001). Irritable bowel syndrome is defined as a chronic and recurrent functional bowel disorder characterized by chronic abdominal pain, discomfort, bloating, and altered bowel behavior in the absence of overt structural abnormalities or infection on routine examination (Ren et al. 2007; Lee 2010). Altered visceral perception and motility are the main pathophysiological factors of IBS (Ait-Belgnaoui et al. 2005). Many studies have shown that patients with MDD have various gastrointestinal motility disorders that often arise from an abnormal physiological response to stimuli such as diet and stress (Maunder and Levenstein 2008; Chrousos 2009).

The mechanism of stress affecting colonic motility in IBS patients is not fully understood. For this reason, its influence on GIT can be followed in experimental animals subjected to acute or chronic stress in laboratory conditions. Multiple parameters – behavioral, metabolic, neurochemical, physiological, endocrine, and immunological can be used to monitor responses in stress models.

In recent decades, the role of brain-gut bidirectional interaction in functional gut diseases has been solidified (Jones et al. 2006; Fukudo and Kanazawa 2011; Al Omran and Aziz 2014; Slyepchenko 2014). These interactions occur via neuronal pathways through the efferent and afferent components of the parasympathetic, sympathetic and enteric nervous systems (ENS) (Furness 2012), a hormonal pathway including the hypothalamic-pituitary-adrenal (HPA) axis (Filaretova 2006) and/or components of the immune system and gut microbiome (Holzer and Farzi 2014).

Disorders of the gut-brain interaction known as functional gastrointestinal disorders, including functional dyspepsia and irritable bowel syndrome (IBS), define the spectrum of disorders associated with chronic or fluctuating gastrointestinal symptoms with no obvious organic structural or biochemical explanation for these symptoms (Drossman 2016; Black et al. 2020). This may be the result of subtle changes in ENS circuits that cannot be detected in routine clinical diagnostics (Avetisyan et al. 2015). In recent years, several shreds of evidence for the involvement of excitatory (acetylcholinergic, substance P – tachykininergic) as well as inhibitory (nitrergic and VIP-ergic) neuro-mediator systems of the ENS in motility in normal and pathological conditions (Steinhof et al. 2014). Tachykinins are neuropeptides expressed mainly in neurons in the gut (Delvalle et al. 2018), and are associated with disturbances in intestinal motility and pain transmission (Carini et al. 2001; Patel et al. 2014) and inhibition of tachykinin pathways offers a potential treatment target in patients with IBS (Szymaszkiewicz et al. 2019). Stimulation of tachykinin/neurokinin-2 (NK2) receptors located in enteric neurons leads to neurogenic inflammation in the ENS through mechanisms that involve intercellular enteric neuron-glia-nociceptor communication (Delvalle et al. 2018). Modern understanding of the composition and function of the ENS and of functional gastrointestinal disorders may lead to the creation of therapies targeting the ENS "second brain in the gut" (Simren et al. 2019).

The present study investigated the effects of NK2 antagonist GR 159897 on the levels of stress hormones and pro- and anti-inflammatory cytokines in the serum of rats subjected to repeated mono- and heterotypic stress with psychogenic characteristics.

Materials and methods

The experiment was performed in accordance with animal welfare regulations and was approved by the Bulgarian Food Safety Agency.

Substances and kits

GR 159897 ($C_{23}H_{27}FN_2O_2S$; CAS 158848-32-9) was purchased from Santa Cruz Biotechnology, Inc. Rat CRH (E-EL-R0270), Rat ACTH (E-EL-R0048), Rat CORT(E_EL_R0160), Rat IL-1alpha_Interleukin 1 Beta (E-EL-R0011), Rat IL-6 (E-EL-R0015) ELISA Kits were purchased from Elabscience.

Animals and experimental protocol

The study was conducted on 30 male Wistar rats (weight 250–280 g, age 5 months). The animals were obtained from the Scientific Laboratory Animal Breeding Center in Slivnitsa (Bulgaria). They were reared in the University vivarium for 1 month at a temperature of 22+2 °C and a humidity of 50 ± 10%, with a normal pelleted diet and water *ad libidum*. The animals were divided into six groups: (1) control; (2) water avoidance stress (WAS) monotypic; (3) WAS + GR 159897 (GR); (4) heterotypic stress (HS); (5) heterotypic stress + GR 159897; (6) sham WAS. Subsets of rats received daily injections (intraperitoneally) of GR 159897 (0.5 mg/kg) in 0.1% dimethyl sulfoxide 15 min before the stress (Kullmann et al. 2017; Delvalle et al. 2018). The controls were injected with saline.

Experimental models of stress

Stress was induced using the water avoidance stress (WAS) method (Mayer et al. 2000; Metz et al. 2001). The experiments were carried out in the morning between 9:00 and 11:00. Rats were subjected to WAS or a sham WAS procedure (no water around the platform). They were placed on a square platform (8 cm, 8 cm, and 9 cm high) mounted in the centre of a white translucent plastic container (50 cm, 35 cm, 33 cm) filled with fresh tap water at room temperature to 1 cm below the surface of the platform in 60 minutes. Chronic stress was applied for 5 consecutive days (in this model, rats are expected to adapt to the stressor to some extent). Because of the above, a chronic heterotypic stress procedure was also conducted, with rats in the group undergoing different types of stress throughout the 5-day experimental protocol: a) WAS for 1 hour; b) immobilization stress for 1 hour; c) cold exposure 4 °C for 1 hour. The stress load on days 1, 3, and 5 was WAS.

Measurement of integral indicators

Body weight, food and water intake, etc. to assess weight change from baseline.

Evaluation of colorectal transit time

Animals were exposed to the stressor and the number of fecal pellets excreted during WAS, sham stress, etc. was recorded to assess stress-induced colonic propulsive motility. A validated procedure was used to assess autonomic

regulation of distal colonic motility upon administration of stressors (Vassallo et al. 1992; Venkova et al. 2010). Post-stress propulsive motility of the distal colon was assessed by measuring the time required to expel a 5 mm diameter rat fecal pellet-like glass bead placed in the distal colon.

Immonological tests

Blood for measuring levels CRH, ACTH, corticosterone and cytokines (IL-1, IL-6, IL-10 and TNF alpha) by ELISA was taken from the heart of anesthetized animals. Tests are performed using an immunoassay kit according to the manufacturer's instructions. Each sample was analyzed in duplicate.

Blood for immunological tests is collected up to 1 hour after the application of the stressor in the case of acute stress, and in the case of chronic stress – after 1 day.

Statistical processing of the results

The primary information was organized and statistically processed using the software products MS Office Excel 2019 and Statgraphics Centurion xvi. Normally distributed data are presented as the mean (±) standard error, and in the absence of a normal distribution, the data will be presented using positional means – median (Me) and interquartile range (IQR). Differences at the $p < 0.05$ level was considered significant. Graphical analysis were performed using the Excel 2019 software package.

Results

Fecal pellet output and body weight were measured daily starting 1 day prior to the stress. Parallel measurements were taken from a control group not subjected to stress. All animals showed similar initial body weight and fecal pellet output (Tabl. 1). Rats subjected to the sham WAS procedure performed similarly to controls, so the results of this group will not be considered.

Table 1. Basal body weight **(**prior to WAS) and on day 6. Fecal pellets output measured daily and mean values for animal subjected to WAS, HS, and controls.

Group	Body weight (BW)		Excreted fecal pellets					
	Basal BW (BW1) mean ± SE	Final BW (BW2) mean ± SE	Day 1 mean ± SE	Day 2 mean ± SE	Day 3 mean ± SE	Day 4 mean ± SE	Day 5 mean ± SE	Mean ± SE
Control	259.17 ± 5.39	266.83 ± 5.34 p = 0.089 vs. BW1	1.6 ± 0.68	1.4 ± 0.4	1.2 ± 0.49	2.0 ± 0.83	1.6 ± 0.93	1.56 ± 0.13
Chronic WAS	253.0 ± 9.06	244.67 ± 8.47 p = 0.72 vs. BW1	7.17 ± 1.30*	6.33 ± 1.3	6.66 ± 0.96	4.17 ± 1.30	3.16 ± 1.10	5.43 ± 0.93 p = 0.003 vs. control
Chronic WAS + GR	242.33 ± 6.44	266.50 ± 6.26 * p = 0.022 vs. BW1	3.83 ± 0.70* p = 0.048 vs. WAS	6.16 ± 0.69 p = 0.91 vs. WAS	5.0 ± 0.96 p = 0.28 vs. WAS	2.0 ± 0.68 p = 0.17 vs. WAS	3.5 ± 0.72 p = 0.64 vs. WAS	3.0 ± 0.68* p = 0.042 vs. WAS
Heterotypic stress (HS)	236.17 ± 10.31	239.17 ± 11.03 p = 0.846	6.17 ± 1.25	n/a†	4.83 ± 1.56	n/a†	4.67 ± 1.43	n/a†
Heterotypic stress + GR	262.17 ± 3.17	283.17 ± 5.31* p = 0.007 vs. BW1	4.83 ± 1.25	n/a†	5.33 ± 0.56	n/a†	4.17 ± 0.70	n/a†

† n/a denotes "not applicable" due to reduced stool consistency and lack of formed pellets.

On day 6, the control animals gained an average of 7.67 g, showing no significant difference between their initial and final body weights. In contrast, animals subjected to WAS lost an average of 8.33 g. However, when stressed rats were treated with GR 159897, their weight increased significantly. In heterotypic stress, the body weight of experimental animals remained almost unchanged, and the administration of GR 159897 resulted in a noticeable increase in weight on day 6 compared to the initial one.

WAS caused an increase in fecal pellet excretion in all animals, whereas exposure to sham stress had no such effect. The output of fecal pellets from the controls was 3.5 times less than the number of fecal pellets produced during 60 minutes of WAS. The pellets in the stressed animals decreased with time, 2.3 times less on day 5. GR 159897 significantly reduced the number of pellets on the first day and the average number of pellets in WAS animals (Table 1, Fig. 1).

The stool consistency of the animals subjected to immobilization stress on day 2 and cold exposure on day 4 was decreased, and there were no formed pellets. Due to the diarrheal stools, the number of fecal pellets could not be counted on days, and it was estimated that heterogeneous stress led to more significant stress-induced changes in colonic propulsive motility. No significant effects were observed on the average amount of fecal pellets on days 1, 3, and 5, nor on the consistency of feces on days 2 and 4 with the administration of GR administered at a dose of 0.5 mg/kg in HS.

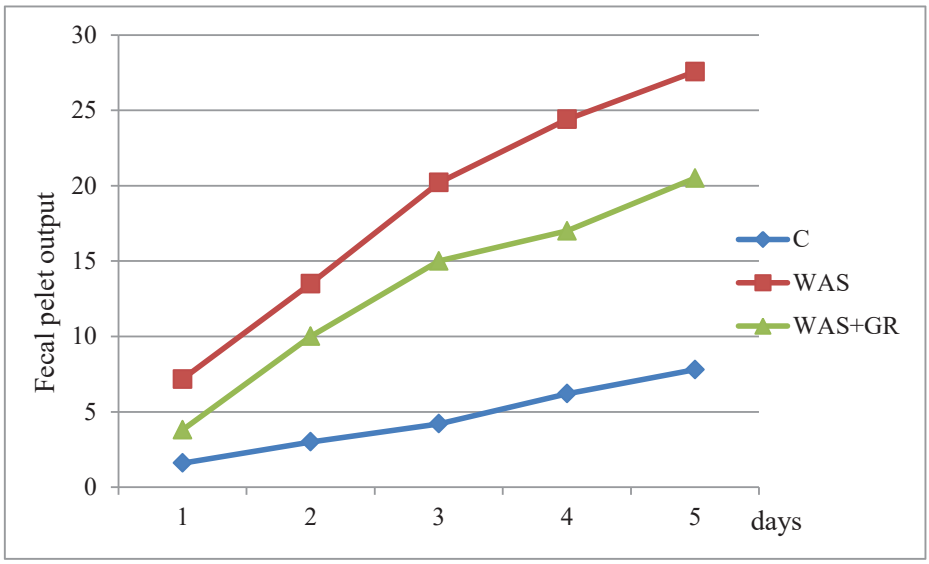

Figure 1. Cumulative fecal pellet output measured by the number of pellets released during 60 min WAS and without stress (control). Data are presented as mean from 6 animals. The statistical significance is presented in Table 1.

In the present study, we assessed systemic levels of stress hormones in response to WAS, HS, and the effect of GR 159897 on their concentration. One-way ANOVA and Fisher's least significant difference (LSD) procedure were used to discriminate among the means. The multiple comparison procedure shows that CRH in WAS group and HS group are significantly different from the control mean ($p < 0.05$). NK2 antagonist GR 159897 significantly reduced CRH serum level in WAS, measured on day 6 (Table 2).

Table 2. Plasma CRH, ACTH, and CORT concentration in rats on day 6.

Groups	Corticotropin-releasing hormone ng/ml	Adrenocorticotropic hormone ng/ml	Corticosterone ng/ml
	Mean ± SEM	Mean ± SEM	Mean ± SEM
Control	4.225 ± 0.568	240.6 ± 38.40	6.84 ± 0.66
Chronic WAS	15.25 ± 2.095*	261.01 ± 26.58	13.33 ± 2.02*
Chronic WAS + GR	11.43 ± 2.374Δ	219.1 ± 34.37	10.08 ± 0.46*
Heterotypic stress (HS)	20.03 ± 0.264*	479.15 ± 107.88*	12.37 ± 5.57*
Heterotypic stress + GR	19.17 ± 0.937	395.38 ± 63.42 Δ	3.32 ± 0.49 Δ

*denotes a statistically significant difference to control; Δ denotes a statistically significant difference to stressed group.

Post-hoc tests for multiple comparisons between the groups revealed a significant increase in plasma ACTH levels in the group subjected to heterotypic chronic stress compared to the WAS and control groups (Table 2). Treatment with GR 159897 reduced ACTH concentrations in both stressed groups, although it had no significant effect in the WAS-subjected rats.

Multiple comparisons of corticosterone levels indicated significant differences in plasma CORT concentrations among the control, WAS, and HS groups. GR 159897 reduced CORT levels in stressed animals (10.08 ± 0.46 ng/ml), but this reduction was not statistically significant to the WAS group (13.33 ± 2.02 ng/ml, $p > 0.05$).

The pro-inflammatory cytokine interleukin-6 was significantly elevated in the serum of animals subjected to WAS (39.60 ± 5.72 ng/ml) compared to the control group (20.82 ± 0.08 ng/ml, $p < 0.05$). Treatment with GR 159897 significantly decreased IL-6 levels to 20.91 ± 0.10 ng/ml ($p < 0.05$). Heterotypic stress had no significant effect on IL-6 concentration (25.99 ± 0.90 ng/ml, $p > 0.05$).

Discussion

This study provides evidence supporting the role of the tachykininergic system in modulating biological stress systems relevant to the pathogenesis of IBS. We have confirmed that chronic water avoidance stress is reproducible animal models of IBS with the involvement of the hypothalamic-pituitary-adrenal axis (Patchev and Patchev 2006; Watanabe et al. 2016; Pang et al. 2024). This well-characterized test is a potent psychological stressor accompanied by an increase in ACTH and cortisol within 30 minutes (Million 1999). We demonstrated that exposure to WAS is an appropriate model for irritable bowel syndrome, better than heterotypic alternating stress of WAS, cold, and immobilization stress. The heterogeneous stress we applied in our experiment led to a significant decrease in the consistency of stool on the days when immobilization and cold stressors were used, which did not allow us to count the number of pellets released and assess the effect of stress and the test substance. Exposure to extreme cold and immobilization, like WAS, are naturalistic models of threat to survival. However, chronic immobilization stress induces depression-related behaviors (Sahin et al. 2020), and cold stress rather induces anxiety-related disorders (El Marzouki et al. 2021). Psychological stress is thought to play a major role in functional gastrointestinal disorders, especially IBD, leading to exacerbation of dyspeptic symptoms and changes in stool frequency and consistency in patients with stress-related functional gastrointestinal disorders (Mönnikesa et al. 2001). Animals subjected

to WAS experienced a decrease in body weight, unlike those in the HS group. WAS also increased fecal pellet excretion in all animals, with the number of pellets decreasing over time, suggesting some adaptation to the stressor.

Substance P, a peptide in the neurokinin family, plays various physiological roles, including neurotransmission, inflammation, pain transmission, and smooth muscle contraction. GR 159897 ((R)-1-[2-(5-fluoro-1H-indol-3-yl)ethyl]-4-methoxy-4-[(phenylsulfinyl)methyl] piperidine) is a highly effective and specific non-peptide antagonist of tachykinin NK2 receptors. In guinea-pig trachea, GR 159897 prevented contractions induced by the NK2 receptor agonist [Lys3,Gly8-R-gamma-lactam-Leu9]-neurokinin A-(3-10) (GR64349). In vivo, GR159897 (0.12 mg/kg i.v.) demonstrated a long duration of action (3 hours) in guinea pigs, effectively counteracting bronchoconstriction caused by GR64349 (Beresford et al. 1995). Walsh et al. (1995) established the anxiolytic-like action of GR 159897. Additionally, Delvalle and Gulbransen (2017) showed that GR 159897 protects against neuroinflammation in the enteric nervous system in the DNBS mouse model of colitis.

Intraperitoneal administration of the NK2 receptor antagonist 15 minutes before stress led to a statistically significant increase in body weight on day 6 in rats subjected to both WAS and HS. We evaluated the effect of GR 159897 on stress-induced colonic propulsive motility by counting the number of fecal pellets excreted during stress. GR 159897 significantly reduced the number of pellets on the first day and the average number of pellets in a model of IBD (WAS animals), without affecting the average number of pellets in HS. Rupniak et al. (2017) investigated the effects of an NK2R antagonist on colorectal pressure and hypotension induced by three NK2 agonist peptides in dogs. The administration of GR 159897 (1 mg/kg IV) abolished the colorectal contractions induced by the agonists, confirming that this response was mediated by the NK2 receptor (Rupniak et al. 2017).

In our study, stress hormones were significantly elevated in rats subjected to WAS and heterotypic stress. The tachykininergic antagonist GR 159897 significantly reduced serum levels of ACTH and corticosterone more than CRH under heterotypic stress. Also, in the WAS the level of CRH was significantly reduced.

The corticotropin-releasing hormone signalling pathways play a key role in response to various stressors and the interaction between the brain and the gut (Taché and Brunnhuber 2008; Holzer and Farzi 2014). CRH modulates gastrointestinal functions through neuronal pathways in the central and the autonomic nervous system (Taché and Bonaz 2007; Larauche al. 2009; Taché 2015). Activation of brain CRH receptors results in delayed gastric emptying and increased colonic motor function under acute psychological, physical, or chemical stress, as well as immune system activation. Recent findings on CRH receptor subtypes highlight the role of medullary CRH-R2 in inhibiting gastric emptying. In contrast, central CRF injection and psychological stress activate CRF-R1, leading to anxiogenic and motility responses in the colon (Martínez and Taché 2001). Given that most knowledge on stress-related GI motility changes comes from acute exposure studies, we examined the effects of chronic homotypic and heterotypic stress. Both stress models showed increased systemic levels of CRH, with a more pronounced increase in HS. Martinez et al. demonstrated that intracerebroventricular injection of a CRH agonist with high affinity for CRH-R1 increased defecation in unanesthetized rats without causing diarrhea. This indicates activation of colonic propulsive activity, as previously established by Tache et al. (1999). Pretreatment with a non-peptide CRH receptor antagonist blocked the

defecation response and reduced fecal pellet output (Martinez et al. 2001). Although, as seen above, increases in CRH mediate many of the gastrointestinal effects of repeated stress by acting on CRH receptors in enteric neurons (Tache et al. 1999; Fukudo 2008), there is growing evidence that corticosterone (Cort) may directly affect neurons in the ENS. Blin et al. (2023) have shown that Cort plays a role in regulating the enteric nervous system and gut functions under stress. They found that changes in colonic motility are partly due to the glucocorticoid-dependent up-regulation of enteric cholinergic neurons. They demonstrated that the activation of CORT receptors during repeated stress increases choline acetyltransferase positive neurons and elevates tissue acetylcholine levels. This leads to an increase in both cholinergic spontaneous contractile responses and enhanced electrically field-stimulated (EFS) contractile responses, as indicated by their sensitivity to atropine. While corticotropin-releasing hormone (CRH)-mediated effects are considered short-term responses leading to acute activation of enteric neurons (Tache et al. 2018), glucocorticoids can preferentially induce long-term changes in neurons via glucocorticoid receptors (Datson 2008).

Inflammatory mediators released during stress can modify components of the enteric nervous system, leading to the development of functional gastrointestinal disorders (Palomo et al. 2015). Observations include increased mast cell infiltration and activation, altered intestinal barrier permeability, and elevated serum levels of pro- and anti-inflammatory cytokines. Molecular interactions have been demonstrated between CRH, which mediates peripheral stress-induced effects on gastrointestinal sensory, motor, mucosal, and immune functions (Larauche et al. 2009) and the pro-inflammatory cytokine IL-6, proposed as a biomarker of irritable bowel syndrome (Dinan et al. 2006). By demonstrating an interactive relationship between CRF and IL-6, leading to potentiation of calcium responses in submucosal neurons and altered colonic secretory and motor activity, O'Malley et al. (2013) elucidated a potential mechanism by which stressful stimuli, combined with ongoing low-grade immune activation, may exacerbate IBS symptoms. We observed an increase in the levels of the pro-inflammatory cytokines IL-1 and IL-6 in the stress models used, alongside elevated levels of CRH. Additionally, GR 159897 had a beneficial effect on these cytokines.

Information on the effects of endogenous substance P (SP) under conditions of stress as well as on the HPA axis in response to stress is limited. Earlier studies of the impact of SP on the HPA axis activity (Larsen et al. 1993; Malendowicz et al. 1996) used peptide antagonists that are specific for SP but bind to all three NK receptors. The research results by Malendowicz et al. (1996) have shown that endogenous SP exerts quite different actions on the HPA-axis response to ether and cold stress. Jessop et al. (2000) investigated the selective NK1 receptor antagonist RP67580 to determine whether endogenous SP inhibits via central NK1 receptors the HPA axis response to acute stress. They found that endogenous SP did not inhibit the initial intensity of the HPA axis response to restrain stress, but did act via neurokinin-1 receptors centrally to reduce the duration of the stress response.

CRH can be secreted outside the brain, where it exerts pro-inflammatory effects by activating mast cells, which are increasingly involved in immunity and inflammation. Substance P is also involved in inflammatory diseases. A study found that substance P, which is released from nerve endings upon stress and tachykininergic receptors mediate the effect of stress on CRH expression in eosinophils (Zheng et al. 2009). CRH can be released in the periphery by eosino-

phils (Overman et al. 2012), immune cells located in the mucosa of the gastrointestinal tract, involved in the initiation and propagation of various inflammatory responses (Hogan et al. 2008). Substance P appears to increase CRH release by activating neurokinin-2 receptors in jejunum segments of chronically stressed (1-hour restraint stress for 10 consecutive days) mice. Zheng et al. summarized that substance P induces CRH release from eosinophils and subsequently activates CRH receptors in mast cells, leading to mast cell degranulation (2009).

It is accepted that IBS is a stress-sensitive disorder, but not only central but also peripheral mechanisms play an important role in the initiation and maintenance of symptoms. Our study suggests that the selective NK2 receptor antagonist GR 159897, which is a small molecule with good penetration into the central nervous system, would affect the changes induced by repeated stress.

It is well-established that IBS is a stress-sensitive disorder, with both central and peripheral mechanisms playing important roles in the initiation and maintenance of symptoms. The results of our study suggest that the selective NK2 receptor antagonist GR 159897, a small molecule with central nervous system penetration, can moderate the changes induced by repeated mono- and heterotypic stress with psychogenic features in rats. The ability of GR 159897 to reduce colorectal contractions, fecal pellet output, stress hormone levels, and proinflammatory cytokines leads us to conclude that NK2 receptor antagonists may be effective in treating conditions characterized by abnormal colorectal motility, such as IBS or inflammatory bowel disease.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: № 18440/03.09.2024.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

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Author contributions

Milena Atanasova – making of ELISA tests, Venka Tsankova and Galia Stavreva – experiment design and precessing of results.

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Data availability

All of the data that support the findings of this study are available in the main text.

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