Bupleurum falcatum Prevents Depression and Anxiety-Like Behaviors in Rats Exposed to Repeated Restraint Stress

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Previous studies have demonstrated that repeated restraint stress in rodents produces increases in depression and anxiety-like behaviors and alters the expression of corticotrophin-releasing factor (CRF) in the hypothalamus. The current study focused on the impact of Bupleurum falcatum (BF) extract administration on repeated restraint stress-induced behavioral responses using the forced swimming test (FST) and elevated plus maze (EPM) test. Immunohistochemical examinations of tyrosine hydroxylase (TH) expression in rat brain were also conducted. Male rats received daily doses of 20, 50, or 100 mg/kg (i.p.) BF extract for 15 days, 30 min prior to restraint stress (4 h/day). Hypothalamic-pituitary-adrenal axis activation in response to repeated restraint stress was confirmed base on serum corticosterone levels and CRF expression in the hypothalamus. Animals that were pre-treated with BF extract displayed significantly reduced immobility in the FST and increased open-arm exploration in the EPM test in comparison with controls. BF also blocked the increase in TH expression in the locus coeruleus of treated rats that experienced restraint stress. Together, these results demonstrate that BF extract administration prior to restraint stress significantly reduces depression and anxiety-like behaviors, possibly through central adrenergic mechanisms, and they suggest a role for BF extract in the treatment of depression and anxiety disorders.

Keywords: Stress, depression, anxiety, tyrosine hydroxylase, Bupleurum falcatum

Major depression is a serious mental disorder with profound personal and public health implications. The illness is characterized by a low mood, low self-esteem, and loss of pleasure. Individuals with depression often report adverse physical symptoms and have an increased mortality rate [23]. Major depression is frequently combined with symptoms of anxiety [19, 21]. Although genetic studies have revealed a heritable component in the development of depression, psychological and social factors, including stress, also increase vulnerability to the disease [24].

Stress is characterized by a combination of physiologic, neuroendocrine, behavioral, and emotional responses to novel or threatening stimuli [10]. One of the main circuits that initiates and maintains the body’s response to stress is the hypothalamic-pituitary-adrenal (HPA) axis. In response to acute stress, multiple hormones are released into the circulation from the HPA axis, including hypothalamic corticotrophin-releasing factor (CRF), pituitary adrenocorticotropin-releasing hormone (ACTH), and adrenal glucocorticoids (GC). Activation of the stress response leads to alterations that improve the ability of an organism to adjust its homeostasis and minimize the potential impact of a threat [8]. Chronic stress system activation, however, may adversely impact brain function [18, 36]. Several animal research models, including the acute restraint stress, have been used to study the HPA axis response to stressful stimuli. Previous studies have shown that repeated restraint stress in rodents alters emotionality and increases anxiety, aggression [30], and depression-like behaviors [10]. An animal model that generates depression and anxiety-like behaviors in response to stressful stimuli is very useful in determining antidepressant and anxiolytic efficacy.

Interestingly, specific herbal extracts and their pharmacological components have been shown to alleviate depression and anxiety-related behavior [44]; however, the underlying mechanisms of these effects are unknown. Bupleurum falcatum (BF), or Chinese Thoroughwax, is widely used in traditional Oriental medicine to treat various psychosomatic disorders, including stress-induced depression [15]. Previous studies have shown that the pharmacological effects of BF on the central nervous system are exerted...
MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley (SD) rats weighing 260–280 g were obtained from Samtaco Animal Co. (Seoul, Korea). The rats were housed in a limited access rodent facility with up to five rats per polycarbonate cage. The room controls were set to maintain the temperature at 22°C ± 2°C and the relative humidity at 55% ± 15%. Cages were lit by artificial light for 12 h each day. Sterilized drinking water and standard chow diet were supplied ad libitum to each cage during the experiments. The animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996, and were approved by the Kyung Hee University Institutional Animal Care and Use Committee. All animal experiments began at least 7 days after the animals arrived.

Preparation of Methanol Extracts of BF and Drugs

*Bupleurum falcatum* (BF) was purchased from Dongwoodang Pharmacy Co., Ltd. (Yeongcheon, Korea). A voucher specimen of BF has been deposited at the herbarium located at the College of Oriental Medicine, Kyung Hee University (No. D0801130 for BF). Five hundred g of BF was cut into small pieces and extracted three times with 2 l of 80% methanol by sonication in a reflux condenser for 24 h at room temperature (25 ± 2°C). The extracted solutions were combined, filtered through Whatman No. 1 filter paper, and concentrated using a rotary vacuum evaporator (Rotavapor R-124; BüCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure. Subsequently it was refrigerated in a recirculating chiller (EYELA FD-800; Tokyo Rikakikai Co., Tokyo, Japan). The BF yield in a powder form was 18.62%.

Experimental Groups

Rats were randomly assigned to weight-matched groups and divided into six groups consisting of seven individuals each as follows: unstressed animals injected with saline instead of BF (0.9% NaCl, i.p., CON group, n = 7), unstressed animals injected daily with 100 mg/kg BF (i.p., BF group, n = 7), restraint-stressed animals injected daily with saline prior to restraint stress (i.p., STR group, n = 7), restraint-stressed plus 10 mg/kg BF group (i.p., STR+BF10 group, n = 7), restraint-stressed plus 50 mg/kg BF group (i.p., STR+BF50 group, n = 7), and restraint-stressed plus 100 mg/kg BF group (i.p., STR+BF100 group, n = 7). The BF or saline was intraperitoneally injected to the animals 30 min before the daily restraint stress for 15 days.

The restraint stress procedure was carried out once daily for 4 h from 10:00 a.m. to 14:00 p.m., as previously described [1]. In brief, rats were forced to be placed in a transparent plastic tube (20 × 7 cm), of which one end is conical shaped and has several 3 mm holes for breathing and the other end is open, for 4 h a day for 15 consecutive days. The animals had ample air but were unable to move within the tubes. The following parameters were measured to monitor the effects of the stress: changes of body weight gains (at the beginning step of restraint stress), and serum CORT levels (after repeated restraint stress). All rat groups except the CON group received the same restraint stresses. The experimental schedules of restraint stress and behavioral examinations are shown in Fig. 1.

Measurement of Forced Swimming Test (FST)

FST is a behavioral test, frequently used to evaluate the activities of potential antidepressant drugs using rodents. Forced immersion of rats in water for an extended period produces a characteristic behavior of immobility [17]. The antidepressant treatments decrease the immobility behavior accompanying with an increase in the escape responses such as climbing and swimming [17]. A transparent plexiglass cylinder (20 cm diameter × 50 cm height) was filled up to a depth of 30 cm with water at 25°C. At this depth, rats could not touch the bottom of the cylinder with their tails or hind limbs. On day 15, the rats in all groups were trained for 15 min by placing them in the water-filled cylinder. On day 16, animals were subjected to 5 min of forced swim, and escape behaviors (climbing and swimming) were determined. The duration of immobility was scored during the 5 min test period. Climbing was defined as upward-directed movements of the forepaws along the side of the swim chamber, and swimming was considered as movements throughout the swim chamber including crossing into another quadrant. Immobility behavior was calculated as the length of time in which the animal did not show escape responses (e.g., total time of the test minus time spent in climbing.
and swimming behaviors). The animal’s behavior was continuously recorded throughout the testing session with an overhead video camera. After the test, the rat was removed from the tank, dried with a towel, and placed back in its home cage. The water in the swim tank was changed between rats.

Measurement of Elevated Plus Maze (EPM)

The EPM test is a widely used behavioral test to assess anxiogenic or anxiolytic effects of pharmacological agents [40]. Animals that conduct anxiety-like behaviors usually show reductions both in the number of entries and in the time spent in the open arms, along with an increase in the amount of time spent in the closed arms in the EPM. On day 16, the elevated plus test was conducted. This apparatus consisted of two open arms (50 × 10 cm each), two closed arms (50 × 10 × 20 cm each), and a central platform (10 × 10 cm), arranged in such a way that the two arms of each type were opposite to each other. The maze was made from black plexiglass and elevated 50 cm above the floor. Exploration of the open arms was encouraged by testing under indirect dim light (2 × 60 W).

At the beginning of each trial, animals were placed at the center of the maze, facing a closed arm. During a 5 min test period, the following parameters were recorded: a) number of open arm entries, b) number of closed arm entries, c) time spend in open arms, and d) time spent in closed arms. Entry by an animal into an arm was defined as the condition in which the animal has placed its four paws in that arm. The maze was cleaned with alcohol after each rat had been tested. The behavior in the maze was recorded using a video camera mounted on the ceiling above the center of the maze and relayed to the S-MART program (PanLab, Barcelona, Spain). Anxiety reduction, indicated by open arm exploration in the EPM, was defined as an increase in the proportion of entries into the open arms relative to total entries into either open or closed arm, and an increase in the proportion of time spent in the open arms relative to total spending time in either open or closed arm. Total arm entries were also used as indicators of changes in locomotor activities of the rats.

Corticosterone (CORT) Measurement

Animals were killed by decapitation one day after behavioral measurements. Blood samples were collected for the determination of serum CORT levels. For this, the unanesthetized rats were rapidly decapitated, and blood was quickly collected via the abdominal aorta. Blood was centrifuged at 4,000 × g for 10 min, and serum was collected and stored at −20°C until use. The CORT concentration was measured by a competitive enzyme-linked immunosorbent assay (ELISA). Using a rabbit polyclonal CORT antibody (OCTESIA Corticosterone kit, Alpco Diagnostics Co., Windham, NH, USA) according to the manufacturer’s protocol. Samples (or standard) and conjugate were added to each well, and the plate was incubated for 1 h at room temperature without blocking. After wells were washed several times with buffers and proper color developed, the optical density was measured at 450 nm using an ELISA reader (MutiRead 400; Author Co., Vienna, Austria).

Corticotrophin-Releasing Factor (CRF) and Tyrosine Hydroxylase (TH) Immunohistochemistry

For immunohistochemical studies, the animals were deeply anesthetized with sodium pentobarbital (80 mg/kg, by intraperitoneal injection) and perfused through the ascending aorta with normal saline (0.9%) followed by 300 ml (per rat) of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, post-fixed over-night, and cryoprotected with 20% sucrose in 0.1 M PBS at 4°C. Coronal sections 30 µm thick were cut through the hypothalamus using a cryostat (Leica CM1850; Leica Microsystems Ltd., Nussloch, Germany). The sections were obtained according to the rat atlas of Paxinos and Watson [28]. The sections were immunostained for CRF and TH expressions using the avidin–biotin–peroxidase complex (ABC) method. Briefly, the sections were rinsed three times for 5 min each in PBS and then incubated with primary goat anti-CRF antibody (1:2,000 dilution; Santa Cruz Biotechnology Inc., CA, USA) and sheep anti-TH antibody (1:2,000 dilution; Chemicon International Inc., Temecula, CA, USA) in PBST (PBS plus 0.3% Triton X-100) for 72 h at 4°C. The sections were washed for 5 min in PBS and then incubated for 120 min at room temperature with biotinylated rabbit anti-goat IgG secondary antibody (for the anti-CRF antibody) and biotinylated goat anti-sheep IgG secondary antibody (for the anti-TH antibody). Both secondary antibodies were obtained from Vector Laboratories Co. (Burlingame, CA, USA) and diluted 1:200 in PBST containing 2% normal serum. To visualize immunoreactivity, the sections were incubated for 90 min in ABC reagent ( Vectastain Elite ABC kit; Vector Labs., Co., Burlingame, CA, USA), washed three times for 5 min in PBS, and incubated in a solution containing 3,3’-diaminobenzidine (DAB; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 0.01% H2O2 for 1 min. Finally, the tissues were washed in PBS, followed by a brief rinse in distilled water, and mounted individually onto slides. Slides were allowed to air dry and were then cover-slipped. Images were captured using the AxioVision 3.0 imaging system (Carl Zeiss, Inc., Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). The sections were viewed at 200 × magnification, and the numbers of cells within 100 × 100 µm² grids were counted by observers blinded to the experimental groups. The cells were obtained according to the stereotactic atlas of Paxinos and Watson [28]. The cells were counted in three sections for each rat.

Statistical Analysis

All measurements were performed by an independent investigator blinded to the experimental conditions. Results in figures are expressed as mean ± standard error of means (SE). Differences within or between normally distributed data were analyzed by analysis of variance (ANOVA) using SPSS (Version 13.0; SPSS, Inc., Chicago, IL, USA) followed by Tukey’s post hoc test. Statistical significance was set at p < 0.05. Statistical analysis of body weight gain was assessed using a one-way ANOVA with a repeated-measure factor of sessions (number of days) followed by the appropriate Tukey’s post hoc analysis. Behavioral data, immunohistochemical data, and CORT concentration analysis were also analyzed by one-way ANOVA followed by Tukey’s post hoc test.

RESULTS

Effect of BF on Repeated Restraint Stress-Induced Body Weight Loss

Rats exposed to repeated restraint stress begin to lose their body weights on the first day of restraint, and this stress-
induced initial reduction of body weight is sustained for a while without restoring to normal level or even exacerbated in some cases [3, 10, 11, 32]. In the present study, we also examined daily body weights for 14 days to identify whether repeated restraint stress (STR group) induced body weight loss (difference between daily weight and starting weight) (Fig. 2). The restraint rats (STR group) gradually gained less body weights for 14 days than did the normal control rats (CON group), even though the reductions of weights were not significant until the 12th restraint stress was applied. Analysis of the body weight values by repeated-measures ANOVA showed significant main effects of day \[F(13,468) = 352.379, p<0.001\] and of treatment group \[F(65,468) = 4.077, p<0.001\], and a significant day × group interaction \[F(5,36) = 6.907, p<0.001\]. Tukey’s post-hoc test revealed a significant reduction in body weight gain from day 12 to 14 in the STR group, as compared with the CON group (p<0.05 on day 12, p<0.01 on day 13, p<0.01 on day 14). During this period, the 100 mg/kg BF-treated rats prior to repeated restraint stress showed significant inhibitions of reductions in body weight gains, as compared with the STR group (p<0.05 on day 12, p<0.01 on day 13, p<0.01 on day 14).

**Effect of BF on Repeated Restraint Stress-Induced Serum CORT Levels**

Acute restraint stress induces a large increase in serum CORT and its levels are gradually decreased as restraint was repeatedly applied to the rats, probably due to adrenal habituation [32]. In the present study, the serum CORT levels were measured in each group after restraint stress exposure for 15 days. The ELISA analysis demonstrated that repeated restraint stress significantly increased the serum CORT concentration in the rats by 188.93% (p<0.05), as compared with the CON group (Fig. 3). It indicated that the repeated restraint was sufficiently stressful despite the evoked CORT response to repeated stress was significantly less than the response to single restraint stress (data not shown). Daily administration of BF slightly inhibited the restraint-induced increase in serum CORT level as compared with the STR group, in spite of little statistical significance. The BF treatment without restraint did not affect CORT concentration in sera.

**Effect of BF on Repeated Restraint Stress-Induced Depression-Like Behavior**

Rats subjected to repeated restraint stress for 15 days exhibited a significant depression phenotype, characterized by increased immobility time during the FST, as compared with saline-treated controls (CON group) (Fig. 4). Statistical analysis of behavioral data from the FST showed that the immobility time in the maze had significant differences among the six experimental groups \[F(5,41) = 8.161, p<0.001\]. The rats in the STR group showed more immobility during the FST, as compared with the CON group (p<0.01; Fig. 4A). However, the rats in the STR+BF100 group showed significant decrease in immobility time during 5 min in the FTS, as compared with those in the STR group (p<0.05), indicating that administration of BF decreased depression-like behavior. In addition, statistical analysis of behavioral data from the FST showed that the climbing behavior in the maze had significant differences among the six experimental groups \[F(5,41) = 3.817, p<0.01\]. The rats in the STR group showed significant decrease in climbing behavior during the FST, as compared with the CON group (p<0.05; Fig. 4B). It was shown that the rats in the STR+BF100 group showed significant restoration in climbing behavior time during
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5 min in the FTS, as compared with those of the STR group (p<0.05). It also indicated that the BF administration significantly restored depression-like despair behavior. However, repeated restraint stress did not induce significant differences of swimming behavior among all groups during the FST [F(5,41) = 0.629, p = 0.678] (Fig. 4C).

Effect of BF on Repeated Restraint Stress-Induced Anxiety-Like Behavior

The effect of BF administration on anxiety-like behavior, expressed by a decrease in open-arm exploration in the EPM test, was also investigated. One-way ANOVA revealed significant differences of the percentage of time spent in the open arms of the maze, evidencing an anxiolytic-like activity of BF extract (Fig. 5). Post hoc comparisons identified significant decreases in the percentage of time spent in the open arms of the maze after repeated restraint stress exposure for 15 days, as compared with the CON group (p<0.05). However, the rats

Fig. 4. Effect of BF on immobility time (A), climbing behavior (B), and swimming behavior (C) in forced swimming test during repeated restraint stress.

Data were analyzed using one-way ANOVA followed by Tukey’s test. *p<0.05, **p<0.01 vs. CON group; #p<0.05 vs. STR group. Vertical bars indicate SE.

Fig. 5. Effect of BF on the percentage of time spent in open-arm exploration (A) and the numbers of entries into open arms (B) during repeated restraint stress.

Data were analyzed using one-way ANOVA followed by Tukey’s test. *p<0.05, **p<0.01 vs. CON group; #p<0.05 vs. STR group. Vertical bars indicate SE.
in the STR+BF100 group showed a significant restoration of the percentage of time spent, that was decreased by repeated restraint, in the open arm of the maze, as compared with those in the STR group (p<0.05; Fig. 5A). Similarly, post hoc comparisons identified a significant decrease in the numbers of entries in the open arms of the maze after repeated exposure to restraint stress for 15 days, as compared with the CON group (p<0.01). The rats in the STR+BF100 group also showed a significant restoration in the numbers of entries in the open arm of the maze (p<0.05; Fig. 5B). Because any significant group differences of the number of closed-arm entries were not observed in the EPM test [F(5,41) = 0.208, p = 0.957], it could be suggested that the observed anxiety-like behaviors of rats with repeated restraint stress were not attributed to the differences of their locomotion activities (Fig. 5B). The BF administration without prior input of repeated stress did elicit an anxiolytic or anxiogenic behavioral activity in this study.

**Effect of BF on Repeated Restraint Stress-Induced CRF-Like Immunoreactivity**

Following the behavioral tasks, CRF-like immunoreactivity was analyzed primarily in the cell bodies of hypothalamic regions including the PVN (Fig. 7). In the rat brains from the STR group, the numbers of CRF-immunoreactive fibers in the PVN were increased by 212.40%. Comparisons of the numbers of CRF-immunoreactive neurons using one-way ANOVA revealed significant differences among the experimental groups [F(5,71) = 15.431, p<0.001]. Post hoc comparisons revealed that the rats with repeated restraint stress exposure showed a significant increase in CRF expression, as compared with the CON group (p<0.001). The number of CRF-immunoreactive neurons decreased significantly in hypothalamic regions in the STR+BF100 group (p<0.01), as compared with the STR group. The increase in CRF immunoreactivity by repeated stress was significantly restored by the BF administration, and the numbers of CRF-immunopositive neurons in the STR+BF100 group was close to those in the CON group.

**Fig. 6.** Representative photographs showing CRF expression in the paraventricular nucleus (PVN) of the hypothalamus and the TH expression in the locus coeruleus (LC) of CON-CRF (A), STR-CRF (B), STR+BF100-CRF (C), CON-TH (D), STR-TH (E), and STR+BF100-TH (F) groups.
The scale bar represents 50 µm.

**Fig. 7.** Effect of BF on the expression of CRF in the paraventricular nucleus (PVN) of the hypothalamus after repeated restraint stress. Values are presented as the mean ± SE of the total number of CRF-like immunoreactive neurons within a 100 × 100 µm grid over the areas at 200× magnification. Significance with Tukey’s test following a one-way ANOVA is indicated as ***p<0.001 vs. the CON; ##p<0.01 vs. the STR group. The scale bar represents 50 µm.
there are, however, reports similar to ours that have demonstrated restraint stress in rodents have produced mixed results. There are repeated restraint stress-induced changes in behavior in comparison with controls. Moreover, pre-emptive BF blocked FST and decreased open-arm exploration in the EPM test in significantly increased the duration of immobility in the present study revealed that repeated restraint stress increased depression and anxiety-like behaviors [34, 38]. We observed a gradual decrease in body weight and increase in serum CORT levels in animals that experienced daily restraint stress. Pre-emptive BF administration restored body weight gain and decreased serum CORT levels in comparison with saline-treated controls, suggesting that BF inhibits the HPA axis-associated psychological effects of repeated restraint stress.

The FST is a valid and reliable behavioral research model of depression in rodents, which is commonly used to determine the efficacy of antidepressant medications [5, 29]. The FST is based on a simple principle: measurement of the duration of immobility when a rodent is exposed to an inescapable situation. The immobility behavior displayed by rats in the FST is reflective of despair and depression in humans. Although the FST provides information about mood (i.e., depression and anxiety) in rodents, it is important to use caution when extrapolating the data to humans. Our results are consistent with previous findings showing that repeated restraint stress increased immobility during the FST [10, 34, 37]. It has been reported that swimming behavior in the FST is associated with serotonergic systems, whereas climbing during the task is under noradrenergic control [5]. In the present study, BF extract reduced immobility and climbing behaviors, but had no effect on swimming in the FST compared with controls. These results suggest that central adrenergic systems mediate the potent antidepressant effects of BF.

The EPM is a valid animal behavioral model of anxiety that is commonly used as a screening tool for putative anxiolytic compounds [26, 39]. The model is based on the rodent’s aversion to open spaces. Anxiety is indicated by a decrease in the proportion of time spent in the open arms of the EPM. In the present study, BF extract treatment prior to repeated restraint stress significantly blocked anxiety-like behaviors. Specifically, pre-emptive BF increased the percentage of time and number of entries into the open arms. Proven anxiolytic agents, including selective serotonin reuptake inhibitors, significantly reduce anxiety-related behaviors in the EPM test [16]. Therefore, our results indicate that BF extract possesses anxiolytic activity.

An increase in the secretion and activity of CRF is associated with the behavioral and physiological manifestations of repeated restraint stress in experimental animals and human clinical populations [6]. Previous studies have shown that the hypothalamic CRF system is involved in the regulation of depression and anxiety-like behaviors induced by restraint stress [33]. Our data suggest that CRF circuits in the hypothalamus were activated following repeated restraint stress and elicited the observed anxiety and depressive behaviors in the stress tests [4]. The expression and secretion of CRF in the PVN of the hypothalamus was significantly greater in the experimental (STR) group compared with the control (CON) group in the present study.

Effect of BF on Repeated Restraint Stress-Induced TH-Like Immunoreactivity

TH-like immunoreactivity was analyzed primarily in the cell bodies of adrenergic regions, including the LC (Fig. 8). In the rat brains from the STR groups, the numbers of TH immunoreactive fibers in the LC was increased by 325.00%. Comparisons of the numbers of TH-immunoreactive neurons using one-way ANOVA revealed significant differences among the groups [F(5,71) = 23.718, p<0.001]. Post hoc comparisons revealed that rats repeatedly exposed to restraint stress showed a significant increase in TH expression as compared with the CON group (p<0.001). The number of TH-immunoreactive neurons decreased significantly in hypothalamic regions in the STR + BF50 group (p<0.01) and STR + BF100 group (p<0.01), as compared with the STR group. The increase in TH immunoreactivity by repeated restraint stress was significantly restored by the BF administration, and the numbers of TH-immunopositive neurons in the STR + BF50 and STR + BF100 groups were close to those in the CON group.

DISCUSSION

The present study revealed that repeated restraint stress significantly increased the duration of immobility in the FST and decreased open-arm exploration in the EPM test in comparison with controls. Moreover, pre-emptive BF blocked these repeated restraint stress-induced changes in behavior.

Previous studies on the emotional effects of repeated restraint stress in rodents have produced mixed results. There are, however, reports similar to ours that have demonstrated

Fig. 8. Effect of BF on the expression of TH in the locus coeruleus (LC) after repeated restraint stress. Values are presented as mean ± SE of the density of TH-like immunoreactive neurons within a 100 × 100 µm grid over the areas at 200× magnification. Significance with Tukey’s test following a one-way ANOVA is indicated as ***p<0.001 vs. the CON; ##p<0.01 vs. the STR group.
study. This is coincident with a previous report showing that alterations in CRF underlie anxiety-like behaviors induced by chronic stress [33]. Therefore, the modulation of the CRF system, typically observed in anxiety and depression related to repeated restraint stress, might be due to activation of the HPA axis [4]. This theory has been supported by some studies in which alteration of HPA axis activity by repeated restraint stress was achieved and thus affects behavioral activity and CRF expression via a hypothalamic mechanism [9]. Thus, it could be suggested that the BF appeared to normalize CRF system balance in the hypothalamus by influencing the HPA axis, resulting in the increased negative feedback. Furthermore, pre-emptive BF significantly blocked the increase in CRF immunoreactivity in the PVN following behavioral testing. These results suggest that CRF modulation in the hypothalamus underlies the antidepressant and anxiolytic effects of BF following repeated restraint stress in rats.

Tyrosine hydroxylase (TH) is an enzyme involved in central stress activation and stress-related psychopathological conditions, including depression and anxiety. The ascending noradrenergic neurotransmitter system that originates primarily in the A6 noradrenergic neurons of the locus coeruleus (LC) is a major circuit in the central nervous system involved in stress responsiveness [25]. TH expression is elevated following exposure to chronic stress in the LC [27], possibly due to a long-term adaptive process in anticipation of exposure to subsequent stress. In the present study, TH immunoreactivity in the LC in response to repeated restraint was greater in the STR group than in the CON group. These data are consistent with previous reports showing that anxiety-like behaviors induced by chronic stress are the results of alterations in central noradrenergic systems [31]. Moreover, we demonstrated that pre-emptive BF extract treatment significantly reduced TH-like immunoreactivity in the LC resulting from repeated restraint stress. Taken together, these findings indicate that BF is capable of attenuating stress, purportedly through hypothalamic CRF modulation and noradrenergic system regulation in the LC in rats. The present results suggest that BF is an effective agent in the treatment of mood disorders and that TH may be a biological target or mechanistic rationale in the development of alternative medical treatments for anxiety and depression.

In summary, BF extract significantly reduced depression and anxiety-like symptoms following repeated restraint stress, purportedly through hypothalamic CRF modulation and noradrenergic system regulation in the LC in rats. The present results suggest that BF is an effective agent in the treatment of mood disorders and that TH may be a biological target or mechanistic rationale in the development of alternative medical treatments for anxiety and depression.

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