Baixiangdan Capsule and Shuyu Capsule Regulate Anger-out and Anger-in: GB1 Mediated GABA Can Regulate 5-ht Level in the Dorsal Raphe Nucleus

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Research

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Abstract

Background: To explore the intervention mechanism of Baixiangdan Capsule(BXD) and Shuyu Capsule(SY) in the treatment of anger-out and anger-in. A kind of GABABR1(GB1) mediated GABA in the dorsal raphe nucleus(DRN) regulating serotonin(5-HT) levels in the Prefrontal Cortex(PFC), Hippocampus and Hypothalamus in Anger-out and Anger-in male rats. To further explore the difference of Baixiangdan Capsule(BXD) and Shuyu Capsule(SY) in the treatment of anger-out and anger-in.

Methods: The anger rat model was established by social isolation combined with resident-intruder paradigm, and behavioral evaluation was used to screen and distinguish anger-out and anger-in model rats. BXD and SY were intervention drugs of anger–out and anger-in rats respectively. On this basis, ELISA was used to detect GABA content in DRN and 5-HT contents in PFC, hippocampus and hypothalamus after different time course (0,1,3,5,7 days) treated with BXD and SY. The co expression of 5-HT and GB1 in DRN was detected by immunofluorescence double labeling technique. Finally, brain stereotactic localization was performed after baclofen, the GB1 specific agonist, and CGP35348, the GB1 specific inhibitor, were injected into the DRN, the 5-HT contents in PFC, hippocampus and hypothalamus was detected by ELISA.

Results: After drug treatment, ABT scores and OFT total distance in BXD group were significantly decreased to the level of control group ($P<0.05$). On the contrary, that in SY group were significantly increased to the level of control group ($P<0.05$). The sugar water preference coefficient of BXD group and SY group was significantly increased to the normal level ($P<0.05$). With the increase of the medication duration, 5-HT levels in PFC, hypothalamus and hippocampus increased, gradually corrected its abnormal decline, and returned to the normal level on the 7th day. Besides, GABA level in DRN decreased, gradually corrected its abnormal increase, and also returned to normal level on the 7th day. A large number of 5-HT positive cells (red) in DRN could be seen on immunofluorescence section, and GB1 positive cells (green) could also be seen. Besides, after the drug intervention, 5-HT level in DRN was elevated to normal level ($P<0.05$). GB1 level in DRN was decreased to normal level in BXD group and SY group ($P<0.05$). Compared with saline injection, 5-HT levels in PFC, hypothalamus and hippocampus in each group was significantly decreased after injection of baclofen into the DRN ($P < 0.05$). On the contrary, 5-HT levels in PFC, hypothalamus and hippocampus in each group was significantly increased after the injection of CGP35348 into the DRN.

Conclusions: BXD and SY can effectively improve the abnormal behavior changes of anger-out and anger-in rats, and the optimal duration of action is 7 days. The improvement way is to correct the following abnormal changes: The significantly increased GABA in DRN combined with a significantly increased GB1 on 5-HT neurons in DRN, which further mediated the synaptic inhibition effect, thereby reducing 5-HT level of 5-HT neurons in DRN, resulting in a significant decrease of 5-HT levels in PFC, hypothalamus and hippocampus. Therefore, GB1 mediated GABA in DRN can regulate 5-HT levels in PFC, hypothalamus and hippocampus, which may be one of the ways that BXD and SY treat anger-out and anger-in.

Highlights

- The co-expression of GB1 and 5-HT in DRN on presynaptic and postsynaptic membranes was found at the level of histocyte.
- Baixiangdan capsule and Shuyu capsule can effectively alleviate the anger-out and anger-in, and the best time of action is 7 days after medication.
- GB1 mediated GABA in DRN can regulate 5-HT level in DRN, and regulate 5-HT levels in the PFC, hypothalamus and hippocampus, which may be one of the ways that BXD and SY treat anger-out and anger-in.

Background

Anger is probably one of the mostly debated basic emotions, owing to difficulties in detecting its appearance during development, its functional and affective meaning, especially in human beings[1]. The enormous impact that anger has had on people and their social interactions has been recorded in many ways and in many places throughout history[2]. In fact,the emotion of anger is defined as a negative emotional response to goal-blockage and unfair behavior by others[3]. Based on the differences in personal characteristics and enduring propensity[4], anger usually has two different opposite ways of expression, anger-in and anger-out[5]. Anger-out is defined as subjective feelings classified as"anger directed outward away from the self", however anger-in is defined as subjective feelings classified as "anger directed toward the self[6]. Anger is a normal human emotion response when encountering a range of unacceptable situations[7]. After all, anger is a subjectively intolerable emotion[8]. In extreme or long-time suffering, anger may lead to aggressive behavior, hostility, violence, anxiety and adverse health consequences[9]. A wide range of psychiatric disorders has been associated with anger, such as major depression disorder[10], post-traumatic stress disorder [11], and premenstrual dysphoric disorder[12]. Episodes of anger are also associated with physical diseases for instance of a transiently higher risk of cardiovascular diseases[13]. Therefore, it is of great significance to study the mechanism of anger and in the prevention of anger-induced diseases.

Baixiangdan capsule (BXD) and Shuyu capsule (SY) are the novel capsule formulation combining several plant extracts that have been usedin traditional Chinese medicine to treat anger-out and anger-in. Analytical studies have shown that the main active components of BXD are paeoniflorin, paeonol, and alpha-cyperone[14-16] , which may have antipyretic, anti-inflammatory, analgesic, and neuroprotective functions [17,18]. SY, a commercially available herbal prescription of traditional Chinese medicine, is composed of four herbal ingredients: Radix Bupleuri (*Bupleurum chinense* DC.), Radix Paeoniae Alba (*Paeonia lactiflora* Pall.), Rhizoma Cyperi (*Cyperus rotundus* Linn.), and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.). Studies have shown that SY can mitigate PMS depression symptoms and that its action mechanism is concentrated in specific cerebral areas[19,20].Paeoniflorin, the main component of Shuyu and paeony extract, has many biological effects, including enhancement of cognitive ability,

improvements in learning disabilities, and nerve protection[21,22]. BXD and SY can respectively treat anger-out and anger-in stably and effectively, but the neural effects of BXD and SY that underlie these actions are unclear.

Human emotion regulation circuit includes several regions of the brain such as the prefrontal cortex (PFC), the amygdala, hippocampus, hypothalamus, anterior cingulate cortex, insular cortex, ventral striatum, and other interconnected structures[23]. Study showed that defensive rage or aggression can typically be elicited by electrical stimulation of sites within hypothalamus in cats and rats[24,25]. The degree of expression of anger in pictures or sounds was positively correlated with the signal intensity of bloodstreams and glucose metabolism in the prefrontal and hippocampal[26], the ventromedial prefrontal cortex activity responded preferentially to anger expressions oriented to self[27]. Thus, PFC, hippocampus and hypothalamus is of great significant in participating the anger regulation[28-30].

Numerous studies have shown that various of neurotransmitters and hormones such as serotonin(5-HT), norepinephrine, dopamine, androgens and estrogens all participate in anger regulation, among these the serotonin is the most important[31,32]. Major serotonergic populations contained in the midbrain dorsal raphe and median raphe nuclei, and then projecting to various regions of the brain such as PFC, hippocampus and hypothalamus[33], participate in regulating cognition, intuition and emotion[34]. Study showed depressed patients with anger attacks may have a relatively greater serotonergic dysregulation than depressed patients without these attacks[23]. But to different anger expression, these is still little known about how 5-HT system was taken part in. GABA is the most important inhibitory neurotransmitter in the central nervous system[35]. About 50% of synapses in the central nervous system use GABA as the neurotransmitter, which can seriously affect the function of prefrontal cortex[36]. GABABR(GB) is a metabolic G protein coupled receptor, which is composed of GB1 and GABABR2(GB2). GABABR1(GB1) is responsible for binding to GABA[37,38], while GB2 is responsible for G protein coupling[39]. GB1 plays an important role in maintaining normal brain function. GABABR1a knockout mice are more likely to suffer from stress and pleasure loss and social escape behavior[40]. The up regulation of GABABR1a expression in dorsal raphe nucleus of socially isolated mice is closely related to abnormal behavior caused by social stress[41]. Thus, it is suggested that 5-HT, GABA and GB1 play an important role in anger and depression, and the specific mechanism needs to be further studied.

To sum up, we speculate that BXD and SY can effectively relieve anger and depression like emotions, which may be through this pathway: GB1 mediated GABA in the dorsal raphe nucleus(DRN) regulate the 5-HT levels in PFC, hypothalamus and hippocampus.To clarify this question, this paper adopts a variety of modern biological techniques to carry out research in a rat model of anger-out and anger-in based on the widely recognized social isolation stress combined with resident-intruder paradigm[42,43].

Materials And Methods

2.1 Animals, groups and treatments.

84 male Wistar rats weighing 180-220g, and 18 male SD rats weighing 120-150g were obtained from Charles River Laboratories (Beijing, China, License No.: SCXK (Beijing) 2012-0001). The wistar rats and SD rats were housed separately with the same condition, temperature at 22 ± 2 ˚C, humidity at 55 ± 10%, noise ≤60dB and 12 h light/dark cycle (lights on at 20:00) with free access to water and food. All behavior tests were performed during the dark phase under a dim red light (<2lux). All procedures performed in this study were in strict accordance with NIH Guide for the Care and Use of Laboratory Animals.

Firstly, we make the model, which were established according to resident-intruder stress combined with social isolation stress protocol that published previously [43]. The wistar rats were used as the resident and normal control, the SD rats were used as the intruders. All procedures carried on in 4 weeks. All animals were kept 1 week for adaption. From the 2nd week, 12 wistar rats were selected randomly into normal group with 4 rats per cages and no stress till the end of the study, the rest of the wistar rats began to be isolated as model group. During the 3rd week, rats in model group were treated with social isolation and resident-intruder stress. The wistar rats in model group was suffered from the induced intruder SD rat into his cages for 15min every day. Intruders were confronted each day with a different resident in a Latin square design. At the end of the 3rd week, 48 rats in model group were divided into anger-out model (AOM) group and anger-in model(AIM) group according to the composite aggression scores from the median analysis. 24 rats in model group with middle scores would be excluded. At the 4th week, the stress was continued. Besides, anger-out Baixiangdan(BXD) group was given Baixiangdan capsule suspension at the dosage of 1.33g/kg (equivalent to 8 times of clinical dose of human), continuing for 7 days. Anger-in Shuyu(SY) group was given Shuyu capsule suspension with the dosage of 1.33g/kg (equivalent to 8 times of clinical dose of human), continuing for 7 days. The control group, AOM group and AIM group were given equal volume of sterilized water, continuing for 7 days. At each end of the weeks, sucrose preference test, open field test and aggressive behavior test (only in model group at the 2nd to 4th week) were performed.

2.2 Behavioral evaluation

2.2.1 Aggressive behavior test (ABT)

Aggressive behavior test has become the most commonly used method for studying depression and aggressive behavior in animals[44]. Although the change of aggressive behavior induced by social isolation combined with resident-intruder paradigm is not defined as anger, it is most similar to human aggressive behavior[45]. In a sense, the generation of human anger is the preparation response to attack[46], and the typical behavior and core symptom of anger emotion[47]- aggression behavior is considered as the standard of anger emotion induction[48].

During the performing of resident-intruder stress, the first 10 min were recorded by camera. After the test, the observers watched the video and note the duration and frequency of the behaviors. Next, "rodent aggression analysis method and device", which has obtained the national invention patent(Chinese patent:CN106472348A,2017-03-08), was used to conduct behavioral evaluation on rats, which could effectively improve the objectivity and accuracy of rodent aggression analysis study and vigorously promote the relevant research level. Composite aggression score = number of attacks + 0.2 × attack duration (s) + number of bites + 0.2 × on-top duration (s) + piloerection[45]. The aggressive behavior was evaluated by blind method, and the video was played back by three persons who were trained uniformly. The results were recorded. The consistency test showed that kappa > 0.95.

2.2.2 Open field test (OFT)

The rats were adapted to the test room for more than 10 minutes[49]. Then the rat was moved into open field box (100cm × 100cm × 60cm) for 5 min. Rat movement was recorded by camera and the Total Movement Distance were calculated by SuperMaze software(Softmaze, Shanghai, China). The open field box should be cleaned by 75% alcohol before testing the next rat.

2.2.3 Sucrose preference test (SPT)

Sucrose Preference Test allowed the rat two bottles of water for freely chosen for 24h[50], one of which contains tap water and the other contains 0.8% sucrose solution. The position of two bottles would be switched to reduce the effect of side bias after 12h of the test. Before and after test, bottles were weighted. Sucrose preference scores were calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake.

2.3 Immunofluorescence

2.3.1 Tissue processing

Animals were administered 75 mg/kg of sodium pentobarbital(Sigma-Aldrich, UK) i.p. After deep anesthesia, the rats were fixed in the supine position with cotton thread Lift the skin of the chest, cut the abdominal cavity along the xiphoid process, cut the diaphragm, cut the thoracic cavity upward, turn over the thymus, fully expose the heart and aorta. The perfusing needle with flattened tip was inserted into the root of aorta from 45° to the right from the left apex. The needle was immediately fixed with an artery clamp to prevent loosening. Gently push the syringe to expand the heart and cut a small opening on the right atrial appendage. Then the normal saline was extracted from the syringe and quickly perfused to the whole body through the perfusion needle until the liquid from the right atrial appendage was clear, about 200ml. After that, 4% paraformaldehyde was infused, first fast and then slowly. Peripheral nerve

stimulation reactions such as limb tremor and tail cocking can be seen, and the perfusion can be stopped until the whole body is stiff, about 200ml. The brain was removed en bloc and post-fixed in 4 % PFA for 2h. The brains were left in 10%, 20% and 30% sucrose solution containing 0.1 M of phosphate buffer in turn for dehydration, then frozen in Tissue-Tek® OCT matrix (TissueTek, Sakura Finetek, UK) in sample preparation positions in cryostats (SLEE, Germany) at −20 °C. Coronal section of DRN at 28 μm thickness were collected in six well plates filled with frozen stock solution (50% glycerin and 50% 0.01 M phosphate buffer saline) and stored at -20 °C.

2.3.2 5-HT and GB1 immunofluorescence double labeling

Allow sections to fix for 30 min at room temperature. Wash the slices 6 times with 1x PBS for 5 min each, then transfer into 1% BSA blocking buffer for 60 min at room temperature. Aspirate the blocking buffer, incubate slices in anti-Serotonin antibody(1:1200, Abcam, USA) and anti-GB1 antibody(1:1000, Abcam, USA) at room temperature for 1 h, then incubate overnight at 4℃. Wash 3 times in 1x PBS for 5min each. Incubate slices in secondary antibody (Rabbit Anti-Goat IgG, 1:300, Abcam, USA; Rabbit Anti-Mouse IgG,1:300, Abcam, USA) for 2 h at room temperature in the dark. Wash 3 times in 1x PBS for 5min each. Pick the slices out from 6 well plates and place on glass slides then cover with anti-fade fluorescence mounting medium. After air-dried away from light, the slides were then viewed under confocal microscope (Zeiss, Germany).

2.3.3 Immunofluorescence intense analysis

The pictures were opened with Zeiss ZEN Lite 2012. Set the area of DRN as “Region of Interest”. Record the value of “Arithmetic Mean Intensity” as the IF intense. Analysis the same position according to The Rat Brain Stereotaxic Coordinates (Paxinos and Watson, 1996) of different specimen.

2.4 Baclofen and CGP35348 microinjection

After behavioral testing, normal saline, baclofen and CGP35348 were injected into DRN respectively. Rats were anesthetized with 75mg/kg sodium pentobarbital i.p. (Sigma-Aldrich, UK) and placed in a stereotaxic apparatus. The method of flat cranial head fixation was used to fix the rat's bilateral inner ear foramen and incisors at three points, adjust the position of the denture, and make the vertical position of the denture 3.3± 0.4mm below the horizontal plane connecting the two ear rods, and the anterior and posterior fontanelles at the same horizontal height. The skin was cut about 1cm along the midline above skull, and holes were drilled on the dorsal skull above the target regions. Then, microinjector was inserted and 0.2ml normal saline or 0.2ml baclofen(1.5mg/ml) or 1ml CGP35348(20mg/ml) were microinjected respectively into DRN(AP -7.8mm, L -2.0mm, V -6.3mm, 20°) of each group for 2 minutes. Stay for 2 minutes and then withdraw the microinjector. After injection, suture the skin and apply gentamycin

sulfate. Brain tissues(PFC, Hippocampus, Hypothalamus) were collected respectively 10minutes after surgery.

2.5 ELISA kits

2.5.1 Tissue processing

Fresh brain tissues were collected at 0, 1, 3, 5 and 7 days after treatment, and the PFC, hypothalamus, hippocampus and DRN were rapidly separated, weighted and stored at -20°C. In addition, 10 minutes after microinjection of normal saline or baclofen or CGRP35348 into DRN, brain removed on ice, then PFC, hippocampus and hypothalamus were quickly separated, weighted and stored at -20°C.

Brain tissues was rinsed with 1x PBS, homogenized in PBS and stored overnight at -20°C. After two freeze-thaw cycles to break the cell membranes, the homogenates were centrifuged at 5000×g for 5 min 4°C. The supernatant was removed and assayed immediately.

2.5.2 ELISA kits

5-HT contents in PFC, hippocampus and hypothalamus were determined by ELISA kit (Cusabio, China) according to the manufacturer's instructions. And GABA content in DRN were determined by ELISA kit (RD,USA) according to the manufacturer's instructions.

2.6 Statistical Analysis

Statistical analyses were carried out using Graphpad Prism 6.0 and results were expressed as Means ± SEM (standard error of the mean). Data were analyzed by independent *t* test between two groups, or one-way ANOVA when more than two groups. Differences were considered to be statistically significant when $P < 0.05$.

Results

3.1 Experiment1:Behavior tests results

ABT score is the main indication of anger-out and anger-in rats model. As shown in Figure 1A and 1B, there was no significant difference in ABT score and ABT latency between groups at baseline ($P > 0.05$). After modeling, rats in model group were divided into anger-out model group(AOM) and anger-in model group(AIM) according to ABT score and latency. After the medication, compared with AOM, ABT score of anger-out BXD(BXD) group was significantly reduced ($P<0.05$) and ABT latency was significantly increased ($P<0.05$). Compared with AIM, ABT score of anger-in SY(SY) group was significantly increased

(P<0.05) and ABT latency was significantly decreased (P<0.05). At the same time, there was still a significant difference between AOM and AIM(P<0.05). OFT total distances (Figure 1C) showed that after modeling, compared with the control group, OFT total distances of AOM significantly increased (P<0.05), while that of AIM significantly decreased (P<0.05), and was significantly lower than that of AOM. After the medication, compared with the control group, the significant difference of AOM and AIM was still maintained (P<0.05). But, compared with AOM, OFT total distances of BXD group decreased significantly (P<0.05) and returned to the level of the control group. Compared with AIM, OFT total distances of SY group was significantly increased (P<0.05) and returned to the level of the control group. SPT scores (Figure 1D) of both AOM and AIM were significantly lower than those of control group after modeling (P<0.05). After the medication, compared with the control group, the significant difference of AOM and AIM was still maintained (P<0.05). SPT scores of BXD group and SY group were significantly increased (P<0.05) and returned to the level of the control group. This study confirms that social isolation combined with resident-intruder paradigm can successfully prepare scientific and stable anger-out and anger-in rats model, which lays the foundation for subsequent experiments. Besides, BXD and SY can effectively improve the behavior of anger-out and anger-in rats.

3.2 Experiment2: The basis for the selection of drug treatment days

5-HT levels in PFC, hypothalamus and hippocampus of both AOM and AIM were significantly lower than that of the control group, and remained at a low level; after the drug intervention, with the increase of the medication duration, the 5-HT levels in PFC, hypothalamus and hippocampus increased, gradually corrected its abnormal decline, and returned to the normal level on the 7th day (Figure2.A,B,C;Figure3.A,B,C). Besides, the GABA level in DRN of both AOM and AIM was significantly higher than that of the control group, and remained at a high level. After the drug intervention, GABA level in DRN decreased with the increase of medication duration, and gradually corrected its abnormal increase, and returned to normal level on the 7th day(Figure 2D;Figure 3D).

3.3 Experiment3: Histologic association of GB1 and 5-HT neurons in DRN

3.3.1 GB1 and 5-HT neurons in DRN immunofluorescence double labeling

Previous studies have found that 5-HT neurons in DRN have nerve fiber connections with PFC, hypothalamus and hippocampus. So, in order to observe the expression of 5-HT neurons and GB1 in DRN and their connections with nerves and the nerve fiber connection between BXD and SY in regulating anger-out and anger-in, we examined the level of tissue cells by immunofluorescence double labeling

method. As shown in Figure 4, 5-HT immunostaining positive cells(red) (5-HT neurons) in DRN of each group rats can also show GB1 immunostaining positive cells(green).

3.3.2 5-HT and GB1 immunofluorescence intensity

Afterwards we semi-quantitatively analyzed the intensity of immunofluorescence. Results showed that intensity in both model groups decreased significantly than that in control group (P<0.05). Besides, after the drug intervention, 5-HT content in DRN was elevated to normal level in BXD group and SY group(P<0.05) (Figure 5A). However, the intensity in both model groups increased significantly than that in control group (P<0.05). Besides, AIM increased significantly than AOM(P<0.05). And, after the drug intervention, GB1 content in DRN was decreased to normal level in BXD group and SY group (P<0.05) (Figure 5B).

3.4 Experiment4: 5-HT levels in PFC, Hippocampus and Hypothalamus were changed after injection of GB1 specific agonist and GB1 specific inhibitors into DRN

Finally, the change of 5-HT levels in PFC, hypothalamus and hippocampus were observed by microinjection of baclofen and CGRP35348 into DRN respectively. As shown in Figure 7, 5-HT concentrations in PFC, Hippocampus and Hypothalamus of rats in each group(Figure 7A, B, C) decreased significantly after injection of Baclofen, a specific agonist of GB1, into DRN($P < 0.05$), compared with the injection of normal saline, whereas 5-HT concentrations in PFC, Hippocampus and Hypothalamus of rats in each group(Figure 7a, b, c) increased significantly after injection of CGP35348, a specific inhibitor of GB1, into DRN($P < 0.05$).

Discussion

Anger-out and Anger-in Rat Model

Social isolation combined with resident-intruder paradigm is a classic modeling method in the rat model of anger-out and anger-in[42,43]. Resident rats are raised alone for a long time, which makes them irritable and aggressive. They can attack the invaders instinctively, so as to protect their territory and generate angry psychology and behavior. Social isolation can induce aggressive behavior in rats[51]. Here we have confirmed that social isolation combined with resident-intruder paradigm could successfully prepare a scientific and stable rat model of anger-out and anger-in, which laid the foundation for subsequent experiments.

Aggression or aggressive behavior, which might be triggered by anger[52], has various associations with anger. Aggression may also be viewed as the behavioral expression of anger[53]. Both anger and

aggression could be seen as a strong and lasting personal trait[54,55], individuals with characteristics of aggression are often accompanied with trait anger and are more likely to generate anger and enmity in the face of offensive or irritating events[56]. Anger may include increased physiological arousal, and an increased predisposition toward aggressive behavior[57,58]. In the close association between anger and aggression, many neurochemical and genomic studies have included Anger-Hostility-Aggression defined by Spielberger as a research object, but not just anger itself [59].

ABT is the main indication of anger-out and anger-in rat model. OFT is often used to measure anxiety-like behavior in rats[60]. OFT and SPT testified the emotional changes that occurred with rats induced aggressive behavior and the complexity of emotions. The generation of anger is often accompanied with a certain degree of anxiety and depression, previous studies also illustrated this[61,62].These three evaluation methods are also used in many researches on attack behavior evaluation[62,63]. The results of this study also confirmed that these three evaluation methods can effectively evaluate the rat model of anger-out and anger-in, and BXD and SY can effectively correct abnormal anger tendency by changing the score of ABT, OFT and SPT. This is consistent with the results of relevant reports[64,65].

Possible Action Targets and Mechanism of BXD and SY Against Anger-out and Anger-in

Many studies have shown that 5-HT is a key neurotransmitter that regulates aggressive behavior. Decreased levels of 5-HT or 5-HIAA in brain tissue can increase the occurrence of aggressive behavior and violence[66]. Activity of 5-HT neurons in DRN increased aggressive behaviors among male mice[67]. On the contrary, studies have also found that overexpression of 5-HT1A receptors weakens the activity of 5-HT neurons in DRN and enhances the aggressive behavior of mice [68]. Studies in knockout mice also showed that increased aggressive behavior was accompanied by decreased 5-HT levels or reduced activity [69-71]. Some scholars think that different basal level of 5-HT and phasic chages may have different role in different types of aggression [72]. In our study(Figure 5A), both anger-out and anger-in rats have a lower level of 5-HT expression in DRN than normal rats, and anger-in rats have a lower level of 5-HT expression than anger-out rats. At the same time, BXD and SY can correct the abnormal decreased 5-HT level in DRN.

DRN contains the largest accumulation of 5-HT neuronal cell bodies in the brain, and it sends afferent projections to several distinct targets including PFC, hippocampus and hypothalamus [73], which was also testified by our preliminary study. The results showed that in anger-out and anger-in rats, 5-HT levels in PFC, hypothalamus and hippocampus decreased in step with that in DRN. Similarly, BXD and SY can also correct the abnormal decrease of 5-HT levels in PFC, hypothalamus and hippocampus (Figure 3A,B,C) , and then regulate the anger-out and anger-in , which need to take 7 days to achieve curative effect(Figure 2A,B,C). However, it has been found that increasing the level of 5-HT in PFC of mice can lead to more anger and aggression[74], which is contrary to this study. The reason may be that the tissue size of the prefrontal region used to detect 5-HT and the selection of the left and right prefrontal regions are

different. The dorsolateral area is mainly involved in cognitive and executive functions, while the ventromedial area is mainly related to positive or negative emotions[75,76].

It can be seen from the above that the release of 5-HT from 5-HT neurons in DRN was significantly reduced in anger-out and anger-in model rats, and the levels of 5-HT projected to PFC, hypothalamus and hippocampus via the fibers was also significantly decreased. BXD and SY can effectively relieve anger-out and anger-in by correcting the above abnormal changes.

GABA is the most important inhibitory neurotransmitter in the central nervous system[35]. About 50% of synapses in the central nervous system use GABA as the neurotransmitter, which can seriously affect the function of prefrontal cortex[77] and participate in the regulation of emotional disorder[36]. A large number of studies have shown that 5-HT and GABA are synaptically linked and mutually regulated[78] . For example, 5-HT can inhibit the release of glutamate and GABA[79]. Excitation of 5-HT neurons in DRN can reduce the degree of stress response in rats[80], and this inhibition can be indirectly regulated by GABA-ergic intermediate neurons in DRN[81,82]. GABA intermediate neurons and 5-HT neurons are synaptically linked[83], and then regulate the activity of 5-HT neurons[84]. The increase of GABA increased its inhibition on 5-HT synthesis[85], and eventually resulted in the decrease of 5-HT content and function. In this study, we found that GABA concentration in DRN increased, BXD and SY can effectively reduce GABA concentration (Figure 3D) and then regulate the anger-out and anger-in, which need to take 7 days to achieve curative effect(Figure 2D).

GABA can regulate 5-HT level in DRN through GABABR(GB) and GABAAR[86]. GB mediate inhibitory effects. In addition, GB can regulate the release of 5-HT[87]. Various pathological processes, including chronic pain, epilepsy and schizophrenia, are often accompanied by changes in GB1 expression and function[88,89]. GB1a knockout mice were more prone to stress pleasure loss and social escape behavior[40]. The expression of GB1a in DRN was up-regulated in socially isolated mice[41]. It will be seen from these studies that GB1 play an important role in maintaining normal brain function. This study found that GB1 expression in DRN was up-regulated in model rats and BXD and SY can significantly down regulate the expression of GB1 (Figure 5B). However, it has been reported that the expression of GB1 in hippocampal neurons of anger model rats is decreased[90], which is negatively correlated with the results of this study. It may be due to the different brain regions detected, so the expression of GB1 is different.

The GABA terminal is in close contact with GABABR/5-HT double-standard neurons[91].We also found that the expression of GB1 in 5-HT neurons in DRN of rats in each group was confirmed by immunofluorescence double labeling technique(Figure 4). Objective conditions for GB1-mediated GABA regulation of the existence of 5-HT neurons in DRN were determined.

Therefore, we can infer that GABA increased significantly in DRN of angry-out and anger-in rats, which further mediated the synaptic effect and exerted inhibitory function by binding with GB1 on 5-HT neurons in DRN, thus reducing the 5-HT level of 5-HT neurons in DRN and leading to a significant decrease 5-HT

levels in PFC, hypothalamus and hippocampus. This is also the intervention target and way of BXD and SY.

GB1 subunit has an agonist binding site[38], that is, GB1 can bind to agonists[92]. Because Baclofen is a GB specific agonist, Baclofen only binds to GB1 and becomes a GB1 specific agonist[93] to activate GB1 signaling pathway. CGP35348 is a specific inhibitor of GB. This study showed that the levels of 5-HT in PFC, hippocampus and hypothalamus of rats in each group decreased significantly after injection of baclofen into the DRN, while the levels of 5-HT in PFC, hippocampus and hypothalamus increased significantly after injection of CGP35348 into the DRN. Therefore, from both positive and negative aspects, it was confirmed that GB1 mediates GABA in DRN to regulate the levels of 5-HT in PFC, hippocampus and hypothalamus, which plays an important role in anger-out and anger-in. And it is the corresponding target of BXD and SY. Both systemic and DRN injection of baclofen enhanced aggressive behavior[94]. Baclofen can promote the expression of GB1, improve the binding ability of GABA to its receptors, and enhance the inhibitory effect of GABA [95], so as to inhibit the release of 5-HT from DRN. These reports are consistent with the results of this study, but other reports are contrary: microinjection of baclofen into DRN can increase the extracellular 5-HT content of medial prefrontal cortex[94], thereby enhancing aggressive behavior, suggesting that the enhancement of 5-HT release in medial prefrontal cortex is synchronous with the increase of aggressive behavior[67]. This may be due to the asynchrony between the total 5-HT contents and extracellular 5-HT contents.

In conclusion, BXD and SY can act on GB1 in DRN, and then affect the effect of GABA, thus affecting the levels of 5-HT in PFC, hypothalamus and hippocampus of anger-out and anger-in rats. That is, GB1 in DRN mediates GABA regulation of 5-HT levels in PFC, hypothalamus and hippocampus, which is the target of BXD and SY in regulating anger-out and anger-in.

Interestingly, BXD and SY can correct the abnormal GB1 mediated GABA regulation of 5-HT levels in PFC, hypothalamus and hippocampus in anger-out and anger-in respectively, and the correction direction is the same. However, BXD and SY can correct the abnormal behavior of anger-out and anger-in rats respectively, but the correction direction is opposite. BXD is composed of Paeoniflorin, volatile oil of Rhizoma Cyperi and Paeonol, the mainly effective component of Paeonia lactiflora pall, Rhizoma Cyperi and Cortex moutan. SY is composed of Bupleurum saponin, Paeoniflorin and volatile oil of Cyperus, the mainly effective component of Radix Bupleuri, Radix Paeoniae Alba and Rhizoma Cyperi. From this we can see that both BXD and SY contain paeoniflorin and volatile oil of Rhizoma Cyperi. Paeoniflorin could ameliorate the symptoms and improve the functional capability of post-stroke depression rats [96]and IFN-α-induced depression mice[97], also can protect against cognitive impairment[98]. The volatile oil of Cyperus rotundus can inhibit depression by regulating the content of 5-HT in brain[99]. However, BXD contains paeonol, SY contains Bupleurum saponin , which is their difference. Paeonol can not only calm hypnosis, but also significantly improve learning memory and anxiety[100]. Saikosaponin can increase the 5-HT level in rat brain, and effectively improve depression like behavior[101]. It can be inferred that Paeoniflorin and Cyperus volatile oil may be inclined to correct the micro mechanism of action; Paeonol may be inclined to

behavioral correction of anger; Bupleurum saponin may be inclined to behavioral correction of depression. Further research is needed to confirm.

Conclusions

BXD and SY can effectively improve the abnormal behavior changes of anger-out and anger-in rats, and the optimal duration of action is 7 days. The improvement way is to correct the following abnormal changes: The significantly increased GABA in DRN combined with a significantly increased GB1 on 5-HT neurons in DRN, which further mediated the synaptic inhibition effect, thereby reducing 5-HT level of 5-HT neurons in DRN, resulting in a significant decrease in 5-HT levels in PFC, hypothalamus and hippocampus. Therefore, GB1 mediated GABA in DRN can regulate the 5-HT level in DRN, and regulate 5-HT levels in the PFC, hypothalamus and hippocampus, which may be one of the ways that BXD and SY treat anger-out and anger-in.

Abbreviations

ABT: Aggressive behavior test
AIM: Anger-in model
AOM: Anger-out model
BXD: Baixiangdan Capsule
DRN: Dorsal raphe nucleus
GB: GABABR
GB1: GABABR1
GB2: GABABR2
OFT: Open field test
PFC: Prefrontal cortex
5-HT: Serotonin
SY: Shuyu Capsule
SPT: Sucrose preference test

Declarations

Ethics approval and consent to participate

The study on animal was approved by the animal ethical committee of Shandong University of Traditional Chinese Medicine(Jinan,China).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Authors' contributions

Jie Gao conceived and designed the work. Haijuan Wang, Xiaoyu Wang and Yinxia Ning contributed to the experiment and analysis of molecular biology, immunology and morphology. Xiaoju Liu contributed to the anger-out and anger-in modeling and took responsibility for the integrity of the work as a whole, from inception to publication. All authors read and approved the final manuscript.

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Figures

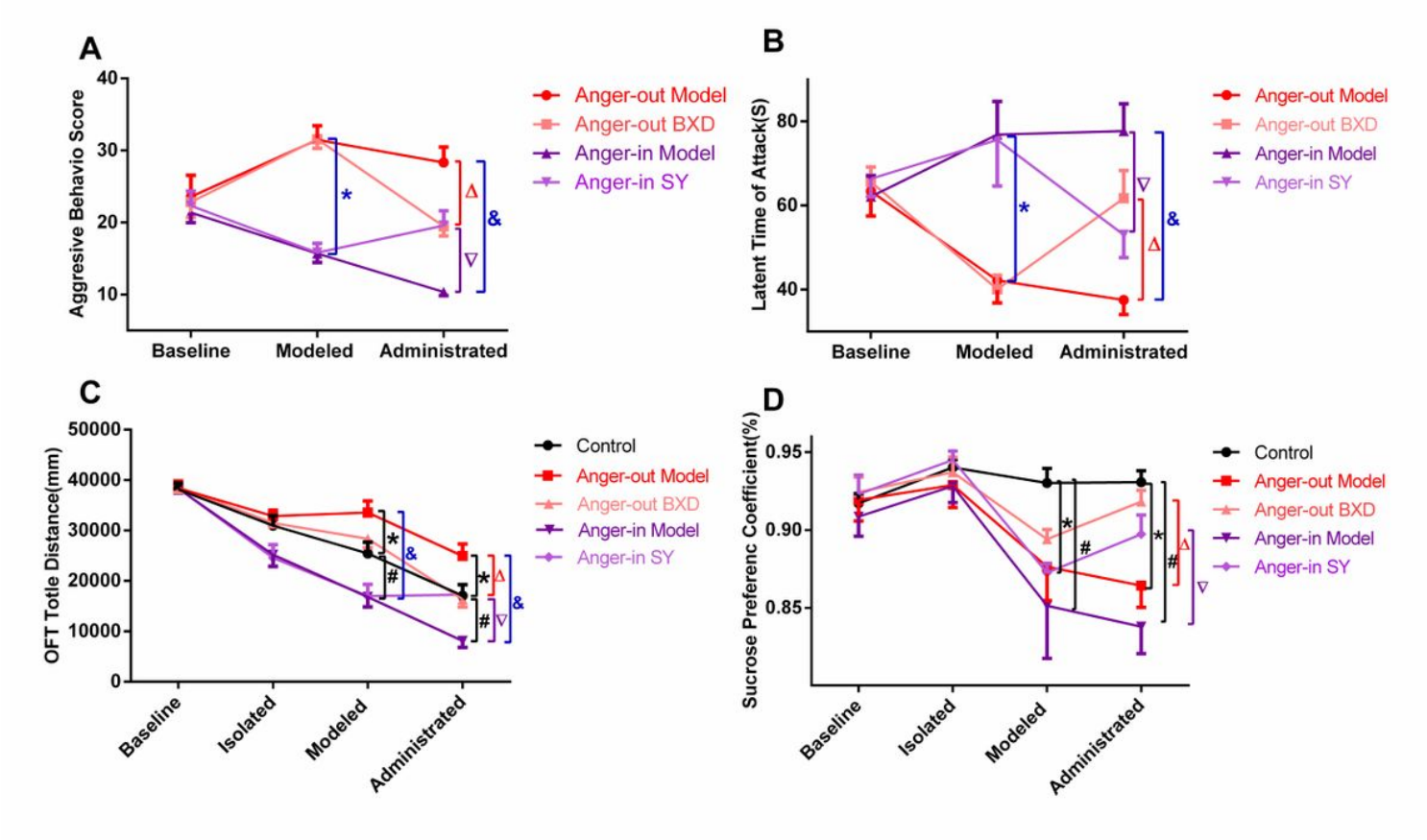

Figure 1

Behavior tests results. A:ABT scores, B:ABT latency, C:OFT totle distance, D:SPT scores. Data were given as Means±SEM, * ,#,&, ∇,Δ, P < 0.05, n = 10.

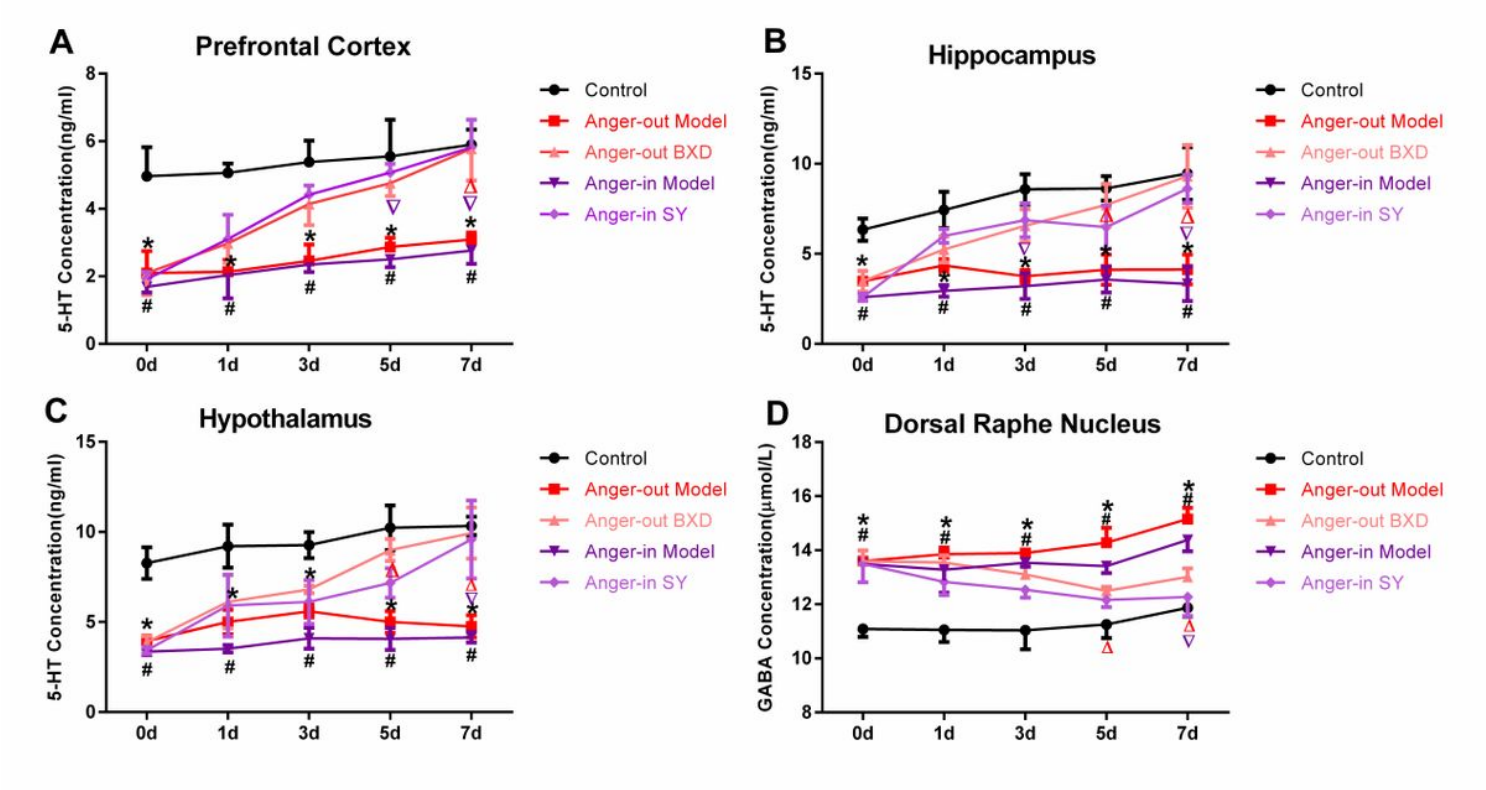

Figure 2

5-HT Levels in PFC, Hippocampus and Hypothalamus, and GABA level in DRN after different days of treatment. 5-HT and GABA levels were determined by detecting the supernatant of homogenized tissue via ELISA kits. Data were given as Means ± SEM, *, #,∇,Δ, $P < 0.05$, n = 10.

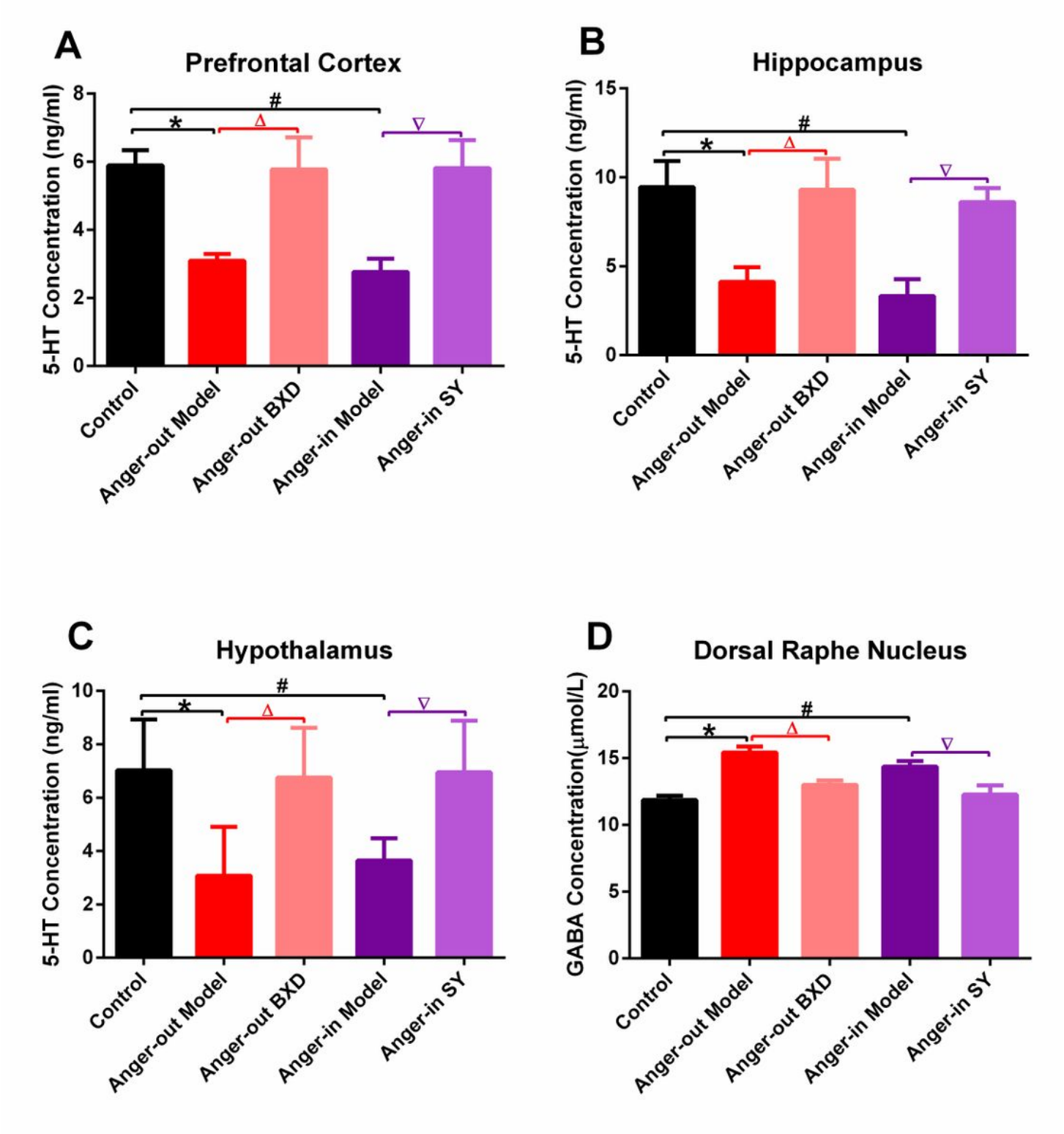

Figure 3

5-HT levels in PFC, Hippocampus and Hypothalamus, and GABA level in DRN after 7 days of drug treatment. 5-HT and GABA levels were determined by detecting the supernatant of homogenized tissue via ELISA kits. Data were given as Means ± SEM, *, #,∇,Δ, $P < 0.05$, n = 10.

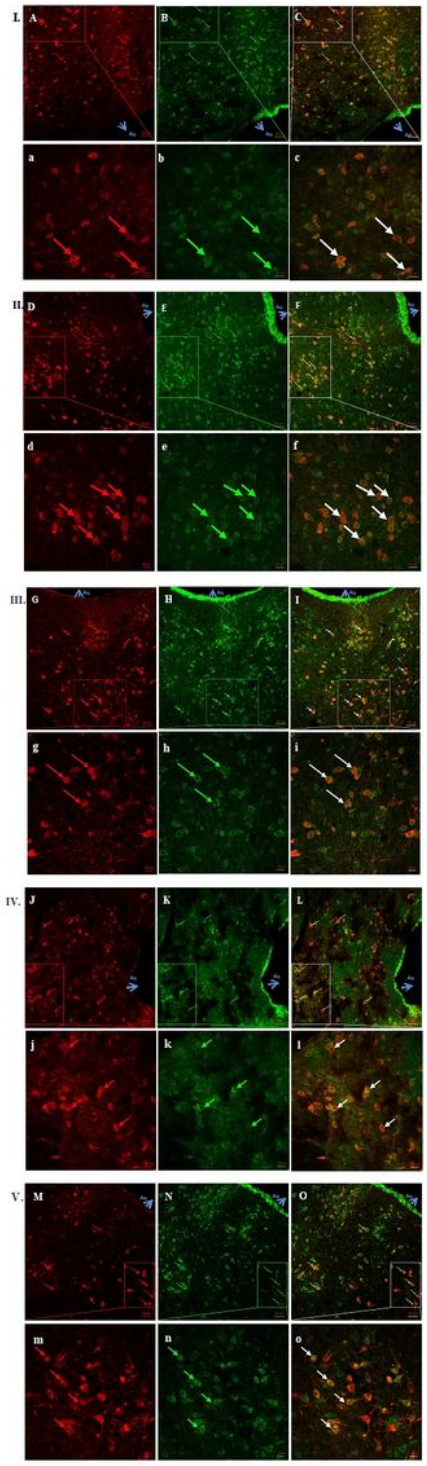

Figure 4

GB1 and 5-HT neurons in DRN immunofluorescence double labeling. After immunofluorescence, we can see the red marked 5-HT neurons in DRN[A(a), D(d), G(g), J(j) and M(m)]. Merged with green marked 5-HT immunofluorescence image in DRN[B(b), E(e),H(h),K(k) and N(n)], we can see red and green double labeled cells(yellow)[C(c), F(f), I(i), L (l) and O(o)]. A(a), B(b), C(c): control group. D(d), E(e), F(f): anger-out model group. G(g), H(h), I(i): anger-out BXD. J(j), K(k), L(l): anger-in model group. M(m), N(n), O(o): anger-

in SY. A-O: The magnification is 200(x200), scale=50mm; a-o: The magnification is 400(x400), scale=20mm.

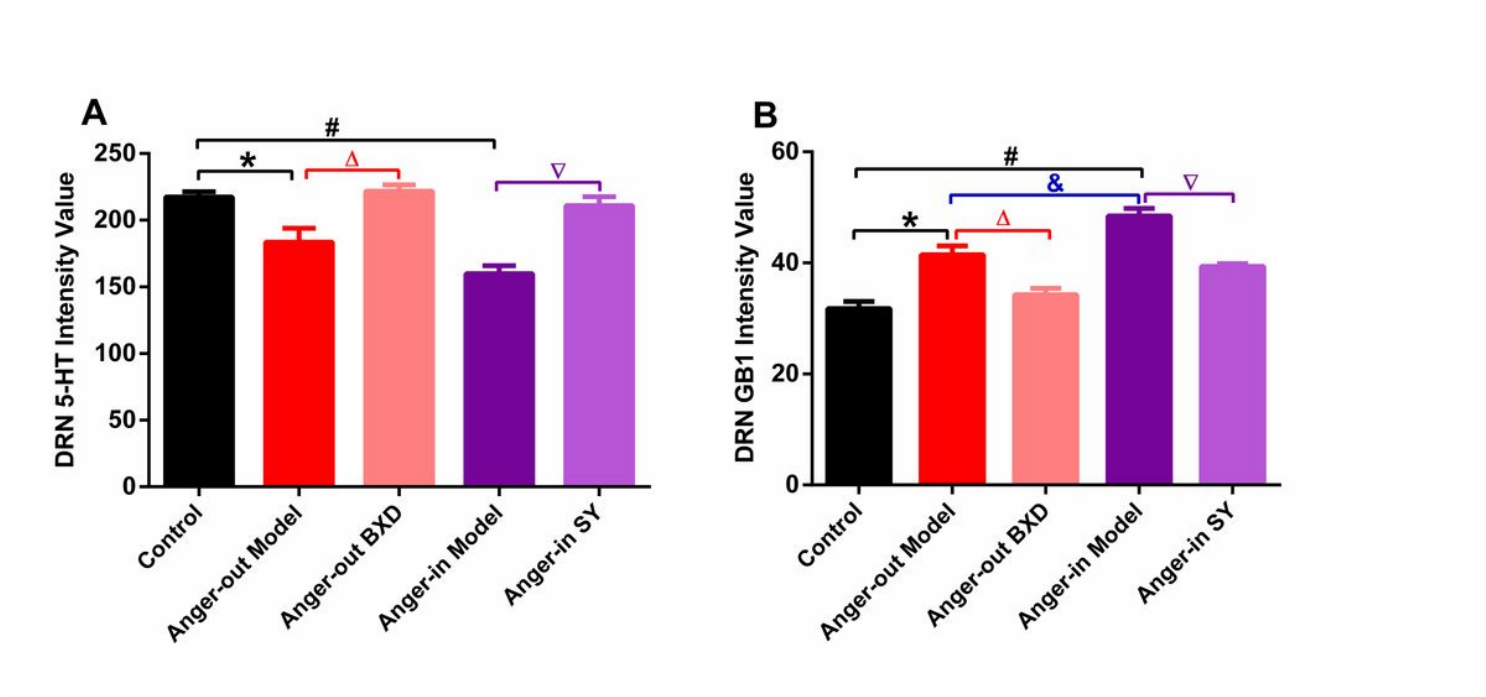

Figure 5

DRN 5-HT and GB1 Immunofluorescence Intensity Value. DRN 5-HT and GB1 were detected by immunofluorescence and the intensity value were analyzed by Zeiss ZEN 2012 software. Data were given as Means±SEM, *,#,&,∇,Δ, $P < 0.05$, $n = 10$.

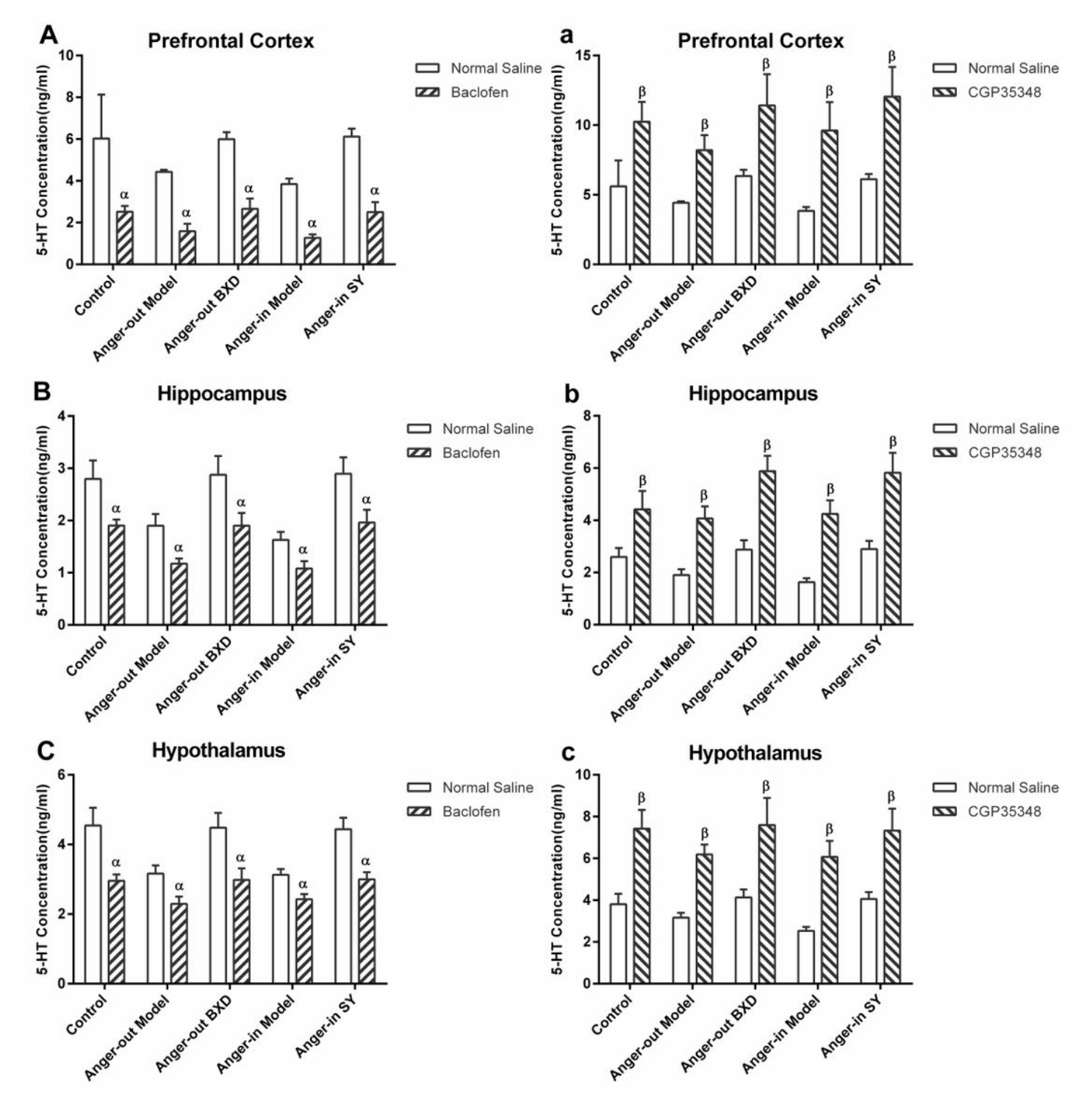

Figure 6

5-HT levels in PFC, Hippocampus and Hypothalamus were changed after injection of GB1 specific agonist or GB1 specific inhibitors into DRN. 5-HT levels in PFC, Hippocampus and Hypothalamus were changed after respectively injection of baclofen(A, B, C) or CGP35348(a, b, c) into DRN of rats in each group. Data were given as Means±SEM, α,β:P < 0.05, n = 10.