Baihe, Dihuang and *Baihe Dihuang Tang* ameliorates PCPA-Induced Insomnia

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Research Article

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Abstract

Clinical evidence from the practice of traditional Chinese medicine (TCM) has shown that Baihe (BH), Dihuang (DH), and their compatibility, Baihe Dihuang Tang (BDT) can treat insomnia. However, the sedative and hypnotic mechanisms of these therapies have not been established. This study evaluated the sedative-hypnotic mechanisms of BH, DH and BDT in terms of their effects on neurotransmitters, the neuroendocrine system and cation-chloride co-transporters in a p -chlorophenylalanine (PCPA)-induced insomnia mice model. The insomnia mice model was established by intraperitoneal injection of PCPA. Mice were randomly assigned into four groups that were treated with BH, DH and BDT (2.25 g/kg, 2.25 g/kg, 4.5 g/kg) and saline solution (wt., 0.9%). Behavioral analyses showed that, compared to mice in the control group, motion activities of mice with insomnia were significantly reduced, sleep latency was prolonged while sleep duration was shortened. Compared to control mice, 5-HT and KCC2 levels were decreased, whereas CRH and ACTH levels were elevated in the insomniac mice. Treatment with BH, DH and BDT ameliorated the symptoms of insomnia and significantly reversed the abnormal levels of the aforementioned neurotransmitters. Notably, BDT effects were the most obvious. These results indicate that BH, DH and BDT improves insomniac symptoms by modifying neurotransmitters levels, the neuroendocrine system and cation-chloride co-transporters.

1. Introduction

Insomnia is a common sleep disorder that is caused by several factors, including physical and psychological stress, chronic pain, and use of certain medications (Atkin et al., 2018; Putnins et al., 2012). This disorder has been declared a public-health epidemic, with about 25% of the population in the United States (US) having been diagnosed with it (Leger and Bayon, 2010). In China, the pooled prevalence of insomnia is 15%, translating to about 2.07 billion people. Treatment for insomnia mainly involves the use of western medicines such as benzodiazepine receptor agonists, non-benzodiazepine receptor agonists, selective melatonin receptor agonists, and sedative antidepressants (Winkler et al., 2014). However, their clinical efficacies remain suboptimal. Prolonged use of chemotherapies results in numerous adverse side effects, such as psychomotor disorders, addiction, tolerance, and rebound effects (Atkin et al., 2018; Uzun et al., 2010), thereby underscoring the need to develop effective sedative-hypnotic medicines against insomnia.

Herbal therapies are effective alternatives for insomnia treatment. Within the previous decade, there was a remarkable progress in the discovery of novel traditional medicines for psychiatric illnesses (Uzun et al., 2010). Due to their superior clinical efficacy, few adverse side effects, insignificant drug dependence and low cost, Traditional Chinese Medicines (TCMs) are preferred to Western medicines (Chen et al., 2011; Hsu and Chung, 2012; Singh and Zhao, 2017). Studies have reported the therapeutic potential of TCMs against insomnia (Shi et al., 2014; Singh and Zhao, 2017; Wang, H. et al., 2020; Yeung et al., 2012). For instance, since the early 3rd century, Baihe Dihuang Tang (BDT) has been used in the treatment of chronic emotion-like disorders. Currently, it is used in the traditional treatment of depression and lily disease (Meng et al., 2018). Clinically, lily presents with palpitation, sleeplessness, loss of appetite, trance and

anxiety (Meng et al., 2018). Moreover, clinical studies have reported the antidepressant properties of BDT (Wei Chen, 2004; Zhen Huang, 2005). There, given the close relationships between insomnia and depression, it has been postulated that BDT can treat insomnia. For instance, in young adults, insomnia is closely associated with uncontrolled self-disgust, anxiety and depression (Ypsilanti et al., 2018). Indeed, depression has been implicated in the pathogenesis of insomnia (Zhong et al., 2020). Qi invigorating TCMs are widely used in treating depression (Lou et al., 2018), while BDT can suppress insomnia-induced negative emotions. Apart from BDT, long term administration of Baihe and Dihuang has been shown to offer satisfactory therapeutic effects against insomnia. However, to the best of our knowledge, mechanisms underlying the sedative and hypnotic effects of Baihe, Dihuang, and Baihe Dihuang Tang have not been reported.

Even though the severity of insomnia is associated with multiple factors, such as dysregulated neurotransmission as well as dysregulated expression of cation-chloride co-transporters (CCCs), the precise pathogenesis of insomnia is not well understood. Regarding neurotransmission, studies have shown that Dopamine (DA), norepinephrine (NE) and serotonin (5-HT), all tyrosine metabolites, regulate the sleep-wake cycle. Serotonin induces sleep, whereas DA and NE promote be awake (Kiehn et al., 2019; Si et al., 2015). Studies have reported under-secretion of 5-HT in the brains of insomniac rats (Wei et al., 2014), contrary to DA and NE, which were over-secreted in the same group of animals (Si et al., 2015; Strekalova et al., 2015; Yun et al., 2015). Insomnia is also linked to deregulated hypothalamic-pituitary-adrenal axis (HPA) (Asarnow, 2020; Gold, 2015; Redeker et al., 2018), one of the main endocrine systems modulating the body's homeostasis to stress. In response to physiological or psychological stress, activated HPA axis induces corticotrophin-releasing hormone (CRH) secretion in the hypothalamus as well as adrenocorticotropic hormone (ACTH) secretion in the anterior pituitary gland (Chu et al., 2020; Chu, L.X. et al., 2021; McEwen, 2008). Thus, serum CRH and ACTH levels are potential biomarkers for the HPA axis activity. Insomniac rats have been shown to exhibit activated HPA axis and upregulated CRH and ACTH expression levels (Bao et al., 2017). Besides the HPA axis, insomnia is associated with Na^+ - K^+ -$2Cl^-$ co-transporter isoform 1 (NKCC1) and K^+-Cl^- cotransporter 2 (KCC2) serum levels, which are members of cation-chloride co-transporters that are responsible for Cl^- homeostasis (Lin et al., 2013). These findings provide a strong basis for evaluating the mechanisms underlying the effects of Baihe, Dihuang and BDT in insomnia therapy.

Overall, we investigated the sedative-hypnotic effects of Baihe and Dihuang either alone or combination, as BDT, on PCPA-induced insomnia in mice models, In addition,we evaluated the secretion levels of monoamine neurotransmitters, expression levels of 5-HT receptors, ACTH and CRH, NKCC1 and KCC2 in the brain to determine the mechanism of action (MOA) of the three drugs against insomnia.

2. Materials And Methods

2.1 Drugs and chemicals

Baihe (BH) and Dihuang (DH) roots were purchased from the Tongrentang Chinese Pharmaceutical Co. Ltd. (Beijing, China). The two herbs were separately ground into a coarse powder using agate pestle. Baihe Dihuang Tang (BDT) was prepared by mixing BD and DH powders in the ratio of 2:1 (BH:DH). The BH, DH and BDT powders were boiled for 60 mins in reflux in 8 water parts (v/w). The procedure was repeated twice, but for 45 mins in the second round. Then, the extract was filtered to remove debris, concentrated to 30 % (w/w) though lyophilization, before storage at 4 ◦C.

Sodium pentobarbital and p-chlorophenylalanine (PCPA) were purchased from Sak chemical technology Co., Ltd. (Shanghai, China) and suspended in 0.5% gum acaia/physiological saline at a concentration of 30 mg/ml.

Mouse ELISA Kits for norepinephrine (NE), dopamine (DA), serotonin (5-HT), adrenocorticotropic hormone (ACTH) and corticotrophin releasing hormone (CRH) were purchased from the IBL International GmbH Company (Hamburg, German). Vectastain ABC were purchased from Vector Laboratories, while the Eastep Super TRNA Extraction Kit was purchased from Promega. The Go Script Reverse Transcription System and GoTaq qPCR Master Mix were purchased from Promega (Wisconsin, USA). All reagents and solvents used in this study were of analytical grade.

2.2 Experimental apparatus

The equipment used for various analyses included the SHA-B constant temperature oscillator (Changzhou, China), UV-254 Ultraviolet Transmitter (Beijing, China), SIGMA 1-15K High Speed Freezer Centrifuge and CFX96 real-time fluorescent quantitative PCR instrument (California, USA), 756MC UV-Vis Spectrophotometer (Shanghai, China), and the MK3FC enzyme-linked immunoassay instrument (Massachusetts, USA).

2.3 Animals

Specific pathogen free (SPF) Male KunMing mice (n=48; 18-22 g) were purchased from the Experimental Animal Center of Hebei Medical University. Each mouse was separately housed at 12/12 h light-dark cycle (lights turned on at 7:00 am and off at 19:00 pm) under (22 ± 2 °C) and (50 ± 10 %) relative humidity. They were fed on adequate food and water. The protocols used in this study were approved by the National Institute of Biological Sciences and Animal Care Research Advisory Committee of Hebei Medical University. Experiments were performed according to the Animal Research Ethics Board of Hebei Medical University guidelines (Approval Number, DWLL2019004).

2.4 Sedative and Hypnotic effects of TCM on normal mice

2.4.1 Test groups and drug dosing

The 48 mice were randomly assigned into 4 groups (n = 12) and given orally administrated with equal doses of the saline solution (control group), Baihe (BH group), Dihuang (DH group) and Baihe Dihunag Tang group (BDT) once daily for 7 days, at dose of 0.2 ml/10g body weight.

2.4.2 Sedative assessment

The open field test (Li et al., 2017) was performed to assess the effects of the herbal medicine on exploratory and locomotor activity of mice. The open field test consisted of a wooden box measuring 40 cm× 80 cm × 80 cm, with opaque walls. The underside was made of 25 equal pieces, divided by white lines. Briefly, each mouse placed at the center of the open box and allowed to explore the box for 3 min. The box was cleaned after each experiment. The experiments were performed in a quiet room.

2.4.3 Pentobarbital-induced sleep test

A subthreshold dose of sodium pentobarbital (i.e., 35 mg/kg) was intraperitoneally injected at 30 min intervals. Mice were considered asleep when they lost righting reflex less than 1 min. The number of mice and time of falling asleep in each group within 30 minutes were recorded.

Moreover, mice in each group were intraperitoneally administered with sodium pentobarbital (45 mg/kg) after 30 min of the last administration of drugs. Sleep latency was defined as the period between drug injection and righting reflex while sleep duration represented the period between loss of righting reflex and recovery. Sleep latency and sleep duration were monitored and recorded (Zhong et al., 2019).

2.5 Sedative-hypnotic effects of BDT on PCPA-induced insomniac mice

2.5.1 Modeling method

Mmice were first acclimatized for one week prior to the experiment. Sixty male mice were randomly and evenly distributed into five groups: blank control, PCPA (model, BH, DH and BDT groups. Except for mice in the control group, mice in the rest of the groups were intraperitoneally administrated with PCPA (300 mg/kg) once a day at 8:00 and 9: 00 a.m. for the first and second day, respectively, to induce insomnia (Hu et al., 2016). Herbal medicines (BH, DH or BDT) were orally administrated 2 days after PCPA injection. Specifically, BH and DH (2.25 g/kg) and BDT (4.5 g/kg) (0.1 ml/10 g of mouse) were intragastrically administrated twice a day (7:00-8:00 a.m., 5:00-6:00 p.m.) for ten days,. Mice in the control and model groups were intragastrically administrated with normal saline (0.1 ml/10 g) twice a day (7:00-8: 00 a.m., 5:00-6:00 p.m.) for ten days. After 10 days of treatment, mice weights were mearsured.

2.5.2 Behavioral analyses

Thirty minutes after herbal treatment, mice were acclimated in the activity cages for 2 minutes and thereafter assessed for explorative behaviors (horizontal and vertical motion) within 3 min (Li et al., 2017). In addition, except for mice in the control group, the other groups were administrated with pentobarbital sodium (i.e., 35 mg/kg) 30 mins after administration of herbal medicines and left to sleep within 30 min. Sleeping was assessed by righting reflex for more than 1 min. Sleep latency and duration were calculated based on the time the mice fell asleep and woke up (Zhong et al., 2019).

2.5.3 Weight changes in mice

Mice were weighed before the experiment, and once a week after PCPA-induced insomnia.

2.6 Plasma and brain samples collection

Plasma and brain tissue samples were collected at the same time of day (between 09:00 and 11:00 a.m.), and after 10 days of treatment. Blood was collected through retro orbital bleeding in heparin (14-17 IU/ml) tubes. Thereafter, 100 µl of plasma from 1 ml of blood centrifuged at 3000 rpm for 1 minute was collected and stored at -80 °C for no more than 24 h before analysis (Chu, L. et al., 2021).

Hypothalamic tissues were collected in tubes on an ice plate with normal cold saline, weighed immediately and stored at -80 °C till use.

2.7 Analysis of neurotransmitters and CRH and ACTH

Immediately after the open test, hypothalamic tissues were extracted after sacrificing the mice, frozen in liquid nitrogen and stored at -80 ∘C prior to analysis. The NE, DA and 5-HT levels in 20 mg of hypothalamus tissues homogenized in 300 µl of normal saline (0.9%) were measured using ELISA kits (Hamburg, German) according to the manufacturer's instructions. The NE, DA and 5-HT levels were expressed in pg/mg wet weight of the tissue.

Corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) levels in plasma were measured using the mouse ELISA Kits for adrenocorticotropic hormone (ACTH) and corticotrophin releasing hormone (CRH) (Hamburg, German) according to the manufacturer's protocols.

2.8 Expression levels of KCC2 and NKCC1

Expression levels of KCC2 and NKCC1 genes were evaluated by qRT-PCR. RNA from hypothalamic tissue cells were extract using the Trizol reagent. The quality of the RNA (5µL) was assessed by gel electrophoresis (1%). Complementary DNA (cDNA) was synthesized from the RNA using the TIAN Script RT Kit, according to the manufacturer's protocol. GAPDH was used as the internal control. The amount of amplified DNA was calculated the Q = 2-ΔCq formulae. Primer sequences used in this study were: NKCC1 (Gene ID: 20496; Access No: NM_009194.3), 5' - TGA TGG GTG TGA ACC ACG AG - 3' for the forward and 3' - GCC CTT CCA CAA TGC CAA AG -5' for the reverse primer; KCC2 (Gene ID: 57138; Access No: AF332064.1), 5' - ACC GTT GTC TTT GTG GGT GT - 3' for the forward and 3' - ATC GGG AAA TTG GGT GGG TC - 5' for the reverse primer, GAPDH (Gene ID: 14433; Access No: GU214026.1), 5' - TGA TGG GTG TGA ACC ACG AG - 3' for the forward and 3' - GCC CTT CCA CAA TGC CAA AG -5' for the reverse primer.

2.5 Statistical analysis

Data were analyzed using SPSS V. 20. Continuous data were presented as mean ± SEM. Differences between multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

3. Results

3.1 Sedative and Hypnotic effects of TCMs on normal mice

Compared to controls, BH, DH, and BDT treatment suppressed the horizontal and vertical motions of the mice, albeit insignificantly ($p > 0.05$) (Fig. 1a-b). However, compared to controls, pentobarbital sodium treatment significantly increased the sleeping rate ($p < 0.05$) (Fig. 1c), reduced sleep latency ($p < 0.05$) (Fig. 1d) and prolonged sleep duration ($p < 0.05$) (Fig. 1e) of mice in all the treatment groups. These findings imply that Baihe, Dihuang, and BDT exhibit synergistic effects when combined with pentobarbital sodium to induce sedation and hypnosis.

3.2 Sedative and hypnotic effects of TCMs on PCPA-induced insomniac mice

Based on horizontal and vertical movements, PCPA treatment was shown to successfully induce motion. Figure 2 shows that insomniac mice exhibited longer sleep latency and shorter sleeping duration ($p < 0.05$). However, compared to the controls, BH, DHH, and BDT treatment decreased horizontal and vertical motions, shortened the sleeping latency and prolonged the sleeping duration of mice ($p < 0.05$). The effect was, however, greater in the BDT group than in the BH and DH groups. These findings demonstrate the sedative and hypnotic properties of BH, DH and BDT, which are potential options for insomnia treatment.

Figure 3 shows that there were no significant differences in the initial weights of mice across the groups. However, compared to controls, mice in the model group gradually lost weight from the first week through the second week ($p < 0.05$). Besides, mice in groups treated with BH, DH and BDT gained more weight than those in the model group from week 1 through to the second week ($p < 0.05$). Notably, the gain was greater for mice in the BDT group ($p < 0.05$).

3.3 Mechanisms underlying the sedative and hypnotic effects of BDT against insomnia

Figure 4 shows that, compared to the controls, PCPA treatment significantly suppressed secretion of 5-HT in the hypothalamus ($p < 0.01$). Contrarily, PCPA treatment elevated the NE and DA levels in the brain ($p > 0.05$). However, BH, DH and BDT treatment significantly supressed the production of NE and DA, but elevated the production of 5-HT in the hypothalamus ($p < 0.05$). Notably, the effect was greater in the BDT group than BH and DH treatment groups ($p < 0.05$).

Compared to the controls, plasma ACTH and CRH levels were significantly elevated in the model group (Figure 5) ($p < 0.05$). However, compared to the model group, plasma ACTH and CRH levels were significantly suppressed in the DH and BDT treatment groups ($p < 0.05$), but not in the BH group ($p > 0.05$). Notably, the effects on ACTH and CRH levels were greater in the BDT group.

Immunohistochemical test (Figure 6) revealed that PCPA treatment modulated the expression levels of both 5-HT_{1A} and 5-HT_{2A} receptors in brain tissues. However, Baihe, Dihuang and BDT treatment restored

the normal expression levels of 5-HT_{1A} receptors but not those of 5-HT_{2A}. Notably, the effect was greatest in the BDT treatment group (Figure 7).

Figure 8 shows that compared to controls, insomnia modulated the mRNA expression levels of KCC2 of mice in the model group ($p < 0.01$), but upregulated that of NKCC1 ($p < 0.01$). However, compared with mice to the model group, BH, DH and BDT treatment enhanced the mRNA expression levels of KCC2 ($p < 0.01$), but suppressed the mRNA expression levels of NKCC1 ($p < 0.05$). Notably, BDT exerted the greatest effect among the three treatments.

4. Discussion

We have shown the synergistic effects of Baihe, Dihuang and BDT when combined with pentobarbital sodium in inducing sleep, shortening sleep latency and prolonging sleep duration. Moreover, BH, DH and BDT treatment increases appetite and brain activity of mice with insomnia as well as the secretion of NE and DA, but suppresses the secretion of 5-HT in the hypothalamus. These herbal drugs also up-regulated the expression of 5-HT_{1A} and down-regulated the expression of 5-HT_{2A} regulated the HPA axis by modulating the secretion of CRH and ACTH and up-regulated the expression of the KCC2 gene. Comparatively, among the three drugs, BDT exerted the greatest effect. These findings exhibit the sedative and hypnotic effects of BH, DH and BDT against insomnia. To the best of our knowledge, this is the first study to report on the effects and MOA of Baihe, Dihuang, and BDT against insomnia.

Sleep is controlled by the circadian rhythm and homeostatic mechanisms that are regulated by the central nervous system (CNS) (Shi et al., 2014) through numerous endogenous neurotransmitters (Zeng et al., 2018). In this study, the sedative and hypnotic effects of BDT were assessed using the validated open field and pentobarbital-induced sleep tests (Barros et al., 1991; Li et al., 2017; Qu et al., 2016; Zhang et al., 2014). The effect of drugs on the CNS can be assessed by evaluating spontaneous motor activities (Barros et al., 1991), while the change in pentobarbital-induced sleep duration can be used to assess the stimulatory or inhibitory effects of drugs on the CNS (Kim et al., 2011). The insomniac mice model in this study was established through intraperitoneal PCPA injection (Wang, H.F. et al., 2020). P-chlorophenylalanine controls the sleeping cycle by regulating 5-HT synthesis and inhibiting tryptophan hydroxylase activity. Dysregulated PCPA secretions disrupts the sleep circadian rhythm, leading to insomnia (Zeng et al., 2018). In this study, Baihe, Dihuang and BDT treatment significantly reduced the sleeping latency and prolonging sleeping duration of mice with PCPA-induced insomnia after pentobarbital sodium-treatment. The open field test is widely used in evaluating locomotor activity and emotional behaviors (e.g., anxiety and stress) of animals (Manchanda et al., 2011). It has been reported that BDT increases locomotor activity of animals (Miao et al., 2019), consistent with our findings (Figure 2). Body weight changes are reliable indicators for the occurrence, development, prognosis and regression of certain diseases (Allison et al., 2012). Clinically, most insomniac patients lose weight, which is attributed to poor appetite (Franz et al., 2007). In this study, compared to controls, insomnia resulted in significant weight loss in the model mice. However, Baihe, Dihuang and BDT treatment prevented

insomnia-related weight loss, with BDT exerting the greatest effect among the three treatments (Fig 3). There, BH, DH and BDT treatment can improve the appetite of insomniac individuals.

In addition, insomnia results in the under-secretion of 5-HT, but increases the secretion of NE and DA. However, Baihe, Dihuang and BDT treatment reversed the abnormal secretion of 5-HT, NE and DA (Figure 4). As mentioned-above, PCPA induces insomnia by inhibiting 5-HT secretion. Primarily, 5-HT promotes the sleep-wake cycle. We found that insomnia modulates the secretion of 5-HT, however, BH, DH and BDT treatment reversed this effect. NE and DA are the central neurotransmitters associated with awareness. High DA levels increases the feeling of excitement (Lopes et al., 2010). Insomnia enhances the secretion of NE and DA (Pan et al., 2017; Sun et al., 2020; Wang, H.F. et al., 2020; Xu et al., 2018), consistent with our findings. However, BH, DH and BDT treatment reversed this phenomenon. These findings suggest that the three TCMs can suppress insomnia induced excitement. Furthermore, sleep is modulated by 5-HT_{1A} and 5-HT_{2A} receptors in the CNS. We found that insomnia down-regulates the expression of 5-HT_{1A} in the hypothalamus, which was reversed by BH, DH and BDT treatment (Figure 7), thus can potentially prolong sleeping duration.

In addition, compared to controls, insomnia increased the expression of CRH and ACTH (Figure 5) in the hypothalamus and pituitary glands, respectively, by over-activating the HPA axis. The findings confirm insomnia associated with HPA axis hyperactivity (Asarnow, 2020; Gold, 2015), which explains the under secretion of monoamines neurotransmitters such as NE and 5-HT under dysregulated HPA axis. Inhibition of the HPA axis activity by 5-HT modulates CRH and ACTH secretion (Zhou et al., 2019). Moreover, 5-HT and NE antagonizes CRH release (Wu et al., 2017). Overall, the secretion of 5-HT was negatively correlated to that of CRH. Consequently, we observed down-regulated and up-regulated expressions of 5-HT and CRH, respectively, in the insomnia mice. However, Baihe, Dihuang and BDT treatment reversed the abnormal expression of CRH and ACTH (Figure 5), implying that oral administration of the three drugs can restore impaired HPA axis functions.

Finally, cation-chloride co-transporters (CCCs) participate in intracellular chloride ion (Cl^-) and extracellular Cl^- homeostasis (Kaila et al., 2014). Among the seven types of CCCs, only KCC2 and NKCC1 are expressed in the central nervous system. KCC2 regulates neuronal Cl^- extrusion activity, whereas NKCC1 regulates Cl^- osmotic stability (Kaila et al., 2014). Dysregulated expression levels of CCCs leads to the pathogenesis of numerous neurological diseases such as insomnia, which is also linked to abnormal Cl^- concentrations (Lin et al., 2013). While, NKCC1 was over-expressed in the hypothalamus of insomniac mice, KCC2 synthesis was significantly down-regulated in the same mice. Therefore, the pathological mechanisms of insomnia may be correlated with abnormal secretion of KCC2 and NKCC1. BH, DH and BDT treatment was found to restore normal expression levels of NKCC1 and KCC2. Therefore, the sedative-hypnotic effects of BH, DH and BDT may be associated with regulated expression levels of NKCC1 and KCC2.

Regarding study limitations, first, measurement of CRH and ACTH secretion levels does not accurately assess HPA activity because they are intermediate products of HPA, which can easily be affected by a negative feedback loop. Second, our variables were only investigated in male mice, which may not be

reflective of reactions in female mice. Therefore, studies should aim at analyzing changes in multiple markers (CRH, ACTH and corticosterone) in both male and female mice.

5. Conclusion

Baihe, Dihuang and Baihe Dihuang Tang ameliorates insomnia by regulating the secretion of NE, DA, 5-HT, NKCC1 and KCC2 in the brain as well as plasma CRH and ACTH levels through the HPA axis.

Declarations

Compliance with Ethical Standards

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Author contributions

Qin Lu and Li-kang Wang designed the concept of the study and they conducted the study. Cairong Qi and Ri Wang analyzed the data. Qin Lu wrote the first draft of the manuscript. Liuxi Chu, Xi Wang and Ping ping Chen edited subsequent versions of the manuscript.

Declarations

Authors declare no conflict of interest. Ethical approach: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

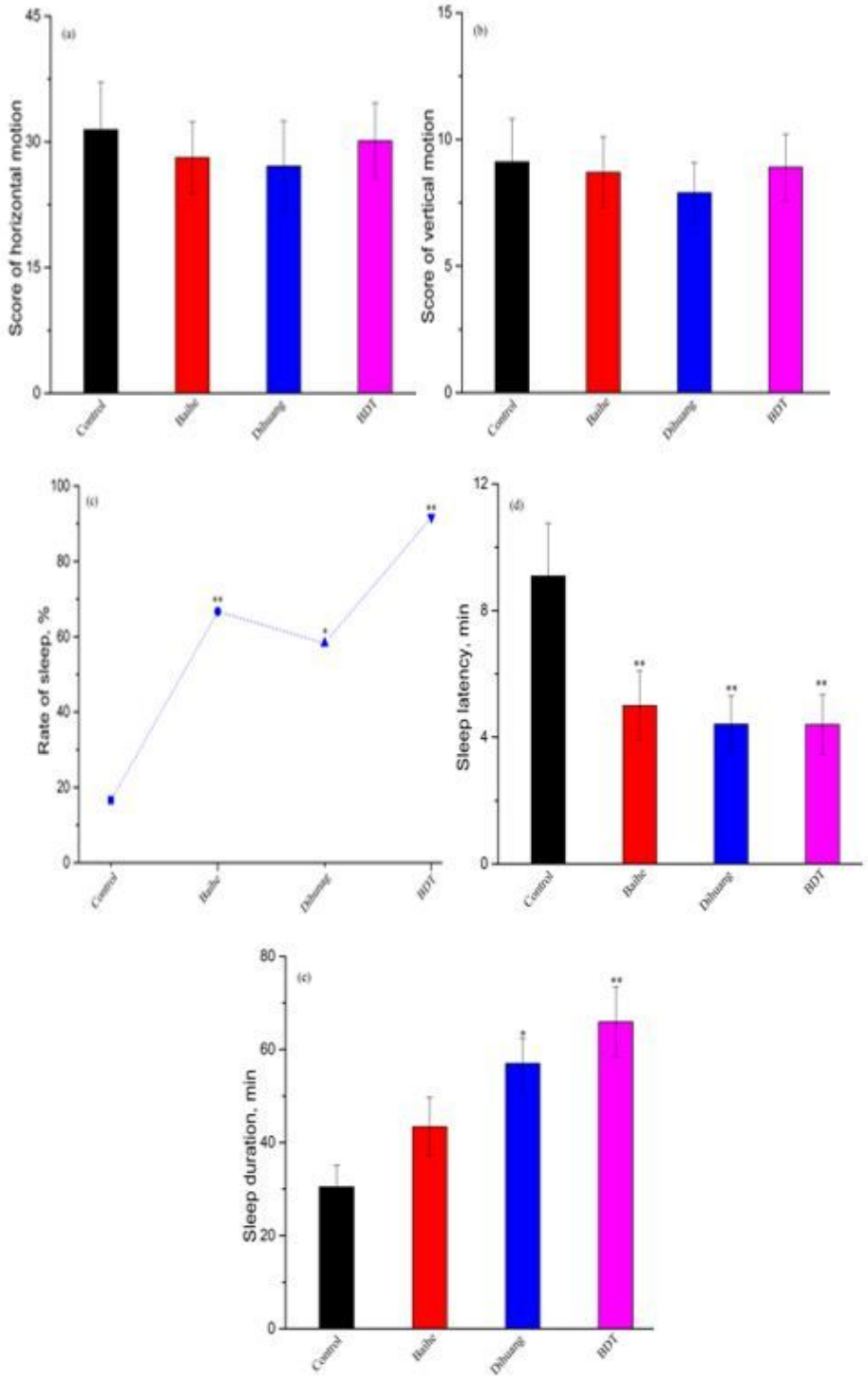

Figure 1

Effect of Baihe, Dihuang, and BDT on sedative and hypnotic effects in normal mice (n=12). Effect of the three TCMs on the sedative influence on the normal mice (a-b). Effect of the three drugs on the hypnotic response to pentobarbital-induced sleep in normal mice. Thirty minutues after administration of sodium pentobartial (35mg/kg and 45 mg/kg, respectively) except for control group. The rate of sleep (c), the

sleep latency (d), and the sleep duration (e) were assessed. Note: The error bar was standard error of mean (SEM), &p < 0.05, &&p < 0.01 vs. control group, *p < 0.05, *p < 0.01 vs. model group.

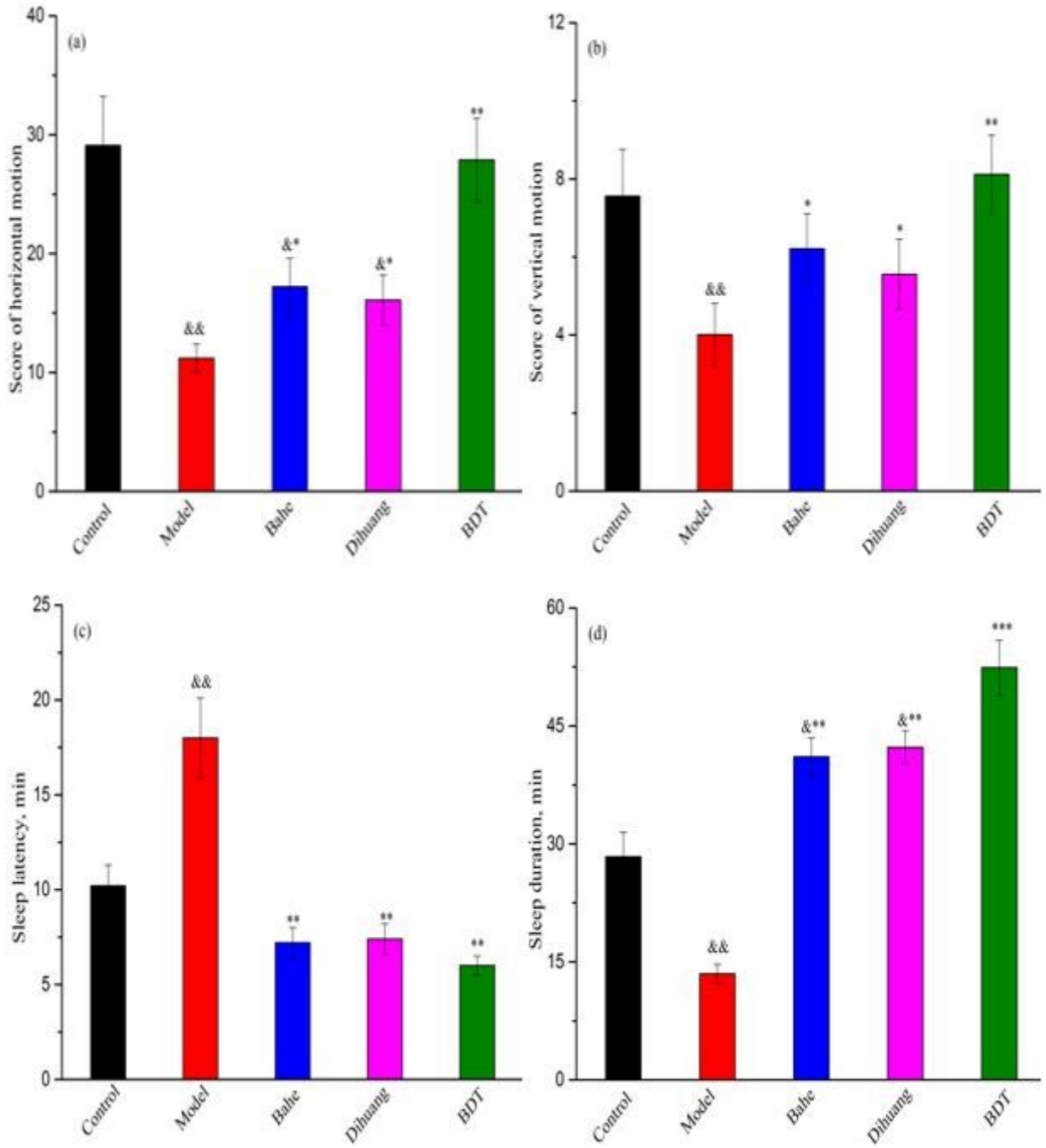

Figure 2

Effects of Baihe, Dihuang, and BDT on sedative and hypnotic influences in insomnia mice (n=12). Autonomous activities (a-b), sleep latency (c), and sleep duration (d) were evaluated. Note: The error bar was standard error of mean (SEM), &p < 0.05, &&p < 0.01 vs. control group, *p < 0.05, *p < 0.01 vs. model group.

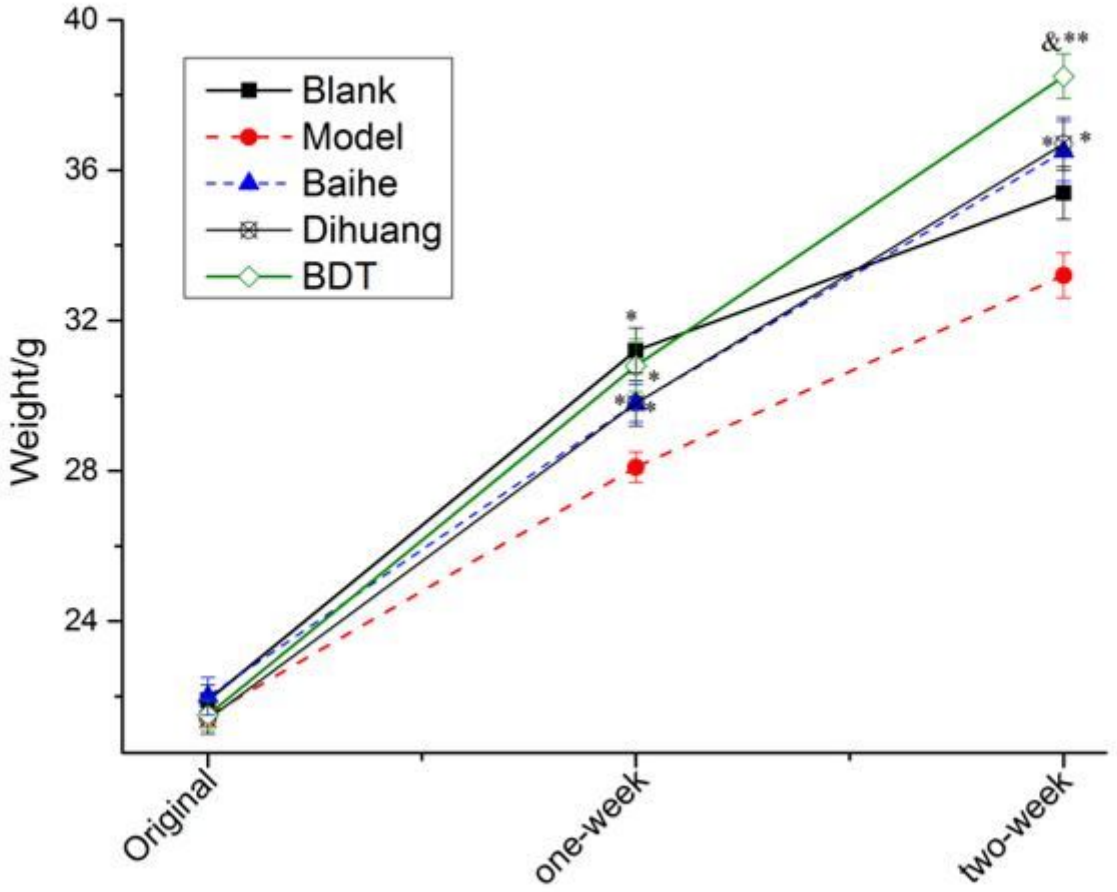

Figure 3

Compariosn of mice' weight in each group every week (n=12). Note: The error bar was standard error of mean (SEM), & $p < 0.05$ vs.control group, * $p < 0.05$, ** $p < 0.05$ vs. model group.

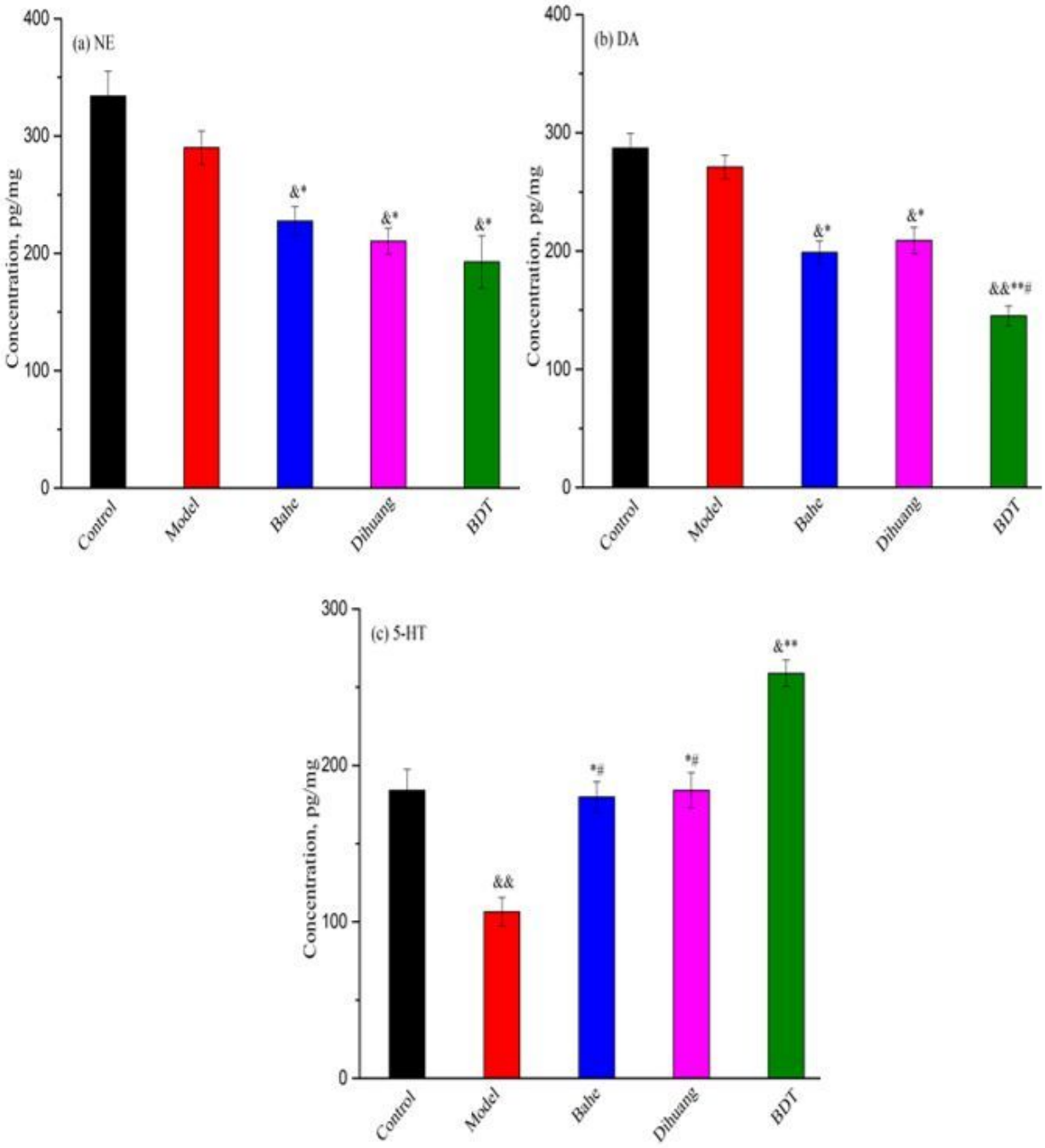

Figure 4

Comaprison of the contents of NE (a), DA (b) and 5-HT (c) in the hypothalamus of mice in each group (n=12). Note: The error bar was standard error of mean (SEM), &p < 0.05, &&p < 0.01 vs. control group, *p < 0.05, *p < 0.01 vs. model group, #p < 0.05 vs. BDT.

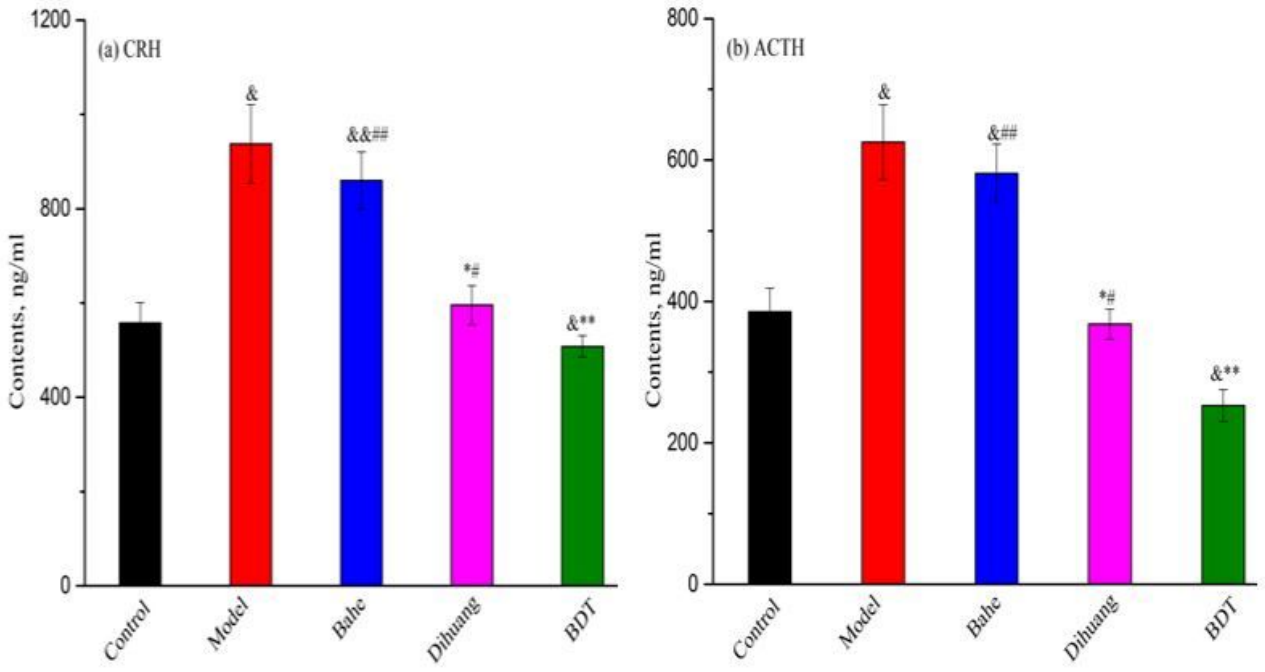

Figure 5

Comparison of the contents of CRH (a) and ACTH (b) in plasma in each group (n=12). Note: The error bar was standard error of mean (SEM), &p < 0.05, &&p < 0.01 vs. control group, *p < 0.05, *p < 0.01 vs. model group, #p < 0.05 vs. BDT.

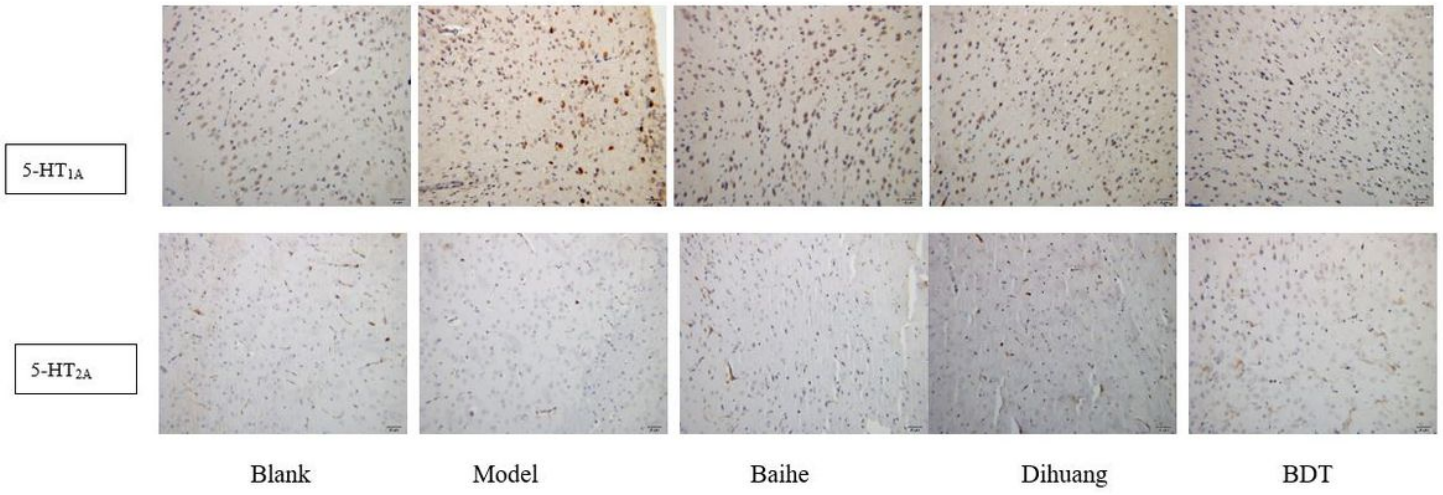

Figure 6

Effects of Baihe, Dihuang, and BDT on expression of 5-HT receptors in hypothalamus of PCPA-induced insomnia mice (n=12). The expression of 5-HT1A and 5-HT2A receptors were shown in the first line and second line, respectively.x

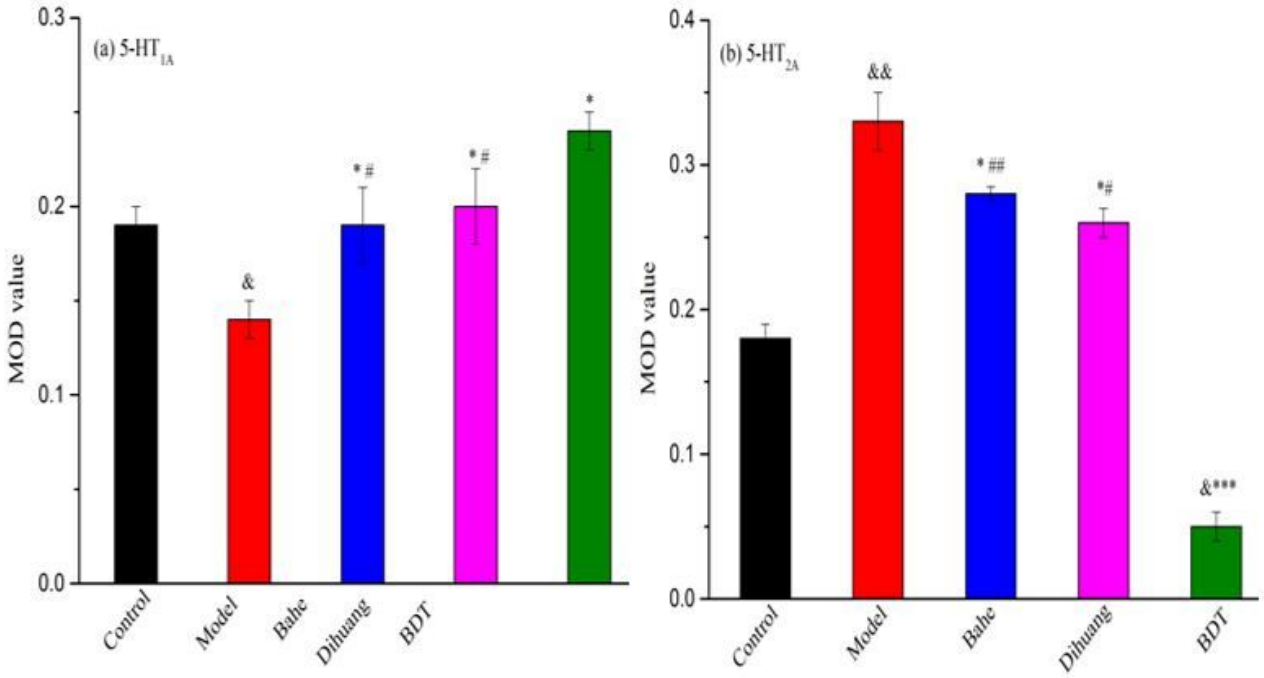

Figure 7

Comparison of protein expression of 5-HT1A (a) and 5-HT2A (b) in the hypothalamus of male mice in each group (n=12). Notes: The error bar was standard error of mean (SEM), & $p < 0.05$, && $p < 0.01$ vs. Control group; * $p < 0.05$, ** $p < 0.01$ vs. Model group; # $p < 0.05$, ## $p < 0.01$, vs. BDT group.

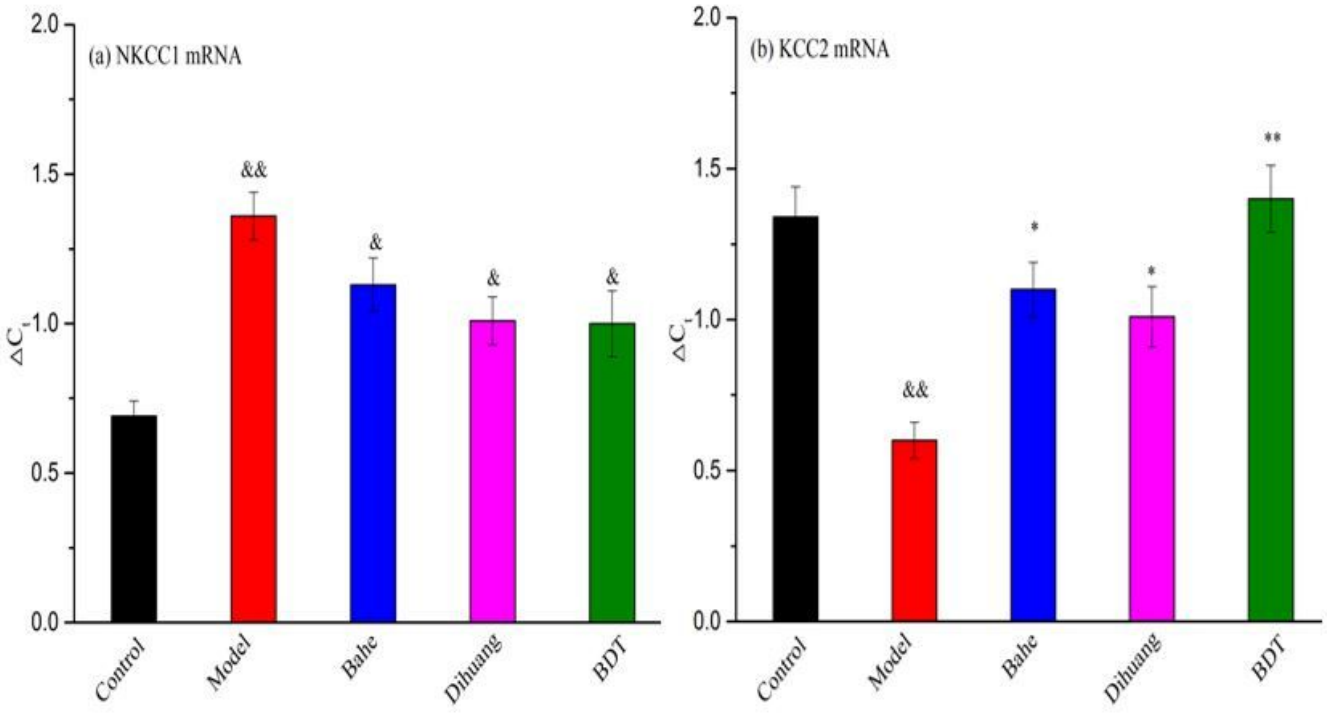

Figure 8

Comparison of NKCC1 (a) and KCC2 (b) gene expression in the hypothalamus of male mice in each group (n=12). Notes: The error bar was standard error of mean (SEM), & $p < 0.05$, && $p < 0.01$ vs. Control group; * $p < 0.05$, ** $p < 0.01$ vs. Model group.

Supplementary Files

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