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Acute serotonergic treatment changes the relation between anxiety and HPA-axis functioning and periaqueductal gray activation

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Abstract

Serotonergic (5-HT) drugs are widely used in the clinical management of mood and anxiety disorders. However, it is reported that acute 5-HT treatment elicits anxiogenic-like behavior. Interestingly, the periaqueductal gray (PAG), a midbrain structure which regulates anxiety behavior - has robust 5-HT fibers and reciprocal connections with the hypothalamic-pituitary-adrenal (HPA) axis. Although the HPA axis and the 5-HT system are well investigated, the relationship between the stress hormones induced by 5-HT drug treatment and the PAG neural correlates of the behavior remain largely unknown. In this study, the effects of acute and chronic treatments with buspirone (BUSP) and escitalopram (ESCIT) on anxiety-related behaviors were tested in an open-field (OF). The treatment effects on PAG c-Fos immunoreactivity (c-Fos-ir) and corticosterone (CORT) concentration were measured in order to determine the neuroendocrine correlates of anxiety-related behaviors and drug treatments. Our results demonstrate that acute BUSP and ESCIT treatments induced anxiogenic behaviors with elevation of CORT compared to the baseline. A decrease of c-Fos-ir was found in the dorsomedial PAG region of both the treatment groups. Correlation analysis showed that the CORT were not associated with the OF anxiogenic behavior and PAG c-Fos-ir. No significant differences were found in behaviors and CORT after chronic treatment. In conclusion, acute BUSP and ESCIT treatments elicited anxiogenic response with activation of the HPA axis and reduction of c-Fos-ir in the dorsomedial PAG. Although no correlation was found between the stress hormone and the PAG c-Fos-ir, this does not imply the lack of cause-and-effect relationship between neuroendocrine effects and PAG function in anxiety responses. These correlation studies suggest that the regulation of 5-HT system was probably disrupted by acute 5-HT treatment.

Keywords: Stress hormone, anxiety, periaqueductal gray, serotonin, hypothalamic-pituitary-adrenal axis

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a prominent endogenous neurotransmitter which has been, implicated in (amongst other functions) the regulation of emotional responses and behaviours in the mammalian brain [1]. Over the past decades, different types of 5-HT drugs have been developed for the treatment of mood and anxiety disorders including social phobia, depression, posttraumatic stress and panic disorders. For instance, selective serotonin reuptake inhibitors (SSRIs) such as escitalopram (ESCIT), citalopram, fluoxetine and paroxetine are widely used in the clinical management of depression, panic and severe anxiety disorders [2, 3]. On the other hand, buspirone (BUSP), a classical 5-HT_{1A} receptor agonist, is effective only in the treatment of generalized anxiety disorder – it is not an effective treatment for other types of severe anxiety [4, 5]. Despite encouraging results, there have been several reports describing patients undergoing acute treatment of either SSRIs [6, 7] or BUSP [8, 9] who have experienced an increase in anxiety levels such as agitation, restlessness and jitteriness, (for review see [10]).

The hypothalamic-pituitary-adrenal (HPA) axis plays an important role in the modulation of anxiety-like behaviour [11, 12]. Earlier studies have consistently shown that a significant correlation exists between the activation of the HPA axis and stress- or anxiety-related behaviour [13]. In particular, abnormal or increased function of the HPA axis has always been found in mood and anxiety disorders [14]. Additionally, the HPA axis and the 5-HT are reciprocally interconnected in terms of their influence on behaviour. It has been shown that activity of the HPA axis can also be stimulated by administration of 5-HT precursors, reuptake inhibitors or receptor agonists, thereby enhancing 5-HT neurotransmission and eventually increasing stress hormone levels via the paraventricular nucleus of the hypothalamus (PVH) [15, 16].

Interestingly, the midbrain periaqueductal gray (PAG), which regulates anxiety and defensive behaviour [17, 18], has robust 5-HT immunoreactive cell fibers and anatomical connections with the PVH [19]. In human and animal models, stimulation of this structure evokes anxiety and panic-like responses, eventually accompanied by activation of the HPA axis [20, 21]. It was shown that the PAG-PVH connection pathway is critical for neuroendocrine regulatory of emotion reaction [17, 20]. This neural circuitry of anxiety is generally affected by a group of 5-HT-containing cells in the dorsal raphe nucleus, which projects its axon to the PAG, one of the pharmacological target regions for anxiolytic action in mediating anxiety and defensive behaviours [22, 23]. Although the reciprocal interaction between the HPA axis and the 5-HT system has been well investigated in numerous studies, the relationship between the stress hormones induced by 5-HT drugs treatment and the PAG neural correlates of the behavioural response remain unknown. Since the PAG modulates anxiety behaviour, studying their correlation will give us important clues into the relationship between anxiety disorders and 5-HT drugs.

In this study, we hypothesized that acute, but not chronic, treatment with BUSP and ESCIT would result in anxiety-like behaviour accompanied by activation of the HPA axis. The behaviour was tested in the open-field (OF) arena and different behavioural measures (time spent in different OF zones, immobility, head-weaving, rearing and self-grooming) were assessed. Levels of stress hormone were also measured 30 min after the OF testing. To unravel the neural mechanism of PAG underlying the anxiety behaviour after 5-HT treatment, different PAG regions were investigated using c-Fos neuronal activation marker. Finally, correlation analysis was performed to measure the relationship between stress hormone, OF behaviour and PAG c-Fos immunoreactivity (c-Fos-ir). We hypothesized that the anxiety-like behavior would be correlated with the HPA axis function and PAG neural activity after acute 5-HT treatments.

2. Methods

2.1. Subjects

Thirty six male albino Wistar rats (Harlan, Horst, the Netherlands) weighing 300-350g were used in this study. Animals were housed individually in standard transparent polypropylene cages on sawdust bedding in a temperature- (20-22 °C), humidity- (60-70%) and light-controlled (12/12-h reversed light/dark cycle) room. Food, standard laboratory chow (Hopefarms, Woerden, the Netherlands), and water were available *ad libitum*. This study was approved by the Animal Experiments and Ethics Committee of Maastricht University.

2.2. Experimental design

Animals were randomly divided into three experimental groups, treated with saline (SAL, n=6), BUSP (n=6), and ESCIT (n=6), in either acute or chronic treatment category. In the acute condition, all animals received a single dose before the OF experiment. In chronic treatment, all animals were given daily injections for 21 days and then tested in the OF arena. All animals were handled regularly in order to habituate them to being picked up and to reduce stress during behavioural testing.

2.3. Drug administration

Escitalopram oxalate (H. Lundbeck A/S, Copenhagen, Denmark) and buspirone hydrochloride (TOCRIS, Cookson Inc., Missouri, USA) were freshly prepared and dissolved in SAL 0.9% NaCl (Braun, Melsungen, Germany). ESCIT (10 mg/kg) and BUSP (3 mg/kg) were injected (s.c. in a volume of 1 ml/kg) 60 min and 120 min before the OF test, respectively. The doses of ESCIT [24] and BUSP [25, 26] were chosen based on previous experimental studies. A week before the actual experimental testing, all animals received 1 ml saline injection 3 times on alternating days in order to habituate the animals to the injection procedure.

2.4. Behavioural testing and evaluation

The OF testing and behavioural analysis were performed according to previous descriptions [25]. In brief, rats were tested in an enclosed OF (square: 100 cm x 100 cm, and height: 40 cm), a clear Plexiglas box with an open top and a dark floor. All behavioural scores were analyzed and calculated for the total 10 min observation of the rats spent in the OF. Observation of animal behaviour was scored by researchers who were blind to the drug treatments and condition of testing.

2.5. Blood sampling and corticosterone radioimmunoassay

One week before the OF testing, blood samples were taken for determination of the baseline concentration of plasma CORT. Blood samples were also taken 30 min after OF testing following acute or chronic treatment with SAL, BUSP, and ESCIT, respectively. All blood samples were withdrawn from the rat tails and directly collected into the heparinized capillary tubes (Microvette, CB300, Sarstedt, Germany) in a cold ice box. Blood samples were centrifuged at 3000 rpm for 5 min at 4°C and the supernatant was thereafter stored at -80°C until analysis. 50 µl of plasma was extracted with 3 ml dichloromethane and vortexed for one minute in order to determine the concentration of plasma CORT. The CORT was then measured directly on 1 ml dried dichloromethane and extracted for radioimmunoassay using CORT-¹²⁵I. The radioimmunological reaction was performed overnight at 4°C, after which a secondary antibody system was used to separate bound and unbound steroid. The entire procedure has previously been described in detail [20].

2.6. *Histological processing*

Two hours after the final testing in the OF or 90 min after blood collection, rats were immediately anesthetized with Nembutal (75 mg/kg) and perfused transcardially with Tyrode and fixative solution containing 4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in 0.1M phosphate buffer (pH 7.6). Brains were removed and post-fixed for 2 hours, and subsequently followed by an overnight 15% sucrose immersion. The brain tissues were cut serially on a cryostat (MICROM, Walldorf, Germany) into 30µm frontal sections and stored at -80°C.

Details of this staining have been previously described [27]. In brief, c-Fos stainings were performed using a primary anti-cFos rabbit polyclonal IgG (diluted 1:20.000 in 0.1% bovine serum albumin and Tris-buffered solution-Triton (TBS-T) solution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for an overnight incubation at room temperature on a constant shaker. After rinsing, all sections were incubated with the secondary antibody (diluted 1:400 in biotinylated donkey anti-rabbit biotin; Jackson ImmunoResearch Laboratories Inc., Westgrove, USA) for 90 min. Next, sections were incubated with an avidin-biotin-peroxidase complex (diluted 1:800 in Elite ABC-kit, Vectastatin; Vector, Burlingame, USA) for 2 h. To visualize the immune complex of horseradish peroxide reaction product, sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB)/ Nickel Chloride (NiCL₂) solution (5 ml DAB solution, 5 ml Tris/HCl, 50 µl NiCL₂, and 3.35 µl hydrogen peroxide). This reaction was stopped after 10 min by thoroughly rinsing all the sections with TBS. Finally, all sections were then mounted on gelatine-coated glasses, dehydrated, and cover-slipped with Pertex (HistolabProducts ab, Goteborg, Sweden).

Systematic quantification of c-Fos-ir cells was performed in different regions of the PAG (Bregma from -6.8 mm to -8.0 mm): dorsomedial PAG (dmPAG), dorsolateral PAG (dlPAG), lateral PAG (lPAG) and ventrolateral PAG (vlPAG). Photographs of the areas of interest were taken at 4X magnification using an Olympus DP70 camera connected to an Olympus AX70 bright-field microscope (analySIS; Imaging System, Münster, Germany). The quantification was based on previously reported method [28]. The boundaries of the areas of interest were delineated and measured according to the Paxinos and Watson rat brain atlas [29]. Similar light intensity and threshold conditions were used for all sections. The counting of the numbers of c-Fos-ir cells was performed using the conventional image analysis program 'Image J' (version 1.38, NIH, USA). Artefacts in the sections were excluded from analysis to ensure the accuracy of measurements. The number of c-Fos-ir cells per mm² was calculated for each area of interest.

2.7. Statistical analysis

All data are presented as mean $\pm$ S.E.M and were analyzed by one-way ANOVA. A Bonferroni post-hoc test was used to analyze group differences in more detail. Two-way ANOVA with repeated measures was performed to analyze the CORT levels. Pearson correlation coefficients were calculated to examine the relationship between variables within a test and between different tests of CORT, PAG c-Fos activity and all behavioural measures in the OF. All p-values < 0.05 were considered significant.

3. Results

3.1. Measures of open-field behaviour

After acute treatment, there was an overall effect in the time that the rats spent in the center ($F(2,13)=10.95$, $p=0.002$) and the corner ($F(2,13)=5.61$, $p=0.02$) zones of the OF tests. Results showed that acute treatment with BUSP and ESCIT significantly reduced the time spent in the center of the OF (Fig. 1B). Meanwhile, only BUSP treated rats spent more time in the corner area (Fig. 1C). Interestingly, a significant reduction in the distance moved ($F(2,14)=6.74$, $p<0.01$) was found after acute treatment of rats.

(Figure 1 about here)

In terms of different behaviours, the treatment effects between groups were significantly different for immobility ($F(2,15)=4.14$, $p=0.04$), rearing ($F(2,15)=5.05$, $p=0.03$), and grooming ($F(2,15)=4.80$, $p=0.03$), and marginally different for head-weaving ($F(2,15)=3.21$, $p=0.073$). Post-hoc analysis revealed that there was an increase in immobility and a reduced frequency of rearing in the BUSP treated animals (Fig. 2A, B). ESCIT treatment decreased the rearing behaviour and marginally increased the frequency of self-grooming ($p=0.067$) (Fig. 2B, D).

(Figure 2 about here)

After chronic treatment, no significant differences were found in the time the rats spent in the center ($F(2,17)=1.412$, n.s.), corner ($F(2,17)=1.924$, n.s.), and wall ($F(2,17)=1.814$, n.s.) zones of the OF field (Fig. 3). In locomotion behaviour, no significant effect was detected in the distance moved ($F(2,17)=1.652$, n.s.).

3.2. *Effects of treatments on plasma corticosterone concentration*

Following acute treatment, no significant effect ($F(2,15)=3.22$, $p=0.073$) on CORT concentration was detected in the animals after OF testing. However, two-way ANOVA with repeated measures revealed a clear increase of CORT after acute BUSP and ESCIT treatments as compared to baseline (see Fig. 4). No differences in terms of CORT concentration were found in the baseline and SAL.

Following chronic treatment, no significant effect on CORT ($F(2,17)=2.424$, $p=n.s.$) was detected in animals after OF test (Fig. 3).

(Figure 3 and 4 about here)

3.3. *Evaluation of c-Fos-ir cells*

After acute treatment, one-way ANOVA with Bonferroni post-hoc test showed a significant difference in the number of c-Fos-ir cells between groups in the dmPAG ($F(2,14)=8.81$, $p=0.004$) and dlPAG ($F(2,14)=4.17$, $p=0.04$), but not for the lPAG ($F(2,16)=3.80$, $n.s.$) and vlPAG ($F(2,15)=2.99$, $n.s.$). A significant decrease in the number of c-Fos-ir cells was found only in the dmPAG region of both the BUSP and ESCIT treated rats as compared with SAL condition (see Fig. 5). Meanwhile, a tendency towards a decrease of c-Fos-ir cells was also shown in the dlPAG after acute BUSP ($p=0.065$) and ESCIT ($p=0.086$) treatments.

Since no significant differences were found for either the anxiety or stress hormone levels after chronic treatment, we did not proceed further with analysis of the PAG c-Fos-ir and correlational study of their interaction.

(Figure 5 about here)

3.4. Correlation analysis of stress hormone, open-field behaviour and PAG c-Fos-ir

The correlations of the CORT concentration, OF behavioural measures and PAG c-Fos-ir are presented in Table 1 (a) and (b). When all animals were included in the analysis, the CORT was negatively correlated with the distance moved and time-spent in the OF center zone, and positively correlated with the immobility and head-weaving behaviour. Interestingly, there was a significant correlation found between the PAG c-Fos-ir and the time spent in the corner and wall OF zones. Meanwhile, the dorsal PAG (dmPAG and dlPAG), except for the lPAG and vlPAG, is positively correlated with the time-spent in the center OF zone. No correlation was found between the CORT and the PAG c-Fos-ir, or for the behavioural measures, particularly immobility, head-weaving, rearing and self-grooming (Table 1 a).

There was a statistically significant difference of correlation matrix when analyzing the SAL, BUSP, and ESCIT independently. In the SAL treated animals, the CORT was negatively correlated with the OF corner and positively correlated with the OF wall zone. Meanwhile, a significant correlation was found between the PAG (dmPAG, dlPAG, lPAG) and the OF behaviour (corner, wall, immobility), except for the dlPAG and the OF wall zone. We found no correlation between the CORT levels and the PAG c-Fos-ir. In addition, the dorsal PAG (dmPAG and dlPAG) was significantly correlated with the lPAG. The data showed no overall correlation for the vlPAG (Table 1 a).

In the BUSP treated animals, the CORT was positively correlated with only the head weaving behaviour, but not with any other OF behaviours and PAG c-Fos-ir. No correlation was found between PAG c-Fos-ir and OF behaviour. Of note, the dmPAG was highly correlated with the dlPAG, whereas the dlPAG was significantly correlated with the lPAG and vlPAG (Table 1 b).

In the ESCIT treated animals, CORT was positively correlated only with the grooming behaviour, but not with any other OF behaviours and PAG c-Fos-ir. Interestingly, the lPAG was positively correlated with the distance moved and velocity; whereas the vlPAG was positively correlated with the distance moved and negatively correlated with the immobility behaviour. It is important to note that, the dmPAG was highly correlated with the dlPAG, whereas the vlPAG was significantly correlated with the dmPAG and dlPAG (Table 1 b).

(Table 1 about here)

4. Discussion

In this study, we aimed to determine the relation between stress hormone and PAG c-Fos-ir and anxiety-like behaviour following acute and chronic administration of BUSP and ESCIT. Our data are consistent with previous clinical and laboratory findings that acute BUSP and ESCIT treatments elicited anxiogenic response accompanied by elevation in plasma CORT concentration. No significant difference was found after chronic BUSP and ESCIT treatments. In analysis of all animals, a significant correlation was found between the stress hormone levels and OF behaviour (time-spent in the OF center and immobility), suggesting a relation between anxiety and CORT. Interestingly, we found that the anxiogenic effects of acute BUSP and ESCIT in the OF test were not correlated with the stress hormone levels. These findings suggest that the extracellular levels of 5-HT release and its receptors regulation were probably disrupted by acute 5-HT treatment. The correlation analysis showed that the anxiety levels induced by BUSP treatment had no neural correlates with the PAG regions. However, in the ESCIT treated animals there was a significant correlation between PAG regions (lPAG and vIPAG) and OF behaviours (distance moved, and immobility). Overall, no significant correlation was detected between the stress hormone and PAG c-Fos-ir, suggesting that the regulation of anxiety in the PAG has no relationship with the neuroendocrine effects.

4.1. Effects of 5-HT treatment on behaviours

Increased immobility and decreased rearing behaviour can be an indication for anxiety and reduced exploratory behaviour [30]. Acute BUSP and ESCIT administration resulted in a transient anxiogenic-like effect (decreased time-spent in the center of the OF). Our data demonstrate that BUSP but not ESCIT suppressed locomotor activity (increased immobility) and decreased thigmotaxic behaviour (time-spent in OF wall zone and rearing) in the OF. These findings are in line with previous studies showing the locomotion suppression and reduced

thigmotaxic activity of BUSP treatment [31, 32]. Furthermore, several reports have shown that patients with acute BUSP treatment have experienced an increase in anxiety levels [8, 9]. Anxiogenic-like effects of BUSP, in particular with regard to the elevated plus-maze [33], the social interaction test [34], and the OF test with diminished exploratory behaviour [25] were also observed in preclinical studies.

For many years, SSRIs have often been the first-line choice for anxiolytic or antidepressant therapy [35]. They have been shown to be effective in several clinical trials associated with depression, social anxiety disorder and generalized anxiety disorder [36, 37]. However, many studies have reported that treatment with acute SSRIs (fluoxetine, citalopram, sertraline, paroxetine) produced anxiety symptoms [6, 38, 39]. Two studies of particular interest have demonstrated that the administration of ESCIT or 5-HT_{1D/1B} receptor agonist sumatriptan enhanced the level of fear response during a stressful simulated public speaking test, and this was accompanied by a remarkable increase of stress hormone [40, 41]. Similarly, there is evidence from numerous animal behavioural studies also pointing to an anxiogenic response. For instance, administration of acute SSRIs induced anxiety-related responses in the elevated plus-maze test [42], the exploration test [43], the social interaction test [44], and the light-dark test [45].

4.2. Effects of 5-HT treatment on stress hormone

It was previously thought that the dysregulation of HPA axis leads to severe mood and anxiety disorders and that normalization of HPA axis, which could be achieved by 5-HT drug treatment, would thus bring relief of anxiety [11, 12]. Activation of the HPA axis is commonly associated with the presence of stress or increased anxiogenic-like behaviour. Our data showed that both BUSP and ESCIT enhanced the release of stress hormone accompanied by anxiety behaviour in the OF. In line with this, a previous study demonstrated that BUSP produced dose-dependent

increases in CORT concentration [46]. Similar findings were also observed with BUSP in which acute treatment increased the basal level of plasma CORT concentration [31, 47, 48]. In addition, some studies have consistently shown that acute administration stimulated the HPA axis while repeated treatments decreased the impact of CORT that lead to attenuation of stress hormone [31, 49, 50]. In SSRIs, acute treatments were similar to the effects of BUSP which induced anxiogenic-like responses with increase of CORT concentration in animals [49, 50].

The release of CORT in response to enhanced 5-HT neurotransmission has previously been shown by direct activation of the 5-HT receptors on the corticotrophin-releasing hormone (CRH) containing neurons in the PVH region [16]. This is supported by evidence of c-Fos-ir in the CRH neurons that are induced by 5-HT compounds. This hormonal response was mediated mainly by the 5-HT_{1A} and 5-HT_{2A/2C} receptor mechanisms at the 5-HT terminal regions in the PVH [16, 51]. Interestingly, previous studies also showed that enhanced 5-HT neurotransmission stimulated the HPA axis with increased concentration of CORT, while its depletion (tryptophan depletion) reduced the CORT [15, 52]. Importantly, these findings showed that both 5-HT and the HPA axis function are inter-related in the pathophysiology of anxiety.

In consideration of the PAG anatomical connectivity, it has been shown that via the injection of biotinylated dextran amine into the PAG, its anterograde label was traced in the parvicellular and magnocellular divisions of the PVH [53]. Furthermore, an electrophysiological study has also demonstrated a connection whereby stimulation of the PAG antidromically evoked a small number of cells in the PVH [54]. These findings indicate that there is a PAG-PVH pathway for the endocrine regulatory integration of an emotional coping reaction. However, contradictory findings exist between the PAG and HPA axis in the context of anxiety for panic disorder. Of particular interest, no activation of the HPA axis was found after electrical stimulation of the

PAG [55]; whereas our previous findings demonstrated that PAG stimulation induced escape behaviour accompanied by increased stress hormone levels [20]. Although these data may seem contradictory, it should be noted that different functional and anatomical interconnections of the PAG underlie distinct functions for anxiety and defensive behaviour (for reviews, see [17, 56]).

4.3. Effects of 5-HT treatment on PAG c-Fos immunoreactivity

In the PAG, 5-HT plays a modulatory role in the regulation of anxiety and defensive behaviours [57]. The present data revealed that 5-HT treatments significantly decreased the number of c-Fos-ir cells only in the dmPAG, but not the dlPAG, lPAG, and vlPAG. This corroborates our previous study showing acute BUSP treatment decreased neuronal activity in the dorsal PAG [28]. Moreover, other studies have also demonstrated a similar reduction of c-Fos-ir in specific regions of the PAG following antidepressant treatments in stress-induced animals [58, 59]. The drug-induced changes in PAG neuronal activation could be caused by either direct or indirect signaling related to neurotransmitter receptors on the PAG, or its projection afferents from the dorsal raphe nucleus (DRN). Importantly, there are substantial amount of 5-HT immunoreactive fibers and different types of 5-HT receptors distributed throughout the PAG [60]. It is noteworthy that the PAG contains robust 5-HT_{1A} and 5-HT_{2A} receptors. Previous studies have shown that intra-dorsal PAG microinjection of 5-HT_{1A} receptor agonist resulted anxiolytic, while microinjection of 5-HT_{2A} receptor antagonist produced aversive behaviour [61]. Since the PAG and DRN are interconnected, it has been shown that microinjection of 5-HT_{1A} drugs into the DRN produced inhibitory electrophysiological responses, while 5-HT₂ induced excitatory responses in the dorsal PAG [62, 63].

Overall, the effects of 5-HT treatment affect a particular receptor subtype. For instance, activation of 5-HT_{1A} receptors usually leads to increased or decreased c-Fos-ir in specific brain

regions [64, 65]. One possible explanation for the decreased c-Fos expression in the PAG would be the inhibitory/deactivation effect of 5-HT_{1A} autoreceptors, as previous studies have shown that acute 5-HT treatment decreased c-Fos-ir in the dorsal PAG in stress-induced animals, while chronic treatment increased its expression [59]. It has been reported that acute SSRI treatment by fluoxetine aggravates panic symptoms by impairing the 5-HT neurotransmission that is mediated by the inhibitory autoreceptors [66]. A further study also showed that neurotoxic lesion of the 5-HT neurons led to decreased c-Fos-ir in the lateral PAG [67]. Thus, these data might possibly explain why acute 5-HT treatment impaired the release of 5-HT which eventually elicited anxiogenic behaviour.

4.4. Effects of treatment on behavioural-neural-endocrine correlates

In the present study, when all animals were included in the analysis, there was a correlation between stress hormone and the OF behaviour (distance moved, OF center zone, immobility and head weaving). However, after acute BUSP and ESCIT treatments, we found no correlation between the stress hormone and the OF anxiogenic behaviour, indicating that the neuroendocrine effects were probably disrupted by acute 5-HT treatments. Although many studies that have demonstrated enhanced 5-HT activated the HPA axis, the mechanisms of neuroendocrine and behavioural correlates with 5-HT treatments remain largely unknown. The lack of correlation between the stress hormone and the OF anxiogenic behaviour after acute 5-HT treatments, does not absolutely imply that there is no relationship between these variables. It is important to note that 5-HT treatment generally affects the entire neurocircuitry of anxiety, and that other brain structures such as the limbic system could also be indirect influence on the PVH neurons which increased stress hormone that eventually lead to anxiogenic behaviour.

Despite the fact that no correlation was demonstrated between the PAG c-Fos-ir and OF behaviour after BUSP treatment, in the ESCIT treated animals there was a significant correlation between PAG regions (lPAG and vlPAG) and OF behaviours (distance moved, and immobility). This finding corroborates data from our previous study showing that ESCIT was effective in reducing the fear-like response (measured by immobility) in the OF arena, but not in the BUSP treated group [24]. The latter study showed that electrical stimulation of the dorsal PAG spontaneously evoked escape behaviour and was subsequently followed by freezing and fear-like behaviour. Thus, the manifestation of this behaviour was mainly regulated in the lPAG and vlPAG, and therefore our correlation analysis showed an association between these regions and the OF behaviour particularly the distance moved and immobility. This notion is strongly supported by the fact that the PAG specifically contains the neural substrates for fear and anxiety that predominantly regulate active defensive behaviours such as fight and flight [17].

In conclusion, our results show that acute, but not chronic, BUSP and ESCIT treatments elicited anxiogenic response with activation of the HPA axis and reduction of c-Fos-ir in the dorsomedial PAG. Although no correlation was found between the stress hormone and the PAG c-Fos-ir, this does not imply the lack of cause-and-effect relationship between neuroendocrine effects and PAG function in anxiety responses. These correlation studies suggest that the regulation of 5-HT system was probably disrupted by acute 5-HT treatment.

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Legends

Figure 1. Effects of acute SAL, BUSP and ESCIT treatments of rats on the measures of distance moved (cm) and duration of time-spent in the different zones of the OF arena. Note: Acute BUSP and ESCIT treatments elicited anxiogenic response with decreased exploratory time spent in the center zone of the OF (*B*). Data represented mean ($\pm$ S.E.M.). * Significant different from the SAL treatment.

Figure 2. Effects of acute SAL, BUSP and ESCIT treatments of rats on a paradigm of different anxiety measures (immobility, head-weaving, rearing and grooming behaviours) in the OF arena. Note: Acute BUSP significantly produced suppression on locomotor activity (*A*) with decreased rearing (*B*), while acute ESCIT showed only decreased rearing (*B*) behaviour. Data represented mean ($\pm$ S.E.M.). * Significant different from the SAL treatment.

Figure 3. Effects of chronic SAL, BUSP and ESCIT treatments of rats on the measures of distance moved (cm) and duration of time spent in the different zones of the OF arena (*A-D*). Concentration of plasma CORT (ng/ml) was measured 30 min after OF testing following 3-weeks chronic treatments of SAL, BUSP and ESCIT of rats. Note: No significant difference was shown. Data represented mean ($\pm$ S.E.M.).

Figure 4. The graphs show the concentration of plasma CORT (ng/ml) 30 min after OF testing with acute treatments of SAL, BUSP and ESCIT, respectively. One week before the OF testing, blood samples were taken for determination of the CORT baseline. Scatter plots display the correlation between the concentration of plasma CORT (ng/ml) and behavioural measures (*B-E*) in the OF arena. Data represent means ($\pm$ S.E.M.). # significant difference from the Baseline value.

Figure 5. The graphs show the number of c-Fos-ir cells per mm² in different regions of the PAG (*A, B, C, D*). Schematic drawing of the PAG regions based on the atlas of the rat brain by Paxinos and Watson (*E*) [29]. Representative low-power photomicrographs (scale bar: 400 µm) of 30 µm-thick sections from the brain of the PAG regions. Boxed areas are shown by the high-power photomicrographs of the dmPAG region (scale bar: 200 µm). The small dark dots represent c-Fos-ir cells. The inset in dmPAG ESCIT treatment shows a representative photomicrograph (100X magnification power) of a typical c-Fos-ir cell. Note: BUSP and ESCIT treatments significantly decreased the number of c-Fos-ir cells only in the dmPAG region except for the dlPAG, lPAG, and vlPAG as compared with the SAL treated condition. Data represent means ($\pm$ S.E.M.) Indication: * Significant difference from the SAL treatment.

Table 1 (a, b). The tables show the Pearson correlation coefficients between the CORT concentration, PAG c-Fos-ir, and different behavioural measures of the OF tests. In table 1 (a), the lower-left side of the table shows data of all animals included in the analysis, while the upper-right side of the table shows data of all SAL treated animals. In table 1 (b), the lower-left side of the table shows data of all BUSP treated animals, while the upper-right side of the table shows data of all ESCIT treated animals.

Figure 1. Hestermann et al.

Figure 2. Hestermann et al.

Figure 3. Hestermann et al.

Figure 4. Hestermann et al.

Figure 4. Hestermann et al.

Figure 5. Hestermann et al.

Figure 5. Hestermann et al.

(E)

Table(s) 1 (a). Hestermann et al.

	CORT	Distance moved	Velocity	Center	Corner	Wall	Immobility	Head Weaving	Rearing	Grooming	dmPAG	dIPAG	IPAG	vIPAG
CORT		.397	.392	.069	-.947*	.963**	-.729	-.221	.228	-.749	.858	.811	.854	.873
Distance moved	-.501*		1.000**	.637	-.366	.308	-.527	.667	.053	-.108	.163	.013	-.100	-.280
Velocity	-.405	.989**		.634	-.360	.303	-.530	.675	.044	-.095	.151	.001	-.112	-.291
Center	-.584*	.767**	.766**		-.282	.185	-.453	.189	-.438	.044	.330	.288	.108	-.263
Corner	.282	-.821**	-.816**	-.667*		-.995**	.840	.330	.041	.662	-.980*	-.962*	-.968*	-.889
Wall	-.470	.826**	.820**	.676*	-.986**		-.814	-.358	.004	-.683	.956*	.943	.967*	.926
Immobility	.519*	-.599*	-.659**	-.632*	.853**	-.791**		-.050	.457	.164	-.972*	-.997**	-.984*	-.852
Head Weaving	.492*	-.308	-.279	-.334	.534*	-.298	.676**		-.021	.521	-.817	-.897	-.923	-.863
Rearing	-.316	.090	.205	.717**	-.481	.600*	-.470	-.211		-.640	.044	-.086	-.035	.110
Grooming	.362	-.099	-.129	-.370	-.173	-.370	-.010	-.356	-.493*		-.830	-.749	-.757	-.725
dmPAG	-.460	.646**	.636**	.642*	-.604*	.695**	-.415	-.348	.406	-.432		.987*	.961*	.815
dIPAG	-.358	.512	.535*	.604*	-.644*	.708**	-.468	-.390	.463	-.383	.948**		.983*	.848
IPAG	-.307	.490	.498*	.483	-.631*	.685**	-.392	-.380	.309	-.284	.901**	.934**		.930
vIPAG	-.146	.433	.435	.426	-.585*	.616*	-.375	-.321	.317	-.270	.809**	.858**	.900**	
All animals (SAL, BUSP, and ESCIT)														

SAL treated animals

** Correlation is significant at the 0.01 level

* Correlation is significant at the 0.05 level

Table 1 (b). Hestermann et al.

	CORT	Distance moved	Velocity	Center	Corner	Wall	Immobility	Head Weaving	Rearing	Grooming	dmPAG	dIPAG	IPAG	vIPAG
CORT		-.525	-.534	-.629	-.909	-.784	.555	-.628	-.700	.838*	-.543	-.565	-.593	-.529
Distance moved	-.574		.998**	.170	-.972*	.908*	-.889*	.068	.168	-.271	.798	.796	.908*	.824*
Velocity	.129	.999**		.172	-.954*	.893*	-.871*	.040	.147	-.256	.785	.786	.916*	.804
Center	-.226	.872	.877		.801	.370	-.485	.558	.870	-.764	.103	.297	-.078	.426
Corner	-.058	-.412	-.707	-.475		-.896	.753	.416	.547	-.574	-.869	-.708	-.923	-.635
Wall	-.020	.381	.654	.441	-.995**		-.955*	.481	.503	-.624	.872	.827	.840	.856
Immobility	.359	-.672	-.757	-.431	.847	-.852		-.342	-.508	.526	-.746	-.800	-.660	-.928**
Head Weaving	.906*	-.575	-.210	-.175	.193	-.242	.652		.810	-.831*	.231	.184	.063	.290
Rearing	.450	.749	.904*	.660	-.459	.374	-.451	.101		-.970**	.399	.486	.045	.564
Grooming	-.501	.088	.203	-.376	-.254	.262	-.612	-.760	.063		-.496	-.549	-.228	-.580
dmPAG	-.470	.311	-.006	-.141	.512	-.523	.005	-.557	.065	.539		.967**	.797	.897*
dIPAG	-.330	.110	.117	-.374	.243	-.269	-.204	-.555	.191	.794	.898*		.722	.951**
IPAG	-.439	-.102	-.142	-.576	.282	-.281	-.138	-.590	-.111	.851	.811	.942*		.641
vIPAG	-.096	.248	.379	-.199	.135	-.190	-.250	-.395	.510	.653	.832	.936*	.770	
BUSP treated animals														

ESCIT treated animals

** Correlation is significant at the 0.01 level

* Correlation is significant at the 0.05 level