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Behavioural, neurochemical and neuroendocrine effects of the endogenous β-carboline harmane in fear-conditioned rats

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Abstract

The putative endogenous imidazoline binding site ligand harmane enhances neuronal activation in response to psychological stress and alters behaviour in animal models of anxiety and antidepressant efficacy. However, the neurobiological mechanisms underlying harmane's psychotropic effects are poorly understood. We investigated the effects of intraperitoneal injection of harmane (2.5 and 10 mg/kg) on fear-conditioned behaviour, hypothalamo-pituitary-adrenal axis activity, and monoaminergic activity within specific fear-associated areas of the rat brain. Harmane had no significant effect on the duration of contextually induced freezing or 22 kHz ultrasonic vocalisations and did not alter the contextually induced suppression of motor activity, including rearing. Harmane reduced the duration of rearing and tended to increase freezing in non-fear-conditioned controls, suggesting potential sedative effects. Harmane increased plasma ACTH and corticosterone concentrations, and serotonin (in hypothalamus, amygdaloid cortex, prefrontal cortex and hippocampus) and noradrenaline (prefrontal cortex) content, irrespective of fear-conditioning. Furthermore, harmane reduced dopamine and serotonin turnover in the PFC and hypothalamus, and serotonin turnover in the amygdaloid cortex in both fear-conditioned and non-fear-conditioned rats. In contrast, harmane increased dopamine and noradrenaline content and reduced dopamine turnover in the amygdala of fear-conditioned rats only, suggesting differential effects on catecholaminergic transmission in the presence and absence of fear. The precise mechanism(s) mediating these effects of harmane remain to be determined but may involve its inhibitory action on monoamine oxidases. These findings support a role for harmane as a neuromodulator, altering behaviour, brain neurochemistry and neuroendocrine function.

Keywords

Imidazoline binding sites, beta-carboline, harmane, fear conditioning, monoamine, HPA axis, brain, rat

Introduction

The β-carboline harmane, a putative endogenous agonist at imidazoline binding sites (Musgrave and Badoer, 2000; Robinson et al., 2003), possesses binding affinity for monoamine oxidase-A (MAO-A) (Herraiz and Chaparro, 2005; Kim et al., 1997) at which it acts as an allosteric inhibitor, and for the gamma-aminobutyric acid ($GABA_{A)}$) receptor (Mousah et al., 1986; Muller et al., 1981; Splettstoesser et al., 2005), where it has an inverse agonistic effect (Evans and Lowry, 2007; McMahon et al., 2006; Petersen, 1983). There is a well-established role for MAO-A and $GABA_A$ receptors in the regulation of emotional processing and a significant body of evidence suggesting that imidazoline binding sites (I_1 and I_2 subtypes) may represent a novel therapeutic target for the treatment of psychiatric disorders (Smith et al., 2009a). Thus, increased understanding of the behavioural and neurobiological effects of harmane is warranted.

Previous studies have demonstrated that systemic administration of exogenous harmane dose dependently reduces immobility in the rat and mouse forced swim test (Aricioglu and Altunbas, 2003; Farzin and Mansouri, 2006) and increases time spent in the open arms of the elevated plus maze (Aricioglu and Altunbas, 2003). Direct administration of harmane into the globus pallidus has been shown to increase time spent in the illuminated sector of the light-dark box without inducing motor impairment (Talalaenko et al., 2006). Furthermore, the anxiolytic-like effect of ethanol is potentiated by pretreatment with imidazoline ligands including agmatine (I_1/I_2 receptor agonist), moxonidine and clonidine (I_1 receptor agonists) and 2-(2-benzofuranyl)-2-imidazoline (2-BFI) (I_2 receptor agonist) in the elevated plus maze (Taksande et al., 2010), suggesting that imidazoline binding site ligands may augment or act synergistically with other receptors to modulate anxiety-related behaviour. Taken together, these data suggest that imidazoline ligands, including harmane, may have anxiolytic and antidepressant-like activity. However, there is a paucity of studies investigating the effects of harmane on conditioned fear responding and associated neurochemical and neuroendocrine alterations which we sought to address here.

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Fear conditioning (FC) paradigms are used to study the expression of fear and formation of fear memory; in particular, the deficits or dysregulation that may lead to anxiety disorders including phobias and post-traumatic stress disorder (PTSD). The neural circuitry underpinning the acquisition and expression of contextual fear is well understood and involves the amygdala, hippocampus and medial prefrontal cortex (mPFC) (Ledoux, 2007; Sanders et al., 2003). Connectivity between the brain-regional components of this fear circuitry and the paraventricular nucleus of the hypothalamus facilitates the neuroendocrine response to fear in the form of hypothalamo-pituitary-adrenal (HPA) axis activation (Herman and Cullinan, 1997; Prewitt and Herman, 1998; Rodrigues et al., 2009).

Multiple neurotransmitter systems have been implicated in coordination of the behavioural response to conditioned fear, with a particularly important role for the monoamines. FC specifically activates the mesoamygdaloid dopaminergic system, increasing the rate of dopaminergic neuron firing, dopamine (DA) content and metabolism in the hypothalamus, amygdala and mPFC (Coco et al., 1992; Goldstein et al., 1994; Inoue et al., 1994; Sasaki et al., 1998). The serotoninergic and noradrenergic systems also play an important role. Conditioned-fear stress induces intense Fos expression in the noradrenergic locus coeruleus and serotonergic dorsal raphe neurons (Ishida et al., 2002). Activation of monoaminergic receptor subtypes or inhibition of neurotransmitter re-uptake alters the rat freezing response in an FC paradigm (Wislowska-Stanek et al., 2008) and in an FC stress test (Mochizuki et al., 2002). Both selective serotonin- and serotonin-noradrenaline reuptake inhibitors are useful first-line agents for most anxiety disorders (Baldwin et al., 2010).

Both I_1 and I_2 binding sites and their putative endogenous ligand harmane are found throughout the limbic system in stress-responsive brain regions (Anderson et al., 2005; Anderson et al., 2006a; Anderson et al., 2006b; Lione et al., 1998;). Our previous work has shown that harmane is capable of modulating neuronal responses to restraint stress in rats (Smith et al., 2009b) and that I_2 binding site ligands potentiate the HPA axis (Finn et al., 2004a) and central noradrenergic (Finn et al., 2002) response to stress. Moreover, agonism of I_1/I_2 binding sites with the putative endogenous imidazoline binding site ligand agmatine has been shown to induce a dose-dependent impairment in the magnitude of learned contextual fear in rats (McKay et al., 2002). In addition, intra-amygdala injections of clonidine, an α_2-adrenoceptor and I_1 binding site agonist, blocked the expression of fear in rats (Schulz et al., 2002). These data suggest an important role for imidazoline binding sites in the modulation of conditioned fear. The aim of the present study was to investigate the effects of harmane on the expression of contextual fear and associated alterations in brain monoaminergic and HPA axis activity in rats.

Materials and methods

Animals

Experiments were carried out on male Sprague-Dawley rats (weighing 290–340 g at time of testing (Charles River, Margate, UK)). Rats were housed singly and maintained at a constant temperature (21±2°C) under standard lighting conditions (12:12 h light:dark, lights on 07:00 to 19:00 h). All experiments were carried out during the light phase between 08:00 and 13:30 h. Food and water were available ad libitum. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under licence from the Irish Department of Health and Children, and in compliance with the European Communities Council directive 86/609.

Drug preparation

Harmane hydrochloride was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany). Harmane was first dissolved in glacial acetic acid (0.2–0.3% final concentration in solution) and made up to a concentration of 10 mg/mL with 0.9% weight/volume (w/v) saline. The stock was then diluted 1:4 to make a 2.5 mg/mL solution. Vehicle-treated control animals received sterile saline (with 0.2–0.3% glacial acetic acid), pH matched at 5.4 with drug solutions. Doses of harmane (2.5 and 10 mg/kg) were determined based on our previous study that demonstrated increases in c-Fos expression in stress-sensitive brain regions (Smith et al., 2009b). These doses were also used to induce antidepressant-like and anxiolytic behavioural responses exhibited in the forced swim test and elevated plus maze, respectively (Aricioglu and Altunbas, 2003). Vehicle or harmane was administered by intraperitoneal (i.p.) injection at 1 mL/kg injection volume.

Experimental procedure

The experimental procedure for induction of conditioned fear was similar to that described previously (Finn et al., 2004b; Finn et al., 2006; Roche et al., 2007). It consisted of two phases, conditioning and testing, occurring 24 h apart. Subjects were randomly assigned to groups and the sequence of testing was randomised in order to minimise any confounding effects of testing procedure or circadian variation. To minimise the potential influence of stress induced by i.p. injection 30 min before testing (re-exposure to the shock chamber), rats received i.p. vehicle injections for four days prior to testing.

On the conditioning day, rats were placed in a Perspex FC/observation chamber (30 × 30 × 30 cm; light intensity: 53 lux) with black sides, and after 15 s they received the first of 10 footshocks (0.4 mA, 1 s duration (LE85XCT scrambled shock generator and timer, Linton Instrumentation, Norfolk, UK)) spaced 60 s apart. Forty-five seconds after the last footshock, rats were returned to their home cage. Controls not receiving footshock were exposed to the chamber for an equivalent 10 min period. A bat detector (Batbox Duet©, Batbox, Steyning, West Sussex, UK) was used to detect ultrasonic vocalisation in the 22 kHz range, and behaviours were recorded for 10 min from a video camera located beneath the observation chamber. The arena was cleaned with Milton® solution between rats and wiped clean with 0.5 % acetic acid solution to remove any remaining urine odor.

The test phase commenced 24 h later when the subject received an i.p. injection of vehicle, 2.5 or 10 mg/kg harmane. This design resulted in six treatment groups: vehicle + NoFC (no fear conditioning) (n = eight), 2.5 mg/kg harmane + NoFC (n = eight), 10 mg/kg harmane + NoFC (n = seven), vehicle + FC (n = eight), 2.5 mg/kg harmane + FC (n = seven) and 10 mg/kg harmane + FC (n = seven). The rats were then returned to their home cage until 30 mins post-injection at which time they were

re-exposed to the chamber in which they had received footshock 24 h previously for a period of 10 min. Rats were decapitated immediately after the 10 min test trial. Trunk blood was collected into chilled 7 mL ethylenediamine tetraacetic acid (EDTA)-treated collection tubes on ice. Plasma was separated from whole blood by centrifugation at 4°C for 15 min at 13,000 g and stored at -80°C prior to measurement of hormones using enzyme immunoassay. Brains were rapidly removed and the hypothalamus, prefrontal cortex (PFC), hippocampus and amygdaloid cortex and were dissected out rapidly on ice, weighed, snap-frozen on dry ice and stored at -80°C prior to high-performance liquid chromatography (HPLC) (see below).

Plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) analysis. ACTH and CORT were measured using enzyme-immunoassay kits suitable for the measurement of these hormones in rat plasma (IDS Ltd., Tyne and Wear, UK). Samples were assayed in duplicate. The limit of detection was 0.46 pg/mL for ACTH and 0.55ng/mL for CORT.

Behavioural analysis. Behaviour was analysed using Observer® software (Noldus, Wageningen, The Netherlands), which allowed for continuous event recording over each 10 min trial. A trained scorer blind to the experimental drug treatments assessed behaviour. The event-recording technique was employed to assess the total duration of freezing (defined as the cessation of all visible movement except that necessary for respiration), 22 kHz ultrasound emission, walking, rearing and grooming. The number of faecal pellets produced during the 10-min conditioning and test trials was also counted. Behaviour from the conditioning trial was also assessed to confirm and validate the aversive response to footshock. As expected, during the conditioning trial footshock significantly increased the duration of freezing behaviour, faecal pellet production and 22 kHz ultrasonic vocalisation whilst decreasing walking and the duration of both rearing and grooming behaviours (data not shown).

HPLC. Each region of interest (the hypothalamus, PFC, hippocampus and amygdaloid cortex) was homogenised by ultrasonication in 1 mL of mobile phase (0.1 M citric acid, 0.1 M NaH_2PO_4, 1.4 mM 1-octane sulphonic acid, 0.1 mM EDTA, 9% methanol; pH 3.5), and homogenates were centrifuged at 4°C for 15 min at 14,000 g. A sample (40 µL) of supernatant was injected onto a Shimadzu HPLC system (SIL9-A) with a reverse-phase C18 column (Licrosorb RP-18 column; Phenomenex, Macclesfield, Cheshire, UK). Electrochemical detection (Shimadzu LECDC6A with glassy carbon electrode and amperometric detector) was used to determine peak heights of monoamines and their metabolites. The electrode was maintained at +0.8 V and the flow rate of the mobile phase through the system was 1 mL/min. Peak heights for standards of 2 ng/20 µL for noradrenaline (NA), 3,4-dihyroxyphenylacetic acid (DOPAC), dopamine (DA), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA (Sigma-Aldrich Ireland, Dublin, Ireland)) were obtained each day prior to injection of samples, and after every 10 samples. N-methyl 5-HT (2 ng/20 µL) was included in the mobile phase as an internal standard. Concentrations of monoamines or their metabolites were expressed as ng neurotransmitter/g of tissue.

Statistical analysis

Behavioural data from the test day and neurochemical and hormone data were analysed using a two-way analysis of variance (ANOVA), with FC and drug treatment as factors. Post hoc analysis was performed by Fisher's Least Significant Difference (LSD) test when appropriate. Behavioural data over time were analysed by two-way repeated-measures ANOVA. All data were tested for normality using Shapiro-Wilk test and for homogeneity of variance using Levene's test. In the event that data were not normally distributed or homogenous for variance before or after transformation, Kruskal-Wallis test was performed to detect significant effects of treatment (FC, drug) followed by Mann-Whitney U tests for pair-wise group comparisons. A p-value of < 0.05 was considered statistically significant. Data are expressed as means + SEM. The statistical software SPSS was used to analyse all data.

Results

Effect of harmane on fear-related behaviour (freezing and 22 kHz ultrasonic vocalisations) in NoFC and FC rats

A two-way ANOVA on the duration of freezing over the full 10 min trial indicated a significant main effect of fear conditioning ($F_{(1,44)} = 15.310$ $p < 0.0001$), but no effect of drug or interaction of factors. Post-hoc analysis revealed a significant increase in the duration of freezing in all three FC groups compared with their respective NoFC counterparts (Figure 1a, $p < 0.001$). A two-way repeated-measures ANOVA on the freezing data analysed in 1-minute time bins over the course of the 10-min trial also indicated a significant main effect of FC ($F_{(1,44)} = 15.310$ $p < 0.001$) but not drug or interaction of factors (Figure 1b). Kruskal-Wallis test with subsequent post hoc testing indicated an increase in the duration of 22 kHz ultrasonic vocalisations ($\chi^2 = 5.5$ $p < 0.05$) over the 10-min trial in vehicle-treated FC rats compared with NoFC counterparts (vehicle+FC: median= 187.8, inter-quartile range= 292.37 vs vehicle+NoFC: median= 0 $p < 0.05$). There was a trend for a harmane-induced of attenuation of this fear-related increase in 22 kHz ultrasonic vocalisation but these effects failed to reach statistical significance (vehicle+FC: median= 187.8, inter-quartile range= 292.37, 2.5 mg/kg+FC: median= 86.9, inter-quartile range= 268.63, 10 mg/kg+ FC: median= 97.2 interquartile range= 221.25).

Effect of harmane on general behaviour in NoFC and FC rats

Statistical analysis (two-way ANOVA) indicated that FC reduced motor activity ($F_{(1,45)} = 21.872$; $p < 0.0001$), defined as the total duration of behaviours which are dependent on conscious motor activity, specifically the sum duration of walking, grooming and rearing, in the 10-min test trial (see Figure 1c). Post hoc analysis revealed a significant reduction in the duration of these active behaviours in all three FC groups, compared with their respective NoFC counterparts (Figure 1c, $p < 0.001$). Harmane, however, did not alter the total duration of active behaviours. Two-way ANOVA

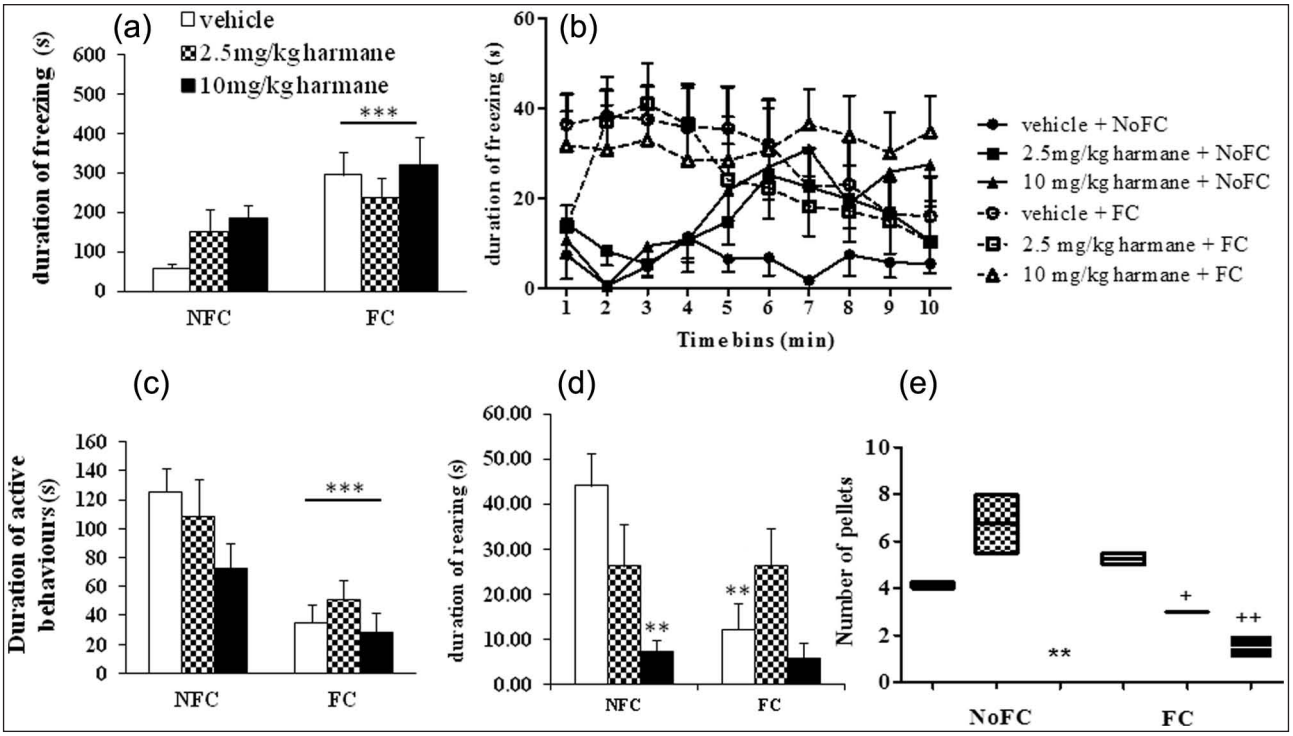

Figure 1. **(a)** The effect of harmane (2.5 or 10 mg/kg, intraperitoneally (i.p.)) on the duration of freezing behaviour in non-fear-conditioned (NoFC) and fear-conditioned (FC) rats in the 10-min test period. Statistical analysis by two-way analysis of variance (ANOVA) (factors: drug, FC). For all panels in figure, data are expressed as means +SEM (n= seven to eight/group) unless otherwise stated. ***$p < 0.001$ compared to NoFC counterparts. **(b)** The duration of freezing behaviour in 1-min time bins over the 10-min test trial. **(c)** The effect of harmane on time spent engaging in motor activity in the 10-min test period. Motor activity was defined as the combined duration of rearing, grooming and walking. Statistical analysis by two-way ANOVA (factors: drug, FC) ***$p < 0.001$ compared to NoFC counterparts (Fisher's Least Significant Difference (LSD) post hoc analysis). **(d)** The effect of harmane on duration of rearing in the 10 min test period. **$p < 0.01$ compared to NoFC-vehicle group (Fisher's LSD post hoc analysis). **(e)** Effect of harmane on the number of faecal pellets produced in NoFC and FC rats. Statistical analysis by Kruskal-Wallis test; pairwise comparisons were made using Mann-Whitney U tests. Data are expressed as median and interquartile range. ** $p < 0.01$ compared to vehicle-treated NoFC controls; + $p < 0.05$, ++ $p < 0.01$ compared to vehicle-treated FC control.

on the duration of rearing indicated a significant effect of FC ($F_{(1,44)} = 4.492$ $p < 0.05$) and drug ($F_{(2,44)} = 6.047$ $p < 0.01$) but no interaction between the two factors. Harmane dose dependently reduced time-spent rearing in NoFC rats compared to vehicle-treated counterparts, with a significant reduction evident at the 10 mg/kg dose (vehicle+ NoFC vs 10 mg/kg harmane+ NoFC, $p < 0.01$) (see Figure 1d). FC was associated with a decrease in the duration of rearing behaviour (vehicle+NoFC vs vehicle+FC, $p < 0.01$), but harmane did not have any significant effect on this reduction.

Effect of harmane on defaecation in NoFC and FC rats

Kruskal-Wallis test revealed a significant effect of treatment ($\chi^2 = 19.707$ $p < 0.001$) on the number of faecal pellets produced. Harmane (10 mg/kg) reduced faecal pellet production in NoFC and in FC rats versus respective vehicle-treated controls (Figure 1e: 10 mg/kg harmane+NoFC vs vehicle+NoFC, $p < 0.01$; 2.5 mg/kg harmane+FC vs vehicle+FC, $p<0.05$ 10mg/kg harmane+FC vs vehicle+FC $p < 0.01$).

Effect of harmane on HPA axis activity in NoFC and FC rats

CORT data were transformed using a natural log transformation before statistical analysis. Two-way ANOVA showed a main effect of drug ($F_{(2,47)} = 19.177$ $p<0.0001$) but not FC or interaction of factors. Harmane (10 mg/kg but not 2.5 mg/kg), increased plasma CORT levels in both FC and NoFC rats compared to their respective vehicle-treated controls (Figure 2a: 10mg/kg harmane+NoFC vs vehicle+NoFC $p < 0.01$, 10mg/kg harmane+FC vs vehicle+FC $p < 0.01$). FC did not alter plasma CORT levels compared to vehicle-treated NoFC controls.

Two-way ANOVA revealed a main effect of drug ($F_{(2,47)} = 19.801$ $p<0.0001$), but not FC or interaction of factors, on plasma ACTH levels. Harmane (10 mg/kg but not 2.5 mg/kg) increased plasma ACTH levels in both FC and NoFC rats compared to respective vehicle-treated controls 10 min following re-exposure to the arena (Figure 2b: 10 mg/kg harmane+NoFC vs vehicle+NoFC $p < 0.01$, 10 mg/kg harmane+FC vs vehicle+FC $p < 0.01$). There was no significant difference between plasma ACTH levels in FC rats compared to levels in NoFC controls.

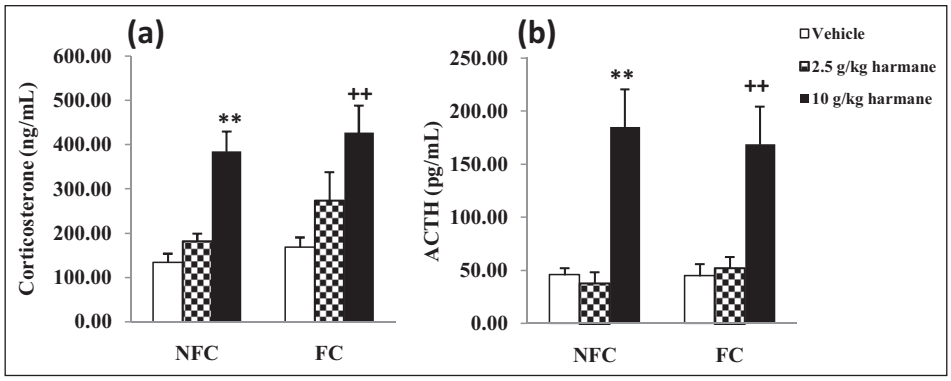

Figure 2. Effect of harmane on (a) plasma corticosterone and (b) plasma adrenocorticotropic hormone (ACTH) levels in non-fear-conditioned (NoFC) and fear-conditioned (FC) rats, 10 minutes following re-exposure to the arena. Statistical analysis by two-way analysis of variance (ANOVA) (factors: drug, FC); Fisher's Least Significant Difference (LSD) post hoc analysis. Data are expressed as means + SEM (n= seven to eight/group). ** $p < 0.01$ compared with vehicle-treated NoFC rats, ++$p < 0.01$ compared with vehicle-treated FC rats.

Table 1. Effect of harmane on serotonin (5-HT) content in the amygdaloid cortex, prefrontal cortex, hypothalamus and hippocampus, noradrenaline (NA) content in the amygdaloid cortex and prefrontal cortex, and dopamine (DA) content in the amygdaloid cortex. Statistical analysis by Kruskal-Wallis test or two-way analysis of variance (ANOVA) (factors: drug, fear conditioning (FC); pairwise comparisons were made using Mann Whitney U or Least Significant Difference (LSD) test post hoc where appropriate. Data are expressed as means ± SEM (n= six to eight/group). Bold values denote $p < 0.05$ compared to their corresponding vehicle-treated controls.

Content (ng/g)	Region	NoFC-Vehicle	NoFC-2.5 mg/kg	NoFC-10 mg/kg	FC-Vehicle	FC-2.5 mg/kg	FC-10 mg/kg
5-HT	Amygdala	219 ± 18	324 ± 52	**479 ± 45**	212 ± 64	**412 ± 42**	**617 ± 69**
	PFC	164 ± 19	**284 ± 52**	**484 ± 18**	136 ± 19	**404 ± 25**	**457 ± 16**
	Hypothalamus	170 ± 29	302 ± 63	**925 ± 234**	170 ± 35	**443 ± 72**	**467 ± 72**
	Hippocampus	147 ± 14	**283 ± 21**	**406 ± 33**	147 ± 20	**308 ± 28**	**418 ± 92**
NA	Amygdala	148 ± 29	125 ± 30	181 ± 17	132 ± 22	**213 ± 27**	**237 ± 20**
	PFC	67 ± 25	108 ± 31	**183 ± 91**	73 ± 19	**163 ± 12**	**185 ± 16**
DA	Amygdala	111 ± 19	174 ± 24	117 ± 33	104 ± 35	275 ± 63	**368 ± 120**

Effect of harmane on 5-HT content in the rat brain

Statistical analysis (two-way ANOVA/Kruskall Wallis where appropriate) revealed significant main effects of drug/treatment on 5-HT content in the amygdaloid cortex ($F_{(2,39)} = 12.148$; $p < 0.0001$), PFC ($\chi^2 = 23.054$ $p < 0.0001$), hypothalamus ($F_{(2,38)} = 11.638$ $p < 0.0001$) and hippocampus ($\chi^2 = 26.650$; $p < 0.0001$) without interaction of factors. Harmane increased 5-HT content in each brain region irrespective of FC. Significant post hoc comparisons are detailed in Table 1.

Effect of harmane on noradrenaline (NA) content in the rat brain

A Kruskal-Wallis test revealed a significant effect of treatment ($\chi^2 = 8.584$; $p<0.05$) on NA content in the amygdaloid cortex. Harmane did not alter NA content in NoFC rats (Table 1). However, harmane increased NA content in FC rats compared with vehicle-treated FC controls (Table 1: 2.5, 10 mg/kg harmane+FC vs vehicle+FC; $p < 0.05$). There was a significant effect of drug ($F_{(2,44)} = 12.365$; $p < 0.0001$) but no effect of FC or interaction of factors on NA content in the PFC. Harmane (10 mg/mg) significantly increased PFC NA content in NoFC rats (Table 1: 10 mg/kg harmane+FC vs vehicle+FC; $p < 0.05$), while both doses of harmane significantly increased levels of NA in the PFC of FC rats (Table 1: 2.5, 10 mg/kg harmane+FC vs vehicle+FC; $p < 0.05$). No significant effects of drug, FC or interaction of factors on NA content were observed in the hypothalamus or hippocampus (data not shown).

Effect of harmane on dopamine (DA) content in the rat brain

An effect of drug ($F_{(2,40)} = 6.321$; $p < 0.01$) and FC ($F_{(1,40)} = 8.876$; $p < 0.01$) on DA content was observed in the amygdaloid cortex using a two-way ANOVA. Harmane did not alter DA content in NoFC in the amygdaloid cortex. FC did not alter DA content compared with vehicle-treated NoFC controls. Harmane (2.5 mg/kg) tended to increase DA content in FC rats compared to vehicle-treated counterparts, although this effect was not statistically significant (Table 1). Harmane (10 mg/kg) increased DA content in

Table 2. Effect of harmane on monoamine turnover in the amygdaloid cortex, prefrontal cortex and hypothalamus. Statistical analysis by Kruskal-Wallis test or two-way analysis of variance (ANOVA) (factors: drug, fear conditioning (FC); Pairwise comparisons were made using Mann Whitney U or Least Significant Difference (LSD) test post hoc where appropriate. Data are expressed as means ± SEM (*n*= six to eight/group). Bold values are $p < 0.05$ compared to their corresponding vehicle-treated controls.

Turnover ratio	Region	NoFC			FC		
		Vehicle	2.5 mg/kg	10 mg/kg	Vehicle	2.5mg/kg	10 mg/kg
DA	Amygdala	1.59 ± 0.6	0.52 ± 0.2	0.4 ± 0.2	1.83 ± 0.5	0.24 ± .005	**0.2 ± 0.05**
	PFC	1.78 ± 0.3	1.08 ± 0.2	**0.43 ± 0.1**	1.66 ± 0.2	**0.6 ± 0.5**	**0.62 ± 0.2**
	Hypothalamus	1.67 ± 0.3	**0.84 ± 0.2**	**0.51 ± 0.2**	**1.04 ± 0.2**	1.01 ± 0.5	**0.44 ± 0.05**
5-HT	Amygdala	2.57 ± 0.3	**0.96 ± 0.3**	**0.27 ± 0.02**	1.97 ± 0.3	**0.53 ± 0.2**	**0.21 ± 0.04**
	PFC	2.02 ± 0.2	**0.99 ± 0.2**	**0.3 ± 0.02**	1.95 ± 0.4	0.86 ± 0.4	**0.46 ± 0.2**
	Hypothalamus	3.1 ± 0.4	**1.31 ± 0.5**	**0.76 ± 0.5**	2.22 ± 0.5	**0.71 ± 0.2**	**0.35 ± 0.04**

DA: dopamine; PFC: prefrontal cortex; 5-HT: 5-hydroxytryptamine.
Effect of harmane on DA and 5-HT turnover in the rat brain

the amygdaloid cortex in FC rats compared with vehicle-treated FC controls (Table 1: 10 mg/kg harmane+FC vs vehicle+FC; $p <$ 0.01).There were no significant effects of drug, FC or interaction between factors on DA content in the PFC, hypothalamus or hippocampus (data not shown).

Effect of harmane on DA turnover in the rat brain

A Kruskal-Wallis test indicated a significant effect of treatment ($\chi^2 = 11.378$; $p < 0.01$) on DA turnover in the amygdaloid cortex. Harmane had no effect on DA turnover in NoFC rats (Table 2). FC did not alter DA turnover in the amygdaloid cortex. Harmane (10 mg/kg) significantly reduced DA turnover in FC rats versus vehicle-treated FC controls (Table 2: 10 mg/kg harmane+FC vs vehicle+FC, $p < 0.01$). A two-way ANOVA revealed a significant effect of drug ($F_{(2,41)} = 10.948$; $p < 0.0001$) on DA turnover in the PFC, with no effect of FC or interaction. Harmane (10 mg/kg) decreased DA turnover in the PFC of NoFC rats compared to vehicle-treated NoFC controls (Table 2: 10 mg/kg harmane+NoFC vs vehicle+NoFC $p < 0.01$). Harmane decreased DA turnover in FC rats versus vehicle-treated FC controls (Table 2: 2.5, 10 mg/kg harmane+FC vs vehicle+FC, $p < 0.05$). A Kruskal-Wallis test detected a significant effect of treatment (χ^2= 13.273 $p < 0.05$) on DA turnover in the hypothalamus. Harmane decreased DA turnover in the hypothalamus in NoFC rats compared to vehicle-treated controls (Table 2: 2.5 or 10 mg/kg harmane+NoFC vs vehicle+NoFC, $p < 0.05$, $p < 0.01$). FC rats had significantly lower DA turnover in the hypothalamus compared with NoFC rats (Table 2: vehicle+NoFC vs vehicle+FC, $p<0.05$). Harmane decreased DA turnover in FC rats versus vehicle-treated FC counterparts (Table 2: 10 mg/kg harmane+FC vs vehicle+FC $p <$ 0.05). There was no significant effect of drug, FC, or interaction between factors on DA turnover in the hippocampus (data not shown).

Effect of harmane on 5-HT turnover in the rat brain

A Kruskal-Wallis test indicated a main effect of treatment ($\chi^2 =$ 26.255; $p < 0.001$) on 5-HT turnover in the amygdaloid cortex. Harmane decreased 5-HT turnover in a dose-dependent manner in NoFC rats versus vehicle-treated controls (2.5, 10 mg/kg harmane+NoFC vs vehicle+NoFC $p < 0.05$ $p < 0.01$). FC did not alter 5-HT turnover. Harmane decreased 5-HT turnover in the amygdaloid cortex in FC rats ($p < 0.01$) compared with vehicle-treated FC controls (2.5, 10 mg/kg harmane+FC vs vehicle+FC, $p < 0.01$). A Kruskal-Wallis test also detected a significant effect of treatment (χ^2 =20.056; $p<0.001$) on 5-HT turnover in the PFC. Harmane decreased 5-HT turnover in NoFC rats versus vehicle-treated NoFC rats (Table 2: 2.5, 10 mg/kg harmane+NoFC vs vehicle+NoFC, $p < 0.01$). FC did not alter 5-HT turnover in the PFC. Harmane (10 mg/kg) decreased 5-HT turnover in FC rats versus vehicle-treated FC controls (Table 2: 10 mg/kg harmane+FC vs vehicle+FC, $p < 0.05$). There was a significant effect of treatment ($\chi^2 = 20.384$; $p < 0.0001$) on 5-HT turnover in the hypothalamus. Harmane decreased hypothalamic 5-HT turnover in NoFC rats versus vehicle-treated counterparts (Table 2: 2.5 or 10 mg/kg harmane+NoFC vs vehicle+NoFC, $p < 0.05$, $p < 0.01$). FC did not alter 5-HT turnover in the hypothalamus. Harmane decreased 5-HT turnover in FC rats versus vehicle-treated FC controls (Table 2: 2.5 or 10 mg/kg harmane+FC vs vehicle+FC, $p < 0.05$, $p < 0.01$). There was no significant effect of drug, FC, or interaction between factors on 5-HT turnover in the hippocampus (data not shown).

Discussion

This work represents the first investigation of the effects of the endogenous β-carboline harmane on the expression of fear and its neuroendocrine and neurochemical correlates in the rat. A single i.p. injection of harmane, 30 min before re-exposure to a context previously paired with footshock, did not alter contextually induced freezing behaviour. Harmane-induced reductions in rearing and defaecation in NoFC rats, coupled with a trend for harmane-induced freezing/immobility in this group, suggests that harmane may in fact have sedative/locomotor-suppressant properties. In addition, we observed increased levels of circulating ACTH and CORT in harmane-treated rats, irrespective of FC and discrete alterations in tissue levels of monoamines and their metabolites in stress-responsive brain regions.

Consistent with the hypothesis that harmane may have sedative properties in rats, harmane significantly reduced time spent rearing, irrespective of FC. This effect was dose dependent in the NoFC rats. Harmane also tended to dose dependently suppress

motor activity (defined as the combined duration of walking, rearing and grooming) in NoFC rats compared to vehicle-treated NoFC controls. Furthermore, on removal of rats from the context arena, the experimenter (blind to pharmacological treatment) observed profound striatal muscle flaccidity in harmane-treated rats, suggesting that harmane may have induced psychomotor retardation. This observation is not surprising since selective I_1 agonists reduced muscular rigidity in the resperine-induced Parkinson's model (Tanabe et al., 2008). The trend observed here for reduced duration of 22 kHz ultrasonic vocalisations in harmane-treated rats may also be due to a reduction in muscle tone. Suppression of 22 kHz ultrasound vocalisation was previously reported by β-carboline analog FG 7142 administered i.p. to rats in an FC paradigm (Jelen et al., 2003). These authors suggest this effect is mediated via $GABA_A$ receptors. Indeed, sedation is known to be mediated by a direct modulation of the α_1-subunit of the $GABA_A$ receptor (Crestani et al., 2002). The β-carboline analog and $GABA_A$ receptor α_1-selective antagonist beta-carboline-3-carboxylate-t-butyl ester (β-CCT) partially reverses zolpidem-induced locomotor sedation (Vinkers et al., 2009). A similar attenuation in diazepam-induced sedation has also been reported for this β-carboline analog (Savic et al., 2009). Thus, harmane binding to $GABA_A$ receptors (Mousah et al., 1986; Muller et al., 1981; Splettstoesser et al., 2005) containing this subunit may explain its propensity to induce locomotor retardation as observed in the present study, effects which complicate interpretation of the effects of harmane on fear expression.

Consistent with the results of the present study, a previous study investigating the role of FG-7142, a β-carboline analog, in a similar contextual FC paradigm in rats, reported no effect on the behavioural and cardiovascular responses to the aversive context (Resstel et al., 2006). We did find that FC increased faecal pellet production (albeit not statistically) on test day, an effect profoundly inhibited by harmane. Reductions in faecal pellet production can be a potential index of anxiolysis. However, harmane also reduced defaecation in NoFC rats and so it is possible that these effects do not, in fact, relate to an anxiolytic effect but rather may result from a local action on cholinergic receptors. Acetylcholinesterase activity and muscarinic receptor binding of homogenates from several brain structures are inhibited by β-carbolines (Skup et al., 1983). This inhibitory effect on cholinergic activity is exerted by a non-competitive inhibition (Skup et al., 1983) and competitive antagonism, respectively (Shi et al., 2001), whereby binding of harmane to peripheral muscarinic receptors and/or acetylcholinesterase may result in the blockade of parasympathetic nerve stimulation to the internal anal sphincter, thus reducing defaecation.

Here, for the first time, we report that harmane (i.p.) robustly increased plasma ACTH and CORT levels in the rat, suggesting that harmane acts centrally to stimulate the HPA axis. It is known that harmane rapidly penetrates the blood brain barrier (Anderson et al., 2006a). Although the pituitary is implicated, whether harmane is acting specifically at this site or by stimulating the synthesis and/or release of corticotrophin-releasing factor (CRF) in the hypothalamus is still to be elucidated. However, we have previously shown that harmane, at the same dose administered here (10 mg/kg i.p.), robustly increases c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN) of naive and restraint-stressed rats, including in CRF-containing PVN neurones (Smith et al., 2009b). As was the case for harmane-induced c-Fos expression in naive vs restraint-stressed rats, harmane (10 mg/kg) in the present study stimulated ACTH and CORT release to the same extent in NoFC vs FC rats. Taken together, these results suggest that the stimulatory effects of harmane on HPA axis activity are retained and not altered in the presence of psychological stress.

The harmane-induced increases in HPA axis activity could result from the alterations in brain monoamines given the important modulatory influence of monoamines on the HPA axis. The central serotonergic system, in particular, appears to be sensitive to administration of harmane as our results demonstrate that 5-HT content was increased in the amygdala, hypothalamus PFC and hippocampus of both NoFC and FC rats, and 5-HT turnover was reduced in the first three of these regions. Five-HT is a potent stimulator of the HPA axis (Heisler et al., 2007), and thus increases in 5-HT content in the hypothalamus may explain the robust harmane-induced elevations in circulating CORT and ACTH levels that were observed in FC and NoFC rats. These data may also tentatively suggest an important role for harmane-evoked alterations in 5-HT in discrete brain regions other than the PVN/hypothalamus in HPA axis activation. However, as the PFC has no direct afferents to the PVN, such a mechanism would likely involve complex circuitry whereby signals are relayed via the bed nucleus of the stria terminalis (BNST) (Spencer et al., 2005).

Administration of harmane also increased NA content in the PFC of NoFC and FC rats. Moderate levels of NA released under control conditions strengthen prefrontal cortical functions via actions at post-synaptic α_{2A}-adrenoceptors, while high levels of NA released during stress impair prefrontal cortical functions via α_1- and possibly β_1-adrenoceptors (Carr et al., 2007). Harmane induced two- to three-fold increases in NA content in the PFC in NoFC and FC rats, which may be sufficient to impair prefrontal cortical function. These data support a previous study which showed that β-carboline analog FG-7142 increases NA release in the PFC of freely moving rats (Dazzi et al., 2002). Interestingly, NA content in the amygdaloid cortex was increased by harmane treatment but only in FC rats. This finding suggests that there is an alteration in the responsivity of the amygdaloid cortex to harmane that is dependent on the emotional state of the rat.

DA is thought to play an important role in learning, memory and the expression of fear. Although DA content was not altered by harmane in any brain region in NoFC rats, it was, however, significantly increased by harmane in the amygdaloid cortex in FC rats. This finding suggests that FC enhances the responsivity of dopaminergic pathways to harmane administration. Harmane reduced DA turnover in the PFC and hypothalamus of both NoFC and FC rats. A harmane-induced reduction in DA turnover in the amygdala was observed only in FC rats, suggesting differential responsivity of dopaminergic metabolism in the amygdala in the presence versus absence of conditioned fear. Despite differential effects of harmane dependent on fear, surprisingly, conditioned fear itself was not associated with alterations in monomaine levels or turnover, with the exception of a decrease in DA turnover in the hypothalamus (Goldstein et al., 1994). Methodological differences between the present study and previous studies where increased 5-HT and DA activation in the PFC during or following expression of conditioned fear were reported (Goldstein et al., 1994) may explain the discrepant results here; these include the precise region of the PFC assayed, sample collection in dark phase vs light phase, differences in footshock regimen, context and period of habituation.

Reduction in monoamine turnover is indicative of a lower ratio of metabolite to neurotransmitter concentration and suggests that 5-HT and DA degradation may have been impaired by harmane. Such an effect is consistent with harmane's activity as an inhibitor of MAO-A (Herraiz and Chaparro, 2005; Kim et al., 1997), an action thought to be mediated through the I_2 binding site (Tesson et al., 1995) where it binds with high affinity: Ki I_2 = 49 nM (Parker et al., 2004). However, residual [^{3}H]harmane binding has been observed in both brain and kidney tissue from MAO-A knockout mice (Anderson et al., 2006b), suggesting that harmane can bind with high affinity to alternative sites. In the brain, harmane may also bind to the I_1 binding site: IC_{50} I_1 = 30 nM (Parker et al., 2004). Harmane and other β-carboline analogs also have pharmacological activity at $GABA_A$ receptors (K_i/IC_{50} of 7 μM), typically exerting an inverse agonistic effect (Evans and Lowry, 2007; McMahon et al., 2006; Petersen, 1983). It is possible that any or all of these receptor types may contribute to the behavioural, neuroendocrine and neurochemical effects observed herein but the present results provide a solid foundation for the design of future studies aimed at investigating the specific target(s) mediating the effects observed here. For example, harmane-induced effects on monoamine turnover could reflect an inhibition of MAO-A-mediated metabolism and/or an increase in monoamine release via its action at $GABA_A$ receptors. Indeed, previous studies using the microdialysis technique have shown that harmane does increase 5-HT and DA efflux in the nucleus accumbens (Baum et al., 1996) and 5-HT efflux in the hippocampus (Adell et al., 1996). The latter study also demonstrated a harmane-induced reduction in 5-HIAA levels, and the authors suggested that this effect was likely a result of MAO-A inhibition (Adell et al., 1996).

In conclusion, these data suggest that under these experimental conditions, harmane, administered exogenously, did not specifically alter the expression of contextually induced fear in rats. However, testing fear expression at a later time-point post-harmane injection or an experiment investigating the acquisition of fear responses may reveal effects distinct from sedation. The differential effects of harmane on monoamine content and turnover in discrete brain regions in NoFC versus FC rats may indicate fear-induced alterations in the responsivity of central monoaminergic pathways to this endogenous ligand. Our results also provide evidence for harmane-evoked stimulation of HPA axis activity in the presence and absence of conditioned fear. Together, these findings support an important role for harmane as a neuromodulator with important effects on behaviour, brain neurochemistry and neuroendocrine function.

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Conflict of interest

The authors declare that there are no conflict of interest.

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