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Protective effects of 5-HT_{1A} receptor agonists against emotional changes produced by stress stimuli are related to their neuroendocrine effects

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1 The effects of the 11β-hydroxylase inhibitor metyrapone on the protective effects of serotonin (5-hydroxytryptamine; 5-$HT)_{1A}$ receptor agonists against emotional changes produced by acute restraint stress were examined in mice.

2 Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity, i.e. total locomotor activity, number and duration of rearing and head-dipping behaviours, and latency to the first head-dipping, using an automatic hole-board apparatus.

3 Treatment with the 5-HT_{1A} receptor agonists flesinoxan (1 mg kg^{-1}, i.p.) and R(+)-2-di-n-propylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (8-OH-DPAT; 1 mg kg^{-1}, i.p.) 24 h prior to exposure to stress significantly suppressed the decrease in various exploratory behaviours that was observed immediately after the exposure to acute restraint stress (60 min). The effects of flesinoxan (1 mg kg^{-1}, i.p.) and 8-OH-DPAT (1 mg kg^{-1}, i.p.) were antagonized by co-injection with N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride (WAY100635; 1 mg kg^{-1}, i.p.), a selective 5-HT_{1A} receptor antagonist.

4 Flesinoxan (1 mg kg^{-1}, i.p.) and 8-OH-DPAT (1 mg kg^{-1} i.p.) significantly increased the plasma corticosterone level, and these effects of 5-HT_{1A} receptor agonists were dose-dependently blocked by pretreatment with metyrapone (12.5 and 25 mg kg^{-1}, s.c.).

5 Metyrapone (25 mg kg^{-1}, s.c.) alone did not modify the stress-induced changes in exploratory behaviours. Pretreatment with metyrapone (12.5 and 25 mg kg^{-1}, s.c.) partly antagonized the protective effects of flesinoxan (1 mg kg^{-1}, i.p.) and 8-OH-DPAT (1 mg kg^{-1}, i.p.) with regard to only the number and duration of head-dipping behaviours.

6 These results suggest that activation of the adrenocortical system *via* 5-HT_{1A} receptors may facilitate some adaptive mechanism(s) involved in the recognition of and/or ability to cope with stressful situations.

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Abbreviations: ANOVA, analysis of variance; β-CCM, methyl-β-carboline-3-carboxylate; FG7142, N-methyl-β-carboline-3-carboxamide; GABA, γ-aminobutyric acid; GRs, glucocorticoid receptors; HPA axis, hypothalamo-pituitary-adrenal axis; 5-HT, 5-hydroxytryptamine; MRs, mineralcorticoid receptors; 8-OH-DPAT, R(+)-2-di-n-propylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide; WAY100635, N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride

Introduction

The hole-board test, which was first introduced by Boissier & Simon (1962; 1964), is a simple method for measuring the response of an animal to an unfamiliar environment. Previously, the hole-board test has been used to assess emotionality, anxiety and/or responses to stress in animals (Rodriguez Echandia *et al*., 1987). Some advantages of this test are that several behaviours can be readily observed and quantified, which makes possible a comprehensive description of the animal's behaviour. To establish a more detailed behavioural analysis, we recently developed an automatic hole-board apparatus (Takeda *et al*., 1998). We expect that this system will make it possible to automatically measure changes in various exploratory activities of animals, and therefore, this system may be a useful tool for objectively estimating various emotional states of animals.

Recent clinical and preclinical studies have suggested that central serotonin (5-hydroxytryptamine; 5-HT) neurotransmission may be involved in the aetiology, expression and treatment of anxiety, impulsiveness and depression (Murphy, 1990). The discovery of multiple 5-HT receptor subtypes and the development of various selective ligands for these receptors offer an opportunity to clarify the roles of 5-HT in these mental disorders and to treat them more effectively (Murphy, 1990; Cowen, 1991; Martin & Humphrey, 1994). 5-HT_{1A} receptors have been of particular interest because they may be involved in the regulation of

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emotional and behavioural processes (Murphy, 1990; Barrett & Vanover, 1993; Artigas *et al*., 1996). Clinical studies involving the 5-HT$_{1A}$ receptor partial agonist buspirone and the full agonist flesinoxan have shown promising results with regard to generalized anxiety disorder and depression (Murphy *et al*., 1991; Grof *et al*., 1993; Pitchot *et al*., 1995). On the other hand, in preclinical studies using various animal models of anxiety, 5-HT$_{1A}$ receptor agonists do not exert anxiolytic activity in some paradigms used to detect the effects of benzodiazepine anxiolytics (Barrett, 1991). These reports indicate the possibility that the reduction in anxiety observed with 5-HT$_{1A}$ receptor agonists in the clinic differs qualitatively from that observed with classical benzodiazepine anxiolytics. Previously, we obtained data to support this hypothesis in studies that compared the effects of benzodiazepine anxiolytics with those of 5-HT$_{1A}$ receptor agonists on various emotional states of naive and stressed mice using our automatic hole-board apparatus (Takeda *et al*., 1998; Tsuji *et al*., 2000). In these experiments, we found that benzodiazepine anxiolytics and 5-HT$_{1A}$ receptor agonists produced quite different effects. The most important findings were that 5-HT$_{1A}$ receptor agonists but not benzodiazepine anxiolytics have protective effects against various emotional changes produced by stress stimuli. In particular, pretreatment with the 5-HT$_{1A}$ receptor agonists flesinoxan and R(+)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (8-OH-DPAT) 24 h prior to stress exposure suppressed the decrease in various emotional behaviours produced by acute restraint stress. These results suggest that 5-HT$_{1A}$ receptor agonists but not benzodiazepine anxiolytics may affect some adaptive mechanism(s) involved in the recognition of and/or ability to cope with stressful situations.

Apart from their anxiolytic and antidepressant effects, 5-HT$_{1A}$ receptor agonists affect neuroendocrine systems, which are involved in stress regulation. Both flesinoxan and 8-OH-DPAT stimulate the hypothalamo-pituitary-adrenal (HPA) axis and enhance the synthesis and secretion of adrenal corticosteroids (Groenink *et al*., 1995; 1996; Van de Kar *et al*., 1998; Vicentic *et al*., 1998), which influence numerous processes in the central nervous system. A growing body of evidence indicates that adrenal glucocorticoids may play a role in mediating the state of emotionality. For instance, it has been reported that exogenous application of corticosterone causes anxiolytic-like effects, whereas the suppression of endogenous corticosterone by adrenalectomy produces anxiogenic-like effects, which can be reversed by corticosterone replacement (Weiss *et al*., 1970; File *et al*., 1979). Thus, it is possible that the neuroendocrine effects of 5-HT$_{1A}$ receptor agonists might be related to their modulatory effects on emotionality.

The aim of the present study was to examine whether the increase in plasma corticosterone levels resulting from activation of the HPA axis is involved in the above-mentioned behavioural effects of 5-HT$_{1A}$ receptor agonists that we previously reported. In particular, the effects of metyrapone, a 11-β-hydroxylase inhibitor that blocks corticosterone synthesis, on the protective effects of 5-HT$_{1A}$ receptor agonists against emotional changes in mice produced by acute restraint stress stimuli were estimated using our hole-board apparatus.

Methods

Animals

Male ICR mice (Charles River, Japan) weighing 25–30 g were housed eight per cage at a room temperature of 23±1°C with a 12-h light-dark cycle (light on 0600 to 0600 h). Food and water were available *ad libitum*. All experiments were carried out between 1300 and 1700 h.

Apparatus

Exploratory behaviours of mice in a novel environment were measured as previously described using an automatic hole-board apparatus (model ST-1, Muromachi Kikai Co., Ltd., Japan) (Takeda *et al*., 1998; Tsuji *et al*., 2000). The apparatus consisted of a grey wooden box (50×50×50 cm) with four equidistant holes 3 cm in diameter in the floor. An infrared beam sensor was installed on the wall to detect the number and duration of rearing and head-dipping behaviours and the latency to the first head-dipping. The distance of movement in the hole-board was recorded by an overhead colour CCD camera; the heads of the mice were painted yellow and the colour CCD camera followed their centre of gravity. Data from the infrared beam sensor and the CCD camera were collected through a custom-designed interface (CAT-10, Muromachi Kikai Co., Ltd., Japan) as a reflection signal. Head-dipping behaviours were double-checked *via* an infrared beam sensor and the overhead colour CCD camera. Thus, only when both the head intercepted the infrared beam and the head was detected at the hole by the CCD camera was head-dipping behaviour counted.

Behavioural procedure

Effects of pretreatment with 5-HT$_{1A}$ receptor agonists on behavioural response of mice to acute restraint stress 24 h later in the hole-board test Groups of animals were injected with flesinoxan, 8-OH-DPAT or saline. Twenty-four hours later, animals were restrained in a plastic snug-fit apparatus (3 cm in diameter and 7 cm in length) (stressed group) or left in their home cage (non-stressed group) for 60 min, and the hole-board test were performed immediately. Each animal was placed in the centre of the hole-board, and allowed to freely explore the apparatus for 5 min. Total locomotor activity, number and duration of rearing and head-dipping behaviours and latency to the first head-dip were automatically recorded. In combination studies, the 5-HT$_{1A}$ receptor antagonist N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride (WAY100635) was co-injected with flesinoxan or 8-OH-DPAT.

Effect of corticosterone synthesis inhibitor on the protective effects of 5-HT$_{1A}$ receptor agonists against changes in various exploratory behaviours of mice produced by acute restraint stress in the hole-board test Groups of animals were injected with flesinoxan, 8-OH-DPAT or saline. Twenty-four hours later, animals were restrained (stressed group) or left in their home cage (non-stressed group) for 60 min, and the hole-board test were performed immediately as previously described. Metyrapone or saline was administered 90 min prior to the injection of flesinoxan, 8-OH-DPAT or saline.

Measurement of the plasma corticosterone concentration

Effects of 5-HT$_{1A}$ receptor agonists and corticosterone synthesis inhibitor on the plasma corticosterone concentrations in mice Mice were decapitated 60 min after the injection of flesinoxan, 8-OH-DPAT or saline, and their blood was collected. Metyrapone was administered 90 min prior to the injection of 5-HT$_{1A}$ receptor agonist or saline. Blood samples were centrifuged at 4°C and 3000 r.p.m. for 15 min, and the plasma was stored at −20°C for future analysis. The corticosterone concentration was measured fluorimetrically in duplicate by the method of Usui *et al*. (1970) with our minor modification (Liu *et al*., 1999). Namely, plasma sample (200 μl) in a 10 ml conical test tube was diluted with 2 ml of distilled water containing 50 μl of 0.1 *N* sodium hydroxide. The mixture was extracted with 5 ml of methylene chloride and the resulting aqueous layer was removed. The methylene chloride layer was washed once with 1 ml of alkaline solution (10 g of sodium sulphate dissolved in 100 ml of 0.1 *N* sodium hydroxide) and then twice with 1 ml of a 0.1 g ml^{-1} sodium sulphate solution. Two ml portion of the methylene chloride extract was transferred to a test tube, and mixed well with 2 ml of a freshly prepared sulphuric acid-ethanol mixture (70 : 30 v v^{-1}). After standing the mixture at 4°C for 45 min, the upper methylene chloride layer was sucked off, and the lower layer was transferred to a rectangular suprasil quartz cell (JASCO, Japan) for fluorimetric determination. Distilled water and 0.05–1 μg ml^{-1} corticosterone solutions were substituted for the plasma extract as the blank and the standard to draw an external standard curve, respectively, in the fluorimetric determination. Correlation coefficients (r) of an external standard curve were >0.997. The fluorimetric determination was carried out on a FP-777 spectrofluorimeter with a xenon lamp (JASCO, Japan). The concentration of plasma corticosterone was estimated from F(468, 520) value, i.e. excitation and fluorescence wavelengths on the monochromators were adjusted to 468 and 520 nm, respectively. These wavelengths are peculiar to 11-hydroxycorticosteroids (Usui *et al*., 1970). In this method, intra- and inter-assay coefficients of variance were 6.8 and 9.6%, respectively, and efficiency of extraction of corticosterone to methylene chloride was 96.5%.

Effects of pretreatment with 5-HT$_{1A}$ receptor agonists and antagonist on the plasma corticosterone concentrations of non-stressed mice 24 h later Mice were decapitated 24 h after the injection of flesinoxan, 8-OH-DPAT, WAY-100635 or saline, and their blood were collected. The plasma corticosterone concentration was measured as previously described. In combination studies, WAY100635 was co-injected with flesinoxan or 8-OH-DPAT.

Effects of pretreatment with 5-HT$_{1A}$ receptor agonists and corticosterone synthesis inhibitor on the plasma corticosterone concentrations of non-stressed mice 24 h later Mice were decapitated 24 h after the injection of flesinoxan, 8-OH-DPAT or saline, and their blood was collected. The plasma corticosterone concentration was measured as previously described. Metyrapone or saline was administered 90 min prior to the injection of flesinoxan, 8-OH-DPAT or saline.

Drugs

The drugs used in the present study were flesinoxan (a gift from Solvay Duphar, The Netherlands), 8-OH-DPAT (Research Biochemicals, U.S.A.), WAY100635 (a gift from Mitsubishi Chemical Co., Japan) and metyrapone (Sigma, U.S.A.). All drugs were dissolved in saline and were injected in a volume of 10 ml kg^{-1}. Metyrapone was injected to mice subcutaneously (s.c.) and other drugs were injected intraperitoneally (i.p.).

Statistical analysis

All of the data are presented as the mean ± s.e.mean. Behavioural data in the hole-board test were analysed and stored in a personal computer using analytical software (Comp ACT HBS, Muromachi Kikai Co., Ltd., Japan). The homogeneity of variances of data was tested with Bartlett's test ($P<0.05$). If the variances of data were not equal, nonparametric Kruskal-Wallis test was used for statistical evaluations ($P<0.05$ and 0.01). Alternately, if the variances of data were equal, one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test was used for statistical evaluations ($P<0.05$ and 0.01).

Results

Bartlett's test for homogeneity of variances

Bartlett's test suggested that the variances of head-dip latency data in the hole-board test except for those in Table 1 were not equal. Statistical results of Bartlett's test (Bartlett statistic, P value) were (22.565, 0.0002), (19.780, 0.0006), (13.643, 0.0011), (27.052, <0.0001), (10.638, 0.0310) and (25.883, <0.0001) in Figures 1, 2, 4, 5 and 6 and Table 2, respectively. Thus, these data were tested with nonparametric Kruskal-Wallis test. Other behavioural data as well as the data of plasma corticosterone concentrations were all not statistically significant in Bartlett's test, indicating that the variances of data were equal. These data were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test.

Effects of pretreatment with 5-HT$_{1A}$ receptor agonists on the behavioural responses of mice to acute restraint stress 24 h later in the hole-board test The effects of pretreatment with 5-HT$_{1A}$ receptor agonists on the behavioural responses of mice to acute restraint stress 24 h later are shown in Figures 1 and 2. A drastic decrease in various exploratory behaviours, i.e. locomotion and number and duration of rearing or head-dipping behaviours, and an increase in the latency of head-dipping were observed immediately after exposure to acute restraint stress. These changes in exploratory behaviours were significantly suppressed by pretreatment with the 5-HT$_{1A}$ receptor agonists flesinoxan (1 mg kg^{-1}, i.p.) and 8-OH-DPAT (1 mg kg^{-1}, i.p.) 24 h prior to exposure to acute restraint stress ($P<0.05$ or 0.01). The effects of flesinoxan (1 mg kg^{-1}, i.p.) and 8-OH-DPAT (1 mg kg^{-1}, i.p.) were antagonized by co-injection with WAY100635 (1 mg kg^{-1}, i.p.), a selective 5-HT$_{1A}$ receptor antagonist ($P<0.05$ or 0.01).

Table 1 Effects of pretreatment with 5-HT$_{1A}$ receptor agonists, antagonist or combinations of these drugs on exploratory behaviours and basal plasma corticosterone concentrations of non-stressed mice

Drugs (mg/kg)	*Locomotion* (cm)	*Rearing*		*Head-dips*			*Corticosterone*
		Counts	*Duration* (s)	*Counts*	*Duration* (s)	*Latency* (s)	(μg/ml)
Saline	2473.2±98.6	36.4±2.4	45.3±2.4	25.1±2.4	510.3±1.3	38.5±8.1	0.133±0.017
Flesinoxan (1)	2420.6±102.3	36.8±1.3	46.7±3.3	30.1±4.7	14.1±2.7	34.7±8.6	0.123±0.012
8-OH-DPAT (1)	2264.9±215.7	31.1±3.3	41.2±4.3	24.1±5.3	10.2±2.4	34.9±6.7	0.125±0.013
WAY-100635 (1)	2235.2±74.6	34.0±2.7	40.9±3.2	25.5±4.5	10.2±2.6	43.6±6.6	0.118±0.013
WAY-100635 (1)+Flesinoxan (1)	2205.2±88.6	30.9±3.5	34.7±4.6	22.0±1.7	10.0±1.9	42.8±11.2	0.132±0.020
WAY-100635 (1)+8-OH-DPAT (1)	2286.4±208.9	34.4±3.4	41.6±3.8	20.9±1.9	9.5±2.0	33.6±6.1	0.122±0.015

Flesinoxan, 8-OH-DPAT, WAY-100635 or saline was injected 24 h prior to the measurement of exploratory behaviours. In combination studies, WAY-100635 was co-injected with flesinoxan or 8-OH-DPAT. Each data represents the means with s.e.mean of eight mice. Data were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test.

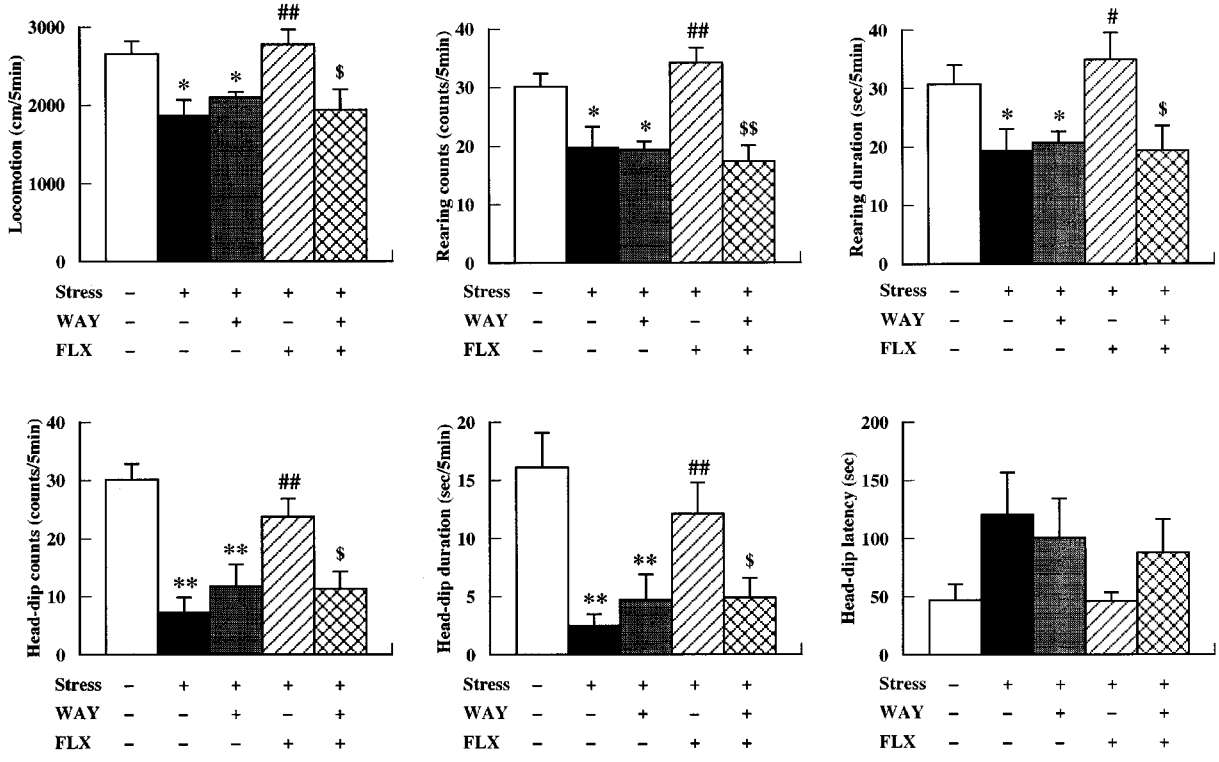

Figure 1 Effects of pretreatment with flesinoxan on the behavioural responses of mice to acute restraint stress 24 h later in the hole-board test. Mice were injected with flesinoxan (FLX; 1 mg kg^{-1}, i.p.) or saline (10 ml kg^{-1}, i.p.). Twenty-four hours later, mice were exposed to acute restraint stress (60 min), and exploratory behaviours on the hole-board were then measured for 5 min. WAY100635 (WAY; 1 mg kg^{-1}, i.p.) was co-injected with flesinoxan. Each column represents the mean±s.e.mean of 8–10 mice. Nonparametric Kruskal-Wallis test was used to test the head-dip latency data. Data of other behavioural parameters were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. *$P<0.05$, **$P<0.01$ vs non-stressed group (open column). #$P<0.05$, ##$P<0.01$ vs stressed group (closed column). \$$P<0.05$, \$\$$P<0.01$ vs flesinoxan plus stressed group (solids column).

Effects of the blockade of corticosterone synthesis with metyrapone on the 5-HT$_{1A}$ receptor agonist-induced increase in plasma corticosterone concentrations The effects of the blockade of corticosterone synthesis with metyrapone on 5-HT$_{1A}$ receptor agonist-induced increase in plasma corticosterone concentrations are shown in Figure 3. A significant increase in plasma corticosterone concentrations was observed 60 min after the injection of flesinoxan (1 mg kg^{-1}, i.p.; Figure 3A) or 8-OH-DPAT (1 mg kg^{-1}, i.p.; Figure 3B) compared to the results in the saline-injected group ($P<0.01$). Treatment with metyrapone alone at doses used in the present study (12.5 and 25 mg kg^{-1}, s.c.) did not modify the basal plasma corticosterone concentrations (data not shown). In contrast, the increase in the plasma corticosterone concentrations produced by 5-HT$_{1A}$ receptor agonists was dose-dependently suppressed by pretreatment with metyrapone (12.5 and 25 mg kg^{-1}, s.c.), and significant effects were observed at 25 mg kg^{-1} ($P<0.05$ or 0.01).

Effects of the blockade of corticosterone synthesis with metyrapone on the protective effects of 5-HT$_{1A}$ receptor agonists against changes in various exploratory behaviours of mice produced by acute restraint stress in the hole-board test The effects of the blockade of corticosterone synthesis

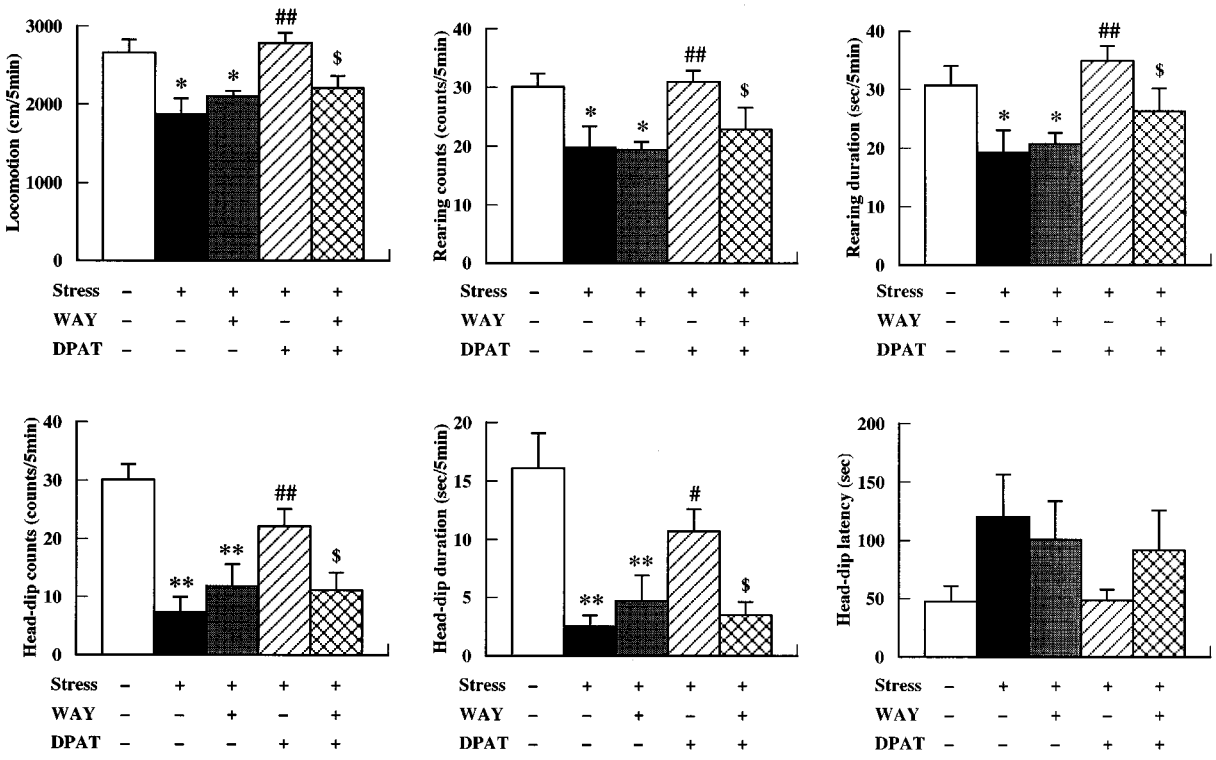

Figure 2 Effects of pretreatment with 8-OH-DPAT on the behavioural responses of mice to acute restraint stress 24 h later in the hole-board test. Mice were injected with 8-OH-DPAT (DPAT; 1 mg kg^{-1}, i.p.) or saline (10 ml kg^{-1}, i.p.). Twenty-four hours later, mice were exposed to acute restraint stress (60 min), and exploratory behaviours on the hole-board were then measured for 5 min. WAY100635 (WAY; 1 mg kg^{-1}, i.p.) was co-injected with 8-OH-DPAT. Each column represents the mean ± s.e.mean of 8–10 mice. Nonparametric Kruskal-Wallis test was used to test the head-dip latency data. Data of other behavioural parameters were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. *$P<0.05$, **$P<0.01$ vs non-stressed group (open columns). #$P<0.05$, ##$P<0.01$ vs stressed group (closed column). \$$P<0.05$ vs flesinoxan plus stressed group (solids column).

Table 2 Effects of pretreatment with 5-HT$_{1A}$ receptor agonists, corticosterone synthesis inhibitor or combinations of these drugs on exploratory behaviours and basal plasma corticosterone concentrations of non-stressed mice

Drugs (mg/kg)	*Locomotion* (cm)	*Rearing* *Counts*	*Rearing* *Duration* (s)	*Head-dips* *Counts*	*Head-dips* *Duration* (s)	*Head-dips* *Latency* (s)	*Corticosterone* (μg/ml)
Saline + Saline	234.9 ± 74.3	30.4 ± 10.5	34.7 ± 3.8	25.6 ± 4.8	11.0 ± 2.1	35.5 ± 7.4	0.127 ± 0.013
Saline + Flesinoxan (1)	2256.2 ± 123.5	30.1 ± 6.2	37.4 ± 3.6	23.6 ± 4.7	13.1 ± 3.8	34.3 ± 6.2	0.130 ± 0.011
Saline + 8-OH-DPAT (1)	2250.0 ± 135.7	28.6 ± 11.1	32.6 ± 4.7	23.8 ± 3.9	9.0 ± 1.6	42.7 ± 23.1	0.139 ± 0.009
Metyrapone (25) + Saline	2223.0 ± 215.1	31.3 ± 6.5	34.4 ± 4.2	27.4 ± 3.5	14.6 ± 2.9	35.6 ± 6.1	0.125 ± 0.012
Metyrapone (25) + Flesinoxan (1)	2106.4 ± 80.7	29.9 ± 10.2	35.7 ± 7.2	21.0 ± 2.8	8.9 ± 2.2	82.2 ± 5.7	0.129 ± 0.008
Metyrapone (25) + 8-OH-DPAT (1)	2282.4 ± 169.5	31.3 ± 7.2	35.8 ± 3.0	20.3 ± 5.6	9.7 ± 3.1	36.9 ± 7.8	0.134 ± 0.013

Flesinoxan, 8-OH-DPAT or saline was injected 24 h prior to the measurement of exploratory behaviours. In combination studies, metyrapone or saline was pretreated 90 min prior to flesinoxan, 8-OH-DPAT or saline injection. Each data represents the mean ± s.e.mean of eight mice. Nonparametric Kruskal-Wallis test was used to test the head-dip latency data. Other data were tested with one way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test.

with metyrapone on the protective effects of 5-HT$_{1A}$ receptor agonists against changes in various exploratory behaviours of mice produced by acute restraint stress are shown in Figures 4–6. Pretreatment with metyrapone (25 mg kg^{-1}, s.c.) alone did not affect the decrease in exploratory behaviours produced by acute restraint stress (Figure 4). In contrast, the restraint stress-induced changes in exploratory behaviours were significantly suppressed by pretreatment with either flesinoxan (1 mg kg^{-1}, i.p.; Figure 5) or 8-OH-DPAT (1 mg kg^{-1}, i.p.; Figure 6) 24 h prior to exposure to acute restraint stress ($P<0.05$ or 0.01). The effects of flesinoxan (1 mg kg^{-1}, i.p.) and 8-OH-DPAT (1 mg kg^{-1}, i.p.) on the number and duration of head dips were dose-dependently antagonized by pretreatment with metyrapone (12.5 and 25 mg kg^{-1}, s.c.) and significant effects were observed at 25 mg kg^{-1} (Figures 5 and 6; $P<0.05$ or 0.01). No other measure was significantly affected.

Effects of 24 h pretreatment with 5-HT$_{1A}$ receptor agonists, antagonists or combinations of these drugs on exploratory behaviours and basal plasma corticosterone concentrations of non-stressed mice Effects of 24 h pretreatment with 5-HT$_{1A}$

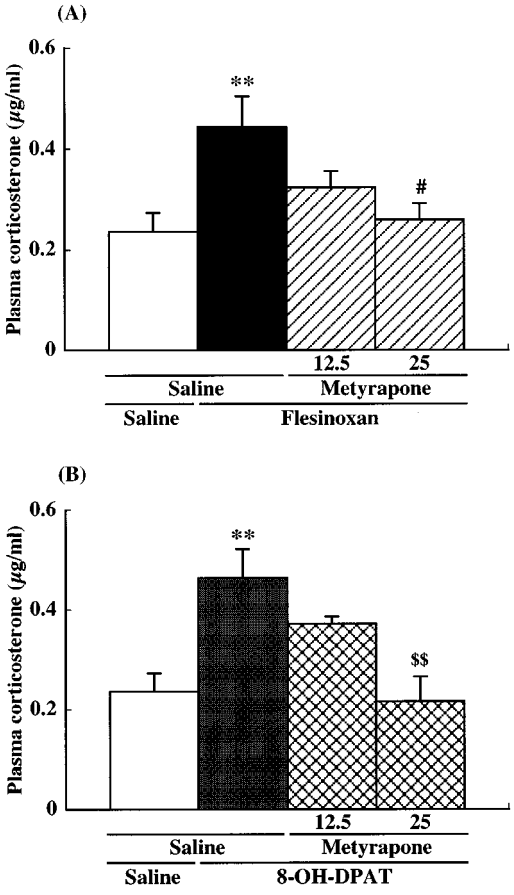

Figure 3 Effects of the blockade of corticosterone synthesis with metyrapone on the 5-HT$_{1A}$ receptor agonist-induced increase in plasma corticosterone concentrations. Mice were pretreated with metyrapone (12.5 or 25 mg kg^{-1}, s.c.) or saline (10 ml kg^{-1}, s.c.) 90 min prior to the injection of flesinoxan (1 mg kg^{-1}, i.p.) (A) or 8-OH-DPAT (1 mg kg^{-1}, i.p.). (B) Each column represents the mean ± s.e.mean of 6 mice. Data were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. $^{**}P<0.01$ vs saline plus saline group (open column). $\#P<0.01$ vs saline plus flesinoxan group (closed column). \$\$Π < 0.01 vs saline plus 8-OH-DPAT group (shaded column).

receptor agonists, antagonists or combinations of these drugs on exploratory behaviours and basal plasma corticosterone concentrations of non-stressed mice were shown in Table 1. None of the exploratory behaviours and plasma corticosterone concentrations of non-stressed mice were affected by pretreatment with flesinoxan, 8-OH-DPAT, WAY-100635 or combinations of these drugs.

Effects of 24 h pretreatment with 5-HT$_{1A}$ receptor agonists, corticosterone synthesis inhibitor or combinations of these drugs on exploratory behaviours and basal plasma corticosterone concentrations of non-stressed mice Effects of 24 h pretreatment with 5-HT$_{1A}$ receptor agonists, antagonists or combinations of these drugs on exploratory behaviours and basal plasma corticosterone concentrations of non-stressed mice were shown in Table 2. None of the exploratory behaviours and plasma corticosterone concentrations of non-stressed mice were affected by pretreatment with flesinoxan, 8-OH-DPAT, metyrapone or combinations of these drugs.

Discussion

The present study clearly demonstrated that 5-HT$_{1A}$ receptor agonists had protective effects against various emotional changes produced by stress stimuli. In particular, pretreatment with the 5-HT$_{1A}$ receptor agonists flesinoxan and 8-OH-DPAT 24 h before exposure to stress suppressed the decrease in various emotional behaviours produced by acute restraint stress. These results are in good agreement with our previous findings (Tsuji *et al*., 2000). Moreover, the effects of 5-HT$_{1A}$ receptor agonists were completely suppressed by the co-injection of WAY100635, a selective 5-HT$_{1A}$ receptor antagonist, suggesting 5-HT$_{1A}$ receptor-mediated actions. We previously found that the exploratory behaviours of non-stressed mice that were habituated to the hole-board apparatus were unaffected by pretreatment with 5-HT$_{1A}$ receptor agonists 24 h beforehand (Tsuji *et al*., 2000). Under these conditions, any emotional abnormality such as anxiety of mice produced by placing them in a novel environment would be reduced by habituation to the hole-board apparatus, indicating that general locomotor and/or exploratory behaviours of mice are not affected by pretreatment with 5-HT$_{1A}$ receptor agonists 24 h beforehand. Moreover, the present study also demonstrated that 24 h pretreatment with 5-HT$_{1A}$ receptor agonists, antagonist or combinations of these drugs did not affect the exploratory behaviours and basal plasma corticosterone level of unrestrained mice. Therefore, the decrease in the behavioural response to restraint stress caused by pretreatment with 5-HT$_{1A}$ receptor agonists may be due to changes in emotional states related to stress stimuli rather than to changes in general motor activity or basal plasma corticosterone level.

The present study shows that the development of emotional resistance to stress stimuli induced by 5-HT$_{1A}$ receptor agonists seems to be partly regulated by adrenal steroid secretion. In fact, an adrenal steroid synthesis inhibitor, metyrapone, reversed the protective effects of flesinoxan and 8-OH-DPAT against emotional changes produced by stress stimuli with regard to only the number and duration of head-dipping behaviours. Moreover, this presumption is supported by the present results that both flesinoxan and 8-OH-DPAT increased plasma corticosterone concentrations, and that these changes in plasma corticosterone levels were completely suppressed by metyrapone. In addition, the present study demonstrated the lack of the effects of 24 pretreatment with 5-HT$_{1A}$ receptor agonists, metyrapone or combinations of these drugs on the exploratory behaviours and basal plasma corticosterone level of unrestrained mice, indicating that present results may be due to changes in emotional states related to stress stimuli rather than to changes in general motor activity or basal plasma corticosterone level. It has been previously reported that there are two distinct types of receptor system for corticosterone, i.e. mineralcorticoid receptors (MRs) and glucocorticoid receptors (GRs), (De Kloet, 1991). Circulating corticosterone readily crosses the blood-brain barrier where it binds directly to both receptors. MRs bind corticosterone with much higher affinity than GRs (Reul & De Kloet, 1985; Sutanto & De Kloet, 1987; Reul *et al*., 1989). Therefore, low circulating corticosterone levels predominantly occupy MRs, whereas GRs become extensively occupied only at high circulating corticosterone levels (Reul *et al*., 1989). The doses of metyrapone used in the present study blocked the increase in plasma corticosterone levels produced

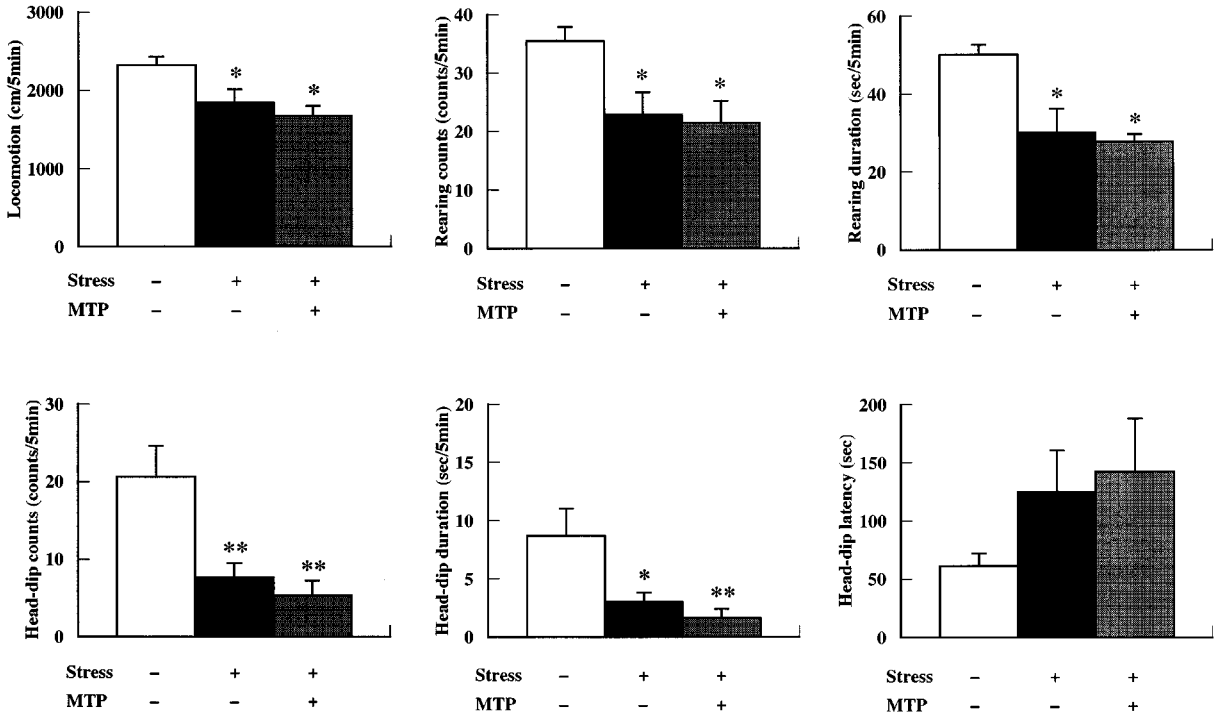

Figure 4 Effects of the blockade of corticosterone synthesis with metyrapone on the behavioural response of mice to acute restraint stress in the hole-board test. Mice were injected with saline (10 ml kg^{-1}, i.p.). Twenty-four hours later, mice were exposed to acute restraint stress (60 min), and exploratory behaviours on the hole-board were measured for 5 min. Metyrapone (MTP; 25 mg kg^{-1}, s.c.) or saline (10 ml kg^{-1}, s.c.) was administered 90 min prior to saline injection. Each column represents the mean$\pm$s.e.mean of 9–10 mice. Nonparametric Kruskal-Wallis test was used to test the head-dip latency data. Data of other behavioural parameters were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. *$P<0.05$, **$P<0.01$ vs non-stressed group (open column).

by 5-HT$_{1A}$ receptor agonists, but basal corticosterone levels were not appreciably affected. This finding suggests that metyrapone may primarily reduce the binding of corticosterone to GRs, whereas the binding to MRs remains at least partly unaffected. Moreover, limbic structures, which are involved in the regulation of emotional states, are known to be loci of corticosteroid receptors, particularly GRs (De Kloet, 1991). Therefore, the behavioural effects observed in our experiments could be related to the activation of GRs. Accordingly, the increase in corticosterone secretion produced by 5-HT$_{1A}$ receptor agonists could, *via* activation of central GRs, modulate the function of the brain circuit involved in the control of emotional states in stressful situations.

A number of investigators have reported results inconsistent with our findings that metyrapone as well as 5-HT$_{1A}$ receptor agonists displayed the antidepressant-like properties in various models of depression (Schipper *et al*., 1991; Van Dijken *et al*., 1992; Baez & Volosin, 1994; Hascoet *et al*., 1994; Healy *et al*., 1999; Khisti *et al*., 2000). However, there are several differences between the current and these previous reports, including dose and time course of drug treatment, and the protocol of behavioural experiments. In the previous study, the doses of metyrapone used were higher than those in the present study, indicating that basal corticosterone levels may be markedly reduced. In contrast, the conditions of metyrapone treatment used in the present study reduced only the enhancement of plasma corticosterone levels produced by 5-HT$_{1A}$ receptor agonists without changing the basal levels. Moreover, it is important to note that preclinical studies of the pharmacokinetics of metyrapone (Maser & Legrum, 1985), 8-OH-DPAT (Perry & Fuller, 1989; Yu & Lewander, 1997) and flesinoxan (unpublished observation, Solvay Duphar B.V., Weesp, The Netherlands) have indicated that these drugs disappear from the body within 24 h after administration. Therefore, under the present conditions, residual drug is unlikely to account for any of the behavioural changes observed. Previously, stress-induced decreases in various behaviours have been widely used as animal models of depression, and the effectiveness of metyrapone and 5-HT$_{1A}$ receptor agonists has been demonstrated in these models (Schipper *et al*., 1991; Van Dijken *et al*., 1992; Baez & Volosin, 1994; Hascoet *et al*., 1994; Healy *et al*., 1999; Khisti *et al*., 2000). However, these previous experiments were performed under conditions in which the administered drugs remained in the body, i.e. behaviours were measured 30–60 min after drug injection. Therefore, the present effects of 5-HT$_{1A}$ receptor agonists or metyrapone are quite new findings and different from those reported in previous reports. Several previous behavioural experiments have inspired the interesting interpretation that disappearance of the behavioural response to stress stimuli reflects the development of stress adaptation (Kennett *et al*., 1985a, b; Ohi *et al*., 1989). Accordingly, the present results imply the possibility that the activation of adrenocortical system *via* 5-HT$_{1A}$ receptors may facilitate some adaptive mechanism(s) involved in the recognition of and/or ability to cope with stressful situations, with the result that the emotional responses to stress stimuli may disappear.

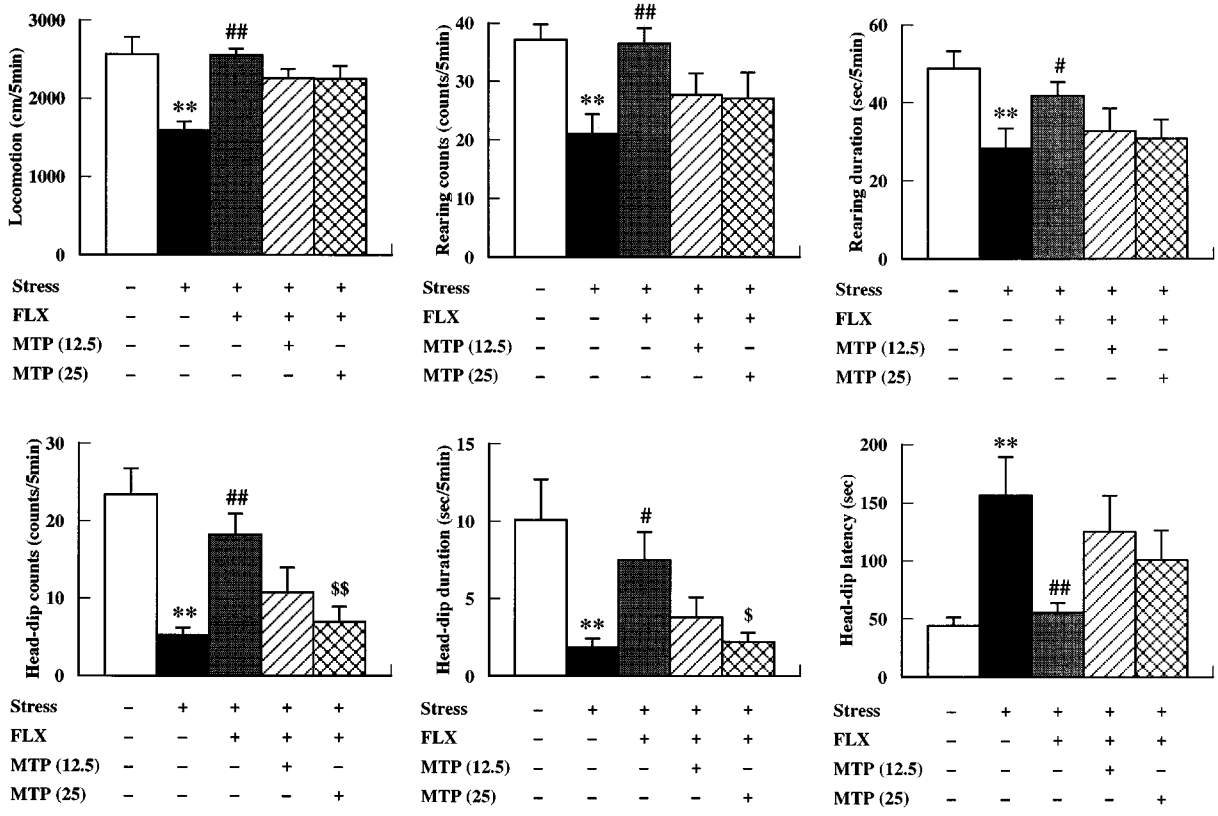

Figure 5 Effects of the blockade of corticosterone synthesis with metyrapone on the protective effects of flesinoxan against changes in various exploratory behaviours of mice produced by acute restraint stress in the hole-board test. Mice were injected with flesinoxan (FLX; 1 mg kg^{-1}, i.p.) or saline (10 ml kg^{-1}, i.p.). Twenty-four hours later, mice were exposed to acute restraint stress (60 min), and exploratory behaviours on the hole-board were then measured for 5 min. Metyrapone (MTP; 12.5 or 25 mg kg^{-1}, s.c.) or saline (10 ml kg^{-1}, s.c.) was administered 90 min prior to flesinoxan injection. Each column represents the mean ± s.e.mean of 9–10 mice. Nonparametric Kruskal-Wallis test was used to test the head-dip latency data. Data of other behavioural parameters were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. $^{**}P<0.01$ vs non-stressed group (open column). $\#P<0.01$, $\#\#P<0.01$ vs stressed group (closed column). $\$P<0.05$, $\$\$P<0.01$ vs flesinoxan plus stressed group (shaded column).

The distinct mechanisms for the development of resistance to stress stimuli by short-term activation of 5-HT$_{1A}$ receptors, which may be partly mediated *via* HPA axis activation, are unclear. However, only some of the behavioural parameters, i.e. number and duration of head-dipping behaviours, were influenced by pretreatment with metyrapone. We previously reported that typical benzodiazepine anxiolytics (diazepam and chlordiazepoxide) and anxiogenics (N-methyl-β-carboline-3-carboxamide (FG-7142) and methyl-β-carboline-3-carboxylate (β-CCM)) have selective effects on head-dipping behaviour in the hole-board test (Takeda *et al*., 1998; Tsuji *et al*., 2000). Both the number and duration of exploratory head-dips were dose-dependently increased by treatment with diazepam and chlordiazepoxide at doses that did not produce sedation. This observation is consistent with previous reports of an increase in the frequency and duration of exploratory head-dips exhibited on a hole-board following the injection of non-sedative doses of either compound (Nolan & Parkes, 1973; Suzuki *et al*., 1990). In contrast, benzodiazepine anxiogenics produced effects on head-dipping behaviour that were opposite to those produced by anxiolytics, i.e. both FG7142 and β-CCM dose-dependently decreased the number and duration of head-dips and increased the latency to the first head-dip. Based on these findings, we suggested that the head-dipping behaviour of mice in the hole-board test is sensitive to changes in the emotional state modulated by benzodiazepine mechanisms. Therefore, the results of the present study indicate that facilitation of the HPA axis *via* 5-HT$_{1A}$ receptor activation might modify the functions mediated by the γ-aminobutyric acid (GABA)$_A$-benzodiazepine receptor complex. In fact, previous studies provide evidence that adrenal glucocorticoids modulate the mRNA levels (Orchinik *et al*., 1995), density or affinity (Miller *et al*., 1988; Bowers & Wehner, 1992) and function (Bowers & Wehner, 1992; Calvo *et al*., 1998) of GABA$_A$ and/or benzodiazepine receptors.

Another possible explanation is the desensitization of presynaptic 5-HT$_{1A}$ autoreceptors. Indeed, it has been previously shown that a single pretreatment with 5-HT$_{1A}$ receptor agonists at the doses used in the present study can produce desensitization of presynaptic 5-HT$_{1A}$ autoreceptors (Kennett *et al*., 1987; Beer *et al*., 1990), although this has not been observed in all studies (Hjorth, 1991; Kreiss & Lucki, 1997). Moreover, several studies using an *in situ* hybridization technique have shown that endogenous corticosteroids suppress 5-HT$_{1A}$ receptor mRNA expression (Chalmers *et al*., 1993; Meijer & De Kloet, 1994; Zhong & Ciaranello, 1995). *In vivo* studies with 5-HT$_{1A}$ receptor agonist-induced hyperphagia (Haleem, 1992) and hypothermia (Young *et al*., 1994), as models of 5-HT$_{1A}$ receptor function, also suggest that corticosterone attenuates presynaptic 5-HT$_{1A}$ receptor

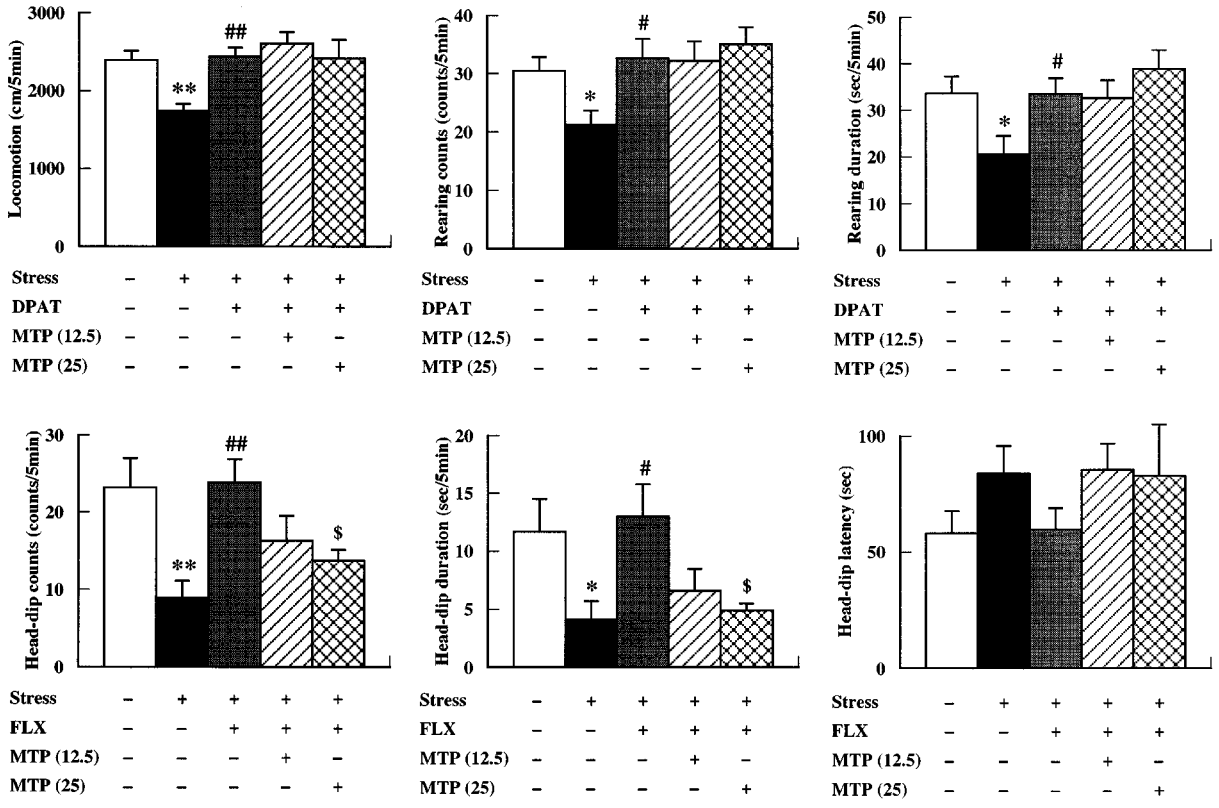

Figure 6 Effects of the blockade of corticosterone synthesis with metyrapone on the protective effects of 8-OH-DPAT against changes in various exploratory behaviours of mice produced by acute restraint stress in the hole-board test. Mice were injected with 8-OH-DPAT (DPAT; 1 mg kg^{-1}, i.p.) or saline (10 ml kg^{-1}, i.p.). Twenty-four hours later, mice were exposed to acute restraint stress (60 min), and exploratory behaviours on the hole-board were then measured for 5 min. Metyrapone (MTP; 12.5 or 25 mg kg^{-1}, s.c.) or saline (10 ml kg^{-1}, s.c.) was administered 90 min prior to 8-OH-DPAT injection. Each column represents the mean with s.e.mean of 9–10 mice. Nonparametric Kruskal-Wallis test was used to test the head-dip latency data. Data of other behavioural parameters were tested with one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. $*P<0.05$, $**P<0.01$ vs non-stressed group (open column). $\#P<0.05$, $\#\#P<0.01$ vs stressed group (closed column). $\$P<0.05$ vs 8-OH-DPAT plus stressed group (shaded column).

function. This suggestion is supported by data from electrophysiological studies (Laaris *et al*., 1995; 1999). The desensitization of presynaptic 5-HT$_{1A}$ autoreceptors would presumably impair the feedback control of 5-HT release at terminals and hence increase 5-HT functional activity (Schlicker *et al*., 1985; Middlemiss, 1986; Sprouse & Aghajanian, 1987). Various lines of evidence have suggested that upregulation of the brain 5-HT system mediates short- and long-term adaptive or coping responses to aversive events such as stress stimuli (Kennett *et al*., 1985a, b; 1986; Ohi *et al*., 1989). We also previously reported the existence of differences in brain 5-HT dynamics between models with and without adaptability to restraint stress (Takeda *et al*., 1996). Marked increases in 5-HT turnover in brain regions were observed in adaptive models, whereas these neurochemical changes were not observed in non-adaptive models. Thus, similar neurochemical changes might occur with a single injection of 5-HT$_{1A}$ receptor agonists. However, changes in functions of not only presynaptic but also postsynaptic 5-HT$_{1A}$ receptor also have been demonstrated 24 h following single administration of 5-HT$_{1A}$ receptor agonists (Forster *et al*., 1994; O'Connell & Curzon, 1996). Thus, the possible involvement of postsynaptic as well as presynaptic 5-HT$_{1A}$ receptor in the expression of the present behavioural effects should also be excluded.

In conclusion, the present results indicate that the protective effects of 5-HT$_{1A}$ receptor agonists against various emotional changes produced by stress stimuli may be partly mediated by adrenal steroids. These results suggest that complex interaction between 5-HT$_{1A}$ receptors and the HPA axis may play a significant role in the regulation of some adaptive mechanism(s) involved in the recognition of and/or ability to cope with stressful situations. Additional neurochemical and/or molecular biological experiments based on the present behavioural findings should help to explain the processes of stress adaptation.

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