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Effect of long-term blockade of CRF_1 receptors on exploratory behaviour, monoamines and transcription factor AP-2

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Abstract

Corticotropin-releasing factor (CRF) holds a central role in reactions to various environmental stimuli. In the present study, the administration of a selective nonpeptide CRF_1 receptor antagonist, CP-154,526, for 6 days, exerted an anxiolytic effect in the elevated zero-maze (EZM) test. CP-154,526 did not affect behaviour in the exploration box when administered acutely, but increased exploration when administered for 5 days, contingently with daily behavioural testing. This effect, although of lesser magnitude, was also present in animals with neurotoxin DSP-4-induced selective denervation of locus coeruleus (LC) projections. When drug administration and behavioural testing were noncontingent in a 2-week administration schedule, CP-154,526 blocked the habituation-induced increase in exploration. This suggests that drug–environment interaction is an important component in the manifestation of the anxiolytic-like effects of CRF_1 receptor blockade. Long-term administration of CP-154,526 had a decreasing effect on noradrenaline (NA) metabolism in the frontal cortex. No manipulation influenced the levels of the transcription factor AP-2 isoforms in the LC area. AP-2 levels correlated positively with 3-methoxy-4-hydroxyphenylglycol (MHPG) in the frontal cortex of vehicle-treated animals. There was a negative correlation between the NA levels in the hippocampus and AP-2 isoforms in the LC area of naive animals. In contrast, in vehicle-treated animals, this correlation was positive. Treatment with CP-154,526, however, made the associations between LC AP-2 levels and hippocampal NA content negative, as was the case in the naive animals. This suggests that CRF_1 receptor blockade counteracts certain mechanisms of habituation, possibly by reducing the LC activity.
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Keywords: Corticotropin-releasing factor (CRF); CRF_1 receptor; CP-154,526; Exploratory behaviour; Monoamine neurochemistry; Locus coeruleus (LC); DSP-4; Transcription factor AP-2

1. Introduction

Corticotropin-releasing factor (CRF) holds a central role in reactions to environmental stimulation. It has been found to mediate changes in the neuroendocrinological functions and behaviour induced by external stimuli. Intracerebroventricular administration of CRF to laboratory animals brings forth a wide range of physiological and behavioural changes comparable with those elicited by stressful stimuli, including increase in heart rate, suppression of exploratory behaviour, reduction in food intake and reproductive behaviour etc. (for a review, see Griebel, 1999).

Of the two CRF receptor subtypes, the CRF_1 receptor has mainly been implicated in mediating the stress-related effects of the CRF system (for a review, see Takahashi, 2001), whereas $CRF_{2\alpha}$ receptor has mainly been associated with ingestive behaviour (Eghbal-Ahmadi et al., 1997, but see also Takahashi et al., 2001). CRF_1 receptor knockout mice have been found to display a reduced anxiogenic response (Timpl et al., 1998; Contarino et al., 1999) while knockout studies represent a chronic state of CRF receptor function inhibition. In contrast, acutely administered selective nonpeptide CRF_1 antagonists have been found to display only limited or no potency in exploration-based anxiety models (Lundkvist et al., 1996; Griebel et al., 1998; Okuyama et al., 1999). Nevertheless, CRF_1 antagonists produce a downright anxiolytic-like activity in animal models involving inescapable stress, conflict procedures,

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social-defeat-induced anxiety and the mouse defense test battery (Griebel et al., 2002). CRF_1 antagonists have also been found to reduce the CRF-enhanced increases in the acoustic startle paradigm (Schulz et al., 1996) and to block the anxiogenic effects of CRF in the elevated plus maze, without affecting anxiety-like behaviour in a vehicle-pretreated group (Okuyama et al., 1999; Zorrilla et al., 2002) when administered acutely. In animal models of depression, CRF_1 antagonists have been found to reduce immobility in the forced swimming test (Griebel et al., 2002; Harro et al., 2001) and, when administered long-term during a chronic mild stress regimen, to improve weight gain and attenuate physical state degradation in rats (Griebel et al., 2002). The number of studies with chronic administration of CRF_1 receptor antagonists has been limited, but available data also show significant anxiolytic and antidepressant effects, with decreases in the defensive withdrawal behaviour (Arborelius et al., 2000) and inhibition of olfactory bulbectomy-induced hyperemotionality (Okuyama et al., 1999). In our laboratory, the repeated administration of the CRF_1 receptor antagonist CRA1000 was found to exert anxiolytic effects in a dose of 1.25 mg/kg, but to block the effect of habituation on exploratory activity in a larger dose, 5 mg/kg (Harro et al., 2001). These data suggest that extensive CRF_1 receptor blockade could disturb normal adaptive behaviour.

Reports of very high levels of CRF_1-like immunoreactivity in noradrenergic brainstem nucleus locus coeruleus (LC) neurons (Sauvage and Steckler, 2001) suggest that the CRF_1 receptor subtype could be an important mediator of CRF-ergic regulation of LC activity. Stress, as well as intraventricular and intra-LC injections of CRF, has been found to increase the discharge rates of LC neurons in rats (Valentino et al., 1983; Curtis et al., 1997; Lejeune and Millan, 2003) and noradrenaline (NA) release in the prefrontal cortex (Curtis et al., 1997). The peptide CRF receptor antagonist α-helical CRF_{9-41}, on the other hand, has been found to attenuate stress-induced increases in NA turnover (Emoto et al., 1993). CRF_1 receptor antagonists have been found to counteract the activation of LC neurons by intracerebroventricular CRF (Schulz et al., 1996; Okuyama et al., 1999; Lejeune and Millan, 2003) or stress (Kawahara et al., 2000; Griebel et al., 2002). We have previously found that repeated treatment with the CRF receptor antagonist CRA1000 increases exploratory behaviour, which was reduced after the denervation of LC projections by treatment with the neurotoxin DSP-4 (Harro et al., 2001). These findings support the notion that the CRF_1 receptor subtype plays a role in the mediation of the functions of the LC.

The interest in molecules affecting transcriptional processes has, during the past few years, increased to learn more about molecular mechanisms underlying mental disorders (Damberg et al., 2001a). The AP-2 family of transcription factors (as measured in the whole rat brain) has been found to be influenced by chronic antidepressant treatment (Damberg et al., 2000), and we recently found that AP-2 levels in the brainstem correlate with monoamine levels in several brain regions (Damberg et al., 2001b). The next logical step appears to be identifying the brain areas in which AP-2 influences monoaminergic systems.

The aims of the present experiments were to study (1) the role of CRF_1 receptors in exploratory behaviour in animals with selective denervation of LC projections and (2) the effects of long-term blockade of CRF_1 receptors on exploratory behaviour, monoamine neurochemistry and AP-2 levels in the LC area.

2. Materials and methods

2.1. Animals

Male Wistar rats (Experiment 1, Finnish Laboratory Animal Center, Kuopio, Finland; Experiment 2, National Public Health Institute, Kuopio, Finland) were housed in groups of three (Experiment 1) or four (Experiment 2) in standard transparent polypropylene cages under controlled light cycle (lights on from 0800 to 2000 h) and temperature (19–21 °C), with free access to tap water and food pellets (diet R70, Lactamin, Sweden). All behavioural testing was carried out between 1 and 7 p.m. The experimental protocol was approved by the Ethics Committee of the University of Tartu.

2.2. Drug administration

In Experiment 1, each dose of neurotoxin DSP-4 (50 mg/kg) was weighed separately, dissolved in distilled water and immediately injected intraperitoneally 1 week before the behavioural experiments. CP-154,526 was suspended in distilled water by adding a few drops of Tween 85. Control animals received vehicle treatment. CP-154,526 was administered intraperitoneally 30 min before behavioural observations, or at about the same time of day in Experiment 2 on days that did not include behavioural testing.

2.3. Exploration box test

The exploration box test has been shown to enable the measurement of different aspects of exploratory behaviour (Harro et al., 1995; Otter et al., 1997). The apparatus was made of metal and consisted of an open area (0.5 × 1 m, height of side walls 40 cm) with a small compartment (20 × 20 × 20 cm) attached to one of the shorter sides of the open area. The open area was divided into eight squares of equal size. In the open area, four objects, three unfamiliar and one familiar (a glass jar, a cardboard box, a wooden handle and a food pellet), were situated in certain places (which remained the same throughout the experiment). The small compartment, which had its floor covered with wood shavings, was directly linked to the open area through an opening (size 20 × 20 cm). The apparatus was cleaned with dampened laboratory tissue after each animal. The explora-

tion test was initiated by placing a rat into the small compartment, which was then covered with a lid. The following measures were taken by an observer: (a) latency of entering the open area with all four paws on it; (b) entries into the open area; (c) line crossings; (d) rearings; (e) exploration of the unfamiliar objects in the open area, and (f) the time spent exploring the open area. To provide an index of exploration, considering both the elements of inquisitive and inspective exploration, the scores of line crossing, rearing and object investigation were summed for each animal. A single test session lasted 15 min and experiments were carried out under dim light conditions.

2.4. Elevated zero-maze test

The elevated zero-maze (EZM) was designed in accordance with the original description (Shepherd et al., 1994) with a few modifications (as in Matto et al., 1997). The EZM was an annular platform (width 10 cm) with a diameter of 105 cm, divided into two opposite open parts and two opposite closed parts (height of the side walls 40 cm). The open parts had borders (height 1 cm). All parts of the apparatus were made of nontransparent plastic, and the apparatus was elevated 50 cm above the floor. For the test, the animal was placed into one of the open parts facing the closed part of the apparatus and was observed for 5 min. Behavioural measures taken included (a) number of open part entries; (b) time spent in the open parts; (c) number of head dips over the edge of the platform; and (d) number of stretched-attend postures. The experiments were carried out under bright light conditions.

2.5. Procedure

In Experiment 1, the rats were observed in the exploration box on five consecutive days. The animals were divided into four groups following a 2 × 2 (Toxin × Drug) design in which every animal received either DSP-4 or vehicle pretreatment, and CP-154,526 or vehicle treatment. All animals receiving CP-154,526 treatment received the drug in a dose of 2.5 mg/kg/day.

In Experiment 2, the animals were divided into five groups, one of which was not handled before sacrifice, while four groups were included in a 2 × 2 design, the two factors being treatment with CP-154,526 or vehicle and behavioural experiments (repeated vs. single). Drug treatment was carried out for 16 days. The "single-tested" rats were used for only one behavioural experiment (the exploration box test on Day 12 of drug treatment), whereas the other groups were studied repeatedly (on Days 1, 5 and 12 of drug treatment in the exploration box test, and on Days 6 and 13 in the zero-maze test).

In Experiment 1, animals were sacrificed immediately after the last behavioural testing, and tissue samples were dissected for determination of the levels of monoamines. In Experiment 2, animals were sacrificed 4 days after the last behavioural testing, during which drug administration was continued; tissue samples were dissected according to the atlas of Paxinos and Watson (1986). Nuclear extracts were immediately prepared for the measurement of transcription factors as described below.

2.6. Biochemical measurement

2.6.1. High-performance liquid chromatography

Monoamines were measured by high-performance liquid chromatography with electrochemical detection. The tissues were disrupted with an ultrasonic homogenizer (Bandelin, Germany) in an ice-cold solution of 0.1 M perchloric acid (10–20 μl/mg) containing 5 mM sodium bisulfite and 0.04 mM EDTA for avoiding oxidation. The homogenate was then centrifuged at 14 000 × g for 20 min at 4 °C, and 20 μl of the resulting supernatant was chromatographed on a Lichospher 100 RP-18 column (250 × 3 mm; 5 μm). The separation was done in an isocratic elution mode at column temperature 30 °C using the mobile phase containing 0.05 M citrate buffer at pH 3.6, 1 mM sodium octylsulfonate, 0.3 mM triethylamine, 0.02 mM EDTA, 1 mM KCl and 6.25% acetonitrile. The measurements were done at electrode potentials of a glassy carbon electrode + 0.6 V vs. Ag/AgCl reference electrode with HP 1049 electrochemical detector (Hewlett Packard, Germany).

2.6.2. Extraction of nuclear proteins

Nuclear proteins were extracted according to a modified protocol of Dignam et al. (1983). Tissue sections of the LC area were homogenized in 3 ml of Buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF). After incubation on ice for 15 min and the addition of 0.25 ml 10% Nonidet P40, the homogenates were centrifuged for 1 min at 17 100 × g at 4 °C. The nuclear pellets were resuspended in 0.5 ml of ice-cold Buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF). Shaking for 15 min at 4 °C was followed by centrifugation at 17 100 × g for 5 min at 4 °C. The aliquots from the supernatants were frozen in liquid nitrogen and stored at − 80 °C. Total protein concentration was determined for all nuclear extracts by the method of Lowry et al. (1951).

2.6.3. Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96-well) were coated with 50 μl (0.06 μg/ml) nuclear extract diluted in 50 mM carbonate–biscarbonate buffer, pH 9.0. The plates were covered with parafilm and incubated overnight at 4 °C. Following the incubation, the antigen solution was removed, 200 μl of blocking buffer (PBS and 1% BSA) was added to each well, and the plates were incubated for 2 h at room temperature. The blocking buffer was removed, and the plates were washed with PBS. Thereafter, the primary antibody (goat polyclonal AP-2α and AP-2β, 15 μl/ml, respectively, Santa Cruz Biotechnology), diluted in blocking buffer, was added (50 μl per well) and the plates incubated overnight at 4 °C.

After incubation, the antibody was removed, and the plates were washed three times with wash buffer I (PBS, 0.05% Tween-20). Thereafter, the secondary antibody (donkey antigoat IgG AP conjugated, SDS), diluted 1:350 in blocking buffer, was added (50 μl to each well), and the plates were incubated for 2 h at room temperature. After the removal of the secondary antibody, the plates were washed three times with wash buffer I and once with wash buffer II (10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5). Thereafter, 50 μl substrate (Phosphate substrate, Sigma, one 5 mg tablet diluted in 5 ml wash buffer II) was added to each well. The reaction continued for 20 min and was terminated by adding 50 μl of 0.1 M EDTA, pH 7.5. The plates were analyzed in an ELISA reader at OD 405/490. The OD of the AP-2 isoforms for each rat was correlated to a value in a standard curve, where known concentrations of antibody are plotted against optical density. The value from the standard curve was then divided with the concentration of the total protein in the nuclear extracts. The quota was used as a relative amount of AP-2α and AP-2β. Samples from each rat were analyzed twice for accuracy.

2.6.4. Statistics

Data from the exploration box tests were analyzed with two-factor ANOVA (Toxin × Drug in Experiment 1 and Testing × Drug in Experiment 2), with repeated measures or one-factor (Drug) repeated-measures ANOVA in Experiment 2. Data from biochemical measurements were analyzed with two-factor ANOVA (Toxin × Drug in Experiment 1 and Testing × Drug in Experiment 2). Subsequent pairwise comparisons were made with Fisher's LSD test.

3. Results

3.1. Experiment 1

3.1.1. The effect of CP-154,526 (2.5 mg/kg) on exploratory behaviour during five consecutive days of treatment in vehicle- and DSP-4 pretreated rats

Two-factor (Toxin × Drug) repeated measures ANOVA revealed a significant DSP-4 pretreatment effect on line crossings, object investigations, enterings, rearings time of exploration and sum of exploratory activity [$F(1,20) = 6.39$, 5.62, 5.35, 4.50, 5.29 and 5.96, respectively, $P < .05$]. A significant CP-154,526 treatment effect was revealed on the latency of entering the open area, line crossings, object investigations and the sum of exploration [$F(1,20) = 4.54$, 4.85, 4.49 and 4.74, respectively, $P < .05$]. The effect of CP-154,526 on rearings and time of exploration remained just above the conventional level of statistical significance [$F(1,20) = 3.99$ and 3.84, respectively, $P = .06$]. There was

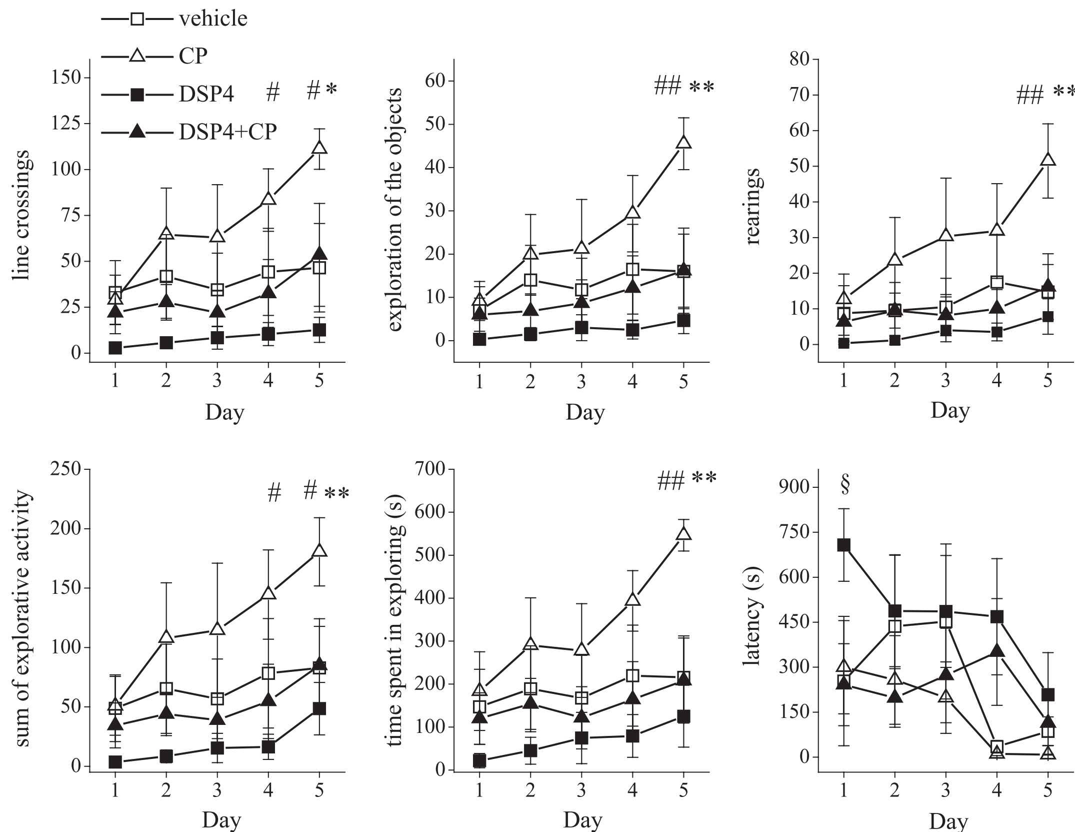

Fig. 1. The effect of DSP-4 pretreatment and CP-154,526 (2.5 mg/kg) on behaviour in the exploration box on five consecutive days (mean ± S.E.M.). * $P < .05$; ** $P < .01$, vehicle vs. CP-154,526; # $P < .05$; ## $P < .01$, CP-154,526 vs. CP-154,526 + DSP-4; § $P < .05$, DSP-4 vs. DSP-4 + CP-154,526.

Table 1
Content of monoamines and their metabolites pmol/mg tissue in the frontal cortex in Experiment 1 (mean ± S.E.M.)

	Vehicle	CP-154,526	DSP-4	DSP-4 + CP-154,526
NA	1.21 ± 0.06	1.13 ± 0.03	0.43 ± 0.03****	0.43 ± 0.04****,####
DA	0.09 ± 0.17	0.09 ± 0.003	0.11 ± 0.01	0.10 ± 0.01
DOPAC	0.38 ± 0.04	0.37 ± 0.03	0.29 ± 0.04	0.27 ± 0.04
HVA	0.17 ± 0.02	0.19 ± 0.01	0.17 ± 0.02	0.16 ± 0.03
(DOPAC + HVA)/DA	6.40 ± 0.69	5.94 ± 0.94	4.54 ± 0.42	3.93 ± 0.56 *
5-HT	0.51 ± 0.05	0.45 ± 0.04	0.50 ± 0.02	0.48 ± 0.04
5-HIAA	1.70 ± 0.08	1.78 ± 0.11	1.18 ± 0.08	1.09 ± 0.02*,#
5-HIAA/5-HT	3.32 ± 0.21	4.15 ± 0.46	2.36 ± 0.13 **	2.33 ± 0.17**,###

$P < .001$ vs. vehicle.
$P < .01$ vs. CP-154,526.
* $P < .05$ vs. vehicle.
** $P < .01$ vs. vehicle.
**** $P < .0001$ vs. vehicle.
# $P < .05$ vs. CP-154,526.
$P < .001$ vs. CP-154,526.
$P < .0001$ vs. CP-154,526.

also a significant effect of repeated testing on the latency of entering, rearings [$F(4,80) = 2.56$ and 5.16, respectively, $P < .05$], line crossings, object investigations, time of exploratory activity and sum of exploratory activity [$F(4,80) = 5.32$, 6.42, 5.69 and 5.94, respectively, $P < .01$] and on Day × Drug effect on line crossings, object investigation and sum of exploratory activity [$F(4,80) = 3.80$, 3.69 and 3.69, respectively, $P < .01$] and rearings [$F(4,80) = 2.51$, $P < .05$], while it remained just above the conventional level of statistical significance on time of exploratory activity [$F(4,80) = 2.22$, $P = .07$]. On the basis of post hoc tests, it was found that no treatment had any effect on the first day of observation (Fig. 1). Starting from Day 2, the differences between the control and DSP-4 animals became apparent as the former's behavioural activity increased. The increasing effect of CP-154,526 on line crossings and sum of exploratory activity became significant on Day 4, and on object investigations, enterings and rearings on Day 5. The effect of CP-154,526 treatment in DSP-4 pretreated animals was lower in magnitude.

3.1.2. The effect of DSP-4 (50 mg/kg) pretreatment and CP-154,526 (2.5 mg/kg) treatment during five consecutive days on monoamine concentrations in the frontal cortex

When using two-factor repeated-measures ANOVA (Toxin × Drug) a significant DSP-4 pretreatment effect in the frontal cortex was found on NA levels [$F(1,20) = 295.0$, $P < .0001$], serotonin (5-HT) turnover [calculated as the 5-HIAA/5-HT ratio; $F(1,20) = 24.6$, $P < .0001$], 3,4-dihydroxyphenylacetic acid [DOPAC; $F(1,20) = 6.16$, $P < .05$], dopamine (DA) turnover [calculated as the (DOPAC + HVA)/DA ratio; $F(1,8) = 8.94$, $P < .05$] and 5-hydroxyindolacetic acid [5-HIAA; $F(1,20) = 14.4$, $P < .01$], while the effect on the frontal cortex DA levels just missed the conventional level of statistical significance [$F(1,8) = 4.33$, $P = .07$]. Post hoc tests revealed significantly lower levels of NA and 5-HIAA and of 5-HT turnover in the vehicle and CP-154,526 groups pretreated with DSP-4 (Table 1).

3.2. Experiment 2

3.2.1. The effect of CP-154,526 (2.5 mg/kg) in the EZM

In the EZM test (performed on Days 6 and 13 of administration of the drug), the repeated-measures ANOVA revealed a significant effect of repeated testing on stretched-attend postures [$F(1,14) = 5.10$, $P < .05$], head dips and time of exploratory activity [$F(1,14) = 18.0$ and 10.4, respectively, both $P < .01$]. All animals revealed significantly lower levels of activity on Day 13 (Fig. 2). This effect remained just above the conventional level of statistical significance

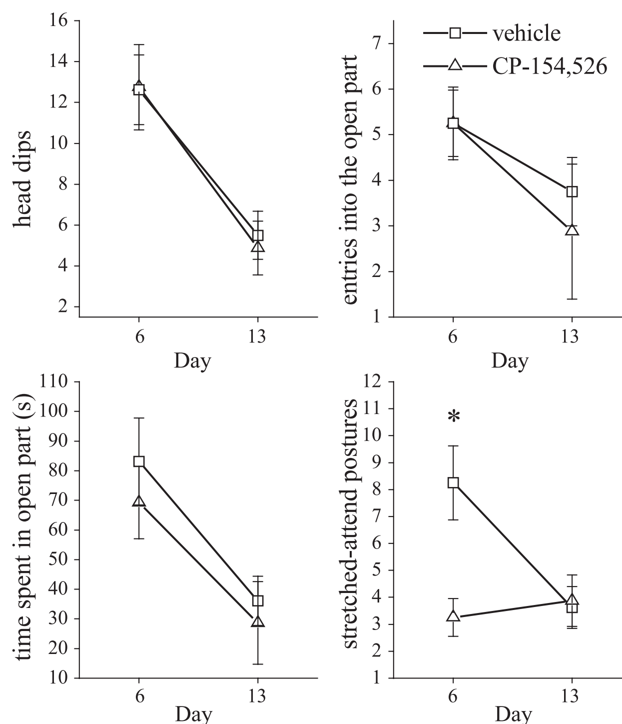

Fig. 2. The effect of CP-154,526 (2.5 mg/kg) on behaviour in the zero-maze (mean ± S.E.M.). * $P < .05$ vs. vehicle.

on enterings into open parts of the apparatus [$F(1,14) = 3.82$, $P = .07$]. In addition, a significant drug effect appeared on the stretched-attend postures [$F(1,14) = 4.86$, $P < .05$], and there was a significant Day × Drug interaction on stretched-attend postures [$F(1,14) = 7.09$, 8.78, respectively, $P < .05$]. Post hoc tests revealed that 6 days of administration of CP-154,526 (2.5 mg/kg) decreased the stretched-attend postures.

3.2.2. The effect of administration of CP-154,526 (2.5 mg/kg) on changes in behaviour in exploration box

In the exploration box test (performed on Days 1, 5 and 12 of administration of the drug), repeated-measures ANOVA on all animals revealed a significant effect of repeated testing on all behavioural measures: latency of entering, line crossing, rearings, time and sum of exploratory activity [$F(2,28) = 4.65$, 4.31, 5.53, 4.84 and 5.09, respectively, all $P < .05$] and investigation of objects [$F(2,28) = 7.54$, $P < .01$]. This effect of repeated testing emerged because the activity of the vehicle group increased (Fig. 3). The administration of CP-154,526 had a significant effect on line crossing, investigation of objects, rearings, time and sum of exploratory activity [$F(1,14) = 4.74$, 4.38, 6.13, 5.02 and 5.11, respectively, $P < .05$], with the CP-154,526 group revealing lower levels of activity. A significant Day × Drug interaction was also revealed on the lastly mentioned measures [$F(2,28) = 3.66$, 4.51, 4.04, 3.35 and 4.08, respectively, $P < .05$]. Post hoc tests revealed that the activity of the CP-154,526 group was lower than that of the vehicle group on the second and third testing (Days 5 and 12 of drug administration, respectively).

3.2.3. The effect of administration of CP-154,526 (2.5 mg/kg) for 12 days on behaviour on the 12th day in the exploration box test

After 12 days of CP-154,526 administration, when single- and repeatedly tested animals were compared, two-factor ANOVA (Drug × Testing) revealed a significant effect of testing on the number of entries into the open part of the apparatus [$F(1,28) = 8.76$, $P < .01$], of the administration of CP-154,526 on line crossing and time of exploration, which just missed the conventional level of statistical significance [$F(1,28) = 3.40$, $P = .076$ and $F(1,28) = 3.06$, $P = .094$, respectively], and similar Drug × Testing interactions on object investigation and time of exploration [$F(1,28) = 4.00$, $P = .055$ and $F(1,28)3.65$, $P = .066$, respectively]. Altogether, the 12-day administration of CP-

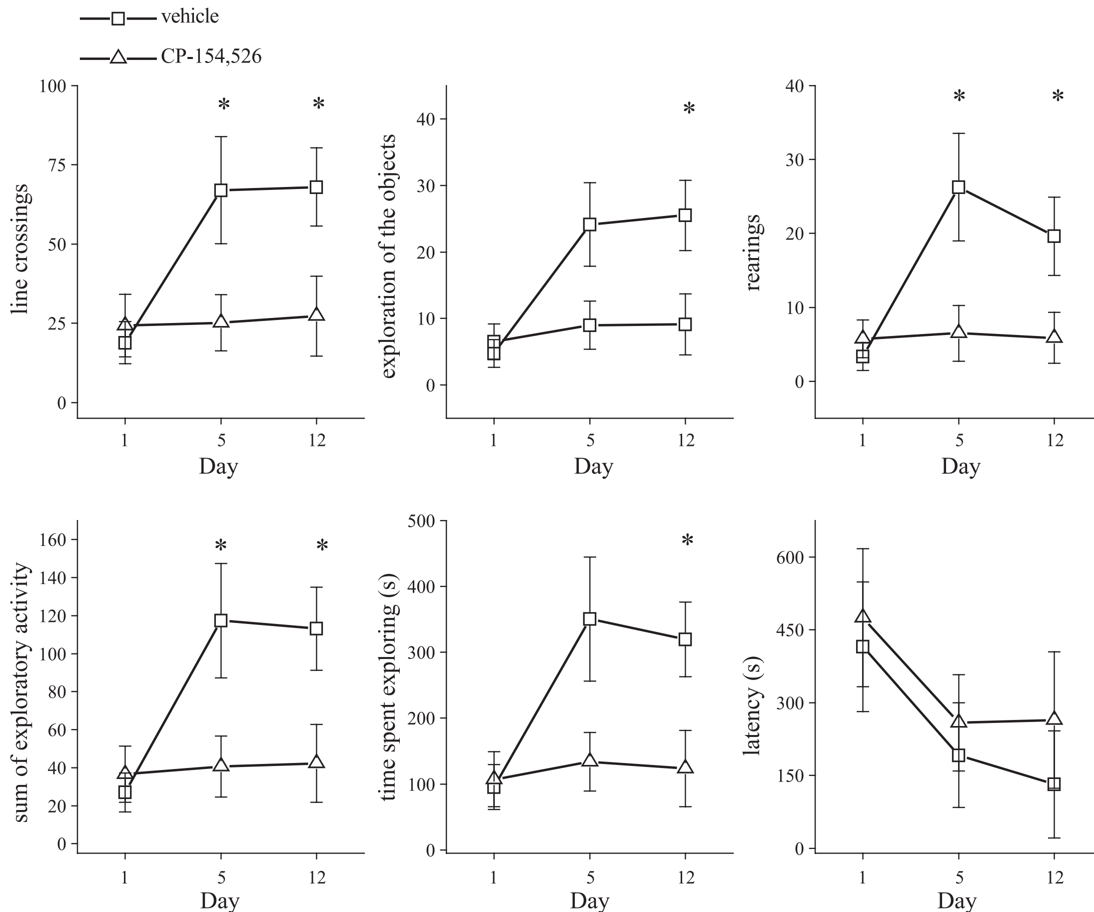

Fig. 3. The effect of chronic administration of CP-154,526 (2.5 mg/kg) on changes in behaviour in the exploration box (mean ± S.E.M.). * $P < .05$ vs. vehicle.

154,526 reduced exploratory activity in animals that had been tested repeatedly during this period (Fig. 4), whereas it had no significant effect on exploratory activity on animals subjected to the experimental apparatus for the first time on test day 12.

3.2.4. The effect of CP-154,526 (2.5 mg/kg) long-term treatment on monoamine concentrations in the frontal cortex, hippocampus and hypothalamus

Two-factor ANOVA (Drug × Testing) revealed a significant effect of CP-154,526 on the 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the frontal cortex [$F(1,28) = 6.81$, $P < .05$] and a similar effect on 5-HT levels in the frontal cortex, which just missed the conventional level of statistical significance [$F(1,28) = 3.75$, $P = .063$]. Thus, MHPG levels were decreased after CP-154,526 administration and there was a trend of decreased 5-HT levels as well (Table 2). Neither the administration of CP-154,526 nor repeated testing had any statistically significant effect on monoamine levels in the hippocampus or hypothalamus.

3.2.5. The association between AP-2 levels in the LC area and NA: the effect of CP-154,526 (2.5 mg/kg) long-term treatment

A two-factor ANOVA (Drug × Testing) revealed no significant effects on transcription factor AP-2 in the LC area (data not shown). For the naive, vehicle- and CP-154,526-treated animals, the AP-2α levels were 7.79 ± 1.53, 6.10 ± 0.53 and 5.61 ± 0.58, respectively (mean AP-2α protein level ± S.E.M.), and the AP-2β levels were 8.83 ± 1.48, 7.49 ± 0.60 and 6.83 ± 0.59, respectively (mean AP-2β protein level ± S.E.M.). For all three groups of animals, the monoamine levels and turnover in the frontal cortex, hippocampus and hypothalamus were analyzed in relation to AP-2 levels in the LC area. As systematic associations with AP-2 levels were acquired with NA and its metabolites only (Table 3), the results of other monoamines will not be presented. In the naive group, NA levels in the hippocampus correlated negatively with both AP-2α and AP-2β levels ($r = -.74$, $P < .05$ and $r = -.80$, $P < .05$, respectively). In the vehicle group, MHPG levels in the frontal cortex correlated positively

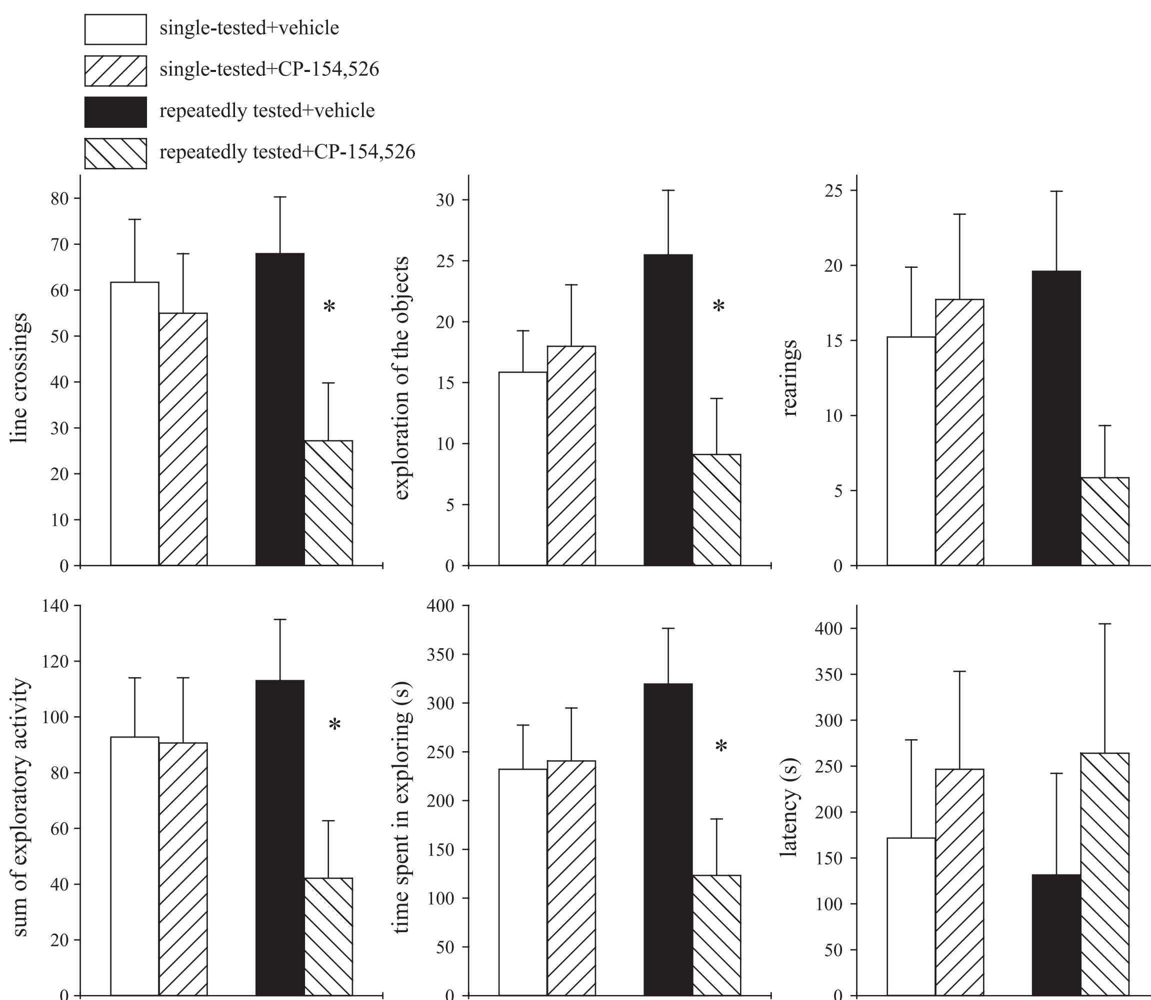

Fig. 4. The effect of 12 days of administration of CP-154,526 (2.5 mg/kg) on behaviour in the exploration box (mean ± S.E.M.). * $P < .05$ vs. the corresponding vehicle group.

Table 2
Content of monoamines and their metabolites pmol/mg tissue in the frontal cortex, hippocampus and hypothalamus, Experiment 2 (mean ± S.E.M.)

	Naive	Single-tested + VEH	Single-tested + CP-154,526	Repeatedly tested + VEH	Repeatedly tested + CP-154,526
Frontal cortex					
NA	2.54 ± 0.15	2.87 ± 0.21	2.52 ± 0.18	2.86 ± 0.21	2.64 ± 0.16
MHPG	0.56 ± 0.08	0.54 ± 0.05	0.46 ± 0.02[#]	0.62 ± 0.05	0.48 ± 0.04[#]
MHPG/NA	0.23 ± 0.03	0.19 ± 0.02	0.19 ± 0.02	0.22 ± 0.02	0.19 ± 0.02
DA	0.54 ± 0.11	0.61 ± 0.12[#]	0.35 ± 0.05	0.48 ± 0.08	0.47 ± 0.05
DOPAC	0.23 ± 0.02	0.24 ± 0.04	0.19 ± 0.02	0.20 ± 0.02	0.21 ± 0.01
HVA	0.19 ± 0.02	0.18 ± 0.01	0.16 ± 0.02	0.19 ± 0.01	0.17 ± 0.01
DOPAC + HVA/DA	1.07 ± 0.28	0.72 ± 0.11	1.16 ± 0.21	1.02 ± 0.18	0.86 ± 0.08
5-HT	2.58 ± 0.37	2.68 ± 0.29	1.89 ± 0.28	2.73 ± 0.46	2.28 ± 0.19
5-HIAA	4.02 ± 0.23	3.86 ± 0.36	4.05 ± 0.27	3.74 ± 0.20	3.73 ± 0.23
5-HIAA/5-HT	1.81 ± 0.29	1.62 ± 0.27[#]	2.73 ± 0.58	1.81 ± 0.40	1.76 ± 0.23
Hippocampus					
NA	1.80 ± 0.21	1.97 ± 0.25	2.03 ± 0.37	2.29 ± 0.37	2.00 ± 0.30
MHPG	0.50 ± 0.08	0.46 ± 0.04	0.36 ± 0.06	0.54 ± 0.04	0.45 ± 0.07
MHPG/NA	0.29 ± 0.04	0.25 ± 0.03	0.48 ± 0.18	0.28 ± 0.05	0.28 ± 0.06
5-HT	0.62 ± 0.25	0.43 ± 0.09	0.59 ± 0.09	0.73 ± 0.13	0.59 ± 0.11
5-HIAA	3.37 ± 0.28	3.22 ± 0.31	3.55 ± 0.56	3.43 ± 0.26	3.49 ± 0.35
5-HIAA/5-HT	8.62 ± 3.04	10.33 ± 2.82	7.23 ± 2.44	5.43 ± 0.98	6.56 ± 1.94
Hypothalamus					
NA	10.23 ± 0.46	11.09 ± 0.70	12.16 ± 0.61	11.87 ± 0.50*	11.29 ± 0.04
MHPG	0.58 ± 0.05	0.61 ± 0.04	0.60 ± 0.04	0.60 ± 0.03	0.58 ± 0.04
MHPG/NA	0.06 ± 0.004	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.004	0.05 ± 0.01
DA	1.35 ± 0.16	1.28 ± 0.19	1.42 ± 0.14	1.40 ± 0.18	1.30 ± 0.14
DOPAC	1.19 ± 0.07	1.05 ± 0.15	1.14 ± 0.20	1.23 ± 0.23	0.90 ± 0.12
DOPAC/DA	0.98 ± 0.15	1.08 ± 0.30	0.89 ± 0.20	1.19 ± 0.47	0.78 ± 0.16
5-HT	1.78 ± 0.15	1.83 ± 0.20	2.03 ± 0.19	1.91 ± 0.21	1.79 ± 0.11
5-HIAA	4.71 ± 0.20	4.55 ± 0.40	4.38 ± 0.30	4.55 ± 0.15	4.33 ± 0.29
5-HIAA/5-HT	2.80 ± 0.32	2.98 ± 0.71	2.32 ± 0.31	2.79 ± 0.56	2.49 ± 0.24

VEH, vehicle.
* $P < .05$ vs. naïve group.
[#] $P < .05$ vs. repeatedly tested + VEH group.

with both AP-2α and AP-2β levels (r=.56, $P<.05$ and r=.64, P=.01, respectively). When, in the latter group, AP-2β levels in relation to NA levels were analyzed in the frontal cortex, the conventional level of significance was just missed (r=.48; P=.06). Furthermore, a positive correlation was found between NA levels in the hippocampus

Table 3
Correlations of AP-2 levels in the LC with NA and its metabolism in the frontal cortex, hippocampus and hypothalamus

	All (n = 40)				Naive (n = 8)				Vehicle (n = 16)				CP-154,526 (n = 16)			
	AP-2α		AP-2β		AP-2α		AP-2β		AP-2α		AP-2β		AP-2α		AP-2β	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
Frontal cortex																
NA	− .16	.35	− .09	.61	− .28	.50	− .24	.57	.31	.24	.48	.06	**− .62**	**<.05**	**− .73**	**.001**
MHPG	**.38**	**<.05**	**.43**	**.01**	.27	.52	.30	.47	**.56**	**<.05**	**.64**	**.01**	.43	.11	.39	.15
MHPG/NA	**.43**	**<.01**	**.43**	**<.01**	.37	.37	.38	.36	.29	.28	.21	.43	**.63**	**<.05**	**.70**	**<.01**
Hippocampus																
NA	− .28	.11	− .19	.27	**− .74**	**<.05**	**− .80**	**<.05**	.38	.15	**.54**	**<.05**	**− .90**	**<.0001**	**− .92**	**<.0001**
MHPG	.03	.88	.01	.97	− .19	.66	− .24	.57	.12	.67	.19	.49	− .01	.98	.15	.65
MHPG/NA	.14	.44	.11	.55	.38	.35	.36	.39	− .35	.20	− .48	.07	.47	.18	**.69**	**<.05**
Hypothalamus																
NA	− .16	.35	− .07	.67	− .18	.72	− .28	.56	− .03	.91	.20	.45	.06	.84	.06	.85
MHPG	.12	.51	.18	.34	− .34	.62	− .43	.52	.52	.06	.48	.09	.14	.66	.31	.32
MHPG/NA	.22	.22	.18	.34	.22	.75	.12	.87	.36	.21	.18	.55	.15	.64	.27	.38

Statistically significant results are marked in bold.

and AP-2β levels in the LC area (r=.54; $P<.05$). In the CP-154,526-treated group, the NA levels in the frontal cortex correlated negatively with both AP-2α and AP-2β levels ($r=-.62$, P=.01 and $r=-.73$, P=.0001, respectively). Moreover, MHPG/NA in the frontal cortex correlated positively with both AP-2α and AP-2β levels (r=.63, P=.01 and r=.70, $P<.01$, respectively), and when analyzing the NA levels in the hippocampus, negative correlations were found with both AP-2α and AP-2β levels in the LC area ($r=-.90$, $P<.0001$ and $r=-.92$, $P<.0001$, respectively). It was also found that MHPG/NA in the hippocampus correlated positively with the AP-2β levels (r=.69, $P<.05$) in the CP-154,526-treated animals.

4. Discussion

While the CRF_1 receptor has been considered as a putative target molecule in the treatment of mood disorders, several contextual variables have been demonstrated to act upon it in paradigms of anxiolytic/antidepressant activity. The aim of the present experiments was to study the effects of CRF_1 receptor blockade in animals with denervation of LC projections and in a chronic treatment regimen.

In Experiment 1, in which one group of animals was treated with the neurotoxin DSP-4, which causes the selective destruction of noradrenergic projections, a clear anxiolytic effect of the CRF_1 antagonist was found when it was administered for five consecutive days of behavioural testing. The increase in exploratory activity of CRF_1 antagonist-treated animals became observable only after repeated administration of the drug. This suggests that CRF_1 receptor blockade does not have an acute anxiolytic effect on exploratory behaviour, which is in agreement with our previous study with another CRF_1 antagonist, CRA1000 (Harro et al., 2001). The neurotoxin DSP-4 [*N*(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine] (Ross, 1976) has been widely used for the selective destruction of noradrenergic projections emerging from the LC. The reduction in exploratory activity observed after DSP-4 was attributed to an increased neophobia because diazepam completely antagonized the antiexploratory effect of the neurotoxin (Harro et al., 1995). In addition, in the present experiment, DSP-4-pretreatment significantly reduced all measures of exploratory activity. However, repeated but not acute administration of CP-154,526 attenuated this effect of DSP-4 by increasing exploratory activity to a similar level as in the vehicle-treated animals (except for latency of entering). Nevertheless, the effect of CP-154,526 was much less pronounced after the denervation of the LC projections than in the vehicle-treated animals, which suggests that these projections are involved in the anxiolytic-like effect of CRF_1 receptor blockade. These results are again consistent with our previous experiments with CRA1000, which, in a dose of 1.25 mg/kg, increased exploratory activity in both vehicle- and DSP-4-pretreated animals, with the strongest effect in vehicle-treated animals (Harro et al., 2001).

In Experiment 2, CP-154,526 had a clear anxiolytic-like effect in the EZM on the risk assessment measure *stretched-attend postures*, which has been considered to be a sensitive criterion in the measurement of the anxiogenic or anxiolytic action of a drug (Dawson and Tricklebank, 1995). This suggests that repeated administration of CP-154,526 in the dose 2.5 mg/kg had the anxiolytic effect also in this experiment. On the other hand, no anxiolytic effects of CP-154,526 administration were revealed in the exploration box test in Experiment 2, when the drug administration was, in most cases, not always followed by behavioural testing. On the contrary, chronic administration of CP-154,526 blocked the increase in exploration that was present with time in the vehicle group. The increase in exploration in the vehicle group was due to habituation with the test apparatus and daily handling, which has previously been shown to increase exploration in rats (Reboucas and Schmidek, 1997; Harro et al., 2001). It has to be noted, however, that in the present experiment, the behavioural testing and drug administration usually occurred noncontingently, with the animal placed back in the home cage in most cases after the administration of the drug. This suggests that drug–environment interaction is important in the manifestation of anxiolytic effects of CRF_1 receptor blockade. This notion is further supported by the fact that without experience of behavioural testing, 12-day treatment with CP-154,526 did not reduce exploration and that CP-154,526 alone had no effect on these activity levels. In conclusion, these results strongly indicate that repeated testing under the influence of CRF_1 receptor blockade prevents habituation-induced increase in exploration. It may also be hypothesized that such a long-term blockade of CRF_1 receptors could have impaired general motor performance because the chronic administration of CRA1000 was recently shown to reduce spontaneous activity in the home cage (Ohata et al., 2002). Still, such an interpretation seems unlikely, as the deletion of the CRF_1 receptor gene has not been found to lead to reduced locomotor activity, but rather to increase activity in anxiety tests (Contarino et al., 1999).

As CRF has been shown to mediate circadian changes in several physiological parameters (e.g., see Buwalda et al., 1997), the effects of CP-154,526 were, in addition to the use of a randomized test procedure, also analyzed according to the daily cycle in Experiment 2, in which some tests were carried out somewhat closer to the dark period in the animal rooms. No systematic effects of CRF_1 receptor blockade on behavioural differences between animals that were tested earlier and later during the day were found.

In previous experiments in our laboratory, the repeated administration of CRA1000 in a high dose led to a blockade of the gradual increase of exploration that was present in the control group (Harro et al., 2001). It was concluded that extensive blockade of CRF_1 receptors may reduce exploratory drive in a relatively familiar environment. It has been

evident for a long time that the administration of CRF increases activity in a familiar environment, and a small dose of CRF (0.01 μg) has been found to increase locomotor activity in a novel environment as well (Sutton et al., 1982). In the light of these findings, it seems that extensive CRF_1 receptor blockade may rather impair the phase of development of environmental habituation, in which higher cognitive processes may also be included, as CRF_1 knockout mice have been shown to display impaired spatial recognition memory (Contarino et al., 1999).

In Experiment 1, DSP-4 significantly reduced NA levels in the frontal cortex, but had no effect on levels of DA or its metabolites. DSP-4 pretreatment also significantly reduced the levels of 5-HIAA in the frontal cortex and of 5-HT turnover ratio, while not having any significant effect on 5-HT levels, which suggests an indirect effect on 5-HT metabolism. Acute intraperitoneal and intracoerulear administration of CP-154,526 has previously been found not to influence monoamine levels in freely moving rats (Millan et al., 2001; Kawahara et al., 2000). In the present studies, the administration of CP-154,526 (2.5 mg/kg) for 5 days did not have any significant effects on monoamines in the frontal cortex, which is in concordance with our similar finding with CRA1000 (Harro et al., 2001). Still, there was a tendency of an increase in 5-HT turnover in the frontal cortex in the vehicle-pretreated but not in DSP-4-pretreated rats after CP-154,526. Of course, it has to be brought to the readers' attention that as the animals in Experiment 1 were sacrificed immediately after the last behavioural testing, the biochemical profiles also embody the interactions of the effects of treatments and behavioural testing. In Experiment 2, also a decrease in levels of MHPG, a metabolite of NA, occurred in the frontal cortex of repeatedly tested CP-154,526-treated rats as compared with the respective vehicle group.

In the present study, no manipulation of the animals had any significant effect on the levels of transcription factor AP-2 isoforms in the LC area. Instead, several correlations between AP-2 levels and NA-related measures were found, but the nature of these associations was dependent upon the treatment of the animals. In addition, great caution has to be applied when interpreting the data due to the small *n*. In our previous studies, the levels of AP-2 in the whole brainstem were found to correlate positively with the NA levels in the frontal cortex (Damberg et al., 2001a,b; our unpublished data). In these previous experiments, animals either had been submitted to a series of behavioural experiments (but not injected) or had received daily intraperitoneal saline injections for 3 weeks. In the present study, where AP-2 levels were measured in the LC area, we have reproduced the positive association with frontal NA in vehicle-treated animals, and a similar trend was found in the hippocampus. Yet, there was a negative correlation between hippocampal NA content and AP-2 levels in naive animals and in animals with chronic CRF_1 receptor blockade. With regard to correlations between LC AP-2 and frontal cortex NA, essentially similar results were obtained. We did not, however, observe a statistically significant negative correlation between LC AP-2 and frontal cortex NA in the naive animals (there were two outliers amongst the total of eight rats), but such a significant negative correlation in the naive animals has indeed been obtained in the frontal cortex in another experiment in our laboratory with a larger group of rats (unpublished). Thus, it seems that in naive rats, there is a negative association between AP-2 and NA, and that CRF_1 receptor blockade reversed the handling stress-induced change in the associations between LC AP-2 levels and brain monoamine content that was revealed in the vehicle-treated animals. This agrees with the notion that CRF_1 receptor antagonists are able to counteract the changes in the brain monoaminergic systems caused by different stressors.

The present study does not suggest the exact mechanism by which these dynamic associations between AP-2 levels and NA-ergic systems are executed. AP-2 has been identified as an important regulator of gene expression in CNS monoaminergic systems, being, for example, necessary for the expression of tyrosine hydroxylase in a cell culture (Kim et al., 2001). However, it has recently been shown that AP-2 is a transcription factor with dual functions. The conditions under which it acts as an activator or repressor are, however, not known (Ren and Liao, 2001). One way of explaining the negative correlations between AP-2 levels and brain NA content in naive animals would be that AP-2, directly or indirectly, exerts a repressor function for genes involved in the formation of NA. If so, manipulations with animals have the potential to switch the role that AP-2 plays in regulating NA-ergic neurons, and this switching is mediated by the release of CRF acting via CRF_1 receptors.

In conclusion, it was found that chronic CRF_1 receptor blockade modulates exploratory behaviour, whereas this modulation is dependent on drug–environment interactions. Long-term blockade of CRF_1 receptors decreased NA metabolism in the frontal cortex but had no effect on transcription factor AP-2 levels in the LC. Yet, it was found to withhold changes in associations between AP-2 levels and NA levels in the frontal cortex and hippocampus that were elicited by handling, which might suggest that CRF_1 receptor antagonists are able to overrun some effects of heightened environmental stimulation. It may also be suggested that AP-2 in the LC area plays a role in the regulation of frontal cortex and hippocampal NA levels.

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