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THE ROLE OF CORTICOTROPIN -RELEASING FACTOR AND LOCUS COERULEUS IN THE REGULATION OF EXPLORATORY BEHAVIOR IN RATS

Master's thesis

Running head: CRF and LC in exploratory behavior

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Abstract

Corticotropin-releasing factor (CRF) holds a central role in reactions to various environmental stimuli. In the studies described herein, the administration of a selective non-peptide CRF_1 receptor antagonist, CP-154,526, for six days exerted an anxiolytic effect in the elevated zero-maze test. CP-154,526 did not affect behavior in the exploration box when administered acutely, but increased exploration when administered for five days contingently with daily behavioral testing. This effect, although of a lesser magnitude, was also present in animals with selective denervation of locus coeruleus (LC) projections induced by neurotoxin DSP-4. When drug administration and behavioral testing were noncontingent in a two-week administration schedule, CP-154,526 blocked the habituation-induced increase in exploration. In rats previously acquainted with the test apparatus, CP-154,526 decreased exploratory behavior after acute administration, and that effect disappeared after long-term treatment. This suggests that drug-environment interaction is an important component in the manifestation of the anxiolytic-like effects of CRF_1 receptor blockade. Interactions of CRF and serotonin (5-HT) systems are involved in anxiety-related behaviors. Brain region specific differences were found between rats with low or high exploratory activity (LE vs. HE rats) in tissue 5-HT and 5-hydroxyindole acetic acid (5-HIAA) levels, and the long-term blockade of CRF_1 receptors had distinct effects in LE and HE rats. Interactions between 5-HT and CRF systems may thus underlie the individual differences in novelty-related behavior. Neither vehicle nor CP-154,526 treatment were found to influence the levels of transcription factor AP-2 isoforms in the LC area. There was a negative correlation between noradrenaline (NA) levels in the hippocampus and AP-2 isoforms in the LC area of naïve animals, which, in contrast, was positive in vehicle-treated animals. 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 naïve animals. This again suggests that CRF_1 receptor blockade counteracts certain mechanisms of habituation, possibly by reducing the LC activity.

Keywords: corticotropin-releasing factor (CRF), CRF_1 receptor, CP-154,526, monoamine neurochemistry, serotonin (5-HT), locus coeruleus (LC), DSP-4, noradrenaline (NA), transcription factor AP-2, exploratory behavior, individual differences.

Kortikotropiini vabastava faktori ning locus coeruleusi roll uudistava käitumise regulatsioonis rottidel

Sisukokkuvõte

Kortikotropiini vabastav faktor (ingl. k. *corticotropin-releasing factor*, CRF) omab olulist osa organismi reaktsioonides erinevatele keskkondlikele stiimulitele. Käesolevalt kirjeldatud uuringutes avaldas CRF_1 retseptori selektiivse mittepeptiidse antagonisti CP-154,526 kuuepäevane manustamine tõstetud nullpuuri testis anksiolüütilist mõju. CP-154,526 ei mõjutanud uudistavat käitumist akuutsel manustamisel, ent suurendas uudistamisaktiivsust manustatuna viie päeva jooksul käitumiskatsele eelnevalt. Sarnane efekt, kuigi väiksemalt määral, avaldus ka loomadel, kelle locus coeruleusi (LC) projektsioone oli kahjustatud selektiivse neurotoksiiniga DSP-4. Ravimi kahenädalasel manustamisel, mil sellele ei järgnenud alati käitumiskatset, blokeeris CP-154,526 uudistamisaktiivsuse katseaparaadiga habituatsioonist tingitud suurenemise. Katseseadmega eelnevalt kokku puutunud loomadel vähendas CP-154,526 akuutsel manustamisel uudistavat käitumist ning see efekt kadus ravimi pikaajalisel manustamisel. Seega näib, et ravimi ja keskkonna vastasmõjudel on CRF_1 retseptorite blokaadi anksiolüütilise mõju avaldumises oluline osa. Nii CRF- kui serotoniini (5-HT) süsteemil on ärevusega seotud käitumistes oluline roll. Erineva uudistamise baastasemega loomadel ilmnesid piirkonnaspetsiifilised erinevused ajukoe 5-HT ja 5-hüdroksüindooläädikhappe (5-HIAA) tasemetes ning krooniline CRF_1 retseptorite blokaad mõjutas neid erinevalt. Seega näivad 5-HT ja CRF süsteemide vahelised interaktsioonid osaliselt olevat aluseks individuaalsetele erinevustele uudistavas käitumises. Ei lahusti ega CP-154,526 manustamine ei mõjutanud transkriptsioonifaktor AP-2 tasemeid LC piirkonnas. Naiivsetel katseloomadel ilmnes hippokampuse noradrenaliini (NA) ja LC piirkonna AP-2 tasemete vahel negatiivne korrelatsioon, mis lahusti-grupi loomadel oli positiivne. CP-154,526 manustamine muutis selle korrelatsiooni negatiivseks, sarnaselt naiivsetele katseloomadele, mistõttu näib et CRF_1 retseptorite blokaad toimib teatud habituatsioonimehhanisme pärssivalt, näiteks LC aktiivsuse vähendamise kaudu.

Märksõnad: kortikotropiini vabastusfaktor (CRF), CRF_1 retseptor, CP-154,526, monoamiinide neurokeemia, serotoniin (5-HT), *locus coeruleus* (LC), DSP-4,

noradrenaliin (NA), transkriptsioonifaktor AP-2, uudistav käitumine, individuaalsed erinevused.

CONTENTS

1. INTRODUCTION

1.1. Corticotropin-releasing factor

Since its identification by Vale and colleagues (Vale et al. 1981), corticotropin-releasing factor (CRF) has been found to hold a central role in reactions to environmental stimulation. Through its double role as a classic hypophysiotropic factor regulating basal and stress-induced release of adrenocorticotropic hormone (ACTH) from the pituitary, and as a direct neuromodulator in the central nervous system it has been found to mediate changes in neuroendocrinological functions and behavior induced by external stimuli. Besides to its high concentrations in the hypothalamic paraventricular nucleus where CRF is produced and secreted, CRF is broadly distributed throughout the whole mammalian central nervous system, being concentrated in corticolimbic regions, such as amygdala, hippocampus, periaqueductal grey, bed nucleus of stria terminalis and prefrontal cortex, as well as the brainstem nuclei locus coeruleus (LC) and dorsal raphe, the origins of ascending noradren(NA)ergic and seroton(5-HT)ergic projections, respectively (Owens and Nemeroff 1991). In line with this organization, extensive literature implies that intracerebroventricular (i.c.v.) administration of CRF to laboratory animals brings forth a wide range of physiological and behavioral changes comparable to those elicited by stressful stimuli, including increase in heart rate, suppression of exploratory behavior, reduction in food intake and reproductive behavior etc. (for a review, see Griebel 1999). Therefore CRF is often considered one of the main "stress molecules" and has been under tense investigation in the context of developing new drugs for treatment of mood disorders for the past decades.

The two CRF receptor subtypes – CRF_1 and CRF_2 - show a different distribution in the central nervous system, with CRF_1 receptors concentrated in the cortex, cerebellum, mesencephalon and pons and to a lesser extent in amygdala and hippocampus, and CRF_2 receptors concentrated in subcortical structures with highest concentrations within the lateral septal nucleus, ventromedial hypothalamic nucleus and choroids plexus, and moderate levels in the olfactory bulb, amygdaloid nuclei, the paraventricular and and supraoptical nuclei of the hypothalamus and raphe nuclei (Chalmers et al. 1995, Radulovic et al. 1998). This distribution is in line with findings of the CRF_1 receptor mostly being implicated in mediating the stress-related effects of the CRF-system (for a review, see Takahashi 2001), whereas the CRF_2 receptor has mainly been associated with ingestive behavior (Eghbal-Ahmadi et al. 1997, but see

also Takahashi et al. 2001). Based on these findings, in the past decade, efforts have been directed toward the development and investigation of non-peptide CRF_1 receptor antagonists for the treatment of stress-related psychiatric disorders, and there are also preliminary findings from a successful open-label study of long-term treatment of depression patients with CRF_1 antagonist R121919 (Zobel et al. 2000). 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, 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 behavior 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). The number of studies with chronic inhibition of CRF_1 receptor function has been limited, but CRF_1 receptor knock-out mice have been found to display a reduced anxiogenic response, showing increased locomotor activity in open field test, increased exploration of the open arms on an elevated plus maze and spending more time in the brightly lit compartment of a light-dark transition box than do wild-type control mice (Timpl et al. 1998; Contarino et al. 1999). When administered long-term during a chronic mild stress (CMS) regimen, CRF_1 receptor antagonist improved weight gain and attenuated physical state degradation and anxiety in rats (Griebel et al. 2002). Other available data also show significant anxiolytic and antidepressant effects on chronic CRF_1 receptor antagonist administration, with decreases in defensive withdrawal behavior (Arborelius et al. 2000) and inhibition of olfactory bulbectomy-induced hyperemotionality (Okuyama et al. 1999). In our laboratory, 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 behavior.

1.2. Locus coeruleus and noradrenaline in stress

Reports of very high levels of CRF_1-like immunoreactivity in NA-ergic brainstem nucleus locus coeruleus neurons (Sauvage and Steckler 2001) suggest that the CRF_1 receptor subtype could be an important mediator of CRF-ergic regulation of LC activity (Valentino et al. 1983). Stress, as well as i.c.v. and intra-LC injections of CRF have 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 increase NA release in prefrontal cortex (Curtis et al. 1997). The peptide CRF receptor antagonist a-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 neurones by i.c.v. CRF (Schulz et al. 1996; Okuyama et al. 1999; Lejeune and Millan 2003) or stress (Kawahara et al. 2000; Griebel et al. 2002). Previously, repeated treatment with the CRF receptor antagonist CRA1000 has been found to increase exploratory behavior which was reduced after denervation of LC projections by treatment with the selective neurotoxin DSP-4 [N(2-chloroethyl)-N-ethyl-2-bromobenzylamine] (Harro et al. 2001). These findings support the notion that the CRF_1 receptor subtype plays a role in mediation of the functions of the LC.

1.3. Serotonin in stress

Serotonin (5-HT) has also been implicated in reactions to stressful stimuli (for review, see Chaouloff et al. 1999), and there are interactions between CRF and 5-HT systems, which may be important for the organism's reactions to environmental challenges. I.c.v. administration of CRF induces 5-HT overflow in the hippocampus (Kagamiishi et al. 2003), and the CRF_1 receptor antagonist CP-154,526 has been found to decrease baseline hippocampal extracellular 5-HT levels (Isogawa et al. 2000). Since hippocampal 5-HT release contributes to anxiety (File et al. 1987), it can therefore be suggested that the reported anxiolytic effects of CRF antagonists (for a review, see Holsboer 1999) are at least partly mediated through the CRF-ergic regulation of hippocampal 5-HT system. For an example, swim stress-induced elevation of hippocampal 5-HT levels was attenuated in mice chronically treated with the selective CRF_1 antagonist NBI30775 (Oshima et al. 2003). Price et al. (1998) have speculated that CRF serves as a neurotransmitter in the dorsal raphe nucleus to regulate the forebrain 5-HT function, in an analogous manner to its abovementioned role in the LC.

1.4. Individual differences in reactions to environmental stimuli

It has been shown previously that rats preselected or bred on the basis of anxiety-related behavior will display consistently different behavioral patterns in many tests of anxiety (Ho et al. 2002; Kabbaj et al. 2000). These behavioral patterns have been accompanied by differences in the activity of 5-HT-ergic system (Giorgi et al. 2003; Thiel et al. 1999). Umriukhin et al. (2002) have found that the rats selectively bred for low anxiety-related behavior in the elevated plus maze show a greater release of 5-HT in hippocampus in response to stressor. The same laboratory has reported that CRF_1 receptor blockade had anxiolytic effect only in rats selectively bred for high anxiety-related behavior (Keck et al. 2001). This suggests that CRF_1 receptor blockade may not have a uniform influence in all animals. It is possible that these effects are linked with stable individual differences that are displayed in exploratory behavior.

1.5. Transcription factor AP-2

The interest in molecules affecting transcriptional processes has, during the past few years, increased in order 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) have been found to be influenced by chronic antidepressant treatment (Damberg et al. 2000) and AP-2 levels in the brainstem have been found to correlate with monoamine levels in several brain regions (Damberg et al. 2001b), that suggest an influence of AP-2 in the regulation of important genes in monoaminergic neurons in the brainstem. The next logical step appears to be specifying the brain areas in which AP-2 influences monoaminergic systems.

1.6. Aims of the experiments

To approach the questions raised above, a series of experiments was carried out, in order to study:

1) the effect of CRF_1 receptor blockade on exploratory behavior in animals with selective denervation of locus coeruleus projections (Experiment 1);

2) the effects of long-term blockade of CRF_1 receptors on exploratory behavior and monoamine neurochemistry (Experiment 2);

3) the effect of long-term CRF_1 receptor blockade on exploratory behavior and 5-HT metabolism ex vivo in rats preselected on the basis of exploratory activity, and the dose-dependency of the eventual effects (Experiments 3a and 3b).

4) the effect of CRF_1 receptor blockade by CP-154,526 treatment on amphetamine-induced behavioral activation in the open field test (Experiment 4);

5) the associations between transcription factor AP-2 in locus coeruleus, medial raphe, dorsal raphe and ventral tegmentum and brain monoaminergic systems in untreated animals (Experiment 5) and in animals with long-term CRF_1 receptor blockade (Experiment 2).

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats (Experiment 1; Finnish Laboratory Animal Center, Kuopio, Finland, Experiments 2, 3a, 3b, 4, 5; National Public Health Institute, Kuopio, Finland) were housed in groups of 3 (Experiment 1) or 4 (Experiment 2, 3, 5 and 6) in standard transparent polypropylene cages under controlled light cycle (lights on from 08:00 h to 20.00 h) and temperature (19°-21° C), with free access to tap water and food pellets (diet R70, Lactamin, Sweden). All behavioral testing was carried out between 1 p.m. - 7 p.m. The experimental protocols were 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 separately weighed, dissolved in distilled water and immediately injected intraperitoneally one week before the behavioral experiments. In all experiments, CP-154,526 was suspended in distilled water by adding a few drops of Tween 85. Control animals received vehicle (vehicle group, Experiments 1, 2, 3, 4) or no treatment (control group, Experiments 2 and 3). CP-154,526 or vehicle were administered intraperitoneally 30 min before behavioral observations, or at about the same time of day in Experiments 2 and 3 on days which did not include behavioral testing.

2.3. Exploration box test

The exploration box test has been shown to enable measurement of different aspects of exploratory behavior (Harro et al. 1995; Otter et al. 1997). The apparatus was

made of metal and consisted of an open area 0.5 x 1m (height of side walls 40 cm) with a small compartment 20 x 20 x 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 x 20 cm). The apparatus was cleaned with dampened laboratory tissue after each animal. The exploration 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 the inner 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 confines (height 1 cm). All parts of the apparatus were made of non-transparent 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 observed for 5 minutes. Behavioral measures taken included: a) number of open part entries; b) time spent in the open parts; c) the number of head dips over the edge of the platform; d) the number of stretched-attend postures. The experiments were carried out under bright light conditions.

2.5. Biochemical measurement

2.5.1. 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 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 14000 x g for 20 min at 4° C and 20 µl of the resulting supernatant was chromatographed on a Lichospher 100 RP-18 column (250 x 3 mm; 5 µm). The separation was done in 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, 1mM KCl and 6,25% acetonitril. The measurements were done at electrode potentials of a glassy carbon electrode +0.6V versus Ag/AgCl reference electrode with HP 1049 electrochemical detector (Hewlett Packard, Germany).

2.5.2. Extraction of nuclear proteins

Nuclear proteins were extracted according to a modified protocol of Dignam and co-workers (1983). Tissue sections of the locus coeruleus, dorsal or medial raphe or vetral tegmentum 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 addition of 0.25 ml 10% Nonidet P40 the homogenates were centrifuged for 1 min at 17 100 x g at 4º 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 4º C was followed by centrifugation at 17 100 x g for 5 minutes at 4º C. The aliquots from the supernatants were frozen in liquid nitrogen and stored at -80º C. Total protein concentration was determined for all nuclear extracts by the method of Lowry et al. (1951).

2.5.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiterplates (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 4º C. Following the incubation the antigen solution was removed and 200 µl of blocking buffer (PBS and 1% BSA) was added to each well and the plates were incubated for two hours 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 4º 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 anti-goat IgG AP conjugated, SDS) diluted 1:350 in blocking buffer was added (50 µl to each well) and the plates were incubated for two hours at room temperature. After removal of the secondary antibody, the plates were washed three times with wash buffer I and once with wash buffer II (10mM 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 minutes and was terminated by adding 50 µl of 0.1 M EDTA, pH 7.5. The plates were analysed in an ELISA reader at optical density (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 OD. The value from the standard curve was then divided by 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 analysed twice for accuracy.

2.6. Procedure

In Experiment 1, rats (n=24) were observed in the exploration box on five consecutive days. The animals were randomly divided into 4 groups following a 2 x 2 (Toxin x Drug) design in which every animal either received 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. Animals were sacrificed immediately after the last behavioral testing and tissue samples were dissected on ice for determination of the levels of monoamines.

In Experiment 2, rats (n=40) were randomly divided into 5 groups, one of which was not handled before sacrifice, while four groups were included in a 2 x 2 design, the two factors being treatment with CP-154,526 (2.5 mg/kg/day) or vehicle and behavioral experiments (repeated vs single). Drug treatment was carried out for 16 days. The "single-tested" rats were used for only one behavioral experiment (the exploration box test on day 12 of drug treatment), whereas 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). Animals were sacrificed four days after the last behavioral testing, during which drug administration was continued; tissue samples were dissected on ice. Nuclear extracts were immediately prepared for the measurement of transcription factors as described above.

In Experiment 3a, rats (n=64) were observed in the exploration box on two consecutive days for determination of baseline activity levels. Then the animals were divided into low exploratory activity (LE) and high exploratory activity (HE) groups on the basis of the median value of the sum of exploratory activity during the second testing, a measure which is a reliable predictor of individual novelty-related activity (our unpublished data). CP-154,526 in doses of 2.5 mg/kg and 10 mg/kg was administered for 14 days, during which animals were tested in the exploration box on days 1, 5 and 12. Two control groups were used, in which animals received either vehicle (vehicle group) or no treatment (control group). Animals were decapitated on day 14, and tissue samples dissected on ice.

In Experiment 3b, rats (n=24) were divided in two groups, one of which was observed in the exploration box on two consecutive days for determination of baseline activity levels. Then the animals were administered either vehicle or CP-154,526 (10 mg/kg) and subjected to the exploration box test once, which was therefore either first or third encounter with the test apparatus.

In Experiment 4, rats (n=16) were observed in the exploration box on two consecutive days for determination of baseline activity levels. Then the animals were tested in the open field test four times with an interval of 3-4 days between testings. The following treatments were applied in a randomized crossover design: vehicle, CP-154,525 (5 mg/kg), amphetamine (0.5 mg/kg), CP-154,526+amphetamine, so that each animal was subject to each treatment once.

Experiment 5 was carried out on two groups of rats (n=21 and 20, respectively), that were taken from their home cages, decapitated immediately, tissue samples dissected on ice and nuclear extracts immediately prepared for the measurement of transcription factors as described above (from medial raphe and locus coeruleus regions in the first group and from the dorsal raphe and ventral tegmentum regions in the second group).

Tissue preparations in all experiments were made according to the atlas of Paxinos and Watson (1986).

2.7. Statistics

Data from the exploration box tests and biochemical measurements were analyzed with two-factor ANOVA (Toxin x Drug in Experiment 1, Testing x Drug treatment in Experiment 2, Baseline Activity x Treatment in Experiment 3a and Testing x Drug in Experiment 3b) with repeated measures or one-factor (Drug) repeated measures ANOVA (in Experiment 2). The post-hoc pairwise comparisons in Experiments 1, 2 and 3 and data analysis from Experiment 4 were made with Fisher's LSD test. In Experiments 2, 5 and 6, Pearson correlation indices were calculated and linear regression analyses computed to study interactions between AP-2 and monoamines.

3. RESULTS AND DISCUSSION

3.1. Experiment 1*

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

Two-factor (Toxin x Drug) repeated measures ANOVA revealed a significant DSP-4 pretreatment effect on line crossings, object investigations, enterings, 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, $p<0.05$). A significant CP-154,526 treatment effect was revealed on 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, $p<0.05$). 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, $p=0.06$). There was also a significant effect of repeated testing on the latency of entering, rearings ($F(4, 80)=2.56$ and 5.16, respectively, $p<0.05$), 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, $p<0.01$) and Day x Drug effect on line crossings, object investigation and sum of exploratory activity ($F(4, 80)=3.80$, 3.69 and 3.69, respectively, $p<0.01$) and rearings ($F(4, 80)=2.51$, $p<0.05$), while it remained just above the conventional level of statistical significance on time of exploratory activity ($F(4, 80)=2.22$, $p=0.07$). 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

* The paper in *Pharmacology Biochemistry and Behavior* presented as Appendix 1 comprises the results and discussion of Experiments 1 and 2 also given herein.

from day 2, differences between control and DSP-4 animals became apparent as the former's behavioral 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.

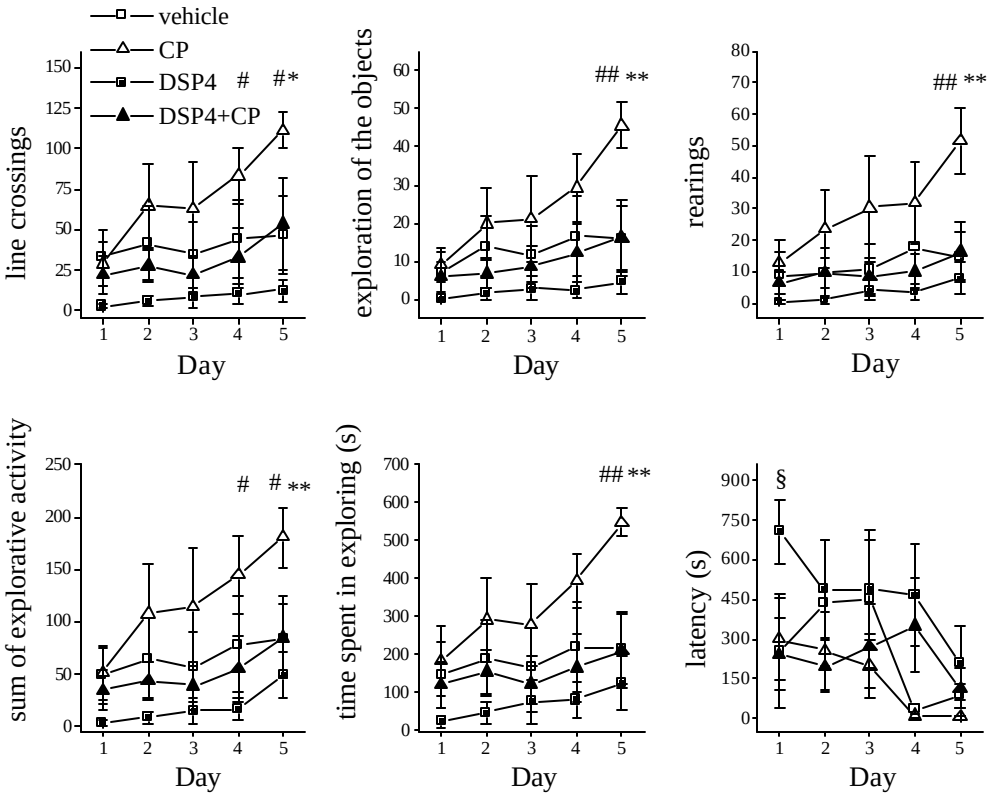

Fig. 1. The effect of DSP-4 pretreatment and CP-154,526 (2.5 mg/kg) on behavior in exploration box on five consecutive days (mean ± SEM). * - $p<0.05$, ** - $p<0.01$ vehicle vs. CP-154,526; # - $p<0.05$, ## - $p<0.01$ CP-154,526 vs. CP-154,526 + DSP-4; § - $p < 0.05$ - DSP-4 vs. DSP-4 + CP-154,526. Data on entries are not shown.

In this experiment, a clear anxiolytic effect of the CRF_1 antagonist was found when it was administered for five consecutive days of behavioral 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 behavior, which is in agreement with our previous study with another CRF_1 antagonist

CRA1000 (Harro et al. 2001). The neurotoxin DSP-4 (Ross 1976) has been widely used for selective destruction of noradrenergic projections emerging from the locus coeruleus. The reduction in exploratory activity observed after DSP-4 was attributed to an increased neophobia, since diazepam completely antagonized the anti-exploratory effect of the neurotoxin (Harro et al. 1995). Also in the present experiment, DSP-4-pretreatment significantly reduced all measures of exploratory activity. Repeated but not acute administration of CP-154,526 attenuated this effect of DSP-4 by increasing exploratory activity to a similar level as vehicle-treated animals (except for latency of entering). Nevertheless, the effect of CP-154,526 was much less pronounced after denervation of the locus coeruleus projections than in 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 stronger effect in vehicle treated animals (Harro et al. 2001).

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 levels in the frontal cortex

When using 2-factor repeated measures ANOVA (Toxin x Drug) a significant DSP-4 pretreatment effect in the frontal cortex was found on NA levels ($F(1, 20)= 295.0$, $p<0.0001$), 5-HT turnover (calculated as the 5-HIAA/5-HT ratio) ($F(1, 20)= 24.6$, $p<0.0001$), 3,4-dihydroxyphenylacetic acid (DOPAC) ($F(1, 20)=6.16$, $p<0.05$), dopamine (DA) turnover [calculated as the (DOPAC+HVA)/DA ratio] ($F(1, 8)=8.94$, $p<0.05$) and 5-hydroxyindolacetic acid (5-HIAA) ($F(1, 20)=14.4$, $p<0.01$) while the effect on frontal cortex DA levels just missed the conventional level of statistical significance ($F(1, 8)=4.33$, $p=0.07$). Post-hoc tests revealed significantly lower levels of NA and 5-HIAA and of 5-HT turnover in vehicle and CP-154,526 groups pretreated with DSP-4 (Table 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 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 intra-coerulear administration of CP-154,526 has previously been found not to influence monoamine levels in freely moving rats

(Kawahara et al. 2000, Millan et al. 2001). In the present studies, administration of CP-154,526 (2.5 mg/kg) for five days did not have any significant effects on tissue monoamine levels in the frontal cortex, which is in concordance with the findings with CRA1000 (Harro et al. 2001). Still, there was a tendency of an increase in 5-HT turnover in the frontal cortex in vehicle 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 behavioral testing, the biochemical profiles also embody interactions of the effects of treatments and behavioral testing.

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

	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<0.05, ** - p < 0.01, *** - p < 0.001, **** - p<0.0001 vs. vehicle; # - p < 0.05, ## - p < 0.01, ### - p < 0.001, #### - p<0.0001 vs. CP-154,526.

3.2. Experiment 2

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

In the elevated zero-maze 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 ($F(1, 14)= 5.10$, $p<0.05$), head dips and time of

exploratory activity (F(1, 14)=18.0 and 10.4, respectively, both p<0.01). All animals had significantly lower levels of activity on day 13 (Fig. 2). This effect remained just above the conventional level of statistical significance on enterings into open parts of the apparatus (F(1, 14)=3.82, p=0.07). Also, a significant Drug effect appeared on stretched-attend postures (F(1, 14)=4.86, p<0.05) and there was a significant Day x Drug interaction on stretched-attend postures (F(1, 14)=7.09, 8.78, respectively, p<0.05). Post-hoc tests revealed that six days of administration of CP-154,526 (2.5 mg/kg) decreased the stretched-attend postures.

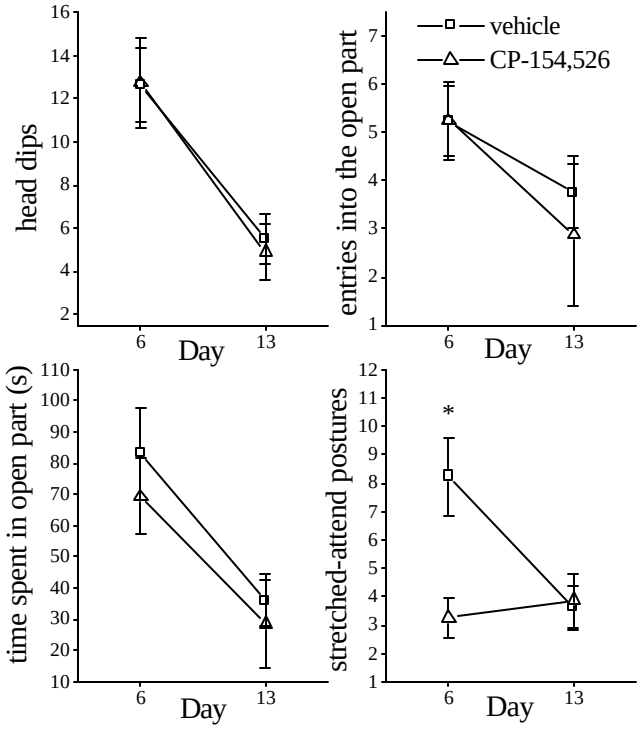

Figure 2. The effect of CP-154,526 (2,5 mg/kg) on behavior in zero-maze (mean ± SEM). * - p<0.05 vs. vehicle.

Thus, CP-154,526 had a clear anxiolytic-like effect in the elevated zero-maze on the risk assessment measure “stretched-attend postures” which has been considered to be a sensitive criterion in 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 an anxiolytic effect also in this experiment.

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

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 behavioral measures: latency of entering, line crossing, rearings, time and sum of exploratory activity ($F(2, 28)=4.65, 4.31, 5.53, 4.84, 5.09$, respectively, all $p<0.05$) and investigation of objects ($F(2, 28)=7.54$, $p<0.01$). This effect of repeated testing emerged since 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 ($F(1, 14)=4.74, 4.38, 6.13, 5.02, 5.11$, respectively, $p<0.05$) with the CP-154,526 group revealing lower levels of activity. A significant Day x Drug interaction was also revealed on the lastly mentioned measures ($F(2, 28)=3.66, 4.51, 4.04, 3.35, 4.08$, respectively, $p<0.05$). Post-hoc tests revealed that the activity of the CP-154,526 group was lower from that of the vehicle group on the second and third testing (days 5 and 12 of drug administration, respectively).

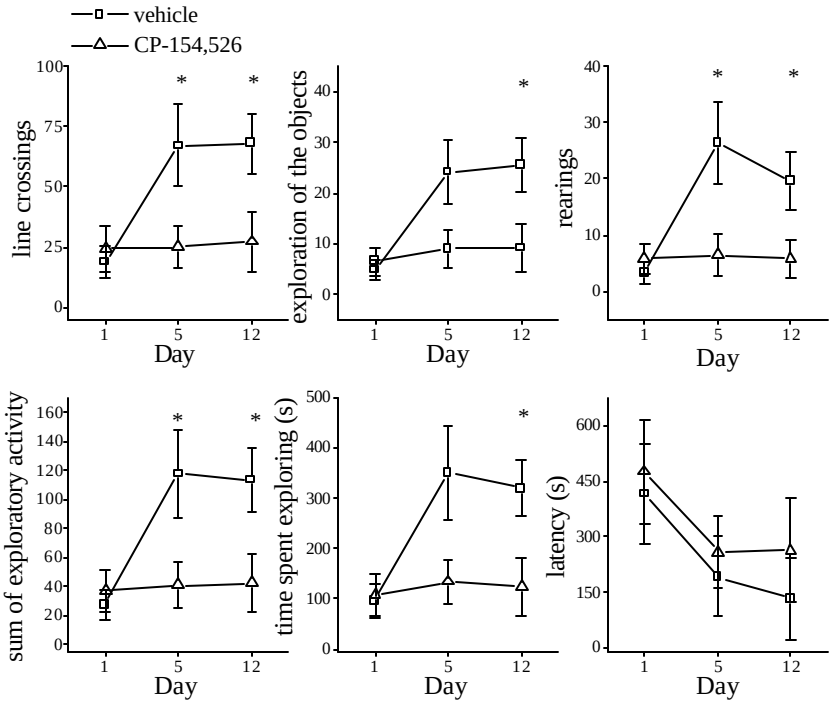

Figure 3. The effect of chronic administration of CP-154,526 (2.5 mg/kg) on changes in behavior in the exploration box (mean ± SEM). * - $p<0.05$ vs vehicle. Data on entries are not shown.

3.2.3. The effect of administration of CP-154,526 (2.5 mg/kg) for 12 days on behavior 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 x Testing) revealed a significant effect of Testing on number of entries into open part of the apparatus ($F(1, 28)=8.76$, $p<0.01$), an effect of administration of CP-154,526 on line crossing and time of exploration, which just missed the conventional level of statistical significance ($F(1, 28)=3.40$, $p=0.076$ and 3.06, $p=0.094$, respectively), and similar Drug x Testing interactions on object investigation and time of exploration ($F(1, 28)=4.00$, $p=0.055$ and 3.65, $p=0.066$, respectively). Altogether, the 12-days administration of CP-154,526 reduced exploratory activity in animals that had been tested repeatedly during this period (Fig. 4), while it had no significant effect on exploratory activity on animals subjected to the experimental apparatus for the first time on test day 12.

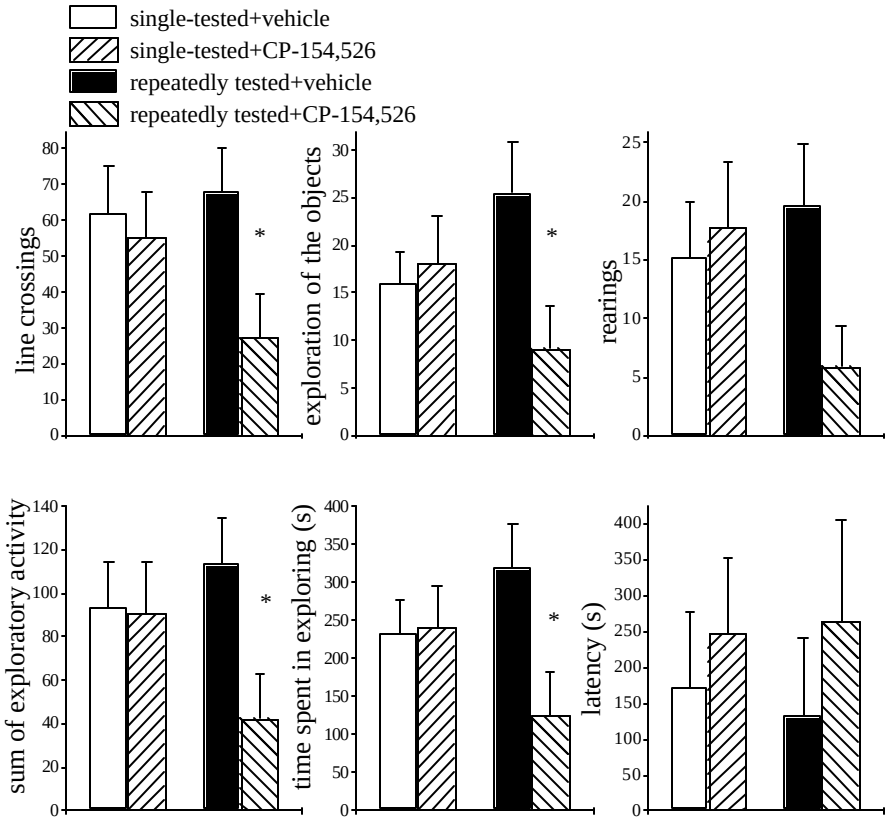

Figure 4. The effect of 12 days of administration of CP-154,526 (2.5 mg/kg) on behavior in the exploration box (mean ± SEM). * - $p<0.05$ vs. the corresponding vehicle group. Data on entries are not shown.

In contrast to the results of Experiment 1 and the elevated zero-maze test in Experiment 2, 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 followed by behavioral testing as it was in Experiment 1. 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 noticed, however, that in the present experiment, the behavioral testing and drug administration usually occurred noncontingently, with the animal placed back to 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 behavioral testing, 12-days 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 suggest that repeated testing under the influence of CRF_1 receptor blockade prevents habituation-induced increase in exploration. In previous experiments in our laboratory, 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 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 (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). It may also be hypothesized that such a long-term blockade of CRF_1 receptors could have impaired general motor performance, since the chronic administration of CRA1000 was recently shown to reduce spontaneous activity in home cage (Ohata et al. 2002). Still, such an interpretation seems unlikely in the present context 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 (for an example, see Buwalda et al. 1997), the effects of CP-154,526 were in addition to the use of a randomized test procedure also analysed according to the daily cycle in Experiment 2, in which some testings were carried out somewhat closer to the dark period in the animal rooms. No systematic effects of CRF_1 receptor blockade on behavioral differences between animals that were tested earlier and later during the day were found.

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

2-factor ANOVA (Drug x Testing) revealed a significant effect of CP-154,526 on 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in frontal cortex ($F(1, 28)=6.81$, $p<0.05$) and a similar effect on 5-HT levels in frontal cortex, which just missed the conventional level of statistical significance ($F(1, 28)=3.75$, $p=0.063$). Thus, MHPG levels were decreased after CP-154,526 administration (Table 2), suggesting a decrease in LC activity after CP-154,526 administration. There was a trend of decreased 5-HT levels as well, which is similar to the finding of Experiment 1. Neither administration of CP-154,526, nor repeated testing had any statistically significant effect on monoamine levels in hippocampus or hypothalamus.

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

A 2-factor ANOVA (Drug x Testing) revealed no significant effects on transcription factor AP-2 in the locus coeruleus area. For the naïve, 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 (mean AP-2α protein level ± SEM) and the AP-2β levels were 8.83±1.48, 7.49±0.60 and 6.83±0.59 (mean AP-2β protein level ± SEM). For all three groups of animals the monoamine levels and turnover in the frontal cortex, hippocampus and hypothalamus were analysed in relation to AP-2 levels in the locus coeruleus 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 naïve group, NA levels in the hippocampus correlated negatively with both AP-2α and AP-2β levels ($r=-0.74$; $p<0.05$ and $r=-0.80$; $p<0.05$ respectively). In the vehicle group, MHPG

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

	naïve	single-tested + vehicle	single-tested + CP-154,526	repeatedly tested + vehicle	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

* - $p<0.05$ vs. naïve group; # - $p<0.05$ vs. repeatedly tested+vehicle group.

Table 3. Correlations of AP-2 levels in the locus coeruleus with noradrenaline and its metabolism in frontal cortex, hippocampus and hypothalamus (Experiment 2). Statistically significant results are marked in bold.

	ALL (n=40)				NAÏVE (n=8)				VEHICLE (n=16)				CP 154,526 (n=16)			
	AP-2α		AP-2ß		AP-2α		AP-2ß		AP-2α		AP-2ß		AP-2α		AP-2ß	
	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	-0.16	0.35	-0.09	0.61	-0.28	0.50	-0.24	0.57	0.31	0.24	0.48	0.06	-0.62	**<0.01**	-0.73	**<0.001**
MHPG	0.38	**<0.05**	0.43	**<0.05**	0.27	0.52	0.30	0.47	0.56	**<0.05**	0.64	**<0.05**	0.43	0.11	0.39	0.15
MHPG/NA	0.43	**<0.01**	0.43	**<0.01**	0.37	0.37	0.38	0.36	0.29	0.28	0.21	0.43	0.63	**<0.05**	0.70	**<0.01**
Hippocampus																
NA	-0.28	0.11	-0.19	0.27	-0.74	**<0.05**	-0.80	**<0.05**	0.38	0.15	0.54	**<0.05**	-0.90	**<0.0001**	-0.92	**<0.0001**
MHPG	0.03	0.88	0.01	0.97	-0.19	0.66	-0.24	0.57	0.12	0.67	0.19	0.49	-0.01	0.98	0.15	0.65
MHPG/NA	0.14	0.44	0.11	0.55	0.38	0.35	0.36	0.39	-0.35	0.20	-0.48	0.07	0.47	0.18	0.69	**<0.05**
Hypothalamus																
NA	-0.16	0.35	-0.07	0.67	-0.18	0.72	-0.28	0.56	-0.03	0.91	0.20	0.45	0.06	0.84	0.06	0.85
MHPG	0.12	0.51	0.18	0.34	-0.34	0.62	-0.43	0.52	0.52	0.06	0.48	0.09	0.14	0.66	0.31	0.32
MHPG/NA	0.22	0.22	0.18	0.34	0.22	0.75	0.12	0.87	0.36	0.21	0.18	0.55	0.15	0.64	0.27	0.38

levels in the frontal cortex correlated positively with both AP-2α and AP-2β levels ($r=0.56$; $p<0.05$ and $r=0.64$; $p=0.01$ respectively). When, in the latter group, AP-2β levels in relation to NA levels were analysed in the frontal cortex, the conventional level of significance was just missed ($r=0.48$; $p=0.06$). Further, a positive correlation was found between NA levels in the hippocampus and AP-2β levels in the locus coeruleus area ($r=0.54$; $p<0.05$). In the CP-154,526 treated group the NA-levels in the frontal cortex correlated negatively with both AP-2α and AP-2β levels ($r=-0.62$; $p=0.01$ and $r=-0.73$, $p=0.0001$ respectively). Moreover, MHPG/NA in the frontal cortex correlated positively with both AP-2α and AP-2β levels ($r=0.63$; $p=0.01$ and $r=0.70$; $p<0.01$ respectively) and when analysing the NA levels in the hippocampus, negative correlations were found with both AP-2α and AP-2β levels in the locus coeruleus area ($r=-0.90$; $p<0.0001$ and $r=-0.92$; $p<0.0001$ respectively). It was also found that MHPG/NA in the hippocampus correlated positively with AP-2β levels ($r=0.69$; $p<0.05$) in the CP-154,526 treated animals.

No manipulation of the animals had any significant effect on the levels of transcription factor AP-2 isoforms in the locus coeruleus area. Still, several correlations between AP-2 levels and NA-related measures were found, the nature of which was dependent upon treatment of the animals. In our previous studies, the levels of AP-2 in the whole brainstem were found to correlate positively with NA levels in the frontal cortex (Damberg et al. 2001b; unpublished data from our laboratory). In these previous experiments, animals had been either submitted to a series of behavioral experiments (but not injected), or received daily intraperitoneal saline injections for three weeks. In the present study, where AP-2 levels were measured in the LC area, we have reproduced the positive association with frontal cortex 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 naïve 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 8 rats), but such a significant negative correlation in naïve animals was later obtained in frontal cortex Experiment 5, using a larger group of rats. Thus, it seems that in naïve 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 locus coeruleus AP-2 levels and brain monoamine content that was revealed in 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. Nevertheless, it should be noticed that great caution has to be applied when interpreting the AP-2 data from Experiment 2 due to the small n.

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 e.g., necessary for the expression of tyrosine hydroxylase in a cell culture (Kim et al. 2001). It has recently, however, 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 naïve 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 AP-2 plays in regulating NA-ergic neurons, and this switching is mediated by release of CRF acting via CRF_1 receptors.

Some of the results in Experiments 1 and 2 with CRF_1 receptor antagonists were unexpected. Thus, while we found that 5-days administration of either CRA1000 (Harro et al. 2001) or CP-154,526 (present results) with contingent behavioral testing increased exploratory behavior in the exploration box test in an anxiolytic-like manner, administration of CP-154,526 (2.5 mg/kg) for two weeks non-contingently with behavioral testing blocked the increase in exploratory behavior that appears after repeated testing of animals. We therefore carried out yet another study of long-term CRF_1 receptor blockade (see Experiment 3 below), to specify its effects on exploratory behavior.

Rats have stable individual differences in their baseline exploratory activity, and an increase by repeated testing is observed only in animals with low baseline. In active explorers, there is rather a tendency of a decrease by repeated testing in the same environment. Thus, it is possible that the effect of CRF_1 receptor blockade on exploration-based behavior depends upon the intrinsic tendency of the rat to explore.

In Experiment 3, rats were preselected on the basis of their exploratory activity before the beginning of the drug treatment.

As the repeated administration of CP-154,526, demonstrating anxiolytic effects of chronic but not acute CRF_1 receptor blockade, brought about tendencies of change in 5-HT metabolism, which nevertheless remained statistically not significant in the two previous independent experiments, we also decided to study 5-HT system in behaviorally preselected animals.

3.3. Experiment 3a

3.3.1. The effect of two -weeks administration of CP-154,526 in the exploration box test

In the exploration box test, a two-factor ANOVA (Baseline Activity x Treatment) with repeated testing revealed a significant Baseline Activity effect on latency, enterings, line crossing, object investigation, rearing, sum of exploratory activity and time of exploratory activity ($F(1, 56)=6.89, 9.25, 14.86, 11.82, 13.15, 14.4713.95$; $p<0.05$, $p<0.01$, $p<0.001$, $p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$, respectively) and a significant Treatment effect on entering ($F(3, 56)=4.65$; $p<0.01$). The Treatment effect on line crossing remained just above the conventional level of statistical significance ($F(3, 56)=2.69$, $p=0.055$). When a one-factor repeated measures ANOVA (Treatment) was assigned to LE and HE groups alone, significant effects of Repeated Testing were revealed in the LE group in object investigation, rearing, sum of exploratory activity and time of exploratory activity ($F(2, 56)=4.28, 6.61, 4.44, 5.56$, $p<0.05$, $p<0.01$, $p<0.05$, $p<0.01$, respectively). The activity of LE animals increased slightly during repeated testings, but there were no differences between treatments in this group. At the same time, significant Treatment effects were revealed in HE group in latency, entering and line crossing ($F(3, 28)=3.91, 6.31, 3.81$; $p<0.05$, $p<0.01$, $p<0.05$, respectively). A significant Repeated Testing effect in HE animals was revealed on latency, line crossing, object investigation, rearing, time of exploratory activity, sum of exploratory activity and time of exploratory activity ($F(2, 56)=3.82, 5.43, 7.21, 8.60, 7.34, 7. 83$; $p<0.05$, $p<0.01$, $p<0.01$, $p<0.001$, $p<0.01$, $p<0.01$, respectively). Also, significant Treatment x Repeated Testing effects were revealed on latency, rearing ($F(6, 56)=3.69, 2.27$; $p<0.01$, $p<0.05$, respectively). The Treatment x Repeated Testing effects remained just above the conventional level of statistical significance for object investigation and sum of exploratory activity ($F(6,$

56)=2.23 2.21, p=0.054, p=0.055) in the HE group. Post-hoc tests showed that the latency of the 10 mg/kg CP-154,526 group increased on first administration (Fig. 5), while both doses of CP-154,526 decreased object investigation and sum of exploratory activity on first administration. The differences in latency, rearing and sum of exploratory activity were the greatest between both drug groups and untreated group on second testing on Day 5. On the third testing on Day 12, the activity of both drug groups had increased to the same level with the control groups and there were no differences between treatments.

The activity of all HE groups decreased on the first day of drug administration as compared to the previous drug-free testing, which is probably attributable to stress caused by injections. The decrease was most pronounced in the 10 mg/kg dose of CP-154,526 HE animals but also in the 2.5 mg/kg CP-154,526 HE group. It should be noted that exploratory activity decreased even in the control group that received no injection. This suggests that the psychological stress elicited in the animals by the first-ever experiment involving injection procedure was similarly anxiogenic as the injection itself. Regarding the LE rats, there was no statistically significant effect of any factor, suggestive that CRF_1 receptor blockade did not have significant effects in this group. During the subsequent testing in the exploration box (on days 5 and 12 of drug administration), the activity of both CP-154,526 treated HE groups was increased, yielding similar levels with vehicle and control HE groups on day 12. The activity levels of vehicle and control HE groups was not changed notably on days 5 and 12 of drug administration. No manipulation significantly affected the activity level of any LE groups. Because just the HE animals were clearly and dose-dependently affected, we hypothetised this be due to a motivation-decreasing effect of CRF_1 blockade in an environment previously made familiar during testing for assignment of animals into LE/HE groups.

The effect of CRF_1 receptor blockade on exploratory behavior is not in a direct disagreement with any previous findings, but is not convergent with them in any obvious way either. It seems that previous experience with the testing environment strongly influences the effect of CRF_1 receptor blockade on behavior in this environment.

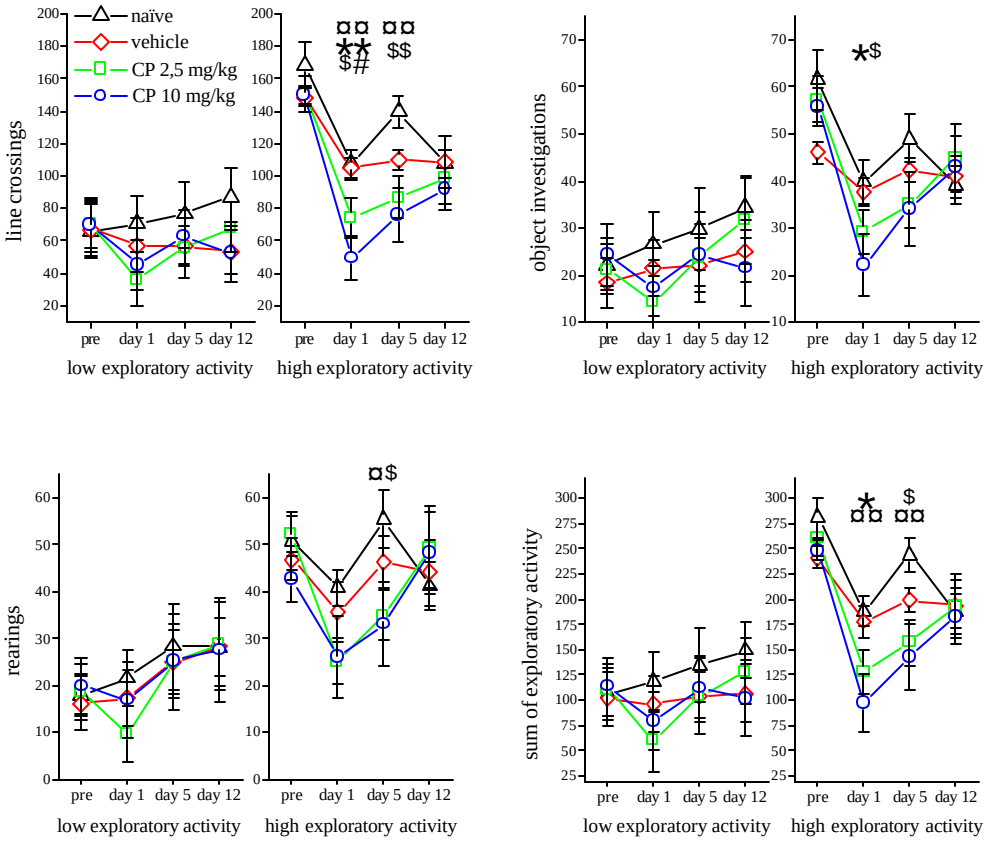

Figure 5. The effect of chronic administration of CP-154,526 (2.5-10 mg/kg) on behavior in the exploration box (mean ± SEM). # - p<0.05 vehicle vs. 2.5 mg/kg CP-154,526; * - p<0.05, ** - p<0.01 vehicle vs. 10 mg/kg CP-154,526. ¤ - p<0.05, ¤¤ - p<0.01 naïve vs. 10 mg/kg CP-154,526. $ - p<0.05, $$ - p<0.01 naïve vs. 2.5 mg/kg CP-154,526. Data on entries, latency and time spent exploring are not shown. pre – second baseline activity measurement.

3.3.2. The effect of CP-154,526 long-term treatment on tissue levels of 5-HT and 5-HIAA[†]

In the frontal cortex, a significant Treatment effect was revealed on 5-HT ($F(3, 56)=2.86$, $p<0.05$). Vehicle treatment had a tendency of decreasing 5-HT content and chronic administration of CP-154,526 significantly elevated 5-HT levels in both doses in LE animals, as compared to the corresponding vehicle group (Fig. 6). A significant Treatment effect, as well as Baseline Activity effect were revealed on 5-HIAA ($F(3, 56)=3.37$, $p<005$ and $F(1, 56)=9.87$, $p<0.01$; respectively). HE animals

[†] The paper submitted to *Brain Research* presented as Appendix 2 comprises the results and discussion of section 3.3.2. herein.

had significantly lower levels of 5-HIAA as compared to the corresponding LE animals and treatment with CP-154,526 dose-dependently elevated the 5-HIAA levels. No treatment had any effect on 5-HIAA in the LE group.

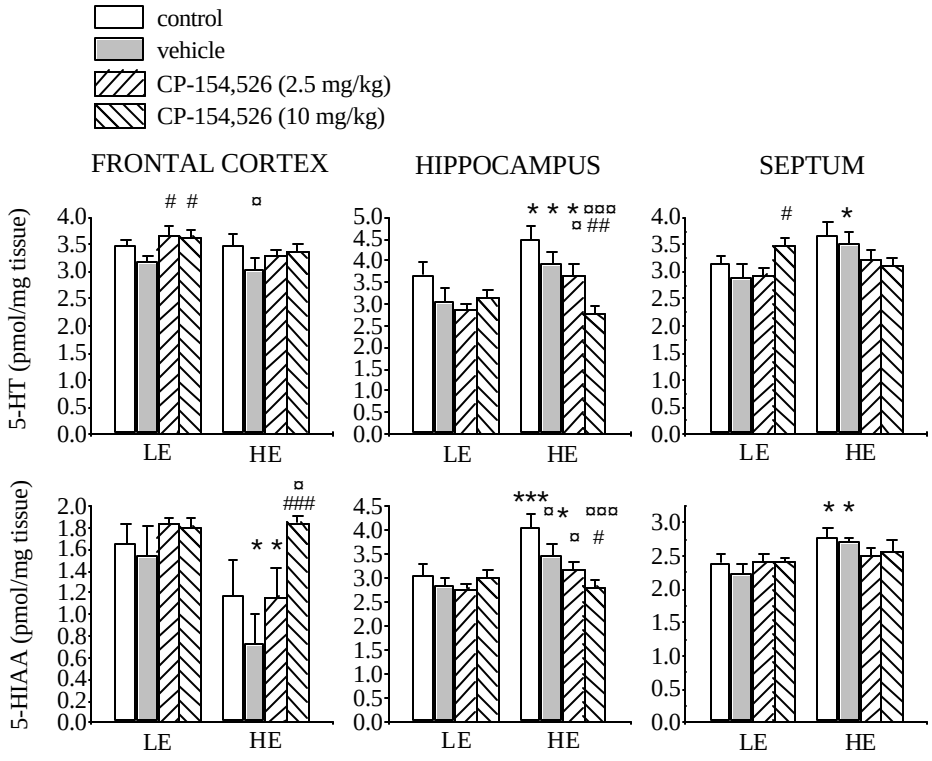

Figure 6. The effect of long-term treatment with CP-154,526 on 5-HT and 5-HIAA in the frontal cortex, hippocampus and septum (mean ± SEM). *, ** - $p<0.05$, $p<0.01$ as compared to the corresponding LE group; #, ### - $p<0.05$, $p<0.001$ as compared to the corresponding vehicle group; ¤, ¤¤¤ - $p<0.05$, $p<0.001$ as compared to the corresponding control group.

In the hippocampus, there was a significant Treatment effect, as well as a Baseline Activity effect on 5-HT ($F(3, 56)=6.67$, $p<0.001$ and $F(1, 56)=8.42$, $p<0.01$; respectively), while Baseline Activity x Treatment interaction remained just above the conventional level of statistical significance ($F(3, 56)=2.53$, $p=0.066$). HE animals had higher levels of 5-HT, except for the 10 mg/kg CP-154,526 group. There were no differences between vehicle and drug treatment in the LE group. Administration of CP-154,526 significantly decreased 5-HT in the HE animals, also showing dose-dependency. A significant Treatment effect, as well as Baseline Activity effect were found for 5-HIAA ($F(3, 56)=4.85$, $p<0.01$) and $F(1, 56)=11.94$, $p<0.01$, respectively).

Also, a significant Baseline Activity x Treatment interaction was revealed on 5-HIAA ($F(3, 56)=3.53$, $p<0.05$). HE animals in both control groups had higher levels of 5-HIAA. The 5-HIAA levels were decreased by vehicle treatment and similarly to 5-HT levels, further decreased by CP-154,526 treatment. No treatment affected 5-HIAA levels in LE animals.

In the striatum, no significant effects were found. In the septum, a borderline Baseline Activity effect and a Baseline Activity x Treatment interaction were found on 5-HT levels ($F(1, 56)=3.48$, $p=0.067$ and $F(3, 56)=2.36$, $p=0.082$, respectively). There was a trend of higher levels of 5-HT in HE animals in the control groups. A significant Baseline Activity effect was revealed on 5-HIAA levels in septum ($F(1, 56)=9.32$, $p<0.01$). HE animals had significantly higher levels of 5-HIAA in the control group. This difference between LE and HE rats remained unchanged after vehicle treatment, but disappeared after CP-154,526 treatment.

These results demonstrate that not only are there differences in the 5-HT-ergic system between rats with low or high exploratory activity, but these traits also influence qualitatively the role of CRF in the regulation of the 5-HT system.

In rats selected on the basis of their rearing behavior in a novel environment, Thiel et al. (1999) found lower tissue levels of 5-HT in the medial frontal cortex, a brain region critical in novelty-related behavior, to be associated with high rearing behavior. Although rearing behavior was also incorporated in the criterion of selection in the present experiment, no such differences in 5-HT levels were found in the frontal cortex in the present study. However, the HE rats displayed lower levels of 5-HIAA in the frontal cortex, indicative of decreased 5-HT metabolism in this region.

CP-154,526 had a dose-dependent increasing effect on 5-HIAA in frontal cortex in HE animals. A statistically unsignificant trend in the same direction in the frontal cortex was described above in Experiments 1 and 2, using 2.5 mg/kg of CP-154,526 and animals not preselected. It is now evident that a higher dose of the drug is required to reveal the full effect of CRF_1 receptor blockade on 5-HT, and that it occurs only in HE animals. In the study by Isogawa et al. (2000), a tendency of increase in frontal cortex extracellular 5-HT after acute CRF_1 receptor blockade was displayed, that remained unsignificant possibly due to the high interindividual variations. Together, these findings suggest that both acute and chronic CRF_1 receptor blockade influence 5-HT-ergic neurotransmission in the frontal cortex, but these effects are highly dependent on individual characteristics of the animals.

In the hippocampus and septum, chronic CRF_1 receptor blockade dose-dependently reduced 5-HT and 5-HIAA in HE rats, in which there were higher levels of both in the control groups, therefore eliminating the difference between LE and HE animals. As both i.c.v. and intra-raphe CRF have been found to decrease 5-HT release in septum in rats (Price et al. 2001) and i.c.v. administration of a CRF antagonist has been found to prevent the swim stress-induced decrease of extracellular 5-HT in septum (Price et al. 2002), these findings support the notion of CRF-ergic regulation of anxious states through septal 5-HT-ergic activity (Price et al. 2002).

Dorsal raphe (DR) 5-HT-ergic neurons that are the main source of 5-HT-ergic input in frontal cortex, are innervated by CRF-immunoreactive fibers and the in vivo administration of CRF has inhibitory effects on DR discharge, while a CRF receptor nonselective antagonist and CRF_1 selective antagonist have been found to attenuate this inhibition (Kirby et al. 2000). Our present findings suggest that in HE rats, which have a significantly lower levels of 5-HT metabolism in the frontal cortex, CRF has a stronger inhibitory effect on 5-HT neurons. The hippocampus and septum are mainly innervated by 5-HT-ergic projections from the median raphe. The different effects of CRF_1 receptor blockade in the regions presently studied in comparison with regions receiving input from the same or different 5-HT-ergic nuclei is indicative of the different roles that CRF-ergic mechanisms play in the modulation of 5-HT system in distinct brain regions.

One has to consider the fact that while CRF_1 receptor blockade eliminates these differences in 5-HT measures, it does not attenuate behavioral differences. It should be noted, however, that we have no idea whether acute CRF_1 receptor blockade, which in Experiment 3 eliminated the difference between LE and HE rats, has a similar effect on 5-HT turnover in HE rats. If so, 5-HT metabolism would simply dissociate from regulation of exploratory behavior in an environment increasingly familiar.

3.4. Experiment 3b

3.4.1. The effect of acute administration of CP-154,526 (10 mg/kg) on exploratory behavior in rats previously acquainted with the test apparatus

In an attempt to specify the rather surprising effects of CP-154,526 on exploratory behavior that were found in Experiment 3a, another experiment was carried out, in which animals (n=24) were either pre-tested in the exploration box test for two days

or not. As a test, both groups of animals were administered either vehicle or CP-154,526 (10 mg/kg) and tested in the exploration box test, that was the first encounter with the test apparatus for half animals and third encounter for the other half. Two-factor ANOVA revealed a significant Drug effect on line crossing and investigation of objects ($F(1, 20)=5.52$ and 4.42, $p<0.05$). A significant Testing effect was revealed on investigation of the objects ($F(1, 20)=4.64$, $p<0.05$) and remained above the level of statistical significance on line crossing ($F(1, 20)=4.24$, $p= 0.051$). Post-hoc tests showed that the single-tested CP-154,526 group had the lowest levels of exploration as compared to all other three groups (Fig. 7). At the same time, there was a drop in some measures of the repeatedly tested CP-154,526 group that remained statistically not significant but nevertheless were similar to that revealed in Experiment 3a, again suggestive that sufficient function of CRF_1 receptors is necessary for normal neotic behavior.

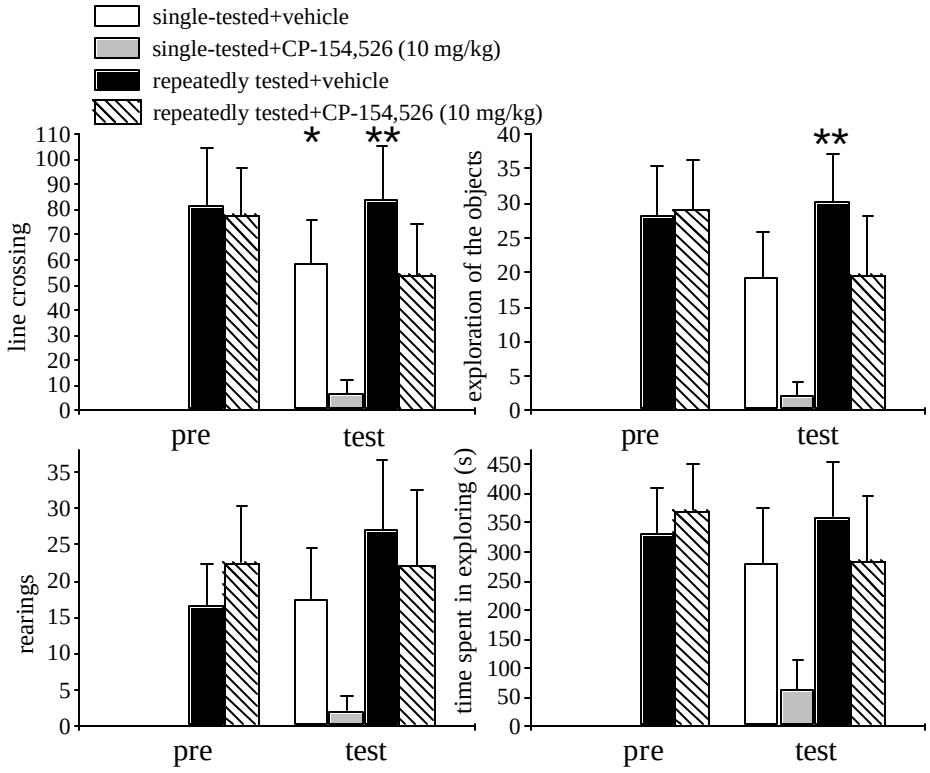

Figure 7. The effect of acute administration of CP-154,526 (10 mg/kg) on exploratory behavior in rats previously acquainted with the test apparatus (mean ± SEM). *, ** - $p<0.05$, $p<0.01$ as compared to the corresponding single-tested+CP group. pre – second baseline activity measurement. Data on enterings, latency and sum of exploratory activity are not shown.

3.5. Experiment 4

3.5.1. The effect of CP-154,526 on amphetamine-induced behavior in open field test

The acute effect of CP-154,526 in HE in the exploration box in Experiment 3 was unexpected, and made us hypothesize that this be due to a motivation-decreasing effect of CRF_1 blockade in an environment previously made familiar as suggested before (Harro et al. 2001). A decrease in DA-mediated motivational mechanisms in a familiar environment could aggravate the effect of anxiety brought about by the injection procedure. Hence we decided to study the effect of CP-154,526 in combination with amphetamine treatment in the open field test.

Amphetamine failed to statistically significantly increase behavioral activity in the dose 0.5 mg/kg either in LE and HE groups as compared to the vehicle treatment (Fig. 8). The administration of 0.5 mg/kg amphetamine increased line crossing, object investigation and time of exploratory activity only in CP-154,526 (5 mg/kg) treated HE animals. However, there was no clear-cut potentiating effect of CRF_1 receptor blockade.

The dose of amphetamine (0.5 mg/kg) was selected on the basis of our previous experiments, in which it has provided a clear psychomotor activation (e.g. Otter et al. 1997). Unfortunately, the effect of amphetamine was smaller and statistically not significant with the given number of rats per group in the present experiment, possibly due to methodological variances. The tendency of increase in activity elicited by amphetamine was apparently not modified much by CP-154,526. It has to be noted, though, that in addition to DA release, amphetamine administration also activates NA system, and partly 5-HT system. It is possible that in the present experiment, those effects interfered with the DA-ergic system. Nevertheless, it should be noted that combination of CRF_1 receptor blockade and amphetamine differentiated the LE and HE animals in the open field test best by increasing the activity of HE animals. This result is interesting in the context of the potentiating effects of CRF administration on sensitisation to psychostimulants (Cador et al. 1993), and suggestive of differences in the reactions of DA-ergic system to CRF_1 receptor blockade in these animals.

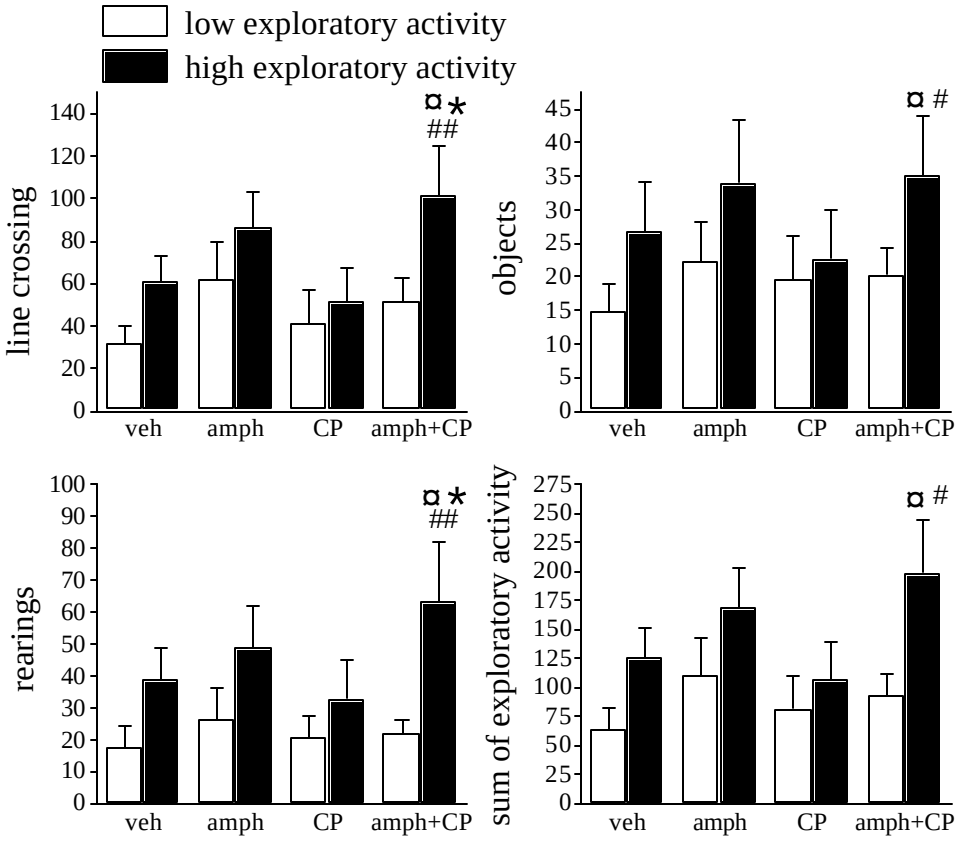

Fig. 8. The effect of amphetamine (0.5 mg/kg) administration on rat behavior in the open field after CRF_1 receptor blockade by CP-154,526 (5 mg/kg) treatment (mean ± SEM). ¤ - $p<0.05$ as compared to the corresponding LE group; * - $p<0.05$ as compared to the corresponding vehicle group; # - $p<0.05$, ## - $p<0.01$ as compared to the corresponding CP-154,526 group. Data on enterings, latency and time spent exploring are not shown.

3.6. Experiment 5

3.6.1. The associations between transcription factor AP-2 in locus coeruleus, medial and dorsal raphe and ventral tegmentum and tissue monoamine levels in frontal cortex, hippocampus, nucleus accumbens, septum, nucleus accumbens and striatum

The regression analyses given in Table 4 show that there were many significant correlations between both AP-2 isoforms and monoamines in different brain regions. Again associations were found between AP-2 levels in the locus coeruleus and NA system. Negative correlations appeared between frontal cortex, hippocampus and striatum NA levels and AP-2 in the LC, which was similar to the results of

Experiment 2, where negative correlations were found in the naïve and CP-154,526 groups. The fact that the present experiment revealed significant negative correlations between AP-2 levels and NA in untreated animals, that remained unsignificant in Experiment 2 with small number of observations, gives additional support to the stress-relieving effect of CP-154,526 treatment hypothesized above.

In the abovementioned study of Damberg et al. (2001b), significant positive associations were discovered between AP-2 and 5-HT-ergic systems in the frontal cortex, septum and hippocampus. While in that study AP-2 levels were measured in the whole brainstem, we studied two brain regions containing populations of 5-HT-ergic neurons, namely medial and dorsal raphe. AP-2 in the medial raphe correlated positively with 5-HT in hippocampus and negatively with 5-HIAA in nucleus accumbens, while AP-2 in the dorsal raphe correlated negatively with both 5-HT and 5-HIAA in the striatum, that is considered functionally similar to the nucleus accumbens. Interestingly, at the same time the DA system activity in nucleus accumbens correlated negatively with AP-2 levels in the medial raphe and positively in the dorsal raphe, suggestive of different control of these two regions over accumbal DA-ergic activity that therefore seems to differ from their control of 5-HT-ergic activity in the limbic structures. Interestingly, negative correlations between dorsal raphe AP-2 levels and NA levels were found in the frontal cortex and hippocampus, that were similar to those found with AP-2 in the locus coeruleus. This result implicitly confirms the 5-HT-ergic control from dorsal raphe over locus coeruleus (for an example, Harro and Oreland, 2001). The AP-2 levels in the ventral tegmentum correlated positively with 5-HT-ergic activity in the frontal cortex and limbic structures. Considering the associations between AP-2 in both raphe regions and the activity of DA system in nucleus accumbens, it seems that there are reciprocal connections between these systems over the control of limbic system activity, as both seem to regulate the activity of the other's main transmitter in the nucleus accumbens. The AP-2 levels in the ventral tegmentum correlated negatively with DA metabolism in the frontal cortex, which is compatible with tegmental control over frontal DA-ergic activity.

The present results again suggest that the transcription factor AP-2 is involved in the regulation of the monoaminergic systems and might therefore be involved in the generation many physiological and psychical states.

4. CONCLUDING REMARKS

While the CRF_1 receptor has been considered 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 on exploratory behavior. It was found that CRF_1 receptor blockade modulates exploratory behavior, while this modulation is dependent on drug-environment interactions, as repeated administration of CP-154,526 either increased exploratory activity, withheld the habituation-associated increase in it or decreased exploratory activity on the first administration of the drug when the test situation was already familiar to the animal. While the present studies reproduced the findings of differences in 5-HT systems in animals with differences in baseline anxiety levels, the most interesting result herein is that long-term blockade of CRF_1 receptors dose-dependently affected 5-HT and its metabolism in distinct brain regions differentially in LE and HE animals. It is therefore apparent that interactions between 5-HT and CRF systems underlie the individual differences in exploratory activity levels. Long-term blockade of CRF_1 receptors also decreased NA metabolism in frontal cortex but had no effect on transcription factor AP-2 levels in locus coeruleus. Yet, it was found to withhold changes in associations between AP-2 levels and NA levels in frontal cortex and hippocampus that were elicited by handling that might suggest that CRF_1 receptor antagonists are able to overrun some effects of heightened environmental stimulation. From the three studies of AP-2, many implications for it's regulation of monoamine systems in distinct brain regions may be drawn.

5. ACKNOWLEDGEMENTS

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Table 4. Transcription factor AP-2 correlations with monoamines in frontal cortex, hippocampus, nucleus accumbens, septum, nucleus accumbens and striatum (Experiment 5). Statistically significant results are marked in bold.

				AP-2α		AP-2ß	
Locus of AP-2 measurement		Locus of monoamine measurement		r^2	*p*-value	r^2	*p*-value
Medial raphe	5-HT system	nucleus accumbens	5-HIAA	-0.59	<**0.01**	-0.58	<**0.01**
		hippocampus	5-HT	0.46	<**0.05**	0.48	<**0.05**
	DA system	nucleus accumbens	DOPAC	-0.40	0.07	-0.37	0.10
		nucleus accumbens	HVA	-0.52	<**0.05**	-0.44	0.05
	NA system	septum	NA	-0.49	<**0.05**	-0.43	0.06
Locus coeruleus	NA system	frontal cortex	NA	-0.44	0.05	-0.57	<**0.01**
		hippocampus	NA	-0.25	0.31	-0.46	<**0.05**
		striatum	NA	-0.25	0.32	-0.48	<**0.05**
		nucleus accumbens	5-HT	0.53	<**0.05**	0.72	<**0.01**
		nucleus accumbens	5-HT turnover	-0.48	<**0.05**	-0.55	<**0.01**
Ventral tegmentum	5-HT system	frontal cortex	5-HT	0.48	<**0.05**	-0.09	0.72
		striatum	5-HIAA	0.53	<**0.05**	-0.01	0.96
		nucleus accumbens	5-HT	0.16	0.51	-0.56	<**0.05**
		nucleus accumbens	5-HIAA	0.51	<**0.05**	-0.14	0.57
	DA system	frontal cortex	DOPAC	-0.55	<**0.05**	0.22	0.48
		frontal cortex	DA turnover	-0.21	0.43	-0.55	<**0.05**
		nucleus accumbens	DOPAC	0.30	0.22	0.53	<**0.05**
Dorsal raphe	NA system	frontal cortex	NA	-0.27	0.28	-0.45	0.06
		hippocampus	NA	-0.56	<**0.05**	-0.53	<**0.05**
	DA system	hippocampus	DA	0.45	0.06	0.46	<**0.05**
		striatum	DOPAC	0.40	0.11	0.41	0.10
		nucleus accumbens	DOPAC	0.46	0.06	0.64	<**0.01**
		nucleus accumbens	DA turnover	0.51	<**0.05**	0.61	<**0.05**
	5-HT system	striatum	5-HIAA	-0.42	0.10	-0.47	0.06
		striatum	5-HT turnover	-0.48	<**0.05**	-0.31	0.22

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To Brain Research

EFFECT OF LONG-TERM CORTICOTROPIN-RELEASING FACTOR 1 RECEPTOR BLOCKADE ON SEROTONIN METABOLISM IN RATS WITH HIGH AND LOW EXPLORATORY ACTIVITY

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Abstract

Interactions of corticotropin-releasing factor (CRF) and serotonin (5-HT) systems are involved in anxiety-related behaviors. We have compared the effect of a selective CRF_1 receptor antagonist CP-154,526 (2.5 or 10 mg/kg for two weeks) on tissue 5-HT and 5-hydroxyindole acetic acid (5-HIAA) in rats with low or high exploratory activity (HE vs. LE rats). Brain region specific differences were found between LE and HE rats, and the long-term blockade of CRF_1 receptors had distinct effects in LE and HE animals. Interactions between 5-HT and CRF systems may thus underlie the individual differences in novelty-related behavior.

Theme: Neural basis of behavior

Topic: Neuropeptides and behavior

Keywords: corticotropin-releasing factor, serotonin, exploratory behavior, individual differences

Both corticotropin-releasing factor (CRF) and serotonin (5-HT) have been implicated in the etiology of stress (for reviews, see [1, 13]), and there are interactions between these systems, which may be important for the organism's reactions to environmental challenges. Intracerebroventricular (i.c.v) administration of CRF induces 5-HT overflow in the hippocampus [10], and the CRF_1 receptor antagonist CP-154,526 has been found to decrease baseline hippocampal extracellular 5-HT levels [8]. Since hippocampal 5-HT release contributes to anxiety [2], it can therefore be suggested that the reported anxiolytic effects of CRF antagonists (for a review, see [7]) are at least partly mediated through CRF-ergic regulation of hippocampal 5-HT system. For an example, swim stress-induced elevation of hippocampal 5-HT levels was attenuated in mice chronically treated with the selective CRF_1 antagonist NBI30775 [15]. Price et al. [17] have speculated that CRF serves as a neurotransmitter in the dorsal raphe nucleus to regulate the forebrain 5-HT function, in an analogous manner to its role in the noradrenergic nucleus locus coeruleus [22].

It has been shown previously that rats preselected on the basis of anxiety-related behavior will display consistently different behavioral patterns in many tests of anxiety [6; 9]. These behavioral patterns have been accompanied with differences in the activity of 5-HT-ergic system [3; 20]. Umriukhin et al. [21] found that the rats selectively bred for low anxiety-related behavior in the elevated plus maze show a greater release of 5-HT in hippocampus in response to stressor. The same laboratory has reported that CRF_1 receptor blockade had anxiolytic effect only in rats selectively bred for high anxiety-related behavior [11].

In our previous study, demonstrating anxiolytic effects of chronic but not acute CRF_1 receptor blockade [14], the repeated administration of CP-154,526 brought about tendencies of elevation in 5-HT metabolism in two independent

experiments, which nevertheless remained statistically not significant. Not excluded, however, that CRF_1 receptor blockade does not have a uniform influence in all animals. It is possible that these effects are linked with stable individual differences that are displayed in exploratory behavior.

The aims of the present experiment were to study the effect of long-term CRF_1 receptor blockade by chronic treatment with CP-154,526, a selective CRF_1 receptor antagonist, on 5-HT metabolism ex vivo in rats preselected on the basis of exploratory activity, and the dose-dependency of the eventual effects.

Male Wistar rats (n=64) were housed in groups of four in transparent macrolone cages under controlled light cycle (lights on from 08:00 h to 20.00 h) and temperature (19-21° C), with free access to tap water and food pellets (diet R70, Lactamin, Sweden). The experimental protocol was approved by the Ethics Committee of the University of Tartu. Rats were pre-tested in the exploration box [4; 16] on two consecutive days for determination of baseline activity levels. Then the animals were divided into low exploratory activity (LE) and high exploratory activity (HE) groups on the basis of the median value of the sum of exploratory activity during the second testing, a measure which is a reliable predictor of individual novelty-related activity (unpublished data). CP-154,526 was suspended in distilled water by adding a few drops of Tween 85 and administered intraperitoneally for 14 days in doses of 2.5 and 10 mg/kg. Two control groups were used that received either vehicle (vehicle group) or no treatment (control group). Animals were decapitated on day 14, and tissue samples dissected on ice. Monoamines were measured by HPLC-ECD as described previously [5]. Data of the monoamine measurements were analysed with two-factor ANOVA (Baseline Activity x Treatment). Subsequent pairwise comparisons were made with Fisher's LSD test.

In the frontal cortex, a significant Treatment effect was revealed on 5-HT ($F(3, 56)=2.86$, $p<0.05$). Vehicle treatment had a tendency of decreasing 5-HT content and chronic administration of CP-154,526 significantly elevated 5-HT levels in both doses in LE animals, as compared to the corresponding vehicle group (Fig. 1). A significant Treatment effect, as well as Baseline Activity effect were revealed on 5-HIAA ($F(3, 56)=3.37$, $p<0.05$ and $F(1, 56)=9.87$, $p<0.01$; respectively). HE animals had significantly lower levels of 5-HIAA as compared to the corresponding LE animals and treatment with CP-154,526 dose-dependently elevated the 5-HIAA levels. No treatment had any effect on 5-HIAA in the LE group.

In the hippocampus, there was a significant Treatment effect, as well as a Baseline Activity effect on 5-HT ($F(3, 56)=6.67$, $p<0.001$ and $F(1, 56)=8.42$, $p<0.01$; respectively), while Baseline Activity x Treatment interaction remained just above the conventional level of statistical significance ($F(3, 56)=2.53$, $p=0.066$). HE animals had higher levels of 5-HT, except for the 10 mg/kg CP-154,526 group. There were no differences between vehicle and drug treatment in the LE group. Administration of CP-154,526 significantly decreased 5-HT in the HE animals, also showing dose-dependency. A significant Treatment effect, as well as Baseline Activity effect were found for 5-HIAA ($F(3, 56)=4.85$, $p<0.01$) and $F(1, 56)=11.94$, $p<0.01$, respectively). Also, a significant Baseline Activity x Treatment interaction was revealed on 5-HIAA ($F(3, 56)=3.53$, $p<0.05$). HE animals in both control groups had higher levels of 5-HIAA. The 5-HIAA levels were decreased by vehicle treatment and similarly to 5-HT levels, further decreased by CP-154,526 treatment. No treatment affected 5-HIAA levels in LE animals.

In the striatum, no significant effects were found. In the septum, a borderline Baseline Activity effect and a Baseline Activity x Treatment interaction were found

on 5-HT levels ($F(1, 56)=3.48$, $p=0.067$ and $F(3, 56)=2.36$, $p=0.082$, respectively). There was a trend of higher levels of 5-HT in HE animals in the control groups. A significant Baseline Activity effect was revealed on 5-HIAA levels in septum ($F(1, 56)=9.32$, $p<0.01$). HE animals had significantly higher levels of 5-HIAA in the control group. This difference between LE and HE rats remained unchanged after vehicle treatment, but disappeared after CP-154,526 treatment.

The present results demonstrate that not only are there differences in the 5-HT-ergic system between rats with low or high exploratory activity, but these traits also influence qualitatively the role of CRF in the regulation of the 5-HT system.

In rats selected on the basis of their rearing behavior in a novel environment, Thiel et al. [20] found lower tissue levels of 5-HT in the medial frontal cortex, a brain region critical in novelty-related behavior, to be associated with high rearing behavior. Although rearing behavior was also incorporated in the criterion of selection in the present experiment, no such differences in 5-HT levels were found in the frontal cortex in the present study. However, the HE rats displayed lower levels of 5-HIAA in the frontal cortex, indicative of decreased 5-HT metabolism in this region.

CP-154,526 had a dose-dependent increasing effect on 5-HIAA in frontal cortex in HE animals. We have previously described a statistically unsignificant trend in the same direction in the frontal cortex in two experiments, using 2.5 mg/kg of CP-154,526 and animals not preselected [14]. It is now evident that a higher dose of the drug is required to reveal the full effect of CRF_1 receptor blockade on 5-HT, and that it occurs only in HE animals. In the study by Isogawa et al. [8], a tendency of increase in frontal cortex extracellular 5-HT after acute CRF_1 receptor blockade was displayed, that remained unsignificant possibly due to the high interindividual variations. Together, these findings suggest that both acute and chronic CRF_1 receptor

blockade influence 5-HT-ergic neurotransmission in the frontal cortex, but these effects are highly dependent on individual characteristics of the animals.

In the hippocampus and septum, chronic CRF_1 receptor blockade dose-dependently reduced 5-HT and 5-HIAA in HE rats, in which there were higher levels of both in the control groups, therefore eliminating the difference between LE and HE animals. As both i.c.v. and intra-raphe CRF have been found to decrease 5-HT release in septum in rats [18] and i.c.v. administration of a CRF antagonist has been found to prevent the swim stress-induced decrease of extracellular 5-HT in septum [19], these findings support the notion of CRF-ergic regulation of anxious states through septal 5-HT-ergic activity [19].

Dorsal raphe (DR) 5-HT-ergic neurons that are the main source of 5-HT-ergic input in frontal cortex, are innervated by CRF-immunoreactive fibers and the in vivo administration of CRF has inhibitory effects on DR discharge, while a CRF receptor nonselective antagonist and CRF_1 selective antagonist have been found to attenuate this inhibition [12]. Our present findings suggest that in HE rats, which have a significantly lower levels of 5-HT metabolism in the frontal cortex, CRF has a stronger inhibitory effect on 5-HT neurons. The hippocampus and septum are mainly innervated by 5-HT-ergic projections from the median raphe. The different effects of CRF_1 receptor blockade in the regions presently studied in comparison with regions receiving input from the same or different 5-HT-ergic nuclei is indicative of the different roles that CRF-ergic mechanisms play in the modulation of 5-HT system in distinct brain regions.

While the present study reproduces the findings of differences in 5-HT systems in animals with differences in baseline anxiety levels, the most interesting result herein is that long-term blockade of CRF_1 receptors dose-dependently affected 5-HT

and its metabolism in distinct brain regions differentially in LE and HE animals. It is therefore apparent that interactions between 5-HT and CRF systems underlie the individual differences in exploratory activity levels.

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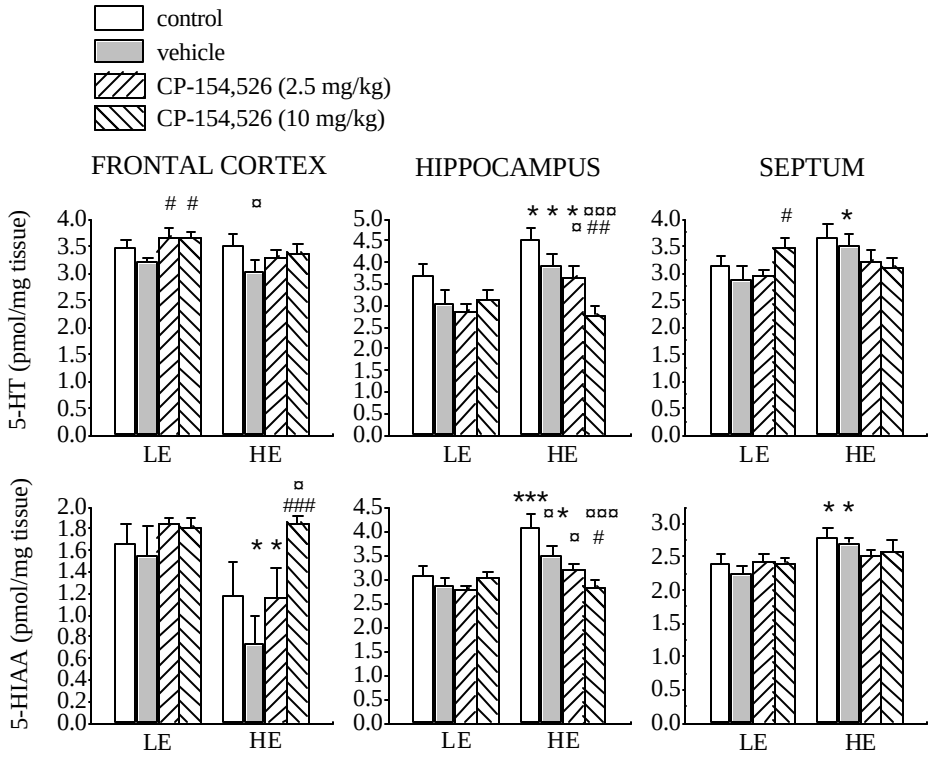

Figure 1. The effect of long-term treatment with CP-154,526 on 5-HT and 5-HIAA in the frontal cortex, hippocampus and septum. *, ** - $p<0.05$, $p<0.01$ as compared to the corresponding LE group; #, ### - $p<0.05$, $p<0.001$ as compared to the corresponding vehicle group; ¤, ¤¤¤ - $p<0.05$, $p<0.001$ as compared to the corresponding control group.