ORIGINAL ARTICLE

Differential effects of stable elevated levels of corticotropin-releasing hormone and systemic corticosterone on various types of rat learning

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Abstract

OBJECTIVES: Stress activates the hypothalamic-pituitary-adrenal (HPA) axis. This activation is executed mainly through the release of corticosteroids from adrenal that subsequently exert negative feedback on corticosterone-releasing hormone (CRH) production. The effects of corticosterone on learning and memory has been studied intensively. Less is known about the effect of CRH on cognitive phenomena.

DESIGN AND SETTING: The present study aimed at studying the separate effects of stress cascade hormones, namely CRH and corticosterone, on learning and memory in a battery of learning tasks.

RESULTS: Long-term administration of CRH led to a transient impairment of spatial performance in the active allothetic place avoidance (AAPA) task requiring cognitive coordination, whilst co-application of CRH and corticosterone resulted in permanent impairment in this task. Corticosterone alone impaired the long-term retention of passive avoidance. CRH alone exerted no effect on the working memory version of the Morris water maze (MWM) and inhibitory avoidance.

CONCLUSIONS: Our results suggest differential effects of stress cascade hormones on various types of behavior.

Abbreviations:

AAPA	- active allothetic place avoidance
ACTH	- adrenocorticotropic hormone
ANOVA	- analysis of variance
CCW	- counter-clockwise
CRH	- corticosterone releasing hormone
CS	- corticosterone
CW	- clockwise
DMP	- delayed matching to place
EDTA	- ethylenediaminetetraacetic acid
HPA	- hypothalamic-pituitary-adrenal
HPLC	- high-performance liquid chromatography
LED	- light-emitting diode
MWM	- Morris water maze

INTRODUCTION

Stressful experiences generally trigger a stress neuroendocrine cascade. The corticosterone releasing hormone (CRH) is critical in playing a central role in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The activation of the HPA axis results in release of corticosteroids from adrenal that subsequently exert negative feedback on CRH production via various brain structures. Excessive exposure to corticosteroids or other factors may lead to damage of the certain brain areas including the hippocampus and by its impairment, an inhib-

itory effect on the hypothalamus and pituitary. This situation results in hyperactivity and deliberation of the HPA axis (Hoschl & Hajek 2001). Therefore, study of deliberation of the HPA axis represents a promising approach in the research of various neuropsychiatric diseases. Animal models show that prolonged exposure to elevated corticosterone levels during stress episodes leads to impairment of neuronal plasticity, particularly in the hippocampus (Dagyte *et al.* 2009; Lussier *et al.* 2009). The hippocampus contains the highest concentration of glucocorticoid receptors in the brain and it plays a critical role in negative feedback in the HPA axis (Chauveau *et al.* 2010). The neurodegenerative changes may lead to deficits in memory and cognition (Swaab *et al.* 2005). It was shown that corticosterone impairs reference memory before working memory in rats. The reasons are unknown but it seems that an acutely elevated corticosterone level is not the cause. It is possible that the deficit is related to suppression of the HPA axis (Coburn-Litvak *et al.* 2003).

Conventionally, it is presumed that neurodegenerative changes are attributable only to long-term highly elevated levels of corticoids. Nevertheless, each component of a neurochemical cascade of the stress response, especially in the case of dysregulation of the HPA axis, could be manifested qualitatively and quantitatively by different means and influence different aspects of animal behavior. We propose that elevated levels of CRH (in combination with corticosterone) may contribute to behavioral changes and memory deficits rather than the influence of corticosterone alone.

CRH regulates adaptive behaviors that occur during stress response but its high levels lead to anxiety, sleep disruption, disturbances in food intake, energy balance and adverse changes in metabolic, cardiovascular and immune functions (Heinrichs & Koob 2004). Similarly HPA axis is less sensitive to stress during pregnancy due to decreasing of CRH production (Russell *et al.* 2008). In addition, CRH dysfunction probably plays a role in various neuropsychiatric and neuroendocrine diseases (Grammatopoulos & Chrousos 2002). CRH and its receptors are abundant in the central nervous system and a variety of peripheral tissues (Grammatopoulos *et al.* 2002), nonetheless, effects of their activation on the memory and cognitive function was studied relatively sparsely.

Therefore, the present study is aimed at investigating the separate and combined effects of long-term stable elevated levels of CRH and corticosterone on cognitive functions in rats. Animals were randomly assigned to three experimental and four control groups before the experiments; here we outline experimental treatments First group was exposed to long-term elevated levels of corticosterone (CS) via subcutaneously implanted corticosterone-releasing pellets. This manipulation resulted in the suppression of CRH secretion (abbreviated as **↑CS**). Second group was exposed to long-term elevated levels of CRH (applied intracerebroventricularly by an osmotic mini-pump). This manipulation resulted in the subsequently increased release of corticosterone (group **↑CRH↑CS**). Third additional group had long-term elevated levels of CRH (applied intracerebroventricularly by an osmotic pump) and were surgically adrenalectomized so that (group **↑CRH**) and their basal level of CS was maintained via corticosterone subcutaneous pellets. There were additional four control groups (*see Methods*). This configuration allowed us to study the separate and collective influences of CRH and corticosterone on cognitive functions and also the delayed effects of elevated levels of CRH and corticosterone on behavior as well.

MATERIAL AND METHODS

Animals

Fifty-four naive adult male Long-Evans rats (3–4 months old, 300–400 g) were used in this study. Animals were obtained from the accredited breeding colony of the Institute of Physiology of The Czech Academy of Sciences. They were housed in pairs in plastic transparent cages (30 × 30 × 40 cm), at a constant temperature (22 °C) with 45% relative humidity, a 12/12 light/dark cycle (with lights on at 7:00), standard laboratory and food and water *ad libitum*. All the experiments took place between 8:00–16:00 (i.e. in the daylight hours). Rats were first habituated to the animal room for 14 days prior to experiments and during this period they were handled for 5 min each day. All experimental manipulations were pursued in accordance with the Animal Protection Code of the Czech Republic, and with EU directive 86/609/EEC and NIH guidelines.

Design of groups

The animals were divided in seven (3 experimental and 4 control) groups:

1) The first group (**↑CS***)* (n=10) was administered with corticosterone (200 mg in a subcutaneous pellet, release time **21 days**, 9.5 mg per animal per day).
2) The second group (**↑CRH↑CS**) (n=11) had an implanted osmotic pump into the right lateral cerebral ventricle which delivered CRH (1.5μg per animal per day) for **28 days**.
3) The third group (**↑CRH**) (n=6) was administered with CRH in equal dosage as the group (**↑CRH↑CS**). Animals in this group were surgically adrenalectomized during anesthesia together with the osmotic pump implantation and brought to basal levels of CS with a subcutaneous pellet (35 μg per subject per pellet (releasing 1.4 mg per animal per day) for **21 days**.
4) Intact control group (n=10) had no surgical or pharmacological treatment.

5–7) There were three additional control groups, in order to exclude the effects of surgical manipulations. Sham-treated animals implanted with a placebo (cholesterol) pellet (n=6). Osmotic pumps

were implanted to animals in another control group delivering artificial cerebrospinal fluid (at the same volume per day as the CRH) into the right lateral cerebral ventricle (n=6). The last group was surgically sham-adrenalectomized (n=6). All groups had identical behavioral procedures. No effects of these surgical manipulations were detected during the behavioral training and there was no difference between control groups.

Unless otherwise stated, all standard chemicals and peptides were purchased from Sigma Aldrich, Czech Republic. Corticosterone and cholesterol subcutaneous pellets were purchased from Innovative Research of America (USA), Osmotic pumps (model 2004) were provided by Alzet (USA).

Surgical procedures

All surgical interventions were done under deep total anesthesia by xylazine (Xylapan 40 mg/kg body weight) and ketamine (Narketan, 40 mg/kg b. w.). Corticosterone in the first group (**↑CS***)* was administered by subcutaneous pellet implanted laterally to the right hypogastric region. Corticosterone pellets were implanted on the first day of the second experimental week in the first period of the experiment. CRH in the groups (**↑CRH↑CS**) and (**↑CRH**) was administered by subcutaneous osmotic pump placed in suprascapular region and delivering the CRH into the right lateral cerebral ventricle. Intracerebroventricular 22-gauge cannulae were fixed *in situ* (AP = –0.92, L=1.8, V=3.2; Paxinos & Watson 2005) in stereotaxic apparatus by means of dental acrylic (Duracrol). Subsequently, the skin was sutured and lidocaine and Septonex were applied locally to prevent pain and infection. Pumps were implanted on the first day of the first week in the first phase of the experiment. Adrenalectomy in the group (**↑CRH**) was pursued via dorsal approach by medial skin incision and paravertebral blunt preparation of muscles. Adrenal glands were then gently removed. This group was substituted by low-dose corticosterone subcutaneous pellet applied as in the group (**↑CS***)*. Adrenalectomy and substitutive pellet implantation was done on the first day of the second experimental week in the first experimental period.

Behavioral tests and instrumentation

The study was divided into following two phases (for overview of study design, see Figure 1). **The first period** lasted 4 weeks. The battery of memory tests was administered to evaluate learning during the last two weeks of the first period. The tests were repeated in the **second period**, which has begun 5 weeks after end of the first period. The reason for this approach was to assess both the acute effect of elevated CRH and CS levels and the long-term consequences of the treatment. The tests included the active allothetic place avoidance (AAPA) task, the Morris water maze (MWM; in two versions) and inhibitory avoidance (step-through task). The testing in each period lasted 10 days (Figure 1). All experimental testing was carried out between 9:00 and 16:00.

Active allothetic place avoidance (AAPA) task

The AAPA (***carousel maze)*** is relatively novel but well established behavioral task requiring spatial learning and cognitive coordination, which is highly dependent upon integrity of hippocampus (Cimadevilla *et al.* 2001) and was used in a number of pharmacological studies so far (Pastalkova *et al.* 2006, Stuchlik & Vales 2009; Stuchlik *et al.* 2009). Cognitive coordination has been defined as the animals' ability to separate spatial stimuli from the environment into a coherent representation of the arena and room, which are brought into conflict by arena rotation. The animals are required to select the room frame as the only relevant one for navigation (as the to-be-avoided sector is defined in this frame) and to ignore the arena frame in order to efficiently manage the task (Wesierska *et al.* 2005).

The AAPA apparatus as described previously (Stuchlik *et al.* 2009) consisted of metallic circular arena (diameter 80 cm), surrounded by a 30 cm high transparent Plexiglas wall and elevated 1 m above the floor of the experiment room containing an abundance of extra-maze landmarks. On a slowly rotating arena (1 rpm) a to-be-avoided 60° sector was defined by a tracking system; entrance of which was punished by a mild electric food-shock (50 Hz, 0.5 s, 0.4–0.7 mA). The animals wore an infrared light-emitting diode (LED) mounted on a rubber jacket worn by the rat. The position of the LED was recorded using a computer-based tracking system (iTrack; BioSignal Group, USA) located in an adjacent room. The tracking system recorded the rat's position every 40 ms and delivered foot shocks upon entrance into the shock sector. If the rat did not leave the sector immediately, additional shocks were given every 1 200 ms, but no more entrances were counted until the rat left the sector for more than 300 ms. The voltage level was individualized for each rat to elicit a rapid escape response but to prevent freezing. In most cases, animals responded appropriately to 0.5 mA and no differences in shock levels were observed between groups. The position time series were stored for off-line analysis (TrackAnalysis, BioSignal Group, USA) (Stuchlik *et al.* 2008). Each animal was tested (without preliminary habituation) in five subsequent daily sessions. Every session lasted 20 min and intervals between sessions were 24 hours. This test was applied as the first test sin both phases of behavioral screening.

Working memory version of Morris water maze (MWM)

In the present study we have employed a working memory version of MWM, in which the platform location is changed every day (Vales *et al.* 2006). The task requires allothetic spatial short-term memory. The MWM (Morris 1984; Stuchlik *et al.* 2007) was a

metallic circular tank (180 cm in diameter, 50 cm high) filled with water (depth 40 cm, 20 °C) with a small randomly placed transparent Plexiglas platform (10 cm in diameter), which is not visible to the swimming animal (submerged 2 cm below water level). MWM tests were performed in a dimly-lit room with many extramaze landmarks. Rats were released facing the wall at quasi-randomly ordered starting points; four starting points were used corresponding to the cardinal compass directions. The release of the rat into the water maze also started the tracking program. A trial stopped when the rat found the platform and climbed upon it. If the rat failed to find the platform in 60 s, the trial was stopped and the rat was lead to the platform by the experimenter. A computer-based tracking system (iTrack, Biosignal Group, USA) with an overhead TV camera recorded the rat's position every 40 ms. The position time series were stored for off-line analysis (TrackAnalysis, Biosignal Group, USA). The rat was allowed to stay on the platform for 10 s and then it was placed to a warmed waiting cage. One daily session consisted of 8 swims (trials) with an inter-trial interval of 1 min. The animals were tested on five subsequent days; and the location of platform was changed every day; the positions of platform were in the center of each quadrant (corresponding to NW, NE, SW, SE) and they were changed pseudo-randomly. Testing in the working memory version of MWM was pursued both in the first and second periods of testing (weeks 4 and 10 of the study; Figure 1).

A modified version of delayed matching to place (DMP) task in the MWM

Delayed matching to place task in the MWM was introduced by Steele & Morris (1999). We have modified this task in the present study to assess both intermediate-term and long term memory by introducing retrieval session at intervals of 1 min and. On day 1 of this testing animals were subjected to two training trials with stable platform location with an inter-trial interval if 1 min. Third trial (following 90 min after) with a platform in the same location was used to asses the intermediate-term memory. Finally, fourth session with platform in the stable location was administered after 24 hours to test the long-term memory. The animals were released from the quadrant opposite to the platform in all trials. This task tested the allothetic orientation, and it required both intermediate- and long-term memory. The task involved already pretrained animals (see previous section) which were capable of selecting the appropriate strategy. This test was administered only in the second phase of screening (week 11; see Figure 1)

Passive avoidance learning: Step-through task

Passive or inhibitory avoidance tasks in general measure the ability of animals to retain information about punishment (i.e., memory) in conditions where the stimuli are of the contextual nature; however the punished area is relatively well-defined in the sensory terms. The task was found to be dependent upon hippocampus in certain configurations and the present study employed it as a test of associative short- and long-term learning and memory (Izquierdo & Medina 1993). The task was pursued in an apparatus consisting of two boxes connected with a guillotine door (6 × 7 cm): one box (30 × 20 × 15 cm) was illuminated and another (25 × 16 × 15 cm) was dark. The test requires remembering of being shocked in the dark compartment and inhibition of spontaneous tendency to enter the dark compartment. The illuminated box was lighted by overhead lamp (60 W) from a distance of 1 m. Both boxes had grid floors, allowing mild constant-voltage AC foot

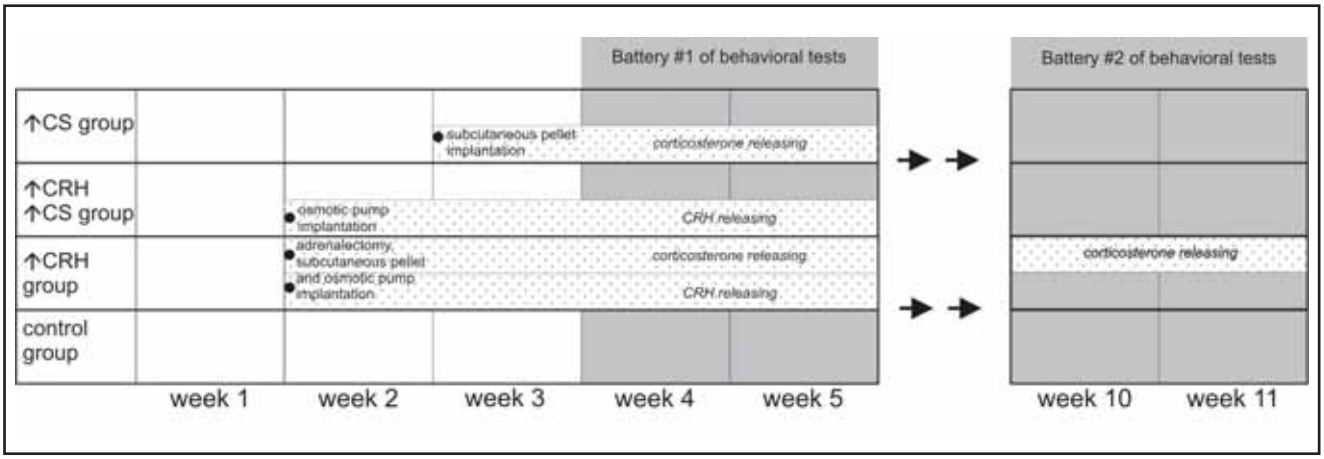

Fig. 1: Schematic diagram of experimental design and manipulations. The study lasted 11 weeks in total. Behavioral screening was administered in two consecutive phases (*shaded areas*), separated by a four-week delay (*bold arrows*). The first phase took place in the final two weeks of hormone treatments. Note that corticosterone (CS) administration in the group ↑**CS** was releasing for 3 weeks, whereas CRH was administered for four weeks, but all hormone application finished at the same time. Second battery of tests was applied in weeks 10 and 11 of the study, four weeks after hormone withdrawal. Basal corticosterone level in the adrenalectomized group ↑**CRH** was maintained until the end of experiment.

shocks (48 V, ~0.1–1.0 mA), which were administered in the dark compartment. The test was performed in two days. The first two sessions were habituation sessions (separated by 60 min) and the footshocks were applied only in the third session (acquisition session, 60 min after second habituation session). The forth session was a short-term memory retention assessment (with no shock; 60 min after conditioning), when the latency to enter the dark compartment was measured. Long-term memory retrieval was tested out at an interval of 24 h after the acquisition session; no shock was applied. If the animal did not enter the dark box within 5 min, the session was stopped. The inhibitory avoidance testing was done only in phase 1 of the behavioral testing (week 5; Figure 1), since the test would be difficult to repeat with the same animals.

Hormones and their application

Corticotropin-releasing hormone (CRH) was purchased from Sigma Aldrich, Czech Republic. Corticosterone and cholesterol subcutaneous pellets (35mg and 200mg per pellet) were purchased from Innovative Research of America (USA), Osmotic pumps (model 2004) were provided from Alzet (USA).

Blood samples were collected from the tail blood, at an interval max. 90 s after beginning of manipulation with a rat. Samples were collected in a separate sterile room at 9:00. The blood was put into a test tube with EDTA. The collected blood was subsequently centrifuged for 15min using the speed of 3 000 rotations per minute. The plasma was then immediately frozen. The samples were kept in the freezer under –80 °C.

The frozen samples were then transported into Institute of Endocrinology, Academy of Sciences, Prague, where they were analyzed with the following procedures under room temperature. For sample analysis the immunoradiometric assay was used for the *in vitro* determination of ACTH, a "sandwich" type of assay, supplied by Immunotech (France). This kit uses three mouse monoclonal antibodies directed against different epitopes of succinylated ACTH. The ACTH in the samples had to be first modified by succinylation, both to ensure binding to antibodies, and to enhance its stability. The acylated samples were first incubated in tubes coated with two different antibodies. Following this incubation, the contents of the tubes were aspirated, and solution containing third antibody, radiolabeled by ^{125}I, was added. After incubation, tubes were aspirated again and after washing twice, bound radioactivity was measured.

Serum cortisone concentrations were determined by a method using high HPLC with UV/VIS detection. Serum sample (200 µl) was diluted with releasing buffer (200 µl) and 4-androsten-3, 17-dione-11β-ol (40 ng per sample) was added as an internal standard. Buffered sample solution was extracted with diethyl ether (2 ml) for 1 min (2 000 rpm) and frozen. The organic phase containing released steroids was decanted and evaporated under the stream of nitrogen. To avoid possible column contamination from free fatty acids, the dry residue from the extract was dissolved in 80% methanol (1 ml, v/v) and n-hexane (1 ml). The mixture was extracted again for 1 min (2 000 rpm). N-hexane-containing phase was removed and discarded. The residual polar phase was evaporated at 55 °C and the dry residue was dissolved in 15 % acetonitrile (50 µl, v/v) and mixed vigorously to rinse the tube walls appropriately. The samples were then centrifuged (2 000 *g*, 3 min, 22 °C) and decanted solution transferred into vials. Simultaneously, blank samples and quality control samples were processed in the same way to avoid possible contamination and to determine procedural losses of individual metabolites.

The results were quantified using calculated calibration curve. The procedure of sample analysis has been described in detail previously (Simunkova *et al.* 2000).

Statistics

In the AAPA task, we measured the **number of entrances** into the punishment sector (reflecting efficiency in avoidance behavior), **maximum time avoided** (a measure of cumulative within-session spatial avoidance), the **time to first entrance** into the punished sector (measuring between-session spatial memory). All these parameters served as spatial performance measures. **Total distanced** walked by a rat in a session was used as a measure of locomotor activity. We also evaluated total session time spent in the clockwise (CW, with respect to the shock sector) quadrant since this variable has been found to uncover different, whilst equally effective spatial strategies in AAPA (Cimadevilla *et al.* 2001). In the MWM, the latency to find the platform was used as an established and informative measure of spatial performance. Different statistical methods were applied in order to match correctly the raw data and their distribution.

1. The AAPA and MWM variables were analyzed by a two-way ANOVA with daily sessions (or swims in the case of modified DMP) as a within-subject factor and groups as the between-subject factor, followed by a Newman-Keuls *post-hoc* test to inspect the differences between experimental group and controls.
2. Number of entrances, time to first entrance, and time spent in the CW quadrant in the AAPA and escape latencies in the water maze were naturally logarithmically transformed prior to analysis to meet the criteria of normal distribution; other AAPA variables were analyzed as "raw" data.
3. Latencies in step-through test, the distribution of which was sharply skewed due to time constraint of the task (5 min max.), a Kruskal-Wallis non-parametric test was used to detect the overall effects of groups, followed by non-parametric Mann-Whitney *post-hoc* tests to compare the differences between groups. In all tests, a value of $p<0.05$ was accepted as statistically significant. All calculation were performed with Statistica v.7 (Statsoft, Czech Republic).

RESULTS

AAPA testing in the first phase

Visual inspection of rats in the arena revealed normal motor and avoidance activity in control groups, whilst in the group **↑CRH↑CS** spatial avoidance was disrupted but locomotion was normal. Illustrative examples of the tracks of animals are shown in Figure 2. Animals treated with corticosterone only (Figure 2B) performed at similar level as controls (Figure 2A), but rats in the group **↑CRH↑CS** exhibited worse performance (Figure 2C). The group **↑CRH** (Figure 2D) was initially performing worse than controls; however, they managed to solve the task with training, having attained the level of control animals. This was later confirmed by statistical analysis, performed here with a two-way ANOVA (sessions x groups). It revealed a significant effect of daily sessions on the number of entrances into the shock sector ($F(3,34) = 9.93$; $p<0.0001$) (Figure 3A). However, a two-way ANOVA found a different degree of training-induced improvement between the groups ($F(4,136) = 82.38$; $p<0.00001$) and sessions vs. groups interaction ($F(12,136) = 2.24$; $p=0.01$). Closer examination by a *post-hoc* Newman-Keuls test specified that performance of the **↑CRH↑CS** group remained lower over the last 3 days compared to the control group. Regarding performance on days 4 and 5 as asymptote, **↑CRH↑CS** rats' asymptotic level was significantly worse than in other groups.

Similar results were obtained for maximum time avoided, however, some differences did not reach the level of statistical significance after *post-hoc testing*. Two-way ANOVA revealed a significant effect of sessions ($F(4,136) = 41.48$, $p<0.0001$), groups ($F(3,34) = 8.66$, $p<0.001$) and a significant interaction ($F(12,136) = 2.05$, $p=0.02$). A Newman-Keuls *post-hoc* test on this parameter indicated that trend to disrupted performance of the **↑CRH↑CS** group on days 4 and 5 (compared to the control group) was not reflected in significant differences in particular sessions. The between-session measure of spatial memory, time to first entrance, increased over the daily sessions ($F(4,136) = 8.20$, $p<0.0001$), but was unaffected by effect of groups ($F(3,34) = 1.51$; $p=0.23$). ANOVA also found a groups vs. sessions interaction ($F(12,136) = 1.97$; $p=0.03$) in this parameter. However, Newman-Keuls *post-hoc* test did not statistically confirm the difference between **↑CRH↑CS** and the control group on any particular day.

Generally, results of AAPA testing have shown that animals with elevated levels of CS and CHR were significantly disrupted in the AAPA task in one but crucial spatially-selective parameter.

AAPA testing in the second phase

The overall number of entrances was considerably lower in the AAPA testing in the second phase than in the first phase as the rats were already familiar with task rules. However, rats slightly improved their performance as

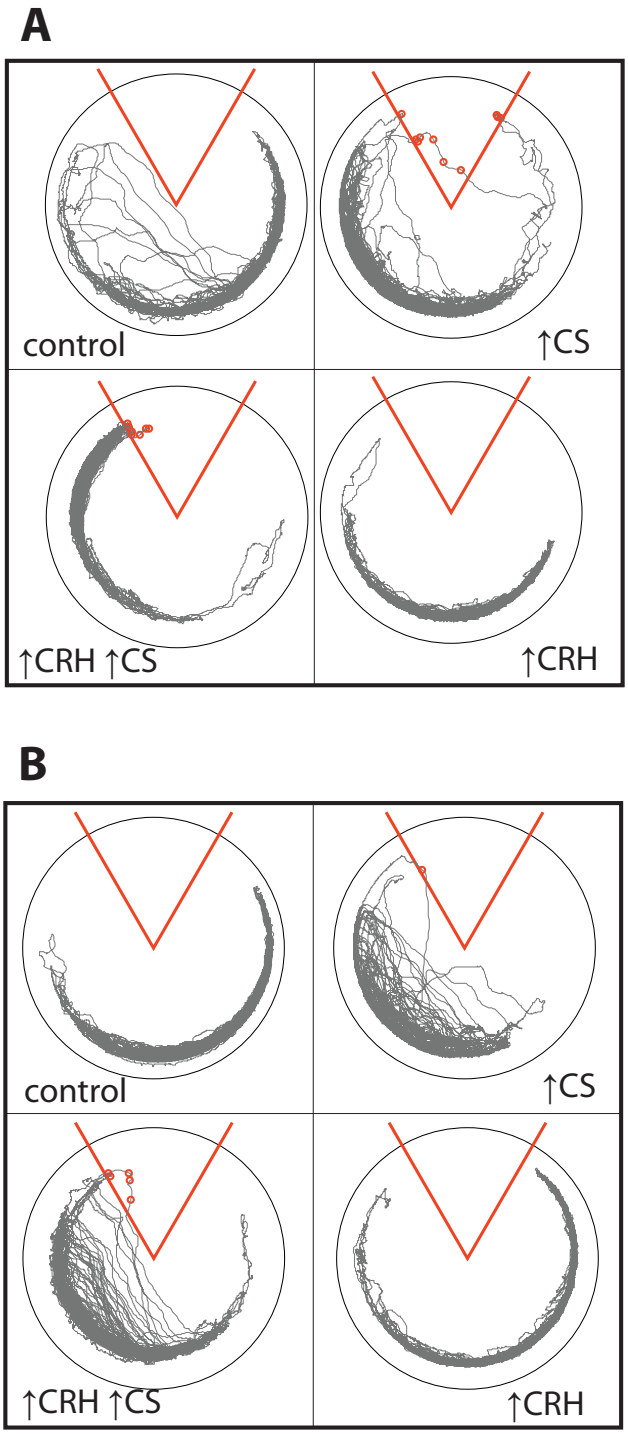

Fig. 2: Representative trajectories collected during complete last session of active allothetic place avoidance (AAPA) in the first phase (**A**) and second phase (**B**). **A**: All groups (**↑CRH**, **↑CS** and controls) except the group **↑CRH↑CS** managed the task in the first phase efficiently. Note that animals with elevated levels of corticosterone (**↑CS**) learned the task similarly as controls. **B**: Second phase of AAPA testing revealed a stable and efficient performance in all groups except persistent deficit in the **↑CRH↑CS** group. The distribution of trajectories over the arena surface is different in various groups (compare trajectories of **↑CS** and control animals (**B**), which exhibited similar efficiency of avoidance, but different distribution of locomotion on the arena; *for explanation, see Methods and Results*). Small circles denote the places of shocks. The shock sector is denoted only virtually, it is not marked on the arena.

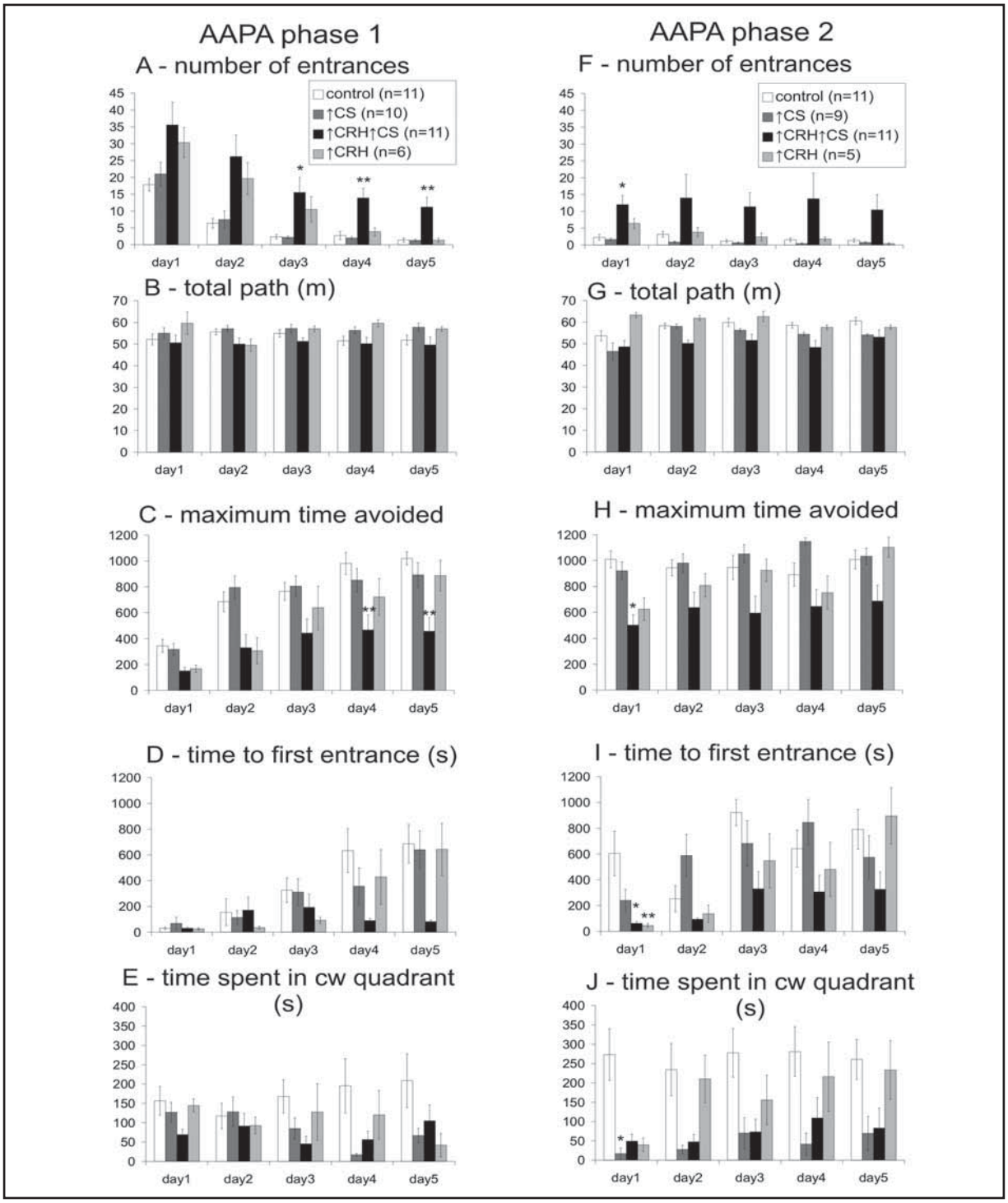

Fig. 3: Performance during the AAPA testing in the first (**left column; panels A-E**) and second phase (**right column; panels F-J**). **(A)** Number of entrances in the first phase. Note the significant impairment in the group **↑CRH↑CS;** all groups exhibited similar level of locomotor activity **(B)**. In maximum time avoided **(C)**, there was a trend of impairment in the group **↑CRH↑CS** in the first phase of testing. Time to first entrance was increasing in all groups (albeit there were high fluctuations) and no significant differences between groups were found **(D)**. **(E)** Time spent in the clockwise-adjacent quadrant (relative to the shock sector), which indicates different locomotion distribution, was not statistically differing between groups in the first phase (*but see below*). **(F)** Number of entrances in the second phase again indicated impairment in the group **↑CRH↑CS** (*see the text of Results*). Statistical significance was, however, detected only for the first session (probably due to high variances in the group **↑CRH↑CS** on subsequnet sessions**)**. There were no significant differences between groups regarding locomotor activity (**G**) in the second phase. Maximum time avoided in the second phase (**H**) was significantly lower in the groupf **↑CRH↑CS** on the first day of testing in phase 2. (**I**) Analysis of time to first entrance showed that it was decreased in the groups **↑CRH↑CS** ($p<0.05$) and **↑CRH** ($p<0.1$) and in the initial session of second phase, suggesting impairment of long-term memory from previous training. (**J**) Analysis of the time spent in cw-adjacent quadrant showed different distribution of locomotion of animals from the **↑CS** group, which spent significantly less time walking in the cw-adjacent quadrant than controls` however, they retained the avoidance efficiency (see F}. Data was analyzed with a two-way ANOVA (sessions x groups, with repeated measures on sessions) followed by s Newman-Keuls *post-hoc* test. Plotted are means ± S.E.M.* $p<0.05$, ** $p<0.01$ compared to control group.

the training proceeded, but the group **↑CRH↑CS** was still significantly impaired in this parameter. A two-way ANOVA revealed a significant effect of sessions ($F(4,128) = 8.38$. $p<0.00001$), groups ($F(3,32) = 10.2$, $p<0.0001$) but no interaction between these two factors ($F(12,128) = 1.47$, $p=0.14$). Closer inspection of the effect of groups, pursued with a Newman-Keuls *post-hoc* test found that the **↑CRH↑CS** group made more entrances than all of the other groups (all $P <0.05$). In more detail, the **↑CRH↑CS** rats significantly differed from controls only on first day of training, while the differences in subsequent sessions were not significant. Analysis of the maximum time avoided provided similar results. A two-way ANOVA found effect of days $F(4,128) = 3.52$, $p<0.009$, group $F(3,32) = 7.30$, $p<0.001$, but no interaction $F(12,128) = 1.54$, $p=0.12$, with the **↑CRH↑CS** group responsible for the significant effect of group. When performing a *post-hoc* test on sessions vs. groups interaction, the **↑CRH↑CS** group was found to differ from controls on day 1.

Additional interesting results were obtained after the analysis of time to first entrance. An ANOVA revealed effect of sessions $F(4,128)=13,13$, $p<0.0001$, groups $F(3,32)=8.16$, $p<0.001$, and interaction $F(12,128)=1.87$, $p<0.05$. A Newman-Keuls *post-hoc* test found that both the **↑CRH↑CS** group and **↑CRH** group were worse than controls on day 1, indicating that these two groups did not actually retain information about the punishment sector location over the delay between the two AAPA testing periods. Animals also distributed their locomotion on the arena surface differently during second AAPA testing. Time spent in the clockwise-adjacent quadrant was affected by group ($F(3,32) = 6.96$; $p<0.001$) but not day ($F(4,128) = 0.62$; $p>0.05$) effect, and there was no interaction $F(12,128) = 0.91$; $p>0.05$). A Newman-Keuls *post-hoc* test revealed that control rats spent more time in the CW quadrant compared to the **↑CS group** ($p=0.003$) or **↑CRH↑CS** group ($p=0.05$). The **↑CS** rats limited their entrances into the CW quadrant particularly during day 1 ($p<0.05$). Locomotor activity varied between days ($F(4,128) = 3.77$, $p<0.01$ and groups $F(3,32) = 7.79$, $p<0.001$, and a *post-hoc* test performed on significant days x group interaction $F(12,128) = 1.90$, $p=0.04$, revealed slight hyperlocomotion in the group **↑CS** compared to **↑CRH** and **↑CRH↑CS** rats but not to the control group.

Working memory version of the MWM

Although the position of the submerged platform changed over days, all animals learned the rule rapidly and there were no differences between the experimental and control groups in the escape latencies (Figure 4A). A two-way ANOVA (sessions x groups) revealed a significant main effect of sessions $F(4,140) = 92.7$, $p<0.0001$ but nor effect of groups ($F(3,35) = 1.39$, $p=0.26$), neither interaction ($F(12,140) = 0.59$, $p=0.84$), reaching their latencies to an asymptotic level on day 2 (*post-hoc* test, $p<0.0001$) (Figure 4B).

In the second-period test of working memory in the MWM, latencies significantly varied between sessions ($F(4,132) = 6.27$, $p<0.0001$) but not between groups ($F(3,33) = 0.44$, $p=0.73$) with no interaction ($F(12,132) = 1.24$, $p=0.84$), i.e., there were not between-group differences (Figure 4B). The effect of sessions in the second period may be due to performance fluctuations rather than a systematic trend of improvement; however, a clear absence of the effect of drugs at both phases of testing suggests that the drugs exerted no influence upon spatial working memory tested in this task.

MWM – modified DMP

All animals were capable of selection of appropriate strategy; the only marked difference in this test was prolonged latency to find the platform in the group with elevated level of corticosterone in the long-term memory test with 24 h delay (Figure 4C). A two-way ANOVA (swims x groups) showed differences between swims ($F(3,75) = 6.51$, $p<0.00001$) but not groups ($F(3,25) = 2.84$, $p=0.06$). However, the ANOVA revealed a significant interaction ($F(9,75) = 5.01$, $p<0.0001$) and a *post-hoc* test found that animals in the group **↑CS** exhibited significantly longer latencies at the 24 h delay (compared to all other groups, $p<0.001$)(Figure 4C). This suggests a relatively selective impairment of long-term memory by elevated levels of corticosterone.

Step-through test

The step-through test was used in the first phase of testing (i.e. under the elevated levels of hormones) and found a relatively selective impairment of memory retention after a 24 h delay in animals exposed to elevated levels of corticosterone (Figure 4D). The Kruskal-Wallis non-parametric test revealed no difference between groups at a 1.5 h interval ($H(3) = 1.96$, $p=0.58$) but detected an effect of groups at the 24 h delay ($H(3)=16.31$, $p=0.001$). Closer examination of this effect with Mann-Whitney U-test confirmed a difference between control and **↑CS** groups ($U=7$, $p<0.01$) at 24 h-delay but failed to show a difference between control rats and groups **↑CRH** and **↑CRH↑CS**. ($U=56$, $p>0.05$) (Figure 4D). These results suggest impairment by corticosterone of the 24 h-delay memory retention, but not a deficit in short-term memory.

Measuring of the levels of hormones,

ACTH and corticosterone levels was assessed from weeks 0, 5, and 10 of the study experiment and results are expressed as normalized values in the Figure 5. Within each group, data were compared to average value of week 0 which was set as 100%. As can be seen in the Figure 5, experimental manipulations changed levels of ACTH only in **↑CRH** group, in which ACTH concentrations decreased below the basal levels in weeks 5 and 10 (** $p<0.01$, * $p<0.05$, respectively). Conversely, corticosterone level was kept persistently increased in **↑CRH** group in weeks 5 and 10. In **↑CRH↑CS** group,

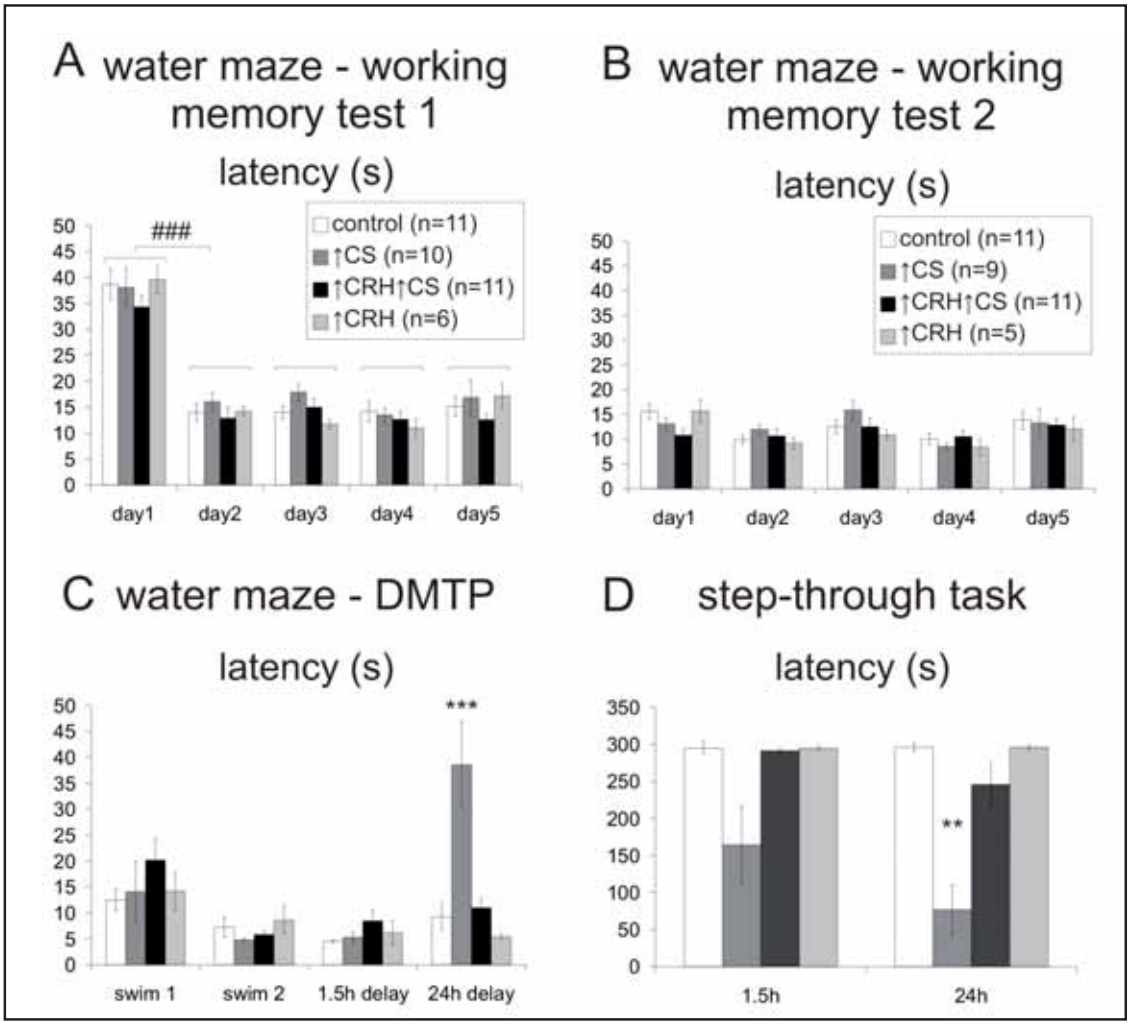

Fig. 4: Performance in working memory (WM) version of water maze (**A,B**), modified DMP (**C**), and passive avoidance (**D**) in the control and experimental groups (means ± S.E.M.). The WM version of water maze revealed no impairment of WM in the first phase (**A**) and second phase of testing (**B**). All groups performed at similar level, there was only striking improvement in all groups from session 1 to session 2; ### $p<0.001$. Data from WM version of the water maze was analyzed with a two-way ANOVA (swims x groups with repeated measures on swims. (**C**) A modified version of DMP adapted for assessment of intermediate and long-term memory (*see Methods and Results*) showed selective impairment of 24-h delay in the group ↑**CS**, suggesting impairment of long-term spatial memory by corticosterone; *** $p<0.001$ compared to control group. The data was analyzed by a a two-way ANOVA (swims x sessions) with repeated measures on the first factor. The negative effect of corticosterone on long-term memory retention was confirmed by passive avoidance step-through task (**D**), which also showed impairment of 24-h memory delay in the group ↑**CS.** ** $p<0.01$. Due to skewed distribution of data, this data was analyzed with a non-parametric Kruskal-Wallis test followed by Mann-Whitney *post-hoc* test.

however, the elevated corticosterone levels were detectable only in week 5.

DISCUSSION

Aim of the present study was to study effect of long-term administration of stable levels of CRH and corticosterone on the behavior of laboratory rats.

Stress and dysregulation of the HPA axis induced changes in hippocampal plasticity; specifically the idea that decreased hippocampal plasticity might be a precipitating factor for neuropsychiatric diseases such a depression. Numerous studies showed that stress impairs hippocampal-dependent spatial memory in the Morris water maze (McEwen & Sapolsky 1995; Sandi *et al.* 2005), in the radial maze (El Hage *et al.* 2006) and the T-maze (Bats *et al.* 2001). Stress can also impair executive functions such as behavioral flexibility (Mizoguchi *et al.* 2000). However, to the best of our knowledge, there is as yet no study aimed at determining the particular role of corticosterone and CRH in spatial memory processes and behavior.

In accordance with previous reports (e.g. Coburn-Litvak *et al.* 2003), results of the present study has shown that chronically elevated corticosterone levels impaired the formation of memory traces in the MWM and passive avoidance and that long-term memory has been more impaired than short-term memory, while

working memory has been relatively spared. Previous studies showed that impairments of spatial working memory were detectable only after a longer-term corticosterone elevation (Coburn-Litvak *et al.* 2003). Nonetheless, another study showed corticosterone reduces both working and reference memory with prolonged treatment (Hoyer & Lannert 2008).

Corticosterone is involved in the structural and functional integrity of the brain, particularly the hippocampus. The effect of corticosterone on learning and memory is more complex. It varies depending on duration of exposition and corticosterone levels as well as on the learning paradigm and stage of learning or memory processes (Klenerova *et al.* 2000; Roozendaal 2003). It is illustrated very often as inverted U-shape dose-response. The hippocampus has a high density of adrenal steroid receptors thereby glucocorticoids regulate several aspects of hippocampal plasticity (Korz & Frey 2003). Corticosterone modulates memory formation by aroused and emotional aspects mainly. It is known to enhance hippocampal dependent spatial memory consolidation (McGaugh & Roozendaal 2002). In this way, the endogenous corticosterone appears to be necessary for acquisition and persistence of associative memories too (Beylin & Shors 2003).

At the other side, many studies documented the negative role of corticosterone in brain functions, especially learning and memory (Belanoff *et al.* 2001; He *et al.* 2008). The chronic application of corticosterone to artificially high levels has been found to induce apical dendritic retraction and debranching in rat CA3c pyramidal neurons (Watanabe *et al.* 1992), while longer durations of the administration resulted in more substantial hippocampal damage (Sapolsky 2000). Moreover, there is a correlation between corticosterone-induced hippocampal morphological changes and learning performance in the MWM (He *et al.* 2008). Yet another study has shown that long-term exposure to elevated corticosterone levels resulted in spatial learning deficits in rats (Bodnoff *et al.* 1995).

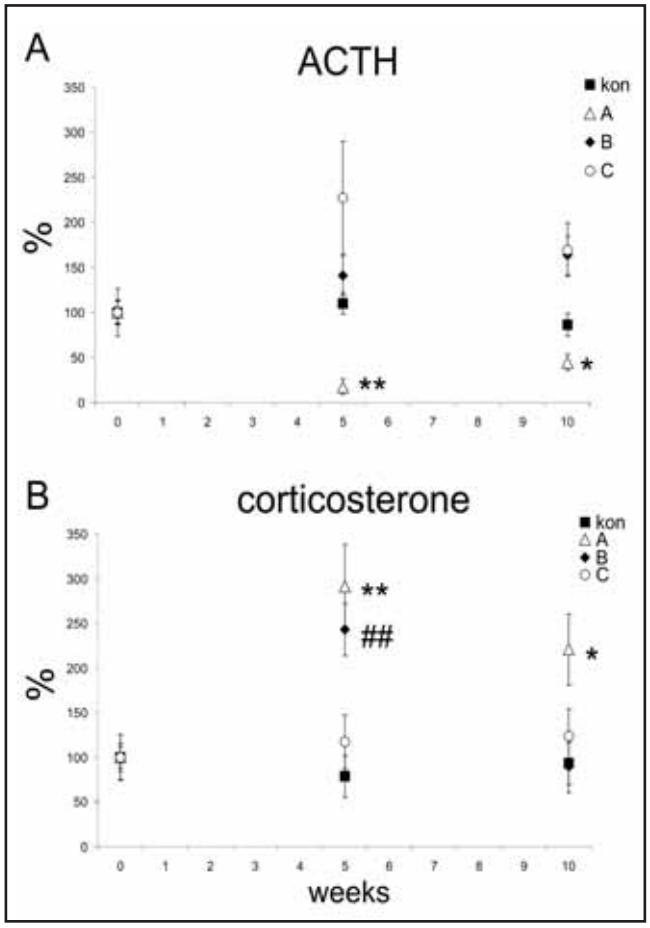

Fig. 5. Levels of ACTH **(A)** and corticosterone **(B)** collected during week 0, 5, and 10 of the experiment and expressed as normalized values. Within each group, data are compared to average value of week 0 which is set as 100%. Experimental manipulations altered level of ACTH only in ↑**CRH** group, in which ACTH dropped below the basal value both in week 5 and 10 (** $p<0.01$, *$p<0.05$, respectively). Conversely, corticosterone level was kept steadily increased in ↑**CRH** group during both measurings (** $p<0.01$, *$p<0.05$, respectively). In ↑**CRH**↑**CS** group, however, the elevated corticosterone was apparent only in week 5 (## $p<0.01$).

In the present study, more interesting results were acquired for animals subjected to the long-term application of CRH. The majority of past studies dealt with the effects of dysregulation in the HPA axis on behavior and cognition used in stressful tasks. We have specifically addressed the question if CRH exerted central effects on learning and memory and we have compared it with the effect of corticosterone alone. The present results show that elevated CRH has no effect on memory trace formation in the MWM and inhibitory avoidance. However, it was found to affect learning of the AAPA task, a spatial test requiring allocentric spatial learning and cognitive coordination. In contrast to the routinely used spatial memory task such as the MWM and the T-maze, this task focuses on testing cognitive coordination and higher cognitive processes and is extremely sensitive to hippocampal integrity (Wesierska *et al.* 2005). As described in results, the locomotor activity did not differ amongst groups in either of the testing phases, which suggests that procedural aspects of the task were unaffected. However, the cognitive parameters were impaired in the first phase in CRH-treated animals. The group with elevated CRH and corticosterone levels performed significantly worse than all the other groups; even on day 5 the animals could not reach control group performance levels. The group with elevated CRH but normal corticosterone levels was impaired in the initial sessions but on day 5 their performance did not differ from that of the controls. Similarly, in the second experimental phase (5 weeks after hormone treatment withdrawal), both groups that had been previously treated with CRH exhibited significant impairment in the first session. The group that had been treated with both CRH and corticosterone did not improve in the successive sessions. The group pre-

viously treated with CRH but not with corticosterone gradually reached the performance level of the control groups (see Figure 3B). Both controls and the animals treated with corticosterone alone performed equally well from the first session and their performance level did not differ from that on day 5 of phase one.

CRH and CRH family proteins modulate cognitive functions and play an integral role in controlling the neural substrates of arousal, emotion and reaction to environmental and physiological challenges (Bosch *et al.* 2009). CRH modulates synaptic efficacy and learning and memory processes in the hippocampus (Hanstein *et al.* 2008; Heinrichs & Koob 2004). CRH systems constitute the crucial pathway for regulation of the HPA axis and CRH was shown to have a prominent role in fear- and anxiety-related behaviors which can be even independent of the HPA axis (Lussier 2009). It was previously reported that stress-induced anhedonia was associated with an increase in CRH gene expression in the hypothalamus (Duncko *et al.* 2001). Similarly, i.c.v. administration of CRH in non-stressed animals under low arousal conditions produces a dose-dependent behavioral activation in a familiar environment. This phenomenon was not blocked by lesions to the pituitary gland or by pretreatment with dexamethasone, suggesting that this effect of CRH was mediated by action in the central nervous system independently of the HPA axis (Koob & Heinrichs 1999). Our observation of negative effects of CRH in the AAPA task supports this view. On the other hand, expression of CRH in the brain region is regulated by glucocorticoid hormones too. Steroid receptor co-activator 1 plays a critical role in this process (Lachize *et al.* 2009). CRH expression itself is strongly regulated in response to stress-induced elevations of glucocorticoids and neurogenic signals. In the core of the HPA-axis, activation of the glucocorticoid receptor represses transcription of CRH as part of negative feedback (Schmidt *et al.* 2009), whereas stress-related noradrenergic and glutamatergic excitatory signals can activate the CRH gene (Duncko *et al.* 2003; Kalin *et al.* 1994). Mice with conditional over-expression of CRH in the entire CNS show stress-induced hypersecretion of stress hormones and increased stress-related behavior reflected in immobility in the forced swim test and tail suspension test (Deussing *et al.* 2008).

It will be imperfection to miss out short discussion about influence and nature of the behavioral tests. Different types of stressor are essentially ingrediented in the behavioral tests. They active the CRH system by different manner too. CRH expression and release is increased by physiologal and psychological stressors in the hypothalamus while in amygdala, preferentially by psychological stress. Spatial maze procedures could induce slight stress responses too. But the stress is inflicted of physical nature not psychological and can lead to reduced fear (Aguilar-Valles *et al.* 2005). A little bit stressful nature of behavioral tests has to be respected in design of experiments. One test could affect the following test. And that is why for example AAPA cannot antedate MWM and more than one test cannot be provided in one day of course.

Using the osmotic pump and subcutaneous pellets does not, of course, allow respecting fact that hormones are pulsatile and circadian factors with extraordinary temporal dynamics. Stress and dysregulation of the HPA axis are a highly complex system and it is difficult to respect all neurobiological aspects relevant for their effects on behavior. This pharmacological approach is not model of stress, but only long-term exposition to CRH and corticosterone and impact of these hormones on the learning and memory functions.

Mechanisms of CRH involvement in learning-induced changes in the nervous system are still unclear. Previous results have shown that direct application of CRH both *in vivo* and *in vitro* failed to exhibit a neurotoxic effect. And that CRH may even prevent cell death caused by oxidative stress in cell cultures (Craighead *et al.* 2000). One possible explanation of the negative effect of CRH upon AAPA task performance, evidenced in the present study, is that this phenomenon might be mediated indirectly. CRH has a significant impact on blood pressure and the brain vascular system. Alterations in blood flow in cerebral capillaries may have affected the extracellular concentration of excitatory amino acids. It was also demonstrated previously that CRH may be a factor affecting neuronal plasticity and may partially, but significantly, contribute to neurotoxicity and neurodegeneration (Craighead *et al.* 2000). CRH was shown to exert a long-term depressant effect on spike activity in the hippocampus (Rebaudo *et al.* 2001). But the biological significance of these phenomena is unclear; however, our results support the view that not long-term elevated levels of CRH may disrupt learning of a spatial task with high demands on hippocampus integrity, i.e. the AAPA task. It is important to point out that CRH is significantly elevated in some CNS disorders, e.g., in the cerebrospinal fluid of depressed patients (Hartline *et al.* 1996).

Our results highlight the importance of the combined effect of CRH and corticosterone in the permanent impairment of cognitive functions measured in the AAPA task. On the contrary, application of CRH alone has caused only a transient impairment in this test. It should be noted that no deteriorating effect of CRH on memory formation was observed in the MWM and passive avoidance task. Nonetheless, chronic application of corticosterone alone impaired long-term memory trace formation in the MWM and passive avoidance task without affecting cognitive coordination and allothetic spatial memory in the AAPA task. Another recent study has showed delayed changes in the volumetric lateralization of the hippocampus after identical procedure, suggesting that these phenomena (lateralization and memory) might be inter-related.

Distinct in results of the tests can also reflect different hippocampal dependence of the behavioral paradigms.

The present paradigm is not model of stress or dysregulation of HPA axis themselves. It represents a long-term exposition to CRH and corticosterone and its impact of these hormones on learning and memory. However elevated corticosterone and CRH play important role in hyperactivity and deliberation of the HPA axis and may work in a synergy *in vivo*. Our results show the long-term negative impact of CRH and CRH with corticosterone on AAPA task learning and suggest that that corticosterone may negatively affect memory formation in both spatial and non-spatial tasks. Long-term elevated levels of CRH *plus* corticosterone lead to disruption of learning of the AAPA task requiring relatively subtle cognitive processes (Wesierska *et al.* 2005), suggesting synergistic effects of such elevated levels on learning and memory functions.

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REFERENCES

1 Aguilar-Valles A, Sánchez E, de Gortari P, Balderas I, Ramírez-Amaya V, Bermúdez-Rattoni F, Joseph-Bravo P (2005). Analysis of the stress response in rats trained in the water-maze: differential expression of corticotropin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. Neuroendocrinology. **82(5–6)**:306–319.

2 Bats S, Thoumas JL, Lordi B, Tonon MC, Lalonde R, Caston J (2001). The effects of a mild stressor on spontaneous alternation in mice. Behav Brain Res. **118**: 11–15.

3 Belanoff JK, Gross K, Yager A, Schatzberg AF (2001). Corticosteroids and cognition. J Psychiatr Res. **35**: 127–145.

4 Beylin AV, Shors TJ (2003). Glucocorticoids are necessary for enhancing the acquisition of associative memories after acute stressful experience. Horm Behav. **43**:124–131.

5 Bodnoff SR, Humphreys AG, Lehman JC, Diamond DM, Rose GM, Meaney MJ (1995). Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. J Neurosci. **15**: 61–69.

6 Bosch OJ, Nair HP, Ahern TH, Neumann ID, Young LJ (2009). The CRF system mediates increased passive stress-coping behavior following the loss of a bonded partner in a monogamous rodent. Neuropsychopharmacology. **34**: 1406–1415.

7 Chauveau F, Tronche C, Piérard C, Liscia P, Drouet I, Coutan M, Béracochéa D (2010). Rapid stress-induced corticosterone rise in the hippocampus reverses serial memory retrieval pattern. Hippocampus. **20**: 196–207.

8 Cimadevilla JM, Wesierska M, Fenton AA, Bures J (2001). Inactivating one hippocampus impairs avoidance of a stable room-defined place during dissociation of arena cues from room cues by rotation of the arena. Proc Natl Acad Sci USA. **98**: 3531–3536.

9 Coburn-Litvak PS, Pothakos K, Tata DA, McCloskey DP, Anderson BJ (2003). Chronic administration of corticosterone impairs spatial reference memory before spatial working memory in rats. Neurobiol Learn Mem. **80**: 11–23.

10 Craighead MW, Boutin H, Middlehurst KM, Allan SM, Brooks N, Kimber I, Rothwell NJ (2000). Influence of corticotrophin releasing factor on neuronal cell death in vitro and in vivo. Brain Res. **881**: 139–143.

11 Dagyte G, Van der Zee EA, Postema F, Luiten PG, Den Boer JA, Trentani A, Meerlo P (2009). Chronic but not acute foot-shock stress leads to temporary suppression of cell proliferation in rat hippocampus. Neuroscience. **162**: 904–913.

12 Deussing JM, Lu A, Steiner MA, VoglAM, Holsboer F, Wotjak CT, Wurst W (2008). Conditional overexpression of corticotropin-releasing hormone in mice, an animal model for understanding the development of corticotropin-relesing hormone-mediated brain pathologyI. Int J Neuropsychopharmacol. **11**: 117.

13 Duncko R, Kiss A, Skultetyová I, Rusnak M, Jezova D (2001). Corticotropin-releasing hormone mRNA levels in response to chronic mild stress rise in male but not in female rats while tyrosine hydroxylase mRNA levels decrease in both sexes. Psychoneuroendocrinology. **26**: 77–89.

14 Duncko R, Schwendt M, Jezova D (2003). Altered glutamate receptor and corticoliberin gene expression in brain regions related to hedonic behavior in rats. Pharmacol Biochem Behav. **76**: 9–16.

15 El Hage W, Griebel G, Belzung C (2006). Long-term impaired memory following predatory stress in mice. Physiol Behav. **87**: 45–50.

16 Grammatopoulos DK, Chrousos GP (2002). Functional characteristics of CRH receptors and potential clinical applications of CRH-receptor antagonists. Trends Endocrinol Metab. **13**: 436–444.

17 Hanstein R, Lu A, Wurst W, Holsboer F, Deussing JM, Clement AB, Behl C (2008). Transgenic overexpression of corticotropin releasing hormone provides partial protection against neurodegeneration in an in vivo model of acute excitotoxic stress. Neuroscience. **156**: 712–721.

18 Hartline KM, Owens MJ, Nemeroff CB (1996). Postmortem and cerebrospinal fluid studies of corticotropin-releasing factor in humans. Ann NY Acad Sci. **780**: 96–105.

19 He WB, Zhang JL, Hu JF, Zhang Y, Machida T, Chen NH (2008). Effects of glucocorticoids on age-related impairments of hippocampal structure and function in mice. Cell Mol Neurobiol. **28**: 277–291.

20 Heinrichs SC, Koob GF (2004). Corticotropin-releasing factor in brain: a role in activation, arousal, and affect regulation. J Pharmacol Exp Ther. **311**: 427–440.

21 Heinrichs SC, Koob GF (2004). Corticotropin-releasing factor in brain: a role in activation, arousal, and affect regulation. J Pharmacol Exp Ther. **311**: 427–440.

22 Hoschl C, Hajek T (2001). Hippocampal damage mediated by corticosteroids-a neuropsychiatric research challenge. Eur Arch Psychiatry Clin Neurosci. **251 (Suppl 2)**: II81–88.

23 Hoyer S, Lannert H (2008). Long-term effects of corticosterone on behavior, oxidative and energy metabolism of parietotemporal cerebral cortex and hippocampus of rats: comparison to intracerebroventricular streptozotocin. J Neural Transm. **115**: 1241–1249.

24 Izquierdo I, Medina JH (1993). Role of the amygdala, hippocampus and entorhinal cortex in memory consolidation and expression. Braz J Med Biol Res. **26**: 573–589.

25 Kalin NH, Takahashi LK, Chen FL (1994). Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus. Brain Res. **656**: 182–186.

26 Klenerova V, Jurcovicova J, Kaminsky O, Sida P, Krejci I, Hlinak Z, Hynie S (2003). Combined restraint and cold stress in rats: effects on memory processing in passive avoidance task and on plasma levels of ACTH and corticosterone. Behav Brain Res. **142(1–2)**:143–149.

27 Koob GF, Heinrichs SC (1999). A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Res. **848**: 141–152.
28 Korz V, Frey JU (2003). Stress-related modulation of hippocampal long-term potentiation in rats: Involvement of adrenal steroid receptors. J Neurosci. **23**: 7281–7287.
29 Lachize S, Apostolakis EM, van der Laan S, Tijssen AM, Xu J, de Kloet ER, Meijer OC (2009). Steroid receptor coactivator-1 is necessary for regulation of corticotropin-releasing hormone by chronic stress and glucocorticoids. Proc Natl Acad Sci USA. **106**: 8038–8042.
30 Lussier AL, Caruncho HJ, Kalynchuk LE (2009). Repeated exposure to corticosterone, but not restraint, decreases the number of reelin-positive cells in the adult rat hippocampus. Neurosci Lett. **460**: 170–174.
31 McEwen BS, Sapolsky RM (1995). Stress and cognitive function. Curr Opin Neurobiol. **5**: 205–216.
32 McGaugh JL, Roozendaal B (2002). Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol. **12**: 205–210.
33 Mizoguchi K, Yuzurihara M, Ishige A, Sasaki H, Chui DH, Tabira T (2000). Chronic stress induces impairment of spatial working memory because of prefrontal dopaminergic dysfunction. J Neurosci. **20**: 1568–1574.
34 Morris R (1984). Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods. **11**: 47–60.
35 Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, Sacktor TC (2006). Storage of spatial information by the maintenance mechanism of LTP. Science. **313**: 141–144.
36 Rebaudo R, Melani R, Balestrino M, Izvarina N (2001). Electrophysiological effects of sustained delivery of CRF and its receptor agonists in hippocampal slices. Brain Res. **922**: 112–127.
37 Roozendaal B (2003). Systems mediating acute glucocorticoid effects on memory consolidation and retrieval.Prog Neuropsychopharmacol Biol Psychiatry. **27**: 1213–1223.
38 Russell JA, Douglas AJ, Brunton PJ (2008). Reduced hypothalamo-pituitary-adrenal axis stress responses in late pregnancy: central opioid inhibition and noradrenergic mechanisms. Ann NY Acad Sci. **1148**: 428–438.
39 Sandi C, Woodson JC, Haynes VF, Park CR, Touyarot K, Lopez-Fernandez MA, Venero C, Diamond DM (2005). Acute stress-induced impairment of spatial memory is associated with decreased expression of neural cell adhesion molecule in the hippocampus and prefrontal cortex. Biol Psychiatry. **57**: 856–864.
40 Sapolsky RM (2000). Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry. **57**: 925–935.
41 Schmidt MV, Sterlemann V, Wagner K, Niederleitner B, Ganea K, Liebl C, Deussing JM, Berger S, Schütz G, Holsboer F, Müller MB (2009). Postnatal glucocorticoid excess due to pituitary glucocorticoid receptor deficiency: differential short- and long-term consequences. Endocrinology. **150**: 2709–2716.
42 Simunkova K, Starka L, Hill M, Kriz L, Hampl R, Vondra K (2000). Comparison of Total and Salivary Cortisol in a Low-Dose ACTH (Synacthen) Test: Influence of Three-Month Oral Contraceptives Administration to Healthy Women. Physiol. Res. **57 (Suppl. 1)**: S193–S199.
43 Steele RJ, Morris RG (1999). Delay-dependent impairment of a matching-to-place task with chronic and intrahippocampal infusion of the NMDA-antagonist D-AP5. Hippocampus. **9**: 118–136.
44 Stuchlik A, Petrasek T, Vales K (2009). A dose-response study of the effects of pre-test administration of beta-adrenergic receptor antagonist propranolol on the learning of active place avoidance, a spatial cognition task, in rats. Behav Brain Res. **200**: 144–149.
45 Stuchlik A, Petrasek T, Vales K (2008). Dopamine D2 receptors and alpha1-adrenoceptors synergistically modulate locomotion and behavior of rats in a place avoidance task. Behav Brain Res. **189**: 139–144.
46 Stuchlik A, Rehakova L, Telensky P, Vales K (2007). Morris water maze learning in Long-Evans rats is differentially affected by blockade of D1-like and D2-like dopamine receptors. Neurosci Lett. **422**: 169–174.
47 Stuchlik A, Vales K (2009). Baclofen dose-dependently disrupts learning in a place avoidance task requiring cognitive coordination. Physiol Behav. **97**: 507–511.
48 Swaab DF, Bao AM, Lucassen PJ (2005). The stress system in the human brain in depression and neurodegeneration. Ageing Res Rev. **4**: 141–194.
49 Vales K, Bubenikova-Valesova V, Klement D, Stuchlik A (2006). Analysis of sensitivity to MK-801 treatment in a novel active allothetic place avoidance task and in the working memory version of the Morris water maze reveals differences between Long-Evans and Wistar rats. Neurosci Res. **55**: 383–388.
50 Watanabe Y, Gould E, Cameron HA, Daniels DC, McEwen BS (1992). Phenytoin prevents stress- and corticosterone-induced atrophy of CA3 pyramidal neurons. Hippocampus. **2**: 431–435.
51 Wesierska M, Dockery C, Fenton AA (2005). Beyond memory, navigation, and inhibition: Behavioral evidence for hippocampus-dependent cognitive coordination in the rat. J Neurosci. **25**: 2413–2419.
52 Zach P, Mrzilkova J, Rezacova L, Stuchlik A, Vales K (2010). Delayed effects of elevated corticosterone level on volume of hippocampal formation in laboratory rat. Physiol Res, in press.