THE BEHAVIORAL PHENOTYPE OF PITUITARY ADENYLATE-CYCLASE ACTIVATING POLYPEPTIDE-DEFICIENT MICE IN ANXIETY AND DEPRESSION TESTS IS ACCOMPANIED BY BLUNTED c-Fos EXPRESSION IN THE BED NUCLEUS OF THE STRIA TERMINALIS, CENTRAL PROJECTING EDINGER–WESTPHAL NUCLEUS, VENTRAL LATERAL SEPTUM, AND DORSAL RAPHE NUCLEUS

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Abstract—Pituitary adenylate-cyclase activating polypeptide (PACAP) has been implicated in the (patho)physiology of stress-adaptation. PACAP deficient (PACAP\textsuperscript{−/−}) mice show altered anxiety levels and depression-like behavior, but little is known about the underlying mechanisms in stress-related brain areas. Therefore, we aimed at investigating PACAP\textsuperscript{−/−} mice in light–dark box, marble burying, open field, and forced swim paradigms. We also analyzed whether the forced swim test-induced c-Fos expression would be affected by PACAP deficiency in the following stress-related brain areas: magnocellular and parvocellular paraventricular nucleus of the hypothalamus (PVN); basolateral (BLA), medial (MeA), and central (CeA) amygdaloid nuclei; ventral (BSTv), dorsolateral (BSTdl), dorsomedial (BSTdm), and oval (BSTov) nuclei of the bed nucleus of stria terminalis; dorsal (dLS) and ventral parts (vLS) of lateral septal nucleus, central projecting Edinger–Westphal nucleus (EWcp), dorsal (dPAG) and lateral (lPAG) periaqueductal gray matter, dorsal raphe nucleus (DR). Our results revealed that PACAP\textsuperscript{−/−} mice showed greatly reduced anxiety and increased locomotor activity compared with wildtypes. In forced swim test PACAP\textsuperscript{−/−} mice showed increased depression-like behavior. Forced swim exposure increased c-Fos expression in all examined brain areas in wildtypes, whereas this was markedly blunted in the DR, EWcp, BSTov, BSTdl, BSTv, PVN, vLS, dPAG, and in the IPAG of PACAP\textsuperscript{−/−} mice vs. wildtypes, strongly suggesting their involvement in the behavioral phenotype of PACAP\textsuperscript{−/−} mice. PACAP deficiency did not influence the c-Fos response in the CeA, MeA, BSTdm, and dLS. Therefore, we propose that PACAP exerts a brain area-specific effect on stress-induced neuronal activation and it might contribute to stress-related mood disorders. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: stress, bed nucleus of stria terminalis, amygdala, hypothalamic paraventricular nucleus, Edinger–Westphal nucleus, dorsal raphe nucleus.

The pathophysiology of stress-related mood disorders is not fully understood yet; however, there is no doubt that the maladaptation of the hypothalamus–pituitary–adrenal (HPA) axis to stress plays a crucial role (Herman et al., 2003; de Kloet, 2008). The key regulator of the HPA axis is the paraventricular nucleus of the hypothalamus (PVN) expressing corticotropin releasing factor (CRF). The pituitary adenylate-cyclase activating polypeptide (PACAP) is known to contribute to the regulation of CRF neurons in the PVN (Das et al., 2007; Kageyama and Suda, 2009). For instance, in the rat i.c.v. administered PACAP activates CREB phosphorylation and c-Fos [product of an immediate early gene, a widely used tool to evaluate neuronal activation (Kovács, 1998)] expression of CRF neurons in the PVN leading to the activation of the HPA axis (Agarwal et al., 2005; Norrholm et al., 2005).

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PACAP is an extensively studied neuropeptide implicated in pleiotropic biological processes (for review see: Vaudry et al., 2009). According to its distribution in stress-related centers in the rat brain in- and outside the PVN (Hannibal, 2002), its overall influence on stress adaptation is also possible. Recent studies demonstrating that transgenic mice lacking the gene encoding for PACAP exhibit depression-like behavior in the forced swim tests (FST), which can be reversed by antidepressant-drug treatment strongly supports this idea (Hashimoto et al., 2009). In humans, Hashimoto et al. (2010) found possible association between major depressive disorder and a single nucleotide polymorphism of the PACAP gene. Other studies also underlie the possible role of PACAP in brain diseases (Hashimoto et al., 2011), as PACAP signaling through its specific PAC1 receptor increases the expression of the product of disrupted in schizophrenia 1 gene (DISC 1) (Hattori et al., 2007). Mutation of disc1 gene was shown to be associated with schizophrenia (Millar et al., 2000) and major depression (Blackwood et al., 2001). Most recently Ressler et al. (2011) published that PACAP levels in blood over-expression in distinct parts of the extended amygdala, viz. in the bed nucleus of the stria terminalis (BST) (see also: Kozicz et al., 1997), of the rat, suggesting the possible role of PACAP in stress adaptation.

Brain areas, such as the extended amygdala (Westenbroek et al., 2003; Badowska-Szalewska et al., 2009; Hammack et al., 2010) and lateral septum (LS) (Ons et al., 2010; Singewald et al., 2011), dorsal raphe nucleus (DR) in the rat (Sawchenko et al., 1983; Liposits et al., 1987) and also in humans (Hornung, 2003) are connected to the PVN, play a role in the regulation of the HPA axis activity, and exhibit robust c-Fos expression upon stress. Regev et al. (2011) in an elegant study in mice have shown that CRF over-expression in distinct parts of the extended amygdala influences anxiety and depression-like behavior in mice. Studies on the central distribution of PACAP in the rat (for review see: Vaudry et al., 2009) reveal dense innervation by PACAP of the extended amygdala, viz. in the bed nucleus of the stria terminalis (BST) (see also: Kozicz et al., 1997), of the rat, suggesting the possible role of PACAP in stress adaptation.

Brainstem stress-centers such as the CRF family member urocortin1 (Ucn1) expressing centrally projecting Edinger–Westphal nucleus (EWcP) (Ryabinin et al., 1999, for review see: Kozicz, 2010; Kozicz et al., 2011) and the serotoninergic DR (Valentino et al., 2010) show c-Fos response to various stressful events both in rats and mice (Kozicz et al., 2001; Gaszner et al., 2004, 2007, 2009; Gardner et al., 2005; Liu et al., 2009). These areas are implicated in stress-related mood disorders as for instance, depressed male suicide victims have nine times higher Ucn1 expression in the EWcP than controls (Kozicz et al., 2008). Moreover, in the DR, post mortem studies show increased 5-HT1A receptor binding in suicide victims (for review see: Savitz et al., 2009). Interestingly, studies on the distribution of PACAP in the rat brain revealed that both the EWcP and DR are densely innervated by PACAP immunoreactive (ir) fibers (Hannibal, 2002).

Based on these data, we put forward the hypothesis that PACAP might exert, at least a modulating role on stress-related centers in the mouse, which would appear both at the level of behavior and immediate early gene expression, that is, c-Fos. In order to test our hypothesis we first assessed behavioral indexes of anxiety and depression-like behavior in PACAP deficient (PACAP−/−) mice (see also: Hashimoto et al., 2001, 2009) in four behavioral tests (open field (OF), FST, light–dark box, and marble burring) compared with their wildtype (PACAP+/+) counterparts. As no extensive morphological data are available in relation to stress reactivity and PACAP deficiency, the second aim of this study was to analyze FST-induced activation pattern of c-Fos in various stress-related brain areas of PACAP−/− and PACAP+/+ mice receiving PACAPergic innervation and/or expressing its receptors [i.e. parts of the extended amygdala: dorsolateral (BSTdl) dorsomedial (BSTdm), oval (BSTov), and ventral (BSTv) BST; central (CeA), basolateral (BLA), and medial (MeA) amygdaloid nuclei; the parvocellular (pPVN) and magnocellular (mPVN); the LS; the dorsal (dPAG) and lateral (lPAG) mesencephalic periaqueductal gray matter; the EWcP and DR] in wildtype and knockout mice subjected to FST paradigm.

**EXPERIMENTAL PROCEDURES**

**Animals**

Thirty-eight in-house bred PACAP−/− male mice and their wildtype (PACAP+/+) male counterparts were used in this study. The generation and maintenance of the knockout mice on the CD1 background have been described previously (Hashimoto et al., 2006). Mice were backcrossed for 10 generations with the CD1 strain, then offspring from the next three generations were used for this study. To avoid possible maternal care quality related litter differences, subjects of each experimental group were taken from four to five different litters at the same age. In addition, Shintani et al. (2002) demonstrated that the PACAP gene deletion does not have a clear litter-size and genetic-background independent influence on the quality of maternal care in PACAP−/− dams. The absence of the PACAP gene was verified by RT-PCR. Animals were kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs at 24–25 °C and provided with standard rodent chow and water ad libitum. The studies were approved by the Ethics Committee on Animal Research of Pécs University based on the European Communities Council Directive of 24 November 1986 and the Law of 1998, XXIII, on Animal Care and Use in Hungary (license No: BA 02/2000-11-2006). All efforts were made to minimize the number of animals used and their suffering.

**Behavioral studies**

Behavioral studies were carried out on naive mice, one animal was exposed to only one behavioral test. Subjects for FST were processed for immunohistological studies.

**Open field test.** Animals (n=7, PACAP−/− and PACAP+/+, respectively) were observed for locomotor activity and anxiety behavior in an open field. After acclimatization to the environment, mice were placed in an open field consisting of a 42×42 cm² box with 21 cm high walls around. The floor was divided into 8×8 areas. Subjects were placed individually in the center always facing the same direction, and they were video-recorded for 5 min.
Recordings were evaluated in a blinded fashion. The locomotor activity was measured by the distance traveled and mobility time. For anxiety, time spent by the walls and in the open areas of the field, the total number of head liftings, rearing, jumping behavior, and grooming activity were also quantified.

**Forced swim test.** The FST described by Porsolt et al. (1977) was carried out using modification by Ghassemi et al. (2009). Each mouse was placed individually in a transparent plastic cylinder (diameter 11.5 cm, height 25 cm) that was filled to the 19 cm mark with water. Mice (n=7, PACAP−/− and PACAP+/+, respectively) were placed into the water and forced to swim for 6 min. The duration of immobility was measured in the last 4 min of the video records. The purpose of this experiment was to study the effect of acute stress on the c-Fos expression; therefore, in this experimental setup we did not expose mice to a pre-test day swim session. The mouse was considered to be immobile when it stopped struggling, passively floated, and kept its head above the water. Water was changed between trials and temperature was maintained at 23±1 °C. Two hours after FST, mice were sacrificed for c-Fos immunohistochemical examination.

**Light–dark box test for anxiety.** The light–dark box test was performed in a plastic white-colored device (40×20×27 cm³) divided by an opaque partition with the 7×7 cm² aperture between two compartments. The bright side was illuminated by a 100 W lamp from above, whereas the other closed compartment was dark. The animals (n=12, PACAP−/− and PACAP+/+ mice, respectively) were placed into the light part of the device facing towards the aperture. The number and time latency of entering the dark compartment, duration of staying there, and the number of peaks into the lift box were measured on 5-min video records of the test. The experiments were done in the morning between 8 and 12 O’clock.

**Marble burying test for anxiety.** In this paradigm mice were placed individually in clear plastic boxes (30×30×28 cm³) containing 24 colored glass marbles (1.5 cm in diameter) evenly spaced on sawdust 5 cm deep, without food and water as described by Njung’e and Handley (1991). The results of marble burying behavior were expressed as the count of glass spheres buried at least to the 2/3 depth into the sawdust within 30 min. In this paradigm 12 mice were tested per group.

**Immunocytochemistry for c-Fos with diaminobenzidine**

Two hours after the end of FST, mice (n=6) were processed for histological examination. To ensure that anesthetic injection was administered exactly 2 h after FST, they were video recorded in FST simultaneously. Animals were injected within a time period of 2 min with i.p. administered Nembutal (sodium-pentobarbital; 100 mg/kg body weight; Sanofi, Budapest, Hungary) 2 h after the end of the FST; and they became unconscious within 2 min. For control (n=6 per group) PACAP-deficient and wildtype mice were sacrificed without FST exposure after identically applied anesthetic injection. Then, two hours after the end of FST, mice (n=12, PACAP−/− and PACAP+/+ mice, respectively) were perfusion with 150 ml of ice-cold 4% paraformaldehyde in 0.2 M Millonig sodium phosphate buffer (pH 7.4) for 2 min, followed by perfusion with 150 ml of ice-cold 4% paraformaldehyde and 20 ml of 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) for 3×10 min. The reaction was developed by 0.02% DAB in Tris buffer with 0.00003% H₂O₂, for 10 min. The reaction was controlled under a stereo microscope and stopped with PBS. After 3×10 min washes in PBS, sections were mounted on gelatin-coated glass slides, air-dried, treated with xylene for 2×10 min, coverslipped with DePex (Fluka, Heidelberg, Germany), and studied with a Nikon Microphot FXA microscope equipped with a Spot RT color digital camera (Nikon, Tokyo, Japan).

**Microscopy, digital imaging and morphometry**

For qualitative purposes, digital images were grayscale and corrected to obtain optimal contrast using Photoshop 7.0.1 (Adobe, San Jose, CA, USA). Per animal, the cell counts positive for c-Fos were determined in five serial sections, each interspaced by 60 μm in the following brain areas according to Paxinos and Franklin (2003): CeA, BLA, MeA, dorsal subdivision of the lateral septal nucleus (dLS), ventral parts of the lateral septal nucleus (vLS), mPvN, pPvN, EWcp, dPAG, IPAG, DR. The anterior division of BST was studied according to parcellation by Dong et al. (2001): quantification of c-Fos nuclei was carried out in the BSTov, BSTdm, BSTd1, BSTv nuclei (see also in: Hammack et al., 2010). Cell counting was carried out on non-edited digital images using ImageJ software (version 1.3.7, NIH, Bethesda, MD, USA).

All data were expressed as mean and standard error of the means, per experimental group. A random selection procedure was maintained throughout all experiments, and all quantitations were performed according to a double-blind protocol, by an observer experienced in the anatomy of the brain, but unaware of the identity of the sections and the aim of the study.

**Statistics**

Statistical analysis was performed using two-way analysis of variance (ANOVA; α=5%) after testing for normality (Shapiro–Wilks test; Shapiro and Wilk, 1965) and homogeneity of variance (Bartlett’s Chi-square test; Snedecor and Cochran, 1989). Post hoc analysis was carried out with Fisher’s test using Statistica (StatSoft, Tulsa, OK, USA) (α=5%). Behavioral data were evaluated by Student’s two-sample t-test (α=5%). Results were expressed as means±standard error of the mean in each group.
RESULTS

Behavioral tests

Forced swim test. In the FST PACAP<sup>−/−</sup> animals showed a 17.9% longer immobility time compared with PACAP<sup>+/+</sup> mice (67.85±2.29% vs. 49.95±4.94%; Student’s t-test, P<0.01), which was in line with literature data indicating depression-like behavior for these mice (Fig. 1a).

Open field test. In the 5 min of open field test PACAP<sup>−/−</sup> mice were almost twice more active (196.85±6.46 s) than wildtypes (101.42±7.90 s) showing their increased mobility time (Fig. 1b; Student’s t-test: P<0.000001). The evaluation of the traveled distance revealed increased mobility of PACAP<sup>−/−</sup> mice as they crossed 40% more areas than the PACAP<sup>+/+</sup> animals (Fig. 1c; Student’s t-test: P<0.001). Knockouts in the first 90-s period spent 37% longer time (Fig. 1d; Student’s t-test: P<0.001) in the corners of the box than the wildtypes. In line with this, we measured that PACAP<sup>−/−</sup> mice spent about 58% less time in the open middle areas (Fig. 1e; Student’s t-test: P<0.01). We did not observe significant differences in the 90–300-s period of the OF test, but there was a tendency that PACAP<sup>−/−</sup> mice spent 10% more time along the walls (P=0.065). Jumping behavior was observed in four of seven knockout mice (1.00±0.43 jumps per animal), whereas none of the wildtype mice showed this behavior, this difference proved to be statistically significant (P<0.05). The other parameters (head lifting, rearing, grooming activity) did not show any significant differences.

Light–dark box test. The 5 min light–dark box paradigm revealed that the PACAP<sup>−/−</sup> mice spent 37.50% less time in the dark compartment than the PACAP<sup>+/+</sup> mice (Fig. 1f; Student’s t-test: P<0.001), suggesting their reduced anxiety level. In contrast, PACAP<sup>−/−</sup> mice entered (11.83±0.83 entries) the dark box by 36.61% higher frequency (Fig. 1g; Student’s t-test: P<0.001) than PACAP<sup>+/+</sup> mice (7.50±0.43), which is explained by their increased locomotor activity. The number of peeks into the illuminated area was 3.14 times more for the wildtype mice than in PACAP<sup>−/−</sup> animals (Fig. 1h; 5.50±1.14 vs. 1.75±0.51; Student’s t-test: P<0.01). The latency of the first entry into the dark chamber did not differ significantly between the two groups (Student’s t-test: P=0.23).

Marble burying test. The marble burying test for anxiety revealed that mice lacking the PACAP gene buried
11.08±1.08 marbles of 24, whereas wildtypes put 19.50±1.05 glass spheres into the sawdust (Fig. 1i). This 44% difference is highly significant (Student’s t-test: \(P<0.001\)), and suggests that PACAP\(^{-/-}\) mice are less anxious in this paradigm as well.

**Immunocytochemistry for c-Fos**

The quantitation of c-Fos expression in stress-related brain centers of PACAP\(^{-/-}\) and PACAP\(^{+/+}\) animals upon FST was carried out in comparison with non-challenged counterparts with the following results:

**The nuclei of the extended amygdala.** The CeA showed an eightfold increase in c-Fos immunoreactivity in response to FST in both wildtype and transgenic mice (Fig. 2b). ANOVA revealed the main effect of stress significant (\(F_{3,24}=50.97, P<0.00001\)), but there was neither significant effect of genotype (\(F_{3,24}=0.79, P=0.38\)) nor interaction between genotype and stress (\(F_{3,24}=1.65, P=0.22\)). In the BLA (Fig. 2c) we found a highly significant stress effect (\(F_{3,24}=80.27, P<0.000001\)), and stress×genotype interaction (\(F_{3,24}=5.51, P<0.04\)). Post hoc test revealed that PACAP-deficient mice showed reduced c-Fos response to FST compared with wildtypes.

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**Fig. 2.** c-Fos immunocytochemistry in the amygdala, representative images. Control WT: non-stressed wildtype; Control KO: non-stressed PACAP\(^{-/-}\); FST WT: wildtype exposed to forced swim stress; FST KO: PACAP\(^{-/-}\) exposed to forced swim stress. Histograms show the number of c-Fos immunoreactive nuclei in (a) for the medial amygdala (MeA), in (b) for central amygdala (CeA), and (c) for basolateral amygdala (BLA). Lettering at the top of columns indicates significant differences between pairs of groups according to Fisher’s post hoc test (\(P<0.05\)). Scale bar: 200 \(\mu m\).
In the MeA nucleus (Fig. 2a) only stress had a pronounced main effect \((F_{3,24}=47.02, P<0.00001)\) without significant genotype effect, or interaction.

In the BSTov (Fig. 3a) we found an eightfold increase upon stress in wildtypes in c-Fos ir cell counts \((P<0.0001)\). PACAP\(^{+/−}\) animals compared with PACAP\(^{+/+}\) mice exerted a blunted c-Fos response \((-63.84\%; P=0.001)\), moreover the stress-induced increase in c-Fos ir did not reach the level of significance in PACAP\(^{+/−}\) mice \((P=0.11)\). ANOVA found the main effect of stress
In line with the BSTov in the BSTdl (Fig. 3b) in PACAP−/− mice the c-Fos expression upon stress was only two times higher than in non-stressed controls (P<0.05). However, wildtypes upon FST (P<0.0001) showed a 4.5-fold rise in c-Fos cell counts in the BSTdl. The stress-related rise in c-Fos positive cell counts was blunted by 40% (P<0.01) in PACAP−/− mice compared with stressed PACAP+/+ mice. ANOVA evaluated the main effect of stress (F_{3,24}=51.05, P<0.00001) and genotype (F_{3,24}=4.93, P<0.05) significant, and there was a strong stress×genotype interaction (F_{3,24}=9.09, P<0.01).

Studies on the BSTdm (Fig. 3c) revealed that stress had a strong effect on c-Fos immunoreactivity (F_{3,24}=40.11, P<0.001), but there was no main effect of PACAP genotype (F_{3,24}=0.64, P=0.43) on neuronal activation and there is only a tendency for stress×genotype interaction (F_{3,24}=3.63, P=0.07). Post hoc tests also supported that in this area the stress-induced c-Fos expression did not depend on the lack of PACAP (P=0.07), as both PACAP−/− (P<0.01) and PACAP+/+ (P<0.0001) mice showed strong c-Fos expression increase upon stress.

In the BSTv (Fig. 3d) ANOVA found significant main effect of stress (F_{3,24}=50.94, P<0.00001), and stress×genotype interaction (F_{3,24}=8.40, P<0.02), but genotype did not have an obvious effect (F_{3,24}=0.02, P=0.88). Post hoc test revealed that both PACAP−/− (P<0.01) and PACAP+/+ (P<0.0001) mice showed a stress-induced c-Fos immunoreactivity, but increase in wildtypes was greater than in knockouts (P<0.05).

The paraventricular nucleus of the hypothalamus. The activity of the pPVN (Fig. 4a) evaluated by c-Fos immunoreactivity was influenced by stress (F_{3,24}=89.16, P<0.000001) and there was a stress×genotype interaction (F_{3,24}=15.97 P<0.01) without significant effect of genotype (F_{3,24}=1.49 P=0.23). However, post hoc compar-

(F_{3,24}=32.52, P<0.0001) and genotype (F_{3,24}=9.62, P<0.01) significant, moreover a strong stress×genotype interaction (F_{3,24}=10.97, P<0.01).

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isons revealed that both PACAP−/− (P<0.01) and PACAP+/+ (P<0.01) mice showed significant stress-induced increase in c-Fos expression, knockouts had a blunted (−36.46%, P<0.01) c-Fos response. Interestingly, we observed a tendency (P=0.08) for baseline c-Fos expression difference.

In the mPVN (Fig. 4b), similarly to the pPVN, ANOVA found the main effect of stress (F3,24=64.09; P<0.0001) significant, moreover there was a strong interaction between stress and genotype (F3,24=15.97; P<0.01), but the genotype did not affect (F3,24=0.69; P=0.41) the c-Fos expression. According to the post hoc comparisons control PACAP−/− mice had 3.2 times more c-Fos positive neurons in the mPVN than PACAP+/+ ones (P<0.04). FST induced an almost 10-fold increase in PACAP+/+ mice (P<0.000001). In the knockouts, however, the elevation was only 87% (P<0.02). The FST-induced c-Fos cell count in the mPVN of knockouts was reduced by 36% (P<0.01) compared with PACAP+/+ mice. It must also be noted that there was a 3.2 times higher baseline (P<0.05) c-Fos expression in PACAP−/− mice than in wildtypes.

The lateral septum. In the dLS (Fig. 5a) stress increased the c-Fos expression (ANOVA: F3,24=37.43; P<0.0001), but neither the effect of genotype (ANOVA: F3,24=0.35; P=0.55) nor interactions (ANOVA: F3,24=1.88; P=0.19) were significant. In contrast, in the vLS (Fig. 5b), we found that stress (ANOVA: F3,24=31.16; P<0.0001) and genotype (ANOVA: F3,24=5.85; P<0.01) had a strong effect without clear interactions (ANOVA: F3,24=2.17; P=0.16). The c-Fos expression in the vLS after stress was about the half in PACAP−/− mice compared with PACAP+/+ counterparts (P<0.02).

The EWcp nucleus. Highly significant effects were observed in this mesencephalic stress center. ANOVA revealed the main effects of stress (F3,24=164.56; P<0.000001) and genotype (F3,24=15.34; P<0.01) significant, moreover there was an obvious stress×genotype interaction (F3,24=23.87; P<0.001). When comparing the
stress reactivity of PACAP⁺/⁻ and PACAP⁻/⁻ mice we observed that their stress-induced c-Fos expression (Fig. 6) significantly differed (P<0.0001). There was an eight-fold increase in c-Fos cell counts (P<0.000001) in PACAP⁺/⁺ mice, in contrast PACAP⁻/⁻ mice displayed only a fourfold increase (P<0.00001).

The periaqueductal gray matter. In the lateral part of the periaqueductal gray matter (PAG) (Fig. 7a), we found that the c-Fos immunoreactivity was dependent on stress (F₃,₂₄=225.14, P<0.000001), and there was a strong stress x genotype interaction (F₃,₂₄=41.45, P<0.000001). The effect of genotype appeared not to be significant in the IPAG (F₃,₂₄=1.59, P=0.22). Two hoc tests found that there is a clear rise in c-Fos expression in both PACAP⁻/⁻ (about two times, P<0.0001) and PACAP⁺/⁺ (10 times, P<0.0000001) mice. Interestingly, the blunted c-Fos response to stress in the knockouts compared with the wild-type (P<0.01) was accompanied by a significant difference in the control groups, as knockouts had about fourfold higher c-Fos cell counts than wildtypes (P<0.00001).

In the dorsal PAG ANOVA found the main effect of stress (F₃,₂₄=49.38, P<0.000001), genotype (F₃,₂₄=9.23, P<0.01), and stress x genotype interaction (F₃,₂₄=5.93, P<0.03) significant. In this area we observed that non-stressed PACAP⁺/⁻ mice had 2.7 times more c-Fos ir neurons in the dPAG (Fig. 7b), than wildtype counterparts. When exposed to FST in wildtypes there was an almost fourfold increase in c-Fos cell counts (P<0.000001), moreover, PACAP⁺/⁺ mice answered with about 50% increase in c-Fos response (P<0.01). Interestingly, after stress, the baseline difference in terms of c-Fos immunoreactivity disappeared, as both genotypes displayed similar c-Fos cell counts after FST (P<0.067).

The DR nucleus. The c-Fos immunoreactivity in the DR (Fig. 8) was influenced both by stress (F₃,₂₄=96.13, P<0.000001) and genotype (F₃,₂₄=40.75, P<0.000001), and ANOVA found a significant stress x genotype interaction (F₃,₂₄=49.11, P<0.000001) as well. Post hoc comparisons revealed that stress resulted in an about 75-times rise in of c-Fos cell counts in wildtypes (P<0.000001). In contrast, although in PACAP⁺/⁻ mice the stress reaction was significant (P=0.02), there was only a fivefold increase in c-Fos cell counts. The comparison of stressed wildtypes and knockouts revealed that the stress-induced c-Fos response of PACAP⁻/⁻ mice was clearly blunted as PACAP⁺/⁺ mice upon FST possessed 4.5-times more (P<0.00001) c-Fos ir nuclei in the DR.

DISCUSSION

Here we show that PACAP⁻/⁻ mice exhibit increased locomotor activity in open field and jumping behavior of PACAP⁻/⁻ mice have also been reported (Hashimoto et al., 2001), which also supports our findings. However, in contrast to the results of Hashimoto et al. (2001), in our 5-min open field session PACAP⁻/⁻ mice spent less time in the central area but more in the peripheral suggesting increased anxiety-like behavior. This contradiction might be explained by the slightly different method, as we did not observe the animals longer than 5 min, whereas in Hashimoto’s work (2001) significant differences appeared from the 30th min of the open field in terms of time spent in the center of the box. In addition, PACAP⁻/⁻ mice are more active in exploring the box (Hashimoto et al., 2001), therefore, the longer time spent at the walls should be interpreted as an increased exploratory behavior and not as a sign of increased anxiety. This idea is also supported by the results of light–dark box and marble burying tests suggesting reduced anxiety in PACAP⁻/⁻ mice.

Reduced anxiety levels were previously recorded in elevated plus maze, novel-object recognition and emergence tests (Hashimoto et al., 2001). To the best of our knowledge this is the first study publishing data on anxiety levels in marble burying and light–dark box paradigms in these mice. In line with previous studies, we have also confirmed that PACAP deficient mice show...
Fig. 6. c-Fos immunocytochemistry in the central projecting Edinger–Westphal nucleus (EWcp), representative images. Control WT: non-stressed wildtype; Control KO: non-stressed PACAP−/−; FST WT: wildtype exposed to forced swim stress; FST KO: PACAP−/− exposed to forced swim stress. Histogram shows the number of c-Fos immunoreactive nuclei. Aq, cerebral aqueduct. Lettering at the top of columns indicates significant differences between pairs of groups according to Fisher’s post hoc test (P<0.05). Scale bar: 200 μm.
reduced anxiety, as they buried fewer marbles, and spent significantly shorter time in the brightly illuminated compartment of the light–dark box. In the latter test, in line with open field results, the increased locomotor activity was also observed because PACAP $^{--}$ mice crossed the opening between compartments more frequently. We also compared the number of peeks into the lit compartment and interpreted this behavior as aborted attempt to enter the lit chamber, representing high anxiety levels (for review see Bourin and Hascoët, 2003). In line with other variables we have found that PACAP $^{--}$ mice are less anxious as they peeked more rarely into

Fig. 7. c-Fos immunocytochemistry in the periaqueductal gray matter (PAG), representative images. Control WT: non-stressed wildtype; Control KO: non-stressed PACAP $^{--}$; FST WT: wildtype exposed to forced swim stress; FST KO: PACAP $^{--}$ exposed to forced swim stress. Histograms show the number of c-Fos immunoreactive nuclei in (a) for lateral part of PAG (IPAG) and (b) for dorsal part of PAG (dPAG). Lettering at the top of columns indicates significant differences between pairs of groups according to Fisher's post hoc test ($P < 0.05$). Scale bar: 100 μm.
the illuminated box and they more frequently cross the opening successfully.

Depression-like behavior in FST accompanied with reduced anxiety in the same animal seems to be contradictory. Although mood- and anxiety disorders are often comorbid diseases (McEvoy et al., 2011) they do not necessarily share common neurobiological substrates. For instance, Regev et al. (2011) recently demonstrated that the local overexpression of CRF in the BSTdl induces depression-like phenotype without influencing anxiety, whereas CRF overexpression in the CeA reduces stress-induced anxiety, but leaves depression like behavior unaffected (Regev et al., 2011). Given the fact that these nuclei, and more specifically their CRF neurons receive PACAP containing innervation in the rat (Hannibal, 2002; Kozicz et al., 1997), PACAP may exert a brain area-specific control on behavior through CRF neurons. Reduced c-Fos expression in the BSTdl in

![Image](image.jpg)

**Fig. 8.** c-Fos immunocytochemistry in the dorsal raphe nucleus (DR), representative images. Control WT: non-stressed wildtype; Control KO: non-stressed PACAP$^{-/-}$; FST WT: wildtype exposed to forced swim stress; FST KO: PACAP$^{-/-}$ exposed to forced swim stress. Histogram shows the number of c-Fos immunoreactive nuclei. Lettering at the top of columns indicates significant differences between pairs of groups according to Fisher’s post hoc test ($P<0.05$). Scale bar: 100 μm.
PACAP deficiency suggests that PACAP could facilitate the activation of these neurons. In the CeA PACAP may be inhibitory, as the lack of the peptide does not reduce the c-Fos expression.

### Activation pattern of stress-related centers upon FST as evaluated by c-Fos

**General considerations.** The product of an immediate early gene c-Fos is a widely used and accepted tool to evaluate acute neuronal activation (for review see: Kovács, 2008). By mapping the c-Fos response by immunocytochemistry 2 h after stress we found that FST was effective in all studied areas to induce a robust rise in the number of c-Fos positive neurons in wildtype mice, which is in line with literature data (Stone et al., 2007, for review see also: Kovács, 1998). The magnitude of c-Fos expression increase upon stress was brain area dependent; for instance, in the dLS we observed only a less than fourfold elevation in c-Fos upon stress, whereas in the DR there was a more than 75-fold rise in c-Fos cell counts upon stress (Table 1). This strongly suggests that the studied brain centers show different sensitivities to FST, which is in line with results of other laboratories obtained in rats (Duncan et al., 1993; Cullinan et al., 1995; Kovács, 1998).

The most important findings of this study are c-Fos response was blunted in PACAP knockouts in several (e.g. BSTov, BSTdl, BSTv, vLS, EWcp, DR, dPAG, IPAG), but not in all (CEA, MeA, BSTdm, dLS) of the studied nuclei upon FST. This suggests that the reduced sensitivity of stress centers in PACAP deficiency might be responsible for the observed robust reduction in anxiety measures in PACAP−/− mice.

In **Table 1** we compare the magnitudes of effects of stress×genotype interactions on c-Fos expression (Table 1). Among the studied nuclei the strongest effects occur in the DR, EWcp, and IPAG. The latter area is involved in descending antinociceptive systems (Heinricher et al., 2009), whereas the DR and EWcp nuclei might have a strong influence on the observed behavioral alterations. Indeed, both serotoninergic neurons in DR and Ucn1 expressing perikarya in the EWcp were shown to contribute to stress-related disorders and anxiety in humans (Kozicz et al., 2008; Savitz et al., 2009; Lowry et al., 2008). The c-Fos expression in the DR serotonin neurons is associated with anxiety-like behavior (Bouwknegt et al., 2007); moreover, urocortinergic neurons in the EWcp might interact with DR serotonin neurons (Kozicz, 2010; Neufeld-Cohen et al., 2010). The DR, EWcp, and IPAG receive

<table>
<thead>
<tr>
<th>ANOVA (F- and P-values)</th>
<th>Relative PACAP immunoreactive fiber density (Hannibal, 2002)</th>
<th>Difference in c-Fos stress response in PACAP−/− mice compared with wildtypes</th>
<th>Fisher’s post hoc comparison of c-Fos cell counts of stressed PACAP−/− and wildtype mice (P-values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress</td>
<td>Genotype</td>
<td>Interaction</td>
<td></td>
</tr>
<tr>
<td>CEA</td>
<td>F=50.97</td>
<td>P&lt;0.00001</td>
<td>F=0.16</td>
</tr>
<tr>
<td>MeA</td>
<td>F=47.02</td>
<td>P&lt;0.00001</td>
<td>F=1.24</td>
</tr>
<tr>
<td>BLA</td>
<td>F=80.27</td>
<td>P&lt;0.00001</td>
<td>F=5.51</td>
</tr>
<tr>
<td>BSTov</td>
<td>F=32.52</td>
<td>P&lt;0.00001</td>
<td>F=10.97</td>
</tr>
<tr>
<td>BSTdl</td>
<td>F=51.05</td>
<td>P&lt;0.00001</td>
<td>F=9.09</td>
</tr>
<tr>
<td>BSTdm</td>
<td>F=40.10</td>
<td>P&lt;0.00001</td>
<td>F=3.62</td>
</tr>
<tr>
<td>BSTv</td>
<td>F=50.94</td>
<td>P&lt;0.00001</td>
<td>F=8.40</td>
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<tr>
<td>dLS</td>
<td>F=37.43</td>
<td>P&lt;0.00001</td>
<td>F=1.88</td>
</tr>
<tr>
<td>vLS</td>
<td>F=31.16</td>
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<td>F=2.17</td>
</tr>
<tr>
<td>EWcp</td>
<td>F=164.55</td>
<td>P&lt;0.000001</td>
<td>F=23.87</td>
</tr>
<tr>
<td>DR</td>
<td>F=96.13</td>
<td>P&lt;0.000001</td>
<td>F=40.75</td>
</tr>
<tr>
<td>dPAG</td>
<td>F=49.38</td>
<td>P&lt;0.00001</td>
<td>F=9.23</td>
</tr>
<tr>
<td>IPAG</td>
<td>F=225.14</td>
<td>P=0.000001</td>
<td>F=1.5971</td>
</tr>
<tr>
<td>mPVN</td>
<td>F=64.09</td>
<td>P=0.000001</td>
<td>F=15.97</td>
</tr>
<tr>
<td>pPVN</td>
<td>F=89.16</td>
<td>P=0.000001</td>
<td>F=1.49</td>
</tr>
</tbody>
</table>

**Table 1.** Summary of statistics on c-Fos cell counts in comparison with relative density of PACAP immunoreactive nerve fibers in respective stress centers (Hannibal, 2002) and with the magnitude of c-Fos reduction in PACAP deficiency (Significant differences highlighted in bold.)
innervation by PACAP fibers (Hannibal, 2002) and express PACAP receptors (Vaudry et al., 2009), thus, the neuro-anatomical basis is given for actions of PACAP on these nuclei.

In other nuclei (e.g. mPVN, pPVN, BSTov, BSTdl, BSTv, BLA) the effect of stress × genotype interaction was less strong (Table 1) but still present. In addition, FST-induced c-Fos response in PACAP−/− mice was remarkably blunted as well, suggesting their possible contribution to the altered behavioral response to stress. The observation that the stress response is blunted in some nuclei suggests that PACAP plays an important role in stress induced activation of these nuclei, and consequently contributes to stress induced anxiety-like behaviors. Upon interpretation of these data one has to consider that developmental compensatory mechanisms are often observed in genetically modified animals; however, Girard et al. (2006) failed to demonstrate such alterations in PACAP−/− mice.

Limitation of data. Upon interpretation of our results, we have to note that although we used well validated techniques, these are not without limitations.

a. Since our model is a developmental PACAP deficient model, possible developmental compensatory mechanisms should be considered. However, Girard et al. (2006) found no developmental compensatory changes in PACAP−/− mice at the level of related neuropeptides (i.e. up-regulation of vasoactive intestinal polypeptide) and their cognate receptors (VPAC or PAC1 receptor expression levels), thus the observed effects are most probably because of lack of PACAP. Compensations at other levels could be present in yet unstudied circuitries, and future studies have to clarify if they are present.

b. Although c-Fos is a widely accepted and well-validated tool to assess neuronal activation, one has to consider the limitations of the technique that it cannot reveal neuronal inhibition (for review see: Kovács, 2008). Such neuronal inhibition could have been also very important in the observed behavioral phenotype in our mouse model.

The BST. In a recent study Hammack et al. (2009) demonstrated in the rat that the expression of PAC1 receptor and PACAP mRNA is increased by chronic stress in the BST, and infusion of PACAP into the BST induces anxiety-like behavior, moreover, the lack of PAC1 receptor causes reduced anxiety-like behavior in mice (Otto et al., 2001). Both BSTov and BSTdl receive dense PACAP containing innervation, and PACAP axon terminals form synaptic contacts with CRF neurons in rats (Köves et al., 1994; Kozicz et al., 1997). PACAP deficiency was accompanied with marked reduction (−63.83% and −40.10%, respectively) in c-Fos expression upon stress in PACAP−/− mice. In follow up studies it has to be established that these reduced stress-induced activation indeed involves CRF-positive neurons in the BSTov and BSTdl. Recently it has been also shown that CRF overexpression in the BSTdl results in depression-like phenotype, without affecting anxiety levels (Regev et al., 2011) further supporting the idea of a CRF-PACAP interaction in the BSTdl. Moreover, it has also to be tested whether PAC1 receptors are expressed by CRF neurons in these nuclei.

The amygdala nuclear complex. The amygdala nuclear complex is also involved in stress adaptation (for review see: McEwen and Gianaros, 2010). Particularly, the extrahypothalamic CRF systems in the amygdala have been shown to be affected by stress in the rat (Nijsen et al., 2001; Kim et al., 2010; Rouwette et al., 2011) and they receive dense PACAP ir innervation (Hannibal, 2002). To our surprise we did not observe significant effects of the lack of PACAP on c-Fos expression in the CeA and MeA in PACAP deficient mice. There was, however, a significant interaction between stress and genotype in the BLA, with slightly (−27%) blunted c-Fos expression according to the post hoc test. This suggests that the BLA and its decreased sensitivity to stress in this area might contribute to the observed reduced anxiety. Although recent evidence suggests that increased CRF overexpression in the CeA might be responsible for reduced anxiety, (Regev et al., 2011) further studies have to characterize possible PACAP-CRF interactions in the CeA. The MeA has been recently reported to be affected by PACAP deficiency in its c-Fos expression upon restraint stress (Tsukiyama et al., 2011). This discrepancy and the low effect of the lack of PACAP on c-Fos might be explained by the different type of stressor used in this study (i.e. FST). Based on these findings it seems that central and medial parts of the amygdala nuclear complex do not play a pivotal role in the observed behavioral and functional morphological changes in PACAP deficient mice.

The PVN. The CRF neurons in the rat PVN are the main regulators of the HPA axis and they show c-Fos expression following PACAP injections into the lateral ventricle (Agarwal et al., 2005), which was attenuated by PACAP antagonists (Grinevich et al., 1997). In stress, Stroth and Eiden (2010) found that PACAP knockout mice show impaired PVN c-Fos activation coupled with blunted corticosterone response in stress. The FST-induced c-Fos activation in the PVN was described by other laboratories in rat (Badowska-Szalewska et al., 2009) and mouse (Stone et al., 2011) as well. Most recently, Tsukiyama et al. (2011) found that upon restraint stress c-Fos expression in the PVN was blunted in PACAP−/− mice, which was concomitant with the attenuation of the corticosterone response. In contrast, in cold exposure and ether stress the corticosterone rise was not affected, therefore, PACAP seemed to have an effect on emotional stress (Tsukiyama et al., 2011). In line with these data we observed a strong effect of interaction between FST and genotype on the c-Fos with blunted c-Fos expression upon stress in PACAP deficient mice.

The EWcp. The EWcp nucleus receives some PACAP containing fibers in the rat (Hannibal, 2002), moreover there are close appositions between PACAP terminals and Ucn1 neurons in the rat EWcp (Kozicz T, unpub-
lished observation). The lack of PACAP reduces the c-Fos reactivity of neurons in the mouse EWcp by 43.46%, which is recruited differentially and specifically by various types of stress in the rat (Gaszner et al., 2004, 2009) and in various mouse strains (Ryabinin et al., 1999). Urocortinergic neurons of the EWcp project mainly to the DR and to the vLS modulating the stress response (Bittencourt et al., 1999; Kozicz, 2007, 2010).

The PAG. The PAG is known to receive PACAP-containing fibers. The dorsal part of the PAG has been implicated in the psychopathophysiology of anxiety disorders (Graeff and Zangrossi, 2010). According to our results there was a clear baseline difference in c-Fos expression, suggesting that this area might be responsible for the behavioral alterations observed in our anxiety tests and emergence test in Hashimoto’s work (2001). Importantly, upon FST exposure, there was no more difference between PACAP−/− and wildtype mice.

The lateral PAG has been extensively studied on the descending nociception control (for review see: Heinricher et al., 2009). It has been shown that PACAP deficient mice show altered c-Fos response in pain models in the PAG (Sándor et al., 2010). In the IPAG-c-Fos we found a strong stress × genotype interaction moreover, in non-stressed mice there is again a baseline difference in neuronal activity, which could explain the known impaired nociceptive behavior in PACAP−/− mice.

The DR nucleus. The comparison of the magnitude of c-Fos response reduction among the nuclei recruited by the lack of PACAP revealed that the strongest effect was observed in the DR with the decrease of 78% in c-Fos cell counts. This is of great interest given that DR serotonergic neurons are pivotal in stress-related anxiety and mood disorders (Bouwknecht et al., 2007; Savitz et al., 2009; Lowry et al., 2008; Valentino et al., 2010). However, according to Hannibal’s (2002) work the rat DR receives only weak PACAP innervation, still, the lack of the peptide has a deep impact on c-Fos reactivity. This suggests that PACAP’s effect is not direct on DR, but relayed by some other brain areas like the EWcp (Kozicz, 2010; Neufeld-Cohen et al., 2010) conveying PACAP-related information to the DR. Future studies on the serotonin systems in PACAP deficiency could help to explain how the DR contributes to the well-known behavioral alterations.

CONCLUSIONS

Although the role of PACAP in stress induced c-Fos activation of the PVN has been shown previously (Grinevich et al., 1997; Agarwal et al., 2005; Stroth and Eiden, 2010; Tsukiyama et al., 2011) this study is the first providing evidence that several stress-related centers besides the PVN show reduced immediate early gene expression to acute stress, and that the effect of PACAP deficiency seems to be brain area specific. Taken together, our data strongly support the notion that PACAP deficiency modulates the functioning of the DR, EWcp, and vLS in stress adaptation leading to increased depression-like behavior in FST and reduced anxiety levels in PACAP deficiency. However, the involvement of all these areas in anxiety and depression-like phenotype have to be further studied, the role of PACAP and its cognate receptors in stress-related mood disorders has been recently established (Hammack et al., in press; Hashimoto et al., 2009, 2010; Pinhasov et al., 2011; Ressler et al., 2011), and our study identified putative brain centers that could be crucial in this process. Future studies are in progress to clarify which neurotransmitter (i.e. serotonin in the DR and PAG) and neuropeptide (i.e. CRF in the extended amygdala, Ucn1 in the EWcp) systems are affected by PACAP signaling, and how their interaction would contribute to the development of stress-related mood disorders.

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