INVESTIGATION OF STRESS ADAPTATION SYSTEMS OF PITUITARY ADENYLATE-CYCLASE ACTIVATING POLYPEPTIDE DEFICIENT MICE IN ACUTE AND CHRONIC MODELS

PhD Thesis

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INTRODUCTION

Stress and depression

János Selye defined the classic concept of stress as a „nonspecific response of the body to any threatening demand" (1936). More recently, McEwen (2000) defined stress as a real or interpreted threat to the physiological or psychological integrity on an individual that results in physiological and/or behavioral responses". The prevalence of mood disorders in the western type of communities is continuously increasing: major depression is now the most common reason for chronic disability causing significant social and financial load on the healthcare systems (WHO, 2016). The complex neurobiological and neurochemical background of depression is not fully understood. Despite the fact that multiple groups of medicines are available for the treatment, there are patients who do not respond to the pharmacotherapy. Additionally, due to the wide range of side effects sometimes it is not possible to introduce the optimal therapeutic strategy. Therefore, to find more effective therapeutical strategies it is essential to deeper understand the pathophysiological background of the psychopathology. To reach this purpose the identification of new key mediators and drug targets is required. This may be possible if the imperative need for new well characterized and more reliable animal models will be fulfilled. Numerous groups neuropeptides and their receptors have been found to exert a tonic regulatory role in stress adaptation-response both in animals and humans. Clinical trials have been lunched to test if newly designed pharmacological agents targeted to the neuropeptide receptors may be applied as useful tools in the management of depression. (Lin 2012, Kormos and Gaszner 2013, Catena-Dell'Osso at al., 2013).

The main goal of my PhD research program was to examine the stress adaptation systems in acute and chronic mouse models with the hope that our new findings may contribute to gain deeper insight into the neurobiological background of depression and may help to identify new therapeutical approaches.

Pituitary adenylate-cyclase activating polypeptide (PACAP) and depression

PACAP belongs to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon family with a 27 and a 38 amino acid isoforms. The primary PAC1 receptor is specific for PACAP, while the VPAC1 and VPAC2 receptors bins PACAP and VIP with similar affinity (Vaudry et al., 2009, Hammack and May 2015). PACAP is a pleiotropic neuropeptide, involved in a wide range of biological functions (Vaudry et al., 2009, Reglődi et al., 2012). In the central nervous system,

besides others it exerts neurotrophic and neuroprotective effects. (Reglődi et al., 2001, Hammack et al., 2008, Hashimoto et al., 2009).

PACAP knockout (KO) mice show depression-like behavioral alteration in forced swim test (FST). This phenotype related to the lack of the functional PACAP gene may be reversed by antidepressant treatment (Hashimoto et al., 2001, 2009). Based on these arose the idea that this animal may be a useful tool to model the genetically inherited predisposition. Hashimoto and co-workers (2010) observe in human studies that some nucleotide polymorphisms in the PACAP gene may be associated with major depression. Other results also support the potential role of PACAP in human psychopathologies (Hashimoto et al., 2011), as the PACAP via PAC1 receptor signaling increased the expression of the DISC1 gene (disrupted in schizophrenia 1) (Hattori et al., 2007). The latter has been shown to be involved in the pathogenesis of both schizophrenia (Millar et al., 2000) and major depression (Blackwood et al., 2001). More recently the gene polymorphisms of PACAP (Ressler at al., 2011) and PAC1 receptor (Almli et al., 2013, Mercer et al., 2016) were found to play an important role in the development of post-traumatic stress disorder in women.

AIMS

1. Studies on anxiety-level and acute stress response of PACAP knockout mice by behavioral tests and c-Fos immunohistochemistry

1.1. Comparison of PACAP KO and wild type (WT) mice in behavioral tests for anxiety- and depression-level assessment.

1.2. Mapping of acute stress-induced neuronal activity pattern by examination of the c-Fos marker. Studies were focused on stress-reactive brain areas which receive PACAPergic innervation and/or express PACAP receptors. Immunohistochemical assessment was performed in WT and PACAP KO mice upon FST in the following brain areas: extended amygdala, such as the dorsolateral (dlBST), dorsomedial (dmBST), oval (ovBST) and ventral (vBST) bed nucleus of the stria terminalis (BST), the central (CeA), basolateral (BLA) and medial (MeA) nuclei of amygdala; the parvocellular (pPVN) and magnocellular (mPVN) divisions of the paraventricular nucleus of the hypothalamus (PVN); the ventral and dorsal lateral septum (LS); the dorsal (dPAG) and lateral (lPAG) periaqueductal gray matter; as well as the centrally projecting Edinger-Westphal (cpEW) and dorsal raphe (DR) nuclei.

2. Behavioral and functional morphological examination of PACAP knockout mice in the chronic variable mild stress (CVMS) model

2.1. Comparison of PACAP KO and WT mice in the two weeks CVMS depression model.

2.2. Examining the effect of the tricyclic antidepressant imipramine and the reversibility of the CVMS-induced effects. The model's validity was evaluated by behavioral (FST), physical (body- and adrenal weight changes), endocrinological [corticosterone (CORT) titer] and functional-morphological tools. To assess the chronic effect of stress, the chronic neuronal activity marker FosB labeling was performed in PACAP containing limbic centers (CeA, MeA, BLA, dmBST, dlBST, ovBST, vBST, the CA1 and CA3 region of the hippocampus, the dentate gyrus (DG), the dLS and the vLS). As these areas and also PACAP influence the stress response of the parvo- and magnocellular divisions of the PVN, FosB was also quantified there. Based on their PACAPergic innervation, FosB expression was also assessed in the anxiety-related dPAG, lPAG (involved in the descending antinociceptive system), and in the cpEW and DR. In the second step, semi-quantitative double label immunofluorescence labelings were performed to examine the function and FosB activity of the a) corticotropin-releasing factor (CRF) expressing ovBST, b) the urocortin1 (Ucn1) containing cpEW as well as the c) serotonin (5-HT) containing DR neurons.

EXPERIMENTAL MODELS AND METHODS

1. Animals

Animals were housed in the animal facility of the Anatomy Department, Pécs University in standard polycarbonate cages. They were reared in a 12 hours light-dark cycle at 24-25 °C. Mice were supplied by drinking (tap) water and standard rodent chow *ad libitum.*

• **Acute stress model:**

38 male PACAP KO mice on CD1 background were examined compared to the same number of WT mice.

• **Chronic stress model:**

36 adult male PACAP KO mice and 33 WT mice were selected. Animals were assigned into 12 groups. Half of mice were subjected to a 14 days CVMS, while the other half was exposed only to a 5 minutes session of FST 10 days prior perfusion. Within both main groups one third of mice were left untreated, one third was subjected to intraperitoneal (i.p.) vehicle injection

(physiological saline) and one third received i.p. imipramine injection treatment (daily 15 mg/bodyweight kg).

2. Behavioral tests

Anxiety tests:

• Open field test

• Light-dark box test

• Marble burying test

Depression test:

• Forced swim test

3. c-Fos immunohistochemistry in the acute model

The protein product of the *c-fos* gene, c-Fos accumulates in the nuclei and is a widely accepted tool to asses the neuronal activity (Kovács 1998). To quantitate this, mice were perfused two hours upon FST and the immunohistochemistry was performed using a polyclonal rabbit c-Fos antiserum and a biotinylated goat anti mouse IgG.

4. Chronic variable mild stress paradigm

In the CVMS model (Willner 2005) animals were exposed both to various daytime stressors and challenges in the dark phase.

Stroger, but short-term daytime stressors:

• Tilted cage

• Laboratory shaker exposure

• Restraint stress

Mild, longer-term nighttime stressors:

• Social isolation stress

• Humid nesting material stress

• Group holding

To minimize the adaptation to the stressors applied, mice were exposed to the stressors in a randomized manner.

Bodyweights were registered on the first and seventh days at the time of changing the nesting material. The last bodyweight measurement was performed on anaesthetized mice on the 15th day just before the perfusion.

Subjects of the stressed groups were exposed on the 14th day to 5 minutes FST. To avoid the acute effect of the FST on controls, mice in the non-stressed groups were exposed 10 days before perfusion to a 5 minutes FST. To avoid a possible chronic effect, mice were subjected to a single FST session without pre-test exposure.

5. FosB immunohistochemistry in the chronic model

To assess the chronic neuronal activity, FosB immunohistochemistry was applied. The staining was performed by a solution of anti-FosB antibodies produced in rabbit and by treatment with biotinylated goat anti-rabbit IgG.

6. Free-floating double-label immunofluorescence for CRF and FosB

To assess the activity of CRF neurons in the ovBST, we applied a primary polyclonal rabbit anti-FosB and a goat anti-CRF antibody. As a secondary, a Cy3-conjugated donkey anti-rabbit and a biotinylated donkey anti-goat antiserum was used with Cy5-conjugated streptavidin.

7. Free-floating double-label immunofluorescence for Ucn1 and FosB

To evaluate the activity of Ucn1 neurons, primary polyclonal rabbit anti-FosB and goat anti-Ucn1 antibodies were applied. As secondary antisera, Alexa Fluor 488-conjugated donkey anti-goat and Cy3-conjugated donkey anti-rabbit antibodies were used.

8. Free-floating double-label immunofluorescence for 5-HT and FosB

In case of the samples for the DR, we used the same protocol as described for Ucn1 and FosB except that we used a mouse monoclonal anti-5-HT serum instead of the Ucn1 serum.

9. Microscopy

Digital images were obtained using a *Nikon Microphot FXA* microscope equipped with a *Spot RT color* digital camera (Nikon, Tokyo, Japan). Immunofluorescence was digitalized by an *Olympus FluoView 1000* confocal microscope.

10. Corticosterone radioimmunoassay

The glucocorticoid hormone content of plasma samples was measured in order to assess the activity of the hypothalamus-pituitary-adrenal (HPA)-axis.

11. Statistics

Acute stress model:

The statistical assessment was performed by two-way analysis of variance (ANOVA) (ANOVA; α=5%). The validity of the ANOVA test was assessed by testing for the normal distribution of data (Shapiro-Wilk test; Shapiro and Wilk 1965) as well as by examination of the within-group variance of homogeneity (Bartlett's Chi-square test; Snedecor and Cochran 1989). *Post hoc* analyses were performed by the Fisher's test using Statistica 8.0 software (α=5%). Behavioral tests were assessed by the Student's two-sample t-test (α=5%). Data were presented as mean of the group with the respective standard error of the mean (± SEM).

Chronic stress model:

All data were expressed as mean and as standard error of the mean for each experimental group. Datasets were tested for normality (Shapiro and Wilk, 1965) and homogeneity of variance (Snedecor and Cochran, 1989). Outlier data beyond the two sigma range were excluded from statistics. Dataset 1 was evaluated by two-way analysis of variance (ANOVA). Dataset 2 was subjected to multifactorial analysis of variance (MANOVA). All post hoc analyses were carried out by Fisher's *post hoc* test, using Statistica 8.0 (α= 5%).

RESULTS AND DISCUSSION

1. Studies on anxiety-level and acute stress response of PACAP knockout mice by behavioral tests and c-Fos immunohistochemistry

Here we show that PACAP KO mice exhibit increased locomotor activity in open field, increased depression-like phenotype in FST, but reduced anxiety in marble burying and light-dark box paradigms. The altered behavioral phenotype of PACAP deficient mice was concomitant with blunted or absent c-Fos response to FST in BST, LS, PVN, cpEW and DR; suggesting that they play an important role in mediating PACAP actions on the animal's mood.

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

1.2.1. General considerations

By mapping the c-Fos response by immunocytochemistry 2 hours 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 4-fold elevation in c-Fos upon stress, while in the DR there was a more than 75-fold rise in c-Fos cell counts upon stress. 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. ovBST, dlBST, vBST, vLS, cpEW, DR, dPAG, lPAG), but not in all (CEA, MeA, dmBST, 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 KO mice.

We compared the magnitudes of effects of stress x genotype interactions on c-Fos expression. Among the studied nuclei the strongest effects occur in the DR, cpEW and lPAG. The latter area is involved in descending antinociceptive systems (Heinricher et al., 2009), while the DR and cpEW nuclei might have a strong influence on the observed behavioral alterations. Indeed, both serotoninergic neurons in DR and Ucn1 expressing perikarya in the cpEW were shown to contribute to stress-related disorders and anxiety in humans (Kozicz et al., 2010; Savitz et al., 2009; Lowry et al., 2008). The c-Fos expression in the DR serotonin neurons is associated with anxiety-like behavior (Bouwknecht et al., 2007); moreover, urocortinergic neurons in the cpEW might interact with DR serotonin neurons (Kozicz, 2010; Neufeld-Cohen et al., 2010). The DR, cpEW and lPAG receive innervation by PACAP fibers (Hannibal 2002) and express PACAP receptors (Vaudry et al., 2009), thus, the neuroanatomical basis is given for actions of PACAP on these nuclei.

In other nuclei (e.g. mPVN, pPVN, ovBST, dlBST, vBST, BLA) the effect of stress x genotype interaction was less strong but still present. In addition, FST-induced c-Fos response in PACAP KO 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.

1.2.2. The bed nucleus of the stria terminalis

PACAP deficiency was accompanied with marked reduction (-63.83% and -40.10%, respectively) in c-Fos expression upon stress in PACAP KO mice in the ovBST and in the dlBST.

1.2.3. The amygdala nuclear complex

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.

1.2.4. The paraventricular nucleus of the hypothalamus

We observed a strong effect of interaction between FST and genotype on the c-Fos with blunted c-Fos response upon stress in PACAP deficiency.

1.2.5. The centrally projecting Edinger-Westphal nucleus

The lack of PACAP reduces the c-Fos reactivity of neurons in the mouse cpEW by 43.46%.

1.2.6. The periaqueductal gray matter

There was a clear baseline difference between PACAP KO and wildtype mice in c-Fos expression, this difference disappears upon FST exposure.

In the lPAG c-Fos we found a strong stress x genotype interaction moreover, in non-stressed mice there is again a baseline difference in neuronal activity.

1.2.7. The dorsal raphe 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.

1.3. Conclusions

Although the role of PACAP in stress induced c-Fos activation of the PVN has been shown previously. This study is the first providing evidence that besides the PVN and MeA several stress-related centers 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 stress (mal)adaptation leading to increased depression and reduced anxiety levels in PACAP deficiency. Our study identified putative brain centers that could be crucial in the observed behavioral alterations.

2. Behavioral and functional morphological examination of PACAP knockout mice in the chronic variable mild stress (CVMS) model

2.1. Model validation

The reduced bodyweight gain proved the effect of CVMS, in agreement with other studies in CD1 mice (Boleij et al., 2014, Rabasa et al., 2015, for review see: Harris 2015). CVMS did not reduce bodyweight in PACAP KO mice, which fits with the findings of Mustafa et al., (2015). Imipramine seems to reduce the bodyweight gain in non-stressed WT mice, which phenomenon was observed by other laboratories both in rats (Lewis et al., 1983) and mice (Zhao et al., 2015). Our data on increased adrenal weights of WT mice also clearly support the effectivity of our CVMS paradigm (for review see: Bali et al., 2015). In contrast to this, CVMS did not induce remarkable growth of adrenals in PACAP KO mice. This might be explained by our new finding that non-stressed PACAP KO mice had greater adrenal weights than wildtype controls. The observation that CVMS did not result in increased adrenal weights correlates with the blunted HPA-axis activity assessed by CORT assays in PACAP KO mice (this study, Storth and Eiden 2010, Tsukiyama et al., 2011, Mustafa et al., 2015). The CVMS-evoked CORT rise was detected in all stressed WT groups further supporting the model's validity. The observation that imipramine did not normalize CORT values in our WT mice may be explained by the known mouse strain-dependency of imipramine efficacy (Ibarguen-Vargas et al., 2008).

As to the behavioral assessment, with respect to the limitations of data by relatively small sample size, the model effectively increased the immobility in FST in WT mice underpinning the effectivity of CVMS. PACAP KO mice showed higher immobility in non-injected controls, however in this study this difference was not statistically significant in contrast to earlier works (Hashimoto et al., 2001, Gaszner et al., 2012). One has to consider that in vehicle treated WT mice the immobility time was relatively high which might be explained by stress caused by intraperitoneal injections.

The stress exposure in our PACAP KO mice caused profound changes in FST behavior. It seems that mild stress paradoxically reduces the depression-like behavior in PACAP KO mice on CD1 background, and the immobility time decreased to the value of imipramine treated control KO mice in line with the findings of Lehmann et al., (2013).

Collectively, WT CD1 animals show the classic physical, endocrinological and behavioral alterations upon stress, supporting the model's validity. It has to be stated that the depression-like phenotype of our PACAP KO mice on CD1 background was not proven in this experiment. Although, there is no doubt that upon CVMS exposure they show blunted reactivity. Therefore, in our ongoing studies additional stress factors are superimposed to the CVMS paradigm to use PACAP mutants to obtain a more reliable tool to study depression (see also: Farkas et al., 2017, Gaszner et al., 2013, 2014).

2.2. Neuronal activation profile as evaluated by FosB expression

In this experimental setup we found that the intraperitoneal injections of vehicle or imipramine may have increased the basal FosB expression in some animals. To avoid the comparisons between these groups, our results were divided into Dataset 1 and 2.

CVMS caused a significant change in FosB expression in 11 brain areas (CeA, MeA, dmBST, ovBST, DG, vLS, pPVN, mPVN, lPAG, cpEW, DR) out of 18 studied in this work. Additionally, two other stress related centers (i.e. CA1 hippocampal area and vBST) were affected by stress × genotype interactions, without the main effect of stress. This further supports the validity of our CVMS model, as more than the half of stress-adaptation centers were affected.

The efficacy of imipramine was limited to four areas (i.e. CeA, MeA, vLS, pPVN) in terms of FosB expression. Additionally, interactions of stress and treatment in the DR, and that of treatment × genotype in the dmBST, vBST, CA1, CA3 areas and the DG affected FosB expression. Post hoc comparisons reveal that the effect of CVMS on FosB was reversed only in the dmBST, the three hippocampal areas and in the DR. The relative ineffectivity of imipramine on FosB in PACAP KO mice may also be explained by the fact, that the FosB increase was almost completely abolished in the studied areas, therefore, the treatment could not further decrease the nearly basal FosB cell counts.

2.3. The lack of PACAP affects FosB expression brain area- and stressor-dependently

The statistical analyses by ANOVA permitted nine post hoc comparisons between non-injected WT and KO mice in CVMS. Out of these, the FosB response was significantly blunted in eight areas. Stress studies on neuronal activity profiles of PACAP deficient mice suggest that PACAP modulates the response of to both CVMS (this study) and FST (Gaszner et al., 2012) in a stressor- and brain area dependent manner: PACAP is required to develop the FosB response to chronic stress in the pPVN, CeA, dmBST, ovBST, vBST, CA1, vLS and DR. Since all the

latter seven limbic structures send afferents to the PVN or periPVN area (Risold and Swanson 1997, Larsen et al., 1996, Hammack et al., 2010, for reviews see Ulrich-Lai and Herman 2009), it is not surprising that the greatest effects were found in the pPVN. Indeed, there is no doubt that PACAP has a deep impact on PVN reactivity (Grinevich et al., 1997, Agarwal et al., 2005, Norrholm et al., 2005, Das et al., 2007, Kageyama and Suda 2009). The lack of PACAP results in functional changes at the PVN accompanied with blunted HPA-axis activity and a somewhat contradictory behavioral phenotype (Storth and Eiden 2010, Tsukiyama et al., 2011, Gaszner et al., 2012, Lehmann et al., 2013).

2.4. CRF neurons in the ovBST do not respond to CVMS in PACAP KO mice

Based on our earlier work we expected that CVMS would affect the neuronal activity in the ovBST-CRF neurons. CVMS increased FosB activity in CRF neurons of WT mice, unlike in PACAP knockouts. The comparison of total FosB and CRF-FosB cell counts reveals that the FosB rise was CRF neuron-specific in the ovBST. Further, the CRF cell counts proved the influence of PACAP deficiency on ovBST-CRF. We propose that the reduced activity of ovBST-CRF in PACAP KO mice contribute the phenotypical anomalies observed at behavioral level.

2.5. Ucn1 in the cpEW is influenced by the lack of PACAP in CVMS

For KO mice, we expected that Ucn1 neurons in CVMS will be affected by the lack of PACAP. We could prove this, as Ucn1 neurons of KO mice did not show increased FosB expression upon CMVS, which is in line with our earlier findings with c-Fos in the cpEW (Gaszner et al., 2012). In addition, Ucn1 immunosignal density was reduced in our WT mice upon CVMS exposure, which finding is in line with earlier works (Korosi et al., 2005, Kozicz et al., 2008a). Based on these, we propose that PACAP may exert a regulatory effect on Ucn1 neurons in the cpEW shaping the stress adaptation response at multiple aspects.

2.6. Increased serotonin content of DR neurons in PACAP mutant mice

One of our main findings in this work is that PACAP KO mice have both increased 5HT content and cell counts in the DR neurons, in addition a strong triple interaction of CVMS × genotype × treatment proves that the DR-5-HT is affected in this model. In line with this, Hashimoto et al., (2001) found that PACAP KO mice have reduced 5-hydroxyindoleacetic acid in the brain. Based on these, we propose that the serotoninergic system is affected by the lack of PACAP, however Hannibal (2002) found only a weak PACAP fiber density in the DR nucleus in the rat.

One explanation for this could be that the effect of PACAP deficiency may be conveyed by urocortins (Kozicz 2010, Gaszner et al., 2012), or CRF. Indeed, DR neurons carry both types of CRF receptorejezetbes which are distributed differentially by stress (Waselus et al., 2009). Based on these we propose that cpEW-Ucn1 and, BST-CRF may convey the effect of PACAP deficiency to the DR. Considering that both cpEW (Kozicz 2010) and the BST has direct connections to the DR (Peyron et al., 1998), cpEW-Ucn1 and BST-CRF neurons innervated by PACAP may set the activity of DR-5HT neurons, modulating the HPA axis activity (Jørgensen 2007) and behavior (Neufeld-Cohen et al., 2010, Kozicz 2010).

2.7. Conclusions

The CVMS exposure is a useful model to study depression in CD1 mice. The lack of PACAP in mice is associated with blunted HPA axis activity, parallel a markedly reduced pPVN neuronal activity is characteristic. The observed blunted CVMS-induced FosB activity of limbic centers, such as that of vLS (Risold and Swanson 1997), DR (Larsen et al., 1996) and CeA, MeA, ovBST, dmBST (for reviews see Ulrich-Lai and Herman 2009, Hammack et al., 2010) having connections to the PVN or peri-PVN may contribute to the decreased HPA-axis reactivity, demonstrating that the presence of PACAP is essential for stress adaptation at the level of higher-order limbic centers also.

To the best of our knowledge this study is the first to show that a) CRF neurons in the ovBST and b) Ucn1 neurons in the cpEW do not response to CVMS in PACAP KO mice, and c) PACAP KO mice show increased basal DR serotonin content. These anomalies collectively may contribute to reduced HPA axis activity and altered depression-like behavior.

Since the main caveats of this study are that a) the behavioral phenotype of our PACAP KO mice on CD1 background was not equivocally depressive and b) the imipramine treatment was insufficient, in our ongoing work additional stress factors are being superimposed on CVMS to develop a reliable depression model to be assessed by serotonin reuptake inhibitor treatment (see also: Farkas et al., 2017, Gaszner et al., 2013, 2014).

SUMMARY OF NEW RESULTS

1. We demonstrated first time that besides the PVN and MeA, several other stress systems show reduced immediate early gene expression in PACAP-deficiency upon **acute stress.** The effect of PACAP-deficiency shows also brain area specificity. Based on these, we propose that PACAP may have a modulatory role in the control of stress (mal)adaptation, which may contribute to increased depression and reduced anxiety. We identified some putative brain areas that could be central in these behavioral anomalies.

2. This is the first study which examines PACAP KO mice in the **CVMS** model including groups subjected to antidepressant treatment, to assess the role of PACAP in the stress adaptation response. The lack of PACAP is associated with reduced HPA axis activity and with markedly reduced neuronal activity in the parvocellular PVN area.

3. The reduced CVMS-evoked FosB activity increase in the vLS, DR, CeA, MeA, ovBST, and dmBST may contribute to the reduced sensibility of the HPA axis. We propose that PACAP is required for the stress adaptation-response of higher order centers also.

4. We published first that a) the ovBST-CRF and b) the cpEW-Ucn1 neurons fail to react to CVMS in PACAP KO mice. We also found that these mice show c) higher basal serotonin content in the DR. These anomalies may contribute to the reduced HPA axis activity and to the depression-like behavior.

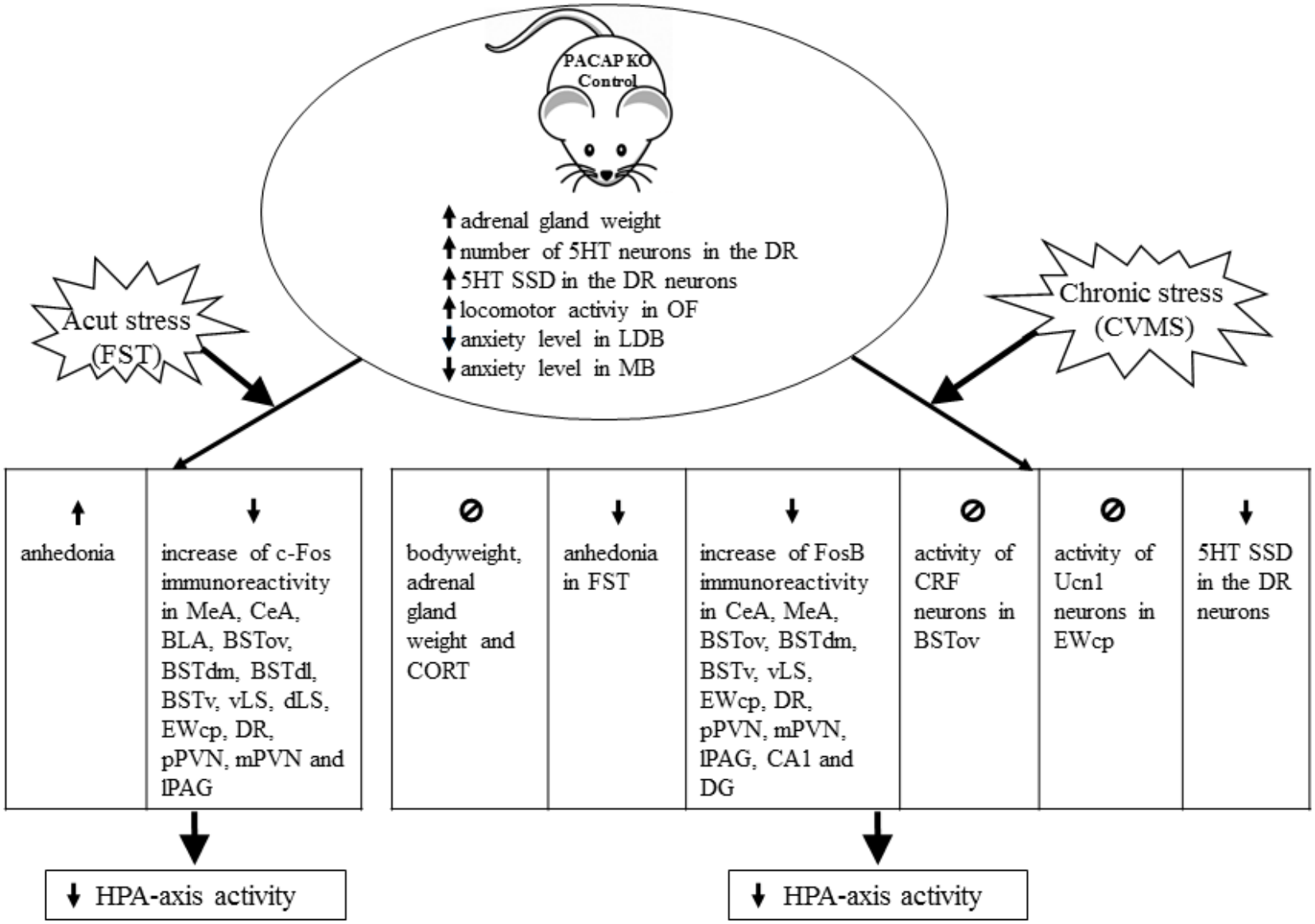
PACAP KO
Control
↑adrenal gland weight
↑number of 5HT neurons in the DR
↑5HT SSD in the DR neurons
↑locomotor activiy in OF
↓anxiety level in LDB
↓anxiety level in MB
Acut stress (FST)
Chronic stress (CVMS)
↑
anhedonia
↓
increase of c-Fos immunoreactivity in MeA, CeA, BLA, BSTov, BSTdm, BSTdl, BSTv, vLS, dLS, EWcp, DR, pPVN, mPVN and lPAG
⊘
bodyweight, adrenal gland weight and CORT
↓
anhedonia in FST
↓
increase of FosB immunoreactivity in CeA, MeA, BSTov, BSTdm, BSTv, vLS, EWcp, DR, pPVN, mPVN, lPAG, CA1 and DG
⊘
activity of CRF neurons in BSTov
⊘
activity of Ucn1 neurons in EWcp
↓
5HT SSD in the DR neurons
↓ HPA-axis activity
↓ HPA-axis activity

Fig. 1. Summary of our most important results. The upper panel summarizes our observations in non-stressed (control) PACAP KO mice in comparison to non-stressed (control) PACAP wild type mice. The left bottom table provides a summary of changes upon acute stress exposure. Effects of chronic stress in the same mouse strain is presented in the right bottom table. Control PACAP KO: not stressed PACAP deficient mice, 5HT: serotonin, DR: dorsal raphe nucleus, OF: open field test, LDB: light dark box test, MB: marble burying test, FST: forced swim test, CVMS: chronic variable mild stress, medial (MeA), central (CeA) and basolateral (BLA) nuclei of amygdala, oval (BSTov), dorsomedial (BSTdm), dorsolateral (BSTdl) and ventral (BSTv) subdivisions of the bed nucleus of the stria terminalis, ventral (vLS) and dorsal (dLS) parts of the lateral septal nucleus, EWcp: centrally projecting Edinger-Westphal nucleus, parvo- (pPVN) and magnocellular (mPVN) parts of the hypothalamic paraventricular nucleus, lateral (lPAG) periaqueductal gray matter, CORT: corticosterone, CA1: cornu Ammonis region 1, DG: dentate gyrus, CRF: corticotropin-releasing factor, Ucn1: urocortin 1, HPA-axis: hypothalamus-pituitary-adrenal-axis)

LIST OF PUBLCATIONS THE THESIS IS BASED ON

Kormos V*, Gaszner B*, Kozicz T, Hashimoto H, Reglődi D, Helyes Z.
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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* These authors contributed equally as first authors.

Kormos V, Gáspár L, Kovács LÁ, Farkas J, Gaszner T, Csernus V, Balogh A, Hashimoto H, Reglődi D, Helyes Z, Gaszner B.
Reduced response to chronic mild stress in PACAP mutant mice is associated with blunted FosB expression in limbic forebrain and brainstem centers.
NEUROSCIENCE (2016) 330:335-358.
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Kormos V, Gaszner B.
Role of neuropeptides in anxiety, stress, and depression: from animals to humans. Review
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Impact factor: 2.546
Independent citations: 116

Cumulative impact factor of publications the thesis is based on: 8.945

Total number of independent citations on publications the thesis is based on: 151

LIST OF OTHER PUBLICATIONS

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Cumulative impact factor of other publications: 24.034

Total number of independent citations of other publications: 73

Citation data are obtained from the *Google Scholar* database (17. 09. 2017).

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