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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$^{-/-}$) 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$^{-/-}$ 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: magno- 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$^{-/-}$ mice showed greatly reduced anxiety and increased locomotor activity compared with wildtypes. In forced swim test PACAP$^{-/-}$ 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 lPAG of PACAP$^{-/-}$ mice vs. wildtypes, strongly suggesting their involvement in the behavioral phenotype of PACAP$^{-/-}$ 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.

Key words: stress, bed nucleus of stria terminalis, amygdala, hypothalmic 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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Abbreviations: ANOVA, analysis of variance; BLA, basolateral nucleus of the amygdala; BSTdl, dorsolateral subdivision of the bed nucleus of the stria terminalis; BSTdm, dorsomedial subdivision of the bed nucleus of the stria terminalis; BSTov, oval subdivision of the bed nucleus of the stria terminalis; BSTv, ventral subdivision of the bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CREB, cAMP responsive element binding; CRF, corticotropin releasing factor; DAB, diaminobenzidine; dLS, dorsal subdivision of the lateral septal nucleus; dPAG, dorsal subdivision of periaqueductal gray matter; DR, dorsal raphe nucleus; EWcp, central projecting Edinger-Westphal nucleus; FST, forced swim test; HPA, hypothalamus–pituitary–adrenal axis; ir, immunoreactive; KO, knockout; lPAG, lateral subdivision of periaqueductal gray matter; MeA, medial nucleus of the amygdale; mPVN, magnocellular part of paraventricular nucleus of the hypothalamus; NGS, normal goat serum; PACAP, pituitary adenylate-cyclase activating polypeptide; PACAP$^{-/-}$, pituitary adenylate-cyclase activating polypeptide deficient; PACAP$^{+/+}$, wildtype; PBS, phosphate-buffered saline; pPVN, parvocellular paraventricular nucleus of the hypothalamus; Ucn1, urocortin 1; vLS, ventral parts of the lateral septal nucleus.

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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 as well as PAC1 receptor polymorphisms have been correlated with severity of posttraumatic stress disorder in female patients.

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-HT_{1A} 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, XXCIII, 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

Behavior 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 cm^2 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.