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Pituitary adenylate cyclase-activating polypeptide (PACAP) in the Central Nucleus of the Amygdala Induces Anxiety via Melanocortin Receptors

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Abstract

Rationale—Anxiety disorders are the most common mental disorders in the United States. Characterized by feelings of uncontrollable apprehension, they are accompanied by physical, affective, and behavioral symptoms. The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor PAC1 (PAC_1R) are highly expressed in the central nucleus of the amygdala (CeA) and they have gained growing attention for their proposed role in mediating the body's response to stress.

Objectives—The aim of this study was to evaluate the anxiogenic effects of PACAP in the CeA and its effects on the hypothalamic-pituitary-adrenal (HPA) axis. Furthermore, the mechanism of action of PACAP in the CeA was investigated.

Methods—PACAP was microinfused into the CeA of rats and its effects in the elevated plus maze (EPM), the defensive withdrawal tests, and plasma corticosterone levels were evaluated. The ability of the melanocortin receptor antagonist SHU9119 to block PACAP effect in the EPM was assessed.

Results—Intra-CeA PACAP exerted a dose-dependent anxiogenic effect and activated the HPA axis. In contrast, PACAP microinfused into the basolateral nucleus of the amygdala (BlA) had no effect. Finally, the anxiogenic effect of intra-CeA PACAP was prevented by SHU9119.

Conclusions—These data prove an anxiogenic role for the PACAP system of the CeA, and reveal that the MC_4R system of CeA mediates these effects. Our data provide insights into this neuropeptide system as a mechanism for modulating the behavioral and endocrine response to stress, and suggest that dysregulations of this system may contribute to the pathophysiology of anxiety-related disorders.

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Keywords

Stress; anxiety; depression; neuropeptide; animal model; MC4; HPA axis

Introduction

Anxiety disorders are among the most common forms of mental illness in the world, affecting 40 million adults in the United States (Kessler et al. 2005). They are characterized by a state of apprehension, tension, and fear resulting from the anticipation of a real or fantasized threatening event or situation, feelings which impair physical and psychological functioning. Anxiety disorders may arise from both genetic and environmental causes which interact with each other to produce the final pathology (Kessler et al. 2005).

The amygdala is a brain area crucial for the regulation of stress-induced fear responses, release of glucocorticoids, and autonomic nervous system activation (Davis 1992). The CeA in particular is a key region for the regulation of anxiety (Kalin et al. 2004; Tye et al. 2011), with several studies indicating this brain region as a potential target for anxiolytic agents (Carvalho et al. 2012; Kang-Park et al. 2004). The CeA integrates sensory information from the environment and sends projections to various effector regions to trigger the appropriate responses (Davis 1992; Davis and Shi 1999; Pitkänen 2000; Zarrindast et al. 2008). Hyperactivity of the CeA has been hypothesized to be a critical factor in the pathophysiology of anxiety- and trauma-related disorders (Etkin et al. 2009; Etkin and Wager 2007; Jiang et al. 2009).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide and neurohormone belonging to the secretin glucagon vasoactive intestinal polypeptide (VIP) family; PACAP-38 (here named “PACAP”) represents the major form of PACAP in the brain. PACAP exerts its effects mainly through the activation of PAC1 receptor (PAC_1R), which binds PACAP with a 1,000-fold higher affinity than VIP; VPAC1 and VPAC2 receptors, on the other hand, bind both PACAP and VIP with similar affinities (Vaudry et al. 2009).

A key role for the PACAP-PAC_1R system in anxiety and stress-related behaviors has been proposed (Dore et al. 2013a; Lezak et al. 2014; Stroth et al. 2011). PACAP is a neurotransmitter at the adrenomedullary synapse and it is required for stress-induced catecholamine release and synthesis (Hamelink et al. 2002; Stroth and Eiden 2010). In the brain, PACAP and its receptor PAC1 are highly expressed in the hypothalamus, particularly in the paraventricular nucleus (PVN) and the supraoptic nucleus, where they stimulate the release of various hypophysiotropic neurohormones, including corticotropin-releasing factor (CRF). PACAP and PAC_1R are, however, also abundantly expressed in extrahypothalamic areas such as the central and the basolateral nuclei of the amygdala (CeA, BlA), the bed nucleus of the stria terminalis (BNST), and the brainstem (Joo et al. 2004).

Several pieces of evidence point at PACAP and PAC_1R as strong stress response mediators. PAC_1R knockout mice exhibit reduced anxiety-like behaviors (Otto et al. 2001), and an association between PACAP/PAC_1R (possibly in the amygdala) and posttraumatic stress

disorder in heavily traumatized patients has been documented (Ressler et al. 2011). Numerous effects of stress have been shown to be mediated by PACAP, as demonstrated by studies showing that stress is unable to increase CRH synthesis in the PVN or plasma corticosterone levels in PACAP knockout mice (Lehmann et al. 2012; Stroth and Eiden 2010). Central administration of PACAP in rodents has been shown to produce stress-like, HPA-activating, and anorectic effects, and PACAP and PAC_1R levels have been reported to be altered in the brain after exposure to stressors (Agarwal et al. 2005; Dore et al. 2013a; Hammack et al. 2009; Legradi et al. 2007; Ressler et al. 2011).

However, the contribution of the different brain regions to the effects observed and importantly the mechanisms by which PACAP exerts its effects are still unclear. Extensive evidence has pointed at the PVN, as well as the BNST, as areas likely involved in the anxiety-modulating effects of PACAP. PACAP indeed increases startled behavior and plasma corticosterone levels when administered into the BNST (Hammack et al. 2009; Lezak et al. 2014); in addition, PACAP administered into the PVN produces elevated face washing, body grooming, decreased locomotor activity, and rearing (Norrholm et al. 2005). Recent studies have begun to propose a role for PACAP in the CeA (Dore et al. 2013a; Missig et al. 2014).

Members of another neuropeptide system, consisting of melanocortins (e.g. α-MSH) and melanocortin receptors (mainly MC4 receptor, MC_4R), are also highly expressed in the CeA. While historically involved mostly in feeding and energy balance regulation, the role of this system in the modulation of anxiety-like behaviors has started to emerge. Central administration of an MC4R agonist was shown to increase CRF transcription as well as plasma corticosterone levels (Lu et al. 2003), and activation of the α-MSH/MC_4R system of the amygdala has been shown to have anxiogenic effects (Kokare et al. 2005; Kokare et al. 2010). Lack of MC4R leads to blunted HPA responses to acute psychological stress (Ryan et al. 2014), and electrical shock increases MC4R mRNA levels in the amygdala and hypothalamus (Yamano et al. 2004). Finally, MC4R antagonism is able to prevent the behavioral consequences of restraint stress and electrical shock (Chaki et al. 2003; Liu et al. 2013; Vergoni et al. 1999), and to reverse isolation -induced anxiety- and depressive-like behavior(Kokare et al. 2010) and single prolonged stress-induced behavioral outcomes (Serova et al. 2013). Whether the PACAP and the melanocortin system interact in the amygdala in the context of anxiety-like behavior is currently unknown. Therefore this study aimed at exploring the role of PACAP in the CeA of rats in the context of anxiety and its mechanism of action.

Experimental Procedures

Subjects

Male Wistar rats, weighing 301–325g at arrival (Charles River, Wilmington, MA), were single-housed in wire-topped, plastic cages in a 12h reverse light cycle, AAALAC-approved, humidity- (60%) and temperature-controlled (22 °C) vivarium. Reverse light cycle allows the testing of rodents during their active phase. Rats had access to corn-based chow (Harlan Teklad LM-485 Diet 7012) and water *ad libitum* at all times. Subject number per experiment follows: EPM dose-response, n= 54 (CeA), n= 37 (BlA); defensive withdrawal, n= 19 (CeA), EPM blockade experiment, n= 29; corticosterone, n= 47 (CeA), n= 33 (BlA).

Animals previously used in the EPM were randomized for the dose previously received and tested again in the Corticosterone experiment (at least 2 weeks later). Rats with incorrect placement of one or both cannulas were not included in the analysis (21 rats for EPM CeA, 14 rats for EPM BlA, 10 rats for the EPM blockade, 21 rats for CeA corticosterone, 15 rats for BlA corticosterone).

Procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and the *Principles of Laboratory Animal Care,* and were approved by Boston University Medical Campus Institutional Animal Care and Use Committee (IACUC).

Drugs

PACAP (PACAP-38) was purchased from American Peptide Company (Sunnyvale, CA), and the melanocortin antagonist SHU 9119 from R&D Systems Inc. (Pittsburgh, PA). Peptides were dissolved in sterile isotonic saline in the presence of 1% bovine serum albumin (Thermo Fisher Scientific, Waltham, MA). SHU9119 has been described to be a potent melanocortin MC3/4 receptor antagonist and MC5 receptor agonist (IC50 = 0.23 ± 0.02 nM at the hMC3R and IC50 = 0.06 ± 0.01 nM at the hMC4R with no cAMP activity at the receptor at 10–5 M concentration of ligand; IC50 = 0.09 ± 0.02 nM and EC50 = 0.12 ± 0.01 nM with 97% of activity at the hMC5R) (Grieco et al. 2007; Hruby et al. 1995). PACAP doses were chosen based on previous reports from our laboratory using intracranial administration (Dore et al. 2013a; Iemolo et al. 2015). For SHU9119 a sub-threshold dose of SHU9119 was chosen based on previous reports using intracranial administration of the drug in the range of 10–100 pmol (Boghossian et al. 2010; Hagan et al. 1999; Iemolo et al. 2015; Liu et al. 2007; Roseberry 2013). A subthreshold of SHU9119 was chosen to ensure no per se activity at any of the melanocortin receptors bound.

Intracranial surgeries, microinfusion procedure and cannula placement

Intracranial surgeries—Rats were stereotaxically implanted with bilateral cannulas as described previously (Iemolo et al. 2013; Sabino et al. 2007). Stainless steel, guide cannulas (24 gauge, Plastics One, Inc., Roanoke, VA) were lowered 2.0 mm above either the CeA or the BlA. Four stainless steel jeweler's screws were fastened to the rat's skull around the cannulas. Dental restorative filled resin (Henry Schein Inc., Melville, NY) and acrylic cement were applied, forming a pedestal firmly anchoring the cannula. The cannula coordinates from the bregma used for the CeA were: AP −2.64, ML ±4.2, DV −6.2 (from skull); the cannula coordinates used for the BlA were: AP −2.64, ML ±4.8, DV −6.5 (from skull), according to (Paxinos and Watson 2007). A stainless steel dummy stylet, which terminated at the end of the guide cannula maintained patency. After surgery, the rats were allowed a 7-day recovery period, during which they were further handled. Supplementary Figure 1 shows CeA and BlA cannula placement (Suppl. Fig. 1A and 1C, respectively) and photomicrographs showing representative injection sites in the CeA and BlA (Suppl. Fig. 1B and 1D, respectively).

Microinfusion procedure—The drugs were microinfused as previously described (Blasio et al. 2013; Dore et al. 2013b; Iemolo et al. 2013). The dummy stylet was removed from the

guide and replaced with a 31-gauge stainless steel injector projecting 2 mm beyond the tip of the guide cannula; the injector was connected via PE 20 tubing to a microsyringe (Hamilton Company, Reno, NV) driven by a microinfusion pump (KD Scientific, Holliston, MA). Microinfusions were performed in 0.5 μl volume per side, delivered over 2 min; injectors were left in place for 1 additional min to minimize backflow. SHU9119 was administered 15 min before PACAP, and 30 min after PACAP administration rats were either tested in the behavioral test or their blood was drawn.

Cannula placement—Cannula placement was verified at the conclusion of all testing. Subjects were sacrificed under anesthesia (isoflurane, 2–3% in oxygen) and injected with 0.5 μl/side of India ink. Brains were removed, frozen in a methyl butane/dry ice bath, and stored at −80°C. Coronal sections of 40 μm were collected using a cryostat and placements were verified under a microscope. Only subjects with correct placements were included in the analyses.

Elevated Plus-Maze Test

The elevated plus-maze test was performed as previously described (Cottone et al. 2009a; Sabino et al. 2009). The black Plexiglas plus-maze apparatus (Blasio et al. 2013; Cottone et al. 2007; 2009b) consisted of four arms (10 cm × 50 cm) positioned at right angles, 50 cm above the floor. Two “closed” arms had 40-cm high walls; two “open” arms had 0.5-cm high ledges. Testing occurred in a dim room with 1.5–2.0 lx of open arm illumination and <1 lx in the closed arms. Rats were kept in a dark anteroom with white noise present (70 dB) for ≥1 h before testing, and white noise was also present during testing. Rats were placed individually onto the center of the maze for a 5-min period. The primary measures were the percent of open arm time [i.e., 100 × open arm/(open arm + closed arm)], a validated index of anxiety-related behavior (Fernandes and File 1996), and the number of closed arm entries, an index of motor activity (Cruz et al. 1994). The test was scored by raters blind to the treatment conditions.

Defensive Withdrawal Test

Rats were acclimated to a room next to the testing for at least 60 min, in the presence of a white noise. The defensive withdrawal test (Cottone et al. 2009b; Zorrilla et al. 2002) apparatus was a walled, black/gray polyvinylchloride open field (106 cm × 92 cm × 77 cm) containing a cylindrical “withdrawal” chamber (2L Pyrex beaker wrapped in black tape). The chamber was located 15 cm from a corner facing the open arena, and testing occurred under room light (~300 lx). Rats were kept in a dark anteroom with white noise present (70 dB) for ≥1 h before testing. For the 10-min test, rats were placed into the withdrawal chamber facing the rear, and behavior was video recorded. The total duration of withdrawal was scored. The test was scored by raters blind to the treatment conditions.

Plasma corticosterone measurement

Plasma levels of corticosterone were determined as previously described (Cottone et al. 2009a; Dore et al. 2013a). Blood was sampled from the rats’ tails and collected in tubes containing 0.5M EDTA pH 8.0 (Gibco, Thermo Fisher Scientific, Cambridge, MA). Plasma was obtained after blood centrifugation, and it was stored at −80 °C until levels of

corticosterone-like immunoreactivity were determined using a commercially available radioimmunoassay kit according to the manufacturer's instructions (MP Biomedicals, Inc., Santa Ana, CA). Intra- and inter-assay coefficients of variation were <10%.

Statistical analysis

PACAP dose-response curve data were analyzed using one-way analyses of variance (ANOVAs). The antagonist blockade experiment was analyzed using a two-way ANOVA with PACAP and Antagonist as between-subject factors. ANOVAs were followed by *post-hoc* comparisons (Dunnett in dose-responses, Fisher's LSD in the antagonist blockade). Significance was set at $p<0.05$. The software/graphic packages used were Systat 11.0, SigmaPlot 11.0, InStat 3.0, and Statistica 7.0.

RESULTS

PACAP produces anxiety-like behavior when administered into the CeA

As shown in Fig. 1A, intra-CeA administration of PACAP significantly affected the % time the rats spent in the open arms of an elevated plus maze (EPM) ($F(4,49)= 6.32$, $p<0.0001$); post-hoc analysis showed that the doses of 0.1, 0.3, and 1 μg/rat were all effective in reducing the % open arm time. As shown in Fig. 1B, PACAP had no effects on the number of closed arm entries (Fig. 1B), an index of motor activity ($F(4,49)= 1.75$, not significant (n.s.)). In addition, as Supplementary Fig. 2 shows, rats administered PACAP intra-CeA (0.3 μg/rat) withdrew more into the sheltered chamber of a defensive withdrawal test than did vehicle-treated rats (~75% reduction; $t(17)=2.02$, $p<0.05$), which is another measure of anxiety-like behavior. Suppl. Table 1 shows the drug effects (in this EPM experiment as well as the following ones) on other measures, such as % open arm entries.

PACAP does not affect anxiety-like behavior when administered into the BlA

Fig. 2A shows that PACAP, administered at doses which were effective intra-CeA, did not affect the % of open arm time when given into the BlA ($F(3,33)= 1.54$, n.s.). Closed arm entries (Fig. 2B) were also unaffected by intra-BlA PACAP treatment ($F(3,33)= 1.31$, n.s.).

PACAP activates the HPA axis when administered into the CeA but not the BlA

Intra-CeA PACAP treatment caused an increase in plasma levels of corticosterone 30 min after administration ($F(3,43)= 3.92$, $p=0.015$), as shown in Fig. 3A; post-hoc analysis showed that the doses of 0.3 and 1 μg/rat were effective in increasing corticosterone levels.

In contrast, as shown in Fig. 3B, intra-BlA PACAP treatment did not affect plasma levels of corticosterone 30 min after drug administration ($F(3,29)= 0.44$, n.s.).

The MC_3R/MC_4R antagonist SHU-9119 blocks intra-CeA PACAP-induced anxiety-like behavior

Fig. 4A shows that the reduction of % open arm time (anxiogenic effect) produced by intra-CeA PACAP, which was administered at the most effective dose (0.3 μg/rat), was blocked by intra-CeA pretreatment with the MC_3R/MC_4R antagonist SHU-9119 (50 pmol/rat) (PACAP x SHU-9119 $F(1,25)= 5.75$, $p<0.05$). Post-hoc analysis showed that PACAP-treated animals

spent significantly less % time in the open arms compared to the vehicle-treated, while the SHU+PACAP-treated animals did not differ from vehicle-treated animals. As shown in Fig. 4B, PACAP, SHU-9119 or their combination had no effect on the number of closed arm entries (PACAP ($F(1,25)= 0.55$, n.s.; PACAP × SHU-9119 ($F(1,25)= 0.71$, n.s.).

DISCUSSION

The main findings of the present study were as follows: *i)* PACAP exhibits a dose-dependent anxiogenic-like effect when microinfused into the CeA, but not the BlA, in male rats; *ii)* PACAP microinfused into the CeA, but not the BlA, elevates plasma corticosterone levels; *iii)* The anxiogenic effect of intra-CeA PACAP is prevented by local infusion of the melanocortin receptor 3/4 antagonist SHU9119; *iv)* PAC_1R is highly expressed in the CeA where it partially co-localizes with MC_4R.

In the elevated plus maze test, rats treated with increasing doses of PACAP injected into the CeA spent a significantly lower percent of time in the open arms compared with vehicle-treated rats, suggesting that drug treatment has an anxiogenic-like effect. Interestingly, the 0.3 μg/rat dose was the most effective one in inducing anxiety-like behavior (44% reduction), which suggests that PACAP at higher doses may lose selectivity. The number of entries in the closed arms, commonly inferred as a measure of motor activity (Cruz et al. 1994), was unaffected by PACAP treatment; this observation confirms that the anxiety-like behavior following PACAP administration into the CeA was not due to a general suppression of motor activity, as we have already shown previously analyzing the number of beam breaks (Iemolo et al. 2015). Importantly, intra-CeA PACAP was able to induce anxiety-like behavior also using a different behavioral task, the defensive withdrawal test. On the other hand, microinfusion of PACAP into the BlA was unable to elicit anxiety-like behavior, suggesting the regional specificity of the observed effects. Even though the hypothesis that doses of PACAP higher than 1 μg/rat may still induce an anxiogenic effect in the BlA cannot be completely ruled out, we consider it unlikely based on published observations that doses of 1 and 3 μg/rat are sufficient to induce anxiety-like and anti-rewarding effects when administered in the entire brain by i.c.v. injection (Seiglie et al. 2015; Telegdy and Adamik 2015). We consider it improbable that the differences in baseline % open arm time between the CeA and the BlA cohorts (~60% vs. ~40%, respectively) may be responsible for the lack of effect of PACAP injected into the BlA; indeed, the BlA % open arm time values under vehicle conditions were still high enough to allow potential reductions to be detected (i.e. a floor effect seems quite unlikely).

Our results are in line with previous studies showing that PACAP and PAC_1R receptor knockout mice are less anxious than the wild-type counterparts and show an anxiolytic profile (Hashimoto et al, 2001; Otto et al, 2001). Pharmacological studies have reported that PACAP produces stress-like effects in rats, such as face washing, body grooming, and wet-dog shakes, when injected either intracerebroventricularly (i.c.v.) or into the PVN (Agarwal et al, 2005; Norrholm et al, 2005). When administered into the BNST, PACAP also increases the startle response (Hammack et al, 2009); recently a nociceptive effect of intra-CeA PACAP has been shown, which was associated with an anxiogenic effect (Missig et al. 2014). However, a higher dose of PACAP was used in that study compared to this one.

Altogether, this evidence strongly indicates a critical role for the PACAP system of the CeA in the behavioral response to stress. A high number of PACAP fibers and PAC_1R positive cells are found in the CeA (Joo et al. 2004); whether the source of PACAP into the CeA is local or not is currently unknown, even though lesions of the lateral parabrachial nucleus have been shown to attenuate PACAP immunoreactivity in the CeA, suggesting that projections from this region could be responsible for part of the PACAP released in the CeA (Missig et al. 2014). The observation that PAC_1R gene expression is elevated in the amygdala of rats following fear-conditioning (Ressler et al, 2011) further suggests a putative role of the endogenous amygdalar PACAP system in anxiety and fear responses. Interestingly, PACAP has been shown to potentiate excitatory transmission in projections from the BlA to the CeA, although those effects were likely mediated by VPAC1 receptor (Cho et al. 2012). Since the present study involved an exogenous administration of PACAP, subsequent studies involving the use of pharmacological antagonists or viral knockdown of the PAC_1R will be needed to establish the role of the endogenous system.

Importantly, we found that administration of PACAP into the CeA (but not the BlA) dose-dependently elevated corticosterone levels in the plasma of rats, suggesting that the activation of extrahypothalamic areas can stimulate the HPA axis. Interestingly, while all three doses of intra-CeA PACAP (0.1, 0.3, and 1 μg) produced anxiogenic effects, only the two higher (0.3 and 1 μg) were effective at increasing the levels of corticosterone. The fact that a significant increase of corticosterone could not be detected at the 0.1 μg dose suggests that variability may have played a role in the apparently differential effect observed, even though the hypothesis that CeA PACAP may just be more potent at inducing its behavioral - compared to endocrine- effects cannot be excluded. I.c.v. administration of PACAP is known to activate the HPA axis (Agarwal et al. 2005; Dore et al. 2013a; Norrholm et al. 2005); more recently intra-BNST PACAP was shown to also increase plasma corticosterone levels, although higher doses appear to be necessary to induce a significant increase (Lezak et al. 2014), suggesting that the CeA may be more sensitive compared to the BNST to the HPA axis-activating effects of PACAP.

PACAP has been proposed as a “master regulator” of the stress response, due to its ability to regulate the HPA axis at multiple levels, including the hypothalamic, pituitary, and adrenal glands (Stroth et al. 2011). Indeed, in PACAP knockout mice, restraint stress is unable to increase CRH mRNA in the PVN or corticosterone levels, and in the same animals social defeat induces less PVN activation and a smaller corticosterone rise compared to wild-type mice (Lehmann et al. 2012; Stroth and Eiden 2010). Noteworthy, the CeA is known to regulate the HPA axis function and glucocorticoid release; electrical stimulation of the CeA increases ACTH release, while lesions of CeA attenuate HPA axis responses to immobilization stress (Beaulieu et al. 1987) and the increase in ACTH secretion following adrenalectomy (Allen and Allen 1974), proving that the CeA participates in the regulation of the HPA axis function in response to stressors.

The CeA, like other limbic structures, has a limited number of direct connections with the hypophysiotropic neurons of the PVN, but it is hypothesized to contribute to the disinhibition of the PVN also via intermediary neurons in the BNST and the lateral septum (Sawchenko et al. 1993). An important question is whether the HPA axis activation produced

by intra-CeA administration of PACAP is responsible for the observed anxiogenic effect or vice versa whether the endocrine effects are secondary to the effects of the peptide on behavior. We have previously shown that, while the CRF receptor antagonist D-Phe-CRF(12–41) is able to prevent i.c.v. PACAP-induced anxiogenic effect, it however does not block PACAP-induced HPA axis activation, suggesting that PACAP-induced endocrine effects are likely not responsible for its anxiogenic effects (Dore et al. 2013a). Since the present study does not help clarify this aspect (as both HPA axis activation and anxiety-like behavior were investigated 30 min after administration), future studies involving a precise time course after PACAP administration and/or using adrenalectomized animals will be needed to fully answer this question.

Pretreatment with the MC_3R/MC_4R antagonist SHU9119 successfully prevented the reduction in % open arm time induced by intra-CeA PACAP. The melanocortin system, and in particular the MC_4R of the amygdala, plays a key role in the regulation of anxiety-like behavior: activation of the α-MSH/ MC_4R system of the amygdala has been shown to produce anxiogenic effects, and the blockade of MC_4R to prevent stress-induced anxiety-like behavior (Kokare et al. 2005; Kokare et al. 2010). Interestingly, it has been demonstrated that manipulation of the MC4R in the medial nucleus of the amygdala (MeA) affects anxiety-like behavior and corticosterone levels (Liu et al. 2013). In this regard, because of the volume of PACAP microinfused into the CeA (0.5 μl/side), we cannot rule out that the solution may have spread into neighbor areas, such as the MeA, to exert its effects; future *in vivo* PACAP studies targeting specifically the MeA will therefore be needed to definitely answer this question. However, it should be noted that lower doses of SHU9119, compared to those described to be required in the MeA, were here able to block the PACAP effects (0.05 nmol vs. 1 nmol), strengthening the notion that the CeA is the site of action.

The melanocortin receptor antagonist used in this study, SHU9119, is widely used as an MC4 receptor antagonist, despite not binding MC4R exclusively; indeed, SHU9119 acts also as an antagonist of MC3R and as an agonist of MC5R (Grieco et al. 2007; Hruby et al. 1995). However, a putative involvement of MC3R in the anxiogenic effects of PACAP can be considered highly unlikely because of the negligible levels of this receptor subtype in the CeA (undetectable by real time PCR, personal observations), in line with previous immunohistochemical and *in situ* hybridization studies showing that the expression of MC_3R is restricted to hypothalamus and brainstem (Liu et al. 2003; Roselli-Rehfuss et al. 1993). In addition, we can also rule out that the SHU9119 blockade of PACAP effects reflects a putative physiological antagonism through activation of MC5R, since in our experiments SHU9119 had *per se* no effect. Therefore, despite the lack of selectivity of the antagonist used represents a limitation of the current study, based on the above we can confidently conclude that MC4R, rather than MC3R and MC5R, mediates the anxiogenic effects of PACAP.

This mechanism is in line with previous observations showing that central administration of PACAP (but not VIP) activates melanocortinergic neurons, and that the effects of PACAP, both in the hypothalamus and in the CeA, on food intake are mediated by MC_4R (Iemolo et al. 2015; Mounien et al. 2006; Mounien et al. 2009). PACAP administered into the CeA has

indeed been shown to reduce food intake (Iemolo et al. 2015). One can hypothesize that PACAP-induced hypophagia may be the consequence of the anxiety-like state; however, since the hypophagia shown previously is not evident until three hours after PACAP treatment while anxiety is already 30 min later, we can exclude this explanation.

In this study, we did not confirm that the anxiogenic effects of PACAP are mediated by the PACAP-selective PAC_1R rather than VPAC receptors. However, the anxiolytic, rather than anxiogenic, profile of VIP may suggest the exclusive involvement of PAC1R in PACAP-induced anxiety (Ivanova et al. 2014), even though the large experimental differences between this study and the VIP study make any conclusion uncertain. Future experiments will be needed to directly ascertain that the effects of PACAP on anxiety-like behavior and the HPA axis are indeed mediated by PAC_1R. In addition, follow-up studies will need to confirm that the PACAP-induced HPA axis activation, like the anxiety-like behavior, is mediated by MC_4R activation.

In summary, our results prove that activation of the PACAP system of the CeA induces anxiety-like behavior via MC_4R, providing novel insights into this neuropeptide system as a mechanism for modulating the behavioral and endocrine response to stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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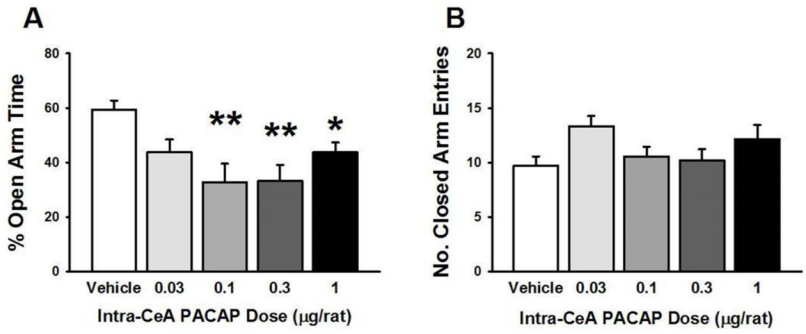

Figure 1.

Effects of PACAP (0–1 μg/rat) microinfused bilaterally into the central nucleus of the amygdala (CeA) on the percent (%) of open arm time (A) and the number of entries in the closed arms (B) of an elevated plus maze. *N*=6–16/group. Data represent Mean ± SEM. * $p<0.05$, ** $p<0.01$ vs. vehicle group.

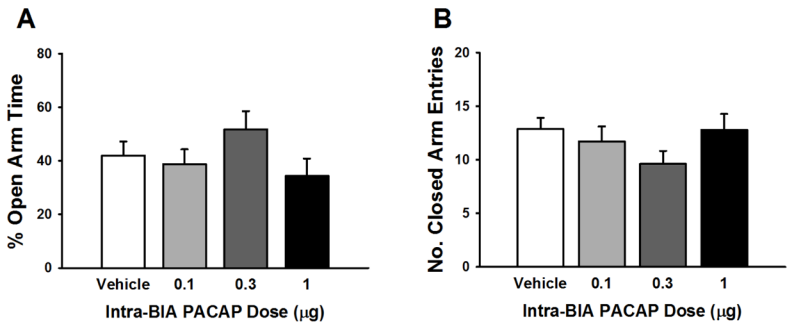

Figure 2.

Effects of PACAP (0–1 μg/rat) microinfused bilaterally into the basolateral nucleus of the amygdala (BlA) on the percent (%) of open arm time (A) and the number of entries in the closed arms (B) of an elevated plus maze. *N*=7–10/group. Data represent Mean ± SEM.

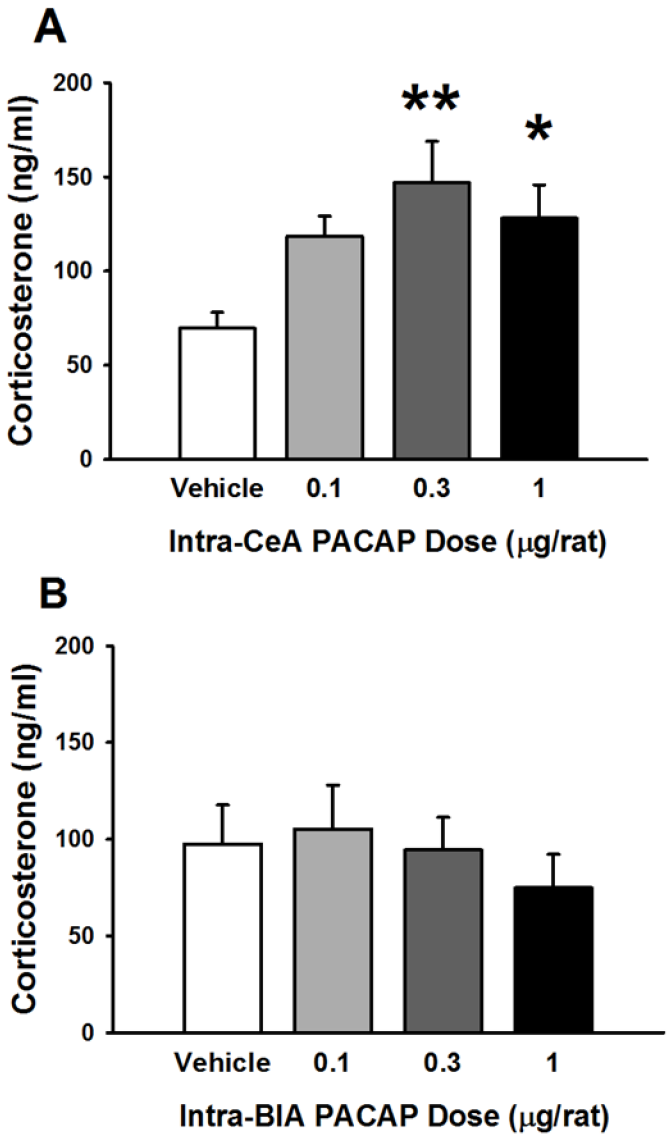

Figure 3.

Effects of PACAP (0–1 μg/rat) microinfused bilaterally either into the central nucleus of the amygdala (CeA, A) or into the basolateral nucleus of the amygdala (BlA, B) on plasma corticosterone levels. *N*=8–14/group (A), 8–9/group (B). Data represent Mean ± SEM. * $p<0.05$, ** $p<0.01$ vs. vehicle group.

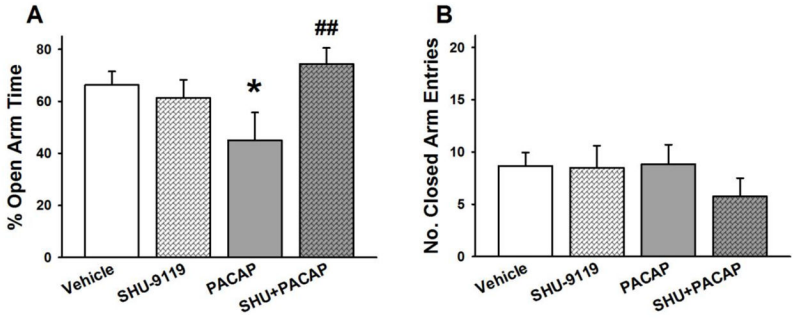

Figure 4.

Effects of PACAP (0, 0.3 μg/rat) and the MC_3R/MC_4R antagonist SHU 9119 (0, 50 pmol/rat, −45 min) microinfused bilaterally into the CeA on the percent (%) of open arm time (A), and the number of closed arm entries (B). *N*=6–9/group. Data represent Mean ± SEM. * $p<0.05$ vs. vehicle group; ## $p<0.01$ vs. PACAP group.