Neurochemistry International 62 (2013) 314–323

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

# Concurrent modulation of extracellular levels of noradrenaline and cAMP during stress and by anxiogenic- or anxiolytic-like neuropeptides in the prefrontal cortex of awake rats

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ARTICLE INFO

*Article history:*
Received 18 June 2012
Received in revised form 6 November 2012
Accepted 18 December 2012
Available online 27 December 2012

*Keywords:*
Noradrenaline
cAMP
Neuropeptides
Microdialysis
Fluorescence derivatization
Rat

ABSTRACT

The purpose of this study was to examine the effects of stress and the role of locally infused anxiogenic-like neuropeptides galanin, CCK-8, vasopressin, substance P and neurokinin A, and anxiolytic-like peptides NPY, nociceptin/orphanin FQ, somatostatin and neurotensin, on modulation of noradrenaline (NA) and cAMP efflux monitored simultaneously by microdialysis in the medial prefronatal cortex of awake rats. Concentrations of cAMP were determined by a newly developed method based on derivatization of cAMP with 2-chloroacetaldehyde followed by HPLC with fluorescence detection. Local infusion of forskolin (10 and 30 μM) dose-dependently increased the cAMP levels to 417% and 1050% of the control group, respectively. Similarly, local infusion of NA (10 μM) increased the cAMP to the peak level of 168%. A 5-min tail pinch and a 10-min swim stress rapidly increased the NA and cAMP levels to 167% and 203% (NA) and 141% and 161% (cAMP), respectively. Infusion of galanin and CCK-8 (0.5 nmol, and 1.5 nmol/0.5 μl) dose-dependently increased NA to the peak levels of 191% and 179% and cAMP levels to 174% and 166%, respectively. The peak levels following infusions of vasopressin, substance P and neurokinin A were 91%, 135% and 86% for NA and 131%, 83% and 76% for cAMP, respectively. Infusions of anxiolytic-like peptides at highest concentrations significantly increased (NPY, 136%) or decreased (nociceptin, 71%; somatostatin, 86%) the NA levels, whereas neurotensin had no effect. The cAMP levels decreased to 86% (NPY, neurotensin), 78% (nociceptin), somatostatin infusion was without effect. The present findings confirmed a close correlation between the stress-induced increases in prefrontal cortical NA and cAMP levels, as well as, concurrent changes in NA and cAMP levels following infusions of galanin and CCK-8 (increased levels) and nociceptin/orphanin FQ (decreased levels). Infusions of other neuropeptides showed a more complex pattern of NA and cAMP responses.



## 1. Introduction

Microdialysis is a well-established in vivo sampling technique for monitoring presynaptic events including neurotransmitter release, reuptake and metabolism (for review, see Kehr and Yoshitake, 2006). In addition, a number of reports have described a feasibility of using microdialysis also for monitoring the postsynaptic actions including sampling the molecules involved in intracellular signalling cascades and being able to traverse, at least partly, into the extracellular fluid (Egawa et al., 1988; Vallebuona and Raiteri, 1993). There is evidence from both in vitro and in vivo studies that intracellular cyclic AMP (cAMP) is able to leak, egress into the extracellular space and most importantly, that there is a linear relationship between the intracellular cAMP concentrations and the efflux rate of cAMP into the extracellular compartment. Thus, the egress of cAMP in cultured cell lines was shown to account for about 15–18% of the total turnover of cAMP and it was concluded that the efflux process was most likely driven by active, energy-dependent mechanisms rather than just passive diffusion (Barber and Butcher, 1980). Similar findings were reported for tissue slices (Lazareno et al., 1985; Stoof and Kebabian, 1981) and for in vivo experiments carried out first, by use of the

*Abbreviations:* aCSF, artificial cerebrospinal fluid; CCK-8, cholecystokinin octapeptide; CNS, central nervous system; GPCRs, G-protein-coupled receptors; IP3/DAG, inositol 1,4,5-triphosphate/diacylglycerol; mPFC, medial prefrontal cortex; NA, noradrenaline; PDE, phosphodiesterase; RIA, radioimmunoassay.

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http://dx.doi.org/10.1016/j.neuint.2012.12.011

push–pull cannula (Korf et al., 1976; Schoener et al., 1979) and then by microdialysis (the year would indicate that it's not "lately", I'd use "then" instead) (Egawa et al., 1988). The subsequent microdialysis studies have demonstrated that the increased production of extracellular cAMP in cortical and hippocampal areas was strongly correlated to increased noradrenergic stimulation. Thus, both noradrenaline (NA) and cAMP levels increased in response to stress caused by immobilization or by intraperitoneal injection of saline (Stone and John, 1992) or by pharmacological means (Egawa et al., 1988; Montezinho et al., 2006, 2007; Mork, 1993; Mork and Geisler, 1995; Stone and John, 1991). Infusion of NA via the probe implanted in the rat frontal cortex caused a dose-dependent increase in cAMP efflux and this effect was blocked by infusion of the β-adrenoceptor antagonist, timolol, indicating that the NA-induced cAMP efflux is mediated primarily by activation of β-receptors (Egawa et al., 1988). This conclusion was confirmed by the following microdialysis studies showing that stimulation of β-adrenoceptors with isoproterenol or forskolin increased the extracellular cAMP levels in the rat medial prefrontal cortex (mPFC) and these effects were significantly inhibited by mood stabilizers such as/including lithium, carbamazepine, or valproate (Montezinho et al., 2006, 2007). These microdialysis data together with a large number of behavioural and pharmacological studies (for review, see Morilak et al., 2005) suggest a role of NA and cAMP in mediating stress and anxiety responses.

Several neuropeptides and their respective receptors are expressed in the central nervous system, including the prefrontal cortex, hippocampus and amygdala, the major anatomical structures implemented in modulation of cognitive functions, as well as affective behaviours (for review, see Ebner et al., 2009; Hökfelt et al., 2000; McGonigle 2012; Ögren et al., 2010). Thus, neuropeptides including galanin, NPY, nociceptin/orphanin FQ, somatostatin and their receptors are predominantly inhibitory being coupled to Gi/o protein-coupled receptors (GPCRs), whereas cholecystokinin octapeptide (CCK-8) and vasopressin receptors are stimulatory Gs or Gq/11 GPCRs and neurotensin, substance P and neurokinin A receptors are Gq/11 GPCRs (Alexander et al., 2011). A body of evidence exists for the role of these peptides in regulation of stress responses, which in turn are strongly linked to the aetiology of affective disorders including depression, anxiety, panic attack, posttraumatic stress disorder, anorexia or fibromyalgia (Ebner et al., 2009; Engin et al., 2008; Feifel et al., 1999; Fernandez et al., 2004; Goeldner et al., 2012; Heilig et al., 1989; McGonigle 2012; Ögren et al., 2010). However, how the neuropeptides may modulate NA release and correspondingly, the extracellular cAMP levels in vivo in relevant neuroanatomical structures is yet not fully elucidated. The purpose of the present study was to investigate, by use of microdialysis, whether local infusions of nine representative neuropeptides with proposed anxiogenic or anxiolytic properties to separate groups of rats could affect the extracellular levels of NA and cAMP and how these potential effects could correlate to the stress-induced responses in NA and cAMP efflux in mPFC of awake rats. The extracellular levels of cAMP in the microdialysis samples were determined by a newly developed high-sensitive and selective liquid chromatographic method based of precolumn derivatization with 2-chloroacetaldehyde and fluorescence detection.

## 2. Material and methods

### 2.1. Chemicals and solutions

Noradrenaline hydrochloride (NA), adenosine 3′,5′-cyclic monophosphate sodium salt (cAMP), sodium potassium chloride, phosphate monobasic monohydrate, sodium phosphate dibasic were obtained from Sigma (St. Louis, MO, USA). Methanol was purchased from Merck (Darmstadt, Germany), EDTA-2Na was obtained from Dojindo (Kumamoto, Japan). Forskolin was obtained from Wako Pure Chemicals (Osaka, Japan). Galanin (porcine) and nociceptin/orphanin FQ were purchased from Bachem (Bubendorf, Switzerland). Neurotensin, CCK-octapeptide (CCK-8), substance P (human, bovine, rat, mouse), neurokinin A (human, porcine, rat, mouse), [$Arg^8$]-vasopressin, neuropeptide Y (human, rat), somatostatin (human, bovine, porcine, rat, mouse) and neurotensin (human, bovine, canine) were purchased from Peptide Institute Inc. (Osaka, Japan). 2-chloroacetaldehyde aqueous solution (40% w/v) was purchased from Tokyo Chemical Industry (Tokyo, Japan), and diluted to 4 M with distilled water immediately before use. All other chemicals were of highest purity available and were used as received. cAMP standards and all other solutions were prepared in distilled and deionized water achieved from Milli-Q system (Millipore, Milford, MA, USA). Stock solutions of cAMP were stored in amber coloured test tubes at 4 °C.

### 2.2. Animals

Adult male Sprague–Dawley rats (SLC, Japan) weighting 260–335 g at the time of the experiment were used in all studies. All animal experiments were approved by the local ethical committee following "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan) and the directives of the "Principles of Laboratory Animal Care" (NIH publication No. 8023). The rats (three animals/cage) were maintained at free access to food and water, on a 12-h light–dark cycle (light at 7:00 AM), room temperature 22 ± 2 °C and humidity 50–55%. All efforts were made to minimise animal suffering and the number of animals used for the study.

### 2.3. Microdialysis and behavioural stimulation

The microdialysis surgery and the following experiments on awake rats were carried out following the protocol described elsewhere (Kehr and Yoshitake, 2006). Briefly, the rats were anaesthetized with pentobarbital (40 mg/kg) and placed into a stereotaxic frame (Narishige Co., Ltd., Tokyo, Japan) in a flat skull position with the incisor bar set to −3.2 mm. The body temperature of the rat was controlled by a rectal thermometer and maintained at +37 °C using a CMA/150 temperature controller (CMA/Microdialysis, Stockholm, Sweden). After exposing the skull, a hole for a probe and two holes for the fixing screws were drilled using a fine trephine drill. A guide cannula (AG for microdialysis only or MI-AG for combined microdialysis and infusion; Eicom, Kyoto, Japan) was implanted into the mPFC at the following coordinates (from bregma and the dural surface): AP +3.3 mm, L −0.5 mm and V −1.2 mm, according to the stereotaxic atlas of Paxinos and Watson, 1997. After implantation, the guide cannula was fixed firmly to the skull with a help of two anchor screws and dental cement. At least 3 days after the surgery, a microdialysis probe (AI type: 0.22 mm o.d., 3.0 mm membrane length, or MI-AI type: 0.22 mm o.d., 3.0 mm membrane length, molecular weight cut-off 50,000 Da; Eicom) was inserted into the respective guide cannula of the awake rat. The microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF) solution (148 mM NaCl, 4 mM KCl, 0.8 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1.2 mM $Na_2HPO_4$, 0.3 mM $NaH_2PO_4$, pH 7.2) at a flow-rate of 1.0 μl/min. After the initial stabilization period of 2–3 h, the microdialysis samples were collected in 20 min intervals. The first four samples were used to estimate basal levels of NA and cAMP, thereafter the rats were exposed to stress or received intracerebral infusions of forskolin (10 and 30 μM) or NA (10 μM) via the microdialysis probe or infusion of the neuropeptide by use of combined microdialysis/infusion probe. For monitoring NA and cAMP responses to stressful stimuli,