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Environmental enrichment increases PACAP levels in the CNS of adult rats

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Abstract

OBJECTIVE: Pituitary adenylate cyclase activating polypeptide (PACAP) is a pleiotropic and multifunctional neuropeptide, widely distributed throughout the body. It is involved in the regulation of various physiological and pathophysiological processes. Numerous studies have shown that PACAP is involved in the development of the central nervous system and has neuroprotective effects. Environmental enrichment is also protective in various injuries, partially through involvement of trophic factors. The interaction between PACAP levels in the brain and environmental effects has not been studied yet. The aim of the present study was to investigate whether environmental enrichment influences PACAP levels of different brain areas in rats.

METHODS: Wistar rats were exposed to enriched environment in adulthood for 3 weeks. PACAP27- and PACAP38-like immunoreactivities were measured with a specific and sensitive radioimmunoassay in homogenates of different brain areas: brainstem, cerebellum, diencephalon and telencephalon.

RESULTS: We found that levels of both PACAP27- and PACAP38-like immunoreactivities showed significant increases in most brain areas after a 3-week-long exposure to enriched conditions. Thus, similarly to several other CNS injuries, enriched environment induced elevation in PACAP levels.

CONCLUSION: As PACAP has strong neuroprotective effects, the elevation observed after exposure to enriched environment is suggested to play a role in the protective effects of such an environment as part of the endogenous neuroprotective machinery in adult rats.

ORIGINAL ARTICLE

INTRODUCTION

The concept of environmental enrichment and its positive effects originate from the neuroscientist, Donald P. Hebb. He described in 1947 that the animals kept as pets i.e. under enriched environment, showed better performance in memory and learning tasks (Hebb, 1947). Numerous experimental data have accumulated regarding environmental factors and their importance since then. Among others, enriched environment has been shown to influence the development of the nervous system (Sale *et al.* 2007; Landi *et al.* 2009; Ortuzar *et al.* 2011). Furthermore, environmental factors have a

major influence on the outcome of different neuronal lesions (Van Praag *et al.* 2000) and so environmental enrichment is a popular strategy to counteract central nervous system injuries. Previously we have shown that environmental enrichment has a protective effect in both neonatal and adult lesions of the retina (Szabadfi *et al.* 2009; Kiss *et al.* 2013) and that enriched environment has positive, protective effects on the neurobehavioral development to counteract early postnatal toxic monosodium glutamate lesions (Horvath *et al.* 2013). Numerous other studies have described that enriched environment can reduce lesions induced by toxic (Schneider *et al.* 2001; Kazl *et al.* 2009; Soeda *et al.* 2010), ischemic (Johansson, 1996; Ronnback *et al.* 2005; Zhu *et al.* 2011) and traumatic (Kovesdi *et al.* 2011) injuries. The mechanism underlying this protective effect includes stimulating synaptic plasticity and neurogenesis, increase of dendritic spines and decrease of apoptotic factors (Nithianantharajah & Hannan, 2006).

Neurotrophic molecules are on top of the list of factors responsible for these plastic changes. It has been shown that environmental enrichment stimulates the expression of brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3) and nerve growth factor (NGF) (Landi *et al.* 2009; Baldini *et al.* 2013; Vazquez-Sanroman *et al.* 2013; Nakamura & Bregman, 2001). Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide with trophic effects in the nervous system and peripheral organs (Vaudry *et al.* 2009). The developmental effects of PACAP have long been known. The expression of PACAP appears very early during development and remains high throughout embryonic and fetal life (Tatsuno *et al.* 1994; Skoglösa *et al.* 1999). PACAP stage-dependently stimulates neuronal proliferation and differentiation, gliogenesis and neuronal migration (Vaudry *et al.* 2009; Waschek *et al.* 2002). The role of PACAP in regeneration and brain plasticity has been suggested also after early developmental periods. Similarly to several other trophic factors, PACAP is upregulated after different injuries of the nervous system (Somogyvari-Vigh & Reglodi, 2004). The underlying molecular mechanism exerted by PACAP and enriched environment overlap at several points (Kiss *et al.* 2011). Therefore, it was not surprising that both protective strategies caused the same degree of amelioration in a model of retinal ischemia in our earlier study (Kiss *et al.* 2011). Mice lacking endogenous PACAP but placed in enriched environment do not show the behavioral alterations typical for PACAP knockout mice, indicating the compensatory effect of the environmental conditions in PACAP deficiency (Ishihama *et al.* 2010; Takuma *et al.* 2014). However, it is not known what changes are induced by environmental conditions on brain PACAP levels. Therefore, the aim of the present study was to investigate whether enriched environment affects expression of PACAP in the brain.

MATERIALS AND METHODS

Experimental animals

A local Wistar rat colony was used for our experiments. Animal housing, care and application of experimental procedures were in accordance with institutional guidelines under approved protocols (No: BA02/2000-15024/2011, University of Pecs following the European Community Council directive). All experimental animals were kept in the same room, under the same illumination and other outside environmental conditions (12hr light-dark cycle, food and water ad libitum).

Environmental enrichment

We applied environmental enrichment in 6 month-old male rats with the following settings similarly to our earlier descriptions (Szabadfi *et al.* 2009). Control animals were kept under regular (standard) circumstances in regular rat cages with 43×30×20 cm dimensions (n=5). Another group was exposed to enriched environmental conditions, at 6 months of age for 3 weeks (n=5). In the larger cage (width: 88×50 cm; height: 44 cm) rats were continuously exposed to intensive multisensory stimulation, so supplemented with a complex environmental enrichment. The cage contained different toys, objects, running tunnels and rotating rods with various shapes, materials (wood, plastic, metal), colors and shades. Half of the objects were changed daily, while the other half were left unchanged to avoid a stressful change of the environment. After the 3-week long environmental enrichment in adulthood the brains were processed for analysis thereafter.

Sample preparation

All animals were sacrificed at the same time. Animals were decapitated under isoflurane anesthesia and brains were removed. Whole hypothalamus, rest of diencephalon (further on referred to as diencephalon), brainstem, cerebellum, and a 5 mm-thick pieces of the telencephalic areas were removed, according to our previous descriptions (Kiss *et al.* 2007). The larger samples (cerebellum and telencephalon) were divided into 2-2 samples. Tissues were weighed and homogenized in ice-cold distilled water. The homogenate was centrifuged (12,000 rpm, 4 °C, 30 min) and the supernatant was further processed for radioimmunoassay (RIA) analysis of PACAP27 and PACAP38 content.

Description of PACAP radioimmunoassays

PACAP38 and PACAP27 RIA procedures were performed as previously described (Jakab *et al.* 2004; Nemeth *et al.* 2007). "88111-3" antiserum was raised against a conjugate of Cys^{23}-PACAP24-38 and bovine thyroglobulin coupled by carbodiimide in rabbit. For PACAP27 measurement "88123-3" antiserum was raised against a conjugate of PACAP27 and bovine thyroglobulin coupled by carbodiimide in rabbit. Ovine PACAP24-38 C-terminal fragment and PACAP27

were iodinated (^{125}I, Institute of Isotopes, Budapest, Hungary) by iodogen and the reaction mixtures were separated on a reverse-phase HPLC column. The mono-iodinated peptides (^{125}I-PACAP 24-38 and ^{125}I–PACAP27) served later on as RIA tracers. Ovine PACAP38 and PACAP27 (Sigma) were used as RIA standards in a range of 0–1,000 fmol/ml. Assays were prepared in 1 ml phosphate buffer (0.05 mol/l, pH: 7.4) containing 0.1 mol/l NaCl, 0.05% NaN_3, 0.25% bovine serum albumin (Sigma). 100 µl antiserums ("88111-3": working dilution: 1:10,000 and "88123-3": working dilution: 1:45,000), 100 µl RIA tracers (5,000 cpm/tube) and 100 µl standards or unknown samples were measured into polypropylene tubes with assay buffer. After 48–72 h incubation at 4 °C, the antibody-bound peptides were separated from the free ones by addition of 100 µl separation solution (10 g charcoal, 1 g dextran, 0.2 g commercial fat-free milk powder in 100 ml distilled water). Following centrifugation (3,000 rpm, 20 min, 4 °C) the tubes were gently decanted and the radioactivity of the precipitates were measured in a gamma counter. PACAP38 and PACAP27 concentrations of the unknown samples were read from calibration curves. PACAP levels were measured as fmol/mg PACAP in the tissue samples. In order to better demonstrate the differences, these values are expressed as relative changes (percentage PACAP fmol/mg), taking the control group results as 100%.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Neuman-Keul's post-hoc analysis. Results were considered significant when $p<0.05$. Levels of significance are indicated as follows: *$0.01<p<0.05$; **$0.001<p<0.01$ and ***$p<0.001$.

RESULTS

In our experiments we could reliably detect PACAP38- and PACAP27-like immunoreactivity (LI) by the sensitive and specific RIA method that was used in our earlier studies (Kiss *et al.* 2007). Similarly to our previous findings and descriptions by others, the absolute level of PACAP38-LI was about 10 times higher than those of PACAP27 (data not shown). In order to compare groups under different conditions, results are given in percentage of control levels, as described in the Materials and methods section.

We measured PACAP-LI in 6-month-old animals after exposure to late (adulthood) environmental enrichment. The 3-week-long environmental enrichment in adulthood preceding sacrifice markedly increased the levels of both forms of the peptide in most brain areas. This was significant in the brainstem, cerebellum, diencephalon and telencephalon in case of PACAP27, and in telencephalon in case of PACAP38 (Figures 1–2).

DISCUSSION

In the present study we showed that enriched environment led to increased PACAP levels in different brain regions in adult rats. Using RIA, we have previously shown that PACAP levels show circadian changes in different brain regions (Jozsa *et al.* 2001) and cerebral PACAP sensitively reacts to fasting as well as castration in rats (Kiss *et al.* 2007; Nemeth *et al.* 2006). As far as the relation between PACAP and environmental conditions are considered, relatively few data are known. Nicot and DiCicco-Bloom (2001) suggested PACAP acts as an environmental sensor and expression changes according to altered conditions.

PACAP is now considered as a trophic factor based on its diverse developmental effects. Levels decline after birth and can be strongly upregulated by various interventions. For example, marked increases have been described following different neuronal injuries (Somogyvari-Vigh & Reglodi, 2004). The observation

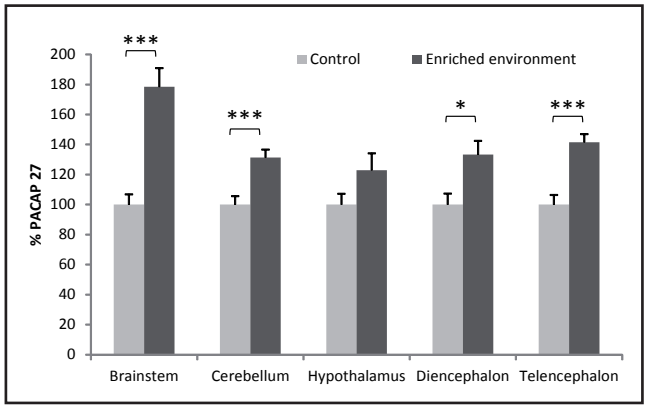

Fig. 1. Percentage of PACAP 27 levels in the CNS of 6-month-old rats. Values of the environmental enrichment group are compared to controls values set at 100%. Percentage changes are expressed as mean±SEM. *$p<0.05$, ***$p<0.001$ control vs enriched environment groups.

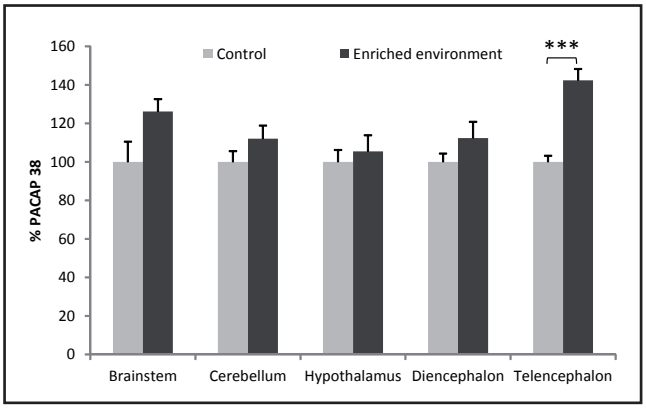

Fig. 2. Percentage of PACAP 38 levels in the CNS of 6-month-old rats. Values of the environmental enrichment group are compared to controls values set at 100%. Percentage changes are expressed as mean±SEM. ***$p<0.001$ control vs enriched environment groups.

that PACAP levels increase in the brain after exposure to enriched conditions in adulthood is thus not surprising. Increases in PACAP levels are in good accordance with its role as a protective and trophic factor. We suggest that some of the protective effects of enriched environment are mediated by increased PACAP expression in adulthood. Similar results were obtained with members of the neurotrophin family, NGF, BDNF and NT-3 following long-term exposure to enriched environment (Ickes *et al.* 2000). The authors suggest that trophic factors provide the biochemical basis for plastic alterations after shifting environmental stimuli (Ickes *et al.* 2000). The importance of BDNF is suggested by several other studies aiming at finding the molecular basis for the positive effects of enriched environment (Landi *et al.* 2009; Gelfo *et al.* 2011; Vedovelli *et al.* 2011). It has even been suggested that BDNF could be a biomarker of the effects of enriched environment (Vedovelli *et al.* 2011). In addition to the neurotrophin family, several other trophic factors have been shown to be altered upon exposure to enriched conditions. The role of IGF has been highlighted in the retinal effects of environmental enrichment (Landi *et al.* 2009). Neuropeptides are less known for these effects and our data are the first to show that PACAP levels increase after exposure to enriched environment. No such effect has been described for members of the structurally related vasoactive intestinal peptide (VIP)/secretin /glucagon peptide family.

In summary, our data support the possible involvement of PACAP in the protective effects of enriched environment in adulthood.

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