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Acute effects on brain cholecystokinin-like concentration and anxiety-like behaviour in the female rat upon a single injection of 17β-estradiol

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ABSTRACT

Background: The neuropeptide cholecystokinin (CCK) has been implicated in the neurobiology of anxiety and panic disorders, as well as in dopamine-related behaviours. Anxiety and panic-disorders are twice as common in females compared to males, but studies of females are rare, although increasing in number. Limited studies have found that CCK fluctuates in limbic regions during the estrous cycle, and that CCK and its receptors are sensitive to estrogen.
Aim/Purpose: The aim of the present work was to study the acute effects of 17β-estradiol on anxiety-like behaviour and on CCK-like immunoreactivity (LI) in the female rat brain (amygdala, hippocampus, nucleus accumbens, and cingulate cortex).
Methods: Four groups of female Sprague–Dawley rats were used: ovariectomized, ovariectomized + 17β-estradiol-replacement, sham, and sham + 17β-estradiol-replacement. The effect of 17β-estradiol-replacement on anxiety-related behaviour was measured in all animals on the elevated plus maze 2–24 h after injection. CCK-LI concentration was measured in punch biopsies by means of radioimmunoassay.
Results: 17β-estradiol decreased anxiety-like behaviour 2 h after administration in ovariectomized and sham-operated animals, as demonstrated by increased exploration of the open arms compared to respective sesame oil-treated controls. This effect was not present when testing occurred 24 h post-treatment. The rapid behavioural effect of 17β-estradiol was accompanied by changes in CCK-LI concentrations in regions of the limbic system including cingulate cortex, hippocampus, amygdala and nucleus accumbens.
Conclusion: Although the interpretation of these data requires caution since the data were collected from two different experiments, our results suggest that estrogen-induced anxiolytic effects may be associated with changes of the CCK-system in brain regions controlling anxiety-like behaviour.

1. Introduction

Cholecystokinin (CCK) is involved in the neurobiology of anxiety and panic disorders in both rodents and humans (Karkanias et al., 1989; Rotzinger et al., 2010), as well as in dopamine-related behaviours (Hökfelt et al., 1980) indicating that CCK plays a role in reward-related behaviour, motivation and addiction, (Lu et al., 2002; Mitchell et al., 2006; Pommier et al., 2002). CCK is highly abundant in the mesolimbic system, e.g. cortex, amygdala, hippocampus and striatum (Hökfelt et al., 1980; Larsson and Rehfeld, 1979; Micevych et al., 1987; Vanderhaeghen et al., 1980). Anxiety and panic-disorders are twice as common in females as in males, and sex-differences in the dopamine system and dopamine-related behaviours such as addiction have been reported (Becker, 1999). However, there are few if any studies that have satisfactorily addressed the involvement of CCK in these diseases in females.

CCK acts through the G-coupled CCK receptor 1 (CCK1-R) and CCK 2 receptor (CCK2-R, (IU-PHAR Classification Monograph, http://www.iuphar-db.org) (Beinfeld et al., 1981; Mercer and Beart, 1997; Mercer et al., 2000) and the anxiogenic properties have been shown to be regulated mainly through CCK2-R. Thus, Fekete et al. (1981) reported that anxiety and fear were aroused by means of injecting CCK-8 into the central nucleus of amygdala in rats, an effect that was found to be blocked by CCK2-R antagonists (Rotzinger and Vaccarino, 2003). CCK1-R on

Abbreviations: Cctx, cingulate cortex; Amy, amygdala; CCK, cholecystokinin; CCK-LI, cholecystokinin-like immunoreactivity; CCK1-R, cholecystokinin receptor 1; CCK2-R, cholecystokinin receptor 2; DG, dentate gyrus; EPM, elevated plus maze; ER, estrogen receptor; h, hours; NAc, nucleus accumbens; ovx, ovariectomized; OVX, ovariectomy.

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the other hand, seems rather to be involved in anxiolysis (Cohen et al., 2004). Pre-pro-CCK is cleaved into smaller biologically active units that can act as neuromodulatory peptides. For instance, CCK-4 and CCK-8 have been found to enhance anxiety- and panic-like behaviour in different animal models (Rex et al., 1997; Wunderlich et al., 2002). Furthermore, recent studies in humans have shown that CCK-4-induced panic is correlated with increased glutamate in the anterior cingulate cortex (aCctx) (Zwanzger et al., 2013).

CCK fluctuates during the estrous cycle in several brain regions (Frankfurt et al., 1986; Hilke et al., 2007) and the concentration of CCK-like immunoreactivity (LI) was found to be lowest during the pro-estrous phase, when the concentration of estrogen in plasma was at its highest level. Limited studies have shown that both CCK and its receptors are sensitive to estrogen (Micevych et al., 1996; Micevych et al., 1997). However, there are still a lot of questions yet to be answered. A deeper understanding of how sex-hormones can affect the CCK system may open new possibilities in our understanding of neuropsychiatric disorders such as anxiety and depression in females.

The classical mechanism of estrogen activation is mediated through the nuclear transcription factor receptors, estrogen receptor (ER)-α (Jensen, 1996) and/or ERβ (Kuiper et al., 1996) and the subsequent regulation of gene expression. However, estrogen also exerts acute effects on synaptic physiology that occur too rapidly to involve changes in gene expression (Kelly and Levin, 2001; Wong and Moss, 1992). These rapid effects have been attributed to extranuclear ERα/ERβ or to novel ERs yet to be characterized. Some studies have reported extra nuclear immunoreactivity for ERα (Milner et al., 2001) and ERβ (Mitra et al., 2003) in dendrites, axons, and glia in the rat brain, e.g. in the hippocampus and hypothalamus. Interestingly, a high degree of extra nuclear ERs in the hippocampus has been found to be co-localized with neuropeptides such as neuropeptide Y (Hart et al., 2007). In addition, we have previously reported acute effects on neuropeptide levels and release (NPY and galanin) in parts of the limbic system already within hours after a single injection of 17β-estradiol (Hilke et al., 2009; Hilke et al., 2005). Taken together, these studies indicate that estrogen can exert both rapid and long-term effects on different neuropeptide systems involved in mood-related behaviour.

With this background we hypothesized that fluctuations of CCK during the estrous cycle might be regulated directly or indirectly by estradiol, or the other way around. Our aim was therefore to study if depletion of estrogen by means of ovariectomy (OVX) and replacement with 17β-estradiol could influence CCK-LI concentration in brain regions involved in mood and reward-related behaviour such as aCctx, amygdala (Amy), hippocampus/dentate gyrus (DG) and nucleus accumbens (NAc). Another question addressed was if these changes in CCK are associated with an influence on anxiety-like behaviour. We believe that our results support our hypothesis that estrogen influences the CCK peptide system in brain regions important for emotional processes and that change in CCK-LI concentration was associated with a decrease in anxiety-like behaviour. Acute effects on CCK-LI concentration were measured by means of radioimmunoassay and anxiety-like behaviour was tested using the elevated plus maze (EPM).

2. Materials and methods

2.1. Animals and surgery

2.1.1. Housing

Female Sprague Dawley rats ($n = 80$, 234 ± 32 g, Universal, Stockholm, Sweden) were kept at the Animal Unit CBR5 of Linköping University Hospital. The animals were housed two and two in each cage at constant room temperature (20 °C), with chow and water during a 12 hour dark and 12 hour light cycle (light on at 6.00 p.m.). The rats were habituated for two weeks prior to surgery. The study and its experimental protocol were designed according to the guidelines of the local ethics committee on animal research in Linköping and according to EU guidelines and were approved by the committee.

2.1.2. Ovariectomy and sham operation

OVX and sham-operation were performed in anaesthetized animals (0.5–1.5% isofluorane, Sigma-Aldrich, St. Louis, MO, USA) and accomplished by the dorsal route. The ovaries were removed from the abdominal cavity and the junction between the fallopian tube and the uterine horn was sutured. A similar procedure was performed in the sham-operated animals except that the uterine horn was not sutured and the ovaries were carefully put back in the abdominal cavity within the perigonadal fat pad without touching them directly. All animals were left for a one week washout period after surgery. No animals were excluded. Groups: A total of 80 rats were used in two separate experiments; 40 rats for studies of CCK-LI concentration in the brain and 40 rats for studies of anxiety-like behaviour by means of elevated plus maze (body weight at decapitation and EPM 250 ± 53 g). In both experiments 40 rats were randomized into 4 groups ($n = 10$ in each group), 1) sham-operated rats with intact ovaries receiving a single dose of 30 μL sesame oil (Sigma-Aldrich) 2) sham-operated rats with intact ovaries receiving a single dose of 10 μg 17β-estradiol in 30 μL sesame oil (Sigma-Aldrich), 3) ovariectomized (ovx) control rats, and 4) ovx rats receiving a single dose of 10 μg 17β-estradiol in 30 μL sesame oil (Sigma-Aldrich). The injections of estradiol or sesame oil in all rats were performed exactly after a one week washout-period to eliminate endogenous estrogen production from the ovaries.

2.1.3. Tissue extraction of CCK

All animals were decapitated 2 h after administration of 17β-estradiol and/or sesame oil respectively (8.00–10.00 A.M.). Punch biopsies were collected from aCctx, Amy, DG and NAc and stored at −80 °C until analysis. Extraction of the punch biopsies was performed according to the following protocol: 0.5 mL MQ water was heated at 100 °C using a heating-block (Grant, Royston, Cambridge, England) and added into the polypropylene vials with the biopsies for 10 min. The tissues were homogenized and centrifuged at 1500 ×g, 4 °C for 10 min. After collecting the supernatants, a second extraction was performed with 0.5 mL of 0.1 M NaOH. In accordance with Ryder et al. (data not shown), we found that this extraction procedure yielded higher concentrations of CCK compared to the commonly used weak acid 0.1 HAc (Ryder et al., 1981). The supernatants were pooled together, lyophilized and stored at −80 °C.

2.2. CCK-LI concentration

The concentration of CCK-LI was measured using a commercial radioimmunoassay (EURIA kit, Euro Diagnostica, Malmö, Sweden). A calibration-curve was made from serial dilutions of 1 mL CCK-8 standard (50 pmoL/L) in 1 mL assay buffer (0.05 M phosphate buffer, pH 7.4, containing 0.25% human serum albumin and 0.02% merthiolate) with the final concentrations of 0.78, 1.56, 3.12, 6.25, 12.5 and 25 pmoL/L. The concentrations of CCK-LI were measured using a rabbit antiserum (anti-CCK-8) diluted in 5.0 mL 0.5 M phosphate buffer, pH 7.4, with 2.5% human serum albumin and 0.5% ProColin with a very low cross-reactivity to gastrin (Rehfeld, 1998).

The lyophilized samples were reconstituted in 1.0 mL assay buffer (0.05 M phosphate buffer, pH 7.4 containing 0.25% human serum albumin and 0.02% merthiolate) and 200 μL of each sample. Controls and standard were mixed with 500 μL anti-CCK-8 and incubated for 50 h at 6 °C. HPLC-purified, ^{125}I cholecystokinin 26-33 sulphate (produced by the Bolton and Hunter method), was used as a radioligand (500 μL, 6472 cpm) and incubated for 100 h at 6 °C. Finally, 100 μL double antibody solid phase (Anti-rabbit-Ig coupled to cellulose particles) was added, mixed and incubated for 60 min at 6 °C and centrifuged at 1700 ×g for 15 min at 4 °C. The supernatant was discarded by aspiration and the radioactivity in the pellets containing the bound fraction of