Daily variation of brain-derived neurotrophic factor and cortisol in women with normal menstrual cycles, undergoing oral contraception and in postmenopause

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Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and is present in the central and peripheral nervous systems (Murer et al., 2001). BDNF is a homodimERIC protein (28 kDa), and its biological activity is mediated by two kinds of cellular receptors, including the NT receptor p75 and a tyrosine kinase receptor trkB, for which BDNF binds with low and high affinity, respectively (Hempstead et al., 1991).

BDNF promotes neurogenesis and neuronal plasticity during brain development and adulthood (Henderson, 1996; Barde, 1999; McAllister et al., 1999; Tapia-Arancibia et al., 2004; Sohrabji and Lewis, 2006). In addition, BDNF is a neurotransmitter that facilitates the rapid depolarization of post-synaptic neurons, induces long-lasting changes in neurotransmitter and neuropeptide production and influences the degree of excitability (Kang and Schumann, 1995).

Several studies have shown an altered production and secretion of BDNF in a variety of diseases. Neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases, appear to be associated with decreased levels of BDNF in the brain (Connor et al., 1997; Parain et al., 1999), although low serum levels of BDNF are thought to characterize major depression (Karege et al., 2002), schizophrenia (Toyooka et al., 1987).
et al., 2002) and eating disorders, such as bulimia and anorexia nervosa (Nakazato et al. 2003; Monteleone et al., 2005). In addition, recent data suggest that plasma BDNF is a biomarker of impaired memory and general cognitive function in ageing women (Komulainen et al., 2008).

BDNF is also expressed and secreted from several tissues in the periphery (e.g. endothelial cells, smooth muscle cells, the miometrium and the endometrium) (Donovan et al., 1995), stored in large quantities in platelets and released by agonist stimulation. The plasma BDNF value is the fraction of BDNF that is available to cross the blood–brain barrier via a rapidly saturable transport system (Pan et al., 1998). BDNF expression is regulated by both endogenous and exogenous factors, including neurotransmitters such as glutamate, acetylcholine and serotonin that can up-regulate BDNF expression, in addition to gamma-amino butyric acid, which is known to act as a down-regulator of BDNF expression (Tapia-Arancibia et al., 2004).

Sex steroids directly affect BDNF expression levels. During the menstrual phase in fertile women, the menopausal status and in the presence of specific exogenous sex steroids the circulating levels of this neurotrophin are modified (Begliomini et al., 2007). In addition, it has been reported that BDNF mRNA levels fluctuate in the rat hippocampus during the oestrous cycle (Gibbs, 1998).

Glucocorticoids are also involved in BDNF regulation (Barbany and Persson, 1992, 1993). A fairly recent study has shown that cortisol exerts a differential influence on BDNF regulation in the rat hippocampus as a function of the specific receptor involved (glucocorticoid or mineralcorticoid receptors) (Hansson et al., 2000). Exogenous stimuli, such as physical activity (Neeper et al., 1991) and light exposure (Castrén et al., 1992), can influence the expression of BDNF, particularly in the rat cortex and hippocampus (Bova et al., 1998). In addition, several studies have indicated a cyclic change in BDNF and trkB expression within a 24-h period (Bova et al., 1998; Schaaf et al., 1998; Dolci et al., 2003).

Recently, it has been hypothesized that BDNF may be involved in the regulation of suprachiasmatic nucleus (SCN) activity. The SCN represents the endogenous oscillator and primary biological clock in mammals (Hastings, 1997). More specifically, the SCN is the central pacemaker that generates and controls the endogenous circadian oscillation of hormones such as cortisol and gonadotrophins (Baker and Driver, 2007).

In a previous study, we discovered a correlation between BDNF and the cortisol circadian rhythm in men following the observation that they follow a similar secretion profile (Begliomini et al., 2008). Therefore, the major aims of this study were to determine whether or not a BDNF-dependent circadian rhythm also exists in women, to determine if BDNF and cortisol diurnal fluctuations are characterized by similar profiles, and to assess whether or not the hormonal status influences BDNF and cortisol diurnal variation by studying BDNF and cortisol plasma concentrations under different hormonal conditions (follicular phase (FP) and luteal phase (LP) of normally menstruating women, hypothalamus–pituitary–ovary axis inhibition by oral contraceptives (OCs) and hypo-oestrogenism caused by menopause).

Materials and Methods

Subjects

Thirty women were enrolled in this study; 10 into each of three groups. The first group (n = 10) consisted of normally menstruating women between 20 and 33 years old (mean ± SD = 25.6 ± 3.2 years) with regular ovulatory cycles. The women in this first test group were not undergoing any hormonal therapies and had normal body mass indices (BMIs) between 20.5 and 23.6 kg/m². The second test group was composed of 10 women (n = 10) between 20 and 32 years old (mean ± SD = 25.7 ± 3.8 years) with normal BMIs between 20.2 and 24.1 kg/m² who were undergoing a continuous combined OC composed of 20 µg of ethinyl-estradiol in addition to 3 mg of drospirenone. The third group was composed of 10 post-menopausal women (n = 10) who were undergoing no hormonal treatments and were 50–68 years old (mean ± SD = 56.8 ± 4.6 years) with normal BMIs between 21.3 and 24.8 kg/m².

Prior to enrolment, the subjects participating in the study provided their written informed consent. In addition, the Local Research Ethics Committee approved this protocol. Each subject was asked to answer a questionnaire to disclose her age, weight, height, and if she was taking regular medication. The subjects were also asked to disclose any known chronic diseases, current illnesses, allergies and family history of endocrinological, psychiatric or neurological diseases. Physical examinations and routine laboratory tests were performed on all test subjects, and these examinations disclosed no abnormalities. Furthermore, none of the subjects were taking any psychoactive medications or anti-inflammatory drugs, and no mood or behaviour disturbances were observed at the time of enrolment.

All fertile women had regular menstrual cycles (28 ± 2 days). Ovulation was determined by assessment of day 21 plasma progesterone levels (values ≥ 10.0 ng/ml were considered ovulatory).

Protocol

The group of normally menstruating women was evaluated both in the FP (days 6–8) and in the LP (days 20–24) of the menstrual cycle. Post-menopausal women and women undergoing OC treatments were evaluated once per study period (days 6–8 of therapy for OC users). After overnight fasting, a blood sample was withdrawn from each subject and analysed for basal evaluation of the plasma BDNF, estradiol and cortisol levels. Furthermore, in order to investigate circadian variations in BDNF and cortisol levels, blood from each subject was collected every 4 h for a total of five samples over a 24-h period. The first blood sample was drawn from the cubital vein of each subject in EDTA-coated tubes (Vacutest Kima s.r.l., Arzergrande, Italy) at 08:00. Subsequently, blood sampling was repeated at 12:00, 16:00, 20:00, 24:00. The tubes were kept on ice, and, after collection, the blood samples were immediately centrifuged at 4°C (2500 × g for 15 min). The plasma was aliquoted and stored at −80°C until the samples were assayed.

BDNF assay

Plasma levels of BDNF were determined using an enzyme-linked immunosorbent assay method (BDNF Emax Immunoassay System, Promega, USA) after appropriate dilution of samples (1:4) using Block and Sample Buffer, according to the manufacturer’s instructions.

Briefly, 96-well flat bottom immunoplates (Iwaki) were coated with anti-BDNF monoclonal antibody (mAb) and incubated at 4°C overnight. After blocking non-specific binding with Block and Sample buffer, standards and samples were added to the plates and incubated with shaking for 2 h at room temperature. Subsequently, after washing with a TBST (Tris Buffered Saline with Tween 20) wash buffer, the plates were incubated for 2 h in the presence of anti-human BDNF mAb. Finally, the plates were incubated with an anti-IgG-HRP conjugate. In the last step of the assay, TMB One solution was added in order to permit colour development. After stopping the reaction with 1 M HCl, the absorbance was read at 450 nm on a microplate reader. The resulting BDNF concentrations were determined according to a BDNF standard curve (ranging
from 7.8 to 500 pg/ml purified BDNF). The entire procedure was performed using a semi-automated Basic Radim Immunoassay Operator (Brio-Radim, Italy) equipped with an optical density microplate reader. A computer system, linked to the Brio, analysed the final results and expressed them in units of pg/ml.

**The cortisol, estradiol and progesterone assays**

Plasma concentrations of cortisol, estradiol and progesterone were determined by use of commercially available radioimmunoassay kits (Radim, Pomezia, Italy). The sensitivities of the assays were 0.90 μg/l for cortisol, 10.0 pg/ml for estradiol and 0.12 ng/ml for progesterone. The respective intra- and inter-assay coefficients of variation were 2.6 and 8.0% for cortisol, 2.1 and 4.2 for estradiol and 4.2 and 7.8 for progesterone.

**Parameters and statistical analysis**

Plasma BDNF and estradiol levels are expressed in units of pg/ml, whereas cortisol levels are expressed in units of μg/l. These data are reported as mean ± standard error of the mean. Statistical analysis was carried out using Graph Pad Prism 4.0. ANOVA and repeated measurements were performed to analyse circadian rhythms, whereas unvaried ANOVA followed by a post-hoc analysis with Bonferroni multiple comparisons was used to test for differences between the groups. A correlation index (Pearson Index) was computed in order to investigate correlations between plasma BDNF and sex steroids throughout the menstrual cycle.

**Results**

**Basal BDNF and estradiol**

In normally cycling women, both the circulating levels of BDNF and estradiol were higher in the LP than in the FP (P < 0.001). In addition, women undergoing OC showed lower plasma estradiol levels than women sampled in the FP (P < 0.01). No significant differences were observed in regard to BDNF levels between women undergoing OC and women in the FP. In menopausal women, BDNF and estradiol levels were significantly lower than those observed in fertile women in the FP (P < 0.001) (Fig. 1). BDNF and estradiol levels were positively correlated in fertile women (r = 0.883, P < 0.0001) (Fig. 2). Interestingly, this correlation was not statistically significant when comparing the results of women undergoing OC and in postmenopause (r = 0.3092 and r = -0.4778, respectively) (Figs. 3 and 4).

**Circadian variations in the FP**

Both the levels of circulating BDNF and cortisol decreased with time during the day, with the highest level observed in the morning (438.6 ± 21.45 for BDNF and 177.7 ± 13.21 for cortisol) while the nadir was observed at midnight (292.6 ± 12.73 for BDNF and 108.2 ± 3.58 for cortisol). In particular, BDNF levels measured at 20:00 were significantly lower than those measured in the morning (P < 0.001 versus 08:00). The trend of decreasing cortisol levels achieved statistical significance when comparing the level at 16:00 to the level at 08:00 (P < 0.01 versus 08:00, Fig. 5).

**Circadian variations in the LP**

No significant variations in the daily BDNF levels were observed in the LP of normally cycling women. Conversely, the cortisol levels showed a decreasing trend in the luteal as well as in the FP, maintaining the same trend starting at 16:00 (P < 0.001 versus 08:00) (Fig. 6).

**Circadian variations in women taking OCs**

In the group of women undergoing OC, the BDNF levels showed a slight decreasing trend during the day, achieving statistical significance at 16:00 (P < 0.01), in a manner that was similar to women in the FP.
The cortisol levels showed a decreasing trend throughout the day, achieving statistical significance at 24:00 in comparison with the values measured during the morning \((P < 0.001 \text{ at } 24:00 \text{ versus } 08:00)\) (Fig. 7).

Circadian variations in post-menopausal women

In post-menopausal women, both BDNF and cortisol plasma levels significantly decreased during the day. This decreasing trend achieves statistical significance at 20:00. \((P < 0.01 \text{ versus } 08:00)\), at which time the cortisol levels were significantly reduced at any sampling time with respect to the cortisol levels that were obtained during the morning \((P < 0.01 \text{ at } 12:00, P < 0.001 \text{ at } 16:00 \text{ p.m.}, 20:00 \text{ and } 24:00 \text{ versus } 08:00\), Fig. 8).

Discussion

The present study determined that plasma BDNF levels show a circadian fluctuation in women, with amplitudes that vary according to the hormonal milieu. In addition, we observed an analogous cyclical pattern upon comparison of the BDNF diurnal changes and the cortisol circadian rhythm.

The evaluation of basal BDNF levels in both fertile (FP and LP) and menopausal women confirmed our previous results, which indicated that the plasma BDNF level varies across the menstrual cycle in fertile women and decreases in post-menopausal women, with a significant correlation index obtained upon comparison to the circulating estradiol and progesterone levels (Begliuomini et al., 2006).

Women undergoing OC showed plasma levels of BDNF in the same range as those measured in fertile women in the FP of the menstrual cycle, although OC treatment significantly reduced the estradiol level. We previously determined that patients with hypothalamic amenorrhea have lower levels of estradiol and BDNF than fertile women during the FP. Thus, a woman undergoing an OC treatment consisting of 0.2 mg of ethinyl-estradiol and 3 mg of drospirenone should maintain BDNF levels in the range expected for fertile women and might even show restored BDNF plasma levels within the physiological values, in patients affected by hypoestrogenism.

Moreover, our data suggest the presence of a BDNF diurnal rhythm in women, similar to that detected in men (Begliuomini et al., 2008). In particular, during the FP of the menstrual cycle and in postmenopause, women showed plasma BDNF values that were higher in the morning.
followed by a substantial decrease throughout the day, with the lowest values observed at midnight. These data clearly indicate a diurnal decreasing trend comparable to that observed for cortisol levels. Similarly, women undergoing OC treatment showed diurnal BDNF changes that were analogous to changes observed in the other groups and BDNF levels that were lower than the highest morning value from 16:00 onward. These findings support also the concept that OC treatment does not affect the daily physiological changes of this neurotrophin.

The decline in BDNF levels during the day may be ascribed to a circadian secretory model. In fact, it has been shown that BDNF has a very short half-life in the blood \( t_{1/2} = 0.92 \text{ min} \) (Poduslo and Curran, 1996); thus, it is conceivable that BDNF is secreted with a pulsatory circadian rhythm that is characterized by a progressive reduction in the amplitude of pulses throughout the day.

Although the BDNF plasma levels differed among these three experimental groups, the feature of its diurnal variation was very similar in all groups. This finding suggests that the reproductive ageing process and estradiol milieu may affect the synthesis and secretion of BDNF without modifying the mechanisms responsible for the generation of its circadian rhythm.

However, women analysed during the LP did not show any diurnal changes in the levels of plasma BDNF, as indicated by the high values observed during the day. We previously demonstrated that, during the mid-LP, a significant increase in plasma BDNF levels occurs, resulting in a positive correlation index with progesterone levels (Begliuomini et al., 2006). Although the precise origin of plasma BDNF remains to be elucidated, it has been suggested that the high plasma BDNF levels observed in the LP may be at least partly due to local production by the corpus luteum and the endometrium (Krizsan-Agbas et al., 2003). This additional peripheral synthesis of BDNF, correlated to progesterone production, increases 2-fold plasma BDNF in comparison with the FP. Thus, it is conceivable that BDNF basal levels and its diurnal variations observed during the FP might reflect the variability of SCN production and secretion, whereas during LP, this variability might be blunted by a greater peripheral BDNF synthesis. This hypothesis is also corroborated by the evidence that women receiving an OC, which inhibits the development of the corpus luteum and of the secretive endometrium, showed daily changes of BDNF levels.

Moreover, recent observations of progesterone and estrogen receptors in the human SCN (Kruitje and Swaab, 2002), coupled with the regulation of the expression of one of the Period genes (Per2) in the SCN of female rats by sex steroids (Nakamura et al., 2005), suggest the intriguing possibility that progesterone may directly influence circadian rhythmicity. Functions such as sleep, body...
temperature and stress response, which are all under SCN control, exhibit a circadian rhythm during the FP, although their circadian rhythms are not observed during the LP (Kirschbaum et al., 1999).

Thus, a direct influence of progesterone on the central rhythm generator of BDNF could be also hypothesized. Indeed, it has been demonstrated that the neuroactive progesterone metabolite allopregnanolone increases the content of BDNF in the hippocampus, amygdala and hypothalamus with a concomitant increase in the release of corticotropin-releasing hormone (CRH) and the generation of elevated serum levels of adrenocorticotropic hormone and corticosterone. These data indicate a direct connection between the levels of progesterone and BDNF and hypothalamus–pituitary–adrenal (HPA) axis activity (Naert et al., 2007). The role of BDNF in response to stress has been extensively probed. Studies have shown that rhythmic variations occur during the day, with the highest levels achieved during periods of activity or when the individual is under stress, although the lowest levels are detected during rest periods (Bova et al., 1998; Berchtold et al., 1999). Furthermore, BDNF may have a role in re-establishing the hypothalamic hormonal pool after a disturbance in the homeostasis, and it may modulate CRH release into the portal system (Givalois et al., 2004).

The present results indicate a correlation between the daily levels of plasma BDNF and cortisol in women and corroborate the hypothesis of co-regulation of cortisol, BDNF and sex steroids in humans. This correlation suggests the fascinating possibility that glucocorticoid and neurotrophic tone may play a synergic role in the homeostasis of cerebral functions.

In addition, the blunted fluctuations of BDNF levels during the LP, coupled with the lack of a correlation with the cortisol rhythm, might be indicative of an additional mechanism that leads to neuroendocrine dysregulation in the second half of the menstrual cycle of susceptible women, thus predisposing them to experience premenstrual symptoms. Indeed, premenstrual dysphoric disorder is associated with an altered regulation of the SCN circadian rhythms and HPA activation (Halbreich, 2003). In addition, the use of OC has been demonstrated to improve symptoms in these patients (Pearlstein et al., 2005; Kroll and Rapkin, 2006). No data are currently available in regard to premenstrual symptoms and circadian fluctuations of BDNF; however, evidence that OC may modify some functions of the SCN, such as BDNF, suggests interesting hypotheses that should be analysed further (Baker and Driver, 2007).

The present study has some limitations that suggest prudent interpretation of the data. For instance, the study groups were small in size, and the luteal testing was scheduled according to the onset of menstruation rather than at the onset of the LH surge, although all fertile women reported regular menses during and at the end of the study. Moreover, the absence of blood samples during the night may have prohibited observation of some important BDNF fluctuations; however, the sleep-wake phases are known to affect the BDNF content in the brain, as indicated by lower levels during sleep and higher levels during wakefulness (Bova et al., 1998). No data are currently available about the effect of time of year and consequent changes to light/dark cycle on BDNF. However, all subjects in the present study have been evaluated in the same season.

In conclusion, the present study indicates that BDNF has a diurnal variation in women, which is somewhat analogous to the fluctuation observed for cortisol. Importantly, the amplitude of the changes in the BDNF levels is modulated by ovarian function. Knowledge of the interactions between BDNF, the HPA axis and sex steroids is essential to improve the understanding of the biology of human homeostasis and adaptation.

**Funding**

This work was partially supported by a grant from the Fondazione Cassa Risparmio di San Miniato, Pisa, Italy.

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Sex hormones influence BDNF and cortisol diurnal variations in women


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Submitted on July 25, 2008; resubmitted on March 31, 2009; accepted on April 7, 2009.