Long-Term Reduction in Anxiety Levels During the Promotion Phase of Mammary Adenocarcinoma Induced by Dimethylbenz (a) Anthracene in Female Sprague-Dawley Rats

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Abstract: *Background/Aims*: It has been reported that long-term neuroendocrine dysregulation of the Hypothalamic-Pituitary-Gonadal (HPG) axis and of the Hypothalamic-Pituitary-Adrenal (HPA) axis precede the appearance of mammary adenocarcinoma induced by 7, 12-Dimethylbenz(a)anthracene (DMBA) in female rats. In the present study, we investigated the hypothesis that DMBA may also induce long-term changes in anxiety levels.

Methods: Female Sprague-Dawley rats were given a single dose of DMBA(75 mg/kg) in 1ml sesame oil, or 1ml of sesame oil(control rats), at 55 days of age. Then,DMBA-treated and control rats were observed, monitoring their oestrous cycle. One month after administering the DMBA, anxiety levels were measured in an open-field test.The test device was an arena with a floor of 100 cm x 100 cm and a wall of 40 cm high, with white floor and walls.The floor was divided into 25 equal squares (16 peripheral and 9 central squares). The statistical analysis was a two-factor analysis of variance.

Results: Overall statistical scores showed that DMBA-treated animals had: a) greater ambulatory activity and more rearing behaviour in the central squares, but not in the peripheral squares, and b) deposited fewer faecal boli.

Conclusions: Greater exploration of the less protected squares (in the centre), lower frequency of defecation and a greater number of rearings show a lower level of anxiety. Changes in HPG axis activity and a decrease in HPA axis activity, as previously reported, may explain the reduction in anxiety levels after treatment with the mammary carcinogen.

Keywords, Exploratory behaviour, mammary adenocarcinoma, female rat.

INTRODUCTION

Female Sprague-Dawley rats develop mammary adenocarcinoma in response to a single intragastric dose of the carcinogen 7, 12-dimethylbenz (a) anthracene (DMBA) [1]. The mechanisms by which DMBA induces mammary tumours in rats have been well characterized. The carcinogen interacts with rapidly proliferating cells in the terminal end buds, forming DNA adducts, which in turn play a role in transforming normal terminal end bud cells into malignant pathways [2-4]. The susceptibility of Sprague-Dawley rats to DMBA peaks at 55-60 days of age and is eliminated by ovariectomy, suggesting that the inducible action of the carcinogen depends on ovarian hormones [5].

Furthermore, the appearance of DMBA-induced adenocarcinoma is preceded by a series of disturbances to the Hypothalamic-Pituitary-Gonadal (HPG) axis and the Hypothalamic-Pituitary-Adrenal axis (HPA) axis during the latency period.

In the HPG axis, the oestrus cycles coincide with blunting of preovulatory surges of plasma LH and FSH [6], an increase in preovulatory surges of plasma 17β-Estradiol [7] and disturbances to Hypothalamic Gonadotropin-Releasing Hormone (GnRH) and its pituitary receptor gene expression patterns, lasting throughout the oestrus cycle [8]. Ovariectomised rats given DMBA 5 days before the ovariectomy will, when given Estradiol *in vivo* one month later, display blunted LH release and a reduction in GnRH release when measured *in vitro* using synaptosomes taken from the mediobasal hypothalamus [9].

In the HPA axis, long-term deregulation of circadian and 17β-Estradiol-induced corticosterone secretion was observed in DMBA-treated rats [10].

Estradiol treatment produces a dose-dependant response with blunting of both spontaneous and Isoproterenol-induced secretion of Melatonin as measured *in vitro* in perifused pineal glands [11].

The literature on the effects of ovarian steroids [12-14] and corticosterone [16, 17] on the regulation of anxiety-

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related behaviour patterns is extensive. One mechanism by which estradiol may have an anxiety-reducing effect behaviour is by increasing serotonin production and secretion in midbrain/brainstem raphe nuclei [18]. Behavioural tests have shown that a selective agonist of the Estrogen Receptor β (ERβ) decreases circulating levels of corticosterone in animals with low anxiety levels, raising the possible interpretation that the behavioural effect of ERβ activation may also be due to a reduction in corticosterone secretion [12].

Given the long-term hormonal dysregulation induced by DMBA, in particular the preovulatory increase in plasma 17β-Estradiol [7] and a flattened circadian rhythm of corticosterone [10] – both playing a key role in regulating anxiety – we hypothesized that these dysregulations may induce changes in levels of anxiety in the course of the development of DMBA-induced mammary adenocarcinoma.

MATERIAL AND METHODS

Animals

The study was conducted in compliance with European regulations and CNRS Animal Welfare guidelines; the animal studies reported were conducted in compliance with accepted standards for humane care. The study was approved by the committee for animal care at the authors' research institute.

One hundred Female Sprague-Dawley rats (Charles River, L'Arbresle, France), 49 to 51 days of age, were used. They were housed (5 per cage), in a controlled temperature environment (21 $\pm$ 1°C) with a 12-hour day/night cycle (lights on from 7am to 7pm). The animals had food and water *ad libitum*.

After 4 or 5 days of acclimatisation, 50 rats were given a single intragastric dose of either DMBA (Sigma, Saint Quentin Fallavier, France) (75 mg/kg) dissolved in 1 ml of sesame oil or 1 ml of the vehicle alone, on the day of oestrous of the oestrous cycle. The stage of the oestrous cycle was determined by vaginal smears, examining morphological changes in vaginal epithelial cells under light microscopy [6]. The rats were treated at age 55-58 days, the peak period for inducing mammary cancer [1]. Then, DMBA-treated and control/vehicle rats were monitored daily, assessing the regularity of their oestrous cycle by vaginal smears. There was a transient disturbance to the oestrus cycle over 4-5 cycles (16-20 days), but it returned to a normal 4-day oestrous cycle, continuing for at least 15 cycles (60 days after administration of DMBA).

Thirty days after the DMBA had been given, at a time when the hormonal imbalances were well established but no palpable mammary tumours could be detected (the first palpable tumours did not appear until 60 days after the DMBA treatment) [6], anxiety was measured, testing in a new environment and comparing the different groups over the 4 different stages of the oestrus cycle: DMBA-treated and control/vehicle rats.

Measuring Anxiety

Groups of five female rats were housed in standard rearing conditions in our animal facility. Sprague-Dawley rats are an albino strain and also have other sight impairments, with varying degrees of prevalence and more prevalent in females [19]. We therefore chose not to use common test procedures for measuring anxiety such as the elevated plus-maze which requires visual perception of depth. The open-field test was used as it minimizes the potential impact of impaired vision. The test stimulates reactions to a new environment and these responses are accepted as parameters measuring anxiety and emotionality in rodents [20]. Naive female rats were tested at 60 ± 3 days. The test device was an arena with a floor of 100 cm x 100 cm and a wall of 40 cm high, with white walls and floor. The floor was divided into 25 equal squares (16 peripheral squares and 9 central squares) delineated by red lines which cannot be seen by rodents as they are unable to discriminate colours. The device was brightly lit (110 lux at floor level). Behavioural testing was done between 0900 h and 14 00 h. A previously reported procedure [21] was used, with the animals placed separately in the arena for the first time.The behavioural parameters recorded were:

Ambulatory activity, counted as the number of lines between squares crossed, separated in two halves (the first 10 minutes and the last 10 minutes of the experiment). The number of lines crossed is a continuous variable. The lines had to be crossed by the animal's body; the tail alone was not counted.

Rearing, counted when the rat stood upright on its hind paws, without the forepaws touching the walls. The number of rearings was a continuous variable.

Leaning, counted when the rat was either sitting or standing on its hind legs and placing one or both forepaws against one of the walls. The number of leanings was a continuous variable.

Defecation, counting the number of animals depositing boli. The proportion of defecating animals was a discontinuous variable.

Ambulatory activity, rearing and leaning were recorded with separate counts for the 16 peripheral squares and the 9 central squares. The rats were observed directly for a 20-minute period of activity, plus any extra time spent grooming and freezing. The behavioural parameters were recorded separately for the first half (10 minutes) and the second half (10 minutes) of the test.

Statistical Analysis

The behavioural parameters were counted and the proportion of rats displaying the behaviour was compared between the first ten minutes and the last ten minutes of the test, using parametric and non-parametric statistics respectively. For continuous measures, testing was done for an oestrous cycle effect using an ANOVA procedure and the two classification factors (DMBA vs. vehicle) and the stage of the oestrus cycle (Diestrous 1, Diestrous 2, Proestrous or Estrous). The $\leq p.05$ value was considered to be the threshold for significance. The size of the statistical effects has been given when available (partial Eta squared, abbreviated $\eta^2 p$, showing that part of the variance attributable to the factor when other non-error sources of variance have been partialled out [22]. Non-parametric statistics (χ^2) were used for the discontinuous measurement (proportion of boli- deposit-

ing animals), and the size of the effect was estimated using Cramer's coefficient [22]. Statistical comparisons were performed between behaviour recorded in the 16 peripheral squares and behaviour in the 9 central squares.

RESULTS

There was no difference in the number of behavioural responses observed in the first 10 minutes and the last 10 minutes. The scores used were then pooled and were the total number of behavioural data and of individuals for the entire period of test (20 minutes).

Ambulatory Activity

We first assessed ambulatory activity in the peripheral squares and found no changes attributable to either the treatment (DMBA vs. vehicle) or the stages of the oestrous cycle; it was the same finding when observations of all four stages were pooled (Fig. **1**).

The oestrous stages did not influence ambulatory activity in peripheral squares, either as a single variable or in interaction with the treatment ($F < 1$). The number of central square crossings appeared to increase but this only reached significance for the di-oestrous 2 group ($p < .05$, $\eta^2p = 0.87$) where it accounted for 8.7 % of the difference observed between DMBA-treated animals and the vehicle controls. A treatment effect was seen when the observations across over all the oestrous stages were pooled ($F = 4.79$, $df = 1.40$, $\eta^2p = 0.11$). The treatment effect on the number of central square crossings was significant, and 11% of the variance between groups could be considered as substantial (Fig. **2**).

Number of Rearings

The total number of rearings in the peripheral squares was not modified by either the treatment, oestrous stages or an interaction between the two (Fig. **3**). The number of rearings in the central squares was influenced by the treatment (DMBA vs. vehicle): the DMBA rats were more active than the control/vehicle rats. The tendency was observed for each oestrous stage but did not reach significance. Pooled observations on all stages showed the oestrous cycle to have a substantial effect, increasing rearing behaviour in the central squares ($F = 6.57$, $df = 1.40$, $\eta^2p = 0.13$) (Fig. **4**).

Leaning

Leaning was observed in peripheral squares only. No effect was found for either treatment, oestrous stages or an interaction between the two.

Defecation

The proportion of treated *vs* non-treated rats depositing boli in the course of the 20 minutes (discontinuous variable) was compared. The proportion of rats depositing boli was lower in the DMBA-treated group than in the control/vehicle group. Pooling data from treated and non-treated rats (Fig. **5**), shows that the number of animals depositing boli was significantly lower in the group given DMBA ($\chi^2 = 4.364$, $df = 1$, $p < .003$). Calculations using Cramer's coefficient (0.33) show that DMBA treatment accounted for $.33^2 = 11$ % of the difference between the DMBA animals and the vehicle animals.

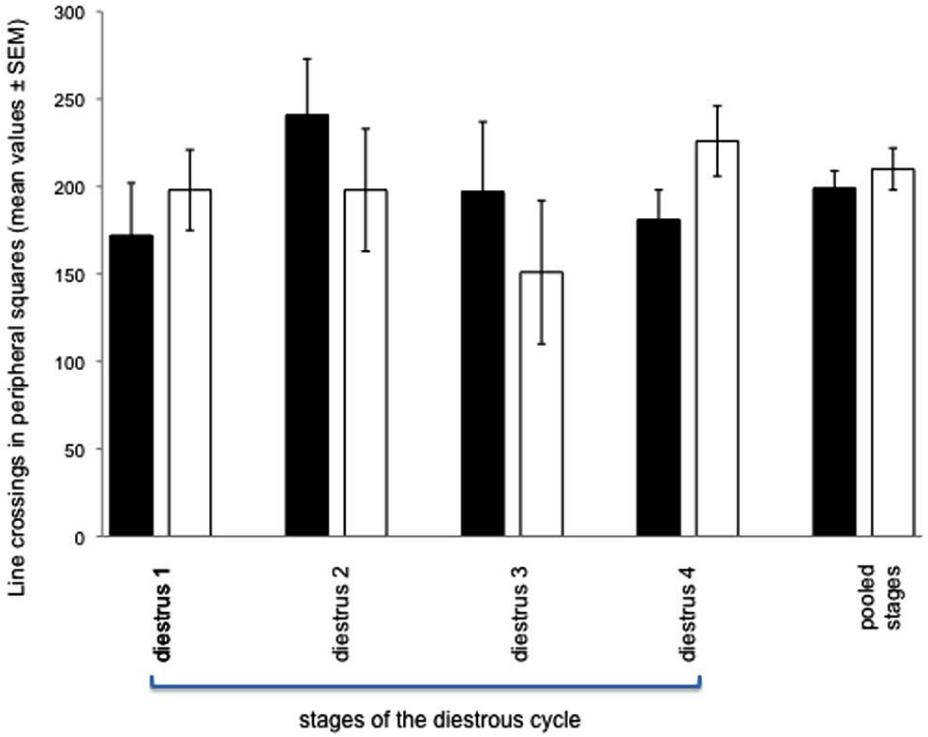

Fig (1). Number of line crossings in peripheral squares by cycling (diestrous 1, diestrous 2, proestrous and estrous stages) and all stages pooled female rats.

rats given DMBA
rats given vehicle

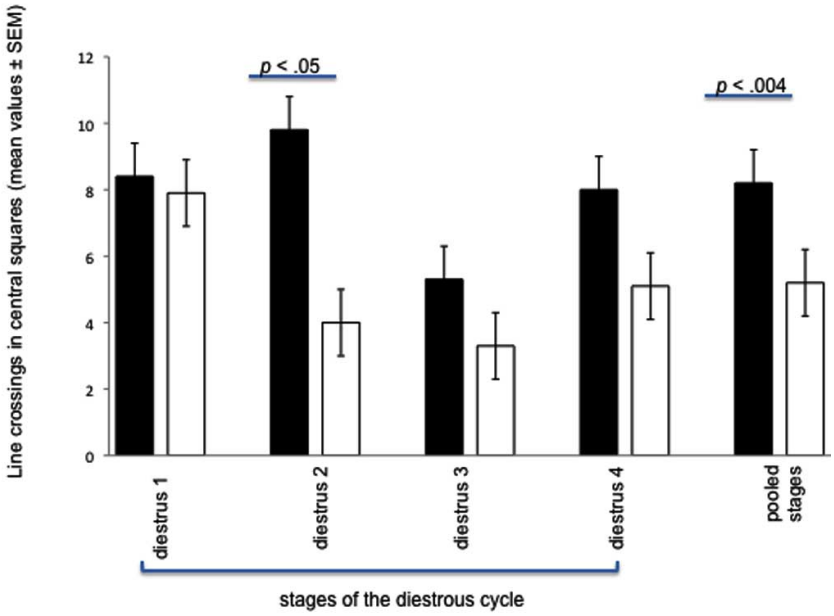

Fig. (2). Number of line crossings in central squares by cycling (diestrous 1, diestrous 2, proestrous and estrous stages) and all stages pooled female rats.

rats given DMBA
rats given vehicle

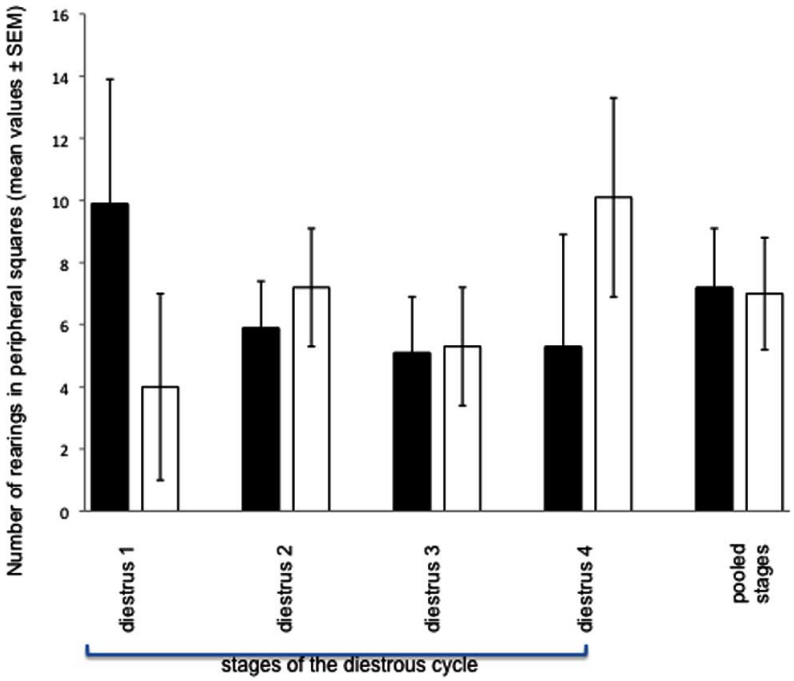

Fig. (3). Number of rearings in peripheral squares by cycling (diestrous 1, diestrous 2, proestrous and estrous stages) and all stages pooled female rats.

rats given DMBA
rats given vehicle

DISCUSSION

The results of the experiment show a marked change in the behaviour of female Sprague-Dawley rats during the promotion phase of DMBA-induced mammary adenocarcinoma. Testing in open-field device showed no DMBA-related change to either ambulatory activity or rearing behaviour in the peripheral squares, but both of these behaviour parameters increased in the central squares for DMBA-treated animals. The proportion of females depositing boli was lower in the DMBA group. An increase in the exploration of the less protected squares that are located in the centre, a decrease in the frequency of defecations and an increase in the number of rearings are signs of a low level of

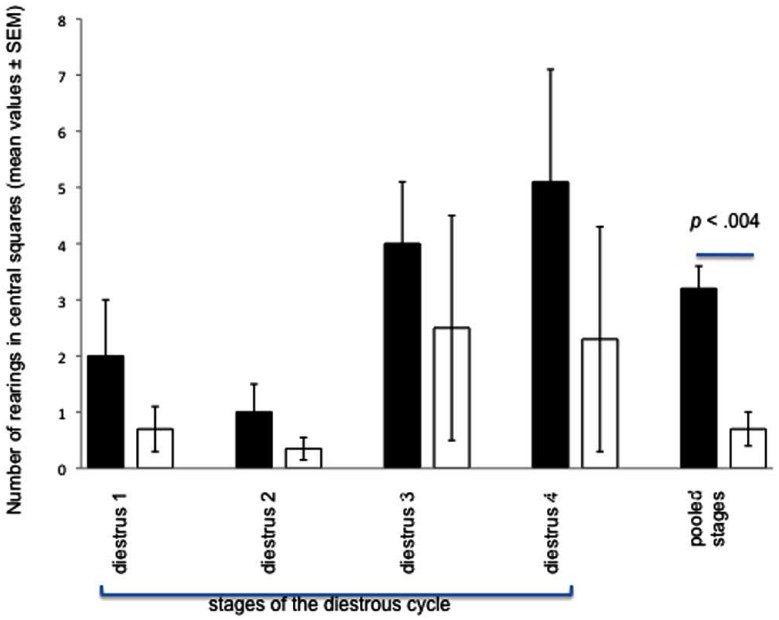

Fig. (4). Number of rearings in central squares by cycling (diestrous 1, diestrous 2, proestrous and estrous stages) and all stages pooled female rats.

rats given DMBA
rats given vehicle

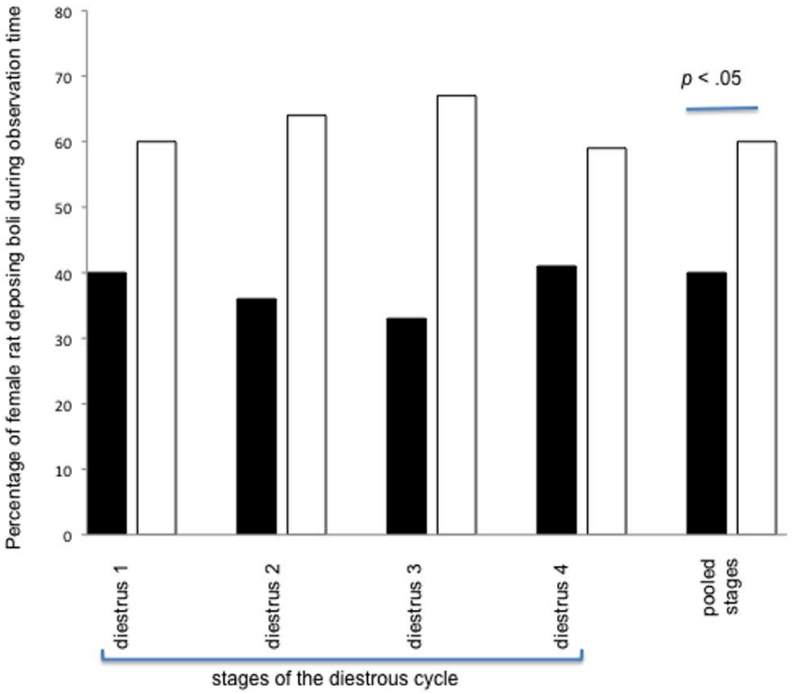

Fig. (5). Percentage of cycling (diestrous 1, diestrous 2, proestrous and estrous stages) and all stages pooled female rats, deposing boli during the observation time.

rats given DMBA
rats given vehicle

anxiety [20, 21], anxiety being measured here in a novel environment where the animal is subjected to bright light and stressful conditions.

These findings for different measurements of anxiety recorded in an open-field situation show a marked reduction in anxiety levels after treatment with the mammary carcinogen. Ambulatory activity showed a tendency to vary in relation to the oestrous cycle, in particular in DMBA-treated rats, but this did not reach significance. No statistical difference could be detected for DMBA treatment or any one stage

of the oestrous cycle, although a statistical difference was found between DMBA and control animals when data on all 4 stages of the oestrus cycle were pooled and considered as a single value.

Similar results were obtained comparing DMBA and non-DMBA-treated animals for the other two indexes: there was an increase in the number of rearings and fewer defecations.

The change in anxiety-related behaviour is probably due to complex neuroendocrine changes occurring along the HPG and HPA axis, as has already been reported [6-11]. Studies have presented extensive data on changes in hormone secretions and HPG axis hormone levels, in particular for 17ß-Estradiol and progesterone with changes occurring during the oestrus cycle, and when oestrogens are given to female rats, leading to changes in exploratory and anxiety behaviour [13, 14].Also, 17β-Estradiol is effective in reducing anxiety and depressive behaviours and enhance sexual behaviour in OVX rats [15]. Estrogens, however, have been reported to have anxiolytic and anxiogenic effects, a dual effect arising from two distinct receptors: ERα and ERβ. The ERα system is thought to play a critical role in reproductive function, whereas the ERβ system is though to play a critical role in mediating anxiolytic effects [12].

Oestrous cycle-related fluctuations of ovarian hormones were not assessed in the present study, but a previous study using the same experimental protocol measured 17ß-Estradiol and Progesterone, as well as estrone, 17-hydroxyprogesterone and testosterone serum levels [7]. It is interesting to note that DMBA treatment stimulated preovulatory surges of 17ß-Estradiol on the afternoon of the proestrous day, but that no significant changes were seen for plasma 17ß-Estradiol or any other steroids or metabolites at any other stage of the oestrous cycle [7].

Our study found no significant evidence for oestrous cycle-related variation in anxiety levels in either control or DMBA- treated animals, but pooled data covering all 4 stages of the oestrous cycle showed a significant reduction in anxiety levels in DMBA-treated animals. The long-lasting reduction in anxiety-related behaviour may be caused by the increase in preovulatory surges of 17β-Estradiol over the previous oestrus cycles.

It has been extensively reported that changes in HPA axis activity can change anxiety levels, a hyper-reactive HPA axis being associated with high anxiety levels [23-25]. In the present experiment, the reduction in anxiety level may be the result of the almost complete blunting of the circadian rhythm of corticosterone secretion [10]. Many studies have shown that the level of activity of the HPA axis determines the level of anxiety, in particular for certain forms of prenatal stress, as seen in experiments with rodents. The exposure of pregnant rats to repeated stress during the last week of pregnancy has been seen to increase both the level of anxiety and HPA axis activity in their adult female offspring, while adult male offspring had no change in anxiety-related behaviour patterns and displayed elevated basal ACTH and a blunted response to stress [26]. Sex-dependent differences in these responses may be related to the absence of testosterone in females; testosterone propionate has recently been shown to reduce the HPA axis stress response and behavioural stress response in female mice [27]. The switch from female-type behaviour to male-type behaviour could be from the direct action of DMBA which has steroidal and morphological effects on the Hypothalamic-Pituitary axis [28, 29].

The rats were obviously tested during the light/day part of the circadian cycle, a time of low HPA axis activity, but it is unlikely that, after DMBA treatment, any increase rather than a decrease in anxiety levels would have been observed if the rats would had been tested during the dark/night part of the cycle, i.e. their period of activity and a time of a high HPA axis activity. Furthermore, corticosterone plasma levels were already lower by the end of the light period in the DMBA-treated rats compared to the controls.

It is interesting to note that human patients with confirmed breast cancer have flattened circadian rhythm of plasma cortisol [30, 31]. In female rats, the flattened circadian rhythm of plasma corticosterone [10], observed during the latency phase of mammary cancer, obviously cannot be caused by any psychological stress related to the development of the disease.

For the moment there is no evidence as to whether a similar flattened circadian rhythm of plasma cortisol and a similar change in anxiety behaviour may appear in the course of the long latency phase of woman breast cancer linked to non-identified carcinogenic factors in the environment.

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