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Age related changes in hypothalamic-pituitary-adrenal axis activity of male C57BL/6J mice

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Abstract

As there is little known about age related changes in the hypothalamic-pituitary-adrenal (HPA) axis of mice, we determined the daily patterns of corticosterone secretion every 2h, together with adrenocorticotrophic hormone (ACTH) release and central HPA axis markers in the morning and evening in 3, 9 and 16 months old male C57BL/6J mice.

We observed that: (i) corticosterone secretion showed a distinct age related circadian pattern. During the light period this was expressed by relative hypercorticism in 9 months old mice and relative hypocorticism in 16 months old mice. ACTH was elevated at 16 months of age; (ii) mineralocorticoid- (MR) and glucocorticoid receptor (GR) mRNA expression in the hippocampus were significantly decreased in 9 months old mice, whereas in 16 months old mice, expression was similar to young animals. Circadian variation was modest in all age groups; (iii) the parvocellular hypothalamic paraventricular nucleus (PVN) expressed very high vasopressin mRNA, which was subject to circadian variation in 3 and 9 months-old mice. Furthermore, significant levels of MR mRNA were expressed in PVN.

In conclusion, basal HPA axis activity and expression of its central regulatory markers are age dependent in mice. This suggests that the capacity to adjust to environmental demands is either a function of age, or depends on different dynamics of the HPA axis.

Introduction

Corticosteroid hormones are potent modulators of neuronal functions. Circulating concentrations of cortisol and corticosterone are regulated by the Hypothalamic-Pituitary-Adrenal (HPA) axis, with arginine-vasopressin (AVP) and corticotrophin-releasing hormone (CRH) as the two main driving hormones from the hypothalamus (Dallman 2000). The central sensors of HPA axis activity in the regulation of feedback and other functions are embodied by the mineralocorticoid- and glucocorticoid receptor (MR and GR), which are expressed in discrete brain regions and in pituitary corticotrophs (Reul and de Kloet 1985).

The secretion of corticosteroid hormones exhibits a circadian pattern and can be induced by stressors (Akana et al. 1986; De Kloet et al. 1998; Windle et al. 1998b). These modes of corticosteroid secretion are regulated by inputs from the suprachiasmatic nucleus (Buijs et al. 1993), prefrontal cortex, amygdala and hippocampus, among others (Spencer et al. 1993). The latter region is an important target for corticosteroid hormones because it expresses high amounts of both MR and GR (De Kloet et al. 1998). Dramatic changes in circadian patterns of corticosteroid hormones have been described in aging and psychiatric diseases like depression and Alzheimer's disease (Hatfield et al. 2004; Peeters et al. 2004). The excessive activity of the HPA axis is generally associated with impaired mental and physical health (Sapolsky 1999; Lupien and Wan 2004) and characterized by increased basal and/or stress-induced levels of corticosteroid hormones and adrenocorticotrophic hormone (ACTH; Van Eekelen et al. 1995; Herman et al. 2001). Although numerous mouse models for a wide range of human stress related disorders have been developed, surprisingly little is known about the impact of age on basal regulation subserving circadian activity of the HPA axis in mice.

In the rat it is known that adjustments of the HPA axis occur in the course of life, that include changes in MR and/or GR protein and mRNA expression (Cai and Wise 1996; Bizon et al. 2001), as well as changes in secretagogue expression, and in adrenal sensitivity to ACTH. Accordingly, we expect to detect also in mice alterations in HPA axis activity that relate to normal life history. These changes may show individual and strain-specific differences as has been reported previously (Bazhanova et al. 2000; Workel et al. 2001).

In this study we have focused on the age dependent changes in circadian HPA axis activity of male C57BL/6J mice, aged 3-, 9- and 16 months. C57BL/6J is the most commonly used inbred mouse strain in research, and the preferential background strain for transgenic mouse models. We estimated corticosterone in blood plasma every 2 hours

to increase the likelihood to detect shifts or irregularities in the circadian pattern. At time points that were expected to coincide with circadian trough and peak concentrations of corticosterone in these mice (Grootendorst et al. 2004), we assessed plasma ACTH concentrations as well as mRNA expression of MR and GR in the hippocampus, and of MR, GR, CRH and AVP in the paraventricular nucleus (PVN) of the hypothalamus.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from Janvier France at the age of 8 weeks. Upon arrival, mice were housed in groups of 8 mice per cage under SPF conditions (TNO, Leiden, The Netherlands). At the age of 3, 9 and 16 months ($n = 16/\text{group}$) they were transported to the animals facilities of the Sylvius Laboratories (Leiden, The Netherlands), acclimatized in a temperature ($21 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) controlled room for two weeks. We chose 16 months as oldest age group, as it was reported that thereafter the survival rate might decrease (Talan and Ingram 1986). All groups were studied at the same time and at the same location, ruling out any differential environmental stimuli at the time of testing. Access to food and water was *ad libitum*; lights were on from 0700 - 1900h (12-12h light-dark cycle). To minimize HPA axis activation, mice were single housed from one day before blood sampling until the end of the experiment. They were also repeatedly handled. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

The circadian secretion of corticosterone was determined in blood samples collected via tail-incision every 2 hours for 24 hours. All age groups were divided in three sub-groups of 5 to 6 animals. As previously described (Durschlag et al. 1996), a small incision at the base of the tail with a razor blade allowed the collection of 50 μl blood, within 90s after opening of the animal's cage. From each mouse, one blood sample was taken every 6 hours. Thus, each time point (with a 2 hour interval) consisted of 5 to 6 mice per group. During the dark period, blood sampling took place under red light conditions.

One week later, mice of each age group were distributed randomly over two groups: decapitation in the morning, 2 hours after lights turned on (0900h) and in the

evening, 2 hours before lights turned off (1700h). Decapitation took place within 15s of opening the animal's cage. Plasma concentrations of corticosterone were determined again as well as plasma ACTH. In the brains we determined the expression levels of molecular markers of HPA axis activity (n = 8 mice/time/age). Brains were snap frozen in isopentane pre-cooled on dry ice/ethanol and stored at -80°C until further use.

Hormone assays

Blood obtained via tail sampling and decapitation was collected individually in capillaries (coated with potassium-EDTA, Sarstedt, Germany), stored on ice and centrifuged with 13000 rpm at 4°C for 10 min. Blood plasma was stored at -20°C . Plasma corticosterone and ACTH concentrations were determined (in $10\mu\text{l}$ and $100\mu\text{l}$ plasma respectively) using commercially available radio immunoassay kits with ^{125}I -corticosterone and ^{125}I -ACTH (MP Biomedicals Inc., CA; USA; sensitivity 3ng/ml and 10pg/ml , respectively).

In situ hybridization

Brains were sectioned at -20°C in a cryostat microtome at $10\mu\text{m}$ in the coronal plane through the level of the olfactory bulb, piriform cortex, hypothalamic paraventricular nucleus (PVN) and dorsal hippocampus. Sections were thaw-mounted on poly-L-lysine coated slides (0.001%), air dried and kept at -80°C until further use.

In situ hybridizations using ^{35}S -labeled ribonucleotide probes (MR, GR, CRH, AVP) were performed as described previously (Schmidt et al. 2003). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense RNA probes were transcribed from linearised plasmids containing exon 2 of mouse MR and GR, the full length coding regions of CRH (rat) and exon C of the rat AVP gene (with 92% homology to mouse). Tissue sections (3 – 4 / slide) were saturated with $100\mu\text{l}$ hybridization buffer containing 20mM Tris-HCl (pH 7.4), 50% formamide, 300mM NaCl, 1mM EDTA (pH 8.0), 1x Denhardt's, 250 $\mu\text{g/ml}$ yeast transfer RNA, 250 $\mu\text{l/ml}$ total RNA, 10mg/ml salmon sperm DNA, 10% dextran sulfate, 100mM dithiothreitol, 0.1% SDS, 0.1% sodium thiosulfate and supplemented with approximately 1.5×10^6 cpm ^{35}S -labeled riboprobe. Brain sections were cover slipped and incubated overnight at 55°C . The next day sections were rinsed in 2 x SSC, treated with RNaseA (20mg/ml) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 x SSC at 65°C for 30 min and dehydrated through increasing concentrations of ethanol. All age groups were assayed

together. Films were apposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY) and developed. For AVP, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4°C for 5 days. Slides were developed, counterstained with Toluidine Blue and examined with a light microscope using both bright and dark field condensers.

Autoradiographs were digitized, and optical density (O.D.) of the areas of interest was quantified using image analysis computer software (analySIS 3.1, Soft Imaging System GmbH). The average density of 4 - 8 measurements for each animal was calculated. For AVP measurement, the area of the parvocellular part of the PVN was determined by light microscopy and the number of radioactive labeled cells was counted.

Statistical analysis

Data are presented as mean \pm S.E.M. The circadian profile of corticosterone was analyzed by analysis of variance (ANOVA; factor: age) with repeated measurements followed by LSD *post-hoc* test. Total corticosterone (AUC: area under the curve) over 24 hours, as well as separately for light and dark periods, were subjected to ANOVA, with age (3, 9 and 16 months) and time of the day (day and night) as fixed factors. Corticosterone and ACTH concentrations and mRNA expression of the various HPA markers collected in the morning and evening were analyzed by ANOVA with age and time of the day (morning, evening) as fixed factors. Significance was accepted at $p < 0.05$.

Results

Circadian rhythm of corticosterone

Mice of all ages showed a circadian rhythm of corticosterone (Figure 1; time $F_{(11,165)}=33.32$, $p < 0.05$) with age dependent characteristics (age $F_{(2,15)}=4.64$, $p < 0.05$). Corticosterone secretion increased from 1400h onwards in all groups with peak values at the end of the light phase and the beginning of the dark phase (between 1600 and 2200h). Interestingly, the course of the circadian rhythm was age dependent (age * time $F_{(22,165)}=3.78$, $p < 0.05$). Corticosterone of 3 months old mice was low during the early light phase (0800 - 1200h; $\pm 20\text{ng/ml}$), increased during the late light phase (1800h) followed by a clear peak at 2000h ($\pm 100\text{ng/ml}$). Thereafter, corticosterone reached baseline resting levels ($\pm 20\text{ng/ml}$) within 2 hours and remained low during the remainder of the dark period.

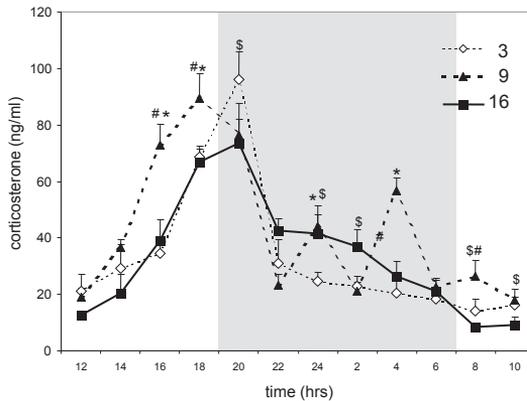


Figure 1

Circadian secretion of corticosterone in ng/ml (mean \pm S.E.M.), measured every 2 hours in blood plasma of 3, 9 and 16 months old male C57BL/6J mice, entrained in a 12-12h light-dark cycle (dark phase from 1900 to 0700h represented by the gray shaded area). P -value < 0.05 : * 3 vs. 9 months; \S 3 vs. 16 months; # 9 vs. 16 months.

The circadian profile of 9 months old mice showed elevated corticosterone already during the early light phase, reached peak corticosterone secretion earlier (at 1600h) than the other two age groups with a prolonged duration (1600 - 2000h). Although corticosterone returned to basal within 2 hours, irregular peaks of corticosterone were found during the dark and early light phase (2200 - 0800h).

The 16 months old mice had low corticosterone levels during the early light phase (0800 - 1200h; ± 10 ng/ml), reaching peak concentrations from 1800 to 2000h (± 75 ng/ml), however, it was at a lower level than the other two age groups. Although corticosterone had decreased at 2200h, it remained elevated during the following period (until 0400h) and dropped at the beginning of the light period to levels below the other two age groups.

Corticosterone: total amount

The total amount of corticosterone changed with age (AUC: age $F_{(2,18)}=8.93$, $p = 0.003$). AUC-values over 24 hours were significantly increased in 9 months old mice ($58.0 \pm 2.0 \times 10^3$) compared to 3 ($49.2 \pm 2.8 \times 10^3$; $p < 0.01$) and 16 months old mice ($45.8 \pm 1.3 \times 10^3$; $p < 0.001$). Separate calculations for the light and dark phase revealed for the dark phase an age-independent corticosterone AUC value. However, age groups differed during the light phase ($F_{(2,18)}=36.45$, $p < 0.0001$): corticosterone was lowest for the 16 months old mice ($19.3 \pm 0.9 \times 10^3$), highest at 9 months ($31.0 \pm 0.9 \times 10^3$) and intermediate at 3 months of age ($25.4 \pm 1.1 \times 10^3$) with significant differences between all groups ($p < 0.001$).

HPA axis activity in the morning and evening

To gain more insight in the regulation of the HPA axis we decapitated the animals at two time points during the day and harvested blood and brain for the detection of hormones and expression of selected mRNAs.

Corticosterone and ACTH

Plasma corticosterone and ACTH concentrations (Table 1) were measured in blood samples, collected by decapitation in the morning (0900h) and towards the evening (1700h). Morning corticosterone was age dependent ($F_{(2,50)}=12.77$, $p < 0.001$) as it was significantly lower in 16 months old mice ($p < 0.05$ vs. 3 and 9 months). All age groups showed increased evening corticosterone ($F_{(2,24)}=8.03$, $p < 0.002$), which was highest at 9 months of age ($p < 0.05$ vs. 3 and 16 months), confirming the circadian measurements. For ACTH, morning and evening values were comparable, but affected by age ($F_{(2,42)}=13.06$, $p < 0.001$); while ACTH was similar in 3 and 9 months old mice, it was increased at 16 months of age at both times ($p < 0.05$; vs. 3 and 9 months).

Table 1:

Basal morning and evening concentrations of corticosterone (ng/ml), ACTH (pg/ml), and expressions of MR, GR, CRH mRNA in the paraventricular nucleus (PVN) of the hypothalamus (arbitrary units of optical density) in 3, 9 and 16 months old C57BL/6J mice.

| | Morning | | | Evening | | |
|----------------|------------|-------------|-----------------------------------|--------------------------------|---------------------------------|-----------------------------------|
| | 3 | 9 | 16 | 3 | 9 | 16 |
| Corticosterone | 16.1 ± 2.2 | 20.5 ± 1.0 | 9.0 ± 0.8 ^{#§} | 52.2 ± 4.6 [†] | 84.7 ± 8.6 ^{*†} | 55.4 ± 4.4 [†] |
| ACTH | 63.7 ± 8.2 | 76.4 ± 14.9 | 156.8 ± 26.6 ^{#§} | 63.0 ± 3.0 | 79.1 ± 14.3 | 134.8 ± 15.7 ^{#§} |
| MR | 21.7 ± 0.7 | 17.8 ± 2.1 | 16.9 ± 1.4 | 25.1 ± 1.5 [†] | 22.5 ± 2.1 [†] | 20.7 ± 1.4 [†] |
| GR | 61.8 ± 5.8 | 57.0 ± 5.8 | 57.5 ± 3.2 | 65.7 ± 5.5 | 54.4 ± 2.8 | 58.4 ± 9.7 |
| CRH | 27.2 ± 4.2 | 31.1 ± 2.1 | 27.2 ± 3.9 | 24.0 ± 6.2 | 33.8 ± 4.4 | 29.3 ± 4.8 |

Bold numbers indicate statistically significant differences. Data are presented as mean ± S.E.M. P -value < 0.05: * 3 vs. 9 months; # 9 vs. 16 months; § 3 vs. 16 months; † morning vs. evening

MR and GR mRNA expression in the hippocampus

Overall hippocampal MR mRNA expression was differentially affected by age and time (Figure 2; age $F_{(8,60)}=3.44$, $p = 0.003$; time $F_{(4,30)}=12.16$, $p = 0.001$). Differences between

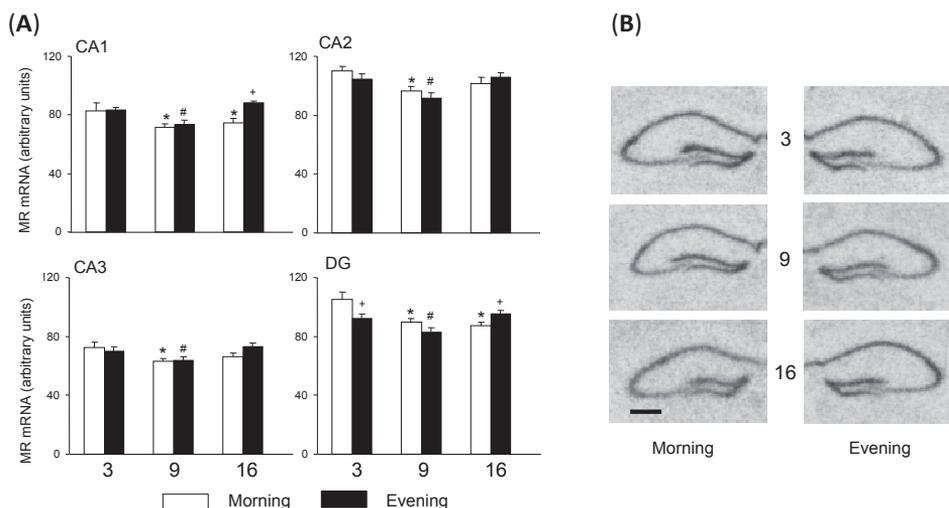
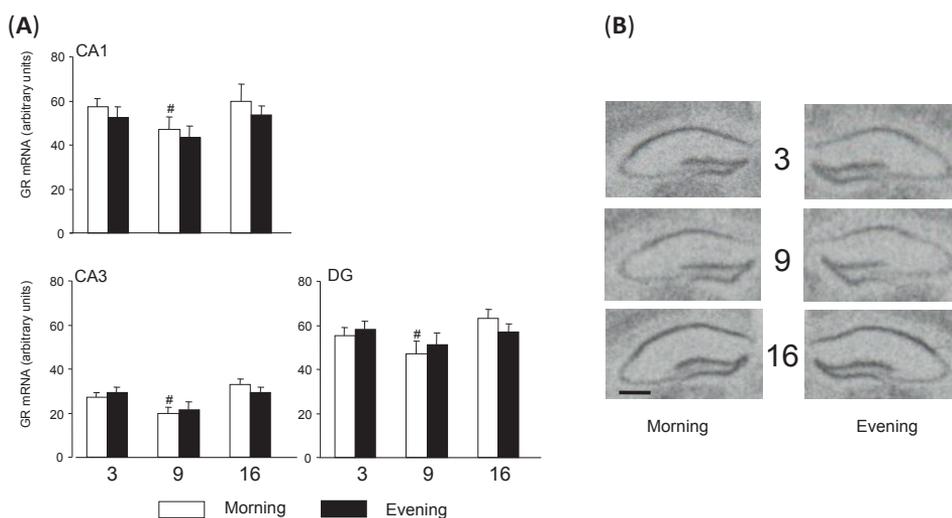


Figure 2

Expression of MR mRNA in the hippocampal subfields CA1, CA2, CA3 and dentate gyrus (DG) of 3, 9 and 16 months old C57BL/6J mice, in the morning and evening hours. **(A)** mean \pm S.E.M. **(B)** Representative photomicrographs; bar = 1 mm; bregma -1.70 mm. P -value < 0.05 : * vs. 3 months; # 9 vs. 3 and 16 months; + morning vs. evening.

groups were in the range of 10 to 15%. The age effect was significant for all subfields (CA1: $F_{(2,33)}=7.755$, $p = 0.002$; CA2: $F_{(2,33)}=7.17$, $p = 0.003$; CA3: $F_{(2,33)}=5.42$, $p = 0.009$; DG: $F_{(2,33)}=9.21$, $p = 0.001$). In the morning, 3 months old mice had the highest MR mRNA expression (3 vs. 9 months: all subfields $p < 0.05$; 3 vs. 16 months: DG - $p < 0.05$). Nine-months old mice had the lowest MR mRNA expression compared to the other two age groups. In the dentate gyrus (DG), we found an interaction between age and time (DG: $F_{(2,33)}=6.07$, $p = 0.006$): in the evening, MR mRNA was lower in 3 months old mice, but increased in 16 months old mice, with no change at 9 months of age. While MR mRNA showed no circadian variation in other subregions of the hippocampus in 3 and 9 months old mice, it was increased in the CA1 in the evening at 16 month of age.

Expression of GR mRNA was about 20 to 30% lower in 9 months old mice (Figure 3; age $F_{(6,66)}=2.57$, $p = 0.027$), compared to 16 months old mice in CA1, CA3 and DG (all $p < 0.05$). Expression of GR mRNA was similar at 3 and 16 months of age. Time of the day did not affect GR mRNA expression.

**Figure 3**

Expression of GR mRNA in the hippocampal subfields CA1, CA3 and dentate gyrus (DG) of 3, 9 and 16 months old C57BL/6J mice, in the morning and evening hours. **(A)** mean \pm S.E.M. **(B)** Representative photomicrographs; bar = 1 mm; bregma -1.70 mm. P -value < 0.05 : # 9 vs. 16 months.

MR, GR, CRH and AVP mRNA expression in the PVN of the hypothalamus

Expression of MR mRNA in the PVN was not affected by age, but was higher in the evening at all ages (time $F_{(1,32)}=6.26$, $p = 0.02$; Table 1). No differences in GR and CRH mRNA expression were found (Table 1).

The strong magno- as well as parvocellular paraventricular expression of AVP mRNA was not affected by age, but by time of the day (Figure 4; $F_{(1,43)}=9.39$, $p = 0.004$), in both the magno- ($F_{(1,41)}=7.71$, $p = 0.009$) and parvocellular part of the PVN ($F_{(1,41)}=6.08$, $p = 0.022$). In both subregions, AVP mRNA was lower in the evening than in the morning of 3 and 9 months old mice ($p < 0.05$), while it was comparable for 16 months old mice.

MR and GR mRNA expression in other brain areas

MR and GR mRNA are also expressed in other brain areas, which are not known to be involved in circadian HPA axis regulation. Therefore, we decided *post hoc* to screen the olfactory bulb for MR mRNA and the piriform cortex for both MR and GR mRNA expression, to detect age- and possible brain-site specific changes of MR and GR mRNA.

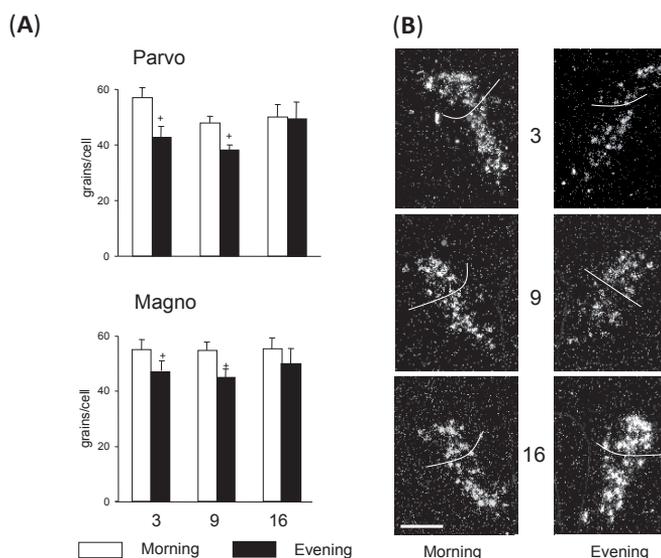


Figure 4

Expression of AVP mRNA in the magno- and parvocellular part of the paraventricular nucleus (PVN) of the hypothalamus of 3, 9 and 16 months old C57BL/6J mice, measured in the morning and evening. **(A)** mean \pm S.E.M. grains/cell. **(B)** Darkfield photomicrographs of AVP mRNA in the morning (left) and evening (right); lines indicate partition between magno- and parvocellular part of the PVN; bar = 0.1 mm. Note the strong expression of AVP mRNA in both the magno- and parvocellular PVN. P -value < 0.05 : ⁺ morning vs. evening.

No significant differences for age or time of the day were detected in any of these brain areas (data not shown).

Bodyweight

Bodyweight showed the expected age related increase ($F_{(2,49)}=205.61$, $p < 0.001$; in gram - mean \pm S.E.M: 3 months 26.3 ± 0.2 ; 9 months 31.3 ± 0.4 ; 16 months 37.6 ± 0.3 ; $p < 0.001$ between all groups).

Discussion

We have characterized several novel elements of HPA axis activity in 3, 9 and 16 months old male C57BL/6J mice during a circadian 12-12h light-dark cycle. Age associated changes notably consisted of shifts in time, amplitude and regularity of corticosterone secretion over time, and elevated ACTH levels in 16 months old mice only. Aging was also reflected in a clearly differential pattern of hippocampal MR and GR mRNA expression, with lowest GR mRNA expression present in the intermediate age group. In the PVN, no age related changes were detected for MR, GR, CRH and AVP mRNA, while MR and AVP mRNA expression showed circadian variation.

Age and circadian variations of hormones

High concentrations of cortisol and corticosterone, interpreted as hypercorticism are frequently reported in aged humans and rats, particularly when determined in blood samples collected during the active period (Meaney et al. 1992; Lupien and Wan 2004). In the present study, we found corticosterone to be highest for the 9 months old and lowest for the 16 months old mice during the light, inactive period with no differences in total AUC corticosterone over the dark, active period. However, if the time course during the active period is taken into account, the prolonged elevation of corticosterone after the peak indicates a transient relative excess of corticosterone at 16 months of age. At this age, a subsequent period of low corticosterone follows, resulting in a total daily exposure of corticosterone similar to that observed in 3 months old mice. These findings suggest that, particularly in the oldest group of mice the total daily exposure of the organism to corticosterone is tightly controlled.

Corticosterone secretion from the adrenals is stimulated by ACTH. Whereas corticosterone showed a clear circadian pattern, we were unable to detect circadian variation of ACTH during the light period. The preferred method of sampling for ACTH would be via intravenous cannulation, but even then, variations in ACTH during the light period have been reported to be marginal in rats (Atkinson and Waddell 1997; Watts et al. 2004). For practical reasons, we had to collect blood via decapitation, which also can be used to detect variations in ACTH (Bradbury et al. 1994; Watts et al. 2004). We guaranteed low basal HPA axis activation via single housing and repeated handling before the start of decapitation similar to Atkinson *et al.* (Atkinson and Waddell 1997). We previously observed that single housing is necessary to obtain low plasma ACTH measurements from decapitated mice (S. Dalm and O.C. Meijer; unpublished data). We

interpret the low variability of the ACTH levels in combination with the clear significant increase in 16 months old mice as an argument for the basal character of the ACTH levels.

The sensitivity of the adrenals to ACTH is part of the mechanism underlying the regulation of circadian corticosterone secretion (Akana et al. 1986). Adequate corticosteroid production is associated with successful aging in rats and humans (Workel et al. 2001; Lupien and Wan 2004), while excessive corticosteroids impair mental and physical health (Sapolsky 1999). In contrast to the reports on age dependent hypercorticism, numerous studies indicate an apparent reduction in the sensitivity of the adrenals to ACTH stimulation, concomitant with 'normocorticism' (Carnes et al. 1994; Van Eekelen et al. 1995; Magri et al. 1997; Workel et al. 2001). Increased morning and evening ACTH of 16 months old mice confirm those findings in humans and certain rat strains. The adrenals of 16 months old mice appear to be hyposensitive, since more ACTH is required to induce either lower or similar corticosterone secretion during the morning and evening compared to 3 months old mice. This suggests that in older animals a minimum corticosteroid level is maintained via an adaptation in the sensitivity of the adrenals towards ACTH. In contrast, at 9 months of age ACTH and corticosterone concentrations point to an adrenal hypersensitivity as was previously observed in the rat (Akana et al. 1986).

Central markers of HPA axis activity

The changes in hormone levels with age were accompanied by variations in central markers of HPA axis activity. We are aware that these mRNA markers provide only an estimate of functional changes across age and the circadian cycle without defining the physiological impact, but they do allow comparison with other studies in mouse and rat.

Like in rats (van Eekelen et al. 1991; Van Eekelen et al. 1995; De Kloet et al. 1998; Sapolsky 1999), hippocampal MR mRNA expression of mice modestly decreased with age but whereas in 9 months old mice expression was decreased by 10 - 15% both in the morning and evening, signal intensity was increased in 16 months old mice up to the level of 3 months old mice during the evening. Similar to young rats (Spencer et al. 1993; Holmes et al. 1997), MR mRNA was decreased in the dentate gyrus of 3 months old mice in the evening. In 9 months old mice, hippocampal GR mRNA expression was decreased by 20 - 30%. No circadian changes were observed. Apparently MR and GR mRNA expression in the hippocampus oscillates as a function of age, with more circadian changes at the level of MR.

Unexpectedly and in contrast to findings in rats (Cizza et al. 1995; Workel et al. 2001) the expression of MR and GR mRNA in the PVN was independent of age. Compared to the hippocampus, MR mRNA expression was lower in the PVN, but clearly detectable. It showed a pronounced circadian rhythm, with increased levels in the evening at all ages. This emphasizes that expression profiles of hippocampal corticosteroid receptor mRNA cannot be generalized to other brain structures nor are they predictive for age related alterations.

CRH is generally considered the principal neural signal controlling (stress-induced) ACTH release whereas AVP is considered to weakly stimulate ACTH release on its own but to markedly amplify the effect of CRH (Kalsbeek et al. 2002; Watts et al. 2004). Both CRH and AVP mRNA expression in the PVN did not change with age. Interestingly, AVP mRNA decreased in the evening in 3 and 9 months old mice but was constant over the day at 16 months of age, which also showed highest ACTH plasma levels. Elevated AVP has been reported in aged humans and rats (Zhou and Swaab 1999; Keck et al. 2000), but although our data may suggest elevated evening levels of AVP mRNA, ANOVA indicated no significant age effect. Novel, and in contrast to rats (Keck et al. 2000; Itoi et al. 2004), is the finding that AVP mRNA is expressed in similar amounts in both parvo- and magnocellular neurons of C57BL/6J mice. Up to now only certain conditions like adrenalectomy in rats are known to induce AVP mRNA in the parvocellular part of the PVN (Grillo et al. 1998; Itoi et al. 2004).

Corticosterone is a regulator of MR, GR (Spencer et al. 1993; Herman and Spencer 1998; Spencer et al. 1998) and CRH and AVP (Sawchenko 1987; Kovacs et al. 2000) mRNA expression. Reciprocally, MR mediates the action of corticosterone on basal HPA axis activity, while GR is mainly involved in the stress-related actions (Ratka et al. 1989; de Kloet et al. 1993b). The reduced hippocampal MR and GR mRNA, therefore, could be either consequence or (part of the) cause of the elevated and irregular secretion pattern of corticosterone in the 9 months old mice. Correlative studies in rat suggest that decreased hippocampal GR expression does not necessarily depend on elevated glucocorticoids, but might be a consequence of aging *per se* (Murphy et al. 2002).

Basal levels of CRH and AVP are under feedback inhibition by corticosterone (Ma and Aguilera 1999). The circadian rise of corticosterone might be associated with the decreased AVP mRNA of 3 and 9 months old mice in the evening. However, at 16 months of age, AVP mRNA remained high in the face of the increased evening corticosterone, together with elevated ACTH. Presumably, the elevated AVP mRNA amplifies the CRH effect on ACTH, thus subserving appropriate corticosterone production from a hyposensitive adrenal. Although the discussion of regulatory mechanisms is challenging

and could be elaborated, it remains speculative, as we did not attempt to perform experimental manipulations of the HPA axis.

Some features of the HPA axis showed remarkable changes during the life of C57BL/6J mice. However, the consequences of these changes in circadian and age dependent patterns of HPA axis activity for the regulation of the stress response and other brain functions like cognitive processes (de Kloet et al. 1999), remain to be elucidated. We and others have shown in rats that early life stress due to maternal deprivation changes HPA axis activity, stress reactivity and cognitive performance throughout life, underlining the importance of an undisturbed development of the HPA axis (Oitzl et al. 2000; Workel et al. 2001). Only if the HPA axis fails to adapt during the aging process, physiological and behavioral processes may be compromised (Everitt and Meites 1989; Meijer et al. 2005).

Concluding, measurement of the aging circadian HPA axis activity in the mouse reveals adaptations at various levels. It appears that there are oscillations in the activity of the various components of the HPA axis rather than linear progressive functional changes. Similar as has been shown in the aged rat, high ACTH was accompanied by low corticosterone secretion. We propose that the adaptive changes in adrenal sensitivity and brain corticosteroid receptor mRNA preserve homeostasis in corticosteroid exposure throughout life.

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