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Research report

Development of divergence in dopamine responsiveness in genetically selected rat lines is preceded by changes in pituitary-adrenal activity

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Accepted 20 December 1995

Abstract

Two pharmacogenetically selected Wistar rat lines have been used as a model for individual variability in behavioral and neuroendocrine responses. As a selection criterion the behavioral responsiveness for the dopamine agonist apomorphine was used, giving rise to the apomorphine-susceptible (apo-sus) and apomorphine-unsusceptible (apo-unsus) rat lines. This selection has been maintained over 16 generations. Recent studies have shown that adult rats of these selection lines also show pronounced differences in responsiveness of the hypothalamic-pituitary-adrenal (HPA) system. In this study we analyzed to what extent the divergence in dopamine phenotype and HPA responsiveness, as observed in adult rats, are linked to possible differences, within both systems, during early postnatal development. Therefore, we measured in neonatal female rats of 10 and 18 days of age several parameters of the dopamine and HPA system which show significant differences in adult rats. These include tyrosine hydroxylase (TH) and dopamine D1 and D2 receptor mRNA levels, which were determined within the nigrostriatal system since this system shows the most pronounced differences between adult rats of both selection lines. As indices of HPA activity we measured CRH mRNA, ACTH and total and free corticosterone plasma concentrations under basal conditions in the morning. Transcripts of the two types of corticosteroid receptors, mineralocorticoid (MR) and glucocorticoid (GR) receptor were measured in hippocampus and paraventricular nucleus. In 10-day-old rats all dopamine and HPA parameters were similar in rats of the two selection lines, except for GR mRNA in the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) of apo-sus rats, which was significantly higher than in apo-unsus rats. Eighteen-day-old apo-sus rats, however, showed significantly higher ACTH, comparable total corticosterone and a trend towards lower free corticosterone plasma levels. This HPA profile resembles the situation in adult apo-sus rats as compared with adult apo-unsus rats. Hippocampal GR mRNA expression and thymus weight were also higher in apo-sus rats. In addition, these rats showed an age-related increase in hippocampal MR mRNA expression, while in apo-unsus rats MR mRNA levels did not change between pnd 10 and 18. The measures of the nigrostriatal dopamine system at day 18 were still similar in rats of both lines. In conclusion, divergence in the dopamine systems of the two pharmacogenetically selected rat lines emerges subsequent to divergence in pituitary-adrenal activity.

Keywords: Ontogeny; HPA-axis; Dopamine system; Selection line; Corticosterone receptor

1. Introduction

Individuals of a normal population show a considerable variation in behavioral and endocrine responsiveness to

environmental stimuli. Several years ago the group of Cools et al. [5] selected two rat lines, representing the two behavioral extremes of a normal Wistar population, to investigate factors involved in these individual differences. Selection was accomplished using an apomorphine-induced gnawing response as a criterion, resulting in an apomorphine-susceptible (apo-sus) rat line consisting of rats with a high gnawing score, and their counterparts the apomorphine-unsusceptible (apo-unsus) rats displaying an extremely low gnawing score. A particular breeding proce-

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ture was followed to maintain the genetic heterogeneity present in the original population. Here we report data on the development of rats of the 16th generation.

Since selective breeding was based on susceptibility of the dopamine system to apomorphine we have previously quantified several parameters indicative of the activity of this system [17]. Adult apo-sus rats showed a significantly elevated tyrosine hydroxylase mRNA expression in neurons of the A9 nigrostriatal and A12 tuberoinfundibular dopamine system. In the caudate-putamen the projection area of the nigrostriatal dopamine pathway, specifically dopamine D2/D3 receptor binding was significantly higher in apo-sus rats. No significant differences were found in the A10 mesolimbic dopamine system. This implies that apomorphine susceptibility is linked to an enhanced responsiveness specifically of the nigrostriatal and tuberoinfundibular dopamine pathways [17].

Exposure to a novel environment resulted not only in a pronounced behavioral difference, i.e. a 50% higher locomotor activity [5], but also in an altered response pattern of hypothalamic-pituitary-adrenal (HPA) activity [16]. Apo-sus rats displayed a higher and prolonged increase in ACTH and free corticosterone release. This prolonged elevated activity of the HPA system appears to be the result of corticosteroid feedback resistance and/or an enhanced dopamine-dependent central drive [16]. In addition, stress-induced prolactin release, which is controlled by dopamine, was significantly attenuated in apo-sus rats. Moreover, apo-sus rats were marked by a higher level of CRH gene expression which resulted in higher ACTH but similar total corticosterone plasma levels under basal conditions. In spite of this adrenal hyporesponsiveness, apo-sus rats showed lower free corticosterone plasma levels due to elevated binding of corticosterone to corticosterone binding globulin (CBG) compared with apo-unsus rats [16].

The enhanced apomorphine susceptibility of adult male apo-sus rats have been maintained throughout many generations thus favouring a genetic basis for these differences. The goal of our study was to determine when in life, being either before birth, during early development or after the age of weaning, the divergence in HPA responsiveness becomes apparent. In other words, is this divergence linked to genetic determinants of dopamine development or is it the consequence of genetically-dependent changes in response to environmental factors during neonatal development which subsequently alter dopamine phenotype? Therefore, several parameters of the dopamine and HPA system, which appeared to be different in adult rats of both selection lines, were measured in neonatal rats. Neonatal rats of two different ages, 10 days and 18 days of age, were tested. Postnatal day (pnd) 10 is within the stress-hyporesponsive period (SHRP) [7,15,20]. Pnd 18 is close to weaning. In these neonatal rats the following parameters were measured: TH mRNA and dopamine D1 and D2 receptor mRNA levels, CRH gene expression in the par-

aventricular nucleus of the hypothalamus (PVN) and plasma ACTH and corticosterone concentrations.

We found no divergence in the dopamine systems of rats of the two selection lines around the age of weaning when the line differences in HPA activity have already developed.

2. Materials and methods

2.1. Animals

Adult male and female Wistar rats of two pharmacogenetically selected rat lines were obtained from the Psychoneuropharmacology Department of the University of Nijmegen, The Netherlands. Selection of the two rat lines was made previously on the basis of their behavioral responsiveness to the dopamine agonist apomorphine. Apo-sus rats are highly susceptible to apomorphine, while the apo-unsus rats, show only a minimal gnawing response. The selection and breeding procedures, which avoid brother-sister crossings, were described in detail by Cools et al. [5]. Retention of genetic selection was tested in rats of the first litter of each generation. Mean gnawing scores of the rats of the first litter of the 16th generation, the generation which was used in this study, were 520 ± 170 for apo-sus rats and 2 ± 0.4 for apo-unsus rats (unpublished observation).

Nine male and nine female apo-sus rats of the 15th generation, with the highest gnawing scores, were placed together to generate the fourth litter of the 16th generation. The same procedure was followed for rats of the apo-unsus lines, however, in this case rats with the lowest gnawing scores were used. After birth the number of male and female pups per litter was counted, and the whole dam was placed in a clean cage at the first day after birth. From this moment on the animals were not handled, nor were the cages cleaned until the day of testing.

Rats were housed under controlled conditions of temperature (21°C) and lighting (12 h light on, 12 h dark cycle, 7.00 a.m. lights on) with food and water available *ad libitum*.

2.2. Testing procedure

Female rats of both lines were randomly assigned to one of the experimental groups. Rats of group one (apo-sus $n = 9$, apo-unsus $n = 12$) were decapitated at pnd 10, while rats of the other group (apo-sus $n = 12$, apo-unsus $n = 15$) were decapitated at 18 days of age. Pups were weighed and subsequently decapitated within 1 minute after handling, between 9.00 a.m. and 1.00 p.m., in a room adjacent to the breeding room.

Brains were quickly dissected, frozen by immersion in isopentane of -60°C and stored at -80°C until sectioning. Trunk blood was collected in EDTA-coated tubes,

centrifuged for 10 min at $3000 \times g$ and stored at -20°C until hormone measurement. Thymus and adrenal glands were removed and weighed.

2.3. Determination of adrenocorticotropin (ACTH)

Plasma ACTH level was measured by radioimmunoassay using a commercially available ACTH antibody directed against the amino acid sequence 5–18 of the ACTH molecule (IgG Corporation, Nashville, TN, USA). The RIA was based on the method described before [21]. Plasma aliquots were chosen which resulted in values around the ED50 values of the standard curves. ACTH_(1–39) was used as a standard and [¹²⁵I]ACTH_(1–39) as tracer. Tracer and sheep antirabbit immunoglobulin G were obtained from Prof. Th. Benraad, University of Nijmegen, The Netherlands. Aliquots of plasma samples (50 μl and 25 μl) were used instead of 100 μl supernatant.

2.4. Determination of total and free corticosterone

Plasma corticosterone was measured by radioimmunoassay using an antiserum raised against corticosterone-21-hemisuccinate bovine serum albumin (Prof. Th. Benraad, University of Nijmegen, The Netherlands) as described previously [24]. Previously, the following cross-reactivities, expressed as percentage on a mass basis, were shown: desoxy-corticosterone, 28.6%; cortisone, 0.2%; cortisol, 0.8%; deoxycortisol, 0.3%; testosterone, 3.8%; progesterone, 22.5% [21]. The sensitivity of the corticosterone RIA is 0.01 $\mu\text{g}/\text{dl}$ sample.

Free corticosterone levels were determined in 200 μl pooled plasma samples (of 4–5 rats). Per group hormone levels were determined in three independent pooled plasma samples. The bound fraction was separated from free corticosterone by ultrafiltration (Beckman GPR centrifuge with a fixed angle rotor) as described previously [11]. The MPS-1 ultrafiltration device (Amicon Corp, MA, USA) contained a YMT membrane and a polyethylene cup to collect the filtrate. During centrifugation which lasted 15 min (4000 rpm ($1700 \times g$)) the temperature within the ultrafiltration device was controlled and kept at $37^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$. The supernatant ($\pm 150 \mu\text{l}$) contains the unbound free corticosterone which was measured in the radioimmunoassay described above for total corticosterone. The supernatant of each pooled sample was measured in triplicate in the RIA.

2.5. Tissue preparation

Serial coronal sections (20 μm) including the caudate putamen, the paraventricular nucleus of the hypothalamus, hippocampus and mesencephalon were cut at a cryostat according to the atlas of Paxinos and Watson [13]. Sections were mounted onto poly-L-lysine-coated slides and stored at -80°C until hybridization.

2.6. In situ hybridization

2.6.1. MR and GR cRNA probes

Different ³⁵S-labelled cRNA antisense probes were employed to hybridize with complementary brain tissue MR and GR mRNA. The antisense MR probe was transcribed from a 513 basepair (bp) rat brain cDNA fragment, which encodes for the last 30 amino acids at the C-terminus of MR plus the adjacent highly specific 3' untranslated region (courtesy of J.L. Arriza, USA). The antisense GR probe was transcribed from a 500 bp cDNA fragment (courtesy of M.C. Bohn, USA), subcloned from a 2.8 kb fragment of the rat liver GR cDNA (courtesy of K.R. Yamamoto, USA) and encoding for the N-terminal region of the GR molecule. Tissue sections were fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, for 10 min. The pre-hybridization and subsequent hybridization procedure described previously for adult brain tissue [23] was used without further modifications. Tissue sections were incubated with 90 μl hybridization mix, probe concentration of 5×10^7 cpm/ml. At a final stage, the sections were exposed for 7–14 days to a Kodak X-OMAT AR film for semiquantitation. Following this, the slides were dipped in Kodak NTB-3 emulsion (diluted 1:1 in 0.6 M ammonium acetate at 43°C) and exposed for another 2–3 weeks. The emulsion-coated sections were used to confirm the cellular localization of the hybridization signal. The control study consisted of hybridization with the receptor-specific MR and GR sense cRNA probes.

2.6.2. D1 and D2 receptor cRNA probes

RNA probes were transcribed in vitro from the full length receptor cDNA, which had been subcloned into pBluescript II KS (+) using appropriate polymerase chain reaction (PCR) primers according to the sequences published by Bunzow et al. [4] and Zhou et al. [27], PCR-amplified with the specific primers flanking the T7 and SP6 RNA polymerase promoters, gel purified and used as a template for in vitro transcription. After transcription the template was degraded with RNase-free DNase I. The [³⁵S]UTP (Amersham, specific activity > 1000 Ci/mmol) labeled antisense cRNA probes were synthesized according to the protocol provided by the manufacturer for the T7/SP6 RNA polymerases (Promega, Madison, WI) with 200 ng of PCR fragment as the template. Sense cRNA probes were used as a control for signal specificity. Preceding the hybridization step sections were fixed, treated with acetic anhydride, dehydrated and defatted as described before [10]. After overnight hybridization with the [³⁵S]UTP-labeled probe (1×10^6 cpm per slide), at 50°C , sections were washed, incubated with RNase A, air-dried and exposed to Kodak X-OMAT films for 14 days.

2.6.3. CRH and TH cDNA probes

A synthetic oligonucleotide encoding rat CRH (CRH, 48 nucleotides; 64–111) or tyrosine hydroxylase (TH, 45

nucleotides; 1442–1487) was prepared and 3' end labelled using α -[35 S]deoxyadenosine triphosphate (specific activity > 1000 Ci/mmol, Amersham) and terminal deoxynucleotidyltransferase (Boehringer Mannheim).

Sections were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min, rinsed twice in PBS and once in $4 \times$ SSC. Subsequently, the sections were placed in 0.25% acetic anhydride in 0.1 M triethanolamine (TEA) for 10 min to reduce nonspecific binding. This prehybridization procedure was completed with a rapid wash with water and dehydration in ethanol and chloroform after which the sections were air-dried. The probe was diluted in a hybridization buffer containing 50% formamide, $4 \times$ SSC, $1 \times$ Denhardt, 1% sarcosyl, 10 mM dithiothreitol, 0.1 M potassium phosphate at pH 7.4, 250 μ g/ml yeast tRNA, 250 μ g/ml herring sperm DNA and 100 μ g/ml polyadenylic acid. Hybridization buffer (90 μ l) was applied to each slide and sealed with a coverslip. For overnight incubation, which was performed at 42°C, the slides were placed in moist containers to prevent dehydration. The following day the coverslips were removed and the sections were washed at 42°C in $4 \times$ SSC containing 0.02% SDS for 30 min, twice in $1 \times$ SSC for 30 min and two times in $0.1 \times$ SSC each for 30 min. Finally, the sections were dehydrated in ethanol and air-dried before being exposed to a Kodak X-OMAT AR film for 14 days at room temperature. The films were used for quantification and distribution analysis. Control sections were treated with RNase A (40 μ g/ml, 30 min at 37°C) prior to hybridization with the probe.

2.7. Computer-assisted image analysis

Optical density was quantified with an OLYMPUS image analysis system (Paes, Nederland), equipped with a Cue CCD camera. Film background was subtracted after shading correction.

2.8. Statistics

Analysis of data was performed with the aid of the statistical package SYSTAT. Data were subjected to an

Table 1
Dopamine D1 and D2 receptor mRNA expression in lateral and medial part of the caudate-putamen of apo-sus and apo-unsus rats measured at 10 and 18 days of age

	APO-SUS		APO-UNSUS	
	D1	D2	D1	D2
<i>PND 10</i>				
Lat. c. putamen	102 \pm 4	ND	101 \pm 4	ND
Med. c. putamen	91 \pm 4	ND	83 \pm 2	ND
<i>PND 18</i>				
Lat. c. putamen	131 \pm 5	685 \pm 127	128 \pm 4	680 \pm 67
Med. C. putamen	107 \pm 5	357 \pm 81	105 \pm 4	346 \pm 44

Four rats per group and 8–10 sections per rat were measured. Data are expressed as mean \pm S.E.M. in arbitrary units of optical density

Table 2

Basal morning total and free corticosterone (μ g/dl) and ACTH (pg/ml) plasma concentrations in apo-sus and apo-unsus rats at pnd 10 (apo-sus $n = 9$, apo-unsus $n = 12$) and pnd 18 (apo-sus $n = 12$, apo-unsus $n = 15$)

	APO-SUS	APO-UNSUS
<i>PND 10</i>		
ACTH (pg/ml)	179 \pm 16	163 \pm 10
Total cort (μ g/dl)	0.15 \pm 0.04	0.14 \pm 0.03
<i>PND 18</i>		
ACTH (pg/ml)	297 \pm 36 *	199 \pm 12
Total cort (μ g/dl)	4.6 \pm 0.4	4.3 \pm 0.5
Free cort (μ g/dl)	0.34 \pm 0.05	0.48 \pm 0.06

Data are expressed as mean \pm S.E.M. apo-sus vs. apo-unsus

* $P < 0.05$.

ANOVA (factors rat-line: apo-sus, apo-unsus; age: pnd 10, pnd 18) followed by post-hoc Tukey's test. For in situ hybridization and RIAs, first the mean of the optical density of brain sections, or triplicates in RIA, were calculated per rat. These values were used to calculate the mean \pm S.E.M. of each group, n refers to the number of animals per experimental group.

3. Results

3.1. Tyrosine hydroxylase and dopamine receptor mRNA expression

Tyrosine hydroxylase (TH) mRNA levels were measured in the substantia nigra as an indication for dopamine synthetic capacity. Quantification resulted in TH mRNA levels of 823 \pm 79 and 860 \pm 43 arbitrary units of optical density in 10-day-old apo-sus and apo-unsus rats respectively. At 18 days of age TH mRNA levels of 1055 \pm 78 and 949 \pm 125 arbitrary units were measured in apo-sus and apo-unsus rats respectively. There were no significant line and/or age differences in TH mRNA content of the substantia nigra. Dopamine D1 receptor mRNA content in the nigrostriatal dopaminergic projection area, separated into lateral and medial caudate-putamen (Table 1) was significantly increased at pnd 18, comparable in both rat lines (age: D1LatC $F_{1,12} = 47.49$, $P < 0.0001$; D1 MedC $F_{1,12} = 24.84$, $P < 0.0001$; Table 1). Dopamine D2 receptor mRNA levels, determined at pnd 18 only were similar in apo-sus and apo-unsus rats.

3.2. HPA-system

3.2.1. Basal plasma hormone levels (Table 2)

Total corticosterone was extremely low at pnd 10 in both groups of rats. The age-related increase in total corticosterone (age: $F_{1,44} = 120.34$, $P < 0.0001$) was comparable in apo-sus and apo-unsus rats and resulted in similar levels in rats of both lines at pnd 18. The free fraction of corticosterone could only be measured at pnd 18, and was slightly but not statistically significant lower

in apo-sus rats (Students *t*-test *t* (df 4), $P = 0.057$). Levels of plasma ACTH were significantly related to the rat line ($F_{1,44} = 5.91$, $P < 0.01$) and age ($F_{1,44} = 16.23$, $P < 0.0001$) and showed a rat line \times age interaction ($F_{1,44} = 5.29$, $P < 0.05$). ACTH concentrations were still comparable in rats of both lines at 10 days of age. However, at 18 days plasma ACTH levels were significantly higher in apo-sus rats only (pnd 10 vs. pnd 18: $P < 0.001$) and also compared with those in apo-unsus rats ($P < 0.01$).

3.2.2. CRH mRNA

After hybridization with the CRH probe, a positive signal was only present in the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN). CRH mRNA values of the quantitative image analysis showed a significant main effect of the rat line ($F_{1,12} = 6.19$, $P < 0.05$) and age ($F_{1,12} = 31.83$, $P < 0.0001$). Apo-sus rats expressed generally higher levels of CRH mRNA. Although a 20% higher CRH mRNA level was detected in apo-sus rats at day 10 (Fig. 1) the post-hoc test did not reveal a statistically significant difference between the rat lines. At pnd 18 levels of CRH mRNA were significantly lower in apo-sus and apo-unsus rats ($P < 0.01$).

3.2.3. Distribution of corticosteroid receptors

3.2.3.1. MR mRNA. Positive hybridization of the MR antisense RNA probe was observed in the CA1–4 pyramidal cell fields and the granular cells of the dentate gyrus of dorsal hippocampus. Non-specific hybridization was assessed with the sense RNA strand which showed a homogenous background staining. The distribution pattern and quantity of MR mRNA expression in the hippocampal subfields was comparable in rats of the two lines at both ages measured (Fig. 2A).

Total hippocampal MR mRNA was significantly higher at day 18 (multivariate ANOVA–Wilks' Lambda $F_{4,13} = 10.36$, $P < 0.001$); apo-sus rats of 18 days of age showed

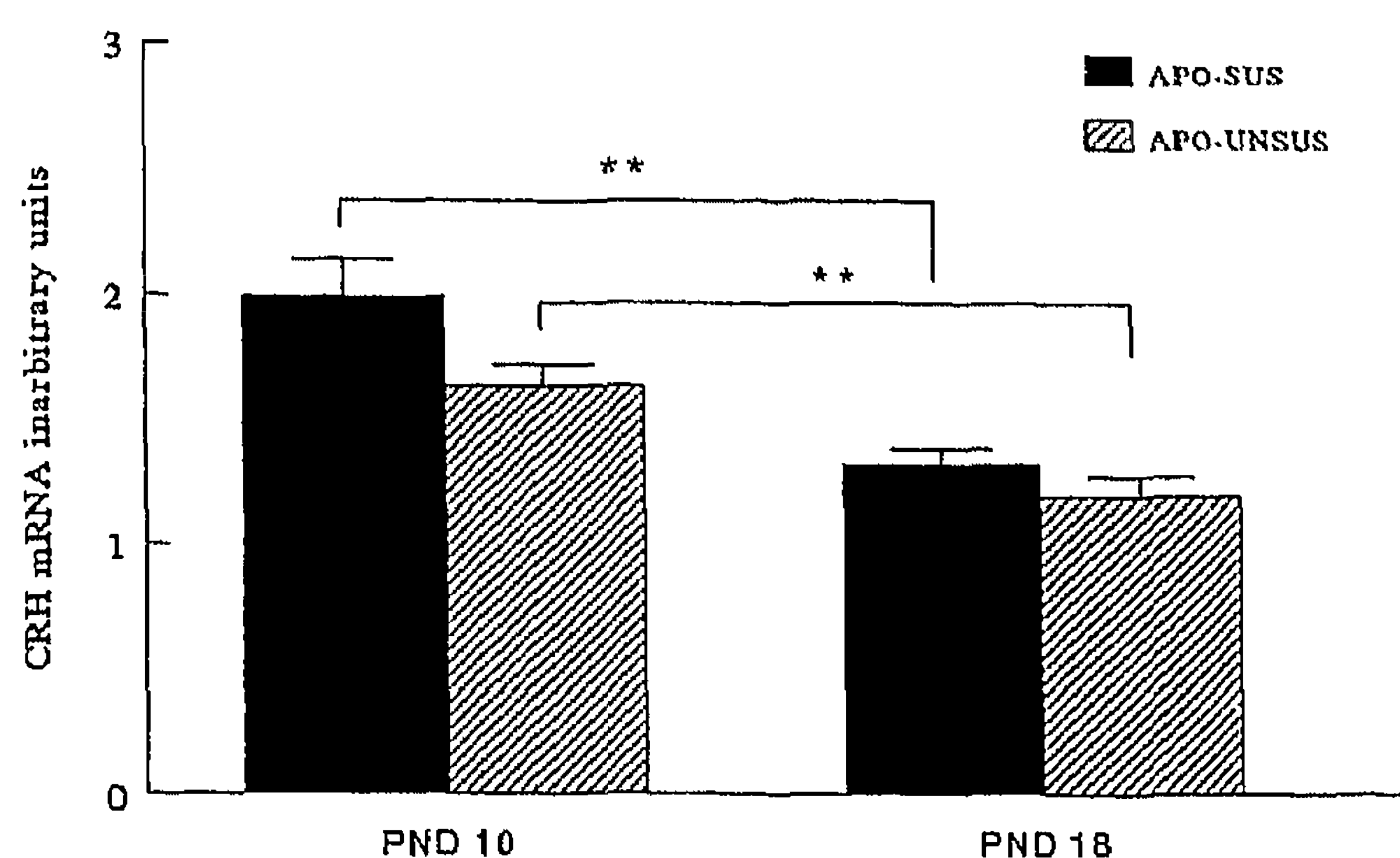


Fig. 1. Hypothalamic paraventricular CRH mRNA expression at two different ages in apo-sus (pnd 10 $n = 4$, pnd 18; $n = 3$ rats) and apo-unsus (pnd 10; $n = 4$, pnd 18; $n = 5$) rats. Five sections per rat were measured. Bars represent mean (\pm S.E.M.) in arbitrary units of optical density reflecting the amount of 35 S-labeled CRH mRNA. ** $P < 0.01$.

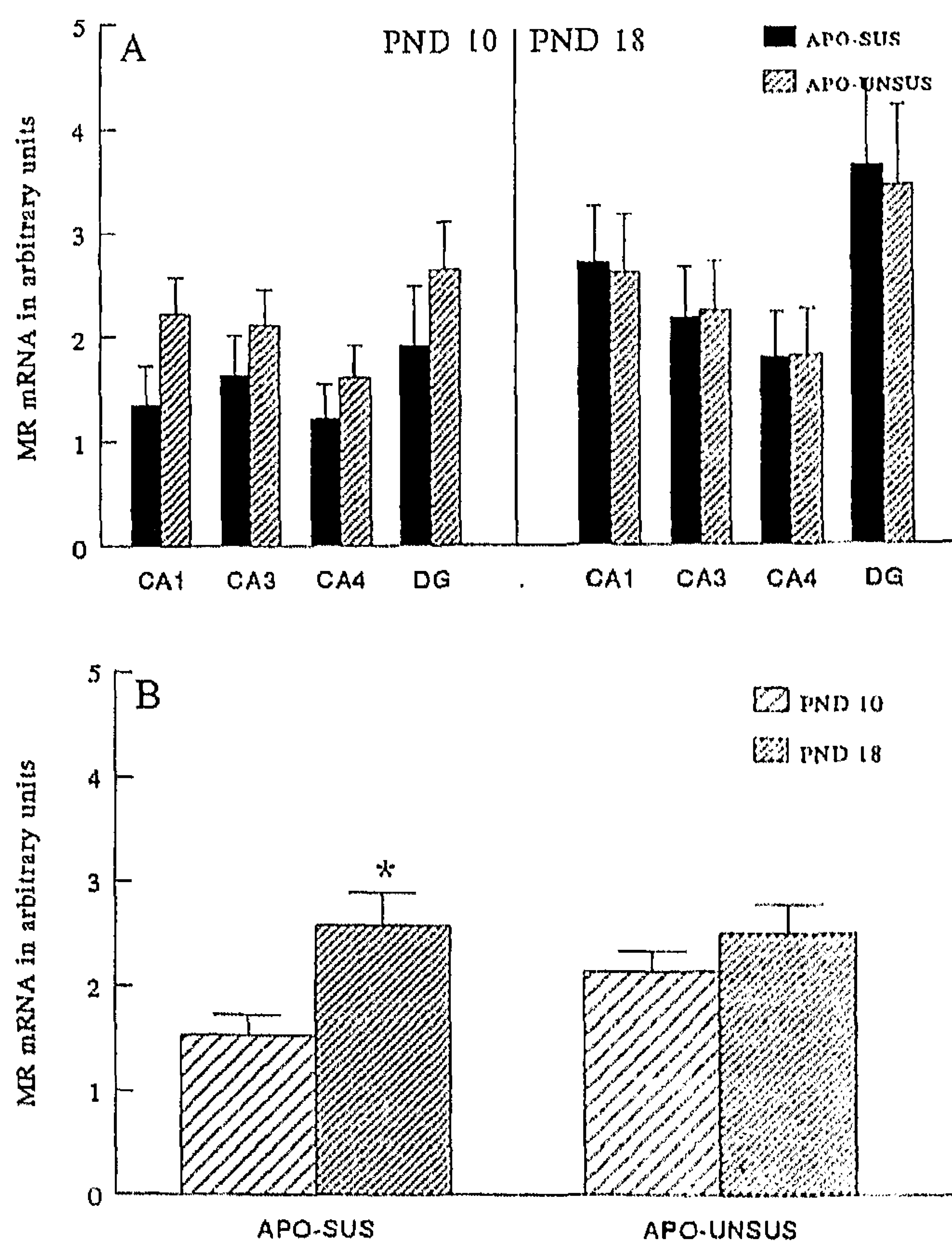


Fig. 2. MR mRNA expression in hippocampal subfields of apo-sus ($n = 5$) and apo-unsus ($n = 5$) rats measured at pnd 10 and pnd 18 (A). Influence of age on MR mRNA expression in each selection line, data were taken from the whole hippocampus (B). Each bar represents mean optical density (\pm S.E.M.) in arbitrary units reflecting the amount of 35 S-labeled MR encoding mRNA. Ten hippocampal loci were measured per rat. * $P < 0.05$ (apo-sus; pnd 10 vs. pnd 18).

an almost 2-fold increase in total MR mRNA levels compared with pnd 10 ($P < 0.05$), while apo-unsus rats showed no age-related difference (Fig. 2B).

3.2.3.2. GR mRNA. Hybridization with antisense GR probes yielded a specific signal in all hippocampal subfields (with the most intense labelling in the CA1 pyramidal cells) and the PVN (Fig. 3A). Quantitative image analysis of autoradiograms revealed a similar pattern of GR mRNA distribution over the hippocampal subfields. Independent of age, apo-sus rats displayed a higher expression of GR mRNA (significant main effects of ratline per subfield. CA3 $F_{1,16} = 6.51$, $P < 0.05$; CA4 $F = 12.42$, $P < 0.01$; DG $F = 6.62$, $P < 0.05$; PVN $F = 16.61$, $P < 0.001$); multivariate ANOVA Wilks Lambda $F_{5,12} = 6.52$, $P < 0.01$). Age significantly influenced GR mRNA (CA4 $F_{1,16} = 4.62$, $P < 0.05$; DG $F = 9.19$, $P < 0.01$, PVN $F_{5,99}$, $P < 0.05$; multivariate ANOVA Wilks' Lambda $F_{5,12} = 24.68$, $P < 0.001$). At pnd10 no statistically significant difference was found between the rat lines in any of the hippocampal subfields (Fig. 3A). However, GR mRNA expression in the PVN of apo-sus rats was significantly higher than in the same area of apo-unsus rats. At pnd 18 apo-sus rats showed a signifi-

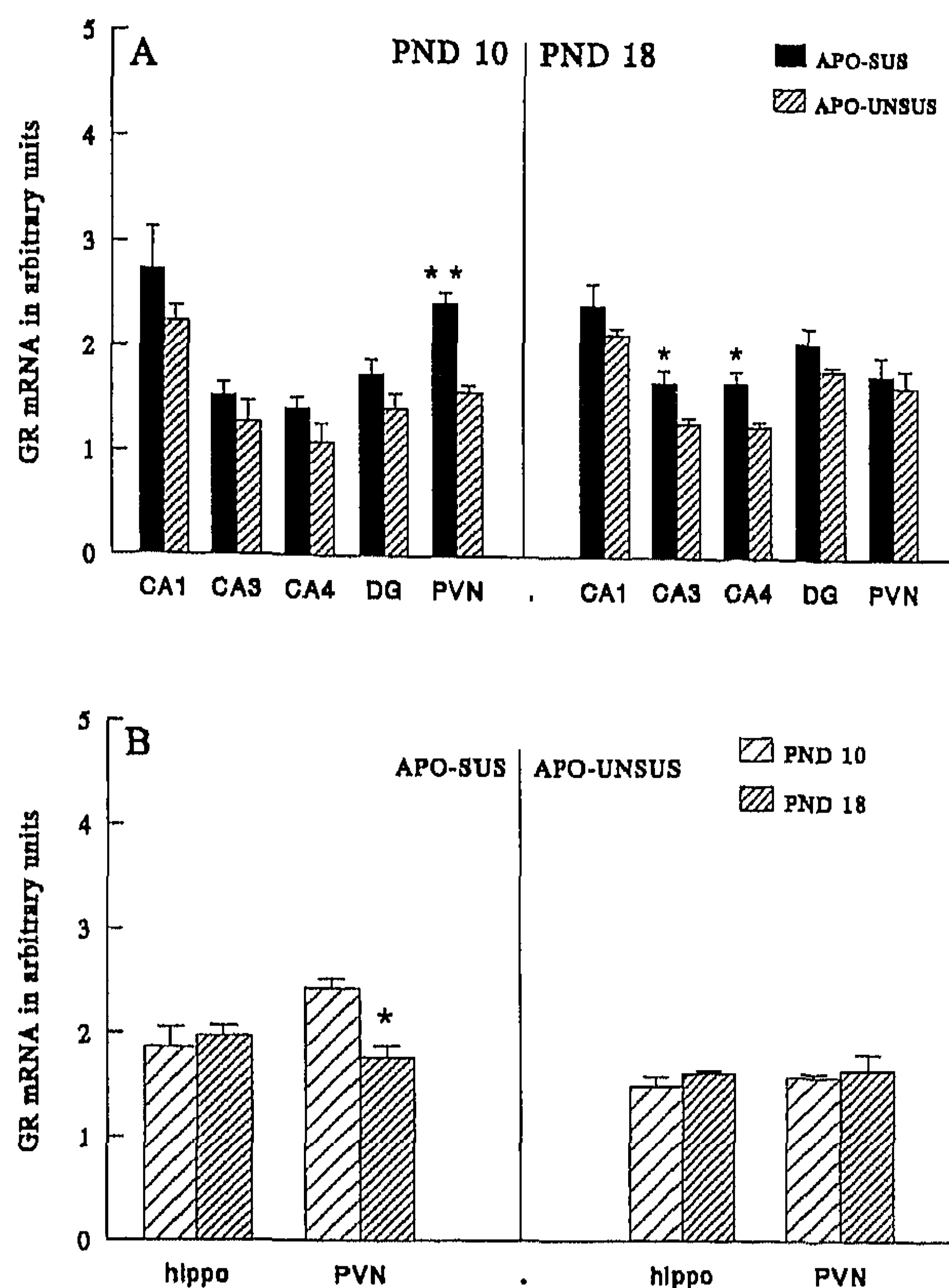


Fig. 3. Quantification of GR mRNA expression in different hippocampal subfields and the PVN at pnd 10 and pnd 18 in apo-sus ($n = 5$) and apo-unsus ($n = 5$) rats (A). Influence of age on GR mRNA expression in each selection line, data were taken from the whole hippocampus (B). Each bar represents mean optical density in arbitrary units \pm S.E.M. reflecting the amount of 35 S-labeled GR mRNA. Per rat ten hippocampal lobes were measured. * $P < 0.05$, ** $P < 0.01$ (Fig. A: apo-sus vs. apo-unsus, Fig. B: pnd 10 vs. pnd 18).

cantly higher ($P < 0.05$) GR signal in the CA3 and CA4 pyramidal cells compared with apo-unsus rats. At this age no difference was found in GR labeling intensity in the PVN of both groups. An interaction between rat-line and age was found for GR mRNA in the PVN ($F_{1,16} = 9.63$, $P < 0.01$); it was significantly lower in apo-sus rats at 18 days than at 10 days of age ($P < 0.05$; Fig. 3B).

3.3. Miscellaneous parameters

Rats of both lines showed an age-related increase in body weight ($F_{1,44} = 101.45$, $P < 0.0001$) and thymus weight (age: $F_{1,44} = 49.17$, $P < 0.0001$). Body weight as well as adrenal weight were comparable in rats of both lines at both ages tested. Apo-sus rats had higher thymus weight ($F_{1,44} = 10.28$, $P < 0.01$), which was significantly higher at pnd 18 compared with apo-unsus rats ($P < 0.01$; Table 3).

4. Discussion

The present study shows that the two rat lines selected for high or low susceptibility to the dopamine agonist

apomorphine both show the previously observed developmental rise in TH mRNA [2], but they do not differ in nigrostriatal TH mRNA, and dopamine D1 and D2 receptor mRNA expression at 10 as well as 18 days of age. This finding is of interest, since these indices of dopamine responsiveness show a profound line difference at adulthood. In a previous study we observed that 2-month-old apo-sus rats have higher levels of D1 receptor mRNA and D2 receptor binding as well as TH mRNA expression in the nigrostriatal and tubero-infundibular pathway, but not in the mesolimbic dopamine projections [17]. Apparently, the divergence in dopamine responsiveness shows some selectivity for the nigrostriatal and tuberoinfundibular systems and it develops post-weaning.

In contrast to dopamine, HPA activity already did begin to change in the third week of life. Here we show that at 18 days of age, but not at 10 days, the plasma ACTH level had become significantly higher in apo-sus rats. This initial elevation in ACTH ultimately leads to the markedly higher basal and stress-induced ACTH levels observed previously in adult rats of this line [16].

Previously, we also found that the increased ACTH levels in apo-sus rats in fact reflected hyporesponsiveness of the adrenal, since basal and stress-induced corticosterone levels are not different in adult rats of the two lines. Apparently, much more ACTH is needed to attain the same corticosterone levels in apo-sus animals [16]. In this study we found that plasma corticosterone level was very low at day 10, which is consistent with the low levels of the steroid hormone commonly measured during the SHRP [7,19]. At day 18 corticosterone plasma level was higher, but did not show a line difference. This increased ratio of ACTH over corticosterone at 18 days is, as pointed out above, a sign of adrenal hyporesponsiveness to the peptide. This suggests, that the relative adrenocortical hyporesponsiveness of apo-sus rats develops pre-weaning. These divergent HPA features seem to precede enhanced nigrostriatal dopamine responsiveness in the rat line pre-disposed to become apomorphine susceptible.

It is of interest which of the measured parameters may be causally related to the increased ACTH level in apo-sus

Table 3
Miscellaneous parameters of 10- and 18-day-old apo-sus and apo-unsus rats

	APO-SUS	APO-UNSUS
<i>PND 10</i>		
Body weight (g)	21.3 \pm 0.9	19.5 \pm 0.5
Adrenal w (mg/100 g b.wt.)	21.9 \pm 2.8	17.8 \pm 1.8
Thymus w (mg/100 g b.wt.)	378 \pm 20	346 \pm 2
<i>PND 18</i>		
Body weight (g)	38.0 \pm 2.0	35.0 \pm 1.8
Adrenal w (mg/100 g b.wt.)	22.5 \pm 1.3	21.1 \pm 1.6
Thymus w (mg/100 g b.wt.)	523 \pm 23 *	445 \pm 3

Data are expressed as mean \pm S.E.M.: apo-sus pnd 10 ($n = 9$), pnd 18 ($n = 12$); apo-unsus pnd 10 ($n = 12$), pnd 18 ($n = 15$)

* $P < 0.01$ apo-sus vs. apo-unsus.

rats at day 18. The first relevant parameter is CRH mRNA. Expression levels were not different between the lines at day 10 and 18, although there is a trend towards higher CRH mRNA in apo-sus rats, which we found previously to reach significance at adulthood [16]. Naturally, line differences in translation efficiency and pituitary responsiveness to CRH [9,14] may occur. Differential developmental patterns of other ACTH secretagogues, such as vasopressin [8,12,18], also cannot be excluded. Alternatively, increased ACTH may result from a diminished corticosterone feedback signal. Adult apo-sus rats have indeed a reduced fraction of free corticosterone level under basal conditions [16], and at day 18 the free steroid level already tended to be lower in apo-sus rats. Furthermore, the thymus weight of the apo-sus rats was significantly higher at 18 days as is the case at adulthood [6,16]. It is known that small increases in biologically active corticosterone readily cause thymus involution. Accordingly, this may imply that already at day 18 resistance to corticosteroid action is developing as a prominent feature of the adult apo-sus animals.

Corticosteroid feedback depends on central drive, plasma levels of free corticosterone, and on the functioning of brain corticosteroid receptors. Previous developmental studies have shown that MR and GR gene transcripts are detectable in brain during neonatal life. Their level remains relatively constant between post-natal days 5 and 15, subsequently higher adult levels are reached at day 30 [3,22]. The present study is generally in support of this age-dependent change. However, in some discrete brain regions critical for neuroendocrine regulation, the developmental receptor mRNA pattern between the two rat lines is different. At day 10 GR mRNA is elevated in the PVN of apo-sus rats followed by a relative decrease as compared to the apo-unsus animals; at day 18 apo-sus GR mRNA is higher in hippocampal CA3 and CA4. Also apo-sus MR mRNA shows a more pronounced developmental rise in hippocampus, perhaps as a preliminary sign for the increased MR binding capacity observed in hippocampus of adult apo-sus rats. Or alternatively, hippocampal MR mRNA level of apo-unsus rats does not increase, resulting in a lower receptor level than would be expected according to the normal developmental pattern. The findings show that potentially there may be differences in corticosteroid receptor function, but it is also obvious that further experiments are needed to substantiate deficient receptor functioning as the basis for corticosteroid feedback resistance of apo-sus animals.

Sex differences in MR and GR as well as TH mRNA expression only start to occur after post-natal day 25 [2,3]. Moreover, there are no sex differences between apo-sus and apo-unsus rats in the gnawing response [5]. Therefore, it is likely that the present study with these female pups is representative for similar developmental changes in male offspring.

The present developmental data of apomorphine selected lines are comparable with data obtained with two rat

lines selected from Wistar stock on the basis of behavioral performance, i.e. the Roman High Avoiders (RHA) and Roman Low Avoiders (RLA). Adult RHA rats showed, similarly to apo-sus rats, a higher ACTH level relative to corticosterone, if compared to their RLA and apo-unsus counterparts, respectively [25,26]. Also in the psychogenetically selected rat lines the expression of differential basal ACTH release appeared around weaning [1,23], preceding the time that differences in behavioral traits, on which the selection of these lines is based, become detectable. Moreover, divergence in apomorphine-induced gnawing behaviour was only present in RHA and RLA rats after 60 days of age (Cools et al., unpublished observation). These findings indicate that the RHA/RLA as well as the apo-sus/unsus lines show delayed divergence in their behavioral and apomorphine selection characteristics which occurs subsequent to development of HPA differences.

In conclusion, the present study shows that divergence in HPA characteristics of apo-sus and apo-unsus lines becomes detectable at 18 days of age and precedes development of divergence in nigrostriatal dopamine system activity.

Acknowledgements

We thank Prof. Th. Benraad and Dr. L. Swinkels, University of Nijmegen, The Netherlands for the supply of the corticosterone antiserum, and sheep antirabbit IgG. The MR clone was a gift from Drs. J.L. Arriza and R.M. Evans (The Salk Institute for Biological Studies, La Jolla, CA). The 2.8 Kb GR clone was provided by Drs. K.R. Yamamoto, R. Miesfield and P.J. Godowski (University of California, San Francisco, CA). We are grateful to Dr. M.C. Bohn, University of Rochester, NY, for providing the 500 bp GR subclone. This study was supported by NWO Grant 900-564-025, the EU Biomed-1 programme BMHI-CT 94-1108 and an NWO-INSERM-sponsored collaborative programme.

References

- [1] Aksentijevich, S., Whitfield, H.J.J., Young III, W.S., Wilder, R.L., Chrousos, G.P., Gold, P.W. and Sternberg, E.M., Arthritis-susceptible Lewis rats fail to emerge from the stress hyporesponsive period. *Dev. Brain Res.*, 65 (1992) 115–118.
- [2] Arbogast, L.A. and Voogt, J.L., Ontogeny of tyrosine hydroxylase mRNA signal levels in central dopaminergic neurons: development of a gender difference in the arcuate nuclei. *Dev. Brain Res.*, 63 (1991) 151–161.
- [3] Bohn, M.C., Dean, D., Hussain, S. and Giuliano, R., Development of mRNAs for glucocorticoid and mineralocorticoid receptors in rat hippocampus. *Dev. Brain Res.*, 77 (1994) 157–162.
- [4] Bunzow, J.R., Van Tol, H.H.M., Grandy, D.K., Albert, P., Salon, J., MacDonald, C., Machida, C.A., Neve, K.A. and Civelli, O., Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature*, 336 (1988) 783–787.

- [5] Cools, A.R., Brachten, R., Heeren, D., Willemsen, A. and Ellenbroek, B., Search after neurobiological profile of individual-specific features of wistar rats, *Brain Res. Bull.*, 24 (1990) 49–69.
- [6] Cools, A.R., Rots, N.Y., Ellenbroek, B. and De Kloet, E.R., Bimodal shape of individual variation in behaviour of wistar rats: the overall outcome of a fundamentally different make-up and reactivity of the brain, the endocrinological and the immunological system, *Neuropsychobiology*, 28 (1993) 100–105.
- [7] De Kloet, E.R., Rosenfeld, P., Van Eekelen, J.A.M., Sutanto, W. and Levine, S., Stress, glucocorticoids and development. In G.J. Boer, M.G.P. Feenstra, M. Mirmira and D.F. Swaab (Eds.) *Progress in Brain Research*, Elsevier Science Publishers B.V., Amsterdam, 1988, pp. 101–120.
- [8] Grino, M. and Burgunder, J., Ontogeny of expression and glucocorticoid regulation of the arginine vasopressin gene in the rat hypothalamic paraventricular nucleus, *J. Neuroendocrinol.*, 4 (1992) 71–77.
- [9] Hoffman, M., Anything goes at the cell biology meeting, *Science*, 255 (1991) 34–35.
- [10] Jongen-Relo, A.L., Docter, G.J., Jonker, A.J., Vreugdenhil, E., Groenewegen, H.J. and Voorn, P., Differential effects of dopamine depletion on the binding and mRNA levels of dopamine receptors in the shell and core of the rat nucleus accumbens, *Mol. Brain Res.*, 25 (1994) 333–343.
- [11] Lentjes, E.G.W.H., Romijn, F., Maassen, R.J., De Graaf, L., Gautier, P. and Moolenaar, A.J., A temperature controlled serum free cortisol assay using the amicon MPS-1 ultrafiltration apparatus and the Abbott TDX, *Clin. Chem.*, 39 (1993) 2518–2521.
- [12] Paulmyer-Lacroix, O., Anglade, G. and Grino, M., Stress regulates differently the arginine vasopressin (AVP)-containing and the AVP-deficient corticotropin-releasing factor-synthesizing cell bodies in the hypothalamic paraventricular nucleus of the developing rat, *Endocrine*, 2 (1994) 1037–1043.
- [13] Paxinos, G. and Watson, C., *The Rat Brain in Stereotaxic Coordinates*, Harcourt Brace Jovanovich Publishers, San Diego, 1986, pp. 1–119.
- [14] Pihoker, C., Cain, S.T. and Nemeroff, C.B., Postnatal development of regional binding of corticotropin-releasing factor and adenylate cyclase activity in the rat brain, *Prog. Neuro-Psychopharmacol. & Biol. Psychiatry*, 16 (1992) 581–586.
- [15] Rosenfeld, P., Suchecki, D. and Levine, S., Multifactorial regulation of the hypothalamic-pituitary-adrenal axis during development, *Neurosci. Biobehav. Rev.*, 16 (1992) 553–568.
- [16] Rots, N.Y., Cools, A.R., De Jong, J. and De Kloet, E.R., Corticosteroid feedback resistance in rats genetically selected for increased dopamine responsiveness, *J. Neuroendocrinol.*, 7 (1995) 153–161.
- [17] Rots, N.Y., Cools, A.R., Béroud, A., Voorn, P., Rostène, W. and De Kloet, E.R., Rats bred for enhanced apomorphine susceptibility have elevated tyrosine hydroxylase mRNA and dopamine D2-receptor binding sites in nigrostriatal and tuberoinfundibular dopamine systems, *Brain Res.*, in press.
- [18] Rundle, S.E. and Funder, J.W., Ontogeny of Corticotropin-releasing factor and arginine vasopressin in the rat, *Neuroendocrinology*, 47 (1988) 374–378.
- [19] Sapolsky, R.M. and Meaney, M.J., Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period, *Brain Res. Rev.*, 11 (1986) 65–76.
- [20] Schoenfeld, N.M., Leatham, J.H. and Rabii, J., Maturation of adrenal stress responsiveness in the rat, *Neuroendocrinology*, 31 (1980) 101–105.
- [21] Sweep, C.G.J., Van der Meer, M.J.M., Hermus, A.R.M.M., Smals, A.G.H., Van der Meer, J.W.M., Pesman, G.J., Willemsen, S.J., Benraad, T.J. and Kloppenborg, P.W.C., Chronic stimulation of the pituitary–adrenal axis in rats by interleukine-1 infusion: in vivo and in vitro studies, *Endocrinology*, 130 (1992) 1153–1164.
- [22] Van Eekelen, J.A.M., Bohn, M.C. and De Kloet, E.R., Postnatal ontogeny of mineralocorticoid and glucocorticoid receptor gene expression in regions of the rat tel- and diencephalon, *Dev. Brain Res.*, 61 (1991) 33–43.
- [23] Van Eekelen, J.A.M., Jiang, W., De Kloet, E.R. and Bohn, M.C., Distribution of the mineralocorticoid and the glucocorticoid receptor mRNAs in the rat hippocampus, *J. Neurosci. Res.*, 21 (1988) 88–94.
- [24] Veldhuis, H.D., Van Koppen, C., Van Ittersum, M. and De Kloet, E.R., Specificity of adrenal steroid receptor system in the rat hippocampus, *Endocrinology*, 110 (1982) 2044–2051.
- [25] Walker, C.D., Aubert M.L., Meaney, M.J. and Driscoll, P., Individual differences in the activity of the hypothalamus-pituitary-adrenocortical system after stressors: use of psychogenetically selected rat lines as a model. In P. Driscoll (Ed.), *Neurobehavioral Dysfunctions*, Birkhauser, Basel, 1994, pp. 276–296.
- [26] Walker, C.-D., Rivest, R.W., Meaney, M.J. and Aubert, M.L., Differential activation of the pituitary-adrenocortical axis after stress in the rat: use of two genetically selected lines (Roman low- and high-avoidance rats) as a model, *J. Endocrinol.*, 123 (1989) 477–485.
- [27] Zhou, Q., Grandy, D.K., Thambi, L., Kushner, J.A., Van Tol, H.H.M., Cone, R., Pribnow, D., Salon, J., Bunzow, J.R. and Civelli, O., Cloning and expression of human and rat D1 dopamine receptors, *Nature*, 347 (1990) 76–80.