Early life experience : neuroendocrine adaptations to maternal absence

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Plasma corticosterone responses reflect the degree of novelty in male and female CD1 mice

Appendix I

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The studies described in this appendix provide methodological considerations for blood sampling via tail incision in mice, a technique that was also used for the determination of the corticosterone responses to swim stress in maternally deprived and control mice described in ***Chapter 4***.

I.1 Abstract

The hypothalamic-pituitary-adrenal axis response to novelty stress is dependent on the type and duration of a certain challenge. Here, we show in CD1 mice that corticosterone secretion increased reflecting the "degree of novelty" (home cage < 5 minutes in cylinder < 60 minutes in cylinder < 1 minute forced swim). The magnitude of the corticosterone response already differed 15 minutes after onset of the stressor, with the more severe challenge causing a faster initiation of the glucocorticoid-related stress response. Furthermore, female mice secreted more corticosterone than male mice under basal conditions and showed a faster and higher increase in response to each novelty.

I.2 Introduction

A variety of physiological [1, 11, 13] and emotional [9, 10, 12, 15] stimuli result in different behavioural coping responses [1, 16, 21]. Any challenge, either real or imagined, evokes a glucocorticoid-related stress response, which serves to restore homeostasis and facilitate behavioural adaptation [17]. Some challenges, however, elicit stronger corticosterone responses than others [6, 10, 12, 13]. In our lab, we generally place rats or mice in a novel environment and estimate plasma corticosterone concentrations at several time points during this novelty exposure [7, 8, 18, 24, 25]. The aim of the present study was to define novelty conditions characterised by differential corticosterone secretion. In future experiments, these should allow to choose the challenge according to the hypothesised change in corticosterone secretion, thus avoiding ceiling effects.

Here, we varied both the kind and duration of novelty. Male and female CD1 mice were placed into a cylinder for 5 or 60 minutes, swam in a large pool with warm water for 1 minute or remained in their home cage. Blood samples were taken sequentially 15, 30 and 60 minutes after onset of novelty, *i.e.* in the proactive phase of the glucocorticoid-related stress response [2, 3]. Depending on the challenge we expected a different amplitude or slope of the corticosterone response and overall higher corticosterone concentrations in female than in male mice.

I.3 Materials and Methods

I.3.1 Animals

Male and female CD1 mice (6 months of age) were housed four per cage in same sex groups with food and water *ad libitum* under a fixed 12 hours light/dark cycle (lights on at 07:00 hours) at

Table I.1: Overview of the challenges with their respective sampling time points

		sample time point (minutes)				n =	
		0	15	30	60	male	female
Basal:		x				20	20
Novel environment:	5'CYL	-	x	x	x	16	16
	60'CYL	-	x	x	x	16	16
	SWIM	-	x	x	x	20	20
Control:	RSC	-	x	x	x	16	16
	RHC	-	-	-	x		13

In this experiment animals were divided over different treatment groups: *Basal*; *Novel environment:* 5'CYL = 5 minutes exposure to a cylinder, thereafter replacement in home cage; 60'CYL = 60 minutes exposure to a cylinder; SWIM = 1 minute forced swimming, thereafter placement under a heating light for 3 minutes and replacement in home cage; *Control:* RSC = repeated sampling control; RHC = repeated handling control. All treatments started at *t*=0 minutes and all handling procedures, with the exception of actual blood sampling, were equal in groups 5'CYL, 60'CYL, SWIM, RSC and RHC. (" x " indicates a blood sample taken, " - " indicates no blood sample taken.)

the animal facilities of the Sylvius Laboratory at Leiden University, The Netherlands. Testing was between 09:00-13:00 hours. Mice were singly housed one week before the experiment started. Experiments were approved by the Local Committee of Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

I.3.2 Evaluation of novelty intensity

The treatment groups are summarised in ***Table I.1***. *Basal:* To determine basal morning corticosterone concentrations, blood samples were collected from male and female mice within two hours after lights on (*n*=20 per sex). *Novel environment:* Mice (*n*=16 per sex and condition) were placed in an upright cylinder (gray PVC; 17 cm high, 9.5 cm in diameter) for 5 minutes before they were returned to their home cage or for 60 minutes (groups 5'CYL and 60'CYL, respectively). Another group of mice (*n*=20 per sex) was placed in the middle of a pool with warm water (140 cm in diameter; 26±1°C) and had to swim for 1 minute (group SWIM). Thereafter, the mouse was placed in a clean cage with tissue bedding under a heating lamp for 3 minutes and returned to its home cage. In all conditions, of each mouse three sequential blood samples were taken 15, 30 and 60 minutes after the start of the challenge. After each blood sample, mice returned to their home cage (5'CYL, SWIM) or cylinder (60'CYL). *Control:* To control for the effects of repeated blood sampling we used two home cage control groups. Repeated sampling control mice (group RSC, *n*=16 per sex) were removed shortly from their home cage, sequential blood samples were taken at *t*=15, 30 and 60 minutes, and after sampling mice returned to their home cage to mimic the handling effects involved for the introduction to a novel environment. In the repeated home cage control group (group RHC, *n*=13 females), we performed the same handling procedures, but without actual tail incisions at *t*=15 and 30 minutes. Only at *t*=60 minutes a blood sample was collected.

I.3.3. Blood collection and corticosterone determination

Blood samples were collected by tail incision, a method that allows estimation of basal concentrations of corticosterone and multiple samples from the same animal [4, 5]. Briefly, the mouse is placed on the grid top of its home cage. A small incision at the base of the tail with a razor blade allows collection of 50-100 µl blood within 10-15 seconds, without anesthesia.

Blood was collected in 100 µl capillaries, coated with potassium-EDTA, and centrifuged for 10 minutes at 13000 rpm at 4°C. Plasma was separated from the pellet and stored at -20°C. Corticosterone was measured with a ^{125}I-corticosterone radio immunoassay (RIA) kit (ICN Pharmaceuticals Inc., New York, U.S.A.).

I.3.4 Data analysis

Data are presented as mean ± S.E.M. Plasma corticosterone concentrations were analysed by ANOVA (factor: sex=2) when appropriate with repeated measures (at 15, 30 and 60 minutes; factors: SEX=2; CONDITION=4: groups RSC, 5'CYL, 60'CYL, SWIM) and by LSD *post hoc* test. Statistical significance was accepted at $P<0.05$.

I.4 Results

Basal corticosterone concentrations were low in both male and female CD1 mice, and as expected significantly higher in female than in male mice ($F(1,38)17.10$, $P<0.001$: male 12.2 ± 1.7 ng/ml; female 33.4 ± 4.8 ng/ml).

Male mice secreted less corticosterone than female mice in response to each challenge ($F(1,128)185.06$, $P<0.001$; ***Figure I.1***). The magnitude of the corticosterone secretion depended on the challenge ($F(3,128)10.29$, $P<0.001$). Corticosterone concentrations increased with time ($F(2,256)274.58$, $P<0.001$), showed a different course in male and female mice (interaction time x sex: $F(2,256)11.45$, $P<0.001$), depending on the experimental condition (interaction time x sex x condition: $F(6,256)3.79$, $P=0.001$). The highest corticosterone concentration was observed for the SWIM condition and the lowest concentration for the home cage control (group RSC).

I.4.1 Male mice

At $t=15$ minutes, corticosterone secretion was significantly increased in relation to the degree of novelty: lowest in the home cage control (RSC: 30.8 ± 7.5 ng/ml; vs. 5'CYL, 60'CYL or SWIM $P<0.001$), followed by mice exposed for 5 minutes to the cylinder (5'CYL: 61.2 ± 6.7; vs. 60'CYL or SWIM $P<0.05$) and mice continuously exposed to the cylinder (60'CYL: 121.2 ± 15.0 ng/ml), with the latter similar to the SWIM group (106.4 ± 17.5 ng/ml). At $t=30$ minutes, corticosterone concentrations were further increased: equally high in groups RSC and 5'CYL, and both significantly lower than 60'CYL and SWIM ($P<0.01$). At $t=60$ minutes, corticosterone concentrations were again further increased and differed between all groups ($P<0.001$ to $P<0.05$), with highest concentrations in the SWIM group.

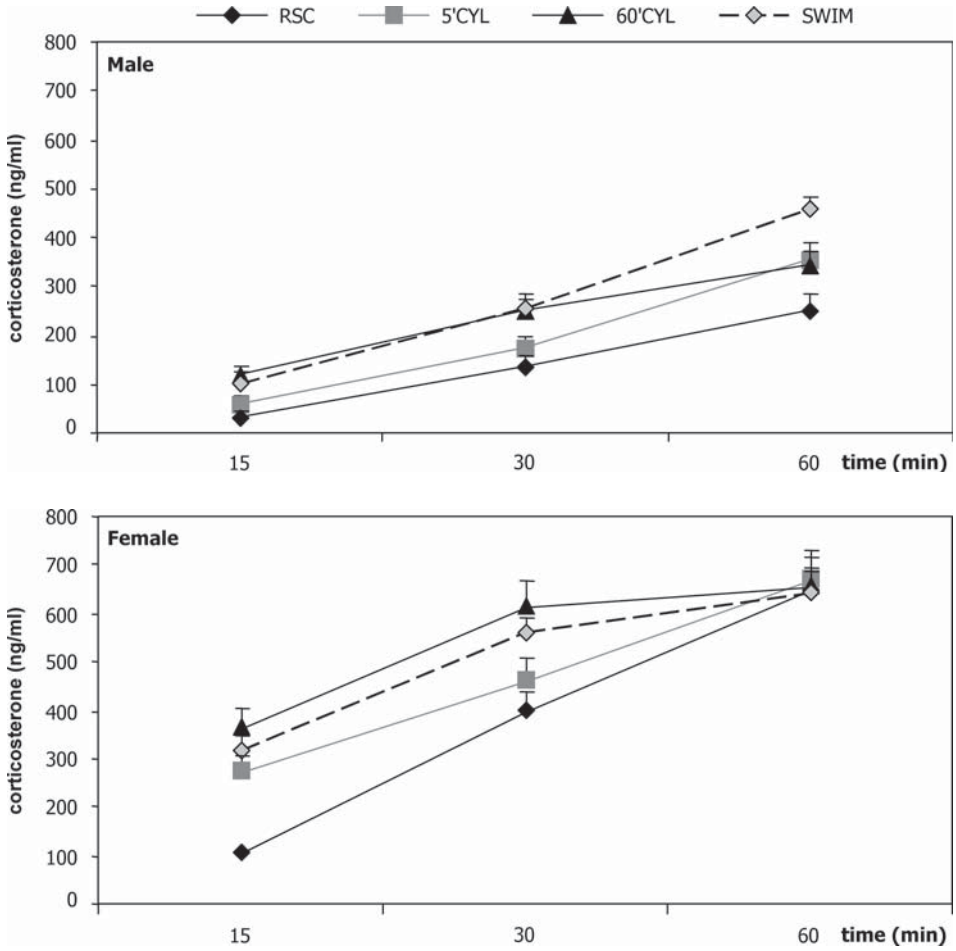

Figure I.1
Novelty-induced corticosterone response for male and female CD1 mice, measured 15, 30 and 60 minutes after initiation of each stressor. For abbreviations see ***Table I.1***. Values are presented as mean ± S.E.M.

I.4.2 Female mice

At *t*=15 minutes, corticosterone was significantly lower in group RSC (101.1 ± 9.2 ng/ml; *P*<0.001) than in groups 5'CYL (272.6 ± 35.1 ng/ml), 60'CYL (364.1 ± 41.7 ng/ml) and SWIM (313.2 ± 33.8 ng/ml). At *t*=30 minutes, corticosterone further increased, but was still lowest in RSC (*P*<0.01) compared to 60'CYL and SWIM. At *t*=15 and 30 minutes, corticosterone of group 60'CYL was significantly higher than of group 5'CYL (*P*<0.05), but similar to group SWIM. At *t*=60 minutes, corticosterone was further increased, but showed the same concentrations in all four groups.

Mimicking the blood sampling procedure without actual tail incision at *t*=15 and 30 minutes, corticosterone concentrations measured in female mice RHC group at *t*=60 minutes (35.5 ± 6.6 ng/ml) were similar to basal conditions.

I.5 Discussion

We differentiated between the "degree of novelty" of a challenge and gender by analysing the plasma corticosterone concentrations in the proactive phase of the glucocorticoid-related stress response [2, 3]. The duration of exposure to the same challenge (*i.e.* 5'CYL versus 60'CYL) affected

the slope of the stress response as corticosterone secretion was initiated faster. This initial faster secretion of corticosterone resulted in more secreted corticosterone after 60 minutes, though ceiling effects were already observed at 60 minutes. One minute forced swimming (group SWIM), followed by three minutes under a heating lamp before returning to the home cage resulted in a similar response as group 60'CYL, but with a higher amplitude in male mice.

Besides challenge effects, female mice also responded differently than males to the challenges. Female mice showed higher corticosterone levels, basal and in response to each of the challenges. Furthermore, female mice seem to reach a plateau of corticosterone secretion where males do not. These observations are consistent with literature, showing that estrogens increase corticosteroid-binding globulin values resulting in higher total plasma corticosterone concentrations [3, 14, 19]. Irrespective of gender, the main factor of group differences indicating the degree of novelty (RSC < 5'CYL < 60'CYL < SWIM) was determined by the shape of the stress response in the proactive phase.

Marquez *et al.* [13] showed in rats that the recovery speed of the stress system (*i.e.* reactive phase) differs after administration of either foot shock or immobilisation. They too found a maximal corticosterone response 60 minutes after initiation of both challenges, but were unable to distinguish between the two stressors in the proactive phase. Combined with our observations these experiments indicate that, despite ceiling effects, different stimuli elicit different proactive and reactive stress responses. Furthermore, we suggest that both proactive and reactive aspects of the stress response may reflect the intensity of a given challenge [6, 10, 12, 13].

It was striking and unexpected to notice that even the repeatedly sampled control group (group RSC) elicited a stress response, though animals returned to their home cages. Rats responded differently to repeated sampling via tail incision, as was shown by Fluttert *et al.* [5]. In their study frequent sampling of 300 µl of blood from handled Wistar rats with an interval of 20-30 minutes only slightly elevated corticosterone levels. Blood samplig via tail incision was therefore considered not to be harmful to the animal [4, 5]. It is possible that the response we observed was not due to the procedure of tail incision itself, but to the frequency of blood sampling. Mice have a much smaller blood volume than rats and we estimated that our mice lost up to 20 % of their total blood volume after three consecutive samples within a time span of 60 minutes. Activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to occur at approximately 12% plasma volume deficit [20, 22, 23]. To assess this possibility a naive group of female mice received the same handling procedures as group RSC, including a virtual sampling using the blunt side of the razor blade, but without actual incision and blood loss at 15 and 30 minutes (group RHC; we expect the same results in males). Only at 60 minutes a blood sample was taken. These handling procedures by itself did not affect circulating corticosterone concentrations. Consequently, it is most likely that dehydration might explain the rise in corticosterone observed in group RSC. Each of the challenges still elicited a faster and higher corticosterone response than group RSC, therefore effects of different challenges on the HPA axis responsiveness were still detectable using repeated sampling. However, considerations have to be made when designing an experiment on

the number of consecutive tail samples and the time frame in which these samples are taken.

In conclusion, this survey demonstrates that different challenges or varying exposure times to the same challenge causes different reaction patterns in both male and female CD1 mice. These effects were observed in the proactive phase of the corticosterone stress response and indicate plasticity for coping with a novel environment.

I.6 Acknowledgements

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