Early life experience : neuroendocrine adaptations to maternal absence

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The role of brain corticosteroid receptors in HPA axis adaptation to repeated maternal separations of newborn mice

Chapter 3

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3.1 Abstract

In previous studies we observed that the CD1 mouse infant's hypothalamic-pituitary-adrenal (HPA) axis readily desensitises to repeated daily 8 hours separations from the dam, but remains responsive to a novelty stressor. The objective of the current study was to examine if this neuroendocrine desensitisation to maternal absence was due to enhanced glucocorticoid feedback. For this purpose the effect of a mineralocorticoid (MR) or glucocorticoid (GR) receptor antagonist was measured on circulating corticosterone levels.

We obtained the following results: (1) The GR antagonist mifepristone amplified at postnatal day (pnd) 5 the infant's corticosterone response to the first maternal separation, but became ineffective if the pups were exposed the preceding days to repeated maternal absence, the procedure that induced desensitisation of the HPA axis. (2) The GR antagonist caused generally a small decrease rather than an increase of basal circulating corticosterone levels during the stress hypo-responsive period at pnds 5 and 8. However, upon a subtle HPA activation achieved by a mild immune challenge, the GR antagonist became active in interfering with glucocorticoid feedback and triggered a profound corticosterone increase. (3) Blockade of the MR by spironolactone resulted in small, but significant increases in circulating corticosterone levels under basal conditions 8 and 24 hours after injection. This MR antagonist-induced increase in corticosterone was also observed after the third separation, but not if animals were separated for the first time. Then, corticosterone actually decreased. (4) During novelty a brief exposure to the MR antagonist enhanced the corticosterone response in the single and repeatedly separated pups, while GR antagonism under those short term conditions was not effective.

In conclusion, the findings exclude an enhanced glucocorticoid feedback as a mechanism underlying the desensitisation of the HPA axis in response to repeated maternal separations and support the role of the GR in maintenance of the stress hypo-responsive period (SHRP). The results also indicate operation of an MR-responsive mechanism during the SHRP that mostly restrains HPA activity under basal, stressful and maternal separation conditions.

3.2 Introduction

Mice have a stress hypo-responsive period (SHRP from postnatal day (pnd) 1-12 [30]), which is characterised by stable low circulating levels of corticosterone. This implies that mild stressors, which trigger a profound ACTH and corticosterone response in adults, do so only weakly in the newborn animal. However, certain stimuli as interleukin-1, an important mediator of the inflammatory response to infection, are able to elicit an ACTH and corticosterone response during the SHRP [9, 11]. Upon separation of the pups from their mother the HPA axis emerges from the SHRP, indicating that the hypo-responsiveness is (partially) dependent on the presence of the mother [5, 12]. In response to this separation, basal levels of ACTH and corticosterone are increased and the HPA axis becomes responsive to mild stressors, which now can trigger a burst

of ACTH and corticosterone release [24, 31, 34].

Repeated daily separations of mother and pups during the SHRP is an established model to study the long-term consequences of early adversity, which induces at adulthood increased HPA axis responsiveness and behavioural fearfulness, at least in a subgroup of animals [10, 13, 16, 20, 22, 23, 27, 40]. The mechanism underlying these lasting effects on brain and behaviour is thought to be triggered by presumed daily activations of the HPA axis induced by repeated maternal separations [15]. However, we recently showed that pups readily adapt to the repeated absence of the dam, since the HPA response to maternal absence already desensitises at the second separation and the response is virtually absent after the third separation period. However, the increased responsiveness to novelty stress is maintained and even enhanced after repeated maternal separation, indicating that the reactivity of the stress system has remained on alert. The desensitisation of the HPA axis response to repeated maternal absence and the response to novelty is reflected in *c-fos* mRNA expression pattern in the paraventricular nucleus of the hypothalamus (PVN). In contrast, we excluded changes of peripheral metabolic factors as glucose or ghrelin as mediators of the desensitisation [***Chapter 2***]. These findings suggest that the neuroendocrine desensitisation to repeated maternal absence is of central origin.

Enhanced glucocorticoid inhibition has long been argued as one of the main causes for the stress hypo-responsiveness during early development [28, 29, 41]. The actions of glucocorticoids on basal and stress-induced HPA regulation are mediated by mineralocorticoid and glucocorticoid receptors (MR and GR, respectively). These receptors are already expressed in the neonatal brain, although their ontogenetic patterns are different. MR mRNA in limbic brain is already relatively abundant at birth and GR mRNA is still rather low [30], but already expressed in the stress regulatory centers [25, 37]. Schmidt *et al.* [32] recently showed that treating 8 days old control mice with an MR antagonist slightly enhanced circulating corticosterone levels. By blocking the GR a profound response of ACTH and corticosterone release was observed. The latter finding proved the significance of glucocorticoid feedback mediated by the GR in maintaining stress hypo-responsiveness during the SHRP [7, 28, 32, 41].

In the current studies the hypothesis was tested that the disappearance of the HPA response to repeated maternal absence during the SRHP is due to an enhanced glucocorticoid feedback. For this purpose MR and GR antagonists were administered to assess the role of corticosterone feedback signals in the maintenance of the low HPA axis activity after the third separation and during exposure to novelty. The results demonstrate that GR is not involved in the desensitisation of the HPA axis upon repeated maternal separation and that central MR-mediated inputs to the PVN remain responsive under these conditions.

3.3 Materials and Methods

3.3.1 Animals

In this study offspring of CD1 mice (obtained from Charles River, The Netherlands) was used.

After a habituation period of two weeks, three females were mated with one male in type 3 polycarbonate cages (820 cm^3) containing sawdust bedding and tissue; food (SRM-A, Hope Farms, The Netherlands) and water (containing 6% HCl) *ad libitum*; lights on from 7:00 to 19:00 hours in a temperature (21 ± 1°C) and humidity (55 ± 5%) controlled room. Pregnant females were individually transferred to clean type 3 polycarbonate cages containing sawdust bedding and tissue to provide nest-building material during the last week of gestation. These females were checked for litters daily between 9:00 and 9:30 hours. If litters were present, the day of birth for that litter was then defined as postnatal day 0 (= pnd 0). On the day after parturition, pnd 1, litters were culled to eight healthy pups (four males and four females).

All animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive 86/609/EEC. The protocols were approved by the Animal Care Committee of the Faculty of Medicine, Leiden University (Leiden, The Netherlands).

3.3.2 Procedures of separation and novelty exposure

Mothers nursing litters selected for maternal separation were removed from their cage and placed in clean type 3 polycarbonate cages at 9:00 hours. The home cage containing the pups was placed in an adjacent room on a heating pad (30 - 33°C) to control for pup body temperature. After 8 hours (at 17:00 hours), the mothers were reunited with their pups and left undisturbed until the next deprivation period or until testing. Non-separated litters were left undisturbed. Novelty exposure occurred by placing the pups individually in a clean novel cage on a heating pad for 30 minutes.

3.3.3 Antagonist administration

Pups were injected subcutaneously, according to the 'experimental designs', with either vehicle, GR antagonist (mifepristone, 100 µg/g body weight) or MR antagonist (spironolactone, 50 µg/g body weight) using either NaCl with 0.4% Tween80 or polyethylene glycol (PEG) as a solvent. Although both antagonists dissolve well in PEG, we chose NaCl with 0.4% Tween80 as an alternative solvent to avoid a too large injection volume in our 5 days old pups (Experiments I – III) as compared to the 8 days old pups in a study previously performed in our lab [32]. Injection volumes for NaCl with 0.4% Tween80 were always adjusted to 2 µl/g body weight, whereas the volumes for PEG were adjusted to 6 µl/g body weight. However, to exclude a possible effect on the activity of the HPA axis we performed an additional study (Experiment IV) comparing both solvents.

3.3.4 Experimental designs

Experiment I: The objective was to investigate the involvement of glucocorticoid feedback mediated by MR or GR in the desensitised response to maternal separation using antagonists for these receptors. Mice were subjected to four different conditions: Mice were either separated for the first time (1st SEP) or separated for the third time (3rd SEP). Two control groups were included to measure the response to injection itself, without or with previous experience to maternal

separation at pnds 3 and 4 (no SEP and 3rd CON, respectively) . On pnd 5, all animals were injected either with vehicle (NaCl with 0.4% Tween80), the MR antagonist or the GR antagonist and marked with Fuchsine staining (100 mg Fuchsine dissolved in 100 ml MiliQ H_2O with 4% phenol and 10% ethanol). Animals were sacrificed 8 hours after injection (17:00 hours). Fixed factors were TREATMENT (no SEP, 1st SEP, 3rd CON and 3rd SEP) and INJECTION (vehicle, MR and GR antagonist).

Experiment II: To determine whether the MR or GR are involved in the apparent sensitised response to novelty after repeated maternal separations, pups were treated with a selective antagonist for these receptors. At pnd 5, one male and one female in each treatment group were not subjected to novelty stress to provide a reference value (control: either 'basal' or 'separated' for 8 hours of maternal care). The remaining pups were injected after 8 hours of maternal separation with vehicle, MR or GR antagonist (dissolved in NaCl with 0.4% Tween80) and immediately thereafter subjected to 30 minutes novelty stress. Pups from the "no SEP" group were directly placed in the novelty cage, whereas the "1st SEP" and "3rd SEP" were subjected to 8 hours maternal absence before placement in the novelty cage. The fixed factors were TREATMENT (no SEP, 1st SEP and 3rd SEP) and INJECTION (no injection (basal), vehicle, MR and GR antagonist).

In Experiment III the effect of the duration of exposure and the age during the SHRP (early or late) on HPA axis activity to either an MR or GR antagonist was investigated. Pups 5 or 8 days of age were injected subcutaneously with vehicle, MR or GR antagonist (dissolved in NaCl with 0.4% Tween80) for 8 or 24 hours. The first injection was administered at 9:00 hours. Animals selected for the 8 hours time point were decapitated the same day at 17:00 hours. Animals selected for the 24 hours time point were injected twice more at 17:00 and 01:00 hours to maintain a blocked MR or GR and were sacrificed the next morning at 9:00 hours. The fixed factors were AGE (pnd 3 and 8), DURATION (8 and 24 hours) and INJECTION (vehicle, MR and GR antagonist).

In Experiment IV the effect of the solvent on HPA axis activity was investigated. Pups 5 days of age were either sacrificed immediately or injected with vehicle or GR antagonist using either NaCl with 0.4% Tween80 or PEG and then sacrificed 8 hours later. The fixed factors were SOLVENT (NaCl with 0.4% Tween80 and PEG) and INJECTION (no injection, vehicle and GR antagonist).

3.3.5 Collection of blood plasma

At the specific time points described in Experiments I to IV, animals were sacrificed by decapitation and trunk blood was collected individually in 1.5 ml EDTA-coated microcentrifuge tubes. Blood samples were kept on ice and centrifuged for 15 minutes at 13000 rpm at 4°C. Plasma was then transferred to 1.5 ml eppendorf tubes. Plasma samples were stored frozen at -20°C until determination of corticosterone and cytokine concentrations.

3.3.6 Hormone and cytokine analyses

Per experiment plasma corticosterone concentrations were measured separately using a

commercially available radio immunoassay (RIA) kit containing ^{125}Iodine labelled corticosterone (MP Biomedicals Inc., USA). Corticosterone concentrations were determined in duplicate from an extended standard curve (0, 6.25, 12.5, 25, 50, 100, 250, 500 and 1000 ng corticosterone/ml), since we noted that the lower boundary provided by the kit was not sensitive enough to measure basal plasma concentrations.

For Experiment IV, plasma C-reactive protein (CRP) concentrations were measured using a using a commercially available ELISA (Life Diagnostics Inc., USA). At the Luminex Core Facility (University Medical Center, Dept. of Pediatrics, Utrecht, the Netherlands) plasma concentrations of several cytokines were determined. The Bio-Plex system employing the Luminex multi-analyte profiling technology (xMAP), allows individual and multiplex analysis of up to a hundred different mediators in a single well containing a sample volume of 50 µl [4].

3.3.7 Statistical analysis

In each experiment every individual group contained animals from at least three different litters (*n*=12). Data of each experiment were analysed by analysis of variance (ANOVA) with their respective main factors and the level of significance set at $P<0.05$. When appropriate this was followed by Tukey's *post hoc* comparisons. The initial analyses included sex as a factor, but once it was determined that sex was not a significant factor, data were collapsed across this variable. All data are presented as mean ± S.E.M.

3.4 Results

3.4.1 <u>Experiment I</u>: To investigate the involvement of glucocorticoid feedback mediated by MR or GR in the desensitised response to maternal separation using antagonists for these receptors.

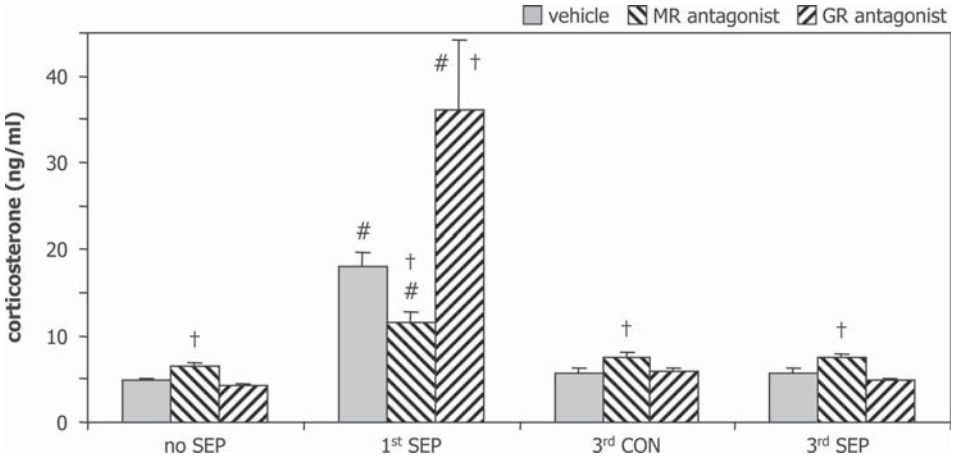

Figure 3.1
Corticosterone response to maternal separation in the presence of either an MR (spironolactone) or GR antagonist (mifepristone) in animals separated from their mother for 8 hours (1st SEP and 3rd SEP), or left with their mother (no SEP and 3rd CON). The 1st SEP group are animals with no previous history of separation, whereas the 3rd SEP group are animals previously exposed to 8 hours separations at pnds 3 and 4. No SEP, which served as a control for the 1st SEP group, was left undisturbed until testing. 3rd CON, which served as a control for the 3rd SEP group, was previously deprived for 8 hours of maternal care at pnds 3 and 4. Injections were given at the start of the 8 hours separation period. Data represent mean ± S.E.M. † $P<0.05$ versus vehicle (within treatment group), # $P<0.05$ versus no SEP.

***3.4.1.1 Corticosterone* (*Figure 3.1*)**

A main effect was observed of treatment (F(3,116)25.57, *P*<0.001) and injection (F(2,116)3.48, *P*<0.05) as well as an interaction between treatment and injection (F(6,116)6.50, *P*<0.001), indicating that the effect of injection depends on the treatment group. Vehicle-treated pups experiencing maternal separation for the first time (1st SEP) showed increased corticosterone levels compared to their controls (no SEP), whereas a third separation did not affect corticosterone levels anymore (3rd SEP versus 3rd CON). Blocking the MR slightly, but significantly increased corticosterone levels in the no SEP, 3rd CON and 3rd SEP groups, while it suppressed corticosterone secretion in the 1st SEP group. Blocking the GR only affected corticosterone levels in the 1st SEP group, resulting in increased secretion. After three times maternal separation MR, but not GR antagonism could affect corticosterone secretion.

3.4.2 Experiment II: The objective was to determine if an altered MR or GR feedback is involved in the sensitised response to novelty after repeated maternal separations by using selective antagonists for these receptors.

***3.4.2.1 Corticosterone* (*Figure 3.2*)**

A main effect was observed of treatment (F(2,133)88.62, *P*<0.001) and injection (F(3,133)38.30, *P*<0.001). *Post hoc* analyses revealed that in control mice corticosterone secretion increased in response to a first maternal separation (no SEP, basal versus 1st SEP, separated; *P*<0.001), but not anymore in response to a third separation (no SEP, basal versus 3rd SEP, separated). However, in all treatment groups, injection plus exposure to a novel environment increased circulating

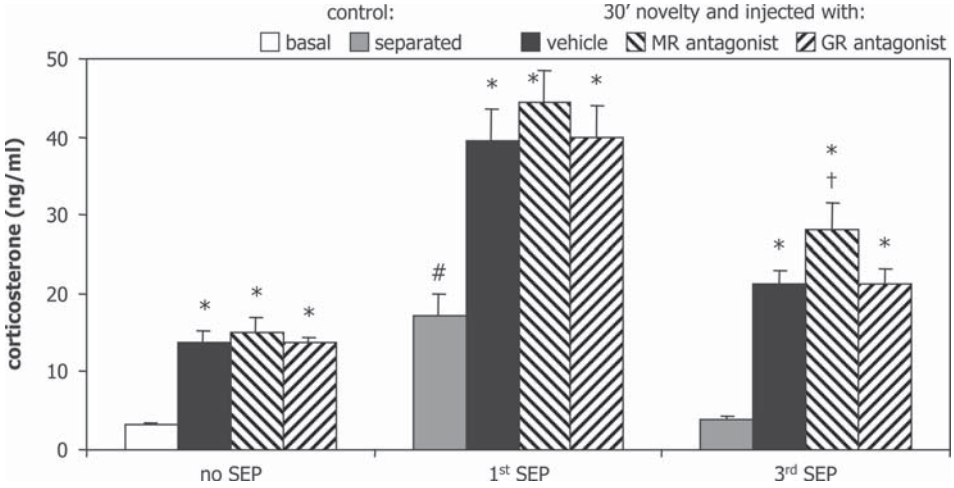

Figure 3.2
Corticosterone response to novelty in the presence of either an MR (spironolactone) or GR antagonist (mifepristone) in animals separated from their mother for 8 hours (1st SEP and 3rd SEP), or left with their mother (no SEP). The 1st SEP group are animals with no previous history of separation, whereas the 3rd SEP group are animals previously exposed to 8 hours separations at pnds 3 and 4. No SEP served as a control for the 1st SEP group and was left undisturbed until testing. Injections were given at the end of the 8 hours separation period right before the 30 minutes novelty exposure. Data represent mean ± S.E.M. * *P*<0.05 versus basal (no SEP) or versus starting levels after 8 hours of maternal separation (1st SEP and 3rd SEP), # P<0.05 versus basal (no SEP), † *P*<0.05 versus vehicle (within treatment group).

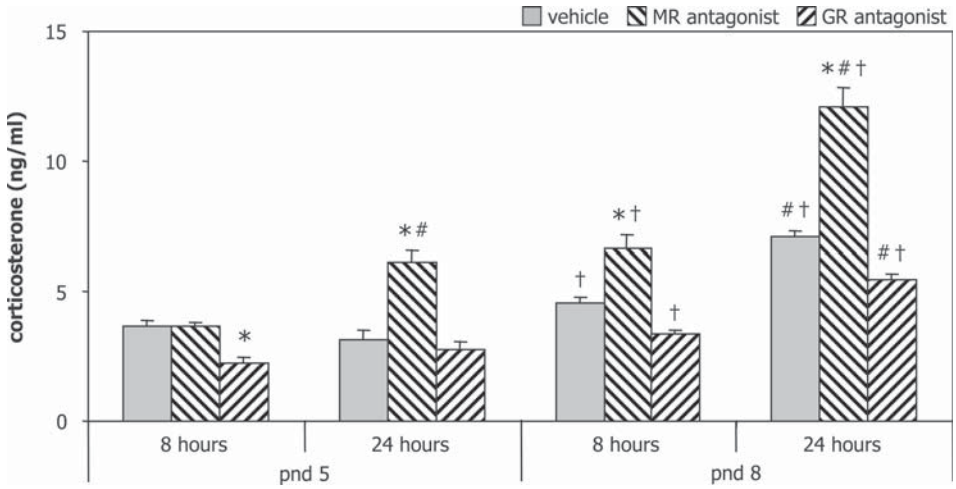

Figure 3.3
Corticosterone concentrations in 5 and 8 days old mice. Mice were treated with either MR (spironolactone) or GR antagonist (mifepristone) dissolved in NaCl with 0.4% Tween80 (vehicle). Data represent mean ± S.E.M., * $P<0.05$ versus vehicle-treated animals, # $P<0.05$ versus 8 hours treatment (within age group), † $P<0.05$ versus pnd 5 (within duration group).

corticosterone levels when compared to control levels ($P<0.001$). Blocking the MR affected corticosterone levels in the 3rd SEP group, resulting in higher corticosterone levels compared to vehicle-treated pups ($P<0.05$). Blocking the GR did not affect corticosterone levels in any of the treatment groups.

3.4.3 <u>Experiment III</u>: The objective was to test the influence of the age of the animal and the duration of antagonist exposure on the amplitude of the pituitary-adrenal response to injection.

3.4.3.1 Corticosterone (***Figure 3.3***)

We observed, besides a main effect of injection ($F(2,150)107.66$, $P<0.001$), that both the age of the pups ($F(1,150)176.51$, $P<0.001$) and duration of treatment ($F(1,150)88.93$, $P<0.001$) significantly

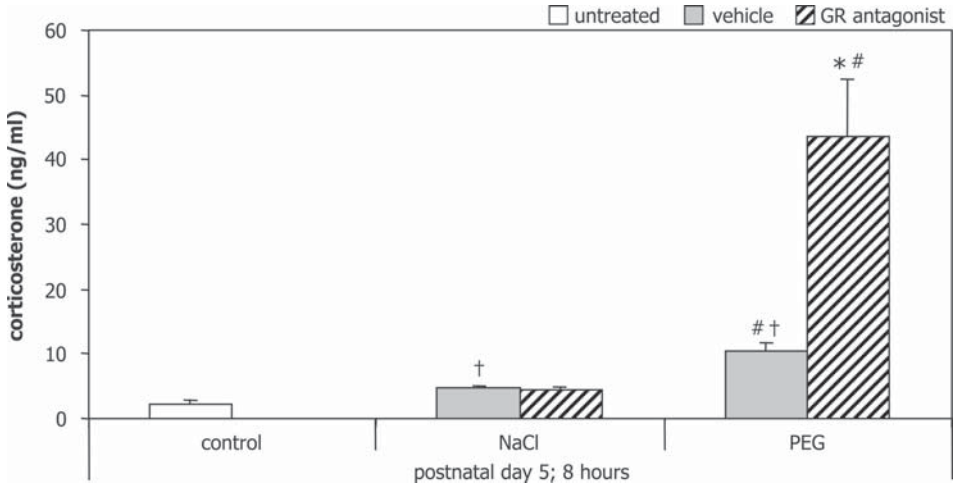

Figure 3.4
Corticosterone concntrations in 5 days old mice after 8 hours treatment with GR antagonist (mifepristone) dissolved in NaCl with 0.4% Tween80 or in PEG. Untreated control animals were naïve to any treatment. Data represent mean ± S.E.M. * $P<0.05$ versus vehicle-treated animals, # $P<0.05$ versus 8 hours treatment (within age group), † $P<0.05$ versus pnd 5 (within duration group).

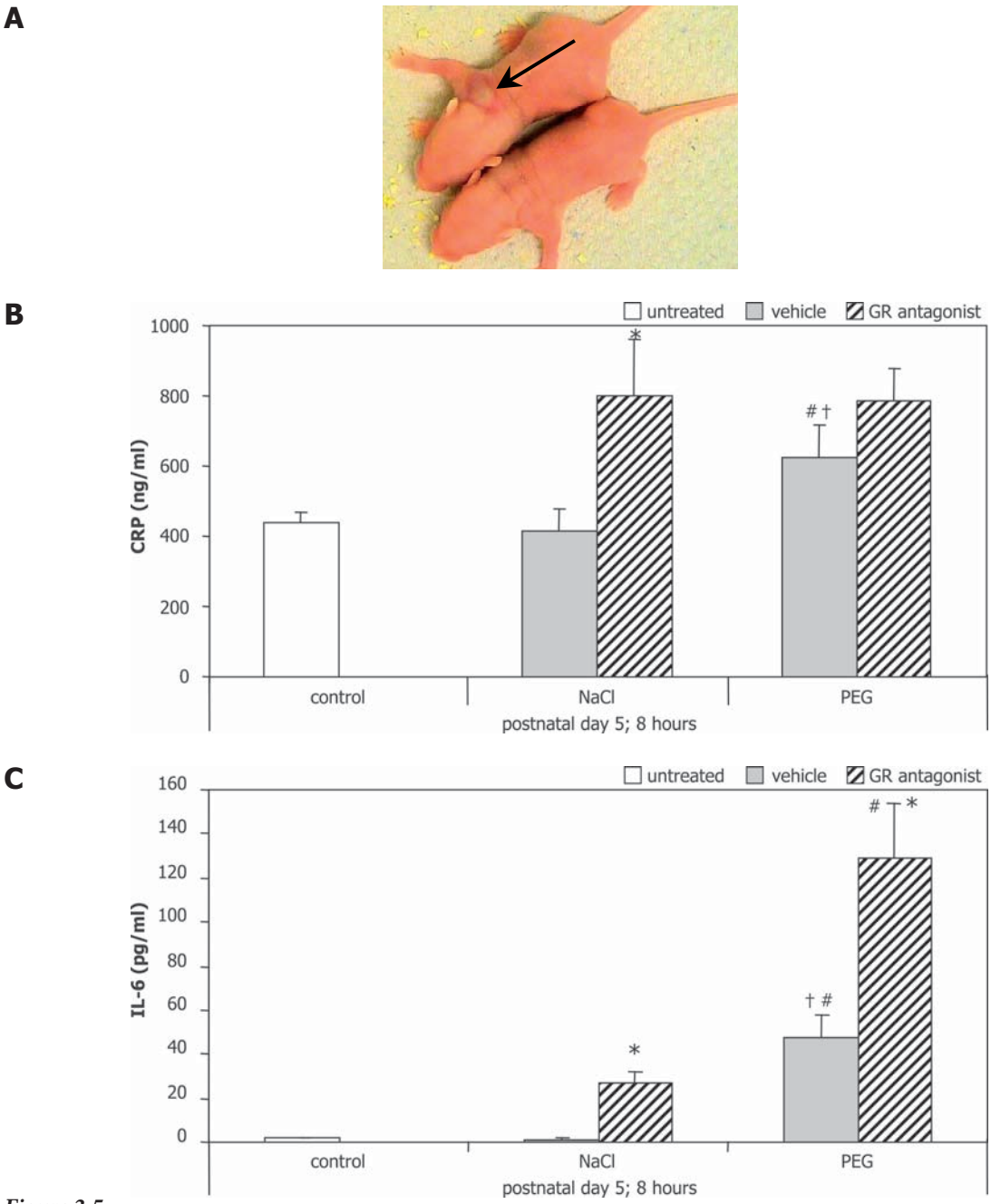

Figure 3.5
(***A***) Example of a mouse pup injected with PEG (arrow points out site of injection) or NaCl with 0.4% Tween80 as vehicle for antagonist delivery. CRP (***B***) and IL-6 (***C***) concntrations after 8 hours treatment with GR antagonist (mifepristone) dissolved in NaCl with 0.4% Tween80 or in PEG. These measurements were performed in the same blood samples as for the corticosterone concentrations of ***Figure 3.4***. Untreated control animals were naïve to any treatment. Data represent mean ± S.E.M. * $P<0.05$ versus vehicle-treated animals, # $P<0.05$ versus 8 hours treatment (within age group), † $P<0.05$ versus pnd 5 (within duration group).

affected corticosterone levels. At pnd 8 the vehicle injection at the 8 hours time point resulted in corticosterone levels that were higher compared the same time point at pnd 5 ($P<0.5$). Furthermore, these levels further increased at the 24 hours time point, whereas this was not observed at pnd 5. Except for the 8 hours time point at pnd 5, the MR antagonist slightly increased circulating corticosterone plasma levels ($P<0.01$). The GR antagonist slightly reduced circulating corticosterone levels ($P<0.05$). Overall, a more pronounced amplitude of the responses was

observed in 8 days old pups as compared to the 5 days old pups.

3.4.4 Experiment IV: The objective was to determine whether the immune challenge of the HPA axis triggered by the injection with PEG as vehicle might interfere with the effect of the MR and GR antagonists.

3.4.4.1 Corticosterone (***Figure 3.4***)

When analysing the corticosterone data we observed both an injection effect (F(1,45)12.01, $P<0.001$) as well as a solvent effect (F(1,45)21.95, $P<0.001$) and an interaction between injection and solvent (F(1,45)12.62, $P<0.01$). When injecting pups with PEG the basal levels of corticosterone after 8 hours increased and this increase was even further enhanced when the GR antagonist was administered ($P<0.001$). However, injecting pups with NaCl with 0.4% Tween80 only slightly raised basal circulating corticosterone levels ($P<0.01$), but this rise was much smaller than when injected with PEG ($P<0.001$). Furthermore, adding the GR antagonist did not further affect these levels.

3.4.4.2 CRP and cytokines (***Figure 3.5***)

Since a reddish circle appeared at the site of injection in the pups that were injected with PEG solvent (***A***) we measured C-reactive protein (CRP) and cytokine levels.

For CRP (***B***) we observed a main effect of solvent (F(1,48)7.54; $P<0.01$), but not of injection. CPR levels in pups injected with PEG were higher relative to NaCl with 0.4% Tween80 as vehicle ($P<0.05$) and compared to uninjected controls ($P<0.05$). Although there were no significant differences in CPR levels in mice receiving either solvent, CPR levels increased significantly in animal receiving the antagonist dissolved in NaCl with 0.4% Tween80, while CRP levels remained high when the it was dissolved in PEG.

For IL-6 (***C***) we also observed a main effect of solvent (F(1,53)19.26; $P<0.001$), but also of injection (F(1,53)37.28; $P<0.001$) and an interaction between solvent and injection (F(1,53)5.24; $P<0.001$). Injection of animals with NaCl with 0.4% Tween80 did not affect basal IL-6 levels, while injection with PEG significantly increased these levels ($P<0.05$). Administration of GR antagonist significantly increased IL-6 levels when it was dissolved in NaCl with 0.4% Tween80 ($P<0.05$). However, IL-6 levels were even further increased when it was dissolved in PEG ($P<0.05$).

3.5 Discussion

The present study shows that the infant's corticosterone response to 8 hours of maternal separation disappears when the procedure is repeated on three consecutive days from postnatal days 3 to 5. However, at the time the corticosterone response to separation is abolished, an additional exposure to novelty still has a profound effect. This finding reinforces our previous observations showing that the pup's HPA axis readily desensitises to repeated separations, but continues to respond to novelty [***Chapter 2***].

In order to test if enhanced corticosterone feedback could explain the abolishment of the pituitary-adrenal response after repeated separations we have administered MR or GR antagonists to the separated pups. The results show that the GR antagonist mifepristone amplified the infant's corticosterone response after the first 8 hour separation, as previously shown also after a 24 hours separation paradigm [32], but became ineffective at the third separation. This finding excludes enhanced glucocorticoid feedback as a mechanism underlying the desensitisation of the HPA response to maternal absence, if the separations of 8 hours were repeated each day.

Enhanced inhibition of the HPA axis during the SHRP via GR has long been argued as the main cause for the hypo-responsiveness during early development [26, 28, 29, 32, 41]. However, in the current studies blockade of GR with the antagonist dissolved in NaCl containing 0.4% Tween80 did under basal conditions not affect circulating corticosterone levels in the 3 to 5 days old neonates, as it did not 24 hours later. The antagonist even quite consistently slightly reduced circulating corticosterone levels ($P<0.05$) at day 5. At first glance our data do not seem to agree with a previous report from this laboratory, demonstrating that GR-mediated feedback maintains the low and stable corticosterone levels characteristic for the SHRP [32]. We have repeated the studies by Schmidt *et al.* under identical conditions both at postnatal day 5 and 8 and neither after a single or two times administration of the GR antagonist a response was measured 8 hours later.

What could be the reason for this paradox? In retrospect, there is no discrepancy. It is likely that in neonatal mice truly basal circulating levels of corticosterone are insufficient to activate the GR. This situation is reminiscent to that in adult animals, where it is since long known that basal levels do not sufficiently occupy the GR and therefore cannot be blocked by a GR antagonist [21]. Thus, it seems that similar to the adult situation GR antagonism only becomes in operation under stress-induced conditions, as this receptor mediates the effects of corticosterone on normalisation and recovery from stress. Hence the conclusion in previous studies that GR mediated feedback maintained hypo-responsiveness is justified, but what could have been the stimulus in the previous studies that had led to the subtle increase in corticosterone levels sufficient to reveal the role of GR-mediated feedback in the SHRP?

Upon repeating the studies we noticed a reddish circle at the site of injection in the pups that were now injected with PEG solvent as used in the previous studies (***Figure 3.5.A***), indicating that the solvent may have influenced the HPA axis by triggering an immune response [2, 42]. Immune system activation of the HPA axis during the SHRP has already been shown in rats [9, 11]. To confirm this we measured the cytokine profile in blood plasma of these mice in response to NaCl with 0.4% Tween80 and PEG alone, or in combination with an anti-glucocorticoid. We measured CRP, an acute phase protein that is elevated in serum as a result of injury, infection or disease [33] and established as a reliable marker for inflammation [19]. As expected the GR antagonist, foreign to the body, triggered an immune response expressed by increased plasma CRP. Surprisingly, PEG alone also induced an immune response, while the NaCl with 0.4% Tween80 vehicle solution did not. Since there is a close reciprocal interaction between the immune and stress system [1], the

observed GR antagonist-induced corticosterone increase with PEG as a solvent has to be judged in the light of an activated HPA axis.

We also measured other cytokines and among these was IL-6, which showed a profound response to PEG. In these experiments we also reproduced that the anti-glucocorticoid in NaCl with 0.4% Tween80 was ineffective 8 hours after. Indeed, also PEG alone caused a slight corticosterone rise apparently sufficient for the anti-glucocorticoid to interfere and to trigger a profound corticosterone response. IL-6 is known to activate the HPA axis, so it appears that a mild inflammatory response is sufficient to uncover the role of glucocorticoid inhibition in the maintenance of the SHRP.

Blockade of the MR gave different results. The corticosterone rise induced by the first 8 hour separation is attenuated, while the MR antagonist produced a small corticosterone response in the pups subjected to three daily separations. A rise in corticosterone was also observed if the animals are older, at 8 days, or when the separation period is extended to 24 hours. Hence, it seems that an MR- rather than a GR-responsive network is still active during repeated maternal absence. Such an MR-responsive pathway could be implicated in the central mechanism underlying desensitisation as was identified in the previous study using *c-fos* mRNA expression in the PVN as a criterion. Such a central pathway may refer to the recent studies of Moriceau *et al.* [17, 18], who showed a switch from a locus coeruleus-olfactory pathway governing attachment to the mother towards the premature development of an amygdala pathway mediating avoidance from adverse conditions. This switch was facilitated by corticosterone, which we showed to be increased under conditions of novelty exposure and inflammation, particular during maternal absence.

The abolished HPA axis response to repeated maternal absence suggests that the pups may be able to predict the return of the mother and thus the reinstatement of maternal care after 8 hours. This situation is reminiscent to experiments showing that 3x 45 seconds stroking of the anogenital region with a wet brush is sufficient to prevent the single 24 hours maternal deprivation induced *c-fos* activation in the PVN and basal increase in plasma ACTH [35, 38, 39]. Subsequent feeding is able to normalise or prevent the rise in corticosterone values [35, 39]. However, in previous studies [***Chapter 2***] we excluded that an altered food intake pattern was the cause of the dynamic changes in the HPA axis upon repeated separations, since circulating glucose and ghrelin levels were not affected. Furthermore, reunion of mother and pups after a prolonged period of separation causes a bout of increased maternal care [14]. This extra attention by the mother also could be involved in the adaptation to repeated separations.

To investigate the role of MR and GR in the novelty response, pups were injected with an antagonist at the end of the 8 hours maternal separation period before applying novelty stressor. This design allowed testing the role of the recently discovered fast non-genomic membrane-mediated MR- and GR-like actions [8, 36]. The data showed that novelty triggered a response both after the first and the third separation, but only the MR antagonist enhanced the novelty-induced corticosterone secretion. GR blockade did not render an effect in our mice, possibly

because the 30 minute time interval is too short for a GR-mediated mechanism to develop.

Interestingly, MR-mediated disinhibition of HPA axis activity is supported by data in both adult rats and mice [3, 6, 21]. The findings therefore suggest that blocking the MR may relieve a tonic inhibition of the HPA axis [7] or more likely inhibits the non-genomic MR-mediated actions in the hippocampus, which would also imply a reduced excitatory outflow from the hippocampus to the GABA-ergic network surrounding the PVN. The latter mechanism, active for MR rather than GR, could explain the rapid enhancement after MR blockade in the novelty-induced stressor. All together, it seems that the MR-mediated control of basal pulsatile HPA activity and its role in sensitivity to stressors is similar in the infant and the adult. Also the GR-mediated actions, which are absent under truly basal activity, become in operation during exposure to mild daily stressors ensuring maintenance of the SHRP.

In conclusion, enhanced GR-mediated feedback underlying the desensitised corticosterone response to repeated maternal separation is unlikely. The previously reported disinhibitory effect on GR antagonists during the SHRP is a striking example how a subtle change in HPA activity characteristic for the SHRP can be amplified when glucocorticoid feedback is inhibited. Finally, it seems that MR-responsive afferents to the PVN are implicated that manage subtle changes in HPA activity during the SHRP under basal conditions and after repeated separations with or without novelty exposure.

3.6 Acknowledgements

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3.7 References

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