# CHAPTER 4

## Developmental and long-lasting consequences of neonatal dexamethasone treatment: impact of early handling

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*Submitted*

## ABSTRACT

Exposure to increased glucocorticoid levels during development is known to have enduring consequences for brain function and behaviour throughout the lifespan. Perinatal dexamethasone administration, a common treatment for prematurity-associated respiratory disorders and an example of such enhanced glucocorticoid exposure, has been shown to result in adverse side effects on the developing nervous system, leading to long-lasting alterations in endocrine and behavioural phenotype.

In the current study we investigated the development of these alterations in male Long Evans rats. Rat pups were injected with dexamethasone or saline on postnatal days 1, 2 and 3. In experiment I, body weight, eye opening and fur development were measured during the postnatal period. In adulthood animals were tested for spatial learning, stress responsiveness, and contextual fear conditioning.

Dexamethasone treatment resulted in growth retardation, an altered pattern of fur development and accelerated eye opening. However, we did not observe alterations in behavioural and endocrine phenotype in adulthood. Therefore, we investigated a potential interaction of the effects of dexamethasone treatment with those of neonatal handling, which was an inevitable component of our experimental design. Thus, we included in experiment II an untreated non-handled control group. We report that these non-handled controls displayed reduced pre-pulse inhibition, motor performance and spatial learning in addition to a prolonged endocrine stress response, compared to handled animals (both saline and dexamethasone-treated).

We conclude that neonatal handling resulted in profound phenotypic alterations throughout the lifespan, potentially protecting against dexamethasone-induced alterations.

## INTRODUCTION

Perinatal life represents a critical period during which an individual is highly susceptible to environmental influences. Adverse infant experiences have been shown to induce profound and long-lasting effects on adult brain function and behaviour (1, 2) that may increase vulnerability for disease development (3-5). These enduring effects of early experiences may be associated with a long-lasting impact on the functioning of the hypothalamic-pituitary-adrenal (HPA) axis (3, 4, 6-8).

During normal early development, the brain appears to be protected from exposure to high levels of endogenous glucocorticoids, since the neonate displays a strongly reduced adrenocortical response to mild stressors, a phenomenon reported in rodents (9-11) and humans (6). This stress hypo-responsive period (SHRP) can only be disrupted when the organism is either exposed to extremely stressful (life threatening) events or when the caregiver is absent. In absence of the mother, corticosterone levels slowly increase and the neonate becomes responsive to mild stressors that would not result in HPA axis activation in her presence (11-13). Emergence from the SHRP, and subsequent exposure to elevated levels of glucocorticoids has profound programming effects on development and is important for shaping the adult phenotype (14-16).

Exposure to exogenous glucocorticoids such as dexamethasone (DEX) can be considered a model for inappropriate glucocorticoid secretion. Interestingly, DEX administration is a common treatment for prematurity-associated respiratory disorders and supposedly a life saving treatment (17). However, as can be expected from neonatal glucocorticoid exposure, this treatment has profound side effects on the developing nervous system, leading to long-lasting alterations in brain function and behaviour both in humans (18) and animals (19). DEX-treated rodents show spatial learning impairments, altered endocrine responsiveness to acute stress and a significantly shortened lifespan (19-21).

In the current study we investigated the development of these alterations in cognitive performance and endocrine stress responsiveness after neonatal glucocorticoid treatment in a rodent model, which is described in experiment I. Interestingly we did not observe the previously reported detrimental effects of neonatal glucocorticoid exposure on adult phenotype. We suggest that neonatal handling, as an inevitable component of the experimental design, might serve as a factor protecting against the adverse effects of neonatal glucocorticoid exposure. This hypothesis was tested in experiment II, using untreated non-handled animals as an additional control group.

## MATERIALS AND METHODS

### Animals

Adult Long Evans rats from our breeding population were used as breeders. Two females were mated with one male for 10 days in type 4 polycarbonate cages (59x38x20cm) containing sawdust bedding and tissues. Food (RM3, Special Diet

Services, Witham, Essex, UK) and water (8 ml 25% HCl /10 L tap water) were provided *ad libitum*. Animals were maintained on a 11-h light : 13-h dark cycle with lights on at 08.30h, in a temperature (21± 2°C) and humidity (55 ± 5%) controlled room. After breeding, pregnant females were individually housed. Females were checked daily for presence of pups. If pups were present, the day of birth for that particular litter was defined as postnatal day 0 (pnd 0). On pnd 1, litters were culled to 8 pups (4 male and 4 female). Cages were cleaned at pnd 10 and after weaning once weekly. 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).

## Experimental Design

Experiment I: To investigate the consequences of neonatal DEX treatment on development and adult phenotype we studied saline (SAL) and DEX treated offspring according to the schedule depicted in fig. 1.

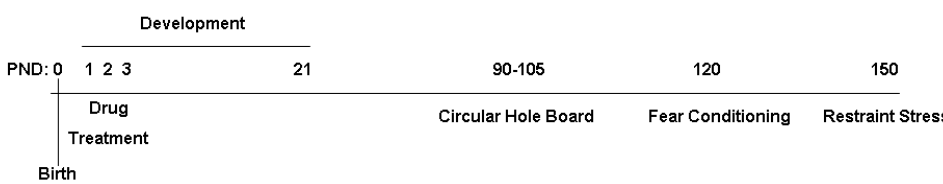


**Fig 1.** Timeline experiment I. PND: postnatal day.

Experiment II: To investigate whether the effects of neonatal handling, induced by daily marking and weighing of the offspring, might have protected against DEX-induced alterations, we extended the experiment with an untreated non-handled (UNT NH) control group. Animals from litters born at the same time in the same breeding colony as the SAL and DEX animals, were included for this purpose at the age of 6 months. These animals were raised and housed under similar conditions as the SAL H and DEX H animals, except for drug administration and daily handling during the postnatal period. They were not subjected to the two behavioural tests in experiment I, and thus kept undisturbed except for a weekly cage cleaning. SAL and DEX treated animals will, in experiment II, be referred to as SAL H and DEX H. Animals were tested according to the schedule depicted in fig. 2.

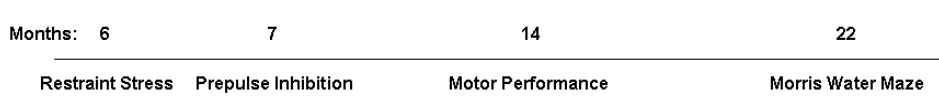


**Fig 2.** Timeline experiment II.

**Drug Treatment**

Male pups were randomly assigned to either the saline (SAL) or the dexamethasone (DEX) group using a within litter design. Pups in the DEX group were subcutaneously (SC) injected with dexamethasone-21-phosphate (Sigma Aldrich, Zwijndrecht, The Netherlands) on pnd 1 (0,5 µg/g body weight), pnd 2 (0,3 µg/g) and pnd 3 (0,1 µg/g). Pups in the SAL group were injected with equivalent volumes of sterile and pyrogen free saline. In order to prevent bias for the dam to show enhanced attention for injected or non-injected offspring, all female littermates were injected with SAL on pnd 1, 2 and 3. All pups were daily marked using a non-toxic, odourless marker in order to discriminate between littermates assigned to either the SAL or DEX group.

**Postnatal development**

Postnatal development was monitored in SAL and DEX treated pups. Body weight, eye opening and fur development were recorded. For eye opening a scale from 0-9 was used indicating: 0 = closed, 3 = occasional/partial opening, 6 = mostly open, 9 = fully open. For fur development a scale from 0-12 was used indicating: 0 = no fur, 3 = fine hairs, 6 = partial fur, 9 = mostly fur, 12 = full fur (adapted from (22)).

*General procedure*

Pnd 1-3: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room, where the holding cage was put on a heating pad. Body weights (BW), eye opening and status of fur development were recorded, followed by marking and injection of the pups. After the procedure (which took ~ 10 minutes per litter) all pups were immediately returned to the home cage.

Pnd 4-21: The daily procedure consisted of removing all pups from the nest followed by transfer to an adjacent room, where the holding cage was put on a heating pad. Body weights, eye opening and status of fur development were recorded, followed by marking of the pups. After the procedure (which took ~ 6 minutes per litter) all pups were immediately returned to the home cage.

Besides these manipulations (which always took place between 9:00 and 13:00h) litters were left undisturbed until weaning on pnd 22, except for a cage change on pnd 10.

**Adult Phenotype**

**Spatial learning: Circular Hole Board**

Spatial learning was assessed between pnd 90 - 105. The Circular Hole Board consisted of a white circular platform (120 cm diameter) with 12 holes (10 cm diameter) equidistantly placed at 12 cm from the edge of the platform. An overhead camera allowed tracking of the behaviour of the animals during testing. Distal spatial cues were mounted on the walls of the testing room for orientation. The paradigm is based on the assumption that rodents are motivated to find a way to escape when being exposed to a light and unprotected place like the hole board. The animals were trained to locate the position of the exit hole that

leads via an escape tunnel to the home cage. All other holes were closed with a lid placed 1 cm below the surface of the platform. This prevented the animal from seeing whether a hole is open or closed until it is in close proximity to the hole. The exit hole was always in the same spatial location; however, the platform was turned between trials to eliminate odour cues. All trials started by putting the animal in a start tube (diameter: 20 cm, height: 30 cm) that was positioned in the centre of the platform and removed to start the trial. If an animal did not find the exit hole within 120 seconds, it was gently guided there by the investigator. The platform was cleaned in between sessions using a 10% ethanol solution to eliminate odour cues. All testing took place between 10.00 and 13.00h.

*General procedure*

In the week prior to hole board training, animals were exposed to 3 sessions of tunnel training to practice manoeuvring through the escape tunnel that will lead to the home cage during hole board training.

On day 1, to familiarize the animals with the platform and the existence of an exit hole plus escape tunnel, animals were allowed to explore the platform during a 3 min free exploration trial (FET) with all holes closed. At the end of the 3 min period, the exit hole was opened and the animal was given another 2 min to locate the exit hole.

On day 2, animals were trained during 6 trials with a 15 min inter-trial interval to find the location of the exit hole.

On day 8, animals were retested to evaluate spatial memory during 2 memory trials, using similar conditions as during training on day 2.

Total distance moved, amount of time spend in different areas/quadrants of the platform, number of holes visited, latency to find exit hole and latency to escape were analyzed using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands).

**Contextual Fear Conditioning**

Contextual fear memory was assessed between pnd 120-130. The shock box (40x40x50 cm) was made of black Plexiglas walls and a stainless steel rod floor, connected to a shock generator. The box was cleaned with a 10% ethanol solution between all sessions to eliminate odour cues. An overhead video camera recorded behaviour of the animals throughout all sessions. All testing took place between 10.00 and 13.00h.

*General procedure*

On day 1 the animal was transferred from the housing room to the adjacent test room where it was placed in the shock box. After 2 min it was exposed to a foot shock of 0.6 mA (duration 2 sec). After the shock the animal remained in the shock box for 2 minutes and was then removed from the box and transferred back to the home cage. 24 hours later (day 2), the animal was re-exposed to the shock box for 4 min, however without receiving a foot shock.

Behaviour of the animal was recorded both during training and re-exposure and analyzed by an observer unaware of treatment conditions using The Observer

9.0 XT (Noldus Information Technology, Wageningen, The Netherlands). The following behaviours were scored (1) freezing (lack of all body movement except those necessary for breathing), (2) scanning (lack of movements, except lateral head movements and movements necessary for breathing), (3) rearing, (4) walking, and (5) sitting. Behaviour was analyzed during 3 distinct time periods: (1) 2 min before shock, (2) 2 min after shock, (3) 4 min re-exposure.



**Endocrine response to restraint stress**

On pnd 150, endocrine HPA axis activation in response to an acute restraint stressor was tested. One day prior to restraint stress a basal blood sample was taken from the tail vein. The next day animals were exposed to 10 min of restraint stress by placing them in a custom made restrainer, which restricts body movements. Blood samples were taken at 2, 5, 10 30, 60, and 120 min.

**Hormone Analysis**

Blood samples were collected in EDTA coated tubes (Microvette CB 300 K2E, Sarstedt, Germany). Samples were kept on ice and centrifuged for 15 min at 13000 rpm at 4°C. Plasma was transferred to Eppendorf tubes and stored at -20°C until further analysis. Plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) concentration were measured using a commercially available radio immuno assay (RIA) kit containing $^{125}$Iodine labelled ACTH or CORT, respectively (MP Biomedicals Inc., USA). All samples were processed in the same assay to exclude inter-assay variability.

**Prepulse Inhibition**

Prepulse inhibition was measured at 7 months of age. Three/four littermates were transferred together to the test room where they were allowed to habituate for 45 min. After habituation they were placed in a startle recording apparatus (SR-LAB, San Diego Instruments, CA, USA), containing a transparent Plexiglas tube (diameter 8.7 cm, length 20.5 cm) mounted on a Plexiglas base. Sounds were presented by a speaker and movement of the animals was detected by a piezoelectric accelerometer mounted below the Plexiglas tube and recorded by a computer. Testing started with a five min habituation session with background white noise of 70 dB[A]. Animals were first presented with six pulse alone trials (117 dB[A]) followed by 39 trials comprising different trial types according to a pseudo-randomized schedule with an inter-trial interval of 10-20 sec. Trial types: 4x background white noise alone, 5x prepulse alone (16 dB[A] above background = 86 dB[A]), 20x prepulse–pulse trials using prepulse intensities of 2, 4, 8, 16 dB[A] above background noise (i.e. 72, 74, 78 and 86 dB[A]), and 10x pulse alone (117 dB[A]). Finally, animals were again exposed to five pulse alone trials. The duration of the prepulses was 20 ms, duration of the pulses was 40 ms. Prepulse to pulse interval was 100 ms. Startle activity was measured during 100 ms after onset of the pulse. The percentage PPI at the different prepulse intensities was calculated as [100-(100 x startle amplitude at prepulse trial)/ (startle amplitude at

startle pulse-alone trial)]. Speakers were calibrated every day. Experiments were performed between 9:00 and 13:00 to minimize circadian influence.

### Motor performance

At 14 months of age all animals were tested in a motor performance test battery. Testing took place over 4 different test sessions on 2 consecutive days.

#### *Balance beam*

The animal was placed on a square metal bridge (2 cm x 2 cm x 40 cm, elevated about 40 cm above the surface) wrapped in anti slip tape. The duration the animal stayed on the bridge was measured to a maximum of 120 s. A pillow was placed beneath the bridge to cushion the animal's fall. Rats were tested twice a day on 2 consecutive days.

#### *Rota-rod*

Motor coordination was measured using the rota-rod test. In this test, the animal was placed on the rota-rod treadmill, attached to a rotating motor. The treadmill consisted of four rotating drums (7 cm diameter, 24 cm above ground), divided by flanges. The first day, rats were familiarized with the apparatus. During 2 habituation trials - one in the morning and one in the afternoon - which lasted 2 min each, the animals were placed on the constantly revolving drum (speed 13 rpm). If a rat fell off during habituation it was placed back immediately. Number of falls during the 2 min habituation periods was recorded. On the second testing day, the animals were tested on an accelerating rota-rod (up to 40 rpm in max 3 min). Latency to fall from the rotating rod was recorded.

#### *Foot-fault test*

This test measures placement dysfunction of the paws and motor coordination. The animals were placed on an elevated wire grid (dimension 100 x 100 cm, grid size 4 x 9 cm) and allowed to explore the grid for 2 minutes. Number of steps and number of errors (foot-faults were counted when a paw fell completely through the bars of the grid) were recorded.

### Water maze performance

At 22 months of age spatial learning in a water maze was tested. Animals were placed, without prior training, in a pool (150 cm diameter) filled with 30 cm of water (21°C) made opaque by adding 3 spoons of latex paint. A platform (10 cm diameter) was hidden 1 cm below the surface of the water and was positioned in the NE quadrant.

Animals were given 2 daily trials (inter-trial interval 15 min) to find the platform on day 1, 2 and 3. On day 4, one more training trial was given, followed by a probe trial without platform present. On day 5, animals were given 3 reversal trials with the platform located in the position opposite (SW) from where it was during training. Trials always started in one of the 3 quadrants where the platform was not located in a pseudo-randomized fashion, following the same order of start position for every animal. If an animal did not reach the platform within 2 minutes,

it was gently guided there by the investigator. Animals were allowed to stay on the platform for 15 sec after finding it or being guided there.

Swim patterns were tracked by an overhead camera and later analysed using EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands). Frequencies and time spent in target and other quadrants, or in close proximity to the platform (or the location where the platform used to be) were analyzed during training and reversal trials, as well as swim patterns during probe trials. After every swim trial, animals were dried with a towel and kept in a clean cage lined with clean dry tissues, placed on a heating pad. All trials took place between 10.00 and 13.00h.

### Survival

Survival of the animals was determined by recording the health span rather than the lifespan. If an animal died without showing signs of pathology, the age of natural death was recorded. However, if an animal did show signs of pathology (i.e. substantial weight loss, impaired locomotion, breathing problems) the animal was sacrificed and age of sacrifice was recorded.

### Data analysis

Data are presented as mean +/- SEM. All data were analysed using repeated measures ANOVA with time (age, trial, day or min) as within and drug treatment and handling as between subject factors, one-way ANOVA, or paired t-test with level of significance set at $p < .05$. Where appropriate main effects and interactions were further investigated using post-hoc analysis with appropriate correction for multiple testing. Corrections for violations of sphericity were conducted where needed. SAL H and DEX H animals were always initially included as separate groups in the analysis. If drug treatment did not significantly contribute to the outcome, data from SAL H and DEX H animals were pooled to investigate the impact of neonatal handling by comparing performance of these 2 groups to UNT NH animals.

## RESULTS EXPERIMENT I

### Postnatal development

Body weight gain is reduced in DEX treated animals during the postnatal period (fig. 3A) and remains lower throughout adult life (fig. 3B) (main effect of drug treatment $F(1,18) = 26.01$, $p < .001$). A significant time x drug treatment interaction was observed ($F(36,648) = 5.62$, $p < .001$). Post-hoc analysis per time point revealed a significant difference between SAL and DEX on all time points starting at pnd 2 ($p < .001$ until week 10, $p < .01$ until week 24, and $p < .05$ at week 26).

Neonatal DEX treatment results in accelerated eye opening (fig. 4A) and an altered pattern of postnatal fur development (fig. 4B). Besides a significant main effect of time ($F(20,520) = 1382.18$, $p < .001$) and drug treatment ($F(1,26) = 168.12$,

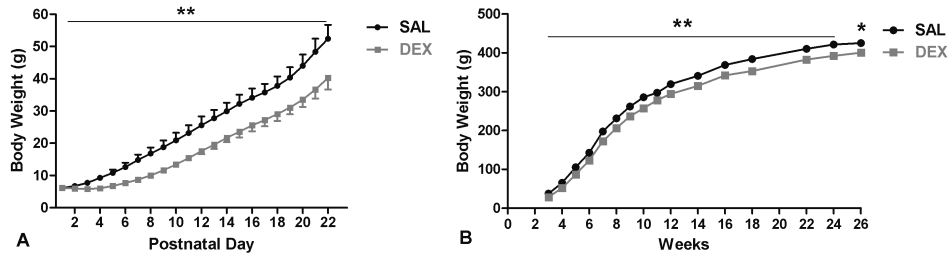


**Fig 3.** Body weight of SAL and DEX treated animals during the postnatal (A) and post-weaning (B) period. DEX treated animals show significantly lower body weight compared to SAL treated animals at all time points starting on pnd 2. ** $p < .01$; * $p < .05$

$p < .001$) a significant time x drug treatment interaction ($F(20,520) = 56.81$, $p < .001$) was observed for eye opening. Post-hoc analysis per time point revealed significant differences between SAL and DEX treated animals on pnd 10, 11, 12 and 13 indicating that DEX treated animals open their eyes at an earlier age.

For fur development we observed, besides significant main effects of time ($F(20,500) = 2059.70$, $p < .001$) and drug treatment ($F(1,25) = 27.70$, $p < .001$), a significant time x drug treatment interaction ($F(20,500) = 40.36$, $p < .001$). Post-hoc analysis per time point revealed that DEX treated animals score significantly higher on pnd 6, 7 and 8, whereas SAL treated animals score significantly higher on pnd 11-20 (all $< .01$ and pnd 20 $p = .044$) leading to full fur development 4 days earlier in SAL compared to DEX treated animals.

### Spatial learning: Circular Hole Board

We analyzed behaviour during all 6 trials and performance during trial 1 and 2 separately. For latency to first visit the exit hole (fig. 5A) and latency to escape

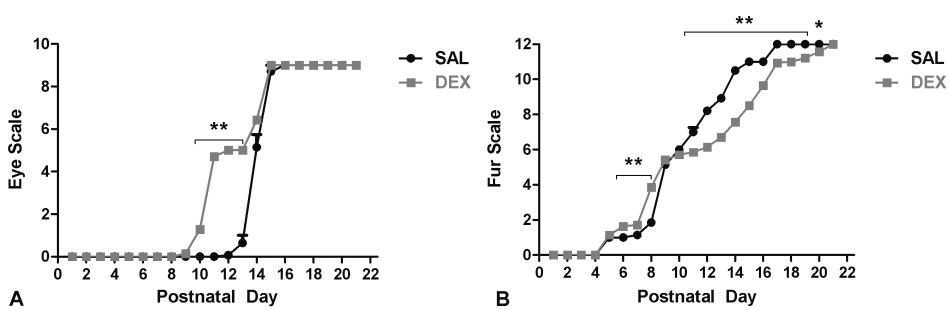


**Fig 4.** Timing of eye opening (A) and fur development (B) in SAL and DEX treated animals during the postnatal period. DEX treated animals show, compared to SAL treated animals, accelerated eye opening and a temporary advantage in fur development. However by pnd 11 SAL treated animals catch up leading eventually to full fur development 4 days earlier compared to DEX treated animals. ** $p < .01$, * $p < .05$

(fig. 5B), significant main effects of time were observed, ($F(2.98,62.47) = 3.08$, $p = .034$) and ($F(3.62,76.09) = 6.60$, $p < .001$) respectively. No effect of drug treatment was observed. For number of holes visited (before the first visit to exit hole, fig. 5C) performance tended to be different between SAL and DEX treated animals ($F(1,21) = 3.28$, $p = .084$). When trial 1 and 2 were analyzed separately, a significant main effect of drug treatment ($F(1,21) = 4.64$, $p = .043$) was observed indicating that DEX treated animals visit significantly fewer holes before going to the one that leads to the escape tunnel, compared to SAL treated animals. Additionally, when the percentage of animals visiting the correct quadrant of the platform during their first hole (any) visit was investigated, we observed that a slightly higher (although not statistically significant) percentage of DEX treated animals search for the exit hole in the correct quadrant compared to SAL treated animals in trial 1 ($\chi^2$ (1) 3.486, $p = .062$; fig. 5D). Behaviour during the memory trial was not different between SAL and DEX treated animals.

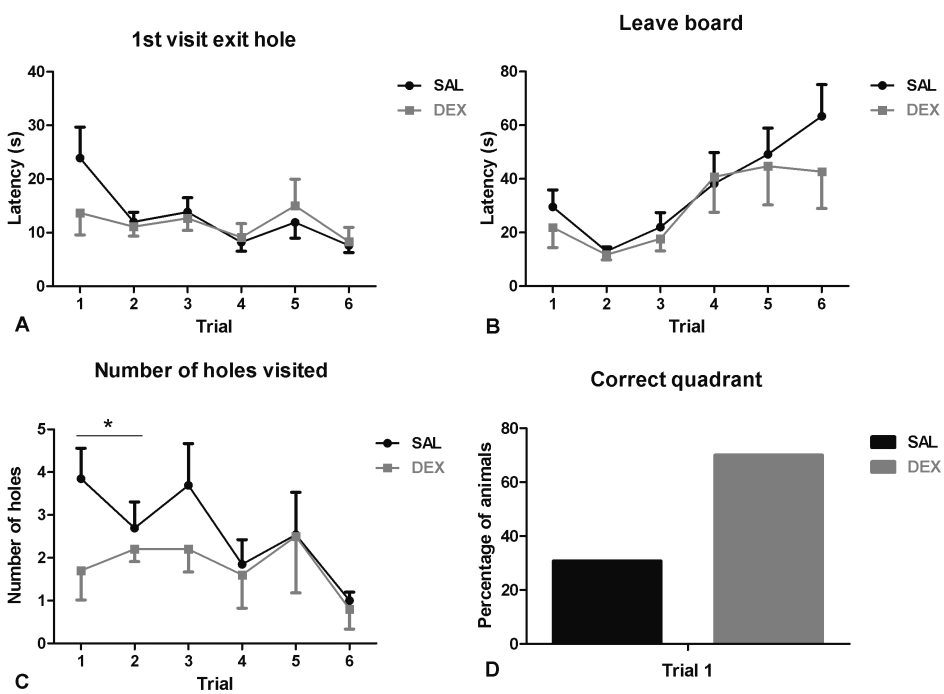


**Fig 5.** Spatial learning on the Circular Hole Board. Latency to first visit the exit hole (A) and latency to leave the platform via exit hole (B) is not different between SAL and DEX treated animals. DEX treated animals visit fewer holes before finding the exit hole compared to SAL treated animals during the first 2 training trails (C). A higher percentage of DEX treated animals visits the correct quadrant of the hole board when visiting a hole for the first time compared to SAL treated animals, although this effect is not statistically significant (D). * $p < .05$.

### Contextual fear conditioning

Freezing, as well as other behaviours measured in the fear conditioning paradigm were not significantly affected by neonatal exposure to glucocorticoids (data not shown).

### Endocrine response to restraint stress

We did not observe DEX-induced changes in ACTH or CORT level under basal conditions or in response to acute restraint stress in adulthood (data not shown).

## RESULTS EXPERIMENT II

### Endocrine response to restraint stress

Since basal and restraint stress-induced ACTH and CORT levels were not different between SAL H and DEX H animals, data from these groups were pooled and analyzed as a handling (H) group. For ACTH a significant effect of time ($F(2.76,63.46) = 62.59$, $p < .001$) and a time x handling interaction ($F(2.76,63.46) = 3.44$, $p = .025$) were observed. The effect of handling did not reach statistical significance ($F(1,23) = 4.06$, $p = .056$).

Post-hoc analysis per time point revealed a significantly lower ACTH level in H animals at t2 ($p = .044$), t5 ($p = .002$), t60 ($p = .048$) and t120 ($p = .000$), while peak level (t30) was not different (fig. 6A). Basal ACTH levels appeared to be higher H animals, although this effect was not statistically significant ($p = .051$).

For CORT level, a significant main effect of time ($F(3.03,87.78) = 331.21$, $p < .001$) and a time x handling interaction ($F(3.03,87.78) = 10.32$, $p < .001$) were observed. Post-hoc analysis per time point indicates that H animals have significantly higher CORT at t30 ($p = .006$) but lower CORT at t120 ($p < .001$) (fig. 6B).

### Prepulse Inhibition

Mean PPI is significantly different between UNT NH animals and animals from the two H groups (SAL H and DEX H) ($F(2,23) = 22.01$, $p < .001$) indicating that

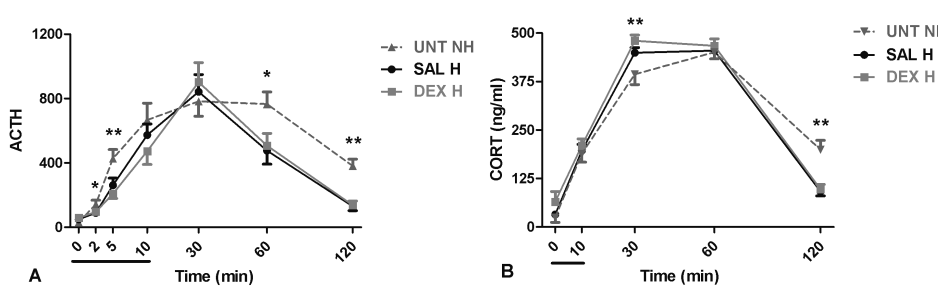


**Fig 6.** Plasma ACTH (A) and CORT (B) levels before, during and after 10 min restraint stress (indicated with horizontal black bar). H animals displayed lower ACTH levels at t2, t5, t60 and t120 compared to NH animals. H animals show higher CORT levels at t30 but lower CORT levels at t120. ** $p < .01$, * $p < .05$

H animals show enhanced PPI compared to UNT NH animals. Post-hoc analysis revealed significant differences between UNT and SAL ($p < .001$) and between UNT and DEX ($p < .001$) (fig. 7).



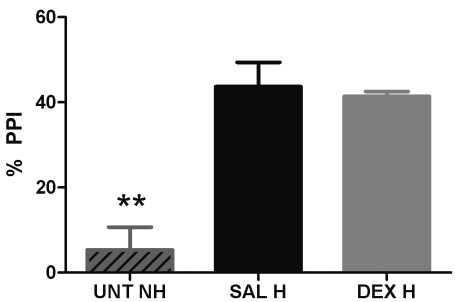


**Fig 7.** Prepulse Inhibition. H animals (SAL H and DEX H) show enhanced PPI compared to UNT NH animals. ** $p < .01$

## Motor performance

### *Balance beam*

Performance (latency to fall) did not differ between treatment groups when analyzed per session. When performance was analyzed within groups to investigate improvement over test sessions, repeated measures ANOVA revealed no improvement in UNT NH animals (fig. 8A). Since SAL H and DEX H did not show differences in performance, data from these groups were pooled. A significant main effect of time was observed ($F(1.67,35.07) = 4.35$, $p = .026$) indicating that performance improved over the different test sessions in H animals (fig. 8B). Moreover a paired t-test showed that performance on day 2 was significantly higher compared to day 1 in H animals ($t = -2.362$ (21), $p = .028$), whereas there was no such effect in UNT NH animals.

### *Rota-rod*

Overall one-way ANOVA revealed that performance during habituation session 1 is comparable for all groups (fig. 9A). During habituation session 2 however, UNT NH animals fall significantly more compared to the two H groups ($F(2,30) = 3.45$, $p = .045$, fig. 9B). All groups fall less frequently during the 2$^{nd}$ compared to the 1$^{st}$ habituation session, however this finding indicates that improvement is stronger in H animals.

Performance on the accelerating rota-rod - on test day 2 - revealed that UNT NH appear to have a shorter latency to fall compared to H animals, although this effect was not statistically significant ($F(2,30) = 3.23$, $p = .054$; fig. 9C).

### *Foot-fault test*

One-way ANOVA indicated that the total number of steps was somewhat lower (although not statistically significant) in DEX H compared to SAL H and UNT NH animals ($F(2,29) = 2.97$, $p = .067$, fig. 10). The error rate was not different between groups (26-27% in all groups).

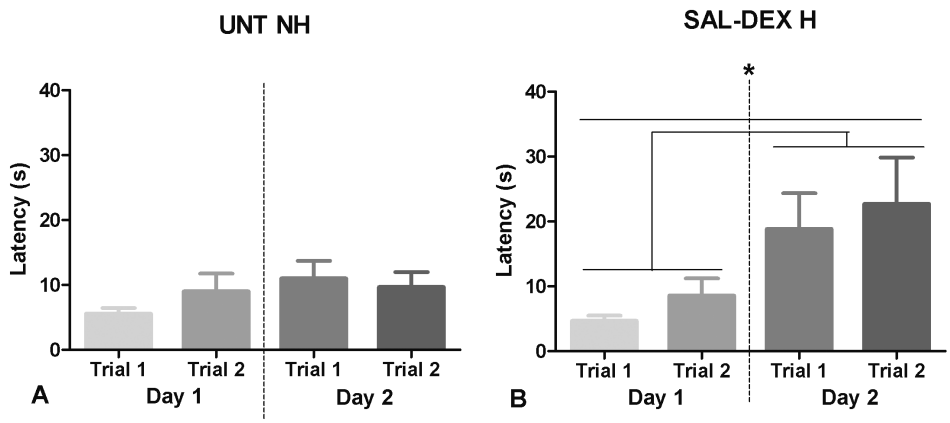


**Fig 8.** Latency to fall from balance beam during 4 sessions over 2 test days. UNT NH (A) animals do not show improvement (increased latency to fall) in contrast to H animals (B) which improve significantly during the different sessions. * $p < .05$

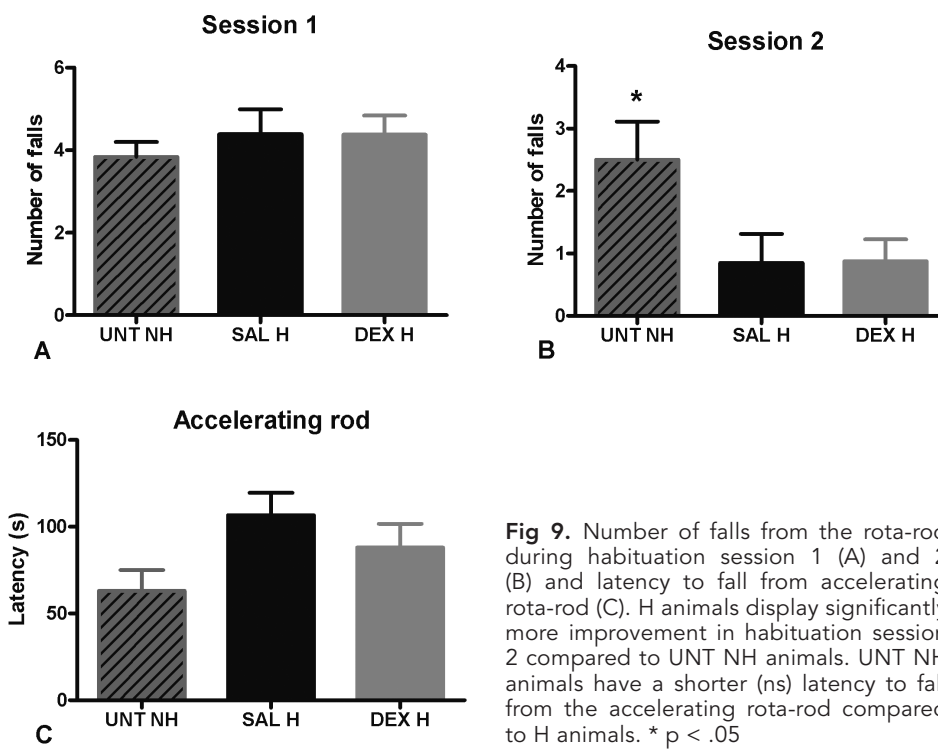


**Fig 9.** Number of falls from the rota-rod during habituation session 1 (A) and 2 (B) and latency to fall from accelerating rota-rod (C). H animals display significantly more improvement in habituation session 2 compared to UNT NH animals. UNT NH animals have a shorter (ns) latency to fall from the accelerating rota-rod compared to H animals. * $p < .05$

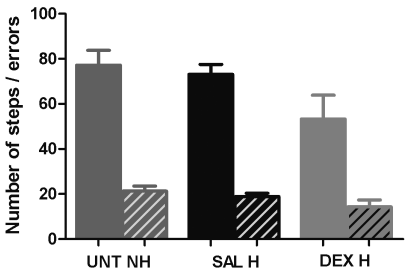


**Fig 10.** Total number of steps and errors in foot fault test. DEX H animals tend to be overall less active compared to SAL H and UNT NH animals, however error rate is comparable between groups.



### Water maze performance

Repeated measures ANOVA revealed a significant main effect of time ($F(3,72) = 14.69$, $p < .001$) and handling ($F(1,24) = 7.55$, $p = .011$) indicating that UNT NH animals show higher latency to reach the platform compared to H animals during spatial learning (day 1-4). Performance during reversal learning on day 5 was not different between groups (fig. 11A). Additionally, on day 1 of spatial learning 65% of UNT NH animals did not reach the platform within 120 seconds and needed to be guided there, compared to only 25% of SAL H and 33% of DEX H animals (fig. 11B). Chi-Square analysis revealed that H animals need significantly less guiding on day 1 compared to UNT NH ($\chi^2$ (1) 6.857, $p = .009$).

### Body Weight

Analysis of body weight of SAL H and DEX H animals shows a significant effect of time ($F(15,240) = 118.89$, $p < .001$) and a time x drug treatment interaction ($F(15,240) = 2.08$, $p = .011$). Post-hoc analysis per time point revealed that the weight of DEX-treated animals is significantly less up to week 44 (all $p < .05$). When age increases further, differences between SAL H and DEX H animals disappear.

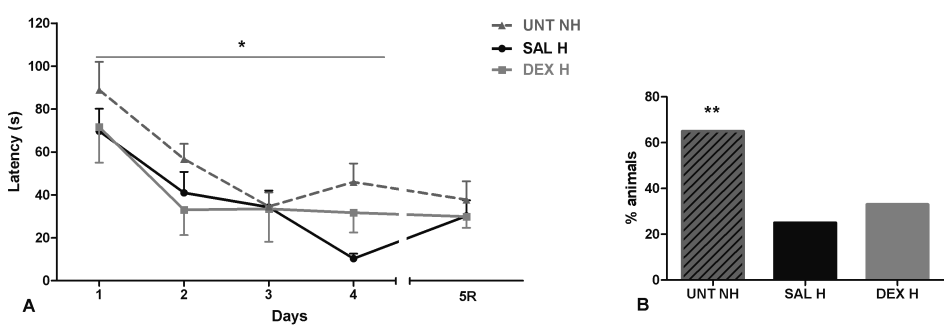


**Fig 11.** Spatial learning in the Morris Water Maze. Latency to reach the platform (A): performance per day (as average of 2 trials/day) on day 1-4 (spatial learning) and day 5 (reversal learning). H animals reach the platform significantly faster compared to NH animals on days 1-4. Performance during reversal learning on day 5 is not different. On day 1 a significantly higher percentage of UNT NH compared to H animals need to be guided to the platform (B). * $p < .05$ ** $p < .01$

When UNT NH animals are included in the analysis, repeated measures ANOVA shows a main effect of time ($F(2.96,71.04) = 199.62$, $p < .001$), without effects of drug treatment or handling (fig. 12).

### Survival

Although DEX H animals appear to die at a younger age and reach 50% survival several months before SAL H and UNT NH animals, Kaplan Meier analysis does not indicate significant differences in survival between treatment groups up to the age of 26 months (fig.13).

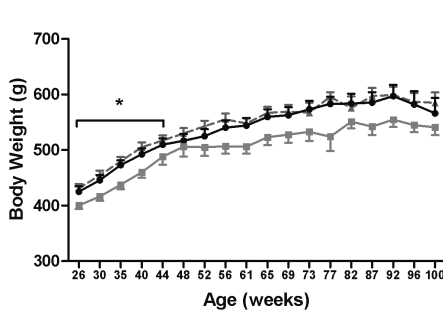


**Fig 12.** Body weights of SAL H, DEX H and UNT NH animals from week 26 – 100. Up to 44 weeks of age, DEX H animals show lower body weight compared to SAL H. However with increasing age differences in body weight disappear. * $p < .05$ (SAL H vs DEX H)

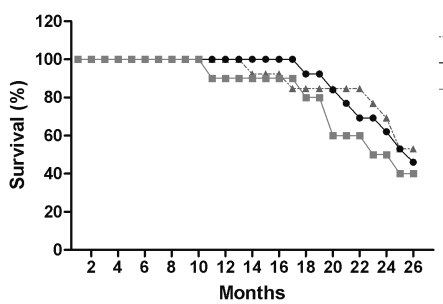


**Fig 13.** Survival curves of UNT NH, SAL H and DEX H animals up to 26 months of age. Although DEX H animals appear to have the steepest survival curve, differences between groups do not reach statistical significance.

## DISCUSSION

The goal of this study was to investigate the impact of neonatal glucocorticoid treatment on development, as well as on adult and aged phenotype in rats. We reported that neonatal DEX treatment resulted in developmental alterations in body weight, eye opening and fur development. However, we did not observe the frequently described alterations in cognitive performance and stress responsiveness in adulthood. Interestingly, the current experimental design consisted, besides manipulations necessary for injections on pnd 1, 2 and 3, of a substantial amount of neonatal handling during the full postnatal period (pnd 1-21) because of daily weighing and marking for discrimination between

individual pups receiving different treatments within the litter. Therefore, we included an untreated non-handled control group - originating from the same cohort of animals - in part II of the experiment to investigate the impact of neonatal handling. Indeed we observed on several parameters (endocrine and behavioural) throughout the lifespan a profound effect of neonatal handling without substantial differences between SAL and DEX-treated animals. Therefore we suggested that the impact of neonatal handling may have interacted with and potentially protected against DEX-induced alterations.

**Neonatal glucocorticoid treatment: impact on development**

The impact of neonatal glucocorticoid treatment on development has been frequently reported. Reduced growth, similar to the findings in the current study, has been demonstrated in human as well as animal studies (21, 23-26). Although we have not investigated the underlying mechanism, others have suggested that DEX prevents adequate growth by inducing protein catabolism during a developmental period normally characterized by low levels of circulating glucocorticoids, thus promoting an anabolic state (27).

Additionally, Vazquez and colleagues suggested that the reduction in weight gain could be attributed to an inability of DEX pups to get milk from the mother due to poor suckling. Their findings also demonstrate that DEX treated rat pups have lower post-weaning food intake (28). Human studies however demonstrate that the reduced growth seen in infants receiving DEX treatment cannot be explained by decreased energy intake or increased expenditure, but may be due to differences in the composition of newly accreted tissue due to a shift in intermediate metabolism (29).

Besides reduced growth, other developmental alterations are associated with neonatal glucocorticoid treatment. We report changes in eye opening and fur development which have also been reported in previous literature (23). The mechanism underlying these findings is still poorly understood. Neurodevelopmental delay following DEX treatment is frequently explained as being the result of inhibition of normal myelination processes (23). Peripherally, the impact of DEX has been often studied in the context of lung development. During normal development, glucocorticoids regulate the degree of proliferation and differentiation. Glucocorticoid-insufficient (CRH knock-out) animals, suffering perinatally from abnormal pulmonary development due to hyper-proliferation, can be rescued by exogenous glucocorticoid treatment (30). In premature infants, glucocorticoid treatment can enhance lung maturation (31) by stimulating differentiation of epithelial cells (32). Exogenous glucocorticoid administration during normal development leads to hypo-proliferation, as well as to pulmonary epithelial maturation (33).

All together, it appears that glucocorticoid exposure enhances maturation and differentiation at the expense of growth and proliferation, as is reviewed by Bolt (34), which is either beneficial or detrimental depending on the developmental context. Similar alterations in other tissues might underlie the developmental alterations observed in the current study.

**Long-lasting effects on adult phenotype**

Many studies have demonstrated long-lasting effects of neonatal DEX treatment on adult phenotype in terms of cognitive performance, hippocampal function, emotionality and stress responsiveness (19, 22, 28). Interestingly we did not observe such effects in the current study. Does this mean that DEX-induced alterations are not as adverse as suggested previously? There might be other factors playing a role in determining phenotypic outcome. Many of the previously mentioned studies have used Sprague-Dawley or Wistar rats as subjects, which might have a different sensitivity for neonatal glucocorticoid exposure, or early-life experiences in general, compared to Long-Evans rats. A comparable phenomenon has been reported in mice, in terms of sensitivity to the programming effects of maternal care on drug self-administration and depression-like behaviour (35). Additionally, besides the use of different strains, there is substantial variation in the treatment regimen among studies investigating the impact of neonatal exposure to glucocorticoids. Dosing and time of treatment varies greatly, as well as post weaning housing conditions. These factors are likely to contribute to determining treatment outcome.

Furthermore, the DEX-induced effects reported previously might be specific for certain behavioural paradigms. In contrast to many studies testing spatial learning using a water maze paradigm (19, 36), we initially used a circular hole board. It is known that the water maze, compared to the hole board results in substantially higher HPA axis activation (37). Altered expression of the glucocorticoid receptor (GR) after neonatal DEX treatment (38), might explain changes in water maze performance between SAL and DEX treated animals without differences on the hole board.

Surprisingly, we observed slightly enhanced performance on certain aspects of circular hole board learning in DEX compared to SAL-treated animals. Moreover, when tested at 22 months of age in the water maze, DEX-treated animals overall do not show learning impairments, although SAL animals appear to continue to improve performance on the last learning day (day 4) in contrast to DEX animals who reach their maximum performance on day 2 without further improvement.

Additionally, the type of stressor appears to determine the DEX-induced effect on the endocrine response. Although DEX-treated animals have been reported to show a blunted CORT response to novelty stress; CORT levels did not differ from SAL-treated animals after experiencing conditioned fear (20). Immobilization stress (used in this study) can be considered a severe stressor; leading to a greater HPA axis activation compared to other stressors (39). This potentially reduced the likelihood to reveal differences between SAL and DEX-treated animals in stress responsiveness, due to a ceiling effect.

However, Felszeghy and colleagues did report a suppressed elevation of both ACTH and CORT in response to restraint stress in adult rats that were neonatally exposed to DEX, but on pnd 1, 3 and 5 (40). Contrarily, other studies have reported a prolonged (rather than blunted) CORT response in DEX compared to SAL-treated animals after crowding stress (23), indicating that the DEX-induced

effect on the endocrine stress response is highly dependent on the type of stressor applied.

Hence, another factor potentially contributing to the somewhat surprising differences in outcome lies in the experimental design. As mentioned before, our design involved a substantial amount of neonatal handling. Besides manipulations necessary for injections on pnd 1, 2 and 3, SAL and DEX treated animals were daily weighed and marked during the full postnatal period in order to discriminate between animals receiving different treatments within the litter. Other studies investigating the long-term effects of neonatal DEX-treatment have used either a between-litter design (21) or a within-litter design with use of another type of marking (41) reducing the amount of daily handling.

The within-litter design was chosen because it has the advantage of having both the genetic contribution as well as the shared maternal environment from a given litter represented in both treatment groups. Daily marking using a non-permanent odourless marker was chosen since it is less invasive compared to a tattoo or toe clip. However, marking did lead to a substantial amount of daily handling of the neonate up to weaning age.

**Impact of neonatal handling**

More than 6 decades ago, it was discovered that brief (3-15 min) daily separations between rodent mother and pup between pnd 1 and 21 had long-lasting impact on adult stress phenotype (42). Follow-up studies demonstrated that these manipulations resulted in HPA axis hypo-responsiveness (10, 43, 44) likely mediated by altered GR expression (45). Additionally, reduced emotionality (43), and increased cognitive performance (46) were reported in handled (H) rats compared to rats raised in undisturbed laboratory conditions, i.e. non-handled (NH).

The data suggest that the effects of H might have potentially compensated for certain DEX-induced alterations. Regarding cognitive performance, H has been shown to improve spatial learning on the circular hole board (47) and in the water maze, an effect that lasts up to old age (48, 49) like we observed in the current study. These findings suggest that the effects of H might have overruled DEX-induced adverse effects on spatial learning.

Interestingly, the effects of H and DEX treatment (20, 22, 44) on endocrine stress responsiveness are suggested to point in the same direction, i.e. they both result in a blunted response and enhanced feedback sensitivity (although likely via different mechanisms). In the current experiment, we observed the frequently described effect of H on the stress response without additional effect of DEX exposure. The effect of H might have overruled a potential HPA suppressing effect of DEX. If both of these individual effects are present, they apparently do not work synergistically, since DEX H animals do not differ from SAL H in their endocrine responsiveness.

Neonatal glucocorticoid treatment has resulted in inconsistent findings regarding PPI phenotype. Ferguson and colleagues report no effect of DEX treatment (50) whereas Hauser and colleagues report an increase in PPI after

prenatal DEX treatment, which was not replicated (51). Additionally, our data indicate no differences between SAL and DEX treated animals. In contrast to other studies demonstrating no effect of H on PPI (52), we report that H enhanced PPI substantially.

Interestingly, other pre-weaning and post-weaning manipulations like maternal deprivation and social isolation (53, 54) have been reported to affect PPI. In this study - besides differences in postnatal experience - there is also a substantial difference in post-weaning manipulation between the H and UNT NH animals up to the age of 6 months (due to extensive behavioural testing of the H animals), which could explain differences in PPI between these groups. However, a study from our laboratory investigating this effect (post-weaning manipulation due to exposure to behavioural testing) indicates no differences between animals with a comparable postnatal experience which are either extensively tested or undisturbed during the post-weaning period (Claessens et al, unpublished data) suggesting that the differences in PPI in the current study can be attributed to early-life experiences.

We did not report DEX induced alterations in motor performance in middle aged animals, in contrast to several human and animal studies showing DEX induced effects on neuro-motor development and performance (55, 56). Findings from human studies have suggested that these impairments cannot be fully attributed to DEX treatment, but that medical and socio-demographic factors other than GC treatment also contribute to the phenotype (57). Although others have suggested that motor performance in aged animals is not affected by H (49), we demonstrate beneficial effects of H on motor learning in middle aged rats, potentially interacting with or compensating for the effects of neonatal DEX exposure.

Finally, we report that DEX treated animals appear to have a steeper survival curve, but in contrast with other rodent studies showing shortening of the lifespan after perinatal DEX treatment (21, 58, 59), this effect did not reach statistical significance. Whether a DEX effect might have interacted with potential beneficial effects of H remains to be investigated. However, we did not demonstrate an overall effect of H on lifespan.

The effects of H are suggested to be mediated via enhancing maternal care (60). It has to be noted that the postnatal manipulations in the current study are not identical to H as it is known in literature. Besides daily periods of brief separation, the offspring was exposed to the procedure of marking and weighing. Altogether, this can be considered a more abundant form of handling, which has been shown to interact with other developmental experiences (61). Unfortunately we have not studied maternal behaviour in the current experiment, but we did observe increased maternal licking and grooming directed at H compared to NH offspring under comparable experimental conditions (Claessens et al, unpublished data). Interestingly, Brabham and colleagues reported that certain (prenatal) DEX induced effects can be normalized by enhanced levels of maternal care during the postnatal period (36). In line with these findings we suggest that enhanced maternal care, as a result of H, is likely to contribute to 'rescuing' the DEX phenotype.

## CONCLUDING REMARKS

We did not observe the frequently reported programming effects of DEX treatment on adult phenotype, while DEX clearly affected the developmental trajectory and body weight. However, we did observe profound and long-lasting effects of H in both SAL and DEX treated animals. Since, to our knowledge, this treatment design (combining H and glucocorticoid treatment) has not been used before, we suggest that H might serve as a protective intervention, potentially compensating for the impact of neonatal DEX exposure. From these findings we cannot conclude that the effects of H have overruled DEX-induced alterations or that the current DEX-treatment would have resulted in a different outcome if administered in a NH context. Whether H can in fact compensate for DEX-induced alterations in adult phenotype remains to be investigated in follow-up studies using appropriate control groups investigating the impact of DEX in a handing and non-handling context.

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