Brain Research

Research Report

Neuroendocrine responses in neonatal mother-deprived rabbits

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A B S T R A C T

To study both short- and long-term adaptation mechanisms activated by rabbits which were separated from their mothers (DLS) for 48 h between postnatal days 9 and 11, we examined plasma corticosterone concentrations before, during, and after DLS as well as the expression of glucocorticoid receptor (GR) in the hippocampus and the adrenal axis responses to a mild stress stimuli or exogenous ACTH injection. At postnatal day 10, plasma corticosterone concentration of DLS rabbits was similar to that of controls, but rose two-fold at day 11 (17.7 ± 1.3 vs. 9.3 ± 1.2 μg/dl, $P < 0.01$) and then decreased at day 12, when suckling was allowed again, to match those of controls with no difference thereafter. At postnatal day 14, both control and DLS rabbits had similar basal corticosterone concentrations (2.2 ± 0.4 vs. 2.3 ± 1.1 μg/dl, respectively) as well as at day 120 (8.8 ± 3.2 vs. 9.7 ± 2.8 μg/dl, respectively). After the standardized stress stimulus, plasma corticosterone concentrations were lower in DLS rabbits than controls at postnatal days 14 ($P < 0.01$) and 120 ($P < 0.05$). At day 120, corticosterone levels rose similarly seven-fold ($P < 0.01$) within 30 min after ACTH administration and remained sustained thereafter in both control and DLS rabbits. Positive immunoreactivity for GR was detected in the hippocampus and in the dorsal medial hypothalamic region at postnatal day 14. The present data suggest that 48 h DLS from postnatal days 9 to 11 results in a modified hypothalamic–pituitary–adrenal axis reactivity later in life.

1. Introduction

Recently, transient doe–litter separation (DLS), a bio-stimulatory technique that improves sexual receptivity and fertility of lactating rabbit does (Theau-Clément et al., 1998; Bonanno et al., 1999, 2002) has been the object of considerable attention. However, there is some concern that the separation and long-term fasting necessarily involved in DLS may induce stress in

Table 1 – Effect of a 48-h mother–litter separation (DLS) between postnatal days 9 and 11 on the pre-weaning growth of young rabbits.

	Postnatal day	Treatment		SEM	P-value		
		DLS	Control		Treatment (T)	Parity (P)	T * P
Kit weight (g)	1[a]	63	65	1.9	ns	ns	ns
	9	164	164	4.4	ns	0.0396	ns
	11	152	191	4.6	<0.001	0.0410	ns
	14	225	255	6.3	<0.0011	ns	ns
	35	733	803	19.9	0.0161	0.0099	0.0387
Kit daily gain (g)	1–9	13.9	13.6	0.47	ns	ns	ns
	9–11	−6.1	13.7	0.46	<0.001	ns	ns
	11–14	16.1	14.2	0.76	ns	ns	ns
	14–35	24.5	26.4	0.80	ns	0.0036	0.0241
	1–35	19.4	21.4	0.57	0.0174	0.0064	0.0284

[a] After litter equalization.

young rabbits. In fact, evidence from a number of studies suggests that stressful events, such as early maternal separation, may program the hypothalamic–pituitary–adrenal (HPA) axis differently through the regulatory effects of corticosteroids on the plasticity in several areas of the central nervous system and alter physiological and behavioral responses to stress later in life (Meaney et al., 1996; Ladd et al., 2000; Lehmann et al., 2002a; Schmidt et al., 2008). Interestingly, in rats, the endocrine response of the HPA axis to neonatal maternal deprivation can be either enhanced or dampened, depending on the postnatal days at which they are separated from the dam (Levine et al., 1991; Plotsky and Meaney, 1993; Liu et al., 1997). Moreover, a window of complete unresponsiveness of the HPA axis to stressful events was found in rats between postnatal days 1 and 12 (van Oers et al., 1997). However, there are still conflicting data on the existence of a similar hypo-responsive period in neonatal rabbits (Pradier and Dalle, 1996; Rovirosa et al., 2005).

To date, no experiment has been done to assess the level of stress caused to young rabbits by early maternal separation and the question whether the DLS exerts any long-lasting effects on postnatal developmental traits has been only marginally addressed by examining pre- and post-weaning growth and mortality rates (Bonanno et al., 2004; Rebollar et al., 2006). Similarly, there have been no studies evaluating the long-term influences of DLS on the physiological responses of rabbits to stress, although there is evidence that early handling can reduce fear reaction to humans (Denenberg et al., 1977; Bilko and Altbäcker, 2000; Pongracz and Altbäcker, 2003). In rabbits, the natural mother–offspring contacts during early development are minimal and limited to few minutes per day during the single suckling episode (Escobar et al., 2000). Thus, in analyzing physiological and behavioral responses of neonates which were separated from their mothers, the rabbit model has the unique advantage of decupling potential confounding mother–offspring interactions.

Therefore, in this study we examined the plasma corticosterone concentrations before, during, and after DLS, from postnatal days 2 to 14, as well as the expression of glucocorticoid receptor (GR) in the hippocampus at day 14 and the adrenal axis responses to a mild stress applied at day 14 or day 120 in order to better understand the short- and long-term adaptation

mechanisms activated in rabbits that were separated from their mothers for 48 h between postnatal days 9 and 11.

2. Results

2.1. Pre-weaning body weights and behavioral evaluation

The number of live-born, still-born, and total-born kits per litter did not differ among groups (8.1±0.87 vs. 7.2±1.4, 0.76± 0.33 vs. 1.2±0.52, and 8.9±0.75 vs. 8.4±1.18 for DLS and controls, respectively). Pre-weaning body weights and growth rates of kits of DLS and control groups are summarized in Table 1. Body weights at postnatal days 1 and 9 as well as daily body gain between days 1 and 9 were similar for both groups. DLS pups were lighter than controls at postnatal days 11, 14, and 35, but there was no difference in the daily weight gains in this period (Table 1).
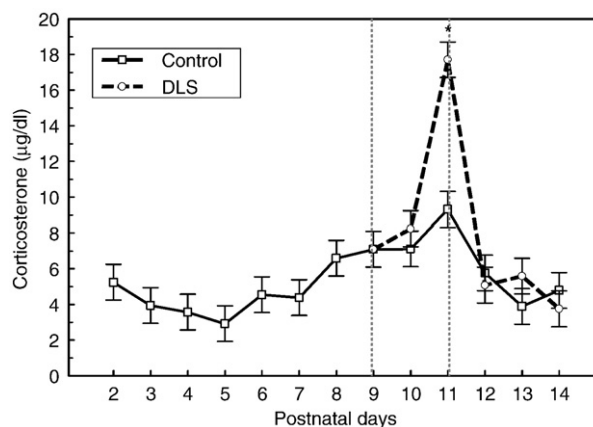
Fig. 1 – Plasma corticosterone profiles in young rabbits from postnatal days 2 to 14. The DLS young rabbits were separated from their mothers for 48 h from postnatal days 9 to 11 (dotted lines); thereafter, the does were allowed free access to the nest-box like the control rabbits. Blood samples were taken by cardiac puncture within 30 s from the capture of each newborn rabbit. Each time point represents the mean± SEM of five young rabbits. Asterisks mark significantly different values (P<0.01) from corresponding controls.

At day 10, after 24 h of separation, DLS litters showed the typical arousal state that normally anticipates the visit of the doe for nursing: pups, freed from the nest material, scratched and licked themselves, and climbed over each other. At day 11, after 48 h of separation, DLS litters were similarly deprived of the nest material, but the kits were more quiet compared with the behavior of the previous day and the majority huddled together and slept, while only few climbed slowly over the other inactive kits. At both days 10 and 11, control litters huddled quietly covered by nest material.

2.2. Corticosterone during postnatal development and maternal separation

In newborn control rabbits, plasma corticosterone concentrations showed a high day-to-day variability ranging from 3 to 8 µg/dl (Fig. 1). In DLS young rabbits, at day 10, mean basal corticosterone concentration was similar to that of controls (Fig. 1), but rose two-fold at day 11 (17.7±1.3 vs. 9.3±1.2 µg/dl; $P<0.01$). The next day, when suckling was allowed again, corticosterone level of DLS rabbits matched that of controls and no difference was observed thereafter up to postnatal day 14.

2.3. Response to acute stress

At postnatal day 14, both control and DLS rabbits had similar basal corticosterone concentrations (2.19±0.4 vs. 2.35±1.1 µg/dl, respectively), but DLS neonate rabbits displayed lower reactivity to the standardized stress stimulus as assessed by the modest change in plasma corticosterone concentrations compared to the four-fold increase ($P<0.01$) in controls 30 min later (Fig. 2). At day 120, basal corticosterone concentrations

did not differ between control and DLS rabbits (8.8±3.2 vs. 9.7± 2.8 µg/dl, respectively). In response to saline injection, plasma corticosterone concentrations rose two-fold ($P<0.05$) in control rabbits, whereas they remained almost unaffected in DLS rabbits during the time frame examined up to 120 min (Fig. 3A). Following ACTH administration, corticosterone levels rose similarly seven-fold ($P<0.01$) within 30 min and remained sustained thereafter in both control and DLS rabbits (Fig. 3B).

2.4. Glucocorticoid receptors

Using a monoclonal antibody, positive staining for GR was evidenced in the hippocampus (Fig. 4) and the dorsal medial hypothalamic area (Fig. 5) of young rabbits belonging to both
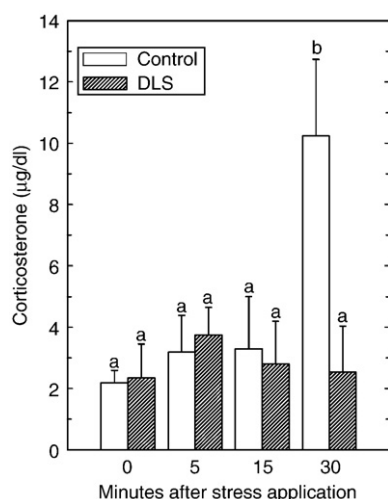

Fig. 2 – Corticosterone (µg/dl) response to a standardized stress induced in control and DLS young rabbits aged 14 days by removing the animal from the cage and placing it in a new closed box for 5 min, and by i.m. saline injection. Blood samples were taken by cardiac puncture just before (basal) and 5, 15, and 30 min after the application of the stress stimulus. Columns represent the mean±SEM of five rabbits for each data point. Different letters above bars identify significantly different values ($P<0.01$).

Fig. 3 – Corticosterone (µg/dl) response to a standardized stress induced in control and DLS young rabbits aged 120 days by i.m. saline injection (panel A) or 30 µg/kg ACTH (panel B). Blood samples were taken by catheterization of the ear central vein just before (basal) and 30, 60, 90 and 120 min after the injection of saline or ACTH. Columns represent the mean±SEM of five rabbits for each data point. Different letters above bars identify significantly different values ($P<0.05$ for panel A, and $P<0.01$ for panel B).
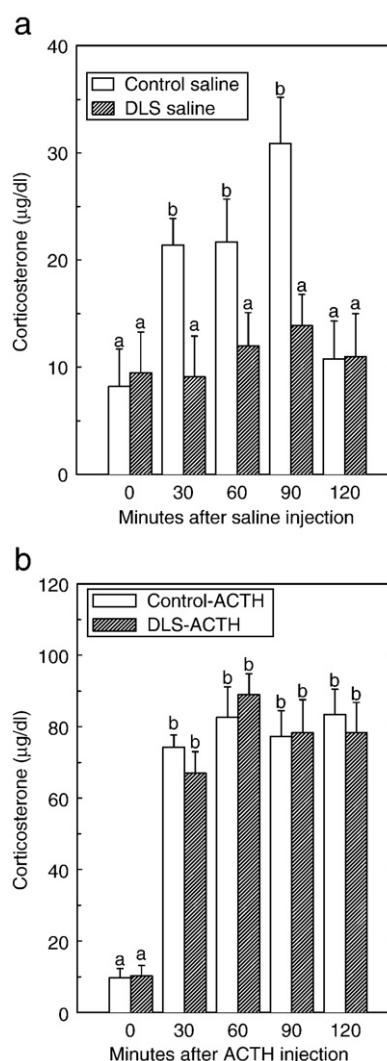
DLS and control groups. Within the hippocampus, the majority of GR immunoreactivity was found in neurons that formed the granular layer (Figs. 4b and d) with a comparable density in DLS and control groups (94.2±1.1 vs. 93.9±0.9, respectively). The number of GR positive cells quantified in the hippocampus within the sub-areas CA1, CA3, and DG outside the granular layer (Figs. 4a and c), was similar in DLS and control groups (5.9±0.3 vs. 7.4±0.7, respectively). The density of GR imnnunoreactive neurons in the hypothalamus (Figs. 4e and f) was higher (P<0.01) in DLS than in control group (21.4± 2.9 vs. 7.8±1.3, respectively).

3. Discussion

To our knowledge, this is the first paper characterizing the function of the hypothalamic–pituitary–adrenal axis in neo-natal DLS rabbits by examining at different ages changes in circulating corticosterone following mild stress stimuli or exogenous ACTH injection. Whereas lactational performance and endocrine responses of nursing rabbit does during
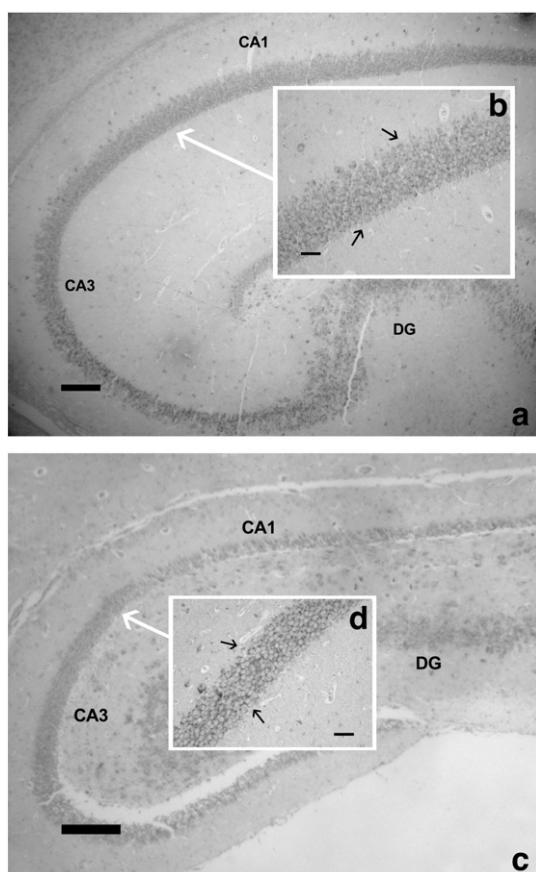
Fig. 4 – Immunohistochemical localization of glucocorticoid receptor (GR) in the dorsal hippocampus of DLS (a) and control (c) young rabbits at postnatal day 14. CA1, CA3, and DG (dentate gyrus) indicate the main hippocampal anatomical regions. The inserts b and d detail GR-positive neurons (arrows) of the granular layer in DLS and control rabbits, respectively. In photographs a and c scale bar=140 μm; in insert micro-photographs b and d scale bar=40 μm.
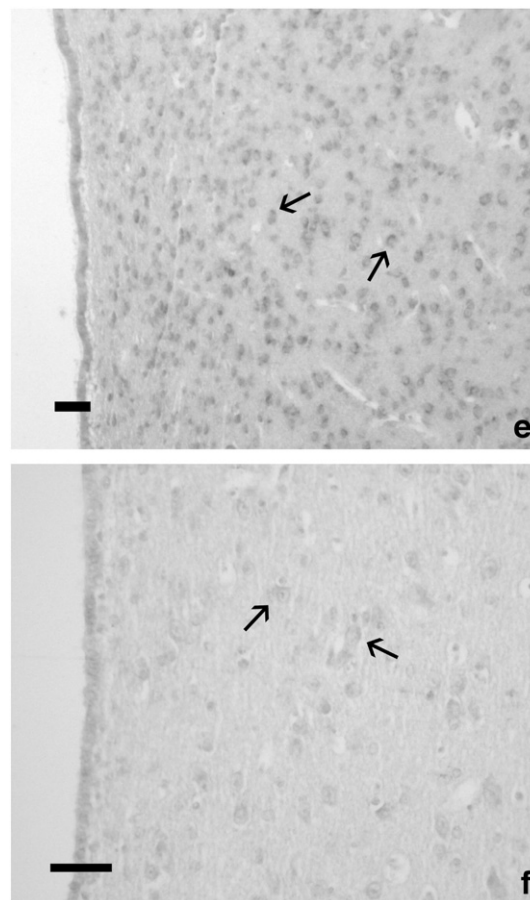
Fig. 5 – Immunohistochemical localization of glucocorticoid receptor (GR) in the dorsal medial hypothalamic region of DLS (e) and control (f) young rabbits at postnatal day 14. The arrows point to GR-positive neurons. Scale bar=40 μm.

transient doe–litter separation have been extensively investi-gated (Ubilla et al., 2000a, b, 2001), no study has focused on the long-term outcome of rabbits which were exposed to DLS in the neonatal period apart from pre- and post-weaning growth and mortality rate. In fact, the present study was designed to answer an important question concerning the practice of temporary DLS never critically evaluated in this species: does early maternal-separation and the associated starvation have any adverse, long-lasting consequences on life history, including neuroendocrine and behavioral disturbances, in the exposed individuals? The answer is intrinsically complex due to a great array of interacting factors that may directly and/or indirectly affect behavior and physiological functions of the individual animal during postnatal development. Undoubtedly, for the altricial young rabbits, a 48 h maternal separation in early postnatal days is a relevant stressful experience that more likely reflects a condition of progressive, forced starvation rather than a lack of normal neonate–mother interactions. Body weight data clearly point out that the omission of the nursing opportunities exerted a significant impact on the growth rate of the young rabbits, which lasted up to adulthood. In fact, newborn rabbits, which are born blind and hairless and with a limited ability to regulate body temperature (Blumberg and Sokoloff, 1998), rely on daily

milk supply provided by suckling for their postnatal growth. On the other hand, both domesticated and wild rabbit does leave their pups immediately after parturition and usually enter the nest for a short period of time once a day during the nocturnal hours only to nurse them (Zarrow et al., 1965; Hudson and Distel, 1982; González-Mariscal, 2007). Therefore, it is not surprising that in rabbits an endogenous feeding entrainable circadian rhythm is active from the first days of life in order to assure maximal milk intake by alerting the pups in time of the periodical presence of the doe (Escobar et al., 2000; Jilge et al., 2001; Jilge and Hudson, 2001).

The adrenal axis of newborn rabbits is active from the earliest days of life as circulating corticosterone concentrations closely matched those found in both growing (Rommers et al., 2004; Rovirosa et al., 2005; Morgado et al., 2008) and adult animals (Brecchia et al., 2006). At postnatal day 10, after 24 h of maternal separation, no difference in corticosterone levels between treated and control groups was appreciable, although newborn rabbits demonstrated the behavioral anticipatory activity associated with the expected nursing and controlled by a circadian rhythm (Jilge et al., 2001). In our experimental model we did not observe the entrained hormonal rhythm of plasma corticosterone described by Morgado et al. (2008) in anticipation to the expected daily meal. However, this discrepancy is likely due to the different nursing scheme adopted in the two experiments. In our case, the free nursing protocol does not allow an exact timing of the fasting interval because the previous meals were not scheduled. Nevertheless, present findings, although indirectly, confirm that young rabbits are well adapted in coping with fasting by maintaining an adequate energy balance during the relatively long intervals among suckling as shown by Escobar et al. (2000). In fact, despite limited suckling, the high fat milk content allows young rabbits to endure fasting for relatively long periods and assures a large increase in body mass during early life which, in turn, positively affects several other vital traits, including survival and reproduction (Coureaud et al., 2000; Rödel et al., 2008). Contrary to what Escobar et al. (2000) described, pups did not show the typical behavioral arousal anticipating the expected nursing after 48 h of maternal separation. Probably, this discrepancy is due to a misalignment in the 24-h synchronization intervals between meals as a consequence of the free milking protocol. When separation was prolonged far beyond that which neonates normally experience (24 h), glucocorticoid secretion increased markedly probably in response to reduced availability of carbohydrates. Similar findings were observed in adult (Dallman et al., 1999) and young (van Oers et al., 1998b) rats as well as in young growing rabbits as a consequence of the restricted feed intake (Rommers et al., 2004), and in adult rabbits following 48 h starvation (Brecchia et al., 2006).

The relatively high blood corticosterone concentrations observed in the second day of maternal separation confirm that the HPA axis of the DLS rabbits was physiologically stimulated by the exposure to a stressor. The stimulus probably involves metabolic signals from prolonged fasting rather than lack of tactile interaction cues with the dam, although this cannot be ruled out in accordance to what found in rats (Stanton et al., 1988; Suchecki et al., 1993; van Oers et al., 1998b). The activation of the HPA axis probably promotes

survival of the DLS starved neonate rabbits by enhancing gluconeogenesis. This hypothesis is further supported by the findings that at postnatal day 12, following re-establishment of regular nursing (and nutrient supply), corticosterone concentrations of DLS rabbits decreased to values comparable to those of controls, suggesting a fast recovery from the stressful condition of forced starvation due to maternal separation.

This delayed, but quick on/off response may have relevant consequences, since optimal HPA axis function and glucocorticoid response are of critical importance for the survival of the animal after exposure to an adverse environment by suppressing immune system overreactions (Levine et al., 1991; Webster and Sternberg, 2004). Moreover, while the effects of excessive corticosteroid secretion on the brain are usually reversible in the adult, during early development they may cause long-lasting consequences by disorganizing normal neural circuits. The potentially harmful role of glucocorticoid in the rabbit is further confirmed by the finding that postnatal dexamethasone treatments between 2 and 7 days of age permanently alter adult phenotype and cause neurological impairment (He et al., 2004).

The dual response of corticosterone during the maternal separation at postnatal days 9–11 confirms that young rabbits do not have an unresponsive phase during the early days of life, an observation in good agreement with the findings reported by Rovirosa et al. (2005), but does not exclude that pups may have such unresponsive phase before postnatal day 7. In fact, according to Pradier and Dalle (1996), the pituitary–adrenal axis of 7-day-old rabbits was less reactive than that of adult rabbits to corticotropin releasing hormone or arginin vasopressine suggesting that a quiescent phase also exists in this species, similarly to that found in laboratory rodents. However, our results do not necessarily conflict with those of Pradier and Dalle (1996) because, during early development, even few days can influence differently the organization of neural circuits and neuroendocrine phenotypes as well.

For the purpose of studying the relevance of early maternal separation in mediating neuroendocrine modulation of the HPA axis, we adopted a psycho-social stress procedure of a saline injection followed, only in young rabbits, by restrain in a closed box which consistently elicited a pronounced elevation in plasma corticosterone. DLS did not affect basal plasma corticosterone levels later in life, at least as evaluated in 14- and 120-day-old rabbits. These findings are in accordance with those found in neonate rats deprived of their mother for 24 h at different ages (Suchecki and Tufik, 1997; van Oers et al., 1998a; Lehmann et al., 2002b). In DLS rabbits, the dynamic neuroendocrine response of the HPA axis to the standardized stress was similar at both 14 and 120 postnatal days. While corticosterone levels rose from two- to three-fold in control rabbits, they remained almost unaffected in DLS rabbits after the stress stimulus in the time frame examined. Thus, at either postnatal day 14 or 120, DLS rabbits were less responsive to stress, as shown by their lower plasma corticosterone level increases compared to those of controls. A similar response to a mild stress was also described by Suchecki and Tufik (1997) in 20-day-old rats maternally deprived at postnatal day 11. In contrast, exogenous ACTH

injection evoked a similar response in both treated and control groups suggesting that the pituitary–adrenal axis of DLS rabbits was not impaired.

In the present study, we have provided the first evidence that DLS up-regulates the expression for glucocorticoid receptors by increasing the number of the hypothalamic immuno-positive neurons in young rabbits, as assessed by immunohistochemistry, but has no apparent effect on the hippocampus where the density of GR-immunoreactive neurons remained unaffected. Glucocorticoid receptors are widely expressed in specific brain areas including the hippocampus and the hypothalamus of all mammalians studied to date (Pryce, 2008), where they regulate neuroendocrine stress response of the HPA axis as well as behavioral, and cognitive functions (de Kloet et al., 2008). In rats, enhanced glucocorticoid secretion due to maternal deprivation during early development leads to long-term alterations in the number for hippocampal GR and in the phenotypes that they mediate by permanently modifying the level of CRH mRNA in different hypothalamic nuclei and hippocampus (Plotsky and Meaney, 1993; van Oers et al., 1997; Ladd et al., 2000). The differential outcome in the expression of GR in the hippocampus and hypothalamus of DLS rabbits compared to maternal-deprived rats may reflect species-specific difference in the neural system that regulates the HPA function in response to an adverse early life event such as mother separation and consequent starvation.

In conclusion, early maternal separation triggers in rabbits specific, long lasting, adaptations of the HPA axis which becomes less responsive to an acute stress. It remains to be explored, however, whether the initial HPA axis response to early maternal separation is caused by metabolic/hormonal signals or rather by psychosocial stimuli or by the endogenous central nervous oscillator.

4. Experimental procedures

4.1. Animals

New Zealand White (NZW) lactating, hybrid does (Provisal) of different parities, aged 5–12 months and weighing 3.8–4.1 kg, were maintained in indoor brick shed facilities under constant photoperiod 16:8 h light–dark cycle. Ambient temperature was maintained between 22 and 24 °C. The animals were raised individually in standard metal cages, outside of which were placed a feeder and a nest-box. Water and food (a commercial rabbit chow containing 11.0 MJ DE/kg dry matter, 17.2% crude protein, and 15.8% crude fiber) were provided *ad libitum*. The does were artificially inseminated (AI) following a 42-day reproduction rhythm. Semen for AI was collected from NZW rabbit males. Each litter size was equalized to eight to nine young rabbits by cross fostering within 1 day after parturition. The day of birth was designated as postnatal day 0. At day 9 post partum, does of same parity were randomly assigned to control or DLS group. Control does had free access to nest boxes for nursing, whereas DLS does were physically separated from their litters by means of a metal screen for 48 h, from day 9 (11:00 a.m.) to day 11 (11:00 a.m.), when the nest boxes were reopened to allow nursing of litters. Free suckling and weaning at 35 days were standard procedures. Kits were weighed individually at postnatal day 1 after equalization, and again at postnatal days 9 (before DLS), 11 (before nursing), 14, and 35 (at weaning). Behavioral activities of DLS and control litters were recorded for 1 h (10:00–11:00 a.m.) at postnatal days 10 and 11.

4.2. Blood sampling and experimental design

Between postnatal days 2 and 14, blood samples were collected daily from newborn rabbits by cardiac puncture using either an insulin or a 2.5 ml syringe provided with a 23 gauge needle. The bleeding procedure took a maximum of 30 s from the moment the kit was removed from the nest-box. Whenever the procedure exceeded this amount of time, the sample was discarded. Casualties due to blood sampling were 1.5%. Upon successful or unsuccessful blood collection, rabbits were marked with an indelible pen mark before being returned to their nest boxes. For each day or time point, a different set of neonate rabbits was used so that each animal underwent only one bleeding throughout the experiment. From 120-day-old rabbits, blood samples were collected via a Teflon catheter (18 gauge, Terumo, Leuven, Belgium) inserted and secured in the central ear vein the day before the experiment. All the blood samples, placed in pre-cooled plastic tubes containing EDTA, were immediately centrifuged at $3000 \times g$ for 15 min and the plasma stored frozen until assayed for hormones. The bleeding sessions were carried out between 10:00 and 11:00 a.m.

To assess the impact of DLS on adrenal axis function of mother-deprived rabbits a total of 40 lactating does were randomly assigned to either control or DLS group (20/group) and their littermates used for the following three trials.

Trial 1. Blood samples were drawn daily by cardiac puncture from postnatal days 2 to 9 (5 rabbits/day) and thereafter from postnatal days 10 to 14 from both control and DLS young rabbits (5 rabbits/day/group).

Trial 2. Acute stress was applied to 14-day-old control and DLS rabbits by means of saline i.m. injection (designed as time 0) followed by confinement for 5 min in an insulated, closed box (30 × 30 × 30 cm) before returning to their nest-boxes. Blood samples were obtained by cardiac puncture (5 rabbits/time point per group) just before (0), and then 5, 15, and 30 min after saline injection.

Trial 3. Female rabbits (120-day-old) of both control and DLS groups (5/group) were subjected to acute stress by injection with either i.m. saline or 30 μg/kg ACTH (Synacthen-Depot, Novartis). Blood samples were collected from unrestrained catheterized animals just prior and then 30, 60, 90, and 120 min after saline or ACTH injection, designed as time 0.

4.3. Hormone assays

Plasma corticosterone concentrations were evaluated by RIA, using CORT kit (ICN Biomedicals Inc., Costa Mesa, CA, USA). The sensitivity of the CORT assay was 0.15 ng/ml. The intra-assay (10 determinations) and inter-assay (10 assays) coefficients of variation were 3.9% and 4.5% respectively. All the samples were assayed in duplicate within the same run to limit inter-assay variability.

4.4. Tissue preparation and immunohistochemistry

For the immunohistochemical detection of corticosterone receptors in the brain, rabbits of both control and DLS groups (3/group) were sacrificed at postnatal days 14. The brains, immediately removed after sacrifice, were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4, PBS) for 24 h at room temperature and then processed for embedding in paraffin following routine tissue preparation procedure.

The brains were frontally sectioned at a thickness of 7 μm and the sections mounted on poly-L-lysin coated glass slides. Every fourth section was reacted with antibody to GR or stained with hematoxylin–eosin for visualization of the anatomical brain structures. All the sections were processed at the same time and under the same conditions. Briefly, each section was de-waxed, re-hydrated in descending grades of alcohol and in distilled water, and rinsed twice with PBS (0.1 M, pH 7.4). The antigen retrieval treatment was carried out by incubating the sections in 10 mM citric acid buffer (pH 6.0) in a microwave oven (750 W, 3 × 5 min). The action of tissue specific endogenous peroxidases was inhibited by treating slides for 10 min with a 0.5% solution of hydrogen peroxide in methanol. All subsequent steps were carried out in moist chambers at room temperature. To prevent non-specific binding of primary antibody, the sections were pre-incubated for 30 min with normal goat serum (1:10, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The primary monoclonal antibody, mouse anti-GR (1:80, GR32 L, Oncogene Research Products, San Diego, CA, USA) was added to sections. After overnight incubation with primary antibody, the sections were washed in PBS and then incubated for 30 min with biotinylated secondary antibody, a goat anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA, USA). After three washes in PBS, sections were exposed to avidin–biotin complex (ABC, Vector Elite Kit, Vector Laboratories) for 30 min and then the reaction site was visualized using a diaminobenzidine-nickel solution (DAB substrate Kit for peroxidase, SK 4100, Vector Laboratories). After washing in PBS, the slides were dehydrated and permanently mounted in Canada Balsam Natural (BDH, Poole, Dorset, England). Positive reactions were recognized as gray/black precipitates. Sections in which the primary antibodies were omitted or substituted by pre-immune mouse gamma globulin were used for the negative control of unspecific staining. These tissue sections were devoid of any significant staining (data not shown).

Tissue analyses were carried out on randomly selected slides using a light microscope (Nikon Eclipse E800, Nikon Corporation, Tokyo, Japan). The corresponding images, captured by a digital camera (Nikon Dxm 1200), were processed using an image analysis system (Lucia, Laboratory Imaging Ltd, Prague, Czech Republic). Three sections per animal of each brain region were analyzed for GR immunoreactivity. In each section, GR-positive neurons were quantified in four 100×100 μm squares. The density of GR-immunoreactive neurons within the given brain area was expressed as the mean number of GR-positive cells per 0.01 mm^2. The settings for image capture were standardized by subtracting the background signals obtained from the matched tissue sections used as negative immunohistochemical controls as previously reported (Dall'Aglio et al., 2006).

4.5. Statistical analysis

Data relative to overall treatment effects on corticosterone were analyzed by ANOVA according to a mixed model that included treatment group, time period, interaction between group and time period, and rabbit within group which was used as the error term. A one-way ANOVA was used to compare the number of GR immunoreactive neurons in each brain area among experimental groups. Comparisons between effects and treatments were performed by Student's t-test. The analysis of pre-weaning growth performance of young rabbits was carried out by means of the GLM procedure of SAS 9.1.2 (2004) according to a linear model including the effect of treatment group (T: DLS and control), does parity (P: 1, 2–3, and >3) and the interaction between group and parity (T * P). All the values are means ± SEM; differences from corresponding controls where $P < 0.05$ were considered as significant.

Acknowledgments

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