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ANTENATAL GLUCOCORTICOIDS BLUNT THE FUNCTIONING OF THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS OF NEONATES AND DISTURB SOME BEHAVIORS IN JUVENILES

G. BURLET,[a,b] B. FERNETTE,[a] S. BLANCHARD,[a] E. ANGEL,[a] P. TANKOSIC,[a] S. MACCARI[b] AND A. BURLET[a]*

[a]*Université Henri Poincaré-Nancy 1, SNCI-EA3453, 38 rue Lionnois, 54000 Nancy, France*

[b]*University of Lille 1, UPRES JE 2365 Lab. Perinatal Stress, USTL, 59665 Villeneuve d'Ascq, France*

Abstract—Antenatal glucocorticoids are highly effective in preventing respiratory distress of premature babies but can induce physiological and behavioral disturbances in young infants as well as in animals. Therefore, the hypothalamic–pituitary–adrenal (HPA) axis of rat neonates, and the consequences on behavioral development of offspring have been studied after five antenatal injections of dexamethasone (DEX) or vehicle. DEX decreased offspring body weight at birth, and significantly delayed the normal growth for the first 3 weeks of life. This paralleled diminished behavioral performances measured on postnatal day 3 (righting reflex) and postnatal day 10 (grasping test). Circulating levels of adrenocorticotrophin (ACTH) and corticosterone were significantly decreased on postnatal day 1 and this was related to a diminution of HPA axis activity shown by the decrease of central expression of corticotropin releasing hormone (CRH) mRNA, immunoreactive content in paraventricular neurons (PVN) and in the median eminence endings were significantly decreased. On the other hand, expression of another secretagogue of ACTH, arginine vasopressin (AVP), was differently affected in the PVN parvocellular neurons of offspring of the DEX group since AVP mRNA increased whereas immunoreactive content of the PVN parvocellular neurons was lowered. Simultaneously, the co-production of AVP and CRH in PVN neurons was stimulated. This can support the view that antenatal DEX reached the fetus and produced some damage which did not parallel that induced by prenatal stress of the pregnant females, especially the low body weight of offspring. The harmful consequence of antenatal DEX treatment was not restrictively due to the blunting of the HPA axis but also to the low body weight, which disturbed behavioral performances for the first weeks of life and could participate in other disorders in adult life. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: pregnancy, CRH expression, AVP expression, behavioral tests, development.

*Corresponding author. Tel: +33-383-683930; fax: +33-383-683939.
E-mail address: arlette.burlet@nancy.inserm.fr (A. Burlet).

Abbreviations: ACTH, adrenocorticotrophin; AVP, arginine vasopressin; BW, body weight; CRH, corticotropin releasing hormone; DEX, dexamethasone; GD, gestational day; GR, glucorticoid receptor; HPA, hypothalamic pituitary adrenal; mAb, monoclonal antibody; ME, median eminence; mPVN, magnocellular subset of paraventricular nucleus; Pab, polyclonal antibody; PBS, phosphate buffer saline; PND, postnatal day; pPVN, parvocellular subset of paraventricular nucleus; PVN, paraventricular nucleus; VEH, vehicle.

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Glucocorticoids are synthesized by the adrenal cortex though activation of the hypothalamic–pituitary–adrenal axis (HPA) and central release of two neuropeptides, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). These are synthesized in the parvocellular neurons of the hypothalamic paraventricular nuclei (PVN) whose axons terminate in the external layer of the median eminence (ME) where they are released into the portal bloodstream. CRH and AVP stimulate thus the secretion from the adenohypophysis of adrenocorticotrophin (ACTH) which is directly responsible for glucocorticoid synthesis. Endogenous glucocorticoids exert in turn a negative feedback control of the HPA axis through activation of different subtypes of glucorticoid receptors (GR) which have been identified in various brain sites (De Kloet, 2000), including the PVN. Glucocorticoids modulate also higher brain functions (Sapolsky et al., 2000; De Quervain et al., 2003), especially during brain maturation (Gould and Cameron, 1996; Bakker et al., 2001). They also exert beneficial effects on maturation of other tissues (e.g. pulmonary) where GR have been located (Ballard, 1980; Berger et al., 1996; Bolt et al., 2001). Thus, glucocorticoids have been especially used to decrease neonatal morbidity because they promote maturation of pulmonary tissue and are highly effective in preventing respiratory distress of premature babies (Liggins and Howie, 1972; Crowley, 1999); in pregnant women, two or three interventions with glucocorticoids are currently used if the risk of preterm delivery persists. The fetus was believed to be under the protection of the placental 11b-steroid-deshydrogenase which metabolizes the excess of circulating glucocorticoids. However, this protection is incomplete and glucocorticoid excess can reach the fetus (Benediktsson et al., 1997) and produce deleterious effects on development and low body weight (BW; French et al., 1999; Kay et al., 2000; Bakker et al., 2001; Matthews, 2001). In animal studies, antenatal glucocorticoids also reduced BW of neonates and produced persistent alterations of HPA function: they decreased circulating plasma corticosterone levels, attenuated hippocampal GR gene expression in adult offspring (Levitt et al., 1996) and also decreased CRH mRNA in the PVN of fetal guinea-pig brain (McCabe et al., 2001), suggesting that the HPA axis function was disturbed. In addition, persistent metabolic disorders such as hypertension, type II diabetes and obesity have been observed (Benediktsson et al., 1993; Nyirenda et al., 1998). Antenatal glucocorticoids can also induce behavioral disturbances in young infants (Trautman et al., 1995; Salokorpi et al.,

1997) and in adult rodents (Brabham et al., 2000; Welberg et al., 2001).

In the present experiments, we investigated some early disturbances induced by antenatal glucorticoids that have not previously been studied, namely, the behavior of very young pups and the status of AVP for the development of HPA axis. The expression of hypothalamic CRH and AVP has been measured in the HPA axis on postnatal day (PND) 1 to evaluate the acute effects of antenatal glucocorticoids. We have also tested behavioral performances for the postnatal first 3 weeks of the young rats since the control of behavior strongly involves the HPA axis in early postnatal life (Francis and Meaney, 1999; Penke et al., 2001).

EXPERIMENTAL PROCEDURES

Animals and treatments

Female Sprague–Dawley rats (250 g; BW) were purchased from IFA CREDO breeding Laboratories (France), housed individually in Macrolon cages and habituated in our animal facility, under constant temperature (23+1 °C) and controlled lighting conditions (12-h light/dark cycle, lights on at 19:00 h), for 2 weeks before starting the experiments. They were provided with rat chow (AO3, UAR, France) and tap water *ad libitum*. Every 2 days, they were weighed and the cages cleaned (change of the wood shavings . . .) and this trained the rats to be manipulated and to avoid the stress induced by handling. They were mated between 10:00 and 18:00 h on the appropriate day of the estrous cycle determined by staining vaginal smears. The efficient fertilization was checked the next morning and this day was designed as day 1 of gestation (GD). On GD15 the females were randomly assigned to a specific 5 day treatment (GD15 to GD 19): the first group of females (n=12) was injected daily s.c. with dexamethasone (DEX; 0.1 mg/kg) in their home cage, and the second group (n=12) received the same volume of vehicle (VEH; 0.350–0.450 ml/injection). DEX injections were prepared from Soludecadron® (Merck Sharp & Dohme-Chibret, Paris, France) provided by the hospital laboratory and VEH solution consisted in the 40-fold dilution of DEX VEH in isotonic saline. This experimental design (DEX doses and timing) was similar to that previously published (Dupouy et al., 1987; Levitt et al., 1996; Brown et al., 1996; Nyirenda et al., 1998; Brabham et al., 2000; Welberg et al., 2001). The females were weighed every day until GD19 and delivery occurred on GD21 to GD22. Twenty-four hours after birth (PND1) litters were culled to 10 pups, the additional pups killed and blood samples and adrenals collected. In these experiments, we did not carry out cross-fostering because previous studies showed that DEX injections did not affect mother's nursing of their pups (Nyirenda et al., 2001), and cross-fostering may produce some complex effects on mother–infant interactions and also modify the consequences of prenatal treatment (Maccari et al., 1995).

All experimental procedures followed the guidelines of Animal Care edited by the CNRS (Centre National de la Recherche Scientifique, France). Efforts were made to minimize the number of animals used and their suffering.

Plasma ACTH and corticosterone assay

Hormones were measured by specific radioimmunoassays, in the plasma collected trunk blood of additional pups decapitated on PND1, in tubes coated with EDTA. After centrifugation (4 °C, 10 min, 2500×g) ACTH (n=20 per group) was assayed with a commercially available Kit (Allegro Kit, Mallinkrodt, France). The sensitivity of the assay was 1 pg per tube of plasma and the intra- and interassay coefficients of variation were 4% and 6%, respectively. Corticosterone (n=15 per group) was assayed with a commercially available kit (125 Biotrack TM Assay System; Amersham Biosciences, UK) measuring total corticosterone (linked and unlinked to corticosterone binding globulin). The sensitivity of the assay was 0.06 ng per tube, with the intra- and interassay coefficient of variation of 5% and 7%, respectively.

Immunocytochemistry of AVP, CRH

Immunocytochemistry was performed and measured by semi-quantitative methods in the brain of additional pups, decapitated on PND1, fixed in paraformaldehyde (1%, pH 7.4) for 2 h, rinsed in phosphate buffer saline (PBS; 0.01 M, pH 7.4) with sucrose (15%) and frozen in liquid nitrogen before sectioning. Frontal sections (12 μm thickness) were collected in continuous serial order onto gelatin-coated slides (two sections per slide). Two brains (VEH and DEX) were collected on the same slide to facilitate the comparison between sections and immunostaining was performed from the organum vasculosum of the lamina terminalis to the caudal neural stalk of the hypophysis.

AVP and CRH were simultaneously stained on the same sections, by an immunofluorescence method using two specific antibodies. AVP antibody was a mouse monoclonal antibody serum (mAb; C3-12) prepared and tested in our laboratory (Robert et al., 1985) and CRH antibody was a polyclonal antibody rabbit serum (Pab), kindly supplied by Dr. Barbanel (UMR CNRS, Montpellier, France), the properties of which have already been reported (Walker et al., 1997). The double staining was performed as follows: a mixture containing AVP–mAb (final dilution 1:400) and CRH-Pab (final dilution 1:600) covered the sections for one night (6 °C). These were rinsed in PBS and covered for 1 h (laboratory temperature) with a mixture containing the fluorescent antibodies (donkey anti-mouse IgG labeled with rhodamin) (Jackson ImmunoReseach, West Grove, PA, USA) and fluorescent goat anti-rabbit IgG (Alexa Fluor; Molecular Probes, USA). After rinsing, the sections were mounted in buffered glycerol and observed with a microscope (Leica DM R, France), equipped with a CCD camera (Kappa DX 30) and a video acquisition data card. We checked previously the staining specificity of the AVP and CRH antibodies by preventing the immunological detection when the specific antibody was replaced by non-immune serum or firstly incubated with synthetic AVP or CRH, respectively (Walker et al., 1997).

In situ hybridization of CRH mRNA and AVP mRNA

In situ hybridization was performed on frozen and unfixed brain of PND1 rats, according to procedures described previously (Walker et al., 1997). The AVP probe was a 27-base oligomer comple-

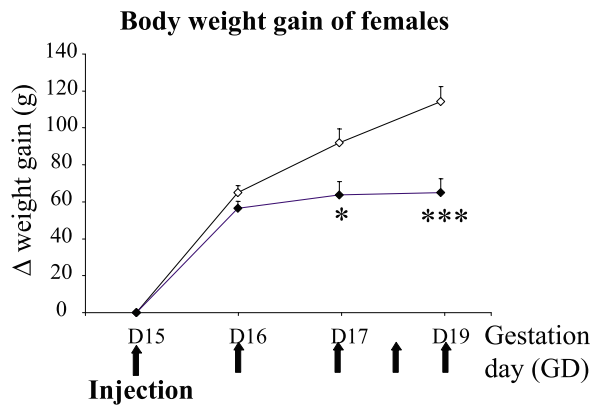

Fig. 1. Effect of treatments on BW gain of pregnant females from GD15 to the day of the last injection (GD19). Each value is the mean±S.E.M. Comparison between VEH (empty lozenges) versus DEX (black circles) groups. * $P<0.05$; *** $P<0.001$.