LONG-TERM EFFECTS OF EARLY POSTNATALLY ADMINISTERED INTERLEUKIN-1ß ON THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS IN RATS

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Objective. Since perinatal stress events are well known to exert long-term influences on the function of hypothalamic-pituitary-adrenal (HPA) axis in rats, to investigate the consequences of exposure to IL-1ß, a potent stimulator of this axis, during early postnatal life.

Methods. Wistar rats were treated twice a day with 0.02 µg human recombinant IL-1ß from day 1-4 of age, while controls received the vehicle only.

Results. IL-1ß-treatment had no significant influence on the mortality and body weight. However, at the end of treatment period on the 4th day of life, the thymus weight was decreased in the IL-1ß-treated group ($P<0.01$), while the adrenals were clearly enlarged ($P<0.0002$). These responses were associated with a nearly 4-fold elevation of the plasma corticosterone (CS) level as compared to vehicle-treated controls ($P<0.001$). At the age of seven months the stimulated CS levels induced by an acute stress (novel environment) were lower in rats treated neonatally with IL-1ß than in controls ($P<0.01$). This functional disturbance was associated with morphological alterations in the parvicellular part of the paraventricular nucleus (PVN) which is the main hypothalamic regulation centre of the HPA axis. A strong reduction of the numerical density of neurons was found in the neonatally IL-1ß-treated rats ($P<0.005$) while the neuronal nuclei were clearly enlarged ($P<0.0005$).

Conclusion. As a part of an infection-induced stress response during critical periods of development, IL-1ß might be capable of inducing a permanent structural malorganization of the PVN and, consequently, functional malprogramming of the HPA axis in rats.

Key words: Interleukin-1ß – Newborn rat – Long-term effects – Paraventricular hypothalamic nucleus (PVN) – Hypothalamic-pituitary-adrenal (HPA) axis.

Nervous, endocrine and immune systems are regarded as a functional unit. A lot of experimental evidence has accumulated concerning the importance of cytokines as modulators of neuroendocrine function. In particular, interleukin-1ß (IL-1ß), released during the course of certain immune and inflammatory processes, stimulates the hypothalamic-pituitary-adrenal (HPA) axis leading to elevated levels of glucocorticoids (GC) which suppress the immune response. This circuit between the immune system and the HPA axis has been considered as an immuno-endocrine feedback loop (Besedovsky et al. 1986). The stimulating effect of IL-1ß on the HPA axis is even present during critical developmental periods in newborn rats (Besedovsky et al. 1991), when their response to acute stress stimuli is physiologically reduced (Sapolsky and Meaney 1986).

Early postnatal environmental events are well known to program the development of the HPA activity in later life. For example, animals exposed to

short periods of neonatal stimulation or handling develop dampened HPA response while maternal separation or physical trauma enhance HPA responsivity to stress in adulthood (for review see MEANEY et al. 1996). Extensive studies by REUL et al. (1994) and SHANKS et al. (1995) revealed that a complex immune challenge, i.e., pre- or neonatal endotoxin exposure, can also lead to permanent alterations of the HPA axis in rats. The underlying mechanisms and specific immune factors possibly responsible are unclear.

The activity of the HPA axis highly depends on its central nervous controllers. The parvicellular division of the hypothalamic paraventricular nucleus (PVN) appears to be the crucial hypothalamic site for regulation of the HPA activity (ANTONI 1986). In general, the morphological and functional development of hypothalamic structures can be altered by abnormal hormone levels during critical differentiation periods (DÖRNER 1976). Steroids are well known to be essential organizers of the HPA axis, as well as the hypothalamic-pituitary-gonadal system, throughout life (DÖRNER 1976; DE KLOET et al. 1986; MC EWEN 1992; MEANEY et al. 1996). Other studies have revealed that proteohormones, like insulin, are also capable of permanently malorganize neuroendocrine systems by malprogramming their own hypothalamic controllers (PLAGEMANN et al. 1992; DÖRNER and PLAGEMANN 1994).

These findings have led us to question whether and how administration of the cytokine and polypeptide IL-1ß during a critical neonatal period of hypothalamic development leads to permanent malprogramming of the HPA axis in rats and, if so, whether the lesion may be due in part to malorganization of the PVN. To investigate this, newborn rats were treated with human recombinant IL-1ß, which is known to be active in rats (SUDA et al. 1990; BESEDOVSKY et al. 1991; RENNER et al. 1995). Some of the animals were investigated immediately after treatment while others were allowed to grow up for investigations in adulthood.

Materials and Methods

Animals: The investigations were performed in the offspring, bred in our institute, of Wistar rats of an outbred colony strain (WIST/SHOE). Virgin female rats were time mated at the age of three months. Under standard laboratory conditions they were singly housed during pregnancy. On the first day of life newborn pups were randomly assigned to the experimental or control group, respectively, and were distributed among mothers. Female offspring were divided into two groups on the first day of life: *Control group* (**Co**) – Newborn rats were treated subcutaneously with 0.1 ml solvent (sterile Tris buffer, pH = 7.8) twice daily (8.00 a.m. and 4.00 p.m.) from day 1 – 4 of life. *Interleukin-treated group* (**IL**) – Newborns were treated subcutaneously with 0.02 µg human recombinant interleukin-1ß (CIBA-GEIGY, Basel, Switzerland) in 0.1 ml solvent twice daily (8.00 a.m. and 4.00 p.m.) from day 1 – 4 of life.

Mortality rates and body weights were registered throughout life. After weaning on day 21 of age rats were housed in groups of four per plexiglas cage. Animals were kept under 12 : 12 h inverse light-dark rhythm (lights on from 5.00 p.m. to 5.00 a.m.). They had free access to standard pellet diet (ALTROMIN), and tap water was provided ad libitum. For testing of sexual behaviour (PLAGEMANN et al. 1997) rats were gonadectomized at the age of three months as described elsewhere (DÖRNER 1976). All animal procedures were in accordance with the guidelines of the German law for protection of animals and were approved by the local Animal Care and Use Committee (G 0297/92).

Corticosterone Determinations: One hour and a half after the last injection on the 4th day of life some of the animals were rapidly decapitated. For the interval between the last injection and decapitation pups were returned to their mothers. Wet weights of adrenals and thymus were determined and the trunk blood was collected using heparinized capillaries.

Plasma levels of corticosterone (CS) were evaluated by a radioimmunoassay: The plasma samples were extracted, mixing 50 µl plasma with 1 ml methanol by vortex and centrifuging at 3000 x g for 10 minutes. 100 µl of the supernatants as well as 100 µl of methanol-solved corticosterone (range 0.36 to 5.77 pmol/100 µl) were evaporated and solved in 500 µl of a 0.2% gelatine-Tris buffer (pH = 8.5) containing the anti-corticosterone serum in a dilution of 1:50000. These samples were gentle mixed for 30 min at room temperature. After this incubation the tubes were giv-

en to an ice-bath and 200 µl of the tracer (15000 dpm H^3-corticosterone) were added and incubated for 16 h at 4 °C. The separation of free from bound was done using the dextran coated charcoal method. The radioactivity of the resulting supernatants was measured by a LKB-scintillationcounter. The antiserum used was raised in rabbits against a corticosterone-3-(carboxymethyl)-oxime bovine serum albumin conjugate (Stahl et al. 1988). Cross reactivities were estimated as follows: cortisol, 11-desoxycortisol, 17α-hydroxyprogesterone, as well as pregnenolone less than 1 %; only progesterone with 16.4 % and 11-desoxycorticosterone with 32.8 % were found to cross-react at a significant level. The intra-assay CV was 5.9 % at 200 nmol/l (n = 11), inter-assay CV was 11.8 % at 173 nmol/l (n = 10).

At seven months of age acute CS stress response was tested between 8.00 a.m. and 9.00 a.m. in all animals by "novel-environment-stress" (Lahti and Barsuhn 1974). After a four week rest period without injections, tests, or any other manipulations except of the daily animal keeping routine the rats were singly brought from their home cages within the animal room to a novel environment (new cages, laboratory room, loud background and bright light). A blood sample was taken immediately by puncture of the retroorbital plexus, and CS was determined as described above.

Morphometrical investigations: The animals were sacrificed by rapid decapitation and wet weights of the thymus and adrenals were determined. The brains were quickly removed from the cranium, weighed, and processed immediately by fixation in 4% formalin solution. After fixation brains were embedded in paraffin. According to Paxinos and Watson (1986) 5 µm-serial-sections were made from plane 22 to plane 26, i.e., including the whole PVN. Sections were stained with haematoxylin-eosin and were then coverslipped. The slides were coded so that they could be examined without knowledge of the subject's treatment. All measurements were performed by the same investigator. Using a digital image analyzing system (VIDAS 2.1, KONTRON), the volume of the PVN was evaluated first. At a final magnification of 150x in each brain the boundaries of the PVN were traced in every slide in which it

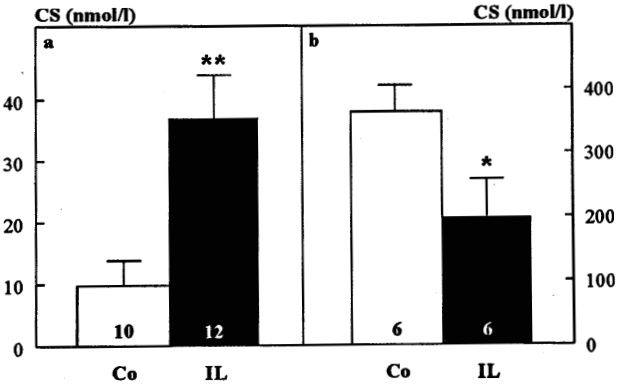

Fig. 1.
Plasma corticosterone (CS) one hour and a half after the last injection of IL-1ß on the 4th day of life (a) **and in response to an acute novel environment stress in adulthood** (b; **means +/- SD; Co = controls; IL = neonatally IL-1ß-treated group; * P<0.01; ** P<0.001).**

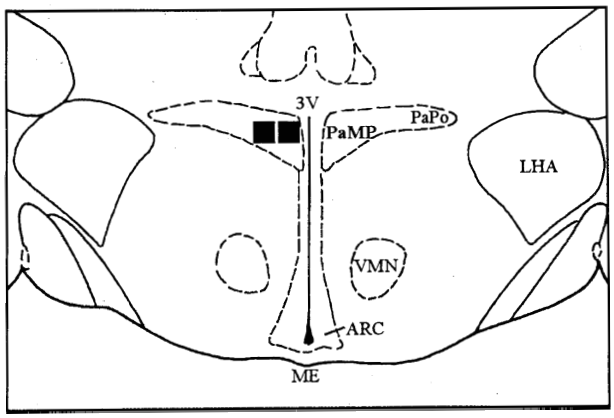

Fig. 2.
Microscopic fields of measurement within the medial parvicellular part of the paraventricular hypothalamic nucleus (■ microscopic field; PaMP – **paraventricular nucleus, medial parvicellular; PaPo – paraventricular nucleus, posterior; 3V – 3rd ventricle; LHA – lateral hypothalamic area; VMN – ventromedial hypothalamic nucleus; ME – median eminence. Plane 26 according to and adapted from Paxinos and Watson (1986).**

appeared. Cross-sectional areas of each tracing of the PVN were quantified and the PVN volume was then calculated by multiplying the sum of the areas by the section thickness. In a second step, within plane 26 cytomorphometric investigations were performed in the medial parvicellular part of the PVN. Within each section investigated two microscopic

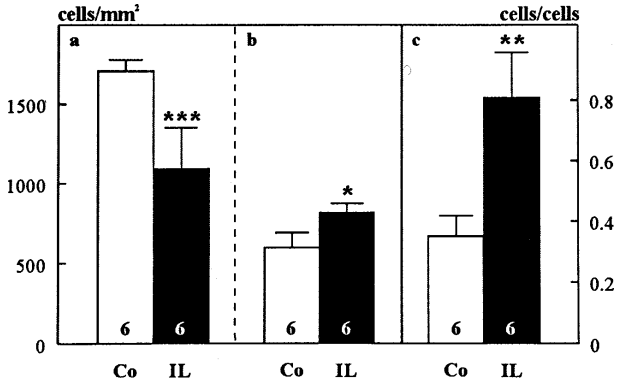

Fig. 3.
Numerical density of neurons (a) and glial cells (b), as well as numerical glia-neuron-ratio (c) within the medial parvicellular part of the PVN in adulthood of neonatally IL-1ß-treated rats (IL) as compared to controls (Co; means +/- SD; * P<0.02; ** P<0.01; *** P<0.005).

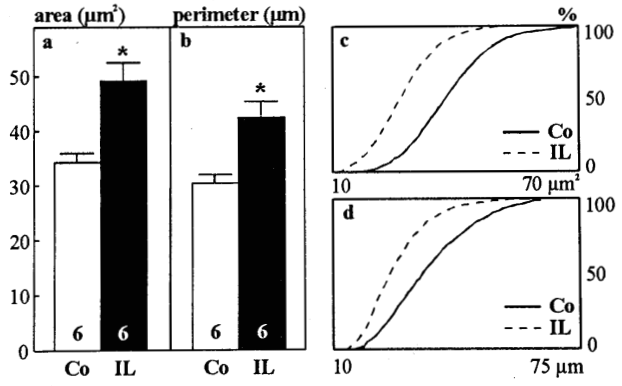

Fig. 4.
Area (a) and perimeter (b) of neuronal nuclei and the corresponding cumulative frequency histograms for area (c) and perimeter (d) of neuronal nuclei within the medial parvicellular part of the PVN in adulthood of neonatally IL-1ß-treated rats (IL) as compared to controls (Co; means +/- SD; * P<0.0005).

fields were situated side by side in each hemisphere of the brain (Fig. 2). Twelve microscopic fields of view were thus evaluated in each brain by measurement of every fourth of successive serial sections. Again, analysis was performed using the digital image analyzer (final magnification: 1500x). The number of neurons and glial cells (numerical density), as well as the area and perimeter of neuronal nuclei were determined (mean number of evaluated neurons per animal and brain, respectively: 177.3 +/- 38.8). Identification of neurons and differentiation between neurons and glial cells involved the following criteria (Bereiter and Jeanrenaud 1979; Hawrylak and Greenough 1995): nuclear content (in contrast to glia neuronal nuclei have a distinct nucleolus within a pale nucleus), nuclear size (glial nuclei in general were smaller than neuronal nuclei) and, facultatively, soma appearance (in contrast to neurons no cytoplasmatic staining appears in glial cells). The staining did not permit the identification of astrocytes, oligodendrocytes and microglia as separate cell types. Therefore, data reported as glia represent all types of glial cells combined. For glial cells, the numerical density was quantified only.

Statistics: Data are presented as means and standard error or standard deviation, respectively, of the values in the number of observations indicated in *Results* or in the legends. For statistical evaluations Chi-square-test, ANOVA followed by two-tailed Student's t-test (with or without Welch correction), and test of linear regression were performed. Significance level was set at P<0.05.

Results

As shown in Table 1, on the fourth day of life 1.5 h after the last injection in the newborn animals a significant decrease of thymus weights in the IL-1ß-treated rats (P<0.01) and a concomitant highly significant increase in the wet weights of the adrenal glands were observed (P<0.0002). This clear enlargement of the adrenals was accompanied by a clear-cut elevation of plasma corticosterone concentrations (P<0.001) in the 4-day-old IL-1ß-treated rats (Fig. 1). By contrast, neither body weight gain (Tab. 1) nor mortality rates were significantly affected by the treatment. Mortality occurred only during the first week of life. Rates of 20.6 % in the control group (6 out of 29 animals) as compared to 10.3 % (3 out of 29 rats) in the IL-1ß-treated group were observed (difference not significant using Chi-square-test).

To evaluate long-term functional consequences of neonatal IL-1ß-exposure we tested the acute stress response in adulthood by determining the plasma corticosterone immediately after an acute novel environment stress. As shown in Figure 1, post-stress CS levels were significantly lower in neonatally IL-

Table 1

Body weight gain and organ wet weights in early postnatal, juvenile and adult age of neonatally IL-1ß-treated rats (IL) as compared to controls (Co). Values are given as means ± SEM. Number of rats is indicated in parentheses.

Parameter	Co	IL
Body weight (g)		
4th day of life:	6.90 ± 0.28 (23)	6.90 ± 0.10 (26)
40th day of life:	120.1 ± 4.53 (6)	117.7 ± 3.06 (6)
180th day of life:	306.5 ± 8.94 (6)	302.8 ± 4.37 (6)
300th day of life:	308.5 ± 17.1 (6)	330.0 ± 5.18 (6)
Adrenal weight (mg)		
4th day of life:	3.19 ± 0.14 (10)	4.00 ± 0.10 (12)**
300th day of life:	61.1 ± 2.33 (6)	69.9 ± 3.39 (6)
Thymus weight (mg)		
4th day of life:	13.0 ± 1.21 (10)	8.52 ± 0.48 (12)*
300th day of life:	214.6 ± 21.3 (6)	224.8 ± 34.7 (6)

* P<0.01 vs controls
** P<0.0002 vs controls

1ß-treated rats as compared to the vehicle-treated controls (P<0.01). The impaired stress response in the IL-group was not associated with statistically significant changes in the wet weights of either the adrenal glands or the thymus (Tab. 1).

The wet weight of the brains was also unaffected by neonatal IL-1ß-treatment (control group: 2.05 ± 0.08 g, n=6; vs. IL-group: 2.08 ± 0.10 g, n=6). Moreover, computer-aided morphometrical investigations of serial sections of the paraventricular hypothalamic nucleus (PVN) showed that the total volume of the PVN was also unchanged (control group: 0.081 ± 0.004 mm^3, n = 6; vs. IL-1ß-treated animals: 0.083 ± 0.004 mm^3, n = 6). However, as shown in Figure 3, evaluation of the numerical cell density within the medial parvicellular division of the PVN (Fig. 2) showed that the number of neurons in neonatally IL-1ß-treated rats was reduced (P<0.005) while the numerical density of glial cells was increased (P<0.02); thus the numerical glia-neuron-ratio was significantly raised in the IL-1ß-treated group (P<0.01 vs. controls). On the other hand, measurements on individual neurons revealed a clear enlargement of neuronal nuclei in the neonatally IL-1ß-treated animals (Fig. 4), estimated by a highly significant increase of the mean area (P<0.0005) and perimeter (P<0.0005) of neuronal nuclei.

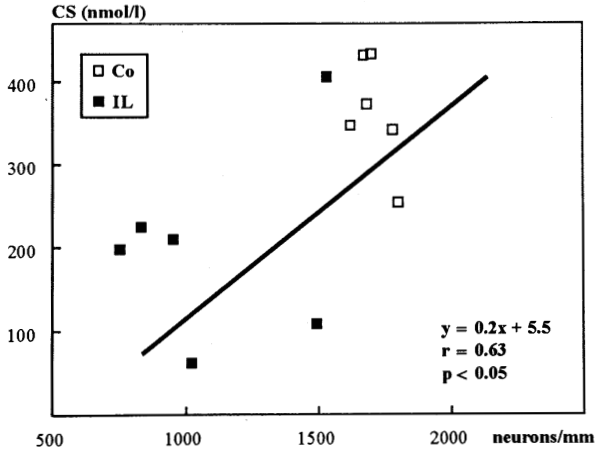

Fig. 5.
Correlation between the mean numerical density of neurons within the medial parvicellular part of the PVN and plasma corticosterone (CS) in acute response to novel environment stress in adulthood of neonatally IL-1ß-treated rats (IL) and controls (Co).

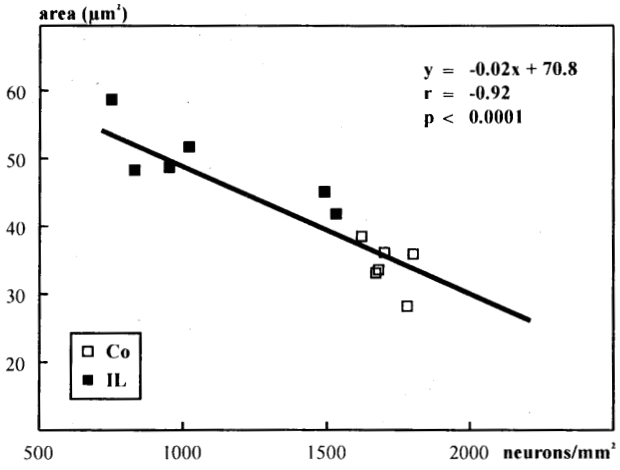

Fig. 6.
Correlation between the mean numerical density of neurons and the mean area of neuronal nuclei within the medial parvicellular part of the PVN in adulthood of neonatally IL-1ß-treated rats (IL) and controls (Co).

Finally, a significant linear correlation was found between the numerical cell density of neurons within the PVN and stimulated CS levels after novel-environment-stress (P<0.05; Fig. 5). Tests of linear regression for morphometrical parameters showed an inverse correlation between the number of neurons within the PVN and the area of their nuclei (P<0.0001; Fig. 6).

Discussion

To our knowledge, this is the first study on hypothalamic morphology in adulthood of rats exposed to a specific cytokine during a critical neonatal period of brain development. Taken together, our data show that neonatal treatment with IL-1ß results in impaired organization of the PVN and hyporesponsiveness of the HPA axis in adulthood.

IL-1ß-administration, in a dose sufficiently low so as to not influence mortality and body weight gain, clearly activated the HPA in neonates. Enlargement of the adrenals and elevation of CS levels are consistent with the observation that the HPA can be activated by this cytokine even at the beginning of the stress hyporesponsive period in newborn rats (Besedovsky et al. 1991; O'Grady et al. 1992). The thymic hypoplasia in the IL-1ß-treated group additionally reflects neonatal hypercorticoidism (Morrissey et al. 1988). It seems noteworthy that after neonatal thymectomy and in congenital athymic mice reduced HPA stress response in adulthood were observed (Deschaux et al. 1979; Daneva et al. 1995).

In adulthood, a decreased HPA response to a moderate stress was observed in the IL-1ß-treated group, confirming investigations in prenatally IL-1ß-exposed animals (Götz et al. 1993). Similar have also been observed in adults after moderate neonatal stimulation or handling (Meaney et al. 1996); interestingly, in the latter group the mRNA level for corticotropin-releasing hormone (CRH) in the parvicellular PVN was strongly diminished (Plotsky and Meaney 1993). On the other hand, the HPA hypoactivity in the IL-1ß-treated group contrasts to findings of increased HPA activity after perinatal immune challenge with endotoxin (Reul et al. 1994; Shanks et al. 1994). However, variability in programming of the HPA responses to stress may derive from several variables such as genetic background, age, the stress model used, the perinatal time point, duration, dose and kind of immune challenge, as well as sex and reproductive stage. Many factors and mechanisms of the neuro-endocrine-immune network are involved in acute immune responses. In the present study only one of them was used, possibly explaining a different outcome as compared to studies in which complex immune challenges were used, e.g., by endotoxin (Reul et al. 1994; Shanks et al. 1995). Thus, prenatal exposure to lipopolysaccharide (LPS) raises the basal CS levels in adult rats, but fails to influence the CS response 30 minutes after novel environmental stress (Reul et al. 1994). By contrast, neonatal exposure to LPS does not affect resting adrenocorticotrophic hormone (ACTH) or CS levels in the adult (Shanks et al. 1995); however, the ACTH and CS response to restraint stress were elevated although in the females the CS response was delayed as compared to males and was not associated with a rise in CRH expression in the PVN. Unfortunately, because of technical and biometrical problems males could not be investigated in our study. However, it must be stressed that our experimental animals were ovariectomized, in order to test their sexual behaviour which was also found to be affected by the neonatal IL-1ß-exposure (Plagemann et al. 1997).

A possible reason for the observed hypoactivity of the HPA in adult rats treated neonatally with IL-1ß could be the impaired organization of the hypothalamus. The neurosecretory neurons in the parvicellular PVN are major participants of the HPA axis regulation. Upon stimulation by stress, these neurons secrete a cocktail of ACTH hormone secretagogues, the most important of which are CRH and arginine-vasopressin (Antoni 1986). From our observations of (a) decreased number of PVN neurons in the IL-group, (b) positive correlation between stimulated CS and neuronal density in the PVN, (c) enlargement of neuronal nuclei in the PVN of the IL-1ß-treated rats, and (d) inverse correlation between the number of PVN neurons and the size of their nuclei it could be suggested that the nuclear enlargement might indicate a compensatory hypertrophy designed to maintain HPA axis activity at a physiological level. Dysplasia of the PVN was not a sign of general brain malorganization in the IL-1ß-treated animals as indicated, e.g., by normal brain weights, normal PVN volumes, and normal cell densities within other central nervous structures, including hypothalamic nuclei other than the PVN (Staudt et al. 1995; Plagemann et al. 1997). In our opinion the findings seem to be consistent with the idea that hypothalamic neurons may be a target for perinatal malprogramming (Dörner 1976), and that this can result in permanent alterations of the HPA axis activity (Meaney et al. 1996). The question arises whether the PVN alterations were induced by IL-1ß itself or

if they rather developed due to the elevated GC concentrations induced by the cytokine neonatally. Abnormal levels of GC during perinatal development can disturb neurogenesis and morphological differentiation of neurons in mammals (DE KLOET et al. 1988). High concentrations in the neonate result in permanent deleterious effects, while low concentrations seem to be essential for normal development (DE KLOET et al. 1988; MC EWEN 1992). On the other hand, various *in vitro* and *in vivo* studies have shown that IL-1ß can be neurotoxic or neuroprotective, depending on the neuronal stage or maturation stage (RELTON and ROTHWELL 1992; BRENNEMAN et al. 1993; RENNER et al. 1995). IL is a potent mediator of cell proliferation, cell differentiation and programmed cell death (DURUM and OPPENHEIM 1989). Peripherally injected IL-1ß can cross the blood-brain barrier (BANKS and KASTIN 1991) and is able to induce Fos-like immunoreactivity, CRH gene expression, and changes of the electrical activity of CRH expressing neurons in the PVN (SUDA et al. 1990; VEENING et al. 1993; SAPHIER and OVADIA 1990). Moreover, IL-1ß induces astrocytosis (GIULIAN and LACHMANN 1985; DA CUNHA et al. 1993), which may disturb neuronal development (MERILL 1992). The reasons for increase of glial cells in adulthood are unclear. Speculatively, it might be a sign of a kind of "prolonged and/or programmed" astrocytosis or might possibly indicate progredient neuronal cell loss.

From a clinical point of view it is widely recognized that patients suffering from major depression show various abnormalities in HPA axis regulation in terms of hyperactivity (HOLSBOER and BARDEN 1996). Moreover, prolonged GC overexposure might be causal for damage of hippocampal neurons, possibly leading to accelerated cognitive ageing (SAPOLSKY 1996). On the other hand, it must be stressed that from experimental and clinical observations hyporesponsiveness of the HPA is suggested as a possible link between atypical depression and rheumatoid arthritis (LICINIO and STERNBERG 1996). In general, HPA hyporeactivity could lead to increased susceptibility to inflammatory and autoimmune diseases. Whether this could be a consequence of malprogramming of the immuno-HPA feedback loop by perinatal infection and IL-1ß, respectively, remains to be elucidated. Early environment might thus determine life-long vulnerability of neuro-endocrine-immune systems (DÖRNER 1976; MEANEY et al. 1996).

In conclusion, our results indicate malorganization of the PVN as a possible reason for malfunction of the HPA axis in adult rats neonatally exposed to IL-1ß as it may occur, e.g., during the course of simple virus infection. Further characterization of the observed phenomena are needed, particularly regarding specific subsets of PVN neurons involved as well as on the dynamics of HPA responses in various types of stress.

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