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Authors
Yi, Su-Jin
Masters, Jeffrey N
Baram, Tallie Z

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Effects of a specific glucocorticoid receptor antagonist on corticotropin releasing hormone gene expression in the paraventricular nucleus of the neonatal rat

Su-Jin Yi [a], Jeffrey N. Masters [b] and Tallie Z. Baram [a]

[a] *Department of Neurology, University of Southern California, Division of Neurology, Childrens Hospital Los Angeles, Los Angeles, CA 90027 (USA) and* [b] *Biotechnology Center, Ohio State University, Columbus, OH 43210 (USA)*

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Mechanisms controlling the synthesis of corticotropin releasing hormone (CRH) in neonatal rats, and the ontogeny of glucocorticoid (GC) feedback control of hypothalamic CRH remain unknown. Specific issues are whether stress induces up-regulation of CRH gene expression during the first postnatal week, and the role of GC feedback, at the hypothalamic level, in the stress-hyporesponsive period. We studied the ontogeny of the negative feedback regulation of CRH gene expression by GC in the paraventricular nucleus (PVN). We implanted chronic cannulae containing a GC-receptor antagonist, RU 38486, in rats on postnatal days 3 to 13. Three days later, animals were sacrificed, and brains were analyzed for CRH-messenger RNA (CRH-mRNA), using semi-quantitative in situ hybridization. Animals implanted with cholesterol-containing cannulae served to evaluate the stressful effect of implantation on CRH-mRNA abundance. The presence of GC receptor messenger RNA (GR-mRNA) in the PVN of neonatal rats was also determined. RU 38486 did not increase CRH-mRNA abundance during the first postnatal week, despite the presence of GR-mRNA in the PVN. Chronic-implantation stress also failed to increase CRH synthesis. CRH gene expression in the PVN was enhanced in infant rats implanted with RU-38486 on postnatal day 9 or later. Cholesterol implantation on days 9, 10 (but not later), resulted in increased PVN-CRH-mRNA. Thus, CRH-mRNA is up-regulated by chronic blockade of GC receptors only subsequent to the eighth postnatal day. Furthermore, such blockade does not affect the response of CRH-mRNA to chronic stress in the neonatal rat.

INTRODUCTION

Corticotropin releasing hormone (CRH) regulates glucocorticoid (GC) secretion from the adrenal cortex via adrenocorticotropic hormone (ACTH)[23]. In adult rats, GCs have an amply documented negative feedback effect on CRH synthesis in the hypothalamic paraventricular nucleus (PVN)[8,22,29], as well as on the peptide's secretion[16]. This effect is at least partially mediated via local GC receptors[10,11,19].

During the first postnatal days, GC receptor function, and the regulation of the hypothalamic–pituitary–adrenal axis have not been defined. In the neonatal rat, both plasma GC levels[13,26] and CRH gene expression in the PVN[2,6] are low. GC receptor messenger RNA (GR-mRNA) has recently been demonstrated in rat fetal brain[9] and neonatal hypothalamus[24].

The response of brain–adrenal axis components to acute and chronic stress in the neonatal rat differs from that in the adult[12,18,21,25,26]. The so-called 'stress-hyporesponsive period' during the first two postnatal weeks has been postulated to result from excess GC negative feedback or immaturity of stress responsive elements at the adrenal, pituitary or hypothalamic levels. Recently, ACTH and corticosterone (CORT) response to acute and repeated stress has been demonstrated on postnatal days 5–10 in neonatal rats[15,25,26].

The purpose of this investigation was to study the ontogeny of GC feedback effect and of stress on CRH synthesis in the PVN. We examined the effect of a specific GC receptor antagonist (RU 38486)[14], implanted adjacent to the PVN, on CRH messenger RNA (CRH-mRNA) abundance during the first two postnatal weeks. We examined the onset of up-regu-

Correspondence: T.Z. Baram, Division of Neurology no. 82, Childrens Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027, USA. Fax: (1) (213) 667-2019.

lation of CRH-mRNA abundance and of plasma CORT by PVN GC receptor blockade. We determined the presence of GC receptor-mRNA in the PVN. Furthermore, we studied the effect of GC receptor blockade on CRH-mRNA up-regulation by chronic, implantation-induced stress.

MATERIALS AND METHODS

Animals

Time-pregnant Sprague–Dawley rats were obtained from Zivic–Miller (Zelienople, PA), kept on a 12 h light/dark cycle and given access to unlimited lab chow and water. Time of birth of pups was determined every 12 h, and day of birth was considered day 0. Litters were culled to 12 pups and mixed among experimental groups.

Study design and data presentation

Implantation (of both sexes) was performed on postnatal days 3, 5, 7, 8, 9, 10, 11, 12 and 13. Experimental groups consisted of: (a) pups implanted with RU 38486; (b) pups implanted with cholesterol-containing cannulae; (c) non-implanted rats. Animals were sacrificed 3 days later, and plasma analyzed for corticosterone (CORT). Plasma CORT was also measured in non-implanted pups aged 7 and 9 days (Fig. 2). Brains were analyzed for CRH-mRNA abundance in PVN.

Cannula preparation and implantation

Methods of cannula preparation and implantation into the PVN of neonatal rats have been described[28]. Briefly, cholesterol or RU 38486 were dissolved in absolute ethanol over a hot-plate. Ethanol was heat-evaporated, and the crystalline residue was utilized. Cannulae were prepared from 30 gauge stainless steel hypodermic tubing (i.d. 0.006 inches) by cutting into appropriate lengths, and were filled by gently tamping one end into the crystalline steroid. The amount of steroid packed into the cannulae and the amount of steroid released from the cannulae into the brain were calculated as follows[28]: cannulae were weighed before (**1**) and after (**2**) packing with steroid or cholesterol, and again when recovered after sacrifice of implanted rats (**3**). Cannulae were then immersed in ethanol to dissolve residual drug, and weighed a final time (**4**). Weight of steroid released into the brain was calculated using the formula:

$$(2-1)-(3-4)$$

Under cold anesthesia, the steroid-containing cannula was placed immediately above the PVN, using a stereotaxic apparatus modified for neonatal rats. Age-dependent coordinates for cannula placement are below:

Age (days)	AP	Vent	Lat	(from bregma)
1	0.5	−5.5	1.0	
3	0.55	−5.6	1.0	
5	0.6	−5.7	1.1	
7	0.65	−5.9	1.2	
10	0.7	−6.4	1.3	
15	0.8	−6.5	1.4	

Following surgery, animals were placed on a heating pad under a heating lamp until their behavior resembled those of naive littermates. The whole litter was returned to the mother as a group. Total time of maternal deprivation was 3–4 h.

Coordinate determination and verification

PVN coordinates were derived as follows: several animals of each age group were implanted with empty cannulae. Cannula placement was examined in some animals immediately and in others, three days later, to control for potential brain growth. Brains were removed and quickly frozen in powdered dry-ice[3]. Twenty μm frozen sections were cut and every eighth section was stained with Cresyl violet. This verification procedure was also followed at the end of each implantation experiment.

Tissue preparation

Neonatal rats were sacrificed three days after implantation. Time of sacrifice was between 08.00 and 09.30 h, to avoid diurnal variability in CRH-mRNA abundance and CORT levels[13,27]. Animals were decapitated rapidly, within 25 s of separation from littermates and mother. Trunk blood was collected, and brains were removed onto dry-ice and stored at −80°C. Coronal sections (20 μm) were mounted on gelatin-coated slides and stored at −80°C.

Preparation of CRH oligonucleotide, and GR-clone probes

A 60 nucleotide synthetic probe corresponding to the codons for the 20 COOH-amino acids of CRH had been generated using an Applied Biosystems DNA Synthesizer[2,4]. The probe was labeled on the 3′-end with [^{35}S]-dATP (New England Nuclear) using terminal deoxy-nucleotidyl transferase (Bethesda Research Lab). A sense-strand and an oligonucleotide probe for somatostatin messenger RNA were similarly generated and labeled as specificity controls[2,4]. A GC receptor-specific clone (originally from Dr. K. Yamada) was obtained from Drs. J.P. Herman and S.J. Watson[7]. Both sense and antisense [^{35}S]-cRNA probes were synthesized using [^{35}S]-UTP (Amersham), and Sp6 and T_7 RNA polymerase respectively. The specific activity of the probes were 1.4×10^9 cpm/ ug.

In situ hybridization procedures

Prior to ISH, slides were brought to room temperature, air-dried and fixed in buffered paraformaldehyde. Following a graded ethanol treatment[2,4] sections were exposed to acetic anhydride–triethanolamine, then dehydrated through 100% ethanol[2,29].

For CRH-mRNA, sections were prehybridized for 1 h, then hybridized for 20 h at 40°C in a humidity chamber, as described elsewhere[2,4]. Serial washes (2×SSC for 15 min ×4 at 40°C, 1× and 0.4×SSC for 30 min each at room temperature) were followed by dehydration and apposition to film (Hyperfilm B-Max, Amersham). Several sections were subsequently dipped in emulsion (NTB-2: Kodak), and developed as previously described[2,4], with the exception that the emulsion was not diluted.

The ISH procedure using GC cRNA probes was modified from that described by Angerer[1] and Simmons[20]. Briefly, acetic anhydride-treated, ethanol dehydrated sections were exposed to 20 μl hybridization solution[20] containing 5×10^7 cpm/ml of labeled probe at 58°C for 15 h. Coverslips were removed in 4×SSC, and the slides treated with 20 μg/ml RNase A for 30 min at 37°C. Serial washes with decreasing concentrations of SSC, containing 1 mM DTT were followed by a high stringency wash (0.1×SSC) at 75°C for 1 h. The slides were then dehydrated through ethanol–0.3 M NH_4Ac and apposed to film.

Sections with the maximal area of PVN, based on Cresyl violet staining, were used for quantitative image analysis. Quantitation and statistical analysis were described previously[2,4]. Briefly, optical density (OD) was determined over PVN and parietal cortex, as background, using the MCID software image analysis system (Imaging Research, Ont., Canada). Each point was derived from 6–12 sections from a minimum of 6 individual rats. Ratios of PVN to parietal cortex were determined as a measure of the abundance of CRH-mRNA, thus eliminating background variability with age. Two-way analysis of variance (treatment and age), was used to compare OD ratios in RU 38486-implanted group to those in cholesterol-implanted or non-implanted controls.

Radioimmunoassay

Plasma CORT was assayed using a commercial RIA kit (ICN, Irvine, CA), as previously described[3]. Assay sensitivity for CORT was 0.05 ng/ml. Intra-assay variability was less than 7%. All samples

were run in the same assay. Significant differences between groups were assessed by analysis of variance.

RESULTS

Effect of implantation on infant rat weight gain

There was no effect of cannula implantation on body weight gain. Fig. 1 shows that neither the stress of implantation (cholesterol group) nor RU-38486 containing cannulae affected body weight for the duration of the experiment. On the day of sacrifice, weights of cholesterol- and RU 38486-implanted rats were not different from those of non-implanted littermates ($P > 0.1$, Fig. 1).

Effect of RU 38486 implantation on plasma CORT levels

Plasma CORT did not differ between the non-implanted and cholesterol-implanted groups at any time point (Fig. 2). The low hormone levels during the first two postnatal weeks are consistent with previous reports[13,21,26]. Plasma CORT levels in RU 38486-implanted pups were higher than those of cholesterol-implanted ones (and of non-implanted controls) starting on postnatal day 8 (day of sacrifice, implanted on day 5, Fig. 2, $P = 0.025$).

Effect of RU 38486 implantation on CRH gene expression in the PVN

CRH-mRNA in rats implanted on days 4, 6, 7, 9 and 10 (sacrificed on days 7, 9, 10, 12 and 13) are shown in Fig. 3. A typical dark-field photomicrograph of ISH for CRH-mRNA is found in Fig. 4. CRH-mRNA abundance in rats implanted on day 3 were indistinguishable from those presented for day 4. Similarly, results

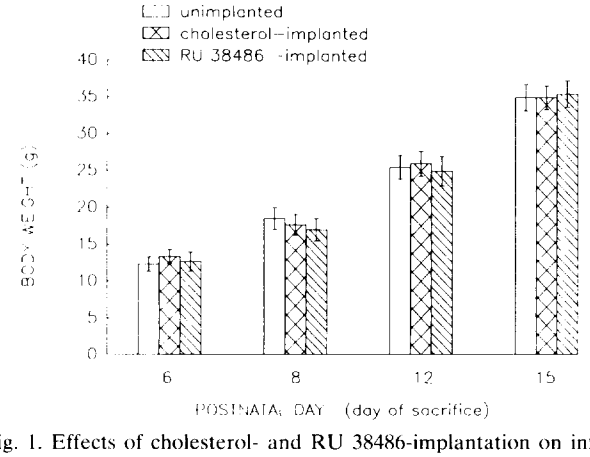

Fig. 1. Effects of cholesterol- and RU 38486-implantation on infant rat body weight gain. Rats were implanted with cholesterol or RU 38486 three days before sacrifice. Each bar represents the mean ± S.E.M. of 6–20 rats. Differences between groups are not significant ($P > 0.05$).

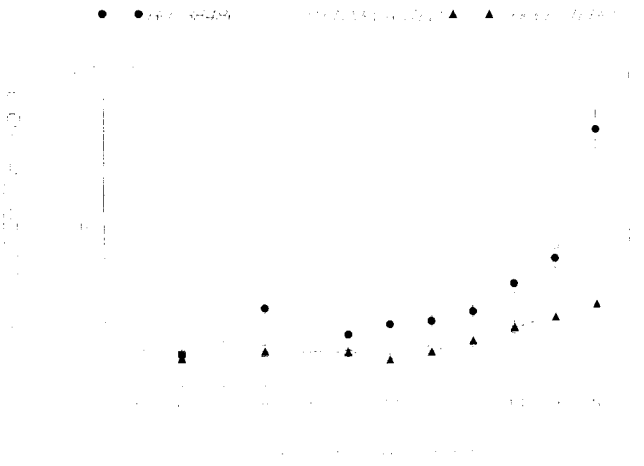

Fig. 2. Plasma corticosterone levels, on day of sacrifice, in cholesterol- or RU 38486-implanted and non-implanted neonatal rats. Starting on postnatal day 8 (i.e. pups implanted on day 5), corticosterone levels of RU 38486-implanted rats were significantly higher than those of cholesterol-implanted controls ($P < 0.05$, ANOVA). Each point represents the mean ± S.E.M. of at least 6 rats.

of implantation on days 5 and 12/13 did not differ from those presented for days 6 and 9/10, respectively. RU 38486 increased CRH gene expression in the PVN when implanted on or subsequent to the 9th postnatal day ($P < 0.05$; Figs. 3, 4). Cholesterol implantation did not cause a significant change in CRH gene expression when implanted on days 2–6 (Fig. 3). Pups subjected to cholesterol-implantation on days 7,9 had a significant ($P = 0.025$) increase in PVN-CRH-mRNA abundance compared to non-implanted controls. Cholesterol im-

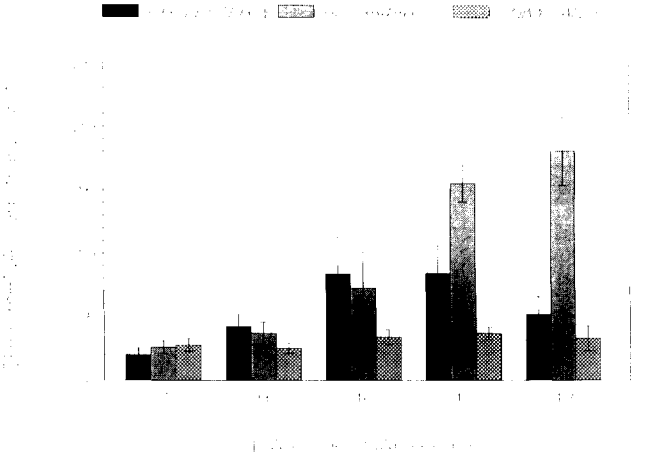

Fig. 3. Effect of cholesterol- and RU 38486-implantation on CRH-mRNA abundance in neonatal rat PVN. Rat pups were sacrificed 3 days after surgery. In pups implanted on days 9, 10 and later (not shown), PVN-CRH-mRNA in the RU 38486 group is significantly increased compared with both the cholesterol and non-implanted controls ($P < 0.05$). Differences between CRH-mRNA levels in cholesterol-implanted and non-implanted groups are significant on day 9 ($P < 0.05$), but not on day 10 ($P = 0.10$). Each bar represents mean ± S.E.M. of at least 6 sections from 6 individual rats.

plantation on day 10 resulted in a smaller increase in CRH-mRNA ($P = 0.10$, cholesterol-implanted vs. non-implanted).

Presence of GR-mRNA in the PVN

GC receptor-mRNA was present in the PVN of the neonatal rat as early as the first postnatal day (not shown). Utilizing ISH, GR-mRNA was confined to the parvocellular subdivisions of the nucleus, similar to the distribution of CRH-mRNA[2,6,22,29] (Fig. 4).

DISCUSSION

We find that during the first postnatal week, neither elimination of GC input into the PVN, nor chronic stress associated with cannula-implantation result in enhanced CRH-mRNA abundance. GC receptor blockade at the PVN starting on days 8–9 results in enhanced CRH synthesis. The increase in CRH-mRNA abundance in rats subjected to implantation stress is transient, and is seen in pups implanted on days 7–9 (sacrificed on days 10–12, Fig. 3).

These results are subject to several interpretations, regarding the development of both the stress response, and the CRH-ACTH-GC feedback loop in the neonatal rat. Elimination of the negative feedback effect of GC in a mature rat results in up-regulation of CRH synthesis[8,10,22,29]. CORT exerts a dose-dependent negative feedback effect on CRH gene expression in the PVN[22]. This effect is at least partially mediated via local GC receptors[10,11,17,19,22]. Our results demonstrate that elimination of GC input into the PVN during the first postnatal week does not increase CRH-mRNA levels. This is in accord with Grino's report that adrenalectomy does not alter CRH synthesis in the 7-day-old rat[5].

This study demonstrates a dysfunction of GC effect on CRH-mRNA at the level of the hypothalamus. Since GC receptors are synthesized in the PVN in the neonatal rat (ref. 24; Fig. 4), absence of GC receptors is an unlikely reason for the lack of CRH-mRNA response to RU 38486 implantation. Alternatively it may be suggested that RU 38486 did not reach PVN GC receptors. This is unlikely for the following reasons: first, we ascertained steroid elimination from the cannulae; second the drug was effective in up-regulating CRH synthesis in older, larger rats, where diffusion distance is greater. Further, both ipsi- and contralateral PVN were affected in this study, as has been reported for adult rats[10,11,19].

In this study CRH-mRNA abundance, determined by semi-quantitative ISH, was used as a measure of CRH synthesis. The technique does not account for possible alteration in messenger RNA stability[1]. Fur-

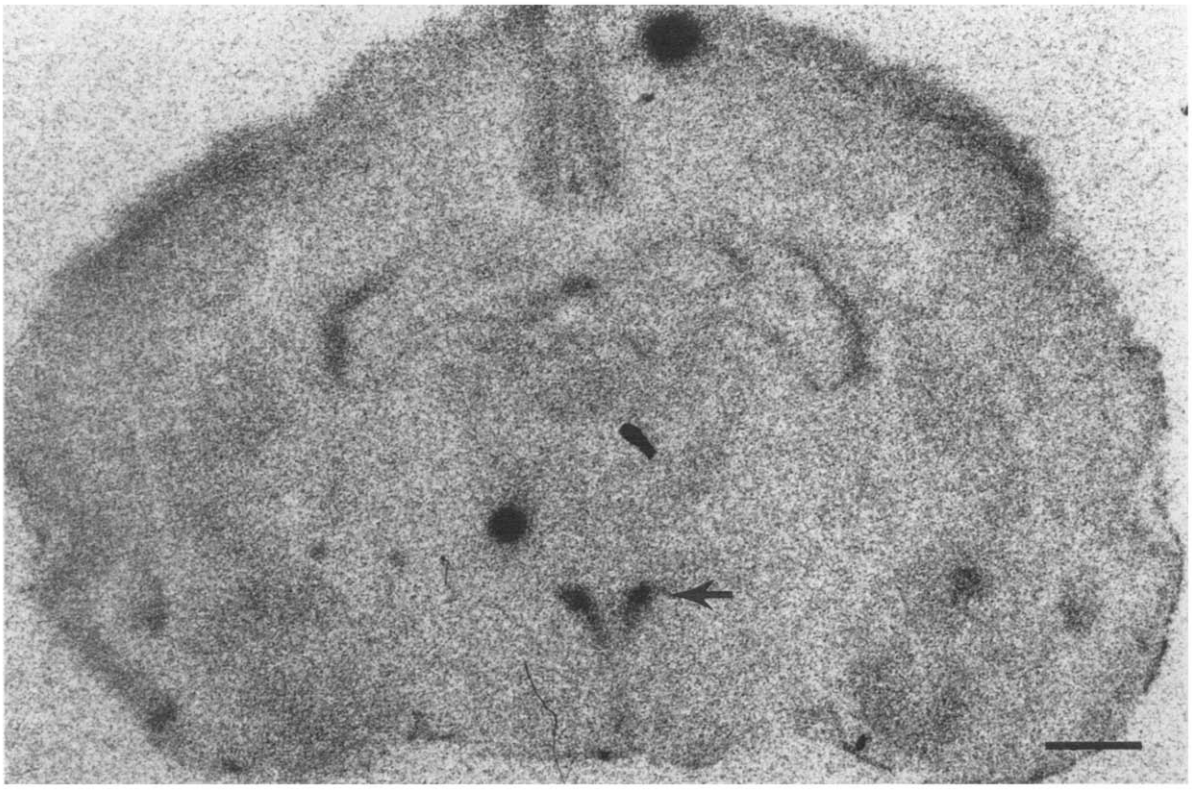

Fig. 4. GC receptor messenger-RNA (GR-mRNA) in the PVN (arrow) of a 7-day-old rat subjected to ISH with GR-mRNA probe. Bar = 1 mm. PVN, paraventricular nucleus.

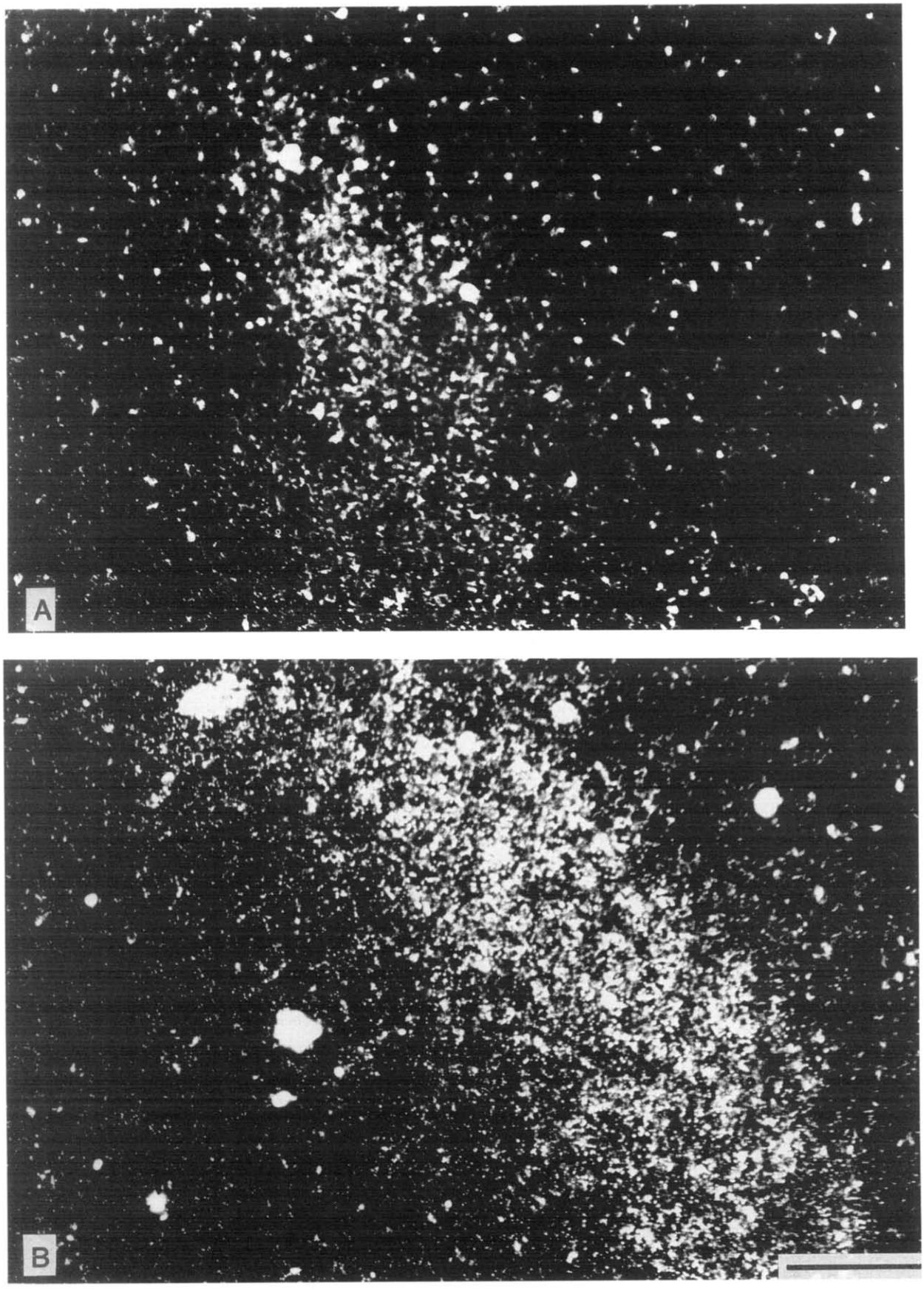

Fig. 5. Darkfield photomicrograph of the PVN of 14-day-old rats implanted with chronic cannulae three days previously and subjected to in situ hybridization for CRH-mRNA. A: cholesterol control. B: RU 38486. Non-implanted pups' CRH-mRNA abundance did not differ from cholesterol-implanted (not shown). Bar = 50 μm.

ther, relatively small (< 30%) changes in CRH-mRNA abundance in the immature rat in response to GC receptor blockade may not be detectible. Such increase in CRH may account for the discrepancy between the effect of GC receptor blockade on CRH-mRNA abundance and on plasma CORT in rats sacrificed on postnatal days 8–11: As is evident from Figs. 2 and 3, RU 38486 implantation during this age increased plasma CORT without significantly altering CRH-mRNA. Alternative mechanisms may involve enhanced pituitary or adrenal sensitivity to circulating CRH and ACTH respectively, or an effect of GC blockade on other ACTH secretagogues, such as vasopressin[15].

Cholesterol containing-cannulae implantation presents both an acute and chronic stressor to the neonatal rat. Maternal separation during surgical procedure was relatively short, and likely did not activate the brain–adrenal axis. Stanton et al.[21], suggested that a one hour maternal separation had no effect on subsequent stress response, and an eight hour deprivation had a modest effect. Further, plasma CORT of maternally deprived but non-implanted littermate controls in the current study were consistent with those reported for non-stressed neonatal rats of similar age[13,21,26]. Cholesterol containing cannulae increased CRH-mRNA in rats sacrificed on days 10–12 but not later, suggesting that for younger pups, carrying a chronic cannula was stressful. It further implied that CRH-mRNA may be affected by chronic stress at this age. Plasma CORT was not increased in these cholesterol implanted pups. The independence of plasma ACTH and CORT from circulating CRH during the second postnatal week has been suggested by Muret[15].

In conclusion, chronic, local blockade of PVN GC receptors by RU-38486, and cannula implantation-induced stress, do not induce CRH gene expression prior to postnatal day 7. Both maneuvers result in up-regulation of CRH-mRNA synthesis by postnatal day 9. Since GR-mRNA is present in the PVN during the first postnatal week, second messenger mechanisms, or those independent of GC-receptors may account for the failure in CRH-mRNA up-regulation.

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