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Methods for Implanting Steroid-Containing Cannulae Into the Paraventricular Nucleus of Neonatal Rats

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Implantation of cannulae into brains of neonatal rats presents methodological difficulties. We discuss such issues as avoiding tissue injury, and describe successful techniques. Cannulae size, methods of preparation, insertion, and securing are evaluated. We present a modified cannula holder applicable to the soft neonatal brain. Application of these methods to the study of glucocorticoid receptors in the neonatal rat hypothalamus is illustrated.

Keywords: Cannula; Rat; Neonate; Paraventricular nucleus; Hypothalamus; Glucocorticoid receptor; Corticotropin releasing hormone

Introduction

Secretion of glucocorticoids (GCs) is stimulated by a variety of stressors acting via corticotropin releasing hormone (CRH) and adrenocorticotropin (ACTH). GCs exert inhibitory feedback effects on the secretion and synthesis of CRH in the hypothalamic paraventricular nucleus (PVN) (Swanson and Simmons, 1989; Dallman et al., 1987; Young et al., 1986) mainly via local GC receptors (Reul and de Kloet, 1985; Kovacs and Mezey, 1987). However, this regulatory mechanism may not be operative during the first postnatal days in the rat (Grino et al., 1989; Baram and Lerner, 1991).

In order to study GC negative feedback mechanisms at the hypothalamic level, we developed a technique for chronic implantation of steroids into the PVN of neonatal rats. There are several difficulties in surgical procedures and implantation in these animals. Issues of susceptibility to anesthesia (Marshall and Wollman, 1985), and maternal cannibalism (Libbin and Person, 1979) have been discussed previously. Further, brains of newborn rats are small and easily damaged by cannulae or electrodes. Stereotaxic atlases delineate postnatal days 1 and 10, but not intervening ages (Sherwood and Timiras, 1970; Paxinos et al., 1991; Valenstein et al., 1969), or describe a single age of a specific rat strain (3-day-old Holtzman rats, Heller et al., 1979). We present techniques devised in our laboratory for determination of PVN coordinates in 1- to 15-day-old rats, for preparation of steroid-containing cannulae applicable to this age, and for minimization of parenchymal injury.

Materials and Methods

Animals

Time-pregnant Sprague–Dawley rats were obtained from Zivic Miller (Zelienople, PA), kept on a 12-hr light/dark cycle and given access to unlimited lab chow and water. Pregnancy was dated by the presence of a vaginal plug (day 0). Animals were checked for presence of pups every 12 hr. Implantation was performed on different postnatal days (days 1 through 15).

Stereotaxic Surgical Procedure

Cold-anesthesia was used. Weighed pups were placed in an ice basket, and when motionless, transferred to a metal top of an ice-filled 400-mL histology jar. They were immobilized in a Kopf neonatal rat stereotaxic apparatus, using blunt-end ear bars. The

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mouth bar was adjusted to maintain bregma and lambda in a horizontal plane. A mid-sagittal incision of skin over the skull was made, and the skull was cleared of connective tissue and blood. A hole on the skull was made using a drill (no. 78, diameter = 0.206 mm) according to the anterior–posterior and medial–lateral coordinates for the PVN from the bregma. The cannula was lowered through a drilled hole, so that steroid-containing cannulae could be inserted immediately above and lateral to the PVN. The cannula was firmly anchored by applying dental cement around it, and released upon drying of the cement.

The cannula holder was fashioned from a mechanical pencil, with the cannula substituting for the graphite [Figure 1 (left panel)]. The pencil shaft was anchored to the original apparatus using heat-sink plastic tubing. Pressing up the pencil-sheath using pincers, resulted in spreading apart of the metal flanges [Figure 1 (right panel)], and release of the cannula with no downward excursion or stress.

Following surgery, animals were placed on a heating pad under a heating lamp until their behavior and mobility appeared normal. They were then transferred back to their mother as a group.

Coordinates for the PVN 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, 3 days after implantation. Brains were removed and quickly frozen in powdered dry ice (Baram and Schultz, 1991). Frozen sections (20-μm) were cut and every eighth section was stained with cresyl violet. This procedure was followed also at the end of each implantation experiment to verify cannula placement.

Cannula Preparation and Filling

Rubin and Barfield's method for preparation of 17β-estradiol-containing cannulae was modified for this study (Rubin and Barfield, 1980). Cannulae were pre-

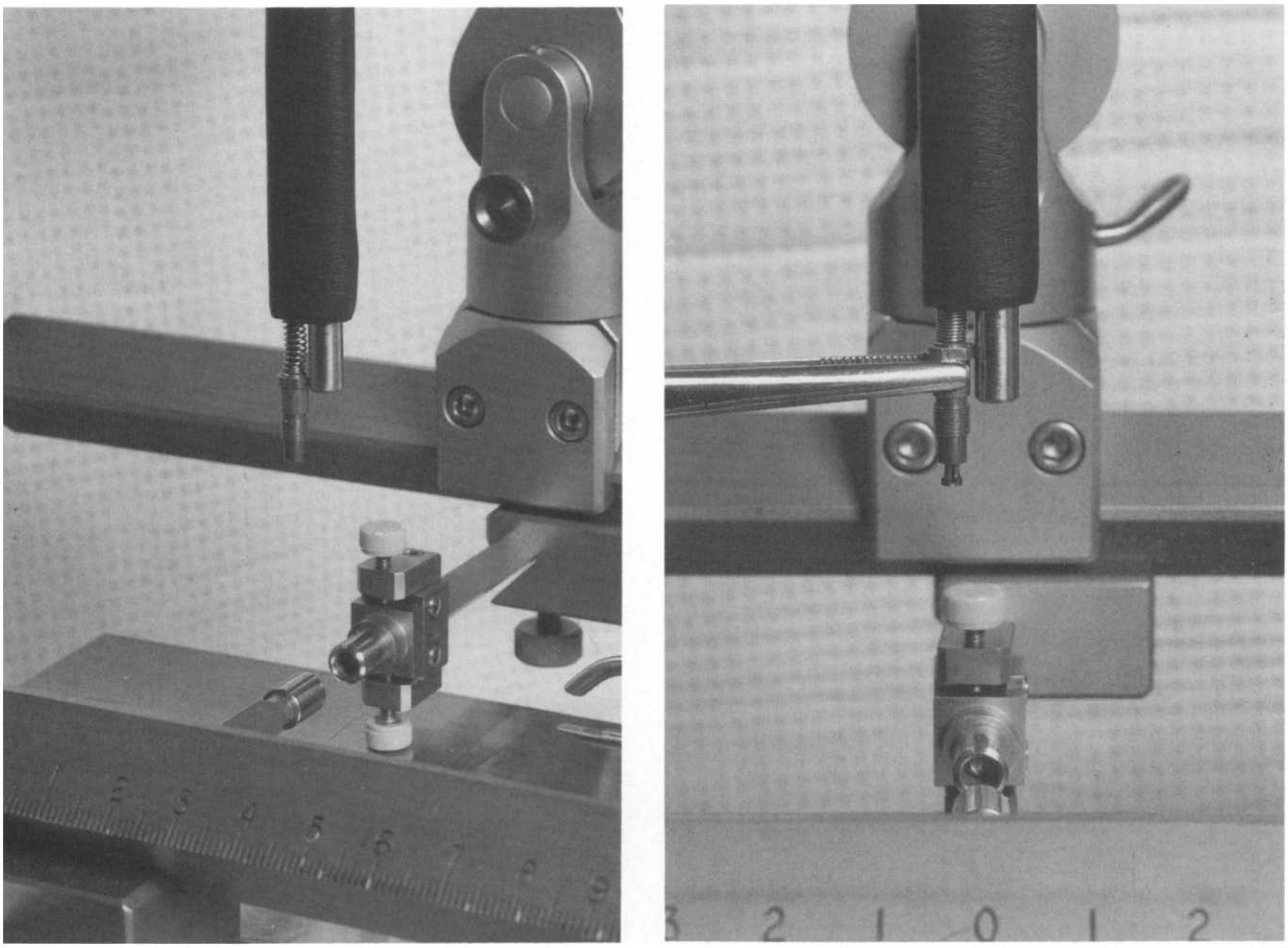

Figure 1. Modified cannula holder designed to eliminate pressure and torque to the infant brain. The cannula holder, a mechanical pencil shaft, is attached to a Kopf infant stereotaxic apparatus using heat-sink tubing (left panel). The tubing (i.d. = 0.5″) was shrunk subsequent to heat application. The cannula is inserted into the center of the closed metal flanges. It is released when the flanges fan open upon elevation of the metal sheath using pincers (right panel).

Table 1. PVN Coordinates for Neonatal Rats

Age (days)	AP	VENT	LAT
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

Abbreviations: AP, anterior-posterior; VENT, ventral; LAT, lateral.

pared from 30-gauge stainless-steel hypodermic tubing; o.d. = 0.012″ (0.3 mm); i.d. = 0.006″ (0.15 mm), by cutting into appropriate lengths (about 12 mm for 10-day-old rats). They were sanded but not polished (cannula-end was blocked by sanding because of the small internal diameter). Cholesterol or RU-38486 (a GC receptor antagonist; Moguilewsky and Philibert, 1984) was placed in a 15-ml glass vial containing absolute ethanol, and the vial was placed on a hot plate (at a low setting) and tightly capped to ensure that the steroid was thoroughly dissolved. The solution was transferred to a glass petri dish and placed on a hot plate to facilitate ethanol evaporation. The crystalline residue was used to fill the cannulae for implantation. Cannulae were filled with 137.50 ± 32.19 μg of drug, by gently tamping one end into the crystalline steroid. For calculating the amount of steroid packed into the cannula, the cannulae were weighed before (1) and after (2) packing with steroid. The outer surface of the cannulae was then cleaned with ethanol. After sacrifice of implanted rats, cannulae were recovered. They were weighed immediately after removal from the animals (3), then after immersion in absolute ethanol for several days to dissolve residual drug (4). The amount of steroid released from the cannula into the brain was calculated using the following formula:

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

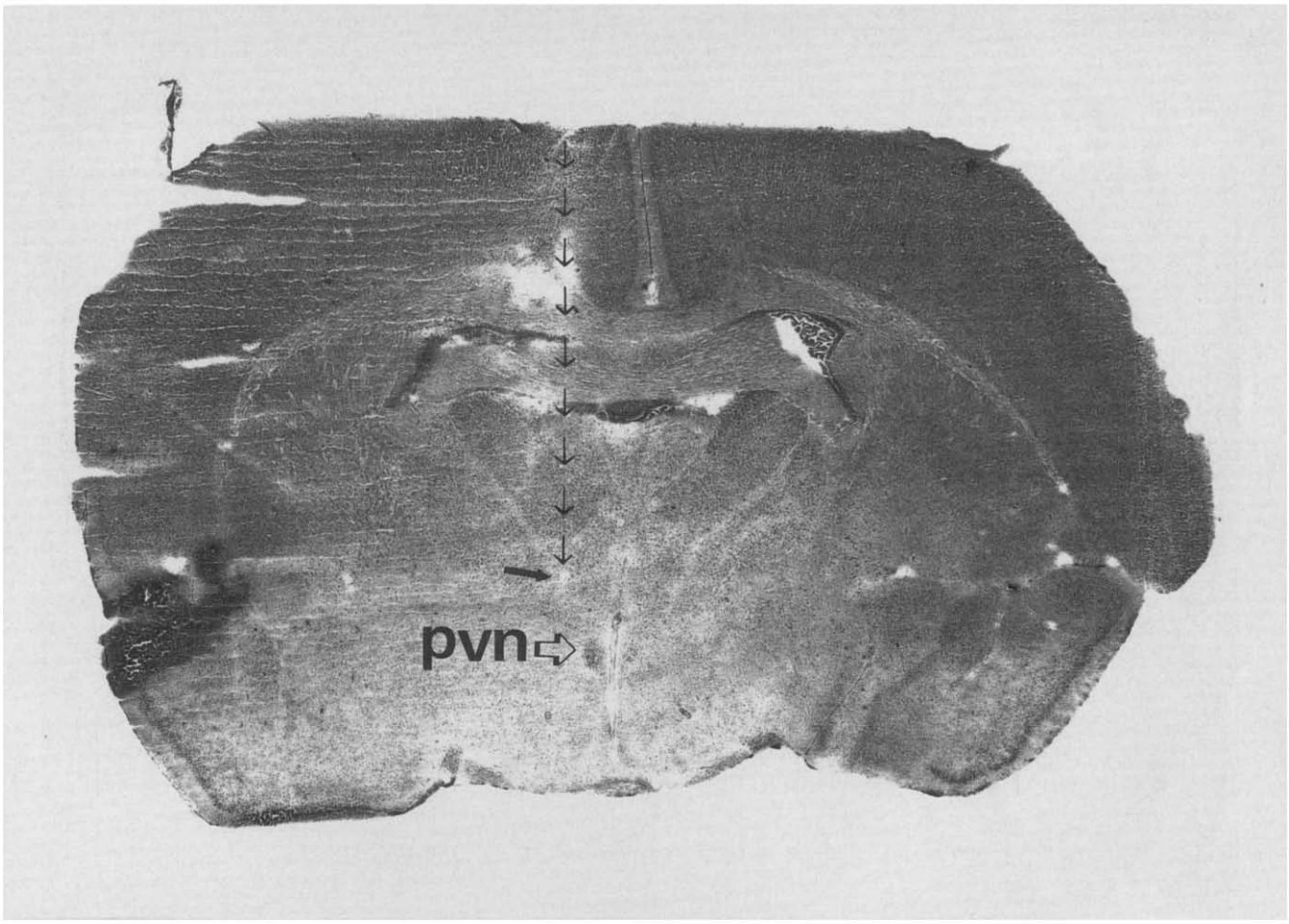

Figure 2. A 20-μm brain section of a 9-day-old rat is shown, stained by cresyl violet, detailing a cannula tract (small arrows) which ends in a small stain-poor area (small arrow) above and lateral to the PVN.

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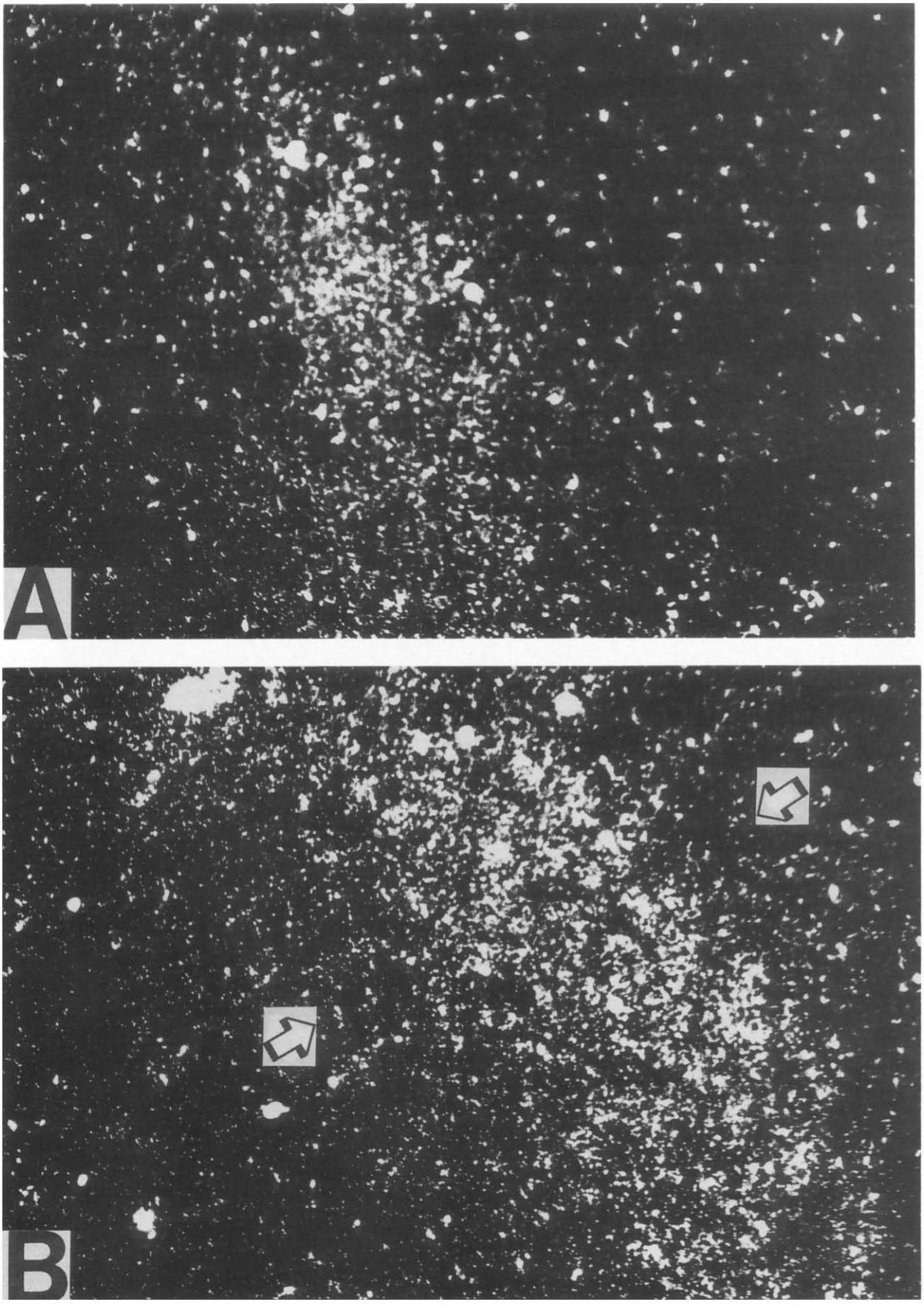

Results

PVN Coordinates for Neonatal Rats

Coordinates are in reference to bregma. They are aimed just above the anterior, lateral PVN (Table 1).

Since some brain growth occurs in a given 72-hr period in neonatal rats, these coordinates aim at the anterior "tip" of the PVN. During the first postnatal weeks, bregma shifts anteriorly in relation to the interaural line (Sherwood and Timiras, 1970). Three days after implantation, the posterior extent of the cannula was in the same vertical plane as the anterior-most PVN (Figure 2).

Drug Release and Parenchymal Injury After Cannula Implantation

Mean and standard deviation of drug released over 72–96 hr was 125.83 ± 31.25 μg. That constituted more than 90% of total drug implanted. Conversely, less than 20% of the drug was released during the first 24 hr subsequent to implantation.

Compared to initial implantation with larger cannulae (i.d. 0.013"), smaller cannulae caused significantly less brain damage. Using a small cannula also allowed more precision in aiming at a small nucleus such as the PVN of neonatal rats (Figure 2).

Impact of Implantation on Infant Weight Gain

Implanted animals continued to gain weight normally, so that body weights of unimplanted, cholesterol-, and RU-38486-implanted rats on the day of sacrifice were not significantly different at any age tested (data not shown).

Effect of RU-38486 Implantation on PVN-CRH Gene Expression

Rats were implanted with either cholesterol- or RU-38486-containing cannulae on postnatal days 3, 4, 6, 7, 9, 10, 11, and 13 (and sacrificed on days 6, 7, 9, 10, 12, 13, 14, and 16). CRH-mRNA abundance in the PVN was measured by in situ hybridization (ISH) technique as described elsewhere (Baram and Lerner, 1991; Yi et al., 1993). RU-38486 increased CRH gene expression in the PVN when implanted on or subsequent to postnatal day 9, compared to either cholesterol or nonimplanted control. A representative dark-field photomicrograph of ISH for CRH-mRNA is found in Figure 3.

Discussion

The described technique for cannula implantation in the PVN of neonatal rats offers several advantages. First, by using smaller cannulae and a specially devised cannula holder, we could minimize parenchymal injury. Second, the technique eliminated the need for screws as anchors: careful clearing of the skull around the implant site of connective tissue and blood, and complete drying of the dental cement prior to caging the pups with their mother, resulted in firmly cemented cannulae for up to 1 week. This technique shortened anesthesia time significantly, decreasing surgical mortality. In addition, potential parenchymal damage from screws was also excluded.

We used cold anesthesia (Ben et al., 1969). Anesthesia is, overall, a stressful experience affecting endocrine systems. Newborn rats are especially difficult to anesthetize with barbiturates and the therapeutic ratio may be close to unity (Done, 1964). With inhalation anesthetics, control over the depth of anesthesia is possible through changes in their inspired concentration. The advantage of inhalant anesthesia, however, can be outweighed in many circumstances by the necessity for suitable administration equipment. Neonatal rats fail to maintain a high, constant body temperature. At this age, they can be chilled on ice, and after becoming motionless, they can be satisfactorily operated upon. They remain in anesthesia up to for 1 hr on an "ice bed." On warming, they become active quickly and are not cannibalized by their mothers. We found that high levels of GC induced by cold stress in neonatal rats returned to control levels within 2 hr (data not shown).

The anesthesia and surgical procedure were well tolerated, as evident from the normal weight gain of implanted animals compared to unimplanted littermates. We implanted either cholesterol or RU-38486 in rats on postnatal days 3 to 13, and sacrificed them 3 days later to analyze CRH-messenger RNA in the PVN using in situ hybridization (Baram and Lerner, 1991; Baram and Schultz, 1991). CRH gene expression in the PVN was enhanced in infant rats implanted with RU-38486 on postnatal day 9 or later (Figure 3). These data indicate that this implantation technique can be applied

Figure 3. Dark-field photomicrograph of the PVN of 14-day-old rats implanted with cannulae 3 days previously and subjected to ISH for CRH-mRNA. (A) CRH-mRNA abundance in the PVN of cholesterol-implanted pups, (B) RU-38486-implanted ones. PVN-CRH gene expression in nonimplanted pups was not different from cholesterol-implanted (not shown). Arrows delineate PVN.

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to study the ontogeny of GC receptor-mediated regulatory mechanisms.

The methods described, and the sequential, age-specific PVN coordinates for neonatal rats may thus prove useful to investigators utilizing this type of experimental paradigm.

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