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Decreased Expression of Fos-Related Antigens (FRAs) in the Hypothalamic Dopaminergic Neurons After Immunoneutralization of Endogenous Prolactin

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In our previous studies we found that administration of exogenous prolactin increased dopamine turnover in the terminal areas of the hypothalamic dopaminergic neurons controlling prolactin secretion from pituitary lactotrophs. In this study we investigated the effect of immunoneutralization of endogenous prolactin on the expression of FRAs in the tuberoinfundibular dopaminergic (TIDA), tuberohypophysial dopaminergic (THDA), and periventricular hypothalamic dopaminergic (PHDA) subpopulations of the hypothalamic dopaminergic neurons.

Female rats were ovariectomized on d 0 of the experiment. At 1000 h of d 10, all animals were injected with 20 µg of 17-beta-estradiol sc to induce a proestrous-like surge of prolactin at 1700 h the next day. At 1000 h on d 11, half of the animals were injected with 200 µL of rabbit anti-rat prolactin antiserum ip, while the controls received normal rabbit serum. Groups of animals were sacrificed for immunocytochemistry in 2 h intervals between 1300 and 2100 h. Double-label immunocytochemistry for FRAs and tyrosine hydroxylase (TH) was performed and the results are presented as percentage of TH-immunoreactive neurons expressing FRAs.

In the control animals, expression of FRAs decreased at 1500 h, gradually increased by 1900 h, but was lower than the basal levels by 2100 h. Expression of FRAs was significantly lower at 1900 h in the PHDA, THDA and TIDA neurons of prolactin antiserum treated rats than in the controls.

These results indicate that elimination of endogenous prolactin from the circulation lowers the activity and/or prevents the reactivation of neuroendocrine dopaminergic neurons at the beginning of the dark phase.

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Introduction

The physiological inhibiting factor of pituitary prolactin secretion is dopamine *(1)* released from three anatomically and functionally distinct hypothalamic neuroendocrine cell populations: the tuberoinfundibular dopaminergic neurons (TIDA) *(2)* of the dorsomedial arcuate nucleus (A_{12}), the tuberohypophysial dopaminergic neurons of the rostral arcuate nucleus (THDA), and the periventricular hypothalamic dopaminergic (PHDA) neurons of the periventricular nucleus (A_{14}). TIDA neurons terminate in the external zone of the median eminence supplying the anterior pituitary gland with dopamine through the long portal vessels, while PHDA and THDA neurons terminate in the intermediate and neural lobes of the pituitary gland, respectively *(3,4)*. A growing body of evidence suggests that not only the well-studied TIDA neurons, but the THDA and PHDA neurons also, contribute to the hypothalamic regulation of prolactin secretion *(5–7)*.

Feedback regulation by dopamine autoreceptors, present in other dopaminergic systems, seems to be less apparent in the neuroendocrine dopaminergic neurons *(8,9)*. Previous data indicate that feedback regulation of TIDA neuronal activity is accomplished by prolactin *(10–12)*. Indeed, prolactin receptors are present in all three populations of hypothalamic dopaminergic neurons *(13)*; however, the relative abundance of prolactin receptors in the different subpopulations is dependent on the ovarian steroid background *(13)*. Our recent findings revealed that administration of exogenous prolactin resulted in increased dopamine turnover *(14)* and increased expression of fos-related antigens (FRAs) in all three hypothalamic dopaminergic neuron populations *(15)*. However, regional differences were apparent, as exogenous prolactin robustly increased expression of FRAs in the TIDA and THDA neurons, while had less effect on the PHDA population in ovariectomized female rats *(15)*.

The immediate early gene *c-fos* is widely used and accepted as a marker of neuronal activation following varying excitatory stimuli *(16)*. However, the tonic neuronal activity of TIDA neurons can be better assessed by detecting the expression of fos-related antigens (FRAs) *(17)*. The term "FRAs" includes at least five different proteins each forming heterodimers with various members of the Jun protein family. These protein dimers bind to the activator protein-1 (AP-1) site of DNA and activate the transcription of target gene(s) *(18,19)*. Our previous publications *(20)* indicate that increase in the expression of FRAs coincides with increased dopamine turnover as well as activation of prolactin receptors in the neuroendocrine dopaminergic neurons *(21)*.

Regulation of prolactin secretion from the pituitary gland is accomplished by several central, peripheral, and environmental factors *(22)*. It is well known that secretion of prolactin from the pituitary gland shows a light-entrained circadian rhythm, which is enhanced by ovarian steroid hormones, especially estrogen. Elevation of prolactin levels occurs near the end of light phase (late afternoon) in nocturnal animals such as rats *(22)*. In female rats, estrogen enhances the amplitude of the circadian prolactin elevation, as seen in the proestrous surge of prolactin *(23)*. Earlier we found that expression of FRAs in the hypothalamic dopaminergic neurons also shows a daily pattern *(24)*. The amplitude of daily changes in FRAs expression was also influenced by ovarian steroid background, i.e., it was decreased in ovariectomized rats *(24)*. However, ovarian steroids may influence both the hypothalamic dopaminergic neurons directly as well as the increased amplitude of daily prolactin release *(22)*. The aim of this study was to differentiate the prolactin-dependent elements in the daily pattern of FRAs expression in the hypothalamic dopaminergic neuron subpopulations. We performed double-label immunocytochemistry detecting FRAs and tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine biosynthesis.

Results

Sampling times in this study were determined based on serum prolactin level measurements in our previous study *(25)*, where the same animal model was used and dopamine turnover in the terminal areas of the hypothalamic dopaminergic neurons were determined. According to these results prolactin levels started to increase at 1500 h, peaked by 1700 h, and decreased to basal levels by 2100 h in the normal-rabbit-serum-treated control rats. Prolactin antiserum treatment abolished the elevation in prolactin levels by 80% *(25)*.

Injection of prolactin antiserum or normal rabbit serum in vivo did not result in immunodetectable rabbit molecules in the mediobasal hypothalamus (Fig. 1). Representative photomicrograph images demonstrate the overall effect of prolactin antiserum treatment on the expression of FRAs in the hypothalamic dopaminergic neurons (Fig. 2). More hypothalamic dopaminergic neurons express FRAs in the normal rabbit serum treated controls (Figs. 2A–D), than in the prolactin antiserum treated rats (Figs. 2A'–D') at 1900 h. The abundance of FRAs immunoreactive nuclei was different between the hypothalamic dopaminergic neuron subpopulations. FRAs immunoreactive dopaminergic neurons were scarce in the periventricular nucleus (Fig. 2A-A'), while more abundant in the rostral (Fig. 2B-B'), dorsomedial (Fig. 2C-C'), and ventrolateral (Fig. 2D-D') portions of the arcuate nucleus. The anatomical boundaries of the areas examined were previously described in details and depicted on schematic drawings *(24,26)*.

For the quantitative analysis of FRAs/TH immunocytochemical assays (Fig. 3), the percentage of TH-positive neurons with FRAs-immunoreactive nuclei was determined among all TH-immunoreactive neurons with visible nuclei in each area and time point. In the periventricular nucleus (Fig. 3A), rostral arcuate nucleus (Fig. 3B), and dorsomedial arcuate (Fig. 3C) nucleus a similar pattern of FRAs immunoreactivity was observed in the dopaminergic neurons. There was a significant decrease in the percentage of FRAs immunoreactive neurons at 1500 h, followed by a gradual increase by 1900h and a moderate decrease by 2100 h (Fig. 3A–C). In prolactin-antiserum-treated rats no increase in FRAs expression could be observed by 1900 h, producing a significant difference between control and prolactin-antiserum-treated rats. In the ventrolateral portion of the arcuate nucleus (Fig. 3D), no significant differences were detected between time points in the normal-rabbit-serum-treated rats. However, prolactin antiserum treatment significantly decreased the level of FRAs expression at 1900 and 2100 h in these neurons as well. The total number of TH-immunoreactive neurons counted in this study are presented in Table 1.

Discussion

Our results indicate that in all hypothalamic dopaminergic neuron populations, the daily pattern of FRAs expression has a prolactin-dependent component at 1900 h, which was abolished by immunoneutralization of endogenous prolactin. This finding confirms the results of our previous study, which, based on dopamine turnover measurements in the terminal areas, concluded that immunoneutralization of prolactin prevents the stimulatory feedback on the hypothalamic dopaminergic neurons *(25)*.

It has been long established that prolactin exerts a negative feedback effect on its own secretion by stimulating the synthesis and release of dopamine *(27)* from the TIDA neurons *(12)*. Prolactin has both short-term and delayed effects on the hypothalamic tuberoinfundibular dopaminergic neurons *(28)*: Prolactin acutely increases dopamine secretory activity by phosphorylation of tyrosine hydroxylase, while as a delayed effect it promotes the transcription of the TH

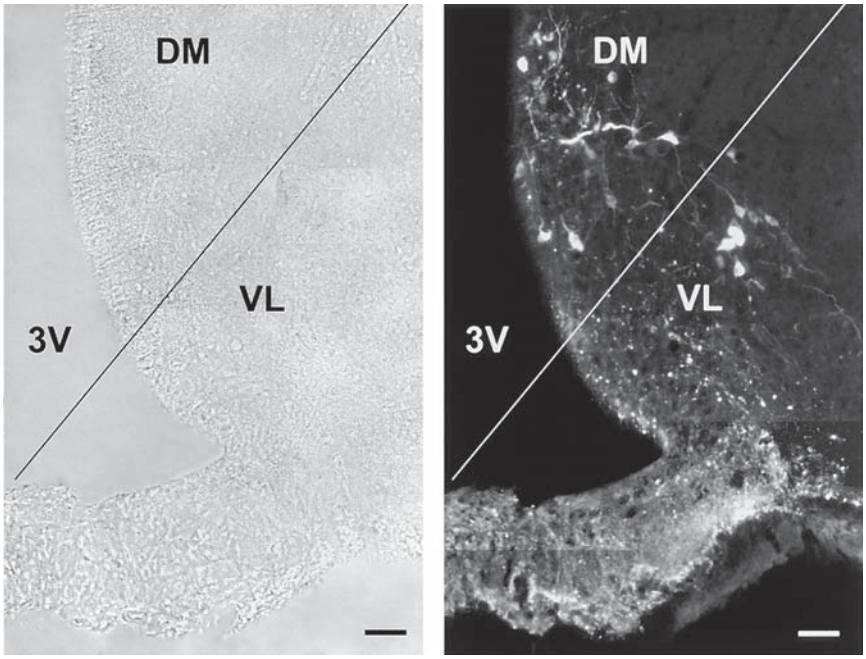

Fig. 1. Representative montage image (obtained with a 40× objective lens) of sections subjected to control stainings. The section shown is the middle portion of the arcuate nucleus of a prolactin antiserum treated rat, sacrificed at 1300 h. Since the animals were injected with anti-prolactin rabbit serum or normal serum, and the anti-FRAs antibody used in our staining protocol was also raised in rabbit, we performed control stainings, in which we omitted the anti-FRAs rabbit serum. The sections were stained according to the same immunocytochemical protocol described in Methods, with the exception of incubation with anti-FRAs rabbit antibody. The right panel (light field, phase contrast) shows the lack of molecules detectable with anti-rabbit biotinylated goat antibodies. The left panel (dark field, TH immunostaining) demonstrates our method of dividing the middle portion of the arcuate nucleus into dorsomedial (DM) and ventrolateral portions (VL). 3V: third ventricle. Bar = 50 μm.

gene *(29)*. Our recent data indicate that such short loop feedback by prolactin is present on all three hypothalamic dopaminergic neuron populations *(13–15,25)*. We have proven the anatomical basis for short prolactin feedback by demonstrating the presence of prolactin receptors on neuroendocrine dopaminergic neurons *(13)*. The incidence of prolactin receptors in the THDA and TIDA neurons is higher than in PHDA neurons, although PHDA neurons also express prolactin receptors in rats with long-term ovarian steroid replacement *(13)*. Administration of exogenous prolactin to ovariectomized rats produced a smaller and delayed effect in the PHDA neurons *(15)*, while in the current study the pattern of FRAs expression in the PHDA neurons was similar to that in the TIDA and PHDA neurons. It should be noted that in the current study the ovariectomized animals received a single injection of estrogen to produce a proestrous-like surge of prolactin the next day, which may have been sufficient to increase the expression of prolactin receptors in the PHDA neurons *(13)*.

We have previously described the presence of a daily rhythm of FRAs expression in TIDA, THDA, and PHDA neurons *(24)* and a rhythm of dopamine turnover in their respective axon terminals in the median eminence, and neural and intermediate lobes of the pituitary gland *(30)*. In this study we examined the time points after the prolactin surge in more detail. Although the overall pattern of FRAs expression was similar to that described previously in proestrous and ovariectomized rats *(24)*, our new results indicate that prolactin-induced activation of the hypothalamic dopaminergic neurons may limit the amplitude of the prolactin surge. Alternatively, prolactin-induced activation of dopaminergic neurons might play a role in the termination phase of the prolactin surge.

It is more difficult to interpret the pattern of FRAs expression in the ventrolateral part of the arcuate nucleus. While no significant difference in FRAs expression can be detected between time points in the ventrolateral arcuate nucleus of normal-serum-treated control rats, prolactin antiserum treatment caused a significant decrease in FRAs expression at 1900 h. This finding indicates that maintained expression of FRAs in these neurons requires prolactin during the afternoon hours. Earlier we found that the tyrosine hydroxylase immunoreactive neurons in the ventrolateral arcuate nucleus express prolactin receptors in great abundance *(13)*; however, the role of these neurons in regulation of prolactin secretion is not understood.

Our findings in this and in our previous study *(25)* suggest that prolactin antiserum has an immunoneutralizing effect

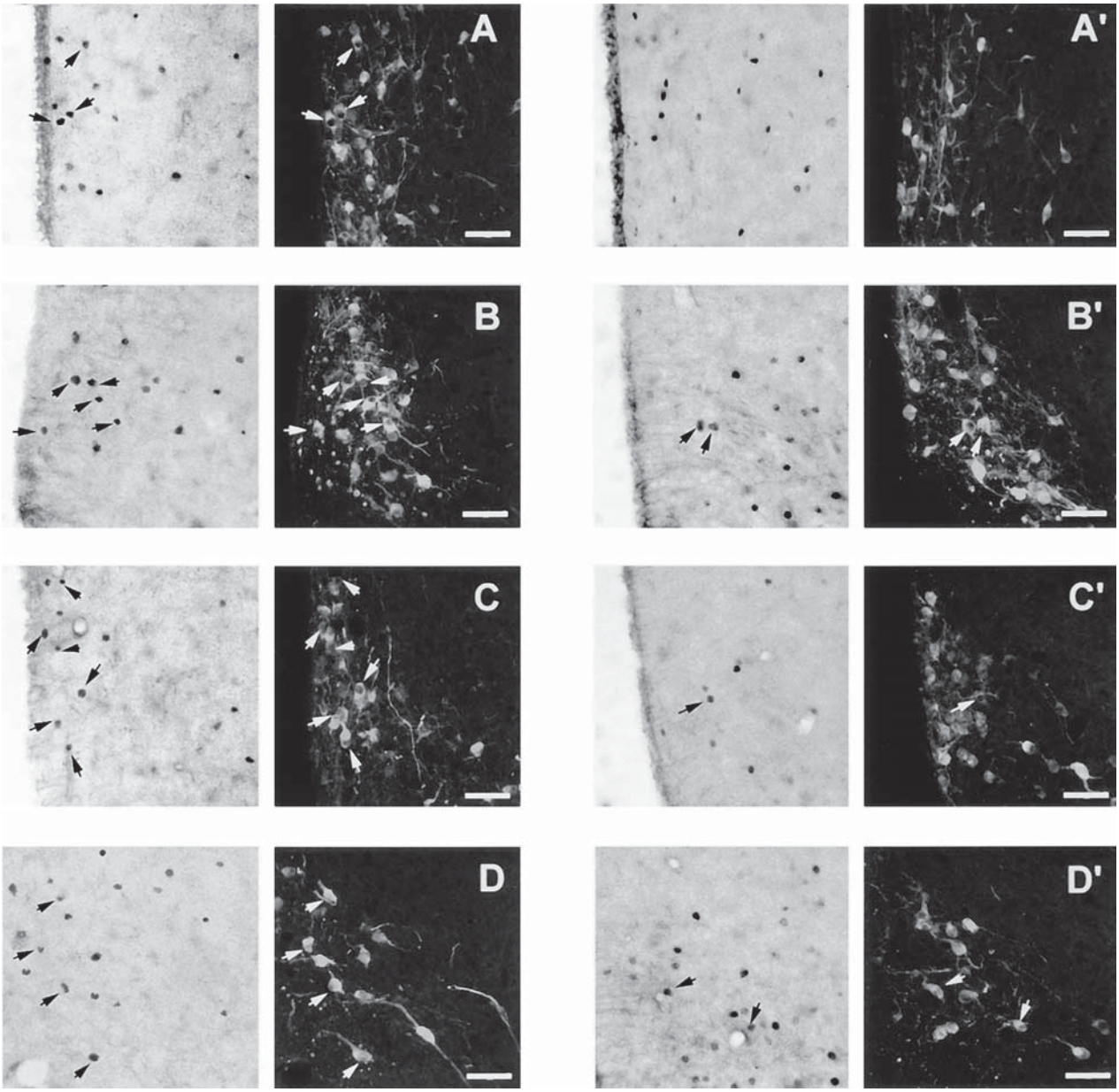

Fig. 2. Representative photomicrographs of double-label immunocytochemistry detecting FRAs (light field, dark dots) and tyrosine hydroxylase (appears as light cells) in hypothalamic dopaminergic neurons from normal-rabbit-serum-treated control (A,B,C,D) and prolactin-antiserums treated (**A',B',C',D'**) rats at 1900 h. with a 40× objective lens. Hypothalamic dopaminergic neurons are located in the periventricular (**A,A'**), rostral arcuate (**B,B'**), dorsomedial arcuate (**C,C'**), and ventrolateral arcuate (**D,D'**) nuclei. Arrows indicate neurons immunoreactive for both FRAs and tyrosine hydroxylase. Bar = 50 μm.

for at least 8 h. This seems to be in conflict with the findings of Lookingland and co-workers *(31)*, which indicate that in female rats a comparable dose of prolactin antiserum immunoneutralizes serum prolactin levels for less than 8 h. However, this disagreement may be attributed to the differing potencies, affinity and binding potencies of the antisera.

Although the central role of TIDA neurons in hypothalamic control of prolactin secretion is acknowledged, the relative contribution and importance of the distinct neuroendocrine dopaminergic neuron subpopulations to the regulation of prolactin secretion is not fully understood. In this study we propose that endogenous prolactin may play a role in the re-activation of all three hypothalamic dopaminergic neuron populations after a prolactin surge.

Materials and Methods

Animals: All in vivo animal procedures were approved by the Animal Care and Use Committees of the respective institutions. On day 0 of the experiment, 2-mo-old female

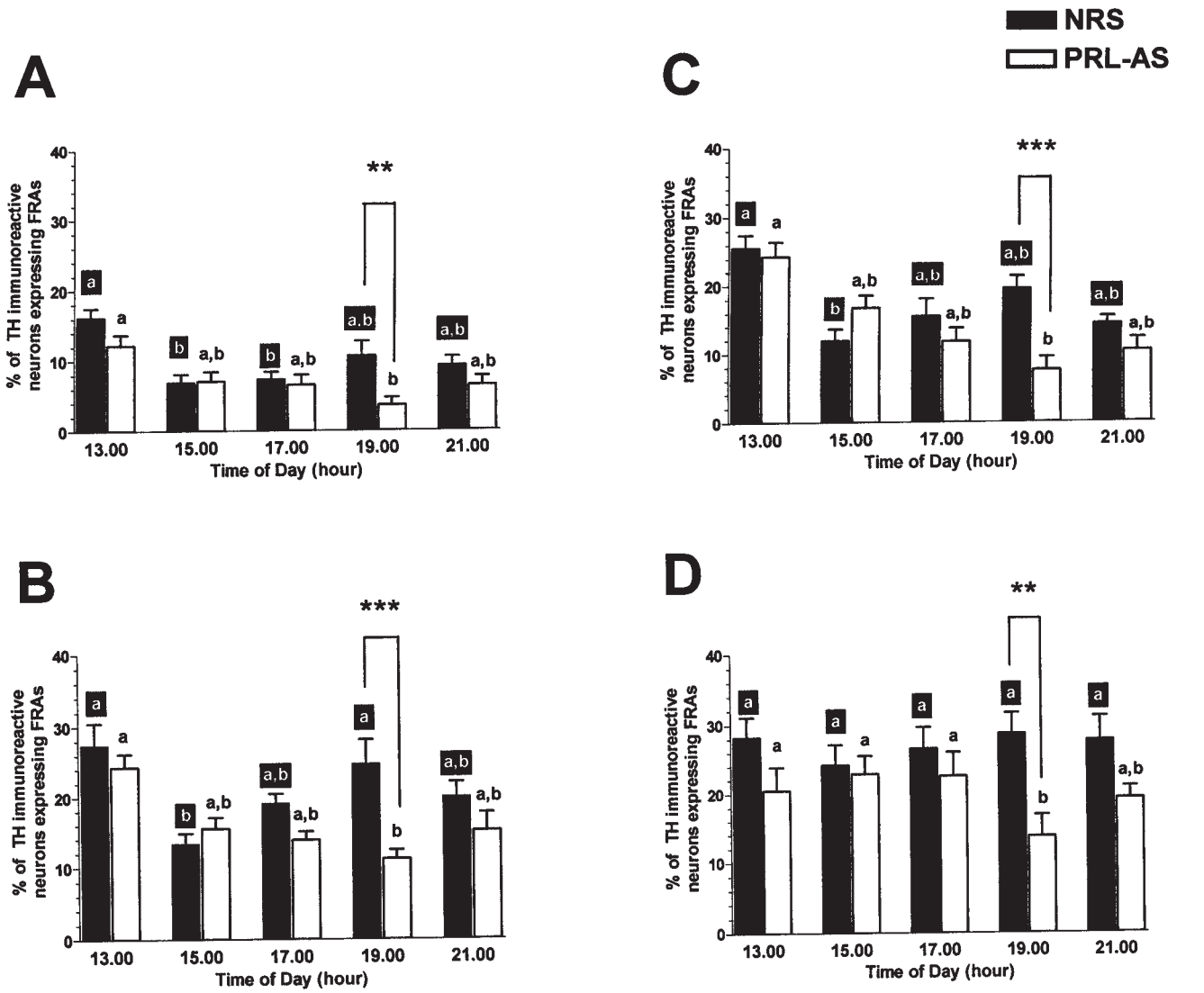

Fig. 3. Relative frequency of FRAs/TH double-labeled neurons among the different subdivisions of the hypothalamic dopaminergic neurons in normal-rabbit-serum-treated control (filled bars) and prolactin-antiserum-treated (empty bars) animals. Hypothalamic dopaminergic neurons in the periventricular (**A**), rostral arcuate (**B**), dorsomedial arcuate (**C**), and ventrolateral arcuate (**D**) nuclei were counted. Bars represent mean frequency ±SE counted from 5 rats/time point. Bars with dissimilar letters indicate significant difference between time points within treatment groups ($p < 0.05$), compared using one-way ANOVA followed by Bonferroni's multiple comparison test. Asterisks indicate significant differences between treatment groups (rabbit antiserum treated versus normal rabbit serum treated) by two-way ANOVA followed by Bonferroni's multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1
Number of Tyrosine Hydroxylase Immunoreactive Cells Counted in Each Area from 50 Animals

Area examined	Total number of TH-IR cells counted ($n = 50$)	Mean ± SE number of TH-IR cells counted/animal
Periventricular nucleus	8283	165.7 ± 6.37
Rostral arcuate nucleus	5102	102.1 ± 4.86
Dorsomedial arcuate nucleus	4188	83.8 ± 4.88
Ventrolateral arcuate nucleus	2309	46.2 ± 2.49

rats were ovariectomized under Halothane (Sigma, St. Louis) anesthesia. On d 10 at 1000 h, all animals were injected with 20 µg of 17-beta-estradiol (Sigma) sc. At 1000 h on d 11, half of the animals were injected with 200 µL of rabbit anti-rat prolactin antiserum (gift of Dr. G.M. Nagy, *19*) ip, while the controls received normal rabbit serum. Groups of animals ($n = 5$) were sacrificed by overdose of Halothane in 2 h intervals between 1300 and 2100 h. The animals were perfused with 50 mL of 0.1 *M* PBS (pH 7.34) through the ascending aorta followed by 100 mL of 4% paraformaldehyde in 0.1 *M* PBS (pH 7.5). The brains were removed, placed in 20% sucrose–PBS until submerged. Hypothalamic blocks were cut into 35 µm free-floating

sections and collected in four parallel sets. Sections were stored in cryoprotectant at –20ºC until immunocytochemistry was initiated.

Immunocytochemistry and Imaging

Sections were rinsed three times in 0.1 *M* PBS and incubated in 10% normal horse serum (in 0.1 *M* PBS containing 0.4% Tritonx100) for 20 min. Then the sections were incubated in the following reagents with 3 × 10 min washes in PBS between steps: anti-FRAs rabbit polyclonal antibody (Sc-835sc. Santa Cruz, 1:800, 24 h), anti-rabbit biotinylated goat IgG (Vector Laboratories, Burlingame, CA, 1:600, 18 h), ABC complex (Vectastain Elite kit, 4.5 µL/mL of A and B, 4 h). Parallel sections were subjected to control stainings, which included (1) incubation of sections with anti-rabbit biotinylated goat antiserum, without application of anti-FRAs rabbit antibody, and (2) incubation of sections with 10% normal goat serum (Vector) instead of 10% normal horse serum. Omission of the FRAs antibody resulted in no staining (Fig. 1). There was no detectable difference in the background staining in the sections incubated with 10% normal horse serum or 10% normal goat serum (data not shown).

The sections were then washed in 0.175 *M* sodium acetate buffer (3 × 20 min) and incubated in a developing solution containing 3,3-diaminobenzidine (D5905, Sigma) 0.5 mg/mL, $NiSO_4$ 25 mg/mL, H_2O_2 1.8 µL/mL in Na acetate buffer, pH 7.5, for 5 min. After rinsing three times in acetate buffer and PBS, the sections were incubated 24 h in anti-tyrosine hydroxylase mouse antibody (T2928, Sigma, 1:10,000). After 3 × 10 min washes in PBS, the sections were incubated 18 h in anti-rabbit donkey IgG conjugated with CY3 (715-165-150, Jackson Immuno-Research, West Grove, PA, 1:400). All antibodies were diluted in Triton-PBS, containing 2% normal horse serum (Intergen Co., Purchase, NY). Three 10-min washes (in PBS) were applied between steps.

After immunocytochemistry the free-floating sections were transferred to distilled water, mounted onto glass microscope slides, air-dried, cleared in xylenes (Fisher Scientific, cat. no. X5-4, a mixture of ortho, meta, and para isomers, may contain some ethyl benzene) and coverslips (Corning) were applied with Permount (Fisher). The slides were coded for a blind (unbiased) collection of data. The sections were viewed with a Leitz DM RX fluorescent light microscope (Leica Microsystems, Inc., Deerfield, IL), equipped with filter system N2.1 (for narrow band green light excitation, short-pass filter BP 515-560 nm, beam splitting mirror RKP 580 nm, long-pass suppression filter LP 580 nm). Light and dark field images were acquired with a Spot camera (Diagnostic Instruments, Sterling Heights, MI). Metamorph image analysis system (Universal Imaging Co., West Chester, PA) was used to overlay images and perform semiautomatic particle counting. The images were edited with Adobe PhotoShop 6.0 (Adobe Systems, Inc., San Jose, CA) for publication.

Statistical Analyses

In general, quantitative data was analyzed using the Prism 3.01 software (GraphPad Software, Inc., CA). One-way ANOVA followed by Bonferroni's post hoc test was used to determine significant differences between time points within experimental groups. Two-way ANOVA was used to determine differences between experimental groups.

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