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# Decline of Vasopressin Immunoreactivity and mRNA Levels in the Bed Nucleus of the Stria Terminalis Following Castration

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**Vasopressinergic (VP) neurons in the bed nucleus of the stria terminalis (BNST) of the rat are regulated by gonadal steroids. Gonadectomy causes the projections of the BNST to lose their VP immunoreactivity gradually over a period lasting more than 2 months. Here we have compared the rate of decline of VP mRNA and VP immunoreactivity in the BNST of adult male rats following castration. In experiment 1, the peak number of VP-immunoreactive cells and the level of VP gene expression were compared in sham-operated controls and at 1, 3, or 8 weeks postcastration. The number of VP-immunoreactive cells was not decreased at 1 week postcastration but was significantly reduced ( $p < 0.0001$ ) at 3 and 8 weeks postcastration. VP gene expression declined more rapidly, and both the total number of labeled cells ( $p < 0.0001$ ) and the average number of grains per cell ( $p < 0.01$ ) were significantly reduced by 1 week postcastration. No VP-expressing cells were detectable at 3 or 8 weeks. The difference in the rate of decline in the number of cells labeled by the two techniques following castration did not appear to be due to colchicine pretreatment. In experiment 2, VP mRNA in the BNST was compared in sham-operated controls or at 1, 3, or 7 d postcastration. A significant decrease ( $p < 0.01$ ) in the average number of grains per cell was detectable by just 1 d following castration, and the number of labeled cells was significantly reduced ( $p < 0.001$ ) by 3 d postcastration. These results indicate that the capacity of BNST cells to synthesize VP responds more dynamically to changes in gonadal steroid levels than do levels of VP immunoreactivity. This difference may reflect the delay between VP gene expression and the processing of VP precursor molecules. Alternatively, gonadal steroids may modulate the release of VP from cells in the BNST.**

Gonadal steroids influence brain physiology and behavior by targeting specific neuronal systems (McEwen and Parsons, 1982). Vasopressin (VP) cells in the bed nucleus of the stria terminalis

(BNST) appear to be a prime target of gonadal steroid actions. In rats, these neurons are sexually dimorphic. Male rats have more neurons in the BNST that express VP mRNA (Miller et al., 1989c) or can be stained immunocytochemically for VP (Van Leeuwen et al., 1985) than female rats. In addition, the VP-immunoreactive projections of these neurons to the septum are denser in males than in females (De Vries et al., 1981, 1983). Gonadal steroids appear to influence the development of these pathways in the early postnatal period (De Vries et al., 1983) and act to maintain the pathways in adulthood (De Vries et al., 1984, 1985, 1986). Castration of adult male rats results in the disappearance of VP-immunoreactive cells in the BNST (Van Leeuwen et al., 1985) and immunoreactive fibers in the septum (De Vries et al., 1984, 1985), although the time course is extremely slow. Approximately 8–12 weeks are needed following castration for the complete disappearance of VP-immunoreactive fibers in the septum (De Vries et al., 1984). While VP-expressing cells in the BNST also disappear following castration (Miller et al., 1989b), the time course of the effects of gonadal steroid removal on VP gene expression has yet to be determined. In this study, we have compared the decline over time of VP mRNA levels and VP immunoreactivity of cells in the BNST following castration.

## Materials and Methods

### *Subjects and general protocol*

**Experiment 1.** Intact male Wistar rats (90 d old) were obtained from Charles River Labs (Wilmington, MA) and were maintained under a 12 hr light/12 hr dark cycle. Food and water were available ad libitum. Animals were killed as sham-operated controls or at 1, 3, or 8 weeks postcastration. Sham-operated controls were killed 1 week postsurgery. All of the animals for immunocytochemistry and some of the sham-operated controls for *in situ* hybridization were treated with colchicine (35  $\mu$ g, i.c.v.) 48 hr prior to death. For immunocytochemistry, rats were perfused through the ascending aorta with 50 ml of 0.9% saline, followed by 5% acrolein in 0.1 M phosphate-buffered saline (pH 7.2) for 5 min (about 300 ml per animal). The brains were removed and sectioned at 50  $\mu$ m on a vibratome. Sections were collected in 0.05 M Tris-HCl. For *in situ* hybridization, brains were removed following decapitation, frozen on dry ice, and sectioned at 20  $\mu$ m on a cryostat. Cryostat sections were thaw-mounted on RNase-free gelatin-coated slides and stored at  $-70^{\circ}\text{C}$  prior to being hybridized.

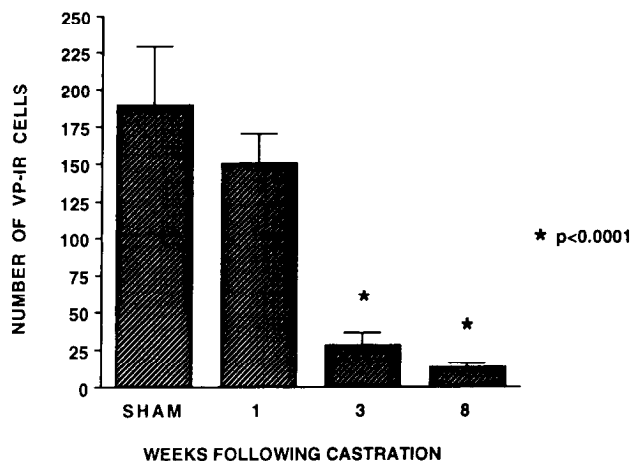
**Experiment 2.** Intact male Wistar rats (90 d old) were obtained from Simonsen Laboratories (Gilroy, CA) and were maintained under a 12 hr light/12 hr dark cycle. Food and water were available ad libitum. Animals were killed as sham-operated controls or at 1, 3, or 7 d postcastration. Sham-operated controls were killed 1 d postsurgery. Brains were collected for *in situ* hybridization and were processed as outlined above.

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**Figure 1.** Total number of VP-immunoreactive neurons identified unilaterally in four atlas-matched sections through the BNST. The number of VP-immunoreactive cells is significantly decreased by 3 weeks postcastration. Data are presented as the mean  $\pm$  SEM.

### Immunocytochemistry

Immunocytochemistry was performed as previously described (De Vries and Al-Shamma, 1990). Free-floating sections were treated with (1) 0.1% sodium borohydride followed by (2) 0.05 M Tris-HCl, pH 7.6 (Tris) and (3) 2% goat serum, 0.9% NaCl, and 0.5% Triton X-100 in Tris (Tris-Triton) for 15 min. Sections were incubated overnight at 4°C in anti-VP (ICN, Lisle, IL) diluted 1:2000 in Tris-Triton. Following incubation, sections were (1) rinsed in Tris-Triton for 15 min, (2) treated for 45 min with goat anti-rabbit IgG serum (ICN) diluted 1:150 in Tris-Triton, (3) rinsed for 15 min in Tris-Triton, (4) treated for 45 min with rabbit peroxidase-antiperoxidase serum diluted 1:300 in Tris-Triton, and (5) rinsed for 15 min in Tris-Triton. These second and third antibody incubation steps were repeated, and sections were then rinsed in 0.9% NaCl in Tris (Tris-NaCl) and reacted with 0.05% 3,3'-diaminobenzidine in Tris-NaCl with 0.01% H<sub>2</sub>O<sub>2</sub> for 20 min. After being rinsed in Tris-NaCl, sections were mounted on slides, air dried, and coverslipped. Specificity of the antiserum was verified by (1) the absence of immunoreactive cells in brain sections from VP-deficient Brattleboro rats and (2) the absence of immunoreactive neurons in sections stained with antiserum that had been reacted with 50  $\mu$ M VP.

### In situ hybridization

*In situ* hybridization procedures were the same as previously described (Miller et al., 1989a). Briefly, sections were postfixed in 4% paraformaldehyde, treated with 0.25% acetic anhydride, dehydrated, delipidated, and air dried. A <sup>35</sup>sulfur-labeled oligonucleotide probe was applied, and sections were coverslipped. The probe used was a 48-base oligonucleotide complementary to the mRNA encoding the last 16 amino acids of the glycopeptide portion of the VP precursor. The probe was 3' end-labeled with terminal deoxynucleotidyl transferase, purified, heat denatured, and diluted to 2.5 pmol/ml in hybridization buffer [50% formamide, 10% dextran sulfate, 300 mM NaCl, 10 mM Tris, 1 mM EDTA, 1  $\times$  Denhardt's (0.02% each BSA, Ficoll, polyvinylpyrrolidone), 0.5 mg/ml yeast tRNA, and 10 mM dithiothreitol] before use. Specific activities were estimated at 4400 Ci/mmol (experiment 1) and 5700 Ci/mmol (experiment 2). A 45  $\mu$ l volume was applied to two brain sections per slide. After overnight incubation, coverslips were removed and sections were washed four times in 1  $\times$  SSC (150 mM NaCl, 15 mM Na citrate) for 15 min at 55°C, followed by two 1 hr room temperature washes in 1  $\times$  SSC. Sections were dehydrated through a graded series of alcohols containing 300 mM ammonium acetate, dipped in Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY; diluted 1:1 with 600 mM ammonium acetate), and exposed for 14 d (experiment 1) or 17 d (experiment 2). Emulsion was developed in D-19 diluted 1:1 with distilled water at 16°C. Sections were counterstained with cresyl violet acetate and coverslipped. All samples from a single experiment were run in a single assay. In order to increase our ability to detect low levels of VP gene expression, the specific activity of the probe and the exposure time were increased in experiment 2.

### Quantification

For immunocytochemistry, sections through the BNST of each animal were anatomically matched using the stereotaxic atlas of König and Klippel (1963). VP-immunoreactive cells were identified in bright-field and counted on the side of colchicine injection in four sections that corresponded to atlas levels 6790–6570. The cells were counted independently by two researchers that were blind to the treatment groups. Data for each group is presented as the mean  $\pm$  SEM of the total number of cells observed. Groups were compared using a one-way analysis of variance followed by a Sheffé *F* test. The criterion for significance was set at  $p < 0.05$ .

For *in situ* hybridization, sections through the BNST of each animal were also matched using the König and Klippel atlas (levels 6790–6570). One section at each of the four atlas levels was read blindly by a single experimenter. Labeled cells were identified in dark-field, and the presence of an underlying neuron was verified using high-magnification bright-field microscopy. Cells were considered to be labeled if the signal-to-noise ratio of the cell was 5 or greater. The total number of cells found unilaterally in the four sections was determined for each animal. The number of grains per cell for up to 10 randomly selected cells per section at each of the four levels (i.e., up to 40 cells/animal) was measured by an automated image analysis system and averaged for each animal. The grain-counting system consisted of a DATA Cube IVG-128 video acquisition board attached to an IBM AT computer. Video images were obtained by a Dage model 65 camera attached to an Olympus microscope (Olympus, New Hyde Park, NY) equipped with a 40 $\times$  objective and dark-field condenser (see Chowen et al., 1991, for a detailed description of the grain counting methodology). Data are presented as the mean  $\pm$  SEM. For each experiment, a one-way analysis of variance followed by a Sheffé test was used to assess differences between the groups. Criterion for significance was set at  $p < 0.05$ .

## Results

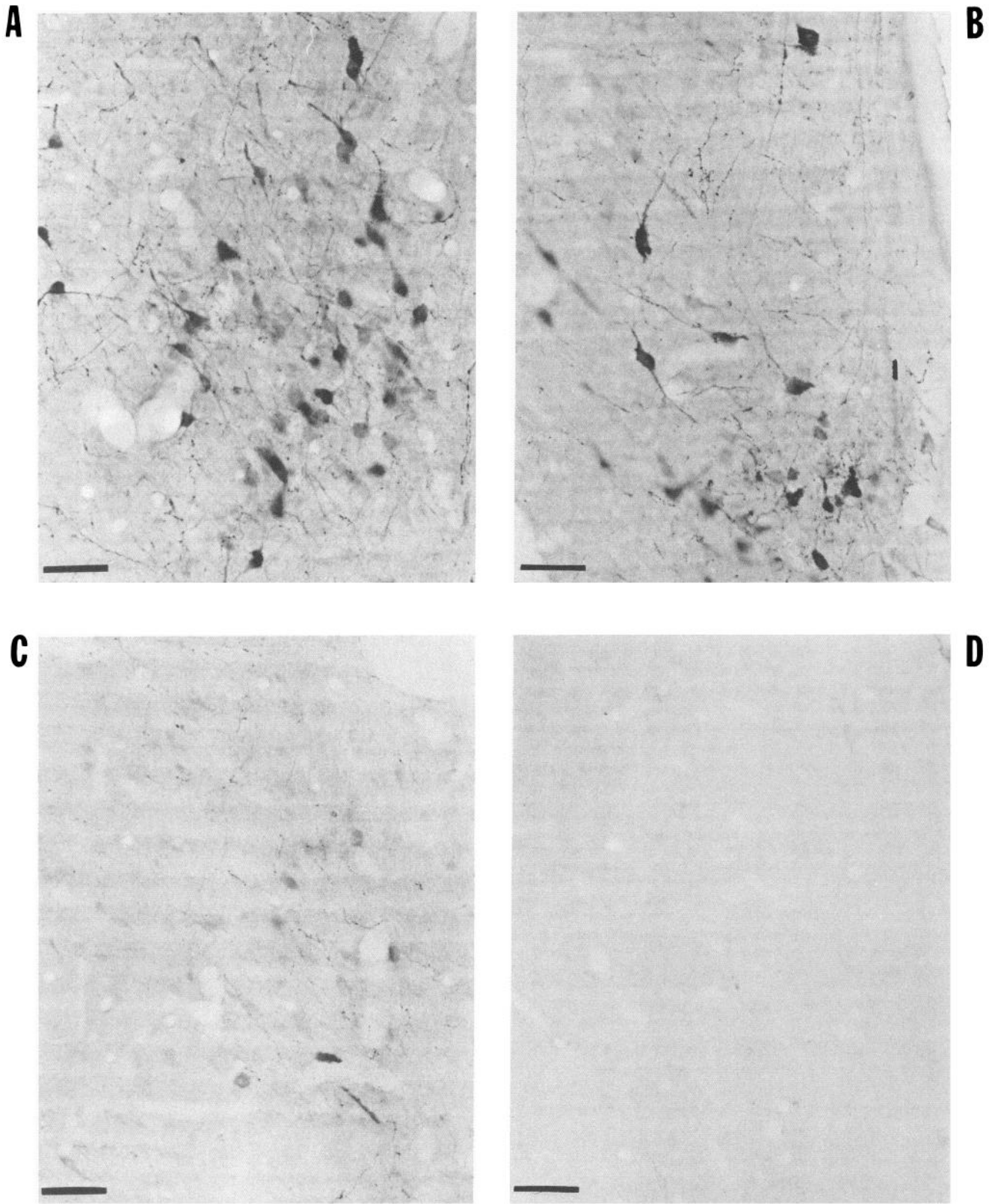
### Experiment 1

As can be seen in Figure 1, the total number of VP-immunoreactive cells found unilaterally in the four sections through the BNST declines following castration. Although animals castrated for 1 week do not differ significantly from sham-operated controls (1 week: 149.5  $\pm$  20,  $n = 6$ ; control: 189.4  $\pm$  39.5,  $n = 6$ ), cell number is significantly decreased ( $p < 0.0001$ ) in animals castrated for 3 weeks (28.5  $\pm$  7.2,  $n = 5$ ). By 8 weeks postcastration ( $n = 6$ ), an average of only 13.3  $\pm$  2.8 stained cells were observed. Figure 2 shows low-power bright-field photomicrographs of VP-immunoreactive neurons in the BNST of a representative control animal (*A*) and animals castrated for 1 (*B*), 3 (*C*), or 8 (*D*) weeks.

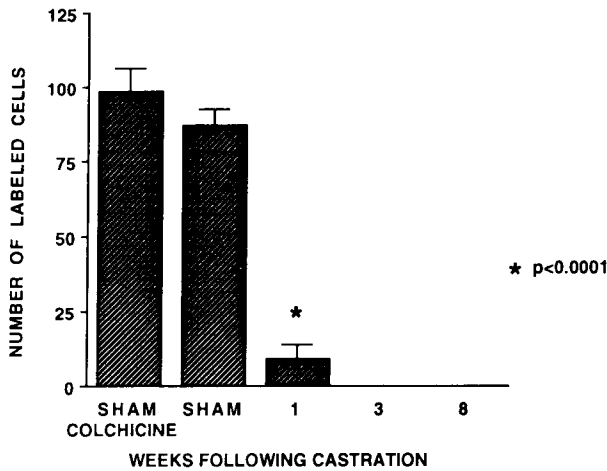
The number of cells expressing VP mRNA in the BNST declines more rapidly following castration than the number of VP-immunoreactive cells. As shown in Figure 3, the total number of labeled cells found unilaterally in the four atlas-matched sections is significantly reduced by 1 week postcastration (98.6  $\pm$  8 vs. 9.0  $\pm$  5.1;  $n = 5$ /group). The remaining cells exhibited a reduced labeling intensity, with 57.2  $\pm$  3.0 versus 31.8  $\pm$  3.0 grains/cell for controls and 1 week castrates, respectively (Fig. 4). No VP-expressing cells were detectable at 3 or 8 weeks postcastration in this experiment ( $n = 5$ /group). By the established criteria, pretreatment of sham-operated controls ( $n = 5$ ) with colchicine did not significantly influence the number of labeled cells or the labeling intensity compared to untreated sham-operated controls.

### Experiment 2

As can be seen in Figure 5, the VP mRNA levels in the BNST are significantly decreased by just 1 d following castration. The average number of grains per cell declined over time from 77.4  $\pm$  3.1 (sham,  $n = 5$ ) to 65.5  $\pm$  1.7 (1 d,  $n = 6$ ), 50.5  $\pm$  2.1 (3 d,  $n = 6$ ), and 40.0  $\pm$  1.0 (7 d,  $n = 6$ ). The average total number



*Figure 2.* Low-power bright-field photomicrographs of VP-immunoreactive neurons in the BNST (level 6670) of a representative sham-operated control animal (*A*) and animals castrated for 1 (*B*), 3 (*C*), and 8 weeks (*D*). Scale bars, 40  $\mu$ m.

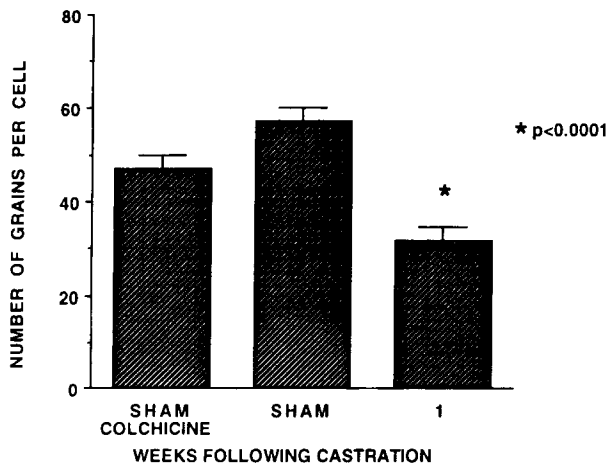


**Figure 3.** Total number of neurons expressing VP mRNA identified unilaterally in four atlas-matched sections through the BNST. The number of labeled cells is significantly reduced by 1 week postcastration. Pretreatment with colchicine does not significantly affect the number of VP-expressing neurons. Data are presented as the mean ± SEM.

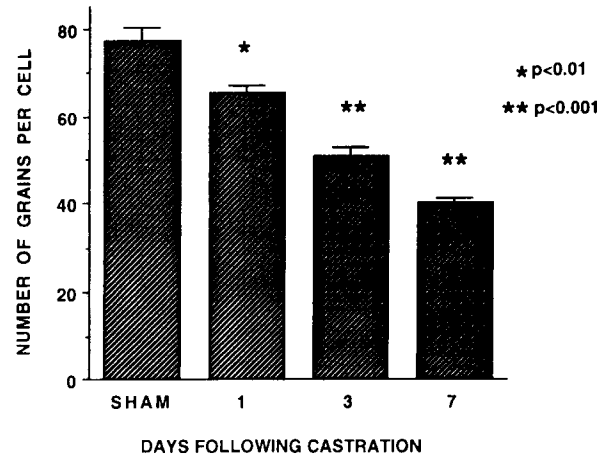
of labeled cells in the four atlas-matched sections is significantly reduced by 3 d following castration and further reduced by 7 d postcastration (Fig. 6). Signal-to-noise ratios of labeled cells averaged 20 in sham-operated controls and 18, 16, and 12 in rats castrated for 1, 3, or 7 d, respectively. Low-power dark-field photomicrographs of hybridized sections through the BNST of representative animals are shown in Figure 7.

**Discussion**

In this article, we report that VP mRNA levels in the BNST respond more dynamically to steroid hormone removal than does VP immunoreactivity. We found that VP gene expression in the BNST was dramatically reduced by 1 week postcastration whereas the number of VP-immunoreactive neurons was not significantly reduced until 3 weeks postcastration. In a second experiment, we observed that VP mRNA levels were significantly decreased by just 1 d following castration. The average



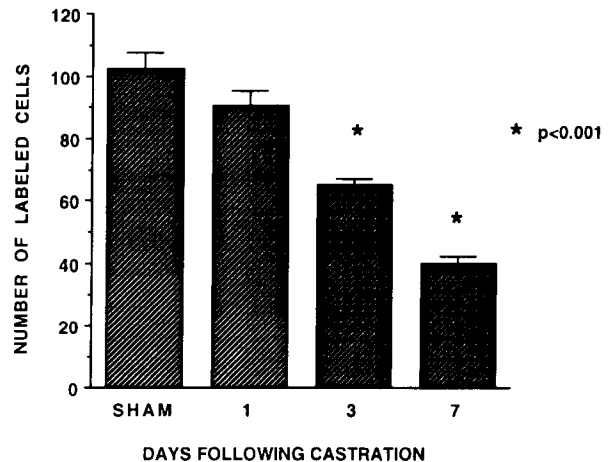
**Figure 4.** Average number of grains per cell expressed by VP neurons in BNST. At 1 week postcastration, cells had significantly fewer grains/cell than did sham-operated controls. Pretreatment of sham-operated controls with colchicine reduced labeling intensity compared to untreated sham-operated controls. Data are presented as the mean ± SEM.



**Figure 5.** Average number of grains per cell expressed by VP neurons at 1, 3, and 7 d postcastration. Labeling intensity is significantly reduced by 1 d postcastration. Data are presented as the mean ± SEM.

labeling intensity was decreased by 15%, 35%, and 48% at 1, 3, and 7 d, respectively. At 3 d postcastration, a significant decline could also be detected in the number of cells expressing VP mRNA. This suggests that mRNA levels may be a more sensitive index than immunoreactivity for assessing the acute effects of steroid hormones on VP neurons in the BNST.

The slower postcastration decline of the VP immunoreactivity than of VP mRNA could be due to colchicine treatment, which was only given to animals used for immunocytochemistry. Although early reports suggested that colchicine treatment did not influence gene expression (Gee and Roberts, 1983; Wolfson et al., 1985), recent studies have indicated that biosynthesis of several transmitter systems is indeed affected by colchicine and the direction of this effect is dependent on both the particular transmitter and the site of synthesis (Cortes et al., 1990). The differential decline of VP immunoreactivity and VP mRNA levels in the BNST could occur if colchicine treatment stimulated VP synthesis and increased peptide content. We assessed the effects of colchicine on VP synthesis by comparing mRNA levels in sham-operated controls with and without colchicine.



**Figure 6.** Total number of labeled cells identified unilaterally in four atlas-matched sections through the BNST at 1, 3, and 7 d postcastration. The number of labeled cells is significantly reduced by 3 d following castration. Data are presented as the mean ± SEM.

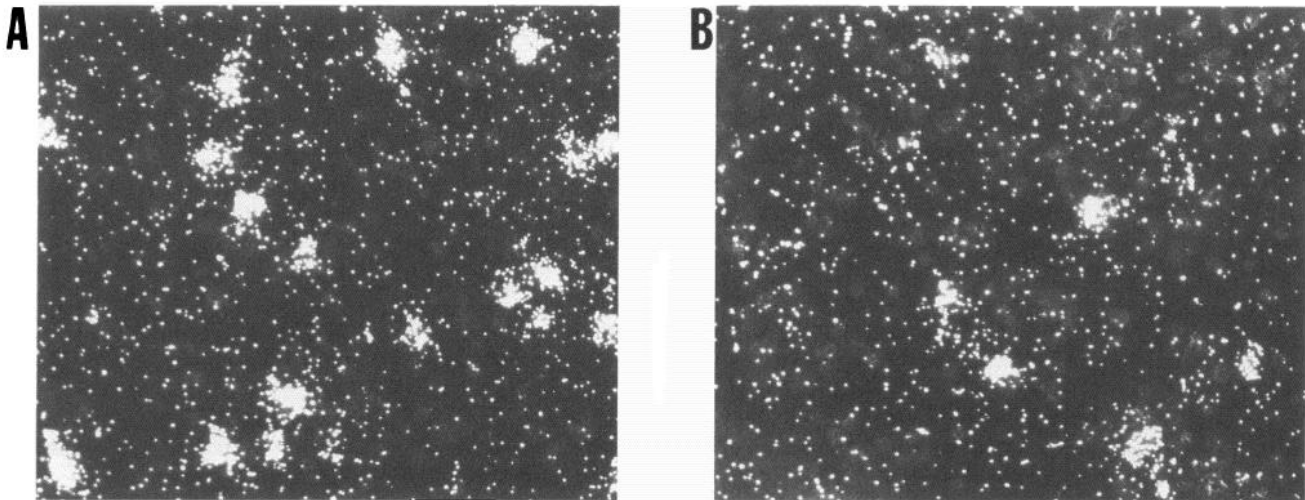


Figure 7. Low-power dark-field photomicrographs of sections (atlas level 6670) hybridized for VP mRNA from a representative sham-operated control (A) and an animal castrated for 7 d (B).

We found that although there was a tendency for reduced labeling intensity, treatment of sham-operated controls with colchicine did not significantly influence VP gene expression in the BNST by our criteria. Unless this tendency would be reversed in castrated rats, these findings suggest that the difference in the rate of decline of VP mRNA and immunoreactivity cannot be attributed to colchicine pretreatment.

VP immunoreactivity could be dependent on several factors including peptide synthesis, release, and degradation. The difference between the decline of VP mRNA levels and the more gradual decline of VP immunoreactivity in the BNST may reflect steroid effects on any of these parameters. Peptide synthesis and processing in the intact animal has been shown to require on the order of several hours (Eipper et al., 1987). This would suggest that factors other than the time necessary for translation and processing contribute to the difference in postcastration decline of VP immunoreactivity and VP mRNA. However, gonadal steroids may also regulate the processing of peptide precursors and alter this expected time frame. Steroid hormones have been shown to influence the processing of other peptides by influencing the activity or levels of processing enzymes. In the paraventricular nucleus, glucocorticoids regulate the expression of peptidyl-glycine  $\alpha$ -amidating monooxygenase (Grino et al., 1990), an enzyme involved in the processing of prepropressophysin. In addition, processing of the prohormone for gonadotropin-releasing hormone is modified by castration (Culler et al., 1988). The strong staining of cell bodies observed at 1 week postcastration indicates that VP is accumulating in the somata after colchicine treatment. Whether this represents *de novo* synthesis or posttranslational processing to immunocytochemically accessible peptide cannot be determined. The rate of processing of the VP precursor may also be reduced following castration. The availability of antibodies directed against spacer peptides of the prepropressophysin molecule (Verbalis et al., 1991) might make it possible to determine whether the rate of processing is reduced following castration.

In addition to potential changes in synthesis and processing, VP transport may be reduced following castration. Recent studies in the magnocellular system have reported that the transport of VP is linked to the rate of synthesis (Roberts et al., 1991). If

VP transport in extrahypothalamic cells is also linked to synthesis, then castration-induced decreases in peptide synthesis would be expected to result in decreases in transport as well. Therefore, it is conceivable that castration-dependent differences in production, processing, and transport might all contribute to the delayed decline of VP immunoreactivity following castration.

Alternatively, VP release from neurons in the BNST may be very low in the basal state such that previously translated peptide may remain stored within the cell and its processes for prolonged periods of time. Early estimates of VP disappearance from the neural lobe of the rat indicated a half-life of approximately 19 d (Burford and Pickering, 1973). Whether VP in extrahypothalamic sites disappears with a similar time course is not known. A slow release is a potential explanation for the gradual (3 month) disappearance of VP immunoreactivity from the terminals (De Vries et al., 1984).

Castration may reduce VP release even further. There is at least indirect evidence that VP release in the ventral septum (presumably from neurons in the BNST) is reduced following castration. In intact rats, injections of pyrogens stimulate VP release in the septum (Cooper et al., 1979), which is linked to suppression of fever (Kasting, 1989). Long-term castrated rats show prolonged fever following challenge with a pyrogen, which may be related to lowered VP release (Pittman et al., 1988). No study has as yet evaluated the effects of short-term castration on central VP release.

Steroids could affect peptide synthesis and release by several different mechanisms. Since VP content of BNST projections appears to depend on the aromatization of testosterone to estradiol (De Vries et al., 1986), and VP-immunoreactive cells within the BNST can also be immunostained for estrogen receptors (Axelson and Van Leeuwen, 1990), steroids could influence synthesis and release by acting directly on the VP cells. As yet, no estrogen response element has been identified in the VP gene. However, steroids can influence VP cells in a variety of ways. In other neuronal systems, for example, steroids can rapidly modulate the excitability of cells via changes in ionic conductance (Moss and Dudley, 1984; Nabekura et al., 1986; Minami et al., 1990). They can modify synaptic inputs by in-

fluencing receptor density (Hammer, 1985; De Kloet et al., 1986; O'Connor et al., 1988) as well as transmitter biosynthesis (Blum et al., 1987; Argente et al., 1990; Chowen et al., 1990). They can even influence the number of axodendritic synapses in adult animals (Miyakawa and Arai, 1987). Theoretically, these steroid effects could independently influence the different processes involved in VP synthesis and release by BNST neurons, which could explain a discrepancy in postcastration decline in VP mRNA and peptide levels.

Slow changes in neuropeptide content following gonadectomy are also seen in other neuronal systems. Although we did not quantify this, we noted that VP neurons in the medial amygdala (MA) showed a similar gradual decline in immunoreactivity as VP neurons in the BNST. VP cells in the MA and BNST are similar in other respects as well, including size and morphology (Caffe and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983), presence of estrogen receptors (Axelson and Van Leeuwen, 1990), sex differences in projections (De Vries and Al-Shamma, 1990), and response to castration (De Vries et al., 1986). One might therefore predict that the rate of postcastration decline in VP mRNA levels in MA cells and BNST cells is also similar.

A gradual decline following castration has also been reported for the neuropeptide content of cholecystokinin (CCK) and substance P cells and fibers in the medial preoptic nucleus (MPN), BNST, and MA of the rat and hamster (Swann and Newman, 1987; Malsbury and Nance, 1989). In male rats, CCK cells in the MA, BNST, and preoptic area show a postcastration decrease in CCK content that lasts for more than 2 weeks and is restored by testosterone treatment (Simerly and Swanson, 1987). The cells in the MA and BNST may be the source of CCK fibers in the MPN, which gradually lose their immunoreactivity over a period of 8 weeks following castration (Malsbury and Nance, 1989). Although there are no data available on the postcastration decline in CCK mRNA levels in males, CCK mRNA levels in females vary across the estrous cycle (Simerly et al., 1988), which suggests that CCK cells may also respond within a day to changes in gonadal steroid levels in males. Conversely, the rate of change in VP mRNA we have observed after castration in males would allow for changes in VP mRNA across the estrous cycle as well.

In conclusion, we have observed that the number of VP-expressing cells in the BNST declines more rapidly following castration than the number of VP-immunoreactive neurons. This difference in time course cannot be attributed to colchicine pretreatment and may reflect the delay between VP gene expression and the production and processing of VP precursor molecules.

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