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# NADPH-d Staining in the Supraoptic and Paraventricular Nuclei Increases in Late Pregnant and Lactating Rats and is Influenced by Ovarian Steroids and Central Oxytocin Levels

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ABSTRACT

NADPH-d Staining in the Supraoptic and Paraventricular Nuclei Increases in Late Pregnant and Lactating Rats and is Influenced by Ovarian Steroids and Central Oxytocin Levels

Naomi Popeski

Previous research has demonstrated that staining for NADPH-diaphorase (NADPH-d) a histochemical marker for nitric oxide synthase is increased in the supraoptic (SON) and paraventricular (PVN) nuclei in late pregnancy. This work was extended to investigate whether increases in staining were evident at other times during pregnancy and lactation. The number of cells in the SON and PVN that stained for NADPH-d in rats on Day 4, 12, 16, and 22 of pregnancy and on Day 4, 12, and 20 of lactation was compared to that in virgin females. In other studies, the influence of ovarian hormones and central oxytocin infusion on NADPH-d staining in these nuclei was examined. Staining in the SON and PVN was compared among ovariectomized (ovex) animals exposed to either a steroid hormone regimen that mimics late pregnancy (estrogen (E) + progesterone (P) treatment followed by P removal), E alone, E + P, or cholesterol alone. A second study compared NADPH-d staining in ovex animals exposed to either chronic E or cholesterol treatment and infused with oxytocin or vehicle into the third ventricle for 7 days.

On the appropriate day, all animals were perfused and the brains were processed for NADPH-d histochemistry. In both the SON and PVN the number of densely stained cells present on Days 12 and 22 of pregnancy and 4 and 12 of lactation was greater than that seen in virgin females. Both the steroid treatment that mimicked late pregnancy and chronic i.c.v. oxytocin infusion in E-primed animals increased NADPH-d staining in ovex animals. These data suggest that, in general, NADPH-d staining in the SON and PVN is increased at times when oxytocin cells in these areas are activated and that the hormonal state associated with late pregnancy is sufficient to up-regulate NADPH-d in these regions.

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Finally, I want to thank Rob Palmer for his love and support. I dedicate this thesis to my parents who have always supported me and who have encouraged me in all of my endeavors.

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Nitric Oxide (NO) is a gaseous molecule that has been implicated in a variety of physiological and pathophysiological processes in both the peripheral and central nervous systems. For example, NO has been shown to regulate blood flow (Bruhwyler, Chleide, Liegeois, & Carreer, 1993; Garthwaite, 1991), and to mediate neuroplasticity (Ramsell & Cobbett, 1996) and neurotoxicity (Dawson, Bredt, Fotuhi, Hwang, & Snyder, 1991). In addition, NO has been involved in neuroprotective (Lei, Pan, Aggarwai, Chen, Hartman, Sucher, & Lipton, 1992; Zhang, White, & Iadecola, 1994) and neuroendocrine functions (Bonavera, Kalra, & Kalra, 1993; Lee & Rivier, 1996; Summy-Long, Bui, Mantz, Koehler, Weiz, & Kadekaro, 1993).

Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), a histochemical marker for the enzyme nitric oxide synthase (NOS) has been used to label neurons that produce NO (Arevalo, Sanchez, Alonso, Carretero, Vazquez, & Aijon, 1992; Hope, Michael, Knigge, & Vincent, 1991). Recent evidence has shown that the number of NADPH-d positive neurons in the cerebellum and the supraoptic (SON) and paraventricular (PVN) nuclei increases in late pregnancy (Weiner, Lizasoain, Baylis, Knowles, Charles, & Moncada, 1994; Woodside & Amir, 1996). In the SON and PVN, Woodside and Amir (1996) found an increase in NADPH-d positive neurons in late pregnant rats (Day 22) relative to ovariectomized and virgin female rats. The mRNA for NOS has also been shown to increase in the SON (Luckman, Huckett, Bicknell, Voisin, & Herbison, 1997) and PVN during lactation (Ceccatelli & Eriksson, 1993). These areas are involved in neuroendocrine function (Armstrong, 1995) including the release of oxytocin (OT) which controls both uterine contraction and milk ejection (Wakerley, Clarke, & Summerlee, 1988) and NO has been implicated in the regulation of OT release (Summy-Long et al., 1993). Thus, changes in the capacity for NO production in the SON and PVN may be of important functional significance for parturition and lactation.

The studies described in this paper elaborate on this previous research by determining more precisely the time course of changes in NADPH-d staining in the SON and PVN through pregnancy and lactation. In other studies, the contribution to these changes of two features of the ovarian and peptide hormonal milieu of late pregnancy was investigated. Because late pregnancy is associated with a dramatic increase in circulating estrogen levels against a background of falling levels of progesterone (Rosenblatt, Siegel, & Mayer, 1979) the effect of mimicking this pattern of hormone exposure on NADPH-d staining in the SON and PVN was examined. In addition, given that late pregnancy is also associated with an increase in activity of oxytocin neurons in the magnocellular areas of the SON and PVN (Jiang & Wakerley, 1995) the effects of increasing central oxytocin levels on NADPH-d staining was also assessed.

Nitric oxide is distinct from other neurotransmitters in that it is not released from vesicles in nerve terminals by an exocytotic mechanism, rather it diffuses from its site of production and has a half-life of only seconds (Vincent & Kimura, 1992). A major effector of NO is soluble guanylyl cyclase. The NO-induced activation of guanylyl cyclase results in a rise in cGMP which activates cGMP-dependent protein kinase that is responsible for many biological functions such as smooth muscle relaxation (Schuman & Madison, 1994), and inhibition of platelet aggregation and adhesion (Radomski, Palmer, & Moncada, 1990). The action of NO is terminated by diffusion away from its targets where it may react quickly with oxygen to produce the inactive anions, nitrite and nitrate.

Nitric oxide is synthesized by a number of isozymes of NOS. NOS catalyses the oxidation of L-arginine to NO and citrulline and can be divided into two distinct classes based on its sensitivity to calcium (Schuman & Madison, 1994). The constitutive isozymes eNOS and nNOS are found in endothelial cells and in neurons respectively, and both are calcium/calmodulin dependent. The cytokine inducible isozyme, iNOS is found in macrophages, neutrophils, hepatocytes, and glia cells and is calcium independent

(Bruhwyler et al. 1993).

All isozymes of NOS depend on NADPH as a cofactor. Several studies have shown that NOS is colocalized with NADPH-d activity (Bredt, Hwang, & Snyder, 1990; Dawson et al., 1991; Vincent & Kimura, 1992). Dawson et al. (1991) have shown that transfection of human kidney cells with NOS cDNA elicits NADPH-d staining, and that the ratio of NOS and NADPH-d staining is the same as seen in neurons. This suggests that NOS accounts for NADPH-d staining and that NADPH-d is a NOS. Thus, histochemical staining for NADPH-d provides a simple technique to stain select populations of neurons expressing NOS throughout the brain.

Studies on the distribution of NOS in the peripheral and central nervous systems have been conducted using immunohistochemistry for NOS (Bredt & Snyder, 1989), in situ hybridization for NOS mRNA (Ceccatelli, Grandison, Scott, Pfaff, & Kow, 1996), and NADPH-d histochemistry (Bredt et al., 1990; Dawson et al. 1991; Hope et al., 1991; Vincent & Kimura, 1992). NADPH-d histochemistry is based on the reduction of soluble nitro blue tetrazolium salt into an insoluble visible formazan by NADPH (Hope & Vincent, 1989) and produces Golgi-like images in a number of discrete populations of neurons (Sagar & Ferriero, 1987) throughout the nervous system.

NADPH-d positive neurons have been localized in several brain regions, including the cortex (Moro, Badaut, Springhetti, Edvinsson, Seylaz, & Lasbennes, 1995), and the hippocampus (Endoh, Maiese, & Wagner, 1994) where NOS activity has been identified in the CA1 region. Studies have also reported NADPH-d staining in the amygdala in response to dehydration (Pow, 1992), and many hypothalamic nuclei have stained for NADPH-d. For example, NADPH-d staining has been demonstrated in the ventromedial hypothalamus (VMH) (Okamura, Yokosuka, McEwen, & Hayashi, 1994; Rachman, Pfaff, & Cohen, 1996), and in the medial preoptic area (MPOA) (Okamura, Yokosuka, & Hayashi, 1994) where NADPH-d positive neurons also show estrogen-receptor

immunoreactivity. NOS activity has also been identified in the magnocellular dorsal region of the SON and in the magnocellular medial subdivision and parvocellular subdivision of the PVN (Arevalo et al., 1992; Vincent & Kimura, 1992).

A wide variety of functions have been attributed to NO. Nitric oxide was first recognized as endothelium derived relaxing factor (EDRF) (Dawson et al., 1991). Garthwaite (1991) has reported that NO is synthesized by stimulated endothelium cells and the amount of NO that was formed accounted for the biological effects of EDRF to control blood flow. It has also been postulated that NO plays a key role in NMDA receptor-mediated neurotoxicity. Evidence has shown that glutamate released in excess acting through NMDA receptors increases intracellular calcium which then activates nNOS to potentiate neurotoxicity (focal ischemia and neurodegeneration) (Choi, 1993). In contrast, other data have shown that NO acts as a neuroprotective agent. Current research has demonstrated that NO has a beneficial effect on the ischemic brain by increasing cerebral blood flow. In addition, NO may serve to protect neurons from glutamate toxicity and NADPH-d positive neurons are resistant to NMDA-induced cell death occurring from neurological insults (Lei et al., 1992).

Recent evidence has demonstrated that NO plays an important modulating role in the neuroendocrine system. Nitric oxide is involved in mediating NMDA induced gonadotropin-releasing hormone (GNRH) secretion from the hypothalamus which then triggers the preovulatory luteinizing hormone (LH) surge from the anterior pituitary (Bonavera et al., 1993; Pu, Xu, Kalra, & Kalra, 1996; Rettori, Belova, Dees, Nyberg, Gimeno, & McCann, 1993). Involvement of NO in controlling the release of luteinizing hormone-releasing hormone (LHRH) is supported by evidence that GNRH neurons are in close proximity to NOS positive neurons suggesting potential contacts between these two groups of neurons (Bhat, Mahesh, Lamar, Ping, Aguan, & Brann, 1995; Grossman, Rossmanith, Kabigting, Cadd, Clifton, & Steiner, 1994).

NADPH-d colocalizes with corticotropin-releasing-hormone (CRH) in hypothalamic parvocellular cells in the PVN (Siaud, Mekaouche, Ixart, Balmefrezol, Givalois, Barbanel, & Assenmacher, 1994). In addition, several lines of research have shown that NOS mRNA is elevated in the PVN in response to immobilization stress and endotoxin injections (Calza, Giardino, & Ceccatelli, 1993; Lee & Rivier, 1996). Because parvocellular cells are important for the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Siaud et al., 1994), these data suggest that the up-regulation of NOS mRNA in the PVN augments the synthesis of NO which may regulate CRH release and subsequently adrenocorticotropin hormone (ACTH) secretion from the anterior pituitary. Consistent with this hypothesis is data showing that NOS inhibitors block stressed-induced c-Fos expression in the PVN in rats exposed to immobilization stress (Amir, Rackover, & Funk, 1997). Other researchers have reported that NO exerts an inhibitory effect on CRH release. Costa and colleagues (1993) have demonstrated that a NO donor inhibited the potassium chloride (KCl) -induced CRF release from the PVN. Moreover, Rivier and Shen (1994), suggested that NO has an inhibitory effect on interleukin-1B, AVP, and OT-induced ACTH secretion. The reported differences of NO's influence on the HPA axis may be a result of the stressor used (pharmacological or physiological).

In addition, NADPH-d staining is observed throughout the magnocellular neurons of the SON and PVN which contain OT or vasopressin (AVP), and a large percentage (95%) of OT neurons stain for NADPH-d (Miyagawa, Okamura, & Ibata, 1994; Sanchez, Alonso, Arevalo, Blanco, Aijon, & Vazquez, 1994). Moreover, several researchers have examined the inhibitory role of NO on OT release from the posterior pituitary (Kadowaki, Kishimoto, Leng, & Emson, 1994; Summy-Long et al., 1993). Kadowaki et al. (1994) have reported that chronic salt loading with simultaneous treatment of intraperitoneal injections of a NOS inhibitor (N-*w*-nitroarginine) enhanced the depletion of OT contents from the posterior pituitary. In addition, Summy-Long et al. (1993) showed that NOS

inhibitors enhanced the rise of OT levels in plasma after only 24 hrs of water deprivation; therefore these data suggest the involvement of NO in modulating peptide release following osmotic stimulation.

Other research has examined the effect of NO on reproductive behavior in rats (Okere, Higuchi, Kaba, Russell, Okutani, Takahashi, & Murata, 1996). Okere et al. (1996) found that the intracerebroventricular (i.c.v.) administration of the NO donor sodium nitroprusside (SNP) to late pregnant rats prolonged parturition and suggested that NO inhibited OT release from the neurohypophysis.

The capacity for NO production varies with the physiological state of the animal. Research has shown that NOS mRNA and NO production may be increased through the stimulation of the hypothalamo-neurohypophyseal system (HNS) by chronic salt loading and dehydration (Kadowaki et al., 1994; Ueta, Levy, Hardial, Chowdrey, & Lightman 1995; Villar, Ceccatelli, Ronnqvist, & Hokfelt, 1994). Villar et al. (1994) have reported that after 5 and 14 days of salt loading, the number of neurons expressing NOS mRNA in the magnocellular subdivisions of the SON and PVN increased. This protocol for salt-loading also augmented the number of NOS-immunoreactive neurons in these brain areas. Pow (1992) also demonstrated intense NADPH-d staining in all magnocellular neurons in the SON in response to dehydration. In addition, dehydration was reported to increase NOS gene expression in the posterior pituitary (Vanhalato & Soinila, 1995). These data support the notion that the synthesis of NOS is up-regulated when the animal is presented with a hyperosmotic challenge.

In some brain areas, the circulating levels of gonadal steroids have been shown to influence the capacity for NO production. Pu et al. (1996) have demonstrated that ovariectomized rats treated with estrogen increased NMDA-induced NOS/cGMP activity in the MPOA and LH release from the pituitary. In addition, Ceccatelli and colleagues (1996) reported that the expression of NOS mRNA was increased in the VMN but not in the SON

and PVN in ovariectomized rats treated with 3 μg/100 g of estradiol benzoate for 7 days. These data are in line with the findings of Okamura et al. (1994) who showed enhanced NADPH-d activity in both the VMN and MPOA after estrogen administration and therefore differences in NADPH-d activity between these nuclei and the SON and PVN may have been due to their sensitivity to estrogen.

Nitric oxide production has been shown to increase during pregnancy. Conrad and colleagues (1993) found an elevation of a NO metabolite, nitrate, in urinary excretion in pregnant rats. Moreover, NO production has been shown to increase in peripheral tissues in late pregnant guinea pigs (Weiner et al., 1994) in which both stimulated and basal release of NO from the uterine artery were increased at the end of pregnancy. Weiner et al. (1994) also demonstrated an increase of NOS activity in the cerebellum in late pregnancy, and after estradiol administration. In addition, NOS mRNA levels in the hypothalamus have been reported to increase in late pregnant rats (Xu, Martin, St. John, Tsai, Summer, Ohara, Kim, & Schrier, 1996). Recent studies have also shown an up-regulation of NOS mRNA gene expression in the SON and PVN during lactation (Ceccatelli & Eriksson, 1993; Luckman et al., 1997). These studies support the view that NOS activity may change as a result of the reproductive state of the animal.

Given that in the SON and PVN, NADPH-d staining has been found to change across reproductive state (Woodside & Amir, 1996), and that NOS mRNA levels have been shown to increase in lactation (Ceccatelli & Eriksson, 1993; Luckman et al., 1997), the purpose of the first investigation was to compare NADPH-d staining in the SON and PVN of female rats at several time points during pregnancy and lactation. In the second experiment, the question of whether gonadal steroid hormones can modulate NADPH-d staining in the SON and PVN was investigated. Finally, the third experiment investigated whether the administration of OT is sufficient to increase NADPH-d staining in the SON and PVN.

## Experiment 1

The purpose of this experiment was to replicate the results of Woodside and Amir (1996) who found an increase in NADPH-d staining in the SON and PVN in late pregnant (Day 22) rats using NADPH-d histochemistry, and to extend these initial findings by comparing NADPH-d staining in the SON and PVN of female rats at various time points during pregnancy and lactation. In addition to late pregnant rats, NADPH-d histochemistry was performed on virgins, rats on Days 4, 12, and 16 of pregnancy, and Days 4, 12, and 20 of lactation.

NOS mRNA and NADPH-d staining have been shown to increase in the SON and PVN as a result of various physiological challenges including osmotic stimulation (Villar et al., 1994; Pow, 1992), late pregnancy ( Woodside & Amir, 1996; Xu et al., 1996), and lactation (Ceccatelli & Eriksson, 1993, Luckman et al., 1997). These data suggest that increases in NOS in magnocellular neurons correlate with their activity. If this were the case, then we would expect increases in NADPH-d staining in late pregnancy when OT activity is associated with uterine contractions (Higuchi, Tadokoro, Honda, & Negoro, 1986), and in early and mid-lactation when OT release stimulates milk ejection (Wakerley et al., 1988), but not in early pregnancy and late lactation when OT cells are less active.

### Method

#### Subjects

Forty-three female Wistar rats from the Charles River Breeding Laboratory (St. Constant, Quebec) were used in this study. Upon arrival in the laboratory, the animals weighed between 220-240 g and were housed in groups of five females. The facility was maintained on a 12/12hr light/dark cycle (lights on from 0800-2000hr) at $20 \pm 2^{0}$ C. The animals were given adlibitum (adlib) access to lab chow (Agway) and to water throughout the experiment.

Experimental groups. Animals were randomly assigned to one of eight different groups: Virgins (n=8), Pregnancy Day 4 (P4, n=5), Day 12 (P12, n=5), Day 16 (P16, n=5), and Day 22 (P22, n=5), and Lactation Day 4 (LA4, n=5), Day 12 (LA12, n=5), and Day 20 (LA20, n=5).

## Procedure

Mating procedure. When vaginal smears indicated the proestrus stage of the estrus cycle, females were placed in a polypropylene cage (43 x 20 x 19 cm) with one male. Pregnancy was verified on the following day if spermatozoa were present in the vaginal smear, and this day was designated Day 1 of pregnancy. Each animal was then returned to the group cage. One week before the estimated date of parturition, the animals in groups P22, LA4, LA12, and LA 20 were housed in individual polypropylene cages with beta chips for bedding. In most animals, parturition occurred in the late afternoon of Day 22 or in the morning of Day 23 of pregnancy. On the day after parturition (Day 1 postpartum), the litters were culled to eight pups and the weights of the lactating females and their litters were recorded. Thereafter, weights of the dams and pups were recorded every second day until the end of the experiment.

Histochemistry. On the appropriate day postconception or postpartum, females were given an overdose of sodium pentobarbital (60 mg/kg; MTC Pharmaceuticals Ltd., Cambridge, Ont.) and were perfused transcardially with 200 ml of ice-cold saline (0.9% NaCl) followed by 300 ml of cold 4% paraformaldehyde in .1 M of phosphate buffer (pH 7.3). The brains were removed and post-fixed for 24 hrs in 4% paraformaldehyde at 4$^{0}$ C. Fifty-micron thick sections throughout the SON and PVN were cut on a vibratome and placed free-floating in Trizma Buffered Saline (TBS; pH 7.3) solution.

Approximately 25 free floating sections were then placed in 5ml of a solution containing a mixture of 5.0 mg of *B*-NADPH, 1.5 mg of Nitro Blue Tetrazolium, 0.3%

Titron X-100, and TBS and were incubated for 75 minutes in a waterbath maintained at 37$^0$ C. Animals from different experimental groups were represented in each assay. Following incubation, sections were washed 3 times for 5 minutes in TBS and then were mounted on gelatin coated slides and cover-slipped with Permount.

Image Analysis. Sections were visualized using a Sony XC77 camera mounted on a light microscope (Labolux Leitz GMBH). Images were captured using NIH image analysis software (1.60b) installed on a Power Macintosh computer (6100). NADPH- d stained cells were counted from the images captured in the TIFF picture files. A previously established criterion was implemented to identify densely stained cells and two independent observers counted the stained cells.

To obtain an estimate of the number of densely stained cells in the SON of each animal, densely stained cells in sections throughout the rostro-caudal extent of the nucleus on one side of the brain were counted and the mean of the five sections with the highest number of densely stained cells was calculated. The coefficient of inter-rater reliability obtained across all sections in the SON was r = .91. For the PVN, only sections in the medial area of the nucleus were counted and an average of all these sections were obtained. The coefficient of inter-rater reliability obtained across all sections in the PVN was r = .93. The resulting data were analyzed using a one-way analysis of variance (ANOVA) followed by pairwise comparisons. All source tables for the ANOVAs are shown in Appendix A.

## Results

Examples of NADPH-d staining observed in the SON of animals at various time points of pregnancy and lactation are shown in Figure 1. The number of densely stained cells in the SON varied significantly across the eight groups ($F(7, 42) = 10.43, p < .01$).

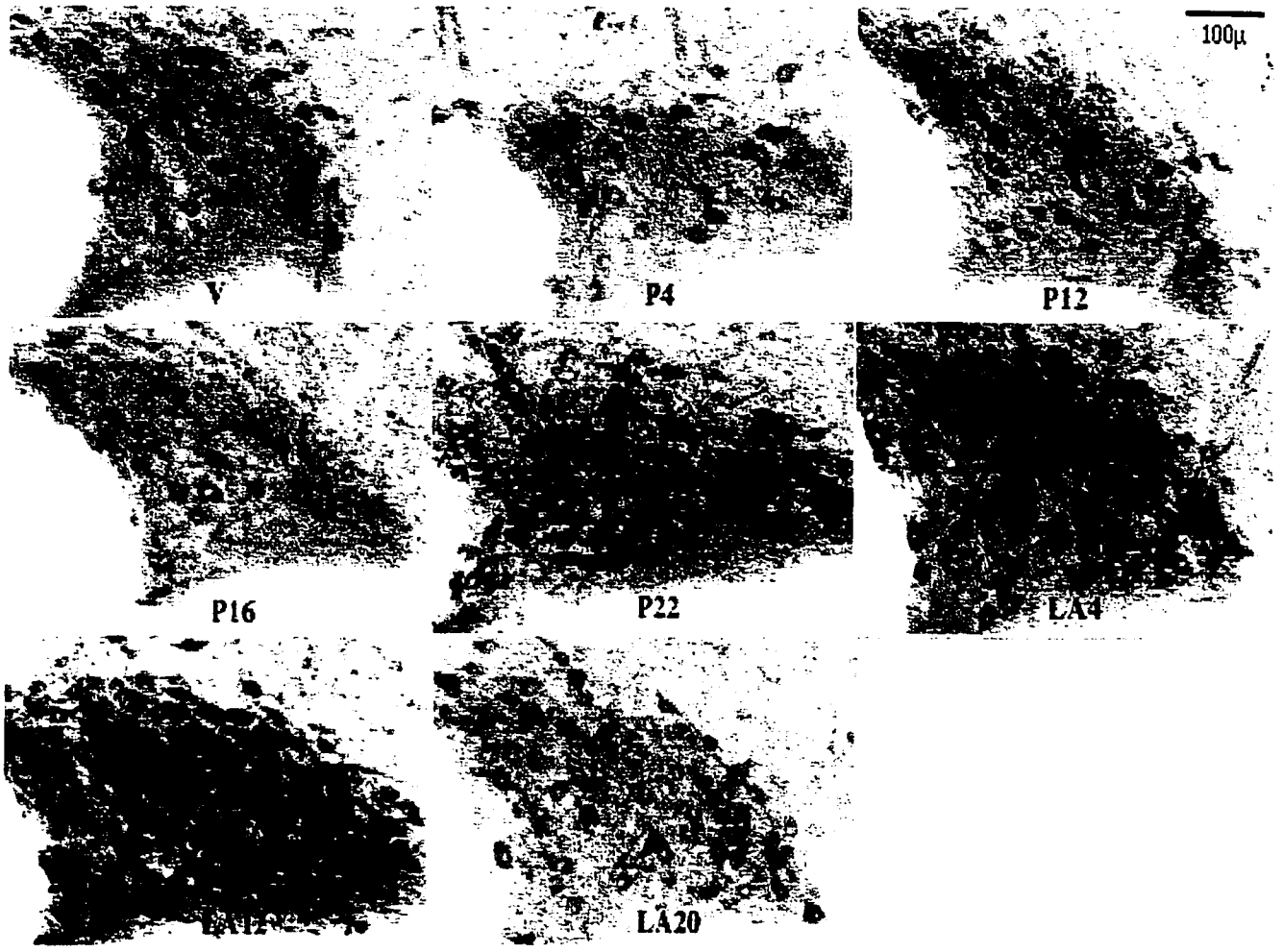


**Figure 1.** Examples of NADPH-d staining in the SON across various days of pregnancy and lactation.

The highest number of densely stained cells in the SON was observed on Day 22 of pregnancy, and Days 4 and 12 of lactation (Figure 2). Pairwise comparisons (Fisher's test) revealed that animals in the P22, LA4, and LA12 groups showed higher densities of staining than those of virgins and animals in the P4 and P16 groups. As expected, NADPH-d staining decreased towards the end of lactation and animals in the LA20 group had less dense staining than females in the LA4 and LA12 groups. In mid-pregnancy (P12), staining was as high as in late pregnancy (P22).

Examples of NADPH-d staining observed in the PVN of animals at various time points of pregnancy and lactation are shown in Figure 3. The number of densely stained cells in the PVN varied significantly across the eight groups ($F(7, 42) = 5.08, p < .01$). The highest number of densely stained cells in the PVN was observed on Day 22 of pregnancy and Days 4 and 12 of lactation (Figure 4). Pairwise comparisons (Fisher's test) revealed that animals in the P22, LA4, and LA12 groups showed higher densities of staining than those of virgin rats and animals in the P4 and P16 groups. As expected, NADPH-d staining decreased towards the end of lactation and animals in the LA20 group had less dense staining than females in the P22, LA4 and LA12 groups. In mid-pregnancy (P12), staining was as high as in mid-lactation (LA12).

## Discussion

The results obtained from the first experiment supported the hypothesis that NADPH-d staining increases in the SON and PVN in late pregnancy (P22), and in early and mid- lactation (LA4 and LA12) and then decreases in late lactation (LA20). These findings replicated those found by Woodside and Amir (1996) who reported a higher number of NADPH-d stained neurons in these areas in late pregnancy, and also extended the investigation of elevated NADPH-d activity into lactation. These data are also consistent with increased levels of NOS mRNA observed in hypothalamic nuclei in late pregnancy

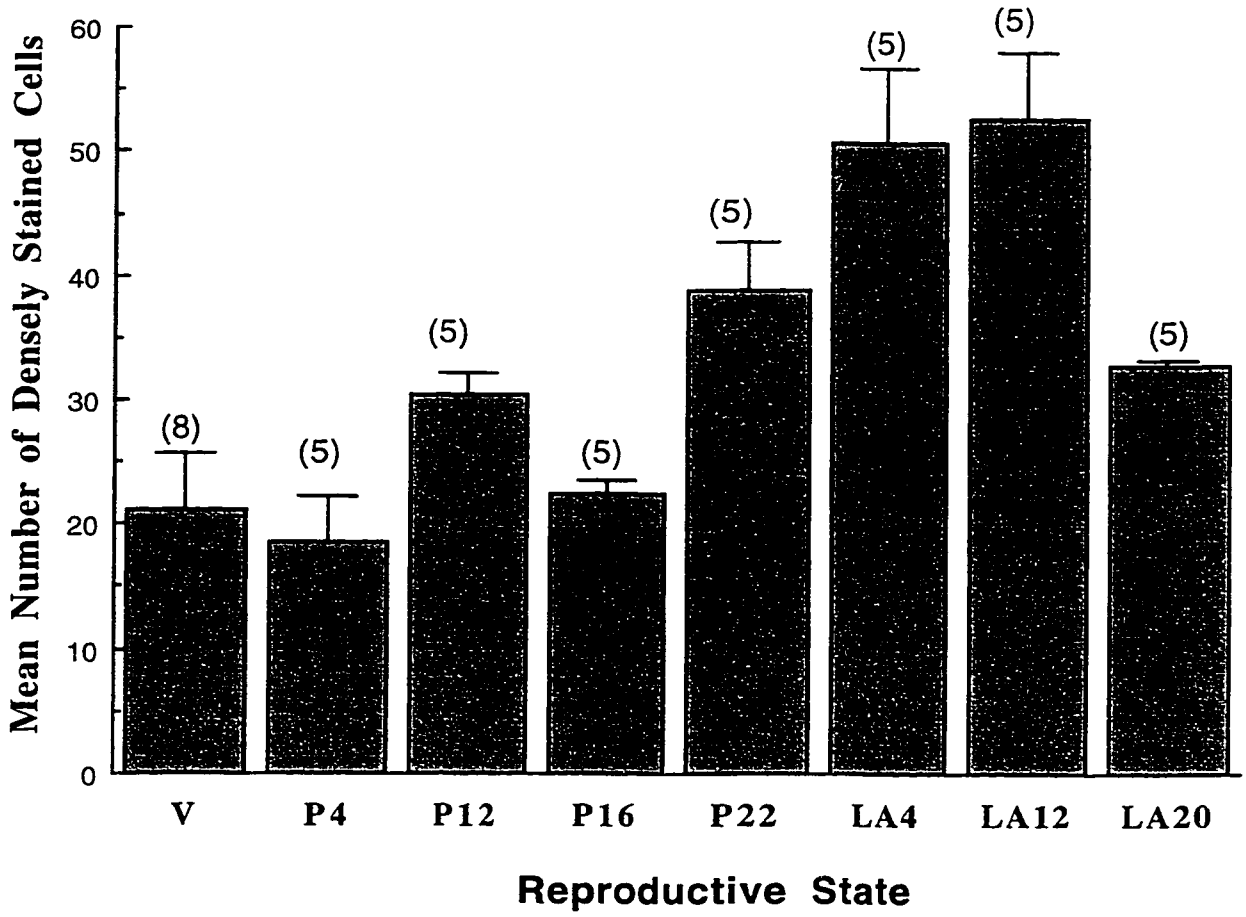


**Figure 2.** Mean number of cells stained for NADPH-d in the SON across various days of pregnancy and lactation. Error bars represent standard errors.

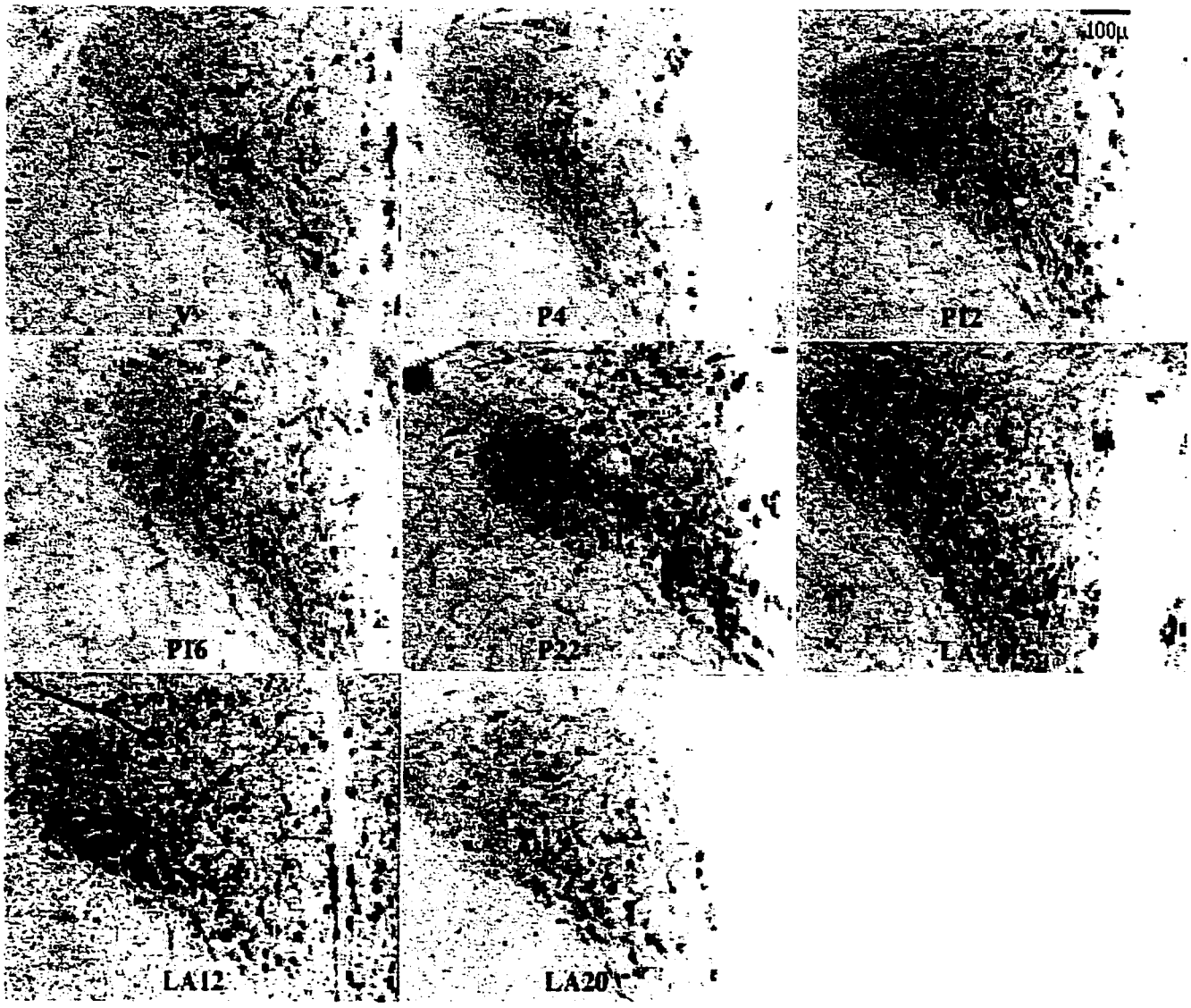


**Figure 3.** Examples of NADPH-d staining in the PVN across various days of pregnancy and lactation.

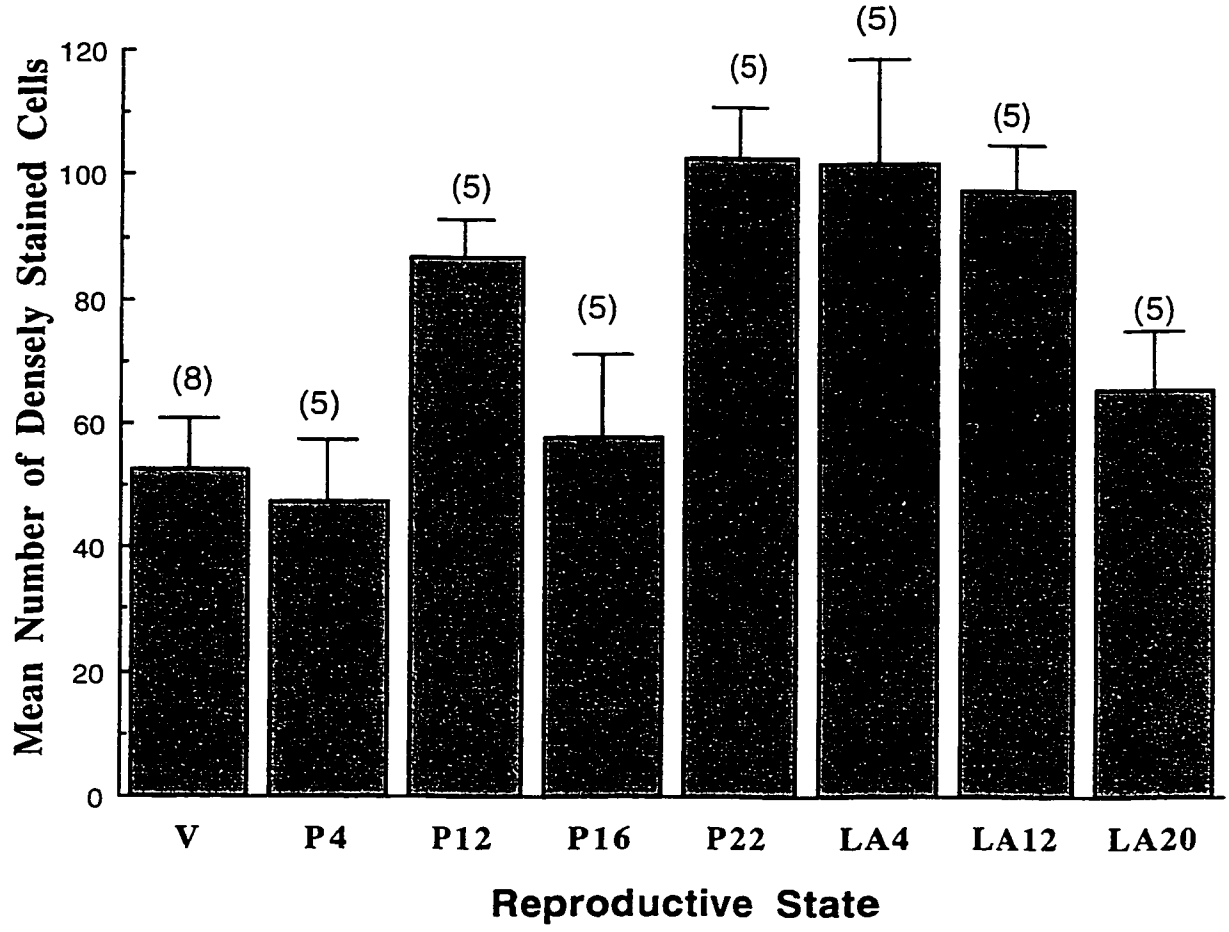


**Figure 4.** Mean number of cells stained for NADPH-d in the PVN across various days of pregnancy and lactation. Error bars represent standard errors.

(Xu et al., 1996) and lactation (Ceccatelli & Eriksson, 1993; Luckman et al., 1997). In sum, it appears that NADPH-d staining increased at a time when OT neurons are active which suggests that NO may be involved in events associated with parturition and milk let-down.

Surprisingly, results revealed an elevated level of NADPH-d stained cells in the SON and PVN of Day 12 pregnant rats (P12). A possible explanation for this result is that these animals were pseudopregnant rather than pregnant. Pseudopregnancy is a state induced by cervical stimulation of proestrus rats that results in bi-daily surges of prolactin (PRL) secretion which are also observed in the first half of pregnancy. Typically pseudopregnancy terminates after 11-12 days with a drop in progesterone and an increase in circulating estrogen levels. Thus, the hormonal state of the female at the termination of pseudopregnancy resembles that of late pregnancy. It seems unlikely, however that all the animals in the P12 group were pseudopregnant because with the exception of one animal, conceptuses were observed in the remaining animals on Days 16 and 22 of pregnancy, and pups were born to all animals in the lactating groups.

Another possible explanation for these data is that it reflects increases in circulating levels of rat placental lactogen (rPL). In the rat, days 11-12 of pregnancy mark the change in the endocrine control of pregnancy from the mother's pituitary to the placental fetal unit and this change is accompanied by increasing levels of rPL. It has been demonstrated that rPL levels are high in the cerebral spinal fluid at mid-pregnancy and share the actions of PRL (Bridges, Robertson, Shiu, Sturgis, Henriquez, & Mann, 1997); levels of which are increased at the end of pregnancy when intense NADPH-d staining has been established.

In sum, the findings indicate that NADPH-d staining changes across pregnancy and lactation. In the next experiment, the ability of ovarian hormones to elicit changes in NADPH-d staining in ovariectomized rats was examined.

## Experiment 2

The results from Experiment 1 indicated that NADPH-d positive neurons were highest in the SON and PVN in late pregnancy and in early and mid-lactation. There is a wide body of evidence that shows that many of the behavioral and neurochemical changes of late pregnancy are elicited by the hormonal state of the animal. In the rat, the end of pregnancy is associated with a drop in circulating progesterone levels and a dramatic increase in circulating estrogen levels (Bridges, 1984). When these changes are induced in ovariectomized female rats using the hormone replacement schedule described by Bridges (1984), they stimulate a rapid onset of maternal behavior (Bridges, 1984), an increase in medial preoptic μ-opiate receptors (Hammer & Bridges, 1987; Hammer, Mateo, & Bridges, 1992), and an elevation of OT mRNA levels in the SON and PVN (Crowley, Insel, O'Keefe, Kim, & Amico, 1995).

These data, together with the recent evidence showing that ovarian hormones increase NADPH-d staining in hypothalamic nuclei, including the VMH and MPOA (Okamura et al., 1994), led us to determine whether the gonadal treatment that mimics the hormonal milieu of late pregnancy (estrogen and progesterone, followed by progesterone withdrawal) would be sufficient to increase NADPH-d staining in the SON and PVN.

### Method

#### Subjects

Sixteen virgin female Wistar rats weighing approximately 220-240 g were obtained from the Charles River Breeding Laboratory (St. Constant, Quebec). General housing conditions were as described in Experiment 1.

Experimental groups. All animals were ovariectomized and randomly assigned to one of four hormone replacement groups. Animals in Group 1 (Ovex) (n=4) received sham implants (cholesterol-filled) and sham implant withdrawal; Group 2 (E) received

estrogen (E) (n=4) and sham implants and sham implant withdrawal; Group 3 (E+P-P) (n=4) received E and Progesterone (P) implants followed by P implant withdrawal which mimics the hormonal state of late pregnancy; and Group 4 (E+P) (n=4) received E and P implants.

Surgical procedure. All animals were ovariectomized one week prior to sham or steroid treatment. Each animal was anesthetized with a ketamine/xylazine mixture (5.7 mg ketamine and .86 mg xylazine/100 g of body weight). The backside of the body was shaved and 70% ethanol was used for sterilization. Bilateral incisions were made in the skin and the dorsal body wall. Each ovary was removed and the wall was sutured with 3-0 silk surgical suture. The overlying skin was clipped with surgical wound clips and a topical antibacterial powder (cicatrin ) was placed on the wound. A seven day postoperative recovery period was given to each subject.

Silastic capsules Hormones were administered via implants which were prepared by cutting silastic tubing (Dow Corning, .078" ID x .125" OD) into a preselected length. The tubes were filled with either cholesterol, 17*B* estradiol (2mm), or crystalline P (30mm) and then were sealed with silastic glue (curing agent and medical grade elastomer mixture). Once sealed, the tubes were rinsed in sterile saline. Twenty-four hours prior to implantation, tubes were incubated in sterile saline at room temperature.

Implantation of silastic capsules. One week following ovariectomy, steroid or cholesterol filled silastic brand silicon tubing capsules were implanted subcutaneously between the scapulae under metofane anaesthetic (Jansen).

Procedure

| | Treatment | | |
|---|---|---|---|
| | Day 1 | Day 3 | Day 14 |
| Ovex | Cholesterol (1x2mm) | Cholesterol (3x30mm) | Cholesterol Removed |
| E | Estrogen (1x2mm) | Cholesterol (3x30mm) | Cholesterol Removed |
| E+P-P | Estrogen (1x2mm) | Progesterone (3x30mm) | Progesterone Removed |
| E+P | Estrogen (1x2mm) | Progesterone (3x30mm) | Anesthetic |

All animals were weighed every day during the hormone treatment to verify that the hormones were operating. Animals in the Ovex, E and E+P-P groups were sacrificed on Day 17 of the hormone treatment. Animals from each group were represented in each assay and the protocols for NADPH-d histochemistry and image analysis were carried out as described in Experiment 1. The coefficient of inter-rater reliability obtained across all sections was r = .96 and r = .95 in the SON and PVN, respectively. The resulting data were analyzed using a one-way ANOVA followed by pairwise comparisons. All source tables for the ANOVAs are shown in Appendix B.

## Results

A one-way ANOVA revealed a significant effect for mean weight change from Day 1 to Day 14 of treatment ($F(2, 9)= 4.84$, $p< .05$) (Figure 5). Pairwise comparisons (Fisher's test) revealed a significant difference between the Ovex and E groups, but no significant differences were found between the Ovex and E+P groups, and the E and E+P groups.

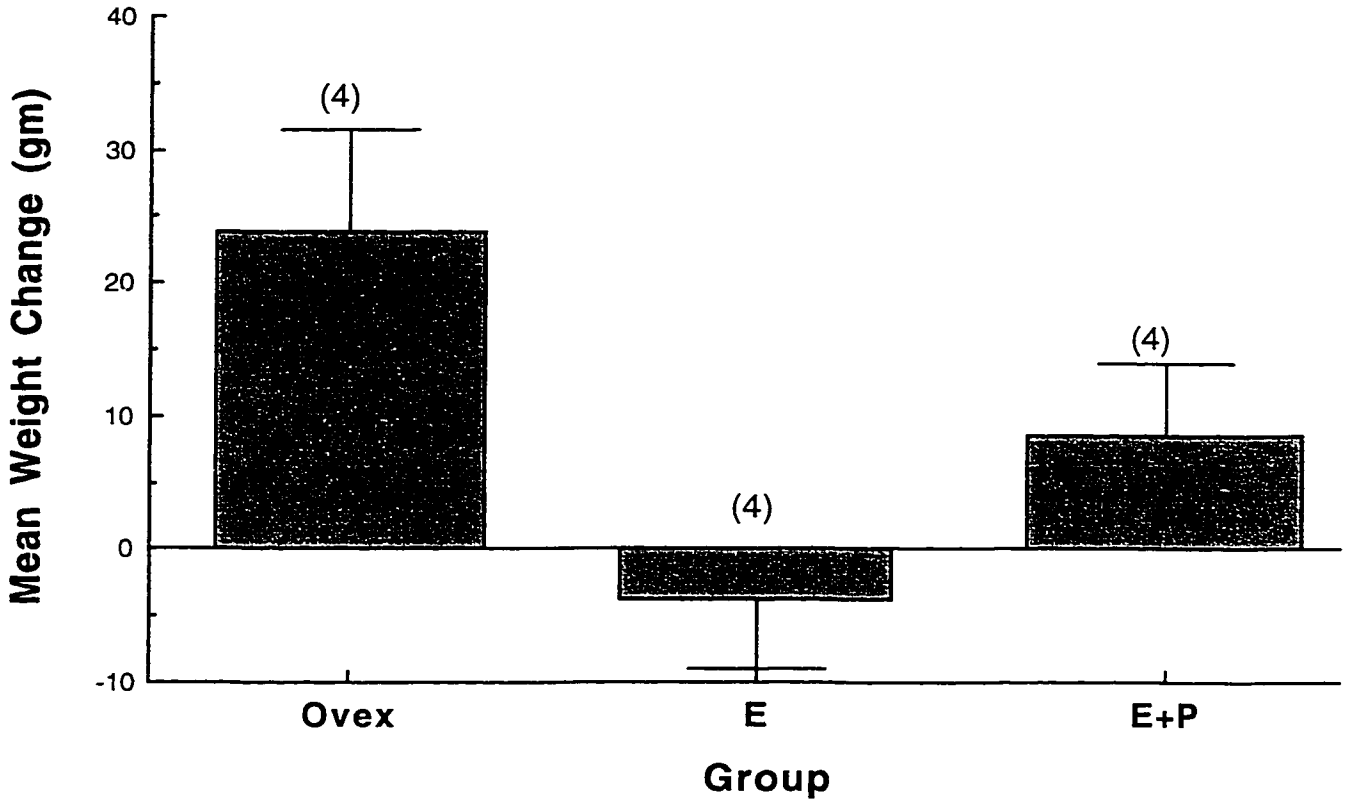


**Figure 5.** Mean weight change from Day 1-Day 14 across hormone condition. Error bars represent standard errors.

Examples of NADPH-d staining observed in the SON of animals in each hormone treatment are shown in Figure 6. The number of densely stained cells in the SON varied significantly across the four hormone treatment groups ($F(3,15) = 5.31$, $p < .05$). The highest number of densely stained cells in the SON was observed in females who received long-term E and P, followed by P withdrawal (Figure 7). Pairwise comparisons (Fisher's test) showed that there were no significant differences among the other three groups.

Examples of NADPH-d staining observed in the PVN of animals in each hormone treatment are shown in Figure 8. The number of densely stained cells in the PVN varied significantly across the four hormone treatment groups ($F(3, 15) = 4.61$, $p < .05$). The highest number of densely stained cells in the PVN was observed in females who received long-term E and P followed by P withdrawal (Figure 9). Pairwise comparisons (Fisher's test) showed that there were no significant differences among the other three groups.

## Discussion

These findings show that the number of neurons showing dense staining for NADPH-d in the SON and PVN is increased in animals maintained in a combination of hormone treatments that mimics that seen in late pregnancy. The change in weight of the animals from Day 1 to Day 14 of treatment clearly demonstrated that the behavior of the animals was associated with their hormonal regimen. These data are consistent with other evidence which reveals that body weight fluctuates in response to gonadal steroid levels (Wade, 1972). Removal of ovarian hormones by ovariectomy results in an increase in food intake and body weight, whereas estradiol replacement has been shown to reverse these effects, resulting in a decrease in body weight (Blaustein & Wade, 1976). Treatment with progesterone in the ovariectomized rat has been shown to be effective when paired only with estradiol in which it suppresses the actions of estradiol on eating and weight gain.

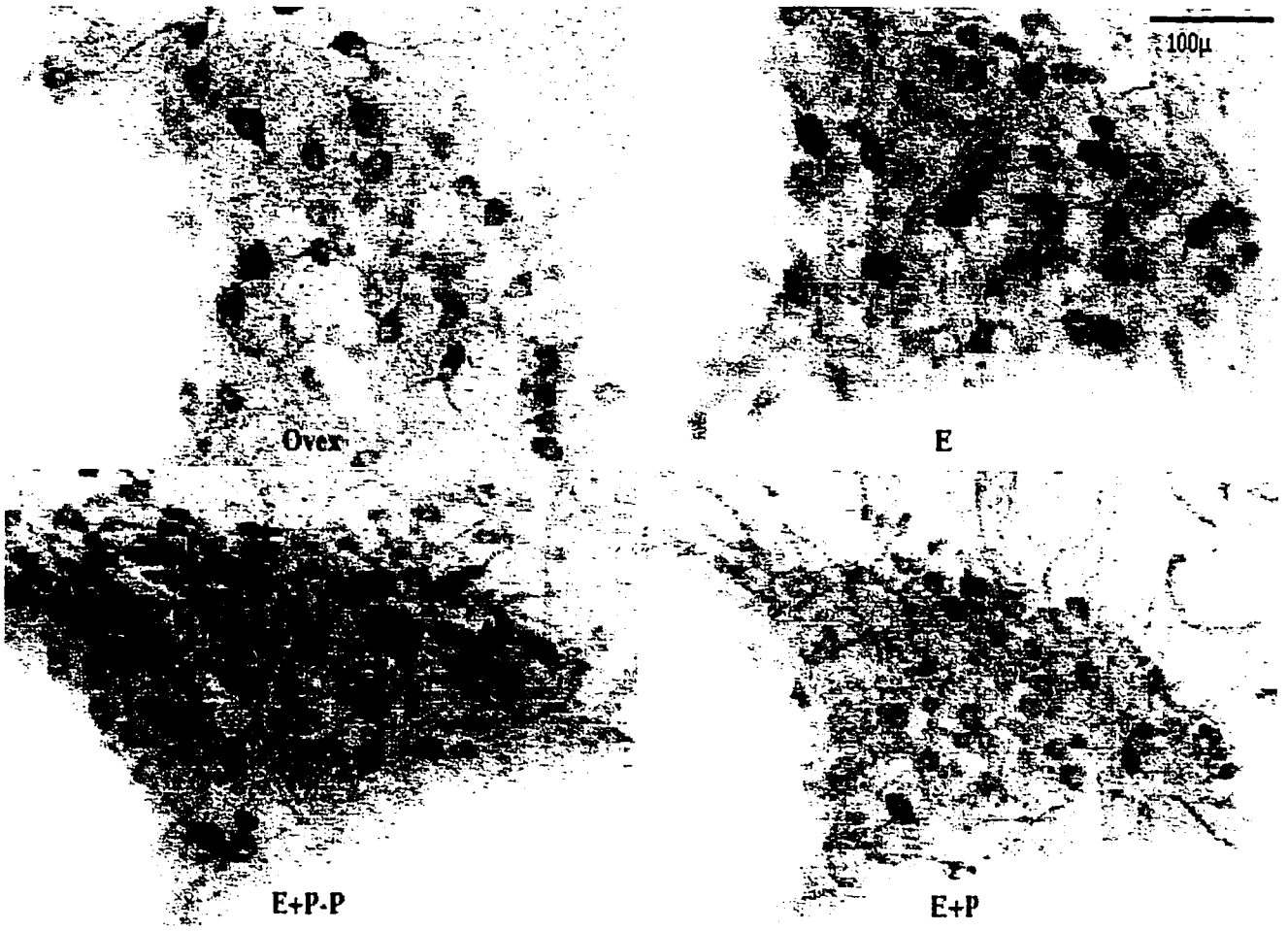


**Figure 6.** Examples of NADPH-d staining in the SON of animals in each hormone condition.

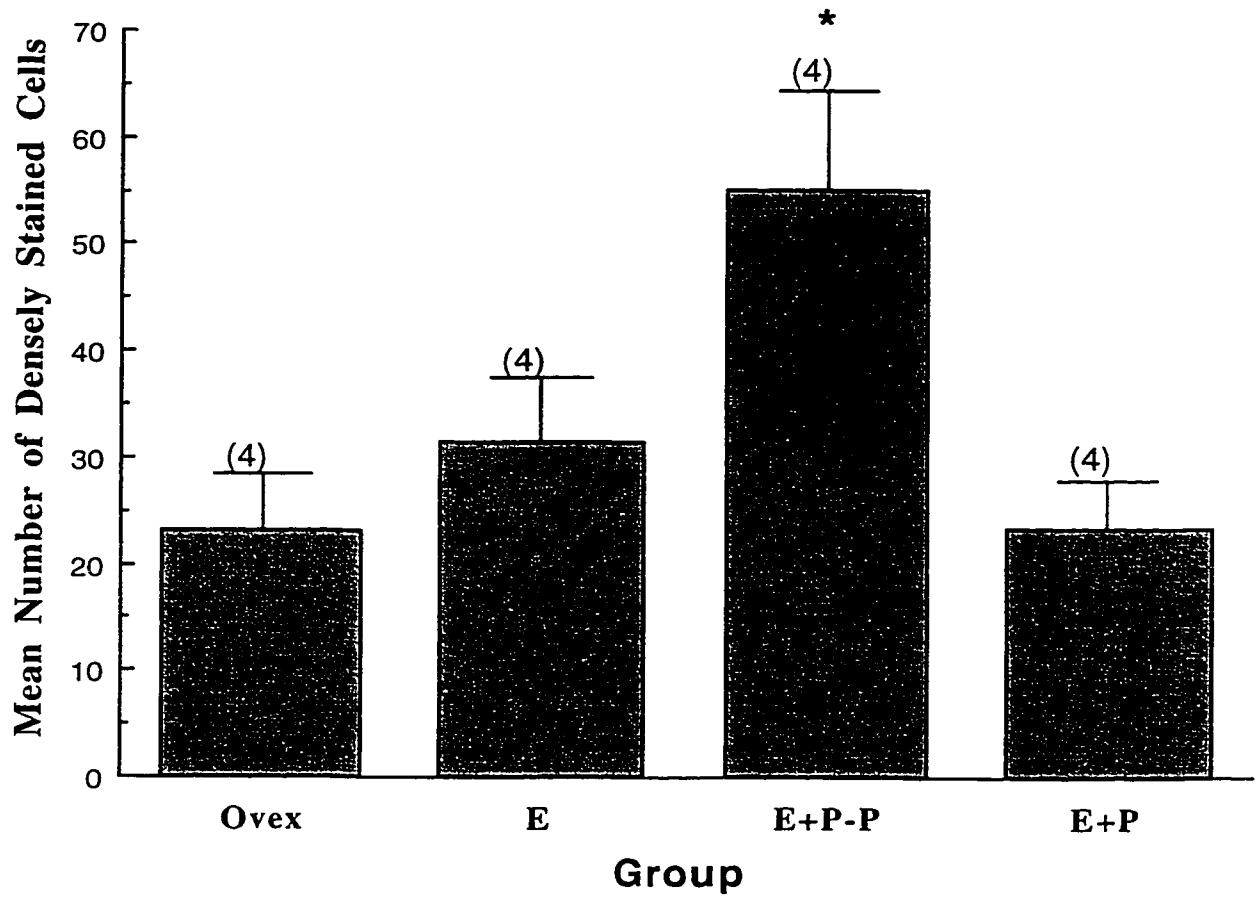


**Figure 7.** Mean number of cells stained for NADPH-d in the SON across hormone condition. Error bars represent standard errors.

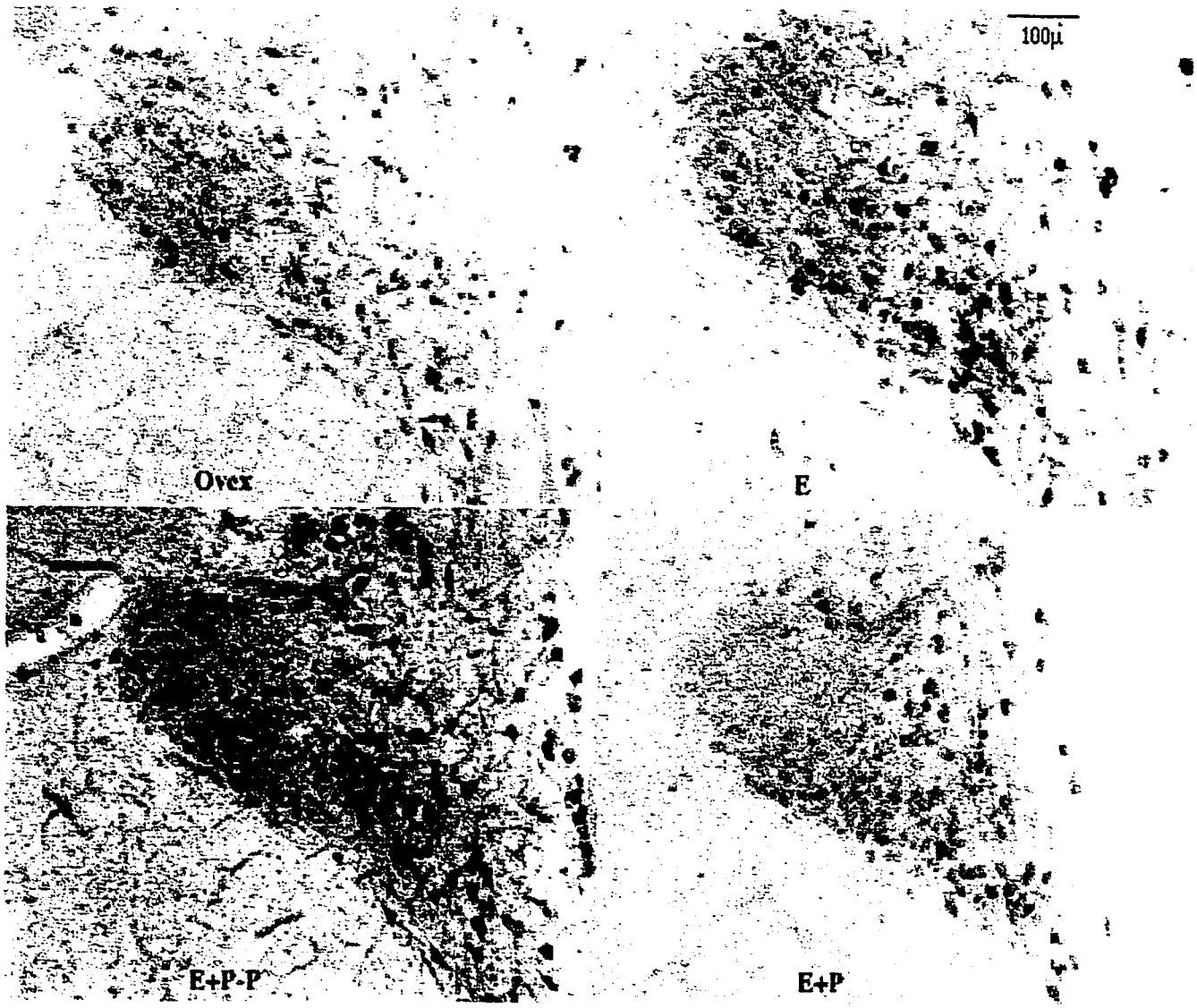


**Figure 8.** Examples of NADPH-d staining in the PVN of animals in each hormone condition.

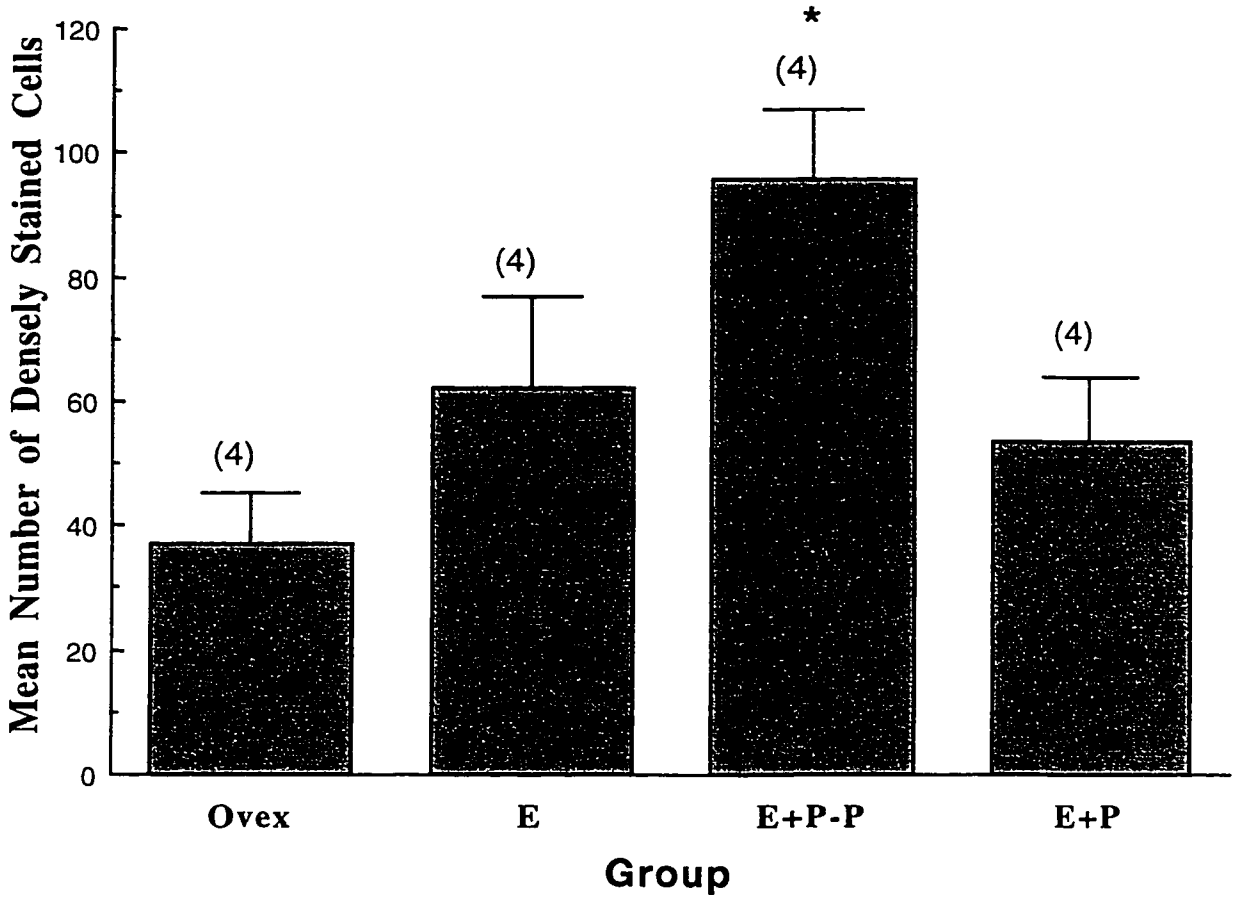


**Figure 9.** Mean number of cells stained for NADPH-d in the PVN across hormone condition. Error bars represent standard errors.

Because the hormonal milieu of late pregnancy has previously been shown to increase mRNA for OT within the SON and PVN (Crowley et al., 1995) and may induce intranuclear OT release, the next experiment addressed the question of whether OT itself would be sufficient to increase NADPH-d staining in these brain regions.

## Experiment 3

Results from Experiment 2, revealed that the number of NADPH-d positive neurons were higher in the SON and PVN after long-term E and P treatment, followed by P removal 72 hours before sacrifice. This hormonal paradigm has also been demonstrated to increase OT mRNA in the SON and PVN, thus suggesting that the hormonal profile of late pregnancy may induce OT release within these hypothalamic nuclei. It is well established that in both the periphery (uterus and myometrium) and the central nervous systems (VMH), OT receptor mRNA expression is up-regulated immediately before parturition, during the second week of lactation, and after E administration; therefore enhancing OT binding (Bale, Pedersen, & Dorsa, 1995; Larcher, Neculcea, Breton, Arslan, Rozen, Russo, & Zingg, 1995). Larcher et al. (1995) reported that ovariectomized rats treated with E showed a rise in OT-receptor mRNA levels in the uterus and proposed that increased OT binding that is observed in late pregnancy may be mediated by estrogen-induced up-regulation of OT receptor gene expression. These data, together with evidence reporting that OT receptors are found on OT neurons in the SON and PVN (Freund-Mercier, Stoeckel, & Klein, 1994) suggest that E may contribute to the increase in OT binding on these brain regions that naturally occurs during late pregnancy and lactation.

Central infusion of OT has been shown to stimulate its own release within the SON (Neumann, Pittman, & Landgraf, 1995). The central infusion of OT has also been demonstrated to induce neuronal-glial and synaptic changes in magnocellular neurons in the SON similar to those observed during parturition and lactation (Montagnese, Poulain, & Theodosis, 1990). Montagnese et al. (1990) administered chronic i.c.v. infusion of OT at a dose of 24 ng/day for 7 days into the third ventricle of intact rats and observed these morphological changes within the SON. The purpose of the third experiment was to investigate whether chronic OT infusion would up-regulate NADPH-d activity in the SON and PVN. Female virgin rats were ovariectomized, implanted with either cholesterol or

estrogen filled implants, and chronically infused with either vehicle or OT into the third ventricle for 7 days. It was hypothesized therefore that animals receiving OT would have an increased number of NADPH-d positive cells in the SON and PVN compared to animals receiving vehicle.

## Method

### Subjects

Twenty-three female Wistar rats from the Charles River Breeding Laboratory (St. Constant, Quebec) were used in this study. General housing conditions were as described in Experiment 1.

Experimental groups. Animals were assigned to one of four groups: ovariectomized females receiving cholesterol-filled implants and vehicle infusion (Ovex, n=6), ovariectomized females receiving cholesterol-filled implants and OT infusion (OT, n=5), ovariectomized females receiving estrogen implants and vehicle infusion (E, n=6), and ovariectomized females receiving estrogen implants and OT infusion (E+OT, n=6).

Ovariectomy surgical procedure. Animals in all groups were bilaterally ovariectomized and the surgical procedure was carried out as described in Experiment 2.

Cannulae implantation. A stainless steel cannula (22 gauge; Plastic One Products) was inserted stereotaxically into the third ventricle (AP: - .80 mm, L: 0, DV: - 6.2 mm below dura (Paxinos & Watson, 1986)) under ketamine/xylazine anesthesia and was fixed to the skull, using four flat head skull screws (1/8 inch) and dental cement (Plastics Product Company). The cannula was connected, via a polyethylene catheter to an osmotic minipump (Alzet, model 2001) delivering fluid at a rate of 1 µl/h. The pump was then implanted under the skin of the back of the animal between the scapulae. The pumps were filled with either vehicle (sterile saline) or OT (Sigma) dissolved to a concentration of 1 ng/µl and infused at a rate of 1 µl/ hour, to yield a daily dosage of 24 ng. The continuous

infusion lasted for 7 days.

Silastic capsules. Hormones were administered as described in Experiment 2.

Procedure

On Day 1 of the experiment, animals in the Ovex and OT groups received one cholesterol-filled implant and animals in the E and E+OT groups received 1 x 2mm E capsule implant. On Day 7, animals in the Ovex, OT, E, E+OT groups underwent cannulae implantation and chronic vehicle or OT infusion began. On Day 14, animals in all groups were sacrificed.

Animals from each group were represented in each assay and the protocols for histochemistry and image analysis were carried out as described in Experiment 1. The coefficient of inter-rater reliability obtained across all sections was $r = .90$ and $r = .95$ in the SON and PVN, respectively. The resulting data were analyzed using a 2 X 2 (Implant X Drug) ANOVA followed by pairwise comparisons. All source tables for ANOVAs are shown in Appendix C.

## Results

Mean weight change from Day 1 to Day 7 of hormone treatment was consistent with results obtained from Experiment 2 (animals receiving cholesterol-filled implants, M= 6.84, SE = ± 1.82; animals receiving E filled implants, M= -5.25, SE= ± 1.97).

Examples of NADPH-d staining observed in the SON of animals in each hormone condition are shown in Figure 10. A two-way ANOVA revealed a significant main effect for Implant ($F(1, 19)= 5.41$, $p<.05$) and a significant interaction of Implant X Drug ($F(1, 19)= 4.69$, $p<.05$) and also revealed that there was no main effect for Drug ($F(1, 19)= 3.16$, $p>.05$) . The highest number of densely stained cells in the SON was observed in females who received E and i.c.v. OT infusion (Figure 11). Pairwise comparisons (Fisher's test) revealed significant differences between the E+OT group and each of the

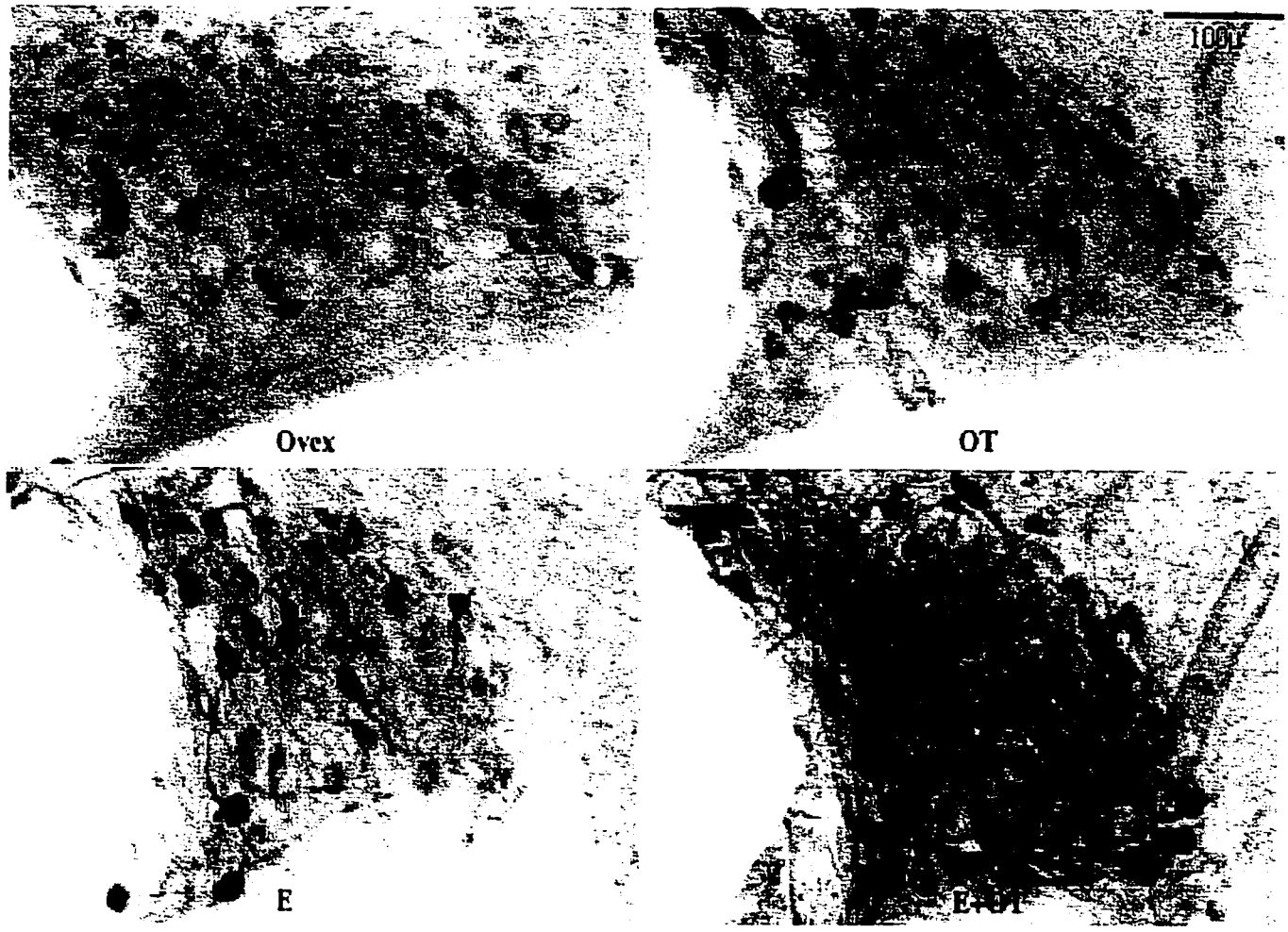


**Figure 10.** Examples of NADPH-d staining in the SON of animals in each hormone condition.

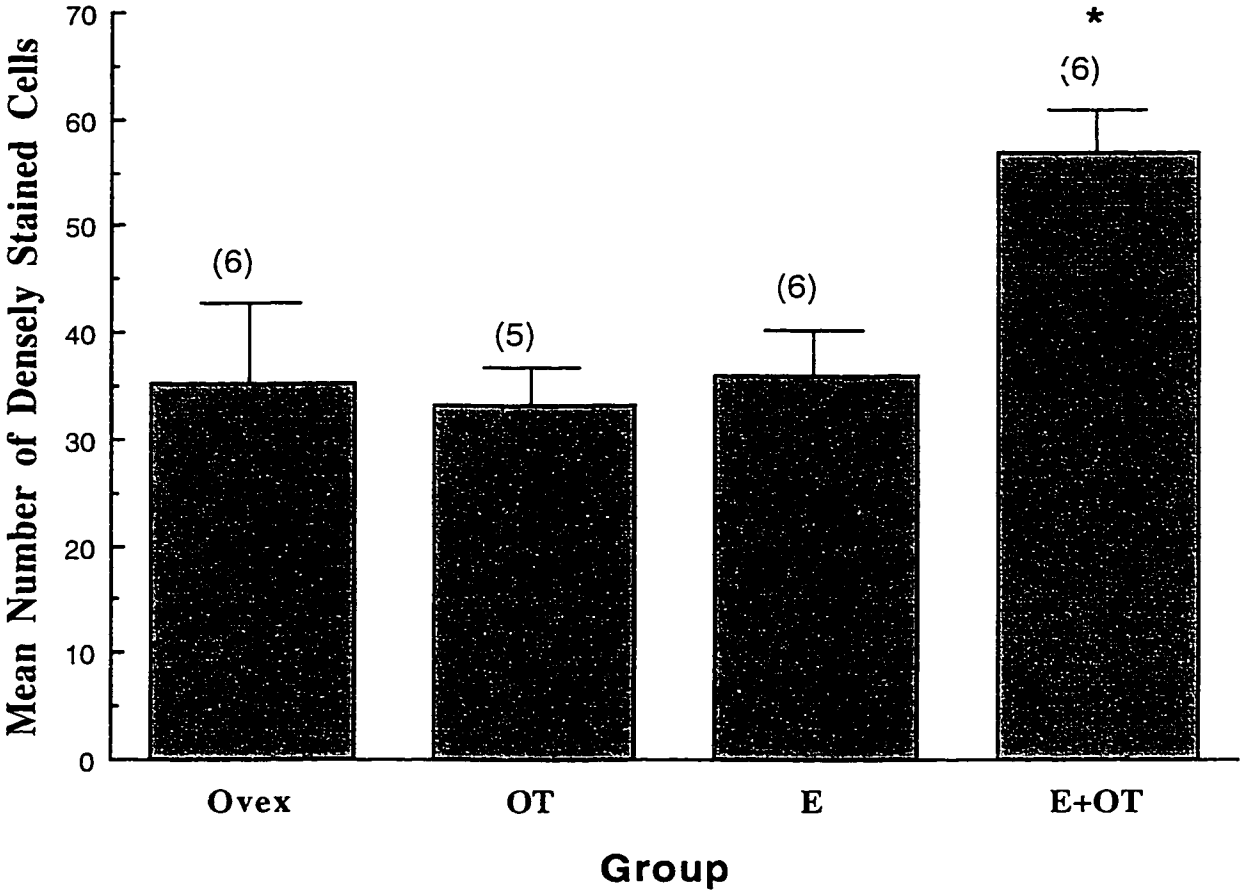


**Figure 11.** Mean number of cells stained for NADPH-d in the SON across hormone condition. Error bars represent standard errors.

other three groups, but there were no significant differences among the Ovex, E, and OT groups.

Examples of NADPH-d staining observed in the PVN of animals in each hormone condition are shown in Figure 12. A two-way ANOVA revealed significant main effects for Implant ($F(1, 19)= 10.07$, $p<.05$), Drug ($F(1, 19)= 4.753$, $p<.05$) and a significant interaction of Implant X Drug ($F(1,19)= 11.82$, $p<.05$). The highest number of densely stained cells in the PVN was observed in females who received E and i.c.v. OT infusion (Figure 13). Pairwise comparisons (Fisher's test) revealed significant differences between the E+OT group and each of the other three groups, but there were no significant differences among the Ovex, E, and OT groups.

## Discussion

Results obtained from this experiment demonstrated that OT is sufficient to elevate NADPH-d staining in the SON and PVN in ovariectomized rats primed with E. Animals in the E+OT group showed higher number of densely stained cells than those in the other three groups which did not differ from each other. Thus it appears that both ovarian steroids and peptides contribute to changes in the capacity for NO production in these hypothalamic nuclei. Although central OT was sufficient to increase NADPH-d staining, these changes were dependent on E priming. This is consistent with other data showing the facilitatory effects of estrogen on the effects of oxytocin. For example, E has been shown to be involved in neurochemical and ultrastructural changes that are normally observed during parturition and lactation such as enhancing electrical activity of OT neurons (Akaishi & Sakuma, 1985), increasing OT receptor density and OT binding on magnocellular and bed nuclei of the stria terminalis (BNST) neurons (Ingram, Cutler, & Wakerley, 1990), and triggering morphological remodelling of the OT system (Theodosis & Poulain, 1993). In a recent study, the administration of E and i.c.v. infusion of OT was

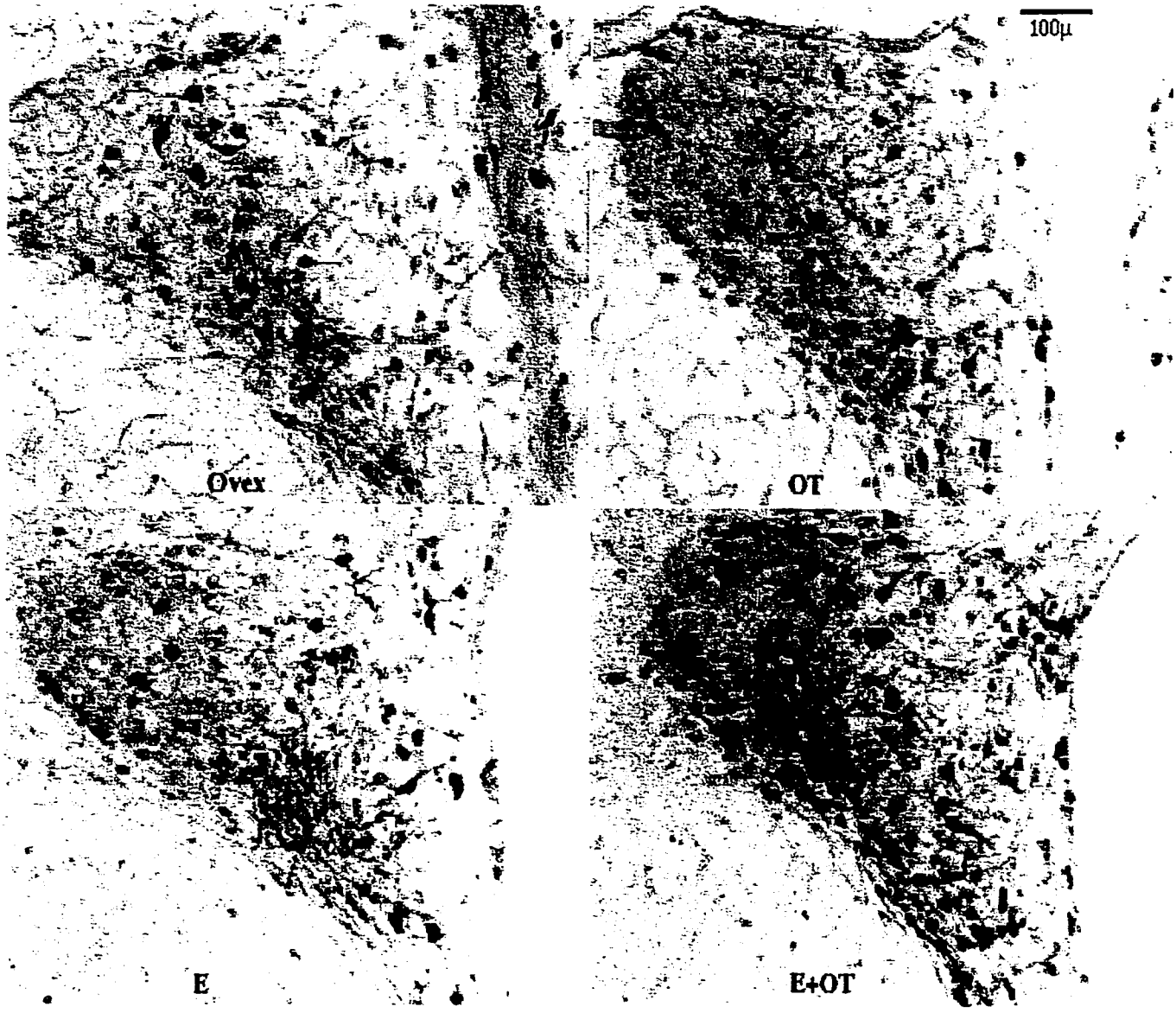


**Figure 12.** Examples of NADPH-d staining in the PVN of animals in each hormone condition.

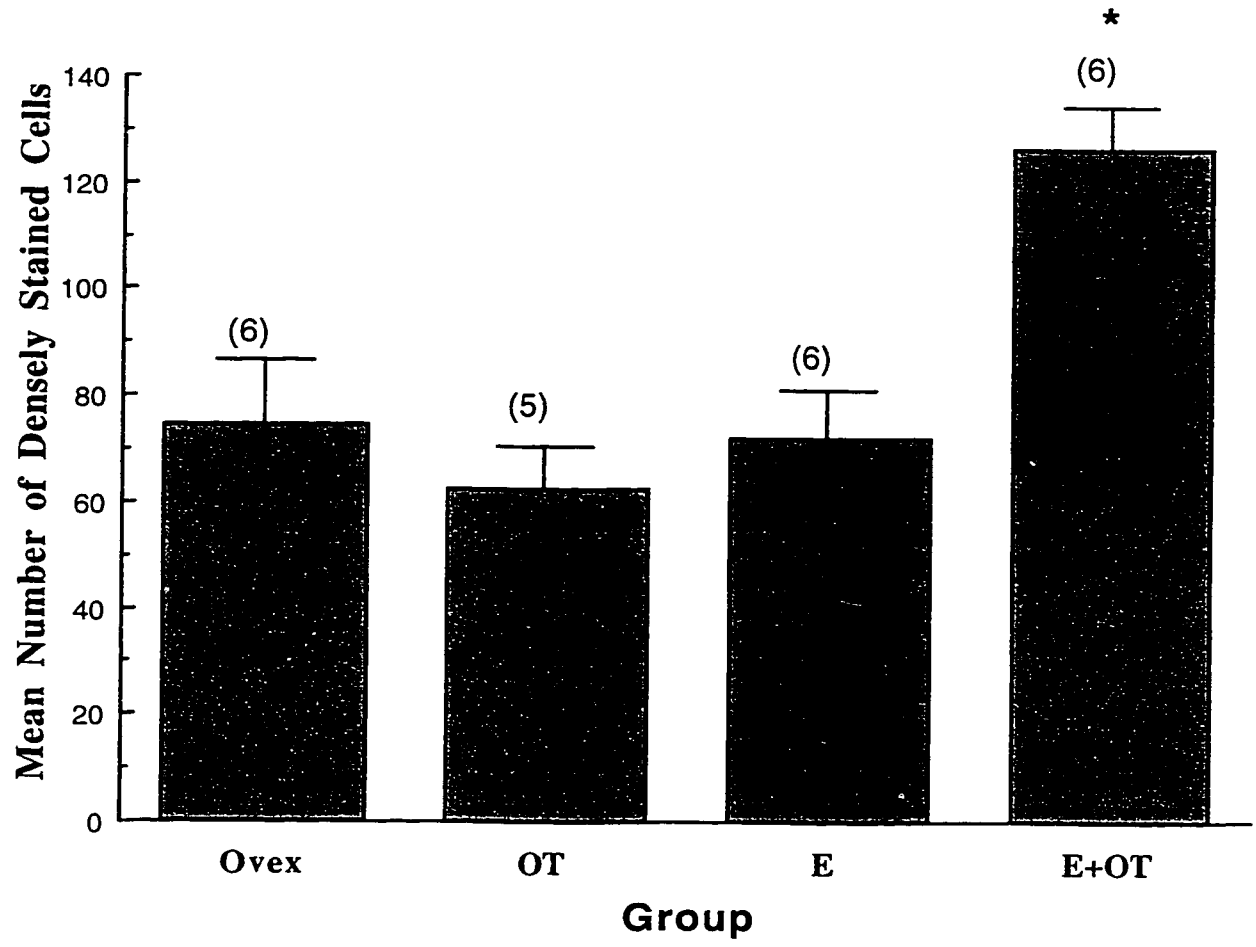


**Figure 13.** Mean number of cells stained for NADPH-d in the PVN across hormone condition. Error bars represent standard errors.

shown to advance the facilitatory response of milk ejection in pre-partum ovariectomized rats (Jiang & Wakerley, 1997) which suggests that E interacts with central oxytocinergic pathways to facilitate the response.

## General Discussion

The experiments described here show that NADPH-d staining in the SON and PVN increases in late pregnancy and in early and mid-lactation. In addition, it was found that these changes can be induced by the administration of ovarian steroids in a pattern that mimics the hormonal milieu of late pregnancy and with OT infusion into the third ventricle if the animals are primed with E.

The finding that the number of cells densely stained for NADPH-d in the SON and PVN was increased in P22, LA4, and LA12 is consistent with previous findings showing elevated NOS mRNA levels and NADPH-d staining in hypothalamic nuclei in late pregnancy (Woodside & Amir, 1996; Xu et al., 1996) and lactation (Ceccatelli & Eriksson, 1994; Luckman et al., 1997). In contrast one report has suggested that fewer cells stain for NADPH-d in the SON and PVN in late pregnancy (Okere & Higuchi, 1996). These conflicting results may reflect differences in days of sampling.

In general, the current results show that NADPH-d staining increases at times when OT neurons in the SON and PVN are known to be active. Similar data from several studies have shown that other physiological stimuli which stimulates this system, such as chronic salt loading, up-regulates the expression of NOS mRNA in the SON and PVN (Kadowaki et al., 1994) and NADPH-d staining in the SON (Pow, 1992). Interestingly, Kadowaki and colleagues (1994) have shown that NOS activity is increased in the posterior pituitary but when this activity is blocked with the administration of a NOS inhibitor, OT release is amplified. In addition, Summy-Long et al. (1993) reported that NOS inhibitors also enhanced the depletion of OT from the posterior pituitary after water deprivation. These data suggest that NO is an inhibitor of OT release when the HNS is stimulated.

NADPH-d staining was also elevated in the SON and PVN of animals in P12. Given that little is known about the oxytocinergic system at this point in pregnancy and in particular whether it is activated at this time, the significance of this increase remains to be

determined.

Experiment 2 and Experiment 3 demonstrated that the manipulation of ovarian hormones and the peptide milieu in female rats modulated NADPH-d staining in the SON and PVN. Exposure to the hormonal schedule E+P-P which stimulates the hormonal state of late pregnancy elevated the number of NADPH-d neurons in the SON and PVN relative to E alone and E+P and suggests that NADPH-d staining is regulated by the pattern of ovarian steroid administration. It is likely that the effects of the E+P-P steroid regimen increased NADPH-d staining through its effects on OT because this pattern of steroid hormone exposure has been shown to increase OT mRNA levels in the SON and PVN (Crowley et al., 1995) which normally peak immediately before parturition (Van Tol, Bolwerk, Liu, & Burbach, 1988) and approach or surpass these levels following the first week of lactation (Wakerley et al., 1988).

Whether this steroid treatment produces its effects on OT directly or acts through a mediator is not yet known. Plasma PRL levels are high in late pregnancy (Amenomori, Chen, & Meites, 1970) and in early (Days 3-7) and mid-lactation (13-17) and then gradually decline as lactation continues (Mattheij, Gruisen, & Swarts, 1979). Moreover, PRL release is stimulated by the same ovarian hormone treatment that increases the mRNA for OT and the increase in NADPH-d staining seen in Experiment 2. PRL- receptor immunoreactive neurons are found in the SON and PVN which suggests a role of PRL in modulating neuronal activity in these areas (Roky, Paut-Pagano, Goffin, Kitahama, Valatx, Kelly, & Jouvet, 1996). To determine whether PRL has central effects on NO synthesis, one would simulate the hormonal milieu of late pregnancy using E+P-P and compare NADPH-d staining between animals in which PRL release is suppressed with the D-2-like agonist, bromocriptine and animals that receive vehicle.

Whichever aspects of the hormonal state of the late pregnant female are critical for stimulating OT synthesis, the results of Experiment 3 demonstrate that infusion of OT into

the third ventricle is sufficient to increase NADPH-d staining in the SON and PVN. An issue that remains to be addressed is where OT is acting. One possibility is that it acts directly on oxytocinergic neurons within the SON and PVN. In addition to its secretion from neurohypophysial terminals, OT is released within the SON and PVN and can stimulate its own release from magnocellular cells during parturition and suckling (Neumann, Russell, & Landgraf, 1993). Furthermore, central infusion of an OT antagonist into the SON of parturient and suckled lactating rats has been shown to attenuate OT release from the SON and from the posterior pituitary (Neumann, Koehler, Landgraf, & Summy-Long, 1994). The presence of OT binding sites has also been demonstrated on magnocellular nuclei of lactating rats and it is possible that these receptors modulate the facilitatory effect of OT on its own release during milk ejection (Freund-Mercier et al., 1994). This idea is supported by evidence showing that intrahypothalamic OT is necessary to trigger the milk ejection reflex (Freund-Mercier, Moos, Poulain, Richard, Rodriguez, Theodosis, & Vincent, 1988).

Although it is probable that the effects of OT infusion seen in Experiment 3 does reflect a direct action of OT on oxytocinergic neurons in the SON and PVN, it is also possible that these effects are produced through OT acting at other brain sites. For example, neurons in the BNST possess OT binding sites and these neurons are excited following i.c.v. injections of OT in lactating rats (Ingram & Wakerley, 1993). The response of BNST neurons to E has also been shown to enhance the sensitivity of the neurons to OT which suggests that the facilitation of the milk ejection reflex may in part be modulated by the estrogen-enhanced responsiveness of the BNST to OT (Wakerley, Jiang, Housham, Terenzi, & Ingram, 1995).

There is considerable evidence to show that circulating E levels modulate OT neurons in the SON and PVN. Until recently, however, only low levels of mRNA for E receptors (ERs) have been observed in these structures (Simerly, Chung, Muramatsu, &

Swanson, 1990; Amico, Crowley, Insel, Thomas, & O'Keefe, 1995). Recent findings have demonstrated however, that ERs in the rat exist as two subtypes, ER-alpha and ER-beta and that the distribution of these subtypes in the brain differs. Although mRNA for ER-alpha is abundant in brain areas such as the VMH and MPOA (Okamura et al., 1994), Shughrue and colleagues (1996) reported high levels of ER-beta mRNA in the SON and PVN which suggests that E may regulate the activity of magnocellular neurons through this receptor subtype.

Another pathway through which E might modulate activity in OT magnocellular neurons in the SON and PVN is through activating neurons which express ERs and project to these brain regions. Voisin and colleagues (1997) combined placing a retrograde tracer into the SON with ER immunocytochemistry and demonstrated that a large number of retrograde-labelled cells expressing ER immunoreactivity were located in the organum vasculosum of the lamina terminalis (OVLT), periventricular nucleus (AVPv), and the medial preoptic nucleus (MPN). Thus suggesting that E may regulate the electrical activity of magnocellular neurons by influencing the activity of neurons projecting to the SON.

In sum the results of the studies in this thesis suggest that the capacity for NO production in the SON and PVN is increased in late pregnancy and in early and mid-lactation and that this increase is dependent on the hormonal state of the animal. This conclusion rests on the assumption that NADPH-d staining is indeed a good marker for NOS. Although most of the evidence supports this hypothesis (Bredt et al., 1990) recent data has shown that in some hypothalamic areas (VMH) only a subpopulation of NADPH-d positive neurons also express NOS mRNA (Rachman et al., 1996). Therefore, a future experiment using NOS immunohistochemistry will be conducted to verify that the neurons staining for NADPH-d in the SON and PVN is NOS.

An obvious remaining question is what is the functional significance of up-regulating the capacity for NO production in the SON and PVN? Luckman and colleagues

(1997) have suggested that the up-regulation of NOS in these neurons during lactation plays a role in reducing the sensitivity of the OT system to various stimuli, including osmotic stimulation (Neumann et al., 1995) and immobilization stress (Higuchi, Honda, Takano, & Negoro, 1988). Luckman et al. (1997) found that NOS mRNA increased not only in the SON and PVN during lactation but also in neurons in the OVLT, subfornical organ (SFO), and median preoptic area (MnPO) which project to these structures. They argue that because NO has been shown to inhibit OT release (Summy-Long et al., 1993) the increase in NOS mRNA levels observed in these forebrain sites in lactation may be involved in attenuating the sensitivity in the forebrain circuit to osmotic stimuli by reducing magnocellular activity. In a future experiment the role of NO in influencing the sensitivity of magnocellular neurons during lactation will be investigated by administering an osmotic stimulus and a NOS inhibitor (N omega-nitro-L-arginine methyl ester (L-NAME)) to both lactating and non-lactating rats and comparing Fos expression in the SON and PVN.

The increased NADPH-d staining may also be associated with the morphological changes seen in the magnocellular neurons of the PVN and SON during dehydration, late pregnancy, and lactation (Calka, Wolf, & Brosz, 1994; Theodosis & Poulain, 1993). Recent studies have implicated NO in structural changes occuring in the posterior pituitary when the system is stimulated. Ramsell and Cobbett (1996) have demonstrated that a NO donor (SNP) which activates soluble guanylate cyclase can induce changes in pituicyte morphology which suggests that NO may act on glia to initiate and maintain structural changes within the HNS when hormone levels are elevated. Moreover, Beagley and Cobbett (1997) administered a NOS inhibitor (L-NAME) intraperitoneally in combination with an acute dehydration stimulus and reported that a high number of axonal profiles were enclosed within astrocytes and that there was less contact between the basal lamina and membranes of the axon terminals. Whether NO might play an analogous role within the SON and PVN which also undergoes similar changes has yet to be determined. One

marker of the morphological changes seen during lactation is the modification in patterns of staining for glial fibrillary acidic protein (GFAP) (Salm, Smithson, & Hatton, 1985). If the increase in the capacity to produce NO in these neurons is important for such changes then inhibiting NOS production with L-NAME should modify GFAP expression.

A future issue that remains to be addressed is whether the changes in NADPH-d staining observed in the SON and PVN in pregnancy and lactation are site specific or if they are present in other brain areas. Preliminary analysis revealed that changes in staining are not present in regions such as the VMH and MPOA, however this question is still under investigation.

In conclusion, NADPH-d staining was increased in late pregnancy and lactation when the activity of the oxytocinergic neurons was high. NADPH-d staining was also increased by the hormonal milieu of late pregnancy, and by chronic OT infusion in E-primed animals which suggests that the pattern of circulating ovarian steroids seen in late pregnancy may up-regulate NADPH-d staining through the intranuclear release of OT. By defining the conditions that induce changes in NADPH-d staining these results indicate several functions during parturition and lactation that an increased capacity to produce NO might serve.

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# Appendix A

Source Tables of Analyses of Variance

## Table 1

Source table for the ANOVA of mean number of cells that stained for NADPH-d in the SON across various days of pregnancy and lactation.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Group | 6405.94 | 7 | 915.13 | 10.43 | .000** |
| Error | 3071.14 | 32 | 87.75 | | |

**$p < .01$

## Table 2

Source table for the ANOVA of mean number of cells that stained for NADPH-d in the PVN across various days of pregnancy and lactation.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Group | 20347.35 | 7 | 2906.76 | 5.08 | .001** |
| Error | 20009.67 | 32 | 571.70 | | |

**$p < .01$

## Appendix B

Source Tables of Analyses of Variance

**Table 3**

Source table for the ANOVA of mean weight change from Day 1 to Day 14 of hormone treatment.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Group | 1521.15 | 2 | 760.58 | 4.84 | .037* |
| Error | 1414.68 | 9 | 157.18 | | |

*$p < .05$*

## Table 4

Source table for the ANOVA of mean number of cells that stained for NADPH-d in the SON across hormone condition.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Group | 2740.64 | 3 | 913.55 | 5.31 | .015* |
| Error | 2066.29 | 12 | 172.19 | | |

*$p < .05$

## Table 5

Source table for the ANOVA of mean number of cells that stained for NADPH-d in the PVN across hormone condition.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Group | 7389.19 | 3 | 2463.06 | 4.61 | .023* |
| Error | 6405.33 | 12 | 533.78 | | |

*$p < .05$

## Appendix C

Source Tables of Analyses of Variance

## Table 6

Source table for the ANOVA of mean number of cells that stained for NADPH-d in the SON across hormone and drug treatment.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Implant | 864.51 | 1 | 864.51 | 5.41 | .031* |
| Drug | 504.91 | 1 | 504.91 | 3.16 | .091 |
| Implant xDrug | 749.16 | 1 | 749.16 | 4.69 | .043* |
| Error | 3033.85 | 19 | 159.68 | | |

*$p < .05$

## Table 7

Source table for the ANOVA of mean number of cells that stained for NADPH-d in the PVN across hormone and drug treatment.

| Source | SS | df | MS | F | P |
|---|---|---|---|---|---|
| *Between groups* | | | | | |
| Implant | 5368.68 | 1 | 5368.68 | 10.07 | .005** |
| Drug | 2534.32 | 1 | 2534.32 | 4.75 | .042* |
| Implant xDrug | 6305.63 | 1 | 6305.63 | 11.82 | .003** |
| Error | 10132.99 | 19 | 533.32 | | |

*$p < .05$

**$p < .01$