Nitric Oxide in Pregnancy and Lactation: Hormonal Regulation and Functional Significance

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ABSTRACT

Nitric oxide in pregnancy and lactation: Hormonal regulation and functional significance

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In female rats, the capacity for nitric oxide (NO) production within the hypothalamus is regulated by reproductive state and by the ovarian and peptidergic hormonal pattern that simulates late pregnancy. The first series of experiments described in this thesis examined the interaction of prolactin and oxytocin in upregulating nitric oxide synthase (NOS) activity and oxytocin gene expression within the hypothalamus in steroid-primed rats. Prolactin was found to upregulate NOS and this effect was mediated by oxytocin receptor activation. In addition, increased prolactin was associated with an increase in oxytocin mRNA within both the paraventricular (PVN) and supraoptic (SON) nuclei. The second set of experiments explored the inhibitory role of NO within the hypothalamic neurohypophysial system. The results demonstrated that NO modulated the activity of neurons in discrete nuclei in the hypothalamus and that this modulation was stimulus specific and varied with reproductive state. Central oxytocin administration induced more Fos expression within the SON and PVN in lactating than in nonlactating rat; but the opposite effect was seen following urethane administration. Inhibiting NO production increased Fos induction following central oxytocin administration in lactating rats whereas in nonlactating rats NOS inhibition decreased Fos-lir following both types of stimulation. The third series of experiments investigated a role for NO in milk ejections and the regulation of established maternal behavior. Data from these studies revealed that central inhibition of NO production disrupted pup retrieval and maternal aggression on

both Day 4 and Day 10 of lactation. Together the experiments described in this thesis show that prolactin and oxytocin interact to upregulate NOS within the PVN and SON. Moreover, the increase in capacity to produce NO during lactation has a restraining effect on the activation of oxytocin neurons by oxytocin. Finally, NO has also been shown to play an important role in maternal retrieval and aggression.

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Chapter 4

Contributions of Authors

For the co-authored paper included in Chapter 2, the candidate, Naomi Popeski made a substantial contribution in designing and implementing the experiment; and in analyzing data from the experiments under the supervision of Dr. Woodside and the co-supervision of Dr. Amir. The in situ hybridization procedure was carried out under the supervision of J. Diorio.

For the co-authored papers included in Chapter 3 and 4, the candidate, Naomi Popeski, made a substantial contribution in designing and implementing the experiment; and in analyzing data from the experiments under the supervision of Dr. Woodside. For Chapter 4, the order of authorship comprises of N.Popeski and B. Woodside.

Chapter 1

General Introduction

Pregnancy and lactation are associated with profound alterations in maternal physiology and behavior. Numerous animal studies have shown that these states are also accompanied by morphological and neurochemical changes in the brain. This thesis focuses on one such neurochemical change: an increase in staining for a marker of the synthetic enzyme for nitric oxide (NO), nitric oxide synthase (NOS), that is observed in the supraoptic (SON) and paraventricular (PVN) nuclei of the rat hypothalamus at the end of pregnancy and throughout lactation. Studies described in Chapter 2 investigate the necessary and sufficient conditions for this change and those in Chapter 3 and 4 investigate its possible functional significance. In the current chapter the biochemistry of NO together with its physiological and behavioral roles are reviewed. Then, in order to provide a context in which changes in the nitrergic system occur, the endocrine and behavioral profiles of pregnant and lactating rats are described together with a review of the morphological and neurochemical changes that occur within the central nervous system at these time.

Nitric Oxide

Nitric oxide, is a free radical gas that has been identified in both the peripheral and central nervous systems. As NO is a gas, it is not released from vesicles in nerve terminals by exocytocis like conventional neurotransmitters. Rather, it diffuses from its site of production. In addition, NO's actions are not terminated by presynaptic uptake or enzymatic degradation, instead it reacts quickly with oxygen to produce the inactive anions, nitrite and nitrate and has an estimated half-life of only seconds (Vincent & Kimura, 1992).

Nitric oxide is synthesized by NOS which catalyses the oxidation of the terminal guanido nitrogen of L-arginine to NO and the by-product, citrulline (Schuman & Madison, 1994) (See Figure 1). Three distinct types of NOS have been identified: the constitutive isozymes eNOS (Mw 133kDa) and nNOS (Mw 150 kDa) and a cytokine inducible form (iNOS; Mw 130kDa) (Schuman & Madison, 1994). The gene sequence for NOS shows extensive homology to that for cytochrome P450 reductase, another electron tranferase enzyme that is found within the brain. Cytochrome P450 requires the same cofactors as NOS but it does not have a binding site for calmodulin and shows a localization pattern distinct from that of NOS (Vincent, 1994).

Endothelial NOS and nNos are found in vascular endothelial cells and in neurons; respectively, whereas iNOS is found in macrophages, neutrophils, hepatocytes, and glia cells. Both eNOS and nNOS are calcium/calmodulin dependent and although iNOS contains a recognition site for calmodulin it is not regulated by changes in the concentration of intracellular Ca^{2+} (Bruhwyler, Chleide, Liegeois, & Carreer, 1993)

Nitric oxide synthase subunits are divided into a reductase domain and an oxygenase domain which contains the catalytic center. This catalytic site includes an iron-containing heme group that is reduced by the transfer of an electron from nicotinamide adenine dinucleotide phosphate (NADPH). The iron binds with molecular oxygen which is then cleaved and one oxygen atom is released as water and the other binds with arginine to yield hydroxyarginine. Subsequent oxidation of hydroxyarginine produces NO, citrulline and water (See Figure 1). In the case of eNOS and nNOS, this reaction requires $Ca+^{2}$/calmodulin, molecular oxygen, NADPH, tetrahydrobiopterin (BH4), and flavins (flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN)) as cofactors.The functions of NO may be inhibited by the scavenger, hemoglobin which binds NO by its active heme –iron site (Alderton, Cooper, & Knowles, 2001).

An important stimulus for activating nNOS in the central nervous system is the activation of NMDA receptors which results in an influx of calcium into the intracellular

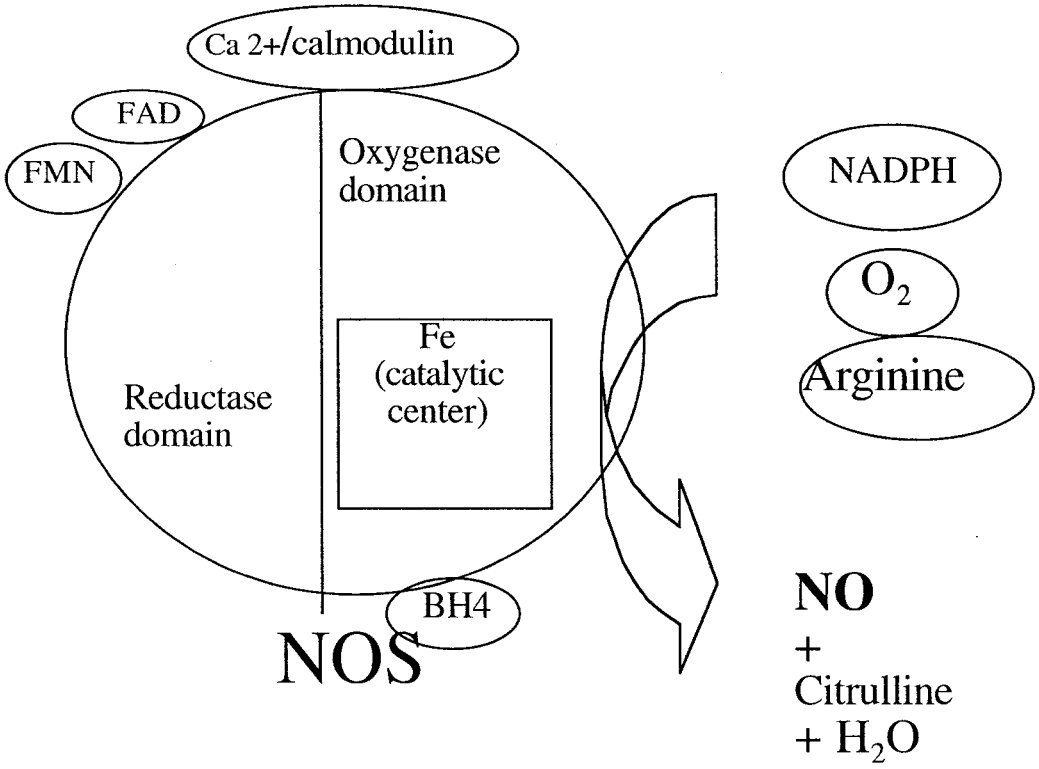

Figure 1. Overall reaction of arginine to NO
(Adapted from:Alderton et al., 2001)

space or releasing calcium from intracellular stores. The influx of calcium results from sustained depolarization of the receptor channels (Bredt, Hwang, & Snyder, 1990). The increase in intracellular calcium activates NOS which results in NO production.

Nitric oxide either diffuses back to the presynaptic terminal and/or astrocytes or remains within the generator cell to stimulate intracellular enzymes, second messenger systems, and ion channels (Bredt & Snyder, 1989; Vincent,1994). Nitric oxide can also bind to iron-sulphur centers of enzymes involved in mitochondrial electron transfer chain and DNA synthesis. The activity of NOS appears to be regulated by the phosphorylation of protein kinase C, calcium/ calmodulin-dependent protein kinase, cAMP-dependent protein kinase and cGMP-dependent protein kinase that occurs primarily on serine residues and have been reported to decrease the catalytic activity of NOS (Kiechle & Malinski, 1993). Interestingly, it has been reported that NOS levels are attenuated following administration of a NO donor, SNP, which suggests that NO at high levels has a negative feedback effect on its own production (Canteros et al., 1996).

A major target of NO within neural tissue is soluble guanylyl cyclase (GC). The NO-induced activation of GC results in a rise in cGMP which activates cGMP-dependent protein kinase which has been implicated in biological functions such as smooth muscle relaxation (Knowles & Moncada, 1992) and inhibition of platelet aggregation and adhesion (Radomski, Plamer, & Moncada, 1990b). Other cGMP modulated processes that may result from actions of NO include regulation of cGMP-dependent serine/threonine protein kinases, cGMP-dependent phosphodiesterases with subsequent cAMP-mediated functions as well as activation of nucleotide gated ion channels (Vincent, 1994). Nitric oxide, concurrent with NADPH, also induces the ADP-ribosylation of proteins. ADP-ribosylation occurs within rat cerebellum following NO production and in platelets ADP-ribosylation is induced by NO donors (Brune &, Lapetina, 1990). Under some circumstances, NO can also interact with superoxide anions to form peroxynitrite anion (ONOO-) which yields toxic hydroxyl free radicals (Bruhwyler et al., 1993) . Peroxynitrite has been implicated in

lipid peroxidation and increased oedema formation in ischemia suggesting a role for NO in inducing neuronal death.

Distribution of NOS

Localization of NOS within the brain has been studied using in situ hybridisation and immunohistochemistry for NOS (Ceccatelli, Grandison, Scott, Pfaff, & Kow, 1996; Bredt & Snyder, 1989); as well as (nicotinamide dinucleotide phosphate diaphorase (NADPH-d) histochemistry (Bredt, et al., 1990; Bredt & Snyder, 1989; Dawson, Bredt, Fotuhi, Hwang, & Snyder, 1991). The NADPH-d histochemical reaction is based on the reduction of the substrate, nitro blue tetrazolium by the reduced cofactor NADPH into an insoluble visible formazan which is bright blue in color (Hope & Vincent, 1989). The latter technique produces Golgi-like staining in discrete populations of neurons throughout the nervous system. Evidence to support the use of NADPH-d as a marker for NOS comes from a number of studies. For example, Dawson et al. (1991) have shown that transfection of human kidney cells with NOS cDNA elicits NADPH-d staining such that the ratio of NOS and NADPH-d staining is the same as that seen in neurons. In addition, Hope et al. (1991) used immunostaining with an antibody specific for NADPH-d to show that neuronal NADPH-d is a NOS.

NOS is found throughout the brain and is perhaps most abundant within the granule cells of the cerebellum and the olfactory bulb. Distributed NO-positive neurons are also found within the cortex (Moro, Badaut, Springhetti, Edvinsson, Seylaz, & Lasbennes, 1995), the striatum and in the CA1 pyramidal neurons of the hippocampus (Endoh, Maiese, & Wagner, 1994). Neurons in the bed nucleus of the stria terminalis (BNST), horizontal limb of the diagonal band of Broca, and the organ vasculosum of the lamina terminalis (OVLT) also stain for NOS as do some of the nuclei within the amygdala (Vincent & Kimura, 1992;

Vincent 1994; Pow, 1992). There is also evidence of NOS staining throughout the hypothalamus including the ventromedial hypothalamus (VMH)(Okamura, Yokosuka, McEwen, & Hayashi, 1994b), medial preoptic nucleus (MPOA)(Okamura, Yokosuka, & Hayashi, 1994a), lateral hypothalamus, nucleus circularis, and, as mentioned previously, in the SON and in the magnocellular and parvocellular subdivisions of the PVN (Vincent & Kimura, 1992).

Functions of NO

NO was first identified as an endothelium derived relaxing factor (EDRF) targeting vascular smooth muscle by increasing levels of cGMP. Palmer et al. (1988), showed that endothelial cells synthesize NO and that the amount of NO was sufficient to account for the biological effects of EDRF. In addition, NOS inhibitors have been shown to constrict the vasculature and increase blood pressure indicating that NO is involved in regulating blood flow.

Nitric oxide has also been implicated in the response to neural insult where it apparently plays both damaging and protective roles. It has been shown, for example, that NO plays a key role in NMDA receptor-mediated neurotoxicity (Choi, 1993). Support for this hypothesis stems from reports that NMDA antagonists or NOS inhibitors prevent NO production and reduce infarct volume following focal ischemia (Garthwaite, 1991). An additional process through which NO may exacerbate neurotoxicity is via NO-mediated DNA damage and consequent activation of a DNA repairing enzyme, poly ADP ribosyltranferase (PARS) Activation of PARS by excessive amounts of NO depletes NAD, the PARS substrate and subsequently adenosine tri-phosphate which is used to resynthesize NAD thus leading to neuronal death from energy deficiency (Zhang, Dawson, Dawson, & Snyder, 1994).

Evidence of a neuroprotective effect of NO comes from studies showing that administration of NO donors or L-arginine reduces damage in several experimental models of stroke. Putative mechanisms include increased vasodilation and hence increase blood supply to ischemic regions (Zang, White, Iadecola, 1994). Other studies have suggested that the neuroprotective effects of NO results from a decrease in activity of NMDA-receptors and hence a decrease in Ca^{2+} influx which results from the reaction of NO with thiol group(s) at the receptor redox modulatory site to decrease Ca^{2+} influx (Lei et al., 1992).

Recent evidence has demonstrated that NO plays an important modulating role in the neuroendocrine system. Nitric oxide has been implicated in NMDA induced gonadatropin releasing hormone (GnRH) secretion from the hypothalamus as well as in surge release of LH (Bonavera, Sahu, Kalra, Kalra, 1993; Pu, Xu, Kalra, Kalra, 1996). Although there is no evidence of colocalization of NOS within GnRH neurons, GnRH neurons are in close proximity to NOS-containing neurons in the OVLT and MPOA (Bhat, Mahesh, Lamar, Ping, Aguan, & Brann, 1995). NOS has also been observed within estrogen receptor containing cells in the VMH (Okamura et al., 1994b) and MPOA (Okamura et al., 1994a) and is upregulated in these areas by estrogen treatment that mimics changes across the estrous cycle. In males, testosterone is apparently necessary to maintain NOS staining within the MPOA (Du & Hull, 1999).

Nitric oxide synthase has also been localized to many different neuroendocrine cells in the PVN and SON, including corticotropin releasing hormone (CRH) (Siaud et al., 1994), vasopressin (Sanchez et al., 1994), angiotensin (Calka & Block, 1993b), somatostatin (Yamada, Emson, & Hokfelt, 1996) and tyrosine hydroxylase (TH) (Blanco, Jirkowski, Juanes, & Vazquez, 1997). Consistent with a functional role for NOS in these systems, activation of these neuroendocrine pathways have been shown to modulate NOS levels within the PVN and SON. For example, activation of the stress axis by treatment with interleukin 1-B (Lee & Rivier, 1998), or restraint stress upregulate NOS levels in these nuclei as does stimulation of the hypothalamic-neurohypophyseal system (HNS) through

chronic salt loading and dehydration (Calza, Giardino. & Ceccatelli, 1993; Kadowaki, Kishimoto, Leng, & Emson, 1994; Ueta, Levy, Hardial, Chowdrey, & Lightan, 1995).

Nitric oxide synthase is also colocalized with oxytocin (OT) within the SON and PVN. There is evidence that NOS is upregulated within OT neurons and within the HNS at times when this system is most active as for example following water deprivation (Pow, 1992) and during lactation (Popeski, Amir, & Woodside,1999), suggesting that NO may regulate activation of the HNS. A study from Summy-Long et al., (1993) found that compared to vehicle treated rats, rats that were treated with a NOS inhibitor and that had been water deprived showed enhanced levels of OT in the plasma; indicating that NO has a negative feedback effect within the HNS.

Several lines of evidence have shown that NOS may exert a permissive or inhibitory effect on corticotropin releasing hormone (CRH) release and neuronal activation within the PVN following stimulation of the stress axis. Amir et al. (1997) found that systemic treatment with NOS inhibitors blocked stressed-induced c-Fos expression in the PVN in rats exposed to immobilization stress. Lee and Rivier (1998) have demonstrated that NOS inhibitors block psychogenic-induced CRH activation within the PVN and ACTH release, suggesting a facilitatory role of NO on HPA activity. In addition, central administration of NO donors have been shown to increase activity within CRH and vaspopressin neurons within the PVN which controls ACTH secretion (Lee, Kim, & Rivier, 1999). In contrast, Turnbull and Rivier (1996) have reported that NO inhibitors regulate activity of the corticotrophs as indicated by enhanced ACTH release in response to systemic injections of proinflammotory cytokines or to vasopressin. Costa and colleagues (1993) have has also demonstrated that a NO donor does not affect basal release of CRH but does inhibit the interleukin-1B and potassium chloride (KCL) -induced CRH release from the PVN. Therefore, evidence that NO exerts a dual influence on activity of the hypothalamic pituitary adrenal axis (HPA) axis may be explained by the type of stimulus (psychogenic or

pharmacological) used to induce the stress response and the parameters used to measure HPA activation.

In addition to being implicated in the function of the neuroendocrine system, NO has also been reported to modulate reproductive behavior in both male and female rats. Injection of NOS inhibitors into the PVN prevents penile erection induced by OT (Argiolas & Melis, 1995) and, when administered into the MPOA, results in a dose-dependent decrease in copulatory behavior (Benelli et al., 1995). It has been suggested that within the MPOA, NO facilitates male sexual behavior by decreasing dopamine reuptake whereas the effect of NOS inhibition on penile erection is apparently mediated by OT. In females, inhibition of NOS prevents progesterone-facilitated lordosis and the NO donor, sodium nitro prusside (SNP) has been shown to facilitate lordosis in rats primed with estrogen but not progesterone (Manni et al., 1994). Other studies using Fos as a neuronal marker of activity have shown that female rats in proestrus that received mounts with intromissions from males displayed more cells that stain for both NADPH-d and Fos in the VMH and magnocellular subdivision of the PVN than rats that received only mounts (Yang & Voogt, 2002).

Nitric oxide has also been implicated in regulating the onset of maternal behavior. Okere et al. (1996b) has demonstrated that both intracerebroventricular and subcutaneous injections of a NO donor, sodium nitroprusside (SNP) to late pregnant rats increased the duration of parturition time and delayed pup delivery. This treatment also disrupted maternal responsiveness immediately postpartum. However, the fact that the administration of OT in combination with the NO donor did not restore postpartum maternal responsiveness suggests that the effect of the NO donor on maternal behavior may have been indirect and associated with difficulties during parturition and with disruption of intrapartum maternal behavior. Other evidence in support of role for NO in the control of maternal behavior comes from studies showing that targeted disruption of the NOS gene (Gammie & Nelson, 1999) eliminates maternal aggression. Similarly,

maternal prairie voles (Gammie, Olaghere-da Silva, & Nelson, 2000) treated with a NOS inhibitor are less aggressive towards male intruders.

Endocrine Profile of Pregnancy and Lactation in Rats

In rats, gestation lasts 22-23 days. Vagino-cervical stimulation experienced during mating provides the afferent input to a neuroendocrine reflex which results in the intiation of bi-daily surges of prolactin (PRL), a pituitary hormone (Butcher, Fugo, and Collins, 1972). Prolactin, initially alone, and, after implantation of the fertilized egg (day 6 post-conception), in synergy with chorionic gonadatropin maintains the corpora lutea and stimulates progesterone release. The bidaily surges of prolactin cease around Day 10 of gestation. At this time, increasing levels of placental lactogen are secreted (Robertson and Friesen, 1981). Two placental lactogens circulate in the dam's serum. Placental lactogen I peaks around Day 10-13 and placental lactogen II is elevated from Day 12 until parturition (Pihoker, Robertson, and Freemark, 1993). These hormones bind to lactogenic receptors including the prolactin receptor and take over the control of progesterone secretion from pituitary prolactin. Thus, at mid pregnancy the endocrine control switches from the mother's pituitary to the placental-fetal unit. At the end of pregnancy prolactin is once again secreted in large amounts from the anterior pituitary and pituitary prolactin levels peak just prior to parturition (Bridges, 1984) (See Figure 2).

As described above prolactin acts in the periphery to stimulate progesterone release (Smith, Freeman, and Neill, 1975), and it also acts at the mammary gland to stimulate glandular development and lactogenesis (Bern & Nicholl, 1968). Prolactin and placental

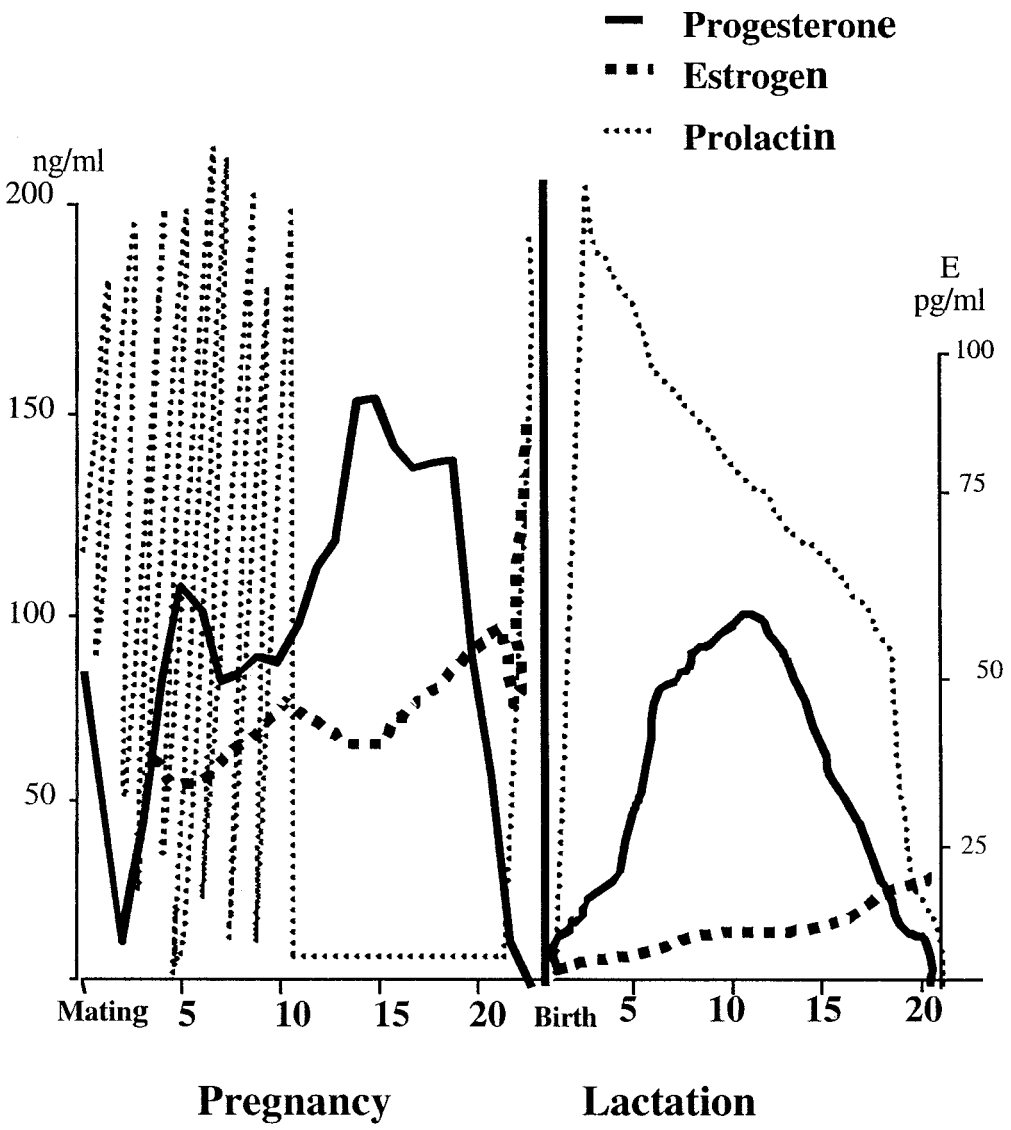

Figure 2. Hormone levels across pregnancy and lactation

lactogens may also act on the brain since they cross the blood-brain barrier via an active transport mechanism (Walsh, Slaby, & Posner, 1987). Moreover, mRNA for the long form of the prolactin receptor is elevated in late pregnancy within the medial preoptic nucleus, dorsal region of the SON and lateral areas of the PVN (Bakowska & Morrell, 1997).

As a consequence of the stimulation by both prolactin and placental lactogens, progesterone levels are high throughout most of pregnancy reaching a peak on Days 19-20 post conception and falling dramatically thereafter (Bridges, 1984)(See Figure 2). This fall in progesterone is necessary in order for parturition to occur. It allows increases in prostaglandin production within the uterus (Jeremy & Dandona 1986), induces gap junction formation between myometrial cells (Garfield, Simms, Kannan, & Daniel, 1978) and enhances the sensitivity of the myometrium to stimulatory agonists during parturition (Gafield, 1984). The fall in progesterone levels may also be facilitatory to the onset of maternal behavior (Bridges, 1984) and to OT activation.

Ovulatory cycles are inhibited during pregnancy, possibly as a function of the negative feedback effects of high levels of progesterone. Estrogen levels increase steadily between Days 10 and 15 post conception, and then rise dramatically to peak at parturition (Bridges, 1984). Thus, as is shown in Figure 2, the onset of parturition is associated with high circulating levels of both estrogen and pituitary prolactin and low levels of progesterone. Interestingly, the prepartum peak in estrogen levels causes a preovulatory surge in gonadotropin releasing hormone and subsequently in luteinizing hormone which stimulates a postpartum ovulation which typically occurs 8 hours after the birth of the litter.

Another peptide hormone oxytocin, (OT), a nonapeptide synthesized within the SON and PVN and released from the posterior pituitary into the circulation plays a critical role in parturition. During parturition, there is an increase in the firing rate of OT neurons (Jiang & Wakerley, 1995) and a dramatic increase in the expression of OT mRNA

(Lefebvre, Giaid, Bennett, Lariviere, & Zingg, 1992), OT receptor mRNA levels (Bale, Pedersen, & Dorsa, 1995), and the number of OT receptors within the uterus (Zingg et al., 1994) and in several areas of the hypothalamus (Yoshimura et al., 1993), as well as an increase in circulating OT levels (Higuchi, Tadokoro, Honda , Negoro, 1986). Oxytocin is also synthesized within the uterus and placenta. Upon the onset of labor, prostaglandins from intrauterine tissues are secreted to trigger uterine contractions which then excite OT cells within both the periphery and the hypothalamus to release OT, resulting in further prostaglandin production and uterine contractions and establishing a positive-feedback loop between the uterus and the hypothalamic OT system (Chan & Chen, 1992).

Recent studies have suggested that OT is also released within discrete nuclei of the brain during parturition, and has central effects which may be important for reproduction. Neumann et al.(1993) demonstrated that administration of an OT receptor antagonist diminished the parturition-related rise in OT release within the SON and reduced the number of pups delivered compared to vehicle-treated controls. Other evidence suggests that OT is released from dendrites and cell bodies within the SON and PVN where it acts on local OT receptors to further amplify its own release in a positive feedback manner (Morris, Christian, Ma, & Wang, 2000; Moos et al., 1984).

Lactation

Parturition is associated with a fall in estrogen and prolactin levels and a shift in the control of both steroid and peptide hormone secretion. Immediately after birth sensory stimulation from the pups, and particularly suckling stimulation drives the females hormonal state. In response to suckling stimulation, prolactin is secreted from the anterior pituitary gland and acts on the epithelial cells of the mammary gland to stimulate milk synthesis (Ollivier-Bousquet, Kann, & Durand, 1993). The amount of prolactin released depends on

the intensity, frequency, and duration of suckling episodes (Grosvenor, Mena, & Schaefgen, 1967; Lee, Haisenleder, Marshall, & Smith, 1989). As is shown in Figure 2, prolactin levels are high in early lactation and decrease as lactation progresses as a function both of changes in the pattern of suckling stimulation and changes in the ability of the hypothalamus to respond to stimulation (Smith & Neill, 1977). Prolatin upregulates its own receptor, thus it is not surprising that both the long and short forms of PRL-R are increased not only in late pregnancy but also in lactation (Pi & Grattan, 1999). In particular PRL-R levels have been shown to increase within the medial preoptic nucleus, ventromedial hypothalamic nucleus, SON and PVN during lactation (Bakowska & Morrell, 1997). The presence of PRL-R in areas other than those directly associated with steroid hormone secretion and milk production suggest possible widespread behavioral and physiological actions of prolactin.

Just as in pregnancy, prolactin stimulates progesterone secretion from the corpora lutea of the ovary during lactation resulting in levels of circulating progesterone that peak around Day 10 postpartum and then gradually decline (Smith & Neill, 1977). Progesterone plays a key role in milk production and also stimulates PRL release.

Lactation is associated with a period of anovulation. GnRH and LH release are suppressed and as a consequence, follicular growth is retarded and estrogen levels remain low until midlactation and then rise slowly (see Figure 2 – only reaching cycling levels in the third week of lactation) (Smith & Neill , 1977). The duration of this anovulatory period depends on the number of young suckled and the energetic state of the female (Woodside, 1991; Woodside & Popeski, 1999).

In addition to PRL, suckling stimulation also triggers OT secretion from the neural lobe of the pituitary by stimulating synchronized high frequency bursting activity of OT neurons within the SON and PVN (Higuchi et al., 1986). Each burst leads to a bolus release of OT into circulation. Oxytocin then acts on myoepithelial cells in the mammary gland to stimulate milk-let down (Lincoln & Paisley, 1982). The biological importance of OT secretion for milk ejection has been demonstrated by elegant studies showing that OT

release is associated with an increase in intramammary pressure resulting in milk ejection (Wakerley & Lincoln, 1973). More recently, it has been shown that OT- deficient female mice fail to transfer milk to their offspring unless exogenous OT is administered (Nishimori , Young, Guo, Wang, Insel, & Matzuk, 1996).

Suckling from the young also stimulates release of OT within the hypothalamus. There is an increase in OT content in microdialysates sampled from the SON and PVN of suckled rats on day 8 of lactation (Neumann, Russell, & Landgraf, 1993). This OT might be released from axon-collateral and from dendrites given that golgi-like immunoperoxidase staining showed the presence of OT in dendrites (Sofroniew & Glasmann, 1981). Further, Neumann et al. (1994) have reported that an OT receptor antagonist infused into the SON suppressed intranuclear and peripheral release of OT during suckling in female rats. In addition, similar treatment reduced the burst amplitude of OT neurons and the frequency of milk-ejections. This line of evidence is consistent with the proposal that during suckling intranuclear OT release may have a positive feedback effects on OT neurons and stimulate further OT release into the neural lobe and facilitate milk-let-down

Oxytocin is found not only in magnocellular neurons of the SON and PVN that send projections to the neurohypophysis, but also in OT parvocellular neurons within the PVN (Swanson & Sawchenko, 1983). The processes of these cells terminate in both hypothalamic and extrahypothalamic sites. Consistent with this OT receptors are localized in the VMH, bed nucleus of the stria terminalis (BNST), MPOA, PVN, and lateral septum (Yoshimura et al., 1993). These findings raise the possibility that OT, like PRL, may play an important role in modulating physiological and behavioral systems other than milk ejection and these possibilities are discussed in later sections of this thesis.

Physiological and Behavioral Changes in Pregnancy and Lactation

Successful reproduction requires that the mother provides her young with the nutrients essential for their growth and development as well as with a supportive intrauterine and postnatal environment. To accomplish this entails changes in both physiological and behavioral systems. Some systems, notably the HPA axis are also changed in late pregnancy and lactation although the functional significance of such a change for the well-being of mother and offspring is perhaps less evident. In this section major features of the changes in energy balance, stress axis and readiness to engage in maternal behavior that occur in pregnancy and lactation are reviewed.

Energy Balance

The nutritional demands of the young change dramatically as they develop. In the first half of pregnancy in rats there is little energetic investment in the fetal placental unit. The modest increase in food intake observed during this time, results primarily from the absence of the anorectic effects of estrogen and the presence of high levels of progesterone and leads to increases in maternal fat stores (Shirling, Ashby, & Baird, 1981). In the second half of pregnancy female rats increase their food consumption to 60% above nonpregnant values and much more of their energy is invested in growth of the young (Cripps & Williams, 1975). In addition, to increasing how much they eat, pregnant rats also show changes in what they choose to eat. When given a choice between diets rich in different macronutrients, pregnant rats tend to avoid carbohydrate-rich diets in the latter half of pregnancy. At the same time they show an increased appetite for salt and calcium to meet

their increased mineral and nutrient requirements (Pike & Yao, 1971; Leshem , Levin, & Schulkin, 2002).

Coincident with these changes in behavior, there are physiological changes that also serve to facilitate nutrient use. For example, there is a doubling of the absorptive area of the gut (Larralde, Fernadez-Otero, & Gonzalez, 1966) as well as an increase in enzymes that facilitate calcium absorption (Pitkin, 1985). In addition, although adipose tissue stores increase throughout pregnancy with a concomitant rise in circulating leptin levels, the ability of leptin to decrease food intake is reduced because leptin binding protein also increases (Seeber, Smith, & Waddell, 2002).

Lactation is much more energetically expensive than pregnancy. To meet this energetic demand female rats increase their food intake, mobilize their energy stores and decrease energy expenditure in brown fat thermogenesis (Trayhurn & Richard, 1985). In spite of increasing food intake to three times the levels seen prior to pregnancy, lactating rats are in negative energy balance as reflected in their loss of adipose tissue and an associated reduction in circulating leptin levels (Woodside, Abizaid & Walker, 2000). The reduction in circulating leptin may contribute to the hyperphagia of lactation by removing the anorectic effects of leptin itself and/or contributing to the increased expression of orexigenic peptides such as neuropeptide Y and agouti-related peptide (AGRP) that is seen in lactating rats (Malabu, Kilpatrick, Ware, Vernon, & Williams, 1994; Chen, Li, Haskell-Luevano, Cone, &Smith, 1999).

Maternal Behavior

In rats, maternal behavior includes nestbuilding, retrieval of pups to the nest, anogenital licking and grooming to stimulate urination and defecation, and nursing. In addition to these pup-directed behaviors, maternal rats also show aggression towards intruders that are introduced into their nest site.

Primiparous rats in the presence of young begin to show these robust behaviors following birth. In contrast, the initial response of virgin rats to young is a lack of maternal behavior, instead they avoid, bury, or attack them (Fleming & Rosenblatt, 1974). After repeated daily exposure to pups, however, virgin females will come to display several features of maternal behavior including nest building, retrieving, licking and crouching over pups (Mayer, Freeman, & Rosenblatt, 1979). This process called "sensitization" occurs after approximately 5-7 days. These responses are not dependent on hormonal changes resulting from pup association because sensitization can occur in ovariectomized and hypophysectomized females (Rosenblatt, 1967).

In addition to repeated exposure to pups, the inhibitory processes that suppress maternal behavior in virgin rats can be overcome by events that are associated with parturition. Interestingly, however, parturition is not a necessary condition for the onset of maternal behavior. For example, primiparous females whose young are delivered by caesarian section still show normal onset of maternal behavior (Moltz, Robbins, & Parks, 1966). In addition there is evidence that maternal responsivity begins prior to parturition. Mayer and Rosenblatt (1984) reported that before Day 22 of gestation, levels of maternal responsiveness were equal or lower than those of non pregnant females. In contrast on Day 22 of pregnancy (3.5 hours before parturition) rats retrieved and tended to foster pups.

A wealth of evidence suggests that it is the hormonal events surrounding parturition, and in particular increases in estrogen and prolactin levels against a background of falling progesterone levels that stimulate the onset of maternal behavior (Rosenblatt & Siegel, 1975; Siegel & Rosenblatt, 1975a; Siegel & Rosenblatt, 1975b; Siegel & Rosenblatt, 1975c; Bridges & Ronsheim, 1990). Moltz et al. (1970) showed that subcutaneous administration of estrogen, progesterone and prolactin to ovariectomized virgin females in a pattern that mimicked the hormonal profile of pregnancy decreased their latency to show full maternal behavior when compared to untreated ovariectomized virgins. Bridges (1984) found similar effects after producing chronic increases in estrogen and progesterone levels

in ovariectomized female rats and then removing the source of progesterone. More recently, it has been shown that steroid and peptide hormones act centrally to stimulate the onset of maternal behavior. The administration of either prolactin or estrogen into the MPOA is sufficient to stimulate the onset of maternal behavior (Bridges, DiBiase, Loundes, & Doherty, 1985; Numan, Rosenblatt, & Komisaruk, 1977). Lesions of the medial amygdala (Fleming, Vaccarino, & Luebk , 1980) or VMH also stimulate maternal behavior (Hansen, 1989). These data are consistent with lesion studies showing that the integrity of the MPOA, is essential for both the onset and maintenance of maternal behavior (Numan et al., 1977). Other brain areas have also been implicated in the control of maternal responsivity including the medial and basolateral amygdala, the ventral BNST, the nucleus accumbens shell and the ventral tegmental area (Numan, 1988;Lee, Clancy, & Fleming, 2000; Stack, Balakrishnan, Numan, & Numan, 2002).

Although the hormone profile of late pregnancy is critical for the rapid onset of maternal behavior once this behavior has been established, it becomes independent of hormonal state. Adrenalectomy, ovariectomy and hypophysectomy have all been shown to be without effect on the maintenance of maternal responsivity (Thoman & Levine, 1970; Moltz & Weiner, 1966; Obias, 1957). Other neurochemicals, however, do influence ongoing maternal behavior. Rubin & Bridges (1984) demonstrated that both pup retrieval and crouching over pups were disrupted following morphine infusion into the MPOA. Consistent with these behavioral effects, Stafisso-Sandoz (1998) reported that morphine treatment reduced Fos expression within the MPOA in maternal rats exposed to pups but the effect of morphine administration was blocked by simultaneous administration of naloxone, an opiate antagonist.

Once established, maternal behavior is also influenced by manipulating the dopamine system. It has been shown that lactating rats treated peripherally with haloperidol or with cis-flupenthixol (FLU), into the nucleus accumben; show a disruption of pup retrieval, and nest-building behavior as well as reduced motor activity (Keer & Stern, 1999).

Stress axis

There is a modest increase in the basal activity of the hypothalamic pituitary adrenal axis in the latter part of pregnancy that is associated with an increase in hippocampal GR receptor density (Johnstone et al., 2000). The lack of pregnancy-associated changes in basal HPA activity are in contrast to the pronounced effects of reproductive state on the stress reactivity of the HPA. Neumann and colleagues (1998) found that exposure to the elevated plus maze and forced swim test, had similar effects on both adrenocorticotropin hormone (ACTH) and corticosterone plasma levels in rats on Day 10 post-conception and in virgin rats. In contrast, females in the last trimester of pregnancy (Day 15-21 post-conception) showed both attenuated hormone responses to stress than virgin rats and reduced CRH receptor density in the anterior pituitary compared to virgin rats.

Basal ACTH and corticostosterone levels are higher and CRF mRNA expression within the PVN is reduced in lactating rats compared to levels seen in pregnant or virgin rats (Walker, Lightman , Steele, & Dallman, 1992). Some researchers have, in fact, argued that the suckling stimulus represents a chronic stressor to lactating females (Walker et al., 1992). In addition, a large number of studies have documented hyporesponsiveness to stress in lactating rats as reflected in a decrease in stress-induced CRH mRNA (Lightman & Young, 1989), ACTH secretion, corticosterone (Stern, Goldman, & Levine, 1973), and, OT (Lightman & Young, 1989), and prolactin levels (Windle et al., 1997) during lactation suggesting alterations in the inputs to stress-responsive hypothalamic neurons. A reduction of neuronal activation as expressed by c- fos mRNA within the PVN has also been demonstrated in lactation in response to various stressors (daCost, Ingram, Wood, & Lightman, 1996). A similar reduction in behavioral responses to stress such as a reduction

in noise-induced freezing (Hard & Hansen, 1985), lower acoustic startle responses (Toufexis et al., 1998), and reduced emotionality when exposed to an open field (Fleming & Luebuke, 1981) or elevated plus maze (Neumann, Torner, & Wigger, 2000) have been demonstrated. In addition, the ability of exposure to a number of stressors and central CRH administration to induce immediate early gene expression in the PVN is reduced in lactating rats (Woodside & Amir, 1996; Da Costa et al., 1997). The hyporesponsiveness to stress of lactating rats is dependent on suckling stimulation from the pups because 48h of separation from the pups results in responses that are similar to those seen in virgin rats (Lightman, 1992).

Hyporesponsivity to stress has also been reported in breast-feeding women. For example. Altemus et al. (1995) showed that breast-feeding women exhibit lower ACTH, cortisol, and epinephrine responses to treadmill stress compared to mothers who bottle fed their babies. In contrast, a study by Heinrichs and colleagues (2001) found that breast-feeding women who were exposed to a psychosocial stressor did not show lower ACTH, norepinephrine, and epinephrine responses than bottle feeding women but did display a short-term reduction in total cortisol response.

Morphological Changes in Pregnancy and Lactation

As mentioned above, OT magnocellular neurons undergo profound changes in their electrical and secretory activities during parturition and lactation (Higuchi et al., 1986). In addition, the oxytocinergic system undergoes extensive synaptic and structural remodeling at these times (Theodosis & Poulain, 1992).

Under basal conditions, OT neurons are separated from each other by neuropil and glia elements and few surface membranes of cells bodies and dendrites are juxtaposed. Throughout most of pregnancy, neurosecretory cells within the SON remain surrounded by astrocytic processes. However on Day 21 of gestation, just hours before the onset of labor, there is a retraction of astrocytic processes around magnocellular OT neurons (Montaganese, Poulain, Vincent, & Theodosis, 1987). Studies by Montagenese at al., (1987) have reported that this change results in a higher incidence of OT neurosecretory somatic profiles directly juxtaposed to somata and/or dendrites of OT secreting neurons. At the same time, there is an increase in "shared synapses", a situation where axonal terminals synapse onto two or more post synaptic neurons. These multiple synaptic contacts are identified on both somata and dendrites but the majority of multiple synapses contact soma-dendritic membranes.

In addition, to the remodelling of synaptic inputs to OT neurons, morphological changes also occur around terminal regions of these neurons. Axons of the OT neurons project to the neurohypophysis where OT is released into the general circulation. As with the soma and dendrites under basal conditions, neurosecretory axons and terminals are covered by astrocytic processes:pituicytes. Under stimulation such as during parturition, the number of neuronal processes surrounded by pituicytes diminishes (Tweedle & Hatton, 1982). This reduction in glia coverage from the perivascular space facilitates the release of OT into the circulation and, presumably the stimulatory role that OT has on uterine contractions.

The neuronal-glial and synaptic plasticity that is observed just prior to parturition is maintained throughout lactation (Montagenese et al., 1987). In addition, increased apposition between dendrites within the SON is observed. This apposition allow gap junctions to form as reflected by increased observations of dye coupling between OT neurons from lactating rats (Hatton, Yang,& Cobbett, 1987). As might be expected the increased frequency of gap junctions is associated with up regulation of connexin-32, a gap junction protein (Micevych, Popper,& Hatton, 1996). It has been proposed that the increase in gap junctions between OT neurons might facilitate the synchronized bursting activity of OT neurons that results in bolus release of OT from the posterior pituitary and stimulation of milk ejection. It may be that the morphological changes that are seen in OT neurons around the time of parturition are, in fact, driven by OT itself. When chronically infused into the third ventricle in a steroid-primed animal, OT can induce morphological changes similar to that observed in physiologically stimulated animals (Montagnese, Poulain, & Theodosis, 1990).

Changes in neuronal juxtapositions and shared synapses are reversed when suckling ceases. If dams are deprived of their pups immediately after birth, the neuronal appositions that developed at the end of pregnancy disappear within 2 days and 'double' synapses disappear in 10 days. On the other hand, the SON can remain structurally modified beyond the time of normal weaning as long as suckling stimulation is prolonged by presenting the rat with new young litters (Montagenese et al., 1987).

The presence, in the SON of adult animals of a number of cell adhesion molecules typically seen only during development may contribute to the continued capacity for remodelling seen in this area of the brain. The adult SON contains the embryonic form of the neural cell adhesion molecule (NCAM), polysialic acid –NCAM, PSA-NCAM (Theodosis, Bonfanti, Olive, Rougon,& Poulain, 1994). The presence of polysialic acid (PSA) on the extracellular domain of NCAM reduces cell adhesion and is considered an important modulator of cell surface interactions. Another cell adhesion molecule,

glycoprotein F3/contactin (F3) is also found within adult OT neurons and in the neurohypophysis (Olive, Rougon, Pierre, & Theodosis, 1995). During development F3 participates in neuronal glial interactions, axonal growth and synaptogenesis (Falk, Bonnon, Girault, & Faivre-Sarrailh, 2002) and can interact with tenascin-C, an extracellular matrix glycoprotein found in astrocytes within the SON (Theodosis, 2002). Interestingly, levels of both PSA-NCAM and F3 are lower in lactating animals (Nothias, Vernier, von Boxberg, Mirman,& Vincent, 1997) than in virgin rats consistent with the notion that new synapse formation induced by stimulation and the consequent withdrawal of glial processes is then reinforced by the suppression of embryonic forms of cell adhesion molecules. PSA-NCAM levels increase once more after weaning.

By far the most well-documented morphological changes in the brain of pregnant and lactating rats are those observed in the PVN and SON, however these nuclei, are not the only sites of morphological changes. A substantial reorganization of the somatosensory cortex takes place during lactation so that a much larger area becomes devoted to the ventral surface surrounding the nipples (Xerri, Stern,& Merzenich, 1994). Increases in expression of the astrocytic protein, glial fibrillary acidic protein (GFAP), and the trophic factor bFGF have been observed within the MPOA (Featherstone, Fleming, & Ivy, 2000) and anterior cingulate cortex of lactating rats (Salmaso, Popeski, & Woodside, unpublished). These findings suggest that remodelling of the maternal brain may extend to areas outside those usually associated with the control of milk let down or even maternal behavior.

Neurochemical Changes in Pregnancy and Lactation

As might be expected, the behavioral and physiological changes that accompany pregnancy and lactation are associated with changes in neurochemical pathways. For example, the anovulation characteristic of lactation is associated with a reduction in GnRH release (Smith & Neill, 1977) and the increase in ingestive behavior with upregulation of the orexigenic peptides NPY (Smith, 1993) and AGRP (Chen et al.,1999) and the down regulation of POMC (Smith, 1993). A correlate of the reduced stress responsivity of lactating females is a reduction in CRF mRNA expression within the PVN (Walker et al., 1992).

Not surprisingly, given the central roles that prolactin and OT play during pregnancy and lactation there are also changes in the neurochemical pathways controlling the release of these peptides. Typically, prolactin regulates its own secretion through a short-loop feedback mechanism in which high prolactin levels induce an increase in dopamine production within the tuberoinfundibular dopamine (TIDA) neurons and an increase in DA release into the hypothalamic hypophysial portal blood system (Reymond & Porter, 1985). Dopamine then binds to its receptors on the lactotrophs to inhibit further prolactin release by decreasing two voltage-activated calcium currents (Goldsmith, Cronin, & Weiner, 1979).

During most of pregnancy, dopamine concentrations are high in both the median eminence and the hypophysial portal blood thus restraining PRL release. At the end of pregnancy and in lactation there is an increase in circulating prolactin levels associated with a reduced sensitivity to its negative feedback effect on TIDA neurons (Grattan & Averill, 1995; Arbogast & Voogt, 1996). Dopamine concentrations in the portal blood are attenuated in lactating rats and suckling suppresses TH activity, and TH gene expression in the TIDA neurons (Wang, Hoffman, & Smith, 1993). Enkephalin has prolactin-releasing activity and is colocalized with dopamine within the arcuate nucleus (Merchenthaler, 1994).

Coincident with a decrease in TH activity in TIDA neurons there is, apparently an increase in enkephalin activity. Moreover, administration of antisera to enkephalin has been shown both to terminate the suckling-induced prolactin increase and prevent the inhibition of dopamine accumulation in the median eminence (Callahan, Klosterman, Prunty, Tompkins, & Janik, 2000). Thus, enkephalin could play a dual role in prolactin release during lactation; reducing the sensitivity of the short-loop feedback regulation and sustaining elevated levels of prolactin in lactating animals.

For the first 19-20 days of pregnancy there is an increase in GABAergic inhibition on OT magnocellular neurons which presumably contributes to the quiescence of the uterus at this time. This is associated with a higher frequency of spontaneous inhhibitory postsynaptic currents (sIPSCs) recorded in vitro from the SON of rats on Day 20 postconception than in virgin rats (Brussaard, Devay, Leyting-Vermeulen & Kits, 1999). Progesterone through its major metabolite, allopregnanolone, apparently makes a dual contribution to the increased inhibitory tone on OT neurons. Allopregnanolone stimulates an increase in alpha-1 subunits within the $GABA_A$ receptor. This change in subunit composition results in greater efficacy of the receptor as reflected by an increase in Cl- currents. At the same time allopregnanolone acts as an agonist at binding sites on the $GABA_A$ receptor complex (Brussaard et al., 1997). On the day of parturition, there is a down regulation in alpha-1 subunit expression possibly as a result of the fall in progesterone levels at the end of pregnancy (Fenelon & Herbison, 1996).

In addition to GABAergic inhibition, endogenous opioid peptides also tonically inhibit the oxytocinergic system during pregnancy. Both enkephalin and dynorphin are synthesized within OT magnocellular neurons in the SON and PVN (Meister, Villar, Ceccatelli, & Hokfelt, 1990). Kappa and mu-opioid receptors have been identified on the cell bodies of these neurons whereas the nerve terminals contain only kappa-opioid receptors (Sumner, Douglas, & Russell, 1992; Herkenham, Rice, Jacobson, & Rothman, 1986). In support of an inhibitory role for endogenous opioids on OT secretion during

pregnancy, administration of an opioid antagonist such as naloxone increases OT secretion in pregnant rats (Douglas et al., 1995). Inhibition of the OT system enhances the accumulation of OT within the neural lobe that can be released during parturition when it is in high demand. The ability of naloxone treatment to stimulate OT release disappears just prior to parturition concomitant with a decrease in sensitivity of the nerve terminals to opioid agonists and a decline in dynorphin content (Douglas, Dye, Leng, Russell, & Bicknell, 1993).

Although, some inhibitory inputs to OT neurons are reduced at parturition, GABA inputs remain important for the timing of OT release during both parturition and lactation. In order to be most efficacious both in stimulating uterine contractions and milk ejections it is critical that OT is released in bursts. Interestingly, OT neurons unlike vasopressin neurons do not show an inherent bursting activity, and the high frequency synchronous bursting observed during suckling, for example, appears to be generated by coordination of inhibitory (GABA) and excitatory (glutamatergic) inputs (Moos, 1995; Moos, Rossi & Richard, 1997). Under basal conditions GABAergic synapses account for approximately 45% of all inputs on OT and vasopressin neurons in the SON (Theodosis, Paut, & Tappaz, 1986). The synaptic remodelling that occurs at parturition and during lactation apparently increases the number of GABAergic synapses on OT neurons. Gies and Theodosis (1994) found a greater frequency of GABAergic terminals making synapses onto somata and dendrites of OT neurons of lactating rats than virgin rats. Interestingly, this effect was not seen on vasopressin neurons.

The increase in GABAergic synapses in the SON during lactation is associated with changes in $GABA_A$ receptors on the postsynaptic membrane reflected in changes in receptor subunits similar to those seen in pregnancy which results in an increase in the inhibitory effect of GABA on OT neurons. Brussaard and colleagues (1999) have shown that the increase in the sIPSCs in the SON that is observed in late pregnancy continues into mid lactation and is associated with an increase in sIPSC decay time. Voisin et al. (1995)

have demonstrated that central administration of a $GABA_A$ receptor agonist, muscimol both inhibits background firing of OT cells, and delays the occurrence of high frequency bursts in OT neurons in urethane-anaesthetized lactating rats.

The activity of OT neurons within the SON during parturition and lactation is also under the influence of excitatory amino acids. As with GABAergic neurons, glutamatergic cell bodies are not found within the SON and PVN. Rather glutamatergic terminals within these nuclei arise from neurons in a number of hypothalamic regions including the organum vasculosum lateral terminalis (OVLT), olfactory nuclei and dorsal hypothalamus (Meeker, Swanson, Greenwood, & Hayward, 1993;Yang, Senatorov, & Renaud, 1994). Anatomical and molecular studies have shown that glutamatergic synapses account for approximately 25% of all inputs on the SON and that there is an increase in glutamatergic synapses during lactation (El Majdoubi, Poulain, & Theodosis, 1997). In addition, NMDA, AMPA, and metabotropic-type glutamate receptors have all been detected within this nuclei and during lactation the frequency of AMPA-mediated miniature excitatory post synaptic currents is doubled in OT but not in vasopressin neurons (Stern, Hestrin, & Armstrong, 2000). Evidence for the functional importance of glutamate for the release of OT during parturition comes from a study by Herbison et al. (1997). These researchers demonstrated a significant increase in glutamate concentrations in dialysates from the SON immediately before the birth of the first pup. A series of studies carried out by Parker and Crowley (1993), suggest that, at least during lactation, NMDA and AMPA/kainate receptor activation act synergistically to stimulate OT release.

Noradrenaline has also been implicated in the stimulation of OT release (Crowley, Shyr, Kacsoh, & Grosvenor, 1987). In cycling rats, noradrenergic inputs innervate cell bodies of both vasopressin and OT magnocellular neurons to a similar extent. However, Michaloudi et al. (1997) demonstrated that the number of noradrenergic varicosities adjacent to only OT cells increases during lactation.Whether this increase represents an

effect of glial retraction around OT neurons or an increase in the number of vasopressin cells that begin to express OT during lactation is not yet clear.

Whatever mechanism results in an increase in noradrenergic inputs on OT neurons, there is ample evidence to suggest that NE plays a role in suckling-induced OT release. For example, Crowley et al. (1987) showed that suckling stimulation increased noradrenaline turnover rate in the rostral PVN and SON of lactating rats. In a later experiment, Bealer and Crowley (1998) found that NE concentrations in dialysates from the PVN increased during suckling concomitant with OT release. Furthermore, administration of alpha or beta-adrenergic receptor antagonists into the SON and PVN has been shown to interfere with suckling-induced central OT release. Other results suggest that NPY acting at the Y1 receptor acts in synergy with NE to stimulate OT release (Kapoor & Sladek, 2001).

As the review presented above demonstrates, pregnancy and lactation are associated with a pattern of coordinated changes in maternal physiology, neural morphology, neurochemistry, and behavior. Recently, evidence has accrued to suggest that at the end of pregnancy and during lactation there are also increases in NOS, and hence an increased capacity for NO production, within peripheral tissue and within the SON and PVN of the hypothalamus.

Weiner and colleagues (1994), have reported that in late pregnant guinea pigs, NO synthase activiy is increased in the heart, kidney, skeletal muscle and in the uterine artery. In addition, Woodside and Amir (1996) found an increase in NADPH-d staining in the SON and PVN of late pregnant rats (Day 22) relative to ovariectomized and virgin female rats. We have also previously shown that compared to virgins and females in early and mid pregnancy the capacity for NO production as measured by NADPH-d increased within the SON and PVN in late pregnant rats (Day 22). Consistent with these effects, both an upregulation of NOS protein and NOS mRNA expression on Day 20 of pregnancy (Xu et al., 1996) within the hypothalamus has also been reported. In contrast, other studies have found that NO production is down-regulated in the SON on either Day 19 (Okere &

Higuchi, 1996b) or Day 22 (Srisawat et al., 2000) of pregnancy assessed by NADPH-d or in situ hybridisation for NOS; respectively. The discrepancies between the findings may be explained in a number of ways. First, it is possible that the amount of NOS mRNA within the SON and PVN in late pregnancy does not correlate with protein content at this time. Second, NOS expression within these brain regions may increase only when OT neurons are activated immediately prior to and during parturition itself. Because the length of pregnancy in rats varies from 22-23 days, simply equating the days of pregnancy may not mean that rats are similarly close to parturition. NOS mRNA (Ceccatelli & Eriksson, 1993) and NADPH-d staining is increased within the SON and PVN during lactation (Popeski et al., 1999) an effect that is dependent on the presence of the suckling stimulus (Otukonyong et al., 2000).

Current Studies

The overall goal of the studies described in this thesis is to understand the relationship between the increase in NOS observed at the end of pregnancy and lactation and the numerous other changes that accompany these states. Thus the extent to which the observed increase in NOS depends on a particular hormonal milieu as well as the functional implications of this change for the stress axis, the milk-ejection reflex, and maternal behavior were investigated.

In the rat, many of the physiological and behavioral changes observed around the time of parturition have been shown to depend not on the birth process itself but on the

hormonal profile of late pregnancy. The dramatic increase in circulating estrogen levels which occurs against a background of falling levels of circulating progesterone in late pregnant females can be simulated in ovariectomized rats by long-term estrogen and progesterone replacement, and subsequent progesterone withdrawal (Bridges, 1984). This treatment induces the onset of maternal behavior (Bridges, 1984); and when combined with OT administration stimulates synaptic remodelling in the SON (Theodosis, et al., 1986a). Such treatment also stimulates prolactin release from the anterior pituitary (Bridges & Ronsheim, 1990), enhances OT mRNA expression within the SON and PVN (Crowley, Insel, O'Keefe, Kim, & Amico, 1995) and increases staining for NADPH-d within the SON and PVN of an ovariectomized female (Popeski et al., 1999). The experiments described in Chapter 2 investigated the possibility that PRL and OT might interact to influence NOS levels in the PVN and SON.

The functional significance of the upregulation of NO production in lactation is the focus of the experiments described in Chapters 3 and 4. In Chapter 3, we investigated the ability of NOS inhibition to modulate neuronal activation within the PVN and SON following exposure to either OT or osmotic stimulation and how such modulation might vary between cycling and lactating rats.

In the experiments described in Chapter 4, the possibility that the increase in NOS seen in magnocellular cells in lactation might contribute to the OT release necessary for milk ejection to occur was examined. Central administration of a NO donor or L-arginine has been shown to inhibit burst activity of OT neurons and reduced the number of milk-ejections (Okere et al., 1996c). Because milk let-down depends on the bolus release of OT, which itself results from synchronous activity of OT neurons, we thought it probable that modulating NO either by increasing its activity through administering a NO donor as

described above or by inhibiting the enzyme through which it is produced, interferes with the synchronous activity of these neurons and thus influences milk-let down.

Further, because milk ejections occur as part of a complex set of behavioral interactions between mothers and their pups and previous research has suggested a role for NO in the onset of maternal behavior and in maternal aggression, the effect of NOS inhibition on this interaction was also examined.

Chapter 2

The supraoptic nuclei (SON) and the magnocellular compartments of the paraventricular nuclei (PVN) of the hypothalamus of late pregnant rats undergo both morphological and neurochemical changes. Among these changes are synaptic remodeling (Perlmutter, Tweedle,& Hatton, 1984) a decrease in glial coverage of cell bodies (Hatton & Tweedle, 1982), and an increase in oxytocin (OT) mRNA (Zingg & Lefebvre, 1988). In addition, we have recently shown that staining for NADPH-d, a histochemical marker for nitric oxide synthase (NOS), is increased in the PVN and SON of late pregnant and lactating rats (Popeski et al., 1999; Woodside & Amir, 1996). Pregnancy per se is not necessary to produce many of these changes, rather they depend on the gonadal steroid state that accompanies late pregnancy. For example, female rats given a hormonal regimen of estrogen combined with progesterone show both an increase in OT mRNA (Crowley et al., 1995) and NADPH-d staining in the PVN and SON following progesterone withdrawal (Popeski et al., 1999). Progesterone withdrawal is also necessary to allow activation of OT neurons at parturition (Antonijevic, Russell, Bicknell, Leng, & Douglas, 2000). The positive correlation observed between the increases in NADPH-d staining and OT mRNA seen close to parturition and following gonadal steroid administration is consistent with results showing an increase in NOS in magnocellular neurons under other conditions of hypothalamic neurohypophysial system stimulation such as salt loading (Villar, Ceccatelli, Ronnqvist, & Hokfelt, 1994).

Nitric oxide synthase colocalizes with OT in the PVN and SON (Yamada, Emson, & Hokfelt, 1996) and a functional relationship between NOS and OT has also been demonstrated. For example, inhibition of NOS results in increased OT release but only under conditions of OT stimulation (Summy-Long et al., 1993). Further, administering the NOS inhibitor, L-NAME, results in increased Fos expression following central OT administration in lactating rats but not in nonlactating rats (Popeski & Woodside, 2001).

Together these data suggest that nitric oxide (NO) may have a restraining effect on the oxytocinergic system but only when the system is chronically stimulated; a state which coincides with NOS upregulation within OT neurons.

Oxytocin receptors are localized on OT neurons in the PVN and SON (Freund-Mercier, Stoeckel, & Klein, 1994) and in estrogen–primed rats, chronic intracerebroventricular OT infusion is sufficient to increase NADPH-d staining in the PVN and SON (Popeski et al., 1999). Intranuclear OT release may therefore play a key role in the regulation of NOS in these nuclei supporting the notion that NO serves a negative feedback function within these cells (Summy-Long et al., 1993). The upregulation of NOS seen in the PVN and SON in late pregnancy may, therefore result from an increase in OT release within these nuclei. Thus, the gonadal steroid profile of late pregnancy may stimulate an increase in OT mRNA resulting in increased intranuclear OT release. In turn, OT within the SON and PVN would then activate OT receptors on OT neurons within these nuclei resulting in an upregulation of NOS. If this were indeed the case, then treating ovariectomized females with a hormonal regimen mimicking late pregnancy and an OT receptor antagonist should eliminate the effect of steroid priming on NADPH-d staining. This hypothesis was tested in Experiment 1.

Administering gonadal steroids to mimic the hormonal profile of late pregnancy results not only in increases in OT mRNA within the SON and PVN but also stimulates prolactin (PRL) release from the pituitary (Bridges & Ronsheim, 1990). Prolactin can enter the brain via an active transport mechanism (Walsh et al., 1987) and PRL receptors have been localized in many areas of the hypothalamus including the PVN and SON (Bakowska & Morrell, 1997). Moreover, a stimulatory effect of PRL on the HNS has been shown in studies documenting an increase in OT release following systemic PRL administration (Parker, Armstrong, Sladek, Grosvenor, & Crowley, 1991) and increases in OT mRNA in hypothalamic explants treated with PRL (Ghosh & Sladek, 1995a; Ghosh & Sladek, 1995b). In Experiments 2 and 3, therefore, we investigated the role of PRL both in the

increase in NADPH-d staining and in the upregulation of OT mRNA seen in the SON and PVN following steroid-priming.

Materials and Methods

Animals

Female Wistar rats from Charles River Breeding Laboratory (St. Constant, Quebec) were used in these studies. 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 ad libitum access to lab chow and to water throughout the experiment. All protocols were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Surgery

Bilateral ovariectomies: Ovariectomies were performed through bilateral dorsal incisions under ketamine/xylazine anesthesia (5.7 mg ketamine and 0.86 mg xylazine/100 g of body weight) and the topical antibacterial powder (Cicatrin^{tm}) was placed on the wound which was closed using wound clips.

Intracerebroventricular (icv) cannulae implantation: Stainless steel cannulae (22 gauge; Plastic One Products) were inserted stereotaxically into the third ventricle (AP: -0.90 mm, L: 0, DV: - 6.4 mm below dura (Paxinos & Watson, 1986) under ketamine/xylazine anaesthesia. L-shaped cannulae were connected, via a polyethylene catheter to osmotic minipumps (Alzet, model 1003D) which were then implanted subcutaneously between the scapulae. Minipumps were primed for six hours at 37 ^{0}C before implantation.

Drug and Hormone Administration

Steroid –priming: The hormone replacement schedule used was shown by Bridges (1984) to produce hormone levels similar to those of pregnancy. All animals were ovariectomized (ovx) one week prior to receiving silastic implants (Dow Corning, 0.078" ID x 0.125" OD) containing either cholesterol or ovarian steroids (17 B estradiol (2mm) or crystalline progesterone 30mm). On Day 1 of hormone treatment, animals received 1 x 2 mm implants of either cholesterol or estrogen. Two days later (Day 3), animals received 3 x 30 mm implants of either cholesterol or progesterone (P). On Day 14, cholesterol or P implants were removed and all animals were sacrificed on Day 17. Animals were sacrificed 72 hours following progesterone withdrawal to mimic the decline in progesterone levels that is observed on Day 20 of gestation (Grattan & Averill, 1991).

OTA treatment: The oxytocin antagonist (OTA($[d(CH_2)_5,Tyr(Me)^2,Orn^8]$-Vasotocin))(Peninsula Laboratories) was dissolved to a concentration of 0.25ng/μl, 1 ng/μl, or 5ng/μl and infused into the third ventricle at a rate of 1μl/ hour for the 3 days following progesterone removal. The range of doses was selected such that the highest dose (5ng/μl) was of the same magnitude that has been used to reduce yawning and penile erection (Melis, Spano, Succu, & Argiolas, 1999). Because the highest dose of the OTA used in Experiment 1 did not completely suppress NADPH-d staining, in Experiment 3, the OTA was dissolved to a concentration of 10ng/μl.

Bromocryptine treatment: Bromocryptine (0.5mg/day) (Sigma) in a volume of 0.1ml was administered subcutaneously at 0900 for the 3 days following progesterone removal. This dose has been previously shown to suppress the circulating levels of prolactin (Walker, Mitchell, & Woodside, 1995).

Prolactin treatment: Subcutaneous PRL (0.5mg) (Sigma) was delivered in a volume of 0.1ml twice daily. This dose has been used to reverse the effects of bromocryptine administration on maternal behavior in steroid-primed female rats (Bridges & Ronsheim,

1990). Central PRL (2μg) was delivered in a volume of 1μl. This dose was used because it has been shown to be sufficient to induce maternal behavior (Bridges, Numan, Ronsheim, Mann, & Lupini, 1990). Both systemic and central injections were administered at 0900h and 1700h for the 3 days following progesterone removal.

Oxytocin treatment: OT (Sigma) was dissolved to a concentration of 1ng/μl and infused at a rate of 1μl/ hour for the 3 days following progesterone removal. This dose was selected because this dose has been shown to increase NADPH-d staining in estrogen-primed ovariectomized female rats (Popeski et al., 1999).

Histochemistry

On the test day, females were overdosed with sodium pentobarbital (60 mg/kg; MTC Pharmaceuticals Ltd.) and were perfused transcardially with 200ml of ice-cold saline (0.9% NaCl) followed by 300ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The brains were removed and post-fixed for 48 hrs in a 30% sucrose, 4% paraformaldehyde solution at 4 ^{0}C. Forty-micron thick sections throughout the SON and PVN were cut on a cryostat and placed free-floating in Trizma Buffered Saline (TBS; pH 7.3) solution. Sections were washed 3 times in TBS. Approximately 25 sections from each animal were then incubated for 75 minutes at 37 ^{0}C in 5ml of a solution containing 5.0mg of β-NADPH, 1.5mg of Nitro Blue Tetrazolium, 0.3% Titron X-100, and TBS. An animal from each experimental group was 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 (Sigma).

Image analysis

For Experiments 1 and 2 sections were visualized using a Sony XC77 camera mounted on a light microscope (Labolux Leitz GMBH). Images were captured using NIH image

analysis software (1.62b) installed on a Power Macintosh computer. NADPH- d stained cells were counted from the images captured in the TIFF picture files. Stained cells in sections throughout the rostro-caudal extent of the SON 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. This method of quantification was used to maintain consistency with previous work (Popeski et al., 1999). Subsequent analyses showed that results remained unchanged if all sections were included.

For the PVN, sections were divided into the magnocellular and parvocellular regions and were counted and then an average over all sections was obtained for each region. The PVN was subdivided based on plate 25 (approximately -1.80 mm from bregma of the rat brain atlas of Paxinos and Watson (1986,) that delineates the magnocellular region (PaLM: paraventricular hypothalamic nucleus, lateral magnocellular and PaV: paraventricular hypothalamic nucleus, ventral) from the parvocellular region (PaMP: paraventricular hypothalamic nucleus, medial parvocellular) (Paxinos & Watson, 1996). To identify densely stained cells; sections from each experimental group across each assay that appeared to be densely stained were selected and the relative density of each cell was recorded. An average of the density of the cells was then calculated to establish the criterion. Densely stained cells in each section were counted by an individual unaware of experimental group assignment.

In situ hybridization for OTmRNA

Fourteen-micron thick coronal sections were cut on a cryostat through the SON until the posterior portion of the PVN. These sections were thaw mounted onto RNAse-free coated slides, desiccated under vacuum and then stored at – 80^0 C until processed. Before hybridization, sections were washed with 4% paraformaldehyde, 2XSSC, 0.25% acetic anhydride in triethanolamine-HCL (PH 8.0), 2XSCC, and then dehydrated by transfer through 50%,70%,95%,100% ethanol, delipidated in chloroform, and then rehydrated in

100% and 95% ethanol; and dried at room temperature. OT mRNA detection was performed with a [^{35}S] ATP-41 base pair oligonucleotide sequence:(5'-GGG CTC AGC GCT CGG AGA AGG CAG ACT CAG GGT CGC AGG CG-3') (written communication from L.Young, Emory University, and the sequence was synthesized at McGill University). The oligo was labeled at the 3' end with terminal transferase (Roche) and $2x10^6$ cpm per slide was used for *in situ* hybridization.. Slides were covered and stored at 37 ^{0}C for 24 hrs. Hybridized sections were then washed 3 times in 1X SSC at 45 ^{0}C and once in 1X SSC at room temperature. Sections were then dehydrated in water, 50%, 70%, and 90% ethanol and air dried overnight. Hybridized slides were apposed to hyperfilm (Amersham) and kept at 4^{0}C for 3 days. The hybridization signal within the SON and PVN was quantified by means of densitometry with an image analysis system (MCID Research, Inc). Means for each animal were based on 5 sections for the SON and 4 sections for the PVN. The data are presented as arbitrary optical density (absorbance) units after correction for background.

Experiments

Experiment 1: *Effects of chronic OTA administration on NADPH-d staining in steroid-primed rats*

All animals were ovariectomized; one group was treated with cholesterol (Ovx+Chol; n=6) and the remainder were treated with the steroidal regimen described above. On Day 14 of the experiment, the steroid-primed rats underwent icv surgery and were assigned to one of four groups for the 72 hours following progesterone removal. These rats received daily icv injections of either Vehicle (saline) (Veh,n=6); 6 ng/day OTA (n=5); 24ng/day OTA (n=6); or 120ng/day OTA (n=6). On Day 17, animals were sacrificed and sections of the SON and PVN were processed for NADPH-d histochemistry.

Experiment 2a: *Effects of bromocryptine administration and subcutaneous PRL replacement on NADPH-d staining*

Ovariectomized steroid-primed rats received either a subcutaneous (sc.) injection of Vehicle (saline) (Veh, n=5), Bromocryptine (BR, n=5), or Bromocryptine and Prolactin (BR+PRL, n=5) for the three days following progesterone withdrawal. On Day 17, all animals were sacrificed and sections of the SON and PVN were processed for NADPH-d histochemistry.

Experiment 2b: *Effects of bromocryptine administration and central PRL or OT replacement on NADPH-d staining*

Ovariectomized, steroid primed rats were assigned to one of four treatment groups: 1) Veh (saline)(n= 5); 2) Bromocryptine (BR, n=4); 3) Bromocryptine and icv Prolactin (BR+ icvPRL,n=5); 4) Bromocryptine + icv Oxytocin (BR+ icvOT, n=4). On the day of ovariectomy, animals in the Veh, BR, and BR+icv PRL groups were implanted with icv cannulae. On Day 14, following removal of the progesterone implants, animals in the Veh, BR, and BR+icv PRL groups received a sc. injection of either vehicle or bromocryptine and an icv injection of saline (1μl) or PRL for the next three days. In addition, on Day 14, following progesterone removal, animals in the BR+ icvOT group were implanted with icv cannulae. For the next seventy-two hours, animals received a sc. injection of bromocryptine in combination with icv OT administration. On Day 17, animals were sacrificed and sections of the SON and PVN were processed for NADPH-d histochemistry.

Experiment 3: *Effects of bromocryptine and PRL replacement on OT mRNA*

All animals were ovariectomized; one group was treated with cholesterol (Ovx+Chol; n=5) and the remainder was treated with the hormonal regimen that was described above. The steroid-treated rats were then assigned to one of four groups for the 72 hours following progesterone removal. These rats received daily sc. injections of 1) Vehicle (saline) (Veh;

n=6) 2) Bromocryptine (BR; n=6), 3) Bromocryptine with PRL sc. replacement (BR+PRL; n=6), or 4) Bromocryptine with PRL sc. and an OTA which was chronically infused into the 3rd ventricle (BR+PRL+OTA; n=6). ICV surgery for the latter group was conducted on Day 14. On Day 17, animals were sacrificed and sections of the SON and PVN were processed for *in situ* hybridization for OT mRNA.

Statistics

For all experiments, data were analysed using a one-way analysis of variance (ANOVA) followed by post hoc comparisons (Fisher PLSD or Tukey HSD) where appropriate. An alpha level of .05 was adopted for all tests.

Results

Experiment 1

In the SON, the number of cells densely stained for NADPH-d varied significantly across the five groups ($F(4,24) = 7.96$, $p < .05$) (Figure 1a). Posthoc pairwise comparisons (Fishers PLSD) showed that rats in the Ovex+Chol group had significantly fewer cells stained for NADPH-d than those in the other groups ($p<.05$). In addition, females that received the highest daily dose of the OTA (120 ng) had significantly fewer positive stained cells than females in the Veh group ($p<.01$).

In the magnocellular subdivision of the PVN we also found that the number of stained cells for NADPH-d also varied significantly across the five groups ($F(4,24)=2.70$, $p=.05$) (Figure 1b). Posthoc tests revealed that rats in the Ovex + Chol group had fewer stained cells than those in the Veh and 6 ng OTA groups ($p<.05$) but did not differ from the 24 ng and 120 ng OTA groups. Overall, there was no significant difference in the number of NADPH-d stained cells in the parvocellular region of the PVN across groups ($F(4,24)= 2.6$, $p>.05$) (Figure 1c). Inspection of Figure 1c, however, suggests

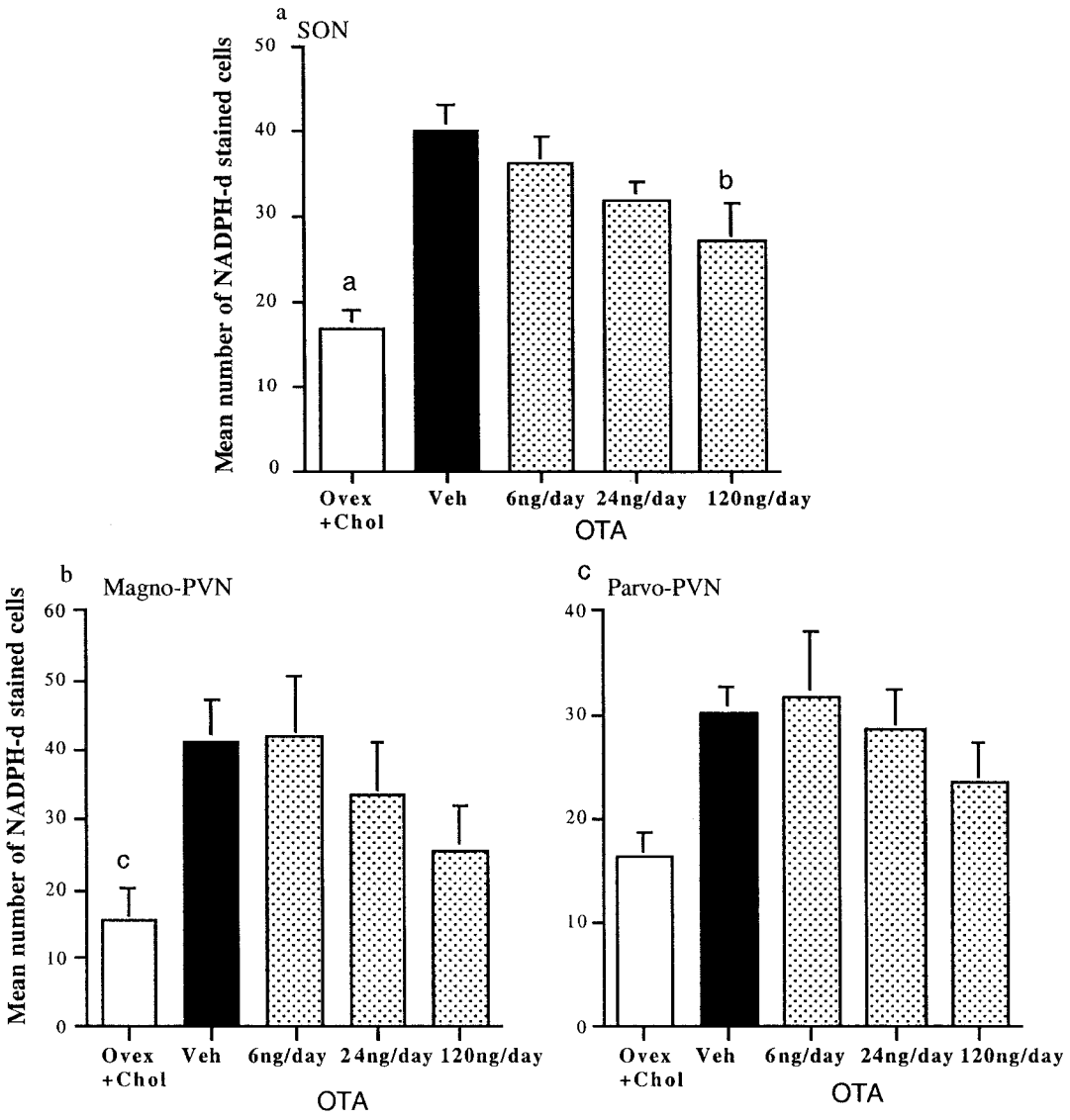

Figure 1. Effects of treatment with various doses of an oxytocin receptor antagonist (OTA) on the mean number of NADPH-d stained cells in the a) supraoptic nucleus (SON) b) magnocellular paraventricular nucleus, and c) parvocellular paraventricular nucleus of steroid-primed rats. The highest dose of the OTA decreased the number of NADPH-d stained cells in the SON and magnocellular portion of the PVN. a $P<.05$,significantly different from all groups; b $P<.05$,significantly different from Veh; c $P<.05$, significantly different from Veh and 6 ng.

that in the absence of any hormone replacement, ovariectomy reduced the number of NADPH-d stained cells.

Experiment 2a

The number of cells stained for NADPH-d in the SON and magnocellular subdivision of the PVN varied significantly across the three groups ($F(2, 12) = 4.06$, $p < .05$); $F(2, 12) = 9.76$, p .01; respectively). Posthoc tests (Fishers PLSD) showed that suppressing PRL levels with bromocryptine reduced the number of cells stained for NADPH-d ($p<.05$). Subcutaneous PRL administration restored the number of NADPH-d positive stained cells in the SON and magnocellular PVN of bromocryptine treated rats (Figure 2a,b). There was no significant effect of treatment on the number of stained cells in the parvocellular subdivision of the PVN ($F(2, 12) = 2.41$, $p > .05$)(Figure 2c).

Experiment 2b

Examples of NADPH-d staining in the SON and PVN as a function of central PRL and OT treatment are seen in Figures 3 and 4. As can be seen in these figures and as presented numerically in Figure 5a,b , there were significant differences in the number of NADPH-d stained cells between groups in both the SON ($F(3,14)=3.82$, $p<.05$) and magnocellular subdivision of the PVN ($F(3,14)=4.52$, $p<.05$). Post hoc (Fisher PLSD) analysis revealed that rats treated with bromocryptine-alone had fewer NADPH-d positive stained cells than animals in the other groups ($p<.02$) which did not differ from each other. There was no significant effect of treatment on the number of stained cells in the parvocellular subdivision of the PVN ($F(3,14)=4.52$, $p<.05$)(Figure 5c).

Experiment 3

Images of *in situ* hybridization for OT mRNA within the SON and PVN are seen in Figure 6. ANOVA showed that the average optical density for OTmRNA in the SON and PVN

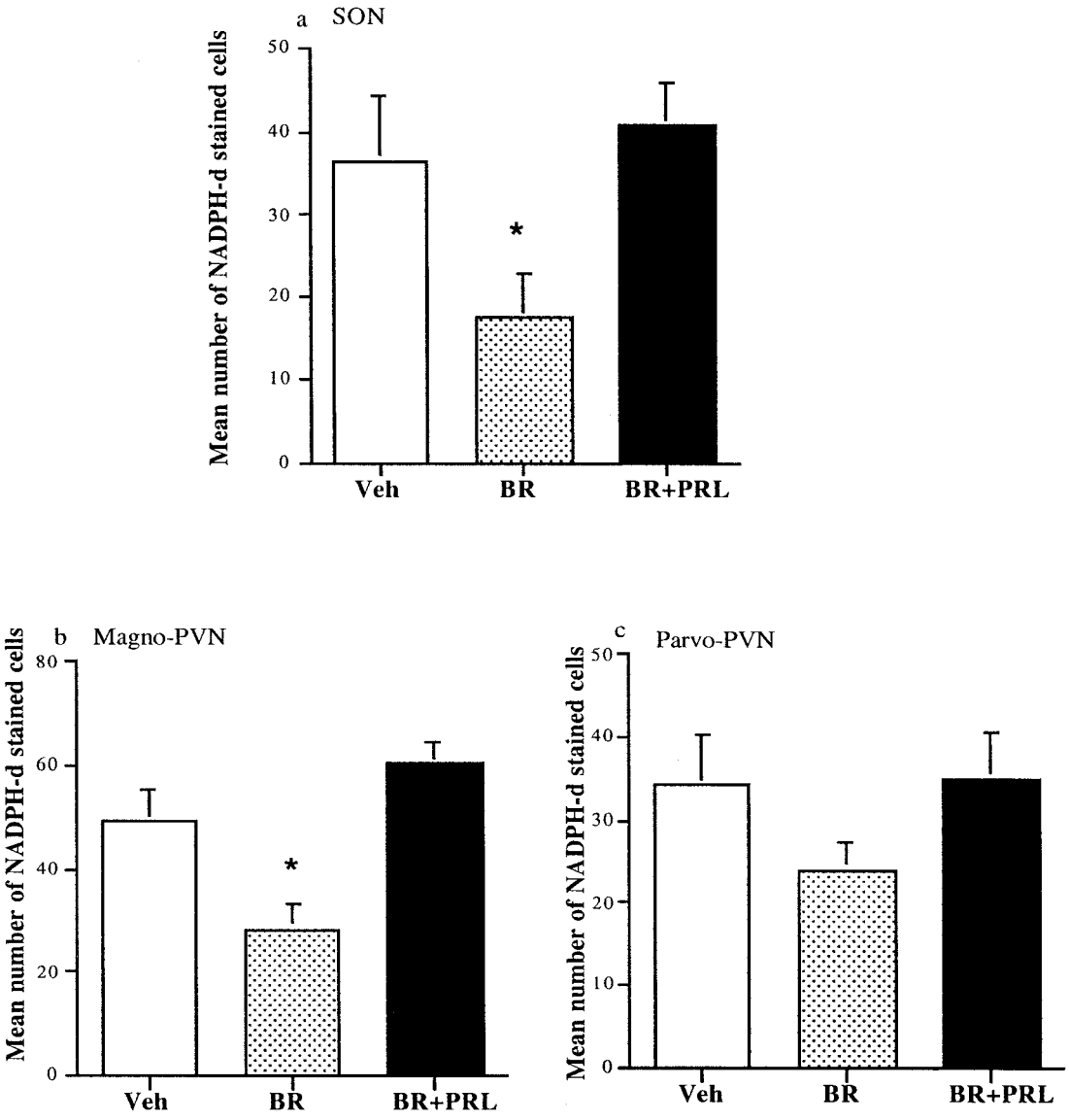

Figure 2. Effects of systemic prolactin replacement on the mean number of NADPH-d stained cells in the a) SON, b) magnocellular paraventricular nucleus, and c) parvocellular paraventricular nucleus in bromocryptine-treated steroid-primed rats. Systemic PRL treatment restored the number of NADPH-d stained cells in the SON and magnocellular portion of the PVN following bromocryptine treatment. * P<.05, significantly different from Veh and BR.

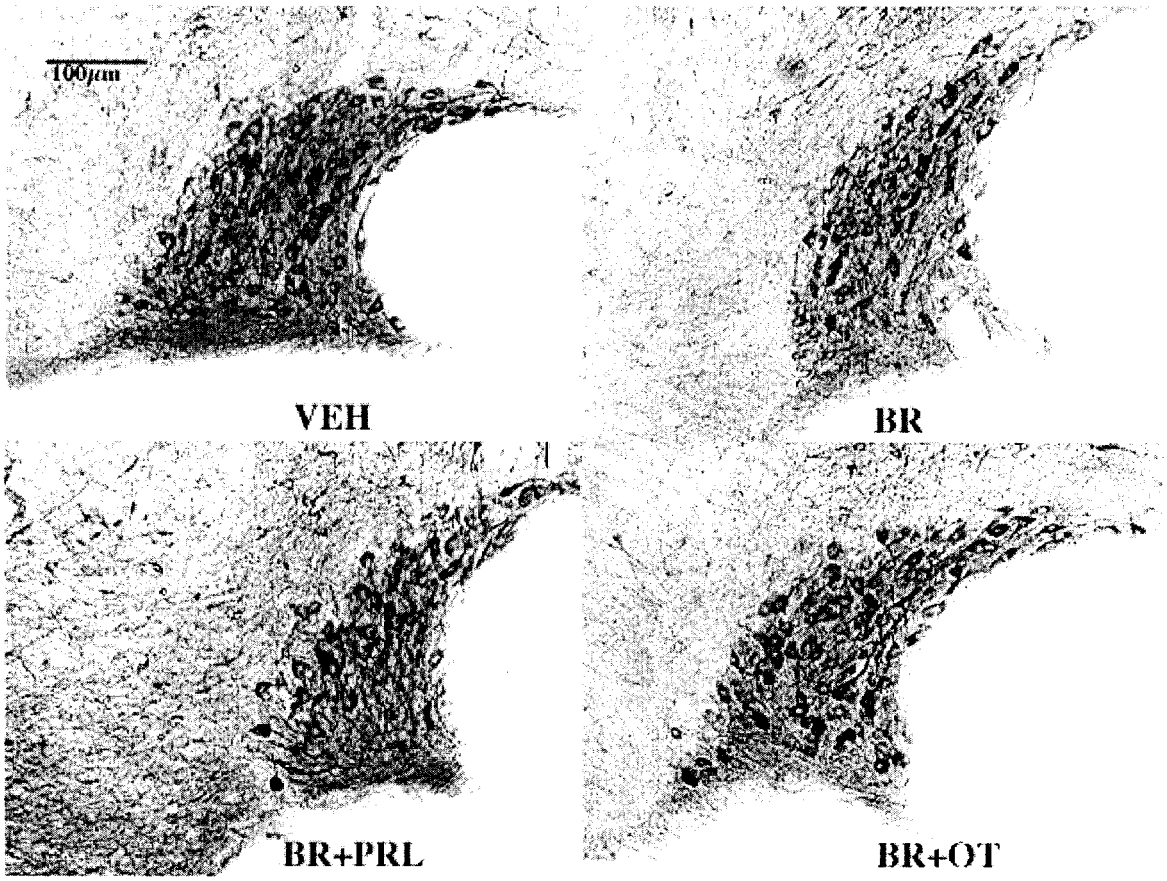

Figure 3. Example of NADPH-d staining in the supraoptic nucleus of the steroid primed rats that received either Vehicle (Veh), Bromocryptine (BR), Bromocryptine and Prolactin (BR+PRL), or Bromocryptine and Oxytocin (BR+OT).

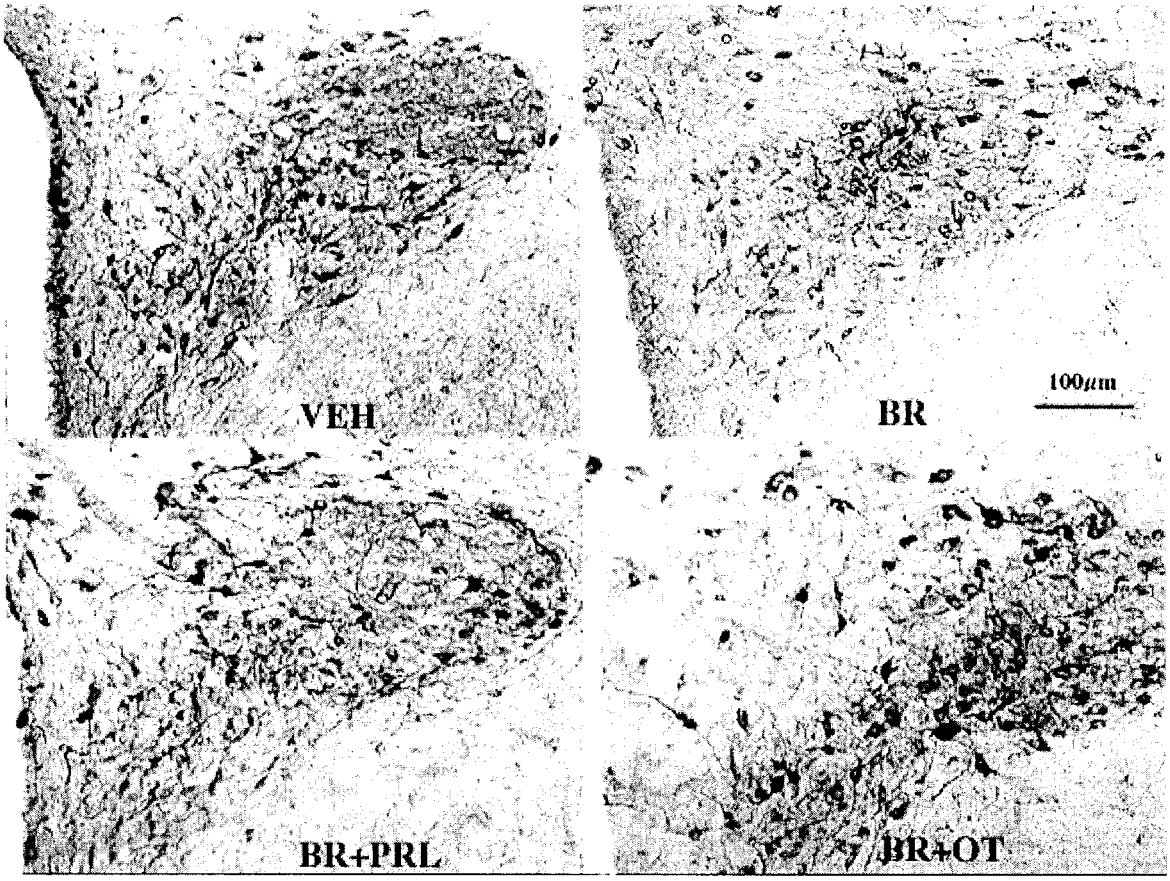

Figure 4. Example of NADPH-d staining in the paraventricular nucleus of the steroid primed rats that received either Vehicle (Veh), Bromocryptine (BR), Bromocryptine and Prolactin (BR+PRL), or Bromocryptine and Oxytocin (BR+OT).

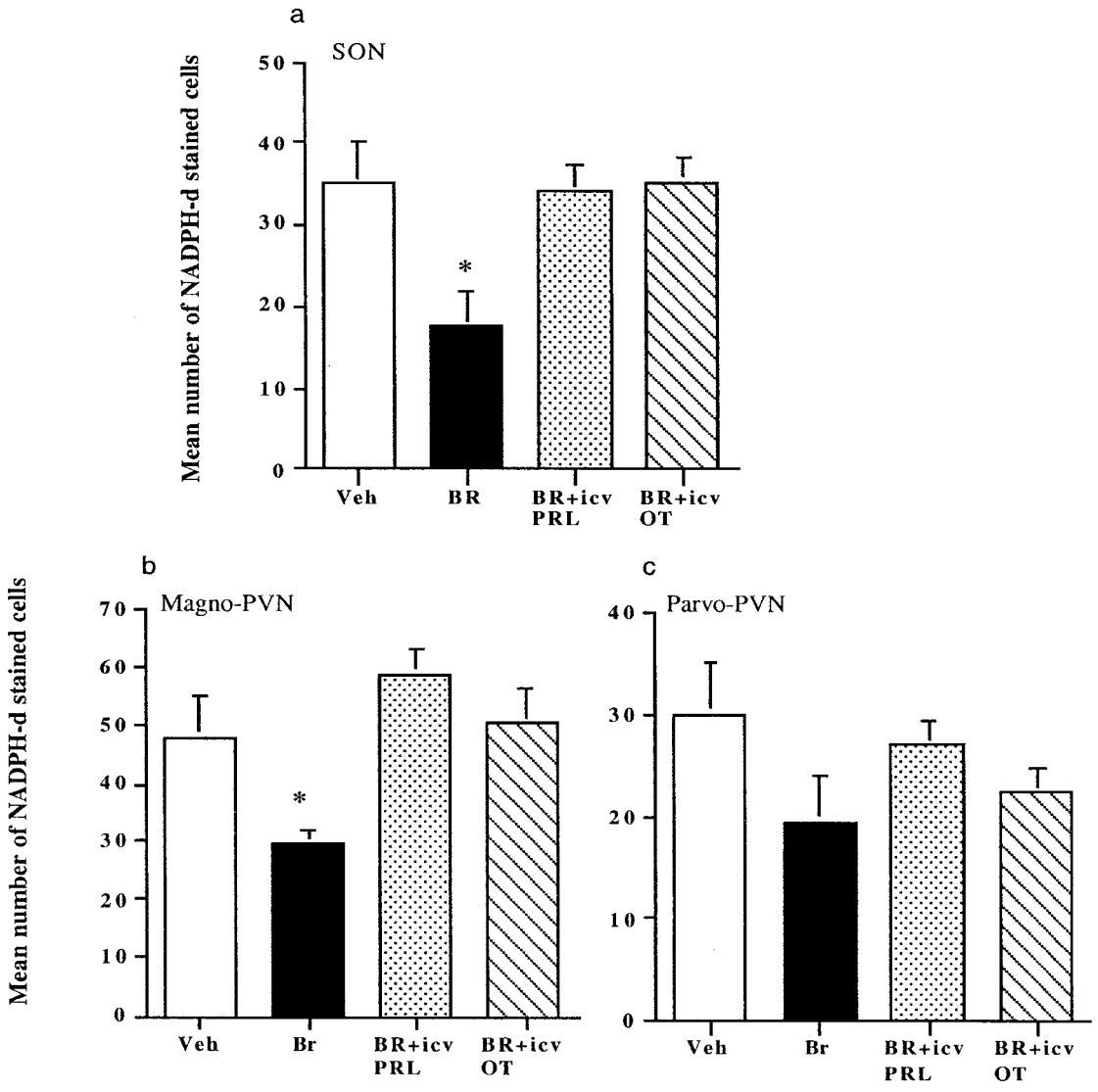

Figure 5. Effects of central prolactin and oxytocin replacement on the mean number of NADPH-d stained cells in the a) SON, b) magnocellular paraventricular nucleus, and c) parvocellular paraventricular nucleus in bromocryptine-treated steroid-primed rats. Both central PRL and OT treatment restored the number of NADPH-d stained cells in the SON and magnocellular portion of the PVN following bromocryptine treatment. * P<.05, significantly different from Veh, BR+icvPRL, and BR+icvOT.

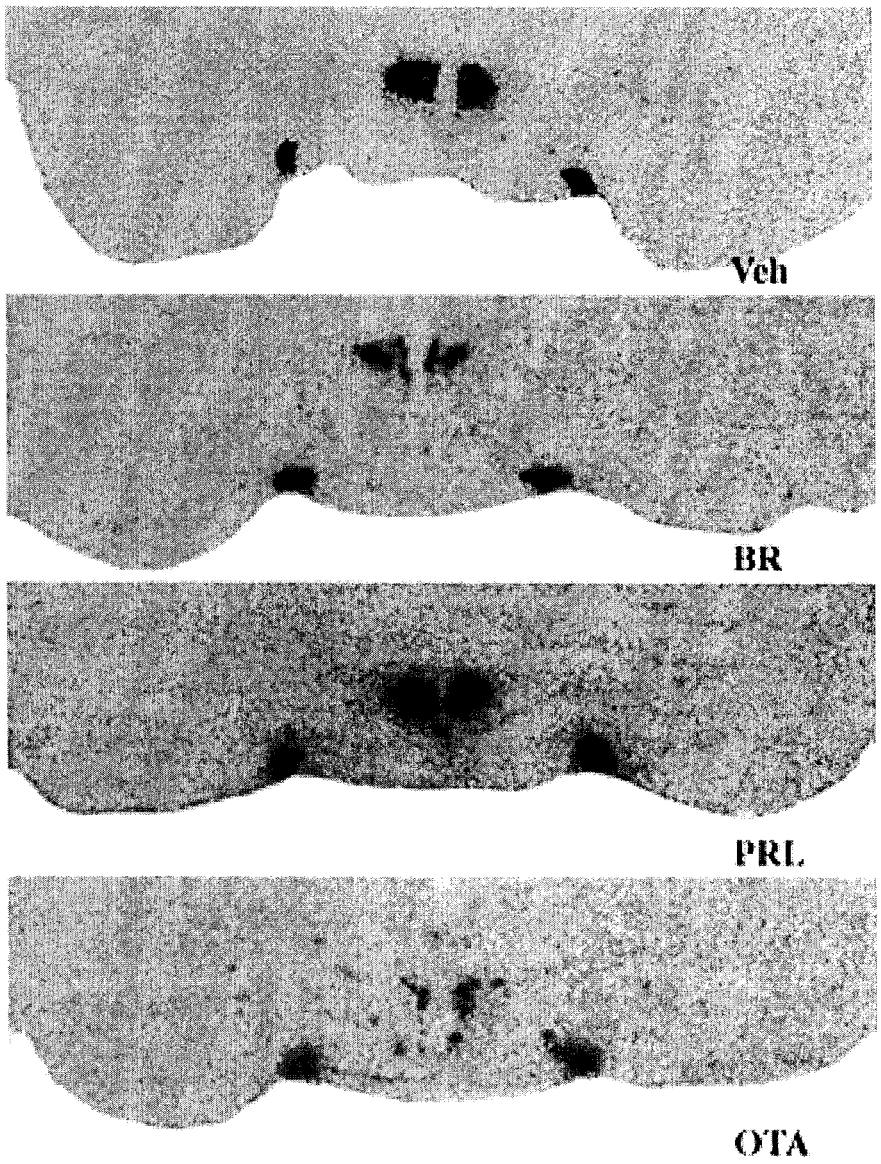

Figure 6. Example of OT mRNA in the supraoptic nucleus (SON) and paraventricular nucleus of ovariectomized rats that received steroid priming and either: Vehicle (Veh), Bromocryptine (BR), Bromocryptine and Prolactin (PRL), or Bromocryptine together with Prolactin and Oxytocin antagonist (OTA).

varied significantly across the groups ($F(4, 24) = 18.76$, $p < .01$); $F(4, 24) = 17.18$, $p < .01$;respectively). Post hocs (Tukey HSD) analysis showed that OT mRNA levels were significantly lower in animals in the Ovex + Chol, BR, and BR+PRL+OTA groups than animals in the Veh and BR+PRL groups ($p<.01$) (Figure 7).

Discussion

The results of these experiments support earlier findings showing that mimicking the gonadal steroid hormone profile of late pregnancy is sufficient to upregulate NOS in the SON and magnocellular compartments of the PVN (Popeski et al., 1999).
They are also consistent with a mediating role of OT in this effect (Popeski et al., 1999). In addition, the current studies extend previous findings by demonstrating both that OT receptor activation plays a key role in the upregulation of NOS and that the effect of steroid priming on NOS in the PVN and SON is mediated not only by OT itself but also by the interaction of OT and PRL.

The results of Experiment 1 demonstrate that central administration of an OT receptor antagonist can reduce the number of cells staining for NADPH-d in the SON and magnocellular compartment of the PVN in rats treated with a hormonal regimen that mimics late pregnancy. These data combined with those of earlier studies demonstrating an increase in NADPH-d staining in the PVN and SON in E-primed ovariectomized females treated with chronic icv OT infusions support a role for OT receptor activation in the regulation of NOS in these areas (Popeski et al., 1999). These findings are also consistent with the hypothesis that NO plays a negative feedback role within the hypothalamic neurohypophysial system (Summy-Long et al., 1993).

As shown in Experiment 2 administration of the D2-like agonist bromocryptine for 3 days following progesterone removal was sufficient to eliminate the upregulation of NADPH-d staining in the SON and in the magnocellular, but not the parvocellular,

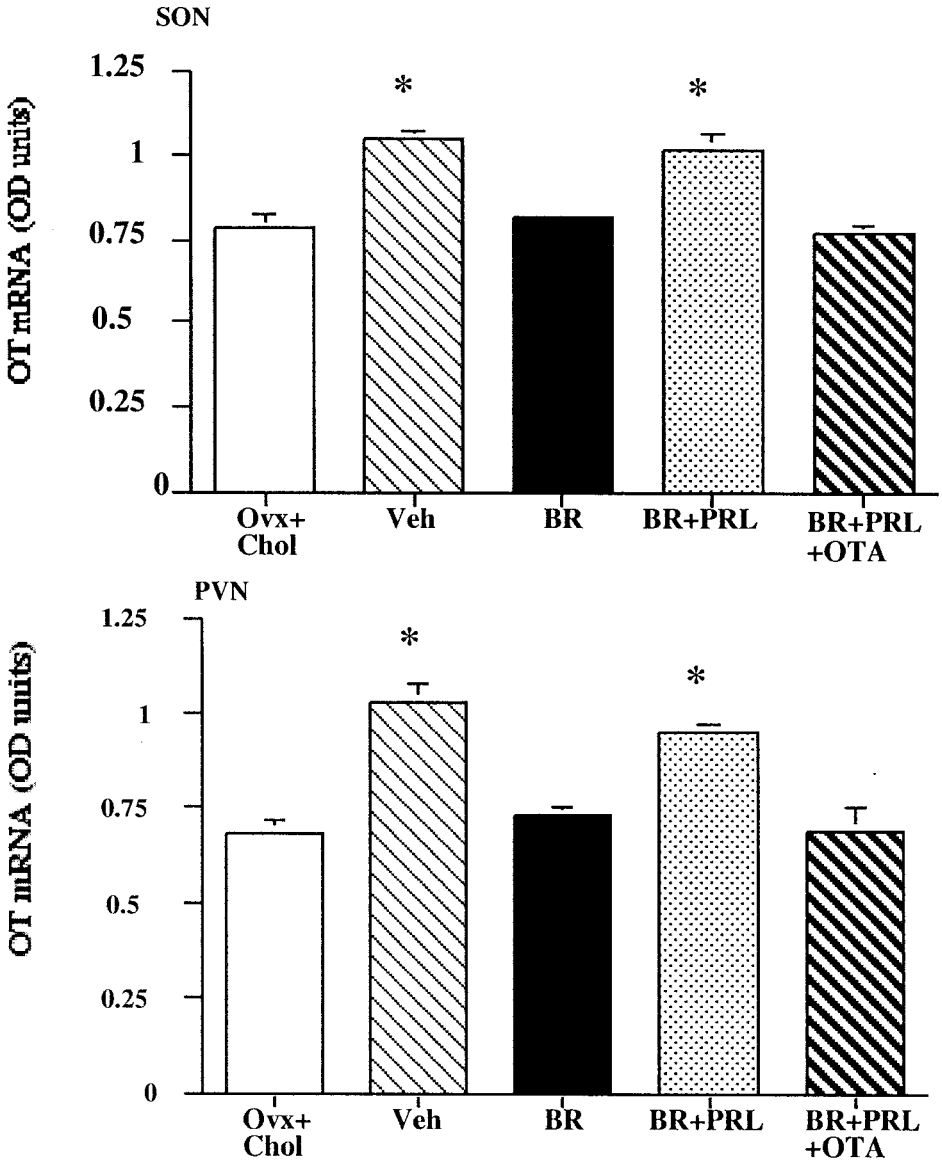

Figure 7. Effects of bromocryptine, and prolactin replacement on OT mRNA (OD units) expression in the a) supraoptic nucleus (SON) and b) paraventricular nucleus (PVN) in steroid-primed rats. Both bromocryptine and OTA treatment suppressed OT gene expression in the SON and PVN in steroid primed rats; while prolactin replacement restored OT mRNA to levels seen in the vehicle group. * $P<.05$, significantly different from Ovx+chol, BR, and BR+PRL+OTA.

subdivision of the PVN in steroid-primed ovariectomized females. Bromocryptine treatment suppresses prolactin release through its action at the D2 receptor (Gardner & Strange, 1998) and its effect on NADPH-d staining could be eliminated by either systemic or central PRL replacement. These data therefore indicate that the effects of bromocryptine are mediated through its ability to suppress prolactin release rather than through other dopamine agonistic effects.

Pituitary PRL release is increased both in late pregnancy (Grattan & Averill, 1990) and by the steroid hormone regimen used in the current experiments (Bridges & Ronsheim, 1990) and reaches the brain through an active transport mechanism (Walsh et al., 1987). Moreover, PRL receptors have been identified within both the PVN and SON of late pregnant and lactating rats (Bakowska & Morrell, 1997; Pi & Grattan, 1999).Together with the fact that both peripheral and icv PRL replacement were effective in increasing NADPH-d staining within these nuclei, this evidence suggests that PRL acts within the brain to promote the upregulation of NOS both in steroid-primed and late pregnant rats. Such a central action of PRL adds to the growing number of behavioral and neurochemical changes that have been attributed to central actions of PRL including stimulation of food intake (Sauve & Woodside, 2001) and maternal behavior (Bridges et al., 1985) and the modulation of stress reactivity (Torner, Toschi, Pohlinger, Landgraf, & Neumann, 2001).

The results of Experiment 2b showed that the effects of bromocryptine treatment on NADPH-d staining could also be reversed by central administration of OT indicating that, although sufficient, PRL is not necessary for the upregulation of NOS in the PVN and SON of steroid-primed rats. In contrast, OT receptor activation does appear to be a necessary mediator of the effects of gonadal steroid priming on NOS because, as shown in Experiment 1, administration of an OTA reduces NADPH-d staining in steroid-primed rats. There are a number of different ways in which the action of OT and PRL on magnocellular neurons could combine so as to increase NOS. It is possible that activation of both OT receptors and PRL receptors can have direct and independent effects on NOS synthesis.

An alternative hypothesis is that the effects of PRL receptor activation on NOS are in fact mediated by OT. Support for this possibility comes from studies showing that, PRL stimulates OT mRNA in hypothalamo-neurohypophysial system explants (Ghosh & Sladek, 1995b; Ghosh & Sladek, 1995a) and OT release from the neural lobe (Parker et al., 1991).

The possibility that PRL actually upregulates OTmRNA in the PVN and SON and that this is in turn, associated with upregulation of NOS was examined in Experiment 3. Here we found that, as previously reported (Crowley et al., 1995), treatment with a steroid regimen that mimicked late pregnancy was sufficient to increase OT gene expression in the PVN and SON. As with NOS, the effect of steroid administration on OT mRNA was eliminated if rats were treated with bromocryptine after progesterone withdrawal and OT mRNA expression was restored if bromocryptine treated rats were given systemic PRL replacement. A role for OT receptor activation in the maintenance of OT mRNA levels was suggested by the fact that PRL replacement was unable to restore OT mRNA in bromocryptine-treated rats in the presence of chronic OTA infusion. These results provide the first *in vivo* evidence of the involvement of PRL in inducing elevated levels of OT mRNA within the SON and PVN.

Although the results of the present study indicate that PRL stimulates OT mRNA, it is also possible that OT plays a role in the increased PRL release that is seen in late pregnancy and after steroid-priming (Johnston & Negro-Vilar, 1988; Arey & Freeman, 1990). It has been shown that plasma OT levels are elevated just prior to the increase in PRL seen when dams are reintroduced to their pups following a 4-hr separation (Samson, Lumpkin, & McCann, 1986). In addition, exogenous administration of OT stimulates PRL release from anterior pituitary cells from estrogen-primed ovariectomized rats (Samson et al., 1986). Thus, the increased intranuclear OT release that stimulates the upregulation of NOS that is observed in late pregnancy might result indirectly from OT first stimulating PRL which then activates OT neurons.

The effects of manipulating PRL and OT on NOS and of prolactin on OT mRNA described in the current studies were all obtained in steroid-primed rats. It is highly probable that the background steroid hormone level is an important component of these effects. It is likely that progesterone withdrawal plays a key role in these effects as it does in the activation of OT neurons at parturition (Antonijevic et al., 2000). In addition, following long term exposure to a combination of estrogen and progesterone, progesterone withdrawal is required for both upregulation of NADPH-d staining (Popeski et al., 1999) and OT mRNA (Crowley et al., 1995) within the SON and PVN. The pattern of exposure to estrogen and progesterone is important for the upregulation of PRL itself, but it may also play multiple roles in modulating the efficacy of both PRL and OT by, for example incresing receptor levels. For example, previous data showed that chronic OT infusion was sufficient to upregulate NADPH-d only if ovariectomized rats were estrogen primed (Popeski et al., 1999). Moreover, PRL is only effective in stimulating maternal behavior in estrogen- primed female rats (Bridges et al., 1990). Considerable more work will be needed to elucidate precisely what type of gonadal steroid exposure is needed to allow the effects that are reported in this paper and to understand the mechanisms through which these effects are produced.

Prolactin and OT are both intimately involved in a number of reproductive processes including lactation. NOS is also upregulated in the hypothalamic neurohypophysial system of lactating rats and NADPH-d staining decreases rapidly following removal of suckling stimulation (Otukonyong et al., 2000). Because suckling stimulates both PRL (Grosvenor & Whitworth, 1974) and OT release (Higuchi, Honda, Fukuoka, Negoro, & Wakabayashi, 1985) it is possible that either or both peptide hormones contribute to the increased expression of NOS in the PVN and SON of lactating rats. In addition, the influence of PRL on OT mRNA and hence OT release may facilitate the positive feedback system for OT that is observed during parturition (Neumann, Douglas, Pittman, Russell, & Landgraf, 1996). It has been reported that mobilization of intracellular calcium from thapsigargin-

sensitive stores can stimulate activity-dependent OT release from dendrites within SON magnocellular neurons (Ludwig et al., 2002). Thus, the dendritic release of OT within hypothalamic nuclei following stimulation of the hypothalamic neurohypophysial system, may act on adjacent neurons to affect their electrical activity or act in an autocrine manner by binding to its own receptors to maintain the sensitivity of the magnocellular OT-secreting neurons.

Intriguingly, both PRL and OT have been implicated in the onset of maternal behavior (Bridges et al., 1985; Pedersen, Ascher, Monroe, & Prange, 1982). Although the effects of PRL on OT mRNA reported here are limited to the hypothalamic neurohypophysial system, it is possible that similar interactions occur within brain structures subserving maternal behavior such as the medial preoptic area (Brooks, 1992).

In conclusion, administration of an OT receptor antagonist and suppression of endogenous PRL both attenuated NADPH-d staining in magnocellular neurons in steroid-primed rats. The effect of prolactin suppression could be reversed either with subcutaneous or central PRL replacement, or with chronic central infusion of OT. Furthermore, suppression of PRL by bromocryptine and central administration of an OTA both decreased OT gene expression within the SON and PVN. These results demonstrate that PRL and OT interact to increase the capacity for NO production within the SON and PVN and suggest that the upregulation of NOS may result from increases in intranuclear OT release. Moreover, these data suggest that PRL and OT interact to modulate OT mRNA expression in the PVN and SON.

Chapter 3

Oxytocin is a nonapeptide hormone that is synthesized within the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus and is released from the posterior pituitary into the general circulation in response to a variety of environmental stimuli including suckling (Higuchi et al., 1986) and osmotic challenge (Evans & Olley, 1988). In addition to its well-established peripheral actions, recent studies have shown that oxytocin acts centrally to modulate both physiology and behavior. For example, the central administration of oxytocin has a facilitatory effect on the proestrus surge of luteinizing hormone (Johnston, Lopez, Samson, & Negro-Vilar, 1990), stimulates penile erection and yawning (Melis, Succu, Iannucci, & Argiolas, 1997) and facilitates the onset of maternal behavior in virgin rats (Pedersen et al., 1982). It also inhibits food intake (Arletti, Benelli, & Bertolini, 1989) and reduces the behavioral and hormonal responses to stress (Windle et al., 1997). One important target of central oxytocin appears to be the oxytocin neurones in the SON because central oxytocin infusion has been shown to facilitate and coordinate the activation of these neurones (Jiang & Wakerley, 1997).

Consistent with these central effects of oxytocin administration, oxytocin receptors have been localized on oxytocin neurones in the PVN and SON (Freund-Mercier et al., 1994). In addition, other brain regions including the medial preoptic area (MPOA), the amygdala, the ventromedial hypothalamic nucleus (VMN), and the bed nucleus of the stria terminalis (BNST) have also been shown to contain oxytocin receptors (Yoshimura et al., 1993). Further, oxytocin release within the SON and PVN during osmotic stimulation (Landgraf, Neumann, & Schwarzberg, 1988) as well as during parturition and suckling has been demonstrated (Neumann et al., 1993). This evidence has led to the suggestion that stimulation of magnocellular oxytocin cells leads to intranuclear oxytocin release which has a positive feedback effect on oxytocin neurones and stimulates further oxytocin release.

Recent evidence suggests that nitric oxide synthase (NOS), the enzyme that synthesizes nitric oxide (NO) (Schuman & Madison, 1994) colocalizes with oxytocin in both the SON and PVN (Miyagawa, Okamura, & Ibata, 1994). Stimuli such as saltloading (Villar et al., 1994) and water deprivation (Ueta et al., 1995) result in an upregulation of NOS in the SON and PVN suggesting that increases in NOS in these nuclei accompany activation of the oxytocin system. However, the functional significance of the increase in NOS is not clear.

Previous studies have shown that NO is an important modulator both of the activity of magnocellular neurones within the SON (Liu, Jia, & Ju, 1997) and of oxytocin secretion (Kadowaki et al. 1994). Electrophysiological studies of neuronal activity within the SON have shown that administering NO donors reduces overall neuronal activity (Liu et al., 1997) and this effect appears to result, at least in part, from an increase in the frequency of inhibitory post synaptic potentials (Ozaki et al., 2000). In addition, depletion of oxytocin content from the posterior pituitary in response to chronic salt loading (Kadowaki et al., 1994) or acute water deprivation (Summy-Long et al., 1993) is enhanced when rats are simultaneously treated with a NOS inhibitor either systemically or intracerebroventricularly. Basal oxytocin release, however, is not affected by inhibiting NOS (Summy-Long et al., 1993). Together, these data have been interpreted to suggest that stimulation of the hypothalamic neurohypophysial system (HNS) leads to an upregulation of NOS and potentially an increase in NO production that has a negative feedback effect on oxytocin release (Kadowaki et al., 1994; Summy-Long et al., 1993).

One mechanism through which NO might act to limit the response of the HNS to stimulation is by modulating the pattern of neuronal activation induced by intranuclear oxytocin release. To investigate this possibility we first used Fos immunohistochemistry to compare the pattern of neural activation in the hypothalamus induced by third ventricle infusions of oxytocin in rats in which the oxytocin system was chronically stimulated and in unstimulated rats, and then determined how this pattern was changed when NOS was

inhibited. It has been well established that during lactation NOS within the SON and PVN is upregulated (Popeski et al., 1999; Luckman, Huckett, Bicknell, & Voisin, 1997), the oxytocin system is more active (Higuchi et al., 1986), and intranuclear as well as hypophysial oxytocin release is increased (Neumann et al., 1993). Therefore in Experiment 1 we compared neuronal activation in the SON, PVN, and MPOA of nonlactating and lactating rats following central oxytocin stimulation using Fos-like immunoreactivity (lir) as a marker of cellular activation. We took this approach because Fos, the protein product of the immediate early gene c-fos has been used extensively to map the response of the hypophysial neurosecretory system to a wide range of stimuli (Fenelon, Theodosis, & Poulain, 1994).

To determine the effects of inhibiting NOS on the effects of central oxytocin administration, in Experiment 2, the induction of Fos-lir in the SON, PVN, MPOA, and BNST was examined in lactating or nonlactating rats given a systemic injection of either L-NAME or vehicle prior to a central injection of either oxytocin or vehicle into the third ventricle.

Finally, to determine whether any effects of the inhibition of NO synthesis on Fos expression that we observed in Experiment 2 would generalize to other stimuli known to activate oxytocin neurones, in Experiment 3, we compared the effect of inhibiting NO synthesis, using L-NAME, on Fos expression in the SON and PVN of lactating and nonlactating rats following urethane administration.

Materials and Methods

Animals

Virgin female Wistar rats from Charles River Breeding Farm, (St. Constant, Quebec) were used in these studies. Upon arrival in the laboratory, the rats weighed between 220-240 g

and were housed in groups of five females. The animal facility was maintained on a 12/12hr light/dark cycle (lights on from 0800-2000hr) at 20± 20 ^{0}C. Rats were given *ad libitum* access to lab chow and to water throughout the experiment. Where appropriate, rats were mated by placing one male rat in a group cage with five females for 2 weeks. Approximately, three days before parturition, each pregnant female was removed from the group cage and placed in a single cage (20 x 45 x 25) with adlib food and water. On the day after parturition (Day 1 postpartum (pp.), litters were culled to eight pups. All protocols were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Surgery

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 (5.7 mg ketamine and .86 mg xylazine/100 g of body weight) anaesthesia on Day 1 pp. in lactating groups and at least 11 days prior to the experimental day in nonlactating groups

Injection Procedure

Manipulations were carried out on Day 12-13 pp. in lactating groups and at least 11 days after surgery in nonlactating rats. To minimize potential stress effects on Fos expression, rats were handled daily. Seven days after surgery each rat received an angiotensin infusion (50 ng/2 μl). If the injection of angiotensin stimulated water intake, then the cannula placement was considered accurate. For three days before the experimental day, rats were

habituated to the injection procedure by placing the injection needle into the cannulae for 2 min each day. Injections were carried out between 1000hr and 1200hr.

Immunocytochemistry

One hour after hormone or urethane injection, each rat was injected with an overdose of sodium pentobarbital and perfused transcardially with 200 ml of ice-cold saline, followed by 300 ml of 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24h. Fifty-micron-thick sections throughout the BNST, MPOA , SON and PVN were cut on a vibratome and placed free-floating in Trizma Buffered Saline (TBS; pH 7.3) solution and then processed for Fos-lir. Each assay included samples from each treatment group, thus interassay variability did not contribute to between group variability. The sections were incubated for 30 min in 3% H2O2 solution in TBS to reduce nonspecific staining and then washed in TBS 3 times for 15 min. Tissue was then incubated for 90 minutes at 4 ^{0}C in blocking serum (.3% Triton X (TTX, Sigma), 3% normal goat serum (NGS, Vector), and TBS) and then incubated in the primary antibody solution. The antibody used was a polyclonal antibody (Ab-5, PC-38, Oncogene Research Products, MA) diluted 1: 130, 000. This antibody, recognizes amino acids 4-17 of the human c-fos epitope. Forty-eight hours later, sections were washed 3 times in TBS for 15 min and then were incubated in secondary antibody (biotinylated rabbit anti-goat, Vector) for 1h, washed 3 times in TBS and incubated in ABC reagent for 2h. Tissue staining was developed using a ABC vector kit (Diminobenzene, nickel intensified). The sections were then mounted on gelatin slides and cover slipped (Permount, Fisher) for analysis.

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. Fos-lir cells were counted from the images captured in TIFF picture files. Stained cells in sections throughout the rostro-caudal extent of the SON 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. For the PVN, only sections in the medial area of the nucleus were used (approximately -1.8 mm from bregma, Paxinos and Watson, 1986). The PVN was subdivided into magnocellular and parvocellular regions. Fos-positive cells in both of these subdivisions in approximately three sections were counted and an average for each region over all sections was obtained. To estimate Fos expression in the MPOA (-.80 mm to -.92 mm from bregma) and BNST (-.30 mm to -.40 mm from bregma), stained cells in approximately three sections were counted and an average of these sections was calculated for each brain region.

Experiments

Experiment 1: Effects of oxytocin on Fos induction in nonlactating and lactating rats. Rats were assigned to one of four experimental groups: Nonlactating-Saline (NL/Sal; N=5), Nonlactating-Oxytocin (OT) (NL/OT; N=4), Lactating-Saline (Lac/Sal; N=4), and Lactating-Oxytocin (Lac/OT; N=5). Lactating females were given an intracerebroventriclar (i.c.v.) injection of either 2 μl of saline or oxytocin (50 ng in 2 μl, Sigma, St.Louis) on Day 12 or 13 pp. Nonlacting females received similar treatments at least 11 days after surgery.

Experiment 2: Effects of L-NAME on Fos induction in central oxytocin -stimulated lactating and nonlactating rats.

2a. Lactating rats were randomly assigned to one of four experimental groups: Saline-Saline (Sal/Sal; N=5), Saline-Oxytocin (Sal/OT; N=4), L-NAME-Saline (LN/Sal; N=5), and L-NAME-Oxytocin (LN/OT; N=4). On Day 12-13 pp. rats were given an i.p. injection of either saline or L-NAME (50 mg/kg) followed 30 min later by an i.c.v. injection of saline or oxytocin (50 ng in 2μl; Sigma, St.Louis).

2b. Nonlactating rats were randomly assigned to one of four experimental groups: Saline-Saline (Sal/Sal; N=6), Saline-Oxytocin (Sal/OT; N=6), L-NAME-Saline (LN/Sal; N=5), and L-NAME-Oxytocin (LN/OT; N=6). Approximately 11 days after surgery, nonlactating rats were given the same treatments as lactating rats.

Experiment 3: Effects of L-NAME on Fos induction in urethane-stimulated nonlactating and lactating rats.

Rats were assigned to one of four experimental groups: Nonlactating-Saline (NL/Sal; N=6), Nonlactating-L-NAME (NL/LN; N=5), Lactating-Saline (Lac /Sal; N=5), and Lactating-L-NAME (Lac/LN; N=6). On the test day (Day 12-13 pp. for lactating females), rats received an i.p. injection of saline or L-NAME (50 mg/kg) followed 30 min later by an i.p. injection of urethane (1.4 g/kg).

Statistics

Data obtained in all three experiments were analysed using 2-way independent groups ANOVA followed, where appropriate, by *posthoc* comparisons using the Fisher PLSD. Homogeneity of variance was assessed in each of the experiments using the Fmax test and where necessary, the α level was adjusted accordingly.

Results

Experiment 1. Effects of oxytocin on Fos induction in nonlactating and lactating rats. Oxytocin administration induced robust Fos expression in both nonlactating and lactating rats in the SON (Fig. 1). Analysis of these data with a 2x2 (Hormone x Reproductive state) independent groups ANOVA showed significant main effects for both hormone ($F(1, 14)= 79.04$, $p<.05$) and reproductive state ($F(1, 14) = 8.14$ $p<.05$) in this nucleus.

Although the analysis revealed no significant interaction between hormone and reproductive state in this area, inspection of the data and subsequent *posthoc* analysis (Fisher PLSD) showed that the effect of reproductive state primarily reflected a greater effect of oxytocin administration on Fos-lir induction in the SON of lactating rats than in that of nonlactating rats.

Oxytocin administration also increased Fos-lir in both the magnocellular and parvocellular subdivisions of the PVN $F(1, 14)= 20.52$, $p<.05$; $F(1, 14)= 18.21$, $p<.05$, respectively; Fig. 1 b and c). There was no significant main effect for reproductive state in either area but a significant trend was seen in the parvocellular subdivision ($F(1,14) = 4.06$, $p=0.06$) which seems to reflect a higher degree of Fos-lir in the saline-treated lactating group.

As is shown in Figure 1d, oxytocin administration induced Fos-lir in the MPOA of lactating rats but had little effect in nonlactating rats. Analyses of these data yielded significant main effects for hormone and for reproductive state. ($F(1,14)= 4.70$, $p< .05$; $F(1,14) = 6.95$, $p<.05$ respectively).

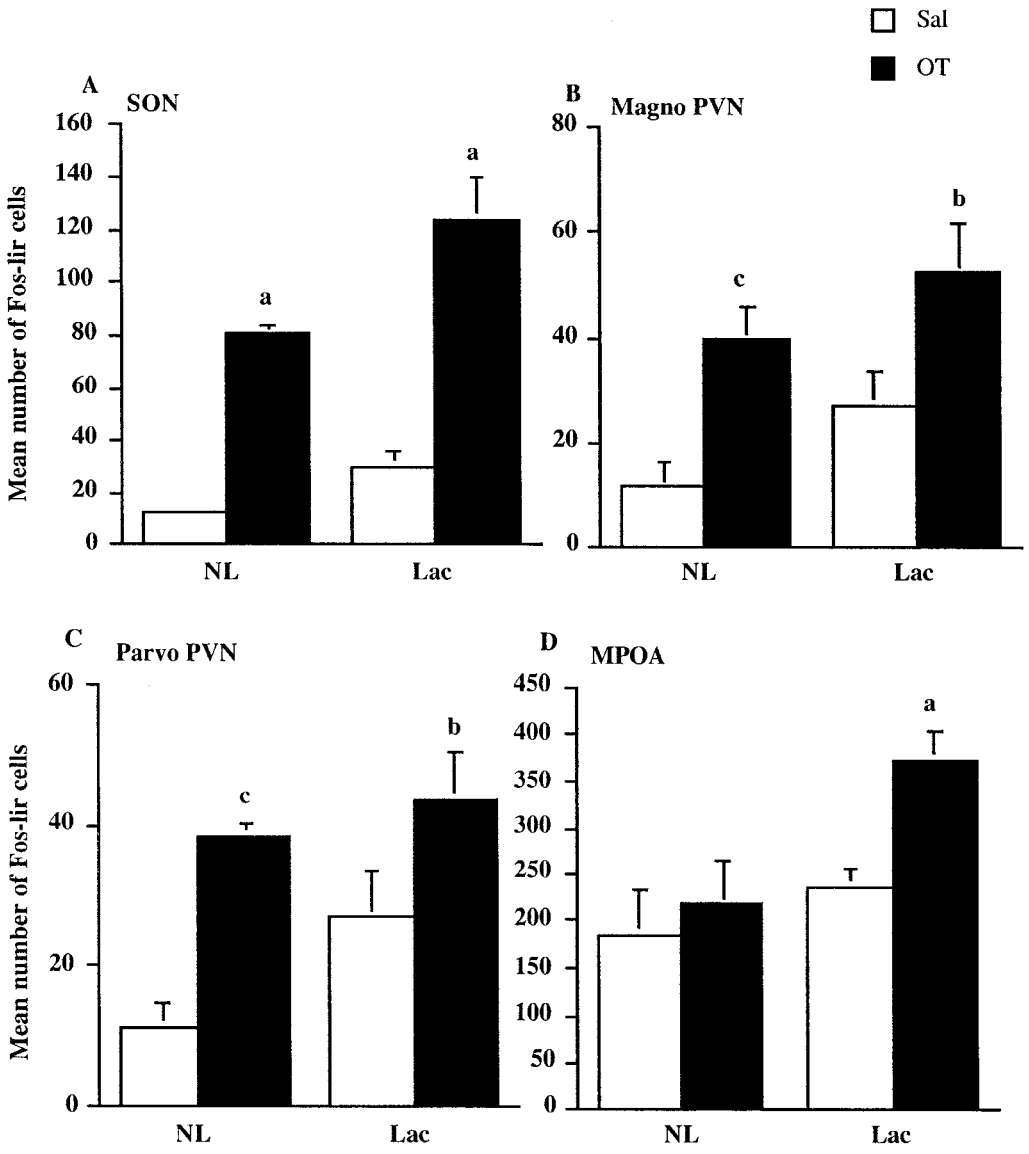

Figure 1: Effects of oxytocin treatment on the mean number of Fos-lir in the SON (panel A), magnocellular PVN (panel B), parvocellular PVN (panel C), and MPOA (panel D) of nonlactating and lactating rats. Oxytocin increased Fos-lir in nonlactating and lactating rats in the SON and both subdivisions of the PVN and only increased Fos expression in the MPOA of lactating rats. (a=significantly different from the other groups, $p<.05$; b= significantly different from NL-Sal and Lac-Sal, $p<.05$; c=significantly different from NL-Sal, $p<.05$)

Experiment 2: Effects of L-NAME on Fos induction in central oxytocin-stimulated lactating and nonlactating rats.

2a. Examples of staining for Fos-lir observed in the SON and PVN of lactating rats that received Sal-Sal, LN-Sal, Sal-OT, or LN-OT are shown in Figures 2 and 3. Counts of mean number of Fos-lir stained cells are shown in Figure 4.

The highest number of Fos-lir stained cells in the SON and both subdivisions of PVN was observed in rats that received L-NAME and oxytocin. Consistent with the results of Experiment 1, oxytocin infusion alone induced a robust Fos response in all three regions and this was augmented by administration of L-NAME prior to central oxytocin infusion in both the SON and parvocellular PVN. Analysis of these data with a 2x2 (Drug x Hormone) independent groups ANOVA showed a main effect for hormone in the SON, and magnocellular and parvocellular subdivisions of the PVN ($F(1, 14)=49.36$, $p<.05$; $F(1, 14)= 21.25$, $p<.05$; $F(1, 14)= 22.81$, $p<.05$; respectively) as well as a main effect for drug in the SON and parvocellular PVN ($F(1, 14)= 5.89$, $p<.05$; $F(1, 14)= 9.22$, $p<.05$; respectively). Subsequent *posthoc* comparisons (Fisher PLSD) revealed that the main effect for drug in the SON and PVN reflected an increase in Fos expression following the co-administration of L-NAME and oxytocin that did not occur when L-NAME was administered prior to saline infusion.

As in Experiment 1, oxytocin infusion increased Fos-lir expression in the MPOA ($F(1,15) = 16.27$, $p<0.05$) but there was no effect of L-NAME in this area (Sal-Sal, $M=205.6 \pm 24.98$; LN-Sal, $M= 212.85 \pm 20.12$; Sal-OT, $M=332.63 \pm 45.02$; LN-OT, $M= 326.24 \pm 29.82$). Similarly in the BNST, central oxytocin infusion increased Fos-lir relative to saline-treated rats ($F(1,15) = 55.01$, $p<0.05$) and L-NAME had no effect on Fos expression (Sal-Sal, $M=55.7 \pm 4.79$; LN-Sal, $M= 61.9 \pm 2.34$; Sal-OT, $M=111.6 \pm 11.58$; LN-OT, $M= 95.8 \pm 4.52$).

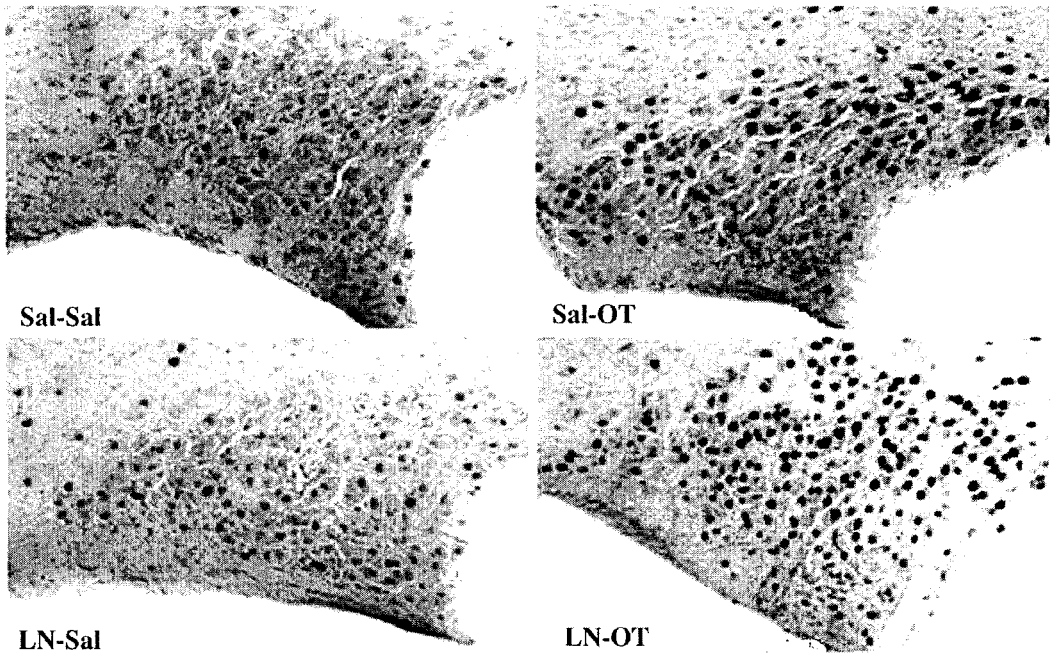

Figure 2: Example of Fos-lir in the SON of lactating rats that received either Saline (Sal-Sal), L-NAME and Saline (LN-Sal), Saline and Oxytocin (Sal-OT), or L-NAME and Oxytocin (LN-OT).

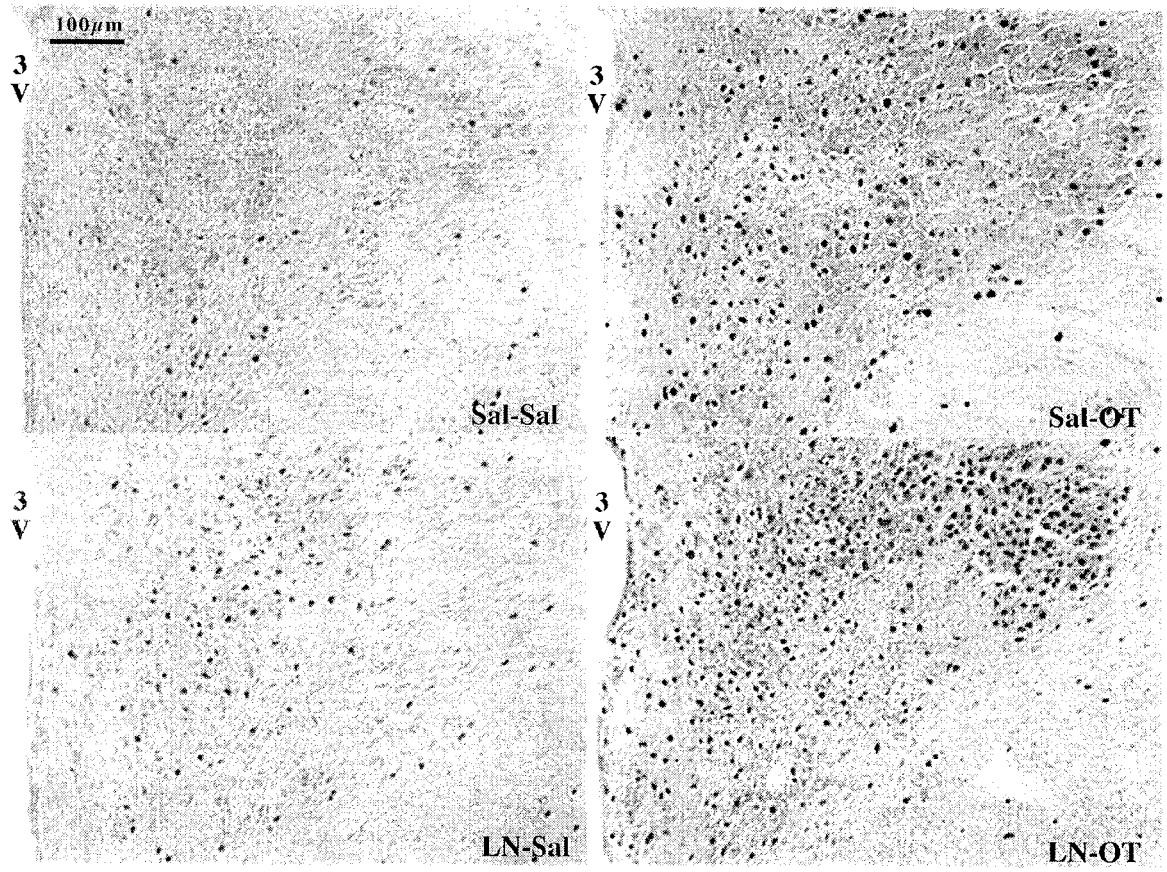

Figure 3: Example of Fos-lir in the PVN of lactating rats that received either Saline (Sal-Sal), L-NAME and Saline (LN-Sal), Saline and Oxytocin (Sal-OT), or L-NAME and Oxytocin (LN-OT).

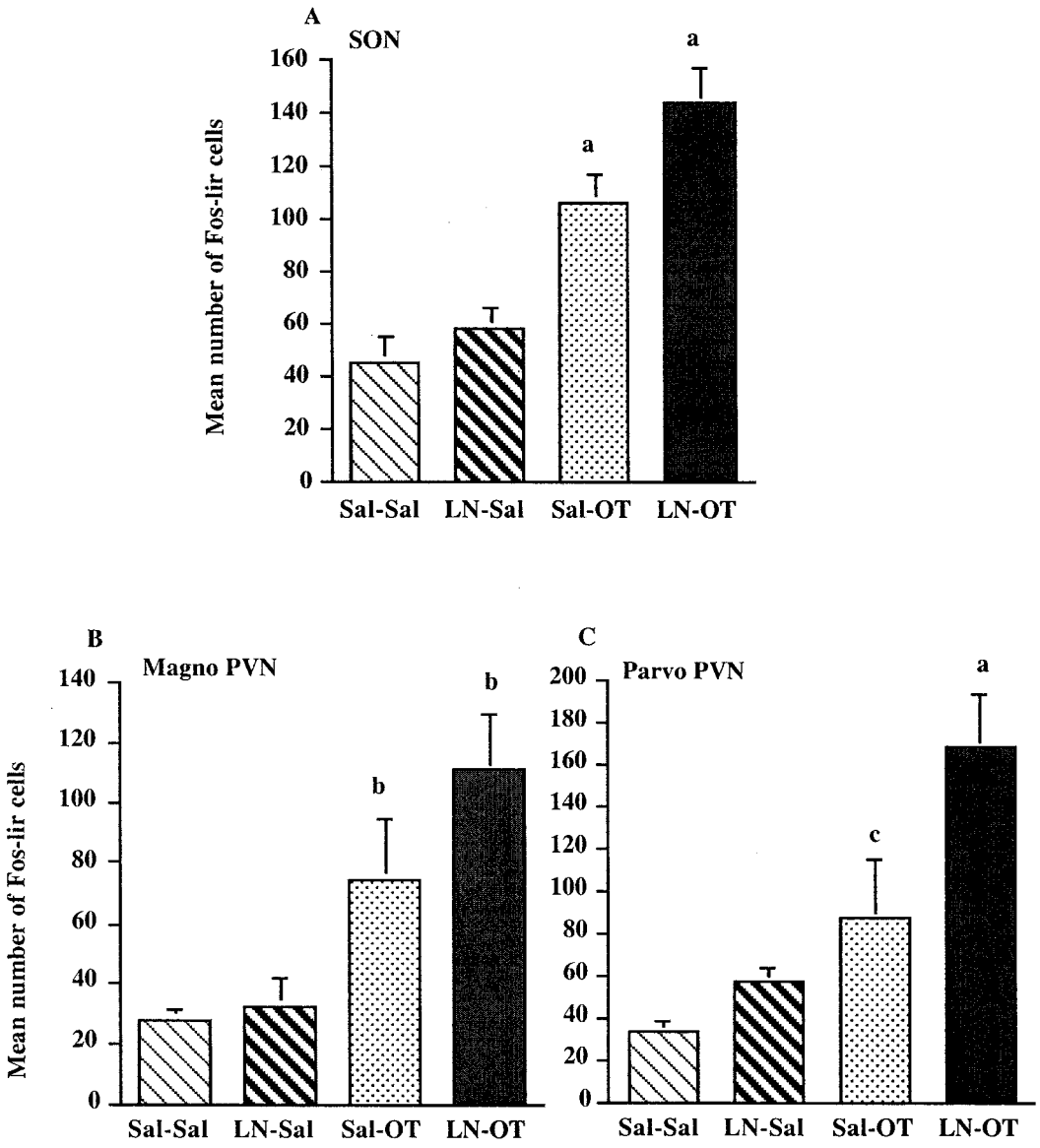

Figure 4: Effects of L-NAME prior to oxytocin treatment on the mean number of Fos-lir in the SON (panel A), magnocellular PVN (panel B), and parvocellular PVN (panel C) of lactating rats. L-NAME prior to oxytocin administration induced more Fos expression in the SON and parvocellular PVN but was not sufficient to significantly increase Fos-lir in the magnocellular PVN compared to Sal-OT-treated rats. (a=significantly different from the other groups, $p<.05$; b= significantly different from Sal-Sal and LN-Sal, $p<.05$; c=significantly different from Sal-Sal, $p<.05$).

2b. Examples of staining for Fos-lir observed in the SON and PVN of nonlactating rats that received Sal-Sal, L-NAME-Sal, Sal-OT or L-NAME-OT are shown in Figures 5 and 6. As seen in Figure 7, oxytocin induced Fos-lir in the SON and the magnocellular and parvocellular PVN in nonlactating rats (significant main effect of hormone $F(1,19)=7.83$, $p<.05$; $F(1,19)=22.61$, $p<.05$; $F(1,19)=11.23$, $p<.05$; respectively). L-NAME administered prior to central oxytocin infusion tended to reduce Fos-lir in both the magnocellular PVN and the SON but did not affect Fos-lir when administered before saline infusion (Drug x Hormone interaction: magnocellular PVN $F(1,19)=4.76$, $p<.05$; SON $F(1,19) = 2.88$, $p=0.10$). There was no main effect for drug in any of the areas examined.

Experiment 3: Effects of L-NAME on Fos induction in urethane-stimulated nonlactating and lactating rats.

Counts of mean number of Fos-lir stained cells for all groups in this experiment are shown in Figure 8. Overall, urethane administration induced more Fos-lir in the SON and magnocellular and parvocellular PVN in nonlactating than in lactating rats. This was reflected in a significant main effect for reproductive state in all the areas examined (SON $F(1, 18)= 37.91$, $p<.05$; magno-PVN $F(1, 17)= 17.64$; parvo-PVN $F(1,17)= 17.48$, $p<.05$). In addition, L-NAME administration reduced Fos-lir in these areas giving rise to a significant main effect for drug in the SON and magnocellular and parvocellular subdivisions of the PVN ($F(1, 18) = 9.75$, $p<.05$; $F(1,17) = 6.59$, $p<.05$; $F(1,17) = 4.13$, $p=.06$; respectively). In the magnocellular and parvocellular subdivisions. of the PVN, L-NAME administered before urethane reduced Fos-lir in nonlactating rats but not in lactating rats (significant Drug x Reproductive state interaction; magno-PVN $F(1,17)=5.69$, $p<.05$; parvo-PVN $F(1,17)=4.41$, $p=.05$). This effect was not seen in the SON.

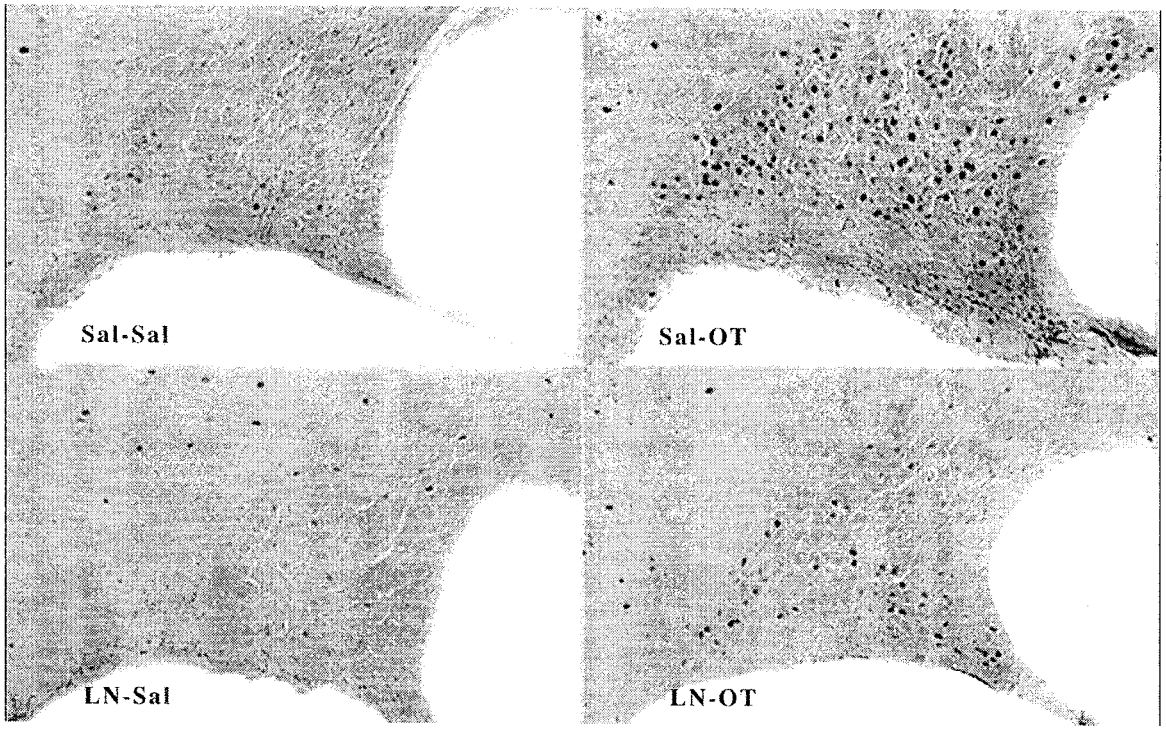

Figure 5: Example of Fos-lir in the SON of nonlactating rats that received either Saline (Sal-Sal), L-NAME and Saline (LN-Sal), Saline and Oxytocin (Sal-OT), or L-NAME and Oxytocin (LN-OT).

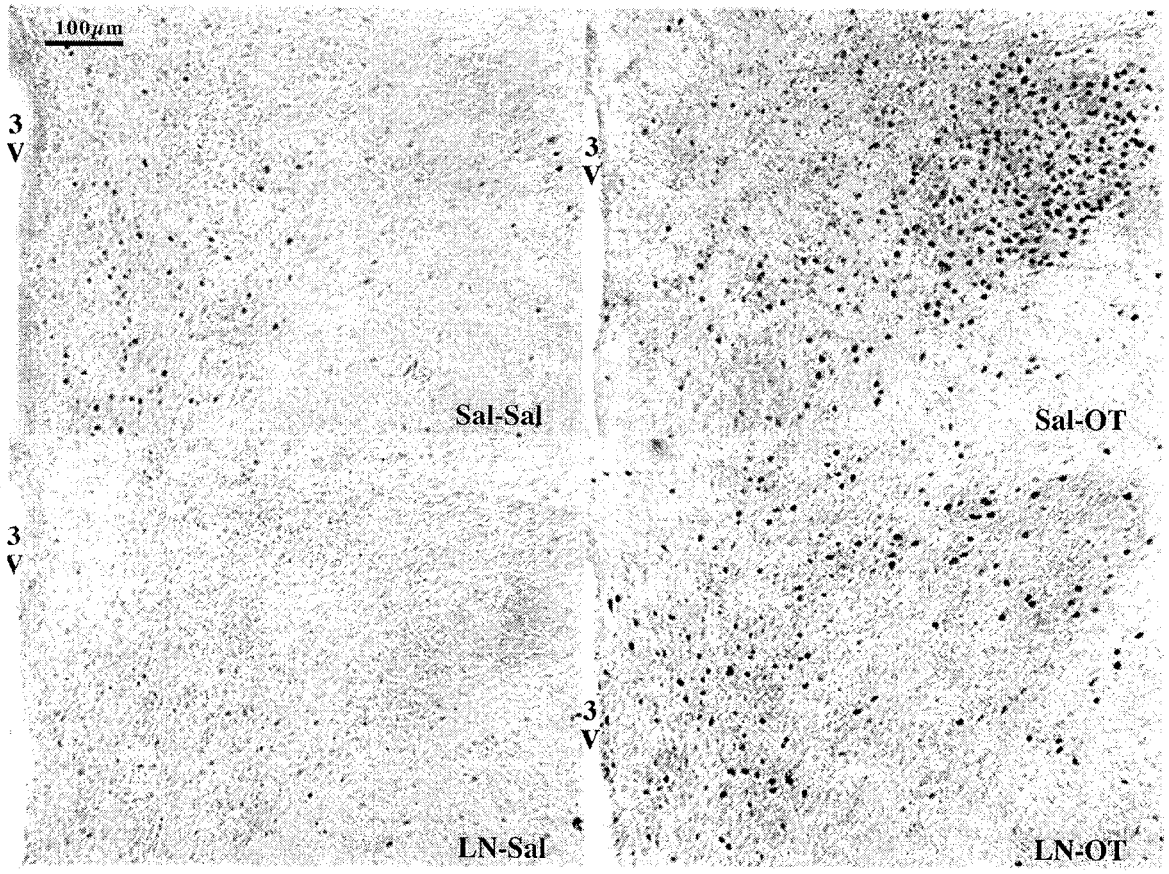

Figure 6: Example of Fos-lir in the PVN of nonlactating rats that received either Saline (Sal-Sal), L-NAME and Saline (LN-Sal), Saline and Oxytocin (Sal-OT), or L-NAME and Oxytocin (LN-OT).

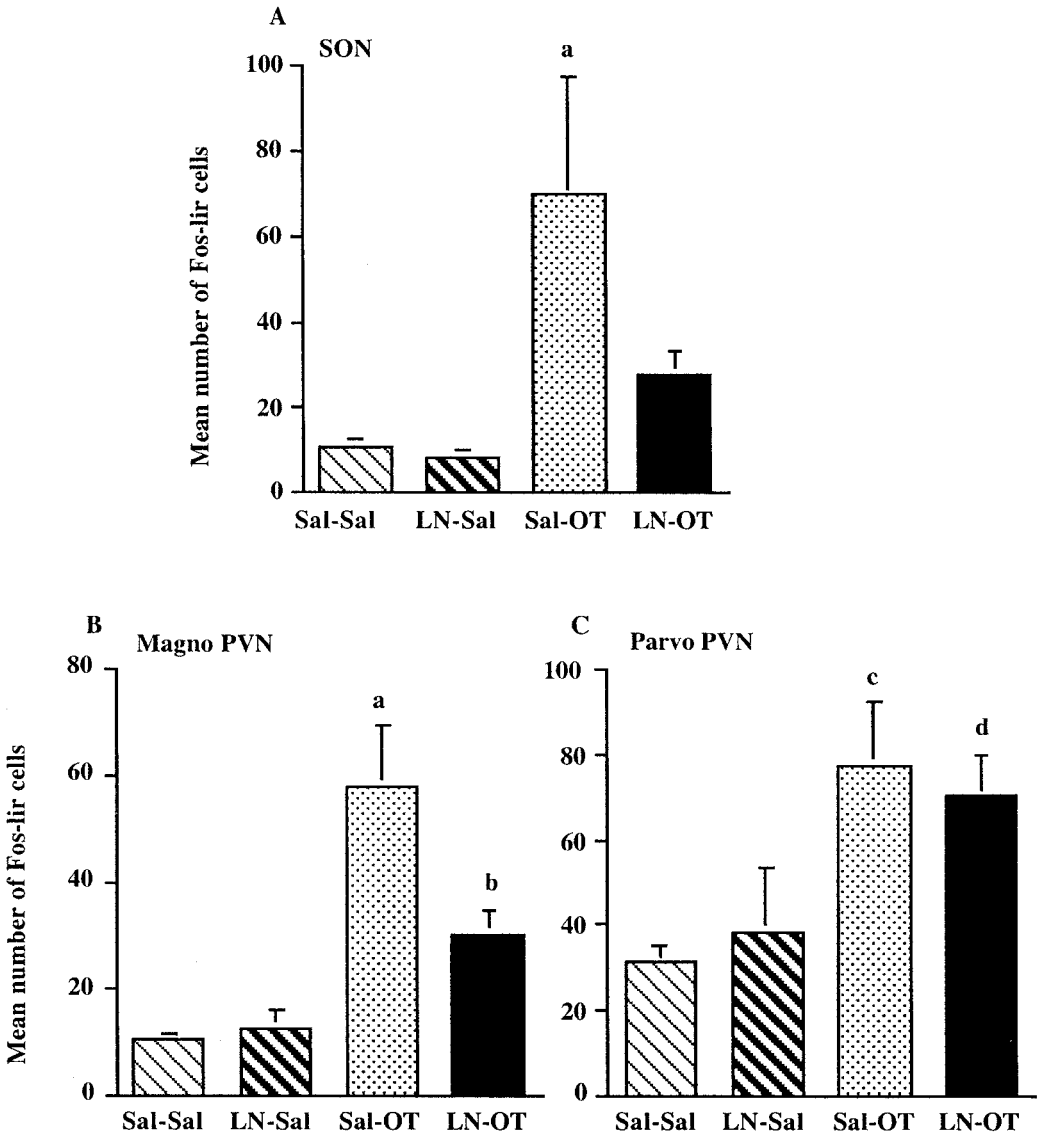

Figure 7. Effects of L-NAME prior to oxytocin treatment on the mean number of Fos-lir in the SON (panel A), magnocellular PVN (panel B), and parvocellular PVN (panel C) of nonlactating rats. L-NAME prior to oxytocin administration reduced Fos expression in the SON and magnocellular PVN but was not sufficient to significantly attenuate Fos-lir in the parvocellular PVN compared to Sal-OT-treated rats. (a=significantly different from the other groups, $p<.05$; b= significantly different from Sal-Sal and Sal-OT, $p<.05$; c=significantly different from Sal-Sal and LN-Sal, $p<.05$; d=significantly different from Sal-Sal, $p<.05$).

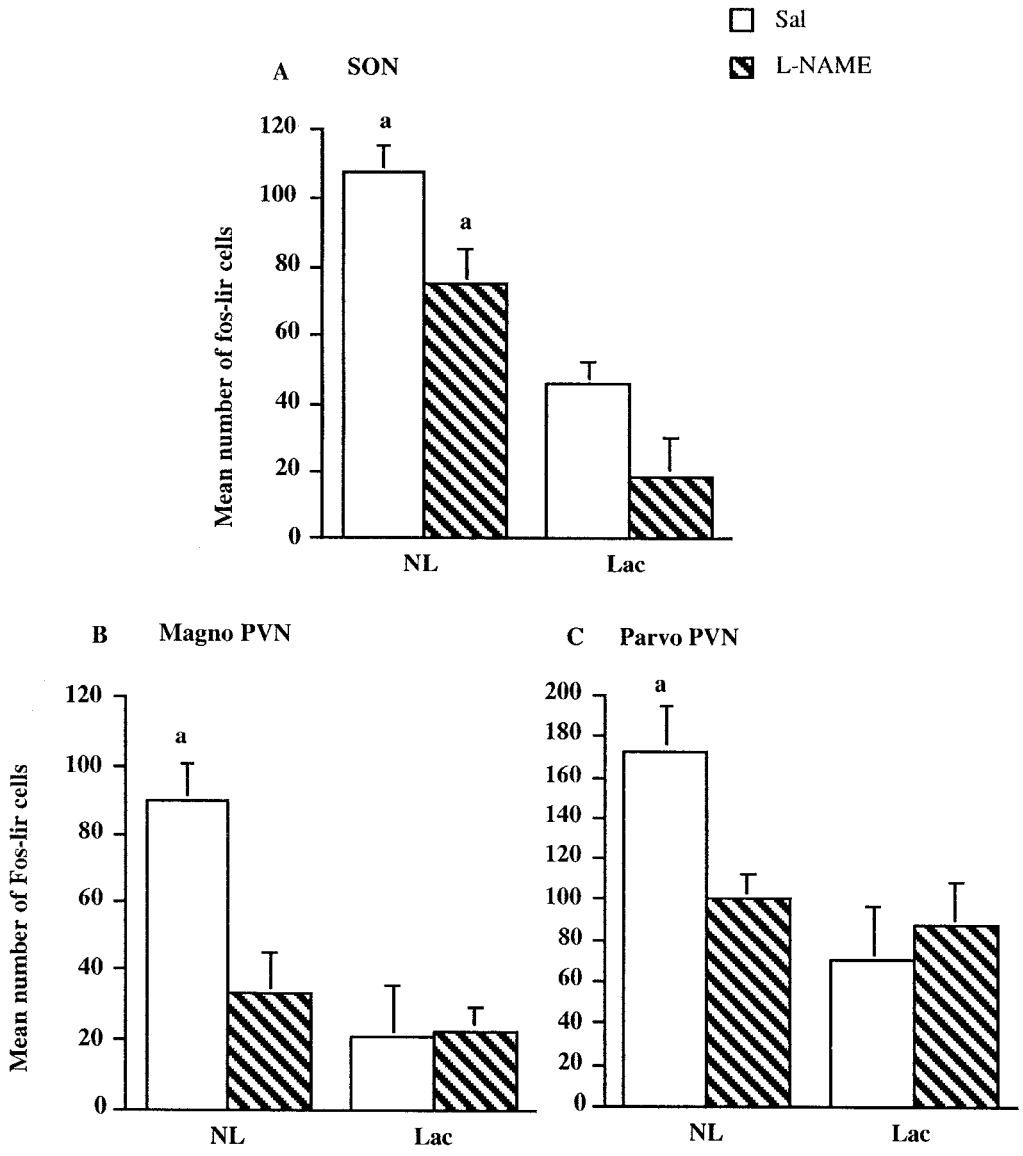

Figure 8. Effects of L-NAME prior to urethane stimulation on the mean number of Fos-lir in the SON (panel A), magnocellular PVN (panel B), and parvocellular PVN (panel C) of nonlactating and lactating rats. L-NAME prior to urethane administration had no affect on Fos expression in any of the brain areas examined of lactating rats but reduced Fos-lir in the SON and both subdivisions of the PVN in nonlactating rats compared to saline-urethane treated rats. (a=significantly different from the other groups, $p<.05$).

Discussion

The results of Experiment 1 showed that central oxytocin administration induced Fos-lir in the SON, and magnocellular and parvocellular subdivisions of the PVN. In the SON, this effect was greater in lactating than in nonlactating rats. Oxytocin administration also increased Fos-lir in the MPOA, but only in lactating rats. In Experiment 2, inhibiting NO synthesis prior to oxytocin administration increased Fos-lir in the SON and PVN of lactating rats but attenuated Fos-lir in these areas in nonlactating rats. The induction of Fos-lir in the MPOA and BNST by oxytocin administration was not affected by L-NAME. In Experiment 3, L-NAME treatment also reduced Fos-lir following urethane administration in nonlactating rats but, with the exception of the SON, there was little effect of combining L-NAME and urethane treatment on Fos expression in lactating rats when compared to the effect of urethane administration alone. The latter data suggest that the ability of L-NAME administration to augment the activational effect of oxytocin in some hypothalamic nuclei of lactating rats does not generalize to all stimuli capable of inducing Fos in these areas.

The neuronal activation induced in the SON and PVN by central oxytocin stimulation, is consistent with a body of evidence demonstrating that central oxytocin administration modulates both behavioural and physiological processes. Given the presence of oxytocin receptors on oxytocin neurones in the SON and PVN (Freund-Mercier et al., 1994), it is reasonable to suppose that at least a portion of the cells showing Fos-lir after oxytocin treatment are oxytocinergic but it is also possible that the activity of nonoxytocinergic cells within the PVN and SON is modulated by central oxytocin infusion. Colocalization studies are needed to describe the phenotype of the neurones in which Fos-lir is induced by oxytocin administration both in magnocellular neurones and in parvocellular PVN where oxytocin neurones are more sparse (Swanson & Sawchenko, 1983). It is also possible that activation of cells within the PVN and SON following oxytocin administration was produced indirectly by oxytocin acting at distal brain sites.

Neurones in the BNST, for example, project to the PVN (Sawchenko & Swanson, 1983), possess oxytocin binding sites (Yoshimura et al., 1993) and are excited following central injections of oxytocin in lactating rats (Ingram & Wakerley, 1993) and thus are one indirect pathway through which central oxytocin administration might activate the PVN and SON.

There was a tendency for oxytocin treatment to induce more Fos-lir in both the SON and PVN of lactating than of nonlactating rats but this effect was most marked in the SON. Central oxytocin administration was also effective in inducing Fos-lir in the MPOA and BNST, but only in lactating rats. These results could be a reflection of the greater density of oxytocin receptors in the hypothalamus during lactation (Insel, 1986). The fact that the Fos response to oxytocin was greater or at least equivalent in the SON and PVN of lactating rats than of nonlactating rats is interesting. Lactating rats have been shown to be hyporesponsive to a variety of stimuli such as immobilization (da Costa et al., 1996), ether stress (Walker et al., 1992), and dehydration (Fenelon, Poulain, & Theodosis, 1993). Moreover, the attenuated response to these stimuli shown by lactating rats is seen both as a reduction in Fos expression in the PVN and SON and as diminished pituitary hormone release including a reduction in oxytocin release (Lightman & Young, 1989). The data obtained from the urethane/saline treated groups in Experiment 3 are an example of such an effect.

The differential responsivity of lactating rats to these two types of stimulation may reflect the fact that urethane administration or immobilization stress initiate a cascade of events; only one step of which is to stimulate oxytocin neurones in the PVN and SON. An attenuation of the response to these stimuli could occur at any of a number of sites along this cascade. Central oxytocin administration, on the other hand, represents a much more specific stimulus which may be acting directly on neurones within the PVN and SON. The results of the current experiment show that the responsiveness of neurones in these areas to oxytocin is not blunted by lactation and thus that the attenuated response to other stimuli

such as urethane observed during lactation results from hyporesponsiveness at an earlier step in the response cascade.

Central oxytocin does not always result in the induction of Fos-lir in the PVN and SON of lactating rats. Persistent suckling, which is accompanied by intranuclear oxytocin release does not stimulate Fos expression in the SON (Fenelon et al., 1993) although an increase in Fos expression in this area is seen when mother and young are reunited after a period of separation (Li, Chen, & Smith, 1999). If, as has been suggested, Fos is typically expressed transiently at the onset of stimulation (Morgan & Curran, 1989) and acts to suppress its own subsequent induction, any chronic stimulus such as suckling and the associated oxytocin release, would not induce Fos expression. It is interesting then that this chronic stimulation does not preclude the ability of exogenous oxytocin to induce Fos-lir.

Results from Experiment 2, demonstrated that the effect of inhibiting NO synthesis prior to central oxytocin infusion on Fos expression depended on the reproductive state of the rat and the brain area examined. The administration of L-NAME and oxytocin to lactating rats enhanced the expression of Fos-lir compared to Sal-OT-treated rats. Nonlactating rats, on the other hand, showed a reduction of Fos-lir in the hypothalamic nuclei when treated with L-NAME and oxytocin than when treated with oxytocin alone. These data therefore suggest that in lactation, NO acts to suppress the responsivity of neurones in the PVN and SON to central oxytocin administration, whereas it facilitates the neural response of nonlactating females to this same stimulus.

These results are consistent with the hypothesis that NO, which is upregulated in lactating (Popeski et al., 1999), dehydrated (Ueta et al., 1995), and salt-loaded rats (Villar et al., 1994), plays a negative feedback role in the HNS when this system is chronically stimulated (Kadowaki et al., 1994). This idea is also supported by the results of previous studies showing that L-NAME administration enhances oxytocin release from the pituitary in dehydrated and salt-loaded rats (Summy-long et al., 1993; Kadowaki et al., 1994). The latter data suggest that at least part of the increased neuronal activation seen in Experiment 2

when L-NAME is co-administered with oxytocin is associated with oxytocin neurosecretory activity.

It has recently been reported that the Fos expression seen in the SON and PVN in suckled females when reunited with their litters after a 12h separation was attenuated following administration of a NO donor (Okere, Kaba, Seto, & Higuchi, 1999). Thus, apparently, NO can function to suppress neuronal activation in response to acute suckling stimulation. Given that suckling stimulation would be associated with an increase in endogenous oxytocin release within the PVN and SON, these data are consistent with the results obtained here in lactating rats. It should be noted, however, that we saw little effect of L-NAME administration alone on Fos-lir perhaps because in the current studies lactating rats were suckled throughout the experiment.

The fact that L-NAME administration also increased Fos-lir in the parvocellular part of the PVN suggests that the ability of NO to suppress neuronal activation following oxytocin administration is not limited to the HNS. It is well established that NADPH-d and NOS mRNA are colocalized with corticotropin releasing hormone (CRH) in the parvocellular region of the PVN (Siaud et al., 1994) and oxytocin neurones are also present in this region of the hypothalamus (Swanson & Sawchenko, 1983). Future colocalization studies will allow us to determine which neuronal populations are affected by L-NAME administration. The fact that Fos-lir in the MPOA and the BNST in response to central oxytocin administration was not affected by pre-treatment with L-NAME suggests site specificity in the ability of NO to modulate the effect of OT on neuronal activity. In addition, these data suggest that the modulation of the response to OT that we see in the PVN and SON is not mediated by an effect in the BNST.

As shown in Experiment 3, the facilitatory effect of inhibiting NOS on Fos-lir in response to oxytocin treatment in lactating rats did not generalize to urethane administration. Fos-lir expression in neither the SON nor in magnocellular and parvocellular subdivisions of the PVN in urethane-stimulated lactating rats was affected by L-NAME treatment. In the

nonlactating rats, on the other hand, administration of L-NAME reduced Fos expression in the SON and magnocellular and parvocellular subdivisions of the PVN. The latter effect is consistent with the results of Experiment 2, and with other studies which have shown that inhibiting NOS decreases the hypothalamic-pituitary adrenal response to various stressors (Amir et al., 1997) and suggest that NO has a facilitative affect on stress-induced Fos immunoreactivity in nonlactating rats. Interestingly, however, a recent report shows that both the Fos immunoreactivity and oxytocin release seen following hypertonic saline injections in virgin rats are increased following NOS inhibition (Srisawat et al., 2000). Since in the latter study the rats were anaesthetised with sodium pentobarbitone, it may be that the state of GABA activation is a critical factor in determining the modulating effects of NO in this system.

In sum, these studies reveal that central administration of oxytocin induces Fos expression in discrete regions of the hypothalamus in lactating and nonlactating rats. The response to oxytocin is greater overall in lactating than in nonlactating rats and is differentially modulated by NO as a function of reproductive state. Furthermore, the effect of inhibiting NO production on Fos expression across reproductive state varies across stimuli. While the results of these studies demonstrate NO modulation of the response to central oxytocin administration and to other stimuli the specific target at which NO produces its effects remain to be addressed.

Chapter 4

In rats, caring for young involves pup-directed behaviors such as licking and grooming, retrieval, and nursing, together with nest building and an increased willingness on the part of the female to attack intruders when introduced into the nest site. Maternal care also entails changes in food and fluid intake as well as metabolism that combine to meet the energetic demands of milk production. These behavioral and physiological adaptations are associated with a wide array of neural and neurochemical changes. Among these changes is an increase in nitric oxide synthase (NOS) within the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus (Popeski et al.,1999; Otukonyong et al., 2000). Nitric oxide synthase is the enzyme responsible for the oxidation of L-arginine into citrulline and the gaseous neurotransmitter nitric oxide (NO). In the studies described below, the functional significance of this increase during lactation was investigated by examining the effect of central inhibition of NO production by administration of the NOS inhibitor N(G)-nitro-$_L$-arginine methyl ester (L-NAME) on various parameters of maternal behavior.

Manipulating NO production either by administration of the NO precursor L-arginine or a NOS inhibitor has been shown to modulate sexual behavior (Benelli et al., 1995), inter-male aggression (Demas et al., 1997), and maternal aggression (Gammie et al., 2000). In addition, NO has been implicated in a variety of physiological processes including vasodilation (Radomski & Moncada, 1993), neuroplasticity (Beagley & Cobbett, 1997; Ramsell & Cobbett, 1996) and neuroendocrine function (McCann, 1996). Within the PVN and SON, NOS colocalizes with corticotropin releasing hormone (Siaud

et al., 1994), oxytocin (OT) (Miyagawa et al. 1994) and vasopressin (Sanchez et al., 1994) and is upregulated under other conditions that, like lactation, are associated with activation of the oxytocinergic system (Kadowaki et al., 1994). Moreover, inhibition of NO production has been shown to increase OT release in stimulated conditions (Summy-Long et al., 1993). Together these results suggest an intimate functional relationship between NO and OT release. Given that milk-ejection is dependent on the bolus release of OT, the role of the increased capacity for NO production during lactation in the timing of milk-ejections was examined. Milk ejections only occur within a complex set of interactions between mothers and their litters; thus several indices of maternal behavior including maternal aggression were also measured in these studies.

Although, rat pups remain wholly dependent on their mother for nourishment for at least the first two weeks postpartum, the behavioral capacities of rat pups develop rapidly during this period. Patterns of maternal care and behavior during this time also change to match the requirements of their young. Thus, the effects of central NO inhibition on milk-ejection and maternal behavior were assessed on both Day 10 and Day 4 postpartum (Experiments 1a and 1b, respectively). Finally, because changes in the effects of NO on maternal behavior might reflect either differential stimulus properties of the pups or differences in the maternal state, in the final study of this series, the effect of L-NAME treatment on the behavior of Day 10 mothers exposed to 4-day old and 10-day old pups was compared (Experiment 1c).

Lesions of the medial preoptic area/ ventral bed nucleus of the stria terminalis (MPOA/vBNST)(Jacobson, Terkel, Gorski, & Sawyer, 1980; Numan, 1996), medial amygdala (MeA) (Lee et al., 2000) periacqueductal gray (PAG) (Lonstein & Stern,

1997), and nucleus accumbens (NAcc) (Lee et al., 2000) have all been shown to produce deficits in maternal behavior. Damage to some of these sites has been shown to have very specific effects on one aspect of behavior. For example, lesioning the PAG reduces the female's ability to adopt the high crouch typical of nursing mothers (Lonstein & Stern, 1997). Damage to other areas however, produces more widespread deficits. MPOA lesions, for example, prevents both the onset of maternal behavior and disrupts ongoing maternal behavior (Lee et al., 2000; Oxley & Fleming, 2000). These brain areas have also been implicated in maternal responsiveness by studies showing that the performance of maternal behavior increases neural activity within these regions as reflected in an increased induction of the protein product of the immediate early gene c-Fos (Numan & Numan, 1994; Numan & Numan, 1997). To gain some insight into where NOS inhibition might be affecting maternal behavior, Fos induction, within several brain regions that regulate maternal responsiveness was compared between Saline and L-NAME treated females in two situations: following a maternal behavior and aggression test on Day 10 pp. (Experiment 2a) and following exposure to an anaesthetised male rat (Experiment 2b).

Methods

Animals

Virgin female Wistar rats obtained from Charles River Breeding Farm, (St. Constant, Quebec) were used in these studies. Upon arrival in the laboratory, the animals weighed between 220-240 g and were housed in groups of five females. The animal facility was maintained on a 12/12hr light/dark cycle (lights on from 0800-2000hr) at 20$\pm$ 2 ^{0}C. Rats were given ad libitum access to lab chow and to water throughout the

experiment. In Experiment 1a,1c,and 2 rats were mated by placing one male rat in a group cage with five females for 2 weeks. Approximately, three days before parturition, each pregnant female was removed from the group cage and placed in a single cage (20 x 45 x 25 cm) with adlib food and water. In Experiments1b 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 observed in the vaginal smear. This day was designated Day 1 of pregnancy. 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 (pp.), litters were culled to eight pups. All protocols were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Surgery

Cannula implantation: A stainless steel cannula (22 gauge; Plastic One Products) was inserted stereotaxically into the third ventricle (AP: - .90 mm, L: 0, DV: - 6.4 mm below dura (Paxinos & Watson, 1986)) under ketamine/xylazine anesthesia (5.7 mg ketamine and 0.86 mg xylazine/100 g of body weight) on Day 1 pp. in Experiment 1 and before mating in Experiments 1b,1c, and 2a.

Injection procedure

Rats were handled daily prior to and after surgery. Seven days post surgery each subject received an intracerebroventricular injection of angiotensin (50 ng in 2 μl). If the injection of angiotensin stimulated water intake, then the cannula placement was considered accurate. To minimize potential stress effects on behavior and Fos expression,

two days before the experimental day, animals were habituated to the injection procedure for 2 minutes each day.

Immunocytochemistry

Each animal was injected with an overdose of sodium pentobarbitol and perfused transcardially with 200 ml of ice-cold saline, followed by 300 ml of 4% paraformaldehyde. Brains were post fixed in a 30% sucrose- 4% paraformaldehyde solution for 48hrs. Forty-micron thick sections throughout the vBNST, MPOA, PVN, paraventricular thalamic nucleus (PVT), and MeA were cut on a cryostat and stored at –80 ^{0}C until processed for Fos-like immunoreactivity (Fos-lir). The sections were incubated for 30 minutes in 3% H2O2 solution in TBS to reduce nonspecific staining and then washed in TBS 3 times for 15 minutes. Tissue was then incubated for 90 minutes at 4 ^{0}C in blocking serum (0.3% Triton X (TTX, Sigma), 3% normal goat serum (NGS, Vector), and TBS) and then incubated in the primary antibody solution (Fos antibody, Ab-5, PC-38 (Oncogene Research Products, MA) diluted 1: 130, 000). Forty-eight hours later, sections were washed 3 times in TBS for 15 minutes and then were incubated in secondary antibody (biotinylated rabbit anti-goat, Vector) for 1 hour, washed 3 times in TBS and incubated in ABC reagent for 2 hours. Tissue staining was developed using a ABC vector kit (Diminobenzene, nickel intensified). The sections were then mounted on gelatin slides and cover slipped (Permount, Fisher) for analysis.

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 (G4). Fos-lir cells were counted from the images captured in the TIFF picture files. To obtain an estimate of Fos expression in the vBNST and MPOA, stained cells in sections from approximately -.80 mm to -.92 mm from bregma (Paxinos and Watson, 1986) were counted and an average of these sections was calculated. For the PVN, only sections in the medial area of the nucleus (-1.80 mm from bregma) were used and an average over all sections was obtained. To obtain an estimate of the number of Fos-lir in the MeA and PVT (-2.56 mm to -2.80 mm from bregma), stained cells were counted and the mean was calculated. To identify stained cells; sections from each experimental group across each assay that appeared to be stained were selected and the relative density of each cell was recorded. An average of the density of the cells was then calculated to establish the criterion. Furthermore, the investigator was blind to the experimental groups when counting cells.

Experiments

Experiment 1a

Lactating female rats (Day 10 pp.) were separated from their pups for 2 hours. Thirty minutes before mothers and their pups were reunited, L-NAME (250μg/5μl; n=11) or Saline (5μl; n=10) was administered into the third ventricle of the female rats. Pups were scattered throughout the cage and mother-litter interactions were videotaped for one hour. Subsequently, a male intruder was introduced into the cage with the mother and her pups and this interaction was recorded for 10 minutes or until the first attack on the male.

Females were measured on several indices of maternal behavior including: first contact of pup, retrieval, anogenital licking, time to first quiescence, time to first milk-ejection, intermilk-ejection interval, number of milk-ejections, time spent with pups, and number and duration of nestbouts. In addition, time to contact the intruder, time spent sniffing the intruder, and whether or not the female showed aggression towards the intruder was measured.

Experiment 1b

The behavioral paradigm used in this experiment was the same as Experiment 1; however, female rats were tested on Day 4 pp. in this experiment. Thirty minutes before mothers and their pups were reunited, either L-NAME (250g/5μl;n=8), D-NAME (250g/5μl;n=5), an inactive isomer of L-NAME or Saline (5μl;n=8) was administered into the third ventricle of the female rats. Further, to determine whether L-NAME impaired any general oromotor capabilities, tootsie rolls were placed inside the cage and retrieval of candy was measured for the L-NAME and Saline-treated females.

Experiment 1c

Day 10 pp. female rats were separated from their pups and 90 minutes later female rats received an icv injection of L-NAME (250μg/5l) or 5μl of Saline. Thirty minutes later, female rats (Day 10 pp.) were reunited with 4-day old pups and their interactions were videotaped for thirty minutes. Thus 2 groups were formed:1) Saline Day 10 with Day 4 pup (Saline10/4pup, n=7); 2) L-NAME Day 10 with Day 4 pup (L-NAME 10/4 pup; n=6). Following the videotaped session, female rats were separated from all pups for fifteen minutes and then were presented with their own 10 day old pups (Saline10/10pup; L-NAME 10/10pup).

Experiment 2a

Brain sections from Day 10 pp. dams from Experiment 1 were processed for Fos-lir one hour following the aggression test. Thus two groups were analysed for Fos-lir: 1) Saline (n=7) and 2) L-NAME (n=6).

Experiment 2b

Two days before the test day, each lactating female (Day 10 pp.) was habituated to the test box (48 cm x 78 cm x 102 cm). On the test day, lactating females received either an icv injection of L-NAME (250μg/5μl) or Saline (5μl). Thirty minutes later, each female with her pups was placed in the test box and was exposed to either an anaesthetized male or to the empty box for 10 min. One hour following this test, brains were processed for Fos-lir. Thus four groups were formed: 1) Sal/male (n=5); 2) Saline/emptybox (n=4); 3) L-NAME/male (n=5); 4) L-NAME/emptybox (n=4).

Statistics

In Experiments 1a,1c, and 2a, Students t test was used to compare behavior and Fos-lir between groups. In Experiment 1b and 2b, one-way Analysis of Variance or Chi Square test for independence were used to analyze behavior. The Kolmogorov-Smirnov two-sample test was used to compare the two distributions that described the nest bout data in Experiment 1a. Fishers exact probability test was used to analyze discrete data in Experiments 1a and 1c.

Results

Experiment 1a

Results from this experiment revealed that L-NAME-treated rats took longer to first contact their pups and had fewer milk-ejections than Saline-treated rats ($t(18)=-2.18$, $p<.05$; $t(19)=2.14$, $p<.05$);respectively (Figure 1a,b). Although not significant, Figure 1(c,d) illustrates that L-NAME-treated females tended to have a more nest bouts ($t(18)=-1.57$, $p=.14$) relative to Saline-treated females. In addition, the duration of the nest bouts for L-NAME treated females were significantly shorter compared to females treated with Saline ($p<0.01$).

As can be seen in Table 1, there was no significant difference between these two groups on several indices of maternal behavior including: time to first quiescence, time from quiescence to first milk-ejection, inter-milk-ejection interval, time spent with pups, and anogenital licking.

There was a tendency for L-NAME-treated females to take longer to contact the male intruder than Saline treated females ($t(19)=-1.58$, $p>.05$)(Figure 2a). Consistent with previous data reported in other species, females that were treated with a NOS inhibitor showed less aggression toward the intruder (27%) compared to Saline- treated females (90%)($p<.05$) even though the females did not differ in the time spent sniffing the intruder male ($t(19)=.10$, $p>.05$)(Figure 2b,c).

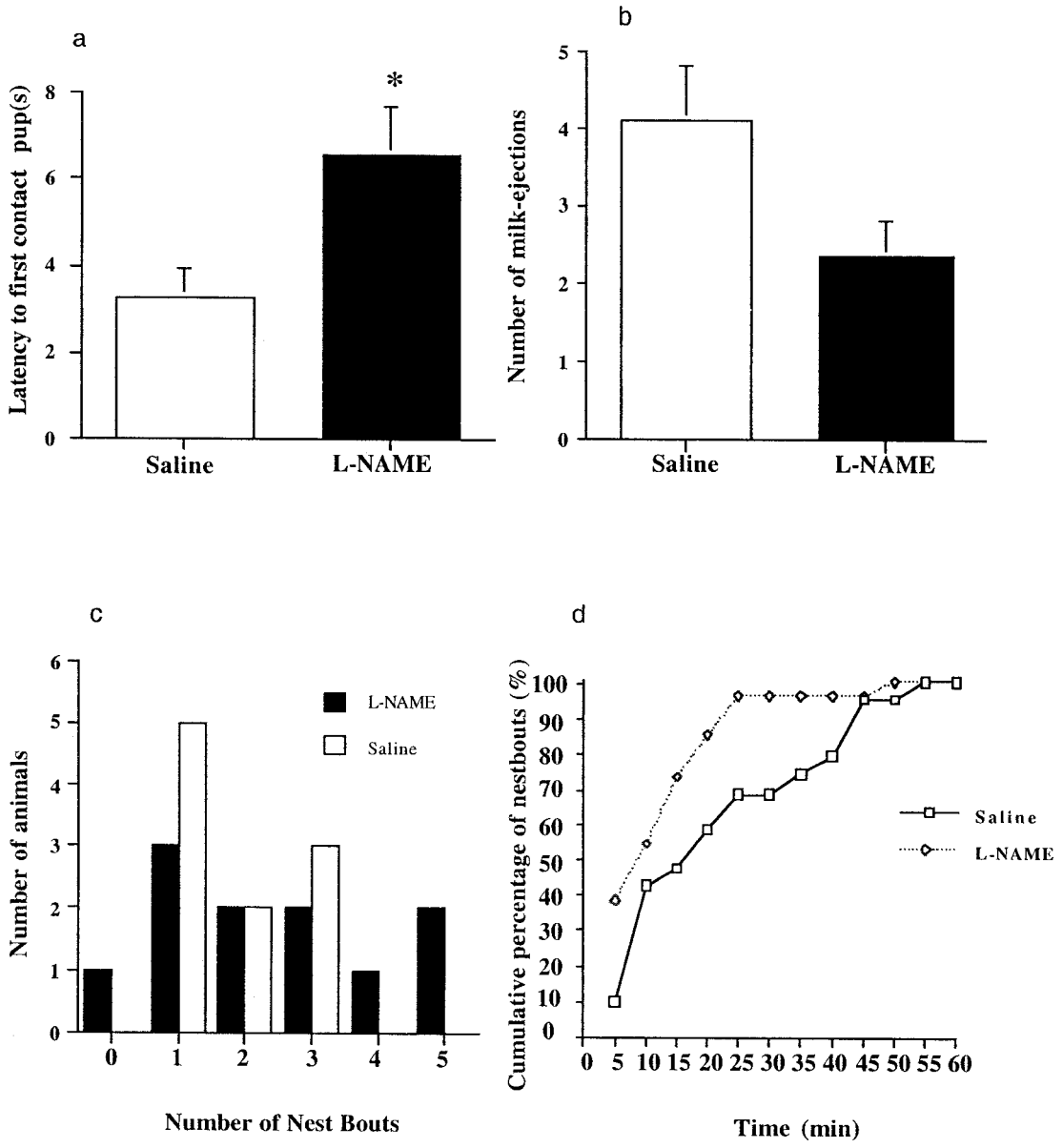

Figure 1. L-NAME impairs the expression of maternal behavior in Day 10 pp. rats. L-NAME treated females (n=11) took longer to contact the first pup (a), had fewer milk-ejections (b), tended to have more nestbouts (c) and spent less time in a nestbout (d) than Saline-treated rats (n==10).

Table 1

Average Time in Seconds of Several Indices of Maternal Behavior as a Function of Drug

	Saline		L-NAME	
Behavior (s)	M	SD	M	SD
Time to first quiescence	1158.6	176.5	1071.6	107.8
Quiescence to 1st ME	401.9	61.9	281.2	62.8
Inter-ME interval	498.9	83.3	617.0	207.4
Total time spent with pups	1843.3	244.3	1208.3	280.3
Anogenital licking	139.0	29.7	153.6	29.2

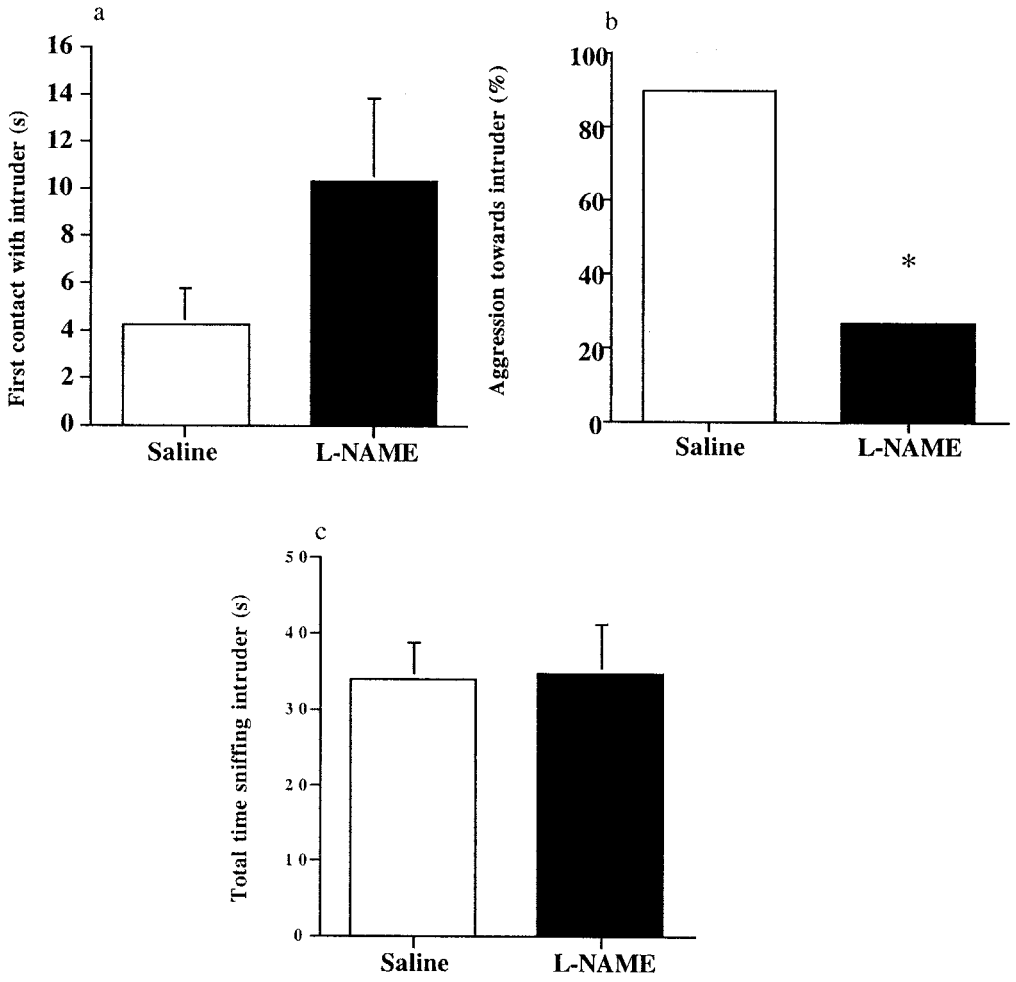

Figure 2. L-NAME impairs the expression of maternal aggression in Day 10 pp. rats. Using a male intruder test, lactating females treated with L-NAME tended to take longer to contact the male (a), and fewer females aggressed towards the male (b). No differences were observed between the two groups in terms of average duration spent sniffing the intruder (c). Bars represent $\pm$ S.E. * $p<.05$.

Experiment 1b

As is shown in Figure 3a, Day 4 pp. L-NAME-treated females took longer to contact their pups than Saline and D-NAME-treated females ($F(2,18)=3.77$, $p<.05$). Notably, none of the L- NAME-treated dams retrieved pups; whereas 75% of Saline-treated dams and 80 % of D-NAME treated dams retrieved pups ($X^2(2, n=21)=11.78$, $p<.01$) (Figure 3b). Because L-NAME treated females did not retrieve their pups, it was impossible to compare other indices of maternal behavior between the three groups. Finally, all Saline and L-NAME treated females retrieved the tootsie rolls.

Consistent with Experiment 1, fewer L-NAME-treated females were aggressive towards the intruder (25%) compared to Saline (75%) and D-NAME treated females (80%) ($X^2(2, n=21)=5.48$, $p=.06$) (Figure 4a). There was a tendency for L-NAME-treated females to take longer to contact the male intruder ($F(2,17)=2.07$,$p<.05$); and as observed previously, there was no difference in the total time the females investigated the male ($F(2,17)=1.579$, $p>.05$) (Figure 4b,c).

Experiment 1c

There was a tendency for Day 10 pp. L-NAME-treated females to take longer to contact 4-day old pups ($t(11)=-1.382$, $p=.09$,1 tailed test) than Saline animals (Figure 5a). Markedly, fewer L-NAME treated females retrieved 4-day old pups compared to Saline treated females ($p<.05$) (Figure 5b) even though females on Day 10 pp. have a great deal of maternal experience. Furthermore, consistent with Experiment 1, Day 10 pp. rats treated with L-NAME took longer to contact their own Day 10 pups than Saline-

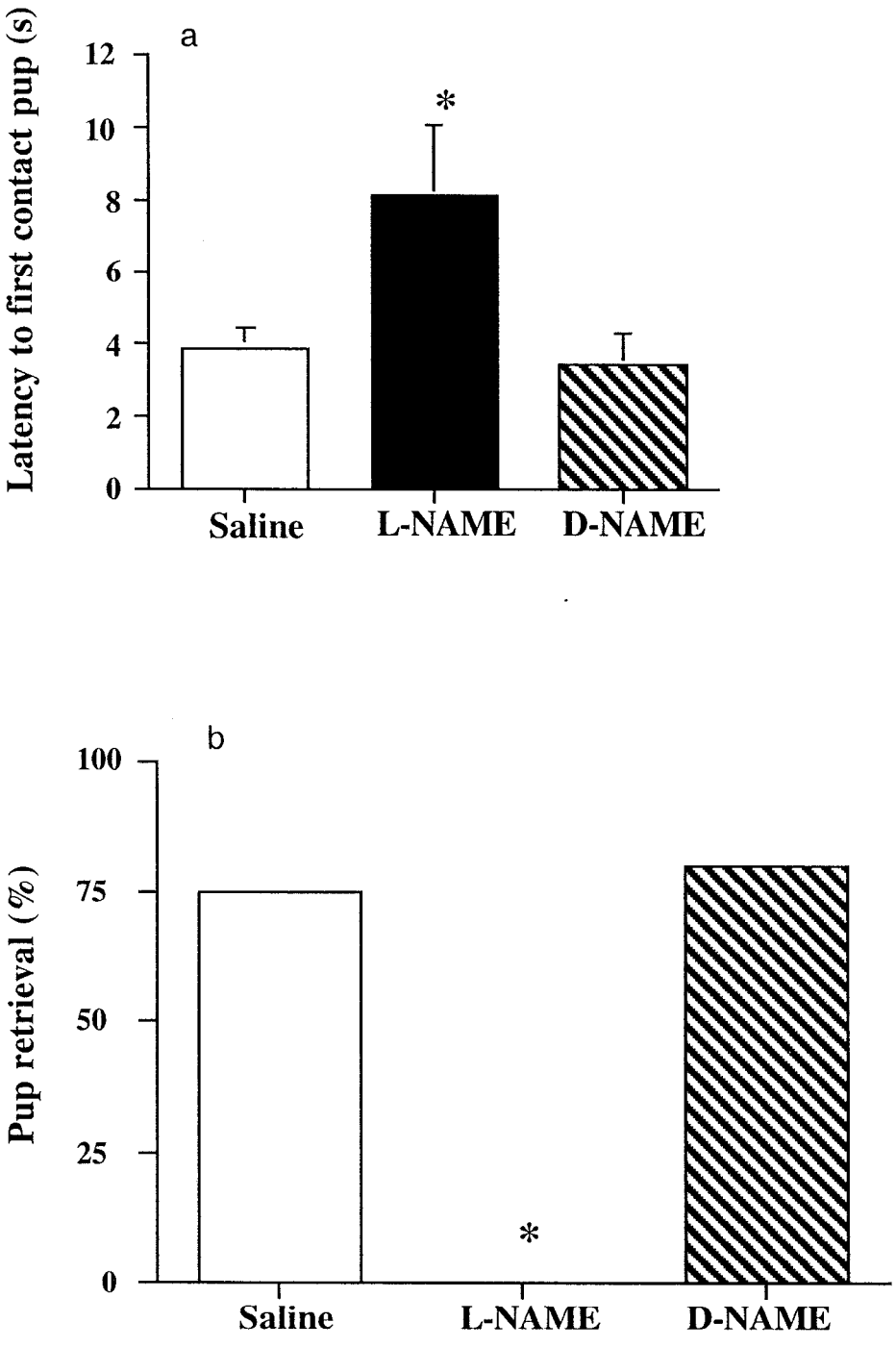

Figure 3. L-NAME impairs the expression of maternal behavior in Day 4 pp. rats. L-NAME (n=8) treated rats took longer to first contact the pup (a) and retrieved fewer pups than both Saline (n=8) and D-NAME (n=5) treated rats (b). Bars represent ± S.E. * $p<.05$.

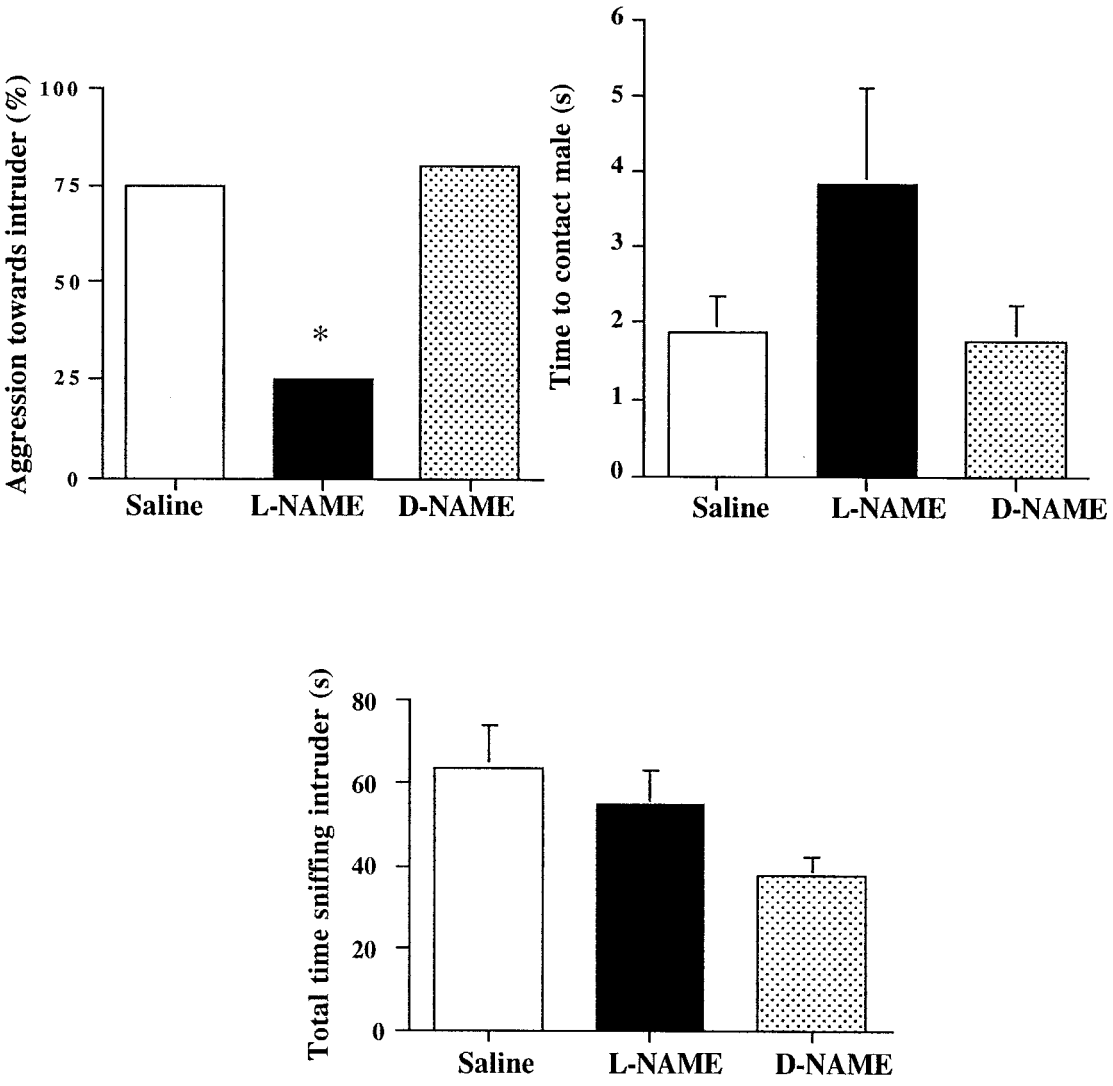

Figure 4. L-NAME impairs the expression of maternal aggression in Day 4 pp. rats. Using a male intruder test, fewer lactating females that were treated with L-NAME aggressed towards the male (a) and females tended to take longer to contact the male then Saline or D-NAME treated animals (b). No differences were observed between Saline and L-NAME treated rats in terms of average duration spent sniffing the intruder (c). Bars represent $\pm$ S.E. * $p<.05$.

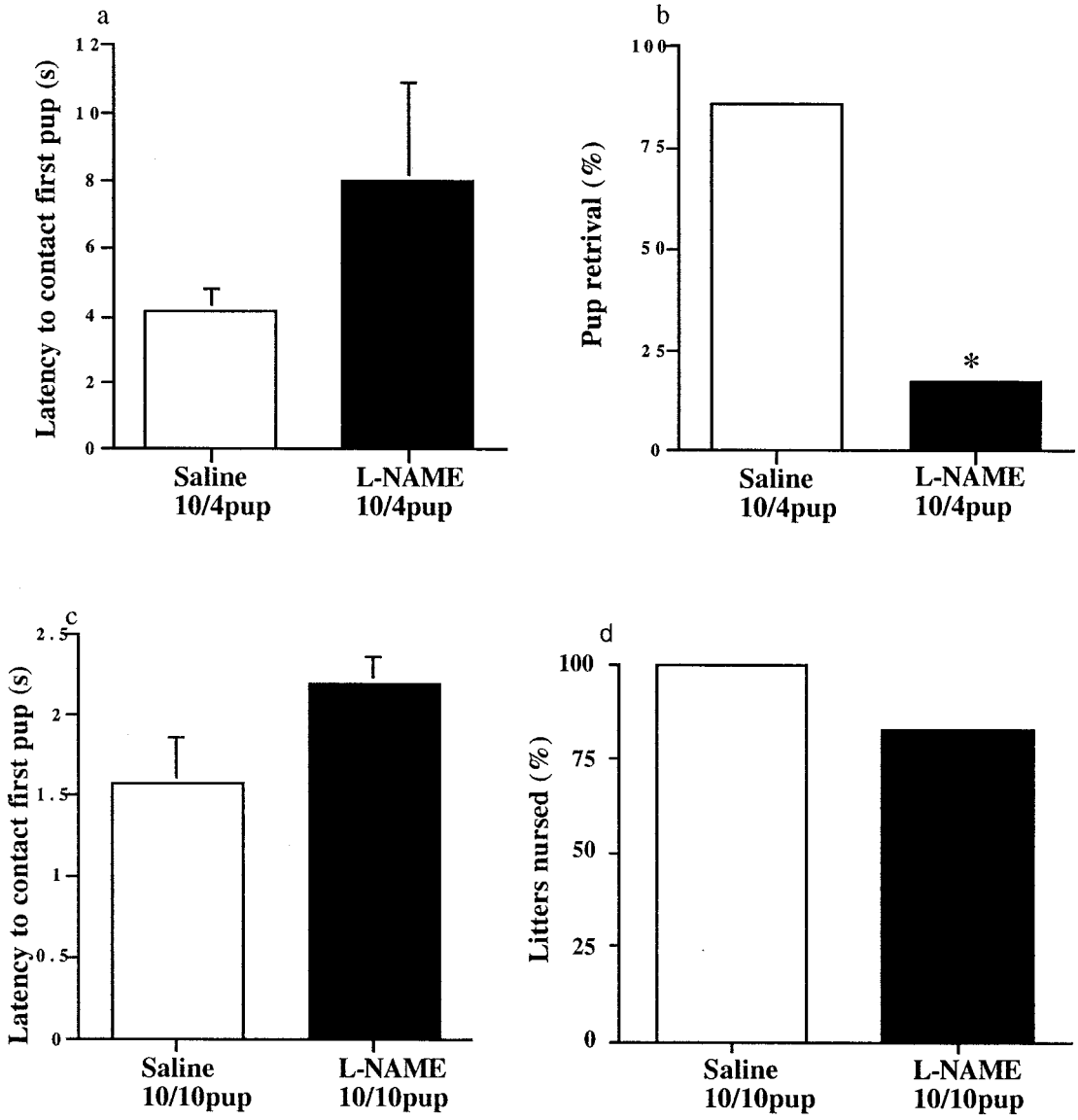

Figure 5. L-NAME impairs the expression of pup retrieval in Day 10 pp. rats. Day 10 pp. L-NAME treated (n=6) rats took longer to contact the first 4-day old pup (a) and retrieved fewer pups than Saline-treated rats (n=7). Day 10 pp. females treated with L-NAME tended to take longer to contact their own 10-day old pup (c) and females in both groups nursed their own litters (d). Bars represent $\pm$ S.E. * $p<.05$.

treated females (t 11)=-1.66, p=.06, 1 -tailed test) (Figure 5c) but both Saline (100%) and L-NAME- treated (83%) females nursed their own litters (Figure 5d).

Experiment 2a

Examples of Fos-lir within the MPOA and vBNST are shown in Figure 6. L-NAME treatment prior to presentation of an intruder significantly reduced Fos expression within the MPOA ($t(11)= 3.36, p<.01$), vBNST ($t(11) =2.76, p<.05$) and PVT($t(10)=2.26, p<.05$) but not within the PVN ($t(11)= .528$, $p>.05$) (Figure 7a-d). There was no significant difference in Fos expression between L-NAME and Saline- treated females within the MeA following aggression when we included all of the animals for the analysis ($t(11)= 1.09, p>.05$). Because this previous comparison was based on all animals including both Saline and L-NAME animals that did and did not show aggression towards the intruder, we were interested to examine whether Fos expression would be different based on the behavior of the female Thus we compared Fos expression of females that aggressed towards the male with those that did not aggress regardless of treatment and we found that females that did not show aggression toward the intruder had less Fos-lir within the MeA ($t (11)= 2.61, p<.05$)(Figure 8a).

Experiment 2b

L-NAME treatment prior to exposure to an anesthetized male reduced Fos expression within the MeA compared to Saline treatment ($F93,14)=5.45$, $p<.05$) (Figure 8b); but had no effect on Fos-lir within the MPOA ($F(3,13)=.559$, $p>.05$, PVN ($F(3,12)=1.39, p>.05$, or PVT ($F(3,14)=1.80, p>.05$).

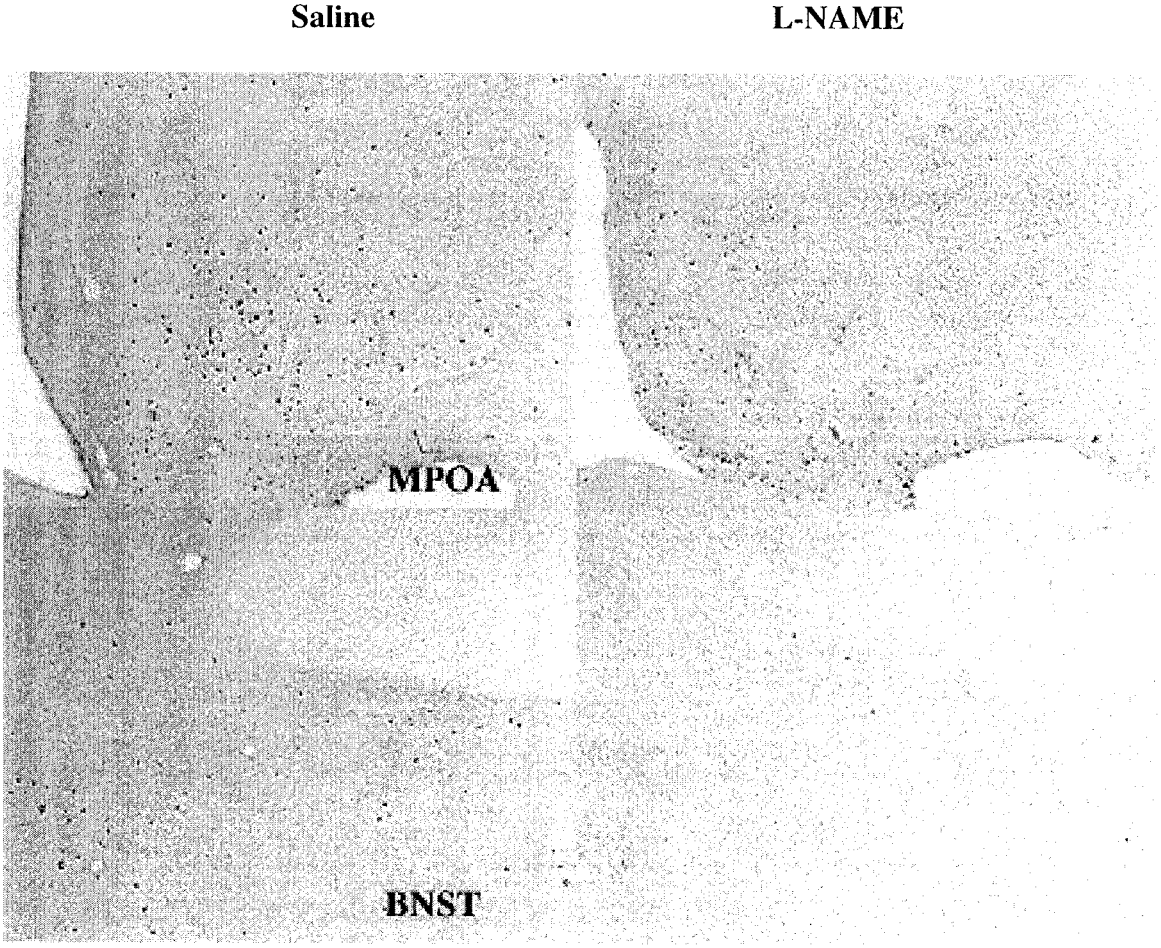

Figure 6. Example of Fos-lir in the medial preoptic area (MPOA) and ventral bed nucleus of the stria terminalis (vBNST) of Day 10 pp. lactating rats treated with Saline and L-NAME following the aggression test.

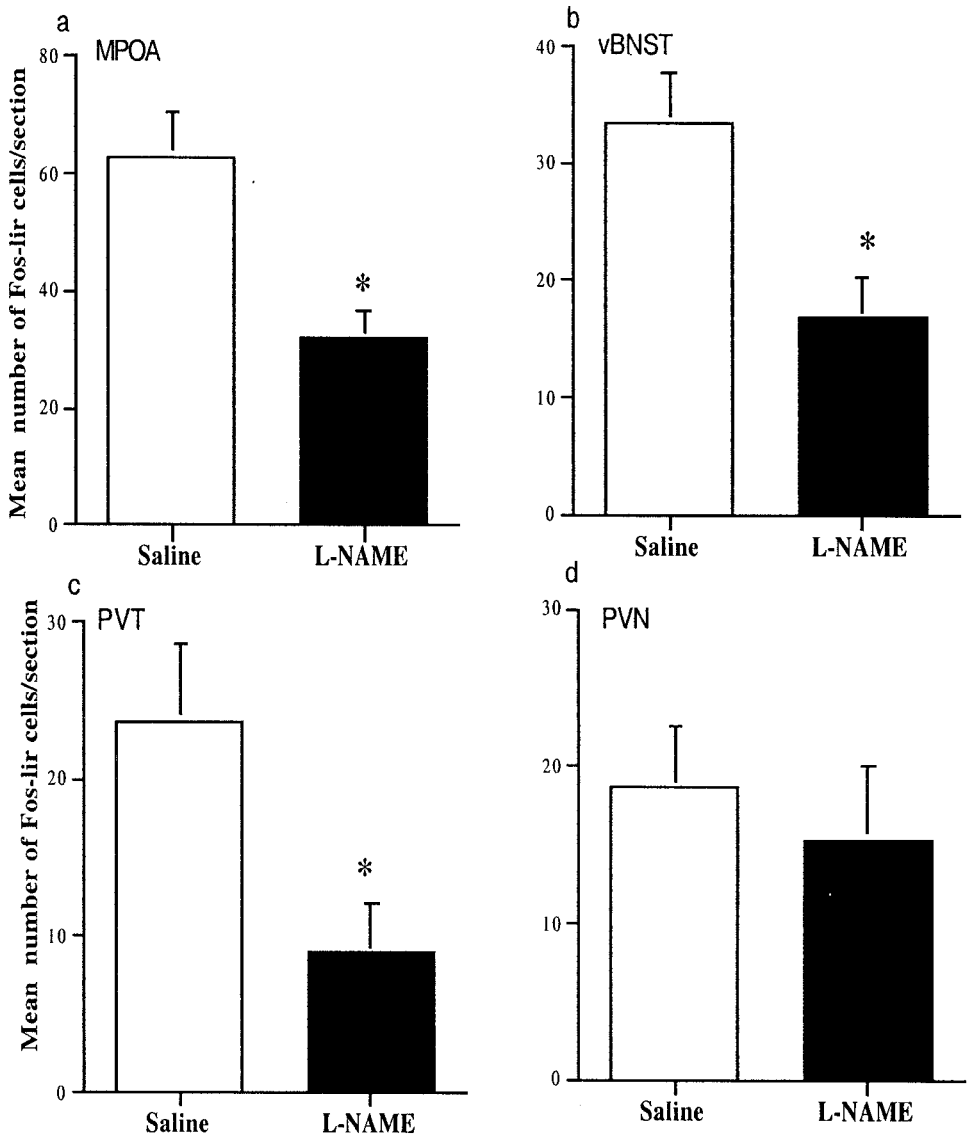

Figure 7. Effects of L-NAME on Fos-lir in Day 10 pp. rats, following the aggression test. L-NAME (n=6) treatment reduced Fos-lir in the medial preoptic area (MPOA) (a) ventral bed nucleus of the stria terminals (vBNST) (b), paraventricular thalamic nucleus (PVT) (c), but not in the paraventricular nucleus (PVN)(d) relative to Saline treated rats (n=7). Bars represent $\pm$ S.E. * $p<.05$.

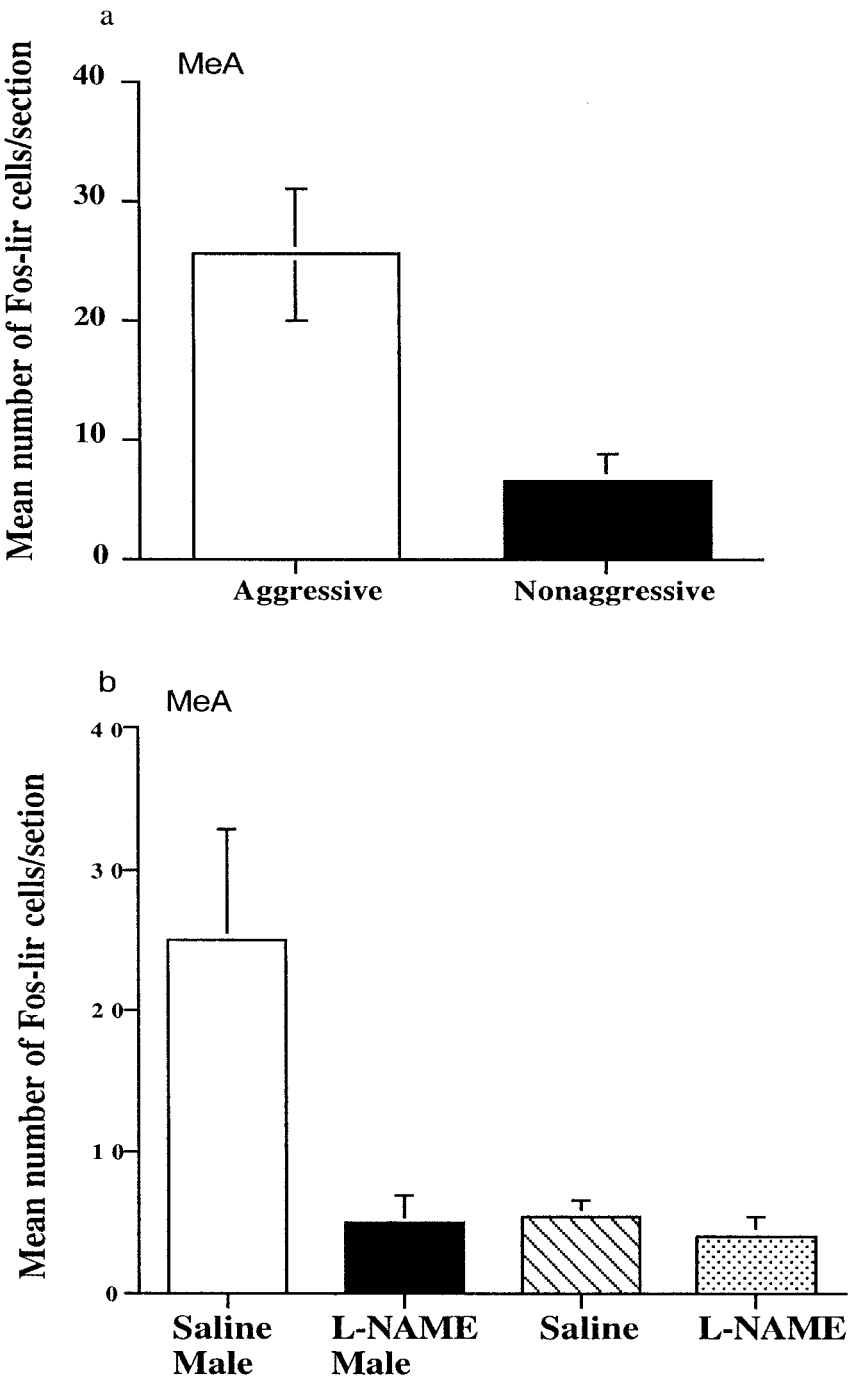

Figure 8. Effects of L-NAME on Fos-lir in the medial amygdala (MeA) of Day 10 pp. rats following exposure to either a mobile or anaesthetized male. L-NAME reduced Fos-lir in the MeA of animals that did not aggress when compared to Saline-treated females that did aggress towards the mobile intruder (a). Exposure to an empty box(n=4 for both Saline and L-NAME)or when treated with L-NAME and exposed to an anaesthetized male (n=5) resulted in less Fos-lir in the MeA compared to Saline-treated rats (n=5) exposed to an anaesthtized male intruder(b).

Discussion

The results from the first series of experiments indicate that inhibiting the capacity for NO production decreases the number of milk-ejections in Day 10 pp. dams. Most surprisingly was the disruption of maternal behavior and the suppression of aggressive behavior toward an intruder male on both Day 4 and 10 pp. Data from Experiment 2 reveals that inhibiting NOS changes the pattern of neuronal activity within discrete brain areas that are involved in regulating maternal behavior.

In Experiment 1a, Day 10 pp. dams that received L-NAME treatment took longer to contact their first pup and had fewer milk-ejections during nursing than Saline-treated dams. Although there were no differences between these two treatment groups on other physiological and behavioral measures such as time to quiescence or time spent with pups that contribute to facilitating a milk-ejection, L-NAME treated females did have more nest bouts and the duration of each nest bout was on average less than Saline treated females. These results then indicate that inhibiting NO production did not interfere necessarily with the neural mechanism that underlies milk-let down but did affect the distributions of the nestbouts with respect to their duration between the two treatment groups. Thus, the short duration of the nest bouts may have contributed the fewer milk-ejections observed in the L-NAME treated group as compared to the Saline–treated group. The above data suggest that milk-ejections are influenced by the activity of the lactating female at her nest-site, therefore the role of NO in the timing of milk-ejections is still unclear. Future studies will examine whether inhibiting NOS activity in anesthetised lactating rats will influence milk let-down.

Lactating females are aggressive toward intruders in their nest site when rearing pups, however, in Experiments 1a and 1b, L-NAME treated dams showed less aggression toward a male intruder then Saline or D-NAME treated dams. Interestingly, the total time of investigation of the intruder was not different between the groups suggesting that the lactating females that received L-NAME treatment did engage in typical exploratory behavior of the male but did not find the male threatening. These data are consistent with those of Gammie et al. (1999) who showed that mice with targeted disruption of the NOS gene and prairie voles that were administered a NOS inhibitor displayed less maternal aggression toward intruders. In addition, Gammie et al. (2000a,b) have shown that citrulline immunoreactivity, which is an indirect marker for NO within the PVN, was elevated following maternal aggression and was reduced following NOS inhibition. Together these data indicate that the central release of NO may play an excitatory role in maternal aggression in rodents.

The most notable effect of central L-NAME administered to Day 4 pp. dams was that it significantly interfered with pup retrieval; specifically L-NAME treatment abolished pup retrieval. This finding is interesting, in that L-NAME treated rats approached their pups but then tended to ignore them for the remainder of the test session. These data are consistent with other manipulations that have contributed to disrupting pup retrieval. For instance, Rubin and Bridges (1984) have demonstrated that lactating females with morphine implants into the MPOA showed an impairment in maternal responsiveness including pup retrieval. Furthermore, blockade of the dopaminergic system has also been shown to interfere with ongoing maternal behavior. Giordano et al. (1990) reported that following administration of haloperidol, a dopamine

receptor antagonist, pup retrieval was impaired in a dose-dependent manner but did not effect pup licking or nursing behavior. In addition, central injections of cis-flu-penthixol, another dopamine receptor antagonist into the NAcc inhibited pup retrieval but did not disrupt the female in performing the arched nursing posture (Keer & Stern, 1999). These studies suggest that deficits in pup retrieval may possibly result from the female not responding to the sensory cues from the pups and/or from disrupting several motivational aspects of maternal behavior.

The results from Experiment 1c demonstrates for the first time that inhibiting NOS disrupts pup retrieval in female rats that are at a stage of lactation in which retrieval is not required to stimulate mother-litter interaction. In contrast to Day 4 pp. dams which are required to search for their pups and gather them to the nest site to facilitate maternal behavior, on Day 10 pp., pups are mobile and typically approach the dam to elicit maternal behavior. Remarkably, central administration of L-NAME to Day 10 pp. females interfered with pup retrieval of 4-day old pups suggesting that NOS inhibition has a specific effect on pup retrieval regardless of whether the female is on Day 4 or Day 10 of lactation. Interestingly, both L-NAME and Saline treated Day 10 pp. females did nurse their own 10-day old pups and together these data indicate that L-NAME treatment impairs pup retrieval but not all pup-directed behavior. These results are consistent with other bodies of work that have reported deficits in pup retrieval following knife cuts of the lateral connections from the MPOA. Numan (1990) has shown that preoptic knife cuts nearly abolished pup retrieval in lactating rats but these females still crouched over and nursed their pups. Similar to our data, even though these dams did not retrieve any pups they could hoard candy indicating that these females had no oromotor impairments.

Inhibiting the capacity for NO production had profound effects on neuronal activation within several brain regions that are involved in regulating the expression of maternal behavior. In Experiment 2a, L-NAME treatment prior to the exposure of an active male intruder reduced Fos expression within the MPOA, vBNST, and PVT compared to Saline-treated females, which is consistent with previous research demonstrating that these regions show increases in Fos expression during maternal responsiveness (Numan & Numan, 1994). However there were no differences in Fos expression within the PVN between the two groups. These data are in contrast to Gammie et al. (2001) in that they found increases in Fos expression in the PVN in lactating mice following maternal aggression. This inconsistency may have occurred from different paradigms that were used to test maternal aggression. In our test, we extracted the male from the female's cage after the first attack on the male or until 10 minutes had passed; whereas the aggression test in the study by Gammie et al. (2001) lasted for 20 minutes regardless of how many attacks occurred. In addition, in the present experiment, Fos-lir was reduced in the MeA of L-NAME treated females but only in animals that did not aggress against the intruder which suggests that the induction of Fos in the MeA is correlational to the behavior expressed by the lactating female towards the male.

The results of the final experiment demonstrated that Fos expression was reduced within the MeA in L-NAME treated dams when the female was presented with a nonthreatening anesthetised male. Interestingly, other data from our laboratory have shown that Fos expression was attenuated within the MeA in L-NAME treated females on Day 6 pp. when presented with pups which were not accessible. Anatomical evidence

suggests that the MeA receives crucial neural input from the olfactory bulbs and is important for the integration of sensory information which involves evaluating the salience of a stimulus (Numan, 1994). Thus, the reduction of Fos-lir we observed in the MeA following inhibition of NO production may have resulted from L-NAME interfering with how the chemosensory input from the male rat was processed.

In these experiments, the deficits in maternal behavior induced by suppressing NO production appear to be limited to behaviorthat involve approaching a distal social stimulus, either pup or male intruder, investigating that stimulus and then responding appropriately. There appears to be only a slight delay in approaching the stimulus, and certainly with respect to the male intruder no deficit in investigation time. Thus, the major deficit appears to involve the inability of the stimulus to elicit the appropriate response – either retrieval or aggression. In this context it is interesting to note that systemic administration of a NOS inhibitor impairs social recognition (Bohme et al, 1993). Further, there is considerable evidence to suggest colocalization of NOS with AVP and OT, both of which have been implicated in social recognition memory in rats. It is possible then that inhibiting NO production has its effects on maternal behavior through one of these peptides.

In these current studies, for the first time we have shown that inhibiting the capacity for NO production disrupts several indices of ongoing maternal behavior across lactation. Central administration of L-NAME reduced both pup retrieval and aggressive behavior towards an intruder and therefore suggests that NO plays a permissive role for these aspects of maternal behavior. Centrally inhibiting NOS activity also reduced the induction of Fos within several brain regions that control the expression of maternal

behavior when presented with either a threatening or non threatening stimulus suggesting that NO is involved in processing chemosensory cues. Thus, together these current experiments indicate that the upregulation of NOS observed in lactation is of important functional significance for ongoing maternal behavior.

Chapter 5

General Discussion

The experiments described in this thesis addressed two major issues: the necessary and sufficient hormonal conditions for the upregulation of NOS in the SON and PVN of female rats and the role that NO plays during lactation. The results from the first series of experiments showed that prolactin is involved in the upregulation of NO that is seen following hormonal priming and that these effects depend on an interaction between PRL and OT. In addition, these studies revealed a novel role for PRL in the modulation of oxytocin gene expression. The findings from the second series of experiments confirmed the inhibitory role that NO plays on neuronal activation of magnocellular cells when the oxytocinergic system is chronically stimulated and that this effect is stimulus specific. In the last series of experiments, a novel role for NO in the control of specific aspects of established maternal behavior was demonstrated.

The schema shown in Figure 1 is based on the results of the first series of experiments described in this thesis. This model suggests that increased prolactin release as a consequence of changes in circulating estrogen and progesterone levels enhances OT mRNA expression within the PVN and SON. This upregulation of OT gene expression is associated with more intranuclear release of OT which in turn activates OTR to lead to an upregulation of NOS.

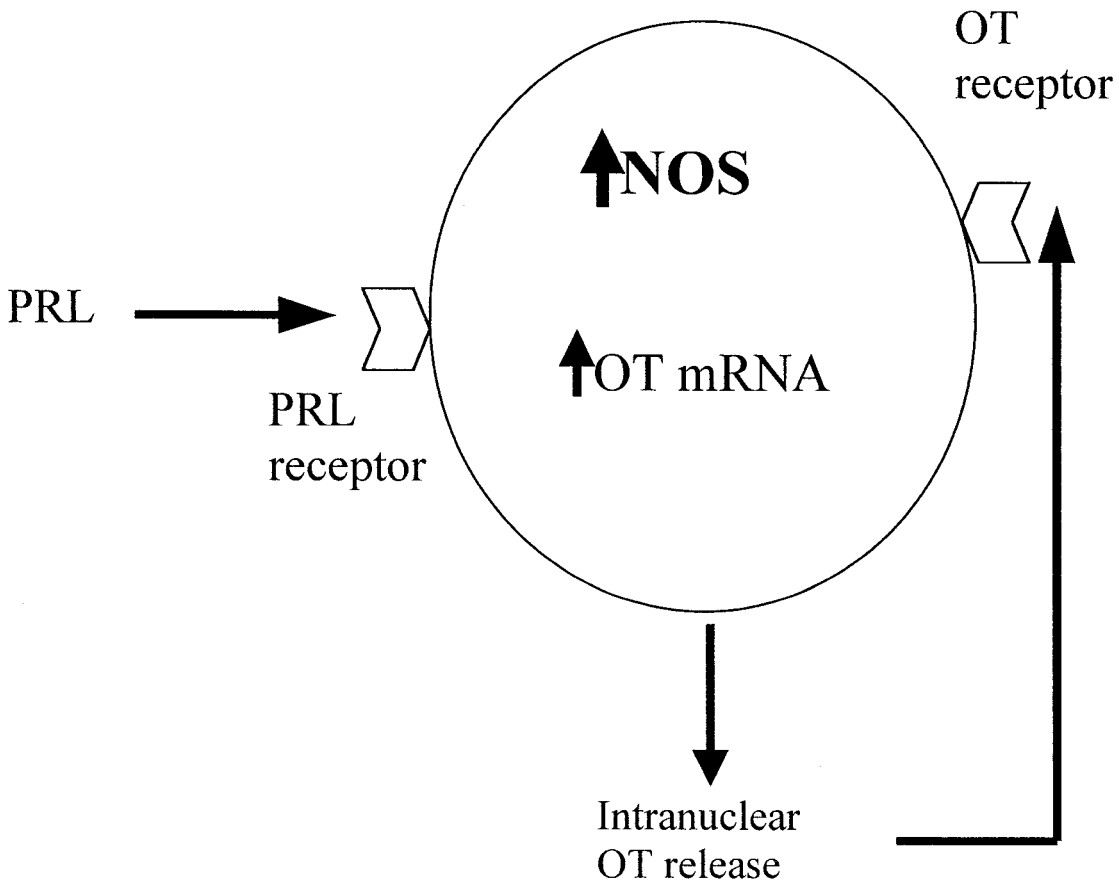

Figure 1. Model of NO production at the end of pregnancy

The idea that OTR activation increases NOS staining is consistent with previous evidence showing OT is released within the PVN and SON in situations in which NOS is upregulated. For example, Neumann and colleagues (1993) have shown that OT is released within the SON during parturition and such release is important in the positive feedback stimulation of OT neurons. The methods used in the current experiments, however, cannot rule out the possibility that central OT injections act outside the PVN and SON to induce the upregulation of NOS within these brain areas.

Many reports suggest that NO regulates OT activity in the SON and PVN when the oxytocinergic system is stimulated such as during water deprivation (Summy-Long et al., 1993; Kadowaki, et al., 1994). Therefore, the production and function of NO during parturition might be related to the activity of OT cells. The upregulation of NOS levels at this time then may aid in regulating the synchronization of bursting activity of OT cells that is important for parturition-related events.

Perhaps the most novel aspect of the model shown in Figure 1 is the proposal that prolactin stimulates the increase in OT mRNA. Although previous results have shown prolactin-stimulated increases in OT mRNA in hypothalamic explants (Ghosh & Sladek, 1991) and systemic prolactin administration increases OT release from the pituitary, the data presented in this thesis are the first to show, in vivo, that PRL upregulates OT gene expression. The possibility of a direct action of prolactin on OT neurons is supported by the fact that prolactin receptor mRNA is found within the PVN and the SON (Bakowska & Morrell, 1997; Pi & Gratton, 1991) and prolactin receptors have been localized to OT neurons (Grattan et al., 2001). How prolactin produces the increase in OT mRNA remains to be investigated. It is possible that activation of the prolactin receptor acts

through the JAK/STAT signalling pathway to increase OT gene transcription directly. Another possibility is that activation of the prolactin receptor enhances the excitability of the OT neurons and it is the resulting increase in firing rate and peptide release that then leads to an increase in OT gene expression. More studies are needed to distinguish between these two possibilities.

The precise role of steroid hormones in prolactin's effects also needs further investigation. Although it is clear that progesterone withdrawal against a background of high levels of estrogen is important for stimulating prolactin release from the pituitary (Bridges, 1984) there is evidence that gonadal steroid priming may also play an important role in facilitating the action of prolactin through other means. For example, central prolactin administration only stimulates maternal behavior in estrogen-primed females (Bridges et al., 1990) suggesting either that estrogen modulates prolactin receptor levels or has some other facilitory effects at the site of prolactin's action.

In addition, although the current studies implicate prolactin in the upregulation of OT gene expression, the source of prolactin that produces this effect in the pregnant rat is not entirely clear. There is ample evidence that prolactin release from the pituitary is increased in late pregnant rats (Bridges, 1984) and that prolactin in circulation is able to gain access to the brain through an active transport mechanism (Walsh et al., 1987) but prolactin mRNA has also been observed within the brain and how such a central prolactin system might contribute to these effects is unknown.

Whatever the source of prolactin, the current data suggest that it is capable of playing a role in a feed forward system that facilitates both intranuclear OT release within the SON as well as OT release from the pituitary and, ultimately, uterine contractions.

The ability of prolactin to facilitate OT actions may not be limited to parturition, however; parvocellular neurons within the PVN also synthesizes OT and project to a number of hypothalamic and extrahypothalamic brain sites (Swanson & Sawchenko, 1983). Whether prolactin increases OT secretion from terminals outside the SON and PVN and in which brain regions OT may act to induce parturition-related events has yet to be determined.

Prolactin and OT have both been implicated in stimulating the onset of maternal behavior and modulating the responsivity of the stress axis. The current studies underline the close relationship between these peptides and show for the first time that that they also act together to upregulate NOS.

A major feature of lactation is a marked blunting of the stress response as reflected by a reduction in Fos-lir following a stressor and an attenuated hormonal response including a reduction in OT secretion (Carter & Lightman, 1987). However during lactation, OT neurons remain responsive to OT itself. Suckling from the pups stimulates OT release centrally from both somata and dendrites (Neumann et al., 1993). OT then acts on its own receptors to further stimulate its own secretion and induce synchronous firing of OT neurons (Moos et al., 1984). Central administration of OT to lactating rats during suckling, increases the basal firing rate of OT cells, the frequency of bursts and the number of spikes of each burst (Freund-Mercier & Richard, 1984) and suggests that central OT administration is a specific stimulus that targets OT neurons to influence their activity. The results of the second series of experiments described here are consistent with these results and show that during lactation, OT administration is also able to induce Fos-lir within cells of the PVN and SON. Moreover, this effect is greater

in lactating than in nonlactating rats probably reflecting the higher levels of OTR seen in lactation. Mezey and Kiss (1991) have shown that many magnocellular vasopressin cells begin expressing oxytocin during lactation. Thus, although the precise phenotypes of the cells in which Fos-lir was induced awaits confirmation by colocalization studies, it is likely that many of them, particularly those in the magnocellular compartments, were indeed oxytocinergic cells.

The pathway through which OT induces Fos-lir within OT neurons is not clear. It is unlikely, however, that this effect is secondary to an increase in firing rate because Luckman et al. (1994) showed that antidromic stimulation of magnocellular neurons was not able to induce Fos-lir whereas central administration of the muscarinic agonist, carbachol did. It is more likely then that Fos-lir is induced as a result of the activation of a second messenger cascade following OTR activation. The functional significance of upregulation of Fos-lir within OT neurons remains to be clarified.

The fact that L-NAME increased the ability of OT administration to induce Fos-lir in the PVN and SON is consistent with the idea that NO has a negative feedback effect within the HNS. NO has been shown to modulate magnocellular neuronal activity through a number of pathways. NO donors can increase the frequency of spontaneous IPSCs suggesting that NO facilitates inhibitory synaptic inputs into SON neurons (Ozaki et al., 2000). Consistent with this hypothesis, it has also been demonstrated that L-NAME enhanced neuronal activity as measured by extracellular discharges in SON neurons from slices of rat hypothalamus in vitro (Liu, Jia, & Ju, 1997). Given that increased firing rate per se does not induce Fos, however, it is unlikely that such pathways play a major role. It is perhaps more likely that NO has its effects on Fos-lir induction by diffusing to

adjacent cells and modulating presynaptic events or alternatively by autocrine actions on non-OT signalling pathways. For example, NO has been shown to modify the NMDA receptor and block NMDA-induced currents. The increase in Fos-lir induction when lactating rats are treated with L-NAME and OT could possibly result from an increase in NMDA receptor mediated effects. Consistent with this idea, systemic administration of an NMDA receptor antagonist reduced both Fos expression within the SON and plasma OT concentration following an injection of hypertonic saline (Onaka & Yagi, 2001).

Interestingly, the permissive effect of NOS inhibition on Fos expression seen following central OT stimulation did not generalize to non lactating rats nor was it seen following urethane administration in lactating rats. L-NAME administration reduced Fos expression both following OT administration in nonlactating rats and following urethane administration in both lactating and nonlactating rats. The differences in effect of NOS inhibition between lactating and nonlactating rats are consistent with previous evidence suggesting that L-NAME administration only increased OT secretion in rats in which the OT system was stimulated by chronic salt loading and in which NOS was upregulated (Kadowaki et al., 1994).

The stimulus specific effects of NOS inhibition in lactating animals may depend on the specific site(s) of action of the two stimuli. OT administration is probably acting largely directly on OT neurons. Urethane administration represents a complex stimulus. It is an osmotic stressor, a stimulus to the HPA and an anaesthetic. Although one consequence of urethane administration is activation of OT neurons this event occurs at the end of a long cascade of events with the potential for modulation by NO at any the levels.

The increased capacity for NO production in lactation then may play a negative feedback role in oxytocinergic activity but only when the OT neurons are directly stimulated. Moreover, the increase in neuronal activation that was observed within the SON and PVN following central NO inhibition may have resulted from the increase in intranuclear OT release.

The functional relationship between NOS and OT in lactating rats raises the obvious question of whether NOS plays a role in the mechanisms subserving milk ejections. Previously, Okere et al. (1996c) demonstrated that central and peripheral injections of a NO donor interfered with the transfer of milk to pups after a 12 hr separation but found no effects of NOS inhibitors. The results of the final set of experiments described also suggest that inhibiting NOS has only modest effects on milk ejections and has more profound effects on maternal behavior itself.

Two specific aspects of maternal behavior: retrieval and maternal aggression were affected by L-NAME treatment. Deficits in these behaviors were observed in females treated on either Day 4 or Day 10 postpartum, suggesting that greater maternal experience does not eliminate these effects. These effects appear to be specific to maternal behaviour, and not to a motoric deficit, because lactating females on Day 4 pp. treated both centrally and peripherally with L-NAME still retrieved candies approximately the same size and weight as the pups (Woodside, MacCaul & Popeski, unpublished).

To test retrieval behavior, it was necessary to present Day 10 dams with Day 4 pups because their own young were already so motorically capable that they approached the dam independently. It is noteworthy that when dams were tested with their own pups

on Day 10 pp. they responded to the pups by grooming them and adopting a nursing posture over them and that the only change in mother-litter interaction observed was a greater frequency of short nest bouts. Indeed, it is likely that the reduction in number of milk ejections recorded in L-NAME treated females on Day 10 pp. resulted from their shorter nest bouts.

Okere (1996a) showed that enhancing NO production, by administering a NO donor during parturition, both disrupted pup delivery and interfered with subsequent maternal behavior but the current results are the first to implicate NO in the maintenance of maternal behavior. Although lesions of the MPOA (Numan et al., 1977) and vBNST (Numan & Numan, 1996) do disrupt ongoing maternal behavior, it has typically proved quite resilient to disruption by neurochemical means. Manipulating gonadal or adrenal steroids or PRL and OT levels are without a major effect on retrieval behavior. (Thoman & Levine, 1970; Moltz & Weiner, 1966; Obias, 1957). Morphine infusion into the MPOA, however, has been shown to produce numerous effects on maternal behavior (Rubin and Bridges, 1984).

The behavioral deficits induced by the inhibition of NO in maternal rats appear to relate to approach, recognition and behaving appropriately toward a social stimulus; whether it is either a pup or an intruder male. Therefore, the ability of NO inhibition to interfere with these aspects of maternal behavior, might be mediated by the effects of NO in brain areas such as the MeA that processes sensory input associated with social stimuli and which is known to project to the MPOA.

Consistent with this notion, it was found that following central L-NAME administration females exposed to either a mobile or anaesthetized male showed less Fos-

lir in the MeA . In addition, Woodside, Service, and Popeski (2002) demonstrated that when females were presented with pups or a nonsocial appetitive stimulus (fruitloops) that could be seen, heard or smelled but not contacted directly, L-NAME treatment reduced Fos-lir induction in the MeA and MPOA in response to the presentation of pups but not of fruitloops. In the context of these results it is interesting that there is an OT projection from the parvocellular PVN to the medial amygdala (Buijs & Swaab, 1979) and that a variety of behavioral effects have been attributed to the action of OT within the MeA.

The MeA and particularly OT in the MeA have been implicated in social recognition because mice with a null mutation of the OT gene (OTKO) fail to recognize conspecifics following a social encounter (Ferguson, Young, & Insel, 2002). Administration of OT into the amygdala of these mice has been shown to restore social recognition (Ferguson, Aldag, Insel, & Young, 2001). The relationship between OT and social recognition in the rat is not as clearly defined as that of the mouse. Administration of large doses of OT (Popik & Vetulani, 1991) have been shown to reduce social recognition in rats. On the other hand, Benelli et al., (1995) reported that a low dose of OT had a memory-improving effect that could be blocked by central administration of an OT receptor antagonist. Therefore, it may be that an optimal level of OT release within the forebrain is required for social recognition.

A number of studies have implicated OT in maternal agression. Lesion studies of the PVN that destroy OT synthesizing neurons have been shown to decrease or increase maternal aggression (Consiglio & Lucion, 1996; Giovenardi, Padoin, Cadore, & Lucion,1988). Electrolytic lesions of the PVN administered on Day 5 pp. resulted in reduced aggression towards the intruder (Consiglio & Lucion, 1996). In contrast, Giovenardi et al. (1998) demonsrated that ibotenic lesions of the parvocellular region of the PVN on the fifth day pp. increased maternal aggression. Similarly, reducing OT synthesis by administration of an antisense to OT increased maternal aggression in rats (Giovenardi et al.,1998). In addition, Elliot et al. (2001) reported that acute treatment with cocaine on Day 6 pp. resulted in elevated levels of OT within the MeA, and resulted in deficits in pup directed behaviour and a reduction in maternal aggression. Together, these latter data suggest that release of OT from parvocellular neurons within the PVN suppresses maternal aggression. If L-NAME increases OT release in neurosecretory OT neurons, a similar effect occurring within the MeA would produce the deficits in maternal aggression found in the current studies. If this were in fact the case, then infusions of an NOS inhibitor directly into the PVN should produce similar effects to those seen following 3rd ventricle administration.

Melis et al.(1997, 1999) has shown that NO and OT also interact to stimulate penile erection and that whereas NO acts within the PVN, OT acts elsewhere. Again, the idea here is that NOS modulates OT release from parvocellular neurons that project into the forebrain.

Although the MeA is part of the maternal circuit, the MPOA is often regarded as the central coordinating structure in the control of maternal behavior (Numan , 1988).

Manipulating NO levels within the MPOA has been shown to effect species-specific behavior. For example, Du and Hull (1999), have shown, in male rats,that NOS in the MPOA is under the control of testosterone and that inhibiting NOS within the MPOA both decreases extracellular dopamine levels and impairs copulatory behavior (Lorrain, Matuszewich, Howard, Du, & Hull, 1996) independent of any effects of NO on penile erection. It is possible that a similar mechanism might underlie the deficits in maternal behavior that were observed following L-NAME administration in the current studies. If so, then administering the NOS inhibitor directly into the MPOA should disrupt retrieval behavior and maternal aggression.

It should be noted that the mechanisms that underlie retrieval behavior and maternal aggresion may not be the same. Whereas a role for OT to act within the MeA to influence maternal aggression has been reported; a role for this peptide to influence retrieval behavior in maternal females has not been found. Although some studies have shown that administration of an OT receptor antagonist can delay the latency to retrieve pups (van Leengoed, Kerker, & Swanson, 1987), these effects are only observed during the onset of maternal behavior and have not been documented once maternal behavior has been established. Furthermore, knife cuts severing lateral connections from the PVN does not interfere with maternal behavior (Numan & Corodimas, 1985) which suggests oxytocinergic pathways from the PVN may not be involved in regulating ongoing maternal behavior.

In summary, the results of this thesis have shown that PRL and OT interact to upregulate NOS within the PVN and SON. This increase in capacity to produce NO has a restraining effect on the activation of OT neurons by OT. NO has also been shown to

play an important role in maternal retrieval and aggression. Whether it does so by influencing OT release from parvocellular neurons that project to areas of the forebrain known to be important for maternal behavior such as the medial amygdala and MPOA remains to be determined.

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Appendix A

Prolactin and oxytocin interaction in the paraventricular and supraoptic nuceli: Effects on OT mRNA and NOS

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Prolactin and oxytocin interaction in the paraventricular and supraoptic nuclei: Effects on OT mRNA and NOS

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Abstract

In these experiments, we investigate the contribution of PRL and OT to the increase in staining for NADPH-d and OT mRNA in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) that is seen at the end of pregnancy and following a steroid- priming regimen that mimics the hormonal profile of late pregnant females. Ovariectomized rats receivedchronic implants of silastic capsules containing estrogen, and progesterone (P) followed by P removal. In Experiment 1 various doses of an oxytocin antagonist (OTA) were administered to rats to investigate whether intranuclear OT release was necessary for NADPH-d staining. In Experiment 2a and b rats received concurrent treatment with bromocryptine (0.5mg/day) to suppress endogenous prolactin release, and either systemic PRL (0.5mg once daily), or PRL (2µg/µl), or vehicle infused twice a day into the third ventricle, or chronic OT infusion (24ng/day) for 3 days following P removal. Brains were then processed for NADPH-d histochemistry. In Experiment 3 the interaction of PRL and OT on OT mRNA within the SON and PVN was examined. NADPH-d staining in the SON and PVN was reduced by the highest dose of the OTA and by bromocryptine treatment. Central prolactin and OT replacement completely restored NADPH-d staining in bromocryptine-treated rats. Finally both bromocryptine and the OTA suppressed OT mRNA levels and PRL replacement restored these levels to that of controls. Together, these data suggest that the increased capacity to produce NO in the SON and PVN during late pregnancy is dependent on PRL stimulating OT gene expression and hence intranuclear OT release.

The supraoptic nuclei (SON) and the magnocellular compartments of the paraventricular nuclei (PVN) of the hypothalamus of late pregnant rats undergo both morphological and neurochemical changes. Among these changes are synaptic remodeling (1), a decrease in glial coverage of cell bodies (2), and an increase in oxytocin (OT) mRNA (3). In addition, we have recently shown that staining for NADPH-d, a histochemical marker for nitric oxide synthase (NOS), is increased in the PVN and SON of late pregnant and lactating rats (4,5). Pregnancy per se is not necessary to produce many of these changes, rather they depend on the gonadal steroid state that accompanies late pregnancy. For example, female rats given a hormonal regimen of estrogen combined with progesterone show both an increase in OT mRNA (6) and NADPH-d staining in the PVN and SON following progesterone withdrawal (4). Progesterone withdrawal is also necessary to allow activation of OT neurons at parturition (7). The positive correlation observed between the increases in NADPH-d staining and OT mRNA seen close to parturition and following gonadal steroid administration is consistent with results showing an increase in NOS in magnocellular neurons under other conditions of hypothalamic neurohypophysial system stimulation such as salt loading (8).

Nitric oxide synthase colocalizes with OT in the PVN and SON (9) and a functional relationship between NOS and OT has also been demonstrated. For example, inhibition of NOS results in increased OT release but only under conditions of OT stimulation (10). Further, administering the NOS inhibitor, L-NAME, results in increased Fos expression following central OT administration in lactating rats but not in nonlactating rats (11). Together these data suggest that nitric oxide (NO) may have a restraining effect on the oxytocinergic system

but only when the system is chronically stimulated; a state which coincides with NOS upregulation within OT neurons.

Oxytocin receptors are localized on OT neurons in the PVN and SON (12) and in estrogen–primed rats, chronic intracerebroventricular OT infusion is sufficient to increase NADPH-d staining in the PVN and SON (4). Intranuclear OT release may therefore play a key role in the regulation of NOS in these nuclei supporting the notion that NO serves a a negative feedback function within these cells (10). The upregulation of NOS seen in the PVN and SON in late pregnancy may, therefore result from an increase in OT release within these nuclei. Thus, the gonadal steroid profile of late pregnancy may stimulate an increase in OT mRNA resulting in increased intranuclear OT release. In turn, OT within the SON and PVN would then activate OT receptors on OT neurons within these nuclei resulting in an upregulation of NOS. If this were indeed the case, then treating ovariectomized females with a hormonal regimen mimicking late pregnancy and an OT receptor antagonist should eliminate the effect of steroid priming on NADPH-d staining. This hypothesis was tested in Experiment 1.

Administering gonadal steroids to mimic the hormonal profile of late pregnancy results not only in increases in OT mRNA within the SON and PVN but also stimulates prolactin (PRL) release from the pituitary (13). Prolactin can enter the brain via an active transport mechanism (14) and PRL receptors have been localized in many areas of the hypothalamus including the PVN and SON (15). Moreover, a stimulatory effect of PRL on the HNS has been shown in studies documenting an increase in OT release following systemic PRL administration (16) and increases in OT mRNA in hypothalamic explants treated with PRL (17,18). In Experiments 2 and 3, therefore, we investigated the

role of PRL both in the increase in NADPH-d staining and in the upregulation of OT mRNA seen in the SON and PVN following steroid-priming.

Materials and Methods

Animals

Female Wistar rats from Charles River Breeding Laboratory (St. Constant, Quebec) were used in these studies. 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 ad libitum access to lab chow and to water throughout the experiment. All protocols were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Surgery

Bilateral ovariectomies: Ovariectomies were performed through bilateral dorsal incisions under ketamine/xylazine anesthesia (5.7 mg ketamine and .86 mg xylazine/100 g of body weight) and the topical antibacterial powder (Cicatrin^{tm}) was placed on the wound which was closed using wound clips.

Intracerebroventricular (icv) cannulae implantation: Stainless steel cannulae (22 gauge; Plastic One Products) were inserted stereotaxically into the third ventricle (AP: - .90 mm, L: 0, DV: - 6.4 mm below dura (19) under ketamine/xylazine anaesthesia. L-shaped cannulae were connected, via a polyethylene catheter to osmotic minipumps (Alzet, model 1003D) which were

then implanted subcutaneously between the scapulae. Minipumps were primed for six hours at 37 ^{0}C before implantation.

Drug and Hormone Administration

Steroid –priming: The hormone replacement schedule used was shown by Bridges (20) to produce hormone levels similar to those of pregnancy. All animals were ovariectomized (ovx) one week prior to receiving silastic implants (Dow Corning, .078" ID x .125" OD) containing either cholesterol or ovarian steroids (17 B estradiol (2mm) or crystalline progesterone 30mm). On Day 1 of hormone treatment, animals received 1 x 2 mm implants of either cholesterol or estrogen. Two days later (Day 3), animals received 3 x 30 mm implants of either cholesterol or progesterone (P). On Day 14, cholesterol or P implants were removed and all animals were sacrificed on Day 17. Animals were sacrificed 72 hours following progesterone withdrawal to mimic the decline in progesterone levels that is observed on Day 20 of gestation (21).

OTA treatment: The oxytocin antagonist (OTA([d$(CH_2)_5$,Tyr(Me)^2,Orn^8]-Vasotocin))(Peninsula Laboratories) was dissolved to a concentration of 0.25ng/µl, 1 ng/µl, or 5ng/µl and infused into the third ventricle at a rate of 1 µl/ hour for the 3 days following progesterone removal. The range of doses was selected such that the highest dose (5ng/µl) was of the same magnitude that has been used to reduce yawning and penile erection (22). Because the highest dose of the OTA used in Experiment 1 did not completely suppress NADPH-d staining, in Experiment 3, the OTA was dissolved to a concentration of 10ng/ µl.

Bromocryptine treatment: Bromocryptine (0.5mg/day) (Sigma) in a volume of 0.1ml was administered subcutaneously at 0900 for the 3 days following

progesterone removal. This dose has been previously shown to suppress the circulating levels of prolactin (23).

Prolactin treatment: Subcutaneous PRL (0.5mg) (Sigma) was delivered in a volume of 0.1ml twice daily. This dose has been used to reverse the effects of bromocryptine administration on maternal behavior in steroid-primed female rats (13). Central PRL (2 µg) was delivered in a volume of 1 µl. This dose was used because it has been shown to be sufficient to induce maternal behavior (24). Both systemic and central injections were administered at 0900h and 1700h for the 3 days following progesterone removal.

Oxytocin treatment: OT (Sigma) was dissolved to a concentration of 1 ng/µl and infused at a rate of 1 µl/ hour for the 3 days following progesterone removal. This dose was selected because this dose has been shown to increase NADPH-d staining in estrogen-primed ovariectomized female rats (4).

Histochemistry

On the test day, females were overdosed with sodium pentobarbital (60 mg/kg; MTC Pharmaceuticals Ltd.) 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 phosphate buffer (pH 7.3). The brains were removed and post-fixed for 48 hrs in a 30% sucrose, 4% paraformaldehyde solution at 4 ^{0}C. Forty-micron thick sections throughout the SON and PVN were cut on a cryostat and placed free-floating in Trizma Buffered Saline (TBS; pH 7.3) solution. Sections were washed 3 times in TBS. Approximately 25 sections from each animal were then incubated for 75 minutes at 37 ^{0}C in 5ml of a solution containing 5.0 mg of β-NADPH, 1.5 mg of Nitro Blue Tetrazolium, 0.3% Titron X-100, and TBS. An animal from each experimental group was 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 (Sigma).

Image analysis

For Experiments 1 and 2 sections were visualized using a Sony XC77 camera mounted on a light microscope (Labolux Leitz GMBH). Images were captured using NIH image analysis software (1.62b) installed on a Power Macintosh computer. NADPH- d stained cells were counted from the images captured in the TIFF picture files. Stained cells in sections throughout the rostro-caudal extent of the SON 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. This method of quantification was used to maintain consistency with previous work (4). Subsequent analyses showed that results remained unchanged if all sections were included.

For the PVN, sections were divided into the magnocellular and parvocellular regions and were counted and then an average over all of sections was obtained for each region. The PVN was subdivided based on plate 25 (approximately -1.80 mm from bregma of the rat brain atlas of Paxinos and Watson (1986,) that delineates the magnocellular region (PaLM: paraventricular hypothalamic nucleus, lateral magnocellular and PaV: paraventricular hypothalamic nucleus, ventral) from the parvocellular region (PaMP: paraventricular hypothalamic nucleus, medial parvocellular) (19). To identify densely stained cells; sections from each experimental group across each assay that appeared to be densely stained were selected and the relative density of each cell was recorded. An average of the density of the cells was

then calculated to establish the criterion. Densely stained cells in each section were counted by an individual unaware of experimental group assignment.

In situ hybridization for OTmRNA

Fourteen-micron thick coronal sections were cut on a cryostat through the SON until the posterior portion of the PVN. These sections were thaw mounted onto RNAse-free coated slides, desiccated under vacuum and then stored at – 80^0 C until processed. Before hybridization, sections were washed with 4% paraformaldehyde, 2XSSC, .25% acetic anhydride in triethanolamine-HCL (PH 8.0), 2XSCC, and then dehydrated by transfer through 50%,70%,95%,100% ethanol, delipidated in chloroform, and then rehydrated in 100% and 95% ethanol; and dried at room temperature. OT mRNA detection was performed with a [^{35}S] ATP-41 base pair oligonucleotide sequence:(5'-GGG CTC AGC GCT CGG AGA AGG CAG ACT CAG GGT CGC AGG CG-3') (written communication from L.Young, Emory University, and the sequence was synthesized at McGill University). The oligo was labeled at the 3' end with terminal transferase (Roche) and $2x10^6$ cpm per slide was used for *in situ* hybridization.. Slides were covered and stored at 37 ^{0}C for 24 hrs. Hybridized sections were then washed 3 times in 1X SSC at 45 ^{0}C and once in 1X SSC at room temperature. Sections were then dehydrated in water, 50%, 70%, and 90% ethanol and air dried overnight. Hybridized slides were apposed to hyperfilm (Amersham) and kept at 4^0C for 3 days. The hybridization signal within the SON and PVN was quantified by means of densitometry with an image analysis system (MCID Research, Inc). Means for each animal were based on 5 sections for the SON and 4 sections for the PVN. The data are

presented as arbitrary optical density (absorbance) units after correction for background.

Experiments

Experiment 1: *Effects of chronic OTA administration on NADPH-d staining in steroid-primed rats*

All animals were ovariectomized; one group was treated with cholesterol (Ovx+Chol; n=6) and the remainder were treated with the steroidal regimen described above. On Day 14 of the experiment, the steroid-primed rats underwent icv surgery and were assigned to one of four groups for the 72 hours following progesterone removal. These rats received daily icv injections of either Vehicle (saline) (Veh,n=6); 6 ng/day OTA (n=5); 24ng/day OTA (n=6); or 120ng/day OTA (n=6). On Day 17, animals were sacrificed and sections of the SON and PVN were processed for NADPH-d histochemistry.

Experiment 2a: *Effects of bromocryptine administration and subcutaneous PRL replacement on NADPH-d staining*

Ovariectomized steroid-primed rats received either a subcutaneous (sc.) injection of Vehicle (saline) (Veh, n=5), Bromocryptine (BR, n=5), or Bromocryptine and Prolactin (BR+PRL, n=5) for the three days following progesterone withdrawal. On Day 17, all animals were sacrificed and sections of the SON and PVN were processed for NADPH-d histochemistry.

Experiment 2b: *Effects of bromocryptine administration and central PRL or OT replacement on NADPH-d staining*

Ovariectomized, steroid primed rats were assigned to one of four treatment groups: 1) Veh (saline)(n= 5); 2) Bromocryptine (BR, n=4); 3) Bromocryptine and icv Prolactin (BR+ icvPRL,n=5); 4) Bromocryptine + icv Oxytocin (BR+ icvOT, n=4). On the day of ovariectomy, animals in the Veh, BR, and BR+icv PRL groups were implanted with icv cannulae. On Day 14, following removal of the progesterone implants, animals in the Veh, BR, and BR+icv PRL groups received a sc. injection of either vehicle or bromocryptine and an icv injection of saline (1µl) or PRL for the next three days. In addition, on Day 14, following progesterone removal, animals in the BR+ icvOT group were implanted with icv cannulae. For the next seventy-two hours, animals received a sc. injection of bromocryptine in combination with icv OT administration. On Day 17, animals were sacrificed and sections of the SON and PVN were processed for NADPH-d histochemistry.

Experiment 3: *Effects of bromocryptine and PRL replacement on OT mRNA*
All animals were ovariectomized; one group was treated with cholesterol (Ovx+Chol; n=5) and the remainder was treated with the hormonal regimen that was described above. The steroid-treated rats were then assigned to one of four groups for the 72 hours following progesterone removal. These rats received daily sc. injections of 1) Vehicle (saline) (Veh; n=6) 2) Bromocryptine (BR; n=6), 3) Bromocryptine with PRL sc. replacement (BR+PRL; n=6), or 4) Bromocryptine with PRL sc. and an OTA which was chronically infused into the 3rd ventricle (BR+PRL+OTA; n=6). ICV surgery for the latter group was conducted on Day 14. On Day 17, animals were sacrificed and sections of the SON and PVN were processed for *in situ* hybridization for OT mRNA.

Statistics

For all experiments, data were analysed using a one-way analysis of variance (ANOVA) followed by post hoc comparisons (Fisher PLSD or Tukey HSD) where appropriate. An alpha level of .05 was adopted for all tests.

Results

Experiment 1

In the SON, the number of cells densely stained for NADPH-d varied significantly across the five groups ($F(4,24) = 7.96$, $p < .05$) (Figure 1a). Posthoc pairwise comparisons (Fishers PLSD) showed that rats in the Ovex+Chol group had significantly fewer cells stained for NADPH-d than those in the other groups ($p<.05$). In addition, females that received the highest daily dose of the OTA (120 ng) had significantly fewer positive stained cells than females in the Veh group ($p<.01$).

In the magnocellular subdivision of the PVN we also found that the number of stained cells for NADPH-d also varied significantly across the five groups ($F(4,24)=2.70$, $p=.05$) (Figure 1b). Posthoc tests revealed that rats in the Ovex + Chol group had fewer stained cells than those in the Veh and 6 ng OTA groups ($p<.05$) but did not differ from the 24 ng and 120 ng OTA groups. Overall, there was no significant difference in the number of NADPH-d stained cells in the parvocellular region of the PVN across groups ($F(4,24)= 2.6$, $p>.05$) (Figure 1c). Inspection of Figure 1c, however, suggests that in the absence of any hormone replacement, ovariectomy reduced the number of NADPH-d stained cells.

Experiment 2a

The number of cells stained for NADPH-d in the SON and magnocellular subdivision of the PVN varied significantly across the three groups ($F(2, 12) = 4.06$, $p < .05$); $F(2, 12) = 9.76$, p .01; respectively). Posthoc tests (Fishers PLSD) showed that suppressing PRL levels with bromocryptine reduced the number of cells stained for NADPH-d ($p<.05$). Subcutaneous PRL administration restored the number of NADPH-d positive stained cells in the SON and magnocellular PVN of bromocryptine treated rats (Figure 2a,b). There was no significant effect of treatment on the number of stained cells in the parvocellular subdivision of the PVN ($F(2, 12) = 2.41$, $p > .05$)(Figure 2c).

Experiment 2b

Examples of NADPH-d staining in the SON and PVN as a function of central PRL and OT treatment are seen in Figures 3 and 4. As can be seen in these figures and as presented numerically in Figure 5a,b , there were significant differences in the number of NADPH-d stained cells between groups in both the SON ($F(3,14)=3.82$, $p<.05$) and magnocellular subdivision of the PVN ($F(3,14)=4.52$, $p<.05$). Post hoc (Fisher PLSD) analysis revealed that rats treated with bromocryptine-alone had fewer NADPH-d positive stained cells than animals in the other groups ($p<.02$) which did not differ from each other. There was no significant effect of treatment on the number of stained cells in the parvocellular subdivision of the PVN ($F(3,14)=4.52$, $p<.05$)(Figure 5c).

Experiment 3

Images of *in situ* hybridization for OT mRNA within the SON and PVN are seen in Figure 6. ANOVA showed that the average optical density for OTmRNA in the SON and PVN varied significantly across the groups ($F(4, 24) = 18.76$, $p < .01$);

F (4, 24) = 17.18, p< .01;respectively). Post hocs (Tukey HSD) analysis showed that OT mRNA levels were significantly lower in animals in the Ovex + Chol, BR, and BR+PRL+OTA groups than animals in the Veh and BR+PRL groups (p<.01) (Figure 7).

Discussion

The results of these experiments support earlier findings showing that mimicking the gonadal steroid hormone profile of late pregnancy is sufficient to upregulate NOS in the SON and magnocellular compartments of the PVN (4). They are also consistent with a mediating role of OT in this effect (4). In addition, the current studies extend previous findings by demonstrating both that OT receptor activation plays a key role in the upregulation of NOS and that the effect of steroid priming on NOS in the PVN and SON is mediated not only by OT itself but also by the interaction of OT and PRL.

The results of Experiment 1 demonstrate that central administration of an OT receptor antagonist can reduce the number of cells staining for NADPH-d in the SON and magnocellular compartment of the PVN in rats treated with a hormonal regimen that mimics late pregnancy. These data combined with those of earlier studies demonstrating an increase in NADPH-d staining in the PVN and SON in E-primed ovariectomized females treated with chronic icv OT infusions support a role for OT receptor activation in the regulation of NOS in these areas (4). These findings are also consistent with hypothesis that NO plays a negative feedback role within the hypothalamic neurohypophysial system (10).

As shown in Experiment 2 administration of the D2-like agonist bromocryptine for 3 days following progesterone removal was sufficient to

eliminate the upregulation of NADPH-d staining in the SON and in the magnocellular, but not the parvocellular, subdivision of the PVN in steroid-primed ovariectomized females. Bromocryptine treatment suppresses prolactin release through its action at the D2 receptor (25) and its effect on NADPH-d staining could be eliminated by either systemic or central PRL replacement. These data therefore indicate that the effects of bromocryptine are mediated through its ability to suppress prolactin release rather than through other dopamine agonistic effects.

Pituitary PRL release is increased both in late pregnancy (26) and by the steroid hormone regimen used in the current experiments (13) and reaches the brain through an active transport mechanism (14). Moreover, PRL receptors have been identified within both the PVN and SON of late pregnant and lactating rats (15,27). Together with the fact that that both peripheral and icv PRL replacement were effective in increasing NADPH-d staining within these nuclei, this evidence suggests that PRL acts within the brain to promote the upregulation of NOS both in steroid-primed and late pregnant rats. Such a central action of PRL adds to the growing number of behavioral and neurochemical changes that have been attributed to central actions of PRL including stimulation of food intake (28) and maternal behavior (29) and the modulation of stress reactivity (30).

The results of Experiment 2b showed that the effects of bromocryptine treatment on NADPH-d staining could also be reversed by central administration of OT indicating that, although sufficient, PRL is not necessary for the upregulation of NOS in the PVN and SON of steroid-primed rats. In contrast, OT receptor activation does appear to be a necessary mediator of the effects of gonadal steroid priming on NOS because, as shown in Experiment 1,

administration of an OTA reduces NADPH-d staining in steroid-primed rats. There are a number of different ways in which the action of OT and PRL on magnocellular neurons could combine so as to increase NOS. It is possible that activation of both OT receptors and PRL receptors can have direct and independent effects on NOS synthesis. An alternative hypothesis is that the effects of PRL receptor activation on NOS are in fact mediated by OT. Support for this possibility comes from studies showing that, PRL stimulates OT mRNA in hypothalamo-neurohypophysial system explants (17,18) and OT release from the neural lobe (16).

The possibility that PRL actually upregulates OTmRNA in the PVN and SON and that this is , in turn, associated with upregulation of NOS was examined in Experiment 3. Here we found that, as previously reported (6), treatment with a steroid regimen that mimicked late pregnancy was sufficient to increase OT gene expression in the PVN and SON. As with NOS, the effect of steroid administration on OT mRNA was eliminated if rats were treated with bromocryptine after progesterone withdrawal and OT mRNA expression was restored if bromocryptine treated rats were given systemic PRL replacement. A role for OT receptor activation in the maintenance of OT mRNA levels was suggested by the fact that PRL replacement was unable to restore OT mRNA in bromocryptine-treated rats in the presence of chronic OTA infusion. These results provide the first *in vivo* evidence of the involvement of PRL in inducing elevated levels of OT mRNA within the SON and PVN.

Although the results of the present study indicate that PRL stimulates OT mRNA, it is also possible that OT plays a role in the increased PRL release that is seen in late pregnancy and after steroid-priming (31,32). It has been shown that plasma OT levels are elevated just prior to the increase in PRL

seen when dams are reintroduced to their pups following a 4-hr separation (33). In addition, exogenous administration of OT stimulates PRL release from anterior pituitary cells from estrogen-primed ovariectomized rats (33). Thus, the increased intranuclear OT release that stimulates the upregulation of NOS that is observed in late pregnancy might result indirectly from OT first stimulating PRL which then activates OT neurons.

The effects of manipulating PRL and OT on NOS and of prolactin on OT mRNA described in the current studies were all obtained in steroid-primed rats. It is highly probable that the background steroid hormone level is an important component of these effects. It is likely that progesterone withdrawal plays a key role in these effects as it does in the activation of OT neurons at parturition (7). In addition, following long term exposure to a combination of estrogen and progesterone progesterone withdrawal is required for both upregulation of NADPH-d staining (4) and OT mRNA (6) within the SON and PVN. The pattern of exposure to estrogen and progesterone is important for the upregulation of PRL itself, but it may also play multiple roles in modulating the efficacy of both PRL and OT by, for example incresing receptor levels. For example, previous data showed that chronic OT infusion was sufficient to upregulate NADPH-d only if ovariectomized rats were estrogen primed (4). Moreover, PRL is only effective in stimulating maternal behavior in estrogen-primed female rats (24). Considerable more work will be needed to elucidate precisely what type of gonadal steroid exposure is needed to allow the effects that are reported in this paper and to understand the mechanisms through which these effects are produced.

Prolactin and OT are both intimately involved in a number of reproductive processes including lactation. NOS is also upregulated in the hypothalamic

neurohypophysial system of lactating rats and NADPH-d staining decreases rapidly following removal of suckling stimulation (34). Because suckling stimulates both PRL (35) and OT release (36) it is possible that either or both peptide hormones contribute to the increased expression of NOS in the PVN and SON of lactating rats. In addition, the influence of PRL on OT mRNA and hence OT release may facilitate the positive feedback system for OT that is observed during parturition (37). It has been reported that mobilization of intracellular calcium from thapsigargin-sensitive stores can stimulate activity-dependent OT release from dendrites within SON magnocellular neurons (38). Thus, the dendritic release of OT within hypothalamic nuclei following stimulation of the hypothalamic neurohypophysial system, may act on adjacent neurons to affect their electrical activity or act in an autocrine manner by binding to its own receptors to maintain the sensitivity of the magnocellular OT-secreting neurons.

Intriguingly, both PRL and OT have been implicated in the onset of maternal behavior (29,39). Although the effects of PRL on OT mRNA reported here are limited to the hypothalamic neurohypophysial system, it is possible that similar interactions occur within brain structures subserving maternal behavior such as the medial preoptic area (40).

In conclusion, administration of an OT receptor antagonist and suppression of endogenous PRL both attenuated NADPH-d staining in magnocellular neurons in steroid-primed rats. The effect of prolactin suppression could be reversed either with subcutaneous or central PRL replacement, or with chronic central infusion of OT. Furthermore, suppression of PRL by bromocryptine and central administration of an OTA both decreased OT gene expression within the SON and PVN. These results demonstrate that

PRL and OT interact to increase the capacity for NO production within the SON and PVN and suggest that the upregulation of NOS may result from increases in intranuclear OT release. Moreover, these data suggest that PRL and OT interact to modulate OT mRNA expression in the PVN and SON.

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Figure Captions

Figure 1. Effects of treatment with various doses of an oxytocin receptor antagonist (OTA) on the mean number of NADPH-d stained cells in the a) supraoptic nucleus (SON) b) magnocellular paraventricular nucleus, and c) parvocellular paraventricular nucleus of steroid-primed rats. The highest dose of the OTA decreased the number of NADPH-d stained cells in the SON and magnocellular portion of the PVN. [a] P<.05,significantly different from all groups; [b] P<.05,significantly different from Veh; [c] P<.05, significantly different from Veh and 6 ng/day.

Figure 2. Effects of systemic prolactin replacement on the mean number of NADPH-d stained cells in the a) SON, b) magnocellular paraventricular nucleus, and c) parvocellular paraventricular nucleus in bromocryptine-treated steroid-primed rats. Systemic PRL treatment restored the number of NADPH-d stained cells in the SON and magnocellular portion of the PVN following bromocryptine treatment. * P<.05, significantly different from Veh and BR.

Figure 3. Example of NADPH-d staining in the supraoptic nucleus of the steroid primed rats that received either Vehicle (Veh), Bromocryptine (BR), Bromocryptine and Prolactin (BR+PRL), or Bromocryptine and Oxytocin (BR+OT).

Figure 4. Example of NADPH-d staining in the paraventricular nucleus of the steroid primed rats that received either Vehicle (Veh), Bromocryptine

(BR), Bromocryptine and Prolactin (BR+PRL), or Bromocryptine and Oxytocin (BR+OT).

Figure 5. Effects of central prolactin and oxytocin replacement on the mean number of NADPH-d stained cells in the a) SON, b) magnocellular paraventricular nucleus, and c) parvocellular paraventricular nucleus in bromocryptine-treated steroid-primed rats. Both central PRL and OT treatment restored the number of NADPH-d stained cells in the SON and magnocellular portion of the PVN following bromocryptine treatment. * P<.05, significantly different from Veh, BR+icvPRL, and BR+icvOT.

Figure 6. Example of OT mRNA in the supraoptic nucleus (SON) and paraventricular nucleus of ovariectomized rats that received steroid priming and either: Vehicle (Veh), Bromocryptine (BR), Bromocryptine and Prolactin (PRL), or Bromocryptine together with Prolactin and Oxytocin antagonist (OTA).

Figure 7. Effects of bromocryptine, and prolactin replacement on OT mRNA (OD units) expression in the a) supraoptic nucleus (SON) and b) paraventricular nucleus (PVN) in steroid-primed rats. Both bromocryptine and OTA treatment suppressed OT gene expression in the SON and PVN in steroid primed rats; while prolactin replacement restored OT mRNA to levels seen in the vehicle group. * P<.05, significantly different from Ovx+Chol, BR, and BR+PRL+OTA.

Figure 1.

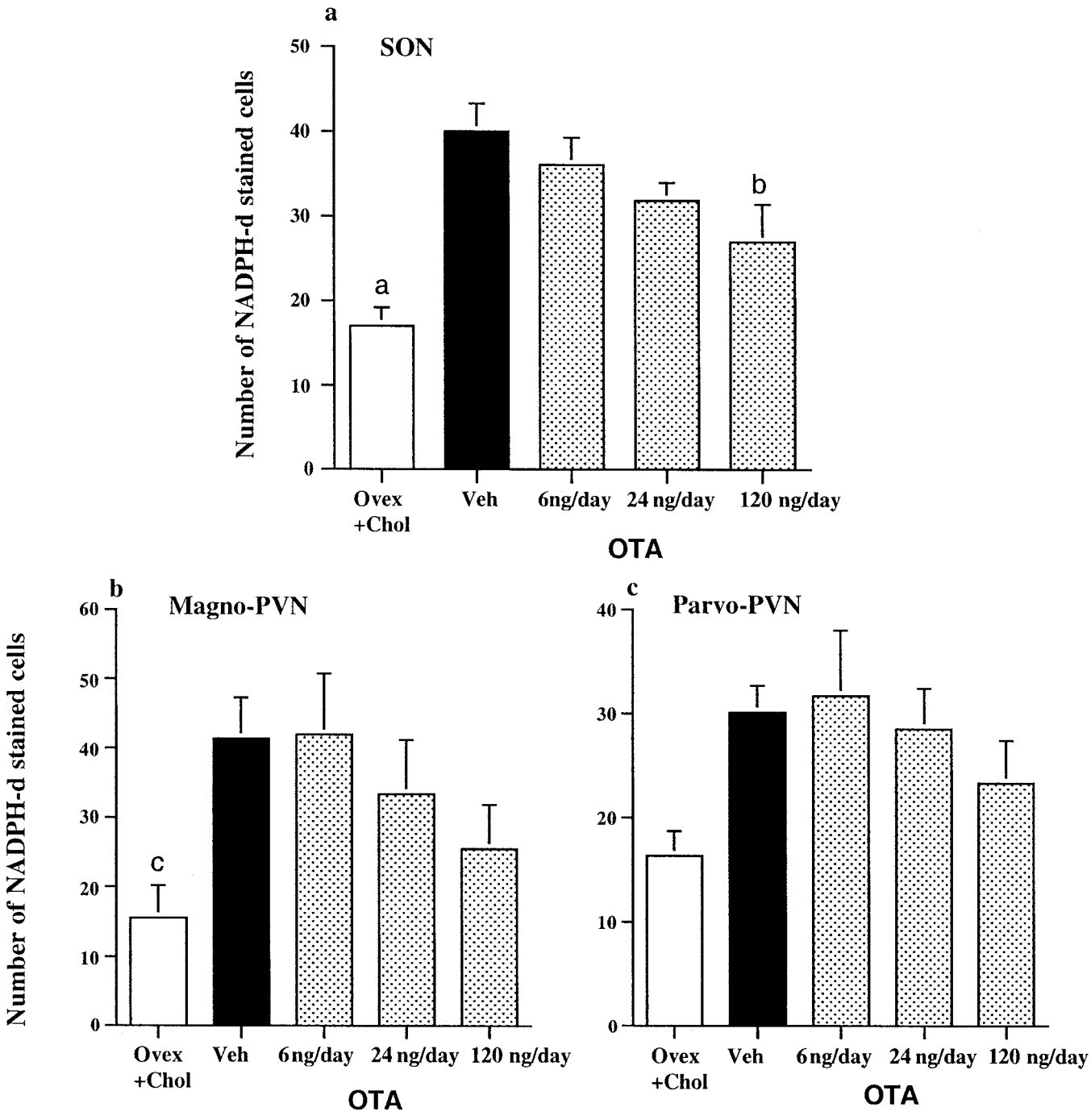

a
SON
Number of NADPH-d stained cells
50
40
30
20
10
0
a
b
Ovex +Chol
Veh
6ng/day
24 ng/day
120 ng/day
OTA
b
Magno-PVN
Number of NADPH-d stained cells
60
50
40
30
20
10
0
c
Ovex +Chol
Veh
6ng/day
24 ng/day
120 ng/day
OTA
c
Parvo-PVN
40
30
20
10
0
Ovex +Chol
Veh
6ng/day
24 ng/day
120 ng/day
OTA

Figure 2.

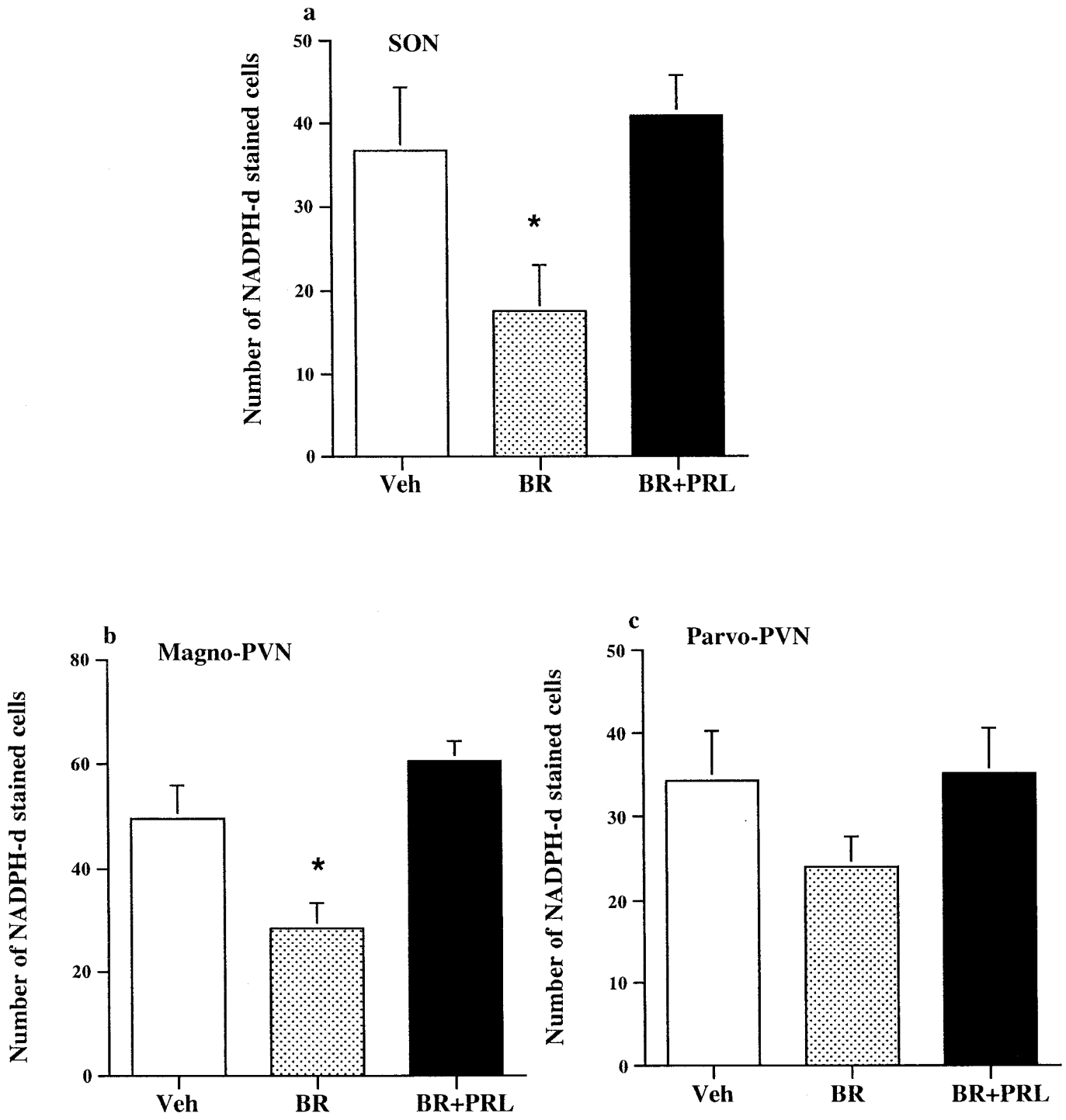

a
SON
Number of NADPH-d stained cells
50
40
30
20
10
0
*
Veh
BR
BR+PRL
b
Magno-PVN
Number of NADPH-d stained cells
80
60
40
20
0
*
Veh
BR
BR+PRL
c
Parvo-PVN
Number of NADPH-d stained cells
50
40
30
20
10
0
Veh
BR
BR+PRL

Figure 3.

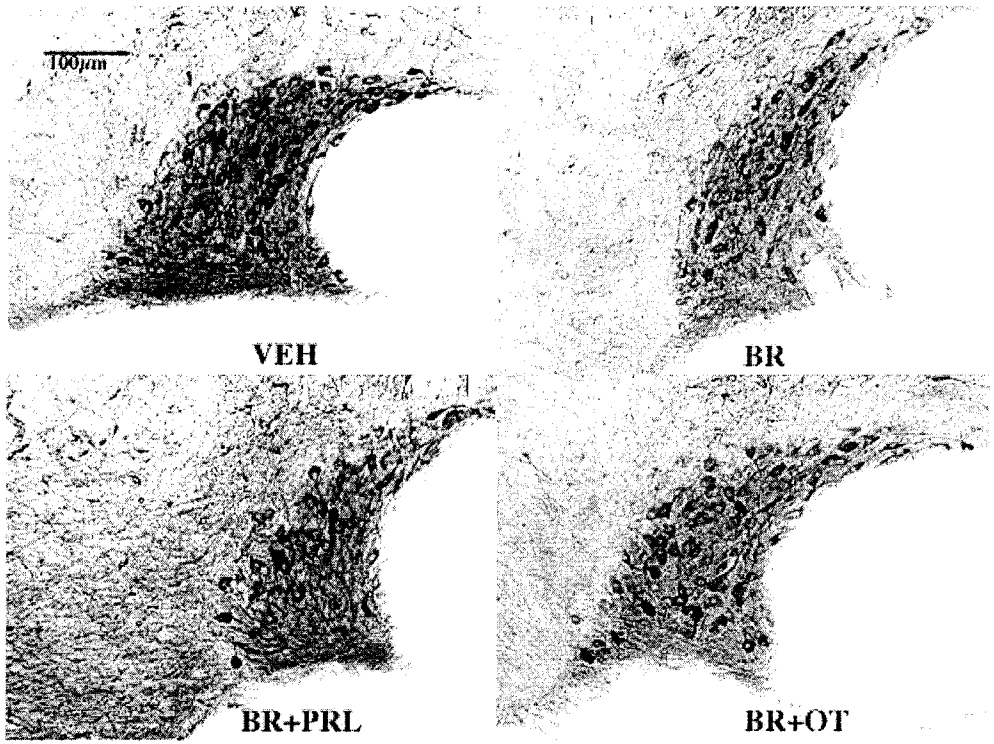


Figure 4.

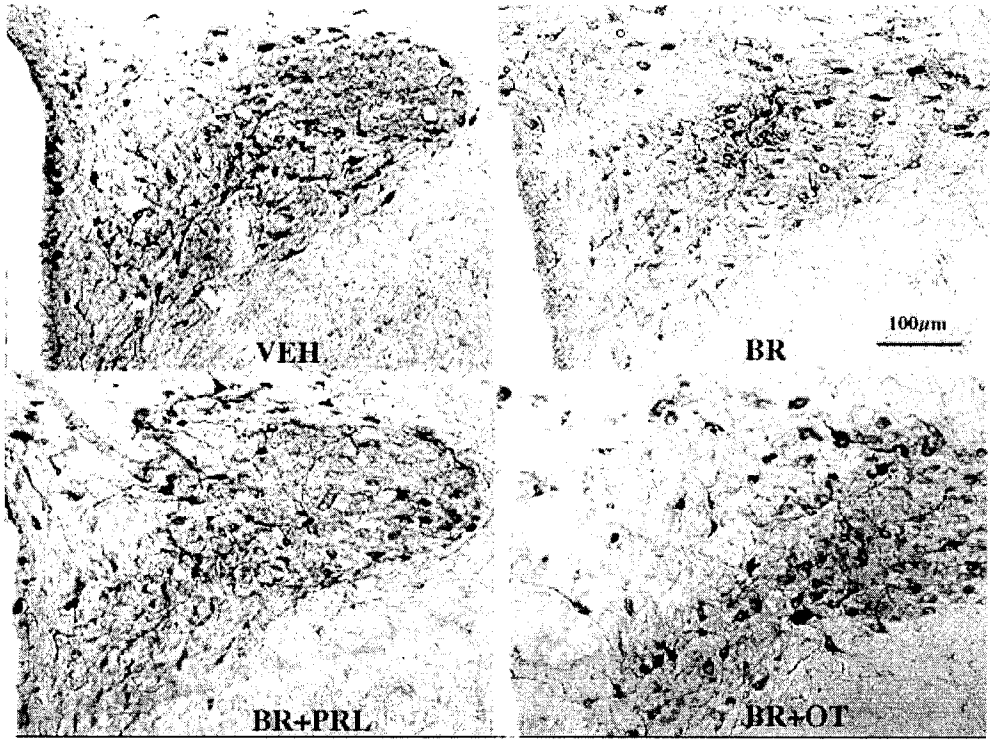


Figure 5.

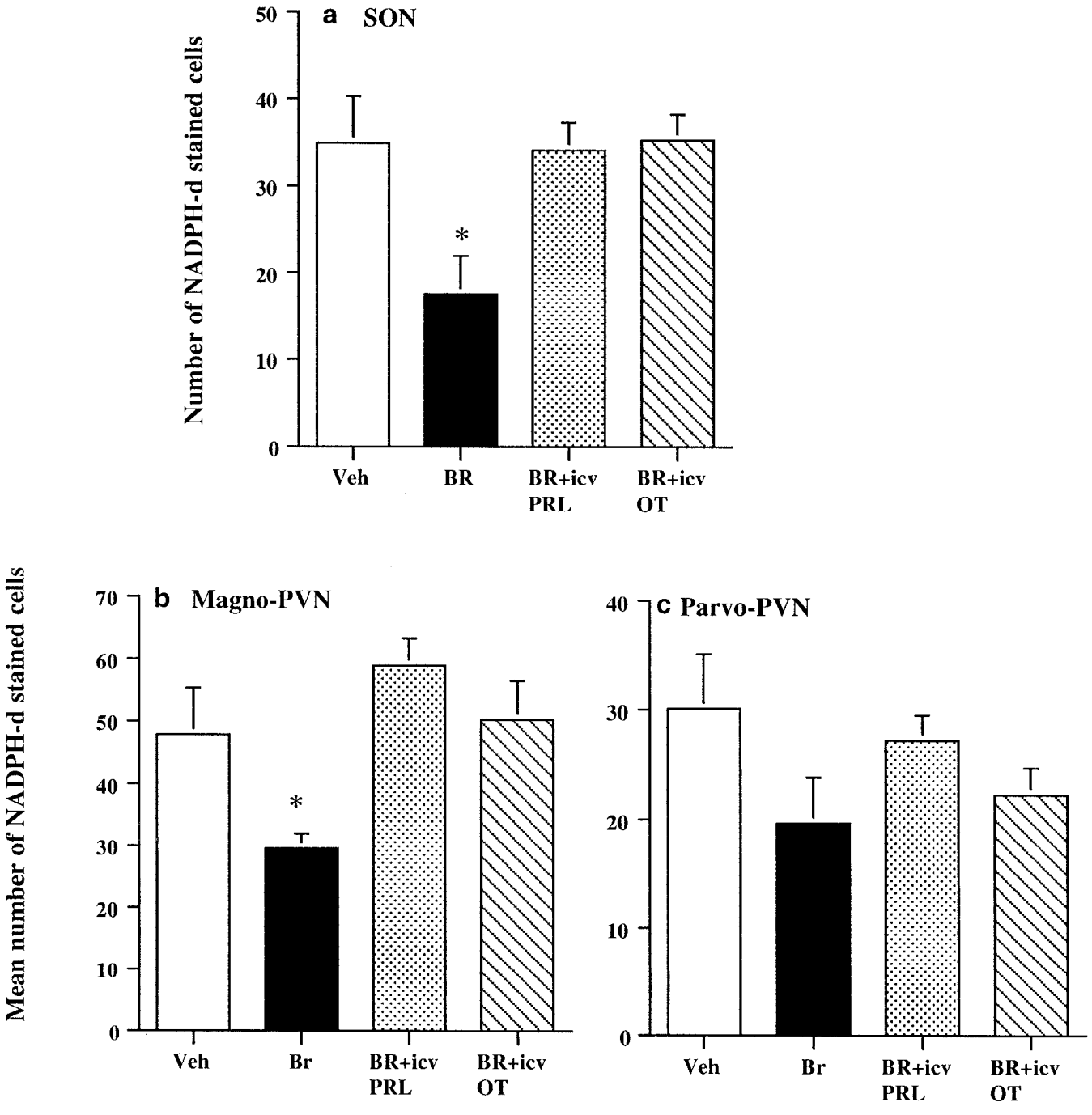

a SON
Number of NADPH-d stained cells
50
40
30
20
10
0
*
Veh
BR
BR+icv PRL
BR+icv OT
b Magno-PVN
Mean number of NADPH-d stained cells
70
60
50
40
30
20
10
0
*
Veh
Br
BR+icv PRL
BR+icv OT
c Parvo-PVN
40
30
20
10
0
Veh
Br
BR+icv PRL
BR+icv OT

Figure 6.

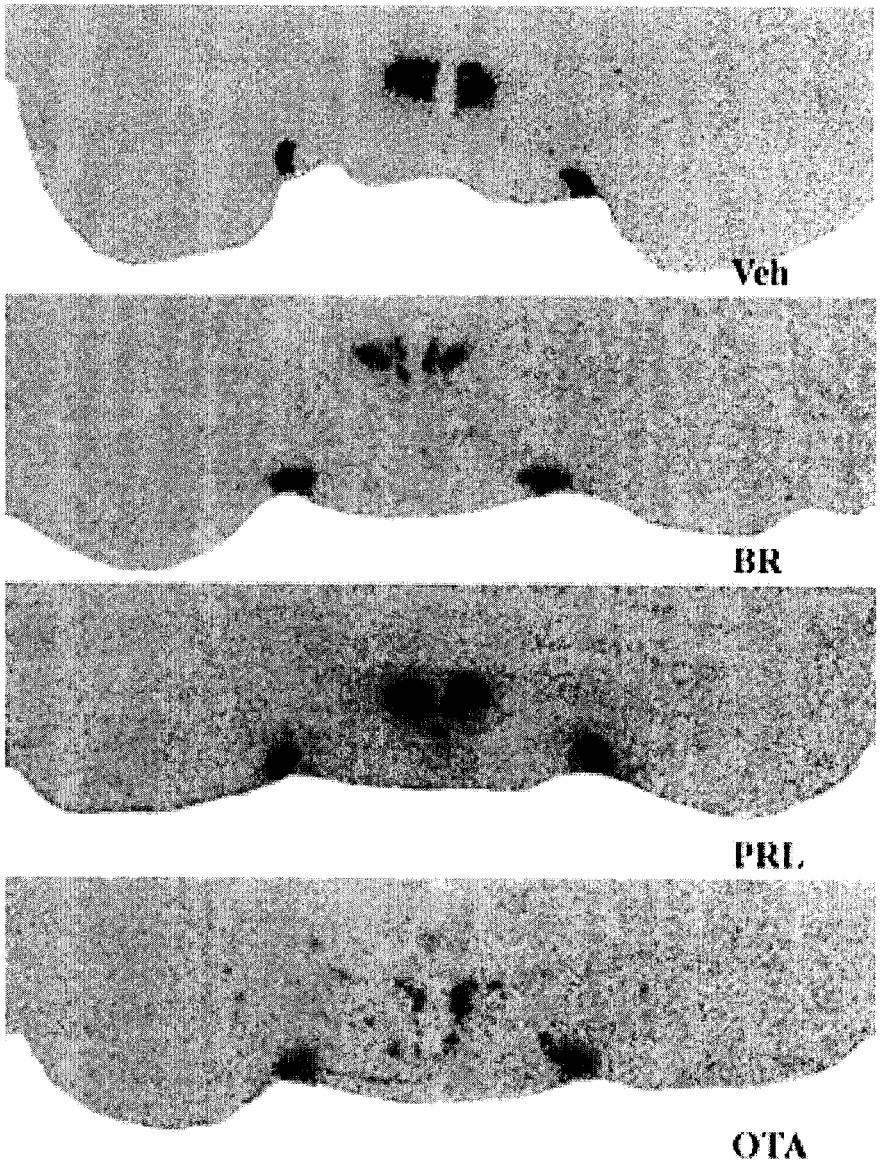
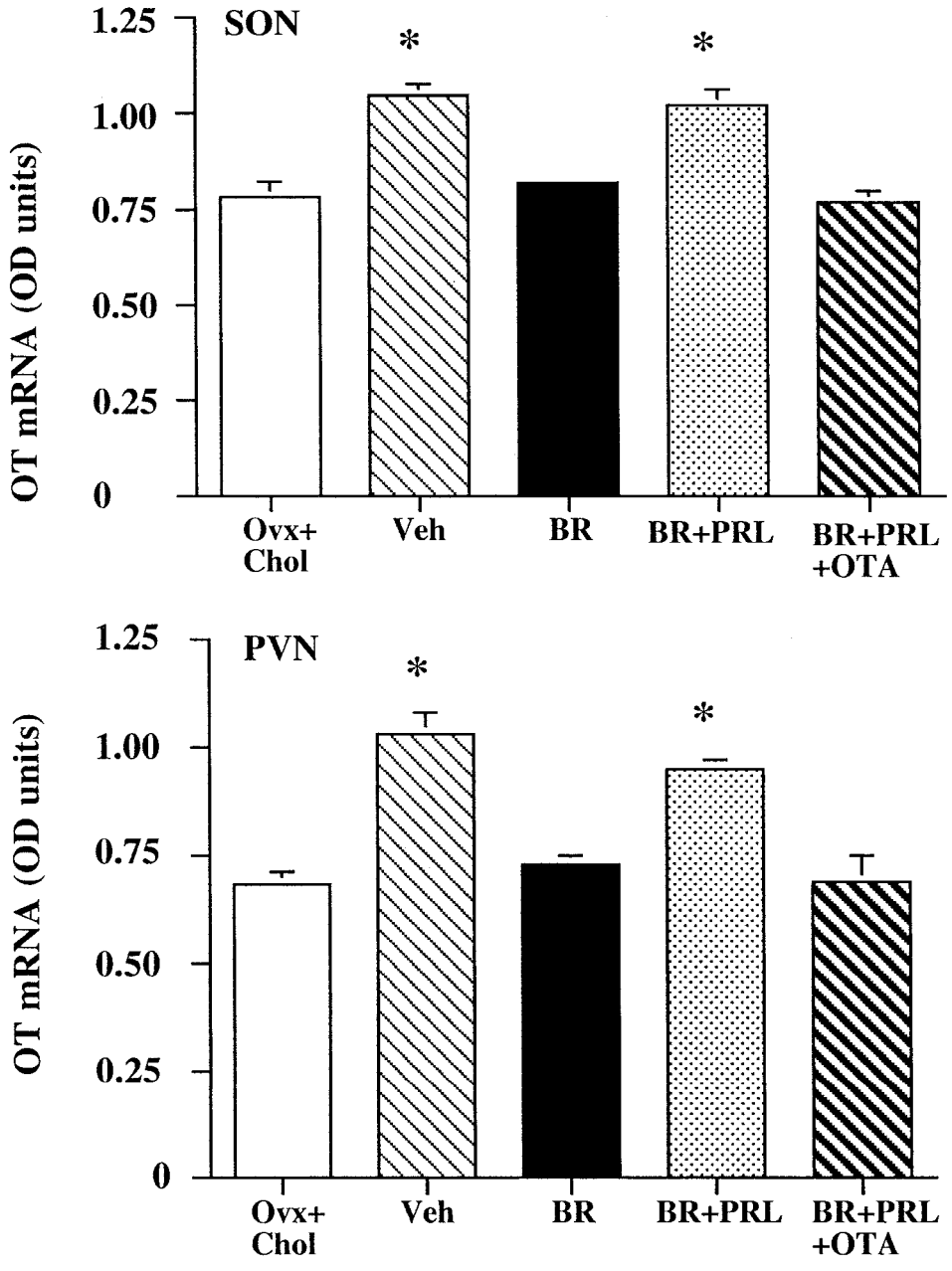


Figure 7.


SON
1.25
1.00
0.75
0.50
0.25
0
OT mRNA (OD units)
*
*
Ovx+ Chol
Veh
BR
BR+PRL
BR+PRL +OTA
PVN
1.25
1.00
0.75
0.50
0.25
0
OT mRNA (OD units)
*
*
Ovx+ Chol
Veh
BR
BR+PRL
BR+PRL +OTA

Appendix B

Effect of NOS inhibition on fos expression within the hypothalamus of female rats following central oxytocin and systemic urethane administration

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Effect of Nitric Oxide Synthase Inhibition on Fos Expression in the Hypothalamus of Female Rats Following Central Oxytocin and Systemic Urethane Administration

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Key words: nitric oxide, lactation, oxytocin, urethane, Fos.

Abstract

Three experiments were carried out to investigate the pattern of neuronal activation induced by central oxytocin administration and its modulation by nitric oxide (NO). First, we compared the induction of Fos-like immunoreactivity (lir) in the supraoptic (SON) and paraventricular (PVN) nuclei and medial preoptic area (MPOA) after central oxytocin administration between nonlactating and lactating rats. Next, we investigated whether NO modulated Fos induction following central oxytocin administration using a nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME). Finally, to determine whether the effects of NOS inhibition on Fos induction would generalize to stimuli other than oxytocin, we compared Fos-lir in the SON and PVN of lactating and nonlactating rats following L-NAME and urethane administration. In the first two experiments, oxytocin (50 ng in 2 µl) or vehicle was administered into the third ventricle. L-NAME (50 mg/kg) was given by an intraperitoneal (i.p.) injection 30 min before oxytocin administration (experiment 2) or an i.p. injection of urethane (1.4 g/kg) (experiment 3). In all experiments, lactating rats were tested on day 12 or 13 postpartum and nonlactating females at least 11 days after surgery or the start of the experiment. Central oxytocin infusion induced Fos expression in the SON and PVN in lactating and nonlactating rats and in the MPOA and bed nucleus of the stria terminalis in lactating rats. Overall, lactating rats that received L-NAME and oxytocin had a greater number of cells showing Fos-lir in both the SON and PVN. Conversely, L-NAME administration reduced Fos-lir in the SON and PVN in oxytocin-stimulated nonlactating rats. In urethane-treated rats, L-NAME administration did not change Fos-lir in lactating rats but reduced Fos-lir in nonlactating rats. These data suggest that the role of NO in modulating the activity of neurones in discrete nuclei in the hypothalamus varies across reproductive state and with the stimulus presented.

Oxytocin is a nonapeptide hormone that is synthesized within the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus and is released from the posterior pituitary into the general circulation in response to a variety of environmental stimuli, including suckling (1) and osmotic challenge (2). In addition to its well-established peripheral actions, recent studies have shown that oxytocin acts centrally to modulate both physiology and behaviour. For example, the central administration of oxytocin has a facilitatory effect on the proestrus surge of luteinizing hormone (3), stimulates penile erection and yawning (4) and facilitates the onset of maternal behaviour in virgin rats (5). It also inhibits food intake (6) and reduces the behavioural and hormonal responses to stress (7). One important target of central oxytocin appears to be the oxytocin neurones in the SON because central oxytocin infusion has been shown to facilitate and coordinate the activation of these neurones (8).

Consistent with these central effects of oxytocin administration, oxytocin receptors have been localized on oxytocin neurones in the PVN and SON (9). In addition, other brain regions, including the medial preoptic area (MPOA), the amygdala, the ventromedial hypothalamic nucleus and the bed nucleus of the stria terminalis (BNST), have also been shown to contain oxytocin receptors (10). Furthermore,

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oxytocin release within the SON and PVN during osmotic stimulation (11) as well as during parturition and suckling has been demonstrated (12). This evidence has led to the suggestion that stimulation of magnocellular oxytocin cells leads to intranuclear oxytocin release which has a positive feedback effect on oxytocin neurones and stimulates further oxytocin release.

Recent evidence suggests that nitric oxide synthase (NOS), the enzyme that synthesizes nitric oxide (NO) (13) colocalizes with oxytocin in both the SON and PVN (14). Stimuli such as saltloading (15) and water deprivation (16) result in an upregulation of NOS in the SON and PVN suggesting that increases in NOS in these nuclei accompany activation of the oxytocin system. However, the functional significance of the increase in NOS is not clear.

Previous studies have shown that NO is an important modulator both of the activity of magnocellular neurones within the SON (17) and of oxytocin secretion (18). Electrophysiological studies of neuronal activity within the SON have shown that administering NO donors reduces overall neuronal activity (17) and this effect appears to result, at least in part, from an increase in the frequency of inhibitory post synaptic potentials (19). In addition, depletion of oxytocin content from the posterior pituitary in response to chronic salt loading (18) or acute water deprivation (20) is enhanced when rats are simultaneously treated with a NOS inhibitor either systemically or intracerebroventricularly. Basal oxytocin release, however, is not affected by inhibiting NOS (20). Together, these data have been interpreted to suggest that stimulation of the hypothalamic neurohypophysial system (HNS) leads to an upregulation of NOS and potentially an increase in NO production that has a negative feedback effect on oxytocin release (18, 20).

One mechanism through which NO might act to limit the response of the HNS to stimulation is by modulating the pattern of neuronal activation induced by intranuclear oxytocin release. To investigate this possibility, we first used Fos immunohistochemistry to compare the pattern of neural activation in the hypothalamus induced by third ventricle infusions of oxytocin in rats in which the oxytocin system was chronically stimulated versus unstimulated rats, and then determined how this pattern was changed when NOS was inhibited. It has been well established that, during lactation, NOS within the SON and PVN is upregulated (21, 22), the oxytocin system is more active (1) and intranuclear as well as hypophysial oxytocin release is increased (12). Therefore, in experiment 1, we compared neuronal activation in the SON, PVN and MPOA of nonlactating and lactating rats following central oxytocin stimulation using Fos-like immunoreactivity (lir) as a marker of cellular activation. We took this approach because Fos, the protein product of the immediate early gene c-*fos*, has been used extensively to map the response of the hypophysial neurosecretory system to a wide range of stimuli (23).

In experiment 2, to determine the effects of inhibiting NOS on the effects of central oxytocin administration, the induction of Fos-lir in the SON, PVN, MPOA and BNST was examined in lactating or nonlactating rats given a systemic injection of either N^{ω}-nitro-L-arginine methyl ester (L-NAME) or vehicle prior to a central injection of either oxytocin or vehicle into the third ventricle.

Finally, in experiment 3, to determine whether any effects of the inhibition of NO synthesis on Fos expression that we observed in experiment 2 would generalize to other stimuli known to activate oxytocin neurones, we compared the effect of inhibiting NO synthesis, using L-NAME, on Fos expression in the SON and PVN of lactating and nonlactating rats following urethane administration.

Materials and methods

Animals

Virgin female Wistar rats from Charles River Breeding Farm (St Constant, Quebec, Canada) were used in these studies. Upon arrival in the laboratory, the rats weighed between 220 and 240 g and were housed in groups of five females. The animal facility was maintained on a 12-h light/dark cycle (lights on from 08.00 to 20.00 h) at 20 ± 20°C. Rats were given access to laboratory chow and water *ad libitum* throughout the experiment. Where appropriate, rats were mated by placing one male rat in a group cage with five females for 2 weeks. Approximately 3 days before parturition, each pregnant female was removed from the group cage and placed in a single cage (20 × 45 × 25) with access to food and water *ad libitum*. On the day after parturition (day 1 postpartum, pp), litters were culled to eight pups. All protocols were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Surgery

A stainless steel cannula (22-gauge; Plastic One Products, Roanoke, VA, USA) was inserted stereotaxically into the third ventricle (anterior-posterior −0.80 mm, lateral 0 mm, dorso-ventral − 6.2 mm below dura under ketamine/xylazine (5.7 mg ketamine and.86 mg xylazine/100 g of body weight) anaesthesia on day 1 pp in lactating groups and at least 11 days prior to the experimental day in nonlactating groups

Injection procedure

Manipulations were carried out on day 12–13 pp in lactating groups and at least 11 days after surgery in nonlactating rats. To minimize potential stress effects on Fos expression, rats were handled daily. Seven days after surgery, each rat received an angiotensin infusion (50 ng/2 µl). If the injection of angiotensin stimulated water intake, then the cannula placement was considered accurate. For 3 days before the experimental day, rats were habituated to the injection procedure by placing the injection needle into the cannulae for 2 min each day. Injections were carried out between 10.00 h and 12.00 h.

Immunocytochemistry

One hour after hormone or urethane injection, each rat was injected with an overdose of sodium pentobarbital and perfused transcardially with 200 ml of ice-cold saline, followed by 300 ml of 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24 h. Fifty µm-thick sections throughout the BNST, MPOA, SON and PVN were cut on a vibratome and placed free-floating in Trizma buffered saline (TBS; pH 7.3) solution and then processed for Fos-lir. Each assay included samples from each treatment group; thus, interassay variability did not contribute to between group variability. The sections were incubated for 30 min in 3% H_2O_2 solution in TBS to reduce nonspecific staining and then washed in TBS three times for 15 min. Tissue was then incubated for 90 min at 4°C in blocking serum (0.3% Triton X; TTX, Sigma, St Louis, MO, USA), 3% normal goat serum (NGS, Vector, Burlingame, CA, USA) and (TBS) and then incubated in the primary antibody solution. The antibody used was a polyclonal antibody (Ab-5, PC-38, Oncogene Research Products, Cambridge, MA, USA) diluted 1 : 130 000. This antibody, recognizes amino acids 4–17 of the human c-*fos* epitope. Forty-eight hours later, sections were washed three times in TBS for 15 min and then were incubated in secondary antibody (biotinylated rabbit antigoat, Vector) for 1 h, washed three times in TBS and incubated in ABC reagent for 2 h. Tissue staining was developed using an ABC vector kit (diminobenzene, nickel intensified). The sections were then mounted on gelatin

slides and cover slipped (Permount, Fisher Scientific Co., Pittsburgh, PA, USA) for analysis.

Image analysis

Sections were visualized using a Sony XC77 camera mounted on a light microscope (Labolux Leitz GMBH, Wetzlar, Germany). Images were captured using NIH image analysis software (v. 1.60b; http://rsb.info.nih.gov/nih-image/) installed on a Power Macintosh computer. Fos-lir cells were counted from the images captured in TIFF picture files. Stained cells in sections throughout the rostro-caudal extent of the SON 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. For the PVN, only sections in the medial area of the nucleus were used (approximately −1.8 mm from bregma). The PVN was subdivided into magnocellular and parvocellular regions. Fos-positive cells in both of these subdivisions in approximately three sections were counted and an average for each region over all sections was obtained. To estimate Fos expression in the MPOA (−0.80 mm to −0.92 mm from bregma) and BNST (−0.30 mm to −0.40 mm from bregma), stained cells in approximately three sections were counted and an average of these sections was calculated for each brain region.

Experiments

Experiment 1: effects of oxytocin on Fos induction in nonlactating and lactating rats

Rats were assigned to one of four experimental groups: nonlactating-saline (NL/Sal; n=5), nonlactating-oxytocin (OT) (NL/OT; n=4), lactating-saline (Lac/Sal; n=4) and lactating-oxytocin (Lac/OT; n=5). Lactating females were given an intracerebroventriclar (i.c.v.) injection of either 2 µl of saline or oxytocin (50 ng in 2 µl, Sigma) on day 12 or 13 pp. Nonlacting females received similar treatments at least 11 days after surgery.

Experiment 2: effects of L-NAME on Fos induction in central oxytocin-stimulated lactating and nonlactating rats

Lactating rats were randomly assigned to one of four experimental groups: saline-saline (Sal/Sal; n=5), saline-oxytocin (Sal/OT; n=4), L-NAME-saline (LN/Sal; n=5) and L-NAME-oxytocin (LN/OT; n=4). On day 12–13 pp, rats were given an i.p. injection of either saline or L-NAME (50 mg/kg) followed 30 min later by an i.c.v. injection of saline or oxytocin (50 ng in 2 µl; Sigma).

Nonlactating rats were randomly assigned to one of four experimental groups: saline-saline (Sal/Sal; n=6), saline-oxytocin (Sal/OT; n=6), L-NAME-saline (LN/Sal; n=5), and L-NAME-oxytocin (LN/OT; n=6). Approximately 11 days after surgery, nonlactating rats were given the same treatments as lactating rats.

Experiment 3: effects of L-NAME on Fos induction in urethane-stimulated nonlactating and lactating rats

Rats were assigned to one of four experimental groups: nonlactating-saline (NL/Sal; n=6), nonlactating-L-NAME (NL/LN; n=5), lactating-saline (Lac/Sal; n=5) and lactating-L-NAME (Lac/LN; n=6). On the test day (day 12–13 pp for lactating females), rats received an i.p. injection of saline or L-NAME (50 mg/kg) followed 30 min later by an i.p. injection of urethane (1.4 g/kg).

Statistical analysis

Data obtained in all three experiments were analysed using two-way independent groups ANOVA followed, where appropriate, by post-hoc comparisons using the Fisher PLSD. Homogeneity of variance was assessed in each of the experiments using the F_{max} test and, where necessary, the α level was adjusted accordingly.

Results

Experiment 1: effects of oxytocin on Fos induction in nonlactating and lactating rats

Oxytocin administration induced robust Fos expression in both nonlactating and lactating rats in the SON (Fig. 1). Analysis of these data with a 2×2 (hormone × reproductive state) independent groups ANOVA showed significant main effects for both hormone [$F(1,14)=79.04$, $P<0.05$] and reproductive state [$F(1,14)=8.14$, $P<0.05$] in this nucleus. Although the analysis revealed no significant interaction between hormone and reproductive state in this area, inspection of the data and subsequent post-hoc analysis (Fisher PLSD) showed that the effect of reproductive state primarily reflected a greater effect of oxytocin administration on Fos-lir induction in the SON of lactating rats than in that of nonlactating rats.

Oxytocin administration also increased Fos-lir in both the magnocellular and parvocellular subdivisions of the PVN [$F(1,14)=20.52$, $P<0.05$; $F(1,14)=18.21$, $P<0.05$, respectively; Fig. 1b,c]. There was no significant main effect for reproductive state in either area, but a significant trend was seen in the parvocellular subdivision ($F(1,14)=4.06$, $P=0.06$) which seems to reflect a higher degree of Fos-lir in the saline-treated lactating group.

As is shown in Fig. 1(d), oxytocin administration induced Fos-lir in the MPOA of lactating rats but had little effect in nonlactating rats. Analyses of these data yielded significant main effects for hormone and for reproductive state [$F(1,14)=4.70$, $P<0.05$; $F(1,14)=6.95$, $P<0.05$, respectively].

Experiment 2: effects of L-NAME on Fos induction in central oxytocin-stimulated lactating and nonlactating rats

Examples of staining for Fos-lir observed in the SON and PVN of lactating rats that received Sal-Sal, LN-Sal, Sal-OT or LN-OT are shown in Figs 2 and 3. Counts of mean number of Fos-lir stained cells are shown in Fig. 4.

The highest number of Fos-lir stained cells in the SON and both subdivisions of PVN was observed in rats that received L-NAME and oxytocin. Consistent with the results of experiment 1, oxytocin infusion alone induced a robust Fos response in all three regions and this was augmented by administration of L-NAME prior to central oxytocin infusion in both the SON and parvocellular PVN. Analysis of these data with a 2×2 (drug × hormone) independent groups ANOVA showed a main effect for hormone in the SON, and magnocellular and parvocellular subdivisions of the PVN [$F(1,14)=49.36$, $P<0.05$; $F(1,14)=21.25$, $P<0.05$; $F(1,14)=22.81$, $P<0.05$, respectively] as well as a main effect for drug in the SON and parvocellular PVN [$F(1,14)=5.89$, $P<0.05$; $F(1,14)=9.22$, $P<0.05$, respectively]. Subsequent post-hoc comparisons (Fisher PLSD) revealed that the main effect for drug in the SON and PVN reflected an increase in Fos expression following the coadministration of L-NAME and oxytocin that did not occur when L-NAME was administered prior to saline infusion.

As in experiment 1, oxytocin infusion increased Fos-lir expression in the MPOA [$F(1,15)=16.27$, $P<0.05$] but there was no effect of L-NAME in this area (Sal-Sal, $M=205.6\pm24.98$; LN-Sal, $M=212.85\pm20.12$; Sal-OT, $M=332.63\pm45.02$; LN-OT, $M=326.24\pm29.82$). Similarly, in the BNST, central oxytocin infusion increased Fos-lir relative to saline-treated rats [$F(1,15)=55.01$, $P<0.05$] and L-NAME had no effect on Fos expression (Sal-Sal,

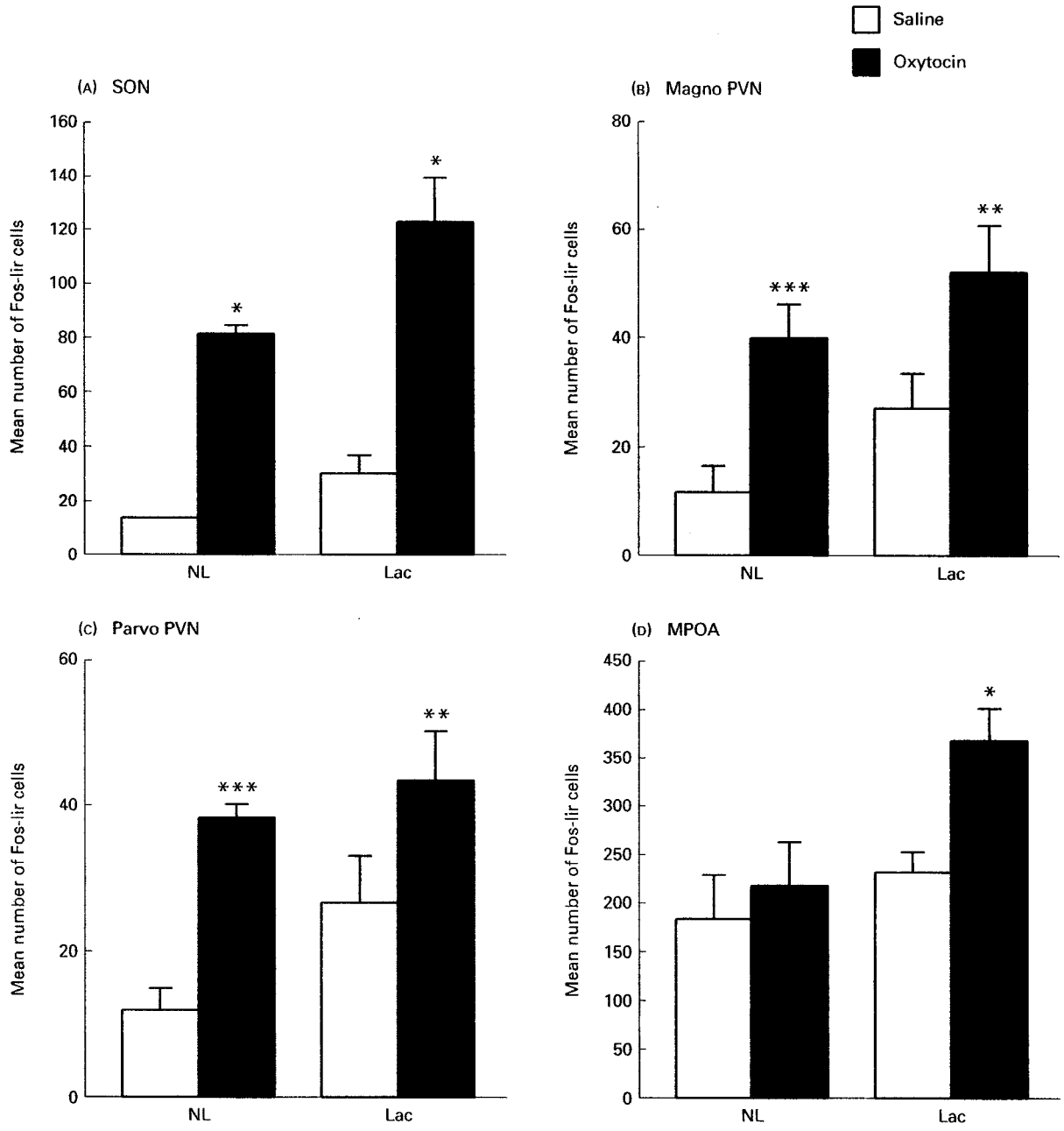

FIG. 1. Effects of oxytocin treatment on the mean number of Fos-like immunoreactivity (lir) in the (A) supraoptic nuclei (SON), (B) magnocellular paraventricular nuclei (PVN), (C) parvocellular PVN and (D) medial preoptic area (MPOA) of nonlactating and lactating rats. Oxytocin increased Fos-lir in nonlactating and lactating rats in the SON and both subdivisions of the PVN and only increased Fos expression in the MPOA of lactating rats. *$P<0.05$, significantly different from the other groups; **$P<0.05$, significantly different from nonlactating-saline (NL-Sal) and lactating-saline (Lac-Sal); ***$P<0.05$, significantly different from NL-Sal.

$M=55.7\pm4.79$; LN-Sal, $M=61.9\pm2.34$; Sal-OT, $M=111.6\pm11.58$; LN-OT, $M=95.8\pm4.52$).

Examples of staining for Fos-lir observed in the SON and PVN of nonlactating rats that received Sal-Sal, L-NAME-Sal, Sal-OT or L-NAME-OT are shown in Figs 5 and 6.

As seen in Fig. 7, oxytocin induced Fos-lir in the SON and the magnocellular and parvocellular PVN in nonlactating rats [significant main effect of hormone $F(1,19)=7.83$, $P<0.05$; $F(1,19)=22.61$, $P<0.05$; $F(1,19)=11.23$, $P<0.05$, respectively]. L-NAME administered prior to central oxytocin

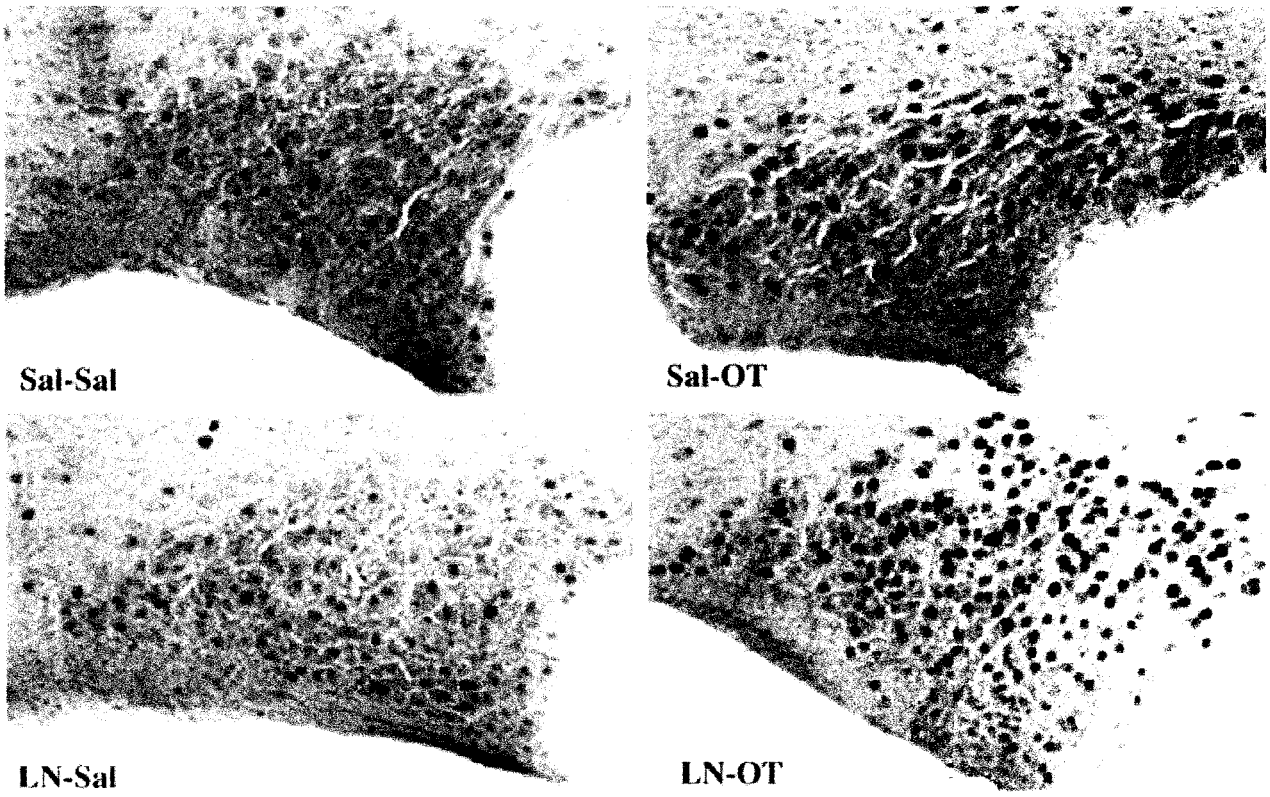

FIG. 2. Example of Fos-like immunoreactivity in the supraoptic nuclei of lactating rats that received either saline (Sal-Sal), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and saline (LN-Sal), saline and oxytocin (Sal-OT) or L-NAME and oxytocin (LN-OT).

infusion tended to reduce Fos-lir in both the magnocellular PVN and the SON but did not affect Fos-lir when administered before saline infusion [drug–hormone interaction: magnocellular PVN $F(1,19)=4.76$, $P<0.05$; SON $F(1,19)=2.88$, $P=0.10$]. There was no main effect for drug in any of the areas examined.

Experiment 3: effects of L-NAME on Fos induction in urethane-stimulated nonlactating and lactating rats

Counts of mean number of Fos-lir stained cells for all groups in this experiment are shown in Fig. 8. Overall, urethane administration induced more Fos-lir in the SON and magnocellular and parvocellular PVN in nonlactating than in lactating rats. This was reflected in a significant main effect for reproductive state in all the areas examined [SON $F(1,18)=37.91$, $P<0.05$; magno-PVN $F(1,17)=17.64$; parvo-PVN $F(1,17)=17.48$, $P<0.05$]. In addition, L-NAME administration reduced Fos-lir in these areas giving rise to a significant main effect for drug in the SON and magnocellular and parvocellular subdivisions of the PVN [$F(1,18)=9.75$, $P<0.05$; $F(1,17)=6.59$, $P<0.05$; $F(1,17)=4.13$, $P=0.06$, respectively]. In the magnocellular and parvocellular subdivisions of the PVN, L-NAME administered before urethane reduced Fos-lir in nonlactating rats but not in lactating rats [significant drug × reproductive state interaction; magno–PVN $F(1,17)=5.69$, $P<0.05$; parvo-PVN $F(1,17)=4.41$, $P=0.05$]. This effect was not seen in the SON.

Discussion

The results of experiment 1 showed that central oxytocin administration induced Fos-lir in the SON, and magnocellular and parvocellular subdivisions of the PVN. In the SON, this effect was greater in lactating than in nonlactating rats. Oxytocin administration also increased Fos-lir in the MPOA, but only in lactating rats. In experiment 2, inhibiting NO synthesis prior to oxytocin administration increased Fos-lir in the SON and PVN of lactating rats but attenuated Fos-lir in these areas in nonlactating rats. The induction of Fos-lir in the MPOA and BNST by oxytocin administration was not affected by L-NAME. In experiment 3, L-NAME treatment also reduced Fos-lir following urethane administration in nonlactating rats but, with the exception of the SON, there was little effect of combining L-NAME and urethane treatment on Fos expression in lactating rats compared to the effect of urethane administration alone. The latter data suggest that the ability of L-NAME administration to augment the activational effect of oxytocin in some hypothalamic

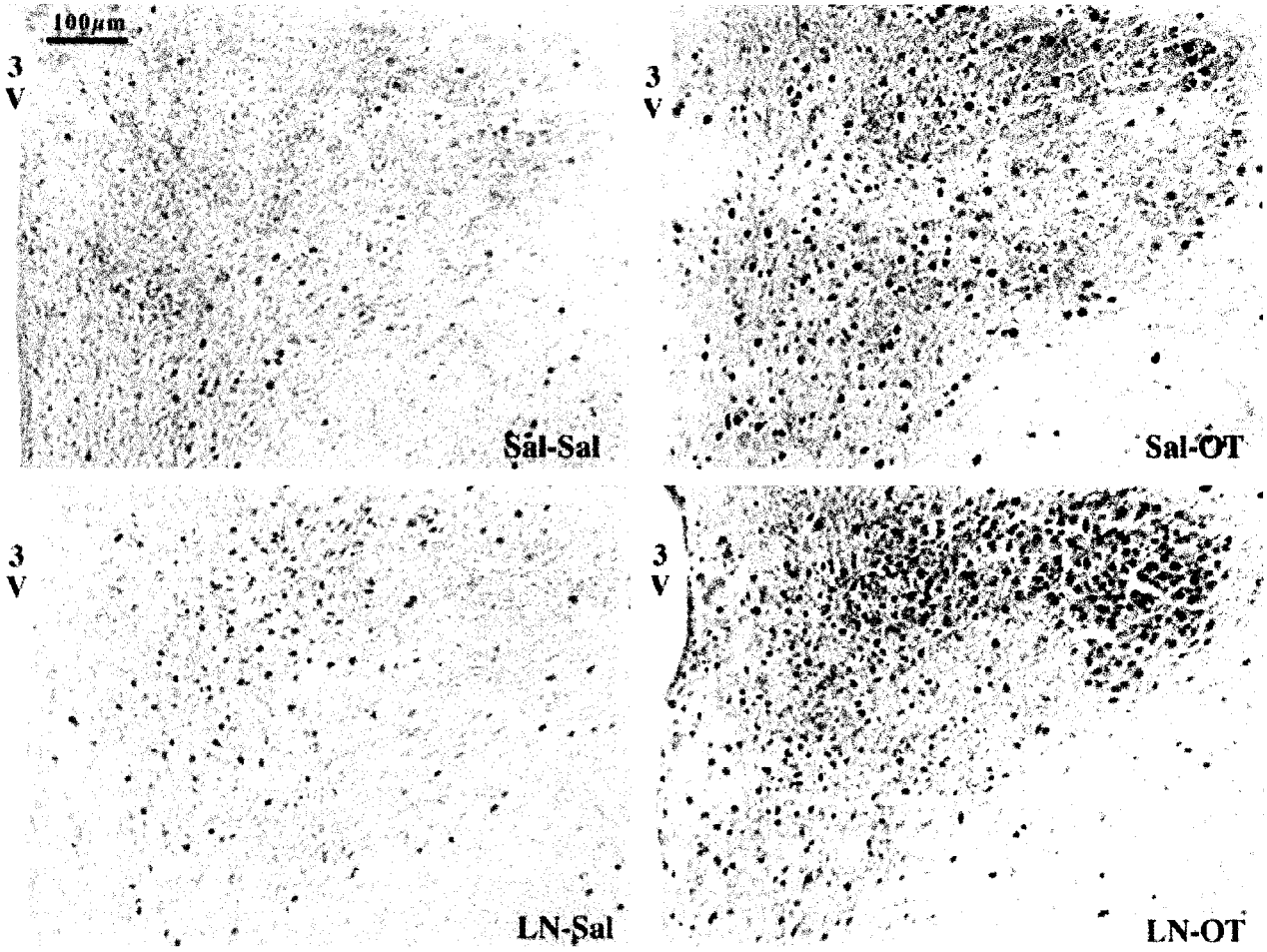

FIG. 3. Example of Fos-like immunoreactivity in the paraventricular nuclei of lactating rats that received either saline (Sal-Sal), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and saline (LN-Sal), saline and oxytocin (Sal-OT) or L-NAME and oxytocin (LN-OT).

nuclei of lactating rats does not generalize to all stimuli capable of inducing Fos in these areas.

The neuronal activation induced in the SON and PVN by central oxytocin stimulation is consistent with a body of evidence demonstrating that central oxytocin administration modulates both behavioural and physiological processes. Given the presence of oxytocin receptors on oxytocin neurones in the SON and PVN (9), it is reasonable to assume that at least a portion of the cells showing Fos-lir after oxytocin treatment are oxytocinergic, but it is also possible that the activity of nonoxytocinergic cells within the PVN and SON is modulated by central oxytocin infusion. Colocalization studies are needed to describe the phenotype of the neurones in which Fos-lir is induced by oxytocin administration both in magnocellular neurones and in parvocellular PVN where oxytocin neurones are more sparse (24). It is also possible that activation of cells within the PVN and SON following oxytocin administration was produced indirectly by oxytocin acting at distal brain sites. Neurones in the BNST, for example, project to the PVN (25), possess oxytocin binding sites (10) and are excited following central injections of oxytocin in lactating rats (26) and thus are one indirect pathway through which central oxytocin administration might activate the PVN and SON.

There was a tendency for oxytocin treatment to induce more Fos-lir in both the SON and PVN of lactating than of nonlactating rats but this effect was most marked in the SON. Central oxytocin administration was also effective in inducing Fos-lir in the MPOA and BNST, but only in lactating rats. These results could be a reflection of the greater density of oxytocin receptors in the hypothalamus during lactation (27). The fact that the Fos response to oxytocin was greater or at least equivalent in the SON and PVN of lactating rats than of nonlactating rats is interesting. Lactating rats have been shown to be hyporesponsive to a variety of stimuli such as immobilization (28), ether stress (29) and dehydration (30). Moreover, the attenuated response to these stimuli shown by lactating rats is seen both as a reduction in Fos expression in the PVN and SON and as diminished pituitary hormone release including a reduction in oxytocin release (31). The data

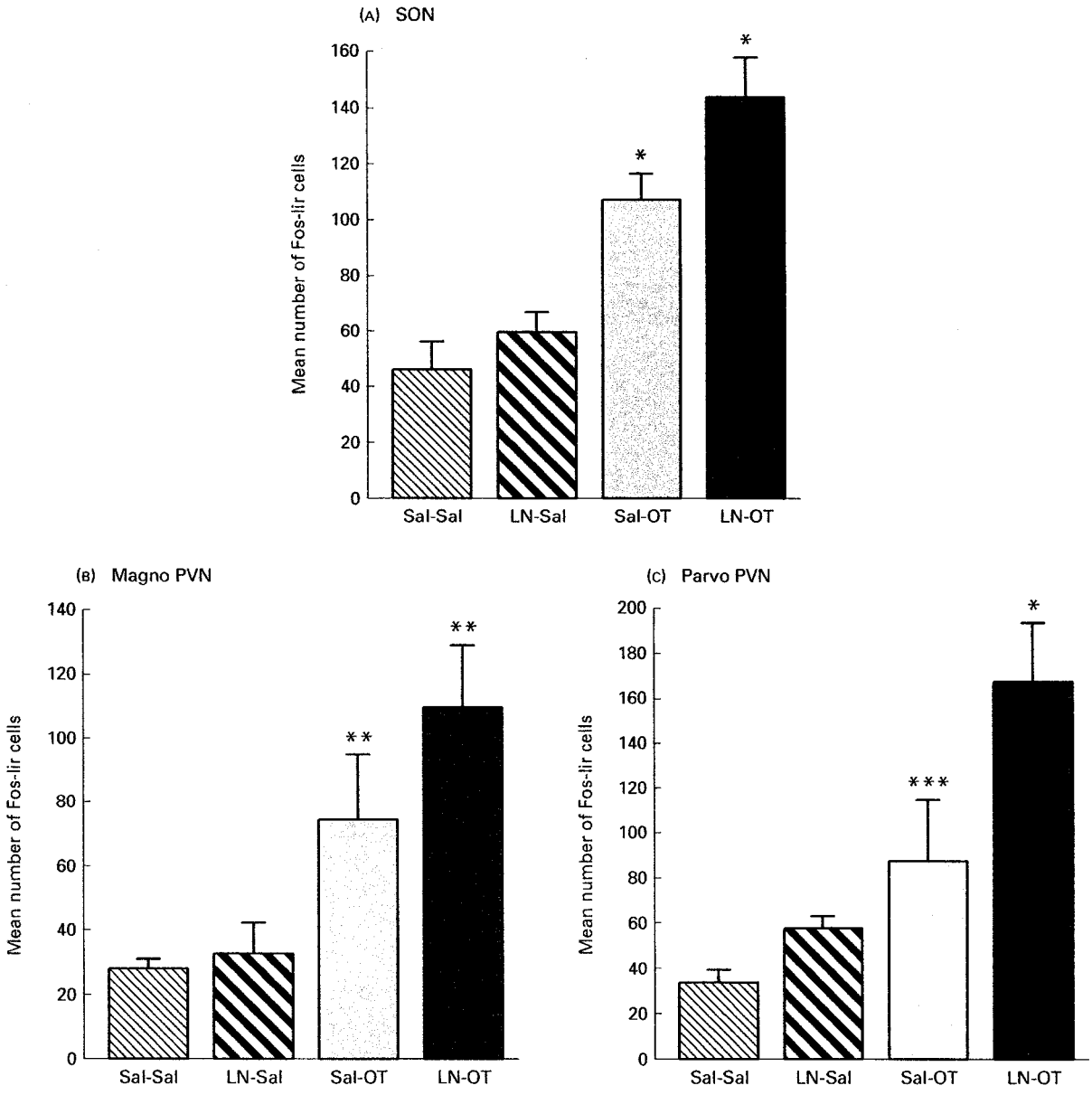

FIG. 4. Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) prior to oxytocin treatment on the mean number of Fos-like immunoreactivity (lir) in the (A) supraoptic nuclei (SON), (B) magnocellular paraventricular nuclei (PVN) and (C) parvocellular PVN of lactating rats. L-NAME prior to oxytocin administration induced more Fos expression in the SON and parvocellular PVN but was not sufficient to significantly increase Fos-lir in the magnocellular PVN compared to saline and oxytocin (Sal-OT)-treated rats. *$P<0.05$, significantly different from the other groups; **$P<0.05$, significantly different from saline (Sal-Sal) and L-NAME and saline (LN-Sal); ***$P<0.05$, significantly different from Sal-Sal.

obtained from the urethane/saline treated groups in experiment 3 are an example of such an effect.

The differential responsivity of lactating rats to these two types of stimulation may reflect the fact that urethane administration or immobilization stress initiates a cascade of events, of which only one step is to stimulate oxytocin neurones in the PVN and SON. An attenuation of the response to these stimuli could occur at any of a number of sites along this cascade. Central oxytocin administration, on the other hand, represents a much more specific stimulus which may be acting directly on neurones within the PVN and SON. The results of the current experiment show that the responsiveness of neurones in these areas to oxytocin is not blunted by lactation and thus that the attenuated response to

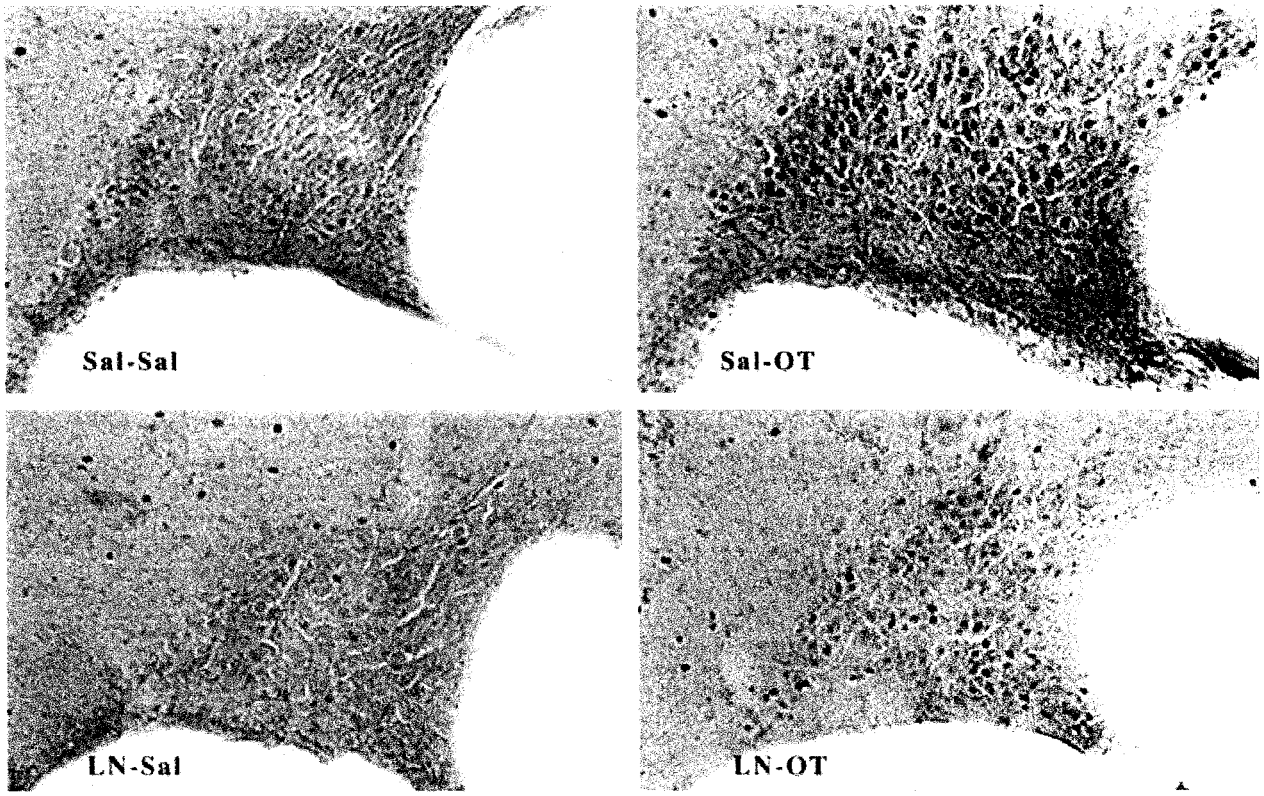

FIG. 5. Example of Fos-like immunoreactivity the supraoptic nuclei of nonlactating rats that received either saline (Sal-Sal), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and saline (LN-Sal), saline and oxytocin (Sal-OT) or L-NAME and oxytocin (LN-OT).

other stimuli such as urethane observed during lactation results from hyporesponsiveness at an earlier step in the response cascade.

Central oxytocin does not always result in the induction of Fos-lir in the PVN and SON of lactating rats. Persistent suckling, which is accompanied by intranuclear oxytocin release, does not stimulate Fos expression in the SON (30) although an increase in Fos expression in this area is seen when mother and young are reunited after a period of separation (32). If, as has been suggested, Fos is typically expressed transiently at the onset of stimulation (33) and acts to suppress its own subsequent induction, any chronic stimulus, such as suckling and the associated oxytocin release, would not induce Fos expression. It is interesting then that this chronic stimulation does not preclude the ability of exogenous oxytocin to induce Fos-lir.

The results from experiment 2 demonstrated that the effect of inhibiting NO synthesis prior to central oxytocin infusion on Fos expression depended on the reproductive state of the rat and the brain area examined. The administration of L-NAME and oxytocin to lactating rats enhanced the expression of Fos-lir compared to Sal-OT-treated rats. Nonlactating rats, on the other hand, showed a reduction of Fos-lir in the hypothalamic nuclei when treated with L-NAME and oxytocin compared to when treated with oxytocin alone. These data therefore suggest that, in lactation, NO acts to suppress the responsivity of neurones in the PVN and SON to central oxytocin administration, whereas NO facilitates the neural response of nonlactating females to this same stimulus.

These results are consistent with the hypothesis that NO, which is upregulated in lactating (21), dehydrated (16) and salt-loaded rats (15), plays a negative feedback role in the HNS when this system is chronically stimulated (18). This idea is also supported by the results of previous studies showing that L-NAME administration enhances oxytocin release from the pituitary in dehydrated and salt-loaded rats (18, 20). The latter data suggest that at least part of the increased neuronal activation seen in experiment 2 when L-NAME is coadministered with oxytocin is associated with oxytocin neurosecretory activity.

It has recently been reported that the Fos expression seen in the SON and PVN in suckled females when reunited with their litters after a 12-h separation was attenuated following administration of a NO donor (34). Thus, apparently, NO can function to suppress neuronal activation in response to acute suckling stimulation. Given that suckling stimulation would be associated with an increase in endogenous oxytocin release within the PVN and SON, these data are consistent with the results obtained here in lactating rats. It should be noted, however, that we saw little effect of L-NAME administration alone on Fos-lir, perhaps because in the

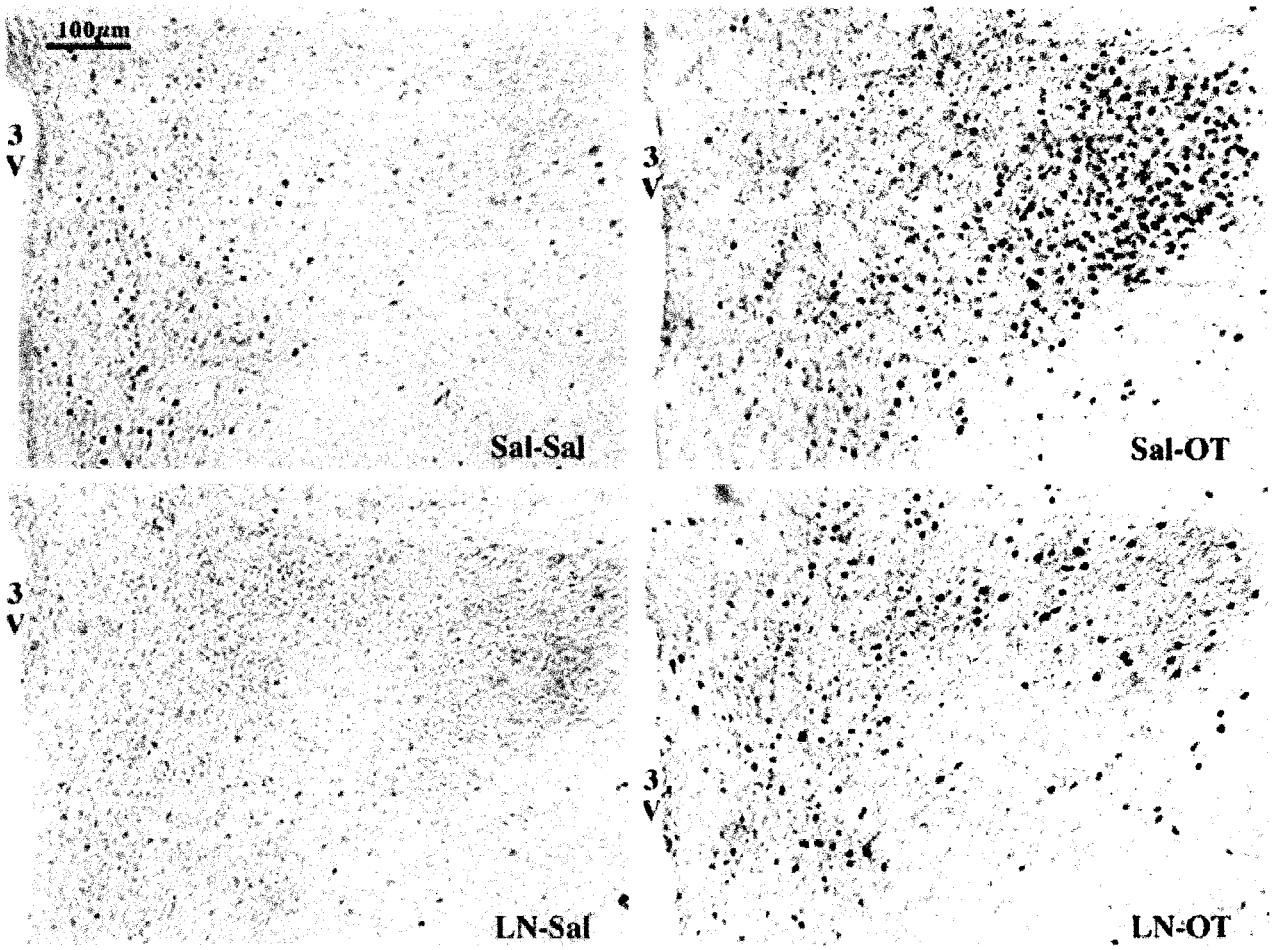

FIG. 6. Example of Fos-like immunoreactivity in the paraventricular nuclei of nonlactating rats that received either saline (Sal-Sal), N^{ω}-nitro-L-arginine methyl ester (L-NAME) and saline (LN-Sal), saline and oxytocin (Sal-OT) or L-NAME and oxytocin (LN-OT).

current studies lactating rats were suckled throughout the experiment.

The fact that L-NAME administration also increased Fos-lir in the parvocellular part of the PVN suggests that the ability of NO to suppress neuronal activation following oxytocin administration is not limited to the HNS. It is well established that NADPH-d and NOS mRNA are colocalized with corticotropin releasing hormone in the parvocellular region of the PVN (35) and oxytocin neurones are also present in this region of the hypothalamus (24). Future colocalization studies will allow us to determine which neuronal populations are affected by L-NAME administration. The fact that Fos-lir in the MPOA and the BNST in response to central oxytocin administration was not affected by pretreatment with L-NAME suggests site specificity in the ability of NO to modulate the effect of oxytocin on neuronal activity. In addition, these data suggest that the modulation of the response to oxytocin that we see in the PVN and SON is not mediated by an effect in the BNST.

As shown in experiment 3, the facilitatory effect of inhibiting NOS on Fos-lir in response to oxytocin treatment in lactating rats did not generalize to urethane administration. Fos-lir expression in either the SON or the magnocellular and parvocellular subdivisions of the PVN in urethane-stimulated lactating rats was not affected by L-NAME treatment. In the nonlactating rats, on the other hand, administration of L-NAME reduced Fos expression in the SON and magnocellular and parvocellular subdivisions of the PVN. The latter effect is consistent with the results of experiment 2, and with other studies showing that inhibiting NOS decreases the hypothalamic-pituitary adrenal response to various stressors (36), and suggests that NO has a facilitative affect on stress-induced Fos immunoreactivity in nonlactating rats. Interestingly, however, a recent study showed that both the Fos immunoreactivity and oxytocin release observed following hypertonic saline injections in virgin rats are increased following NOS inhibition (37). Since, in the latter study, the rats were anaesthetized with sodium pentobarbitone, it may be

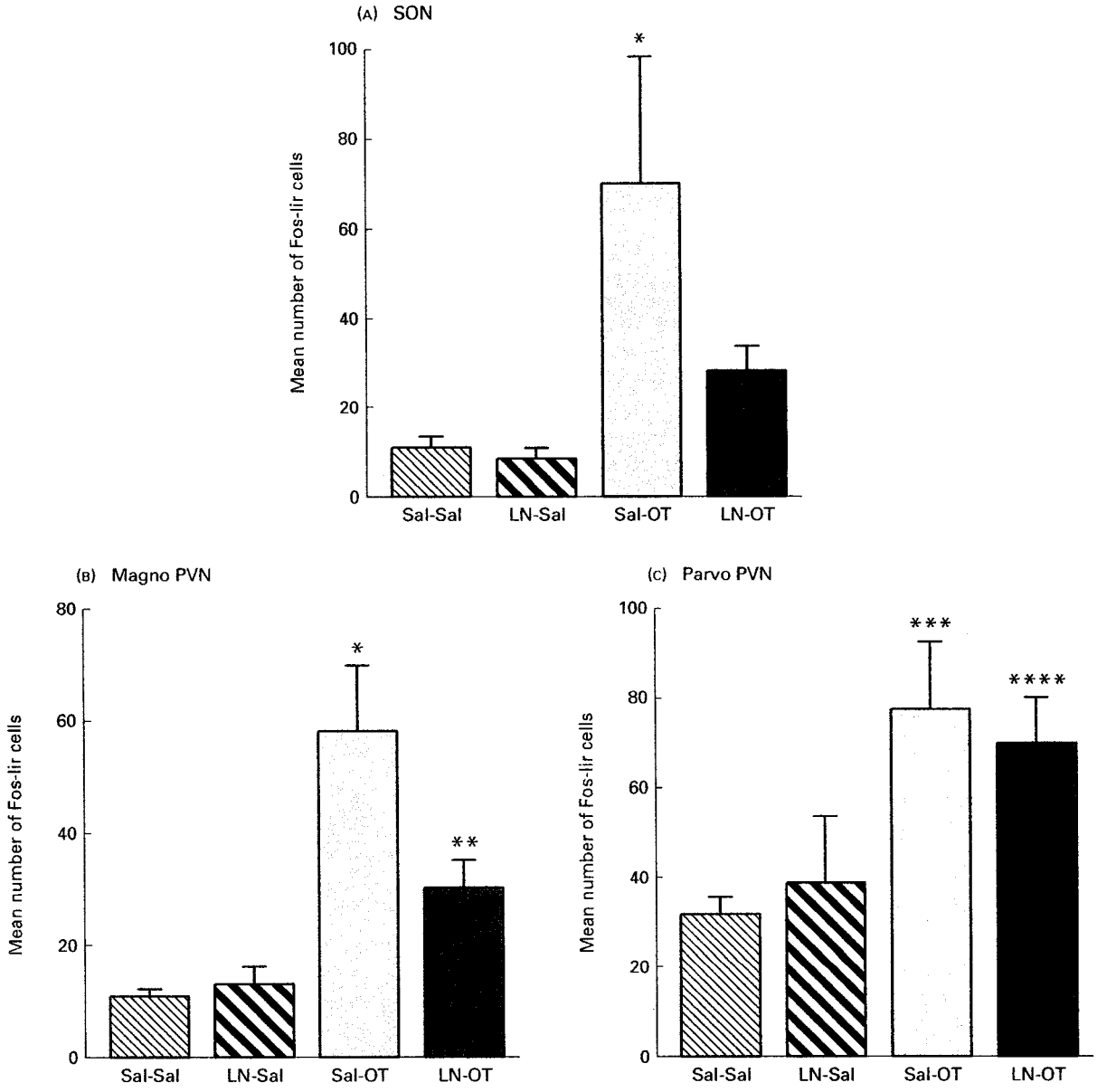

FIG. 7. Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) prior to oxytocin treatment on the mean number of Fos-like immunoreactivity (lir) in the (A) supraoptic nuclei (SON), (B) magnocellular paraventricular nuclei (PVN) and (C) parvocellular PVN of nonlactating rats. L-NAME prior to oxytocin administration reduced Fos expression in the SON and magnocellular PVN but was not sufficient to significantly attenuate Fos-lir in the parvocellular PVN compared to saline and oxytocin (Sal-OT)-treated rats. *$P < 0.05$, significantly different from the other groups; **$P < 0.05$, significantly different from saline (Sal-Sal) and Sal-OT; ***$P < 0.05$, significantly different from Sal-Sal and L-NAME and saline (LN-Sal); ****$P < 0.05$, significantly different from Sal-Sal.

that the state of GABA activation is a critical factor in determining the modulating effects of NO in this system.

In summary, these studies reveal that central administration of oxytocin induces Fos expression in discrete regions of the hypothalamus in lactating and nonlactating rats. The response to oxytocin is greater overall in lactating than in nonlactating rats and is differentially modulated by NO as a function of reproductive state. Furthermore, the effect of inhibiting NO production on Fos expression across reproductive state varies across stimuli. While the results of these studies demonstrate NO modulation of the response to central oxytocin administration and to other stimuli, the specific target at which NO produces its effects remain to be addressed.

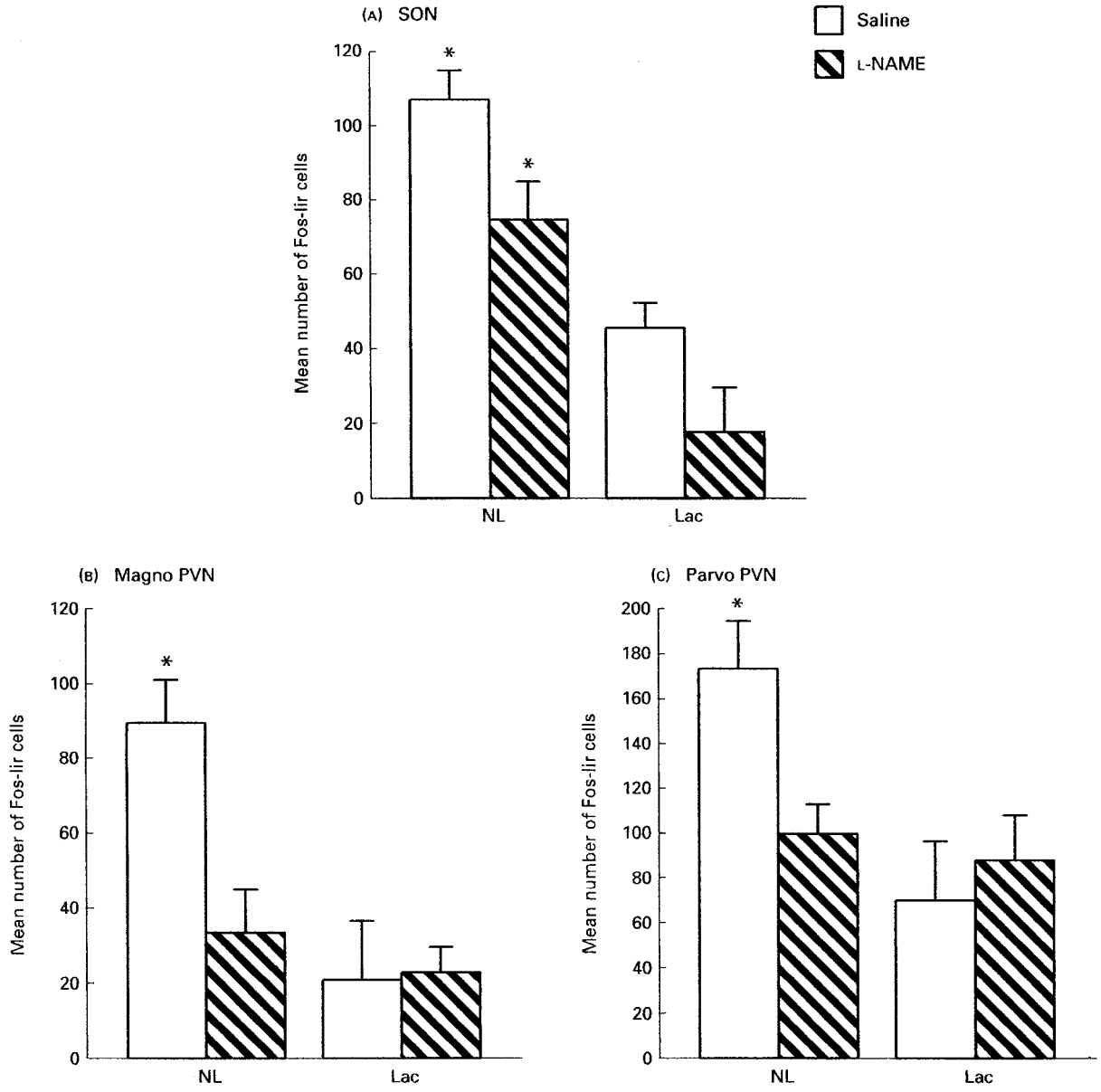

FIG. 8. Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) prior to urethane stimulation on the mean number of Fos-lir in the (A) supraoptic nuclei (SON), (B) magnocellular paraventricular nuclei (PVN) and (C) parvocellular PVN of nonlactating and lactating rats. L-NAME prior to urethane administration had no affect on Fos expression in any of the brain areas examined of lactating rats but reduced Fos-like immunoreactivity (lir) in the SON and both subdivisions of the PVN in nonlactating rats compared to saline-urethane treated rats. *$P<0.05$, significantly different from the other groups.

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