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Nitric Oxide Synthase Activity and Expression are Decreased in the Paraventricular Nucleus of Pregnant rats

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1. Introduction

Pregnancy is associated with increased heart rate, increased blood volume, decreased vascular sensitivity to endogenous vasoconstrictors and decreased total peripheral resistance and arterial blood pressure (DeSwiet, 1988; Lindheimer and Katz, 1992). Increased synthesis of nitric oxide (NO) by the vascular endothelium likely contributes to vasodilation and decreased vasoconstrictor responses in pregnant animals, including humans. However, peripheral NO mechanisms cannot fully account for changes in blood pressure regulation in pregnancy (Brooks et al., 2001; McLaughlin and Conrad, 1995; Sladek et al., 1997). In addition to changes in the peripheral vasculature, the central nervous system (CNS) homeostatic systems for control of both blood volume (Lindheimer and Davison, 1995) and the autonomic nervous system (Brooks et al., 1997; Greenwood et al., 2001; Masilamani and Heesch, 1997; O'Hagan and Casey, 1998) are reset and contribute to the hemodynamic profile characteristic of normal pregnancy. In addition to its peripheral actions, NO is an important neuromodulator in hypothalamic nuclei involved in control of blood volume and cardiovascular regulation (Badoer, 2001; Coote, 1995).

Nitric oxide is a highly diffusible gaseous signaling molecule that is synthesized on demand by the enzyme, nitric oxide synthase (NOS). Once formed, NO diffuses from nerve terminals and/or cell bodies to act as a nontraditional neurotransmitter/neuromodulator influencing cells in the vicinity of its release (Esplugues, 2002). Neuronal NOS (nNOS) is the predominant isozyme responsible for the formation of NO in the SON and PVN (Bhat et al., 1996; Gingerich and Krukoff, 2005). In both the SON and PVN, nNOS is expressed in magnocellular neurons that synthesize and release vasopressin and oxytocin. In addition, nNOS is expressed in autonomic related parvocellular neurons in the PVN that project to the rostral ventrolateral medulla (RVLM) and/or the intermediolateral cell column of the spinal cord and play an important role in control of sympathetic nerve activity and blood pressure (Badoer, 2001; Swanson and Sawchenko, 1983; Stern, 2004; Stern and Zhang, 2005). NO tonically inhibits neuronal activity in hypothalamic magnocellular and pre-autonomic neurons mainly due to pre-synaptic facilitation of GABAergic neurotransmission (Horn et al., 1994; Li et al., 2003; Stern, 2004). Regulation of nNOS is dependent on neuronal activity (Kadekaro, 2004) and changes in expression and activity of nNOS in the hypothalamus have been implicated in many physiological and pathophysiological adaptations in neurohumoral control of the circulation (DiCarlo et al., 2002; Krukoff, 1999; Mueller et al., 2006; Patel et al., 1996; Zhang et al., 1998b).

Although plasma volume is increased by approximately 40% and plasma osmolality is decreased by 10 mOsm during pregnancy, plasma vasopressin levels are no different than in the nonpregnant state. The osmotic threshold for vasopressin secretion is decreased, but sensitivity to osmotic stimuli and to changes in blood volume and arterial pressure is not changed by pregnancy (Lindheimer and Davison, 1995; Summy-Long and Kadakaro, 2001; Ward et al., 1991). Thus, resetting of the osmotic threshold for vasopressin secretion contributes to the maintenance of the expanded blood volume in pregnancy. Regulation of sympathetic nerve activity is also changed by pregnancy. While arterial baroreflex sympathoexcitation is blunted, baroreflex sympathoinhibition is slightly augmented in term pregnant rats (Masilamani and Heesch, 1997). Independent of the arterial baroreflex, baseline GABAergic inhibition of the RVLM is greater in pregnant compared to nonpregnant rats (Kvochina et al., 2007). Increased baseline inhibition at the level of the RVLM likely contributes to attenuated baroreflex sympathoexcitation in pregnant rats. However, increased GABAergic inhibition of the RVLM does not explain the fact that baseline sympathetic tone to the heart (Cohen et al., 1988; Lucini et al., 1999) and vasculature (Greenwood et al., 2001; Masilamani and Heesch, 1997) is slightly elevated in pregnant animals, including humans. In male rats, inhibition of neuronal activity in the PVN reduces arterial pressure and renal sympathetic nerve activity, suggesting that the PVN may contribute to baseline sympathetic tone (Dampney et al., 2000; Zhang and Patel, 1998a). Therefore it is possible that changes in sympathoexcitatory output from pre-autonomic neurons in the PVN could contribute to the maintenance of baseline sympathetic outflow in pregnancy and that adaptations in NO mechanisms may play a role.

There are conflicting reports in the literature regarding expression of nNOS and nNOS activity in term pregnancy. Some studies have provided evidence for increased nNOS in the hypothalamus of term pregnant animals (Popeski et al., 1999; Woodside and Amir 1996; Xu et al., 1996). In contrast, others have reported no change or decreased nNOS in the PVN and/or SON of term pregnant animals (Daubert et al., 2007; Okere and Higuchi, 1996; Srisawat et al., 2000). Differences in methods for assessing nNOS, stage of the cycle in the nonpregnant control group, and the day of pregnancy studied in these experiments could contribute to the conflicting results.

Experiments in the current study tested the hypothesis that in near-term pregnant rats, nNOS is decreased in the PVN and SON, which would be consistent with decreased tonic inhibition of these regions. nNOS activity (NADPH-diaphorase staining), nNOS protein, and mRNA for nNOS in the PVN and SON were evaluated in a controlled set of experiments in similarly treated near-term pregnant and nonpregnant female rats. Experiments in cycling nonpregnant rats were performed in the estrus stage of the cycle when ovarian hormones are consistent and low (Butcher et al., 1974; Haim et al., 2003). Experiments in near-term pregnant rats were performed on the morning of day 21 which is 1.5–2 days prior to expected delivery (Francis et al., 2002; Kobayashi et al., 1999) and is characterized by elevated estrogen and progesterone levels (Bridges, 1984; Concas et al., 1998; Garland et al., 1987; Kobayashi et al., 1999).

2. Results

2.1. Effect of pregnancy

Table 1 contains a summary of baseline information for the 37 rats used in these experiments. All nonpregnant rats were in the estrus stage of the estrous cycle. Near term pregnant rats weighed significantly more than nonpregnant rats. Number of fetuses and average fetal weights are consistent with those previously reported for day 21 of pregnancy in the rat (Ericsson et al., 2007).

2.2. Effect of pregnancy on NADPH-diaphorase staining

Fig. 1 contains individual photomicrographs of the PVN and SON of representative rats. In both nonpregnant and pregnant rats, maximum NADPH-diaphorase staining in the PVN and SON was present at rostral-caudal levels similar to previous experiments (DiCarlo et al., 2002; Mueller et al., 2006; Zhang et al., 1998b). In the PVN diaphorase staining appeared to be less abundant in the pregnant compared to the nonpregnant state. Mean data from eight nonpregnant and eight pregnant rats are shown in Fig. 2. The average number of NADPH-diaphorase positive cells was greater in the PVN compared to the SON in both groups. In the PVN pregnancy was associated with a decreased number of NADPH-diaphorase positive cells, whereas the average number of NADPH-diaphorase positive cells in the SON was not different between pregnant and nonpregnant rats.

2.3. Effect of pregnancy on nNOS protein content

Micro punches were obtained from the PVN and SON regions of 4 nonpregnant and 4 pregnant rats. Histological examination of the forebrain slices verified that punches were obtained from the correct anatomical locations. Fig. 3 contains representative examples of Western blots from the PVN of one nonpregnant and one pregnant rat. Single bands of the appropriate molecular weight for nNOS and GAPDH (internal standard) indicate the specificity of antibodies used in the current experiments. To correct for potential differences in protein recovery and/or loading, the level of nNOS was normalized to the level of GAPDH for the same punch. Compared to other pregnant rats, one pregnant rat had substantially lower expression (~50% less) of both GAPDH and nNOS in the PVN, most likely indicating less starting material in the punch. For consistency this rat was eliminated from analysis, although statistical results were similar whether or not this rat was included. Mean data are shown in Fig. 4. Although data are from a limited number of animals, these results indicate that nNOS protein content was greater in the PVN than in the SON of both groups. In the PVN only, pregnancy was associated with significantly less nNOS protein compared to the nonpregnant state.

2.4. Effect of pregnancy on nNOS mRNA expression

Micro punches were obtained from the PVN and SON of 7 nonpregnant and 6 pregnant rats. Using real time RT-PCR, relative expression of nNOS mRNA in nonpregnant and pregnant rats was calculated within each region. In the pregnant group, successful experiments were completed on PVN punches from 5 rats and on SON punches from 5 rats. Fig. 5 contains examples of PCR amplification curves (A), melt peaks (B), and agarose gels of PCR products for nNOS and the housekeeping gene GAPDH (C) from PVN micropunches in one nonpregnant and one pregnant rat. Mean data for the PVN indicate that relative to nNOS mRNA in nonpregnant rats, nNOS mRNA in pregnant rats was decreased (Fig. 5D). In contrast, relative expression of nNOS in the SON was not different in pregnant (0.92 ± 0.21) compared to nonpregnant rats (1.0 ± 0.33) (Not shown). Thus, the effect of pregnancy on mRNA for nNOS was similar to the effects of pregnancy on NADPH-diaphorase staining and nNOS protein content.

3. Discussion

3.1. Purpose

Neuronal NOS (nNOS) is the major isozyme of NOS present in neurons in the hypothalamus (Gingerich and Krukoff, 2005) and thus was the focus of this study. Experiments assessed indices of nNOS in hypothalamic regions involved in control of vasopressin secretion and sympathetic nerve activity. Results indicate that in the PVN, near-term pregnancy was associated with decreased nNOS message, nNOS protein, and NADPH-diaphorase staining, an index of nNOS activity in the hypothalamus (Bhat et al., 1996). Thus, down regulation of

nNOS mRNA translates to decreased nNOS protein which likely contributes to decreased nNOS activity. The end result would be less NO available for modulation of neuronal activity in the PVN of pregnant compared to nonpregnant rats. In contrast, pregnancy had no effect on nNOS in the SON. The lack of effect of pregnancy on nNOS in the SON could be related to the presence of different neuronal populations within the PVN and SON. Although both regions contain neurosecretory magnocellular neurons, in the PVN pre-autonomic projection neurons involved in control of sympathetic nerve activity also express nNOS (Nylen et al., 2001; Stern, 2004). In our experiments assessment of nNOS mRNA, nNOS protein, and nNOS activity in the PVN included both magnocellular and parvocellular neurons. Thus, if changes in nNOS in the PVN were primarily in pre-autonomic neurons, this could explain observed effects in the PVN, but not the SON. Another possibility is related to the fact that, similar to other studies (Mueller et al., 2006; Popeski et al., 1999; Woodside and Amir, 1996), nNOS activity and expression were lower in the SON compared to the PVN. Therefore we cannot exclude the possibility that lower baseline expression of nNOS in the SON compared to the PVN could make it more difficult to detect a decrease in the SON due to pregnancy.

3.2. Decreased NO inhibition of vasopressin neurons could contribute to “normal” vasopressin levels in pregnant rats

The increased blood volume and decreased plasma osmolality characteristic of pregnancy would be expected to decrease vasopressin release. However, baseline plasma levels of vasopressin are not altered by pregnancy. Although the mechanisms for resetting of vasopressin secretion are not completely understood, chorionic gonadotropin and relaxin, which are elevated early in pregnancy, likely play a role (Lindheimer and Davison, 1995; McKinley et al., 1997). Also, circulating levels of angiotensin II are elevated in pregnancy (DeSwiet, 1988; Hanssens et al., 1991). Chronic elevations of plasma angiotensin II act at circumventricular organs to preferentially release vasopressin over oxytocin. This effect appears to be related to withdrawal of baseline NO-mediated inhibition of vasopressin neurons at a time when baseline NO-inhibition of oxytocin neurons is unchanged (Kadekaro, 2004). Thus, it is possible that, despite the presence of hypo-osmolality and hypervolemia – both stimuli for decreasing vasopressin – as pregnancy progresses decreased tonic NO inhibition of the PVN could contribute to maintenance of “normal” plasma vasopressin levels.

3.3. Decreased NO inhibition of pre-autonomic neurons in the PVN could contribute to elevated baseline sympathetic nerve activity in pregnant rats

Decreased NO results in decreased GABAergic inhibition of pre-autonomic neurons in the PVN and has been associated with increased baseline sympathetic nerve activity (Zhang et al., 2001; Zhang et al., 2002). Since term pregnancy is associated with increased baseline sympathetic outflow (Greenwood et al., 2001; Masilamani and Heesch, 1997), we considered that similar mechanisms in the PVN could contribute to maintenance of baseline sympathetic nerve activity in pregnant animals. Preliminary experiments in our laboratory found that increases in arterial pressure and renal sympathetic nerve activity in response to GABA_A receptor blockade in the PVN were less in pregnant compared to nonpregnant rats, suggesting decreased baseline GABAergic tone in the PVN in term pregnancy (Kvachina et al., FASEB J 19: A616, 2005). The current experiments indicate that nNOS is also decreased in the PVN of pregnant rats. Functionally, decreased inhibition of renal sympathetic nerve activity at the level of the PVN is consistent with previous experiments suggesting that sympathetic innervation of the kidney contributes to expanded blood volume in term pregnant rats (Patel and Zhang, 1993). Thus the PVN may play a role in maintaining sympathetic nerve activity in pregnancy due to a decreased inhibitory influence of NO and GABA on pre-autonomic neurons.

3.4. Effects of ovarian hormone treatments on nNOS in the SON and PVN

There is general consensus that nNOS is increased in both the PVN and SON during lactation (Ceccatelli and Eriksson, 1993; Luckman et al., 1997; Okere and Higuchi, 1996; Popeski et al., 1999), with no change in nNOS in these regions at midterm pregnancy (Luckman et al., 1997; Okere and Higuchi, 1996; Srisawat et al., 2000). However, there are conflicting reports regarding expression of nNOS and nNOS activity in term pregnant rats. Review of the literature regarding the effects of ovarian hormone treatments on nNOS in the SON and PVN may provide important insights to resolve conflicting reports. In vitro exposure of a rat hypothalamic slice culture to 17 β estradiol resulted in decreased nNOS immunoreactivity in both magnocellular and parvocellular neurons in the PVN (Gingerich and Krukoff, 2005). Treatment of ovariectomized female rats with high doses of 17 β estradiol decreased NADPH-diaphorase staining in both the PVN and SON, while low dose 17 β estradiol treatment was without effect on nNOS in these regions (Ceccatelli et al., 1996; Wang and Morris, 1999). In contrast, nNOS activity in both the SON and PVN is increased following ovarian hormone treatments designed to mimic conditions very late in pregnancy (estradiol + progesterone treatment followed by 72 h of progesterone withdrawal) (Popeski et al., 1999). Blockade of oxytocin and/or prolactin receptors decreased NADPH-diaphorase staining in rats treated with the ovarian steroid priming/progesterone withdrawal regimen (Popeski et al., 2003). The authors propose that the increased capacity to produce NO in the SON and PVN with this hormone treatment (and in the antepartum period of pregnancy) is dependent on progesterone withdrawal followed by prolactin stimulation of oxytocin gene expression and oxytocin release.

Taken together, results of these studies suggest that exposure to relatively high levels of estrogen decreases nNOS activity in the PVN and SON. Very late in rat pregnancy (~last 24 h), progesterone levels drop, while estrogen remains elevated. Hormone treatments designed to mimic very late pregnancy (at the time of parturition) result in increased nNOS activity in the SON and PVN. Importantly the increase in nNOS activity following progesterone withdrawal appears to be dependent on prolactin and oxytocin secretion.

3.5. Studies evaluating effects of pregnancy on nNOS in the SON and PVN

A confounding factor to the interpretation of results from studies on the effects of pregnancy is that different conventions are used to define the day of pregnancy. For clarity of this discussion, the day of pregnancy has been adjusted when needed to follow the convention used by our laboratory whereby the day of confirmed mating (vaginal sperm or a mating plug) is defined as day 1 of pregnancy. In the rat, estrogen is increased significantly during the last half of pregnancy and remains elevated at the time of parturition (Bridges, 1984; Garland et al., 1987). Plasma progesterone levels increase early in pregnancy and remain elevated until approximately 24 h prior to parturition, at which time they decrease to nonpregnant levels (Bridges, 1984; Concas et al., 1998; Garland et al., 1987; Kobayashi et al., 1999). The precipitous drop in progesterone within the last day of pregnancy is followed by increased prolactin secretion immediately prior to parturition (Andrews and Grattan, 2004; Grattan and Averill, 1991; Kobayashi et al., 1999) and increased oxytocin secretion in association with parturition (Kobayashi et al., 1999). In the current experiments, brain tissue was harvested on the morning of day 21, which would be 1.5 to 2 days prior to parturition (Francis et al., 2002; Kobayashi et al., 1999). At this time plasma progesterone levels would still be elevated above nonpregnant levels and prolactin and oxytocin would not have risen yet.

In experiments using homogenates of the entire hypothalamus, Xu et al. (1996) reported increased nNOS mRNA and nNOS protein and increased hypothalamic and plasma vasopressin on what appears to be analogous to our day 21 of pregnancy. Others report that plasma vasopressin is no different from nonpregnant control levels through day 21 of pregnancy in the rat (Barron et al., 1985; Ohara et al., 1998). In dogs (Olsson et al., 2003), sheep (Kendrick et

al., 1991) and cows (Landgraf et al., 1983) plasma vasopressin and oxytocin increase only during labor and/or delivery. Therefore, it is difficult to reconcile the reported day of pregnancy with the increased vasopressin levels in the study by Xu and colleagues. Woodside and colleagues reported increased NADPH-diaphorase staining in the SON and PVN of pregnant rats on the day of parturition, which they correlated with low progesterone and increased oxytocin secretion (Popeski et al., 1999; Woodside and Amir, 1996). As discussed above, one possible interpretation of these studies is that following the antepartum prolactin surge, increased activity of oxytocin and possibly vasopressin neurons in the SON and PVN could have resulted in neuronal activity dependent increases in nNOS activity.

In contrast to the studies discussed above, and similar to our results, other laboratories have reported decreased nNOS in the PVN and SON of near-term pregnant rats. For example, compared to nonpregnant and mid-term pregnant rats, NADPH-diaphorase staining in the PVN decreased in days 19–21 pregnant rats and then increased post-partum (Okere and Higuchi, 1996). In another study, nNOS mRNA was decreased in the SON and tended to decrease in the PVN of late pregnant rats prior to changes in plasma oxytocin levels. In parturient rats, nNOS mRNA in the SON was significantly greater than in the late pregnant rats (Srisawat et al., 2000). Thus, in both of these studies at times in late pregnancy prior to increased oxytocin secretion, nNOS expression and activity were decreased in the SON and/or PVN. However, during parturition, a time when oxytocin secretion had increased, nNOS expression and activity in the SON and PVN were increased (Okere and Higuchi, 1996; Srisawat et al., 2000). These results are consistent with the notion that studies demonstrating increased nNOS in the PVN and SON of late pregnant rats may have been performed at a time point very near parturition. If this were the case, up-regulation of nNOS mRNA in the antepartum period would likely be related to increased neuronal activity of neurosecretory neurons.

3.6. Estrogen receptors in the SON and PVN

The mechanism for decreased nNOS expression and activity in near-term pregnant rats was not addressed in the current study, but may involve alterations in ovarian hormones in pregnancy. While expression of progesterone receptors is sparse (Francis et al., 2002), the beta subtype of the estrogen receptor (ER- β) is highly expressed in the PVN and SON (Shughrue et al., 1997). In neurosecretory magnocellular neurons ER- β is associated primarily with vasopressin, rather than oxytocin containing neurons (Alves et al., 1998; Forsling et al., 2003). Interestingly, nNOS is also more highly expressed in vasopressin compared to oxytocin neurons (Stern, 2004). Within the PVN, immunohistochemical labeling for ER- β is more robust in parvocellular subnuclei which contain pre-autonomic neurons, compared to magnocellular subnuclei (Bingham, 2006; Stern and Zhang, 2003). Thus it is possible that the elevated levels of estrogen in pregnancy could affect nNOS activity, vasopressin secretion and sympathetic nerve activity through ER β mechanisms within the PVN.

3.7. Significance

These experiments demonstrated that, compared to estrus stage nonpregnant rats, nNOS in the PVN was down-regulated in near-term pregnant rats (day 21). nNOS message, nNOS protein, and nNOS activity were all decreased. Total nNOS was evaluated in these experiments, so decreased nNOS expression and activity in pregnant rats could be due to decreases within magnocellular neurons, parvocellular neurons, or both. Since NO is a readily diffusible gaseous neuromodulator, the neuronal source does not strictly determine which neuronal type might be influenced by NO within a brain region (Esplagues, 2002). Less tonic inhibition of both magnocellular and pre-autonomic neurons is consistent with physiological adaptations that occur in pregnancy. Decreased NO inhibition of vasopressinergic magnocellular neurons in the PVN during pregnancy could contribute to maintenance of “normal” baseline vasopressin secretion in the presence of decreased plasma osmolality. Moreover, decreased NO in the PVN

could result in decreased release of GABA and relative disinhibition of pre-autonomic sympathoexcitatory neurons. Consistent with this idea, preliminary experiments from our laboratory suggest that tonic GABAergic inhibition of pre-autonomic neurons in the PVN is decreased by pregnancy. This could contribute to elevated baseline sympathetic nerve activity in pregnant animals despite the increased plasma volume which would tend to suppress sympathetic outflow. Thus decreased NO within the PVN may play an important role in pregnancy associated adaptations in control of blood volume and sympathetic nerve activity.

4. Experimental procedure

4.1. Experimental animals

All protocols were approved by the University of Missouri-Columbia Animal Care and Use Committee, and were based on the guidelines in the NIH *Guide for the Care and Use of Laboratory Animals*. Food (Formulab Diet, 0.28% sodium, Purina, St. Louis, MO,) and water were available *ad libitum* and animals were maintained on a 12 h light-12 h dark cycle at 20–21 °C. The estrous cycle was monitored in female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) by examining daily vaginal smear cytology. Pregnant rats were either mated in-house or purchased from Harlan. On the day of estrus, rats assigned to the pregnant group in-house ($n=8$) were placed overnight with a fertile male. Presence of sperm in the vaginal smear on the following morning was defined as day 1 of pregnancy. Timed-pregnant rats ($n=10$) were purchased from Harlan and the presence of a mating plug in the cage on the morning after exposure to a fertile male was defined as day 1 by our laboratory. Experiments were performed on day 21 in near-term pregnant rats (parturition is on day 22 or 23). Average number of fetuses and average fetal weight were determined at the end of all experiments in pregnant rats. There were no differences between in-house bred and purchased timed-pregnant rats, verifying equivalent stages of pregnancy. All nonpregnant rats ($n=19$) exhibited normal estrous cycles and experiments were performed on the day of estrus in rats age-matched to the pregnant group.

4.2. NADPH-diaphorase studies

NADPH-diaphorase histochemical staining was used as an index of NOS activity in neuronal tissue (Bredt et al., 1990; Dawson et al., 1991; Hope et al., 1991; Vincent and Hope, 1992). Forebrain tissue was obtained from pregnant and nonpregnant rats to assess differences in NOS activity as described previously for studies using other animal models (DiCarlo et al., 2002; Mueller et al., 2006; Zhang et al., 1998b). NADPH-diaphorase histochemistry utilizes the properties of the enzyme NOS that catalyzes the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble and visible formazan. There are at least eight other enzymes in the central nervous system that could contribute to NADPH-diaphorase activity (Blottner et al., 1995). However, rats in the current experiments were perfused with paraformaldehyde and NOS is the only one of these enzymes which is not inactivated by formaldehyde. In addition nNOS is the major NOS isozyme in the hypothalamus of rats and distribution of immunostaining with a monoclonal antibody for nNOS is identical to NADPH-diaphorase staining in the hypothalamus (Bhat et al., 1996). Therefore, in the current experiments NADPH-diaphorase staining is a reliable index of nNOS activity.

Pregnant and nonpregnant rats were anesthetized deeply with pentobarbital (50 mg/kg, i.p.; Nembutal; Abbott Laboratories) and perfused transcardially with 100–150 ml of heparinized saline followed by 200–250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Following perfusion, the brain was removed and postfixed at 4 °C for 4 h in 4% paraformaldehyde solution. The brain was then placed in 20% sucrose for 24 h before sectioning on a cryostat. Sections (30 μ m) were collected in 0.1 M phosphate buffer containing 0.1 mg/ml nitroblue tetrazolium, 0.3% Triton X-100, and 1.0 mg/ml β -NADPH and then placed in an oven at 37 °C for 1 h. Sections were rinsed in phosphate buffer and mounted onto slides.

With the aid of a microscope nNOS activity in neurons within the PVN and supraoptic nucleus (SON) was estimated by counting the number of cells that were stained positively (dark blue staining) for NADPH-diaphorase. Counts from three independent observers, blinded to the condition (nonpregnant or pregnant), were averaged for each brain region. Cells were counted at the same coronal level for each brain region in each individual rat. The number of cells counted in three adjacent sections were within 5% of each other. Thus the average number of cells per section was taken as an index of NOS positive cells within a given brain region.

4.3. Analysis of nNOS protein

Western immunoblot analysis of nNOS protein was performed on separate groups of pregnant ($n=4$) and nonpregnant rats ($n=4$). Animals were anesthetized as above (pentobarbital, 50 mg/kg, i.p.). Brains were rapidly removed and frozen in an isopentane bath on dry ice. Coronal slices (1 mm) were made through the forebrain using a rodent brain matrix (ASI). Sections containing the PVN or SON were identified and tissue punches were collected using a stainless steel, blunt 20 gauge Hamilton needle. Micropunches containing the PVN or SON were collected from the section of forebrain containing the junction of the optic tracts and optic chiasm. The punch containing the SON was obtained just lateral to the optic chiasm on the ventral surface of the brain slice. The punch containing the PVN was taken lateral to the dorsal aspect of the 3rd ventricle. Punches were frozen at -80°C without buffer until used. Slices were examined histologically and compared to a standard rat brain atlas (Paxinos and Watson, 1997) to verify proper punch location.

Single frozen micropunches of the PVN or SON were first solubilized in Laemmli buffer (60 μL , 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris-Cl, pH 6.8, 160 mM DTT, 0.001% bromophenol blue, 6 M urea). One third of each punch (20 μL) was loaded onto a 4–12% acrylamide gradient SDS gel (Nupage Bis-Tris, Invitrogen) and electrophoresed in 3-(N-morpholino) propane sulfonic acid (MOPS)-SDS buffer (50 mM MOPS, 50 mM Tris base, 3.4 mM SDS, 1 mM EDTA, Invitrogen) under denaturing conditions. It was then transferred to polyvinylidene difluoride membrane (Hybond-P, Amersham) in 25 mM Tris base buffer and 0.7 M glycine. Membranes were blocked with 5% nonfat milk in Tris-buffered saline-Tween 20 (TBS-Tween; 50 mM Tris base, 200 mM NaCl, 0.1% Tween 20) for two h. Membranes were then incubated with primary antibody against nNOS (1:3,333; Cat#610309, Lot#13; BD Transduction Laboratories) at room temperature overnight. Immunoblots were washed six times with TBS-Tween and then incubated for one h in a horseradish peroxidase conjugated secondary antibody against the primary antibody host species (1:2,000; horseradish peroxidase-conjugated anti-mouse; Cat#A6782, Lot#040K9190; Sigma) at room temperature. Membranes were then washed, and labeled proteins were detected by enhanced chemiluminescence (ECL reagents, Amersham) exposure to radiographic film (Hyperfilm ECL, Amersham). Densitometry was performed by acquiring digital images of radiographic film (Kodak EDAS 290, Kodak) of the bands of interest. All blots were blocked again at room temperature for one h and incubated with a primary antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10,000; Cat#MAB374; Lot#21042494; Chemicon International) overnight. Labeled protein bands were detected as described above.

4.4. Analysis of nNOS mRNA

Micropunches of the PVN and SON were obtained from nonpregnant ($n=7$) and pregnant ($n=6$) rats as described above. Lysis buffer (100 μL) was added immediately to each tube and the punch was homogenized. Paramagnetic oligo (dT) polystyrene beads (Dynabeads oligo (dT)₂₅, Dynal) were used to isolate Poly (A)+RNA. Beads were resuspended and first-strand cDNA synthesis was performed in a volume of 28 μL (Invitrogen). Three microliters were removed from each sample and used for a reverse transcriptase negative control (RT-). Reverse transcriptase (1 μL ; 200 U; SuperScript III; Invitrogen) and RNase inhibitor (1 μL ; 40 U; RNase-

OUT; Invitrogen) were added to each RT+ sample which contained 20 mM dithiothreitol (DTT), 0.5 mM deoxynucleotide triphosphate (dNTP; 0.5 mM each dATP, dCTP, dGTP, dTTP). Samples were incubated in a 42 °C water bath for 50 min, with gentle mixing every 10 min throughout incubation. Samples were then incubated at 70 °C (15 min) to heat inactivate the RT enzyme. Residual RNA was degraded in all RT+ samples by addition of RNase H (1 µl; 2 U; Invitrogen) and incubation at 37 °C (20 min). Real time PCR reactions were performed in duplicate on samples for mRNA's of nNOS and GAPDH, using a Cepheid SmartCycler and Sybr green intercalating dye (Takara SYBR Premix Ex Taq (2×)). Primers were purchased from IDT Integrated DNA technologies. Primer sequences for nNOS were: forward = AAT GGA GAC CCC CCT GAG AAC; reverse = TCC AGG AGG GTG TCC ACC GC. GAPDH primer sequences were: forward = CCA GTA TGA TTC TAC CCA CGG CAA; reverse = ATA CTT GGC AGG TTT CTC CAG GCG. GAPDH primers were used for RT- reactions. Total reaction volume was 25 µl: 12.5 µl SYBR premix; 8.5 µl HyPure molecular biology grade water (HyClone); 1 µl forward primer (10 pmol for nNOS and 5 pmol for GAPDH); 1 µl reverse primer (10 pmol for nNOS and 5 pmol for GAPDH); and 2 µl cDNA template. The PCR reaction consisted of an initial 1 min denaturing step at 95 °C. This was followed by 95 °C denaturation (30 s), 60 °C annealing (30 s), and 72 °C extension (30 s) steps for 40 cycles. A final 72 °C extension step (300 s) completed the amplification protocol. Brief exposure to 95 °C (15 s), then 60 °C (15 s) was followed by a gradual increase in temperature from 60 °C to 95 °C to determine melt temperature for PCR products.

To verify amplification of the correct product, 10 µl of the PCR product plus 2 µl of loading dye (Sigma) were electrophoresed (Horizon 11.14 apparatus, Gibco-BRL Life Technologies; power supply, E-C Apparatus) on 2% agarose gel, visualized with ethidium bromide, photographed (Kodak DC 290 camera) and saved digitally (Kodak 1D analysis software). A 123 bp DNA ladder (Invitrogen) was included on each gel for estimation of amplicon size. A prominent band of the approximate molecular weight for nNOS (281 bp) and GAPDH (624 bp) amplicons verified amplification of the desired target.

4.5. Data analysis

The number of cells per section with NOS activity as assessed by NADPH-diaphorase histochemistry was expressed as mean $\pm$ s.e.m. nNOS protein expression was normalized to GAPDH expression for each sample (see below) and is presented as the mean $\pm$ s.e.m.

Analysis of the digitized images of the immunoblots was performed using an IBM compatible computer and 1D Image Analysis software (Kodak). Mean intensity of the bands of interest was used as an index of protein content. GAPDH was used as an internal standard to control for small differences in punch size and sample loading. Data from the immunoblot experiments are expressed as relative densitometric units with normalization to GAPDH.

For real time PCR, cycle thresholds (CT's) were determined using a Cepheid SmartCycler and Sybr Green intercalating dye for each tissue punch. Data for nNOS and GAPDH from a given tissue punch was used only if the reverse transcriptase negative control (RT-) and a PCR reaction containing no template showed no amplification of product. If data were obtained from more than one punch from the PVN or SON of a given animal, the CT's were averaged, and statistical analysis was performed on one value per brain region per animal. Within each brain region, expression of nNOS in the pregnant group, relative to expression in the nonpregnant control group was determined using the $2^{-\Delta\Delta CT}$ method for analysis of relative changes in gene expression (Livak and Schmittgen, 2001).

Student's *t*-tests for independent groups were used for comparisons of body weights, and relative expression of nNOS mRNA between nonpregnant and pregnant rats. Two way ANOVA was used for comparisons among groups and brain regions for NADPH-diaphorase

staining and nNOS protein. When a statistically significant interaction was determined by the two-way ANOVA, posthoc pairwise comparisons were made using the Student-Newman-Keuls method. Statistical tests were performed using a commercially available software package (SigmaStat, SPSS Inc., Chicago, IL). A probability value $P \leq 0.05$ was considered significant.

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Fig. 1. Photomicrographs of NADPH diaphorase staining in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of nonpregnant (NP) and term pregnant (P) rats. (A) PVN from a nonpregnant rat. (B) PVN from a pregnant rat. (C) SON from a nonpregnant rat. (D) SON from a pregnant rat. Scale bar=300 μ m.

Fig. 2.

Mean data representing NADPH-diaphorase staining in the PVN and SON of non-pregnant (NP, $n=8$) and pregnant rats (P, $n=8$). Compared to the SON, the PVN contained a greater number of NADPH-diaphorase stained cells (#) [Region effect; $F(1,28)=168$, $P<.001$]. Group effect [$F(1,28)=45$, $P<.001$] and region X group interaction [$F(1,28)=21.8$, $P<.001$] were statistically significant. Pregnancy was associated with a decreased number of diaphorase positive cells in the PVN ($*P<.001$), while diaphorase staining in the SON was not different between nonpregnant and pregnant rats.

Fig. 3.

Protein expression in the PVN of non-pregnant and pregnant rats. Left: Immunoblots of PVN micropunches taken from one nonpregnant (NP) and one pregnant rat (P) for nNOS protein. The estimated size of the nNOS protein was similar to the predicted weight of 155 kDa. Right: Immunoblots of GAPDH from the same PVN micropunches. The estimated size of GAPDH protein was similar to the predicted weight of 36 kDa.

Fig. 4. Mean data representing nNOS protein expression in the PVN and SON from nonpregnant (NP, $n=4$) and pregnant (P, $n=3$) rats. Compared to the SON, the PVN had higher expression of nNOS protein (#) [Region effect; $F(1,11)=73.4$, $P<.001$]. Region X group interaction was statistically significant [$F(1,11)=8.9$, $P<.013$]. In the PVN, P rats had a significantly lower expression of nNOS compared to NP rats (* $P=.05$).

Fig. 5. Effects of pregnancy on mRNA for nNOS in the PVN. (A) Examples of amplification curves for GAPDH (dashed lines) and nNOS (solid lines) in a nonpregnant (black) and a pregnant (grey) rat. (B) The first derivative of melt temperatures resulted in single peaks demonstrating one PCR product for GAPDH (solid lines) and nNOS (dashed lines) for PCR reactions shown in (A). (C) Agarose gels of PCR products (duplicates) in (A), demonstrating prominent bands for GAPDH (~625 bp) and nNOS (~281 bp) amplicons (black arrowheads). (D) Mean data representing relative expression of nNOS mRNA in the PVN of nonpregnant (NP, $n=7$) and pregnant rats (P, $n=5$). * $P<0.05$.

Table 1

Body weights for nonpregnant (NP) and 21 day pregnant (P) rats, stage of cycle (NP group) and fetal number and weight (P group)

Group (n)	Weight (g)	Stage	# Fetuses	Average fetal wt. (g)
Nonpregnant (19)	241±4.0	Estrus	----	----
Pregnant (18)	352±7.9 [*]	Day 21	13.1±0.65	4.1±0.07

Body weight was greater in pregnant rats.

* Pregnant compared to nonpregnant rats, $P<0.05$.