THE PHYSIOLOGICAL ROLE OF ENDOGENOUS NITRIC OXIDE IN THE MAGNOCELLULAR NEUROSECRETORY SYSTEM

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DECLARATION

The studies outlined in this thesis were undertaken in the Department of Biomedical Sciences, University Medical School, Edinburgh, under the supervision of Professor Gareth Leng. This dissertation has not been submitted for any other qualification at any other university. All of the work was performed by the author, except otherwise indicated.

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ABSTRACT

The thesis examines the regulation of neuronal nitric oxide synthase (nNOS) mRNA expression in magnocellular neurosecretory neurones under physiological circumstances including pregnancy, hyperosmotic challenges and prolonged stimulation of the axons of the magnocellular neurones, and the functional role of nitric oxide (NO) in regulating oxytocin and vasopressin neuronal activities, and the release of oxytocin. The studies have included measurement of mRNA expression by *in situ* hybridization, *in vivo* electrophysiological studies combined with intrasupraoptic nucleus retrodialysis, Fos immunocytochemistry studies, and functional studies involving measurement of oxytocin secretion.

Expression of nNOS mRNA in the magnocellular neurones in the supraoptic (SON) and paraventricular nuclei (PVN) has been confirmed to be regulated in response to chronic osmotic stimuli, suggesting an important involvement of NO in this situation. It has been demonstrated that an induction of this gene is not a rapid response since an increase of NOS gene expression in the magnocellular neurones in the SON and PVN has been found at 6 h after acute hypertonic saline intraperitoneal injection, but not at 4 h after injection.

Neuronal activity of oxytocin and continuously firing vasopressin neurones in the SON was inhibited by local application of NO donor, and was increased by local administration of NOS inhibitor. Nitric oxide donor inhibited neuronal activity of phasic vasopressin neurones, while NOS inhibitor did not alter neuronal activities of these neurones. Blocking the central production of NOS by NOS inhibitor enhanced the expression of Fos in the magnocellular neurones in the SON and PVN following high doses, but not low doses of hypertonic saline. In addition, inhibition of NOS potentiated the release of oxytocin evoked by electrical stimulation of the axons of the magnocellular neurones. Furthermore, prolonged electrical stimulation of the axons of the magnocellular neurones for 2 h produced a down-regulation of nNOS mRNA expression in the magnocellular neurones in the SON. Thus NO, generated in

an activity-dependent manner, appears to act as a feedback inhibitor of oxytocin release at the cell bodies and the terminals of oxytocin neurones. However, although NOS expression is up-regulated in conditions of chronic demand for oxytocin, the present experiments indicate that this does not reflect a coupling of spike activity to increased NOS mRNA expression.

In experiments in virgin rats, central administration of NO donor attenuated the release of oxytocin in response to acute intraperitoneal hypertonic saline and systemic administration of NOS inhibitor potentiated oxytocin release induced by acute intraperitoneal hypertonic saline but not cholecystokinin. Blocking the central production of NO with NOS inhibitor further enhanced the release of oxytocin in response to acute intraperitoneal and intravenous hypertonic saline. In contrast, in experiments in 21-day pregnant rats, systemic administration of NOS inhibitor was unable to enhance the release of oxytocin evoked by acute intraperitoneal and intravenous hypertonic saline. Furthermore, down-regulation of nNOS mRNA expression in the magnocellular neurones in the SON was found in 22-day pregnant rats, which was followed by an increase in the expression of nNOS mRNA in the magnocellular neurones in the SON during parturition. These results indicate a functional down-regulation of the influence of central released NO on the responsiveness of oxytocin neurones to osmotic stimulation during late pregnancy. The decrease in NOS activity in late-pregnancy may release the magnocellular secretory system from this inhibition, making the oxytocin system more excitable at this time.

The thesis provides new information on the mechanism by which NO influences oxytocin secretion. Nitric oxide exerts a predominantly inhibitory effect on the magnocellular oxytocin and vasopressin neurones in the SON and may serve within a negative feedback loop to control the production and release of oxytocin. Neuronal NOS gene has been shown to be a late response gene and an increase of nNOS mRNA expression does not reflect a direct coupling to spike activity. The endogenous NOS system has a potent restraining influence on oxytocin neurone

excitability. A functional down-regulation of this system in the magnocellular neurosecretory system occurs at the end of pregnancy, which may play a key role in the osmoresponsiveness of oxytocin neurones during late pregnancy.

LIST OF ABBREVIATIONS

AP-1	= activator-protein 1
AP-2	= activator protein 2
ADP	= adenosine diphosphate
ATP	= adenosine triphosphate
ANOVA	= analysis of variance
a.c.	= anterior commissure
AV3V	= anteroventral third ventricle region
aCSF	= artificial cerebrospinal fluid
ABC	= avidin-biotin complex
$adddH_2O$	= autoclaved deionized double distilled water
b.s.a.	= bovine serum albumin
Ca^{2+}	= calcium ion
CaM	= calmodulin
CCK	= cholecystokinin
cDNAs	= complementary deoxyribonucleic acids
cNOS	= constitutive nitric oxide synthase
CRF	= corticotropin-releasing factor
cpm	= count per minute
cAMP	= cyclic adenosine 3′, 5′-monophosphate
CRE	= cAMP response element
cGMP	= cyclic guanosine 3′, 5′-monophosphate
CYT	= cytochrome
COX	= cyclooxygenase
$dddH_2O$	= deionized double distilled water
dUTP	= deoxy uridine triphosphate
DAPs	= depolarizing after-potentials
DAB	= diaminobenzidine
DIG	= digoxigenin
dpm	= disintegrations per minute
EDRF	= endothelium-derived relaxing factor
eNOS	= endothelial nitric oxide synthase
EPSPs	= excitatory postsynaptic currents
FAD	= flavin adenine dinucleotide
FMN	= flavin mononucleotide
FRAs	= Fos related antigens
GPCR	= G-protein-coupled receptor
GABA	= gamma-aminobutyric acid
GTP	= gaunosine 5′-triphosphate
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
IEG	= immediate early gene
ISH	= *in situ* hybridization
iNOS	= inducible nitric oxide synthase
i.d.	= inner diameter
i.c.v.	= intracerebroventricular

i.v.	= intravenous
i.p.	= intraperitoneal
AMG	= medial amygdaloid nucleus
Mp	= medial parvocellular
MnPO	= median preoptic nucleus
mRNA	= messenger ribonucleic acid
nNOS	= neuronal nitric oxide synthase
NADPH	= nicotinamide adenine dinucleotide phosphate
NO	= nitric oxide
NOS	= nitric oxide synthase
NO_2	= nitrite
NANC	= non-adrenergic, non-cholinergic
NF-1	= nuclear factor 1
NF-κB	= nuclear factor κB
NTS	= nucleus tractus solitarii
L-NNA	= N^{ω}-nitro-L-arginine
L-NAME	= N^{ω}-nitro-L-arginine methylester
SNAP	= *S*-nitroso-*N*-acetyl-penicillamine
NMDA	= *N*-methyl-D-aspartate-type receptors
L-NMMA	= N^{ω}-monomethyl-L-arginine
o.c.	= optic chiasm
OVLT	= organum vasculosum of the lamina terminalis
o.d.	= outer diameter
PBN	= parabrachial nucleus;
PVN	= paraventricular nucleus
PEG	= polyethylene glycol
Pm	= posterior magnocellular
PP	= posterior pituitary;
PSD-95	= postsynaptic density protein of 95 kDa,
PDZ	= postsynaptic density protein of 95 kDa, Drosophila discs large protein and Zonal occludens protein 1
RIA	= radioimmunoassay
RPM	= round per minute
$[Na^+]$	= sodium concentration
SNP	= sodium nitroprusside
sGC	= soluble guanylate cyclase
SFO	= subfornical organ
IPSPs	= spontaneous inhibitory postsynaptic currents
SON	= supraoptic nucleus
SIN-1	= sydnonimine-1
H_4B	= tetrahydrobiopterin
V3	= third ventricle
PDEs	= phosphodiesterases
VLM	= ventrolateral medulla
ZI	= zona incerta

CHAPTER 1

GENERAL INTRODUCTION

Nitric oxide (NO), a simple diatomic molecule, has long been known as an atmospheric pollutant arising from automobile exhaust, aircraft emissions and industrial fumes (Johnson *et al.*, 1992). The first reports of endogenous formation of NO in mammalian cells appeared in 1987 (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). This molecule has since been implicated in a diverse number of physiological systems and pathophysiological states. In medicine, NO-generating substances (*e.g.* glyceryl trinitrate) have been used for their vasodilating actions in the management of angina pectoris for many years.

In the cardiovascular system, endogenous NO was identified as endothelium-derived relaxing factor (EDRF) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), which is released from the endothelial cells when stimulated by a variety of vasodilator substances such as acetylcholine, bradykinin and adenosine triphosphate (ATP), causing relaxation of the underlying smooth muscle (Furchgott and Zawadzki, 1980). Nitric oxide also inhibits platelet aggregation (Azuma *et al.*, 1986; Bredt *et al.*, 1991b) and adhesion (Radomski *et al.*, 1987).

In the immune system, NO is responsible for the tumoricidal and bactericidal actions of macrophages. Nitric oxide is produced by activated macrophages, which contributes to their cytotoxic action by inhibiting the cell proliferation that is necessary to raise the effectiveness of immune responses. Nitric oxide is a non-specific effector capable of killing or inhibiting the growth of many pathogens and tumor cells (for reviews see Nathan and Hibbs, 1991).

Considerable evidence has indicated that NO is also a novel type of neurotransmitter in the peripheral and central nervous systems. Nitric oxide is unlike classical neurotransmitters that are stored in synaptic vesicles, released by exocytosis upon membrane depolarization, and act at receptor proteins on adjacent neuronal membranes. Nitric oxide is a lipophilic molecule with a very short half-life that readily diffuses across cellular membranes, and is not stored in synaptic vesicles or in association with the plasma membrane. Instead, NO is synthesized on demand and

diffuses out of neurones and into target cells, where it interacts with specific molecular targets. The putative receptor for NO is iron in the heme of soluble guanylate cyclase (sGC), which can influence guanosine 3′, 5′-monophosphate (cGMP) formation (for reviews see Bredt and Snyder, 1992).

In the peripheral nervous system, NO generated in response to non-adrenergic, non-cholinergic (NANC) neurotransmission has been implicated in the relaxation of the corpus cavernosum smooth muscle of the penis, inducing penile erection (for reviews see Argiolas and Melis, 1995). Nitric oxide released from NANC neurones also causes gastric relaxation, intestinal peristalsis, and sphincter relaxation, inducing gastrointestinal motility (Lefebvre, 1997; Li and Rand, 1990). Nitric oxide may also act to regulate renal sympathetic nerve activity (Sakuma *et al.*, 1992).

In the central nervous system, the existence of NO was first suggested by the observation of Garthwaite *et al.* in 1988 (Garthwaite *et al.*, 1988) that dissociated cerebellar neuronal cultures released a factor with properties resembling NO, as well as by the demonstration of NO forming activity in the brain (Bredt and Snyder, 1989; Knowles *et al.*, 1989). Possible functions for NO as a neurotransmitter are suggested by the localization of the enzyme responsible for NO formation, nitric oxide synthase (NOS) in the brain (Bredt *et al.*, 1991a; Bredt *et al.*, 1990; Vincent and Kimura, 1992), which is now known to be identical to nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase (Dawson *et al.*, 1991; Hope *et al.*, 1991). It has been proposed that NO may be a messenger in the brain that can contribute to both retrograde and anterograde signalling at the synapse (for reviews see Garthwaite and Boulton, 1995).

1.1 Mechanism of NO production

1.1.1 Biosynthesis of NO

Nitric oxide is composed of one atom each of nitrogen and oxygen, and is called a *radical gas* because of its characteristics as an uncharged molecule with an unpaired electron. Nitric oxide can diffuse freely across membranes, and is highly reactive, with a very short half-life of less than 30 s. After release, NO decays spontaneously into nitrite (NO_2) (Ignarro, 1990; Ignarro *et al.*, 1993). Nitric oxide is synthesized by nitric oxide synthase (NOS), a cytochrome (CYT) P450-like structure (Bredt *et al.*, 1991b), in an unusual reaction that converts the semi-essential amino acid L-arginine and oxygen to N^{ω}-hydroxyl- L-arginine first (Stuehr *et al.*, 1991) and then into L-citrulline and NO (Fig. 1). The precise biosynthetic pathway is still not clear, but it involves electron oxidation of the guanidino nitrogen of L-arginine to NO. This reaction requires molecular oxygen and the guanidine nitrogen of L-arginine as the substrates, nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor, and flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (H_4B), heme and Ca^{2+}/calmodulin as cofactors. Electrons donated by NADPH are transported by FAD and FMN to heme when calmodulin (CaM) is bound to the enzyme (Fig. 2). Tetrahydrobiopterin is essential for the coupling of NADPH-dependent O_2 activation to NO synthesis. Finally, an atom of oxygen binds with the terminal guanidine nitrogen of L-arginine to form NO (for reviews see Mayer and Hemmens, 1997).

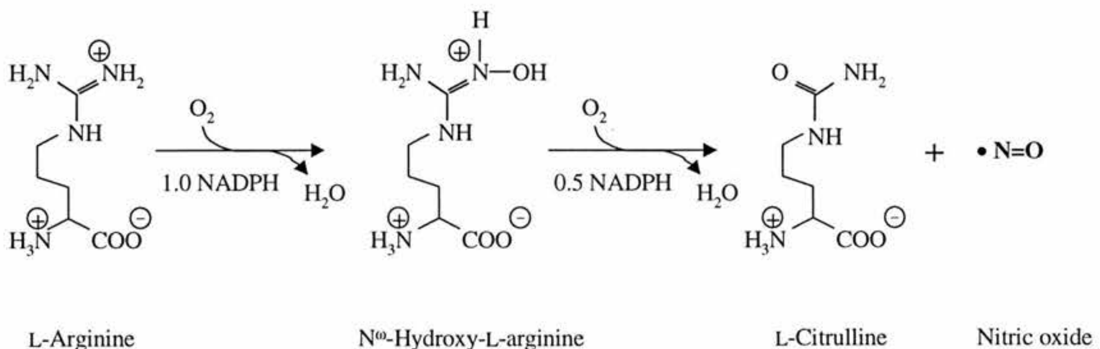

Figure 1. The nitric oxide synthase reaction. (From Mayer and Hemmens, 1997)

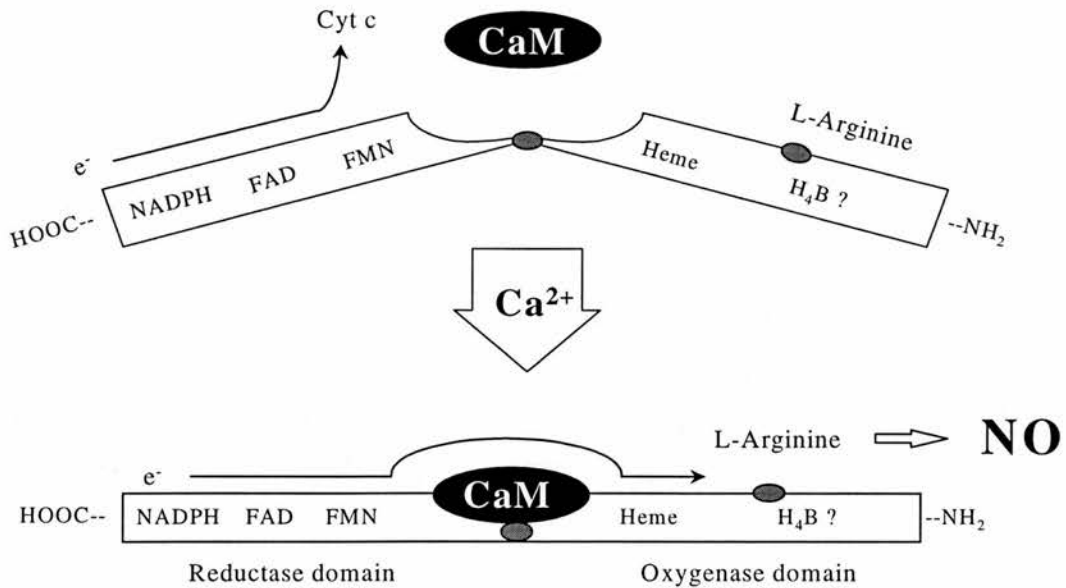

Figure 2. Schematic representation of NO synthesis by NOS. When calmodulin (CaM) is not bound to the enzyme (top), electrons, donated by NADPH, do not flow to the oxygenase domain and are accepted by cytochrome c and other electron acceptors. In the presence of CaM (bottom), L-arginine is converted into NO and L-citrulline. 'Coupled' NO synthesis requires H_4B, a co-factor whose function in NO synthesis is not entirely clear. Abbreviations: CaM, Calmodulin; Cyt c, cytochrome oxidase; FAD, Flavin adenine dinuceleotide; FMN, Flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; H_4B, tetrahydrobiopterin. (From Iadecola, 1997)

1.1.2 Classification of NOS subtypes

In mammalian cells, several NOS isoforms exist, which share significant sequence similarity and which contain conserved putative cofactor binding sites (Fig. 3). The sequences differ at the N-terminal extension, which appears not to be essential for catalysis, and which probably functions in the intracellular localization of the enzyme. There are two distinct categories of NOS, classified as either Ca^{2+}-dependent (constitutive NOS or cNOS) or Ca^{2+}-independent (inducible NOS or iNOS) (Nathan, 1992). Constitutive NOS and iNOS exist as dimeric and tetrameric complexes respectively. Each unit contains two identical domains with four

prosthetic groups (FAD, FMN, H_4B and heme). The C-terminal reductase domain contains binding motifs for FAD, FMN and NADPH, and N-terminal oxygenase domain contains the consensus sequences for H_4B, the heme binding site and the potential phosphorylation sites. The reductase and oxygenase domains are linked by a calmodulin binding site which facilitates electron transfer from NADPH and the flavins to the heme group during NO production.

Both categories of NOS are strictly dependent on calmodulin (CaM), which triggers the electron flow between the reductase and oxygenase domains. Calmodulin binds just at the N-terminal side of the reductase domain. An important difference between cNOS and iNOS is that cNOS synthesizes NO only when the intracellular Ca^{2+} concentration is elevated and calmodulin is bound to the enzyme (Fig. 2), whereas iNOS produces NO continuously and independently of intracellular Ca^{2+}. Calmodulin is bound to inducible NOS, and is fully active at resting cellular levels of calcium (30-70 nM) (Abu-Soud and Stuehr, 1993). By contrast, constitutive NOS only binds calmodulin at stimulated intracellular concentrations of Ca^{2+}. Thus, constitutive NOS, calmodulin binding and activation respond to physiological changes in Ca^{2+}. Consequently, constitutive NOS produces NO in small and highly regulated bursts that are well suited for the molecular messenger function of NO (Garthwaite and Boulton, 1995). In contrast, iNOS produces large amounts of NO continuously for long periods, a feature that is responsible for the cytotoxicity of NO (Gross and Wolin, 1995).

The cNOS isoform does not require new protein synthesis, whereas iNOS is transcriptionally regulated (Xie *et al.*, 1992). Three isoforms of NOS are named after the tissue in which they were first purified and cloned, neuronal NOS (nNOS or type I), macrophage or immunological NOS (iNOS or type II), and endothelial NOS (eNOS or type III) (Griffith and Stuehr, 1995). These isoforms are encoded by three distinct genes and are regulated by diverse signaling pathways. Across species, amino acid sequences of NOS isoforms are more than 90% conserved for nNOS and eNOS, and are more than 80% identical to iNOS. The eNOS and nNOS isoforms are

constitutively expressed and regulated by Ca^{2+}/calmodulin, whereas iNOS is not normally expressed but is inducible in many cell types by selected immunological stimuli (Nathan, 1992; Nathan and Xie, 1994). Recent evidence has suggested that the level of expression of eNOS and nNOS can be subject to up- and down-regulation (Kadowaki *et al.*, 1994; Sessa *et al.*, 1994; Uematsu *et al.*, 1995; Weiner *et al.*, 1994).

The classification of the NOS family, summarized in Table 1, is based on physical and biochemical characteristics, and prototypical locations. Indeed, nNOS is not only expressed in brain and spinal cord but also expressed in skeletal muscle (Kobzik *et al.*, 1994) and the airway epithelium (Kobzik *et al.*, 1993; Kowalski *et al.*, 1998). eNOS, originally found in vascular endothelial cells, is also found in platelets (Radomski *et al.*, 1990) and certain neuronal populations in the brain (Dinerman *et al.*, 1994). In addition, iNOS, while mainly expressed in macrophages, appears also to be present constitutively in bronchial epithelium (Kobzik *et al.*, 1993).

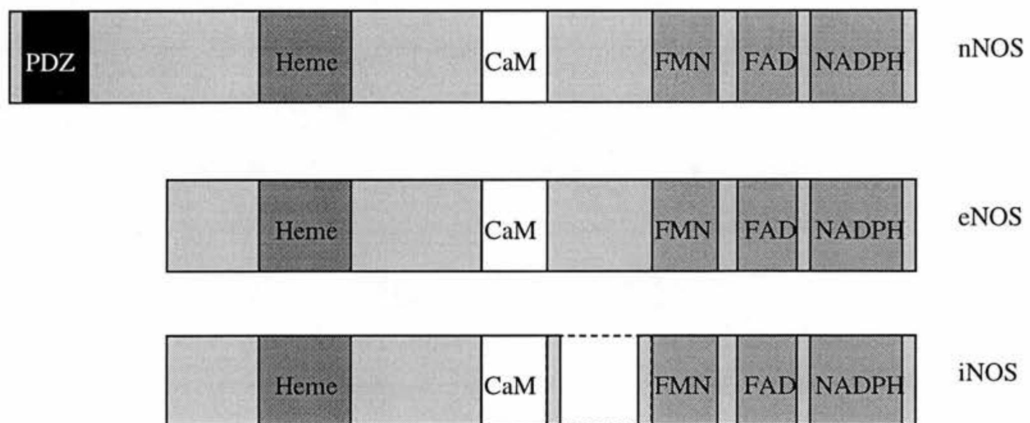

Figure 3. Schematic alignment of the NOS proteins. nNOS has a unique amino-terminal PDZ motif. Binding sites: CaM, Calmodulin; FMN, flavin adenine mononucleotide; FAD, flavin adenine dinucleotide; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS. (From Brenman and Bredt, 1997)

Table 1. Isoforms of nitric oxide synthase

Isoform	Endothelial NOS (eNOS) (Type III NOS, NOS-3, cNOS)	Neuronal NOS (nNOS) (Type I NOS, NOS-1, cNOS, bNOS)	Inducible NOS (iNOS) (Type II NOS, NOS-2, macNOS, hepNOS)
Primary regulation	Ca^{2+}/calmodulin	Ca^{2+}/calmodulin	Inducible by cytokines, Ca^{2+} independent, CaM subunit Gene expression
Subcellular location	Mainly soluble	Soluble/particulate in varying proportion	>90% particulate
NO output	Low (pmolar)	Low (pmolar)	High (μmolar)
Function	Cell signalling	Cell signalling	Cytotoxic, Cytostatic, Cytoprotective
Molecular weight (kDa)	135	150-160	130-135
Major tissues/cell locations	Endothelial cells, syncytiotrophoblasts, colon interstitial cells,	Brain, nitrergic neurones, skeletal muscle, kidney, macular densa cells, pancreatic islet cells	Macrophages, hepatocytes, chrondrocytes, adenocarcinoma cells, vascular smooth muscle, endothelial cells, mesangial cells

Adapted from Moncada *et al.,* 1997, Pollock *et al.,*1995 and Griffiths *et al.*, 1998.

1.1.3 Synaptic function of NO

Although NO was first identified as the EDRF, it is now clear that the brain is the primary source of NO in the body, and the major isoform present in the hypothalamus is nNOS (Bhat *et al.*, 1996). Depending on the location of the enzyme, the activity of nNOS is coupled to one of two main types of physiological stimuli: *postsynaptic neurotransmitter receptor stimulation* leading to Ca^{2+} influx or mobilization, *and action potentials in presynaptic nerves* eliciting Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Garthwaite and Boulton, 1995). Postsynaptically, the activity of nNOS is tightly regulated by Ca^{2+} through the binding of the ion to calmodulin. At resting cytoplasmic Ca^{2+} concentrations (<100 nM), calmodulin is largely free of Ca^{2+} and so does not bind to nNOS. The binding of glutamate to *N*-methyl-D-aspartate-type receptors (NMDA) leads to postsynaptic Ca^{2+} influx, which would activate nNOS resulting in the formation of NO (Garthwaite and Boulton, 1995). After formation at presynaptic or postsynaptic sites, NO diffuses out to act on neighbouring cellular elements. Thus, NO can operate as an intercellular messenger, which represents a potential line of communication from postsynaptic to presynaptic elements, and *vice versa*, as well as between presynaptic fibers or postsynaptic structures (Garthwaite, 1991).

In contrast to eNOS, nNOS does not associate directly with membranes, but is associated with membranes by the amino-terminal, which possesses a PDZ domain (postsynaptic density protein of 95 kDa, Drosophila discs large protein and Zonal occludens protein 1)(Fig. 3). Synaptic localization is regulated by the amino terminus of nNOS, which encodes a PDZ protein motif as shown in Fig. 3 (Brenman *et al.*, 1996). PDZ domains are modular protein motifs implicated in signal transduction in neurones and other cells (Sheng, 1996). Indeed, the PDZ domain of nNOS appears to link the synthase to protein complexes at the postsynaptic density that contain NMDA receptors. The NR2 subunit and certain NR1 splice forms of the *N*-methyl-D-aspartate (NMDA) receptor contain a carboxy-terminal tSXB motif (where S is serine, X is any amino acid, and V is valine) that can interact with the PDZ domain

of the postsynaptic density protein of 95 kDa, PSD-95 (Kornau *et al.*, 1995). Neuronal NOS binds *via* its N-terminal PDZ domain to PSD-95 through a PDZ-PDZ binding interface (Brenman *et al.*, 1996). PSD-95 can mediate the assembly of a tight ternary complex containing nNOS and NMDA receptor subunits. This macromolecular complex probably underlines the particular sensitivity of nNOS to activation of the NMDA type of glutamate receptor.

1.2 NO signal transduction

To mediate a signal between cells, most molecules (*e.g.* hormones and neurotransmitters) act through specific protein receptors associated with the membranes. By contrast, NO diffuses from the cell that generates it and into target cells (Table 2). Because of its chemical reactivity, there may be multiple targets for NO. The best-characterized "receptor" of NO is iron, contained in certain proteins as a heme group or as an iron-sulphur complex. Nitric oxide exerts its effects by binding to iron-containing enzymes and either activating or inactivating the enzymes. When NO binds to the iron in the heme group of soluble guanylate cyclase (sGC), the enzyme is activated and catalyses the formation of cyclic guanosine 3′, 5′-monophosphate (cGMP) which then activates other cellular processes (Bredt and Snyder, 1989; Garthwaite *et al.*, 1989; Johns, 1991). Like sGC, cyclooxygenase (COX) is a heme-containing enzyme that can directly bind NO, and NO may serve as an activator for the synthesis of prostaglandins from arachidonic acid in a cGMP-independent manner (Rettori *et al.*, 1993; Rettori *et al.*, 1992; Salvemini *et al.*, 1996). When NO binds to iron-sulfur centers of nonheme-containing enzymes, it causes a loss of enzyme activity (Nathan, 1992). Nitric oxide also potently enhances adenosine diphosphate (ADP) ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by attaching an ADP-ribose group to it, thereby blocking the production of adenosine triphosphate from glycolysis (Brune *et al.*, 1994; Brune and Lapetina, 1989; Kots *et al.*, 1992; Zhang and Snyder, 1992).

Soluble guanylate cyclase (sGC)/cGMP

Soluble guanylate cyclase (sCG) exists as a heterodimer composed of α and β subunits (Schmidt *et al.*, 1993). Three α and β subunit isoforms (α1-3, β1-3) and subunit splice variants have been identified (Garbers, 1992). Although each subunit contains the cyclase domain located within the carboxy-terminus and the heme-binding domain located at the amino-terminus, the catalytic activity apparently depends on both subunits together with the presence of Mg^{2+} (Murad, 1994). Only one molecule of heme is bound per sGC molecule at the heme binding site co-ordinated with a histidine residue (His 105) and cysteine residues (Cys 78, Cys 214) of the β subunit. Nitric oxide appears to activate sGC by interaction with the heme group (Schmidt *et al.*, 1993) and rapidly forms a nitrosyl-heme complex with sGC which may induce a conformational change of sGC that reveals the catalytic site to gaunosine 5′-triphosphate (GTP) (Traylor and Sharma, 1992). The enzymatic reaction of sGC is then stimulated, and GTP is converted into the second messenger cGMP (Humbert *et al.*, 1990; Schmidt, 1992; Schmidt *et al.*, 1993; Stone and Marletta, 1994). Regulation of physiological events by cGMP is accomplished by interaction of the nucleotide with target proteins, including cGMP-gated ion channels, cGMP-dependent protein kinases and cGMP-regulated cyclic nucleotide phosphodiesterases (Garthwaite, 1991; Garthwaite and Boulton, 1995; Lincoln and Cornwell, 1993; McDonald and Murad, 1996). This means that cGMP can alter cell function through protein phosphorylation or through mechanisms indirectly related to protein phosphorylation. Cation channels of retinal photoreceptor cells are gated by binding of cGMP that allows Ca^{2+} entry the cells, which is important for visual transduction (Finn *et al.*, 1996). cGMP activation of cGMP-dependent protein kinase may be involved in reduction of Ca^{2+}concentration, *via* Ca^{2+}sequestration and extrusion, by phosphorylating and activating Ca^{2+}-ATPase or components associated with the transporter (Lincoln and Cornwell, 1993; Schmidt *et al.*, 1993) causing smooth muscle relaxation and platelet inhibition. cGMP can also control the breakdown of itself and another nucleotide, cAMP, by stimulating or inhibiting specific subtypes of cyclic nucleotide phosphodiesterases (PDEs). cGMP-inhibited

PDE has been found to enhance cAMP-mediated relaxation of smooth muscle and platelet inhibition (Lincoln and Cornwell, 1993). cGMP-stimulated PDE-induced reduction in cAMP levels has been found to suppress Ca^{2+} in hippocampal pyramidal neurones (Doerner and Alger, 1988). Termination of signal transduction by hydrolysis of cGMP to GMP depends on the GMP-specific PDEs.

It has thus been proposed that sGC is the main target for NO in the brain (Bredt and Snyder, 1994). The $\alpha 1$ and $\beta 1$ subunits of sGC are widely distributed in most neurones throughout the brain, with different levels of expression. Both subunits are expressed markedly in the glomerular layer of the olfactory bulb, dorsal and ventral striatum, and several regions in the brainstem (Furuyama *et al.*, 1993). Interestingly, low levels of expression of GC mRNA have been demonstrated in the hypothalamus (Matsuoka *et al.*, 1992). There is a paucity of cGMP in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in non-stimulated rats (Southam and Garthwaite, 1993). Cyclic GMP localizes within a dense fibre network in both the PVN and SON and also in small cells, perhaps astrocytes in the SON, rather than being present in the magnocellular neurones (Bredt *et al.*, 1990). It has been reported recently that NO potently regulates hypothalamic cGMP levels (Bhat *et al.*, 1996). The release of cGMP from the posterior pituitary has been reported following depolarization (O'Dea *et al.*, 1978; Vale and Hope, 1982) and from the SON following osmotic stimulation (Carter and Murphy, 1989). More recently, it has been proposed that heme-containing enzymes other than guanylate cyclase, such as cyclo-oxygenase, may be targets for NO in the hypothalamus (Rettori *et al.*, 1993; Rettori *et al.*, 1992).

In general, NO-producing neurones seem unlikely to be influenced by released NO because the high concentration of intracellular calcium necessary to trigger NO release prevents the activation of the sCG. Therefore, NO release from one cell type will act on sGC in another cell type in a paracrine function within a volume delimited by the half-life and diffusion of NO (approximately 200 μm), causing elevation of intracellular cGMP (Southam and Garthwaite, 1993; Wood and Garthwaite, 1994). This hypothesis may not apply to magnocellular neurones of the

PVN and SON, which express high levels of both the α1 and β1 subunits of sGC (Furuyama *et al.*, 1993). Therefore, the released NO from a stimulated neurone may activate sGC within the same neurone and any neighbouring magnocellular neurones in a paracrine and autocrine function.

Table 2. Molecular targets of NO

Interaction sites or modification types	**Target molecules**	**Functional consequences**
Heme	Soluble guanylyl cyclase Cyclooxygenase I & II Cytochrome P450	Increased cGMP Increased prostaglandin E_2 Impaired detoxification
Nonheme-iron	Cytosolic aconitase Mitochondrial aconitase Complex I and II Ferritin/transferrin Ribonucelotide reductase	Inhibition of glycolysis, activation of IRF to regulate iron metabolism Inhibition of glycolysis Inhibition of respiratory chain Iron loss and lipid peroxidation Inhibition of DNA synthesis
Nitrosylation	NMDA receptor Protein kinase C GAPDH Ca^{2+}-dependent-K^+-channel G proteins Tyrosine phosphatase Albumin t-PA Glutathione	Block of Ca^{2+} influx Inhibition of phosphorylation Inhibition of glycolysis, enhance ADP-ribosylation Activation, vasorelaxation Activation, causing NF-κB translocation Activation of $p56^{lck}$ NO carrier activity Vasodilation and antiplatelet activities Activation of the hexose monophosphate shunt
Superoxide ($ONOO^-$ formation)	Zn,Cu-superoxide dismutase α-Tocopherol in LDL DNA strand break GAPDH	Tyrosine nitrations Oxidation PARS activation Inhibition of glycolysis
Deamination	DNA	Mutations and strand breaks, PARS activation

Abbreviations: IRF, iron-responsive factor; complex I & II, NADH-succinate oxidoreductase and NADPH-ubiquinone oxidoreductase; NMDA, *N*-methyl-D-aspartate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear factor κB, a transcription factor; t-PA, tissue-type plasminogen activator; $ONOO^-$, peroxynitrite; LDL, low density lipoproteins; PARS, poly (ADP-ribose) synthetase. (From Zhang and Snyder, 1995)

1.3 Molecular biological features of neuronal NOS (nNOS)

Neuronal NOS (nNOS), originally isolated from rat (Bredt *et al.*, 1990) and porcine cerebellum (Mayer *et al.*, 1990), is also expressed in skeletal muscle (Nakane *et al.*, 1993) and pulmonary epithelium (Asano *et al.*, 1994). Neuronal NOS in the brain is mostly soluble, and migrates with a molecular mass of 150-160 kDa on SDS-polyacrylamide gel electrophoresis (Bredt *et al.*, 1990; Mayer *et al.*, 1990).The cDNAs encoding nNOS have been cloned from rat (Bredt *et al.*, 1991b), mouse (Ogura *et al.*, 1993) and human brains (Nakane *et al.*, 1993). The cDNA sequences of rat and human nNOS predict proteins of 160 and 161 kDa, respectively, which is in good agreement with the purification results. Neuronal NOS seems to be a highly conserved enzyme between species; 93% amino acid identity exists between the rat and human species (Nakane *et al.*, 1993). The human nNOS gene is localized to chromosome 12 (Marsden *et al.*, 1993).

Although nNOS is constitutively expressed in various cells, expression of nNOS is subject to regulation as suggested by a number of recent observations. In particular, nNOS gene expression has been reported to be up-regulated in various brain areas by estrogen (Ceccatelli *et al.*, 1996; Weiner *et al.*, 1994), water deprivation (Ueta *et al.*, 1995b), chronic salt loading (Kadowaki *et al.*, 1994; Villar *et al.*, 1994b), lactation (Ceccatelli and Eriksson, 1993; Luckman *et al.*, 1997), stress (Calza *et al.*, 1993), axotomy (Verge *et al.*, 1992), hypophysectomy (Villar *et al.*, 1994a), gonadectomy (Ceccatelli *et al.*, 1993), and occlusion of the middle cerebral artery (Zhang *et al.*, 1994). Conversely, down-regulation of nNOS gene expression has been reported following food deprivation (O'Shea and Gundlach, 1996; Ueta *et al.*, 1995a). Thus, nNOS expression can be regulated under some circumstances, but the molecular mechanism of this regulation remains to be elucidated.

Little information is available on the regulation of nNOS expression. One of the interesting features of the nNOS genes is that nNOS mRNA transcripts are highly diverse, originating from different first exons spliced to a common second exon. At

least two alternatively spliced transcripts of human and murine nNOS genes and at least three alternatively spliced transcripts of the rat nNOS gene arising from alternative splicing promoters and differing in their 5′-untranslated regions have been described (Brenman *et al.*, 1996; Min *et al.*, 1997; Xie *et al.*, 1995). The use of multiple alternative promoters in conjunction with alternative splicing may allow spatial and temporal regulation of transcription of the nNOS gene. The putative promoter regions of human nNOS contains consensus sequences that encode putative functional domains for the binding of several transcription factors, such as activator protein 2 (AP-2), acute-phase reactants, nuclear factor 1 (NF-1) and NFκB, providing a possible means of transcriptional regulation of the nNOS gene in response to physiological or pathological perturbations (Hall *et al.*, 1994). Neuronal NOS might also be regulated post-transcriptionally by altered mRNA stability and post-translationally by modulation of protein stability of protein phosphorylation (Nathan and Xie, 1994). Post-translational modifications might provide a powerful mechanism for down-regulation of NOS activity. Enhanced phosphorylation of nNOS decreases nNOS catalytic activity that might be a useful strategy for neuroprotection (Dawson *et al.*, 1993).

1.4 The magnocellular neurosecretory system

The magnocellular neurosecretory system plays major roles in body fluid homeostasis maintenance, in parturition, in milk ejection during lactation, and in stress responses. This system originates from the magnocellular neurones of the supraoptic (SON) and paraventricular hypothalamic nuclei (PVN) of the hypothalamus where oxytocin and arginine vasopressin are synthesized and stored in neurosecretory granules, in separate neurones. The neurosecretory granules are then transported along axons that project through the pituitary stalk and terminate within the posterior lobe of the pituitary gland (Brownstein *et al.*, 1980). In response to appropriate stimuli, action potentials arise as a result of synaptic input to the cell bodies and are transmitted down the axons, causing exocytosis of the neurosecretory granules by triggering an influx of calcium into the nerve terminals. Either oxytocin

or vasopressin is then released from the nerve terminals into the general circulation (Hatton, 1990). The amount of release has been shown to correlate with the frequency and pattern of the action potentials (see Bicknell & Leng, 1981 and Renaud & Bourque, 1991 for reviews).

Oxytocin and vasopressin are structurally related nonapeptide hormones, differing at only two amino acid residues. Oxytocin and vasopressin mediate their physiological roles through interaction with specific G-protein coupled receptors (oxytocin, vasopressin V_{1a}, V_{1b} and V_2), which have been classified on the basis of both the second messenger system coupled to the receptors and the affinity of a series of vasopressin and oxytocin analogues with enhanced selectivity for a certain receptor type, and by sequencing of their genes. Vasopressin V_{1a} receptors are found in liver and smooth muscle cells from blood vessels. V_{1b} and V_2 receptors are found in adenohypophysis and kidney, respectively while oxytocin receptors are found in uterus and mammary gland. Vasopressin V_{1a} and oxytocin receptors are also localized in discrete brain nuclei (Barberis and Tribollet, 1996). Recently, vasopressin V_{1b} receptor mRNA has been detected in the kidney, thymus, lung, spleen, uterus and breast, and in numerous areas of the rat brain (Lolait *et al.*, 1995). Molecular cloning has confirmed that vasopressin and oxytocin receptor subtypes are members of the seven-transmembrane domain, G-protein-coupled receptor (GPCR) (Kimura *et al.*, 1992). Oxytocin appears to act through a single receptor that signals through the phosphoinositol pathway, while vasopressin effects are mediated by two classes of receptors linked to separate transduction systems. Vasopressin V_1 receptors signal vascular muscle contraction by means of the phosphoinositol pathway, whereas vasopressin V_2 receptors utilize the cyclic AMP system to produce the antidiuretic effect in renal tubules.

1.4.1 Morphology of the magnocellular neurosecretory system

The PVN contains both large (magnocellular) and small (parvocellular) neurones. There are eight distinct subdivisions within the PVN, three of which are magnocellular and the rest are parvocellular (Swanson and Kuypers, 1980). The anterior and medial parts of the magnocellular division contain primarily oxytocin neurones. The posterior part of the nucleus, the largest subdivision, consists of two distinct populations located in the medial and the lateral areas (Hatton *et al.*, 1976). Oxytocin neurones are primarily found in the medial area, and vasopressin neurones in the lateral area. The SON contains only large (magnocellular) neurones: oxytocin neurones are mainly located posterodorsally and vasopressin neurones are mainly located posteroventrally (Swaab *et al.*, 1975; Vandesande *et al.*, 1975). The magnocellular neurones of the SON and PVN each have between one to five and one to three simple dendrites, respectively, together with one axon arising directly from the soma or from a primary dendrite (Armstrong *et al.*, 1982; Armstrong *et al.*, 1980; Swanson and Kuypers, 1980). Almost all of the axons project to the posterior pituitary gland. Some axons display collateral branches, most of which terminate in the hypothalamus close to the SON and PVN (Hatton *et al.*, 1985; Mason *et al.*, 1984). Some vasopressin-synthesizing neurones, originating from the parvocellular, not the magnocellular neurones of the PVN, project to the median eminence (Vandesande *et al.*, 1977), the brain stem and spinal cord (Buijs, 1978; Kannan and Yamashita, 1983) instead of to the neural lobe of the pituitary gland. In the SON, most of the dendrites course ventrally toward the ventral glial lamina along the base of the nucleus, where they form a layer of dendritic bundles, intermingled with glial cell bodies and glial processes (Randle *et al.*, 1986).

Although oxytocin and vasopressin are predominantly released directly into the systemic circulation, the central release of oxytocin and vasopressin has also been reported. Oxytocin and vasopressin are released by exocytosis from the axonal swellings (Nordmann and Dayanithi, 1988), the axonal collaterals (Hatton *et al.*,

1985; Mason *et al.*, 1984) and the dendrites of magnocellular neurones in response to various stimuli (Ludwig, 1998; Pow and Morris, 1989).

1.4.2 Afferent pathways to the magnocellular neurosecretory system

The magnocellular neurosecretory system receives inputs from several forebrain regions including the olfactory bulb, the amygdala, the septum and structures associated with the lamina terminalis of the third ventricle which include the subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT) and the median preoptic nucleus (MnPO) (Hatton, 1990; Sawchenko and Swanson, 1983). The SFO and OVLT, so-called "circumventricular organs", lack an effective blood-brain barrier and are characterized by the presence of dense vascularization and the outer membranes of the endothelial cells that line blood vessels in these regions possess fenestrations. These areas send direct efferent projections to the magnocellular neurosecretory system (Fig. 4) (Armstrong *et al.*, 1996; Sawchenko and Swanson, 1983). The SFO and OVLT also have indirect connections with the magnocellular neurones *via* synapses in an intervening nucleus, the MnPO (Oldfield *et al.*, 1991b; Oldfield *et al.*, 1991c). The SFO, OVLT and MnPO have been suggested to contain osmoreceptors, which participate in the control of osmoregulatory hormone release (Bourque *et al.*, 1994). Angiotensin II may mediate an excitatory input from the SFO to the SON (Jhamandas *et al.*, 1989) and the anteroventral third ventricle region (AV3V) region (Tanaka *et al.*, 1987). The SFO sends an angiotensinergic input that evokes a predominantly excitatory effect on vasopressin neurones (Jhamandas *et al.*, 1989; Renaud, 1996). The MnPO exerts a $GABA_A$ receptor-mediated inhibition of supraoptic neurones in the rat (Nissen and Renaud, 1994; Renaud *et al.*, 1993). Glutamatergic innervation and GABAergic afferents from the OVLT to the SON exert excitatory and inhibitory influences, respectively (Renaud *et al.*, 1993; Yang *et al.*, 1994).

Noradrenergic inputs from the brainstem to the magnocellular nuclei arise from the A1 cell group of the ventrolateral medulla (VLM), and the A2 cell group of

the caudal part of the nucleus tractus solitarii (NTS). The A6 cells of the locus coeruleus also innervate the PVN, but predominantly the parvocellular division (Sawchenko and Swanson, 1982). The A1 and A2 cells send afferent inputs to both parvocellular and magnocellular neurones of the PVN and to the SON. The terminals anterogradely labelled from A1 pathways are preferentially concentrated around vasopressin neurones in the SON, primarily on their dendrites (Shioda *et al.*, 1992), while the anterogradely labelled fibers from A2 cells innervate oxytocin neurones of the SON (Cunningham and Sawchenko, 1988). Some A2 cells also send a direct projection to the neurohypophysis (Garten *et al.*, 1989). The supraoptic oxytocin and vasopressin neurones are excited after stimulation of the A2 cells (Raby and Renaud, 1989). Non-noradrenergic cells in the NTS project to the A1 cell group (Sawchenko and Swanson, 1982). The NTS appears to mediate activation of oxytocin neurones directly, whereas stimuli that activate vasopressin neurones are likely to be indirectly mediated *via* a non-noradrenergic projection to the A1 cells and then to the vasopressin neurones (Raby and Renaud, 1989). Afferent inputs from baroreceptors, stretch receptors, chemoreceptors and gastrointestinal distention are conveyed in the vagus nerves and glossopharyngeal nerves to the NTS (Cunningham and Sawchenko, 1991). Activation of chemoreceptors may activate a cholinergic pathway from the NTS to the A1 cells, resulting in stimulation of vasopressin release. Activation of baroreceptors and stretch receptors may activate GABA neurones in the NTS that innervate the A1 cells, resulting in inhibition of vasopressin release (Bisset and Chowdrey, 1988). The caudal NTS also receives direct information from the area postrema (McKinley *et al.*, 1990). Signals from the vaginal and uterine distention receptors, somatic sensory receptors and nociceptors are carried to the A1 cell group and the caudal NTS *via* the dorsal horn of the spinal cord (Menetrey and Basbaum, 1987; Menetrey *et al.*, 1983) by way of the spinoreticular and spinosolitary tracts, respectively.

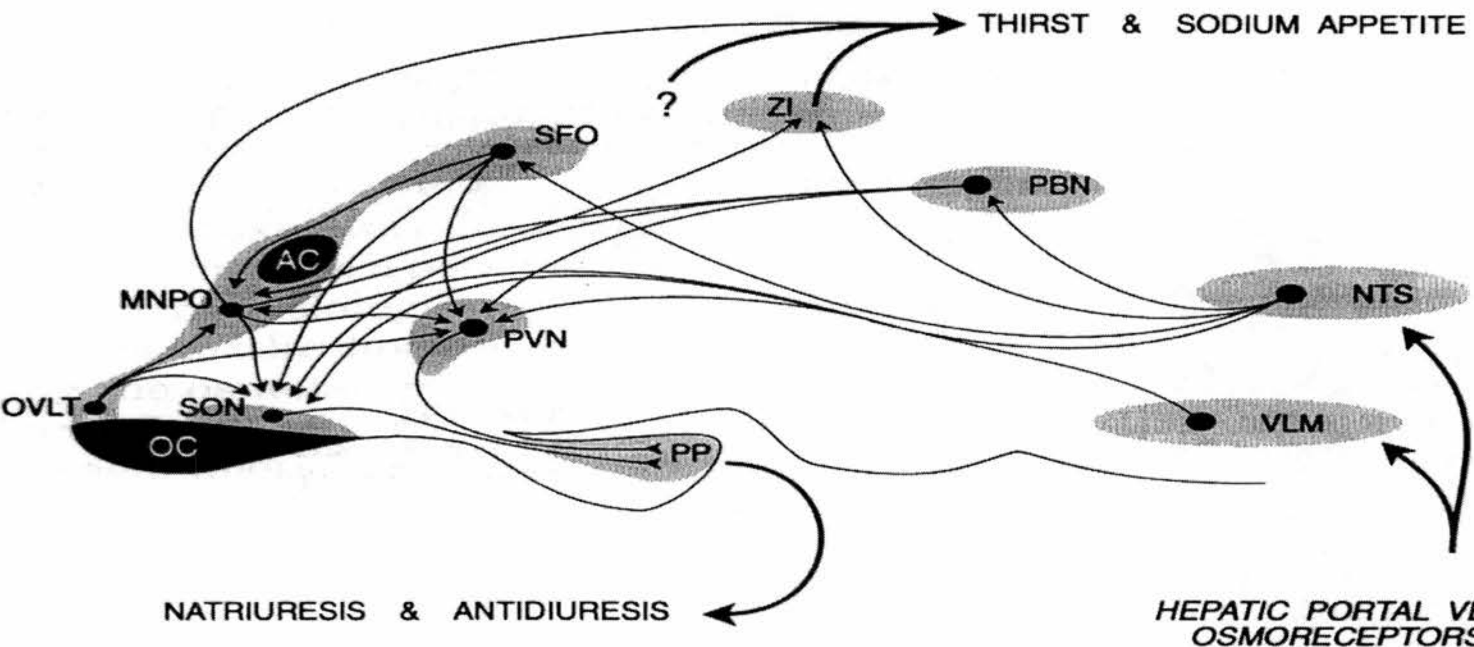

Figure 4. Schematic illustration of selected neural pathways subserving osmoregulation. Abbreviation: a.c., anterior commissure; MnPO, median preoptic nucleus; NTS, nucleus tractus solitarius; o.c., optic chiasm; OVLT, organum vasculosum lamina terminalis; PBN, parabrachial nucleus; PP, posterior pituitary; PVN, paraventricular nucleus; SFO, subfornical organ; SON, supraoptic nucleus; VLM, ventrolateral medulla; ZI, zona incerta. (From Bourque *et al.*, 1994)

1.4.3 Co-localization of neuroactive substances within the magnocellular neurosecretory system

Oxytocin and vasopressin are typically synthesized and secreted by separate magnocellular neurones (Mohr *et al.*, 1988; Vandesande *et al.*, 1975; Young and Lightman, 1992). Exceptionally, under strong stimulation, the expression of oxytocin and vasopressin and their mRNAs can be found to be co-localized in some magnocellular neurones of the SON and PVN (Kiyama and Emson, 1990; Trembleau *et al.*, 1993).

In the magnocellular neurones of the SON and PVN, not only oxytocin and vasopressin, but also a wide variety of messenger molecules have been found to co-localize. Vasopressin neurones primarily express tyrosine hydroxylase, galanin, dynorphin, angiotensin II, Leu-enkephalin and peptide histidine-isoleucine, and to a minor extent CCK, Met-enkephalin and thyrotropin-releasing hormone, whereas oxytocin neurones mainly contain CCK, Met-enkephalin and corticotropin-releasing factor (CRF), and to a minor extent galanin, dynorphin, Leu-enkephalin and thyrotropin-releasing hormone (Brownstein and Gainer, 1977; Brownstein and Mezey, 1986; Brownstein *et al.*, 1980; Meister *et al.*, 1990b; Mezey and Kiss, 1985; Sawchenko *et al.*, 1984; Vanderhaeghen *et al.*, 1981; Villar *et al.*, 1990). In addition, vasopressin has been found to co-localize with nitric oxide synthase (NOS) (Calka and Block, 1993b; Sanchez *et al.*, 1994; Torres *et al.*, 1993), and oxytocin also coexists with NOS (Miyagawa *et al.*, 1994; Sanchez *et al.*, 1994; Torres *et al.*, 1993) in the magnocellular neurones of the SON and PVN.

Regulation of coexisting peptide or mRNA levels within magnocellular neurones may be influenced by several systemic challenges known to target oxytocin and vasopressin synthesis and secretion (Lightman and Young, 1987; Meister *et al.*, 1990a; Sherman *et al.*, 1986). Salt-loading is the best challenge that up-regulates several mRNAs of co-localized peptides, including vasopressin (Herman *et al.*, 1991; Lightman and Young, 1987), oxytocin (Lightman and Young, 1987), dynorphin

(Lightman and Young, 1987; Sherman *et al.*, 1988), CCK (Sherman *et al.*, 1988), tyrosine hydroxylase (Young, III *et al.*, 1987), galanin (Meister *et al.*, 1990a) and nitric oxide synthase (Kadowaki *et al.*, 1994).

1.4.4 Electrophysiology of the magnocellular neurones

The stimulus-secretion coupling at the axon terminals of magnocellular neurones depends upon the electrical activity of these neurones. The amount of release correlates with the frequency and pattern of the action potentials (Bicknell and Leng, 1981; Renaud and Bourque, 1991).

Electrical stimuli applied to the neural stalk evoke action potentials in the axons of magnocellular neurones, which are propagated antidromically to the cell bodies (Leng, 1981). These antidromic action potentials have been used to identify putative neurosecretory neurones *in vivo* (Poulain and Wakerley, 1982; Renaud and Bourque, 1991). In the rat, the majority of both oxytocin and vasopressin neurones fire in a slow, irregular pattern under conditions of low demand for neurohypophysial hormones. One of two patterns of activity, either regular continuous firing or phasic bursting, in which silent pauses punctuate prolonged bursts, has been revealed under certain stimulation *in vivo* (Poulain and Wakerley, 1982). The former pattern is strongly associated with oxytocin neurones and the latter with vasopressin neurones. However, there have been exceptions reported, particularly in lactating rats, when the continuous firing pattern is periodically interrupted by a brief (<5 s) high-frequency bursts of activity immediately preceding periodic milk ejections (Lincoln and Wakerley, 1974). This profile is associated with oxytocin neurones only. Oxytocin and vasopressin neurones are best distinguished *in vivo* by their firing patterns following gastric, cardiovascular and suckling stimuli. The firing rate of vasopressin neurones is unaffected by suckling. In response to haemorrhage or osmotic stimulation, the firing rate of vasopressin cells is increased and eventually they adopt a phasic bursting pattern of activity, while oxytocin neurones display a continuously elevated firing rate under these conditions (Brimble and Dyball, 1977; Poulain *et al.*,

1977). The electrical activity of single neurones in the SON, identified antidromically as projecting to the posterior pituitary, can be identified as oxytocin neurones by virtue of their continuous firing pattern, and by their excitatory response to intravenously injected cholecystokinin (Renaud *et al.*, 1987) and identified as vasopressin neurones by their phasic firing pattern, and by their inhibitory response to intravenously injected phenylephrine (Harris, 1979).

In vitro, oxytocin and vasopressin neurones can be distinguished by their different responses to certain neurochemicals (or stimuli), and by the presence of phasic bursting mostly correlated with vasopressin neurones (Armstrong *et al.*, 1994; Cobbett *et al.*, 1986). The continuous firing pattern is often associated with both cell types (Andrew and Dudek, 1984; Armstrong *et al.*, 1994). The membrane properties of oxytocin and vasopressin neurones are generally similar *in vitro*. Depolarizing after-potentials (DAPs) are more often exhibited by, but are not exclusive to, vasopressin neurones (Armstrong, 1995). However, its presence is not a signature for cell type (Armstrong *et al.*, 1994). The summation of DAPs supports a plateau potential underlying the burst of phasic firing activity in magnocellular neurones (Andrew and Dudek, 1984). Furthermore, oxytocin and vasopressin neurones can be distinguished by their different membrane responses to sustained outward rectification (Stern and Armstrong, 1995). The sustained outward rectification is present only in oxytocin neurones.

1.4.5 Physiological and pharmacological regulation of neurohypophysial hormone release

1.4.5.1 Cholecystokinin

The polypeptide cholecystokinin (CCK), in particular the C-terminal octapeptide (CCK_{26-33} or CCK8), has a wide distribution throughout the brain and spinal cord as well as in the gut (Hokfelt *et al.*, 1991; Hokfelt *et al.*, 1988). CCK is expressed highly in the magnocellular neurosecretory system, and is co-localized

mainly with oxytocin and to a minor extent with vasopressin (Meister *et al.*, 1990b; Vanderhaeghen *et al.*, 1981). Systemic administration of CCK selectively induces oxytocin release into the circulation in a dose-dependent manner (Verbalis *et al.*, 1986a), while vasopressin release is unaffected or inhibited (Blackburn and Leng, 1990). This release is in accordance with a rapid but transient increase in the electrical activity of the oxytocin neurones and unresponsiveness or inhibition in the activity of the vasopressin neurones (Renaud *et al.*, 1987; Ueta *et al.*, 1993). These responses to systemic CCK have been used to identify and differentiate between the oxytocin and vasopressin neurones in *in vivo* electrophysiological studies. Oxytocin has also been found to be released within the SON in response to either systemic or central CCK administration (Neumann *et al.*, 1994).

Two distinct types of CCK receptor, CCK_A and CCK_B, are present in both the central nervous system and peripheral tissues (Hill *et al.*, 1987). CCK_A receptors are predominantly located in the periphery, but are also present in the area postrema and the NTS, whereas CCK_B receptors are found mainly in the brain (Crawley *et al.*, 1991; Menetrey *et al.*, 1984). Though CCK_B receptors are present in the SON and PVN (Gaudreau *et al.*, 1983), the excitation of oxytocin cells by systemic administration of CCK appears to be a consequence of peripheral actions, since CCK poorly penetrates the blood-brain barrier. Systemic administration of CCK has been shown to activate neuronal activity as determined by Fos protein expression in the area postrema and in sensory parts of the dorsal vagal complex (Monnikes *et al.*, 1997), catecholaminergic neurones in the A1 and A2 cell groups (Luckman, 1992; Luckman *et al.*, 1993; Rinaman *et al.*, 1995) and oxytocin neurones (Hamamura *et al.*, 1991; Verbalis *et al.*, 1991). The administration of a selective CCK_A antagonist, but not a selective CCK_B antagonist, abolishes CCK-induced excitation of oxytocin neurones (Luckman *et al.*, 1993). Administration of a CCK_A receptor antagonist, but not of a CCK_B antagonist, blocks oxytocin release following CCK, while it also prevents CCK-induced Fos protein expression in the SON, PVN, area postrema and dorsal vagal complex (Luckman *et al.*, 1993; Miller *et al.*, 1993). Thus, systemic

CCK acts on CCK_A receptors located either on gastric vagal afferent fibers, or in the area postrema.

Systemic CCK also induces noradrenaline release in the dorsal region of the SON where oxytocin neurones predominate (Kendrick *et al.*, 1991; Onaka *et al.*, 1995b). The CCK-induced oxytocin release is not abolished by destruction of the so-called AV3V region, which includes the OVLT and the MnPO (Blackburn and Leng, 1990). However, this response is abolished by gastric vagotomy (Verbalis *et al.*, 1986a) and attenuated by destruction of the area postrema (Carter and Lightman, 1987). Thus, the actions of systemically administered CCK on oxytocin neurones are mediated by vagal afferents or through the area postrema to the NTS.

Systemic administration of CCK, gastric distention from feeding, and nauseants such as lithium chloride, copper sulphate and apomorphine, all selectively release oxytocin in rats (McCann *et al.*, 1989; Renaud *et al.*, 1987; Verbalis *et al.*, 1986a; Verbalis *et al.*, 1986b) and produce a parallel inhibition of gastric motility and food intake (McCann *et al.*, 1989). These latter effects are not mediated by the peripheral release of oxytocin and may be relayed by the central oxytocinergic projection from the PVN to the dorsal motor nucleus of the vagus (Flanagan *et al.*, 1992a; Flanagan *et al.*, 1992b; Olson *et al.*, 1992).

1.4.5.2 Pregnancy and parturition

Oxytocin has a physiological role in both parturition and milk-ejection, or milk let-down, but there is little evidence for a major role of vasopressin in such conditions. The release of oxytocin is enhanced by two reflex mechanisms. The first is associated with tactile stimulation of the nipple during suckling, the *milk-ejection reflex*. The second is associated with stretch receptors in the lower genital tract, particularly the uterine cervix during the expulsion stage of parturition, the *Ferguson reflex* (see Leng *et al.*, 1999 for reviews).

Oxytocin gene expression in the SON and PVN is increased (Crowley *et al.*, 1993; Schriefer, 1991; Zingg and Lefebvre, 1988; Zingg and Lefebvre, 1989) or unchanged (Brooks, 1992; Douglas *et al.*, 1998; Spinolo *et al.*, 1992) during pregnancy, possibly reflecting the counteraction between inhibitory actions of progesterone and the stimulating action of relaxin.

During pregnancy, an up-regulation of $GABA_A$ subunit receptor mRNA expressed in oxytocin neurones of the SON and PVN has been revealed, followed by down-regulation on the day of parturition (Fenelon and Herbison, 1996). High concentrations of progesterone, secreted from the corpus luteum, have been found in early pregnancy and this persists until shortly before the end of pregnancy. Parturition is associated with a relative increase in circulating oestrogen levels against a background of falling levels of progesterone. Progesterone is essential for establishing and maintaining pregnancy in mammals, as progesterone receptor antagonist can induce preterm labour (for reviews see Garfield *et al.*, 1998b). One of its functions includes maintenance of uterine quiescence by decreasing uterine sensitivity to oxytocin (Burgess *et al.*, 1992). Furthermore, in ovariectomized rats, administration of progesterone inhibits oxytocin neurone electrical activity (Negoro *et al.*, 1973). It has been shown recently that progesterone inhibits oxytocin binding to oxytocin receptors *in vitro* (Grazzini *et al.*, 1998).

A peptide hormone relaxin, produced from the ovary, is present throughout the second half of pregnancy until term (Sherwood *et al.*, 1980) to prepare the birth canal for parturition and sensitizing the uterus to oxytocin. The plasma concentration of oxytocin is elevated and sustained throughout pregnancy (Douglas *et al.*, 1993), which may arise from the actions of ovarian relaxin upon the SFO and OVLT (Geddes and Summerlee, 1995; McKinley *et al.*, 1997). Systemic administration of relaxin increases the electrical activity of oxytocin and vasopressin neurones and increases oxytocin and vasopressin secretion (Parry *et al.*, 1994; Way and Leng, 1992), while ovariectomy during pregnancy decreases oxytocin secretion (Way *et al.*, 1993). However, these effects are either reduced or not observed in late-pregnant rats

(Parry *et al.*, 1994). The excitation of vasopressin neurones by relaxin results in antidiuresis causing plasma volume expansion and a decrease in plasma osmolality in order to satisfy the increased vascular needs of the developing placenta and foetus. These changes are maintained by a comparable reduction in osmotic thresholds for drinking and neurohypophysial hormone release (Barron *et al.*, 1985; Durr *et al.*, 1981; Koehler *et al.*, 1993). Despite this decrease in the set-point for osmoregulation and volume homeostasis, there is no change in sensitivity of the magnocellular neurosecretory system to osmotic stimulation in late pregnancy (Durr *et al.*, 1981; Koehler *et al.*, 1993). This new balance is reflected in the sustained hyponatremia and hypervolemia of pregnancy. However, a reduction of the osmoresponsiveness of the oxytocin system has also been reported, indicating no change in the osmotic threshold for oxytocin release in late pregnancy (Russell *et al.*, 1992).

Two distinct opioid mechanisms regulate oxytocin secretion (Russell *et al.*, 1993), while there is no significant action of either morphine treatment or of endogenous opioids on vasopressin secretion. Functional κ-and μ-receptors are present on the cell bodies and dendrites of oxytocin neurones, but only κ-receptors are present at the nerve terminals. Therefore, both μ- and κ-receptor agonists can act centrally to inhibit oxytocin neurones but only κ-receptor agonists (and not μ-agonists) can act at the secretory terminals (Bicknell *et al.*, 1988). Met-enkephalin, which is co-secreted with oxytocin, is a δ-receptor agonist, but extended forms of Met-enkephalin are active at neurohypophysial κ-receptors (Leng *et al.*, 1994). At the neural lobe, oxytocin secretion is restrained by co-secreted opioid peptides including dynorphin (released by both vasopressin and oxytocin terminals) and Met-enkephalin (released by oxytocin terminals), acting through κ-receptors (Leng *et al.*, 1994; Zhao *et al.*, 1988). This appears to be principally an auto-inhibitory mechanism. In mid-pregnancy, oxytocin secretion is powerfully restrained by this auto-inhibition (Douglas *et al.*, 1993; Russell *et al.*, 1995), which may contribute to the accumulation of oxytocin stores in the neural lobe in advance of parturition. By the end of pregnancy oxytocin nerve terminals become desensitized to endogenous opioid restraint, which may contribute to the high secretion of oxytocin during

parturition. At this time, a separate endogenous opioid system, acting upon μ-receptors, actively restrains the electrical activity of oxytocin neurones (Douglas *et al.*, 1995; Russell *et al.*, 1995). Oxytocin release within the supraoptic and paraventricular nuclei is elevated during parturition as well as during suckling, but not during late pregnancy. Intranuclear oxytocin release is unrestrained by endogenous opioids during parturition. This intranuclear release of oxytocin may act by local positive-feedback stimulation of oxytocin neurones to excite further oxytocin release in the brain and into blood during both parturition and lactation (Neumann *et al.*, 1993).

Systemic and central administration of morphine, an opioid agonist, delays the course of established parturition, with the delay accompanied by reduced plasma oxytocin levels and overcome by treatment either with naloxone, a non-selective opioid antagonist, or by infusion of oxytocin (Bicknell *et al.*, 1988). Evoked oxytocin release is potentiated by naloxone in late pregnancy, before and during parturition but not in virgin rats (Hartman *et al.*, 1986). The process of parturition is hastened by administration of naloxone. Thus, an endogenous opioid mechanism inhibiting oxytocin release appears to be active during late pregnancy, immediately before and during parturition. This mechanism does not operate earlier in pregnancy or during normal lactation and is not seen in virgin rats (Leng *et al.*, 1988).

On the last day of pregnancy, increased local production of prostaglandin by the uterus and decreased production of progesterone have been shown. The fall in progesterone removes both a restraining influence on the oxytocin neurones and an inhibitory effect on uterine tone, and also results in increased oxytocin receptor expression in the uterus. It is likely that local prostaglandin, but not oxytocin, is a physiological trigger for parturition. Mice lacking the gene encoding the receptor for prostaglandin F are unable to deliver normal fetuses at term (Sugimoto *et al.*, 1997). Parturition is initiated when prostaglandin interacts with the prostaglandin receptor in ovarian luteal cells to induce luteolysis. After corpus luteum collapse, a positive-feedback loop between the uterus and the hypothalamic oxytocin system is

established. Local prostaglandin production triggers uterine contractions, which excite the oxytocin neurones. The released oxytocin triggers further prostaglandin production and further uterine contraction (for reviews see Leng & Brown, 1997). Oxytocin is secreted in response to stretching of the uterine cervix and hastens expulsion of the fetus and the placenta. Oxytocin stimulates uterine contractions by action on the uterine oxytocin receptors. A dual action of oxytocin appears in the uterus, a uterotonic action on myometrium and a prostaglandin-releasing action on endometrial/decidual cells (Chan, 1980).

The expression of oxytocin receptors has been demonstrated in the uterus, mammary gland, pituitary and brain including the ventromedial hypothalamus, the bed nucleus of the stria terminalis, the ventral pallidum, the paraventricular nucleus, and the dorsal part of the supraoptic nucleus (Adan *et al.*, 1995). A marked increase of oxytocin receptor density is found in the endothelium and myometrium at term pregnancy and reaches a maximum during labour (Alexandrova and Soloff, 1980; Fuchs *et al.*, 1982). The level of oxytocin receptor mRNA at the cervix also increases at term with a further marked increase at parturition (Fuchs *et al.*, 1996). An increased sensitivity of the myometrium to oxytocin has been established at term that could be the result of both up-regulation of oxytocin receptors and an increase in the fraction of receptors coupled to signal transduction components (Strakova and Soloff, 1997).

Oxytocin is important for the normal progress of parturition to increase the contractility of the uterus near term, although observations of transgenic mice lacking oxytocin have demonstrated that parturition can proceed in the absence of oxytocin (Nishimori *et al.*, 1996; Young, III *et al.*, 1996). During parturition, as during suckling, bursts of activity of oxytocin neurones becomes highly synchronized, and these bursts are correlated with abdominal contractions and pup delivery in conscious rats (Summerlee, 1981). The pulsatile release of oxytocin from the pituitary gland is a consequence of bursting activity of oxytocin neurones that is important for advancing the progress of parturition. Background secretion of oxytocin is also increased during

the expulsive phase (Higuchi *et al.*, 1985; Higuchi *et al.*, 1986a). Oxytocin is also released within the SON and PVN during parturition, which may act by local positive feedback stimulation of oxytocin neurones to excite further oxytocin release in the brain and into blood (Neumann *et al.*, 1993).

The process of parturition appears to involve the contractions of the uterus induced by oxytocin release, causing movement of fetuses along the uterus to the cervix, and also the contractions of abdominal muscles to expel the foetus from the birth canal, which requires the integrity of the pelvic nerves. An increase of oxytocin secretion from the neurohypophysis during parturition is probably in response to afferent inputs arising from the uterus and cervix that are carried from the brainstem in part by noradrenergic fibres from the nucleus tractus solitarii (NTS) (Antonijevic *et al.*, 1995; Menetrey and Basbaum, 1987; Menetrey *et al.*, 1983), some of which project directly to the oxytocin neurones. They are also activated by uterine contractions, not just by the distention of the birth canal that results from fetal expulsion (Douglas *et al.*, 1995). Destruction of the pelvic nerve which projects to the NTS or the NTS itself is associated with a prolonged duration of labour (Higuchi *et al.*, 1986b; Higuchi *et al.*, 1987).

1.4.5.3 The milk-ejection reflex

Oxytocin is essential for suckling-induced milk-let down in lactation. In the absence of oxytocin, particularly in transgenic 'oxytocin-knockout' mice, milk ejection does not occur (Nishimori *et al.*, 1996; Young, III *et al.*, 1996). Oxytocin (and vasopressin) mRNA expression is increased in the SON during lactation (Zingg and Lefebvre, 1988), and the plasma concentrations of oxytocin, induced by suckling, become higher than normal (Higuchi *et al.*, 1985; Meyer *et al.*, 1987). Oxytocin released from the neurohypophysis acts at oxytocin receptors on the myoepithelium which surround the alveoli to promote contractions in the mammary gland causing milk let-down.

In the lactating rat, suckling evokes periodic bursting activity (high frequency bursts of spikes) in the oxytocin neurones at intervals of 1-10 min (Wakerley and Lincoln, 1973). These bursting activities occur synchronously in most or all oxytocin neurones of the SON and PVN (Belin and Moos, 1986; Belin *et al.*, 1984), leading to the release of a bolus of oxytocin into the circulation that is large enough to produce contraction of the mammary myoepithelial cells and to cause milk ejection.

Central administration of oxytocin enhances the frequency of occurrence and the amplitude of bursts (Freund-Mercier and Richard, 1984) whereas an oxytocin antagonist has the opposite effect (Lambert *et al.*, 1993). Moreover, local release of oxytocin has been reported to occur within the PVN and SON during the milk-ejection reflex (Landgraf *et al.*, 1992; Moos *et al.*, 1989a). It has thus been speculated that oxytocin release within the hypothalamus is necessary for the bursting activity of oxytocin neurones and their excitability, which exerts a facilitatory role and provides a positive feedback signal to promote bursting of the whole oxytocin neurone population (Freund-Mercier and Richard, 1984; Freund-Mercier and Richard, 1981; Lambert *et al.*, 1993). These bursting activities are accompanied by structural plasticity of exclusively the oxytocin neurones involving a further increase in membrane apposition and increased numbers of double synapses which may contribute to the synchronized electrical activity in oxytocin neurones (Hatton and Tweedle, 1982; Theodosis *et al.*, 1986). An enlargement of oxytocin neurone nucleoli in the PVN during lactation has also been reported (Russell, 1983).

As well as the magnocellular nuclei, certain limbic structures, including in particular the bed nucleus of the stria terminalis and the ventrolateral septum, may play an important part in the central action of oxytocin on the milk-ejection reflex. In lactating rats, oxytocin excites neurones in these structures *in vitro* (Ingram *et al.*, 1990; Ingram and Moos, 1992) and also facilitates the bursting activity of oxytocin neurones (Moos *et al.*, 1991; Moos and Richard, 1989b).

1.4.5.4 Osmoregulation

Vasopressin plays a critical role in maintaining body fluid and electrolyte balance by promoting water reabsorption by the kidney (Abramow *et al.*, 1987). In the rat, however, oxytocin also contributes to sodium balance and mediates natriuresis (Conrad *et al.*, 1993; Verbalis *et al.*, 1991). Indeed, the release of oxytocin and vasopressin is regulated by changes in plasma osmolality (Dunn *et al.*, 1973; Negoro *et al.*, 1988; Verbalis *et al.*, 1986). Therefore, the concerted osmotic control of vasopressin and oxytocin secretion represents a powerful osmoregulatory response in the rat to regulate body fluid balance and counteract ionic disturbance in rats through the effects of diuresis and natriuresis (Bourque *et al.*, 1994).

At the physiological osmotic set-point, approximately 295 mosm/kg in rats (Dunn *et al.*, 1973), both vasopressin and oxytocin neurones tend to display slow (2-3 Hz) irregular firing (Poulain and Wakerley, 1982), consistent with the presence of low, but detectable, plasma concentrations of vasopressin and oxytocin. Under resting conditions, these low levels of vasopressin and oxytocin in the blood are sufficient to contribute to basal antidiuresis (Conrad *et al.*, 1993; Verbalis *et al.*, 1991) and natriuresis (Dunn *et al.*, 1973), respectively. A reduction in plasma osmolality by systemic administration of water or hypotonic saline inhibits magnocellular neurones (Brimble and Dyball, 1977) and suppresses oxytocin and vasopressin release (Rosella-Dampman *et al.*, 1987; Stricker and Verbalis, 1986), which promotes compensatory sodium retention and water excretion. Elevation in plasma osmolality without changes in blood volume by systemic hypertonicity, in contrast, dramatically increases the firing rate and eventually induces a phasic bursting pattern of activity of putative vasopressin neurones (Poulain *et al.*, 1977), and also increases the firing rate of oxytocin neurones which in these cases fire continuously rather than in bursts (Brimble and Dyball, 1977; Poulain *et al.*, 1977). Systemic administration of hypertonic saline also enhances the release of oxytocin and vasopressin (Brimble and Dyball, 1977; Dunn *et al.*, 1973; Landgraf *et al.*, 1988), which promotes sodium excretion and water retention. In addition, there is

enhanced release of oxytocin and vasopressin within the SON following systemic administration of hypertonic saline (Ludwig *et al.*, 1994a). Direct administration of hypertonic saline into the SON or PVN *via* a microdialysis probe evokes oxytocin and vasopressin release within the SON and PVN (Hattori *et al.*, 1990; Landgraf and Ludwig, 1991; Ludwig *et al.*, 1994b) as well as into the blood (Hattori *et al.*, 1990). In addition, dehydration produced by salt-loading (giving a 2% sodium chloride in place of drinking water) or water deprivation results in an increased plasma sodium concentration or osmolality which, in turn, elevates plasma oxytocin and vasopressin concentrations (Balment *et al.*, 1980; Jones and Pickering, 1969; Van Tol *et al.*, 1987) and depletes the neural lobe (George, 1976; Jones and Pickering, 1969; Van Tol *et al.*, 1987). Since substitution of hypertonic saline with small permeant molecules such as mannitol causes a similar activation without any change in extracellular sodium, oxytocin and vasopressin neurones are activated in response to an increase in plasma osmolality rather than an increase in plasma sodium concentration (Brimble *et al.*, 1978).

The osmosensitive sites are located centrally and possibly also peripherally to regulate sodium and water balance in a manner that maintains the osmotic pressure of the extracellular fluid near an ideal set-point (Bourque *et al.*, 1994). Peripheral osmoreceptors may be located in the region of the hepatic portal vein, a strategic site for early detection of systemic osmolality changes from ingested foods and fluids. Osmosensitive information derived from peripheral osmoreceptors is transmitted to the central regions that participate in the control of osmoregulatory reflexes (Fig. 4), the NTS and the VLM, *via* the hepatic branch of the vagus nerve (King and Baertschi, 1992; Kobashi *et al.*, 1993). The NTS and VLM send direct projections to magnocellular neurones of the SON and PVN (Day *et al.*, 1984; Raby and Renaud, 1989). The osmosensitive information appears to be relayed to a number of rostral structures including the MnPO (Saper and Levisohn, 1983), the zona incerta and the parabrachial nucleus (Kobashi *et al.*, 1993; Yamashita *et al.*, 1980).

Changes in systemic osmolality are detected centrally within forebrain structures associated with the lamina terminalis including the OVLT, MnPO and SFO (McKinley *et al.*, 1996). Alterations in plasma tonicity or in the concentrations of blood-borne hormones which play a role in blood volume and electrolyte homeostasis, including angiotensin II, atrial natriuretic peptide and relaxin can be detected by neurones in the SFO and OVLT (McKinley *et al.*, 1996). Neurones in the SFO and OVLT possess many binding sites for angiotensin II (McKinley *et al.*, 1990). The AT_1 subtype of the angiotensin receptor is the specific membrane receptor for angiotensin II in the SFO and OVLT (Lenkei *et al.*, 1997). AT1 receptors are directly involved in the osmotically induced release of vasopressin (Hogarty *et al.*, 1994). Neurones in the SFO that project to the SON and PVN are activated, as identified by Fos expression, after systemic osmotic stimulation (Oldfield *et al.*, 1994). Thus, angiotensin II, generated both peripherally and centrally, may play an important role in the osmotic control of vasopressin release by acting at the SFO and AV3V.

Neurones in the SFO, MnPO, OVLT are also activated, as identified by Fos expression, after systemic osmotic stimulation (Oldfield *et al.*, 1994; Oldfield *et al.*, 1991a) and water deprivation (McKinley *et al.*, 1994). Some of the activated neurones, particularly in the OVLT, project to magnocellular neurones in the SON (McKinley *et al.*, 1994; Oldfield *et al.*, 1994). In addition, destruction of the anteroventral third ventricle region (AV3V) region, which includes the OVLT and MnPO, attenuates the increase in plasma oxytocin concentration in response to hypertonic injection (Blackburn *et al.*, 1987), produces deficits in osmotically regulated vasopressin release (Chiaraviglio and Perez, 1984), disturbs the ability to excrete sodium during water deprivation (Bealer *et al.*, 1983b), attenuates natriuresis response to hypertonicity (Bealer, 1983a; McKinley *et al.*, 1992), disrupts drinking response to systemic administration of angiotensin and hypertonic saline (Lind and Johnson, 1982), and produces deficits in the pressor responses to angiotensin II, leading to progressive accumulation of sodium (Bealer, 1982; Hartle and Brody, 1984), suggesting that osmotic control of most osmoregulatory responses is achieved

through central pathways originating from neurones located within these structures, particularly the OVLT. Furthermore, it has been suggested that some neurones of the OVLT are intrinsically osmoresponsive. Increase in the osmolality of the superfusion media by addition of NaCl or mannitol prompts a depolarization of OVLT neurones that project to the SON *in vitro* (Nissen *et al.*, 1993) and also increases the firing frequency of neurones in the OVLT (Vivas *et al.*, 1990). In addition, local hypertonic stimulation of the OVLT excites magnocellular neurones in the SON and PVN *in vivo* (Honda *et al.*, 1987). From intracellular recording experiments, hyperosmotic stimulation of the OVLT *in vitro* has been shown to evoke an increase in the frequency of spontaneous excitatory postsynaptic potentials (EPSPs) and in the firing rate of neurones in the SON (Richard and Bourque, 1992; Richard and Bourque, 1995). Therefore, it is likely that osmoresponsive neurones located within the OVLT that project to the magnocellular system are essential for osmoregulation of oxytocin and vasopressin release.

The SON and PVN lie inside the blood-brain barrier, but are densely vascularized with a large capillary surface area. In these areas, changes of osmotic pressure of the extracellular fluid in these regions closely match that of the plasma. Oxytocin and vasopressin neurones respond directly to changes in osmotic pressure (Mason, 1980; Oliet and Bourque, 1993). Both continuous-firing and phasic-firing neurones in the SON are activated in response to application of hypertonic saline to the immediate neighbourhood of neurones in the SON by microtap *in vivo* (Leng, 1980). In addition, intracellular recordings of neurones in the SON *in vitro* have shown that hypertonic solutions cause direct membrane depolarization (Abe and Ogata, 1982; Bourque, 1989; Mason, 1980). These intrinsic responses result from changes in the activity of mechanosensitive cation channels (Oliet and Bourque, 1993; Oliet and Bourque, 1996). These channels are inactivated when the cell membrane is stretched due to cell swelling caused by hypotonicity, thereby hyperpolarizing the neurone. In contrast, hyperosmotic stimulation causes cell shrinking, resulting in an enhancement of a voltage-independent inward cationic current, and provokes membrane depolarization.

Indeed, intrinsically generated changes in membrane potential in magnocellular neurones may be subthreshold for spike discharge during osmotic stimulation (Bourque *et al.*, 1994). An increase in firing rate is provoked by osmotic stimulation of the OVLT from a selective modulation in the frequency of glutamatergic EPSPs (Richard and Bourque, 1995). In addition, an acute electrolytic lesion of the AV3V silences all neurones in the SON. Neurones are not spontaneously active and are not excited by hypertonic stimuli, however, an increase of neuronal activity in response to hypertonic stimuli is restored by local application of glutamate (Leng *et al.*, 1989; Leng *et al.*, 1988; Russell *et al.*, 1988). It is likely that the intrinsic osmosensitivity of magnocellular neurones combines with osmotically regulated excitatory synaptic drive, derived from osmosensitive neurones in the OVLT, to modulate the firing rate of magnocellular neurones, which in the rat, contributes to the regulation of natriuresis and diuresis through effects of oxytocin and vasopressin (Bourque *et al.*, 1994). Therefore, the sensitivity of the neurohypophysial responses to osmotic stimuli *in vivo* requires concurrent activation of intrinsic and extrinsic mechanisms in osmoregulation.

1.5 Distribution of neuronal nitric oxide synthase

Nitric oxide is a highly reactive and readily diffusible substance, and it has proved difficult to localize NO in tissue and measure NO formation directly (Bredt & Snyder, 1992). The sites of action of NO in brain tissue are therefore preferably localized by the identification of the synthesizing enzyme NOS, by using NADPH-diaphorase histochemistry, immunohistochemistry with antibodies to nNOS, and/or *in situ* hybridization with antisense probes complementary to nNOS mRNA (Beesley, 1995; Bredt *et al.*, 1991a; Bredt *et al.*, 1990; Dawson *et al.*, 1991; Vincent and Kimura, 1992). These methods detect an enzyme associated with the NOS molecule, the NOS molecule and NOS mRNA, respectively.

The NADPH-diaphorase histochemical technique is based on the presence of an enzyme that can catalyze the NADPH-dependent conversion of a soluble tetrazolium salt, the best known is nitroblue tetrazolium, to an insoluble, visible

formazan. This establishes that NOS catalytic activity, which requires NADPH, is responsible for the NADPH-diaphorase histochemical activity. The distribution of NOS neurones appears identical to that of neurones staining for NADPH-diaphorase (Bredt *et al.*, 1991a; Dawson *et al.*, 1991), and purified brain NOS has NADPH-diaphorase activity (Hope *et al.*, 1991). The NADPH-diaphorase staining is found in the soma, in the cytoplasm but not in the nucleus, in the dendrites, axons and terminal fields. The NADPH-diaphorase localizes in the nuclear envelope, inner and outer membranes of mitochondria, Golgi apparatus, and endoplasmic reticulum in both oxytocin and vasopressin neurones in the SON and astrocytes (Calka *et al.*, 1994).

NADPH-diaphorase containing neurones are distributed throughout the brain, particularly in the olfactory bulb, the basal forebrain, the medulla, the hypothalamus, the striatum, the hippocampus, the cerebellum, the visual system and the cortex (Bruhwyler *et al.*, 1993; Vincent and Kimura, 1992). In the hypothalamus NADPH-diaphorase staining has been found, especially in the SON and PVN, the OVLT, the preoptic nucleus, the lateral hypothalamic area, the ventromedial and dorsomedial nuclei, the arcuate nucleus and various parts of the mammillary region (Vincent and Kimura, 1992; Yamada *et al.*, 1996). Neuronal NOS (nNOS) is the major nitric oxide synthase isoform in the hypothalamus (Bhat *et al.*, 1996).

High concentrations of nNOS, nNOS mRNA and the electron donor for the enzyme reaction, NADPH are present in the magnocellular neurones in the SON and PVN as well as the posterior pituitary gland (Bredt *et al.*, 1990; Pasqualotto *et al.*, 1991; Pow, 1992; Sagar and Ferriero, 1987; Vincent and Kimura, 1992). The posterior pituitary receives a widely distributed NO innervation, which originates in the magnocellular neurosecretory system (Vanhatalo and Soinila, 1995).

It has been shown that nNOS and NADPH-diaphorase are co-localized with neurotransmitters and the messenger molecules in the SON and PVN. Co-localization of NADPH-diaphorase with vasopressin (Calka and Block, 1993b;

Sanchez *et al.*, 1994; Torres *et al.*, 1993), oxytocin (Miyagawa *et al.*, 1994; Sanchez *et al.*, 1994; Torres *et al.*, 1993), somatostatin (Alonso *et al.*, 1992), angiotensin II (Calka and Block, 1993a), dynorphin (Murakami, 1994), CRF (Siaud *et al.*, 1994) and Met-enkephalin (Murakami, 1994) in the neurosecretory nuclei has been reported. Neuronal NOS protein also coexists with oxytocin, CCK and galanin in the SON, and with enkephalin, oxytocin and CRF in the PVN (Yamada *et al.*, 1996).

1.6. Role of NO in biological functions integrated within the magnocellular neurosecretory system

1.6.1 The role of NO in the regulation of neurohypophysial hormone secretion

The hypothesis that NO may play a role in osmoregulation has been led by the study of Sagar and Ferriero in 1987 (Sagar and Ferriero, 1987). They found an increase in NADPH-diaphorase staining in rat posterior pituitary gland after 8-days of salt loading. Up-regulation of NOS mRNA expression has also been found in the PVN and SON after chronic salt loading (Kadowaki *et al.*, 1994; Villar *et al.*, 1994b). Dehydrated rats also show an increase of NADPH-diaphorase activity and NOS mRNA in both the PVN and SON (O'Shea and Gundlach, 1996; Pow, 1992; Ueta *et al.*, 1995b). Furthermore, an involvement of NO under chronic hyperosmotic stimulation is suggested in studies in mice and rats with inherited diabetes insipidus. Homozygous (*di/di*) mice have nephrogenic diabetes insipidus and do not respond to vasopressin that is secreted in increased amounts, while homozygous Brattleboro (*di/di*) rats have hypothalamic diabetes insipidus and a deficiency in the secretion of vasopressin (Valtin *et al.*, 1965). An increase in the amount of nNOS protein in the PVN and SON but not in the OVLT, MnPO and SFO of female homozygous (*di/di*) mice and homozygous Brattleboro (*di/di*) rats has been reported (Wang and Morris, 1996). In addition, up-regulation of nNOS mRNA expression in the SON and PVN has recently been reported in female homozygous Brattleboro (*di/di*) rats (Yamamoto *et al.*, 1997). After hypophysectomy, up-regulation of NOS mRNA expression has been revealed in magnocellular neurones in the SON and PVN (Villar *et al.*, 1994a).

Furthermore, NO appears to be involved in the plasticity of ultrastructural changes in the neurohypophysis in response to hypertonic saline, since the contact between the basal lamina and the membrane of axon terminals in neurohypophysis of rats injected with the NOS inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME) and hypertonic saline was less than those of rats that received hypertonic saline alone. Rats receiving both L-NAME and hypertonic saline also had more axonal profiles enclosed within neurohypophysial astrocytes than rats received hypertonic saline alone (Beagley and Cobbett, 1997). These anatomical findings are complemented by functional studies. Increases in NO-forming activity in the posterior pituitary have been reported after chronic osmotic stimulation (Kadowaki *et al.*, 1994; Pow, 1994).

The NOS system in the PVN and SON can also be modulated by non-osmotic-stimuli, such as hypotension, haemorrhage and hypovolemia. Isotonic hypovolemia, induced by polyethylene glycol (PEG) without changes of plasma [Na^+], resulting in increased nNOS mRNA expression in the SON and PVN (Ueta *et al.*, 1998), accompanied by dense staining for NADPH-diaphorase in the PVN and SON. Increased expression of Fos protein in NADPH-diaphorase-positive cells has been reported in the SON and the magnocellular and the medial parvocellular parts in the PVN after PEG-induced hypovolemia (Ueta *et al.*, 1998). In addition, Fos expression in NADPH-diaphorase positive neurones in the SON and PVN is increased in hypotensive conscious rats following haemorrhage and sodium nitroprusside infusion (Petrov *et al.*, 1995). Furthermore, nNOS mRNA expression is increased in the caudal ventrolateral medulla, dorsal medulla, and hypothalamus, primarily in the PVN (both parvo- and magnocellular divisions) and SON of spontaneously hypertensive rats (Plochocka-Zulinska and Krukoff, 1997).

It has been demonstrated that central blockade of NOS by i.c.v. administration of the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA) to rats deprived of water for 24 h increased the plasma concentration of oxytocin, but not vasopressin (Summy-Long *et al.*, 1993), and attenuated drinking and decreased glucose utilization in the SFO and MnPO (Kadekaro *et al.*, 1994). In conscious rats,

central administration of L-NAME further enhanced oxytocin release, but not vasopressin, in response to a painful stimulus (needle prick), isotonic saline, and moderate, but not strong, hypertonic stimulation (Kadekaro *et al.*, 1997). Central administration of either the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) or the NO precursor L-arginine increased the basal release of vasopressin in conscious rats (Ota *et al.*, 1993). Intracerebroventricular administration of L-NAME increased plasma concentration of vasopressin and oxytocin in normovolaemic rats, and further enhanced increased oxytocin release, but not vasopressin, in response to hemorrhage (Kadekaro *et al.*, 1998). Central administration of L-NAME potentiated basal release of oxytocin and vasopressin in conscious rats. In rats i.c.v. administered with angiotensin II and L-NAME, plasma concentration of oxytocin, but not vasopressin, was significantly greater than in rats that received either angiotensin II or L-NAME alone. Furthermore, Angiotensin II-induced water intake was attenuated by L-NAME (Liu *et al.*, 1997). In conscious rabbits, the basal release of vasopressin was increased by i.v. infusion of either L-NAME or sodium nitroprusside (SNP). Infusion of L-NAME did not alter increased vasopressin release in response to i.v. infusion of either hypertonic saline or SNP (Goyer *et al.*, 1994). In pentobarbital anaesthetised rats, the basal release of vasopressin was increased after i.c.v. injection of L-arginine and was decreased after i.c.v. injection of L-NAME. Central administration of L-NAME also reduced vasopressin release in response to hypotension induced by SNP (Cao *et al.*, 1996). Systemic administration of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) enhanced oxytocin and vasopressin release in salt-loaded rats, but not in normallly hydrated rats (Kadowaki *et al.*, 1994). It is clear that NO has an inhibitory effect on the release of oxytocin, whereas the effect of NO on vasopressin release remains controversial.

In *in vitro* studies, KCl-evoked vasopressin release from rat hypothalamic explants comprising the PVN and SON was attenuated by L-arginine and the NO donors, sydnonimine-1 (SIN-1) and SNP. The effect of L-arginine was reversed by the presence of L-NMMA, and reduced by the NO scavenger hemoglobin (Yasin *et al.*, 1993). A transient increase of basal release of vasopressin from posterior

pituitary explants was found after incubation of the glands with L-NAME and hemoglobin. L-arginine (but not D-arginine) reduced the stimulatory influence of L-NAME on both vasopressin and oxytocin release (Lutz-Bucher and Koch, 1994). Furthermore, SIN-1 also reduced KCl-stimulated vasopressin release. Nitric oxide may regulate the release of oxytocin and vasopressin by acting on either the cell bodies in the SON and PVN or their nerve terminals in the neurohypophysis.

In *in vitro* electrophysiological studies, an inhibitory effect of NO on neuronal activity of both phasic and non-phasic neurones in the rat SON has been shown, as SNP and L-arginine inhibited neuronal activity, whereas L-NAME and hemoglobin enhanced neuronal activity of the SON neurones (Liu *et al.*, 1997). Nitric oxide may control SON neurons through NMDA receptors *via* a cGMP-independent pathway, since the NO donors SNP and isosorbide dinitrate suppressed NMDA-evoked depolarization, whereas the NO scavenger hemoglobin and the NOS inhibitor L-NNA enhanced NMDA-evoked depolarization. Furthermore, L-NNA also attenuated an NMDA-driven increase in intracellular concentration of cGMP (Cui *et al.*, 1994).

It has been recently found that NO preferentially potentiates GABAergic synaptic inputs into SON neurones without influencing glutamatergic inputs *via* cGMP-independent mechanisms, since application of SNAP increased the frequency, but not the amplitude, of spontaneous inhibitory postsynaptic currents (IPSPs) recorded from the SON neurones, representing presynaptic GABA release, while SNAP had no effect on excitatory postsynaptic currents (EPSPs), representing presynaptic glutamate release (Ozaki *et al.*, 1999). Hemoglobin and L-NNA did not alter spontaneous IPSPs. The SNAP-evoked IPSPs were reduced by hemoglobin, indicating that the effect of SNAP was mediated through NO. Furthermore, application of a membrane-permeant analog of cGMP, 8-bromo cGMP, inhibited both the amplitude and the frequency of the IPSPs and EPSPs, suggesting that the action of NO on IPSCs in the SON may be mediated *via* cGMP-independent mechanisms. Hence, NO increases GABA release by acting at presynaptic terminals of GABAergic neurones rather than by a direct effect on SON neurones. In the rat

PVN magnocellular neurones, spontaneous IPSPs were evoked by SNAP, bubbled NO solution or L-arginine while spontaneous IPSPs were not altered by the NOS inhibitor L-NAME. However, NMDA-evoked IPSPs were attenuated by either L-NAME or by $GABA_A$ receptor antagonists, indicating that NO potentiated GABA inputs *via* postsynaptic as well as presynaptic mechanisms (Bains and Ferguson, 1997b). Furthermore, parvocellular neurones, but not magnocellular neurones, in the PVN are depolarized by bath application of NO, SNAP and L-arginine, and also by bath application of a membrane-permeant cGMP analogue, 8-bromo-cGMP (Bains and Ferguson, 1997a). Taken together, NO may exert a predominantly inhibitory action on SON and PVN neurones *via* presynaptic as well as postsynaptic mechanisms.

In the SFO neurones, an inhibitory effect of NO on neuronal activity has been found to be mediated by cGMP (Rauch *et al.*, 1997). Nitric oxide derived from SNP inhibited the spontaneous electrical activity of the SFO neurones. Application of 8-Bromo-cGMP mimicked the inhibitory effect of SNP. Furthermore, firing rate of SNP-sensitive neurones in the SFO was suppressed by L-arginine and increased by L-NMMA. It has been reported recently that that hyperosmotic stimuli directly influence glutamatergic excitatory synaptic inputs to the neurones in the SON *in vitro* by an action on the presynaptic terminals, as the frequency of the spontaneous EPSPs induced by application of hypertonic saline was almost completely blocked by both a non- N-methyl-D-aspatate (NMDA) antagonist and a non-selective glutamate antagonist, but not altered by an NMDA antagonist (Inenaga *et al.*, 1997).

1.6.2 Involvement of NO in pregnancy and parturition

In late-pregnant and parturient rats, but not in mid-pregnant rats, a decrease of the density and number of NADPH-diaphorase-positive cells in the SON and PVN and a reduction in the NOS enzyme activity in the posterior pituitary has been reported (Okere and Higuchi, 1996a). By contrast, an increase in the number of cells showing dense staining for NADPH-diaphorase increase in the SON and PVN has

been demonstrated in mid-pregnant (Popeski *et al.*, 1999) and in late-pregnant rats (Popeski *et al.*, 1999; Woodside and Amir, 1996). Other authors have reported an increase in NOS protein and NOS mRNA expression in the hypothalamus obtained from 20 day pregnant rats, assessed by western blot and northern blot, respectively (Xu *et al.*, 1996). However, an increase in NOS mRNA expression in the SON, the PVN and the lamina terminalis has not been revealed in 15 day pregnant rats (Luckman *et al.*, 1997). Central administration of a NO donor in parturient rats significantly delays the progress of parturition and inhibits the expression of maternal behaviour (Okere *et al.*, 1996b), which can be restored by central administration of oxytocin. Systemic administration of a NO donor prolongs the duration of parturition probably by interfering with foetal expulsion, while a NOS inhibitor has no effect (Okere *et al.*, 1996b).

Down-regulation of the NOS system in the brain in late pregnancy and during parturition is consistent with observed changes in the uterine NOS system (Natuzzi *et al.*, 1993; Sladek *et al.*, 1993; Yallampalli *et al.*, 1994; Yallampalli *et al.*, 1993). Staining for NADPH-diaphorase present in multiple uterine structures increases during pregnancy and decreases during parturition (Natuzzi *et al.*, 1993). Uterus NOS activity decreases in parturient rats (Natuzzi *et al.*, 1993) and rabbit (Sladek *et al.*, 1993). In addition, the uterus posses an L-arginine-NO relaxation system, which inhibits contractility during pregnancy but not during labour. During pregnancy, but not during labour, NO generated from the uterus inhibits the spontaneous contractility of the uterus, causing substantial relaxation, which can be reversed by administration of a NOS inhibitor and soluble guanylate cyclase (Yallampalli *et al.*, 1993). In women in pre-term labour, a NO donor glyceryl trinitrate continuously delivered by transdermal patches is effective in suppression of uterine contractions and in prolonging gestation (Lees *et al.*, 1994). Furthermore, uterine NO and cGMP formation are low in virgin rats, substantially increased during pregnancy, and decreased during labour and immediately *postpartum*. The uterine production of NO and cGMP is inhibited by oestrogen (Yallampalli *et al.*, 1994). The NO-cGMP system also exists in the human uterus, NO produced locally inhibits spontaneous

contractility of the uterus. The relaxation responsiveness to NO is elevated during pregnancy and decreased during labour (Buhimschi *et al.*, 1995). Thus, the uterine NOS system is up-regulated during pregnancy to maintain uterine contractile quiescence, and down-regulated in late pregnancy and during parturition. A rise in estrogen at term is thought to inhibit NO and cGMP generation from this system, increase uterine contractility and facilitate the initiation of labour. In contrast to findings of Yallampalli and colleagues, an increase in the calcium-dependent NOS activity (nNOS and eNOS) measured indirectly by citrulline production has been found in various tissues following treatment with oestrogen, but not progesterone, in pregnant guinea pigs (Weiner *et al.*, 1994), which suggests that the changes in NOS expression and activity are mediated by oestrogen. In addition, NADPH-diaphorase staining in the SON and PVN is increased following chronic oxytocin infusion in oestrogen-implanted rats and by manipulation of ovarian steroid hormones involving administration of chronic oestrogen and progesterone, which followed by progesterone withdrawal to mimick the hormonal milieu of late pregnancy in ovariectomized rats (Popeski *et al.*, 1999).

1.6.3 Involvement of NO in lactation

During lactation, nNOS mRNA expression is increased in both magnocellular and parvocellular neurones in the PVN (Ceccatelli and Eriksson, 1993). The number of cells that show dense staining for NADPH-diaphorase also increases in the SON and PVN (Popeski *et al.*, 1999). The increase in NOS mRNA expression appears to occur mainly in oxytocin neurones rather than vasopressin neurones as shown by double *in situ* hybridization (Luckman *et al.*, 1997). In addition, NOS mRNA expression is increased in the SFO, MnPO and OVLT (Luckman *et al.*, 1997). Central administration of a NO donor but not a NOS inhibitor causes a disruption of milk transfer from lactating rats to their pups after a 12 h period of separation (Okere *et al.*, 1996). *In vivo* electrophysiological studies have reported that central administration of a NO donor or L-arginine disrupts, whereas a NOS inhibitor permits, the milk-ejection associated bursts of oxytocin neurones, as well as the

magnitude of milk-ejection bursts, without altering mammary gland sensitivity. Systemic administration of a NO donor inhibits the contraction of the mammary gland myoepithelial cells, since NO abolishes intramammary pressure in response to oxytocin or electrical stimulation of the neurohypophysial stalk in anaesthetized lactating rats (Okere *et al.*, 1996). In conscious lactating rats, central administration of NO donor inhibits Fos expression, in the PVN, SON, periventricular and preoptic nuclei while a NOS inhibitor attenuates Fos expression in the PVN of urethane-anaesthetized rats (Okere *et al.*, 1999). Other authors have reported that systemic administration of L-NAME potentiates Fos expression in the SON and PVN induced by oxytocin i.v. infusion in conscious lactating rats (Woodside and Popeski, 1999).

1.7 c-*fos* immediate early gene product as a marker of neuronal activity

The expression of the immediate early gene c-*fos* and its protein product Fos has been widely used as a marker of neuronal activity (Hughes and Dragunow, 1995; Kaczmarek and Chaudhuri, 1997). The basal level of c-*fos* mRNA and Fos expression are relatively low in most cell types (Cohen and Curran, 1989). Following stimulation, transcription of c-*fos* is induced rapidly and transiently, and Fos is translated (Morgan and Curran, 1988). In neurones, Fos is synthesized following depolarization and voltage-gated enhancement of Ca^{2+} influx (Morgan and Curran, 1986). Once synthesized in the cytosol, Fos goes through extensive post-translational modifications, including phosphorylation and translocation back to the nucleus, where it influences the expression of the late-response genes. Fos is associated with Jun, another protein product of the immediate early gene c-*jun*, through the leucine zipper domain to form a dimerized complex. The Fos-Jun heterodimer represents one form of transcription factor, activator-protein 1 (AP-1), which selectively binds to the consensus sequence 5′-TGACTCA-3′, known as the AP-1 binding site, in the promoter region of the target genes to regulate transcription (Curran and Morgan, 1987; Sonnenberg *et al.*, 1989). Fos can also recognize and bind to other DNA sequences, one of which is 5′-TGACGTCA-3′ known as CRE (cAMP response element) which differs by only one base from AP-1 (Macgregor *et al.*, 1990). CRE is

present in the promoter region of the vasopressin gene (Mohr and Richter, 1990). The regulatory effects of Fos heterodimers appear rather complex *in vivo*, ranging from activation to repression of transcription. Fos itself acts as a negative regulator of its own promoter, to repress transcription of c-*fos* gene (Konig *et al.*, 1989; Sassone-Corsi *et al.*, 1988; Wilson and Treisman, 1988).

Fos is reliably produced following synaptic activation, allowing its use for showing the activation of magnocellular neurones under a variety of stimulated conditions such as systemic administration of hypertonic saline (Ceccatelli *et al.*, 1989; Sharp *et al.*, 1991; Xiong and Hatton, 1996), dehydration (McKinley *et al.*, 1994; Oldfield *et al.*, 1994), systemic administration of CCK (Luckman, 1992; Onaka *et al.*, 1995a) and parturition (Luckman, 1995; Luckman *et al.*, 1993). Hypertonic stimuli, dehydration and parturition are not specific to either cell types, whereas CCK activates only oxytocin neurones. Interestingly, suckling in the lactating rat, a potent stimulus for oxytocin neuronal activation and oxytocin release, is not inefficient in inducing Fos expression (Fenelon *et al.*, 1993). In addition, antidromic stimulation of oxytocin and vasopressin neurones, that does not cause transynaptic activation, is unable to induce Fos (Luckman *et al.*, 1994).

In response to hypertonicity, Fos expression is induced in the SON and PVN in parallel with enhanced release of oxytocin and vasopressin from the neurohypophysis (Sharp *et al.*, 1991; Verbalis *et al.*, 1995). Prolonged hypertonic stimulation by chronic salt loading has been found to up-regulate the expression of oxytocin and vasopressin mRNA in the SON and PVN (Lightman and Young, 1987). Intraperitoneal injection of hypertonic saline induces Fos-immunoreactivity in the SON, PVN and structures associated with the lamina terminalis of the third ventricle, including the SFO, OVLT and MnPO (Ceccatelli *et al.*, 1989; Giovannelli *et al.*, 1990; Giovannelli *et al.*, 1992) with a corresponding up-regulation of *c-fos* mRNA expression in these areas (Hamamura *et al.*, 1992). Although the pattern of neuronal activity induced by hypertonic saline may determine the expression of Fos, the precise role of activity in induction of Fos is questionable.

1.8 Aims

The experiments were undertaken to investigate the role of NO in the magnocellular neurosecretory system. We were particularly interested in exploring changes of nNOS mRNA expression in the magnocellular neurones, as this has been thought to be involved in the major events known to regulate the release of oxytocin such as hyperosmotic stimulation, salt-loading, pregnancy and parturition. In particular, we wished to test the following hypotheses:

1. Nitric oxide, produced by oxytocin neurones, is a negative-feedback inhibitor of oxytocin secretion by its actions in the supraoptic nucleus and/or at the posterior pituitary?

2. This action is particularly important in osmoregulation? To test these hypotheses we undertook a series of experiments to study the role of the NO system in the regulation of oxytocin release; both in response to osmotic stimuli, and in response to other, non-osmotic stimuli. We used CCK as a stimulus for oxytocin secretion which activates oxytocin neurones through a different pathway to that through which osmotic stimuli activate oxytocin neurones.

3. Changes in this pathway underlie the altered osmoreceptiveness of oxytocin neurones in pregnancy?

CHAPTER 2

GENERAL METHODS

2.1 Animals

Adult female Sprague-Dawley rats weighing 250-350 g (Bantin & Kingman, UK) were used throughout this work. Rats were housed up to five per cage under standard conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1°C). Food (standard breeder diet, Bantin & Kingman, UK) and water were available *ad libitum*, except where otherwise specified. Rats were acclimatised in the MFAA unit for 1-2 weeks after arrival before use in experiments or being mated.

To obtain pregnant rats, adult female rats were caged individually with a stud male in mesh floored breeder cages. If a vaginal plug of semen appeared on the underlying tray the next morning then that day was designated as day 0 of pregnancy. After mating, rats were housed separately until the day of experiment. The number of pups was counted post mortem and data were excluded if less than four pups were found.

2.2 Non-recovery catheterization of the femoral vessels

To withdraw serial blood samples for determination of oxytocin and sodium concentration the left femoral artery and vein were catherised. A sufficient number of catheters (approximately 5-15 cm in length, OD 0.75 mm, size 3FG, Portex Ltd., Kent, UK) were prepared prior to surgery. The leading edges of catheters were trimmed to 45° to ease insertion and connected to 1 ml plastic syringes (Steriseal poly-vu syringes, Worcestershire, UK) filled with heparinised saline. Heparinised saline (50 IU/ml) was prepared by a 1:100 dilution of a 5000 units/ml heparin solution (Multiparin, CP Pharmaceutical Ltd., Wrexham, UK) in isotonic saline solution (Steriflex No. 1, FL (manufacturing) Ltd., England, UK).

Surgery

On the day of experiment, rats were intraperitoneally injected with urethane (1.25 g/kg ethyl carbamate, 25% w/v solution, Sigma Chemical Company Ltd., Dorset, UK) which usually produced a sufficient level of anaesthesia by checking withdrawal reflex that occurs in response to a noxious and usually painful stimulation of the skin or subcutaneous tissues and muscle. If necessary, supplement doses were given. Rats were placed in the supine position and an incision (approximately 1-2 cm) made just below the second inguinal nipple. The sheath which encapsulates the femoral artery, vein and nerve was exposed and gently opened for several millimetres. The femoral artery and vein were then separated from each other and the femoral nerve by blunt dissection. Two ligatures (EP 1 suture silk, Davis and Geck, Hampshire, UK) were then placed around the vessels. The first ligature was placed as distal from the inguinal ligament as possible and tightened to occlude the vessel. A loose ligature (half-hitch) was then formed but left several millimetres approximately to the first ligature and a small clamp was placed over the vessel as close to the ligament as possible. The vessel was then semi-transected with fine scissors between the two ligatures. The tip of the catheter was inserted into the vessel and pushed forwards in the direction of the ligament, this being facilitated by opening the incision with fine forceps. The loose ligature was then tied round the vessel and catheter to prevent blood loss while simultaneously advancing the catheter into the vessel. The clamp was then removed and the catheter lay below the vessel until the tip of the catheter lied below the inguinal ligament. If blood was easily drawn back along the catheter, the ligature was then tied and two further ligatures were applied to secure the catheter. Once catheters for both vessels were in place, the surgical wound was closed with 2-3 stitches using suture silk (EP3, Davis and Geck, UK).

To infuse hypertonic saline solution i.v., the right femoral vein was catheterised using the same procedure as for catheterization of the left femoral vessels.

Blood sampling

To determine the oxytocin concentration, 300 µl blood samples were taken *via* a femoral arterial catheter into 1 ml heparinised syringes, then transferred into pre-numbered 1.5 ml eppendorf tubes and immediately centrifuged at 13,000 RPM for 2-3 minutes (Micro Centaur, MSE, UK). Plasma samples (approximately 150 µl) were separated from the blood cells, aliquoted into separate eppendorf tubes and stored at -20°C until assay for oxytocin concentration. The remaining blood cells were resuspended in 150 µl isotonic saline solution to equal the amount of plasma removed. This solution was then returned to the rat as quickly as possible *via* the femoral vein catheter.

To measure sodium concentration at the same time, a further 300 µl of blood was taken from the femoral artery and treated in the same manner as above. Plasma samples were aliquoted into separate eppendorf tubes for oxytocin concentration measurements and sodium concentration measurements before frozen storage (4°C).

2.3 Intracerebroventricular (i.c.v.) infusion

To allow central drug administration into the right lateral ventricle, implantation of intracerebroventricular cannulae was performed on urethane anaesthetised rats. The scalp was shaved and the head of the rat was placed in a stereotaxic frame. A longitudinal midline incision through the skin was made with a scalpel. The dorsal surface of the skull was exposed by retracting the underlying tissue and scraping away the periosteum. A 1 mm diameter hole was drilled at the following co-ordinates: 1.6 mm right lateral, 0.6 mm caudal from Bregma for insertion of a 22-gauge stainless steel guide cannula (Plastics One, USA). The i.c.v. guide cannula (length 4.5 mm) was brought down vertically through the hole so the tip was 3.5 mm below the surface of the skull. Two additional holes (1.2-1.3 mm diameter) were drilled on either side of the midline lateral to the cannula placement for insertion of 2 stainless steel screws (3.2 mm × 10 BA). Dental cement (Simplex

Rapid, Associated Dental Products Ltd., Swindon) was then poured over the stainless steel screws and around the guide cannula to a depth of approximately 3 mm to hold the guide cannula in place. A cap was inserted into the guide cannula until the start of the experiment. After the implantation of the cannulae, all rats were allowed to rest for at least 2 h before the experiment was performed.

Before the experiment, an infusion cannula (28-gauge, length 5.5 mm, tip 4.5 mm below the surface of skull) was introduced into the right lateral ventricle *via* the guide cannula. Solutions were administered through the infusion cannula *via* a polythene tube (1.0 mm o.d., 0.5 mm i.d., Portex, R&J Wood, Paisley, UK) connected to a 100 μl gas tight microsyringe (Hamilton, Sigma, UK).

2.4 Dorsal approach to the pituitary stalk

On the day of experiment, rats were anaesthetised with urethane and their left femoral vein and artery cannulated for blood sampling. The head of the rat was placed in a stereotaxic frame and the skull exposed and levelled by ensuring that bregma and lambda were in the same horizontal plane. A 1 mm diameter guide hole was drilled on the midline, 0.3 mm caudal to Bregma. A concentric stimulating electrode (SNEX-100X, Clarke Electromedical Instruments, England, UK) was placed down the guide hole until it reached the base of the skull. The electrode was then lifted by about 0.1 mm to locate the electrode in the neural stalk of the pituitary gland. After that, the stimulator (Grass stimulator) was set to deliver trains of biphasic pulses (20 Hz, 10 s on 10 s off, 1 mA peak to peak) for 2 h. Blood samples were collected before and after stalk stimulation in order to check the effectiveness of stalk stimulation. Data were excluded from experiments of stalk stimulation if plasma concentration of oxytocin was not increase following stalk stimulation.

2.5 Intra-supraoptic nucleus retrodialysis

In 1997, Ludwig and Leng developed the intra-supraoptic nucleus microdialysis technique combined with *in vivo* extracellular recording to investigate the effect of local drug application on the activity of oxytocin and vasopressin neurones. This technique was used in this thesis to deliver drugs directly to specific areas of the brain.

The microdialysis probe

An in-house designed U-shaped dialysis probe consisting of a 1.3 mm U-shaped dialysis fibre (molecular weight cut-off 5 kDa, 0.2 mm o.d., 3 mm total membrane length, Spectra/Por RC Hollow Fibers®, Spectrum Med. Inc., Houston, TX, USA) glued with cyanoacrylate adhesive on to the ends of two pieces of parallel side-by-side 24-gauge stainless steel tubing (approximately 1.5-2 cm length) which were connected to a syringe pump *via* polythene tubing (0.8 mm o.d., 0.4 mm i.d.; Portex).

The microdialysis probe was perfused at 3 μl/min with artificial cerebrospinal fluid (aCSF; pH 7.2, composition (mM): NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; $MgCl_2$, 1.18) or with aCSF including a drug (see experiment). As the dialysis membrane is semipermeable and the concentration of drug in the dialysate higher than in the tissue, the membrane allows the passage of the drug into the extracellular compartment.

Recovery rate of vasopressin with these probes has been estimated previously as 1.60% *in vitro* (Landgraf and Ludwig, 1991) and 0.5% *in vivo* when implanted into the lateral septum (Engelmann *et al.*, 1992). *In vitro* recovery of oxytocin has also been estimated as 1.99% for $[^3H]$ oxytocin and 1.62% for $[^{125}I]$ oxytocin (Neumann *et al.*, 1993).

The ventral surgical approach

(Set up by Dr. Mike Ludwig)

Under urethane anaesthetised conditions, a femoral vein and the trachea of female Sprague-Dawley rats were cannulated. The pituitary stalk and the right SON were exposed by a transpharyngeal approach described previously (Leng and Dyball, 1991). Briefly, animals were laid supine in a stereotaxic frame. An incision was made through the lower jaw between the lower incisors. The tongue was retracted ventrally through the split jaw until the entire soft palate and epiglottis was clearly visible, and secured with a retractor anchored to the frame. The lower incisors were pulled apart to give clear access to the soft palate. The soft palate was removed using a cautery probe to expose the posterior pharyngeal wall. The hard palate (approximately 3 mm) was then removed with a dental drill. At the rostral end of the pharynx, the presphenoid and basisphenoid bones and a venous sinus within the basi-sphenoid bone were revealed. The sinus appeared as a purple smudge across the sphenoid bone.

To expose the neural stalk that lies beneath the sinus, a shallow hole was made at the midline about 2 mm caudal to the sinus. The hole was filled with bone wax to prevent blood leakage from the sinus and drilling was continued caudally further down and rostrally, packing it with bone wax at regular intervals until an opaque colour was clearly seen in the sinus. After the cavity reaches the sinus, it was then drilled deeper. The last thin layer bone was gently and carefully removed using fine forceps and a minute spatula with a slightly hooked end formed from a disposable needle. The rostral adenohypophysis and the neural stalk were then exposed.

To expose the right supraoptic region, the lateral wing of the palatine bone and medial pharyngoid plate were cleared of muscle, and drilled away to expose the trigeminal nerve. The SON lies laterally to the suture between the pre-sphenoid and basi-sphenoid bones. The trigeminal nerve was removed from this region with two pairs of fine forceps. The venous sinus, which runs alongside the sphenoid bone, was

then compressed rostrally and caudally with small pledgets of tissue wedged against the sphenoid bone. The supraoptic region was identified by the density of vasculature that is often visible beneath the dura.

After removal of the dura with fine scissors, an in-house designed U-shaped dialysis probe (described previously) was bent to position the loop of the membrane flat onto the exposed ventral surface of the brain on the ventral glial lamina of the SON. A glass micropipette (filled with 0.15 M NaCl, 20-40 MΩ resistance, GC150F-10, 1.5 mm O.D, 0.86 mm I.D., borosilicate glass capillaries, Clarke Electromedical Instruments) was introduced into the centre of the loop of the dialysis membrane. A stimulating electrode (SNEX-200X, Clarke Electromedical Instruments) was placed on the neural stalk of the pituitary gland and set to deliver single matched biphasic pulses (1 ms duration, <1 mA peak to peak) for antidromic identification of SON neurones. Electrical stimuli applied to the neural stalk evoke action potentials in the axons at a constant latency following the stimulus. These evoked spikes are propagated antidromically to the cell bodies, and the latency reflects the conduction velocity and length of the axon.

2.6 Oxytocin Radioimmunoassay

The radioimmunoassay (RIA) that was used throughout this work was modified from a previous study (Higuchi *et al.*, 1985). The oxytocin content in unextracted plasma samples from individual rats could be determined by using antibodies to a synthetic oxytocin-bovine serum albumin (b.s.a.) conjugate which was raised in the rabbit and purified by Higuchi and his co-workers. Specificity of the anti-oxytocin antibody was determined by Higuchi *et al.* (1985) by comparing the inhibition of the binding of ^{125}I-labelled oxytocin to antibody with arginine-vasopressin, arginine-vasotocin, and melanocyte-stimulating hormone-release inhibiting factor. The cross-reactivity of these peptides is less than 0.005%.

This antibody (Higuchi rabbit anti-oxytocin antibody) was added to solutions containing either duplicate aliquots of rat plasma (containing unknown concentration of oxytocin) or triplicate aliquots of known concentrations of standard oxytocin in a assay buffer. Mixtures were left to incubate for 24 h to reach equilibrium. A fixed amount of ^{125}I-oxytocin (calculated to contain approximately 6-8,000 cpm) was then added to all tubes and left for 48 h incubation. The amount of ^{125}I-oxytocin bound to the rabbit anti-oxytocin antibody was inversely proportional to the concentration of oxytocin in the standard or plasma sample. The rabbit anti-oxytocin-^{125}I-oxytocin conjugate was then precipitated out of solution using a second antibody (Donkey anti-rabbit gammaglobulin). To further aid precipitation and visualisation, a suspension of white Pansorbin cells was added prior to centrifugation. The supernatant was then aspirated and the radioactivity of the precipitates was measured with a gamma counter. Oxytocin concentration of the unknown samples was determined by comparing the unknown precipitate radioactivity to precipitate radioactivity of known standard oxytocin contents.

2.6.1 Materials

Assay Buffer

The assay buffer was prepared in distilled water and contained:

0.025% w/v Sodium Dihydrogen Orthophosphate 2-hydrate ($NaH_2PO_4.2H_2O$ AnalaR, BDH Ltd, Poole Dorset, UK)

0.119% w/v Disodium Hydrogen Orthophosphate (Na_2HPO_4 AnalaR, BDH)

0.1% w/v Sodium Azide (NaN_3 AnalaR, BDH)

0.5% w/v Bovine Serum Albumin (BSA RIA grade, Sigma)

Standard Oxytocin

Standard oxytocin obtained from the National Institute for Biological Standards and controls (Herts, UK) was dissolved in assay buffer to make a desired concentration (50 ng/ml), aliquoted and kept in a -20°C freezer.

First Antibody Buffer

First antibody buffer was an assay buffer containing normal rabbit serum (SAPU, Lanarkshire, Scotland, UK) at a dilution of 1:400 to aid the precipitation of the anti-oxytocin-^{125}I-oxytocin conjugate. This dilution was the lowest concentration of normal rabbit serum that produced the highest percentage of binding of ^{125}I-oxytocin in the precipitates and was determined from trial assays containing standards diluted in buffer and rat plasma. Total binding of assays was 40.76 ± 2.15 % of the total count of radioactivity.

First Antibody Solution

Rabbit anti-oxytocin antibody THF-3 was kindly donated by Dr. T. Higuchi and stored at -20°C (diluted 1:200 in assay buffer). The final dilution used in assays was 1:200,000 (1 μl/ml) in first antibody buffer. Anti-oxytocin serum at this dilution bound 40-50% of the ^{125}I-labelled oxytocin.

Second Antibody

Donkey anti-rabbit gammaglobulin (A-PPT1; IDS, Tyne and Wear, UK) was stored at 4°C. The dilution used in assays was 1:25 in assay buffer.

Lyophilised ^{125}I-Oxytocin

Lyophilised ^{125}I-oxytocin (New England Nuclear, Boston, MA) was resuspended in distilled water aliquoted and stored at -20°C. A concentration calculated to give 3.7 KBq/ml radioactivity in assay buffer was found to give between 6,000-8,000 cpm/50 μl/min when counted by a gamma counter (LKB-Wallac 1272 Clinigamma with a single detector consisting of a NaI crystal in a 3″ aluminium tube of wall thickness 0.25 mm, Wallac Oy, Finland).

Standardised Pansorbin Cells

Standardised pansorbin cells (10% w/v in phosphate buffer containing 0.1% w/v sodium azide, Novabiochem (UK) Ltd, Nottingham, UK) stored at 4°C. The dilution added to assays was 1:25 in assay buffer.

2.6.2 Methodology

To quantify the unknown oxytocin concentration of experimental rat plasma samples, a standard curve of precipitate radioactivity against known standard oxytocin concentration was compiled. On the first day of assay, serial double dilutions (in assay buffer) of a stock oxytocin aliquot were made to produce a range of concentrations from 2.4-2,500 pg/ml.

Three other standards were also prepared for construction of the curve and assessing the sensitivity and binding of the assay: -

TC, contained an aliquot of ^{125}I-oxytocin only, to determine total count of radioactivity added,

NSB, contained ^{125}I-oxytocin and second antibody but no first antibody or unlabelled oxytocin, to determine non specific binding of radioactivity,

BO, contained aliquots of ^{125}I-oxytocin trace, first and second antibody but no unlabelled oxytocin, to determine the maximum percentage binding of radioactivity.

On the day of assay, plasma samples were removed from the freezer as required, thawed to 4°C and centrifuged for 1-2 min at 13,000 RPM before being aliquoted into the appropriate tubes. The assay was performed in pre-numbered 0.75 ml polystyrene tubes (LP2 tubes, Luckman Ltd, Sussex, UK). Standards were assayed in triplicate. Intra-assay, inter-assay standards and rat plasma samples were assayed in duplicate.

Day 1

The experimental procedure for the addition of reagents to the appropriate tubes was as follows:

TC	Nothing
NSB	50 µl assay buffer, 50 µl first antibody buffer
BO	50 µl assay buffer, 50 µl first antibody solution
Standards	50 µl first antibody solution, 50 µl standard
Samples:	50 µl first antibody solution, 50 µl plasma sample

Solution was mixed well, covered with aluminium foil and kept overnight at 4°C.

Day 2

50 µl of ^{125}I-oxytocin was added to all tubes, mixed well, recovered in foil and kept at 4°C for 2/3 days.

Day 4/5

50 µl of second antibody solution was added to all tubes except TC, mixed well, re-covered in foil and kept at 4°C for 24 h.

Day 5/6

50 µl of standardised pansorbin cells was added to all tubes except TC. Five minutes later, all tubes were centrifuged at 3,000 rpm at 4°C for 30 min (Minifuge 2, Heraeus-Christ, GMBH, Osterode, Germany). The supernatant was aspirated and the radioactivity of each precipitate was measured for 1-3 min in a gamma counter.

2.6.3 Calculation of radioimmunoassay results

A standard curve of precipitate radioactivity against known standard oxytocin concentration was constructed automatically by Wallac Ultroterm 2 software. Oxytocin contents of intra-assay, inter-assay standards and rat plasma samples were then extrapolated from the standard curve.

Assay Sensitivity

The sensitivity of an assay is the minimum detection limit of an assay. It provides information about the minimum acceptable sample concentration and was calculated using the following equation:

$$\frac{[BO-(2\times SD)] - NSB}{BO\text{-}NSB}$$

SD is the standard deviation of the ^{125}I-oxytocin bound in the three BO tubes. The value obtained from this equation was then compared to the % of BO radioactivity bound by each standard (2.4-2,500 pg/ml) calculated using the following equation:

$$\frac{\text{mean standard cpm} - NSB}{\text{mean BO} - NSB}$$

The sensitivity of the assay was taken at the point where the value obtained using the first equation was greater than those values obtained from the second equation and converted to a concentration in pg/ml. The assay sensitivity was 3.84 ± 0.66 pg/ml at 93.25 ± 1.10 % of total binding.

Inter- and Intra-assay variation

Known concentrations of standard oxytocin (10, 50, 100, 250 and 500 pg/ml) were included in each assay run to determine inter- and intra-assay variation. Duplicates of each intra-assay standards were added at the beginning and at the end of assays that allowed an intra-assay variation to be determined. The inter-assay variation could be determined by adding the intra-assay standard solutions of oxytocin from the previous assay and a comparison between the two assays was made. The intra-assay and inter-assay coefficients of variation were 13.51 ± 1.97 % and 16.66 ± 4.06 %, respectively.

2.7 Fos immunohistochemistry

Fos which is the 380 amino-acid protein product of the immediate early gene (IEG), *c-fos* (Curran *et al.*, 1984), can be visualised by immunohistochemistry using an indirect method (Douglas *et al.*, 1995) and an avidin-biotin complex (ABC) method (McKinley *et al.*, 1994)). In our methodology, Fos immunoreactivity was localised by both methods using the N-terminally selected Rabbit Polyclonal anti Fos (*c-fos* Ab-2, Oncogene Sciences, Cambridge Bioscience, UK) as a primary antibody. This antibody was raised against a peptide (S G F N A D Y E A S S S R C) which corresponds to residues 4 to 17 of human Fos and reacts with human and rodent cellular forms of Fos (De Togni *et al.*, 1988). This antibody offers a high specificity with little cross-reactivity with Fos related antigens (FRAs) (Hoffman *et al.*, 1992). Nuclear immunolabelling was completely blocked by preabsorption of this antibody with the synthetic 4-17 residues of human Fos but not affected by preabsorption of the antiserum with the non-immunogenic residues 128-152 (McKinley *et al.*, 1994; Patronas *et al.*, 1998; Rinaman *et al.*, 1997).

Initially, brain sections were soaked with hydrogen peroxide solution to deactivate endogenous peroxidase and then incubated in normal sheep serum solution to preblock nonspecific sites. They were then incubated with a rabbit polyclonal primary antiserum specific for Fos (*c-fos* Ab-2) for 48 h, followed by incubation with a secondary antibody. For the indirect method, the sections were incubated with a goat anti-rabbit IgG-peroxidase complex for 24 h. For the ABC method, the sections were incubated with biotinylated goat anti-rabbit IgG for 1 h followed by incubation for 1 h with Avidin-biotinylated horseradish peroxidase complex (ABC). Finally, the sections were immersed in a glucose oxidase-nickel diaminobenzidine (DAB) solution to label peroxidase. Fos immunoperoxidase labelling was clearly demonstrated in the nucleus as a permanent, dense purple/black colour product deposited around each bound antibody.

2.7.1 Materials

Heparinised Saline

Heparinised saline (5 IU/ml) was prepared by adding 1 ml of a 5000 units/ml heparin solution (Multiparin, CP Pharmaceutical Ltd., Wrexham, UK) to 1,000 ml isotonic solution (Steriflex No. 1, FL (manufacturing) Ltd., England, UK).

4% Paraformaldehyde in 0.1 M Phosphate Buffer

4% Paraformaldehyde solution was prepared in double distilled water and contained:

1.15% w/v Disodium Hydrogen Orthophosphate 2-hydrate ($Na_2HPO_4.2H_2O$ AnalaR; BDH),

0.272% w/v Sodium Dihydrogen Orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$ AnalaR; BDH).

4.0% w/v Paraformaldehyde (CH_2O; Sigma)

This solution was then adjusted to pH 7.3-7.4 with 1 M HCl or 1 M NaOH.

Phosphate Buffer (PB)

Phosphate buffer (1 M) was prepared in double distilled water and contained:

11.5% w/v Disodium Hydrogen Orthophosphate 2-hydrate ($Na_2HPO_4.2H_2O$ AnalaR; BDH),

2.72% w/v Sodium Dihydrogen Orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$ AnalaR; BDH).

This solution was then adjusted to pH 7.3-7.4 with 1 M HCl or 1 M NaOH.

To make 1,000 ml of 0.1 M Phosphate buffer, 100 ml of 1 M phosphate buffer was added to 900 ml double distilled water.

Washing Solution (PB-T)

Washing solution for the indirect method was 0.1 M Phosphate buffer (pH 7.3-7.4) containing 0.3% v/v Triton X-100 (iso-Octylphenoxypolyethoxyethanol,

BDH). Washing solution for the ABC method was 0.1 M Phosphate buffer (pH 7.3-7.4) containing 0.2% v/v Triton X-100 (BDH).

Preincubation Buffer

Preincubation buffer was a washing solution containing 1% v/v Normal Sheep Serum (SAPU, Lanarkshire, Scotland, UK).

Hydrogen Peroxide Solution

Hydrogen peroxide solution was 0.1 M phosphate buffer (pH 7.3-7.4) containing:

0.3% w/v Hydrogen Peroxide (H_2O_2 AnalaR; BDH)

20% v/v Methanol (CH_3OH AnalaR; BDH).

Primary Antibody

Rabbit Polyclonal anti Fos (*c-fos* Ab-2, Oncogene Sciences) was diluted 1:1,000 in preincubation buffer.

Secondary Antibody

Secondary antibody for the indirect method was goat anti-rabbit IgG-peroxidase complex (PI 1000, Vector, UK) diluted 1:1000 in 0.1 M PB containing 0.3% Triton X-100 (BDH) and 1% Normal sheep serum (SAPU).

Secondary antibody for the ABC method was made in PB-T and contained 1% v/v biotinylated anti-rabbit immunoglobulin and 3% v/v normal goat serum (Vectastain Elite ABC Kit rabbit IgG's, Vector Laboratories, Peterborough, UK).

Avidin-biotinylated horseradish peroxidase complex (ABC)

ABC solution was made in PB-T and contained:

2% v/v Avidin DH

2% v/v Biotinylated horseradish peroxidase

(Vectastain Elite ABC Kit rabbit IgG's, Vector Laboratories)

This solution was left to incubate for at least 30 minutes prior to use.

Acetate Buffer (0.2 M)

Acetate buffer (0.2 M) was made in double distilled water and contained: 1.64% w/v Sodium Acetate Anhydrous (CH_3COONa; BDH).
This solution was then adjusted to pH 6.0 using acetic acid.

Glucose Oxidase-Ni DAB Solution

Glucose oxidase-Ni DAB solution contained:
0.4% w/v Glucose ($C_6H_{12}O_6$ AnalaR; BDH)
0.08% w/v Ammonium Chloride (NH_4Cl AnalaR; BDH)
0.003% w/v Glucose Oxidase (b-D-glucose: oxygen 1-oxidoreductase; EC 1.1.3.4, $O(CHOH)_4CHCH_2OH$, type VII-S from *Aspergillus niger*; Sigma)
2.5% w/v Di-Ammonium Nickel (II) Sulphate 6-Hydrate ($(NH_4)_2SO_4NiSO_4.6H_2O$ AnalaR; BDH)
0.025% Diaminobenzidine (DAB) (3,3′,4,4′-Tetraaminobiphenyl, tetrahydrochloride, $C_{12}H_{14}N_4.4HCl$; Sigma)

To make up 100 ml of glucose oxidase-Ni DAB solution, nickel sulphate, glucose and ammonium chloride were dissolved in 50 ml of 0.2 M acetate buffer and DAB was dissolved in 50 ml of double distilled water. After that, nickel solution and DAB solution were mixed well and filtered. The glucose oxidase was added immediately prior to use.

Stop Solution

Stop Solution (0.1 M Acetate Buffer) was made by diluting 0.2 M acetate buffer with an equal volume of double distilled water.

Chrome Alum Gelatine

Chrome alum gelatine solution was made in double distilled water and contained:
1% w/v gelatine powder (BDH)

0.1% w/v chromic potassium sulphate (Chromium (III) potassium dodecahydrate, $CrK(SO_4)_2.12\ H_2O$, BDH)

2.7.2 Methodology

In our methodology, an indirect method and an ABC method were used for Fos immunohistochemistry and both were performed with gentle agitation on a rotational shaker (R100/TW, Luckman Ltd., Sussex, UK).

2.7.2.1 Indirect method

After decapitation, rat brains were removed from the skulls, covered with powdered dry ice until frozen and then stored at -70°C. Frozen brains removed from a -70°C freezer were sectioned coronally (15 μm thickness) through the level of the SON and PVN, which corresponded to the stereotaxic atlas of Paxinos and Watson (1982), on a cryostat at optimal temperature (-12 to -16°C). Sections were thaw-mounted onto chrome alum gelatine subbed slides (4 sections per slide) and kept in slide boxes stored in a -70°C freezer. Marker slides for orientation were collected every ninth section and counterstained with toluidine blue.

Day 1

After warming up to room temperature, slides were selected and the sections were framed using PAP pens (Bayer Diagnostics, Hampshire, UK). The slides were placed in racks and the sections fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.3) for 30 min and then washed in 0.1 M PB (2×5 min, 1×10 min). Endogenous peroxidase was then deactivated with hydrogen peroxide solution for 15 min and washed off with washing solution for 15 min. Non-specific staining was then blocked by preincubation buffer containing 0.3% Triton X-100, 1% normal sheep serum in 0.1 M PB for 1 h. Excess liquid was drained off and the slides were placed in flat slide boxes containing filter paper soaked with 0.1 M PB. An excess of primary antibody solution containing Ab-2 Fos antibody at 1:1,000 diluted in preincubation

buffer was pipetted onto each section on all slides. The slide boxes were sealed and the sections incubated for 48 h at 4°C. Additional primary antibody solution was applied to prevent drying out of the solution the next morning and early on day 3.

Day 3

Excess primary antibody was washed out using washing buffer containing 0.3% Triton X-100 in 0.1 M PB (8×5 min). After draining off any excess liquid, the slides were replaced in the flat slide boxes. An excess of secondary antibody solution, goat anti-rabbit peroxidase complex, was pipetted onto sections and incubated in the sealed slide boxes for 24 h at 4°C.

Day 4

Early the next morning, additional secondary antibody solution was applied as required. The sections were washed in PB-T (2×10 min) and rinsed with 0.1 M acetate buffer for 2 min. The sections were then incubated with the glucose oxidase-Ni DAB solution for approximately 10 min. Progress of the reaction was followed by viewing under a light microscope. Once the reaction had worked and some nuclei appeared black, the reaction was terminated with stop solution for 5 min, and then a rinse with PB for 5 min to prevent excessive background developing. The sections were then dehydrated through serial concentrations of ethanol (70%, 90%, 95%, 100% and 100%) and then into xylene. The slides were finally coverslipped using DPX mountant (BDH).

2.7.2.2 Avidin-biotin complex (ABC) method

Rats were deeply anaesthetised with sodium pentobarbitone (Saggatal, Rhône Mérieux Ltd., Essex, UK) at a dose of 50 mg/kg, i.p.. An incision was made through the abdomen just below the rib cage. The thoracic cavity was opened by making two horizontal cuts through the rib cage and the cut thoracic wall was then folded headward to expose the heart. A blunt 23-guage hypodermic needle connected to a perfusion pump (Minipuls 2, Gilson, Anachem House, Luton, UK) *via* polythene

tubing filled with heparinised saline was quickly inserted upward through the apex of the left ventricle, passed through the aortic valve and the tip of the needle located in the ascending aorta. The needle was then clamped in place with a hemostat across the ventricle. Rats were perfused with 30 ml of heparinised saline at a flow rate of 10 ml/min. Immediately after starting the pump, the right atrium was cut to allow an escape route for the blood and perfusion fluid. After the atrium effluent was clear, rats were perfused with 250-300 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3-7.4) at a flow rate of 10 ml/min. The brains were removed and post-fixed in fixative overnight at 4°C. Brains were then transferred into 30% sucrose in fixative overnight and stored in 30% sucrose in 0.1 M PB at 4°C for 24 h or until the brains sank.

Day 1

Sections (50 μm thickness) were cut on a sledge microtome equipped with a freezing platform throughout the brain area of interest (SON and PVN). Free-floating sections from each brain were collected in a petri dish containing 0.1 M PB and then washed (4×15 min) in washing solution (0.2% Triton X-100 in 0.1 M PB). [Triton X-100 is a detergent that increases tissue impregnation.] Endogenous peroxidase was then deactivated with hydrogen peroxide solution for 15 min and washed off with washing solution (3×10 min). Non-specific staining was then blocked by preincubation buffer containing 1% normal sheep serum for 30 min. After that the sections were incubated in Ab-2 Fos antibody (Rabbit polyclonal; Oncogene Sciences, Cambridge Bioscience, UK), at 1:1,000 diluted in preincubation buffer, for 48 h in 7 ml labelled plastic bottles (Bijou container, Sterilin) at 4°C.

Day 3

Excess Ab-2 Fos antibody was washed off from the sections with PB-T (3×10 min). Fos immunoreactivity was visualised by the avidin-biotin peroxidase technique. To localise the antibody-antigen complex, sections were incubated in secondary antibody solution (1% biotinylated anti-rabbit immunoglobulin and 3% normal goat serum in PB-T) for 1 h at room temperature. After that, the sections were

washed in PB-T (3×10 min) and then left to incubate in ABC complex solution (2% Avidin DH and 2% Biotinylated horseradish peroxidase in PB-T) at room temperature for 1 h. The sections were washed in PB-T (2×10 min). After that the sections were rinsed with 0.1 M acetate buffer for 5 min. They were then incubated with the glucose oxidase-Ni DAB solution for approximately 10 min. Progress of the reaction was followed by viewing under a light microscope. Once the reaction had worked and some nuclei appeared black, the reaction was terminated by replacing the glucose oxidase-Ni DAB solution with stop solution for 5 min, and then a rinse with PB for 5 min to prevent excessive background developing. The sections were then mounted onto the chrome alum gelatine subbed slides in serial order and left to dry overnight. The sections were then dehydrated through serial concentrations of ethanol (70%, 90%, 95%, 100% and 100%) and then into xylene. Slides were finally coverslipped using DPX mountant (BDH).

Quantitative Analysis

The slides were coded by covering identifying numbers with masking tape and assigned random numbers before quantification to prevent bias. The sections were viewed under a light microscope (×10 objective, Wang 6000 series microscope) and Fos positive nuclei were counted in the SON and PVN on both the left and right sides. The animal mean for each region and the group means ± SEM were then calculated.

2.8 *In situ* Hybridization

In situ hybridization histochemistry (ISH) has become a powerful technique for the detection and localisation of specific messenger ribonucleic acids (mRNA) within morphologically preserved cells or tissue preparations (Lewis *et al.*, 1985). In our methodology the expression of neuronal nitric oxide synthase (nNOS) and oxytocin gene was demonstrated within fixed brain tissues using chemically synthesized oligonucleotide probes as described previously (Kadowaki *et al.*, 1994; Luckman *et al.*, 1997). These probes comprised short single stranded DNA sequences (ssDNA) complementary to designated sequences of nNOS mRNA (Bredt *et al.*, 1991b) and oxytocin mRNA (Ivell and Richter, 1984). Their small sizes (45 bases for nNOS probe and 25 bases for oxytocin probe) give them good penetration properties, which is considered to be one the main factors for successful *in situ* hybridization. nNOS and oxytocin probes were labelled using terminal deoxynucleotidyl transferase (TdT) with either [α-^{35}S]dATP (Davies *et al.*, 1995; Kishimoto *et al.*, 1993) or digoxigenin-dUTP (Lewis *et al.*, 1990), respectively.

Brain sections were fixed with paraformaldehyde solution to reduce the activity of tissue nucleases and preserve the structure of native mRNA without disrupting tissue morphology. The sections were washed in phosphate buffer saline solution, acetylated in triethanolamine solution containing acetic anhydride in order to reduce non-specific DNA binding to charged particles. The sections were then dehydrated through a series of ethanol solutions (70%, 80%, 95%, 100%), delipidated in chloroform to improve probe access, and rehydration in 95% ethanol. They were then hybridized for 17 h at 37°C in hybridization buffer containing the ^{35}S-labelled nNOS probes (100,000 dpm/μl) or the DIG-labelled oxytocin probes (2 fmol/μl). The slides were then washed stringently in 1 × SSC at 55°C, which is important to remove unhybridized probe. The slides for radioactive *in situ* hybridization were rinsed in ammonium acetate to remove salts and then in 70% ethanol. After air drying, they were exposed to autoradiographic film for 3 weeks followed by dipping in emulsion for 4 weeks. The film and emulsion dipped slides

were photographically developed and fixed. Film autoradiography provides information on the intensity of the hybridization signals and the regional distribution of nNOS mRNAs while photographic emulsion provides information concerning the cellular localization of nNOS mRNA transcripts.

The slides for non-radioactive *in situ* hybridization were taken from 1 × SSC for immunological detection. They were incubated in blocking solution for 30 min and covered with anti-DIG-alkaline phosphatase solution (1:500) for 1 h. The slides were then left to incubate with colour reaction solution overnight. The non-radioactive hybridization signal for oxytocin mRNA was clearly shown in the cytoplasm as a brown colour product, which allowed localization of oxytocin mRNA transcripts with cellular resolution.

2.8.1 Oligonucleotide Probes

Oligonucleotide probes must be species specific and only recognise a designed mRNA sequence especially for gene family homologies such as oxytocin (Ivell and Richter, 1984) and vasopressin (Land *et al.*, 1982) which contain extensive sequence similarities. The stability of the hybridized sequences is determined by the probe length, GC:AT ratio, the number of mismatched base pairs and by temperature and salt concentration during the hybridization washes (Lewis *et al.*, 1985).

2.8.1.1 Antisense Oxytocin oligonucleotides

Antisense oxytocin oligonucleotides were obtained from Oswel DNA Service, Department of Chemistry, University of Edinburgh. They were prepared by an automatic DNA synthesiser and partially purified by gel filtration and dissolved in distilled water (16 OD units per ml or 0.2 µmole/ml). The sequence of the antisense oxytocin oligonucleotide, complementary to bases 912-936 of oxytocin mRNA (Ivell and Richter, 1984) (MW=9555), is shown below:

5′- CTC GGAGAA GGC AGA CTC AGG GTC G -3′

2.8.1.2 Antisense neuronal Nitric oxide synthase (nNOS) oligonucleotides

Three 45-mer antisense nNOS oligonucleotides (070, 071 and 073) which correspond to rat nNOS sequence (Bredt *et al.*, 1991b) were kindly donated by Dr S.M. Luckman from the Babraham Institute, Cambridge, UK. They were dissolved in distilled water (33 ng/μl or ~2 pmol/μl).

nNOS 070 (complementary to bases 223-267 of nNOS mRNA (5′-noncoding region), MW=16900)
The sequence of antisense nNOS 070 oligonucleotide is shown below:

5′- AGA GTT CTG TCG CCT CTG TCT TGA CGT CAA CTT GGC GTC ATC TGC -3′

nNOS 071 (complementary to bases 4714-4758 nNOS mRNA (3′-noncoding region), MW=17102)
The sequence of antisense nNOS 070 oligonucleotide is shown below:

5′- AGG CAG AGC AGG GCC ACT CGG TTC AGC CGT GTG TGT CCG AAT TTA -3′

nNOS 072 (complementary to bases 1662-1706 of nNOS mRNA, MW=16946)
The sequence of antisense nNOS 070 oligonucleotide is shown below:

5′- CCT TTG TTG GTG GCA TAC TTG ACA TGG TTA CAG ATG TAG TTG AAC -3′

2.8.2 Materials

1. Slides

Slides were soaked in strong cleaning solution overnight, washed with tap water throughout the day, rinsed 3 times in autoclaved deionised double distilled water (adddH$_2$O), dipped in 80% alcohol to degrease and left to dry in a 37°C oven. Slides were then coated with chrome-alum gelatine and baked in a 37°C oven overnight. Coated slides were stored in clean dry slide boxes with silica gel packets.

2. Glassware and plasticware

All glassware was baked at 200°C for 12 h, and all plasticware was wrapped with tinfoil and then autoclaved.

3. Solutions

All solutions were made in DEPC-treated H_2O, except for Tris buffer and T/E buffer.

Diethyl pyrocarbonate treated water (DEPC-treated H_2O)

0.1% v/v Diethyl pyrocarbonate (DEPC, Sigma), inhibitor of RNAses, was added to the deionized double distilled water.

Strong Cleaning Solution

200 g potassium dichromate was dissolved in 2000 ml distilled water and then 200 ml concentrated sulphuric acid was added while the flask was immersed in cold water.

Chrome Alum Gelatine

Chrome alum gelatine solution was made in DEPC-treated H_2O and contained:

1% w/v gelatine powder (BDH)

0.1% w/v chromic potassium sulphate (Chromium (III) potassium dodecahydrate, $CrK(SO_4)_2.12\ H_2O$, BDH)

0.2 M Phosphate Buffer Saline Solution (PBS)

Stock A: 0.2 M Na_2HPO_4 stock solution was made in DEPC-treated H_2O and contained:

3.56% w/v Disodium Hydrogen Orthophosphate 2-hydrate ($Na_2HPO_4.2H_2O$ AnalaR; BDH),

1.7% w/v Sodium Chloride (NaCl AnalaR; BDH)

Stock B: 0.2 M $NaH_2PO_4 \cdot H_2O$ stock solution was made in DEPC-treated H_2O and contained:

2.76% w/v Sodium Dihydrogen Orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$ AnalaR; BDH).

1.7% w/v Sodium Chloride (NaCl AnalaR; BDH)

To make 1,000 ml of 0.2 M Phosphate buffer, 190 ml of *Stock B* was added to 810 ml of *Stock A*. This solution was then adjusted to pH 7.2-7.4 with 1 M HCl or 1 M NaOH.

0.1 M PBS was made by diluting 0.2 M PBS with an equal volume of DEPC-treated H_2O.

4% Paraformaldehyde in 0.1 M PBS (pH 7.4)

4.0% w/v Paraformaldehyde (CH_2O; Sigma) was dissolved in 0.1 M PBS and then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

Triethanolamine (TEA) solution for acetylation

Triethanolamine solution was made in DEPC-treated H_2O and contained:

0.25% v/v Acetic anhydride ($C_4H_6O_3$, Sigma)

1.332% v/v Triethanolamine (1.12 g/ml Triethanolamine, $C_6H_{15}NO_3$, Sigma)

0.877% w/v Sodium chloride (NaCl AnalaR, BDH)

300 m M Ammonium Acetate

2.312% w/v Ammonium acetate (BDH) was dissolved in DEPC-treated H_2O.

1 M Tris stock solution

12.12 g Trizma-Base (Tris[hydroxymethyl]aminomethane, $C_4H_{11}NO_3$, Sigma) was dissolved in 80 ml adddH$_2$O. Concentrated HCl was added slowly while stirring until desired pH was reached.

desired pH:	Approximate amount of concentrated 1 M HCl
pH 7.4	7 ml
pH 7.6	6 ml

pH 8.0 4.2 ml

0.5 M EDTA stock solution (pH 8.0)

1.861 g EDTA (ethylenediaminetetraacetic acid, disodium, dihydrate, $C_{10}H_{14}N_2O_8Na_2.H_2O$, Sigma) was dissolved in 40 ml adddH$_2$O. To help dissolve of this solution 10 M NaOH or 2 g NaOH pellets were added.
adddH$_2$O was then added to make up to 100 ml. The solution was adjusted to pH 8.0 with 1 M HCl or 1 M NaOH.

0.2 M EDTA (pH 8.0)

4 ml of 0.5 EDTA (pH 8.0) was added to 6 ml adddH$_2$O.

Tris/EDTA Buffer (10 mM Tris (pH 7.4) / 1 mM EDTA (pH 8.0))

100 ml of T/E buffer contained:
1 ml 1 M Tris (pH 7.4) stock solution
200 μl 0.5 M EDTA (pH 8.0) stock solution

RNase Buffer (10 mM Tris 8.0, 0.5 M NaCl and 1 mM EDTA)

RNase buffer was made in DEPC-treated H_2O and contained:
0.14% w/v Tris 8.0 (Sigma)
2.9% w/v Sodium chloride (NaCl AnalaR, BDH)
0.04% w/v EDTA (ethylenediaminetetraacetic acid, disodium, dihydrate, $C_{10}H_{14}N_2O_8Na_2.H_2O$, Sigma)

RNase solution (20 μg/ml)

11.2 μl of RNase A (100 mg/ 7 ml: 100 units/mg RNase enzyme solution A type XA, Sigma) was added to 8 ml RNase buffer.

Standard sodium citrate (SSC)

20×SSC stock solution was made in DEPC-treated H_2O and contained:
17.54% w/v Sodium chloride (NaCl AnalaR, BDH)

8.82% w/v Tri sodium citrate ($Na_3C_6H_5O_7.H_2O$ AnalaR, BDH)

The solution was adjusted to pH 7.0 with a few drops of a 10 N NaOH.

To make dilutions: 1×SSC 50 ml 20×SSC was added to 950 ml DEPC treated H_2O

2×SSC 100 ml 20×SSC was added to 900 ml DEPC treated H_2O

4×SSC 200 ml 20×SSC was added to 800 ml DEPC treated H_2O

Herring sperm single stranded DNA

10 mg single stranded deoxyribonucleic acid (ssDNA, from herring sperm, Sigma) was dissolved in 1 ml DEPC-treated H_2O.

Dextran sulphate

50% w/v Dextran sulphate sodium salt (Sigma) was made in DEPC-treated H_2O.

Deionized Formamide

50 ml Formamide ($HCONH_2$, BDH) was added to 5 g of mixed-bed, ion-exchange resin (AG501-X8(D), Bio-Rad), stirred for 30 minutes at room temperature and then filtered twice.

Prehybridization buffer

Prehybridization buffer contained all components of a hybridization buffer except probe and dextran sulphate. 10 ml prehybridization buffer contained:

2 ml	20×SSC
200 μl	50×Denhardt's solution (Sigma)
500 μl	10 mg/ml Herring sperm ss DNA (Sigma)
5 ml	Deionized Formamide (BDH)
30 μl	2-Mercaptoethanol (C_2H_6OS, Sigma)
2.270 ml	DEPC-treated H_2O

Hybridization Solution

Hybridization solution was made in DEPC-treated H_2O and contained:

5 ml	20×SSC
500 μl	50×Denhardt's solution (Sigma)
1250 μl	10 mg/ml ss DNA (Sigma)
5 ml	50% Dextran sulphate (Sigma)

Hybridization buffer

Hybridization buffer contained:

4700 μl	Hybridization solution
5000 μl	Deionized Formamide (Sigma)
30 μl	2-mercaptoethanol (Sigma)

The volume of radioactive labelled probe added was calculated to give 100,000 dpm/40 μl of hybridization buffer.

The non-radioactive labelled probe was diluted in hybridization buffer to a concentration of 2 fmol/μl concentration.

Glycogen-EDTA mixture

200 μl 0.2 M EDTA (pH 8.0) was added to 1 μl 20 mg/ml Glycogen solution (Boehringer Mannheim).

4 M Lithium Chloride

4 M Lithium Chloride was made by diluting 8 M Lithium Chloride (LiCl for molecular biology, Sigma) with an equal volume of DEPC-treated H_2O.

Soaking solution

4×SSC was added to an equal volume of Formamide.

1 M Magnesium Chloride

20.33% w/v magnesium chloride ($MgCl_2$ AnalaR, BDH) was made up in adddH_2O.

2M Sodium Chloride

11.688% sodium chloride (NaCl AnalaR, BDH) was made up in adddH$_2$O.

Buffer 1 (100 mM Tris HCl (pH 7.5), 150 mM NaCl)

100 ml of buffer 1 was made up in adddH$_2$O and contained:

10 ml 1 M Tris HCl (pH 7.5)

7.5 ml 2 M Sodium Chloride (NaCl AnalaR, BDH)

Blocking solution

Blocking solution was buffer 1 containing 0.1% Triton® X-100 (for molecular biology, Sigma) and 2% normal sheep serum (SAPU).

Anti-digoxigenin antibody solution

Anti-DIG-alkaline phosphatase (Sheep anti-Digoxigenin alkaline phosphatase [Fab fragment], Boehringer Mannheim) was diluted 1:500 in Buffer 1 containing 0.1 % Triton® X-100 and 1 % normal sheep serum.

Buffer 2 (100 mM Tris HCl (pH9.5), 100 mM NaCl, 50 mM $MgCl_2$)

100 ml of buffer 2 was made up in adddH$_2$O and contained:

10 ml 1 M Tris HCl (pH 9.5)

10 ml 2 M Sodium Chloride

5 ml 1 M $MgCl_2$

Colour substrate solution

Colour substrate solution contained:

45 μl NBT solution (75mg/ml nitroblue tetrazolium salt in dimethylformamide, Boehringer Mannheim)

35 μl BCIP solution (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in dimethylformamide, Boehringer Mannheim)

2.4 mg Levamisole (Sigma)

10 ml buffer 2

Buffer 3 (10 mM Tris HCl (pH 8.1), 1 mM EDTA)

100 ml of buffer 3 was made up in adddH$_2$O and contained:

1 ml 1 M Tris HCl (pH 8.1)

200 μl 0.5 M EDTA

2.8.3 Methodology

Tissue preparation

Rat brains were frozen in powdered dry ice immediately after removal. The brains were wrapped with foil, kept in pre-numbered plastic bags and then stored at -70°C. The brains were sectioned coronally, in a frozen state, on a cryostat at -12° to -16°C. Coronal sections (15 μm) through the hypothalamus at the level of the SON and PVN, guided by Paxinos and Watson (1982), were cut, thaw-mounted onto chromic acid cleaned chrome alum gelatine subbed slides and then kept in desiccated slide boxes stored at -70°C. Every ninth section for orientation was collected and counterstained with toluidine blue.

Brain Paste Standards

Rat brains were dissected and homogenised in 1 ml distilled water containing a few drops of mineral oil to prevent bubbling. Equal volumes of brain paste (approximately 1 ml) were transferred to 10 flat bottom tubes. Two microlitres of adddH$_2$O was added to 2 μl [α-^{35}S]deoxy-ATP and mixed well. One microlitre of this solution was taken and added to brain paste standard 1. An equal volume of dddH$_2$O was then added to the remaining diluted radioactivity and 1 microliter of this solution was then added to brain paste standard 2. This procedure was followed until ten serial dilutions of the standards were made. After adding known amounts of diluted radioactive [α-^{35}S]deoxy-ATP to each tube, the brain paste standards were mixed well, centrifuged to remove air bubbles, frozen on dry ice and carefully removed from the tube. After that the standards were fixed onto cryostat chucks with

Tissue Tek (O.C.T. compound, Miles, USA), sectioned (15 μm thickness) and thaw-mounted onto slides. Five serial concentrations of [α-^{35}S]deoxy-ATP were placed on one slide so two sets of slides were needed for the 10 serial concentration sections. Slides were kept in desiccated slide boxes and stored at -70°C. Two sections from each standard were also taken into separate scintillation vials containing 3 ml scintillation fluid (Ultima Gold MR, Packcard Instrument Company, USA) and radioactivity was counted for 1 min by a scintillation counter (Rackbeta Liquid Scintillation Counter, LKB, Wallace). The mean disintegrations per minute (dpm) emitted by each standard was then computed.

Probe Labelling methods

1. 3′ end labelling with [α-^{35}S]deoxy-ATP of Antisense nNOS oligonucleotides

The following was added to an autoclaved eppendorf tube:

3 μl Antisense nNOS oligonucleotide probes (2 pmol/μl nNOS 070, 071 or 072)

5 μl tailing buffer (Pharmacia Biotech Limited, Herts, UK)

39 μl adddH$_2$O

1 μl [α-^{35}S]deoxy-ATP (Dupont NEN, New England Nuclear, Boston, MA)

2.1 μl Terminal deoxynucleotidyltransferase (19.2 units/μl TdT, Pharmacia Biotech)

This reaction mixture was incubated for 1 h at 37 °C with gentle agitation. The reaction was stopped by adding 5 μl of 0.5 M EDTA on ice. To separate labelled probe from the free label, the mixture was then added along with 500 μl T/E buffer to a Sephadex G-25 column (NAP® 5 Columns DNA grade, Pharmacia Biotech), which was equilibrated with 10 ml T/E buffer. T/E buffer was allowed to saturate the gel bed completely. A further 1 ml T/E buffer was added and the eluted solution was collected every 3 drops (approximately 100 μl) in pre-numbered eppendorf tubes. Two 1 μl aliquots of each eluted solution were added to separate scintillation vials containing 3 ml scintillation fluid (Ultima Gold MV) and then counted for 1 minute by a scintillation counter (Rackbeta Liquid Scintillation Counter, LKB, Wallace). As the labelled probe is larger than the free label, then the labelled probe was eluted

before free label. The tubes containing the labelled probe were identified by the first peak of detected radioactivity and free label appeared as the second peak. The incorporation of ^{35}S-dATP was acceptable if the radioactivity was greater than 70,000 cpm/μl.

The known counting efficiency for Ultima Gold is 95% so all counts were corrected to 100% efficiency. The mean disintegrations per minute (dpm) emitted by the tubes containing labelled probe were then assumed to represent 100% counting efficiency, and multiplied by the remaining number of μl of labelled probe.

2. Tailing with DIG-dUTP of Antisense Oxytocin oligonucleotide

The following was added to an autoclaved eppendorf tube.

4 μl 5×Reaction buffer (1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml bovine serum albumin; pH 6.6, Boehringer Mannheim)

4 μl $CoCl_2$ solution (25 mM, Boehringer Mannheim)

1 μl DIG-dUTP (1 mM, Boehringer Mannheim)

0.5 μl Antisense oxytocin oligonucleotide (200 pmol/μl)

1 μl dATP (10 mM in Tris buffer; pH 7.5, Boehringer Mannheim)

8.5 μl adddH$_2$O

1 μl Terminal Transferase (50 units, Boehringer Mannheim)

The reaction mixture was mixed well, centrifuged briefly and then incubated at 37°C for 15 min. The reaction was stopped by adding 2 μl of the glycogen-EDTA mixture on ice. The tailed oligonucleotide was purified from the labelling reaction mixture by ethanol precipitation. This involved adding 2.5 μl of 4 M LiCl and 75 μl of prechilled (-20°C) 100% ethanol and incubating for at least 30 min at -70°C or 2 hours at -20°C. This solution was then centrifuged (13,000 ×g) for 15 min at 4°C, the supernatant was discarded and the pellet washed with 50 μl ice-cold 70% ethanol and re-centrifuged (13,000 ×g) for 5 min at 4°C. The supernatant was again discarded and the pellet dried under a vacuum. The dry pellet was dissolved in 200 μl adddH$_2$O and stored at -20°C.

To estimate the concentration of the newly labelled probes, a side by side comparison of the newly DIG-labelled oligonucleotide with a known concentration of DIG-labelled control oligonucleotide was performed. Serial dilutions of both probes were prepared and spotted on a piece of Nylon membrane (0.45 μm pore size, positively charged (pH 3-10), Boehringer Mannheim). Probes were then fixed to Nylon membrane by cross-linking with UV light or by baking for 30 min at 120°C. Subsequently, the membrane was colorimetrically analysed by incubation in a diluted antibody solution (Anti-DIG-alkaline phosphatase in blocking solution) and a colour substrate solution. Direct comparison of the intensities of the newly DIG-labelled oligonucleotide and the DIG-labelled control allowed us to estimate the labelling yield. Dots that gave the most similar intensities between the newly DIG-labelled oligonucleotide and the DIG-labelled controls were selected then the dilution factor was calculated. The concentration of the newly DIG-labelled probe was calculated by dividing the concentration of DIG-labelled control by the dilution factor.

Prehybridization

Slides were removed from the -70°C freezer and left for 1 h to warm up to room temperature. The selected slides were placed in racks and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min, rinsed in 0.1 M PBS and washed in 0.1 M PBS for 10 min, followed by acetylation in triethanolamine solution for 10 min to increase tissue permeability. The sections were then dehydrated through 70%, 80%, 95% and 100% ethanol (5 min each), delipidated in chloroform for 10 min and partially rehydrated in 95% ethanol (10 min). After air drying the slides for radioactive ISH were placed in a humidified chamber containing filter paper soaked with soaking solution. The slides for the non-radioactive protocol were incubated in prehybridization buffer for 1 h at 37°C. Prehybridization treatment is carried out to facilitate probe penetration, to reduce nonspecific annealing or binding of the labelled probe to proteins or nucleic acids. Excess solution was drained off and then placed in a chamber containing filter paper soaked with soaking solution.

To confirm hybridization, control experiments were performed by treatment of sections with RNase prior to hybridization with the labelled probe to determine whether this pretreatment abolished the hybridization signal. Control slides treated with 40 μl RNase (20 μg/ml) were incubated for 1 h at 37°C in a separate chamber containing filter paper soaked with RNase buffer to prevent contamination. After incubation, slides were washed in adddH$_2$O, air dried and then placed in a chamber containing filter paper soaked with soaking solution.

In situ hybridization

1. Radioactive *In situ* Hybridization

The hybridization procedure for the ^{35}S-labelled nNOS probes was performed as previously described (Kadowaki *et al.*, 1994). The labelled probe was diluted in hybridization buffer, such that 40 μl aliquots of buffer diluted probe would contain 100,000 dpm of activity (2,500 dpm/μl). Forty microlitres of hybridization buffer containing radiolabelled probe (40 μl) was then pipetted onto each section. Incubation chambers were then sealed and incubated for 17 h at 37°C.

To verify the specificity of the oligonucleotide probe, control experiments were performed by incubating the sections with a 100-fold molar excess of unlabelled oligonucleotide probe in the presence of ^{35}S-labelled probe to determine whether 'cold' oligonucleotide probe blocked the signal obtained with labelled probe. The total amount of the labelled nNOS probe (mole/μl) was calculated then multiplied by 100 to give the amount of 'cold' nNOS probe to be added to the hybridization buffer containing labelled probe. Forty microlitres of hybridization buffer containing radiolabelled probe and 100-fold excess of 'cold' nNOS probe was then pipetted onto each section. Slides were then incubated for 17 h at 37°C.

2. Non-radioactive *In situ* Hybridization

DIG-labelled probes were diluted in hybridization buffer to a concentration of 2 fmol/μl. Prehybridization buffer was drained from the slides and the hybridization buffer containing DIG-labelled probe (40 μl) was pipetted onto each section.

To check oligonucleotide probe specificity, control experiments were performed by incubating the sections with 100-fold molar excess of 'cold' oligonucleotide probe in the presence of DIG-labelled probe. The unlabelled oxytocin oligonucleotide was diluted in hybridization buffer containing DIG-labelled oxytocin to a concentration of 200 fmol/μl. Forty microlitres of hybridization buffer containing 2 fmol/μl DIG-labelled oxytocin and 200 fmol/μl unlabelled oxytocin probe was then pipetted onto each sections. Slides were then incubated for 17 h at 37°C.

Post-hybridization

After hybridization, the excess solution was drained off. The slides were briefly rinsed three times in 1xSSC at room temperature to remove excess buffer and unhybridized probe, washed three times for 30 min in 1xSSC and washed for a further 1 h in 1xSSC at room temperature. The slides from the radioactive *in situ* hybridization were air-dried, dipped in 300 mM ammonium acetate, 70% ethanol and then air-dried. After air-drying, slides were placed in autoradiographic cassettes and exposed to Hyperfilm-βmax autoradiography film (Amersham, UK) together with brain paste standards for 3 weeks at 4°C. The exposed film was developed in D19 developer (Kodak) for 5 min, and then fixed (Ilford Hypam rapid fixer, 2x5 min). After that, the slides were dipped in Ilford G-5 emulsion (Mobberley, Cheshire, UK) diluted 1:1 with adddH$_2$O for the cellular localization of nNOS mRNA. The emulsion-coated slides were exposed for a further 4-5 weeks to visualize NOS cellular signal, and then developed (Kodak D19 developer) for 5 min and fixed (Ilford Hypam rapid fixer twice 5 min each). The developed sections were

counterstained with cresyl violet, dehydrated in a graded ethanol series, and coverslipped in DPX mountant.

The slides from the non-radioactive *in situ* hybridization were taken from the final 1xSSC wash, washed twice for 10 min with buffer 1 with gentle agitation on a rotational shaker (R100/TW; Luckman Ltd., Sussex, UK) and then covered with blocking solution for 30 min at room temperature. The blocking solution was then decanted and the sections were incubated overnight in an incubation chamber with anti-DIG antibody solution. After incubation, the sections were then washed twice with buffer 1 for 10 min followed by buffer 2 for 10 min with gentle agitation. The sections were then covered with approximately 200 μl of colour substrate solution and incubated in an incubation chamber overnight in the dark. When the colour had developed with high resolution and low background, the colour reaction was stopped by incubating the slides in buffer 3 and then briefly dipped in distilled water. Slides were then mounted using Aqua-Mount (BDH).

Quantitative Analysis

To prevent bias slides were coded by covering identification numbers with masking tape and assigned random numbers before quantification. The relative amount of nNOS mRNA in the brain nuclei of interest was assessed by the silver grain density of autoradiographic films exposed to the nuclei as viewed under the light microscope (×5, ×10 objective, Wang 6000 series microscope) attached to a high performance CCD video camera (Cohu). A fixed counting area of a circular frame was set for each nucleus measurement. Grain areas in the nucleus of interest and the background from areas immediately outside the nucleus of interest were then measured and computed by NIH Image analysis system version 1.58 on an Apple McIntosh computer. The NIH-Image public domain image processing software package was developed by Wayne Rasband. The value of silver grain density per unit area was obtained by subtracting the mean grain area of the background measurements from each specific nucleus measurement, and dividing by the area

measurements. Each brain paste standard was measured twice, and corresponding background measurements, from the area immediately outside brain paste standard, subtracted. A graph was then plotted of logarithmic radioactivity against grain density for all the standards. Acceptable grain density values lay on the straight portion of the curve to eliminate as many variables between each run of ISH as possible. For each identified brain area, the mean grain density per unit area of each rat was calculated and the group mean grain density per unit area calculated.

2.9 Statistics

Statistical analysis was performed using the SigmaStat software package on a P.C. computer. The Kolmogorov-Smirnov test (with Lilliefors'correction) was used to test data for normality of the estimated underlying population. Parametric tests were used for comparisons of samples from normally distributed populations. Non-parametric or distribution-free tests were performed on ranks of the observations, were used when the populations were not normal. To compare data within a group the parametric paired *t*-test and the non-parametric Wilcoxon-Signed Rank test were used. To compare data between two independent groups the parametric *t*-test and the non-parametric Mann-Whitney Rank Sum test were used. For comparisons among three or more groups, the parametric one way analysis of variance (ANOVA) was used to compare the effect of a single factor on the mean of two or more groups. Kruskal-Wallis ANOVA on ranks was the non-parametric test used to compare effects of a single factor on the rank of two or more groups. The parametric two way ANOVA was used to compare the effect of two different factors on the means of two or more groups. To test the effect of treatment with more than one measurement on the same individuals, the parametric one way repeated measures ANOVA was used to compare the effect of a single series of treatments. The parametric two way repeated measures ANOVA was used to compare the effect of two factors where one or both factors were a series of treatments. The Friedman one way repeated measures ANOVA on ranks was the non-parametric test to compare the effect of a single series of treatments. If a significant difference was found, several multiple comparison

procedures or *post-hoc tests*, were performed to determine which groups were different and the size of the difference. Results were considered significantly different at $P \leq 0.05$.

CHAPTER 3

NITRIC OXIDE AND OSMORESPONSIVENESS OF THE OXYTOCIN SYSTEM IN PREGNANCY

3.1 Introduction

During late pregnancy in rats, plasma osmolality decreases by approximately 10 mosmol/kgH_2O, plasma [Na^+] decreases by 5.8 mEq/l while blood volume expands to nearly twice that of virgin rats (Barron *et al.*, 1985; Barron *et al.*, 1984; Durr *et al.*, 1981). These changes are maintained by a decrease in the osmotic threshold for drinking and for the secretion of vasopressin (Barron *et al.*, 1985; Durr *et al.*, 1981; Koehler *et al.*, 1993). Although the set-point for osmoregulation is decreased and volume homeostasis is increased, there is no change in the sensitivity of the magnocellular neurosecretory system to osmotic stimulation in late pregnancy (Durr *et al.*, 1981; Koehler *et al.*, 1993). By contrast, the osmoresponsiveness of the oxytocin system in urethane-anaesthetised late pregnant rats has been reported to be reduced, since i.p. injection of similar amounts of hypertonic saline produced a large increase of plasma concentration of oxytocin in virgin rats, but not in late-pregnant rats (Russell *et al.*, 1992). It has also been proposed that, during late pregnancy, the osmotic sensitivity for oxytocin release is increased, since i.v. infusion of hypertonic saline after removal of opioid inhibition by naloxone produced a large increase of oxytocin release in pregnant rats compared to virgin rats (Russell *et al.*, 1992). Moreover, the studies of Kohler *et al.* (1993), using s.c. injection of hypertonic saline, indicate a decrease in the threshold for osmotic stimulation of oxytocin secretion in pregnancy. Therefore, the hypoosmolality, hyponatremia and hypervolaemia of pregnancy are perceived as normal by the vasopressin system enabling retention of water. For oxytocin, which has natriuretic actions, a reduced threshold implies stimulation of excretion of Na^+ at the lower osmolality, which would deplete body Na^+; whereas in pregnancy body fluid volume and Na^+ content are increased. However, the mechanisms responsible for the altered osmoregulation are obscure.

An endogenous opioid mechanism inhibiting oxytocin release appears to be active during late pregnancy, since administration of naloxone at this time increases the plasma concentration of oxytocin and decreases the oxytocin content of the posterior pituitary (Douglas *et al.*, 1993; Hartman *et al.*, 1986; Leng *et al.*, 1988).

However, the reduction of the oxytocin secretory response to osmotic stimulation in late-pregnant rats is independent of endogenous opioids, since naloxone enhanced the increase in plasma concentration of oxytocin in response to hypertonic saline more in virgin rats than in pregnant rats (Russell *et al.*, 1992). Naloxone also enhanced the release of oxytocin evoked by electrical stimulation of the region anterior and ventral to the third cerebral ventricle (AV3V region) more in virgin rats than in pregnant rats, suggesting a decreased excitatory coupling of the AV3V input to oxytocin neurones in pregnancy that was similarly independent of endogenous opioid peptides (Bull *et al.*, 1994). Thus, an increase in endogenous opioid inhibition of oxytocin secretion is not responsible for the reduction of osmoresponsiveness of oxytocin neurones in late pregnancy.

Changes in endogenous NOS in the magnocellular neurosecretory system have been reported in pregnancy, parturition and lactation. In late-pregnant and parturient rats, a decrease in the density and number of NADPH-diaphorase-positive cells in the SON and PVN together with a decrease in the specific activity of NOS in the posterior pituitary have been found (Okere and Higuchi, 1996a). By contrast, other authors have reported an increase in NADPH-diaphorase staining in the SON and PVN in late pregnancy (Popeski *et al.*, 1999; Woodside and Amir, 1996) that persists into lactation (Popeski *et al.*, 1999). Up-regulation of nNOS protein and nNOS mRNA expression in the hypothalamus was found on day 20 of pregnancy (Xu *et al.*, 1996). However, there was no change in NOS mRNA expression in the SON, PVN and lamina terminalis on day 15 of pregnancy (Luckman *et al.*, 1997). These observations suggest that the endogenous NOS system may play an important role in modulating the osmoregulation of the oxytocin neurosecretory system during pregnancy. Therefore, we tested the hypothesis that changes in the expression of NOS in oxytocin neurones contribute to the changes in the osmoresponsiveness of oxytocin neurones during pregnancy.

3.2 Aims

- To study the role of NO in osmoregulation of oxytocin release in urethane-anaesthetised virgin rats.
- Does the altered osmoresponsiveness of the oxytocin neurosecretory system in the pregnant rats reflect a change in the NOS system?
- To investigate the expression of the oxytocin and the neuronal NOS genes in the hypothalamic magnocellular neurones of rats during pregnancy and parturition.

3.3 Materials and methods

3.3.1 Animals

Experiments were carried out on age-matched female Sprague-Dawley rats (250-300 g). They were housed under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1°C) with free access to food and water. Pregnant rats were obtained by leaving virgin rats with stud males overnight. Day 0 of pregnancy was determined by the appearance of a vaginal plug of semen that had been shed into the mating cage on the following morning after mating. The rats were then housed individually with food and water *ad libitum*.

3.3.2 Experimental procedure

1. Effect of NOS inhibitor on hypertonic- and CCK-stimulated oxytocin release in virgin rats

On the day of the experiment, rats were anaesthetised with urethane to allow cannulation of a femoral vein and artery. The surgical methods are described in General Methods 2.2.

In the first series of experiments, rats were given either the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA, Sigma, 10 mg/kg, i.p.) or isotonic saline. In a second series

of experiments, rats were given either the NOS inhibitor N^{ω}-nitro-L-arginine methylester.HCl (L-NAME, Sigma, 50 mg/kg, i.p.) or isotonic saline. Both L-NNA and L-NAME are L-arginine analogues that are competitive with L-arginine in binding the arginine-binding site of the constitutive NOS. After 4 h, all rats were intravenously injected with cholecystokinin (CCK, 20 μg/kg, i.v., cholecystokinin-(26-33)-sulphated; Bachem Ltd, Saffron Walden, Essex, UK) followed by intraperitoneal injection of hypertonic saline (4 ml/kg 1.5 M NaCl, i.p.). Blood samples were collected from a femoral artery cannula before and after CCK administration and hypertonic saline injection and immediately centrifuged. Plasma was then separated and stored at -20°C until assayed for oxytocin by specific RIA as described previously in General Methods 2.6. The remaining blood cells were resuspended in isotonic saline at the same volume as the plasma taken and returned *via* the left femoral vein. Plasma sodium ($[Na^+]$) concentration in the first and the last plasma samples was determined using a Corning 455 flame photometer.

2. Effect of central administration of a NO donor on oxytocin release in virgin rats

A femoral vein and artery of urethane-anaesthetised virgin rats were cannulated. A 22-gauge stainless steel guide cannula was placed into the right lateral cerebral ventricle, at the following coordinates: 1.6 mm lateral, 0.6 mm caudal from Bregma and 4.5 mm below surface of the skull. The guide cannula was held in place with two stainless screws and dental cement. The cap was inserted into the guide cannula until the start of the experiment. Following surgery, all rats were left for at least 2 h before starting of the experiment. For full details of surgery refer to General Methods 2.3.

Before the experiment, the infusion cannula was introduced into the lateral cerebral ventricle *via* the guide cannula. The infusion cannula was connected to a polythene tube attached to a microsyringe filled either with the NO donor sodium nitroprusside (SNP, Sigma, 10 nmol/ml) or isotonic saline.

Rats were given an i.c.v infusion of 25 nmol SNP (10 nmol/μl) or isotonic saline (5 μl at 2 μl/min followed by 20 μl at 0.7 μl/min) immediately after a hypertonic saline injection (4ml/kg 1.5 M NaCl, i.p.). Blood samples were taken from the left femoral artery before and at regular intervals (10 min) during i.c.v. infusion of SNP or isotonic saline. Blood samples were immediately centrifuged. Plasma was then separated and stored at -20°C until assayed for oxytocin by specific RIA and measurement of plasma sodium concentration.

3. The effect of NOS inhibitor on hypertonic saline-stimulated oxytocin release in late pregnancy

A femoral vein and artery of urethane-anaesthetised virgin and 21 day pregnant rats were cannulated. They were given the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA, Sigma, 10 mg/kg, i.p.) or isotonic saline. After 4 h the rats received hypertonic saline (virgin: 4 ml/kg 1.5 M NaCl, i.p., pregnant: 5 ml/kg 2 M NaCl, i.p.). The volume of hypertonic saline given to late pregnant rats was greater than virgin rats in order to produce a similar plasma [Na^+] between virgin and late pregnant rats since the reduced plasma [Na^+] is found during pregnancy. Intravenous (i.v.) naloxone (Sigma, 5 mg/kg) was given 40 min after the hypertonic saline injection. Serial plasma samples were collected from the femoral artery cannula before and after hypertonic saline and naloxone administration (to investigate the relationship between the mechanisms of endogenous opioids and nitric oxide), and stored at -20°C until assayed for oxytocin by specific RIA and measurement of plasma sodium concentration.

4. The effect of NOS inhibitor on oxytocin release following intravenous infusion of hypertonic saline in late pregnancy

On the experimental day, the left femoral artery and both femoral veins of virgin and day 21 pregnant rats were cannulated under urethane anaesthesia. All rats received either N^{ω}-nitro-L-arginine (L-NNA, Sigma, 10 mg/kg i.p.) or isotonic saline

solution 4 h before blood sampling. Hypertonic saline (2.0 ml) was infused through the right femoral vein for 1 h in virgin and pregnant rats (2.0 M NaCl and 2.6 M NaCl, respectively). Serial plasma samples were collected from the left femoral artery before and at regular intervals (10 min) during the infusion, and stored at -20°C until assayed for oxytocin by specific RIA and measurement of plasma sodium concentration.

5. NOS gene expression in the SON and PVN in pregnancy

Age-matched virgin, 16 day pregnant, 22 day pregnant and parturient rats were used to study nNOS mRNA expression. Rats were killed by decapitation in the morning for virgin, day 16 and day 22 of pregnancy. Parturient rats were decapitated 2 h after the birth of the first pup. Their brains were rapidly removed and frozen on dry ice, stored at -70°C and later processed for study of the expression of nNOS mRNA by using radioactive *in situ* hybridization histochemistry.

6. Oxytocin gene expression in the SON and PVN in pregnancy

To study the expression of oxytocin mRNA, age-matched virgin, 22 day pregnant and parturient rats were used. Virgin and 22 day pregnant rats were killed by decapitation in the morning. Parturient rats were decapitated 2 h after the birth of the first pup. Their brains were rapidly removed and placed on dry ice, stored at -70°C and later processed for study of the expression of oxytocin mRNA by using non-radioactive *in situ* hybridization histochemistry.

3.3.3 Radioimmunoassay

The specific radioimmunoassay for oxytocin is described in General Methods 2.6.

On the day of assay, plasma samples were thawed and centrifuged. Specific anti-oxytocin antiserum (Higuchi *et al.*, 1985) was added to triplicate aliquots of standards (ranging from 2.4 to 2500 pg/ml) and duplicate aliquots of plasma samples. Mixtures were left for 24 h before adding a fixed amount of ^{125}I-oxytocin (between assayed, approximately 6-8,000 cpm) to all tubes. Mixtures were left for 2-3 days. To precipitate the first antibody-^{125}I-oxytocin complex, second antibody (Donkey anti-rabbit gammaglobulin) was added to all tubes and left for 24 h. To aid visualization of the precipitate standardized Pansorbin cells (Novabiochem (U.K.) Ltd., 0.1% w/v solution in phosphate buffer) was added to mixtures prior to centrifugation. Supernatants were then aspirated. The remaining precipitates were counted in a gamma counter (LKB-Wallace 1272 Clinigamma). All samples in each experiment were measured in a single assay in order to avoid interassay variance.

The concentration of oxytocin in the standard or plasma sample was inversely proportional to the amount of ^{125}I-oxytocin bound to the rabbit anti-oxytocin antibody. Oxytocin concentration of the unknown samples could be determined by comparing the unknown precipitate radioactivity to precipitate radioactivity of known standard oxytocin contents.

3.3.4 *In situ* Hybridization

The full methodology for *in situ* hybridization is described in General Methods 2.8.

Brain sections

Coronal sections (15 μm) through the hypothalamus at the level of the SON and PVN were cut using a cryostat and thaw-mounted onto gelatin-coated glass slides. Slides were then stored in desiccated slide boxes at -70°C before *in situ* hybridization.

Probes

Three 45-mer antisense oligonucleotide probes for NOS which were complementary to bases 223-267 (5′-noncoding region), 4714-4758 (3′-noncoding region) and 1662-1706 of the rat neuronal NOS (nNOS) sequence (Bredt *et al.*, 1991b) were used. Probes were labelled at the 3′ end with [α-^{35}S]deoxy-ATP (NEN) using terminal deoxynucleotidyltransferase (Pharmacia).

An antisense oligonucleotide probe for oxytocin which was complementary to bases 912-936 of oxytocin mRNA (Ivell and Richter, 1984) was used. The probe was labelled by 3′ end tailing with DIG-dUTP using terminal transferase (Boehringer Mannheim).

In situ hybridization

Sections were fixed at room temperature with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min, then rinsed in 0.1 M PBS and washed in 0.1 M PBS (10 min), followed by acetylation in triethanolamine solution (0.25% acetic anhydride in 0.1 M triethanolamine -0.15 M NaCl) for 10 min to increase tissue permeability. The sections were then dehydrated through 70%, 80%, 95% and 100% ethanol (5 min each), delipidated in chloroform for 10 min, and partially rehydrated in 95% ethanol (10 min). After air-drying the slides were placed in the humidified chamber.

Radioactive *in situ* hybridization

The sections were hybridized for 17 h at 37°C in hybridization buffer (4xSSC, 50% formamide, 1xDenhardt's solution, 500 µg/ml sheared salmon sperm DNA, 10% dextran sulphate and 0.3% mercaptoethanol) containing the ^{35}S-labelled NOS oligonucleotide probes at a concentration of 2500 dpm/µl. After hybridization, the excess solution was drained. The slides were briefly rinsed three times in 1xSSC at room temperature, washed three times for 30 min 1xSSC and were washed for a

further 1 h in 1xSSC at room temperature. The slides were dipped in 300 mM ammonium acetate, 70% ethanol and then air-dried. The slides were then placed in autoradiographic cassettes and exposed to Hyperfilm-βmax autoradiography film (Amersham, UK) together with brain paste standards. The film was left to expose for 3 weeks at 4°C. The films were developed in D19 developer (Kodak) for 5 min, then fixed (Ilford Hypam rapid fixer, 2x5 min). After that, the sections were dipped in Ilford G-5 emulsion-coated slides (Mobberley) diluted 1:1 with autoclaved water. The emulsion-coated slides were exposed for a further 4-5 weeks to visualize NOS cellular signal, and then developed (Kodak D19 developer) for 5 min and fixed (Ilford Hypam rapid fixer twice 5 min each). The developed slides were counterstained with cresyl violet, dehydrated in a graded ethanol series, and coverslipped in DPX mountant.

Non-radioactive *in situ* hybridization

The sections were prehybridized with prehybridization buffer for 1 h at 37°C and then hybridized for 17 h at 37°C in hybridization buffer containing the DIG-labelled oxytocin probes at a concentration of 200 fmol/μl. After hybridization, the excess solution was drained. The slides were briefly rinsed three times in 1xSSC at room temperature, washed three times for 30 min 1xSSC and were washed for a further 1 h in 1xSSC at room temperature. The slides were taken from the final 1xSSC into buffer 1 (100 mM Tris HCl (pH 7.5), 150 mM NaCl) for 10 min (×2) and then incubated with blocking buffer (buffer 1 containing 0.1% Triton® X-100 and 2% normal sheep serum) for 30 min. The sections were incubated overnight with anti-DIG antibody solution (1:500). After incubation, the sections were washed with buffer 1 (2×10 min), followed by washing in buffer 2 (100 mM Tris HCl (pH9.5), 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. The sections were then incubated overnight in the dark with colour substrate solution (buffer 2 containing 340 μg/ml nitroblue tetrasolium and 170 μg/ml BCIP). The colour reaction was stopped by incubating the slides in buffer 3 (10 mM Tris HCl (pH 8.1), 1 mM EDTA) and then

briefly dipped in distilled water. The slides were then mounted using Aqua-Mount (BDH).

Double *in situ* hybridization

The sections were prehybridized with prehybridization buffer for 1 h at 37°C and then hybridized for 17 h at 37°C in hybridization buffer containing the DIG-labelled oxytocin probes at a concentration of 200 fmol/μl and the ^{35}S-labelled NOS oligonucleotide probes at a concentration of 2500 dpm/μl. After hybridization, the excess solution was drained. The slides were briefly rinsed three times in 1xSSC at room temperature, washed three times for 30 min 1xSSC and were washed for a further 1 h in 1xSSC at room temperature. The slides were then processed for non-radioactive ISH followed by radioactive ISH. The slides were taken from the final 1xSSC into buffer 1 for 10 min (×2) and then incubated with blocking buffer for 30 min. The sections were incubated overnight with anti-DIG antibody solution (1:500). After incubation, the sections were washed with buffer 1 (2×10 min), followed by washing in buffer 2 for 10 min. The sections were then incubated overnight in the dark with colour substrate solution. The colour reaction was stopped by incubating the slides in buffer 3, briefly dipped in distilled water and then air-dried. The slides were then placed in autoradiographic cassettes and exposed to Hyperfilm-βmax autoradiography film (Amersham, UK) together with brain paste standards. The film was left to expose for 3 weeks at 4°C. The films were developed in D19 developer (Kodak) for 5 min, then fixed (Ilford Hypam rapid fixer, 2x5 min). After that, the sections were dipped in Ilford G-5 emulsion-coated slides (Mobberley) diluted 1:1 with autoclaved water. The emulsion-coated slides were exposed for a further 4-5 weeks to visualize NOS cellular signal, and then developed (Kodak D19 developer) for 5 min and fixed (Ilford Hypam rapid fixer twice 5 min each). The developed slides were then washed in distilled water and mounted using Aqua-Mount (BDH).

Quantitative Analysis

The amount of nNOS mRNA in the SON and PVN was assessed by silver grain density of autoradiographic films of the SON, PVN, SFO and AMG viewed under the light microscope, ×5 and ×10 objective. Grain density was quantified by NIH Image analysis system version 1.58 on an Apple McIntosh computer. The value of silver grain density per unit area was obtained by subtracting background measurements over adjacent tissue from each specific tissue measurement, and dividing by the area measurements taken. Each brain paste standard was measured twice, and also corresponding background measurements. A logarithmic relationship was then plotted for radioactivity against the grain density of the standards. Acceptable grain density values lay on the straight portion of the curve. For each identified brain area, the mean grain density per unit area for each rat was calculated and the group mean grain density per unit area calculated.

Specific radioactivity of labelled probes was ranged from 482.25 to 1753.13 Ci/mmol.

3.3.5 Statistical Analysis

All data are reported as means ± S.E.M.. Data were analyzed statistically using SigmaStat by a one-way repeated measures ANOVA for differences with time and a two-way repeated measures ANOVA for differences between groups followed by Dunnett's method, Bonferroni's method or Student-Newman-Keuls method. To compare data between and after treatment within a group the paired *t*-test was used. Differences between groups were compared using one way ANOVA followed by Student-Newman-Keuls method. $P < 0.05$ was considered statistically significant.

3.4 Results

1. Effect of NOS inhibitors on hypertonic saline- and CCK-stimulated oxytocin release in virgin rats

In two series of experiments, rats received either the NOS inhibitor (L-NNA or L-NAME) or isotonic saline followed by injections of CCK and hypertonic saline. There was no difference in the basal plasma [Na^+] or the basal plasma oxytocin concentrations between groups (Fig. 5, 6). Therefore, L-NNA and L-NAME had no significant effect on basal plasma [Na^+] or on the basal release of oxytocin.

A significant increase in the plasma concentration of oxytocin occurred in all rats 10 min after i.v. injection of CCK ($P<0.05$, Fig. 5, 6). In the first series of experiments, after CCK injection, plasma oxytocin concentrations of L-NNA pre-treated rats (n=8) and isotonic saline pre-treated rats (n=6) were increased by 30.15 ± 5.06 and 30.57 ± 6.14 pg/ml, respectively (not significantly different; Fig. 5). In a separate series of experiments, a similar increase of plasma oxytocin concentration after CCK injection was demonstrated. CCK increased the plasma oxytocin concentration by 28.72 ± 3.85 pg/ml in L-NAME pre-treated rats (n=8) and 19.02 ± 5.19 pg/ml in isotonic saline pre-treated rats (n=8), respectively (Fig. 6). Again, there was no significant difference in CCK-evoked oxytocin release between L-NAME pre-treated rats and isotonic saline pre-treated rats.

In all rats, injection of hypertonic saline produced a significant increase of plasma [Na^+] ($P<0.01$). There was no significant difference between rats pre-treated with isotonic saline and rats pre-treated with L-NNA or L-NAME following hypertonic saline administration (Fig. 5, 6). In the first series of experiments, plasma [Na^+] increased by 23.25 ± 4.57 mM (L-NNA pre-treated rats, n=8) and 21.00 ± 3.91 mM (isotonic pre-treated rats, n=6). In separate experiments, plasma [Na^+] increased by 5.13 ± 1.37 mM (L-NAME pre-treated rats, n=8) and 4.63 ± 1.17 mM (isotonic pre-treated rats, n=8).

A significant increase in the plasma concentration of oxytocin occurred in all rats 30 min after i.p. injection of hypertonic saline ($P<0.05$, Fig. 5, 6). In the first series of experiments, after hypertonic saline was administered, the plasma oxytocin concentrations in L-NNA pre-treated rats (n=8) and isotonic saline pre-treated rats (n=6) were increased by 190.46 ± 42.94 and 69.37 ±18.28 pg/ml, respectively. The oxytocin release following hypertonic saline was significantly greater in L-NNA pre-treated rats than in isotonic saline pre-treated rats ($P<0.05$, Fig. 5), despite similar increases in plasma [Na^+]. In a separate series of experiments, hypertonic saline increased the plasma oxytocin concentration by 490.76± 144.70 in L-NAME pre-treated rats (n=8) and by 163.68 ± 28.67 pg/ml in isotonic saline pre-treated rats (n=8), respectively. Again, in L-NAME pre-treated rats the osmotically-induced oxytocin release was significantly greater than in isotonic saline pre-treated rats ($P<0.05$, Fig. 6), despite similar increases in plasma [Na^+]. Thus, both of the NOS inhibitors (L-NNA and L-NAME) significantly potentiated the secretion of oxytocin induced by i.p. hypertonic saline, but had no significant effect on CCK-evoked oxytocin release.

2. Effect of central administration of a NO donor on oxytocin release in virgin rats

Urethane-anaesthetised rats were given an i.c.v infusion of 25 nmol SNP (10 nmol/μl) or isotonic saline (5 μl at 2 μl/min followed by 20 μl at 0.7 μl/min) immediately after a hypertonic saline injection (4ml/kg 1.5 M NaCl, i.p.).

There were no differences in the basal plasma [Na^+] and basal plasma oxytocin concentrations between groups. A significant increase in plasma [Na^+] upon hypertonic saline injection was shown in all rats ($P<0.01$, Fig. 7). Plasma [Na^+] increased by 11.17 ± 1.30 and 10.43 ± 2.69 mM in SNP-infused rats (n=6) and vehicle-infused rats (n=8), respectively; there was no significant difference in plasma [Na^+] between SNP-infused rats and vehicle-infused rats following hypertonic saline administration.

In all rats, i.p. injection of hypertonic saline induced a significant increase in the plasma concentration of oxytocin ($P<0.05$, Fig. 7). In vehicle-infused rats, the plasma concentration of oxytocin increased by 186.06 ± 45.00 pg/ml (n=8). In SNP-infused rats, the plasma concentration of oxytocin increased by 71.65 ± 27.33 pg/ml (n=6). However, this increase was significantly less in SNP infused rats than in vehicle-infused rats ($P<0.05$, Fig. 7), despite similar increases in plasma [Na^+]. Thus, centrally administration of the NO donor SNP significantly attenuated hypertonic saline-induced oxytocin release.

3. The effect of NOS inhibitor on hypertonic saline-stimulated oxytocin release in late pregnancy

Adult virgin rats and late-pregnant rats received either the NOS inhibitor (L-NNA) or isotonic saline. There was no difference in the basal plasma [Na^+] or the basal plasma oxytocin concentrations among the groups (Fig. 8, 9). Therefore, L-NNA had no effect on basal plasma [Na^+] or basal oxytocin secretion in either virgin or late-pregnant rats.

A significant increase in plasma [Na^+] following hypertonic saline injection was observed in all rats ($P<0.005$, *t*-test, Fig. 8, 9). The increase in plasma [Na^+] induced by i.p. injection of hypertonic saline in virgin rats pre-treated with L-NNA (7.60 ± 1.27 mM) or isotonic saline (10.10 ± 2.00 mM) was not significantly different from that in late-pregnant rats pre-treated with L-NNA (14.17 ± 1.31 mM) or isotonic saline (12.00 ± 2.03 mM).

In all rats, oxytocin release was significantly increased 30 min after i.p. injection of hypertonic saline. In virgin rats, this response was significantly greater in rats pre-treated with L-NNA than in rats pre-treated with isotonic saline ($P<0.05$, Fig. 8). The plasma concentration of oxytocin increased at 30 min by 57.75 ± 12.02 pg/ml and 30.93 ± 6.36 pg/ml in virgin rats pre-treated with L-NNA (n=13) or isotonic

saline (n=12), respectively. By contrast, in late-pregnant rats pre-treatment with L-NNA had no significant effect on hypertonic saline-induced oxytocin release (Fig. 9).

Intravenous injection of naloxone significant enhanced osmotically-induced oxytocin release in all rats. In virgin rats, this response was significantly greater in rats pre-treated with L-NNA than in rats pre-treated with isotonic saline ($P<0.05$, Fig. 8). The plasma concentration of oxytocin increased by 709.37 ± 85.99 pg/ml and 388.82 ± 95.86 pg/ml in virgin rats pre-treated with L-NNA (n=13) and virgin rats pre-treated with isotonic saline (n=12), respectively. By contrast, in late-pregnant rats pre-treatment with L-NNA had no significant effect compared to pre-treatment with isotonic saline; in these groups the plasma oxytocin concentration was increased by 658.15 ± 148.40 pg/ml and 556.54 ± 171.95 pg/ml, respectively (Fig. 9). Thus, in virgin rats but not in late pregnant rats, pre-treatment with L-NNA resulted in a significant enhancement of naloxone-evoked oxytocin release, following hyperosmotic stimulation.

4. The effect of NOS inhibitor on oxytocin release following intravenous infusion of hypertonic saline in late pregnancy

There were no differences in the basal plasma [Na^+] and basal plasma oxytocin concentrations between virgin and late-pregnant rats that received either L-NNA or isotonic saline (Fig. 10-12). Therefore, L-NNA had no effect on basal plasma [Na^+] and basal oxytocin secretion in either virgin or late-pregnant rats.

In all rats, plasma [Na^+] gradually increased throughout the course of the hypertonic saline i.v. infusion (Fig. 10, 11). A significant increase in plasma [Na^+] following hypertonic saline i.v. infusion was shown in all rats ($P<0.05$). The increases in plasma [Na^+] between the start and the end of the infusion were 25.75 ± 2.80 mM (virgin rats pre-treated with L-NNA, n=8), 21.25 ± 2.23 mM (virgin rats pre-treated with isotonic saline, n=8), 20.63 ± 1.78 mM (late-pregnant rats pre-treated with L-NNA, n=8) and 18.13 ± 1.76 mM (late-pregnant rats pre-treated with

isotonic saline, n=8) (Fig. 12B). There were no significant differences between groups.

In all rats, the release of oxytocin was increased significantly in parallel with the increase in plasma sodium concentration following hypertonic saline i.v. infusion (P<0.05, Fig. 10, 11). The increase of plasma oxytocin concentration in L-NNA treated virgin rats (673.98 ± 118.13 pg/ml, n=8) was significantly greater than in isotonic saline treated virgin rats (408.03 ± 79.66 pg/ml, n=8) following i.v. infusion of hypertonic saline for 50 min (P<0.05, Fig. 10). By contrast, in late-pregnant rats, pre-treatment with L-NNA had no significant effect on oxytocin release in response to i.v. infusion of hypertonic saline. (Fig. 11). The differences in plasma concentrations of oxytocin following i.v. infusion of hypertonic saline between pre-treatment with L-NNA and isotonic saline for virgin and pregnant rats are shown in Fig. 12A. In virgin rats, but not in pregnant rats, L-NNA pre-treatment resulted in a further increase in Δ plasma oxytocin concentration in parallel with the increase in plasma sodium concentration following i.v. infusion of hypertonic saline.

5. NOS gene expression in the SON and PVN in pregnancy

There were strong hybridization signals for nNOS mRNA over the supraoptic nucleus (SON), magnocellular region of the paraventricular nucleus (PVN), subfornical organ (SFO) and medial amygdaloid nucleus (AMG). In the SON, a decreased nNOS mRNA signal was revealed (Fig. 14C). The silver grain density over the SON of 22-day pregnant rats was significantly less than in the other groups, with no differences for the PVN, SFO or AMG (Fig. 13A). Neuronal NOS mRNA expression measured as film density over the SON of 22-day pregnant rats (n=8) significantly less than in the virgin rats (n=8) and 16-day pregnant rats (n=8) by 24.25 % and 25.47 %, respectively (P<0.05, Fig. 13A). The grain density measurement of nNOS mRNA expression in the SON of parturient rats (n=9) was significantly greater than in the 22-day pregnant rats (n=8) by 23.61 % (P<0.05, Fig. 13A). By contrast, the nNOS mRNA signal was unchanged in the SON and in other

areas measured including the PVN, SFO and AMG of 16-day pregnant and parturient rats compared to virgin rats (Fig. 14-16).

6. Oxytocin gene expression in the SON and PVN in pregnancy

Oxytocin gene expression in the SON and PVN was demonstrated using non-radioactive *in situ* hybridization. The signal for oxytocin mRNA was densely located in the cytoplasm of neurones. Oxytocin mRNA containing neurones were markedly located at the dorsal cap of the SON (Fig. 17A, B, C). The distribution of the oxytocin gene expression in virgin, 22-day pregnant and parturient rats was evidently similar in the SON. In the PVN, oxytocin mRNA-containing neurones were clearly shown in the magnocellular part of the PVN (Fig. 17D, E, F) and the expression pattern of the oxytocin gene in virgin, 22-day pregnant and parturient rats was evidently similar. In the SON, double *in situ* hybridization revealed that nNOS mRNA was present in both oxytocin and vasopressin neurones (Fig. 18). However, the results are not consistent so we are unable to quantify the expression of nNOS mRNA in oxytocin neurones compared to vasopressin neurones in different stages of pregnancy.

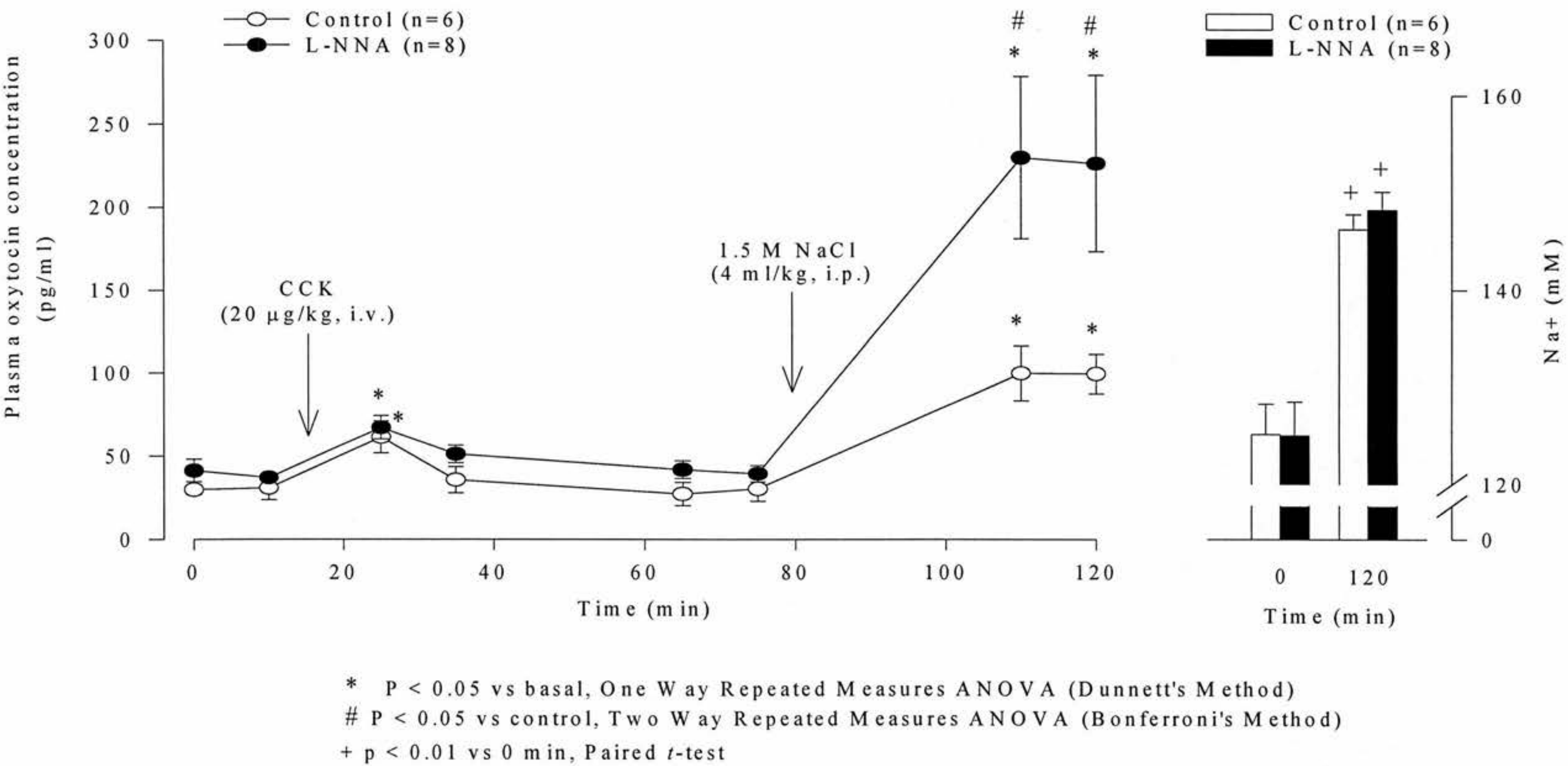

Figure 5. Plasma oxytocin concentration in response to CCK and hypertonic saline injection in N^{ω}-nitro-L-arginine (L-NNA) treated (4 h before time 0) and control virgin rats. Values are means ± SEM. Note that L-NNA significantly enhanced plasma oxytocin concentration in response to hypertonic saline injection (left), while plasma $[Na^{+}]$ increased similarly in the two groups (right).

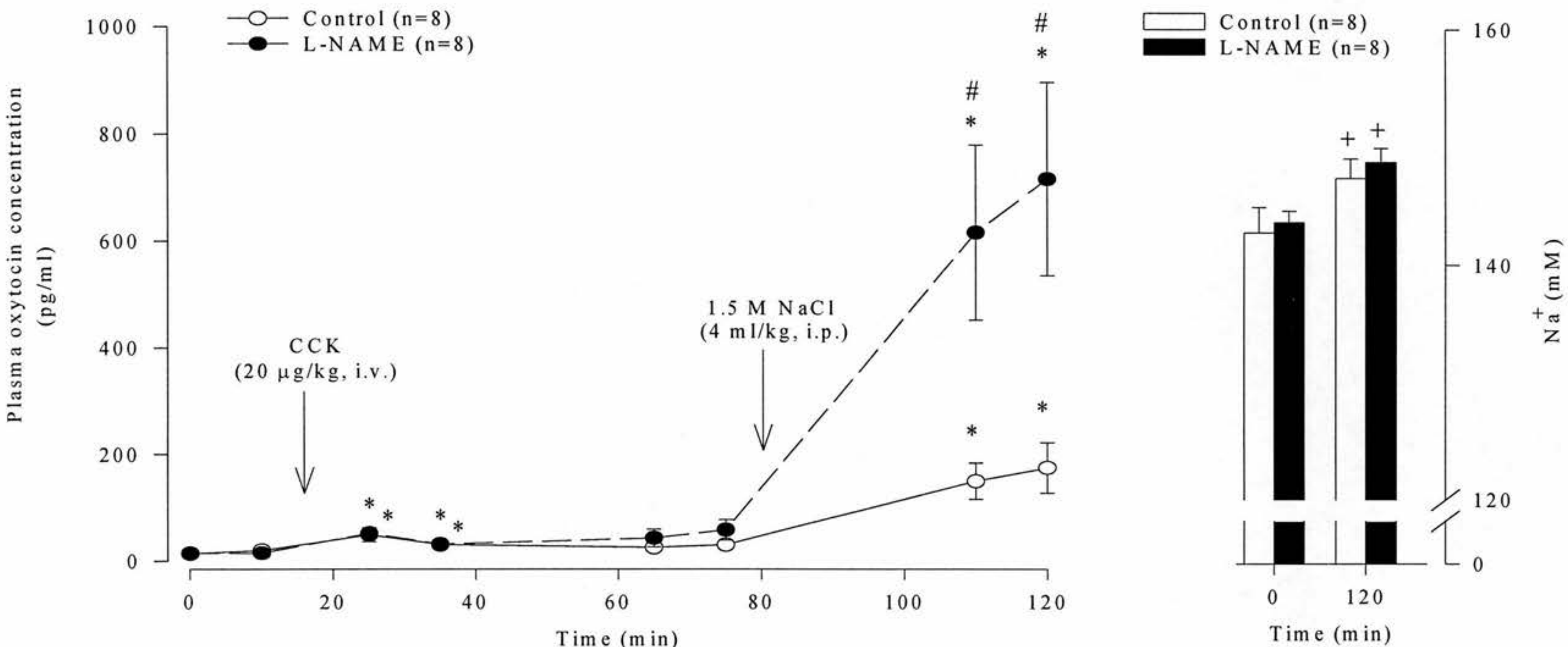

Figure 6. Plasma oxytocin concentration in response to CCK and hypertonic saline injection in N^{ω}-nitro-L-arginine methylester (L-NAME) treated (4 h before time 0) and control virgin rats. Values are means ± SEM. Note that L-NAME significantly enhanced plasma oxytocin concentration in response to hypertonic saline injection (left), while plasma [Na^{+}] increased similarly in the two groups (right).

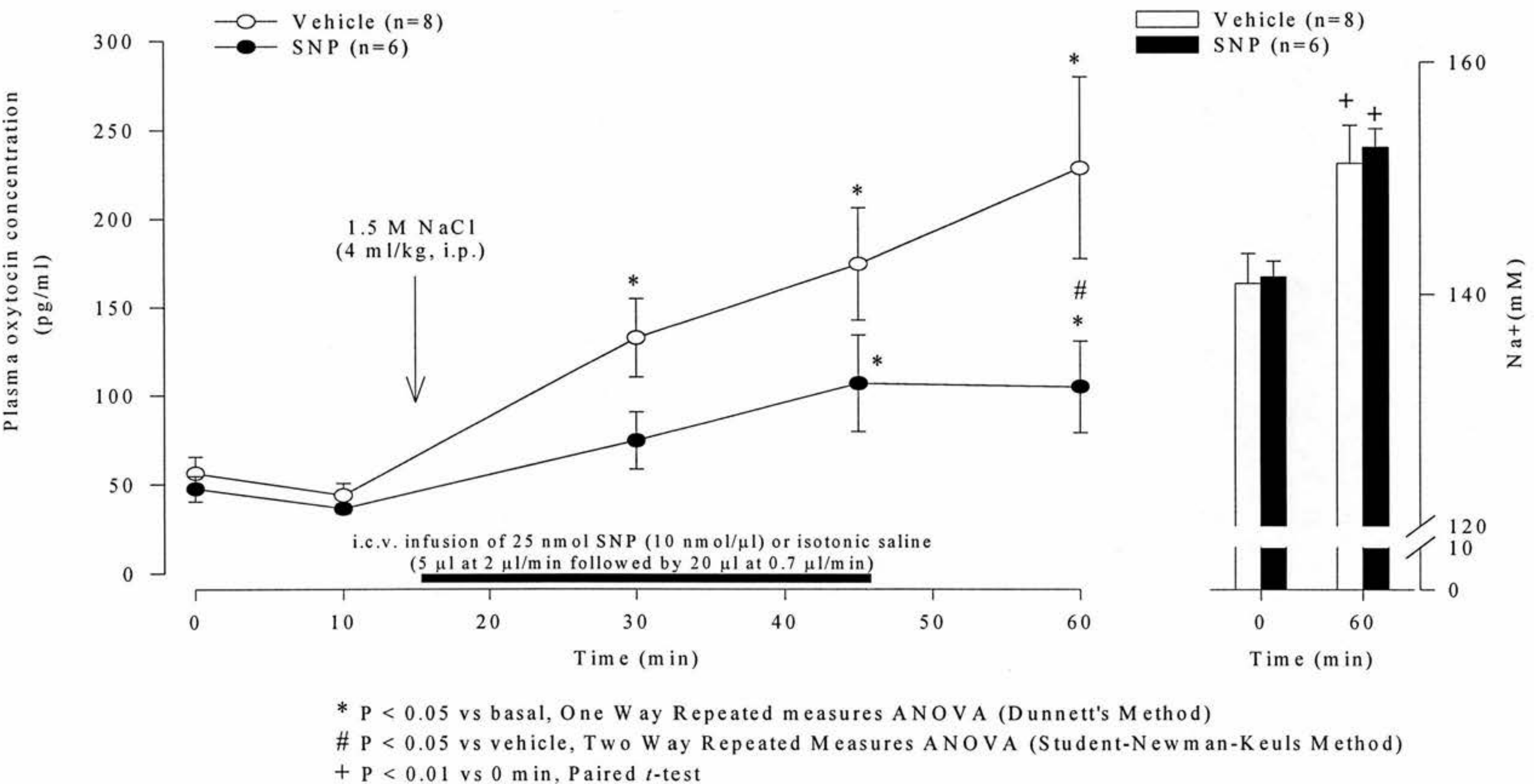

Figure 7. Plasma oxytocin concentration in response to i.c.v. administration of SNP and vehicle in hypertonic saline treated virgin rats. Values are means ± SEM. Note that SNP significantly decreased plasma oxytocin concentration in response to hypertonic saline injection (left), while plasma [Na^+] increased similarly in the two groups (right).

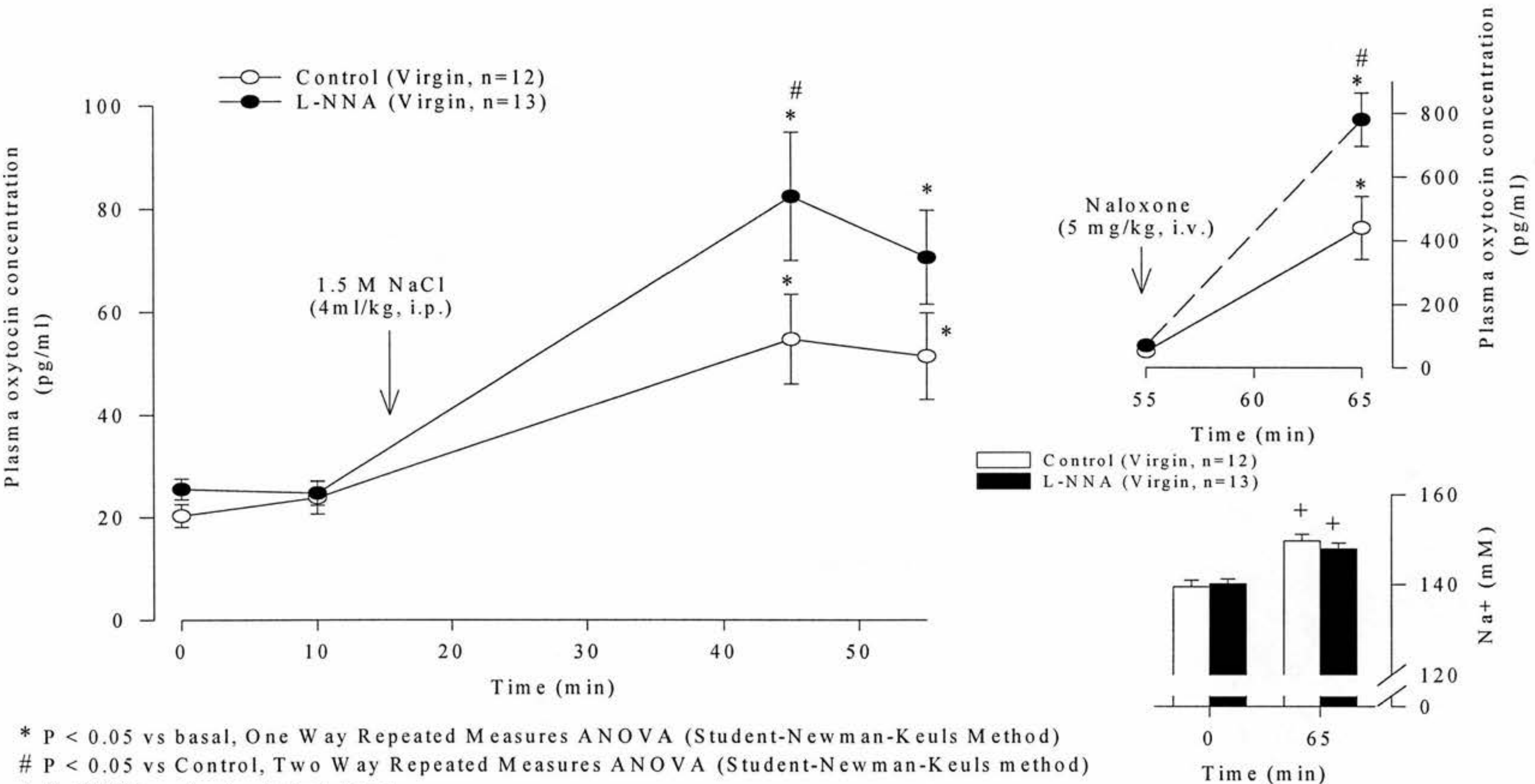

Figure 8. Plasma oxytocin concentration in response to hypertonic saline injection in virgin rats pre-treated with either N^{ω}-nitro-L-arginine (L-NNA) or isotonic saline. Values are means ± SEM. Note that significant enhancement of plasma oxytocin concentration in response to hypertonic saline injection (left) and naloxone administration (upper right) was observed in virgin rats pre-treated with L-NNA compared to virgin rats pre-treated with isotonic saline, while plasma [Na^+] increased similarly in the two groups (bottom right).

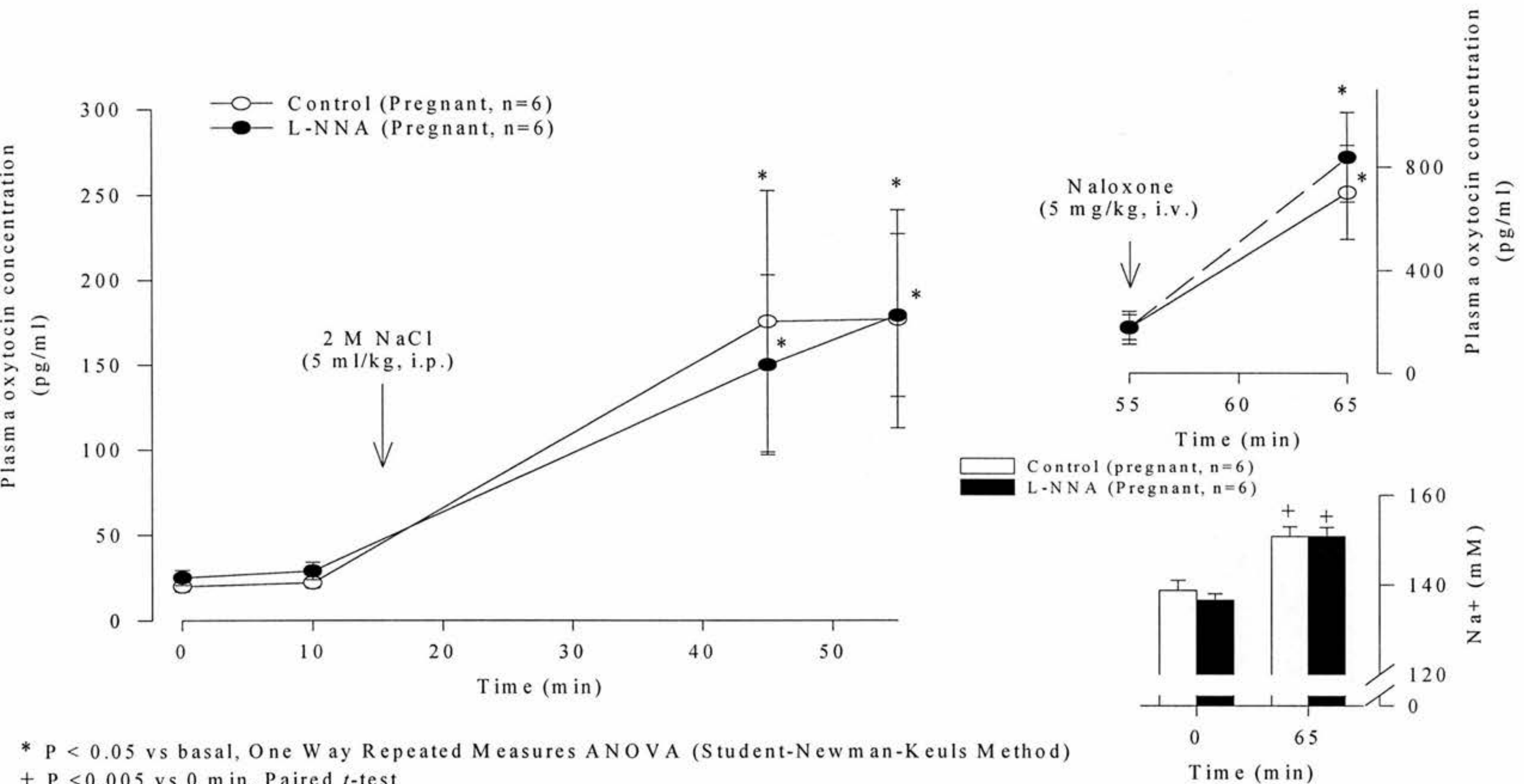

Figure 9. Plasma oxytocin concentration in response to hypertonic saline injection in late pregnant rats pre-treated with either N^{ω}-nitro-L-arginine (L-NNA) or isotonic saline. Values are means ± SEM. Note that there was no significant difference in plasma oxytocin concentration in response to hypertonic saline injection (left) and naloxone administration (upper right) between late pregnant rats pre-treated with L-NNA and isotonic saline. Plasma $[Na^+]$ increased similarly in the two groups (bottom right).

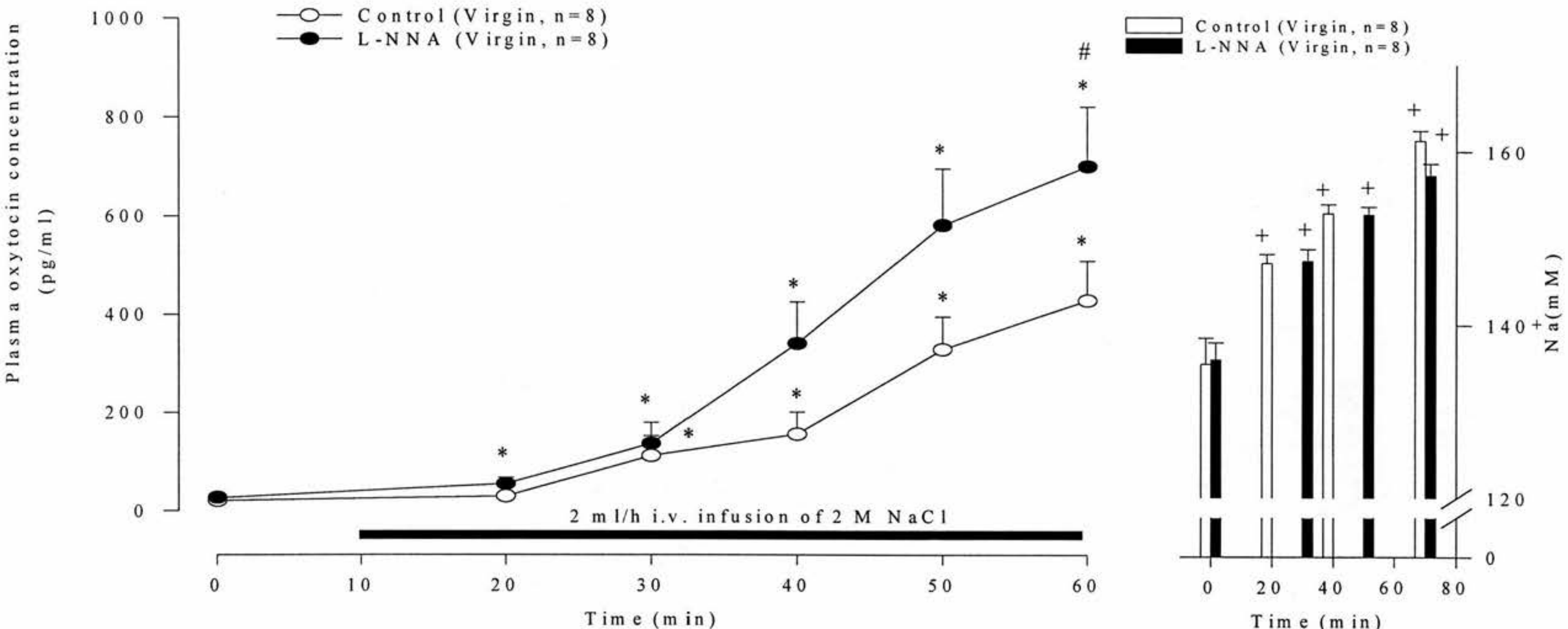

Figure 10. Plasma oxytocin concentration in response to isotonic saline or N^{ω}-nitro-L-arginine (L-NNA) in hypertonic saline infused (i.v.) virgin rats. A significant enhancement of plasma oxytocin concentration in response to i.v. infusion of 2 M hypertonic saline (at a rate of 2 ml/h) was observed in virgin rats pre-treated with L-NNA compared to virgin rats pre-treated with isotonic saline (left). An increase in plasma $[Na^+]$ following i.v. infusion of hypertonic saline was measured in all rats and there was no significant difference between groups (right). Values are means ± SEM.

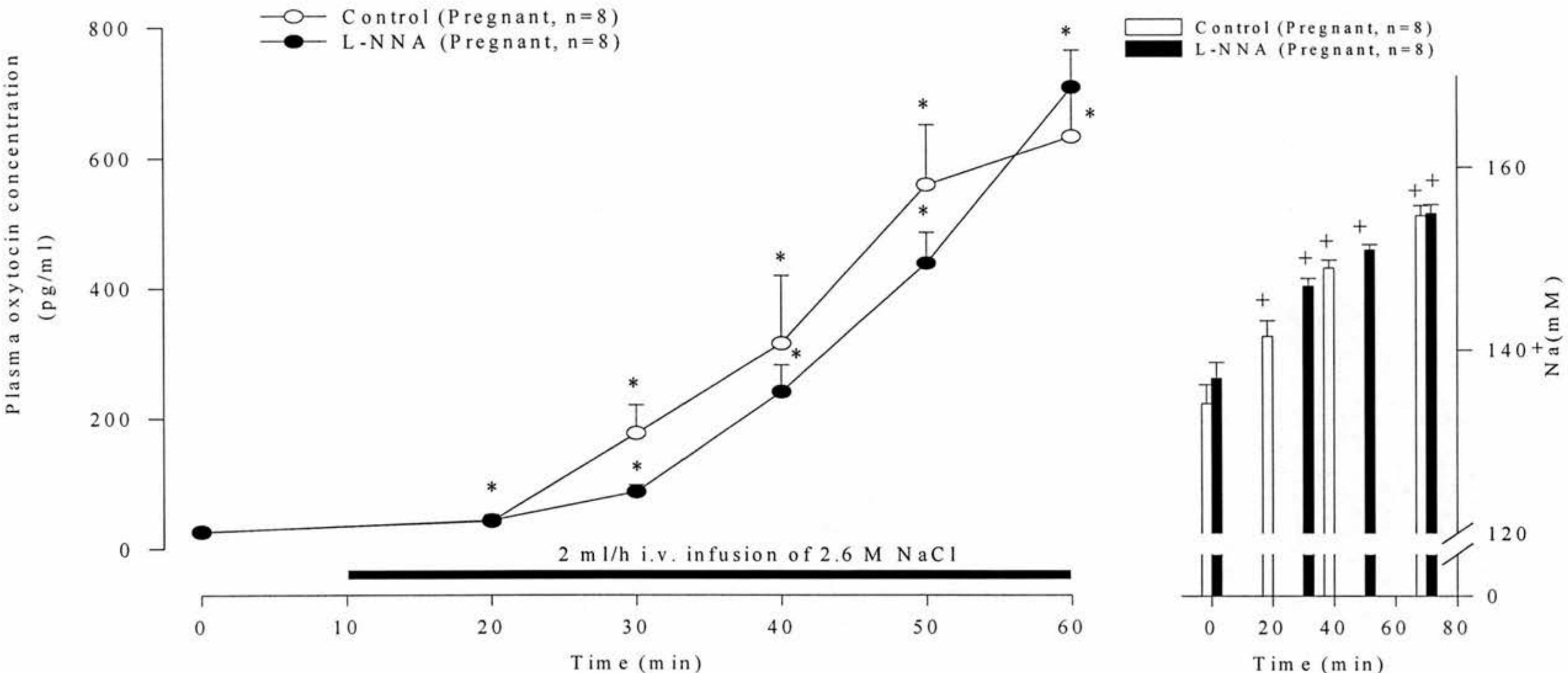

Figure 11. Plasma oxytocin concentration in response to isotonic saline or N^{ω}-nitro-L-arginine (L-NNA) in hypertonic saline infused (i.v.) pregnant rats. There was no significant difference in plasma oxytocin concentration in response to i.v. infusion of 2.6 M hypertonic saline (at a rate of 2ml/h) between late pregnant rats pre-treated with L-NNA and isotonic saline (left). An increase in plasma [Na^+] following i.v. infusion of hypertonic saline was measured in all rats and there was no significant difference between groups (right). Values are means ± SEM.

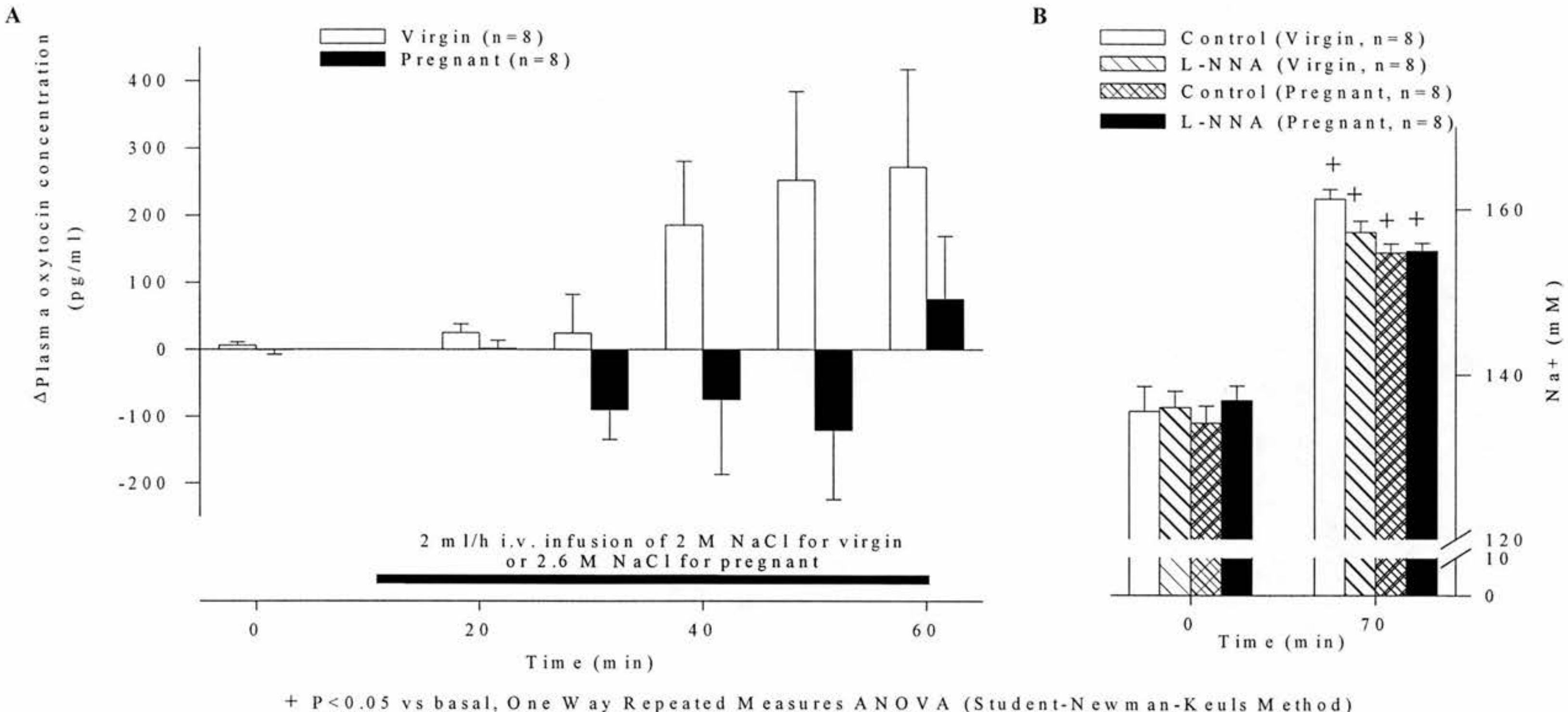

Figure 12. Differences in plasma oxytocin concentrations following hypertonic saline infusion (i.v.) between pre-treatment with N^{ω}-nitro-L-arginine (L-NNA) and isotonic saline in virgin and late pregnant rats. Values for Δ plasma oxytocin concentration are means ± SEM differences between L-NNA and isotonic saline pre-treatment. In virgin but not in late pregnant rats, an increase in Δ plasma oxytocin concentration following L-NNA and i.v. infusion of hypertonic saline was observed (A). An increase in plasma [Na^+] following i.v. infusion of hypertonic saline was measured in all rats and there was no significant difference between groups (B). Values for plasma [Na^+] are means ± SEM.

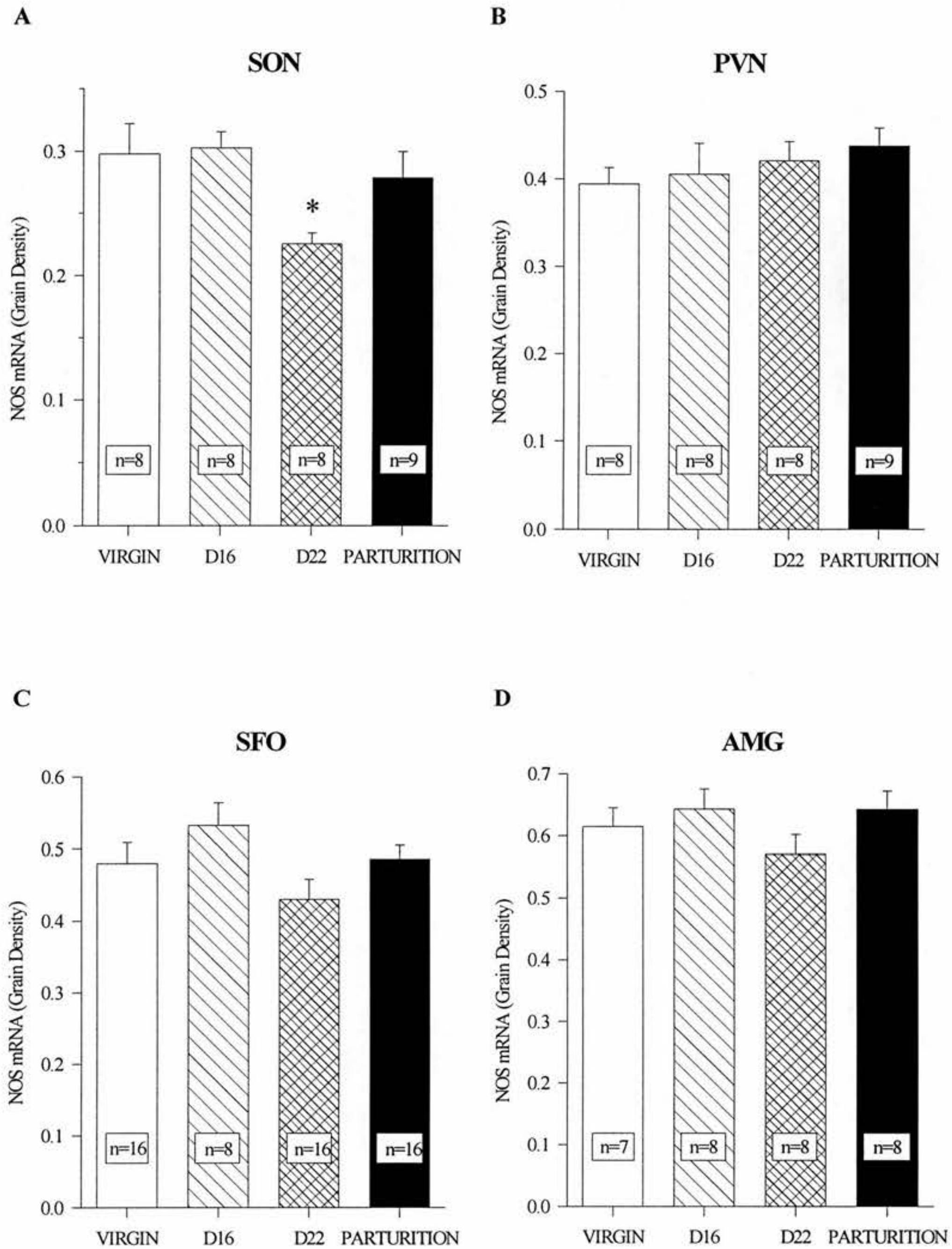

Figure 13. Neuronal NOS mRNA expression measured as film grain density over the SON, PVN, SFO and AMG. A significant decrease in nNOS mRNA expression was observed in late pregnant rats (day 22) compared to virgin rats in the SON (**A**) (*, P<0.05, One Way ANOVA; Dunn's Method). There was no significant change in NOS mRNA expression in the PVN (**B**), SFO (**C**) and AMG (**D**). Values are means ± SEM.

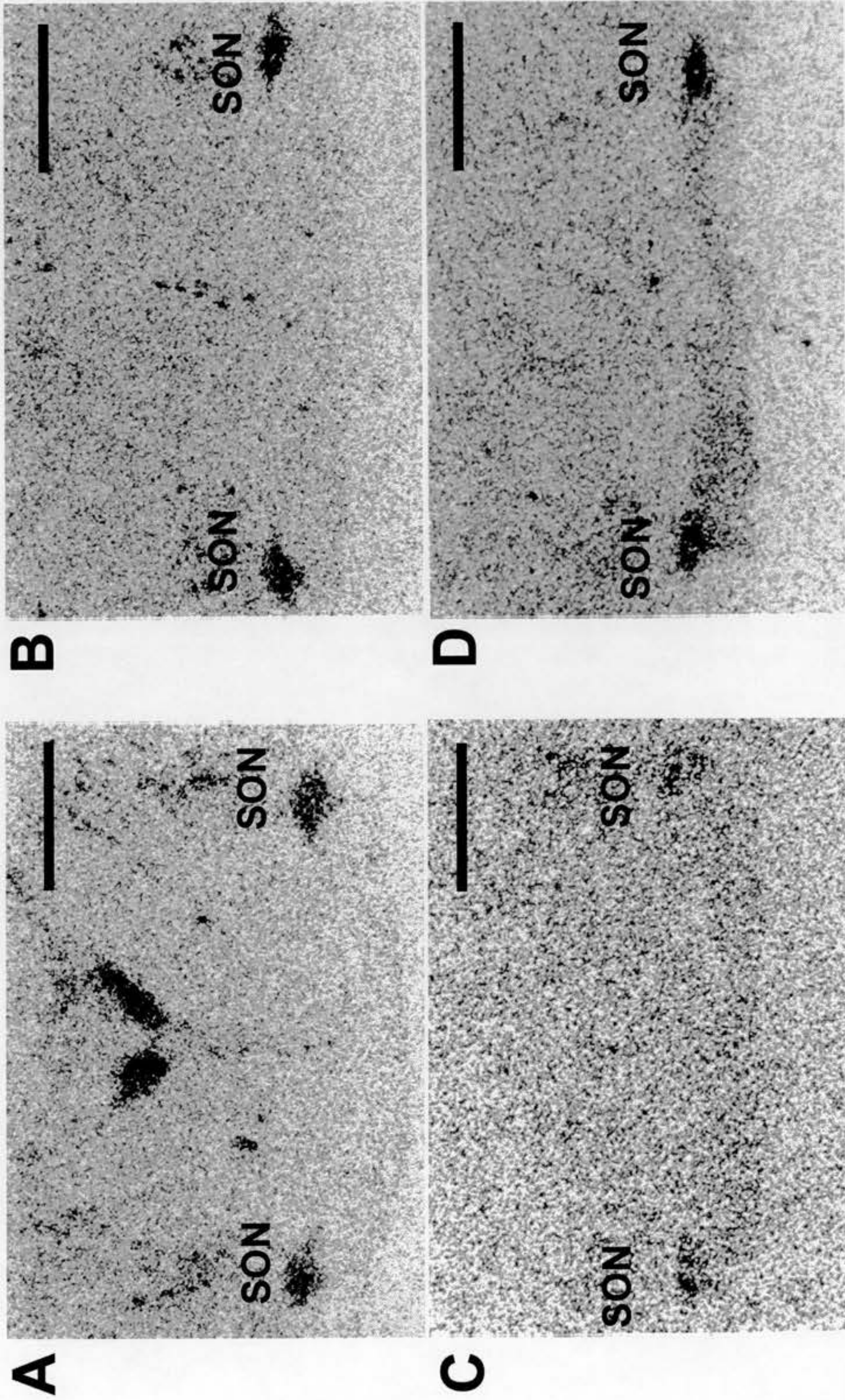

Figure 14. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA. NOS signal in the SON of 22 day pregnant rats **(C)** was lower than in virgin **(A)**, 16 day pregnant **(B)** and parturient rats **(D)**. SON; supraoptic nucleus. Bar=1mm.

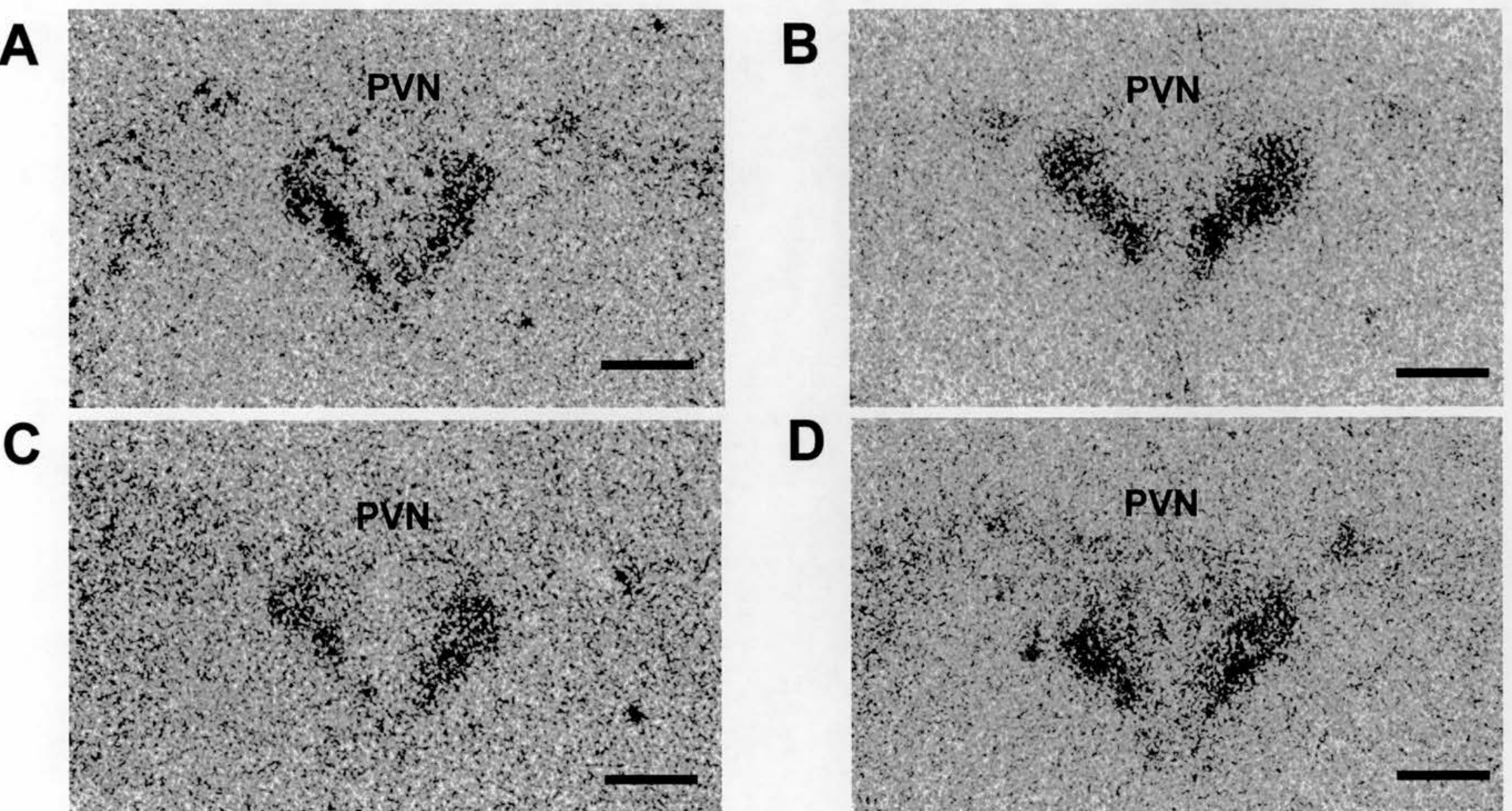

Figure 15. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA. NOS signal in the PVN of 22 day pregnant rats (**C**) was similar to virgin (**A**), 16 day pregnant (**B**) and parturient rats (**D**). PVN; paraventricular nucleus. Bar=500 µm.

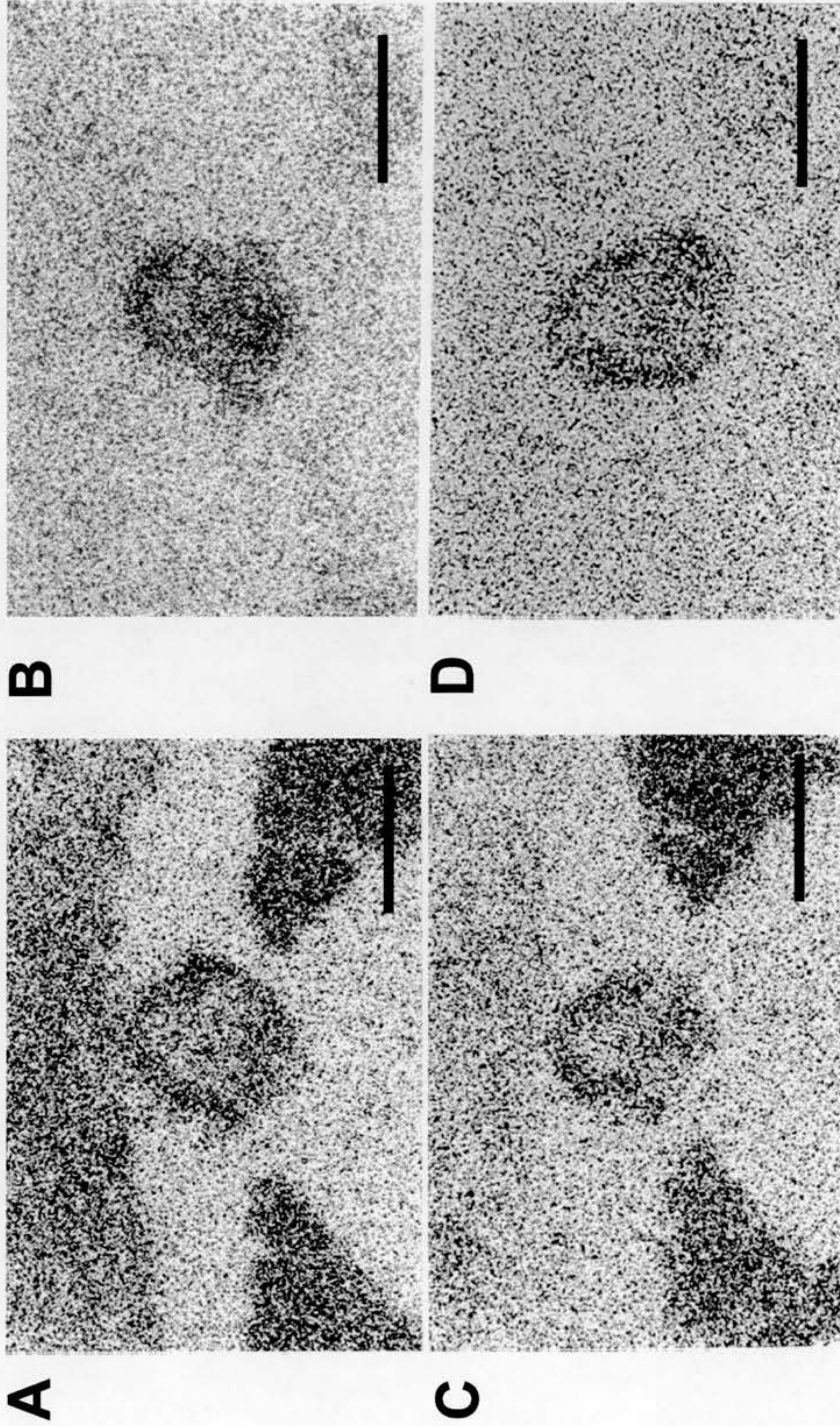

Figure 16. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA. NOS signal in the SFO of 22 day pregnant rats (**C**) was similar to virgin (**A**), 16 day pregnant (**B**) and parturient rats (**D**). Bar=500 µm.

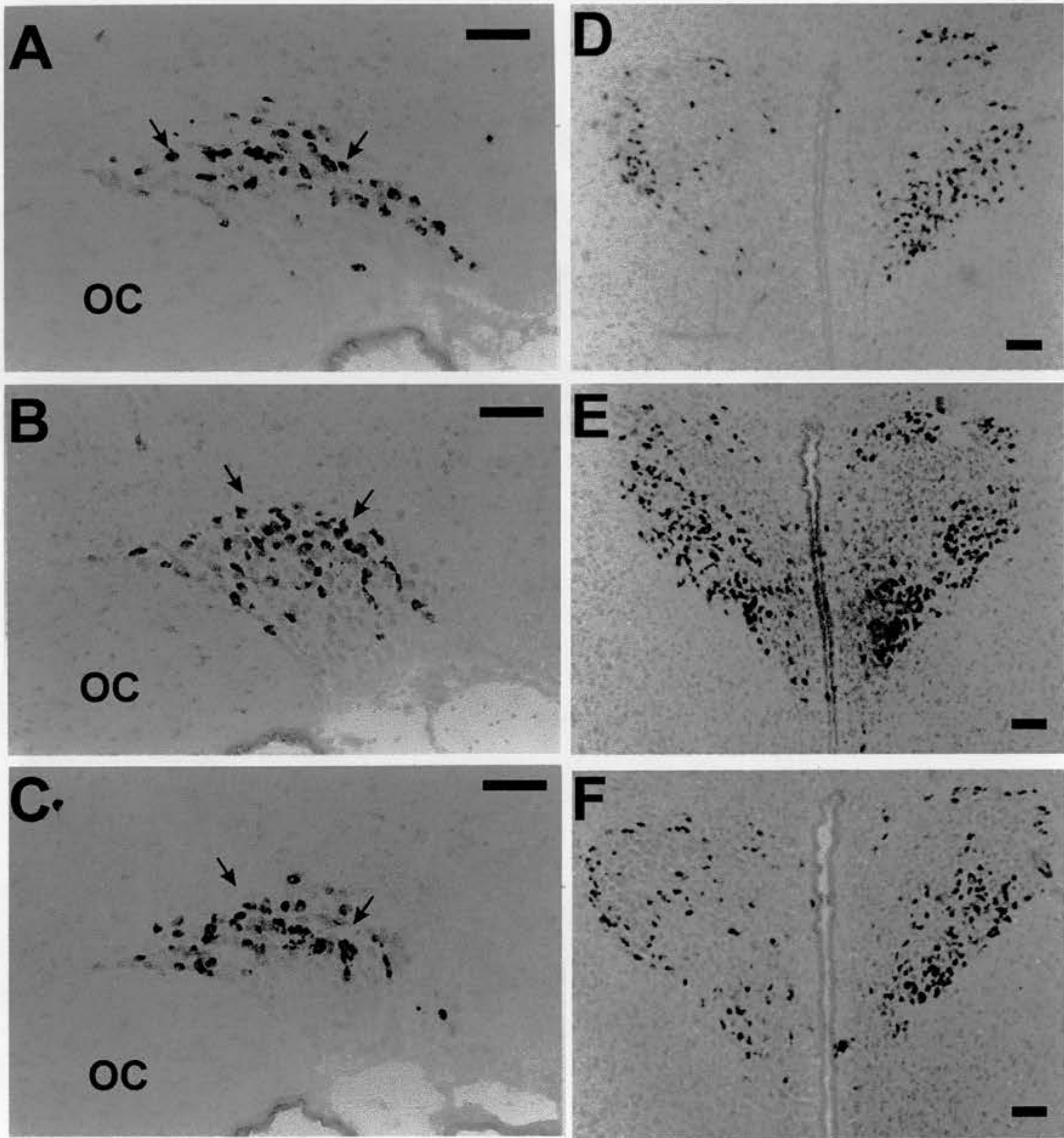

Figure 17. Photomicrographs showing oxytocin mRNA-containing neurones in the SON and PVN of virgin **(A, D)**, 22 day pregnant **(B, E)** and parturient rats **(C, F)**. Sections of the SON and PVN were hybridized with a digoxigenin-labelled probe against rat oxytocin mRNA. OC; optic chiasm, V3; third ventricle. Bar=100 μm.

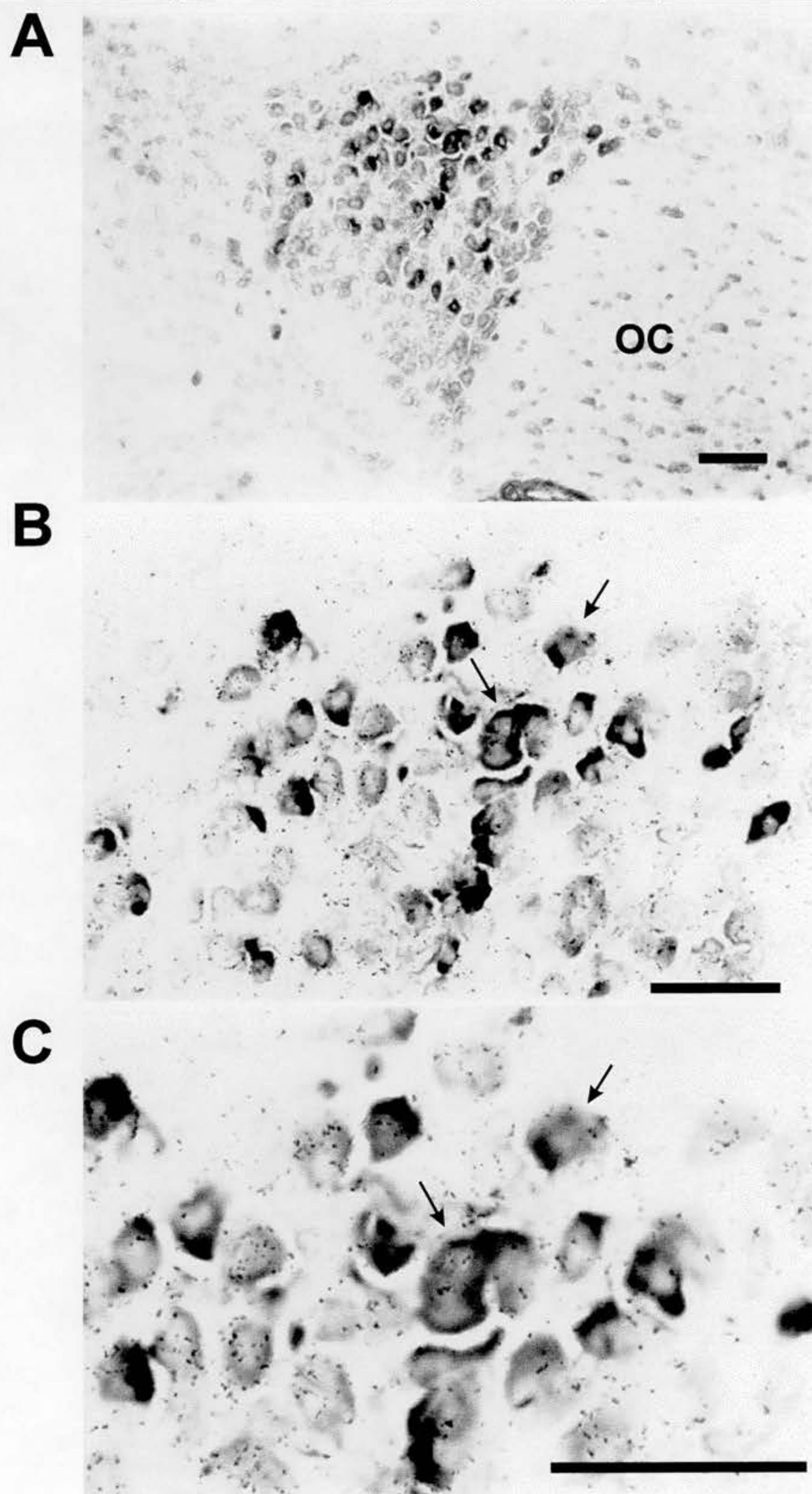

Figure 18. Photomicrographs showing double *in situ* hybridization using a radioactive labelled nNOS probe and a digoxigenin-labelled oxytocin probe. Oxytocin mRNA-containing neurones in the SON were shown in **A**. Double-labelled neurones (arrows) were shown in **B** and **C**. OC; optic chiasm. Bar=50 μm.

3.5 Discussion

The role of NO in osmoregulation of oxytocin release in urethane-anaesthetised virgin rats.

The present results demonstrated that the basal release of oxytocin and plasma [Na^+] were unaffected by systemic administration of either L-NNA or L-NAME in urethane-anaesthetised rats (Fig. 5, 6), suggesting that, at least under urethane anaesthesia, endogenous NO does not regulate the basal secretion of oxytocin. Our results are consistent with previous findings in conscious normally hydrated rats, in which systemic injection of L-NNA had no effect on the depletion of oxytocin and vasopressin from the posterior pituitary (Kadowaki *et al.*, 1994). However, other authors have reported that centrally-produced NO inhibited basal release of oxytocin but not vasopressin in conscious rats (Kadekaro *et al.*, 1997, Liu *et al.*, 1997). By contrast, in pentobarbitone-anaesthetised rats, NO formed centrally has been reported to stimulate basal vasopressin release (Cao *et al.*, 1996). Two possibilities may account for the discrepancies: first, the effect of L-NNA and L-NAME on the basal release of oxytocin may be obscured by the effects of urethane. Alternatively, we need to consider the route by which the NOS inhibitor was applied. Although L-NNA and L-NAME are able to cross the blood-brain barrier (Dwyer *et al.*, 1991; Traystman *et al.*, 1995) when administered systemically, L-NNA and L-NAME could also have effects on the cardiovascular system, causing an increase in blood pressure (Ishii *et al.*, 1990). An increase of blood pressure (induced for instance by i.v. administration of phenylephrine) transiently inhibits the neuronal activity of vasopressin neurones in the SON, and generally has no effect on the firing rate of oxytocin neurones in the SON, resulting in a decrease in vasopressin release but little effect on oxytocin release (Harris, 1979). Vasopressin release, but not oxytocin release, is potentiated by hypotension caused either by haemorrhage (Wakerley *et al.*, 1975) or in response to i.v. infusion of SNP, which does not cross the blood-brain barrier (Cohen and Burke, 1979). Central inhibition of NOS inhibited an increase of vasopressin secretion in response to SNP-induced hypotension (Cao *et*

al., 1996). It is not expected that any increase in blood pressure following systemic administration of L-NNA and L-NAME will alter the basal release of oxytocin.

Interestingly, the present results in virgin rats showed that blockade of NOS with systemic administration of either L-NNA or L-NAME further enhanced the secretion of oxytocin induced by hypertonic saline, but had no effect on CCK-evoked oxytocin release (Fig. 5, 6). These findings suggest that endogenous NO may selectively inhibit the release of oxytocin in conditions of sustained intense stimulation. It appears that there is a different effect of NO on CCK-induced oxytocin release and oxytocin release evoked by hyperosmotic stimulation. However, hypertonic stimulation is a much more effective stimulus than CCK; there is a gradual and sustained increase in the firing rate of oxytocin neurones following hyperosmotic stimulation (Brimble *et al.*, 1978), whereas CCK evokes a rapid but transient excitation of oxytocin neurones (Renaud *et al.*, 1987). However, we cannot distinguish whether NO directly inhibits oxytocin neurones, or acts indirectly by affecting adjacent presynaptic terminals from the forebrain circuitry of fluid balance and not on noradrenergic pathways from the nucleus tractus solitarii, a relay centre for vagal afferents that are activated by systemic CCK.

Our observations on the effects of i.p. injection of L-NNA or L-NAME on the oxytocin secretory response to hypertonic saline are similar to the results from i.p. injection of L-NNA on the depletion of oxytocin in the posterior pituitary in response to salt loading in conscious rats (Kadowaki *et al.*, 1994), and of i.c.v. injection of L-NMMA and L-NAME during dehydration in conscious rats (Summy-Long *et al.*, 1993). In addition, central blockade of NOS by L-NAME increased oxytocin release in response to moderate, but not strong, hyperosmotic stimulation in conscious rats (Kadekaro *et al.*, 1997). The moderate hyperosmotic stimulus (15 ml/kg 0.45 M NaCl, s.c.) used by Kadekaro *et al.* (1997) provided a similar Na^+ load (6.75 mM/kg) to the doses of hypertonic saline that we used in our experiments (4 ml/kg 1.5 M NaCl, i.p.), 6 mM/kg. Further investigation in our studies demonstrated that i.c.v. infusion of SNP also inhibited the secretion of oxytocin induced by hypertonic saline

in urethane-anaesthetised rats (Fig. 7). Therefore, our findings support the hypothesis that NO may play a role as a modulator in osmoregulation of oxytocin secretion. In particular, NO may serve within a negative-feedback loop to restrain release of oxytocin in conditions of intense stimulation.

It has been proposed that NO may originate from either oxytocin or vasopressin neurones, as an increase in NADPH-diaphorase activity and an up-regulation of NOS mRNA expression have been found in the SON and PVN during dehydration induced by salt loading and water deprivation (Kadowaki *et al.*, 1994; O'Shea and Gundlach, 1996; Pow, 1992; Ueta *et al.*, 1995b; Villar *et al.*, 1994b). Nitric oxide may also originate from the neurohypophysis, and from the forebrain structures of the lamina terminalis (the subfornical organ, median preoptic nucleus and organum vasculosum of the lamina terminalis) since an increase in number and/or intensity of NADPH-diaphorase staining neurones in these areas was shown after chronic salt loading and dehydration, respectively (Ciriello *et al.*, 1996; Sagar and Ferriero, 1987).

Nitric oxide may inhibit the release of oxytocin in response to hyperosmotic stimulation by acting at the SON, the PVN or at the axon terminals of magnocellular neurones. Our findings in chapter 4 (Fig. 23) showed that NO can inhibit the activity of the cell bodies of SON neurones. Another site of action of NO appears to be the neurohypophysis since L-NAME appears to prevent the ultrastructural changes in the neurohypophysis induced by injection of hypertonic saline (Beagley and Cobbett, 1997). Kadekaro *et al.* (1994) postulated that NO may act at axon terminals to inhibit oxytocin release during dehydration since NO did not alter the increase in glucose utilization in the SON, PVN and neurohypophysis, which indicates that NO did not alter the metabolic/electrical activity of this system during dehydration. Furthermore, NO increased the rate of glucose utilization in the subfornical organ and preoptic nucleus, suggesting that NO may exert an inhibitory action on synaptic activation of oxytocin neurones by increasing the firing rate of these neurones during water deprivation. There may therefore be multiple sites of production and action of NO in

the modulation of the responses of magnocellular oxytocin neurones to osmotic stimulation.

Does the altered osmoresponsiveness of the oxytocin neurosecretory system in pregnant rats reflect a change in the NOS system?

In late pregnancy, plasma osmolality and plasma [Na^+] are reduced (Barron *et al.*, 1985; Durr *et al.*, 1981) and the osmoresponsiveness of the oxytocin neurosecretory system is also reduced (Russell *et al.*, 1992). Therefore, we investigated the influence of endogenous NO on the responsiveness of the oxytocin system to osmotic stimulation in late pregnancy. We found that NO did not alter the basal release of oxytocin in either virgin or pregnant rats under urethane anaesthesia.

To produce similar increases of plasma [Na^+], pregnant rats were i.p. injected with 2.5 M hypertonic saline while virgin rats received 1.5 M NaCl. Unlike previous studies (Russell *et al.*, 1992), in which oxytocin secretion was measured following similar increases in plasma [Na^+] in pregnant and virgin rats, in the present study plasma [Na^+] was increased to similar values in pregnant and virgin rats. The robust oxytocin secretory responses in pregnant rats in the present study are likely to be a consequence of the greater plasma [Na^+] attained (Fig. 8, 9). A significant enhancement of oxytocin release by L-NNA was shown in virgin rats, but not in late-pregnant rats. Thus, the inhibitory influence of endogenous NO on hypertonic saline-induced oxytocin release is down-regulated in late pregnancy. Reduced inhibition by NO in late pregnancy reflects the reduced excitability of oxytocin neurones shown in previous studies with less effective hyperosmotic stimulation (Russell *et al.*, 1992), or AV3V stimulation (Bull *et al.*, 1994).

The similarity of the enhancement of oxytocin release in response to hypertonic saline by blockade of NOS with L-NNA (Fig. 5, 6) or blockade of opioid receptors with naloxone (Shibuki *et al.*, 1988) prompted us to investigate the relationship between these two mechanisms. In accord with previous studies

(Douglas *et al.*, 1993; Summy-Long *et al.*, 1990), naloxone enhanced oxytocin release in response to hyperosmotic stimulation in both virgin and late-pregnant rats (Fig. 8, 9). Pre-treatment with L-NNA did not alter the increase in oxytocin release after naloxone in late pregnancy, in contrast with a greater effect of naloxone on oxytocin secretion after L-NNA in virgin rats. These differences between late-pregnant and virgin rats suggest that the involvement of NO in osmoregulation of the oxytocin system is not due to opioid inhibition, and *vice versa.* An opioid inhibition of oxytocin release seems to amplify the inhibitory influence of NO on oxytocin release in response to hypertonic saline. In agreement with our findings, the preferential inhibition by NO of oxytocin release (Summy-Long *et al.*, 1993) may be an additional mechanism, but independent of an inhibitory effect of opioid peptide, since oxytocin release induced by i.c.v. administration of L-NAME, but not by naloxone, was attenuated by inhibition of prostaglandin production by the cyclo-oxygenase inhibitor indomethacin (Summy-Long *et al.*, 1996). Thus, an involvement of NO in the osmoregulation of oxytocin system is independent of the inhibitory influence of endogenous opioids.

In addition to elevating plasma osmolality (Dunn *et al.*, 1973), i.p. injection of hypertonic saline produces signs of visceral illness and pain (Verbalis *et al.*, 1986b). Therefore, the release of oxytocin induced by i.p. injected hypertonic saline may result from activation of afferent neural pathways associated with nausea, stimulated by either local peritoneal irritation or *via* chemoreceptors in the area postrema (Verbalis *et al.*, 1986a). Although our studies were in anaesthetised rats, to avoid these effects we also administered hypertonic saline *via* i.v. infusion instead of i.p. injection. Pregnant rats were i.v. infused with 2.6 M NaCl and virgin rats received 2 M NaCl at the rate of 2 ml/h. Similar changes in plasma [Na^+] were found in both groups. In both virgin and late-pregnant rats, plasma concentration of oxytocin increased in dose-dependent manner in parallel to the increase in plasma [Na^+] following i.v. infusion of hypertonic saline (Fig. 10, 11). Our results demonstrated that L-NNA enhances the secretion of oxytocin in response to hypertonic saline infusion, confirming that endogenous NO restraints oxytocin

secretion stimulated by i.v. hypertonic saline infusion is attenuated in late pregnancy. Similar to the findings in late-pregnant rats given the high dose of i.p. hypertonic saline, L-NNA did not enhance the oxytocin response to i.v. hypertonic saline in late pregnancy. Therefore, the osmoresponsiveness of the oxytocin neurosecretory system in the pregnant rats is not restrained by the NOS system when driven by strong osmotic stimulation. As a result, when appropriately and strongly stimulated the oxytocin secretory response to osmotic stimulation in pregnant rats is at least as great, and can be greater, in pregnant compared with virgin rats.

Our experiments provided additional evidence that nNOS mRNA expression in the SON can be modulated by naturally-occurring changes in the reproductive state, since the expression of nNOS mRNA in the SON was reduced at the end of pregnancy and increased again during parturition (Fig. 13A). These findings are consistent with the data reported by Okere and Higuchi (1996a) who found fewer NADPH-diaphorase-positive cells in the SON and PVN together with a decrease in the specific activity of NOS in the posterior pituitary in 19-21 day pregnant and parturient rats, but not in mid-pregnant rats. By contrast, other authors have reported that the number of NADPH-diaphorase positive cells in the SON and PVN is increased in mid-pregnant rats (Popeski *et al.*, 1999) and 22 day pregnant rats (Popeski *et al.*, 1999; Woodside and Amir, 1996). Another study demonstrated an up-regulation of nNOS protein and nNOS mRNA expression in the hypothalamus of 20 day pregnant rats assessed by western blot and northern blot, respectively (Xu *et al.*, 1996). There are two possibilities to explain these discrepancies: first, there is likely to be a delay between changes in nNOS mRNA expression and nNOS protein expression. Second, expression of the nNOS mRNA, described as a late response gene (Chapter 5), may vary over the last days of pregnancy, so selection of the time points selected in each experiment may give different results. Alternatively, the expression of nNOS mRNA may be regulated by nNOS protein in a negative feedback way.

No increase in nNOS mRNA expression was found in either the SON or the PVN in mid-pregnancy (Fig. 13A, B), consistent with the findings of Luckman *et al.* (1997). However, after a decrease in expression in late pregnancy, there was an acute increase in expression in the SON during parturition (significant increase from late pregnancy levels, Fig. 13A). This indicates that the activation of oxytocin neurones during parturition may stimulate nNOS mRNA expression.

In conclusion, this study indicates a functional down-regulation of the influence of endogenous NO on the excitability of oxytocin neurones to osmotic stimulation during late pregnancy. In virgin rats, the endogenous NOS system has a potent restraining influence on oxytocin cell excitability. Thus, the decrease in the inhibitory effect of NO due to a down-regulation of the NOS system in the magnocellular neurosecretory system and a decrease in NOS activity in the posterior pituitary in late pregnancy (Okere and Higuchi, 1996a) may cause the oxytocin system to become more excitable, resulting in the capacity for greater release of oxytocin at term.

Down-regulation of the NOS system in the magnocellular neurosecretory system at the end of pregnancy in the rat may play a physiological important role in the timing of the onset and/or progress of parturition. During parturition NOS mRNA expression is activated, possibly reflecting activity-dependent regulation of expression at this time.

CHAPTER 4

NITRIC OXIDE SIGNALLING IN THE MAGNOCELLULAR NEUROSECRETORY SYSTEM

4.1 Introduction

Nitric oxide may regulate the release of oxytocin and vasopressin by acting on either the cell bodies in the SON and PVN, or on their nerve terminals in the neurohypophysis. Electrophysiology studies suggest that NO may modulate the activity of SON neurones, since the activity of both phasic and non-phasic neurones in the SON was inhibited by NO (Liu *et al.*, 1997). However, the mechanisms responsible for this inhibitory influence of NO are still unclear. Nitric oxide may control the activity of the SON neurons through NMDA receptors at postsynaptic sites, as NO suppressed NMDA-evoked depolarization (Cui *et al.*, 1994). Alternatively, NO may increase GABA release by acting at presynaptic terminals rather than by a direct effect on SON neurones, since NO potentiated the spontaneous inhibitory postsynaptic currents (IPSPs), but not excitatory postsynaptic currents (EPSPs) recorded from SON neurones *in vitro* (Ozaki *et al.*, 1999). In the PVN, NO potentiated GABA inputs *via* postsynaptic as well as presynaptic mechanisms, since NO evoked spontaneous IPSPs recorded from PVN neurones *in vitro* (Bains and Ferguson, 1997b).

We therefore tested the hypothesis that the NO responsible for inhibition of oxytocin release co-exists with either oxytocin or vasopressin, and that NO may be involved in controlling the release of oxytocin and vasopressin by acting at the level of the cell bodies of the magnocellular neurosecretory neurones and the terminals of these neurones in the neurohypophysis.

4.2 Aims

This study examined the specific role of NO on the release of oxytocin from the magnocellular neurosecretory system. We were particularly interested to determine the involvement of NO in controlling neurohypophysial hormone release at the level both of the cell bodies, and the nerve terminals. Furthermore, we also investigated the expression of neuronal NOS gene in the hypothalamic magnocellular

neurones of rats after prolonged electrical stimulation of the axons of magnocellular neurosecretory neurones.

4.3 Materials and methods

4.3.1 Animals

Experiments were carried out on virgin female Sprague-Dawley rats, weighing 250 to 300 g. They were housed under standard laboratory conditions (12-hour: 12-h dark-light cycle, ambient temperature 20 ± 1°C) with free access to food and water.

4.3.2 Experimental procedure

1. The effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on Fos expression in the SON and PVN in rats treated with low doses of hypertonic saline

Rats were anaesthetised with sodium pentobarbitone (Saggatal, Rhône Mérieux Ltd.) at a dose of 50 mg/kg, i.p.. They were treated with either the irreversible NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA, 10 mg/kg, i.p., Sigma, (Dwyer *et al.*, 1991)) or vehicle. After 4 h, rats received either hypertonic saline solution (2 ml/kg 1.5 M NaCl, i.p.) or isotonic saline solution. Rats were decapitated 90 min later. Brains were removed, frozen with crushed dry ice and kept at -70°C. Frozen brains were coronally sectioned (15 μm) through the SON and PVN on a cryostat and processed for Fos immunocytochemistry using the indirect method.

2. The effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on Fos expression in the SON and PVN in rats treated with high doses of hypertonic saline

Sodium pentobarbitone anaesthetised rats were treated with either the irreversible NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA, 10 mg/kg, i.p., Sigma) or

isotonic saline solution, then left for 4 h. Rats were then injected with hypertonic saline solution (4 ml/kg 1.5 M NaCl, i.p.). Rats injected with isotonic saline solution followed by 1.5 M NaCl were included as positive controls (Hamamura *et al.*, 1992). After 90 min, the rats were perfused transcardially with isotonic saline solution, followed by fresh 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were removed, post-fixed 2-5 h in the same fixative, cryoprotected in 30% sucrose in fixative overnight at 4°C and then left in phosphate-buffered 30% sucrose (v/v) at 4°C until the brains sank. Brains were then coronally-sectioned (50 μm) through the SON and PVN on a freezing microtome and serially collected in PB. Sections were processed for Fos immunocytochemistry using the ABC method.

3. The role of NO in oxytocin release following electrical stimulation of the neural stalk

Rats were anaesthetised with urethane to allow cannulation of femoral veins and arteries on the day of the experiment (General Methods 2.2). The pituitary stalk of each urethane-anaesthetised rats was exposed by the transpharyngeal approach as described in General Methods 2.5. A stimulating electrode was placed on the neural stalk of the pituitary gland to deliver biphasic pulses (10 Hz, 1 mA peak to peak). The neural stalk was stimulated for 3 min before and 2, and 4 h after administration of either L-NNA (Sigma, 10 mg/kg, i. p.) or isotonic saline solution. Blood samples were taken from the left femoral artery before and immediately after stalk stimulation and immediately centrifuged. The plasma was then separated and stored at -20°C until assayed for oxytocin by specific RIA. The remaining blood cells were resuspended in isotonic saline at the same volume as the plasma taken and returned *via* the left femoral vein.

4. NOS mRNA expression in the SON and PVN following prolonged neurohypophysial stalk stimulation

Rats were anaesthetised with sodium pentobarbitone (Saggatal, Rhône Mérieux Ltd., 50 mg/kg, i.p.). A femoral artery and vein were cannulated for blood sampling and reinfusion. Rats were divided into three groups; control, sham-operation and stalk stimulated group. Control group: the skull was exposed only. Sham-operation group: the pituitary stalk was approached dorsally and the electrode was implanted in the stalk without current application. Stalk-stimulated group: the pituitary stalk was approached dorsally and the electrode was implanted in the neural stalk, and stimuli applied for 2 h (20 Hz, 10 s on, 10 s off, 1 mA peak to peak). The surgical method is described in General Methods 2.4. After stimulation, the rats were left a further 2 h before decapitation. Brains were removed and quickly frozen with crushed dry ice and kept at -70°C. Frozen brains were then cryostat sectioned (15 μm) coronally and processed for study of the expression of nNOS mRNA by using radioactive *in situ* hybridization histochemistry.

To verify the correct placement of the electrode, blood samples were collected 5, 10 min before and 15, 20 min after the start of stalk stimulation. Plasma samples were kept at -20°C until the day of oxytocin assay.

5. The role of NO in the electrical activity of oxytocin and vasopressin cells

The experiments were carried out on urethane-anaesthetised rats. A femoral vein and the trachea were cannulated. The pituitary stalk and right SON were exposed by a transpharyngeal surgery as described in General Methods 2.5. A combined intrasupraoptic nucleus retrodialysis/*in vivo* extracellular recording technique (Ludwig and Leng, 1997) was used to investigate the effect of local nitric oxide on the activity of identified oxytocin- and vasopressin-cells. An in-house designed U-shaped dialysis probe (total membrane length, 2.0 mm; Spectra/Por RC Hollow Fibers®, Spectrum Med. Inc., (Ludwig *et al.*, 1994a)) was bent to position the loop of

the membrane flat onto the exposed ventral surface of the brain on the ventral glial lamina of the supraoptic nucleus after removal of the meninges. A glass micropipette (filled with 0.15 M NaCl, 20-40 MΩ resistance) was introduced into the centre of the loop of the dialysis membrane to record the extracellular activity of single neurones in the SON. A stimulating electrode (SNEX-200X, Clarke Electromedical) was placed on the neural stalk of the pituitary gland and set to deliver single matched biphasic pulses (1 ms duration, <1 mA peak to peak) for antidromic identification of SON neurones. Oxytocin cells were distinguished from vasopressin cells by their firing pattern and by their responses to i.v. injection of 20 μg/kg cholecystokinin (CCK, cholecystokinin-(26-33)-sulphated; Bachem Ltd). All continuously active neurones were identified as oxytocin neurones by a transient excitation, and as vasopressin neurones by no effect or short-term inhibition after CCK injection (Leng *et al.*, 1991; Renaud *et al.*, 1987)).

Artificial cerebrospinal fluid (aCSF; pH 7.2, composition (mM): NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; $MgCl_2$, 1.18) was dialysed onto the SON at a flow rate of 3 μl/min throughout the experiment. Dialysis fluid was changed to aCSF containing the NO donor sodium nitroprusside (SNP; 10mM, 100 mM, Sigma), the NO precursor L-arginine (100 mM, Sigma) or the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA; 10 mM, Sigma). Dialysis was maintained until a clear effect was observed, or for 30 min where no effect was seen.

The firing rates of identified oxytocin- and vasopressin-cells were recorded using the Spike2 software package (Cambridge Electronic Design, Cambridge, UK). The mean firing rate (spikes/s) of each cell and for phasic cells, the activity quotient, were calculated for the 5 min period immediately before and for successive 5 min periods after drug retrodialysis.

4.3.3 Fos immunocytochemistry

The full methodology is described in General Methods 2.7.

Indirect method

Brains were cryostat sectioned coronally (15 μm) through the SON and PVN guided by the stereotaxic atlas of Paxinos and Watson (1982) and thaw mounted on chrome alum gelatine subbed slides. Sections were stored in desiccated slide boxes at -70°C.

Cryostat sections were fixed in 4% paraformaldehyde in 0.1 M PB for 30 min and washed in 0.1 M PB. Endogenous peroxidase was blocked with hydrogen peroxide solution for 15 min. Slides were then washed with PB-T (0.1 M PB containing 0.3% Triton X-100). Non-specific binding was blocked by preincubation buffer containing 1% normal sheep serum for 1 h. Sections were incubated for 48 h with a polyclonal antibody raised in rabbits against rat Fos (*c-fos* Ab-2; Oncogene Sciences) at a dilution of 1:1000 in preincubation buffer containing 1% v/v normal sheep serum. After 48 h, sections were washed with PB-T. Sections were then incubated for 24 h with secondary antibody (goat anti-rabbit IgG-peroxidase complex) at a dilution of 1:1000 in preincubation buffer containing 1% v/v normal sheep serum. Sections were washed with PB-T and rinsed with 0.1 M acetate buffer for 2 min. Sections were then incubated with the glucose oxidase-Ni DAB solution until the reaction had worked and some nuclei appeared black. The reaction was terminated with stop solution for 5 min, and then a rinse with PB for 5 min. Sections were then dehydrated through serial concentrations of ethanol (70%, 90%, 95%, 100% and 100%) and then into xylene. Slides were finally coverslipped using DPX mountant (BDH).

ABC method

Paraformaldehyde fixed brains were coronally sectioned (50 μm) through the SON and PVN using a freezing microtome. Sections were collected in 0.1 M PB and processed for Fos immunonocytochemistry.

Free-floating sections were washed in PB-T (0.2% Triton X-100 in 0.1 M PB). Endogenous peroxidase was then deactivated with hydrogen peroxide solution for 15 min and washed off with PB-T. Non-specific staining was then blocked by preincubation buffer containing 1% normal sheep serum for 30 min. After that the sections were incubated for 48 h in Ab-2 Fos antibody (Rabbit polyclonal; Oncogene Sciences), at a dilution of 1:1,000 in preincubation buffer. Slides were washed with PB-T. The antibody-antigen complex was localized by incubating sections in secondary antibody solution (1% biotinylated anti-rabbit immunoglobulin and 3% normal goat serum in PB-T) for 1 h and washed in PB-T (3×10 min). Slides were then left to incubate in ABC complex solution (2% Avidin DH and 2% Biotinylated horseradish peroxidase in PB-T) for 1 h. Sections were washed in PB-T (2×10 min). After that sections were rinsed with 0.1 M acetate buffer for 5 min. They were then incubated with the glucose oxidase-Ni DAB solution for approximately 10 min until some nuclei appeared black, the reaction was terminated with stop solution for 5 min, and sections then rinsed with PB for 5 min. Sections were then mounted onto the chrome alum gelatine subbed slides in serial order and left to dry overnight. Sections were then dehydrated through serial concentrations of ethanol (70%, 90%, 95%, 100% and 100%) and then into xylene. Slides were finally coverslipped using DPX mountant (BDH).

The number of neurones in the SON and PVN expressing Fos immunoreactivity was counted under a light microscope (×10 objective lens).

4.3.4 Radioimmunoassay

The full methodology for specific radioimmunoassay for oxytocin is described in General Methods 2.6.

On the day of assay, plasma samples were thawed and centrifuged. Specific anti-oxytocin antiserum (Higuchi *et al.*, 1985) was added to triplicate aliquots of standards (ranging from 2.4 to 2500 pg/ml) and duplicate aliquots of plasma samples. Mixtures were left for 24 h before adding a fixed amount of ^{125}I-oxytocin (approximately 6-8,000 cpm) to all tubes. Mixtures were then left for 2-3 days. Second antibody (Donkey anti-rabbit gammaglobulin) was added to all tubes and left for 24 h. Standardized Pansorbin cells (Novabiochem (U.K.) Ltd., 0.1% w/v solution in phosphate buffer) was added prior to centrifugation. Supernatants were then aspirated before precipitates were counted in a gamma counter (LKB-Wallace 1272 Clinigamma). All samples in each experiment were measured in a single assay to avoid interassay variance.

The concentration of oxytocin in the standard or plasma sample was inversely proportional to the amount of ^{125}I-oxytocin bound to the rabbit anti-oxytocin antibody. Oxytocin concentration in the unknown samples was determined by comparing the unknown precipitate radioactivity to that of known standard oxytocin contents

4.3.5 *In situ* Hybridization

The full methodology is described in General Methods 2.8.

Brain sections

Brains were coronally sectioned (15 μm) through the hypothalamus at the level of the SON and PVN using a cryostat and thaw-mounted onto gelatin-coated

glass slides. Slides were then stored in desiccated slide boxes at -70°C before *in situ* hybridization.

Probes

Three 45-mer antisense oligonucleotide probes complementary to bases 223-267 (5′-noncoding region), 4714-4758 (3′-noncoding region) and 1662-1706 of the rat neuronal NOS (nNOS) sequence (Bredt *et al.*, 1991b) were used. Probes were labelled at the 3′ end with [α-^{35}S]deoxy-ATP (NEN) using terminal deoxynucleotidyltransferase (Pharmacia).

In situ hybridization

Sections were fixed with 4 % paraformaldehyde in 0.1 M PB (pH 7.4) for 30 min, then rinsed in 0.1 M PBS and washed in 0.1 M PBS (10 min), followed by acetylation in triethanolamine solution (0.25% acetic anhydride in 0.1 M triethanolamine-0.15 M NaCl) for 10 min. The sections were then dehydrated through 70%, 80%, 95% and 100% ethanol (5 min each), delipidated in chloroform for 10 min, and partially rehydrated in 95% ethanol (10 min). After air-drying the slides were placed in a humidified chamber. The sections were covered with hybridization buffer (4xSSC, 50% formamide, 1xDenhardt's solution, 500 μg/ml sheared salmon sperm DNA, 10% dextran sulphate and 0.3% mercaptoethanol) containing the ^{35}S-labelled NOS oligonucleotide probes at a concentration of 2500 dpm/μl and left to hybridize for 17 h at 37°C.

After the hybridization process, the excess hybridizing solution was drained off. The slides were briefly rinsed three times in 1xSSC at room temperature, washed three times for 30 min in 1xSSC and were washed for a further 1 h in 1xSSC at room temperature. The slides were air-dried and dipped in 300 mM ammonium acetate, 70% ethanol and then air-dried. The slides were then placed in autoradiographic cassettes and exposed to Hyperfilm-βmax autoradiography film (Amersham, UK) together with brain paste standards. The film was left to expose for 3 weeks at 4°C.

The films were developed in D19 developer (Kodak) and then fixed (Ilford Hypam rapid fixer). After that, the sections were dipped in Ilford G-5 emulsion-coated slides (Mobberley) diluted 1:1 with autoclaved water. The emulsion-coated slides were exposed for a further 4-5 weeks to visualize the NOS cellular signal, and then developed (Kodak D19 developer) for 5 min and fixed (Ilford Hypam rapid fixer twice 5 min each). The developed sections were counterstained with cresyl violet, dehydrated in a graded ethanol series, and coverslipped in DPX mountant.

Quantitative Analysis

The amount of nNOS mRNA in the SON and PVN was assessed by silver grain density of autoradiographic films of the SON, PVN, SFO and AMG viewed under the light microscope, with ×5 and ×10 objectives. Grain density was quantified by the NIH Image analysis system version 1.58 on an Apple McIntosh computer. The value of silver grain density per unit area was obtained by subtracting background measurements from each specific tissue measurement, and dividing by the area measurements. Each brain paste standard was measured twice, and corresponding background measurements taken. A logarithmic relationship was then plotted for radioactivity against the grain density of the standards. Acceptable grain density values lay on the straight portion of the curve. For each identified brain area, the mean grain density per unit area for each rat was calculated and the group mean grain density per unit area calculated.

Specific radioactivity of labelled probes was ranged from 536.82 to 866.42 Ci/mmol.

4.3.6 Statistical Analysis

All data are reported as means ± S.E.M.. Data were analysed statistically by *t*-test, one-way ANOVA and two way ANOVA for comparison differences between groups, a one-way repeated measures ANOVA for differences with time and a two-way repeated measures ANOVA for differences between groups followed by Dunnett's method or Student-Newman-Keuls method (SigmaStat). The parametric paired *t*-test and the non-parametric Wilcoxon-Signed Rank test were used for comparison differences between before and after treatment within group. $P < 0.05$ was considered statistically significant.

4.4 Results

1. The effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on Fos expression in the SON and PVN in rats treated with low doses of hypertonic saline

Fos was clearly localised in neuronal nuclei of magnocellular neurones in the SON and PVN but not in surrounding areas. Compared to vehicle/isotonic saline-injected controls (V/IS), there was a marked increase in Fos-immunoreactivity in the SON and PVN 90 min after administration of low dose hypertonic saline (2 ml/kg, 1.5 M NaCl, i.p.) in isotonic saline pre-treated rats (V/HS)(Fig.21A, B and Fig. 22A, B). In rats pre-treated with the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA, 10 mg/kg, i.p.) followed by a low dose of hypertonic saline (L-NNA/HS), intense Fos-immunoreactivity in the magnocellular neurones of the PVN was observed when compared to vehicle/isotonic saline-injected rats (V/IS)(Fig.22A, D). Pre-treatment with L-NNA did not significantly alter Fos–immunoreactivity in the magnocellular neurones of the SON and PVN in response to systemic injection of either isotonic saline or low dose hypertonic saline (Fig. 21C, D and Fig. 22C, D).

In vehicle pre-treated rats injected with a low dose of hypertonic saline (V/HS, n=7) the mean number of Fos-positive neurones in the SON was significantly

greater than in isotonic saline-injected rats (V/IS, n=6)(58.59 % increase; P<0.05, Fig. 19). Significantly more Fos-positive cells were also found in the magnocellular region of the PVN in response to the low-dose hypertonic saline (V/HS, n=7) than in the V/IS group (n=7)(45.82 % increase; P<0.05, Fig. 20).

In rats pre-treated with L-NNA injected with a low dose of hypertonic saline (L-NNA/HS, n=7) the mean number of the magnocellular neurones expressing Fos in the PVN was significantly greater than in vehicle-injected rats (V/IS, n=7)(55.44 % increase; P<0.05, Fig. 20). However, L-NNA pre-treatment did not alter the number of magnocellular PVN neurones expressing Fos immunoreactivity in response to low-dose hypertonic saline since there was no significant difference between the number of Fos-positive neurones after L-NNA pre-treatment (L-NNA/HS, n=7) and vehicle pre-treatment (V/HS, n=7) (Fig. 20). The number of Fos-positive neurones in the SON from L-NNA-pre-treated rats that were injected with a low dose of hypertonic saline (L-NNA/HS, n=6) was increased, but not significantly different, compared with V/IS rats (n=6, 61.07 % increase). Pre-treatment with L-NNA did not significantly alter the mean number of Fos-positive cells in the SON in response to low-dose hypertonic since there was no significant difference between rats pre-treated with L-NNA (L-NNA/HS, n=6) and rats pre-treated with vehicle (V/HS, n=7) (Fig. 19).

The mean number of Fos-positive nuclei in the SON and the magnocellular PVN of L-NNA pre-treated rats injected with isotonic saline (L-NNA/IS, n=7) was not significantly increased compared with V/IS rats (n=7). There was no significant difference in the mean number of Fos-positive neurones in the SON and magnocellular part of the PVN between the L-NNA/HS group and the L-NNA/IS group (Fig. 19, 20).

Thus, pre-treatment with L-NNA did not significantly alter the basal number of Fos-positive cells in the SON or in the magnocellular part of the PVN, and had no

effect on the number of magnocellular neurones of the SON and PVN expressing Fos immunoreactivity in response to a low dose of hypertonic saline.

2. The effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on Fos expression in the SON and PVN in rats treated with high doses of hypertonic saline

Extensive immuno-staining for Fos was seen in neuronal nuclei of magnocellular neurones of the SON and PVN 90 min after i.p. injection of 1.5 M NaCl (4 ml/kg) in both NOS inhibitor pre-treated (10 mg/kg L-NNA, i.p.) and vehicle pre-treated rats (Fig. 24). Significantly more magnocellular neurones in the SON expressed Fos in L-NNA pre-treated rats (n=8) than in vehicle-injected positive control rats (n=8)(23.77 % increase; $P<0.05$, Fig. 23). The number of Fos-positive cells in the magnocellular part of the PVN of rats pre-treated with L-NNA followed by a high dose of hypertonic saline was not significantly increased compared with control rats (Fig. 23).

Thus, pre-treatment with L-NNA enhanced the expression of Fos in the SON of rats given a high dose of hypertonic saline, suggesting an inhibitory action of NO on the activation of the neurones following strong hypertonic stimuli. No significant effect of pre-treatment was observed in rats given a lower dose of hypertonic saline.

3. The role of NO in oxytocin release following electrical stimulation of the neural stalk

In this experiment, the neural stalk of urethane-anaesthetised rats was stimulated for 3 min (10Hz, 1 mA) before, 2 and 4 h after administration of either the L-NNA or vehicle. Pre- and post-stimulation samples for oxytocin RIA were taken immediately before and after stalk stimulation. In all rats, the plasma oxytocin concentration in pre-stimulation samples did not alter following L-NNA or vehicle administration, suggesting that NO had no effect on the basal release of oxytocin. In vehicle pre-treated rats (n=5), stalk stimulation induced an increase in plasma

oxytocin concentration by 2.12-, 1.75- and 1.93-fold before, 2 h and 4 h after vehicle administration. The increases in plasma oxytocin release following stalk stimulation were significant before and 2 h after vehicle administration ($P<0.05$, Fig. 25A). There was no statistically significant difference between pre- and post-stimulation samples taken 4 h after vehicle administration. In L-NNA pre-treated rats (n=7), stalk stimulation increased plasma oxytocin concentration by 2.69-, 3.52- and 2.86-fold before, 2 h and 4 h after L-NNA administration, which were all significant increases ($P<0.01$, Fig. 25B). The oxytocin concentration in the post-stimulation sample 4 h after administration of L-NNA, was significantly greater than in the post-stimulation samples taken before and 2 h after L-NNA administration ($P<0.05$, Fig. 25B).

In control rats (n=5), the increase in oxytocin release following stalk stimulation at 2 and 4 h was not significantly altered by vehicle administration (Fig. 26). By contrast, in L-NNA treated rats (n=7), the stimulated oxytocin release increased progressively following L-NNA treatment. The stimulation of oxytocin release was significantly enhanced 4 h after L-NNA administration compared to the control group ($P<0.05$, Fig. 26).

L-NNA did not affect basal oxytocin release. L-NNA enhanced oxytocin release evoked by electrical stimulation of the axons of magnocellular neurosecretory neurones, suggesting an inhibitory action of NO on the release of oxytocin following action potentials propagated along the axons. Thus NO, generated in an activity-dependent manner, appears to act as a feedback inhibitor of oxytocin release at the terminals of oxytocin neurones.

4. NOS mRNA expression in the SON and PVN following prolonged neurohypophysial stalk stimulation

To verify the position of the stimulating electrode and to establish the effectiveness of stalk stimulation, plasma oxytocin concentrations were measured before and after the start of stalk stimulation. The basal oxytocin release in control,

sham-operated and stalk-stimulated rats were not significantly different from each other (Fig. 27).

Under sodium pentobarbitone anaesthesia, neurohypophysial stalk stimulation (20 Hz, 10 s on, 10 s off, 1 mA peak to peak) for 15 min caused a dramatic increase in the plasma oxytocin concentration from basal by 46.44-fold ($P<0.05$, n=8, Fig. 27). In control and sham-operated rats, the plasma concentration of oxytocin did not significantly change.

Strong nNOS mRNA hybridization signals were observed in the SON, the magnocellular region of the PVN, SFO and AMG. A decreased nNOS mRNA signal was revealed by silver grain density in the SON of prolonged stalk-stimulated rats (Fig. 29C). The nNOS mRNA signal in the magnocellular part of the PVN, SFO and AMG of prolonged stalk-stimulated rats was not significantly different from control rats (Fig. 30, 31).

Prolonged stalk stimulation (n=8) resulted in a significant decrease in silver grain density (nNOS mRNA) in the SON compared to sham-operated rats (n=7)(28.88 % increase; $P<0.05$, Fig. 28A). By contrast, there was no change in nNOS gene expression in the PVN, SFO and AMG after prolonged stalk stimulation.

5. The role of NO in the electrical activity of oxytocin and vasopressin cells

The firing rates of oxytocin cells and continuously firing vasopressin cells were increased after local administration of the NOS inhibitor L-NNA, and were reduced after local administration of the NO donor SNP or the NO precursor L-arginine (Fig. 32, 33). L-arginine similarly tended to inhibit the spontaneous activity of both types of cell. L-NNA had a significant excitatory effect on the spontaneous activity of both oxytocin cells and vasopressin cells ($P<0.05$ and $P<0.0005$ vs basal, respectively, Fig. 34). The mean spontaneous firing rate of five oxytocin cells tested increased by 0.83 ± 0.20 spikes/s and the mean spontaneous firing rate of twelve

vasopressin cells tested increased by 1.78 ± 0.36 spikes/s following retrodialysis of 10 mM L-NNA. In addition, the spontaneous firing rate of both oxytocin cells and vasopressin cells was inhibited by SNP in a dose-dependent way (Fig. 35). The firing rate of oxytocin cells and vasopressin cells was significantly inhibited after 100 mM SNP administration ($P<0.05$, $P<0.0001$, respectively). The mean spontaneous firing rate of the four oxytocin cells decreased by 3.01 ± 1.11 spikes/s and the mean spontaneous firing rate of the twelve continuously firing vasopressin cells decreased by 7.01 ± 0.63 spikes/s following retrodialysis of 100 mM SNP.

In each of four phasic vasopressin cells, 100 mM SNP retrodialysis onto the SON for 45 min caused a significant decrease of activity quotient, firing rate and mean burst length ($P<0.05$, Fig. 36). After 45 min of 100 mM SNP administration, the activity quotient of phasic vasopressin cells decreased by 0.58 ± 0.20, the mean spontaneous firing rate decreased by 4.38 ± 1.62 spikes/s and the mean burst length decreased by 115.01 ± 65.16 s. Three phasic cells were recorded during retrodialysis of 10 mM L-NNA onto the SON for 40 min. No significant change in neuronal activity was observed (Fig. 37).

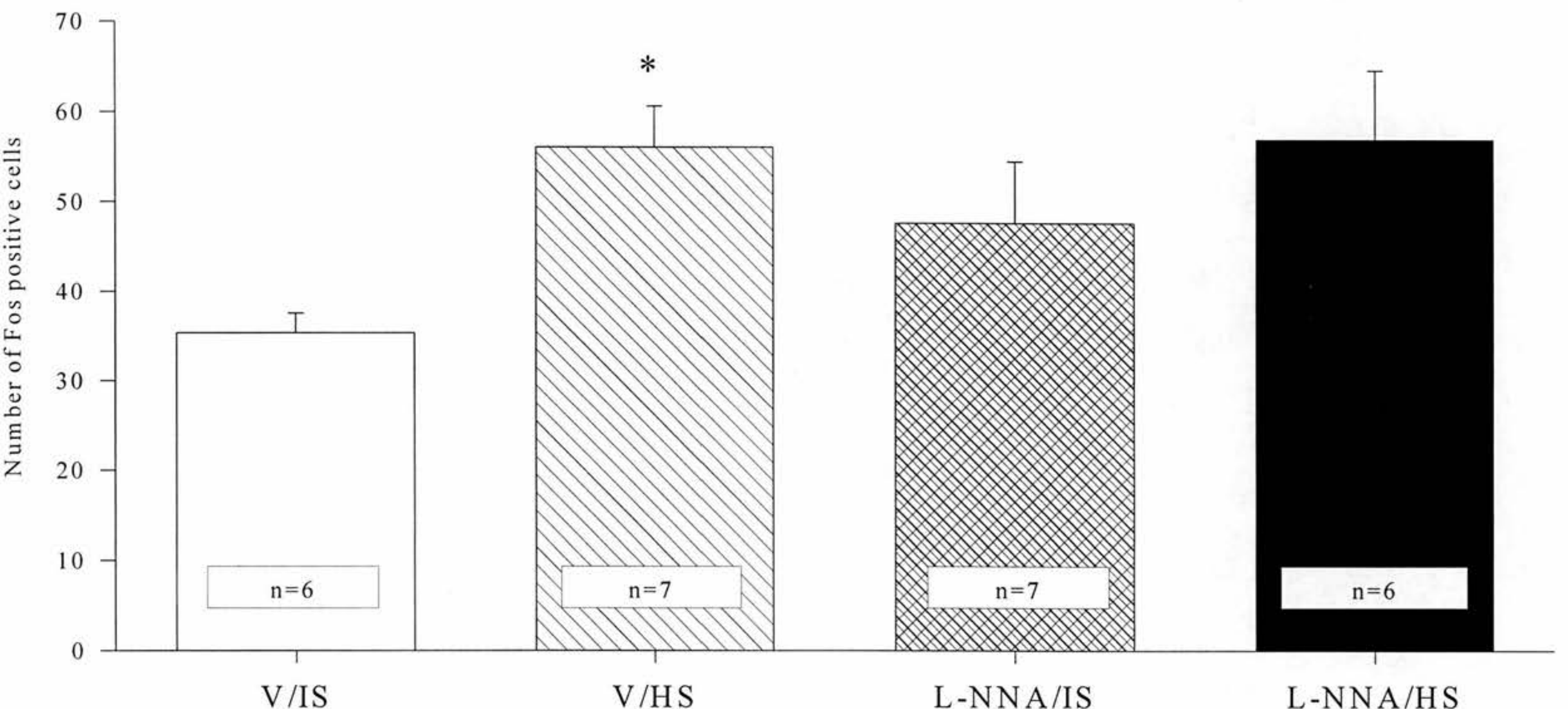

Figure 19. Expression of Fos protein in the magnocellular neurones of the SON of rats pre-treated with either N^{ω}-nitro-L-arginine (L-NNA) or vehicle (V) followed by either hypertonic saline (HS, 2 ml/kg 1.5 M NaCl, i.p.) or isotonic saline (IS). The number of Fos positive neurones in the SON of rats pre-treated with vehicle followed by hypertonic saline (V/HS) was significantly higher than in V/IS rats (* $P<0.05$, difference from V/IS, Two Way ANOVA, Student-Newman-Keuls Method). Values are means ± SEM.

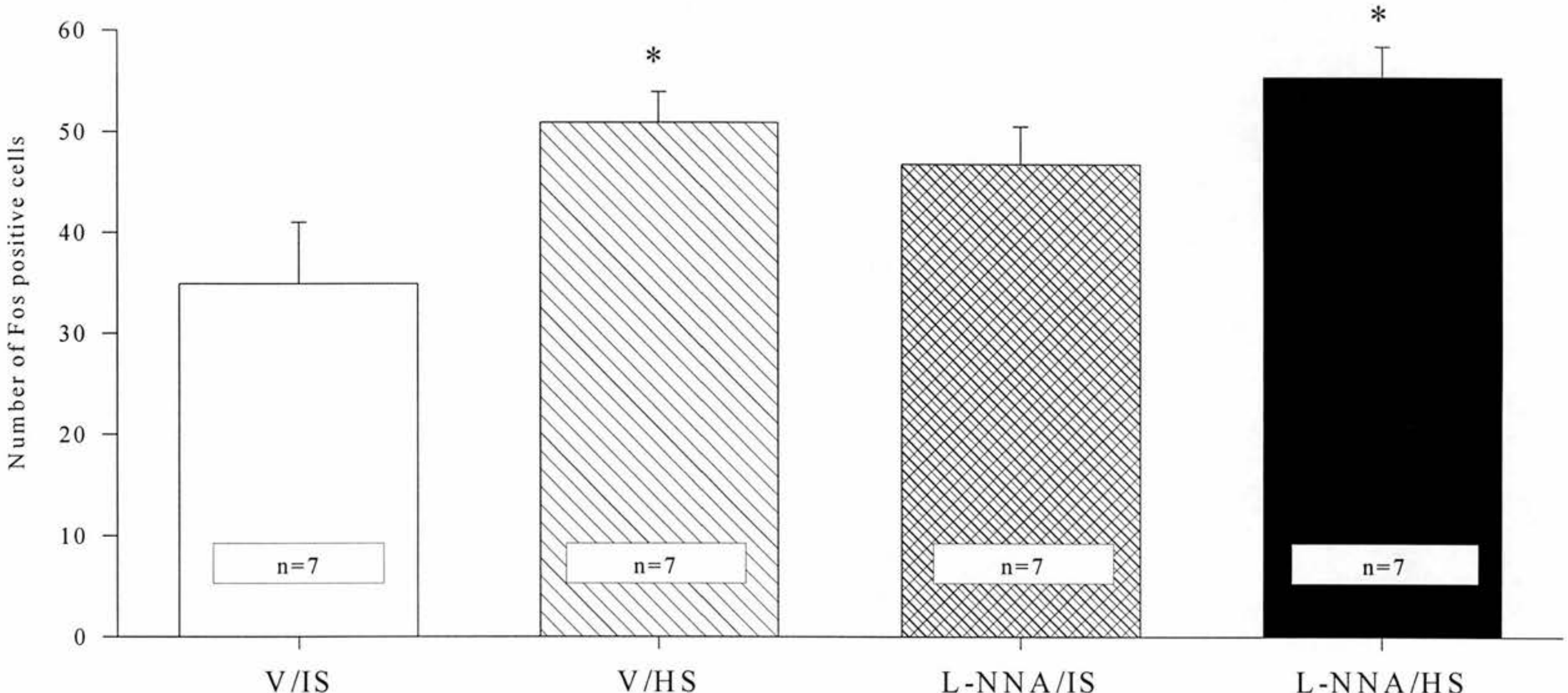

Figure 20. Expression of Fos protein in the magnocellular neurones of the PVN of rats pre-treated with either N^{ω}-nitro-L-arginine (L-NNA) or vehicle (V) followed by either hypertonic saline (HS, 2 ml/kg 1.5 M NaCl, i.p.) or isotonic saline (IS). Significant increases in number of Fos positive neurones in the PVN of rats pre-treated with either L-NNA or vehicle followed by hypertonic saline (L-NNA/HS and V/HS) were observed when compared to V/IS rats (* $P<0.05$, difference from V/IS, Two Way ANOVA, Student-Newman-Keuls Method). Values are means ± SEM.

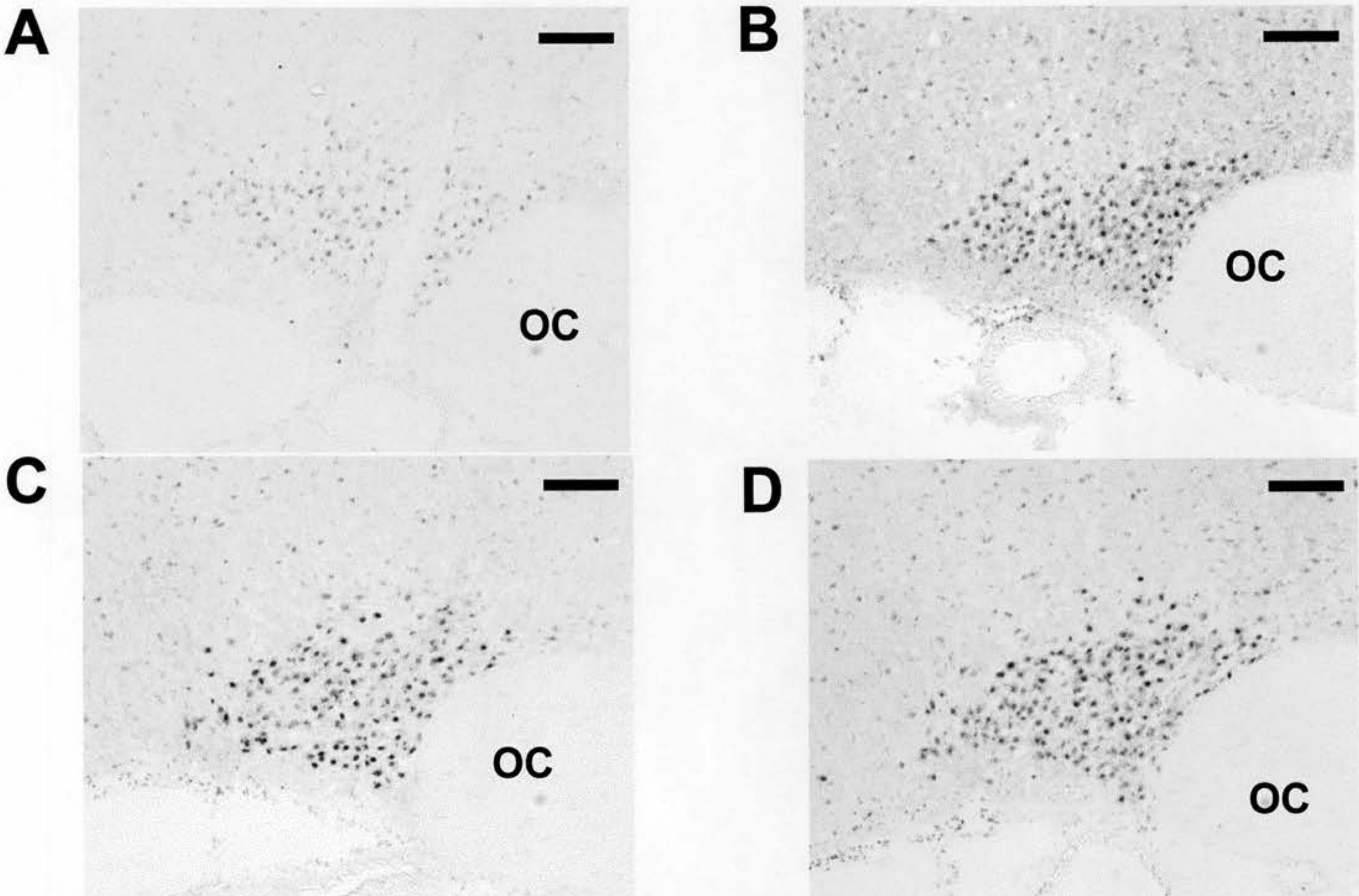

Figure 21. Photomicrographs of Fos expression in the magnocellular neurones of the SON. **A**, rats were pre-treated with vehicle followed by isotonic saline (V/IS). **B**, rats were pre-treated with vehicle followed by hypertonic saline (2 ml/kg 1.5 M NaCl, i.p.) (V/HS). **C**, rats were pre-treated with N^{ω}-nitro-L-arginine (L-NNA) followed by isotonic saline (L-NNA/IS). **D**, rats were pre-treated with L-NNA followed by hypertonic saline (L-NNA/HS). Sections of V/HS rats (**B**) showed higher Fos-positive cells than sections of V/IS rats (**A**). OC; optic chiasm. Bar=100 μm.

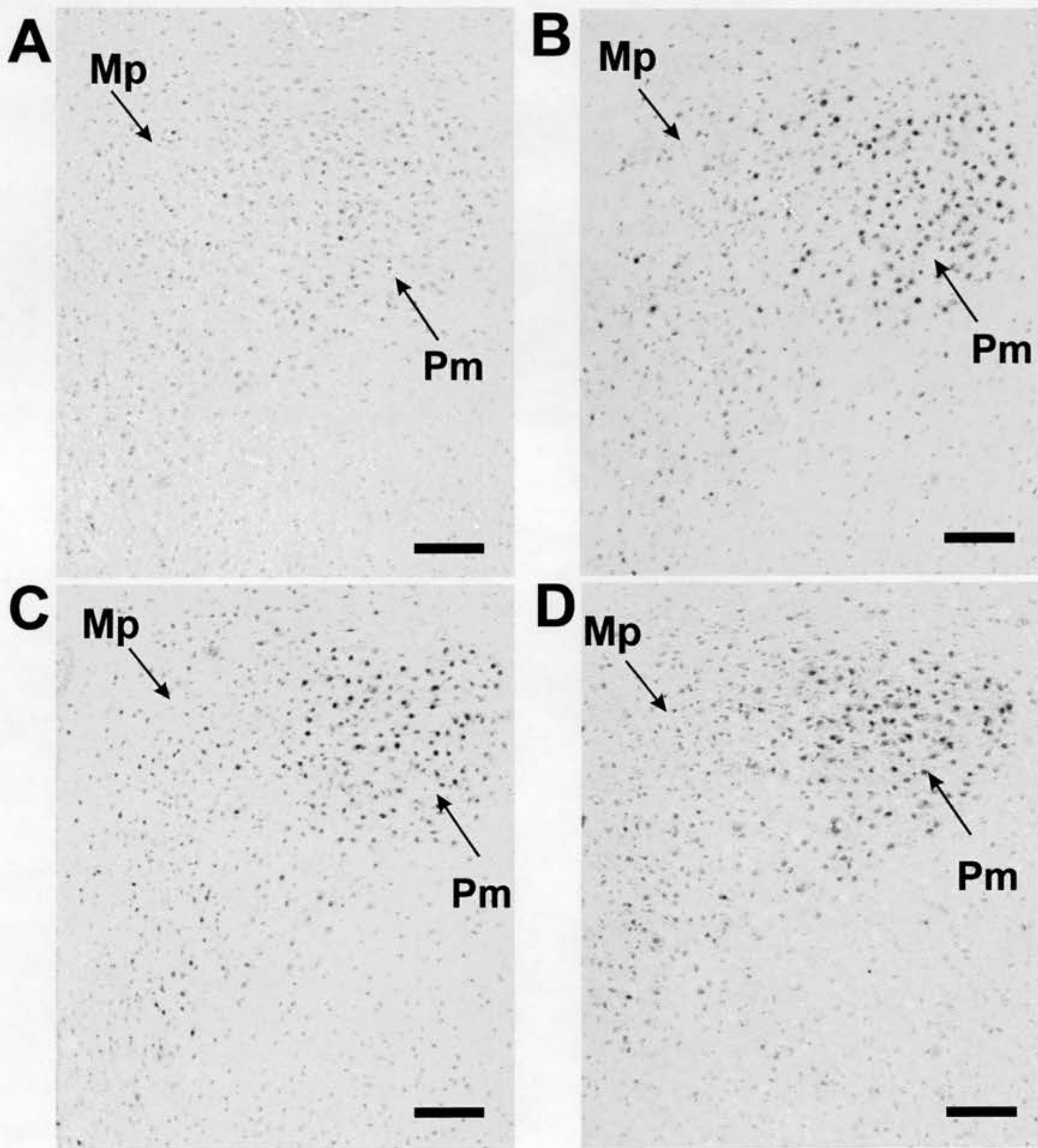

Figure 22. Photomicrographs of Fos expression in the magnocellular neurones of the PVN. **A**, rats were pre-treated with vehicle followed by isotonic saline (V/IS). **B**, rats were pre-treated with vehicle followed by hypertonic saline injection (2 ml/kg 1.5 M NaCl, i.p.) (V/HS). **C**, rats were pre-treated with N^{ω}-nitro-L-arginine (L-NNA) followed by isotonic saline (L-NNA/IS). **D**, rats were pre-treated with L-NNA followed by hypertonic saline (L-NNA/HS). Sections from V/HS (**B**) and L-NNA/HS rats (**D**) showed more Fos-positive cells than sections from V/IS rats (**A**). Mp; medial parvocellular, Pm; posterior magnocellular. Bar=100 μm.

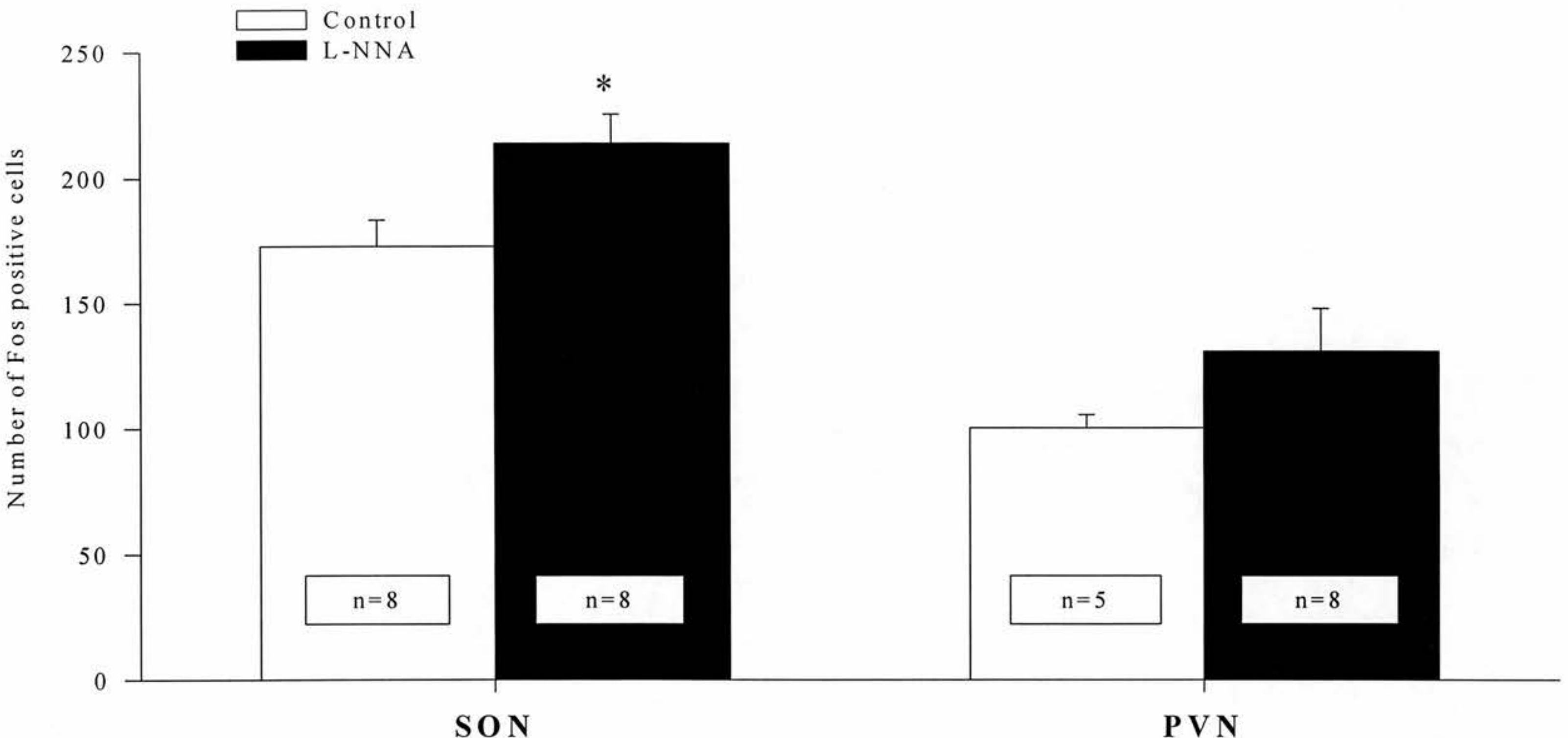

Figure 23. The effect of N^{ω}-nitro-L-arginine (L-NNA) on Fos expression in the magnocellular neurones in the SON and PVN of rats treated with a high dose of hypertonic saline (4ml/kg, 1.5 M NaCl, i.p.). A significant increase in number of Fos positive neurones was observed in the SON of L-NNA pre-treated rats compared to vehicle pre-treated rats (*, $P<0.05$, difference from control group, *t*-test). Values are means ± SEM.

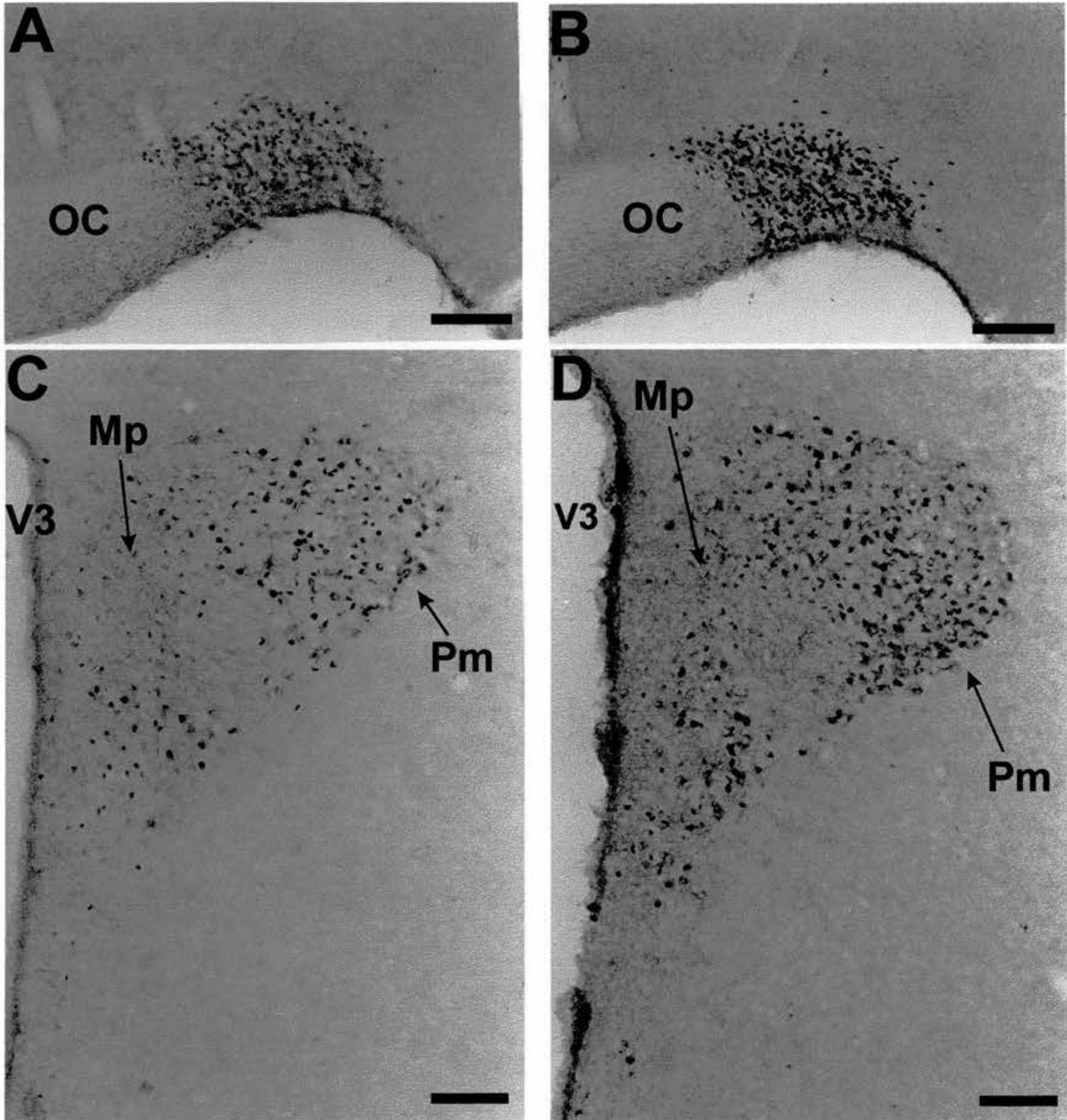

Figure 24. Photomicrographs of Fos expression 90 min after hypertonic saline injection (4ml/kg 1.5 M NaCl, i.p.) in the SON (**A**, **B**) and PVN (**C**, **D**). **A** and **C** were pre-treated with vehicle; **B** and **D** were pre-treated with L-NNA. In the SON, there was more Fos-positive cells from rats pre-treated with L-NNA (**B**) than from rats pre-treated with vehicle (**A**). Mp; medial parvocellular, Pm; posterior magnocellular, OC, optic chiasm; V3, third ventricle. Bar=100 μm.

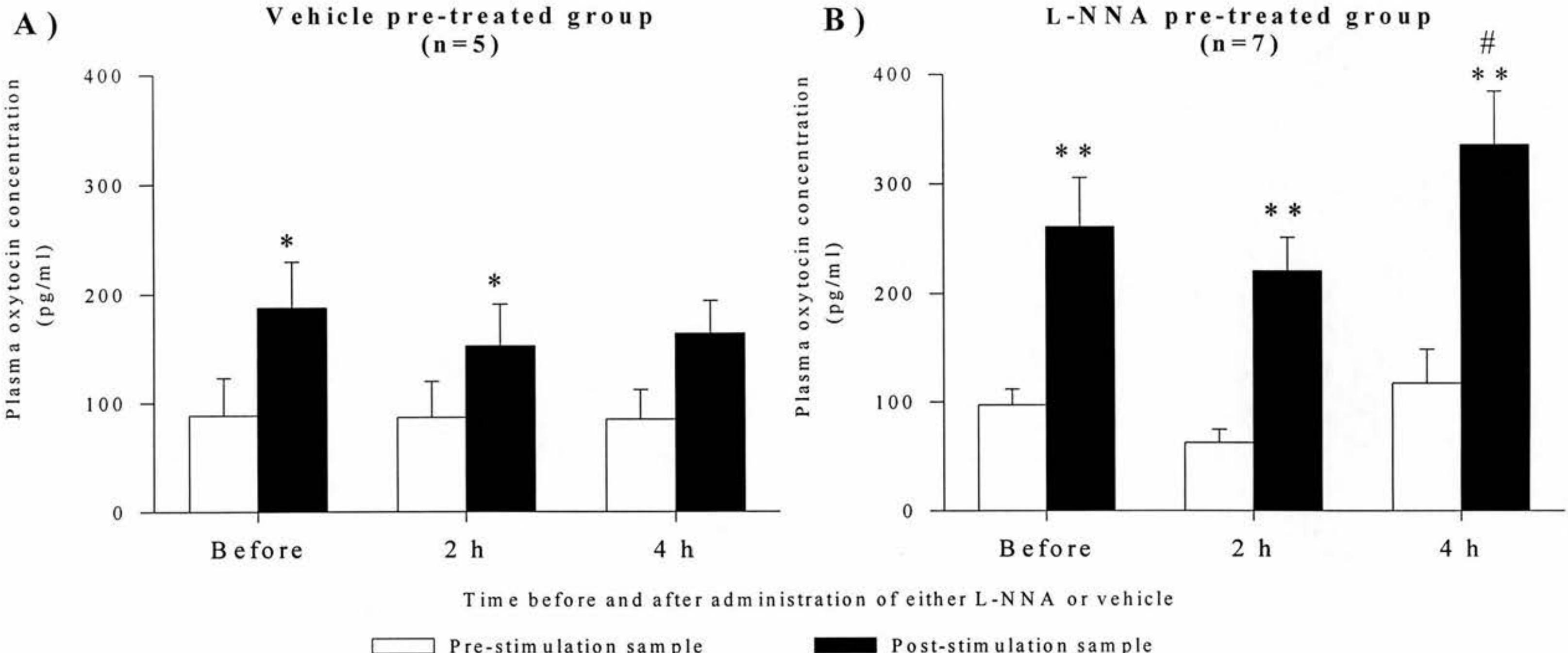

Figure 25. Effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on plasma oxytocin concentration following neural stalk stimulation *in vivo*. The neural stalk of urethane-anaesthetised rats was stimulated for 3 min (10Hz, 1 mA) before, 2 and 4 h after administration of either L-NNA or vehicle. Initially (Before), a significant increase of plasma oxytocin concentration following stalk stimulation was found in both groups (**A**)(**B**)(*, ** $P<0.05$ and $P<0.01$, paired *t*-test, respectively). After 4 h, a significant increase of plasma oxytocin concentration following stalk stimulation was found in L-NNA pre-treated rats but not vehicle pre-treated rats. Oxytocin concentration in post-stimulation samples taken 4 h after L-NNA administration was significantly higher than post-stimulation samples taken before and 2 h after L-NNA administration (**B**) (#, $P<0.05$ vs after stalk stimulation before L-NNA injection, One way repeated measures ANOVA (Student-Newman-Keuls method). Values are means ± SEM.

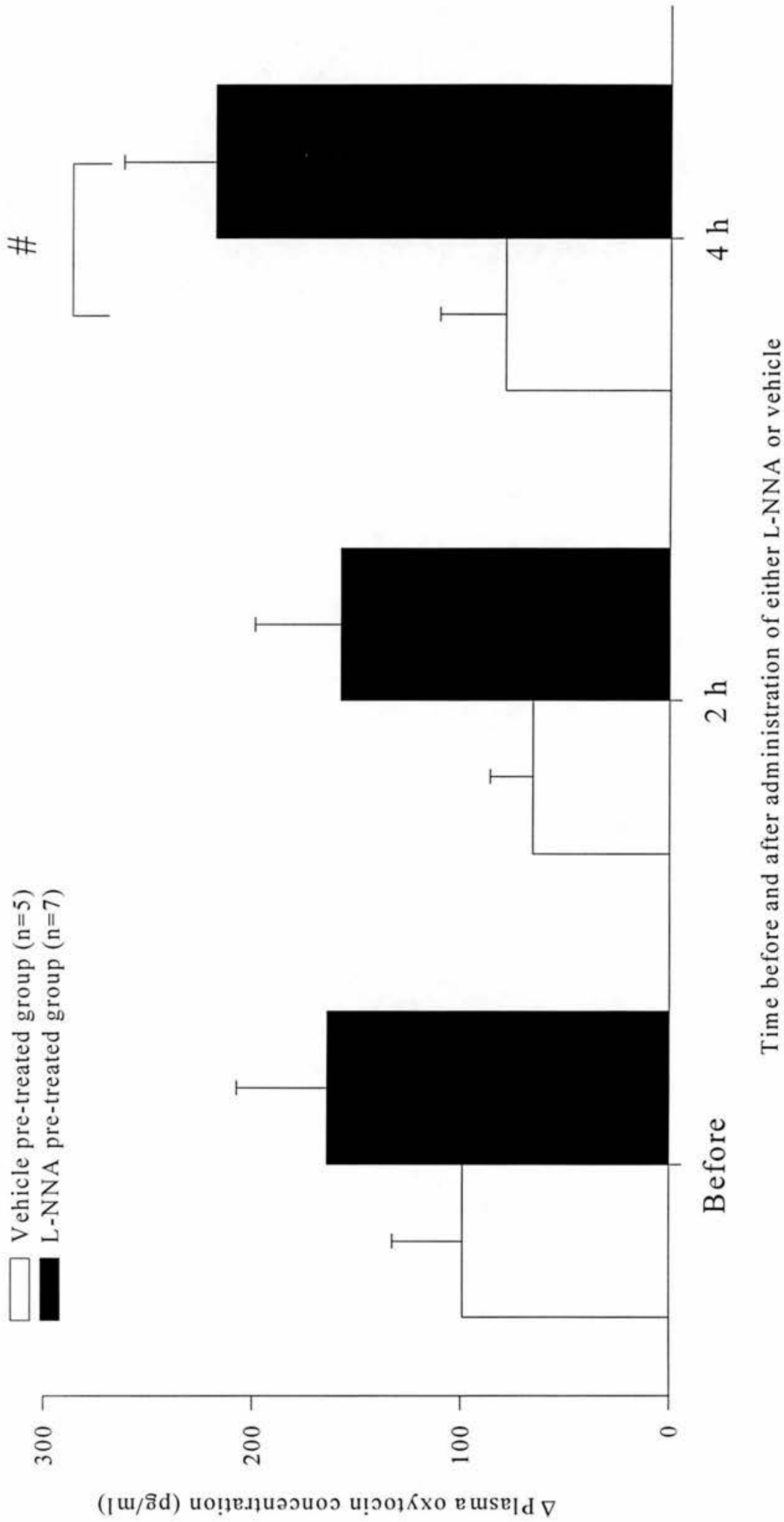

Figure 26. Effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on oxytocin release following neural stalk stimulation *in vivo*. A significant difference for change of plasma oxytocin concentration was shown in 4 h L-NNA pre-treated rats compared to 4 h vehicle pre-treated rats (#, $P<0.05$, *t*-test). Values are means ± SEM.

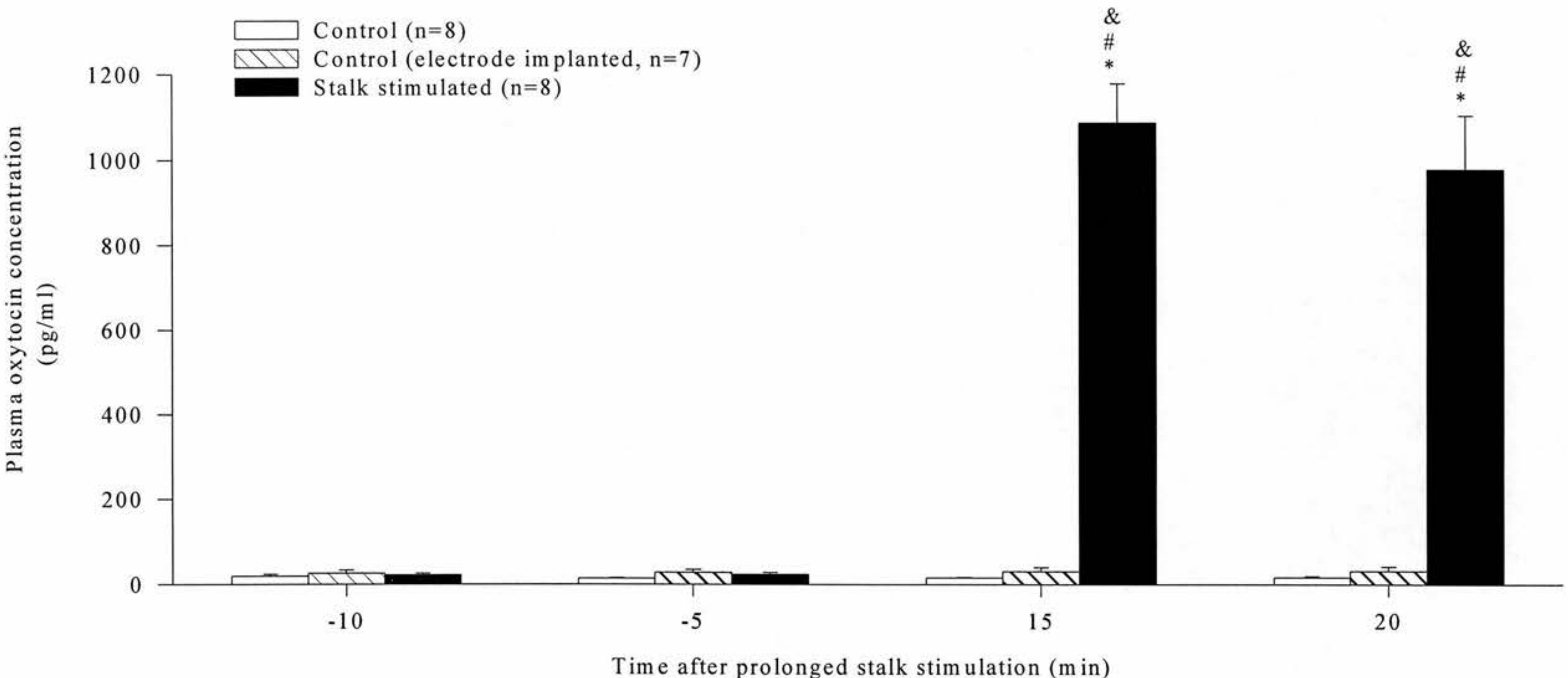

Figure 27. Plasma oxytocin concentration in response to neurohypophysial stalk stimulation (20 Hz, 10 s on, 10 s off, 1 mA peak to peak) *in vivo*. Note that basal oxytocin release in control, control (electrode implanted) and stalk stimulated rats were not significantly different. In stalk stimulated rats, oxytocin release increased significantly after stalk stimulation compared to basal (*, $P<0.05$). Values are means ± SEM.

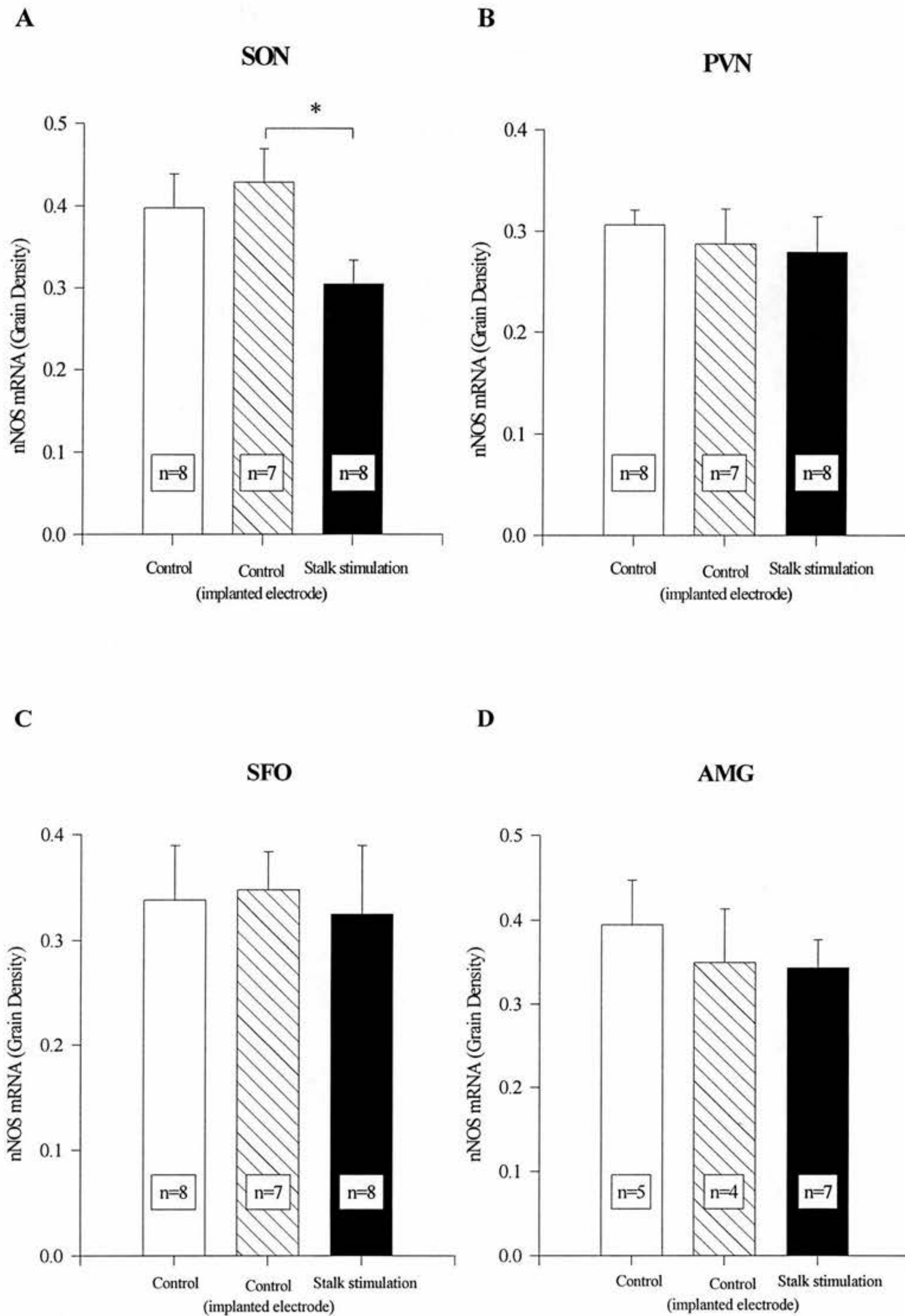

Figure 28. Neuronal NOS mRNA expression measured as film grain density over the SON, PVN, SFO and AMG. A significant decrease of nNOS mRNA expression was observed in the SON after prolonged stalk stimulation compared to control with implanted electrode (*, $P<0.05$, *t*-test). There was no significant change in NOS mRNA expression in the PVN (**B**), SFO (**C**) and AMG (**D**). Values are means ± SEM.

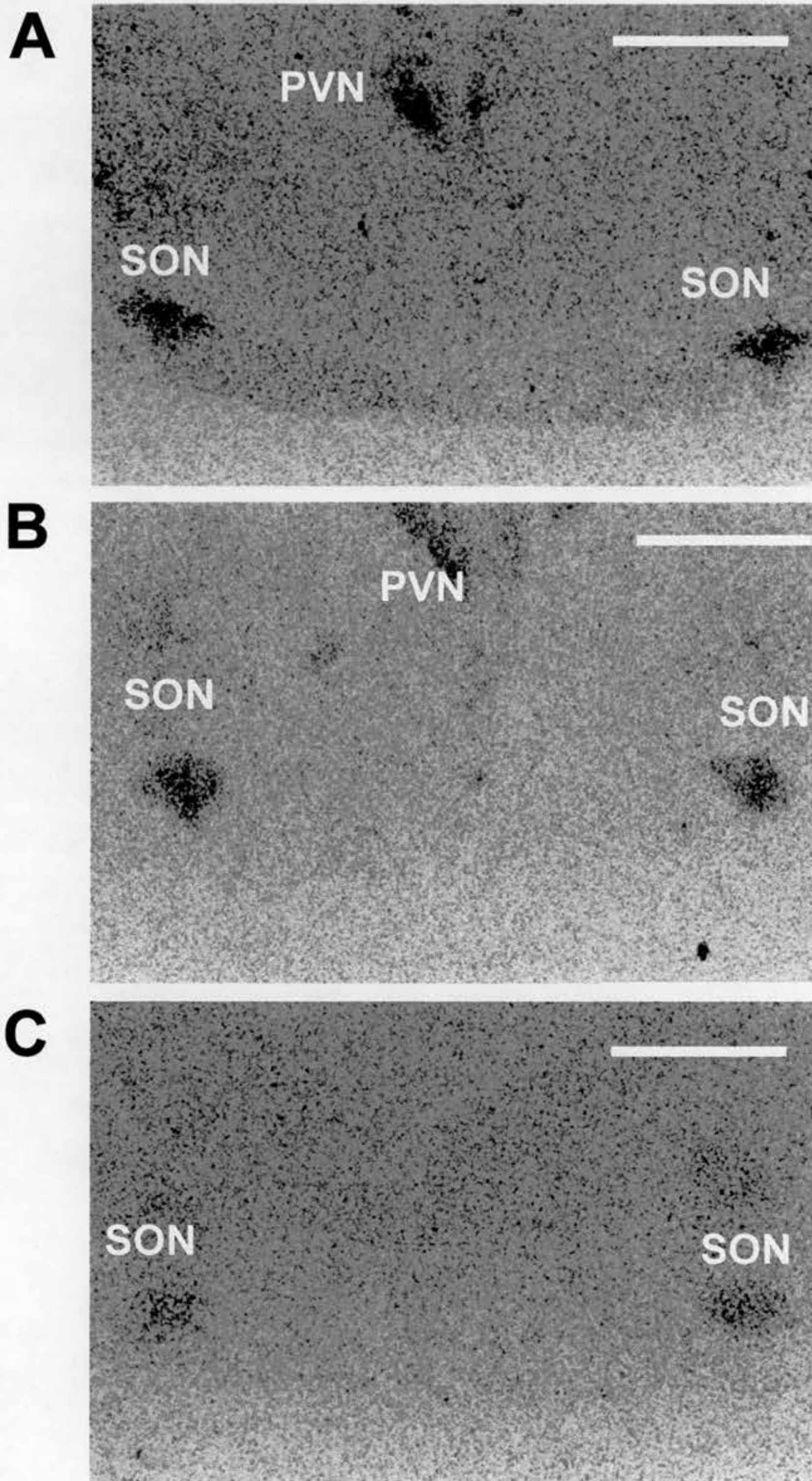

Figure 29. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the SON. Neuronal NOS mRNA signal in the SON in prolonged neurohypophysial stalk stimulated rats (**C**) was lower than in control rats with an implanted electrode (**B**). PVN; paraventricular nucleus, SON; supraoptic nucleus. Bar=1 mm.

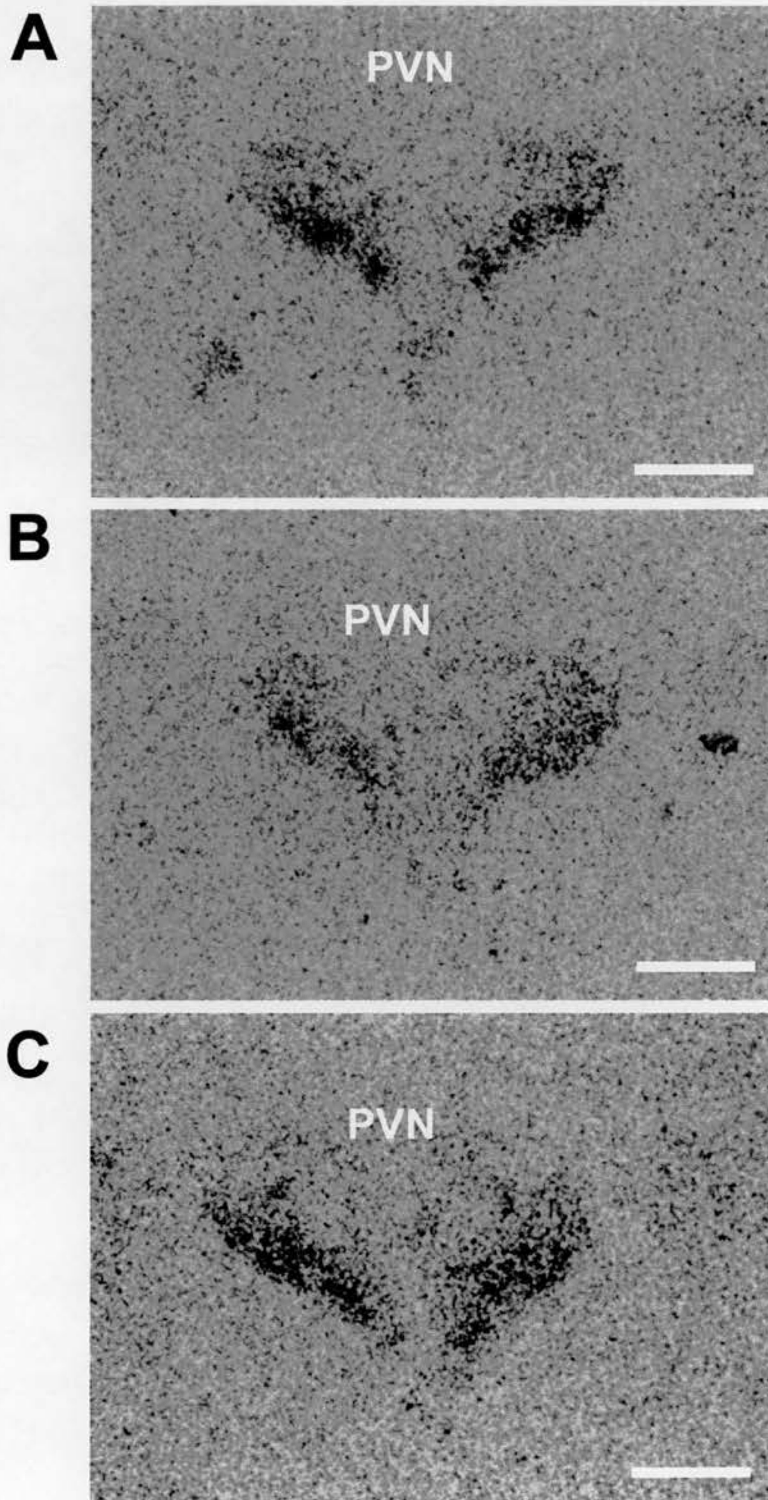

Figure 30. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the PVN. Neuronal NOS mRNA signal in the PVN in prolonged neurohypophysial stalk stimulated rats (**C**) was similar to control rats with and without an implanted electrode (**B**, **A**). PVN; paraventricular nucleus, SON; supraoptic nucleus. Bar=500 μm.

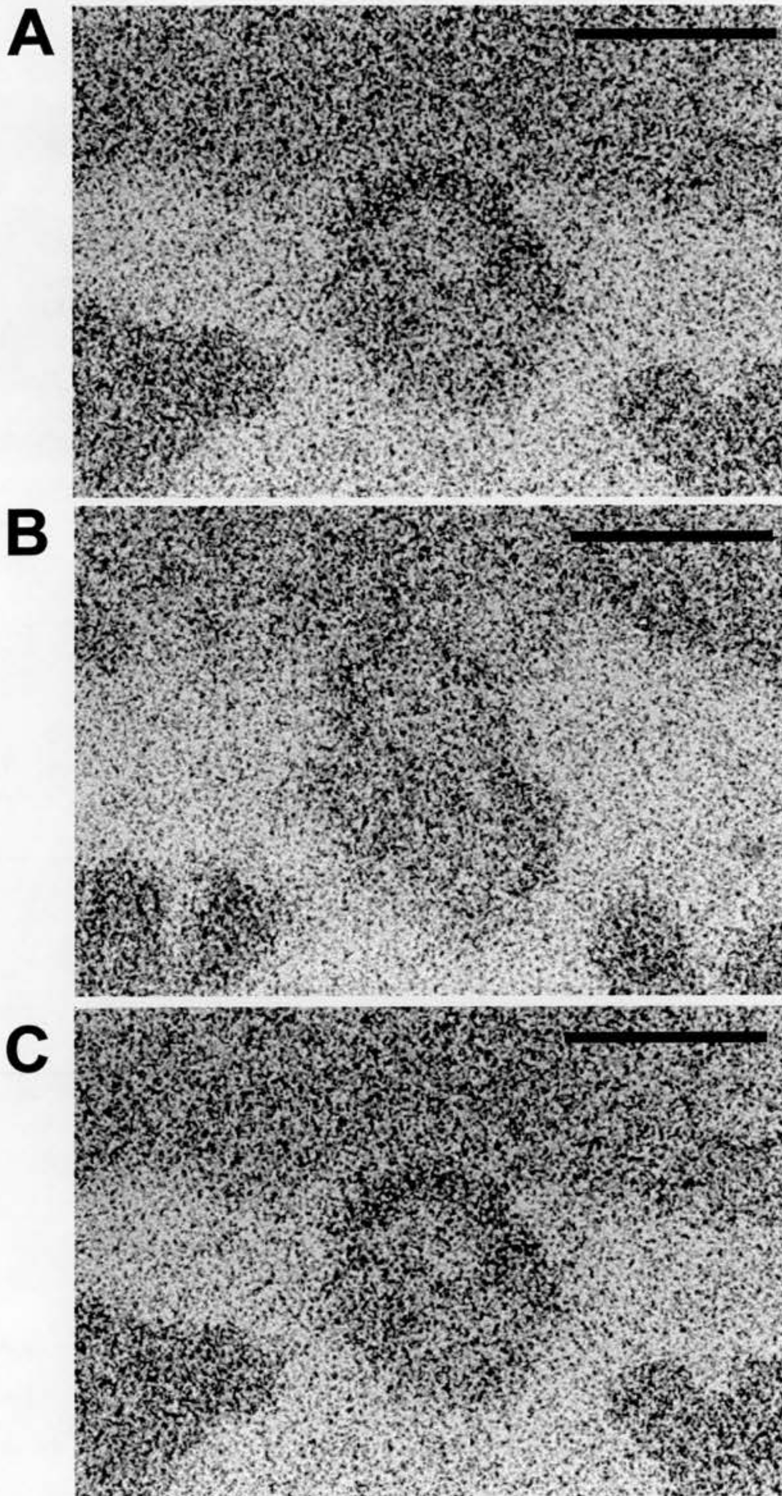

Figure 31. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the SFO. Neuronal NOS mRNA signal in the SFO in prolonged neurohypophysial stalk stimulated rats (**C**) was similar to control rats with and without implanted electrode (**B, A**). Bar=500 μm.

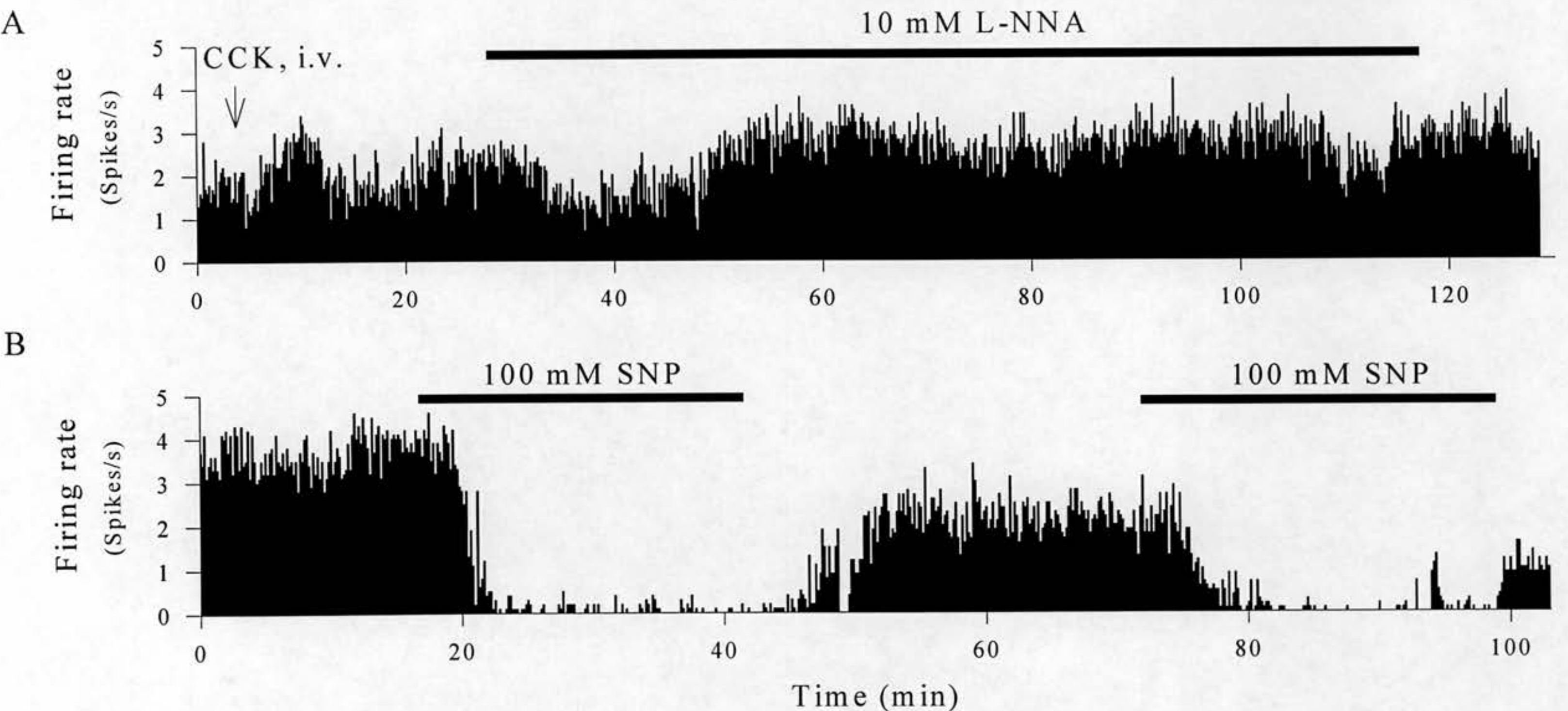

Figure 32. Effects of L-NNA and SNP retrodialysis onto the SON on activity of oxytocin neurones.

A, the spontaneous firing rate (in 10 s bins) of an oxytocin neurone identified by its transient increase in firing rate following CCK (2 μg/kg, i.v.). L-NNA(10 mM) administered over 80 min induced a small sustained increase in the firing rate of this neurone.

B, SNP (100 mM) administered twice over 20 min onto the same neurone inhibited this neurone for >20 min before spontaneous recovery of activity.

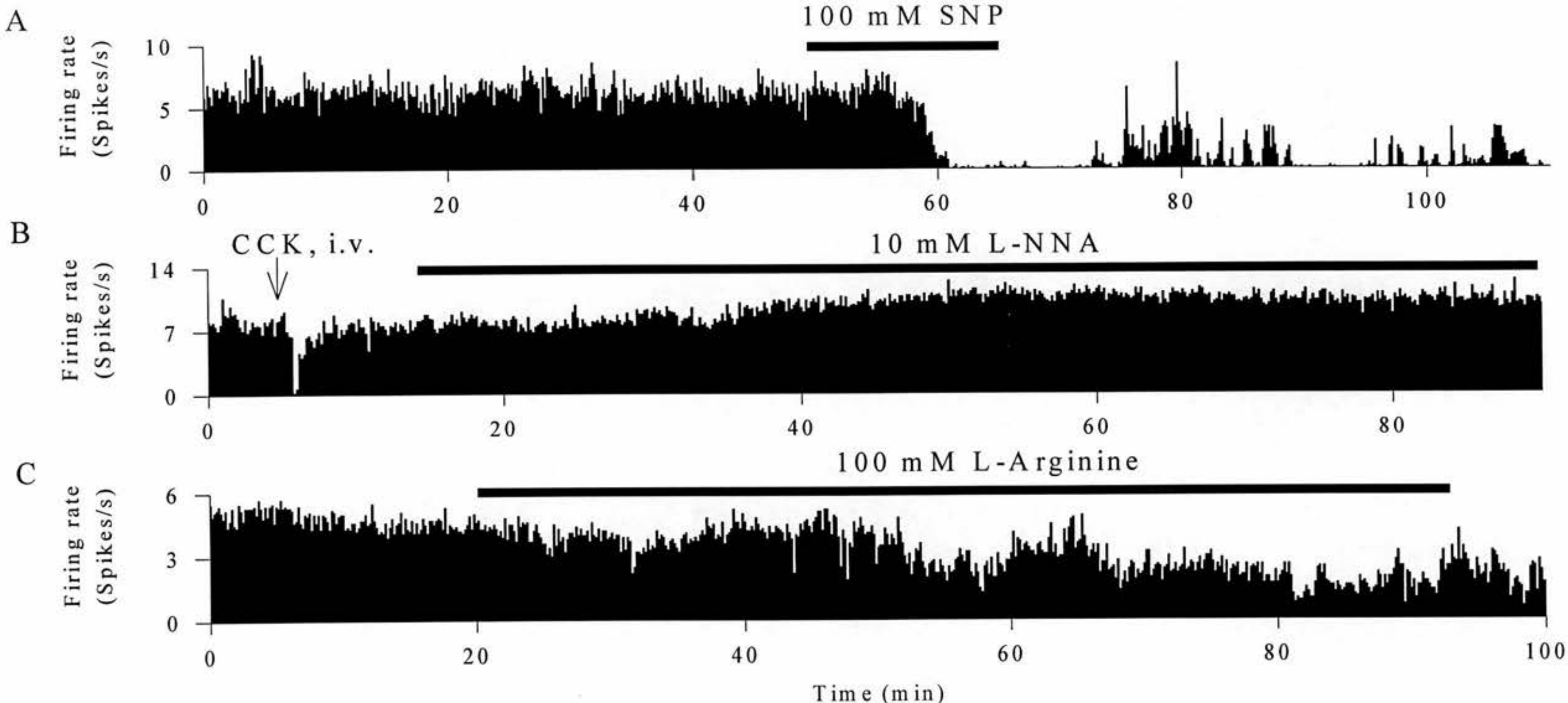

Figure 33. Effects of SNP, L-NNA and L-arginine retrodialysis onto the SON on activity of continuously firing vasopressin neurones.
A, SNP (100 mM) administered over 15 min onto the same neurone repeatedly inhibited this neurone.
B, An inhibitory response to i.v. CCK identified this as a vasopressin neurones; L-NNA (10 mM) administered over 60 min induced an increase in the firing rate of this neurone.
C, Inhibition of a continuously firing vasopressin neurone after administration of L-arginine over 70 min.

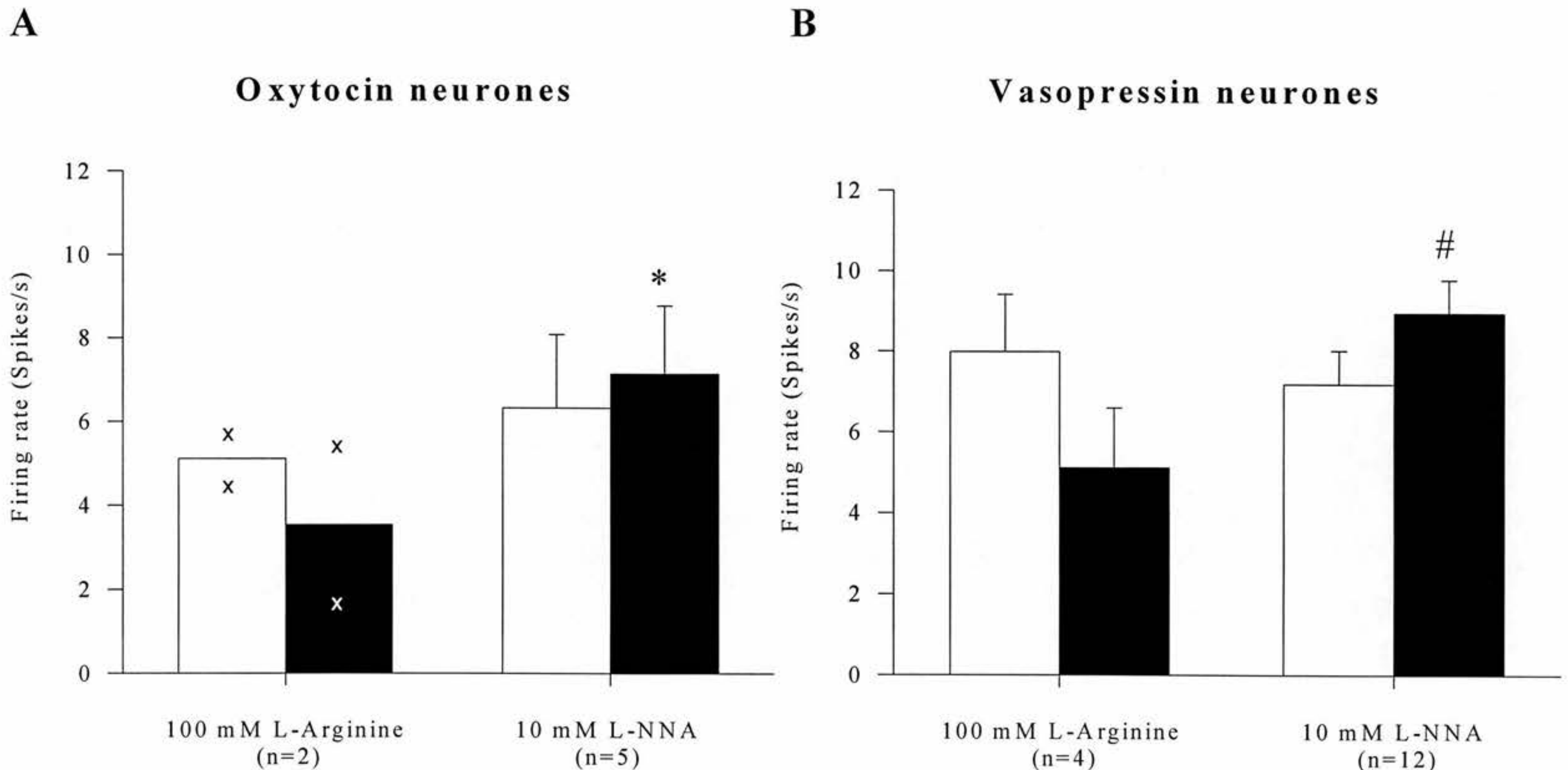

Figure 34. Effects of L-arginine and L-NNA retrodialysis onto the SON on firing rate of oxytocin and vasopressin neurones (Mean firing rates of all analysed cells ± SEM). L-arginine similarly tended to inhibit the spontaneous activity of both types of cells. L-NNA had a significant excitatory effect on the spontaneous activity of both types of cell (*, $P<0.05$ vs basal, Paired *t*-test and #, $P<0.0005$, Wilcoxon Signed Rank test). □ represents 5 min period before and ■ represents successive 5 min periods after drug administration.

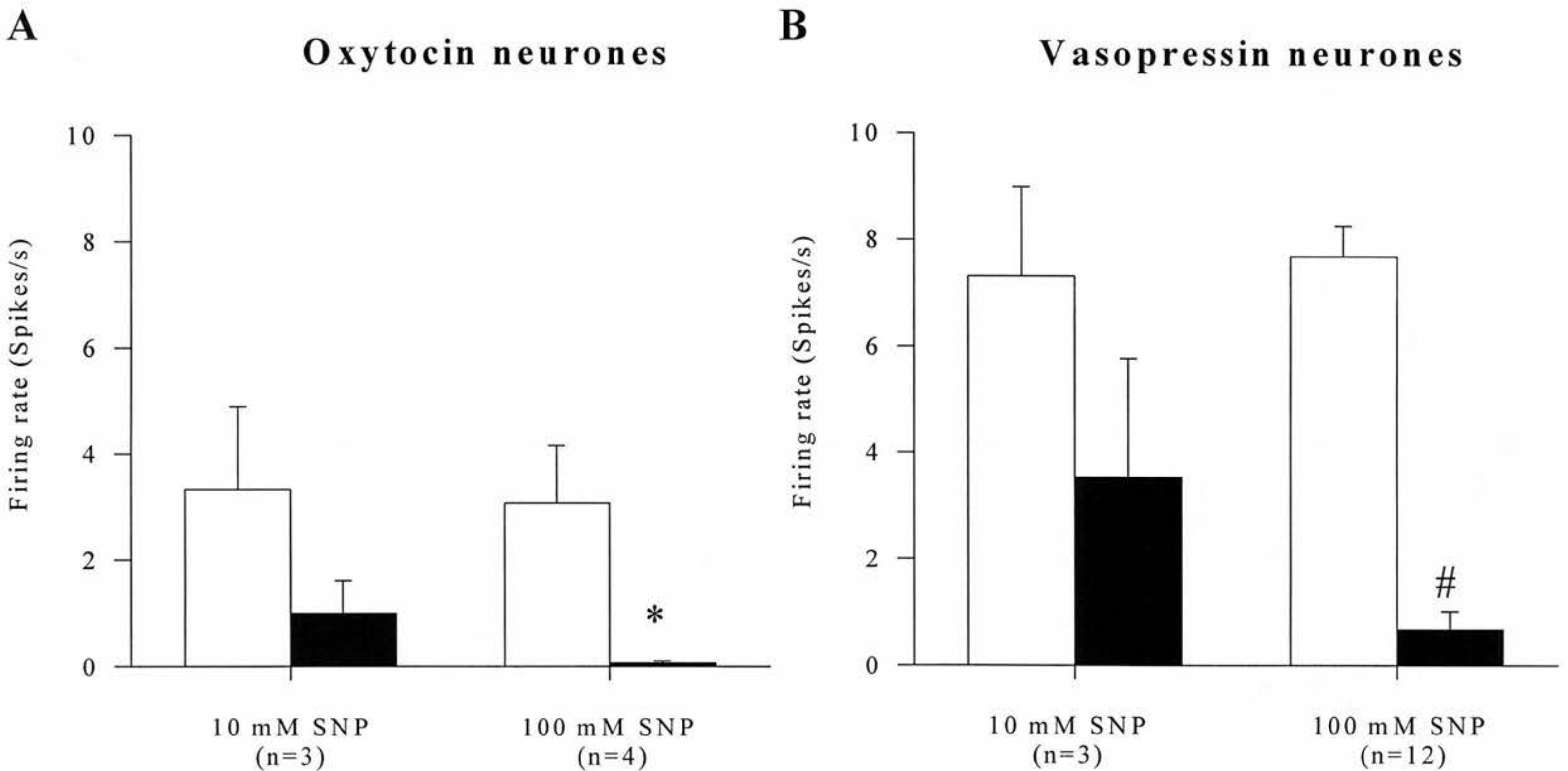

Figure 35. Effects of SNP retrodialysis onto the SON on firing rate of oxytocin and vasopressin neurones (Mean firing rates of all analysed cells ± SEM). The spontaneous firing rate of both oxytocin and vasopressin neurones was inhibited by SNP in a dose-dependent way (*, $P<0.05$, One Way ANOVA; Student-Newman-Keuls Method, #, $P<0.0001$, paired *t*-test). □ represents 5 min period before and ■ represents successive 5 min periods after drug administration.

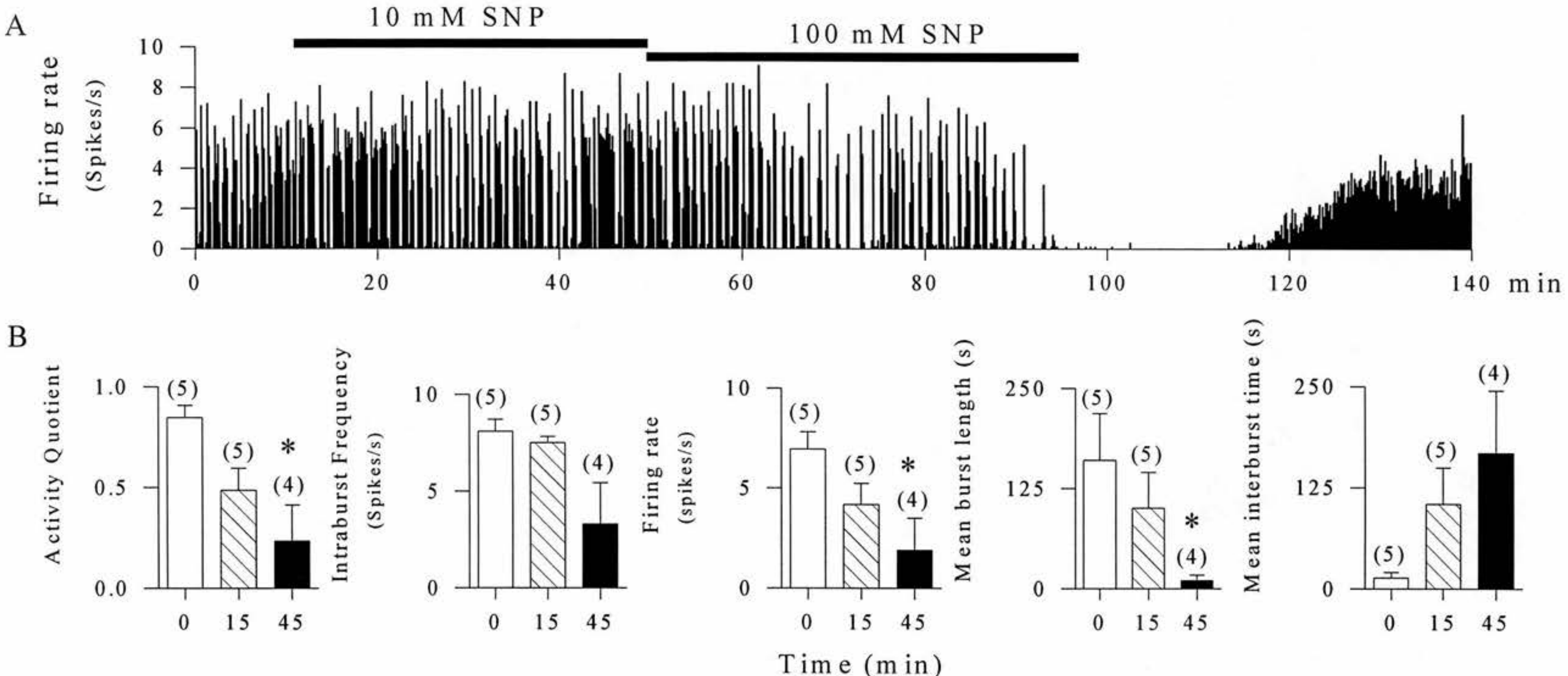

Figure 36. Effects of SNP retrodialysis onto the SON on activity of phasic vasopressin neurones.

A, this neurone was silenced after administration of 100 mM SNP over 30 min, but not after administration of 10 mM SNP. SNP inhibited this neurone for >20 min before recovery of spontaneous activity.

B, a significant decrease of activity quotient, firing rate, and mean burst length of phasic vasopressin neurones was observed after administration of 100 mM SNP for 45 min (*, $P < 0.05$, One Way ANOVA; Dunnett's method). Numbers in parentheses indicate the numbers of rats. Values are means ± SEM (5 min bins).

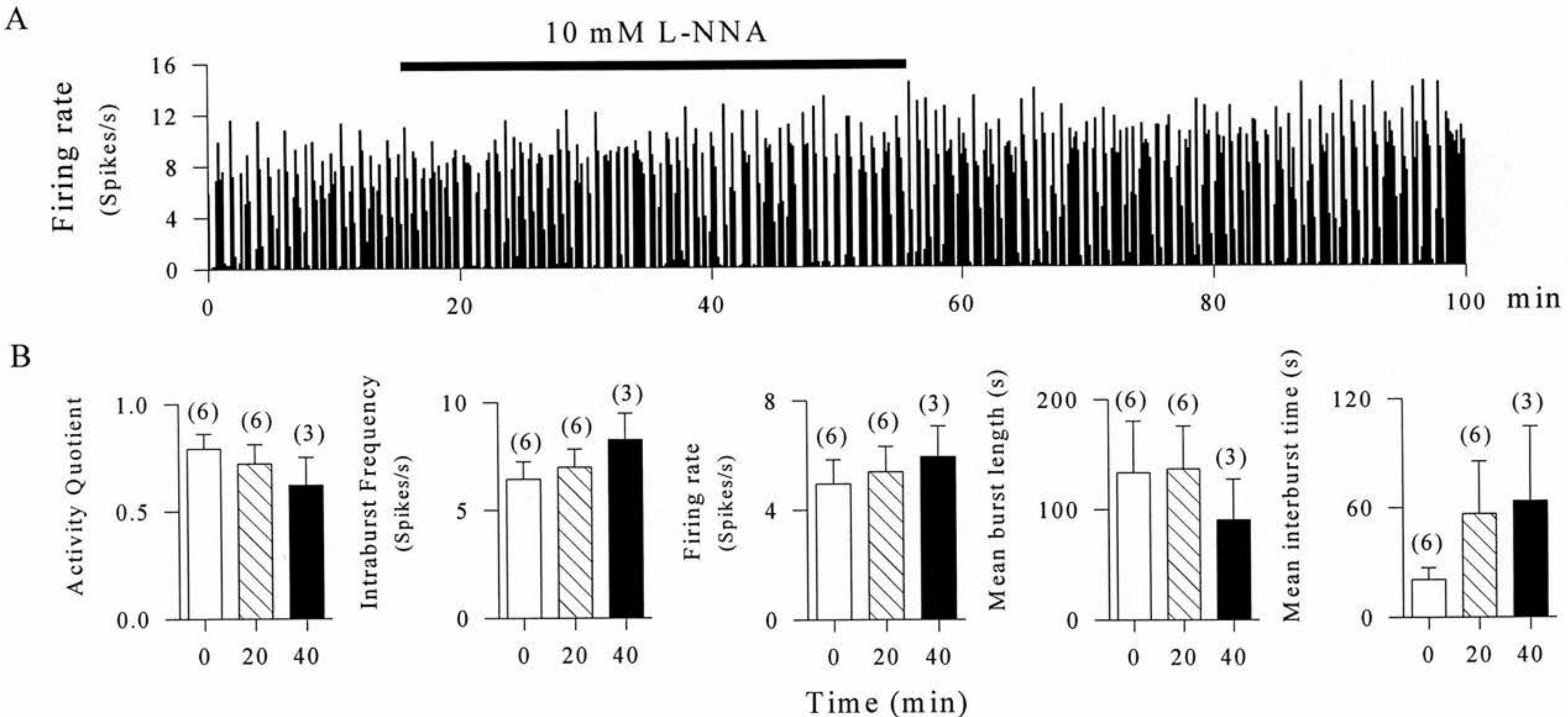

Figure 37. Effects of L-NNA retrodialysis onto the SON on activity of phasic vasopressin neurones.
A, Retrodialysis of L-NNA (10 mM) over 40 min did not significantly change the activity of this neurone.
B, activity quotient, intraburst frequency, firing rate, mean burst length and mean interburst interval of phasic vasopressin neurones did not show significant differences after administration of 10 mM L-NNA for 20 and 40 min. Numbers in parentheses indicate the numbers of rats. Values are means ± SEM (5 min bins).

4.5 Discussion

The effect of the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) on Fos expression in the SON and PVN in hypertonic saline treated rats

In the present experiments, expression of Fos was studied in the SON and PVN following intraperitoneal injection of hypertonic saline in rats pre-treated with L-NNA. In addition to inhibiting NOS activity (Dwyer *et al.*, 1991), L-NNA may reduce NO formation by inhibiting re-uptake of L-arginine (Westergaard *et al.*, 1993). The result of both actions would be reduce the availability of NO. In this study, systemic administration of L-NNA alone did not alter significantly the expression of Fos in either the SON or PVN in pentobarbital-anaesthetised rats (Fig. 19, 20), suggesting that NO does not regulate basal Fos expression in the SON and PVN. By contrast, other authors have recently reported that i.p. injection of L-NAME reduced urethane-induced Fos expression in the SON and PVN (Woodside and Popeski, 1999). Urethane dehydrates the rat by elevating plasma osmolality (Hartman *et al.*, 1987), and urethane induced more Fos-positive neurones than pentobarbitone (Takayama *et al.*, 1994). Therefore, Fos expression in the SON and PVN following osmotic stimuli is modulated by NO.

Corresponding to previous findings (Giovannelli *et al.*, 1992; Hamamura *et al.*, 1992), injection of either 2 ml/kg 1.5 M NaCl or 4 ml/kg 1.5 M NaCl but not isotonic 0.15 M NaCl, caused increased Fos expression in the SON and PVN in a dose-dependent manner. We chose 90 min after hypertonic saline administration to assess Fos expression (Hamamura *et al.*, 1992) as this time point gives that maximal number of Fos-positive neurones (Giovannelli *et al.*, 1992). Interestingly, Fos expression in the SON induced by high doses of hypertonic saline (4 ml/kg 1.5 M NaCl, i.p.) but that not induced by low doses of hypertonic saline (2 ml/kg 1.5 M NaCl, i.p.) was enhanced by systemic administration of L-NNA (Fig. 19, 23), suggesting an inhibitory effect of NO on the activity of the SON neurones at high levels of activity. This finding parallels our previous results in Chapter 3 that the

release of oxytocin induced by high doses of hypertonic saline (4 ml/kg 1.5 M NaCl, i.p.) was enhanced by the NOS inhibitor, L-NNA and L-NAME (Fig. 5, 6), whereas CCK-induced oxytocin release was not altered by L-NNA and L-NAME. These data are in accord with the findings of Kadekaro *et al.* (1997) who demonstrated that plasma oxytocin release in response to similar doses of hypertonic saline was potentiated by L-NAME in conscious rats. Other authors have demonstrated an inhibitory role of NO on KCl-evoked vasopressin release by acting directly on the cell bodies in the SON and PVN *in vitro* (Yasin *et al.*, 1993). Thus, NO exerts a predominantly inhibitory effect directly on the cell bodies in the SON and PVN, and may serve within a negative-feedback loop to restrain the production and release of oxytocin in condition of intense stimulation.

The role of NO in oxytocin release following electrical stimulation of the neural stalk

The present experiments provide the first evidence for an inhibitory effect of NO on the release of oxytocin at the nerve terminals of magnocellular neurones *in vivo*. Systemic administration of L-NNA did not alter the basal release of oxytocin (Fig. 25). As expected, electrical stimulation of the axons of magnocellular neurones caused an increase in the plasma concentration of oxytocin before drug treatment but also 2 h after administration of either vehicle or L-NNA (Fig. 25). At 4 h after pre-treatment with either vehicle or L-NNA, a significant increase in evoked oxytocin release was found in rats pre-treated with L-NNA. Our results parallel the previous *in vitro* study of Lutz-Bucher and Kuch (1994) who reported that NO attenuated KCl-evoked the release of vasopressin from pituitary explants.

Application of high frequency electrical stimuli to the neural stalk of the pituitary evokes action potentials conducted antidromically to the cell bodies and conducted orthodromically to the nerve terminals (Dyball *et al.*, 1988). An influx of calcium into the nerve terminals following orthodromically action potentials causes an increase of intracellular calcium, which will activate NOS enzyme to promote the production of NO. Once released, NO is thought to act by stimulating cytoplasmic

guanylate cyclase, resulting in the elevation of cGMP (Southam and Garthwaite, 1993; Wood and Garthwaite, 1994), with a half life of a few seconds. Cyclic GMP can regulate physiological events by interaction with target proteins, including cGMP-gated ion channels and cGMP-dependent protein kinases and cGMP-regulated cyclic nucleotide phosphodiesterases (Garthwaite, 1991; Garthwaite and Boulton, 1995; Lincoln and Cornwell, 1993; McDonald and Murad, 1996). This means that cGMP can alter cell function both through protein phosphorylation and through mechanisms not directly related to protein phosphorylation. It has been shown recently that cGMP and NO itself directly inactivated NOS activity in medial basal hypothalamus explants and homogenates, providing an ultra-short loop negative feedback (Canteros *et al.*, 1996).

Depolarization induced by high concentration of potassium chloride (KCl) also stimulates the release of oxytocin, vasopressin and co-exiting neuropeptides from hypothalamic explants and isolated neural lobe (Cazalis *et al.*, 1987). Vale and Hope (1982) demonstrated that the release of cGMP, cAMP and vasopressin from the rat isolated neural lobe was evoked by high concentration of KCl, suggesting that KCl probably enhanced the production of NO, which in turn binding to the target molecule sGC resulting in an increase of cGMP (Moncada *et al.*, 1989; Southam and Garthwaite, 1993; Wood and Garthwaite, 1994).

Consistent with our functional studies, Beagly *et al.* (1997) have postulated that NO may play a role in the maintenance of structural relations in the neurohypophysis. Neurohypophysis of rats injected with L-NAME and hypertonic saline had significantly less contact between the basal lamina and the membrane of axon terminals and a significantly larger number of axonal profiles enclosed within piuicytes than those of rats injected only with hypertonic saline. The activation of the magnocellular neurones caused increased NO production and that NO generated by these neurones activates sGC in the pituicytes to initiate altered pituicyte morphology through cGMP dependent processes.

NOS mRNA expression in the SON and PVN following prolonged neurohypophysial stalk stimulation

The present study showed for the first time that prolonged electrical stimulation of the axons of the magnocellular neurones for 2 h (20 Hz, 10 s on, 10 s off, 1 mA peak to peak) significantly down-regulated the expression of nNOS mRNA in the rat SON (Fig. 28A). However, the NOS mRNA expression in the PVN, SFO and AMG did not change after prolonged neural stalk stimulation (Fig. 28B, C, D). Down-regulation of nNOS mRNA following prolonged neural stalk stimulation in the present study was unexpected as an increase of NOS mRNA expression and NADPH-diaphorase staining in the SON and PVN has been demonstrated following hyperosmotic stimulation (Kadowaki *et al.*, 1994; Pow, 1992). Thus, although NOS expression is up-regulated in conditions where there is a chronic demand for oxytocin secretion (Kadowaki *et al.*, 1994; Ueta *et al.*, 1995b; Villar *et al.*, 1994b), the present experiments indicate that this does not reflect a coupling of spike activity to increased NOS mRNA expression.

Application of electrical stimuli to the neural stalk of the pituitary evoked the release of oxytocin as expected (Fig. 25A, 27), and this was enhanced by inhibition of NO production (Fig. 26). The physiological circumstances in which NO inhibits oxytocin secretion from the neurohypophysial nerve terminals are not yet clear, although recent evidence suggests that this system may be activated during conditions of chronic demand for oxytocin secretion such as dehydration. However, this is not the case during prolonged neural stalk stimulation. The important point is that increased spike activity in the cell bodies alone (from antidromic stimulation) did not stimulate the nNOS gene, but instead decreased nNOS mRNA content. Up-regulation of nNOS gene may therefore be stimulated by pathways activated through synapses on the neurones, and not as a consequence of electrical activity. The changes of nNOS mRNA expression in the SON indicate that the NOS system in the SON plays a long term as well as an acute neuromodulatory role in the rat magnocellular neurosecretory system.

It has been shown recently that a high concentration of SNP directly inactivates NOS activity when added to medial basal hypothalamus explants and homogenates, providing an ultra-short loop negative feedback on the activity of NOS (Canteros *et al.*, 1996). As in the case of SNP, cGMP also blocked NOS activity when incubated with medial basal hypothalamus explants and homogenates. Nitric oxide activated sGC in the SON, PVN and neurohypophysis, leading to the production and liberation of cGMP (Snyder and Bredt, 1991), mediates many of the actions of NO. This cGMP might diffuse within the NOS-containing neurones and reduce the activity of NOS. Therefore, high concentrations of NO evoked by prolonged electrical stimulation of the axons of the magnocellular neurones may block NOS activity in the NOS-containing neurones. Nitric oxide itself probably inhibits NOS activity by changing the conformation of the heme group in NOS (Canteros *et al.*, 1996). Therefore, high concentrations of produced NO could suppress the release of NO from NOS-containing neurones. Furthermore, high concentrations of cGMP released by NO may be taken up by NOS-containing neurones and suppress NOS activity.

The role of NO in the electrical activity of oxytocin and vasopressin cells

Application of intra-supraoptic nucleus retrodialysis of SNP, L-arginine and L-NNA coupled with the single cell recording technique provided the first information of the effect of local NO on the activity of oxytocin and vasopressin neurones *in vivo*. The present results demonstrate an inhibitory effect of NO on the neuronal activity of both oxytocin and vasopressin neurones in urethane-anaesthetised rats. Both oxytocin and vasopressin neurones increased their firing rate in response to L-NNA (Fig. 34). The firing rate of oxytocin and vasopressin neurones was decreased by SNP but not significantly decreased by L-arginine (Fig. 35). Our present results are consistent with the *in vitro* electrophysiological study of Liu *et al.* (1997) who reported inhibitory effects of NO on neuronal activity of both phasic and non-phasic firing neurones in the SON.

There are two mechanisms that may explain the inhibitory action of NO on oxytocin and vasopressin neurones; first, NO inhibits NMDA receptor-mediated glutamatergic excitatory inputs by acting at postsynaptic sites of the SON neurones. Cui *et al.* (1994) has reported that NO suppressed the membrane depolarization evoked by the glutamate receptor agonist NMDA in SON neurones *in vitro*. Alternatively, NO may potentiate inhibitory synaptic inputs to SON neurones by acting at presynaptic GABA neurones. Nitric oxide increased the frequency, but not the amplitude, of spontaneous inhibitory postsynaptic currents (IPSPs) recorded from the SON neurones *in vitro*, representing presynaptic GABA release, while NO had no effect on excitatory postsynaptic currents (EPSPs), representing presynaptic glutamate release (Ozaki *et al.*, 1999). These two mechanisms are mediated *via* cGMP-independent pathways. The mechanism that may explain the inhibitory action of NO on PVN neurones may be different from those of the SON neurones. In the rat PVN, NO potentiated GABA inputs *via* postsynaptic as well as presynaptic mechanisms, since NO increased both the amplitude and the frequency of spontaneous IPSPs recorded from the PVN type I neurones (putative vasopressin or oxytocin) *in vitro* (Bains and Ferguson, 1997b).

In conclusion, the results from this chapter provide information about the mechanisms by which NO influences oxytocin and/or vasopressin secretion. Nitric oxide exerts a predominantly inhibitory effect on the neuronal activity of oxytocin and vasopressin neurones in the SON. Endogenous NO inhibits oxytocin release, and nNOS co-existing with either oxytocin or vasopressin generates NO in an activity-dependent manner. NO may serve within a negative feedback loop to control the production and release of oxytocin by acting at both the cell bodies and the nerve terminals of oxytocin neurones to prevent over stimulation of oxytocin. However, although NOS expression is up-regulated in conditions of chronic demand for oxytocin secretion, the present experiments indicate that this does not reflect a coupling of spike activity to increased NOS mRNA expression.

CHAPTER 5

nNOS mRNA EXPRESSION IN THE MAGNOCELLULAR NEUROSECRETORY SYSTEM AFTER ACUTE HYPERTONIC STIMULATION AND CHRONIC SALT LOADING

5.1 Introduction

The hypothesis that NO plays an important role in osmoregulation of vasopressin and oxytocin release was initiated by the study of Sagar and Ferriero in 1987. They found an increase of NADPH-diaphorase staining in the rat posterior pituitary gland after 8-days of salt loading. Up-regulation of nNOS mRNA expression was also found in the SON and PVN after chronic salt loading (Kadowaki *et al.*, 1994; Villar *et al.*, 1994b). Dehydrated rats also show an increase of NADPHd activity in the SON (Pow, 1992) and nNOS mRNA expression in the SON and PVN (Ueta *et al.*, 1995b), however, it is not clear that acute hypertonic administration can regulate nNOS gene expression as chronic salt loading and dehydration do. The anatomical findings of changes of NOS system within the magnocellular neurosecretory system during chronic hyperosmotic stimulation are complemented by functional studies. An increase in NO-forming activity in the posterior pituitary gland has been reported after chronic osmotic stimulation (Kadowaki *et al.*, 1994). Under normal salt–water balance conditions, endogenous NO tonically inhibits secretion of vasopressin and oxytocin (Kadekaro *et al.*, 1997; Liu *et al.*, 1997; Liu *et al.*, 1998). Centrally-produced NO selectively inhibits oxytocin release in response to a painful stimulus (needle prick) and moderate osmotic stimulation (Kadekaro *et al.*, 1997), in dehydrated rats (Summy-Long *et al.*, 1998; Summy-Long *et al.*, 1993) and in angiotensin II-treated rats (Liu *et al.*, 1997). In parallel, systemic inhibition of NOS enhanced oxytocin and vasopressin release in salt-loaded rats (Kadowaki *et al.*, 1994). Furthermore, NO inhibits stimulated vasopressin secretion from rat hypothalamic explants *in vitro*, without affecting basal secretion (Yasin *et al.*, 1993). Nitric oxide also inhibits the basal release of vasopressin from the pituitary gland *in vitro* (Lutz-Bucher and Koch, 1994). However, the effect of NO on vasopressin release remains controversial since there is also evidence that centrally produced NO can stimulate vasopressin secretion in rats *in vivo* (Ota *et al.*, 1993).

Although many studies have demonstrated that osmotic stimuli cause a marked increase in NOS activity and in the expression of nNOS mRNA in the PVN

and SON (Kadowaki *et al.*, 1994; Pow, 1992; Ueta *et al.*, 1995b; Villar *et al.*, 1994b), there is no direct evidence about whether the expression of nNOS mRNA in the PVN, SON and structures of the laminae terminalis is modulated by acute hypertonic saline administration In the present study we examined the time course changes in nNOS mRNA in the SON, PVN and structures in the laminae terminalis following acute hypertonic administration using *in situ* hybridization histochemistry.

5.2 Aims

- To consider the possible role of NO in osmoregulation.
- To investigate the up-regulation of NOS gene expression in the SON and PVN after chronic salt loading.
- To examine whether acute hypertonic saline administration has a rapid effect on the expression of the NOS gene in the SON, PVN and structures of the lamina terminalis.

5.3 Materials and Methods

5.3.1 Animals

Female Sprague-Dawley rats, weighing 250-300 g, were used throughout the experiments. Before the experimental manipulation, all rats were housed under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1°C) with free access to food and water, or otherwise, as stated in the experiments.

5.3.2 Experimental procedure

1. Neuronal NOS gene expression in the SON, PVN and SFO in salt-loaded rats

Rats were divided into two groups. The control group received food and water *ad libitum*. The salt loading group received free access to food and 2% NaCl

solution as the only source of water. After four days of salt loading, rats were decapitated, their brains were rapidly removed and frozen on dry ice, before storing at -70°C and processing for *in situ* hybridization histochemistry.

2. Time course of the effects of acute hypertonic saline administration on nNOS gene expression in the SON, PVN, and structures of the lamina terminalis.

Rats were given hypertonic saline (4 ml/kg 1.5 M NaCl; i.p.) or isotonic saline. They were decapitated after 2h, 4h or 6h. and the brains were rapidly removed. The brains were then frozen on dry ice and kept at -70°C and processed for *in situ* hybridization histochemistry.

5.3.3 *In situ* Hybridization

The full methodology is described in General Methods 2.8.

Brain sections

Brains were coronally sectioned (15 μm) on a cryostat through the level of the structures of the lamina terminalis (the OVLT, MnPO and SFO), SON and PVN, using the stereotaxic atlas of Paxinos and Watson (1982) and thaw-mounted onto gelatin-coated glass slides. Slides were then stored in desiccated slide boxes at -70°C before *in situ* hybridization.

Probes

Three 45-mer antisense oligonucleotide probes for nNOS which were complementary to bases 223-267 (5′-noncoding region), 4714-4758 (3′-noncoding region) and 1662-1706 of the rat nNOS sequence (Bredt *et al.*, 1991b) were used. Probes were labelled at the 3′ end with [α-^{35}S]deoxy-ATP (NEN) using terminal deoxynucleotidyltransferase (Pharmacia).

In situ hybridization

Sections were fixed with 4 % paraformaldehyde in 0.1 M PB (pH 7.4) for 30 min, then rinsed in 0.1 M PBS and washed in 0.1 M PBS (10 min), followed by acetylation in triethanolamine solution (0.25% acetic anhydride in 0.1 M triethanolamine-0.15 M NaCl) for 10 min. The sections were then dehydrated through 70%, 80%, 95% and 100% ethanol (5 min each), delipidated in chloroform for 10 min, and partially rehydrated in 95% ethanol (10 min). After air-drying the slides were placed in a humidified chamber. Sections were covered with hybridization buffer (4xSSC, 50% formamide, 1xDenhardt's solution, 500 μg/ml sheared salmon sperm DNA, 10% dextran sulphate and 0.3% mercaptoethanol) containing the ^{35}S-labelled NOS oligonucleotide probes at a concentration of 2500 dpm/μl and left to hybridize for 17 h at 37°C.

After the hybridization process, excess hybridizing solution was drained off. Slides were briefly rinsed three times in 1xSSC at room temperature, washed three times for 30 min 1xSSC and were washed for a further 1 h in 1xSSC at room temperature. Slides were air-dried and dipped in 300 mM ammonium acetate, 70% ethanol and then air-dried. Slides were placed in autoradiographic cassettes and exposed to Hyperfilm-βmax autoradiography film (Amersham, UK) together with brain paste standards. The film was left to expose for 3 weeks at 4°C. The films were developed in D19 developer (Kodak) and then fixed (Ilford Hypam rapid fixer). After that, sections were dipped in Ilford G-5 emulsion-coated slides (Mobberley) diluted 1:1 with autoclaved water. Emulsion-coated slides were exposed for a further 4-5 weeks to visualize NOS cellular signal, and then developed (Kodak D19 developer) for 5 min and fixed (Ilford Hypam rapid fixer twice 5 min each). Developed sections were counterstained with cresyl violet, dehydrated in a graded ethanol series, and coverslipped in DPX mountant.

Quantitative Analysis

The amount of nNOS mRNA in the regions of interest was assessed by silver grain density of autoradiographic films of the SON, PVN, SFO and AMG viewed under the light microscope, ×5 and ×10 objective. Grain density was quantified by NIH Image analysis system version 1.58 on an Apple McIntosh computer. The value of silver grain density per unit area was obtained by subtracting background of each specific tissue measurement, and dividing by the area measurements. Each brain paste standard was measured twice, and also corresponding background measurements. A logarithmic relationship was then plotted for radioactivity against the grain density of the standards. Acceptable grain density values lay on the straight portion of the curve. For each identified brain area, the mean grain density per unit area of each rat was calculated and the group mean grain density per unit area calculated.

Specific radioactivity of labelled probes was ranged from 367.01 to 1457.18 Ci/mmol.

5.3.4 Statistical Analysis

Data are expressed as means ±SEM. Groups were compared using the *t*-test (SigmaStat). $P < 0.05$ was considered statistically significant.

5.4 Results

1. Neuronal NOS gene expression in the SON, PVN, and SFO in salt-loaded rats

At the end of the experiment, the rats given 2% NaCl for 4 days had a mean body weight (n=8) of 243.46 ± 2.60 g, while the mean body weight of control rats (263.88 ± 2.79 g, n=8) was significantly greater (P=0.0001, *t*-test).

Strong hybridization signals for nNOS mRNA were observed in the SON, the magnocellular region of the PVN, the SFO and the medial amygdaloid nucleus (AMG). An increase of NOS mRNA signal was apparent as an increase in silver grain density in the SON and the magnocellular part of the PVN of chronically salted-loaded rats (Fig. 39A, C). The neuronal NOS mRNA signal was unchanged in the SFO (Fig. 39E) and AMG of chronic salt loaded rats compared to control rats.

Up-regulated nNOS mRNA expression was revealed as a significant increase in mean silver grain density per unit area, in the SON and magnocellular region of the PVN compared to their respective controls after 4 days of salt loading (n=8 in each group, P<0.05 and P<0.005, *t*-test, respectively, Fig. 38). After 4 days of salt loading (n=8), there was a significant 1.53-fold and 1.61-fold increase in the expression of nNOS mRNA in the SON and magnocellular region of the PVN, respectively. There was no significant change in nNOS gene expression in other areas measured after 4 days salt loading, including the SFO and AMG at this time (Fig. 38).

2. Time course of the effects of acute hypertonic saline administration on nNOS gene expression in the SON, PVN, and structures of the lamina terminalis.

At the end of the experiment, the rats given 1.5 M NaCl (i.p.) 2h, 4h and 6h previously had mean body weights of 240.38 ± 3.35, 243.13 ± 3.44 and 242.50 ±

3.77 g, respectively (n=8 for each group). There was no significant difference when compared to the mean body weight of control rats (241.40 ± 1.91 g, n=15).

Hybridization signals for nNOS mRNA were intense in the OVLT, MnPO, SFO, SON, PVN and AMG. An increase of NOS mRNA signal was revealed by analysis of silver grain density in the SON, and in the magnocellular part of the PVN 6 h after administration of 1.5 NaCl (Fig. 41D, 42D) and in the MnPO 4h following administration of hypertonic saline, but not after 6 h (Fig. 43C). Neuronal NOS mRNA signal was similar in the OVLT, SFO and AMG 2h, 4h and 6h after hypertonic saline administration (Fig. 44-45).

Up-regulation of nNOS mRNA expression was evident as a significant increase in mean silver grain density per unit area, in the SON and magnocellular region of the PVN 6 h after hypertonic saline administration and in the MnPO after 4 h compared to isotonic saline administration ($P<0.05$, *t*-test, Fig. 40). Quantitation of the autoradiographic image analysis obtained from the SON and magnocellular part of the PVN 6 h after 1.5 NaCl (i.p.) showed a significant 1.21-fold and 1.16-fold increase in nNOS mRNA expression in the SON and magnocellular region of the PVN, respectively (n=8, Fig. 40A, B). The expression of nNOS in the MnPO showed a significant 1.25-fold increase following 4 h administration of hypertonic saline (n=7, Fig. 40D). In the OVLT, we were unable to demonstrate a significant up-regulation of nNOS gene 2 h, 4h and 6 h after hypertonic saline administration. However, the expression of nNOS mRNA in the OVLT does increase (not significantly) 4h after hypertonic saline injection (n=8, Fig. 40E). There was no change in nNOS gene expression in the SFO and AMG 2, 4 and 6 h after hypertonic saline administration.

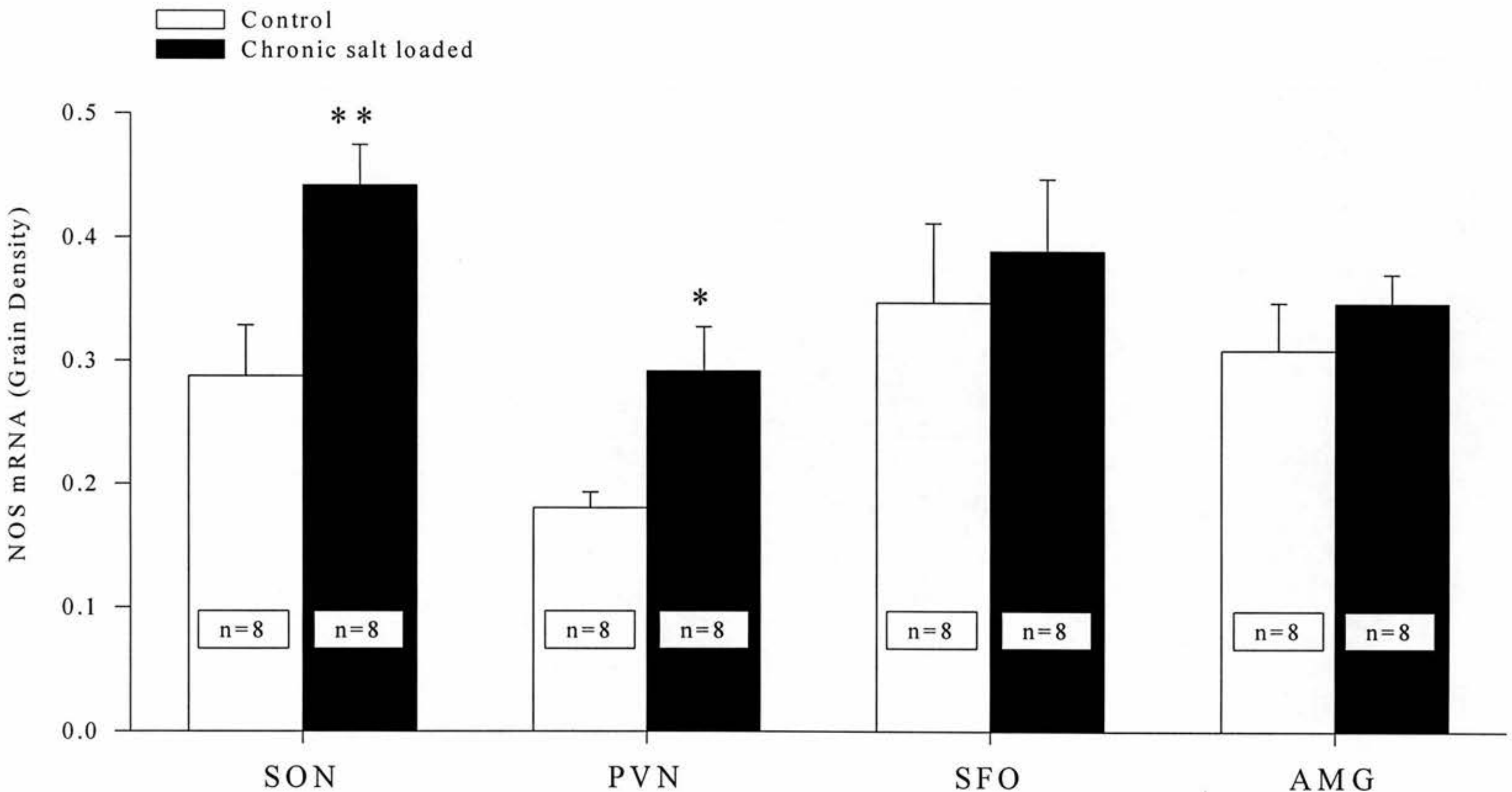

Figure 38. Neuronal NOS mRNA expression measured as film grain density over the SON, PVN, SFO and AMG. After chronic salt loading (4 days), a significant increase in nNOS mRNA expression was observed in both the PVN and SON (*, ** $P<0.05$, $P<0.005$, *t*-test, respectively). There was no significant change in nNOS mRNA expression in the SFO and AMG. Values are means ± SEM.

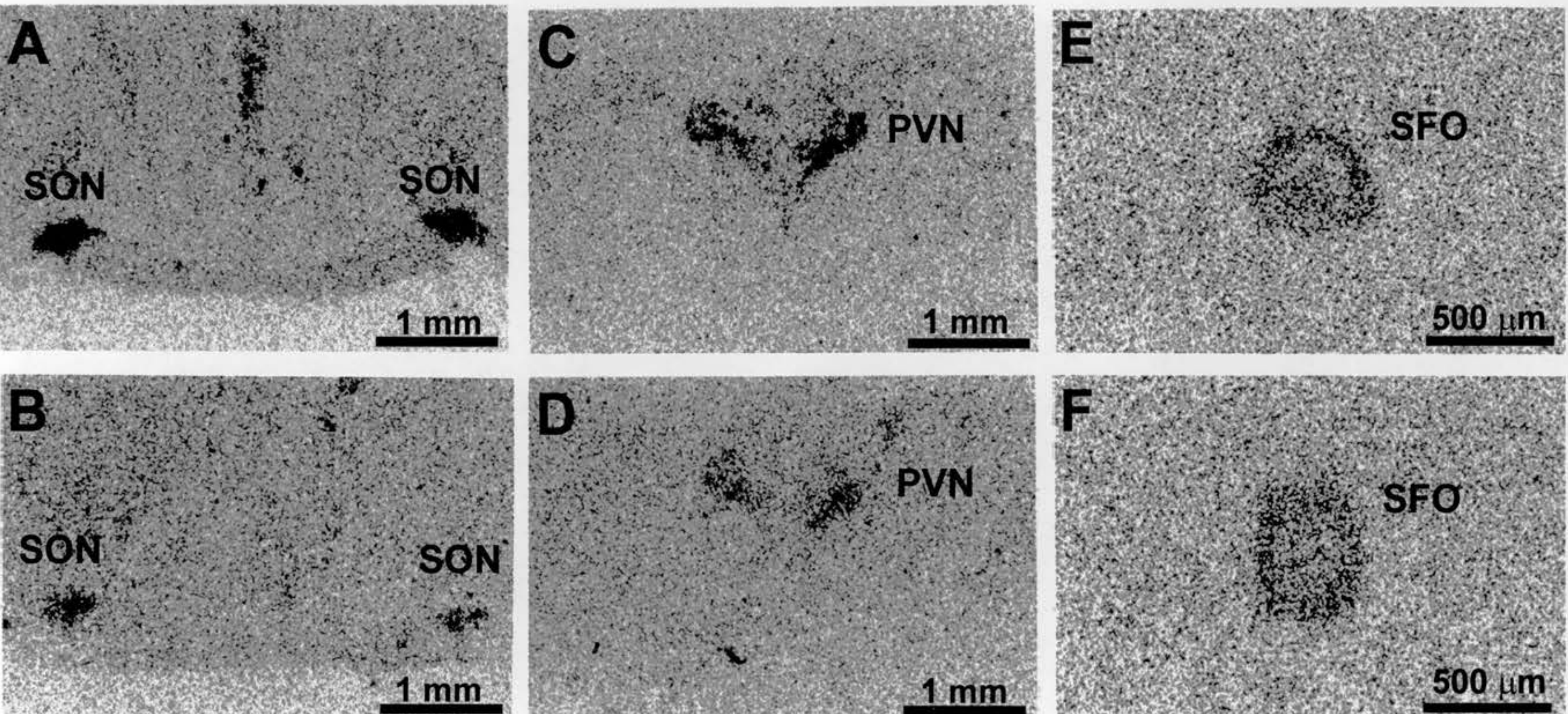

Figure 39. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA. Neuronal NOS signal in the SON of 4 days salt loaded rats (**A**) was greater than in control rats (**B**). The PVN of 4 days salt loaded rats (**C**) also showed more signal than control rats (**D**). nNOS signal in the SFO of 4 days salt loaded rats (**E**) was similar to control rats (**F**). SON; supraoptic nucleus, PVN; paraventricular nucleus, SFO; subfornical organ.

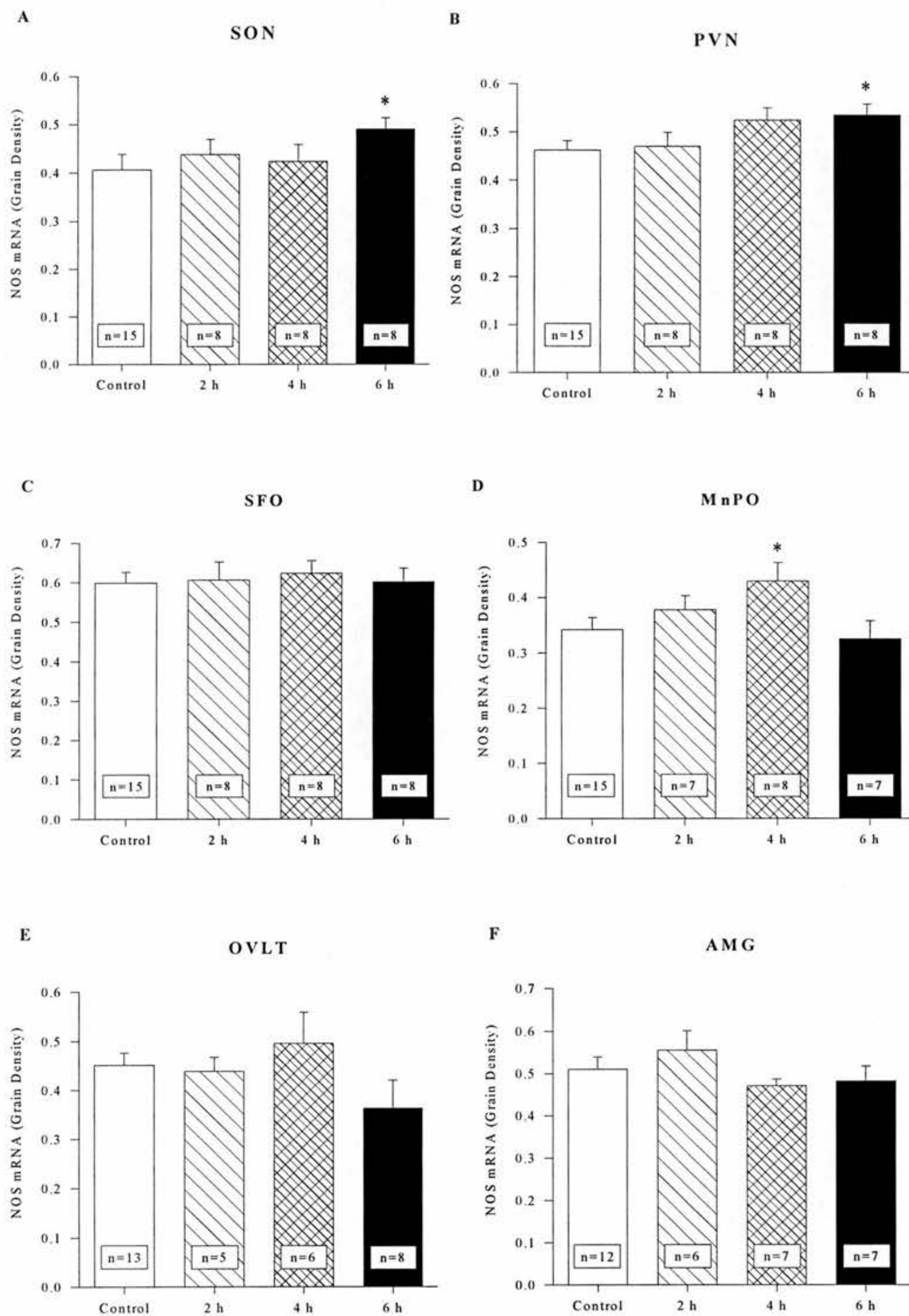

Figure 40. Neuronal NOS mRNA expression measured as film grain density over the SON, PVN, SFO, MnPO, OVLT and AMG. Significant increases in nNOS mRNA expression were observed in both the PVN and SON 6h after hypertonic saline administration (4 ml/kg, 1.5 M NaCl, i.p.) and in the MnPO 4 h after hypertonic saline administration compared to the control group (* $P<0.05$ vs control, *t*- test). There was no significant change in nNOS mRNA expression in the OVLT, SFO or AMG. Values are means ± SEM.

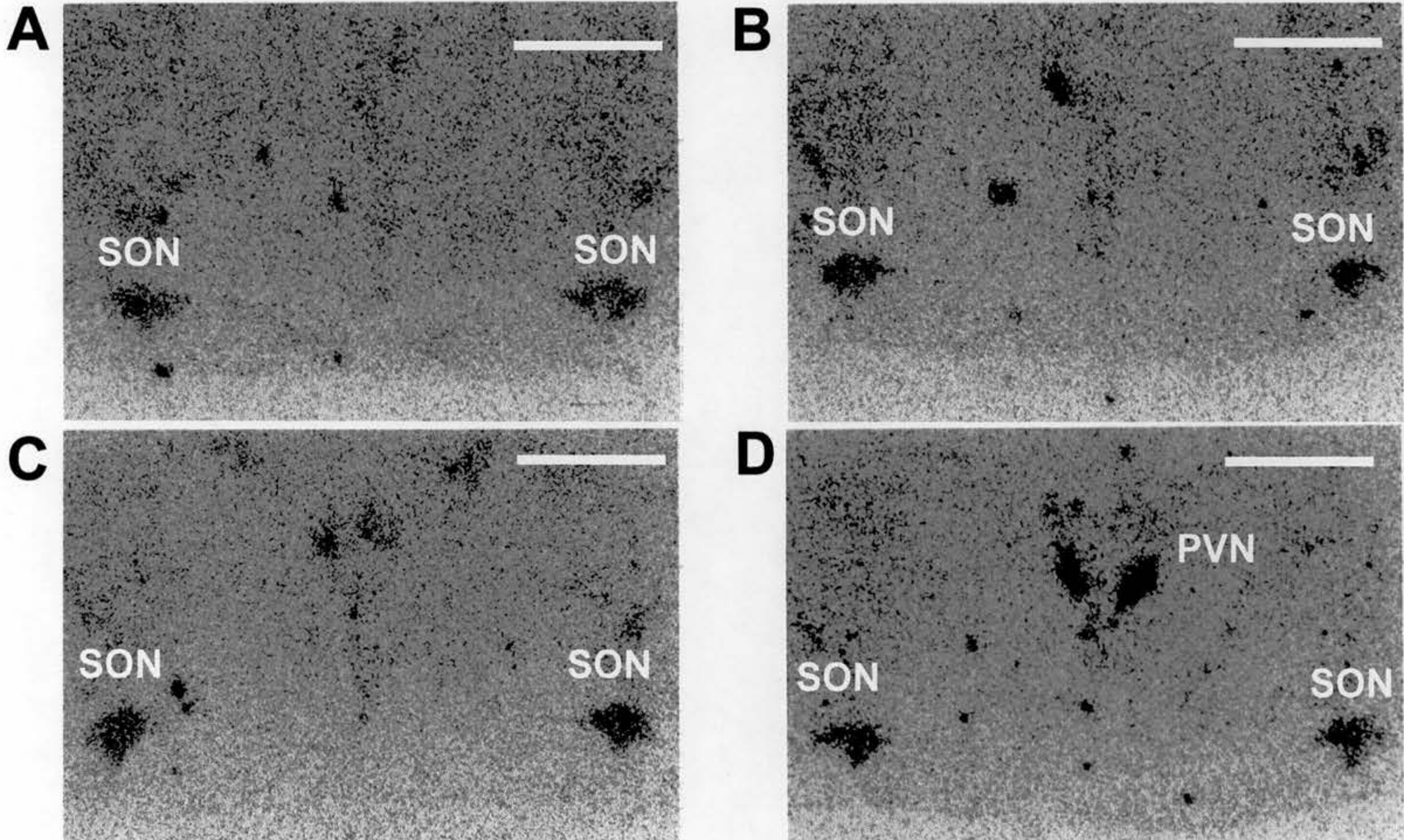

Figure 41. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the SON. Neuronal NOS signal in the SON of 6 h hypertonic saline treated rats (**D**) was greater than in control rats (**A**). Neuronal NOS signal in the SON of 2h and 4h hypertonic saline treated rats (**B, C**) was similar to control rats (**A**). SON; supraoptic nucleus, PVN; paraventricular nucleus. Bar=1 mm.

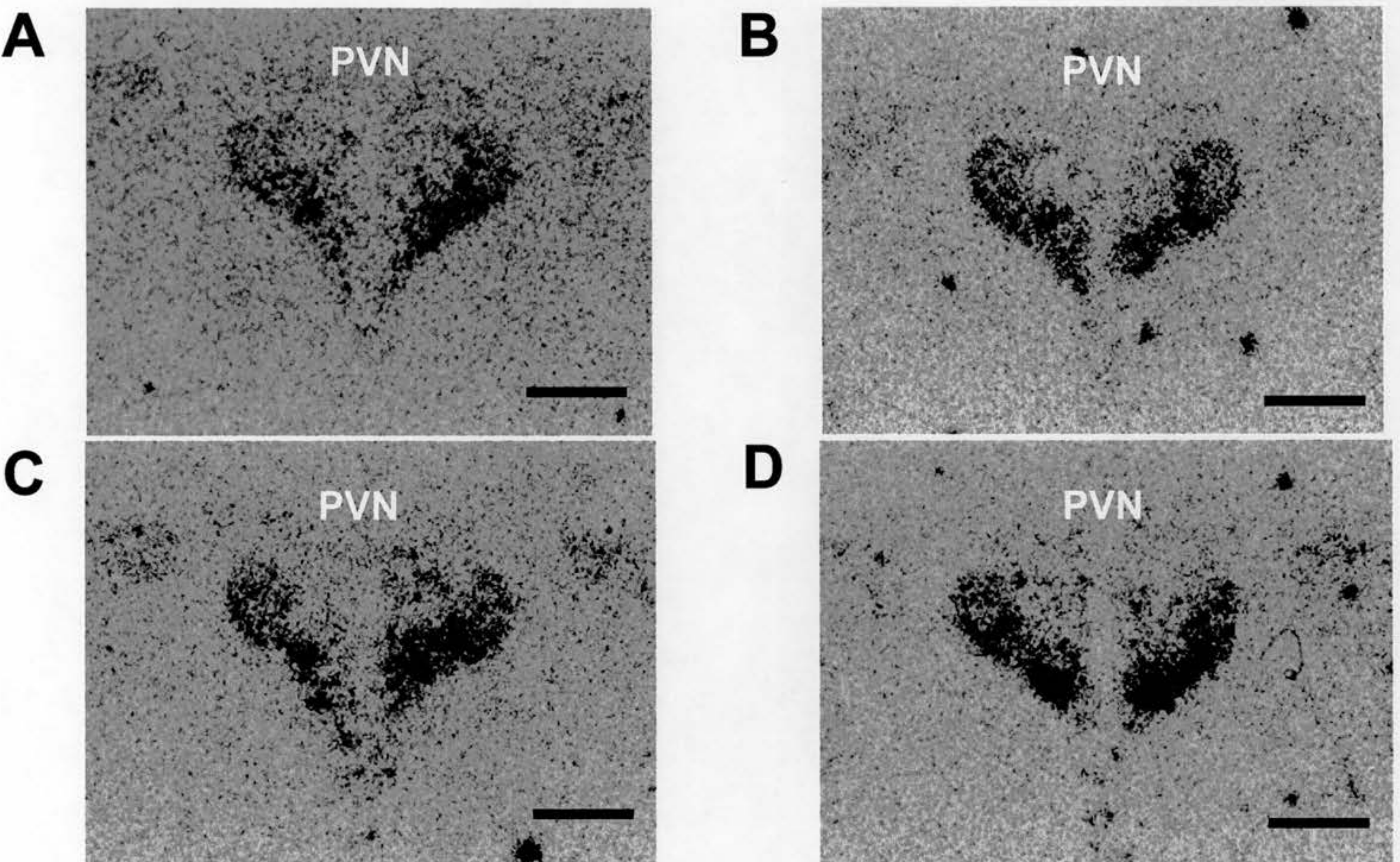

Figure 42. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the PVN. Neuronal NOS signal in the PVN of 6 h hypertonic saline treated rats (**D**) was greater than in control rats (**A**). Neuronal NOS signal in the PVN of 2h and 4h hypertonic saline treated rats (**B, C**) was similar to control rats (**A**). PVN; paraventricular nucleus, SON; supraoptic nucleus. Bar=500 μm.

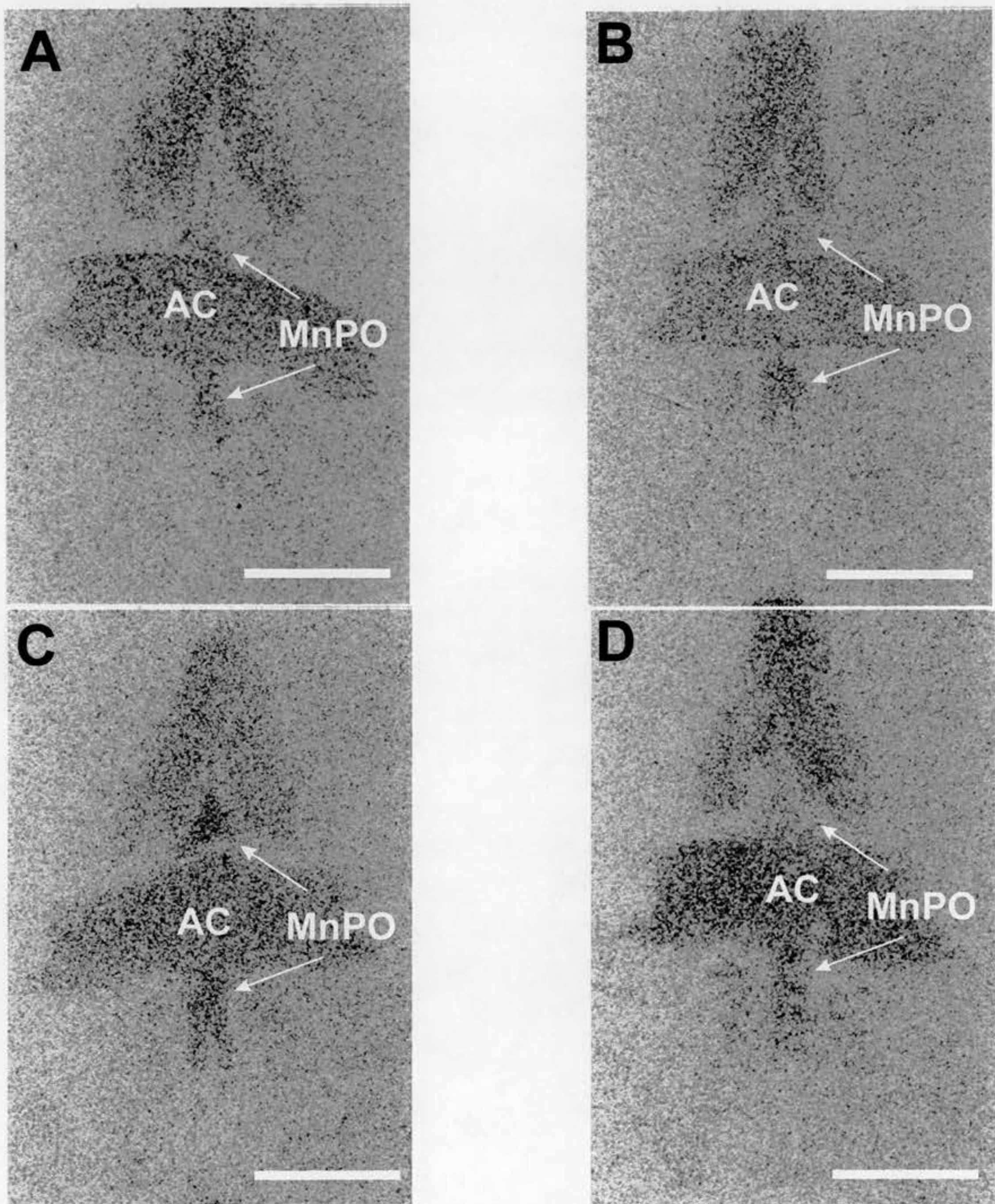

Figure 43. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the MnPO. Neuronal NOS signal in the MnPO of 4 h hypertonic saline treated rats (**C**) was greater than in control rats (**A**). Neuronal NOS signal in the MnPO of 2h and 6h hypertonic saline treated rats (**B, D**) was similar to control rats (**A**). MnPO; median preoptic nucleus, AC; anterior commissure. Bar=1 mm.

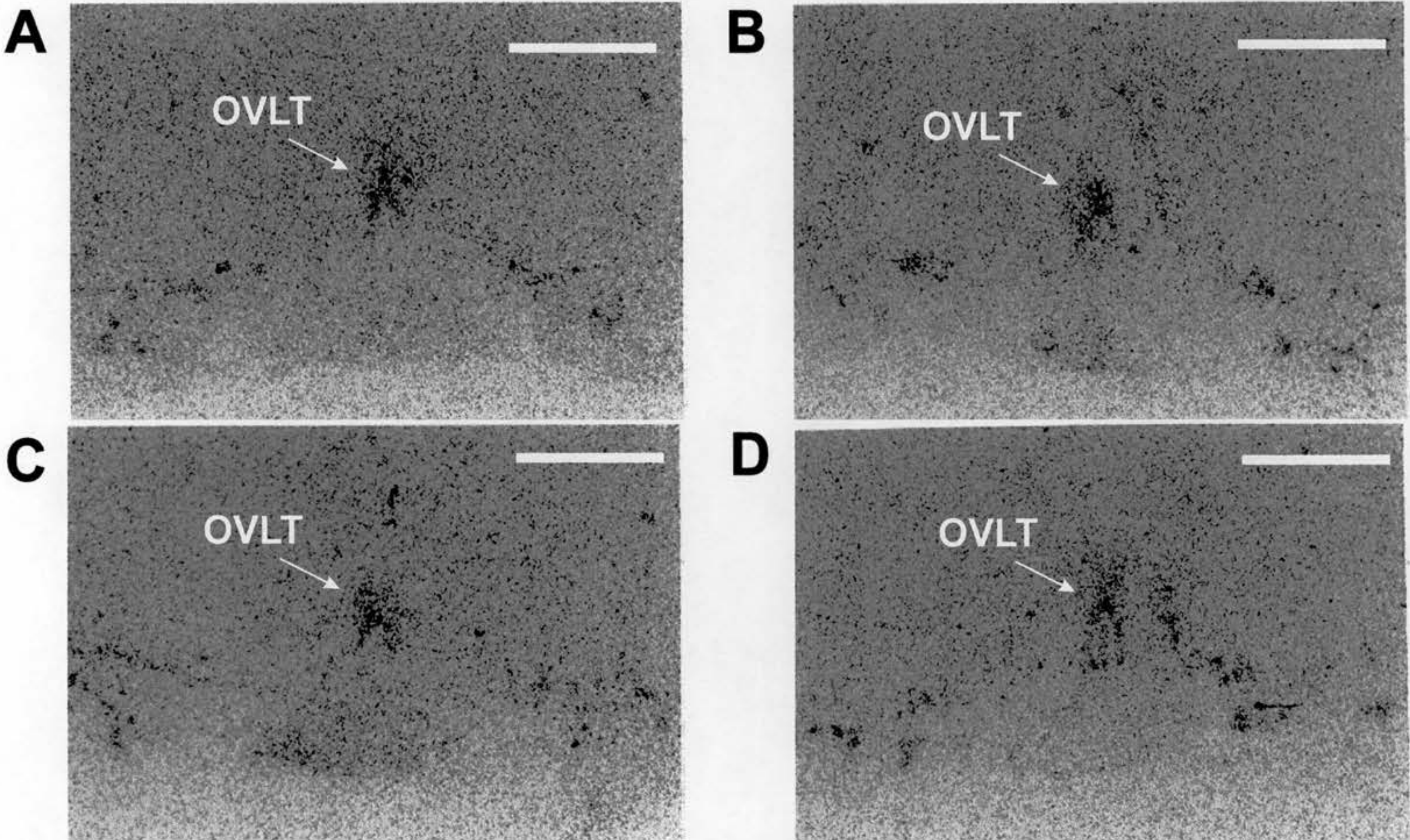

Figure 44. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the OVLT. Neuronal NOS signal in the OVLT of 2h, 4h and 6h hypertonic saline treated rats (**B, C, D**) was similar to control rats (**A**). OVLT; organum vasculosum of the lamina terminalis. Bar=1 mm.

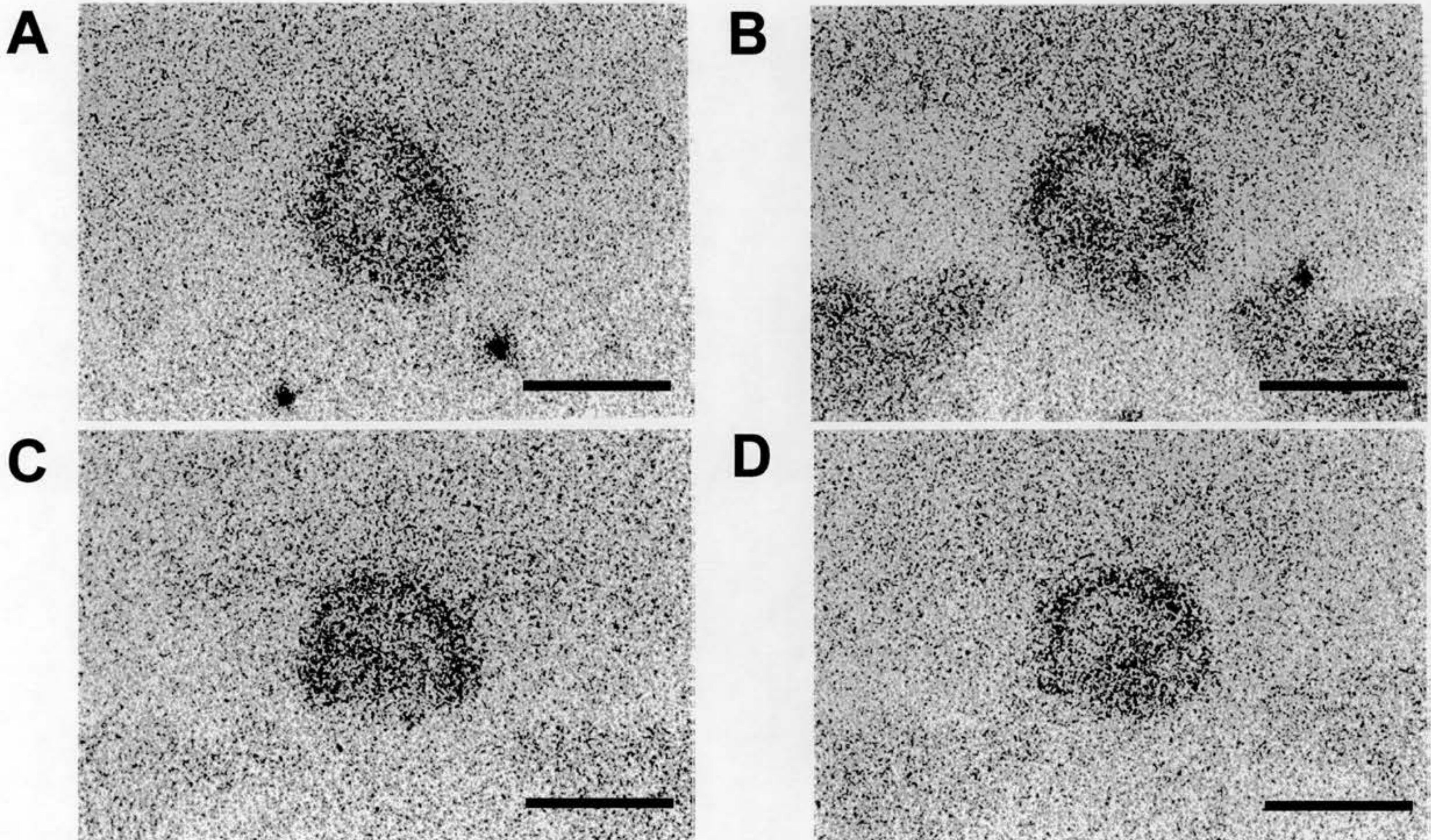

Figure 45. Film autoradiographs of sections hybridized with a ^{35}S oligonucleotide probe against rat neuronal NOS mRNA in the SFO. Neuronal NOS signal of 2h, 4h and 6h hypertonic saline treated rats (**B, C, D**) was similar to control rats (**A**). Bar=500 μm.

5.5 Discussion

Dehydration may be produced by either chronic salt-loading (giving a 2% NaCl solution in place of drinking water) or water deprivation. Dehydration produces an increase in plasma osmolality which leads to depletion of oxytocin and vasopressin from the neural lobe, and elevation of plasma oxytocin and vasopressin concentrations (Balment *et al.*, 1980; George, 1976; Jones and Pickering, 1969; Shoji *et al.*, 1994; Van Tol *et al.*, 1987). A number of studies have demonstrated that dehydration can up-regulate the expression of vasopressin and oxytocin mRNA in the SON and PVN (Amaya *et al.*, 1999; Ding *et al.*, 1994; Herman *et al.*, 1991; Lightman and Young, 1987; McCabe *et al.*, 1990; Meister *et al.*, 1990a; Suemaru *et al.*, 1990; Van Tol *et al.*, 1987). Dehydration also up-regulates the expression of tyrosine hydroxylase, galanin, dynorphin, cholecystokinin and nitric oxide synthase mRNAs in the SON and PVN (Kadowaki *et al.*, 1994; Lightman and Young, 1987; Meister *et al.*, 1990a; Sherman *et al.*, 1988; Villar *et al.*, 1994b; Young, III *et al.*, 1987). Translated peptides from these genes co-localize with oxytocin and vasopressin in the SON and PVN (Brownstein and Gainer, 1977; Brownstein and Mezey, 1986; Brownstein *et al.*, 1980; Meister *et al.*, 1990b; Mezey and Kiss, 1985; Sawchenko *et al.*, 1984; Vanderhaeghen *et al.*, 1981; Villar *et al.*, 1990).

As expected, the expression of nNOS mRNA in the rat SON and PVN was up-regulated after 4 days of salt loading (Fig. 38). However, nNOS mRNA expression in the SFO was not significantly altered after 4 days of salt loading. In the present study, we observed a 1.53-fold and 1.61-fold increase in nNOS mRNA expression in the SON and PVN, respectively, after 4 days of salt loading, indicating that magnocellular neurones of the SON and PVN respond similarly to the elevated plasma osmolality. This indicates that the expression of the nNOS gene is sensitive to a chronic osmotic stimulus.

An up-regulation of nNOS gene expression in the SON and PVN following chronic salt loading has been previously demonstrated by Kadowaki *et al.* (1994) and

Villar *et al.* (1994b). Villar and his colleagues demonstrated an increase of nNOS expression and NOS-immunoreactive cells in the SON and PVN after 5 days and 14 days of salt loading, and the expression of nNOS mRNA in the SON and PVN appeared to reach a peak after 5 days of salt loading (Villar *et al.*, 1994b). Kadowaki and her colleagues showed an up-regulation of nNOS mRNA in the SON and PVN after 4 days of salt loading, which was accompanied by an increase in NADPH-diaphorase activity in the SON and PVN, and NO-forming activity in the posterior pituitary (Kadowaki *et al.*, 1994). Water deprivation induced an increase in NADPH-diaphorase activity in the SON (Pow, 1992) and in the expression of nNOS mRNA in the SON and PVN (O'Shea and Gundlach, 1996; Ueta *et al.*, 1995b). In addition, it has been reported that NADPH-diaphorase activity is increased in the posterior pituitary after chronic salt loading (Sagar and Ferriero, 1987). In rats with inherited diabetes insipidus, which are chronically stimulated by a hyperosmotic condition due to a deficiency in the secretion of vasopressin, an increase in the number of nNOS-immunopositive cells (Wang and Morris, 1996) and an up-regulation of nNOS gene expression in the SON and PVN (Yamamoto *et al.*, 1997) have been reported. The up-regulation of NOS activity, nNOS-immunopositive cells and the expression of nNOS mRNA in the SON and PVN, and increase of NO-forming activity in the posterior pituitary following chronic hyperosmotic stimulation, which is expected to cause an increase in local NO concentration, may serve as a negative-feedback loop to prevent overstimulation of oxytocin and vasopressin secretion following the prolonged elevation of osmolality. Therefore, local NO may provide "fine tuning" in the control of oxytocin and vasopressin release by acting either at the posterior pituitary or at the level of the SON and PVN.

The present study has shown that acute administration (i.p.) of hypertonic saline resulted in an increase in nNOS expression in the SON and PVN 6 h later (Fig. 40A,B). There was also an up-regulation of nNOS mRNA expression in the MnPO 4 h after administration of hypertonic saline (Fig. 40D). Acute hypertonic saline administration causes rapid and transient increases in plasma osmolality and plasma vasopressin and oxytocin (Brimble and Dyball, 1977; Dunn *et al.*, 1973). These

results indicate that an acute osmotic challenge has a stimulatory effect on nNOS gene expression, but, the nNOS mRNA response was 4 to 6 h later than the plasma oxytocin and vasopressin response. Although the nNOS gene is of the constitutive type, several studies have revealed that nNOS gene expression is regulated by physiological manipulations including chronic osmotic stimuli (Kadowaki *et al.*, 1994; Ueta *et al.*, 1995b; Villar *et al.*, 1994b) and non-osmotic stimuli (Ueta *et al.*, 1998). The late response of the nNOS gene suggests the complexity of the mechanisms underlying the induction of nNOS gene transcription, which include the second messenger system, primary and secondary gene transcriptions, and the maturation process of nNOS mRNA. On the other hand, the time delay for the response of the nNOS gene might merely reflect the difficulties in measuring a small increase in a large pool of mRNA, inasmuch as the larger the pool size, the longer it takes to double in size.

The involvement of NO within central osmoresponsive neurones during a hyperosmotic challenge has been demonstrated in the present study as an increase in nNOS mRNA expression in the MnPO 4 h after administration of hypertonic saline (Fig 40D). We also found that the expression of the nNOS gene in the OVLT was increased (but not significantly), while the expression of nNOS mRNA in the SFO was not altered 4 h after administration of hypertonic saline (Fig. 40E, C). In addition, nNOS gene expression in the SFO was not altered by 4 days of salt loading (Fig. 38) and 48 h water deprivation (Ueta *et al.*, 1995b). Nonetheless, nNOS mRNA was strongly expressed in the SFO, so we cannot exclude the possibility that induction of NOS expression that was not detected because of the difficulties in measuring a small increase in a large pool of nNOS mRNA in the SFO.

Ciriello and his co-workers found increased NADPH-diaphorase activity in the SFO, OVLT and MnPO following 3 days of water deprivation (Ciriello *et al.*, 1996). Nitric oxide is believed to be synthesized within these structures since NOS protein and NADPH-diaphorase positive cells have been identified in these areas (Bredt *et al.*, 1991a; Vincent and Kimura, 1992). The SFO, MnPO and OVLT are

interconnected and form a local neural circuit for the processing of afferent information regarding plasma osmolality and the plasma concentration of angiotensin II (AII), which play a major role in body fluid and electrolyte balance (see Johnson and Thunhorst, 1997 and McKinley *et al.*, 1996 for reviews). Central NO inhibits the basal release of oxytocin and vasopressin and selectively inhibits oxytocin release following AII administration (Liu *et al.*, 1997). Nitric oxide is thought to be related to AII-induced drinking since AII-induced drinking was significantly attenuated by a NOS inhibitor (Liu *et al.*, 1997; Liu *et al.*, 1998). The neurones of the SFO and OVLT send direct and indirect (*via* the MnPO) connection to the SON and PVN (McKinley *et al.*, 1990). Luckman and colleagues have recently shown that some neurones of the lamina terminalis (the OVLT, MnPO and SFO) that send afferent projections to the SON contain nNOS (Luckman *et al.*, 1997).

An up-regulation of nNOS mRNA in the MnPO following acute hypertonic administration observed in the present study may reflect the function of NO as a neuromodulator within the central osmoresponsive circuitry that controls the release of oxytocin and vasopressin. Some neurones in the OVLT and SFO that project to the MnPO are activated, as identified by Fos expression, after systemic osmotic stimulation (Oldfield *et al.*, 1994). The increase in the activity of presynaptic neurones in the SFO and OVLT may evoke NO release from the postsynaptic MnPO neurones, which in turn, release NO to inhibit further release of specific neurotransmitters in this central osmoresponsive circuitry.

CHAPTER 6

GENERAL DISCUSSION

This thesis described investigations of the physiological role of endogenous NO in the magnocellular neurosecretory system *in vivo*. The thesis aimed to determine the role of NO in osmoregulation of oxytocin release, to investigate the sites of action of NO in controlling oxytocin release, to address the question of whether the changes in the NOS system may reflect the reduction of osmoresponsiveness in late pregnancy, and to examine the expression of nNOS mRNA in the SON and PVN during late pregnancy, and after prolonged stalk stimulation and hypertonic stimulation.

Nitric oxide has been shown to be involved in the physiological regulation of oxytocin secretion during water deprivation and salt loading in conscious rats (Kadowaki *et al.*, 1994; Summy-Long *et al.*, 1993). The involvement of NO in osmoregulation of oxytocin release in virgin rats under urethane anesthesia was confirmed in this thesis. Systemic blockade of NOS by either L-NNA or L-NAME did not alter the basal release of oxytocin. However, endogenous NO may preferentially inhibit the release of oxytocin in conditions of intense stimulation, as systemic administration of the NOS inhibitors L-NNA and L-NAME enhanced hypertonic saline-induced oxytocin release, but did not alter CCK-evoked oxytocin release (Chapter 3). Further support for the role of NO in inhibiting oxytocin release in response to hypertonic saline was found where i.c.v. administration of the NO donor SNP resulted in decreased oxytocin release induced by hypertonic saline. The inhibitory influence of endogenous NO on osmoregulation of oxytocin release is independent of the inhibitory effect of endogenous opioids, as the non-selective opioid receptor antagonist naloxone enhanced oxytocin release in urethane-anaesthetized rats pre-treated with either L-NNA or vehicle. Systemic administration of L-NNA increased the expression of Fos protein in the SON in pentobarbital-anaesthetized rats after high doses of hypertonic saline, but not after low doses of hypertonic saline (Chapter 4). Thus, this thesis provides additional evidence of an inhibitory effect of NO on neuronal activity of the SON neurones at high levels of activity in a manner that is independent of endogenous opioids, which in turn determines the amount of oxytocin release. This postulate is supported by the

experiments in conscious rats where central blockade of NOS by L-NAME potentiated oxytocin release in response to moderate hypertonic saline (similar doses of hypertonic saline to those used in this thesis), but not strong hypertonic saline (Kadekaro *et al.*, 1997). However, the mechanisms by which NO influence oxytocin release and the sites of action of NO are still unclear.

Elevation in plasma osmolality following acute hypertonic saline administration, salt loading or water deprivation dramatically increases the firing rate of oxytocin and vasopressin neurones (for reviews see Renaud and Bourque, 1991), and subsequently increases the release of oxytocin, vasopressin and probably NO. As mentioned before, up-regulation of NOS protein, NADPH-diaphorase staining and NOS mRNA in the SON and PVN was found in these conditions leading to increase in local NO concentration in the SON and PVN (Kadowaki *et al.*, 1994; Ueta *et al.*, 1995b; Villar *et al.*, 1994b). Additionally, NADPH-diaphorase also increased in the SFO, OVLT and MnPO (Ciriello *et al.*, 1996), and the neurohypophysis under conditions of dehydration (Sagar and Ferriero, 1987). Dehydration causes morphological changes in the axonal processes of the magnocellular neurones (Beagley and Hatton, 1992; Dellmann *et al.*, 1988). Nitric oxide plays a potential role in morphological plasticity of the neurohypophysis in response to fluctuations of fluid balance (Beagley and Cobbett, 1997). It has been reported that NO is involved in two major forms of long-lasting neuronal plasticity; long-term potentiation in the hippocampus (Bon *et al.*, 1992; Haley *et al.*, 1992) and long-term depression in the cerebellum (Daniel *et al.*, 1993; Shibuki and Okada, 1991). Thus, a similar function in morphological plasticity in these areas may be attributed to NO and may explain the observed effects of NO on the release of oxytocin. In general, locally produced NO may diffuse out of the magnocellular neurones in the SON and PVN and act retrogradely on presynaptic terminals to modulate the release of oxytocin and vasopressin in response to hypertonicity. It is possible that NO modulates the release of oxytocin and vasopressin from neurones that are activated in response to an elevation in plasma tonicity. Nitric oxide may participate in the autocrine or paracrine regulation of oxytocin and vasopressin production by acting on target molecule sGC

in the same cells or neighboring cells. Thus, the production of NO is increased in the SON and PVN in order to mediate the physiological response required to re-establish homeostatic balance.

The response of oxytocin neurones to changes in systemic osmolality is a result of the combination between excitatory synaptic drive from the osmosensitive neurones in the AV3V region and endogenous osmosensitivity of oxytocin neurones (Bourque *et al.*, 1994). Since the overall effect of NO on hormone release is thought to be inhibitory, it is possible that NO may act throughout the AV3V region to modulate the sensitivity of the magnocellular neurosecretory system. This is suggested by the increase of NADPH-diaphorase expression in the SFO, OVLT and MnPO that has been shown in dehydrated rats (Ciriello *et al.*, 1996). In accord with the findings in the thesis, nNOS mRNA expression in the MnPO was up-regulated 4 h after hypertonic saline administration, although there was no change in the OVLT and SFO after hypertonic saline injection. From the previous findings, dehydration did not alter the expression of nNOS mRNA in the SFO (Ueta *et al.*, 1995b).

The sites of action of NO to exert an inhibitory effect on oxytocin release have been described in this thesis. Nitric oxide may serve as an important intermediary in the regulation of neuronal excitability of oxytocin and vasopressin neurones in the SON and NO may serve as an inhibitory regulator on oxytocin release by acting at the nerve terminals. This thesis provides the first evidence *in vivo* that NO may potentially play an important role in modulating the electrical activity of oxytocin and vasopressin neurones in the SON. Local application of L-NNA increased the electrical activity of oxytocin and vasopressin neurones in the SON (Chapter 4). In addition, local production of NO by SNP inhibited neuronal activity of both types of cells *in vivo*. Indeed, the ability of NO to inhibit neuronal activity of oxytocin and vasopressin neurones in the SON has been demonstrated previously in the slice preparation (Liu *et al.*, 1997). Therefore, the release of oxytocin and vasopressin is attenuated by NO, since the amount of oxytocin and vasopressin release can be determined by the rate and pattern of firing of oxytocin and

vasopressin neurones (Bicknell, 1988). Furthermore, NO has been found to inhibit vasopressin release from hypothalamus explants (Yasin *et al.*, 1993). The possible mechanisms responsible for this inhibition have been examined by *in vitro* electrophysiological studies. Nitric oxide inhibits the electrical activity of SON neurones, probably by acting at postsynaptic sites of SON neurones, since NO suppressed NMDA-evoked membrane depolarization of SON neurones *in vitro* (Cui *et al.*, 1994), and also by acting at presynaptic GABA neurones, as the frequency of IPSPs, but not EPSPs, recorded from the SON neurones *in vitro* was increased by NO (Ozaki *et al.*, 1999). In magnocellular neurones of the PVN, NO modulates inhibitory transmission in response to NMDA receptor activation, as NO stimulates GABA release and also increases GABA's postsynaptic efficacy (Bains and Ferguson, 1997b). Supporting these possibilities are the findings that local administration of NO *via* microdialysis caused an increase of GABA and glutamate release within the PVN (Horn *et al.*, 1994). The excitation of inhibitory GABA neurones, the increased postsynaptic efficacy of GABA in the PVN, the excitation of GABA release, and the inhibition of postsynaptic efficacy for NMDA in the SON, may help to explain earlier observations where NO exerts an inhibitory action on oxytocin release in response to hyperosmotic stimulation.

Nitric oxide may also act at the nerve terminals of oxytocin neurones. Systemic administration of L-NNA enhanced stalk stimulation-evoked oxytocin release *in vivo* (Chapter 4). Furthermore, NO inhibited vasopressin release from neurohypophysis explants (Lutz-Bucher and Koch, 1994). Thus, the NO responsible for inhibition of oxytocin and vasopressin release co-exists with either oxytocin or vasopressin and is generated in an activity-dependent manner. Nitric oxide thus appears to act as a feedback inhibitor of oxytocin release at the terminals of oxytocin neurones.

Although nNOS is thought to be a constitutively expressed enzyme, recent data suggest that nNOS mRNA expression in the SON and PVN is dynamically regulated in conditions of chronic demands of oxytocin *e.g.* water deprivation and

salt loading (Kadowaki *et al.*, 1994; Ueta *et al.*, 1995b; Villar *et al.*, 1994b). We have found that the nNOS gene is a late response gene, as an up-regulation of NOS mRNA expression in the SON and PVN was found 6 h after acute hypertonic saline administration (Chapter 5). Furthermore, nNOS mRNA expression in the MnPO was increased 4 h after hyperosmotic stimulation. However, an increase of nNOS expression in the SON does not reflect a direct coupling to spike activity, since the expression of nNOS mRNA in the SON was reduced after prolonged stalk stimulation (Chapter 4). Moreover, down-regulation of nNOS mRNA expression in the SON has been found during late pregnancy (Chapter 3). Thus, nNOS mRNA expression in the SON and PVN can be regulated under some circumstances, but the molecular mechanisms responsible for the dynamic regulation of nNOS transcription are poorly understand.

We have found for the first time that NO activity is reduced during late pregnancy in a manner that is independent of endogenous opioids. The potentiation of oxytocin release stimulated by a high dose of hypertonic saline by systemic blockade of NOS by L-NNA disappeared in late-pregnant rats, while naloxone enhanced oxytocin release in rats pre-treated with either L-NNA or vehicle. Furthermore, the expression of nNOS mRNA is reduced during late pregnancy and increased during parturition (Chapter 3). An attenuation of physiological function of NO during late pregnancy coincides with a time when oxytocin neurones are quiet (for reviews see Leng *et al*, 1999). Thus, reduced NO activity may be a consequence of reduced activity in oxytocin neurones at this time. In other words, oxytocin neurones are quiet but have a reduced amount of NOS protein at term so when they are excited there is no restraint, resulting in an enhanced secretory capacity of the oxytocin system at term. Moreover, the NOS system present in the uterus may be important in maintaining uterine quiescence during pregnancy. Nitric oxide production is decreased in the uterus and increased in the cervix at term. Since NO relaxes smooth muscle this results in a coordinated increased excitability of the uterus, and changes in the cervix leading to dilation, essential for the process of labour (Garfield *et al.*, 1998a). A down-regulation of NOS system in the SON and in

the uterus at term may initiate labour. By the end of pregnancy, inhibitory influences onto oxytocin neurones are also decreased, as a down-regulation of $GABA_A$ subunit receptor mRNA in oxytocin neurones of the SON and PVN has been revealed and the potentiation of GABA actions *via* allopregnanolone diminishes with the collapse of progesterone secretion (Brussaard *et al.*, 1997; Fenelon and Herbison, 1996). Oxytocin nerve terminals in the neural lobe become desensitized to endogenous opioid restraint and the electrical activity of oxytocin neurones is reduced by a separate endogenous opioid system, acting upon μ-receptors (Russell *et al.*, 1995). These changes in inhibitory components during late pregnancy may contribute to the increase of electrical activity of oxytocin neurons at the time of parturition (Summerlee, 1981) and facilitate the release of oxytocin with a bursting pattern of firing of oxytocin neurones during parturition (Bicknell *et al.*, 1988). The changes in afferent inputs to cell bodies and nerve terminals of oxytocin neurones in virgin, late-pregnant and parturient rats are summarized in figure 46.

In summary, a physiological role of NO in regulating the functional activity of the magnocellular neurosecretory system has been established. The plasticity of the NOS system in the SON and PVN has been demonstrated following both artificial stimuli *e.g.* hyperosmotic stimulation and electrical stimulation of the neural stalk, and physiological stimuli *e.g.* reproductive state. It is likely that NO is produced locally at the SON and PVN and at the neurohypophysis, when oxytocin and vasopressin neurones are activated by either artificial stimuli or physiological stimuli. Endogenously generated NO may function in a negative feedback loop to inhibit electrical activity of oxytocin and vasopressin neurones, and to restrain the release of oxytocin. Evidence presented in this thesis indicates that endogenously generated NO participates in the regulation of oxytocin release in response to hyperosmotic stimulation in a manner that is independent of opioid inhibition. Furthermore, the functional down-regulation of endogenous NO action due to a reduction of nNOS system activity in the SON in late pregnancy has been demonstrated, which may increase the excitability of the oxytocin system, resulting in the capacity for greater release of oxytocin at term. The findings presented in this thesis contribute to

understanding the function of endogenous NO in the magnocellular neurosecretory system during hyperosmotic stimulation and pregnancy.

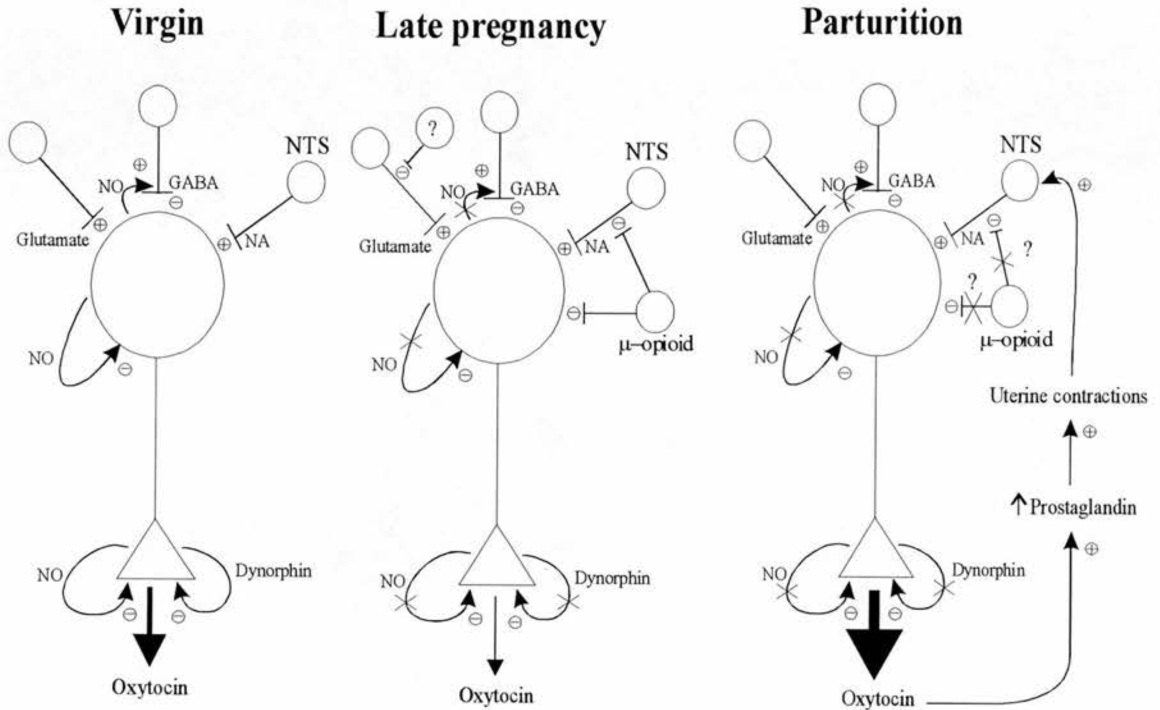

Figure 46. Schematic representing changes in afferent input to cell bodies and nerve terminals of oxytocin neurones in virgin, late-pregnant and parturient rats. Nitric oxide, produced by oxytocin neurones, has a potent inhibitory influence on oxytocin release in virgin rats. During late pregnancy, the inhibitory effect of NO on oxytocin release is decreased, in parallel with a desensitization of κ-poioid restraint in oxytocin nerve terminals and a reduction of the electrical activity of oxytocin neurones by μ-opioid, causing less oxytocin release. During parturition, these inhibitory components are decreased while the excitatory influence onto oxytocin neurones becomes more active, causing a large amount of oxytocin release. Abbreviations: NTS, nucleus tractus solitarius; NO, nitric oxide; GABA, gamma-aminobutyric acid; NA, noradrenaline.

Further studies:

As mentioned above, nNOS mRNA expression in the SON and PVN is up-regulated following hyperosmotic stimulation. In parturient rats, an up-regulation of nNOS mRNA expression in the SON was demonstrated in this thesis. During lactation, the expression of nNOS mRNA in the PVN (Ceccatelli and Eriksson, 1993) and in the structures of the lamina terminalis including the SFO, MnPO and OVLT has been revealed (Luckman *et al.*, 1997). Neuronal NOS mRNA expression increased in oxytocin neurones, but not vasopressin neurones, in the SON in lactating rats (Luckman *et al.*, 1997). Hyperosmotic stimuli cause a sustained continuous firing pattern of oxytocin cell activity, inducing continuous release of oxytocin. Conversely, suckling evokes a bursting pattern of oxytocin cell activity, and a comparable activity has been reported during parturition, causing pulsatile release of oxytocin (for reviews see Poulain and Wakerley, 1982). Milk ejection is related to this bursting activity of oxytocin neurones and a sudden but transient rise in intramammary pressure, which indicates oxytocin release. Since NOS co-exists with oxytocin, if the bursting activity activated nNOS to produce NO, the pattern of the release of endogenous NO should be similar to that of oxytocin secretion. In contrast, NO is likely to be produced continuously during hyperosmotic stimulation while release of NO may be found during parturition and suckling. The inhibitory effect of locally produced NO on oxytocin release induced by hyperosmotic stimulation is clearly demonstrated in this thesis and other authors' findings (Kadowaki *et al.*, 1994; Summy-Long *et al.*, 1993). In contrast, locally-produced NO may not affect the pulsatile release of oxytocin evoked by suckling, since an increase of endogenous NO may occur following the burst during the background non-burst activity of oxytocin cells; however, NO may contribute to the post-burst quiescence. Furthermore, chronic systemic or acute central administration of L-NNA had no effect, while exogenous NO produced by SNP reduced milk transfer from mother rats to pups, which was reversed by systemic injection of oxytocin (Okere *et al.*, 1996). Central administration of L-NNA permitted, while SNP inhibited the induction of neuronal

burst activity recorded from SON oxytocin neurones and intramammary pressure during suckling (Okere *et al.*, 1996).

During lactation, the sensitivity of oxytocin neurones and vasopressin neurones to osmotic stimuli is reduced, although the plasma osmolality and the threshold for oxytocin and vasopressin release return to control levels (Higuchi *et al.*, 1988; Koehler *et al.*, 1993). The reduction of osmosensitivity of the oxytocin system during lactation corresponds to an up-regulation of nNOS expression in the SON, PVN and lamina terminalis. Thus, endogenous NO within these areas may be involved in the reduced osmosensitivity during lactation. Further studies of the physiological role of endogenous NO during parturition and lactation may explain the importance of endogenous NO in the magnocellular neurosecretory system.

Further issues raised by this thesis need to be investigated. First, NO inhibits neuronal activity of both oxytocin and vasopressin neurones *in vivo*, but the mechanisms of this inhibition are unclear. To gain a better understanding of the mechanisms of the inhibitory actions of NO we need to investigate the involvement of NO in the modulation of inhibitory transmission resulting from the activation of GABA neurones, the increase of the efficacy of the $GABA_A$ receptors or the decrease of the efficacy of glutamate NMDA receptors at postsynaptic sites using *in vivo* electrophysiology. Second, studies of the osmoregulation of oxytocin release in nNOS gene knockout mice may give a better idea of the mechanisms that control nNOS transcription and subsequent protein levels. These studies may provide further details for the mechanisms of the inhibitory role of NO on oxytocin and vasopressin neurones. Third, although we have shown that nNOS mRNA expression in the SON is down-regulated during late pregnancy, we do not know whether this down-regulation is preferentially found in oxytocin neurones or vasopressin. This question can be addressed by using double *in situ* hybridization. The results from this investigation may indicate which types of SON cells produce less NO during parturition. Lastly, the causes of the changes in nNOS expression in magnocellular

neurones in pregnancy have not been investigated: are these a result of the sex steroid changes in pregnancy?

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R. Srisawat, P.M. Bull, A.J. Douglas, J.A. Russell and G. Leng (1998). Down-regulation of nitric oxide signalling in the supraoptic nucleus (SON) in late pregnancy. European Journal of Neuroscience.10 (Suppl. 10), 28.

OTHER PRESENTATION
(included in meeting abstract books)

1. PRESENTATION AT INTERNATIONAL MEETING

R. Srisawat, P.M. Bull, J.A. Russell and G. Leng. Nitric oxide as an inhibitory modulator of oxytocin stimulation in the rat. Poster presentation at the XXVIIth Meeting of Experimental Neuroendocrinology Society in Lille, France, September 2-5, 1998.

R. Srisawat, M. Ludwig and G. Leng. Inhibitory action of nitric oxide on the magnocellular neurosecretory system. Poster presentation at the 4th International Congress of Neuroendocrinology, Kitakyushu, Japan, October 12-16, 1998.

R. Srisawat, P.M. Bull, A.J. Douglas, J.A. Russell and G. Leng. Reduced nitric oxide synthase (NOS) gene expression anf NOS action in relation to oxytocin neurones at the end of pregnancy. Poster presentation at the Maternal Brain an international meeting on neurobiological and neuoendrocrine adaptation and disorders in pregnancy and post-partum. Bristol, UK, July 6-9, 1999.

R. Srisawat, M. Ludwig, J.A. Russell and G. Leng. Nitric oxide modulation of oxytocin release following electrical stimulation of the axons of magnocellular neurosecretory neurones. Poster presentation at 1999 World Congress on Neurohypophysial Hormones, Edinburgh, Scotland. August 28-September 2, 1999.

2. PRESENTATION AT NATIONAL MEETING

R. Srisawat, A.J. Douglas and G. Leng. Nitric oxide synthase (NOS) gene expression after acute hypertonic stimuli and chronic salt. Poster presentation at Annual Meeting of the British Neuroendocrine Group, Cambridge, UK, September 14-16, 1997.

15.08 DOWN-REGULATION OF NITRIC OXIDE SIGNALLING IN THE SUPRAOPTIC NUCLEUS (SON) IN LATE PREGNANCY.

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We examined the expression of nNOS mRNA in the rat SON using *in situ* hybridization. A significant decrease of nNOS mRNA expression was observed in late pregnant rats (day 22) compared to virgin rats (late pregnancy: 0.23±0.009 mean grain density/unit area, n=8, virgin: 0.30±0.024, n=8, $p<0.05$, one way ANOVA; Dunn's method). We also investigated the influence of endogenous NO on the responsiveness of the oxytocin system to osmotic stimulation. The NOS inhibitor, Nω-Nitro-L-Arginine (L-NA; 10 mg/kg i.p) or saline was administered to urethane-anaesthetized virgin and late pregnant rats. After 4 h, rats were injected with hypertonic saline (virgin:4 ml/kg 1.5 M NaCl, late pregnant: 5 ml/kg 2 M NaCl, i.p.). Plasma oxytocin concentration was measured by specific radioimmunoassay. In all rats, oxytocin release was significantly increased by injection of hypertonic saline. In virgin rats, this response was significantly elevated in rats pre-treated with L-NA, whereas in pregnant rats pre-treatment with L-NA had no significant effect. The study demonstrates down-regulation of nNOS activity in the magnocellular system of the SON in late pregnancy.