Neural mechanisms involved in the regulation of oxytocin secretion in late pregnant rats

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Declaration:

The studies outlined in this thesis were undertaken in the Department of Physiology, Medical School, University of Edinburgh and the Laboratory of Neuroendocrinology, at the Babraham Institute in Cambridge. This dissertation is not substantially the same as any I have submitted for a degree or diploma, or any other qualification at any other University. It is the result of my own work and collaborations with others are specifically indicated as such.

Irina A. Antonijevic

May, 1995

ICH KENNE DICH, Du bist die tief Gebeugte,
Ich, der Durchbohrte bin Dir untertan.
Wo flammt ein Wort, das für uns beide zeugte ?
Du - ganz wirklich, ich - ganz Wahn.

(Paul Celan)

THE UNIVERSITY *of* EDINBURGH

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Abbreviations

AMPA	=	RS-α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid
AV3V	=	Area anterior and ventral to the third ventricle
BNST	=	Bed nucleus of the stria terminals
CCK	=	Cholecystokinin
EPSP	=	Excitatory postsynpatic potential
icv	=	intracerebroventricular(ly)
im	=	intramuscular(ly)
IMP	=	Intramammary pressure
ip	=	intraperitoneal(ly)
ir	=	immunoreactive
IUP	=	Intrauterine pressure
iv	=	intravenous(ly)
LH	=	Luteinising hormone
LHRH	=	Luteinising hormone-releasing hormone
MePN	=	Median preoptic nucleus
NMDA	=	N-Methyl-D-aspartic Acid
NPY	=	Neuropeptide Y
NTS	=	Nucleus tractus solitarii
OVLT	=	Organum vasculosum of the lamina terminalis
PDGF2α	=	Prostaglandin F2α
PVN	=	Paraventricular nucleus
sc	=	subcutaneous(ly)
s.e.m.	=	standard error of the mean
SFO	=	Subfornical organ
SON	=	Supraoptic nucleus
TH	=	Tyrosine hydroxylase
VLM	=	Ventrolateral medulla

Abstract

Neural mechanisms involved in the regulation of oxytocin secretion in late pregnant rats

In the present work, I have investigated the role of oxytocin, a peptide hormone synthesised in the paraventricular and supraoptic (SON) nuclei of the hypothalamus and secreted from axon terminals in the posterior pituitary, in the initiation and maintenance of parturition in rats. Furthermore, I have studied afferent pathways involved in the secretion of oxytocin at the end of pregnancy, using immunocytochemical detection of Fos, the protein product of the immediate early gene c-*fos*.

First, I have shown that delivery in rats, that has been disrupted by a systemic injection of morphine, which prevents pituitary oxytocin release, can be restored by pulsatile but not continuous administration of physiological doses of oxytocin, indicating a critical role of pulsatile oxytocin for the normal progress of delivery. Following from there, I have shown that labour and delivery can be induced in late pregnant rats with pulsatile administration of oxytocin, while systemic administration of peptide oxytocin antagonists can delay the onset of delivery and prolong its progress. Using Fos immunocytochemistry, I have demonstrated that Fos expression is increased in the SON and in putative afferent neurones, including those in the nucleus tractus solitarii (NTS) in the brainstem, in parturient compared to prepartum rats. Similarly, Fos expression in these areas can be induced by a systemic injection of cholecystokinin, that is known to excite magnocellular oxytocin neurones and hormone release, indicating the involvement of NTS neurones in the regulation of oxytocin secretion. Following from there, I have shown that during oxytocin-induced labour, Fos expression in the NTS and the SON is increased even before delivery of pups, suggesting that vagino-cervical stimulation is not a prerequisite for activation supraoptic neurones at term.

Using double-immunocytochemistry, I have shown that in response to pulsatile oxytocin one third of NTS neurones immunoreactive for Fos contain tyrosine hydroxylase (TH), the rate limiting enzyme for catecholamine synthesis. In addition, I have observed an increase in the number of TH-containing neurones during oxytocin-induced labour and delivery. This increase in TH immunoreactivity during induced labour is reflected by an increase in TH mRNA expression, as assessed by radioactive *in situ* hybridisation for TH. Conversely, in untreated rats, TH mRNA expression in the NTS is elevated on the day of term before delivery but declines to significantly lower levels at the time of parturition. Furthermore, I

used a subcutaneous injection of progesterone to delay the onset of delivery. Such treatment also impaired the progress of delivery and was associated with a reduced expression of Fos in the SON and of TH immunoreactivity in the NTS. Using *in situ* hybridisation for TH, I found significantly lower TH mRNA expression in the NTS of late pregnant rats treated with progesterone compared to rats given vehicle, consistent with the hypothesis that catecholaminergic pathways from the NTS to the SON are involved in the secretion of oxytocin during parturition. Finally, we have recorded electrical activity of supraoptic neurones in term pregnant rats during oxytocin-induced labour. Since firing rate of supraoptic neurones increased concomitantly with uterine pressure and cervix contractions during a four hour oxytocin pulse treatment, these data support the hypothesis that oxytocin administration to late pregnant rats, via augmented uterine activity, stimulates supraoptic neurones and hence contributes to the initiation of pituitary oxytocin secretion.

General Introduction

Once upon a time ...,

nearly one hundred years ago, the first report on the uterotonic effects of pituitary extracts was published (Dale 1906), followed after some years by the observation that such extracts, when given to lactating animals, lead to an increase in intramammary pressure and milk-ejection (Dale 1909, Ott & Scott 1910) due to the contraction of myoepithelial cells in the mammary glands. Some years later, two active principles were identified in the posterior pituitary: one causing an increase in uterine activity and intramammary pressure, was termed oxytocin, meaning "fast delivery", and the other, causing an increase in blood pressure, was referred to as vasopressin (Kamm *et al.* 1928).

in the same magnocellular neurone at times of increased hormone release, e.g. during lactation (Mezey & Kiss 1991) and chronic dehydration (Kiyama & Emson 1990).

In the past, magnocellular neurones in the SON and PVN have often been considered to form a single functional unit, thus disregarding a) the difference in their position within the hypothalamus and b) the fact that the SON consists primarily of magnocellular neurones, while in the PVN magnocellular neurones form three main clusters within a network of a variety of peptide-producing parvocellular neurones (Kiss *et al.* 1991, Swanson & Kuypers 1980, Swanson & Sawchenko 1983). Also, the SON is close to the ventral surface of the brain and hence to the subarachnoid space, while dendrites of magnocellular PVN neurones project towards the subependymal layer of the third ventricle (Hatton 1990). Since in the PVN, unlike in the SON, magnocellular neurones are found within close proximity to parvocellular neurones, the possibility of a mutual influence between these neurones has been suggested (Swanson & Sawchenko 1983) and such differences between the SON and PVN might explain recent observations that magnocellular neurones in the two nuclei are not equally activated in response to stress (Jezova *et al.* 1993), pain (Smith & Day 1994), dehydration (Roberts *et al.* 1993) and the milk-ejection reflex (Fenelon *et al.* 1994, Lambert *et al.* 1993).

Beside the classical nonapeptides, an increasing number of additional peptides are found in magnocellular oxytocin neurones (including cholecystokinin (CCK), corticotropin-releasing factor, met-enkephalin, dynorphin and thyrotropin-releasing factor) and vasopressin neurones (including galanin, tyrosine hydroxylase, dynorphin and leu-enkephalin) and at least some of these peptides can be released from axon terminals in the posterior pituitary (Bondy *et al.* 1989b, Leng *et al.* 1994, Martin *et al.* 1983, Meister *et al.* 1990, Vanderhaeghen *et al.* 1981), though studies *in vitro* (Bondy *et al.* 1989b, Meister 1993) and *in vivo* (Leng *et al.* 1994) indicate that the amount of the co-released peptides is much smaller than that of the nonapeptides. This observation, together with the description of functional CCK (Bondy *et al.* 1989a) and kappa opioid receptors in the neural lobe (Bondy *et al.* 1989b, Sumner *et al.* 1992, Van de Heijning *et al.* 1991a) points to an involvement of the co-expressed peptides in the local regulation of oxytocin and vasopressin release from neurosecretory axon terminals in the neural lobe. Furthermore, co-expression of peptides and peptide receptors in magnocellular hypothalamic neurones is altered by ovarian hormones (Levin & Sawchenko 1993) and during different functional states (Meister *et al.* 1994, Shea & Gundlach 1993) supporting a physiological implication of this co-localisation.

The oxytocin gene and its regulation

Oxytocin and vasopressin genes, that have been detected in many mammals, show a similar structural organisation and are believed to have evolved millions of years ago by duplication from a common ancestor gene (Ivell & Richter 1984a, Mohr & Richter 1993). Both genes consist of three exons (A, B, and C), that encode the hormone precursor, including the respective neurophysin, separated by two introns (Ivell & Richter 1984a). Despite the similarities between oxytocin and vasopressin with regard to the gene organisation and peptide structure, the promoter regions of the two genes show little homology between them, but a remarkable homology across species (Ivell & Richter 1984a). In the oxytocin gene, a composite hormone response element and an oestrogen-responsive element, both of which can enhance gene expression *in vitro* (Adan *et al.* 1993, Mohr & Schmitz 1991), have been detected. Similarly, *in vivo* experiments suggest a modulatory role of gonadal steroids on oxytocin mRNA expression (Crowley *et al.* 1995, Kawata *et al.* 1991): thus, beginning in mid-pregnancy, an increase in the hypothalamic oxytocin mRNA content has been described by some authors (Van Tol *et al.* 1988, Zingg & Lefebvre 1988b), though not by others (Douglas *et al.* 1993b). More recently, oxytocin mRNA expression in the SON and PVN has been shown to be unchanged after sustained treatment with oestradiol and progesterone, but to increase by nearly 200%, when progesterone is withdrawn (Crowley *et al.* 1995), a situation similar to that at the end of pregnancy. Since at the end of pregnancy and throughout lactation, the oxytocin transcript shows an increased polyadenylation (Zingg & Lefebvre 1989), possibly indicating a more efficient translation (Zingg *et al.* 1988a), an increase in oxytocin mRNA content would not be a prerequisite for the observed high peptide content in the neural lobe at term (Fuchs & Saito 1971). However, magnocellular oxytocin neurones in the rat do not express nuclear gonadal steroid receptors (Bethea *et al.* 1994, Fox *et al.* 1990, Rhodes *et al.* 1981a, Sar 1988) and thus the exact mechanism of steroid effects on oxytocin gene expression in magnocellular neurones remains unclear.

Unlike sustained stimulation of oxytocin secretion, e.g. during chronic dehydration (Brimble *et al.* 1978, Crowley & Amico 1993), or labour and lactation (Higuchi *et al.* 1985), an acute stimulus applied to supraoptic neurones does not result in a significant change in oxytocin mRNA expression (Sumner *et al.* 1989).

Functional aspects of oxytocin

The best known functions of pituitary oxytocin include the induction of uterine contractions during labour and delivery (Du Vigneaud *et al.* 1954, Fuchs & Poblete 1970) and the milk let-down effect, following contraction of myoepithelial cells in the mammary gland in response to suckling (Ott & Scott 1910, Lincoln *et al.* 1973, Cunningham & Sawchenko 1991). This increased secretion of oxytocin during labour and lactation is reflected by the increased posterior pituitary content of oxytocin in term pregnant rats (Fuchs & Saito 1971, Kumaresan *et al.* 1979). Since a) in late pregnancy the neural lobe content of vasopressin is increased to a similar extent as that of oxytocin (Fuchs & Saito 1971) and b) this accumulated excess is secreted during labour and delivery (Fuchs & Saito 1971, Kumaresan *et al.* 1979), both neurohypophysial hormones might play a role for parturition. Indeed, vasopressin can stimulate uterine activity in rabbits and humans (Fuchs 1969, Fuchs & Poblete 1970), yet so far, vasopressin has been primarily implicated in the regulation of body fluid homeostasis and arterial blood pressure (Honda *et al.* 1990, Leng *et al.* 1988b).

Reproductive functions of pituitary and central oxytocin

In humans, the reflex release of oxytocin in response to vaginal distension by the foetus during the expulsive phase of labour was first described by Ferguson (Ferguson 1941, see below for more details) and has since then been observed in most mammals.

During suckling and parturition in rats, there is a burst-like discharge of oxytocin neurones (Lincoln & Wakerley 1974, Summerlee 1981), that is reflected by the intermittent release of large amounts of oxytocin (Higuchi *et al.* 1986b, Lincoln & Wakerley 1974). This bursting activity is associated with increased intranuclear oxytocin release in the SON and the PVN (Moos *et al.* 1992, Neumann *et al.* 1992, 1993), possibly from dendritic processes of supraoptic oxytocin neurones (Pow & Morris 1989).

Administration of an oxytocin antagonist into the SON during parturition and lactation impairs the progress of delivery (Neumann, personal communication) and the milk-ejection reflex (Lambert *et al.* 1993, Neumann *et al.* 1994), indicating reduced pituitary oxytocin release. In addition, during osmotic stimulation, neither bursting activity of oxytocin neurones (Leng *et al.* 1993b) nor a significant increase in intranuclear oxytocin release are observed (Moos *et al.* 1992), despite the increased release of oxytocin from the pituitary (Hamamura *et al.* 1992, Honda *et al.* 1990) and hence the intranuclear release of oxytocin could play a key

role for the generation of burst-like oxytocin neurone activity and hormone release during delivery and lactation.

Pituitary oxytocin release has also been shown in response to vaginal stimulation during sexual activity and following manual stimulation (Argiolas & Gessa 1991, De Wied *et al.* 1993, Dreifuss *et al.* 1976) in female rats, and in male rats during ejaculation (Argiolas & Gessa 1991, De Wied *et al.* 1993, Insel 1992b). During the menstrual cycle in humans (Shukovski *et al.* 1989) and during the rat oestrous cycle (Windle & Forsling 1992) variations in plasma oxytocin concentrations have been described, though the implication of these observations remains to be examined.

Besides being secreted into the general circulation, central oxytocin release, particularly within the limbic system (including the bed nucleus of the stria terminalis: BNST) and the hypothalamus, is stimulated during parturition (Neumann *et al.* 1992) and suckling (Moos *et al.* 1991, Neumann & Landgraf 1989) and has been implicated in lordosis and maternal behaviour in female rats (Richard *et al.* 1992) and penile erection and copulatory behaviour in male rats (Argiolas & Gessa 1991, De Wied *et al.* 1993). Interestingly, in mice maternal behaviour can be induced by both subcutaneous and intracerebroventricular administration of oxytocin (McCarthy 1990).

In female rats, increased oxytocin immunoreactivity is observed in the hypothalamus at term and following ovariectomy and oestrogen-supplementation, indicating the involvement of gonadal steroids in the regulation of oxytocin synthesis (Jirikowski *et al.* 1988, 1989). Also, at term an increased apposition of oxytocin-immunoreactive neurones to the basement membrane of arterioles in the hypothalamus has been described (Blanco *et al.* 1991), pointing to a facilitated secretion of central oxytocin into the cerebral blood stream at the end of pregnancy. This increased oxytocin secretion could contribute to the stimulation of prolactin release from the anterior pituitary, critical for lactation. Though the effect of oxytocin on prolactin release is small in the presence of a dopaminergic tone (Mogg & Samson 1990), at times when this inhibitory influence is reduced, e.g. experimentally (Arey & Freeman 1989) or at the end of pregnancy (Soaje & Deis 1994) and during lactation (Arbogast & Voogt 1991b, Hoffman *et al.* 1994, Wang *et al.* 1993), oxytocin is a potent stimulator of prolactin release (Arey & Freeman 1989).

Non-reproductive functions of pituitary and central oxytocin

Pituitary oxytocin in rats is also released in response to stressful (Jezova *et al.* 1993, Harbuz & Lightman 1988, Lightman & Young 1989, Patel *et al.* 1991) and painful stimuli (Onaka & Yagi 1991, Smith & Day 1994) and food intake (Verbalis *et al.* 1986). The latter effect, which is mediated by CCK, released from the duodenum in response to gastric dilatation, is mediated by the vagus nerve (Fraser & Davison 1992) and involves in the periphery the CCK_A type receptor (Miller *et al.* 1993b). In response to an intravenous injection of CCK, an increase in electrical activity of supraoptic oxytocin neurones (Hamamura *et al.* 1991b) and in plasma oxytocin concentrations (Luckman *et al.* 1993b) have been described and these effects involve activation of putative afferent, catecholaminergic neurones in the brainstem (Luckman 1992). Oxytocin has also been shown to induce natriuresis at physiological concentrations (Windle & Forsling 1991), which might explain its release, along with vasopressin, in response to an acute hyperosmotic stimulus and during chronic dehydration (Brimble *et al.* 1978, Verbalis *et al.* 1991a).

Within the CNS, oxytocin has been suggested to facilitate extinction of learned avoidance behaviour, thus being antagonistic to the mnestic effects of vasopressin (De Wied *et al.* 1993, Insel 1992b), yet on the other hand oxytocin injections into the medial preoptic area can improve social recognition, an effect similar to that described for vasopressin (Popik *et al.* 1991). In addition, both oxytocin and vasopressin, along with many other peptides, act as neurotransmitters in the brain (Swanson & Kuypers 1980, Sawchenko & Swanson 1982b) and possibly in the spinal cord (Reiter *et al.* 1994) and the dorsal root ganglia (Kai-Kai *et al.* 1986).

Extracerebral sites of oxytocin actions

Apart from the CNS, oxytocin synthesis has been described in reproductive organs including the ovaries in rats, sheep and cows (Ho *et al.* 1992, Ivell *et al.* 1990, Ivell & Richter 1984b), the pregnant and non-pregnant uterus in humans (Chibbar *et al.* 1993, Kimura *et al.* 1992, Miller *et al.* 1993a) and in pregnant rats in the uterus (Lefebvre *et al.* 1992b), the amnion and the placenta (Lefebvre *et al.* 1992a, 1993). However, in the rat uterus the actual peptide content is very small compared to that of the hypothalamus (c.f. Lefebvre *et al.* 1992b and Fuchs & Saito 1971) and hence the physiological significance of such oxytocin production requires further investigation. Similarly, a mismatch between the high oxytocin mRNA and small peptide content has been found in rat testis and rat and bovine ovaries (Ang

et al. 1994, Foo *et al.* 1991, Ungefroren *et al.* 1994a). This observation led to the hypothesis that a post-transcriptional block could be a common feature in gonadal tissues with regard to the oxytocin and vasopressin genes and could represent a mechanism through which over-expression of the nonapeptides, and hence unwanted effects, are prevented (Ungefroren *et al.* 1994a).

Besides the presence in gonadal tissue, oxytocin receptors have been identified in the rat thymus and since their concentration decreases following mating (Caldwell *et al.* 1993), peripheral oxytocin might also be involved in the modulation of immune functions.

Oxytocin receptors - Distribution and regulation in the CNS

The effects of oxytocin, both within the CNS and in the periphery, are mediated by specific oxytocin receptors. Within the CNS, in the hypothalamus and the limbic system, autoradiographic studies have demonstrated oxytocin binding sites (Dreifuss *et al.* 1992, Kremarik *et al.* 1991, Tribollet & Dreifuss 1981, Tribollet *et al.* 1990), which are regulated by gonadal steroids (Patchev *et al.* 1993, Schumacher *et al.* 1990). Though in the SON oxytocin binding has only been detected after pretreatment with an oxytocin antagonist, that probably leads to an up-regulation of oxytocin binding sites (Freund-Mercier *et al.* 1994), many studies suggest the presence of functional oxytocin receptors within the SON and PVN, at least in term pregnant (Neumann, personal communication) and lactating rats (Lambert *et al.* 1993, Neumann *et al.* 1994). Furthermore, using *in situ* hybridisation techniques oxytocin receptor mRNA has been detected in regions of the magnocellular hypothalamus (Yoshimura *et al.* 1993). Also, oxytocin can act via the vasopressin receptor, namely the V_{1b} vasopressin receptor, as shown by recent data on the stimulatory effect of oxytocin on ACTH release from the anterior pituitary, that are prevented by a specific V_{1b} vasopressin receptor antagonist (Schlosser *et al.* 1993).

In the hypothalamus and the BNST, the oxytocin receptor density (Coirini *et al.* 1991, 1992, Dreifuss *et al.* 1992, Jirikowski *et al.* 1989) and oxytocin receptor mRNA expression (Bale & Dorsa 1995) increase in response to combined treatment with oestrogen and progesterone. This is in agreement with the hypothesis that at the end of pregnancy, after priming with progesterone and oestrogen, central oxytocin is involved via oxytocin receptors in the initiation of maternal behaviour (Caldwell *et al.* 1987, Jirikowski *et al.* 1988, 1989, Pedersen *et al.* 1982) and the facilitation of magnocellular oxytocin neurone activity (Belin & Moos 1989, Lambert *et al.* 1993).

Afferent projections to the magnocellular hypothalamus

Afferent projections from the forebrain and from within the hypothalamus

Unlike parvocellular PVN neurones, that receive direct projections from most hypothalamic and many forebrain areas (Swanson & Kuypers 1980, Swanson & Sawchenko 1983), magnocellular neurones in the hypothalamus receive an afferent input from a limited number of areas in the forebrain only, including the subfornical organ (SFO), the organum vasculosum in the lamina terminalis (OVLT) , the BNST and the olfactory bulbs (Smithson *et al.* 1989), and from within the hypothalamus, like the dorsomedial, median preoptic (MePN) and arcuate nuclei (see Fig. A). Most of these projections to magnocellular neurones show a preference for either oxytocin (the dorsomedial and arcuate nuclei and the BNST) or vasopressin neurones (the SFO), but equally innervate the preferred type of neurone in both the PVN and SON (Swanson & Kuypers 1980, Swanson & Sawchenko 1983). One exception is the projection from the olfactory bulbs, that seems to exclusively innervate supraoptic neurones (Hatton & Yang 1989).

In addition, some dopaminergic fibers have been detected immunohistochemically in the SON and are believed to derive from the tuberoinfundibular and periventricular hypothalamic nuclei (Buijs *et al.* 1987, Decavel *et al.* 1987, Lindvall *et al.* 1984). Dopamine agonists reduce, but do not prevent, the suckling-induced oxytocin release (Crowley *et al.* 1987) and, as shown by more recent studies, this effect is mediated by dopamine D_2 receptors, while stimulation of central D_1 receptors excites supraoptic oxytocin neurones (Crowley *et al.* 1991, Parker & Crowley 1992). Following a systemic injection of CCK, that induces pituitary oxytocin release, no change in intranuclear dopamine concentrations has been detected in the SON, and only a small increase has been described in the PVN (Kendrick *et al.* 1991), indicating that the dopaminergic innervation of the SON does not play a key role for oxytocin release in response to CCK. Since in the tuberoinfundibular dopaminergic system, that restrains prolactin release, gene expression of TH, the rate limiting enzyme in the dopamine synthesis, is suppressed during lactation (Wang *et al.* 1993), when prolactin secretion is enhanced, it is unlikely that in late pregnant and lactating rats dopamine-mediated inhibition of oxytocin release plays an important role.

Also, histaminergic projections from the tuberomammillary nuclei to the SON and PVN have been described (see Hatton 1990) and recent experiments have shown stimulation of vasopressin release via H_1 histamine receptors *in vitro* (Armstrong & Sladek 1985), and

pituitary oxytocin release following intracerebroventricular injections of histamine *in vivo* (Kjaer *et al.* 1994, Knigge & Warberg 1991).

With regard to the SON, a number of inputs, including the projection from the lateral septum, the amygdala and the diagonal band of Broca, do not enter the SON proper, but terminate dorsal of the SON in the perinuclear zone (Hatton 1990, Jhamandas & Renaud 1986, Tribollet *et al.* 1985), where, amongst others, GABA-immunoreactive neurones are located (Herbison 1994, Theodosis *et al.* 1986b). Thus, it has been suggested that stimulation of neurones in the diagonal band of Broca excites GABA neurones in the perinuclear zone of the SON, which in turn inhibit supraoptic vasopressin (Jhamandas & Renaud 1986) and possibly oxytocin neurones (Wuarin & Dudek 1993).

The functional importance of afferent projections from the forebrain to magnocellular neurones and/or the perinuclear zone, some of which are reciprocal, is well documented for the regulation of plasma osmolality (Honda *et al.* 1990) and arterial blood pressure and blood volume (Wall & Ferguson 1992) and, though to a lesser extent, the milk-ejection reflex (Ingram & Moos 1992, Moos *et al.* 1991). Thus, the release of neurohypophysial hormones in response to hyperosmotic stimulation is critically dependent on an intact afferent projection from the OVLT (Leng *et al.* 1989a) and the nucleus medianus, which in turn receives extensive projections from the SFO (Brimble *et al.* 1978, Honda *et al.* 1990, Leng *et al.* 1988b, Tanaka *et al.* 1987). In contrast, ablation of the region anterior and ventral to the third ventricle (AV3V), including the OVLT and the nucleus medianus, does not prevent the release of oxytocin in response to suckling and delivery (Russell *et al.* 1988, 1989a), indicating that inputs other then those from the AV3V region are critical for the secretion of oxytocin during lactation and parturition.

Both the SFO and the OVLT belong to the group of circumventricular organs, which lack an effective blood-brain barrier and these two structures are receptive to changes in plasma osmolality (Honda *et al.* 1990, McKinley *et al.* 1992, Miselis 1981). During pregnancy, there is a resetting of the plasma osmolality-vasopressin relationship: thus, pregnant rats (and humans) show normal plasma vasopressin concentrations despite a slight decrease in the plasma osmolality, that would normally inhibit vasopressin secretion (Lindheimer *et al.* 1985), possibly because the threshold for vasopressin and oxytocin release in response to osmotic stimulation is reduced (Koehler *et al.* 1994).

In response to suckling, projections from the BNST to the magnocellular hypothalamus (Ingram *et al.* 1992, Moos *et al.* 1991) and connections between the hypothalamic

magnocellular nuclei (Moss & Richard 1989, Moos *et al.* 1992) facilitate activity of oxytocin neurones and oxytocin release and this seems to be the case also during delivery (Neumann *et al.* 1992, 1993). The extensive connections between the magnocellular nuclei, including direct projections from the PVN to the ispilateral SON and the contralateral PVN (Silverman *et al.* 1981, Tribollet & Dreifuss 1981) and between both SON (Takano *et al.* 1990) are believed to be of importance for the milk-ejection reflex. Thus, magnocellular oxytocin neurones, that for most of the time display a slow and irregular activity with 1-10 spikes/sec, show prior to each milk ejection a high frequency discharge of about 80 spikes within 2-4 sec, that involves synchronised activity of most oxytocin neurones from all four magnocellular nuclei (Belin & Moos 1986, Poulain & Wakerley 1982). This coordinated activity results in the release of a large pulse of oxytocin from the posterior pituitary (Dyball 1971, Lincoln & Wakerley 1974). Similarly, during parturition the birth of each pup is preceded by a high frequency discharge of magnocellular oxytocin neurones in the PVN (Summerlee 1981), that is reflected by peak oxytocin plasma concentrations (Higuchi *et al.* 1986b). Following extensive lesioning of afferent projections from the forebrain to the magnocellular hypothalamus and of inter-hemispheric connections between the magnocellular nuclei, the burst amplitude in response to suckling is greatly reduced (and hence milk ejection impaired). In contrast, such deafferentation does not disrupt the synchronisation of bursts (Moos & Richard 1989), suggesting that inputs from the forebrain are facilitatory, but not crucial, for the generation of burst-like activity of magnocellular oxytocin neurones.

Afferent projections from the hindbrain

Following bilateral lesions made caudal of the magnocellular nuclei including the mesencephalic lateral tegmentum, the milk-ejection reflex is completely prevented (Dubois-Dauphin *et al.* 1985a, 1985b). However, anterogradely labelled neurones from the mesencephalic lateral tegmentum were not detected in the SON or the PVN (Dubois-Dauphin *et al.* 1985a), suggesting that the neural organisation of the milk-ejection reflex involves afferent pathways in addition to those in the mesencephalic areas.

Injections of retrograde tracer into the hypothalamus result in the greatest density of labelled cells in the brainstem, at the level of the area postrema, mainly in the ispilateral nulceus of the tractus solitarii (NTS) and both the ispi- and contralateral ventrolateral medulla (Cunningham & Sawchenko 1988, Sawchenko & Swanson 1982a). Both regions contain noradrenergic neurones, the A2 cell group in the NTS and the A1 cell group in the

ventrolateral medulla, and excitatory projections from these neurones to the hypothalamus, including magnocellular neurones, are well documented (Cunningham & Sawchenko 1988, Day & Sibbald 1988, Day 1989). Bilateral injections of a neurotoxin, selective for catecholaminergic fibers, into the magnocellular hypothalamus greatly reduce plasma oxytocin concentrations in response to suckling (Crowley *et al.* 1987), indicating the importance of an afferent catecholaminergic input for pituitary secretion in lactating rats. Furthermore, administration of an α_1-adrenergic agonist can induce burst-like activity of magnocellular oxytocin neurones *in vitro* (Wakerley & Ingram 1993). Since oestrogen treatment of ovariectomised rats attenuates α_2-adrenoreceptor-mediated autoinhibition of hypothalamic noradrenaline release (Karkanias & Etgen 1993), at times of high oestrogen concentrations, e.g. the end of pregnancy and early lactation, a reduced autoinhibition would favour an excitation of hypothalamic neurones to small amounts of catecholamines.

Normally, the excitatory effects of noradrenaline on oxytocin and vasopressin neurones are mediated by α_1-adrenergic receptors (Parker & Crowley 1993a, 1993b, Willoughby *et al.* 1987) and are potentiated by administration of α_2-adrenergic antagonists (Day *et al.* 1985), while β_1-adrenergic agonists inhibit suckling-induced oxytocin release (Moos & Richard 1979). Recent data have shown that the excitatory effects of catecholamines on neuronal activity and pituitary oxytocin release in lactating rats can be greatly enhanced by co-administration of an α_1-adrenergic agonist together with the glutamate receptor agonist AMPA (Parker & Crowley 1993b) or neuropeptide Y (NPY) (Parker & Crowley 1993a). Thus, peptides co-localised within catecholaminergic neurones, e.g. NPY, that has been detected within a subset of noradrenergic NTS neurones that project to the PVN (Sawchenko *et al.* 1985), could play a critical role for the burst-like release of oxytocin during parturition and in response to suckling.

Afferent projections from catecholaminergic neurones in the brainstem

In the brainstem, noradrenergic neurones constitute the largest population of catecholaminergic cells, extending rostro-caudally from the obex, just rostral of the area postrema, to the pyramidal decussatio in the caudal brainstem (Cunningham & Sawchenko 1988). Adrenergic cells are located almost exclusively rostral to the area postrema, in the dorsolateral and ventromedial medulla, the C1 and C2 region, respectively (Kalia *et al.* 1985), and innervate mainly parvocellular neurones in the medial and dorsal hypothalamus

(Cunningham & Sawchenko 1988, Sawchenko & Swanson 1982a). Dopaminergic neurones are found in the caudal part of the medulla, lateral to the central canal and ventral to the NTS (Kalia *et al.* 1985). Unlike neurones in the A1 cell group, which project to a number of regions including hypothalamic vasopressin, but not oxytocin, neurones (Sawchenko & Swanson 1982a, Swanson *et al.* 1981), A2 neurones innervate in the hypothalamus preferentially oxytocin neurones (Cunningham & Sawchenko 1988), while, as indicated by electrophysiological data, the input to vasopressin neurones is indirect and consists of two limbs, a peptidergic projection from the A2 to the A1 cell group (Thor & Helke 1989) and a noradrenergic projection from the latter to the hypothalamus (Cunningham *et al.* 1991, Raby & Renaud 1989a, 1989b). Indeed, we and others have shown recently using retrograde tracers, that CCK-induced stimulation of pituitary oxytocin secretion involves activation of a noradrenergic projection from the NTS to the SON and PVN (Onaka *et al.* 1995b, Rinaman *et al.* 1994). Likewise, afferent noradrenergic projections that travel in the ventral bundle are critically important for normal sexual behaviour in female rats (Hansen *et al.* 1980).

On the other hand, it has been shown that lesions of ascending noradrenergic pathways abolish the immobilisation stress-induced oxytocin release in male rats only, while having no effects in females (Carter & Lightman 1987a), indicating a sexual dimorphism in rats with regard to the involvement of a noradrenergic projection from the hindbrain for stress-induced oxytocin release.

Gonadal steroid regulation of hypothalamic catecholamines

As pointed out in the previous paragraph, noradrenaline release from terminals of ascending catecholaminergic pathways that reach the hypothalamus through the ventromedial forebrain bundle is modulated by gonadal steroids (Carter & Lightman 1987a). In female rats, basal levels of noradrenaline release in the hypothalamus are very low and not influenced by gonadal steroids or behaviour, such as feeding and locomotion (Etgen *et al.* 1992). However, during mating (Etgen *et al.* 1992) and following central application of oxytocin (Vincent & Etgen 1993), noradrenaline release in the ventromedial hypothalamus is significantly increased in female rats, in which lordosis behaviour has been facilitated by pretreatment with oestrogen and progesterone, while lesions of the ventral noradrenergic bundle abolish steroid-facilitated lordosis behaviour (Hanson *et al.* 1980).

Also, at the time of the LH surge, when female rats are receptive and pregnancy can be induced, noradrenaline release in the medial preoptic area is increased (Demling *et al.* 1985,

Rance *et al.* 1981) and this increased release is reflected by an increased expression of TH mRNA in catecholaminergic neurones in the ventrolateral medulla (Liaw *et al.* 1992c), that project to the hypothalamus (Wright & Jennes 1993). The 'gating effects' of noradrenaline, that are modulated by gonadal steroids, promote neuronal responses to stimuli, which would remain subthreshold without steroid priming (Etgen *et al.* 1992, Tetel *et al.* 1994). Since oestrogen attenuates the α_2-adrenoreceptor-mediated autoinhibition of noradrenaline release (Karkanias & Etgen 1993) and facilitates presynaptic noradrenaline release in the hypothalamus (Vincent & Etgen 1993) and depolarisation of hypothalamic neurones (Kow *et al.* 1991), oestrogen might enhance the stimulatory effects of noradrenaline with regard to neuroendocrine responses, such as the release of LH at proestrous and of oxytocin at term.

Afferent projections from peptidergic neurones in the brainstem

The NTS receives viscerosensory and somatic information from the periphery (Van Giersbergen *et al.* 1992), including the uterus (Ortega-Villalobos *et al.* 1990) and is reciprocally connected with higher autonomic centres in the forebrain, namely the hypothalamus and parts of the limbic system (Riche *et al.* 1990, Swanson & Sawchenko 1983).

In the NTS, a variety of putative neurotransmitters have been detected (Van Giersbergen *et al.* 1992), and for many of these substances functional binding sites within the NTS have been demonstrated. Some of the neuropeptides and biogenic amines, including enkephalin (Sawchenko *et al.* 1990), NPY (Sawchenko *et al.* 1985), CCK (Kawai *et al.* 1988), neurotensin (Van Giersbergen *et al.* 1992) and dynorphin (Ceccatelli *et al.* 1992), are co-localised with catecholamines in NTS neurones, while other substances, e.g. inhibin-β, somatostatin (Sawchenko *et al.* 1990) and nitric oxide (Ohta *et al.* 1993), are found primarily in non-catecholaminergic subpopulations of the NTS. Since firstly, neurones in the NTS containing inhibin-β (Sawchenko *et al.* 1988a, 1990) and somatostatin (Sawchenko *et al.* 1988b, 1990) project to the magnocellular hypothalamus and secondly, it has been shown that inhibin-β plays a role for suckling-induced oxytocin release (Plotsky *et al.* 1988), while somatostatin modulates electrical activity of supraoptic oxytocin neurones (Raby *et al.* 1988), peptidergic projections from the NTS could, beside the catecholaminergic cell groups, play a role for the regulation of oxytocin secretion during labour, delivery and lactation.

Furthermore, in the CNS CCK receptors have been localised amongst other regions in the hypothalamus, where primarily CCK_B receptors are found (Honda *et al.* 1993, Meister *et al.* 1994), and in the NTS and the area postrema, which contain both CCK_A and CCK_B receptors (Hill & Woodruff 1990). In the SON and PVN, CCK_B receptor gene expression is increased, along with CCK peptide gene expression and synthesis during dehydration (Honda *et al.* 1993, Meister *et al.* 1994). Also, CCK receptor density increases in association with magnocellular activity (Day *et al.* 1989, Shea & Gundlach 1993) and since CCK peptide is also present in NTS neurones (Kawai *et al.* 1988), it might be involved in the regulation of magnocellular oxytocin (and vasopressin) neurone activity.

Pregnancy and parturition

Pregnancy and parturition - Oxytocin

Although the first function of oxytocin that was observed and published regarded its uterotonic effects (Dale 1906), today more is known about the role of oxytocin for milk ejection. This might be partly due to the fact that the milk-ejection reflex is not abolished by anaesthesia (Lincoln *et al.* 1973), while it seems unlikely that parturition would occur spontaneously in anaesthetised rats. However, apart from these technical limitations, the greater interest in the regulation of oxytocin secretion during suckling might reflect long-standing doubts about a physiological significance of oxytocin for parturition (Smith 1932). These doubts were strengthened by the lack of a dramatic rise in plasma oxytocin concentrations during human labour (Chard 1989, Fuchs *et al.* 1982) and were maintained despite 1) the common use of oxytocin infusions for the induction and augmentation of labour in humans and 2) the more than two fold greater oxytocin peptide content in the posterior pituitary of term pregnant compared to virgin rats (860 vs 350 mU oxytocin/gland, Kumaresan *et al.* 1979) and the release of this accumulated excess into the general circulation in the course of delivery (Fuchs & Saito 1971, Kumaresan *et al.* 1979).

Early animal studies showing that hypophysectomy had no effect on the onset or the progress of delivery seemed to support the view that oxytocin was not involved in parturition (Smith 1932). However, following removal of the neurohypophysis, neurosecretory oxytocin and vasopressin axons are able to regenerate within days and thus hormone release into the general circulation is not greatly impaired by such surgical intervention (Villar *et al.* 1994). In contrast, hypothalamic lesions including the ventromedial hypothalamus and the medial forebrain bundle, increase the length of gestation and disrupt delivery (Averill & Purves 1963,

Gale & McCann 1961), suggesting that hypothalamic neurones and their afferent inputs are involved in the initiation of parturition.

The role of oxytocin for parturition was further disputed when experiments using repeated injections of oxytocin antibodies to late pregnant rats showed no impairment of parturition (Kumaresan *et al.* 1971, Schriefer *et al.* 1982). Eventually, the notion of a physiological importance of oxytocin during delivery was strengthened by the demonstration of a dramatic rise in the endo- and myometrial oxytocin receptor density at term (35-150 fold compared to mid-pregnancy or non-pregnant conditions, respectively, Alexandrova & Soloff 1980, Fuchs *et al.* 1982, Fuchs 1987), resulting in a dramatic increase in uterine sensitivity to oxytocin (Fuchs & Poblete 1970). This increase in uterine oxytocin receptor density at term is dependent on the rise in plasma oestrogen concentrations (and fall in plasma progesterone) at the end of gestation (Alexandrova & Soloff 1980, Fuchs *et al.* 1982, 1983, see Fig. B & C). There is no similar increase in the density of vasopressin receptors, which are also present in the uterus of rats (Chan *et al.* 1990) and humans (Maggi *et al.* 1991a) and which can mediate uterine contractions (Maggi *et al.* 1991a). However, vasopressin can also bind, though with a lower affinity than oxytocin, to the myometrial oxytocin receptor (Chan *et al.* 1990) and thus both neurohypophysial hormones might be involved in the generation of labour at term.

In rats, some reports have shown a heterogeneity of myometrial oxytocin receptors (El Alj *et al.* 1980) and in particular an increase in the oxytocin receptor density only in the circular myometrial layer (Crankshaw 1986, Tuross *et al.* 1987), but no change for the oxytocin receptor density in the longitudinal muscle at term. However, the physiological implication of a possible heterogeneity of myometrial oxytocin receptors remains to be clarified. Also, there is evidence for a pharmacological distinction between the endometrial and the myometrial oxytocin receptor in rats: thus, only stimulation of the endometrial subtype results in an increased release of prostaglandins, while the myometrial receptor mediates uterine contractility (Chan *et al.* 1993).

Beside the greater oxytocin receptor density in the term pregnant uterus, that suggests a role for oxytocin during labour and delivery, a more detailed analysis of oxytocin release showed not only a gradual increase in plasma oxytocin concentrations at term, but superimposed on the background secretion, intermittent peaks of the plasma oxytocin concentration (80 pmol/ml in rats, Higuchi *et al.* 1986b). In humans, the frequency and amplitude of these peaks increase progressively during labour and delivery, reaching a maximum during the expulsive phase (Dawood 1989, Fuchs *et al.* 1991). The physiological

relevance of such pulsatile release of oxytocin for the progress of delivery is demonstrated by the greater effectiveness of intermittent compared to continuous administration of oxytocin at inducing and augmenting uterine contractions (Randolph & Fuchs 1989, Dawood 1989). In addition, intermittent stimulation of uterine activity does not endanger the foetus(es) in the same way as a sustained increase in intrauterine pressure would do (Dawood 1989, Fuchs *et al.* 1991). Although doubts about the importance of oxytocin remain, particularly within the medical profession, some clinical trials have demonstrated the greater effectiveness of pulsatile compared to continuous administration of oxytocin for the induction of delivery (Dawood 1989).

Taken together, these recent advances have helped to establish the importance of oxytocin for the progress of labour, though we still lack a coherent picture of the mechanisms involved in initiating labour and delivery and hence the reflex release of oxytocin.

Pregnancy and parturition - The initiation of labour

Rats deliver between 8-18 pups after 22 days of pregnancy, usually in the second half of the light phase. Expulsion of pups is preceded by increased uterine activity for 2-4 hours, intensive grooming and nest building activity (Fuchs 1969) and once it has started it is normally completed within 90 min (Fuchs & Poblete 1970, Leng *et al.* 1988a). Despite some species differences with regard to the neuroendocrine aspects of pregnancy, the key features involved in maintaining pregnancy and initiating parturition are similar, at least for the rat, the sheep, in which most pioneering work was done, and the human. The maintenance of pregnancy in mammals is dependent on high plasma progesterone concentrations, that reach a maximum in the last trimester (Csapo & Wiest 1969, Csapo *et al.* 1980). During pregnancy, progesterone is produced in the corpus luteum and in the placenta, the latter of which is the main source of progesterone in the last two trimesters in the human (Itskovitz & Hodgen 1988). In contrast, in rats the corpus luteum remains the main source (Csapo & Wiest 1969). However, following ovariectomy of rats on day 18 of pregnancy, placental progesterone synthesis is sufficiently increased to maintain pregnancy (Csapo & Wiest 1969). High concentrations of progesterone during pregnancy (50-150 ng/ml in rats) keep the uterus in a state of relative quiescence and insensitivity to oxytocin (Burgess *et al.* 1992, Itskovitz & Hodgen 1988, Lau *et al.* 1993). Thus, a crucial step for the initiation of uterine activity is the increase in the ratio of plasma oestrogen/progesterone concentrations before term, resulting from either a decline in plasma progesterone, e.g. in rats (Fuchs *et al.* 1976, Howard & Wiest

1972), or an increase in oestrogen concentrations, e.g. in humans (Mazor *et al.* 1994, Mitchell & Wong 1993) and sheep (Levine & Nathanielsz 1993, Thorburn & Challis 1979). Under the influence of oestrogen, there is an increase in the activity of hydroxysteroid-dehydrogenase, an enzyme located in the corpus luteum that degrades progesterone (Fuchs *et al.* 1976, Mitchell & Wong 1993, Seong *et al.* 1992, Wahawisan & Gorell 1980). Furthermore, in the placenta progesterone is metabolised (Howard & Wiest 1972), as well as converted into oestrogen, contributing to the oestrogen dominance at term (Chan & Leathem 1975, Pepe & Rothchild 1972).

Under the rising plasma oestrogen concentrations, the secretion of prostaglandins (and in the rat particularly of prostaglandin F2α (PGF2α) from decidua cells) is increased (Chwalisz *et al.* 1991, Fuchs *et al.* 1981, Fuchs 1987) and contributes to the rise in oxytocin receptor density in the decidua at term (Fuchs 1987), which in turn is a prerequisite for the greatly enhanced production of prostaglandins seen in response to oxytocin in late pregnancy (Fuchs *et al.* 1981, Fuchs 1987). Prostaglandins are important for the initiation of uterine activity (Chan 1977), the expression of oxytocin receptors (Chan 1987) and the formation of gap junctions in the myometrium (Chan *et al.* 1988, 1991, Puri & Garfield 1982, Saito *et al.* 1985). Though the recently described increase in oxytocin mRNA in the endometrium of late pregnant rats and humans (Chibbar *et al.* 1993, Lefebvre *et al.* 1992b, Miller *et al.* 1993a) opens up the possibility that local oxytocin could initiate the increase in prostaglandin synthesis and release, in rats the actual oxytocin peptide content in the uterus is negligible compared to that in the hypothalamus (2 mU vs 700 mU, c.f. Lefebvre *et al.* 1992b and Fuchs & Saito 1971) and hence the physiological significance of uterine oxytocin in the rat awaits further investigation.

Under the oestrogen dominance at term, there is also an enhanced formation of gap junctions (Garfield *et al.* 1982, Puri & Garfield 1982) and of myometrial oxytocin receptors (Fuchs et al 1983) and α_1-adrenergic receptors (El Alj *et al.* 1989, 1993, Legrand *et al.* 1987), all of which are crucial for the generation of strong and coordinated uterine contractions in response to oxytocin during labour and delivery. At the same time, the number of myometrial β_2-adrenoreceptors that are coupled to adenylate cyclase and mediate uterine quiescence, decrease (El Alj *et al.* 1989, Legrand *et al.* 1987, Viva *et al.* 1992). The expression of other receptors, including those for serotonin, epidermal growth factors and

angiotensin, which have uterotonic actions, are also favoured by oestrogen (Bigsby & Young 1994, Soloff 1989).

More recently, a number of substances with oxytocic properties, including endothelins (Nakamura *et al.* 1993, Maggi *et al.* 1993), serotonin, histamine (Soloff 1989) and neuropeptide Y (Fallgren *et al.* 1989) have been described in the circulation at term and might play a role in the initiation of uterine activity. Conversely, there are a number of substances with antioxytocic properties, including vasoactive intestinal peptide (Soloff 1989), relaxin (Downing & Sherwood 1985, Sherwood *et al.* 1985) and nitric oxide (Yallampalli *et al.* 1993), which might contribute to the fine tuning of the initiation of labour. Since most of these substances have vasoactive properties and arterial vasoconstriction, e.g. induced by stimulation of the hypogastric and ovarian nerves, is followed by uterine contractions (Hutchison *et al.* 1994), some of these substances might regulate uterine activity via their effects on uterine blood flow. Furthermore, relaxin controls uterine activity indirectly by inhibiting oxytocin release form the posterior pituitary (Jonas & Summerlee 1986).

Beside uterine activity, active cervical contractions have recently been reported during delivery in the human (Olah *et al.* 1993) and might, together with oxytocin, contribute to the effacement of the cervix (Khalifa *et al.* 1992), which is a prerequisite for the normal outcome of delivery. In addition, prostaglandins and in the rat, relaxin that is released from the corpus luteum at the end of pregnancy, are involved in the softening of the cervix at term (Downing & Sherwood 1985).

Pregnancy and parturition - The foetal contribution to the initiation of labour

A foetal contribution to the initiation of parturition is best established in sheep: the maturation of the foetal hypothalamic-pituitary-adrenal axis results in increased foetal plasma cortisol concentrations, which induce placental enzymes capable of metabolising progesterone to oestrogen (Myers & Nathanielsz 1993, Thorburn & Challis 1979). Thus, maturation of the foetal hypothalamo-pituitary axis initiates the increase in plasma oestrogen while decreasing plasma progesterone, which, as described above, is a prerequisite for the initiation of parturition in mammals. In the human, foetal cortisol does not seem to influence labour and delivery (Bonica & McDonald 1990, Thorburn & Challis 1979), but foetal oxytocin concentrations are significantly increased at term (Chard 1989) and since oxytocin crosses the placental barrier (Chard 1989), foetal oxytocin might contribute to the rise in maternal plasma oxytocin concentrations and hence to the initiation of uterine activity.

Unlike the sheep and the human, that usually give birth to one infant, the rat uterus contains many foetuses and thus a foetal contribution to the onset of delivery would involve a coordinated influence of the entire litter. Studies on gestation length after aspiration of foetal hypothalami (Swaab *et al.* 1977) or injections of oxytocin antibodies (Schriefer *et al.* 1982) or dopamine (to inhibit foetal oxytocin secretion, Schriefer *et al.* 1980) into the foetuses have not yielded conclusive results, and thus, in rats the role of foetal cortisol and oxytocin for the initiation of parturition remains controversial. In addition, in rat foetuses the release of oxytocin following stimulation, e.g. by hypertonic saline (Almazan *et al.* 1989) or ovarian steroids (Miller *et al.* 1989), is not fully developed at birth and hence a significant contribution of the foetus to the increase in maternal oxytocin concentrations appears unlikely.

Though our understanding of the mechanisms initiating parturition is still incomplete, I would like to suggest that the increased ratio of the plasma oestrogen/progesterone concentration in the last days of pregnancy allows a progressive augmentation in uterine activity, that enhances PGF2α production and release (Chan 1977). Prostaglandins, in turn, further stimulate myometrial oxytocin receptor expression and hence increased uterine sensitivity to oxytocin, creating a positive feedback loop (Fuchs *et al.* 1982) that will eventually lead to an increased neurosecretory activity of hypothalamic magnocellular neurones.

Pregnancy and parturition - Afferent innervation of the uterus and the cervix

Until recently, the role of uterine afferents has attracted little attention, possibly due to reports on a functional denervation of the uterus at term (Morizaki *et al.* 1989). Uterine afferents, that are unmyelinated or thinly myelinated fibers travel with the hypogastric (Steinman *et al.* 1992) and the pelvic nerves (Berkley *et al.* 1988, Bonica & McDonald 1990, Robbins *et al.* 1992) and enter the spinal cord at the lumbal and sacral level (L1-L4 for the hypogastric nerves and L5-S1 for the pelvic nerves, Berkley *et al.* 1988, 1993, Steinman *et al.* 1992). The sensory information is then relayed in the brainstem to higher autonomic centres (Hubscher & Berkley 1994, Ortega-Villalobos *et al.* 1990). Electrophysiological recordings from afferent nerves (Berkley *et al.* 1988, Peters *et al.* 1987, Robbins *et al.* 1992), as well as from neurones in the brainstem (Hornby & Rose 1976, Hubscher & Berkley 1994) and from neurones in the PVN itself (Akaishi *et al.* 1988, Negoro *et al.* 1973a, 1973b), have clearly shown a responsiveness of these neurones to vagino-cervical and uterine distension throughout

pregnancy and particularly at term. The physiological importance of these afferents for normal reproductive functions is suggested by the observation that lesions of the pelvic nerves interfere with the induction of pseudopregnancy (Carlson & Feo 1965), the foetus-expulsion reflex (Higuchi *et al.* 1987) and the reflexive ovulation following caesarean section (Cunningham *et al.* 1992). Furthermore, the uterus, the cervix (Steinman *et al.* 1992) and the vagina are heavily innervated by both parasympathetic and sympathetic fibers (Nance *et al.* 1988), the former of which travel predominantly with the pelvic nerves (nervi erigentes), while the latter travel with both the pelvic and the hypogastric nerves (Peters *et al.* 1987, Wray 1993). Postganglionic fibers, derived from parasympathetic ganglia in the parametria and from the lumbar sympathetic chain, enter the uterus along with blood vessels and reach into the endometrium and the myometrium (Peters *et al.* 1987, Vera *et al.* 1994). Since stimulation of the hypogastric nerve causes constriction of uterine arteries, that is followed by an increase in intrauterine pressure (Hutchison *et al.* 1994), such neurogenic mechanisms could play a role for the initiation of uterine contractions at term. In addition, some sensory innervation of the uterus and the cervix is provided by the vagus nerve (Ortega-Villalobos *et al.* 1990), that terminates in the caudal part of the NTS and the dorsal motor nucleus of the vagus in the caudal medulla oblongata (Hubscher & Berkley 1994).

Afferent projections from the female reproductive organs to the brainstem and the hypothalamus

Lesions of the vagus nerve eliminate the response of NTS neurones to uterine stimulation and alter those to vaginal and cervical stimulation (Hubscher & Berkley 1994). However, such lesions do not impair the response of putative neurosecretory oxytocin neurones in the PVN to vaginal distension (Akaishi *et al.* 1988), indicating that pathways in addition to those involving the NTS can convey sensory innervation from pelvic reproductive organs to the hypothalamus. Since vaginal distension modulates activity of neurones located in the ventral medulla, and particularly in the nucleus paragigantocellularis (Hornby & Rose 1976), that projects to the hypothalamus (Van Giersbergen 1992), neurones in the nucleus paragigantocellularis could represent an alternative relay station for the transmission of sensory information from the uterus to the hypothalamus.

Electrical activity, evoked in peripheral and central afferent neurones by vaginal stimulation, are modulated by gonadal steroids, with the greatest responsiveness to such stimulation at times of high plasma oestrogen concentrations (Berkley *et al.* 1988, Robbins *et*

al. 1992), while progesterone administration inhibits, via a central site of action, the reflex release of oxytocin following vaginal distension (Roberts 1971). Since progesterone concentrations are high during pregnancy, an inhibitory effect of progesterone on oxytocin release could play an important role in the prevention of premature oxytocin secretion. Systematic studies on electrical activity evoked in neurosecretory oxytocin neurones in the PVN during different reproductive states and following treatment with gonadal steroids have shown that the percentage of cells excited by vaginal distension in mid-pregnancy, when plasma progesterone concentrations are high, is significantly smaller than that at the end of parturition (6.9% vs 72%) (Negoro *et al.* 1973b), when progesterone levels are very low (Csapo *et al.* 1980). Furthermore, spontaneous activity of magnocellular oxytocin neurones is higher at the end of pregnancy than in mid-pregnancy, and spike activity at term can be further increased in a majority of cells by vaginal distension (Negoro *et al.* 1973b). The modulation of magnocellular neurone activity by gonadal steroids is further demonstrated by the reduced spike frequency following vaginal probing in late pregnant rats that have been ovariectomised and pretreated with progesterone, but increased spike frequency in response to vaginal probing following pretreatment with oestrogen (Jiang & Wakerley 1994). In addition, the stimulatory effect of oxytocin on electrical activity of paraventricular oxytocin neurones *in vitro* is only observed at term, while in mid-pregnancy administration of oxytocin to the preparation inhibited most of the recorded cells (Kawarabayashi *et al.* 1993).

Taken together, a high plasma progesterone concentration seems to be associated with reduced activity of putative oxytocin neurones. However, progesterone treatment of ovariectomised rats does not result in the same decrease in cell activity as seen in mid-pregnancy (Negoro *et al.* 1973a), suggesting that besides progesterone, other factors contribute to the inhibition of spontaneous activity of neurosecretory oxytocin cells in mid-pregnancy.

Pregnancy-related changes in the hypothalamus and the neurohypophysis - Oxytocin synthesis

At the end of pregnancy, the rat neural lobe contains about twice as much oxytocin (and vasopressin) peptide than that of virgin rats (Fuchs & Saito 1971, Kumaresan *et al.* 1979), and this increased synthetic activity is associated with elongated poly(A) tails of both nonapeptide mRNAs in pregnant and suckled rats, indicating increased stability and hence

possibly a more efficient translation of the transcripts (Carter & Murphy 1991, Zingg & Lefebvre 1989, Zingg *et al.* 1988a).

Besides magnocellular neurones, other oxytocin-containing neurones, that are involved in the initiation of maternal behaviour (Numan 1988), including neurones in the ventromedial hypothalamus, the medial preoptic area and the BNST (Caldwell *et al.* 1987, Jirikowski *et al.* 1988, 1989) show enhanced oxytocin immunoreactivity at the end of pregnancy and during lactation.

Gonadal steroids, and particularly oestrogens, have been implicated in the up-regulation of oxytocin synthesis in the hypothalamus (Jirikowski *et al.* 1988) and, as mentioned before, oxytocin mRNA expression in the SON and PVN increases by nearly 200%, when progesterone is withdrawn (Crowley *et al.* 1995). Thus, progesterone withdrawal at the end of pregnancy, which is considered a critical step in the initiation of parturition, could contribute to the observed neuronal excitation and increased hormone release at term. Though the oxytocin gene contains steroid responsive elements (Adan *et al.* 1993, Burbach *et al.* 1990, Miller *et al.* 1989a), the lack of nuclear gonadal steroid receptors in rat hypothalamic oxytocin neurones, including magnocellular neurones (Fox *et al.* 1990, Rhodes *et al.* 1981a, Sar 1988) suggests an indirect action of gonadal steroids on magnocellular oxytocin neurones at term.

Pregnancy related changes in the hypothalamic-neurohypophysial system - Oxytocin secretion

During the last trimester of pregnancy and during parturition, administration of the opioid-antagonist naloxone results in greatly increased plasma (Bicknell *et al.* 1988c, Douglas *et al.* 1993b, Russell *et al.* 1989b) and intranuclear (in the SON) oxytocin concentrations (Neumann *et al.* 1992). Since the potentiating effect of naloxone on oxytocin release from isolated neural lobes declines in the course of pregnancy (Bicknell *et al.* 1988c, Douglas *et al.* 1993b), the increased inhibition of oxytocin release by endogenous opioids seen during pregnancy (Bicknell *et al.* 1988c, 1993) is exerted primarily at the hypothalamic level and might, by preventing afferent inputs to excite magnocellular neurones, contribute to the described accumulation of oxytocin in the neural lobe during pregnancy (Fuchs & Saito 1971, Kumaresan *et al.* 1979).

Though the origin of endogenous opioids that mediate this inhibition is not fully clarified, possible candidates are β-endorphin-synthesising neurones in the mediobasal hypothalamus, that project to the oxytocin-dense part of the SON (Sawchenko *et al.* 1982c) and show an increased peptide content at the end of pregnancy (Petraglia *et al.* 1985).

In the magnocellular hypothalamus, both mu (Mansour *et al.* 1988) and kappa opioid binding sites (Sumner *et al.* 1992) have been demonstrated, while in the neural lobe only kappa opioid binding sites, which are found on oxytocin-terminals (Herkenham *et al.* 1986), have been detected (Mansour *et al.* 1988). Since dynorphin, a kappa opioid receptor agonist, is co-expressed (Watson *et al.* 1982) in secretory granules containing vasopressin in the posterior pituitary, dynorphin, co-released with vasopressin, could play a role for the regulation of oxytocin release from axon terminals in the neural lobe (Van de Heijning *et al.* 1991b). In addition to the direct effects of opioids on oxytocin neurones, morphine has recently been shown to prevent CCK-induced noradrenaline release in the SON via a presynaptic site of action within or close to the SON (Onaka *et al.* 1995a) and similarly, the increasing opioid restraint on magnocellular oxytocin neurone activation in the course of pregnancy involves a presynaptic action on catecholaminergic afferents (Bicknell *et al.* 1993). Since an opioid-inhibition of prolactin release in term pregnant rats is dependent on the normal decline of plasma progesterone concentrations (Soaje & Deis 1994), a similar mechanism could contribute to the (opioid) control of preterm release of oxytocin.

Beside opioid peptides, the classical "inhibitory" transmitter GABA has been implicated in the control of oxytocin secretion, since GABA-containing synapses contact supraoptic oxytocin neurones (Theodosis *et al.* 1986b). Some of these GABA-containing presynaptic terminals contact two postsynaptic elements (so called "double synapses"), and interestingly, these have only been observed on oxytocin neurones and most commonly in lactating rats, in which both the incidence of "double synapses" and the proportion of such synapses that are immunoreactive for GABA are increased compared to virgin rats (Theodosis *et al.* 1986b). Also, oestrogen-induced plasticity of GABAergic synapses in the hypothalamus of rats has been observed (Parducz *et al.* 1993).

In the SON and its perinuclear zone, $GABA_A$ receptors containing the α_1- and α_2-subunits are found, and in the case of the SON they have been located on both types of neurones (Fenelon & Herbison 1994, Herbison 1994). Since progesterone and its metabolite allopregnanolone are potent agonists at the $GABA_A$ receptor (Zhang & Jackson 1994),

particularly that containing the α_1–subunit (Paul & Purdy 1992), and since plasma progesterone concentrations are elevated during pregnancy but decline at term, the increased neurosecretory activity of oxytocin neurones might involve a decreased $GABA_A$ receptor-mediated inhibition of supraoptic neurones. Furthermore, evidence from experiments using the patch-clamp technique indicates that peptide secretion from axon terminals in the neural lobe is also modulated by progesterone and allopreganolone acting on $GABA_A$ receptors (Zhang & Jackson 1993, 1994) and the description of a GABAergic innervation of pituicytes has led to speculations about a possible involvement of GABA in the regulation of glial retraction at the end of gestation (Buijs *et al.* 1987).

Pregnancy related changes in the hypothalamo-neurohypophysial system - Morphological changes

During delivery magnocellular hypothalamic oxytocin neurones show a high frequency discharge prior to the delivery of each pup (Summerlee 1981), that resembles the bursting activity observed in response to suckling (Lincoln & Wakerley 1974, Lincoln *et al.* 1973). In contrast, during increased oxytocin secretion in response to CCK and hypertonic saline such burst-like discharge is not seen (Leng *et al.* 1993b, Renaud 1987) and hence it has been suggested that prolonged or repeated stimulation, that could induce structural adaptations within the magnocellular hypothalamus, is required for such activity. However, during chronic dehydration, a condition accompanied by increased secretion of oxytocin and vasopressin (Brimble *et al.* 1978, Hamamura *et al.* 1992) and by structural alterations within the hypothalamus and the neurohypophysis similar to those at the end of pregnancy and during lactation (Perlmutter *et al.* 1984, Theodosis *et al.* 1986a), no bursting activity of oxytocin cells is observed (Leng *et al.* 1993b, Moos *et al.* 1992).

Morphological changes seen at the end of pregnancy and during chronic dehydration include at the level of the hypothalamus an increase in both the extent and the incidence of direct appositions between somata of oxytocin neurones (Theodosis *et al.* 1986a) due to retraction of glial elements and interneuronal coupling, that is mostly restricted to neurones of the same peptide content (Cobbett *et al.* 1985, Theodosis *et al.* 1986a). Furthermore, the incidence of "double-synapses", that permit transmission of information to more than one postsynaptic cell, is increased at term (Theodosis *et al.* 1984, 1986a, Perlmutter *et al.* 1984).

Similarly, in the neural lobe structural changes that involve predominantly oxytocin terminals occur, namely a retraction of pituicyte processes that normally surround neurosecretory elements and the pericapillary basal lamina, thus allowing greater access of axon terminals to the basal lamina of the fenestrated capillaries and hence facilitating oxytocin secretion into the general circulation (Tweedle & Hatton 1982, 1987). Possible mechanisms causing these structural changes include a direct effect of increased plasma osmolality (Perlmutter *et al.* 1984) and a mediation by adrenergic receptors, that are located on pituicytes (Bicknell *et al.* 1988a, Garten *et al.* 1989, Saavedra 1985). However, an increase in plasma osmolality is an unlikely cause for the changes seen at the end of pregnancy, since plasma osmolality is decreased rather than increased during gestation (Lindheimer *et al.* 1985). An involvement of catecholamines on the other hand seems more likely, since the neural lobe receives a) a central noradrenergic input from the catecholaminergic A2 cell group in the dorsomedial medulla oblongata (Garten *et al.* 1989), which also sends an excitatory projection to hypothalamic magnocellular neurones (Raby & Renaud 1989b) and b) a sympathetic innervation from the cervical superior ganglia (Alper *et al.* 1980, Saavedra *et al.* 1985). Furthermore, increased plasma concentrations of circulating catecholamines during labour (Abboud *et al.* 1982, Bonica & McDonald 1990) and in response to the milk-ejection reflex (Clapp *et al.* 1985) could, due to the proximity of pituicyte processes to blood vessels, easily interact with adrenergic receptors located on these processes. Though these alterations in both the hypothalamus and the posterior pituitary can occur *in vitro* within a few hours of a stimulus (Perlmutter *et al.* 1984), they normally develop during the last days of gestation and are most pronounced during delivery and lactation (Theodosis *et al.* 1984). After weaning, as well as after the end of chronic dehydration, these changes are completely reversed, however, the time required for such restoration increases with repeated stimulation (Chapman *et al.* 1986, Theodosis *et al.* 1984).

Unlike morphological alterations, that occur in response to chronic dehydration and during pregnancy and are hence likely to be a prerequisite rather than the cause for burst-like activity, increased intranuclear oxytocin release, that is observed during parturition and lactation (Moos *et al.* 1992), might play a crucial role for the facilitation of bursting activity (Moos & Richard 1989). The sources of such intranuclear release of oxytocin include dendritic processes in the SON and axon collaterals that terminate in the periventricular zone of the SON (Pow & Morris 1989, Theodosis 1985). In addition, oxytocin terminals of so far

unknown origin, have been shown to synapse on supraoptic oxytocin neurones (Theodosis 1985) and might contribute to the enhanced intranuclear release of oxytocin.

Recently, a reciprocal excitation of supraoptic oxytocin neurones has been shown in lactating, but not male rats (McKenzie *et al.* 1995), which could further facilitate and enhance synchronised bursting activity of neurones, e.g. in response to suckling and during parturition.

Immediate early genes and their protein products as markers of neuronal activation in the CNS

Detection of neuronal activation has for many years been assessed using intra- and extracellular electrophysiological recordings. Recently, a new technique has been described that allows detection of neuronal activation by immunocytochemical staining for Fos, the protein product of the immediate early gene c-*fos* (Sagar *et al.* 1988, Sheng & Greenberg 1990), that was first described in carcinoma cells and hence was initially classified as an oncogene (Sheng & Greenberg 1990). Unlike late response genes, including most genes encoding enzymes, hormones and neurotransmitters, whose expression is induced over a time frame of hours and whose transcription is thought to be the specific response of a cell to trans-synaptic activation, transcription of immediate early genes following an extracellular stimulus is activated rapidly (within minutes) but transiently, since the protein products of these genes negatively regulate their own promotor (Sassone-Corsi *et al.* 1988).

Today many immediate early genes are known beside the first described c-*fos* and c-*myc* genes and they have been grouped into families, including the Fos family (with c-*fos*, fos-B and Fos-related antigens Fra-1 and Fra-2) and the Jun family (with c-*jun*, *jun*-B and *jun*-D) (Sheng & Greenberg 1990). Protein products of the c-*fos* and c-*jun* families interact with each other to form heterodimeric transcription factor complexes, which then bind with high affinity and specificity to DNA elements of the consensus sequence 5'-TGACTCA-3' and stimulate the transcription of nearby promotors. The -TGACTCA- sequence, which was first identified as a phorbol ester-inducible promotor element and the binding site for a transcription factor complex, was termed AP-1 (activator protein 1). All of the different combinations of dimers formed by members of the Fos and Jun families can bind to the AP-1 sequence, however, the affinity of a Jun/Fos heterodimer is much greater than that of a Jun/Jun homodimer (Sheng & Greenberg 1990). Though normally Fos/Jun complexes are believed to activate transcription of genes containing AP-1 sites, under certain circumstances *fos*-B/Jun complexes have been implicated in the repression of transcription (Gizang-Ginsberg & Ziff 1994).

The initial mode of action for the induction of c-*fos* is an influx of calcium through voltage-dependent Ca^{2+} channels in response to a depolarising stimulus, which results in detectable cellular concentrations of c-*fos* mRNA within 10 min that return to undetectable concentrations within 60 min, demonstrating the transient expression of these genes (Hamamura *et al.* 1991b). Since Fos, the protein product of the c-*fos* gene, reaches the highest concentration in the nuclei of supraoptic neurones within 60-90 min after a stimulus and remains detectable for about four hours (Verbalis *et al.* 1991b), immunocytochemical detection of Fos protein has become a useful tool to study activation of neurones.

Nowadays, antibodies against a number of immediate early gene products are available, however, the polyclonal anti-Fos antiserum was one of the first antibodies that allowed reliable immunocytochemical detection of an immediate early gene protein (Hunt *et al.* 1987) and has ever since been used extensively as a marker for neuronal activation (Fenelon *et al.* 1993, Hoffman *et al.* 1993, Hunt *et al.* 1987, Luckman 1992, Verbalis 1991b). The antibody used for immunocytochemical detection of Fos protein is directed against the N-terminal amino acids 2-17, which show the least homology between members of the Fos family, and does not cross react with FRA proteins, it is specific for Fos (Hunt *et al.* 1987).

In cortical neurones, a relationship has been demonstrated between bursting activity, as seen during kindled seizures, and the induction of c-*fos* mRNA expression (Labiner *et al.* 1993) and in neurosecretory hypothalamic neurones, induction of c-*fos* has been associated with hormone release (Hoffman *et al.* 1993). However, as recently demonstrated, this is only true when hormone release is trans-synaptically stimulated (Luckman *et al.* 1994). Since under most physiological circumstances, increased secretion of oxytocin (and vasopressin) is mediated by trans-synaptic stimulation, normally hormone release is associated with Fos expression in the hypothalamic magnocellular system (Verbalis *et al.* 1991b, Hoffman *et al.* 1993). In contrast, the reflex release of oxytocin during normal suckling is not accompanied by increased Fos expression in the SON (Fenelon *et al.* 1993). Whether, as has been proposed, the overall increase in cell activity in response to suckling is not sufficient to activate c-*fos* expression (Leng *et al.* 1993b) and/or whether in suckled rats there is an increased threshold for the induction of the c-*fos* gene, as suggested by experiments showing a reduced secretion of oxytocin in response to certain stimuli in suckled compared to non-suckled rats (Higuchi *et al.* 1988, Koehler *et al.* 1994, Lightman 1992), remains to be clarified. Recently, it has been shown that Fos expression is induced in the SON in response to the milk-ejection reflex when pups have been removed for at least 48 hours prior to the

suckling stimulus (Smith *et al.* 1994). Thus, while continuous application of a stimulus seems ineffective at inducing Fos expression, the same stimulus can, when applied acutely and after a period of relative quiescence, induce Fos expression in neurosecretory neurones and this might be due to the habituation of neurones with regard to the induction of Fos, in response to repeated stimulation (Melia *et al.* 1994).

Apart from neurosecretory neurones themselves, Fos expression is also observed in putative afferent neurones in the rat brainstem and detection of c-*fos* mRNA has been used to identify neural circuits mediating the release of neurohypophysial hormones (Hamamura *et al.* 1991a, 1991b, 1992). Thus, following osmotically induced oxytocin and vasopressin secretion, c-*fos* mRNA expression has been detected in the anteroventral third ventricle region and the SFO, areas that are critical for oxytocin secretion in response to such stimulation (Hamamura *et al.* 1991a, 1992). Likewise, systemic administration of CCK, a stimulus for oxytocin, but not vasopressin secretion (Verbalis *et al.* 1991b), results in increased Fos immunoreactivity in putative afferent neurones in the brainstem, including the NTS (Luckman 1992).

Since Fos protein is a regulator of gene transcription, Fos expression in the magnocellular hypothalamus in response to a stimulus associated with peptide release, might be followed by an increase in gene expression of the released peptide. Yet, neither the oxytocin nor the vasopressin gene contain the consensus AP-1 binding site (Icard-Liepkalns *et al.* 1992), but a region that differs from that sequence by one basepair (TGACCA in the oxytocin and TGAATCA in the vasopressin gene, Leng *et al.* 1993b), to which binding of Jun/Fos complexes has not been demonstrated. However, binding of a variety of Fos/Jun complexes to an AP-1 site, that differs from the consensus AP-1 site by one base (a 'T' rather than a 'C' at the centre of the palindrome) has been demonstrated in the case of the tyrosine hydroxylase (TH) gene (Gizang-Ginsberg & Ziff 1994, Icard-Liepkalns *et al.* 1992).

In summary, numerous studies have shown a correlation between increased Fos expression and neuronal activation, and in the case of the hypothalamic magnocellular system an association with increased hormone release, and thus immunocytochemistry for Fos is a powerful tool for the investigation of neuronal activation, as long as limitations of this technique are taken into consideration when interpreting the data. Particularly the combination of immunocytochemistry for Fos with a second immunocytochemical procedure to characterise a Fos-immunoreactive cell in terms of the transmitter produced, has proven useful to map putative afferent pathways activated by a given stimulus.

Aims of the present study

Our understanding of the initiation and maintenance of parturition remains incomplete. Thus, the onset of labour is often unpredictable and therefore encourages medical doctors (and some of the pregnant women) to use an infusion of oxytocin to induce delivery at a "convenient" time. However, such treatment does not contribute much to the clarification of physiological mechanisms involved in inducing and maintaining labour and delivery. Here, our aims were three fold: firstly to examine the involvement of pituitary oxytocin in the initiation and maintenance of labour and delivery in rats, secondly to identify putative afferent neurones mediating the increased release of oxytocin at the end of pregnancy and thirdly to investigate their regulation.

We began by examining the effects of oxytocin administration on the progress of delivery in rats in which endogenous oxytocin has been inhibited by the opioid agonist morphine. In addition, we used immunocytochemical detection of Fos, the protein product of the immediate early gene c-*fos*, a relatively novel technique, to assess whether the known activation of supraoptic neurones during a physiological, but strong stimulus like delivery of pups, was reflected by increased expression of this immediate early gene.

We then employed another stimulus for pituitary oxytocin release, an injection of CCK, and, using again Fos immunocytochemistry, we thought to identify common pathways involved in oxytocin secretion.

Furthermore, we performed double immunocytochemistry to identify neurones immunoreactive for Fos in terms of the transmitters produced and thus to map neuronal circuits involved in oxytocin secretion during late pregnancy and parturition.

Finally, we tried to validate some of our conclusions concerning the activation of supraoptic neurones at term, by recording electrical activity of supraoptic neurones in late pregnant rats during induced labour.

Fig. A **Afferent projections to magnocellular neurones in the hypothalamus (modified from Cunningham & Sawchenko 1991)**
Direct afferent projections to magnocellular neurones in the hypothalamus (in the SON and PVN) are derived from the brainstem, including the NTS and noradrenergic cells in the ventrolateral medulla (A1 cell group), and the forebrain, including the SFO, OVLT and the median preoptic nucleus (MePN) in the anterior hypothalamus. The SFO and OVLT which lack an effective blood-brain barrier and are susceptible to changes in plasma osmolality, are part of the osmoreceptor complex, that controls, via pituitary oxytocin and vasopressin release, body fluid homeostasis. Neurones in the brainstem, and particularly the NTS, relay sensory afferents from the periphery to higher autonomic centres, including magnocellular neurones in the hypothalamus. Neurotransmitters, including noradrenaline (NE), inhibin-β (Iβ), somatostatin (SS), enkephalin (Enk) and neuropeptide Y (NPY), have been detected in fibers projecting from the brainstem to the hypothalamus, including magnocellular neurones.

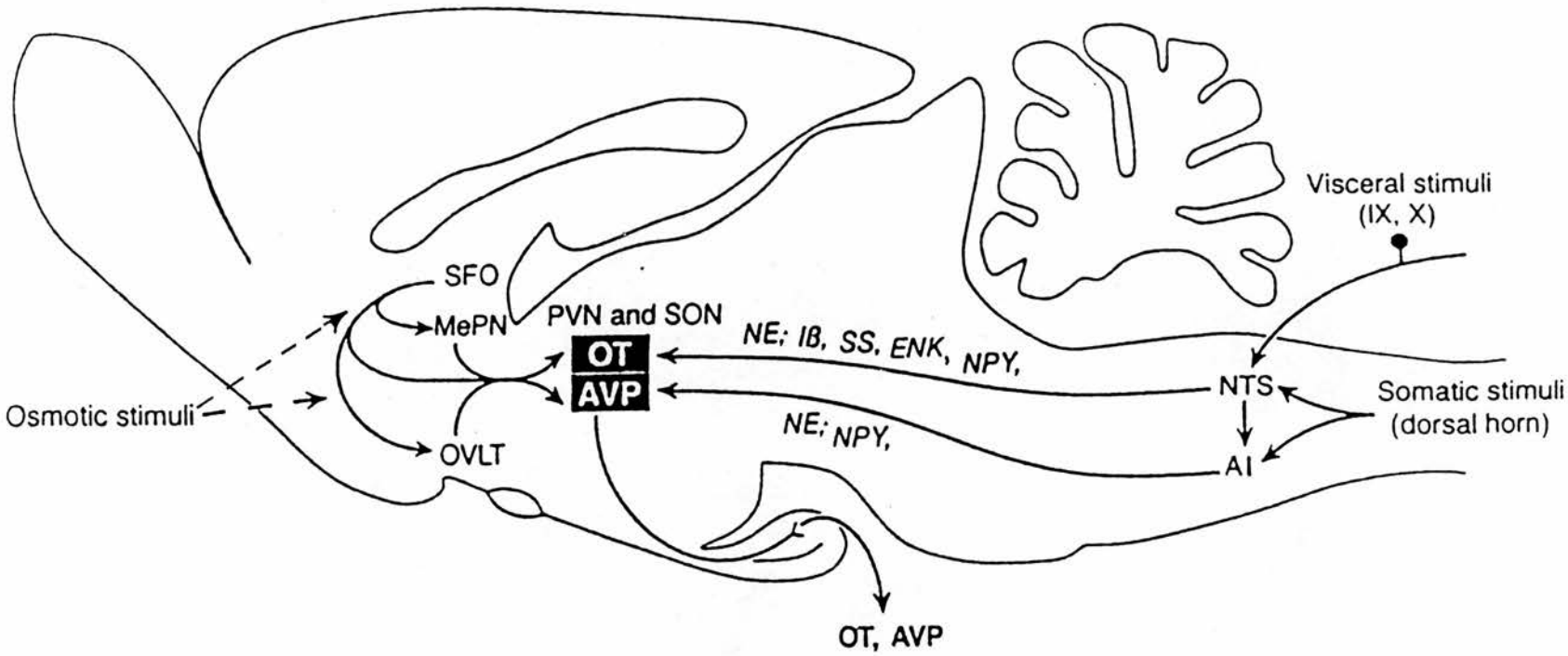
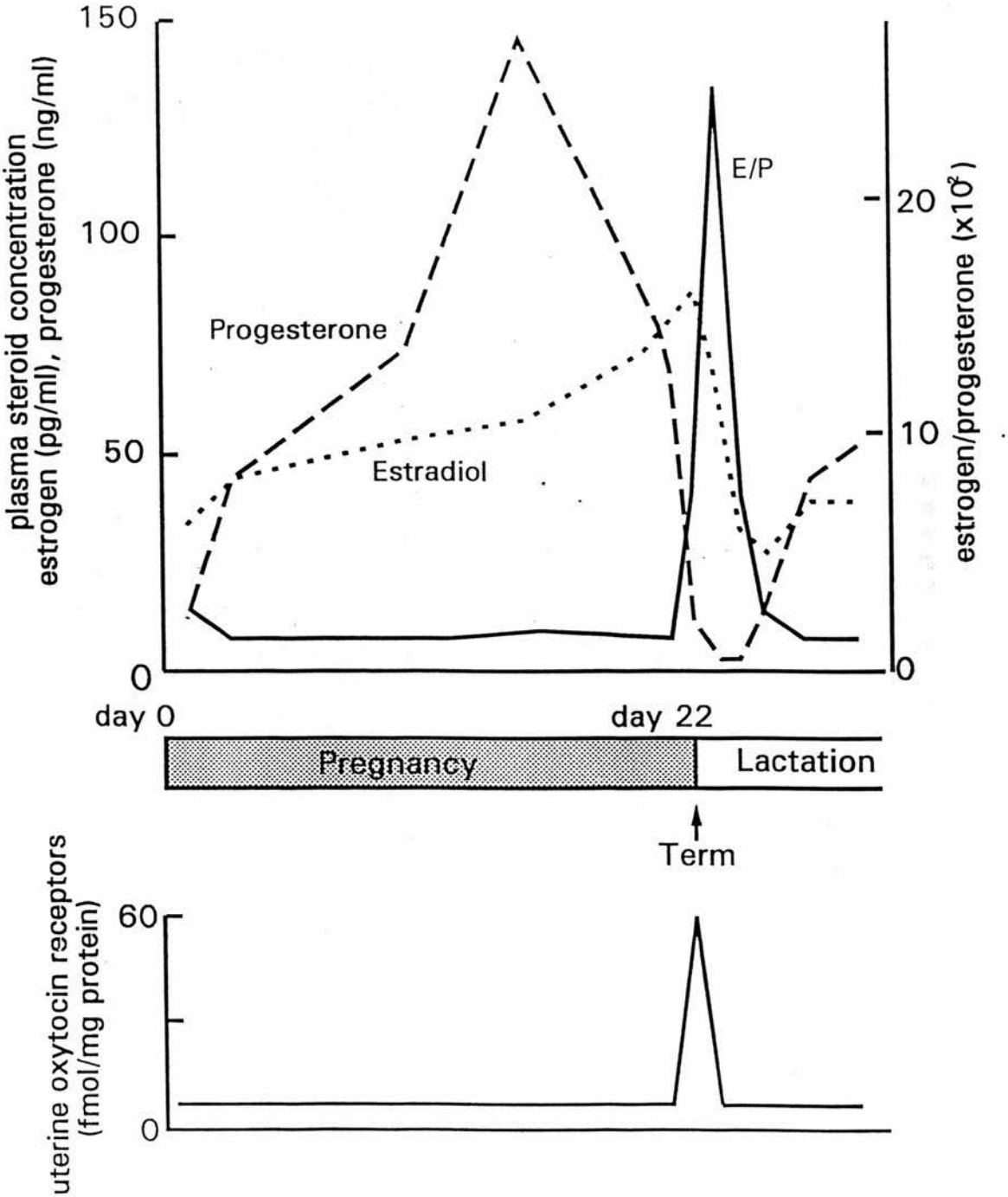

Visceral stimuli
(IX, X)
SFO
MePN
PVN and SON
OT
AVP
NE; IB, SS, ENK, NPY,
NTS
Somatic stimuli
(dorsal horn)
Osmotic stimuli
NE; NPY,
OVLT
AI
OT, AVP

Fig. B **Gonadal steroid and oxytocin receptor concentrations during pregnancy**
Top: Parturition is preceded by a fall in plasma progesterone and subsequent rise in the plasma oestrogen/progesterone ratio
Oestrogen and progesterone plasma concentrations (left y-axis) and the ratio of the plasma concentration of oestrogen/progesterone (right y-axis) during pregnancy in the rat
Bottom: Uterine oxytocin receptor concentrations increase dramatically at term in the rat
(data from S.A. Way, PhD thesis 1992, drawing by S. Dye)


150
100
50
0
plasma steroid concentration
estrogen (pg/ml), progesterone (ng/ml)
Progesterone
Estradiol
E/P
20
10
0
estrogen/progesterone (x10²)
day 0
day 22
Pregnancy
Lactation
Term
60
0
uterine oxytocin receptors
(fmol/mg protein)

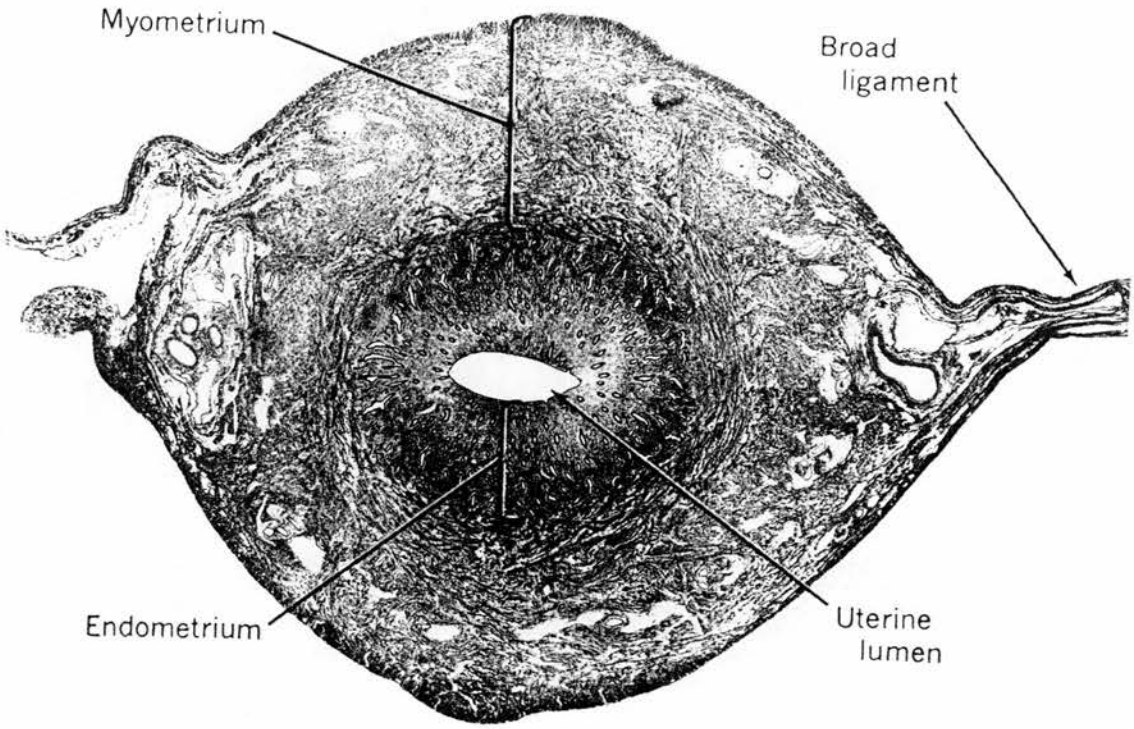
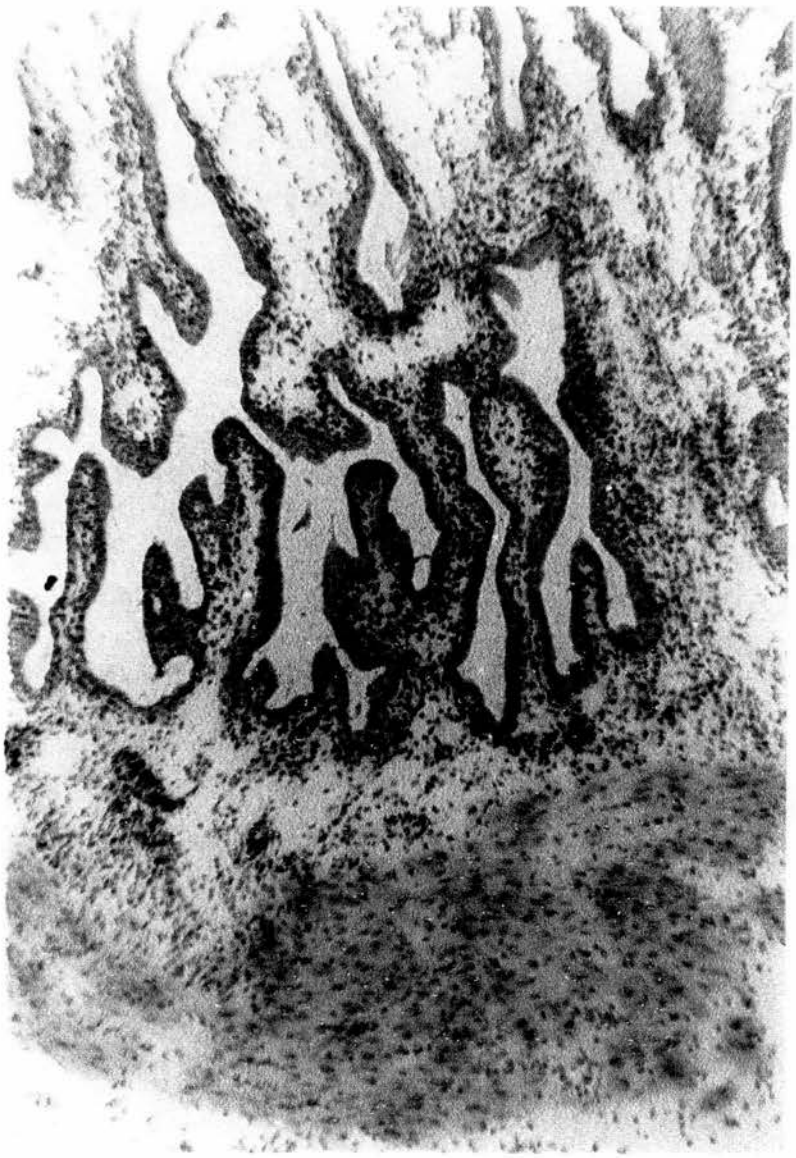


Fig. C **The rat uterus**
Top: Circular section through the rat uterus (modified from Bloom 1975)

Right: Higher magnification view of the endo- and myometrial layer of a term pregnant rat uterus stained with eosin and haematoxylin


endometrium
myometrium
100 μm

General Materials and Methods

Animals and Surgery

For all experiments on pregnant rats date mated female rats (Wistar rats in Babraham and Sprague-Dawley rats in Edinburgh) of 280-450 g body weight at term were used. For mating, virgin rats (200-220 g body weight) were left overnight with experienced males, and all female rats in which sperm was detected in vaginal smears the next morning were kept from then on in single cages with food and water *ad libitum*. Wistar rats were kept on a 14 h L:10 h D (lights on 05.00-19.00 h) schedule, Sprague-Dawley rats on 12 h L: 12 h D (lights on 08.00-20.00 h). For experiments on cycling rats, daily vaginal smears were taken and examined under the light microscope for at least two consecutive four day oestrous cycles, before rats were used for experimental purposes.

Besides female rats, we performed one experiment in male rats (see chapter two) of similar weight to virgin females (200-300 g body weight) from the Babraham colony (Wistar rats). For experiments requiring intravenous drug application in conscious rats, animals were implanted with a jugular vein cannula (inner diameter 0.5 mm, outer diameter 1.0 mm) either

on day 19 of pregnancy under halothane anaesthesia (Wistar rats) or on day 20 or day 21 under brief ether anaesthesia (Sprague-Dawley rats). In the morning of the experiment, normally the day of expected term (day 21 of pregnancy in both strains of rats), the cannulae were flushed with 0.1 ml heparinised saline (50 IU heparin/ml saline) before any injection and then connected to polyethylene tubing (same diameter as above, volume 0.1 ml) to allow drug application with as little disturbance of the animals as possible.

On the day of the experiment (day of term, see above), animals were assessed every 5-10 min for signs of labour (stretching, straining, vaginal bleeding), pup delivery and maternal behaviour (nest building, excessive grooming, licking of external genitals, licking of the pups during and after delivery, pup retrieval). Onset of delivery was defined as the time when the first pup was fully expelled and the time of birth for each following pup was recorded until at least the eighth pup. During the dark period the observation was continued in red light.

At the end of each experiment rats were killed by cervical dislocation and after laparotomy the uteri were stripped of remaining pups and placentae.

For immunocytochemistry and *in situ* hybridisation, the brains and/or uteri were removed, immediately frozen under crushed dry ice and stored at -80°C until processed. To optimise conditions for double immunocytochemistry some animals were deeply anaesthetised with sodium pentobarbitone (50 mg/kg body weight iv) and perfused transcardially with chilled 0.9% saline and then 4% paraformaldehyde in phosphate buffer. The brains were postfixed and then kept frozen at -80°C until processed for immunocytochemistry.

The use of two strains of rats should not give rise to any concern as during the long-standing collaboration between the two laboratories we never noticed any striking difference between the two strains regarding parameters like gestation length, time of onset of delivery, the progress of delivery and litter size. In contrast, similar results obtained in both laboratories should strengthen the validity of our data.

Drugs

Cholecystokinin (CCK26-33, sulphated; Sigma, UK) was dissolved in sterile 0.9% saline (1 mg/ml) and injected intravenously at a concentration of 20 μg/kg body weight, the injected volume being 100 μl/100 g body weight.

Morphine sulphate and naloxone HCl (Sigma, UK) were dissolved in sterile 0.9% saline and injected at a concentration of 1 mg/kg and 5 mg/kg body weight, respectively, in a volume of 100 µl/100 g body weight.

Oxytocin (Syntocinon, Sandoz) was dissolved in sterile 0.9% saline and injected at a concentration of either 33 mU/ml (= 66 ng/ml), 330 mU/ml (= 660 ng/ml) or 660 mU/ml (= 1320 ng/ml), respectively, the injected volume being 0.03 ml per injection.

Two peptide oxytocin antagonists were used, desGly9,d(CH2)5[Tyr(Me)2Thr4]OVT (OVT16) (Manning *et al.* 1989) and the compound F382® (des-Gly9-[D-Trp2,alloIle4,Orn8]dC6OT, Ferring, Sweden) generously provided by Dr. M. Manning, Medical School of Toledo, Ohio, USA, and Dr. P. Melin, Ferring Research Institute, Malmö, Sweden. The effective dose (ED) was defined as the dose (in nmol/kg body weight) that reduces the response to 2x units of agonist to equal the response to 1x unit (1.7±0.3 nmol/kg for OVT16 (Manning *et al.* 1989) and 2.9±0.2 nmol/kg for F382 (P. Melin, personal communication)); both peptide oxytocin analogues were dissolved in 0.9% saline containing 0.03 mM HCl and were given in a volume of 0.05 ml/100 g body weight per injection, at a concentration of 30 or 60 µg/kg body weight.

Commercially available progesterone (Intervet, UK, 5 mg per rat, dissolved in 0.3vol% cresol, 89.7vol% arachis oil and 10vol% benzylalcohol) or vehicle (0.2 ml per rat) was injected subcutaneously into the right outer calf.

Tissue preparation

For immunocytochemistry brains were cut into coronal sections either on a sliding microtome (30 µm) in the case of *in situ* perfusion-fixed brains, or on a cryostat (15 µm), for non-perfused brains. Sections were collected, in 0.1 M phoshate buffered solution, throughout the SON (extending from the anterior commissure to the median eminence) and through the brainstem (from the pyramidal decussation to the widening of the fourth ventricle rostral of the area postrema). We counted immunoreactive cells on every fifth section from the respective areas (a minimum of 8 cryostat and 8 microtome sections per area), thus ensuring that sections from similar rostro-caudal positions for all animals were analysed, so that a variation in cross section area and section volume is controlled for. For *in situ* hybridisation of uterine tissue, circular sections (15 µm) were cut under RNAse free conditions.

All cryostat sections (from both the brain and the uterine) were fixed in 4% paraformaldehyde in phosphate buffer for 30 min prior to the immunocytochemistry or *in situ* hybridisation procedure.

Immunocytochemistry

In the first instance, endogenous peroxidase was deactivated with a solution of 0.1 M phosphate buffer, 20% methanol and 0.2% Triton-X (15 min at room temperature).

For Fos immunocytochemistry the sections were preincubated for 60 min in 0.1 M phosphate buffer containing 0.3% Triton-X and 1% normal sheep serum, then incubated with a polyclonal antiserum raised in rabbits against the N-terminal amino acids 2-17 of the rat Fos protein (kindly provided by Drs. G.I. Evan and D. Hancock, Imperial Cancer Research Fund, London, UK), used at a dilution of 1:10 000 at 4°C for 24 h. The second antibody (IgG made in goats, Vector, UK) coupled to a peroxidase complex was used at 1:500 for 24 h.

For immunocytochemical detection of tyrosine hydroxylase (TH, the rate limiting enzyme for the synthesis of catecholamines), sections through the brainstem were first preincubated for 60 min in 0.1 M phosphate buffer containing 0.3% Triton-X, 1% normal horse serum and 0.5% bovine serum albumin and were then incubated with a mouse monoclonal TH antibody (1:4000, purchased from Chemicon International, Temecula, CA, USA) for 24 h at 4°C. Sections were then incubated with a biotinylated horse anti-mouse antiserum (at 1:2000 for 2 h at room temperature, Vector, UK) and then with a commercial streptavidin-biotinylated horseradish peroxidase complex (1:200, Amersham, UK) for 2 h at room temperature.

In both protocols, the antigen-antibody complex was visualised using the nickel intensified (glucose-amino-oxidase) 3',3'-diaminobenzidine method (Shu *et al.* 1988), which results in either a dark purple nuclear staining in the case of Fos or in dark cytoplasmic staining in the case of TH. The sections were then dehydrated in graded alcohols, cleared in xylene and coverslipped.

Immunoreactive cells were counted with the identity of the sections coded, using a microscope with a x10 objective and a brightfield condenser. To test the specificity of the increase in Fos expression in the brainstem and the SON in response to a treatment, Fos-immunoreactive nuclei were also counted on some cryostat sections containing the subfornical organ (SFO) and the area postrema. These areas are part of the group of circumventricular organs which lack an efficient blood-brain barrier and are susceptible to changes in plasma

osmolality and blood borne substances. Every third section was collected of the respective areas and a minimum of five sections per area were analysed.

Double immunocytochemistry

For Fos and vasopressin double immunocytochemistry the sections were first stained for Fos as described above. Then the sections were washed and preincubated with 2% normal horse serum and 0.5% bovine serum albumin for 1 h. The first antibody, a mouse monoclonal anti-vasopressin antiserum (a gift from Dr. F.R. Robert, INSERM, Hopital Henri Mondor, 94101 Creteil, France, Robert *et al.* 1985) was used at a concentration of 1:100 000 for 40 h followed by the second antibody, a biotinylated anti-mouse IgG raised in horse, used at a concentration of 1:400 for 2 h at room temperature. After another 2 h incubation with the streptavidin-biotinylated horseradish peroxidase complex (1:200, Amersham, UK) the staining was visualised using H_2O_2 and 3',3'-diaminobenzidine, resulting in a light brown reaction product over the cytoplasm that can be clearly distinguished from the dark purple nuclear Fos staining.

For Fos and TH staining, the sections were first stained for Fos as above, then washed and preincubated with 2% normal horse serum and 0.5% bovine serum albumin for 1 h. After that, sections were incubated with a mouse monoclonal TH antibody (1:4000, purchased from Chemicon International, Temecula, CA, USA) for 24 h at 4°C. For visualisation of the antibody, the streptavidin-biotinylated peroxidase complex method was used. After incubation with the TH antibody, the sections were first incubated with a biotinylated horse anti-mouse IgG (at 1:2000 for 2 h at room temperature, Vector, UK) and then with a commercial streptavidin-biotinylated horseradish peroxidase complex (1:200, Amersham, UK) for 2 h at room temperature. The antigen-antibody complex was visualised using the 3',3'-diaminobenzidine method, resulting in a light brown reaction product over the cytoplasm that can be clearly distinguished from the dark purple nuclear Fos staining.

In the brainstem, double- and single-labelled cells were counted in at least 42 alternate sections per animal to survey the catecholaminergic cell groups of the NTS and of the ventrolateral medulla (the A2/C2 and A1/C1 cell groups, respectively), while TH immunoreactive cells located in the dorsal motor nucleus of the vagus were not counted, as the majority of these neurones at the level of the area postrema contain dopamine, unlike at the caudal level of the A2/C2 region, where the majority of TH immunoreactive cells contain

noradrenaline (Kalia *et al.* 1985). The counts were divided into seven groups of six sections each, so that each group extended rostro-caudally over a subdivision of 0.36 mm. The obex, just rostral of the area postrema (Paxinos & Watson 1986), was taken as reference point zero.

For Fos and oxytocin double immunocytochemistry the sections were first stained for Fos as described above, but using a biotinylated second antibody (1:200, Vector, UK) and the streptavidin-biotinylated horseradish peroxidase complex (1:200, Amersham UK). The staining was visualised using the nickel-intensified (glucose-amino-oxidase) 3',3'-diaminobenzidine method.

Sections were then washed and preincubated with 1% normal sheep serum for 1 h, before incubation with a polyclonal antiserum, raised in rabbits, against oxytocin (kindly given to us by Dr. T. Higuchi, Kochi Medical School, Japan), for 24 h at a concentration of 1:50 000 at 4°C. The second antibody, made in goat against rabbit IgG and coupled to a peroxidase complex, was used at a concentration of 1:500 for 24 h (Vector, UK). Cellular staining was visualised using the 3',3'-diaminobenzidine method, resulting in a light brown reaction product over the cytoplasm that can be clearly distinguished from the dark purple nuclear Fos staining.

***In situ* hybridisation**

In situ hybridisation for TH mRNA

Coronal sections (15 µm) were cut through the brainstem on a cryostat under RNAse free conditions, mounted on gelatinised slides and stored at -80°C until used.

A synthetic oligonucleotide probe complementary to the bases 1223-1252 (30 mer) of the rat TH gene (Grima *et al.* 1985) was 3' end labelled with [35S]dATP (1000-1500 Ci/mmol, New England Nuclear NEG 034H) using 50 U terminal desoxynucleotidyl transferase (Pharmacia, UK) resulting in a specific activity of approximately 2.8 x 10^5 dpm/ng. This oligonucleotide probe sequence has been used previously to map the distribution of tyrosine hydroxylase mRNA expression in rat brain and adrenal (Kiyama *et al.* 1990). The radiolabelled probe was purified by gel filtration on a Sephadex G-50 column. Frozen sections were warmed to room temperature, fixed with 4% paraformaldehyde in 0.1 M phoshate buffer for 20 min and rinsed in 0.1 M phosphate buffered saline (PBS). Sections were then pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% sodium chloride for 10 min, dehydrated through a series of graded alcohols and left to air dry. The oligonucleotide probe was diluted in hybridisation buffer (50% deionised formamide, 4 x SSC, 10% dextran

sulphate, 1 x Denhardt's solution, 250 µg/ml sheared salmon testis DNA, 0.3% ß-mercaptoethanol to a final concentration of 1.7 x 10^3 dpm/µl (equivalent to 0.06 pmol/µl), and 350 µl of diluted probe applied to each slide (6-10 sections per slide). Following an overnight hybridisation at 37°C, sections were washed once in 1 x SSC at room temperature, then three times in 1 x SSC at 55°C (30 min each) and finally one more time in 1 x SSC at room temperature for 60 min. Sections were briefly rinsed in dH_20, followed by 70% ethanol/300 mM ammonium acetate and then absolute alcohol. Sections were allowed to air dry before either being exposed to hyperfilm ß-MAX (Amersham, UK) for 4 weeks at room temperature, or being dipped in Ilford K-5 nuclear track emulsion and exposed for 5 weeks in dark tight boxes at room temperature. Emulsion dipped slides were photodeveloped with Ilford K-5 (1:5 in distilled water, at 4°C), counterstained with methylene blue, to allow visualisation of cells, and coverslipped. The hyperfilm ß-MAX was developed in Kodak D-19 (1:5 in distilled water, 5 min at 20°C) and fixed in Ilford Hypam (1:4 in distilled water, 10 min at 20°C). Signal specificity was assessed by incubating some sections with the radiolabelled probe in the presence of a 25-fold excess of unlabelled probe.

For the quantitative analysis of TH mRNA expression an automated image analysis system, the Joyce Loebl µMagiscan, was used. The average optical density per cell (silver grains/cell) in the NTS and the ventrolateral medulla were measured in a minimum of 20 cells (4 x 5 cells from four different sections) per area per rat. On most sections, the area covered by the screen of the image analysis system (dorsal and lateral of the central canal and in the ventrolateral medulla), contained five or less sections, so that the cells included in the analysis were usually all or most of the cells containing silver grains per half section.

In situ hybridisation for oxytocin mRNA

Uterine circular sections (15 µm) were cut on a cryostat under RNAse free conditions, mounted on gelatinised slides and stored at -80°C until used.

A synthetic oligonucleotide probe (27 mer) complementary to a sequence on exon C of the rat oxytocin gene (912-939, Ivell & Richter 1984) was 3' end labelled with [35S]dATP (1000-1500 Ci/mmol, New England Nuclear NEG 034H) using 50 U terminal desoxynucleotidyl transferase (Pharmacia, UK) resulting in a specific activity of approximately 4.3 x 10^4 dpm/ng. This oligonucleotide probe sequence has been shown to hybridise with oxytocin mRNA in rat brain, uterus and placenta and its specificity has been

verified using Northern blot analysis (Lefebvre *et al.* 1992a, 1992b, Zingg & Lefebvre 1988b). The radiolabelled probe was purified by gel filtration on a Sephadex G-50 column. Frozen sections were warmed to room temperature, fixed with 4% paraformaldehyde in 0.1 M phoshate buffer for 20 min and rinsed in 0.1 M phosphate buffered saline. Sections were then pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% sodium chloride for 10 min, dehydrated through a series of graded alcohols, then delipidated with chloroform for 10 min, partially rehydraded and finally left to air dry. The oligonucleotide was diluted in hybridisation buffer (50% deionised formamide, 4 x SSC, 10% dextran sulphate, 1 x Denhardt's solution, 250 µg/ml sheared salmon testis DNA, 0.3% ß-mercaptoethanol to a final concentration of approximately 2 x 10^3 dpm/µl (equivalent to 0.05 pmol/µl) and 20 µl of diluted probe applied to each section (and two sections/slide). Following an overnight hybridisation at 37°C, sections were washed once in 1 x SSC at room temperature, then three times in 1 x SSC at 55°C (30 min each) and finally one more time in 1 x SSC at room temperature for 60 min. Sections were briefly rinsed in distilled dH_20, followed by 70% ethanol/300 mM ammonium acetate and then absolute alcohol. Sections were allowed to air dry before either being exposed to hyperfilm ß-MAX (Amersham, UK) for 26 h at 4°C, or being dipped in Ilford K-5 nuclear track emulsion and exposed for 26 h in dark tight boxes at 4°C. Emulsion dipped slides were photodeveloped with Ilford Phenisol (1:5 in distilled water, 5 min at 20°C), counterstained with haematoxylin eosin and coverslipped. The hyperfilm ß-MAX was developed in Kodak D-19 (1:5 in dH_2O, 5 min at 20°C) and fixed in Ilford Hypam (1:4 in distilled water, 10 min at 20°C). Signal specificity was assessed by incubating some sections with the radiolabelled probe in the presence of a 25-fold excess of unlabelled probe.

For the quantitative analysis of oxytocin mRNA expression an automated image analysis system, the Joyce Loebl µMagiscan, in combination with a M17 Vickers microscope, was used. Measurements of optical density, assessed in a minimum of 4 areas per endo- and myometrium, respectively were transformed using a standard curve into dpm/mm^3 endo- and myometrium.

Extracellular electrophysiological recording from supraoptic neurones and measurement of uterine and/or cervix pressure on the day of expected term

Date mated day 21 pregnant Wistar rats were deeply anaesthetised with sodium pentobarbitone (initially 20-30 mg/kg body weight intraperitoneally) and implanted with a jugular vein cannula and a tracheal cannula. Then animals were carefully laparotomised by a medial longitudinal incision (approximately 20 mm) in the abdominal wall. One horn of the pregnant uterus was taken out of the abdominal cavity and kept moist with 37°C warm sterile water. An small incision (5 mm) was made mid-way between the ovarian and the cervical end in the uterine wall at the site of a pup and then a purse-string-suture was made around the incision using an atraumatic needle and a sterile chromic collagen thread (Ethicon, 6/0, UK). Then a deflated latex balloon (condom tip) was inserted and pushed towards the ovarian end until it was located in between two pups. The balloon was then carefully inflated with 1.5-3 ml sterile water to ensure the correct location and then deflated. The uterine incision was closed and the polyethylene tubing attached to the balloon was carefully fixed with the thread to the uterine wall before being reinserted into the abdominal cavity. For the implantation of a second balloon in the other uterine horn the procedure was repeated in exactly the same way using the same abdominal incision. Finally, the balloons were inflated with 1.5-4 ml sterile water and attached to the pressure recorder. In some rats we introduced an additional balloon of the same size as above into the cervix. In that case the deflated balloon was introduced through the vagina and inflated just above the cervix (the correct length has been tested in pilot experiments under vision). At the end of the experiment the location of all inserted balloon was examined prior to deflation.

Then the SON and the neural stalk were exposed by ventral surgery performed by Dr. G. Leng as previously described (Dyball & Leng 1986, Leng 1981). Briefly, a bipolar stainless-steel stimulating electrode was placed upon the neural stalk and a glass micropipette, filled with 0.15 M NaCl, was introduced into the supraoptic nucleus under direct visual control. Single neurones, recorded extracellularly from the supraoptic nucleus region were identified antidromically as projecting to the neural lobe and as putative oxytocin neurones by their continuous pattern of discharge and by their transient and significant increase in firing rate in response to an intravenous injection of CCK (20 μg/kg body weight), while vasopressin neurones were identified by a lack of such excitation and by their phasic activity. For antidromic stimulation a matched biphasic stimulus was used (2 ms pulses, <1 mA peak-to-peak).

Statistical analysis

Unless indicated otherwise, non-parametric tests and a two-tailed hypothesis were used for statistical comparisons. Data are expressed as means±s.e.m.. In the case of two groups, the Mann Whitney U-test was used, while the Kruskal-Wallis test, followed by Dunn's post-hoc test was used for multiple comparisons. A difference was considered significant for a two-tailed value of $p<0.05$.

For statistical analysis of the progress of delivery, the time between birth of pups (interbirth intervals) was recorded and the comparison calculated either for the cumulative between a number of pups or for individual intervals. Since the time between the birth of the first and the second pup is very variable, this first interbirth interval was excluded from subsequent analysis. For the same reason, drugs, administered to parturient rats, were injected in all but one experiment after the birth of the second pup.

Chapter 1

The maintenance of normal parturition in the rat requires neurohypophysial oxytocin

During delivery in rats 30-40% of the accumulated pituitary oxytocin content (about 350 mU, Fuchs & Saito 1971, Kumaresan *et al.* 1979) is released into the general circulation. The physiological importance of this release is demonstrated by the disruption of the normal progress of delivery following inhibition of oxytocin secretion by administration of central or systemic opioid receptor agonists to parturient rats and by the restoration of such disrupted delivery by an infusion of oxytocin (Bicknell *et al.* 1988c, Leng & Russell 1989c, Russell 1989b). During pregnancy endogenous opioids restrain the release of oxytocin at the level of the hypothalamus and the pituitary (Bicknell *et al.* 1988c, 1993, Douglas *et al.* 1993a), however the opioid inhibition of oxytocin release from terminals in the neural lobe declines gradually towards term (Douglas *et al.* 1993a), while in the hypothalamus an endogenous opioid tone remains present on magnocellular oxytocin neurones throughout delivery (Bicknell *et al.* 1988c, Neumann *et al.* 1992). Despite this opioid restraint during delivery in rats, burst-

like discharge of neurosecretory neurones has been observed preceding the birth of each pup (Summerlee 1981) and this electrical activity is reflected by intermittent peak plasma concentrations of oxytocin up to 80 pg/ml (Higuchi *et al.* 1986b), indicating pulsatile hormone release. In humans, such pulsatile release of oxytocin has been described throughout the last trimester of pregnancy (Chard 1989), with an increase of the pulse frequency and amplitude in the course of labour and a maximum during the expulsion of the foetus (Fuchs *et al.* 1991).

Since in rats, an injection of the opioid receptor agonist morphine during delivery significantly reduces oxytocin secretion and greatly prolongs delivery of subsequent pups (Russell *et al.* 1989b), we used such treatment to investigate the effectiveness of different patterns of oxytocin administration at restoring the normal progress of parturition in rats. Furthermore, we used Fos immunocytochemistry to examine whether expression of this immediate early gene product, that is induced in magnocellular hypothalamic neurones in response to non-physiological or pharmacological stimuli, including haemorrhage, an injection of hypertonic saline (Fenelon *et al.* 1993) and an injection of CCK (Hamamura *et al.* 1991b), is also induced during parturition, which is a physiological stimulus for the secretion of oxytocin.

Materials and Methods

Animals

Date mated pregnant Wistar rats from the Babraham colony were implanted with a jugular vein cannula under brief halothane anaesthesia on day 19 of pregnancy. In the morning of day 21 of pregnancy, the day of expected term, the cannulae were flushed with 0.1 ml heparinised saline (50 IU heparin/ml saline) and connected to polyethylene tubings filled with isotonic saline or oxytocin. Rats were continuously observed for signs of labour (stretching, straining, vaginal bleeding), pup delivery and maternal behaviour (nest building, excessive grooming, licking of external genitals, licking of the pups during and after delivery, pup retrieval). During the dark phase the observation was continued in red light.

Experimental design

One group of animals was killed on the day of expected term but prior to any signs of labour and delivery (prepartum group, n=5). Another group of rats was allowed to deliver pups without any treatment (controls, n=9). All other rats received a single injection of morphine sulphate (Sigma, 1 mg/kg body weight, in 0.05 ml saline/100 g body weight)

immediately after the birth of the second pup. Then rats were randomly assigned to one of the following treatment groups:

- the naloxone group (n=6) was given a single injection of naloxone HCl (Sigma, 5 mg/kg body weight, in 0.1 ml saline/100 g body weight)
- the morphine group (n=10) was given a continuous infusion of saline (6 µl/min) for 90 min
- the oxytocin infusion groups were given an infusion of oxytocin (Syntocinon, Sandoz) at a higher or lower rate (0.4 mU oxytocin/min, n=9, or 0.1 mU oxytocin/min, n=10)
- the oxytocin pulse groups were given the equivalent of the higher or lower infusion rate of oxytocin in pulses (2 mU oxytocin in 0.03 ml each 5 min, n=5, or 1 mU oxytocin in 0.06 ml each 10 min, n=9 or 0.5 mU oxytocin in 0.03 ml each 5 min, n=6)

Time of birth for each pup was recorded until 90 min after delivery of the second pup, when animals were deeply anaesthetised with sodium pentobarbitone (50 mg/kg body weight iv) and *in situ* perfusion-fixed with 4% paraformaldehyde in phosphate buffer. The brains were postfixed and kept frozen at -80°C until processed for Fos immunocytochemistry.

Fos immunocytochemistry

Coronal sections (30 µm) though the hypothalamus and the brainstem were cut on a sliding microtome and processed for standard Fos immunocytochemistry according to Shu *et al.* 1988 (see general materials and methods section). Fos immunocytochemistry was performed on brains of 4-7 rats per group for the following groups: prepartum, parturient, morphine only, morphine plus oxytocin pulses (1 mU oxytocin each 10 min) and morphine plus an infusion of oxytocin (0.1 mU oxytocin/min). For each rat Fos-immunoreactive nuclei were counted under the light microscope on every fifth section (on average 11-15 sections per area) through the hypothalamus (SON and PVN) and the brainstem (including the area postrema and the dorsal vagal complex (DVC), the latter consisting of the NTS and the dorsal motor nucleus of the vagus nerve), the locus coeruleus and the nucleus of the hypoglossal nerve. The counts were averaged for each area in each animal and then group means±s.e.m. were calculated.

In addition, sections through the hypothalamus of one parturient rat were processed for double-immunocytochemistry for Fos and oxytocin to examine whether during parturition c-*fos* expression was indeed detected in magnocellular oxytocin neurones.

Statistical analysis

The cumulative times between delivery of pups for experimental and control groups were compared using the Mann-Whitney U-test. The first birth interval, which is usually the longest, was excluded from statistical analysis because it is highly variable between animals.

The incidence of Fos-immunoreactive nuclei was compared between each experimental group and the control parturient or prepartum group for each respective area using the Mann-Whitney U-test. For $p<0.05$, based on a two-tailed hypothesis, a difference was considered significant.

Results

The progress of delivery in control rats and rats injected with morphine, morphine + naloxone or morphine+oxytocin

All animals that were allowed to deliver started to give birth between 12.00 h on day 21 and 14.37 h on day 22. Though a total of 85 rats had been implanted with a cannula, sixteen rats were excluded due to either a small litter size (less than 7 pups/litter, n=2), the unreliability of the implanted cannula (n=2), or the missed onset of delivery, assigned to the time of the birth of the first pup (n=12).

The remaining 69 rats had on average 11 ± 0.7 pups per litter. Delivery of the first pup was preceded by a period of 1-4 hours of straining and stretching movements of mother rats and licking of the vaginal orifice. Most rats started to bleed from the vagina shortly before the onset of delivery.

Once the first pup was expelled, mother rats that were given a saline injection engaged in normal maternal behaviour, including licking of pups, crouching and retrieving of pups, nest building activity and eating of the placentae, which were delivered in between pups. In control animals, the birth intervals shortened in the course of delivery and all but one of nine control animals delivered all pups within 90 min of the birth of the second pup (and thus before being killed). In contrast, most morphine-treated rats did deliver only 6 pups within 90 min of the first injection and hence we used the cumulative time between the birth of pups 2-6 for statistical comparison between the treatment groups.

Control animals (n=9) gave birth to pups 2-6 in 56±6.1 min (Fig. 1), while rats given a single injection of morphine took significantly longer to deliver pups 2-6 (82.5±3.6 min, n=10, U-test, $p<0.05$). Furthermore, morphine-treated rats showed for 30-60 min after the morphine injection a complete lack of maternal behaviour (they failed to lick and retrieve pups, they did not engage in nest building activity and they did not eat the placentae). These adverse effects of morphine were completely reversed by an injection of naloxone. Thus, morphine + naloxone treated rats delivered pups 2-6 in a time similar to controls (42±5.6 min, n=6) and displayed normal maternal behaviour throughout delivery.

When the single injection of morphine was followed by an infusion of oxytocin at a rate of 0.4 mU/min the progress of delivery was not different from controls and significantly shorter than in morphine only treated rats (time between pups 2-6: 56.6±7.5 min, n=10, U-test, $p<0.05$), while a lower dose oxytocin infusion (0.1 mU/min) following the morphine injection was not effective at restoring delivery (time between birth of pups 2-6: 68±6.3 min, n=10, not significantly different from morphine only treated rats).

In contrast, when the lower dose of oxytocin was administered in pulses, 1 mU each 10 min, following morphine, the time between pups 2-6 was significantly shorter than in morphine-treated rats (43.3±5.1 min, n=6, U-test, $p<0.05$) and than in rats given morphine plus the same dose of oxytocin as an infusion (see above, U-test, $p<0.05$) or as pulses each 5 min (see below, U-test, $p<0.05$), while there was no significant difference to control animals. Also, these animals showed normal maternal behaviour, including licking of pups, crouching and retrieving of pups and eating the placentae. Thus, oxytocin at a phsyiological dose of 1 mU each 10 min was able to fully reverse the effects of morphine on the progress of delivery and maternal behaviour.

Using a different pulse pattern, a pulse of oxytocin each 5 min, we found that similar to the continuous administration of oxytocin, only the higher dose (2 mU oxytocin each 5 min = equivalent to the higher dose oxytocin infusion of 0.4 mU/min) was effective at restoring the normal progress of delivery (time between pups 2-6: 53±9.8 min, n=5), while 0.5 mU oxytocin each 5 min could not significantly improve the prolonged delivery following an injection of morphine (time between pups 2-6: 78±8.4 min, n=6, U-test, $p<0.05$ compared to controls).

In summary, the prolongation of delivery and the impairment of normal maternal behaviour following a systemic injection of morphine to parturient rats can be restored either by naloxone, by a higher dose of oxytocin given as a continuous infusion or with oxytocin

given at physiological doses but in pulses each 10 min. Conversely, pulses given at 5 min intervals, even at a higher dose, were less effective.

Fos-immunoreactive nuclei throughout the brain of parturient rats (Table 1)

Control animals were killed before any signs of labour (prepartum) or 90 min after the birth of the second pup (parturient), *in situ* perfusion-fixed and the brains processed for Fos immunocytochemistry.

Table 1

Fos-immunoreactive (ir) nuclei in the brain of prepartum and parturient rats

area	prepartum	parturient
anterior olfactory nuclei	+	+++
lateral septal nuclei	(+)	++
bed of the stria terminalis (BST)	-	+
anterior hypothalamus	+	++
medial preoptic area (MPOA)	+	++
primary olfactory cortex	+	++
medial preoptic nucleus (MePO)	+	++
ventromedial hypothalamus (VMH)	+	++
dorsomedial hypothalamus (DMH)	+	++
arcuate nucleus	+	++
supramammillary nuclei	++	++
mammillary nuclei	(+)	++
central nucleus inferior colliculus	+	++
central grey	+	++
parabrachial nuclei	(+)	++
medial vestibular nucleus	+	++

- : no Fos-ir nuclei, (+) : few Fos-ir nuclei, + : some Fos-ir nuclei, ++ : many Fos-ir nuclei, +++ : very many Fos-ir nuclei

Fos-immunoreactive nuclei were widely distributed throughout the brain (Table 1, Fig. 2) in prepartum and parturient rats and though there seemed to be a slight increase in the number of Fos-immunoreactive nuclei in most areas in rats killed during (parturient) compared to rats killed before parturition (prepartum), the difference was not very pronounced.

Unlike in the areas listed above, we observed a marked increase in the incidence of Fos immunoreactive nuclei in the brainstem and the magnocellular hypothalamus of parturient rats compared to those killed prior to delivery. Therefore, we confined counting of Fos-immunoreactive nuclei to areas which are either likely to be involved in the secretion of oxytocin during delivery (including the SON and the PVN in the hypothalamus and putative afferent neurones in the dorsal vagal complex) and areas in which the increase in Fos expression seemed most marked (including the area postrema, the locus coeruleus and the nucleus of the hypoglossal nerve, that contains motor neurones of the tongue).

Fos-immunoreactive nuclei in the SON and PVN of the hypothalamus (Fig. 3 & 4)

In the SON, the number of Fos-immunoreactive nuclei was low before delivery and increased significantly during parturition (U-test, $p<0.05$). Animals that were given morphine after the birth of the second pup had significantly fewer Fos-immunoreactive nuclei per section SON compared to control parturient animals (U-test, $p<0.05$). When the progress of delivery was fully or partly restored with pulses or an infusion of the lower dose of oxytocin (1 mU oxytocin each 10 min or 0.1 mU oxytocin/min), Fos expression in the SON remained reduced and thus, rats given morphine and oxytocin pulses or an infusion of oxytocin showed Fos expression in the SON similar to that in morphine only treated rats and significantly less than the control parturient group (U-test, $p<0.05$). An injection of naloxone restored Fos expression in the SON to normal parturient levels (67 ± 11 Fos-immunoreactive nuclei/section SON vs 58 ± 13 in control parturient rats, data not shown).

In the PVN, the number of Fos-immunoreactive nuclei did not show a significant increase in parturient compared to prepartum rats, nor was it significantly affected by morphine and/or oxytocin treatment.

Double-immunocytochemistry for Fos and oxytocin on sections through the hypothalamus revealed that Fos-immunoreactive nuclei were detected in oxytocin-immunoreactive neurones in both the SON and PVN, though not all oxytocin neurones were also immunoreactive for Fos, particularly in the PVN (Fig. 5), and furthermore in the latter Fos-immunoreactive nuclei were not confined to the oxytocin cell population.

Beside the magnocellular hypothalamus, we observed Fos expression throughout the hypothalamus in both prepartum and parturient rats, but failed to find a marked difference in the number of Fos-immunoreactive nuclei in these areas between groups and therefore we assessed Fos expression only qualitatively in these areas (Table 1, Fig. 1).

Fos-immunoreactive nuclei in the brainstem (Fig. 4 & 6)

In the dorsal vagal complex, Fos expression was low before delivery and increased significantly during parturition (U-test, $p<0.05$). An injection of morphine had no significant effect on Fos expression in the dorsal vagal complex, but Fos expression remained at the elevated level seen in control parturient rats regardless of whether the progress of delivery was fully reinstated with pulses of oxytocin or continued to be slow in response to an infusion of oxytocin at a dose of 0.1 mU/min.

In the area postrema, Fos expression was apparent before delivery and did not show a significant further increase during parturition and, similar to the PVN, Fos immunoreactivity was not affected by morphine nor by subsequent administration of oxytocin.

Fos-immunoreactive nuclei in the locus coeruleus and the nucleus of the hypoglossal nerve (Fig. 7)

In two other areas in the hindbrain, we observed a increase in Fos expression in parturient compared to prepartum rats: the locus coeruleus and the nucleus of the nervus hypoglossus (Fig. 7). While in the former, Fos expression was not significantly reduced following morphine and thus not increased in response to the restoration of delivery with oxytocin, in the nucleus of the hypoglossal nerve Fos expression decreased significantly in response to morphine, but was not different from control parturient rats when pulses of oxytocin (1 mU each 10 min) were given that fully restored delivery. On the other hand, in rats given the lower dose oxytocin infusion (0.1 mU/min), which was not fully effective at restoring the normal progress of delivery, Fos expression in the nucleus of the hypoglossal nerve remained reduced and similar to that in morphine only treated rats.

Discussion

Here, we have shown that in rats, in which pituitary oxytocin secretion has been inhibited by an injection of morphine (Russell *et al.* 1989b), administration of oxytocin in pulses at a physiological dose is highly effective at restoring the disturbed progress of delivery. In contrast, to reinstate delivery with a continuous infusion of oxytocin a four fold higher dose of oxytocin was needed. Similarly, when pulses of oxytocin each 5 min rather than each 10 min were given, a higher dose of oxytocin was required to reinstate normal delivery. Thus, the greatest effectiveness of physiological doses of oxytocin, injected intermittently each 10 min, at restoring delivery that has been disrupted by morphine indicates that the pattern of oxytocin

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release is critical for the normal progress of delivery. This is in agreement with observations in humans, in whom pulsatile secretion of oxytocin has been reported to occur during labour and delivery (Fuchs *et al.* 1991). Also, in humans intermittent oxytocin injections are effective at inducing delivery at much lower doses than that required with an infusion of oxytocin (Dawood 1989), indicating the physiological significance of such pulsatile release at inducing and maintaining labour and delivery.

Since systemic administration of pulsatile oxytocin was highly effective at restoring delivery, these data indicate, as has been suggested from studies performed *in vitro* (Russell *et al.* 1989b) that uterine responsiveness to oxytocin has not been impaired by systemic morphine. Furthermore, such pulsatile release of oxytocin, which is associated with transient but high plasma concentrations of the peptide (Higuchi *et al.* 1986b), might be important for the generation of forceful uterine contractions and at the same time the prevention of a desensitisation of the uterus to the effects of oxytocin, that are observed after prolonged continuous administration of the peptide (Engstrom *et al.* 1988).

In the second part of our study, we have demonstrated that Fos, the protein product of the immediate early gene c-*fos*, which has been used widely to examine neuronal activation in response to a variety of non-physiological stimuli (Curran & Franza 1988, Hoffman et al 1993), and that in the magnocellular hypothalamus has been shown to be associated with hormone release from the posterior pituitary (Luckman *et al.* 1993b, Hamamura *et al.* 1991b, Verbalis *et al.* 1991b), is also induced in response to a physiological stimulus like spontaneous parturition. Though we have observed Fos expression in parturient rats in a number of areas in the brain, not all of these areas are directly involved in parturition. Conversely, Fos expression in some areas, including the olfactory nuclei and cortex, the anterior hypothalamus and the BST, in late pregnant rats might reflect the initiation of maternal behaviour at term (Insel 1992b). Since we were primarily interested in the control of pituitary oxytocin secretion at term, we examined Fos expression quantitatively only in areas known to be involved in oxytocin secretion, namely the magnocellular hypothalamus and the NTS.

Neurones in the anterior hypothalamus, including the preoptic area, and the BNST have been implicated in rats in the initiation of maternal behaviour (Numan 1988, Numan & Numan 1994), which depends on steroid priming with progesterone followed by oestrogen as seen in the last trimester of pregnancy. Furthermore, central, but not systemic administration of oxytocin can induce maternal behaviour in oestrogen-primed rats (Pedersen *et al.* 1982) and

similarly, vagino-cervical stimulation, that is associated with increased Fos expression in parvocellular hypothalamic neurones (Flanagan *et al.* 1993), can induce maternal behaviour in multiparous rats (Yeo & Keverne 1986). Thus, the observation that systemic oxytocin administration that restored delivery following the disruption by morphine, also reinstated maternal behaviour, that was impaired following an injection of morphine, might be due to a central release of oxytocin in response to the expulsion of pups and hence following activation of sensory pathways from the uterus to the forebrain. This is supported by data in mice, in which subcutaneous as well as intracerebroventricular administration of oxytocin can induce maternal behaviour (McCarthy 1990).

Fos was also observed before and during delivery in neurones of the OVLT, which are part of the osmoreceptor complex (Honda *et al.* 1990, Leng *et al.* 1988b), and in which Fos has been shown in response to osmotic stimulation (Hamamura *et al.* 1991a). Unlike the release of oxytocin in response to osmotic stimulation that is significantly reduced by ablation of the region anterior and ventral to the third ventricle (AV3V), including the OVLT (Blackburn & Leng 1990a), such lesions do not greatly disturb the progress of parturition (Russell *et al.* 1989a), supporting the assumption that these neurones are not part of the afferent pathway mediating the reflex release of oxytocin during delivery. Conversely, activation of a number of neurones throughout the hindbrain and the mesencephalon in late pregnant and parturient rats indicates, as one might expect, that labour and delivery stimulates a variety of sensory and motor afferents. Thus, neurones in areas including the central grey might be activated in response to the pain associated with delivery (Morgan *et al.* 1991), while activation of neurones in the olfactory bulbs and the pyriform cortex could reflect olfactory stimulation by the newborn (Argiolas & Gessa 1991).

In the PVN, Fos expression was apparent in rats before delivery of any pups, and though the distribution of Fos immunoreactive nuclei in the PVN before delivery was most prominent over the parvocellular parts, we cannot exclude the possibility that some magnocellular PVN neurones also expressed Fos prior to parturition. In contrast, Fos expression in the SON was low prior to delivery but significantly increased in rats killed during parturition. The primarily dorsal distribution of Fos-immunoreactive nuclei in the SON in parturient rats suggests the activation of oxytocin neurones and sections taken from one animal for double immunocytochemistry confirmed the presence of Fos-immunoreactive nuclei in supraoptic oxytocin neurones, though some vasopressin neurones also seemed to be activated. Since morphine inhibits oxytocin secretion (Russell *et al.* 1989b) and the progress of parturition,

while exogenous oxytocin was highly effective at restoring parturition, the lack of Fos expression in the SON of morphine-treated rats seems to reflect reduced secretion. The reinstatement of delivery with oxytocin was not associated with normal Fos expression in the SON, while in the dorsal vagal complex Fos expression was not affected by the disruption nor the restoration of delivery, indicating that morphine acts rostrally of the dorsal vagal complex and possibly at, or close to, supraoptic neurones. Indeed, it has been suggested that opioids could regulate supraoptic oxytocin neurones via a presynaptic site of action on catecholaminergic afferents (Bicknell *et al.* 1993, Onaka *et al.* 1995a).

Thus, the increased incidence of Fos-immunoreactive nuclei in the magnocellular hypothalamus and in putative afferent neurones in the dorsal vagal complex of parturient rats reflects the increased plasma concentration of oxytocin during delivery of pups (Higuchi *et al.* 1986b, Douglas *et al.* 1993a). Since putative afferent neurones involved in mediating the release of oxytocin during delivery were activated only at the time of parturition, this is consistent with the hypothesis that delivery of pups initiates the positive feedback stimulation of pituitary oxytocin release via neurones in the dorsal vagal complex (Ortega-Villalobos *et al.* 1990). Although this study does not provide conclusive evidence that neurones in the dorsal vagal complex that are activated during delivery mediate the reflex release of oxytocin, the importance of the afferent projection from neurones in the NTS to magnocellular hypothalamic nuclei for neurohypophysial hormone release is well established (Day 1989, Raby & Renaud 1989a, Sawchenko & Swanson 1982b, Onaka *et al.* 1995b, Rinaman *et al.* 1994).

Unlike the SON, that contains predominantly neurosecretory neurones, the PVN is a more heterogeneous nucleus, containing both magnocellular and parvocellular neurones (Sawchenko & Swanson 1982a, Swanson & Sawchenko 1983), the latter of which project to the dorsal vagal complex and thus might form a neural circuit between the hypothalamus and the brainstem, which could account for the maintenance of Fos expression in the dorsal vagal complex in rats in which vagino-cervical stimulation was prevented. In addition, activation of this circuit might have comprised an excitatory pathway from the locus coeruleus, and in particular from noradrenergic cells within this nucleus, which project to the parvocellular neurones in the PVN (Sawchenko & Swanson 1982a) and in which Fos expression was greater in parturient compared to prepartum rats and not significantly reduced following an injection of morphine. Alternatively, morphine might not have completely prevented pituitary oxytocin secretion and/or uterine activity might have been maintained, though possibly at

reduced force, by factors other than pituitary oxytocin, e.g. prostaglandins (Chan 1987, Chan *et al.* 1988), and such mechanisms might have contributed to the maintenance of Fos expression in the NTS and the parvocellular PVN. Furthermore, the inhibitory effect of an intravenous injection of morphine to parturient rats on oxytocin release lasts about 50 min and thus can explain the gradual reinstatement of parturition after the initial complete abolition of further births.

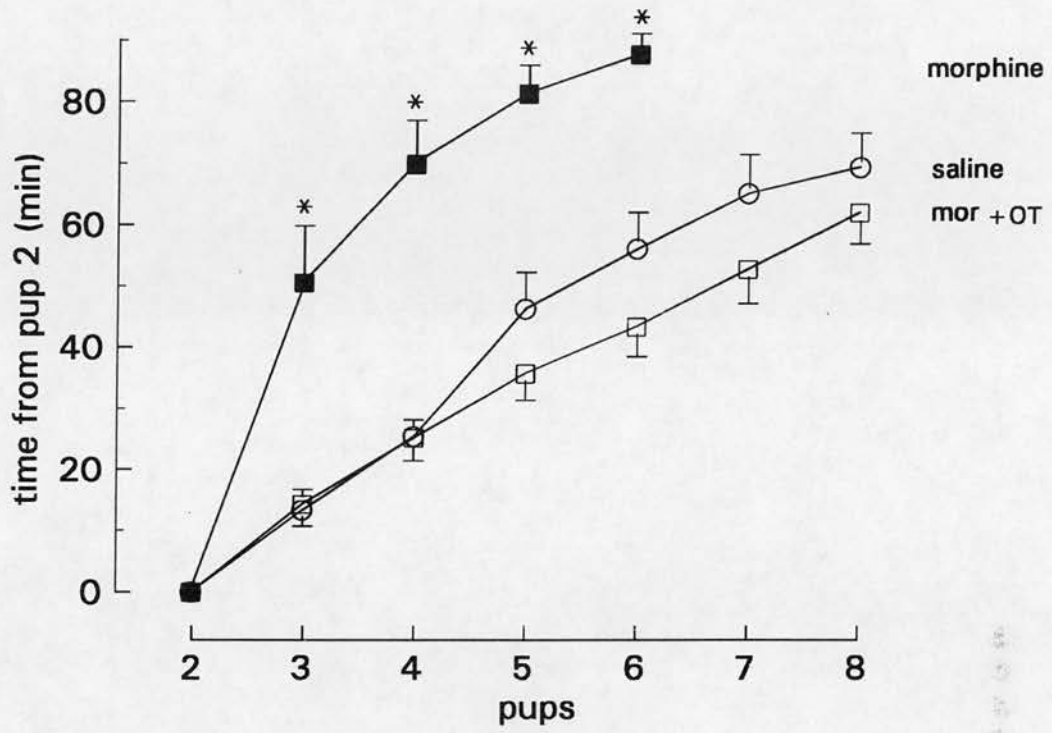
In contrast, in the nucleus of the nervus hypoglossus, where afferent fibers from the tongue terminate, Fos expression increased during delivery, possibly reflecting the licking behaviour of the mother, which is part of normal maternal behaviour during parturition. Thus, disruption of maternal behaviour by systemic morphine resulted in the significant reduction of Fos expression compared to control parturient animals in this nucleus, but was restored when delivery and maternal behaviour were reinstated with pulses of oxytocin.

In summary, it appears that pituitary oxytocin is important for the normal progress of delivery and that the pattern of this release is critical for the efficient biological action of oxytocin in sustaining the progress of delivery. Furthermore, we have for the first time shown that a physiological stimulus for pituitary oxytocin release, namely delivery of pups, is associated with an increased expression of the immediately early gene product Fos in magnocellular neurones and putative afferent neurones in the brainstem. Besides these areas, which show an increase in Fos expression at the time of parturition, we have described a number of nuclei throughout the brain in which Fos expression is observed before and during delivery of pups. This results indicates activation of a number of sensory and motor neurones during parturition, that are not necessarily directly involved in mediating oxytocin secretion.

Fig. 1.1. Effects of morphine on the progress of delivery

Top: Cumulative time (min) between pups 2-8 in rats injected with saline, morphine or morphine and oxytocin (values expressed as means±s.e.m.)
Rats were given either an injection of isotonic saline (open circles, n=9), or morphine (1 mg/kg body weight) followed by isotonic saline (filled squares, n=10) or morphine followed by oxytocin pulses (1 mU each 10 min, open squares, n=9).
In morphine-treated rats, which delivered on average only 6 pups prior to killing (90 min after the birth of the second pup) delivery was significantly slower than in saline treated rats.
*$p<0.05$, U-test, compared to the saline-treated group

Bottom: Comparison of the time between delivery of pups 2-6 (means±s.e.m.) in different treatment groups
Rats were given isotonic saline (open bar, n=9) or an injection of morphine (1 mg/kg body weight) followed by either an infusion of saline (filled bar, n=10), or a single injection of naloxone (5 mg/kg body weight iv, shaded bar, n=6), an infusion of 0.1 mU oxytocin/min or pulses of 0.5 mU oxytocin each 5 min (narrow cross-hatched bars, n=10, 6), or pulses of 1 mU oxytocin each 10 min (wide cross-hatched bar, n=6), or an infusion of 4 mU oxytocin/min or pulses of 2 mU oxytocin each 5 min (right hatched bars, n=9, 5).
In rats given only morphine and rats given morphine and pulses of 0.5 mU oxytocin each 5 min, the time between pups 2-6 was significantly longer than in control rats (†$p<0.05$, U-test). In contrast, rats given morphine followed either by an injection of naloxone, or low dose pulses of oxytocin (1 mU oxytocin each 10 min) or the higher dose of oxytocin (0.4 mU oxytocin/min as an infusion or as pulses) gave birth to four more pups in significantly less time than morphine-treated rats (*$p<0.05$, U-test).


time from pup 2 (min)
0
20
40
60
80
2
3
4
5
6
7
8
pups
morphine
saline
mor +OT
*

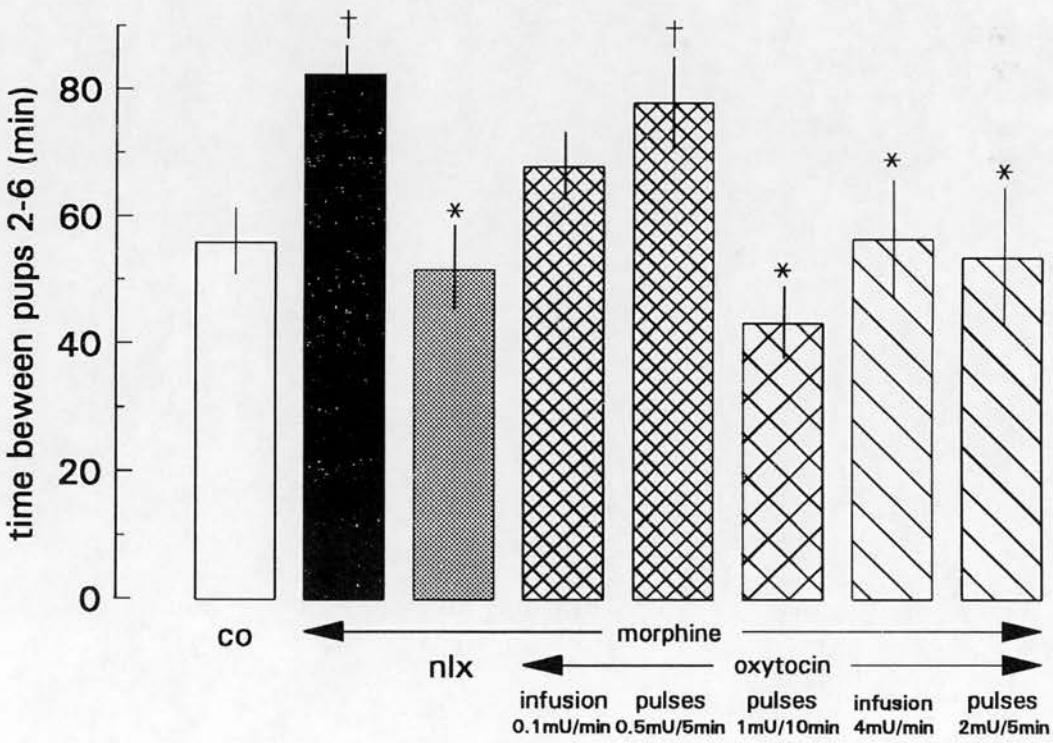
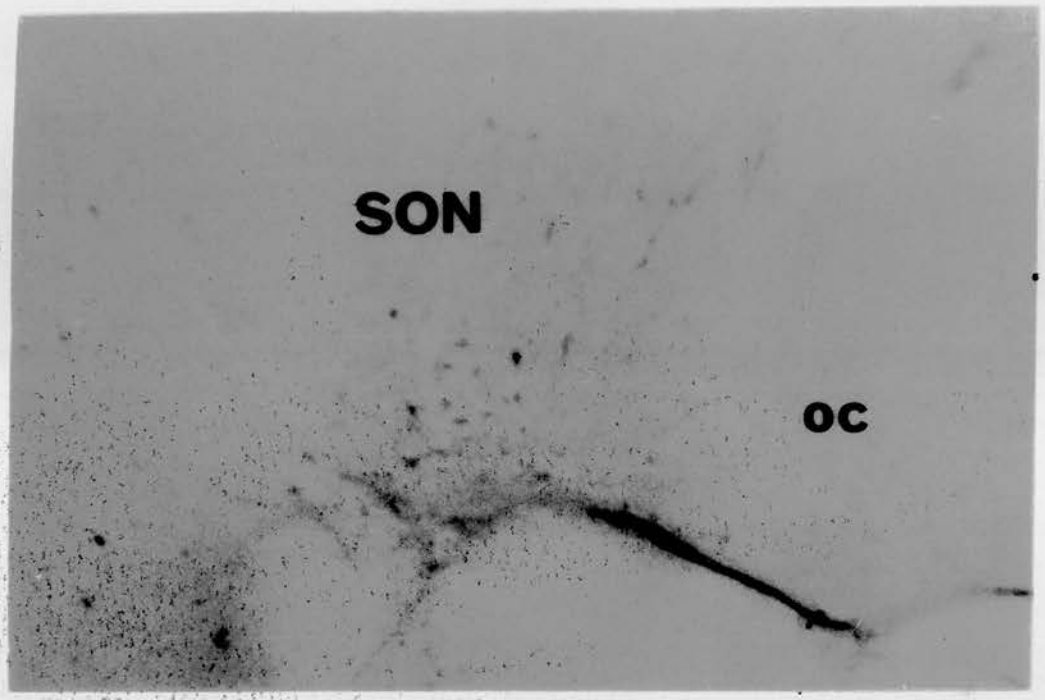
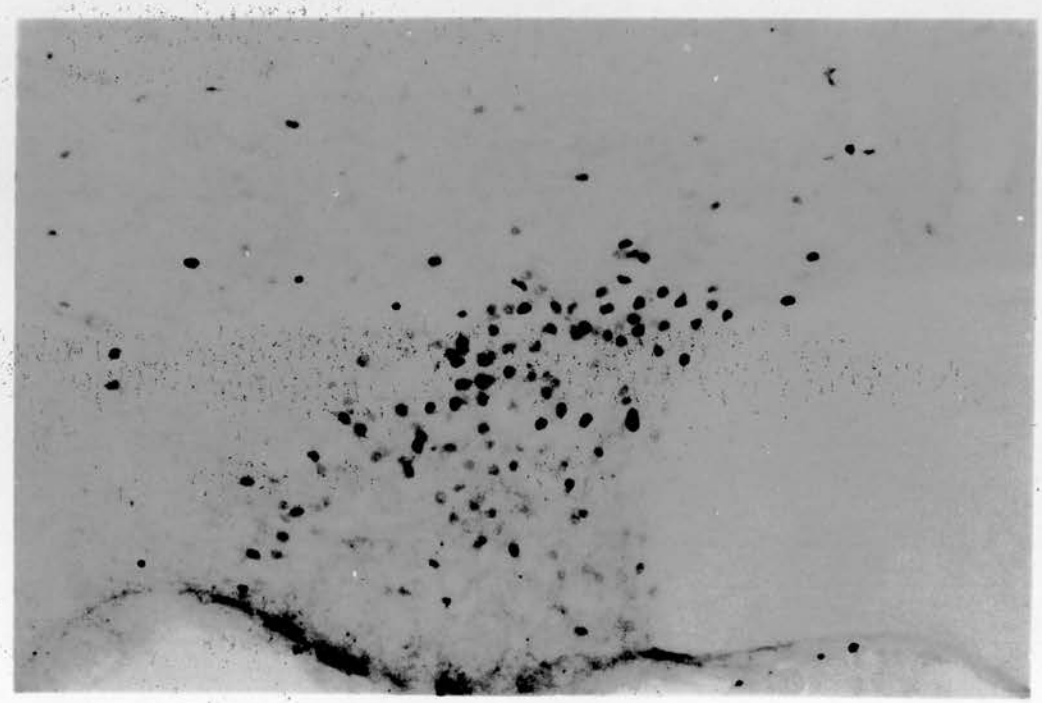

time beween pups 2-6 (min)
0
20
40
60
80
†
*
co
nlx
morphine
oxytocin
infusion 0.1mU/min
pulses 0.5mU/5min
pulses 1mU/10min
infusion 4mU/min
pulses 2mU/5min

Fig. 1.2. Fos-immunoreactive nuclei in the SON before and during parturition
Control rats were killed by an overdose of sodium pentobarbitone (50 mg/kg body weight iv) and *in situ* perfusion fixed with 4% paraformaldehyde either in the morning of the day of expected term (day 21 of pregnancy) but before delivery of any pups (prepartum, **Top**) or 90 min after the birth of the second pup (parturient, **Bottom**). The brains were cut into 30 μm coronal sections through the SON, at the level of the optic chiasm, and processed for standard Fos immunocytochemistry (values are means±s.e.m.).
Prepartum rats showed significantly fewer Fos-immunoreactive nuclei in the SON than parturient rats.
oc = optic chiasm, scale bar 100 μm

prepartum



parturient



100 μm

Fig. 1.3. Fos-immunoreactive nuclei in the NTS before and during parturition
Control rats were killed as described above and the hindbrains cut through the rostro-caudal extent of the NTS (from the decussatio of the pyramids to the rostral end of the area postrema) into 30 μm coronal sections and processed for standard Fos immunocytochemistry.
As in the SON, prepartum rats (**Left**) showed significantly fewer Fos-immunoreactive nuclei in the NTS than parturient rats (**Right**). Scale bar 100 μm

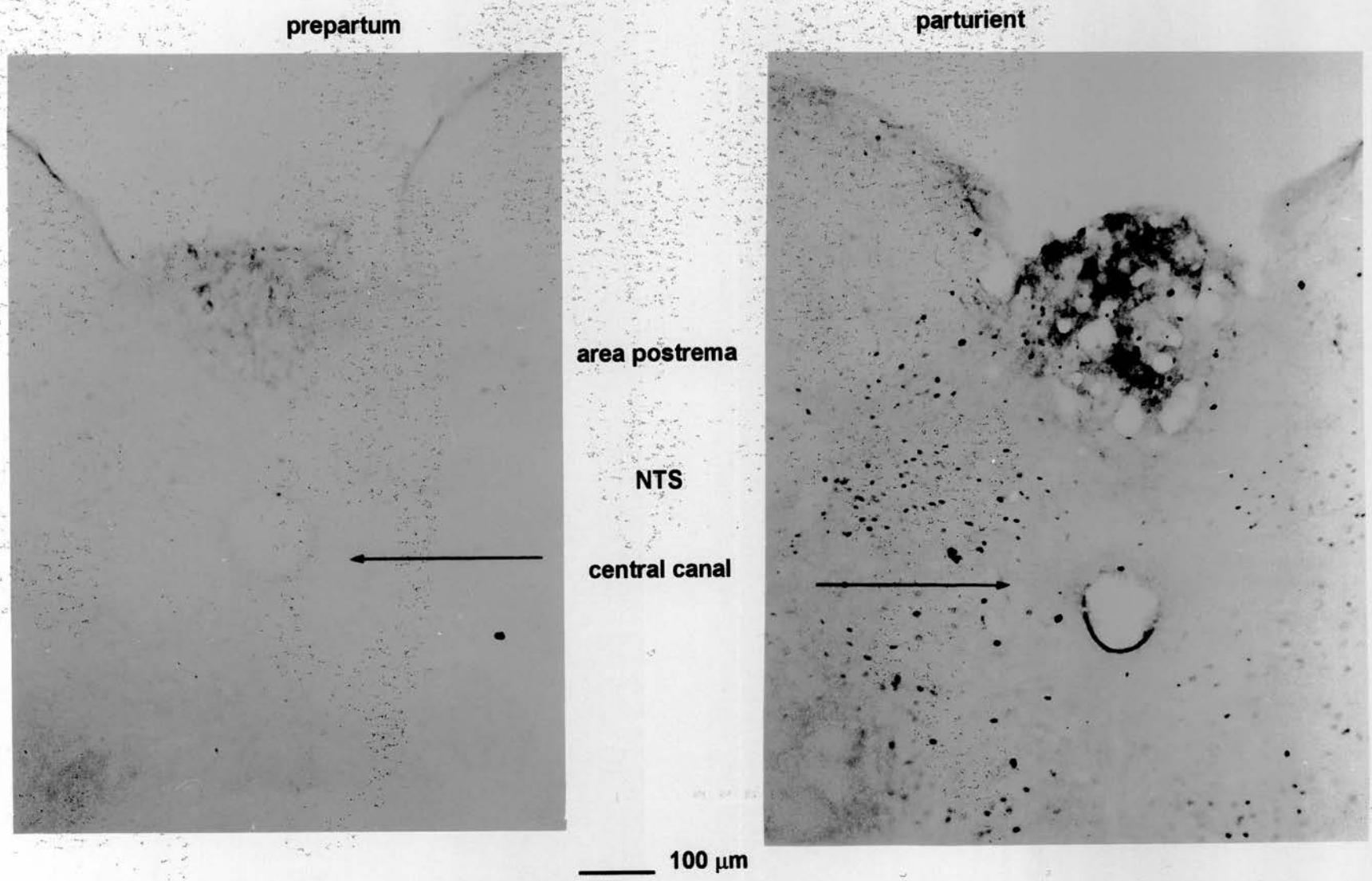

prepartum
parturient
area postrema
NTS
central canal
100 μm

Fig. 1.4. Effects of morphine and morphine plus oxytocin pulses on Fos expression in the hypothalamus and the brainstem during delivery

Rats were given after the birth of the second pup and for 90 min either isotonic saline (control parturient, open bars), an injection of morphine followed by an infusion of saline (morphine, filled bars), morphine plus pulses of 1 mU oxytocin each 10 min (morphine+OT pulses, wide cross-hatched bars) or morphine plus an infusion of oxytocin (1 mU oxytocin/min, mor+OT infusion, narrow cross-hatched bars). Rats were killed 90 min after the birth of the second pup and the brains processed for standard Fos immunocytochemistry (n=4-7 per group). The numbers of Fos-immunoreactive nuclei/section were counted in the hypothalamus (the SON and PVN) and the brainstem (the dorsal vagal complex (DVC) and the area postrema).

In the SON and DVC, but not the PVN and area postrema, the incidence of Fos-immunoreactive nuclei/section was significantly higher in control parturient than prepartum rats (†$p<0.05$, U-test, means$\pm$s.e.m. number of Fos-immunoreactive nuclei in rats killed prepartum are indicated as shaded area within each chart). Morphine and morphine plus oxytocin treated (regardless whether oxytocin was given as an infusion or in pulses) parturient rats showed significantly fewer Fos-immunoreactive in the SON than control parturient rats (*$p<0.05$, U-test), while in the PVN, the DVC and the area postrema there was no significant difference between the parturient groups.

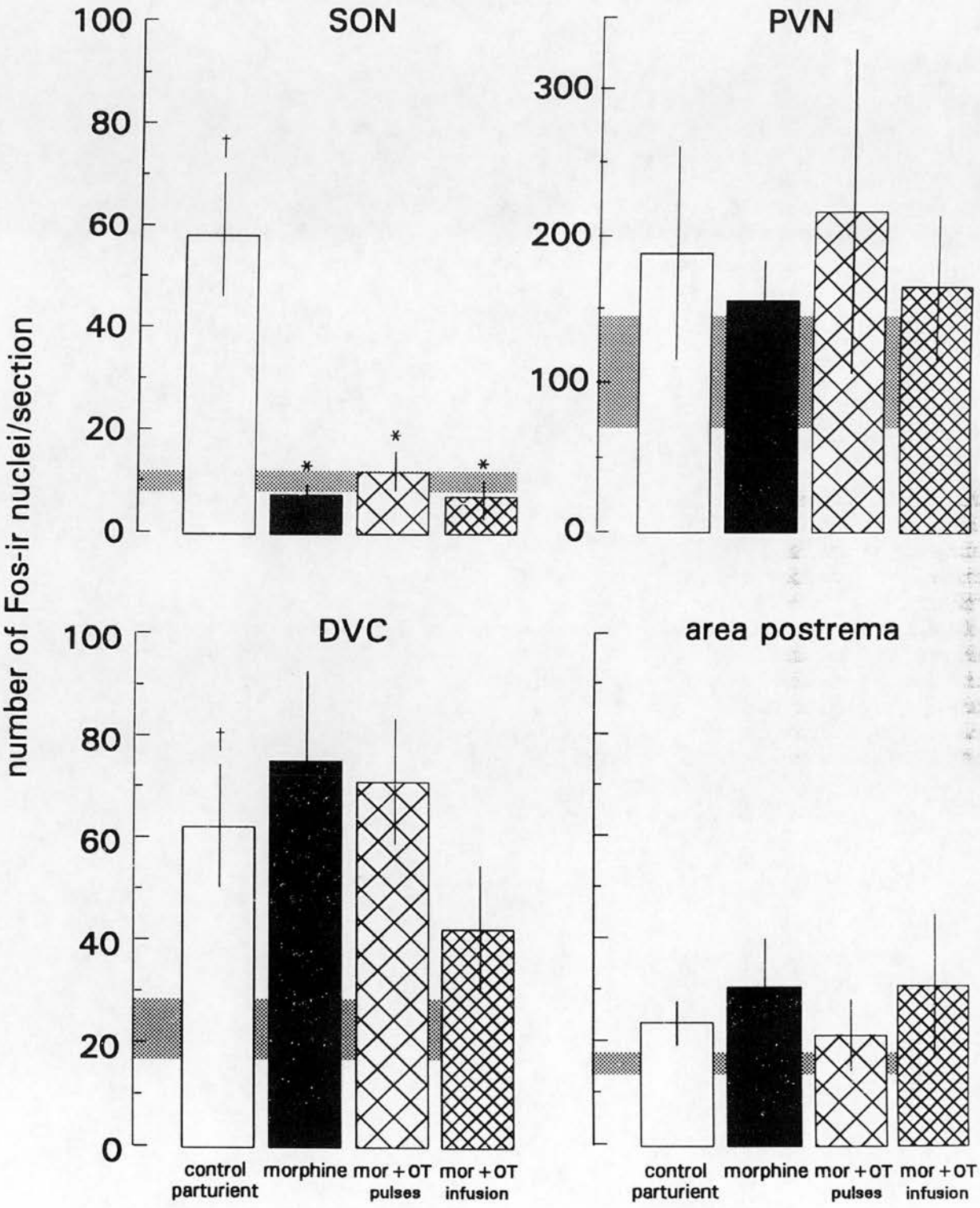

SON
PVN
DVC
area postrema
number of Fos-ir nuclei/section
100
80
60
40
20
0
300
200
100
control parturient
morphine
mor + OT pulses
mor + OT infusion

Fig. 1.5. Effects of morphine and morphine plus oxytocin pulses on Fos expression in the locus coeruleus and the nucleus of the hypoglossal nerve

Rats were treated as described in Fig. 1.4., killed 90 min after the birth of the second pup and the brains processed for standard Fos immunocytochemistry (n=4-7 per group). Fos-immunoreactive nuclei/section were counted in the locus coeruleus and the nucleus of the hypoglossal nerve in the medulla oblongata.

In both areas, the incidence of Fos-immunoreactive nuclei/section was significantly higher in control parturient (open bars) than prepartum rats (†$p<0.05$, U-test, means$\pm$s.e.m. number of Fos-immunoreactive nuclei in rats killed prepartum are indicated as shaded area within each chart).

In the locus coeruleus of morphine (filled bar) and morphine plus oxytocin treated rats (cross-hatched bar) the number of Fos-immunoreactive nuclei per section was not significantly different from normal parturient rats, while in the nucleus of the hypoglossal nerve, morphine-treated rats (filled bar) showed significantly fewer Fos-immunoreactive nuclei compared to control parturient rats (*$p<0.05$, U-test). In rats in which delivery was fully restored with pulses of oxytocin (wide cross-hatched bar), Fos expression in the nucleus of the hypoglossal nerve was not significantly different from that in control parturient rats, but higher than in rats given morphine plus an infusion of oxytocin (narrow cross-hatched bar).

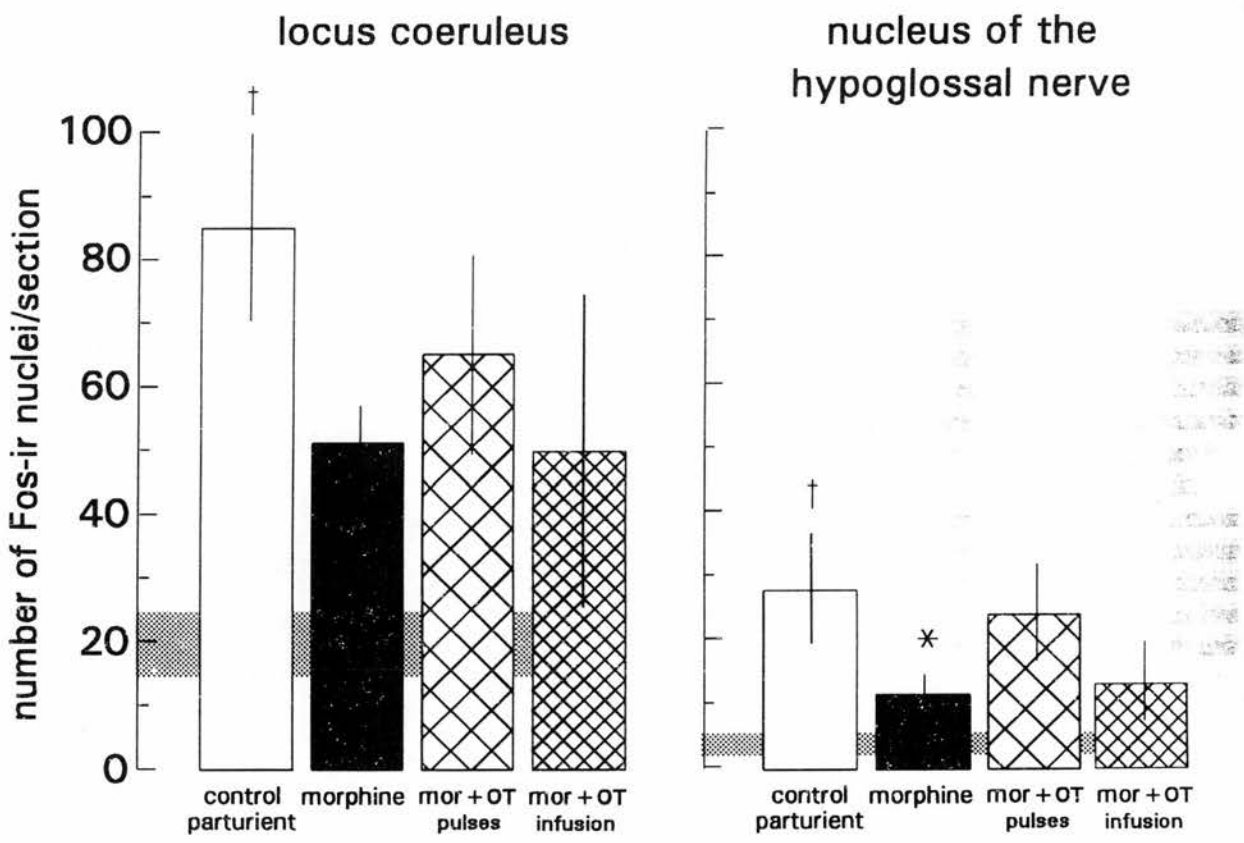
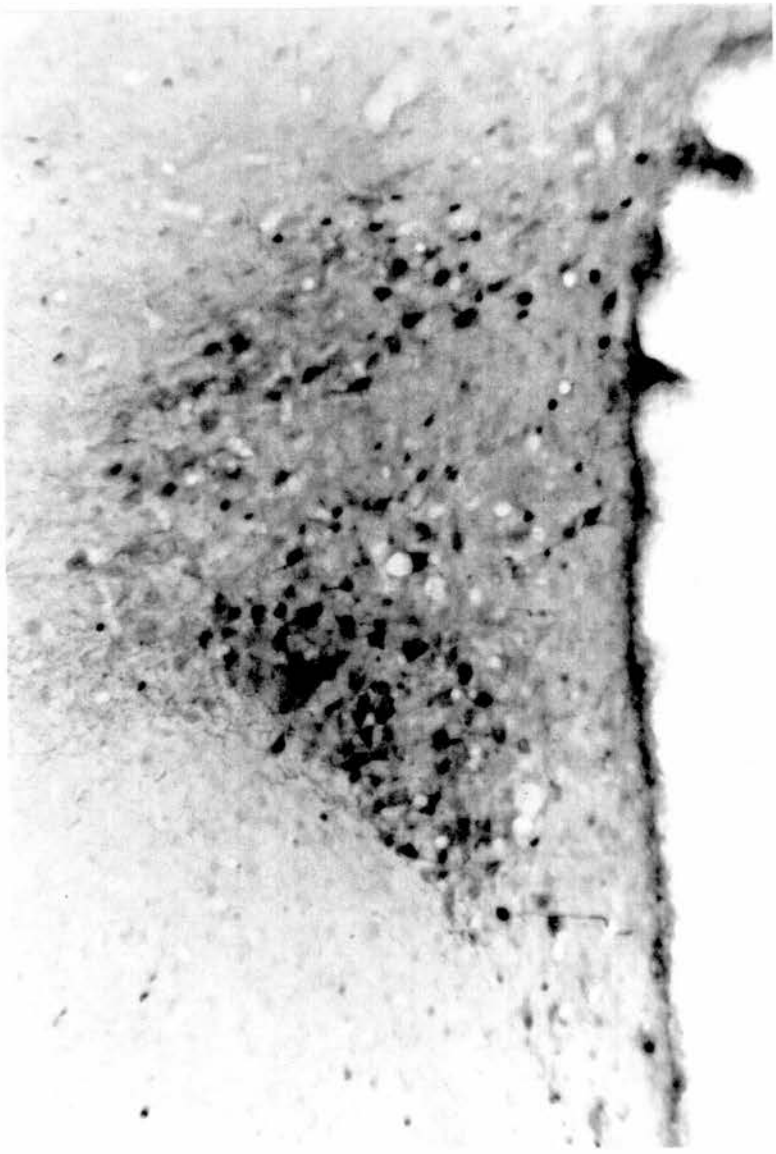

locus coeruleus
nucleus of the hypoglossal nerve
number of Fos-ir nuclei/section
100
80
60
40
20
0
†
*
control parturient
morphine
mor + OT pulses
mor + OT infusion

Fig. 1.6. Fos and oxytocin immunoreactivity in the PVN of a parturient rat
Coronal sections (30 μm) through the PVN of control parturient rats were processed for double immunocytochemistry for Fos and oxytocin.
Fos-immunoreactive nuclei (dark purple nuclear staining) were detected in PVN neurones stained for oxytocin (light brown cytoplasmic staining), however, Fos expression was also observed in other neuronal subpopulations. Also, not all oxytocin neurones in the PVN expressed Fos during parturition.
Third ventricle on the right side of the photograph. Scale bar 100 μm.

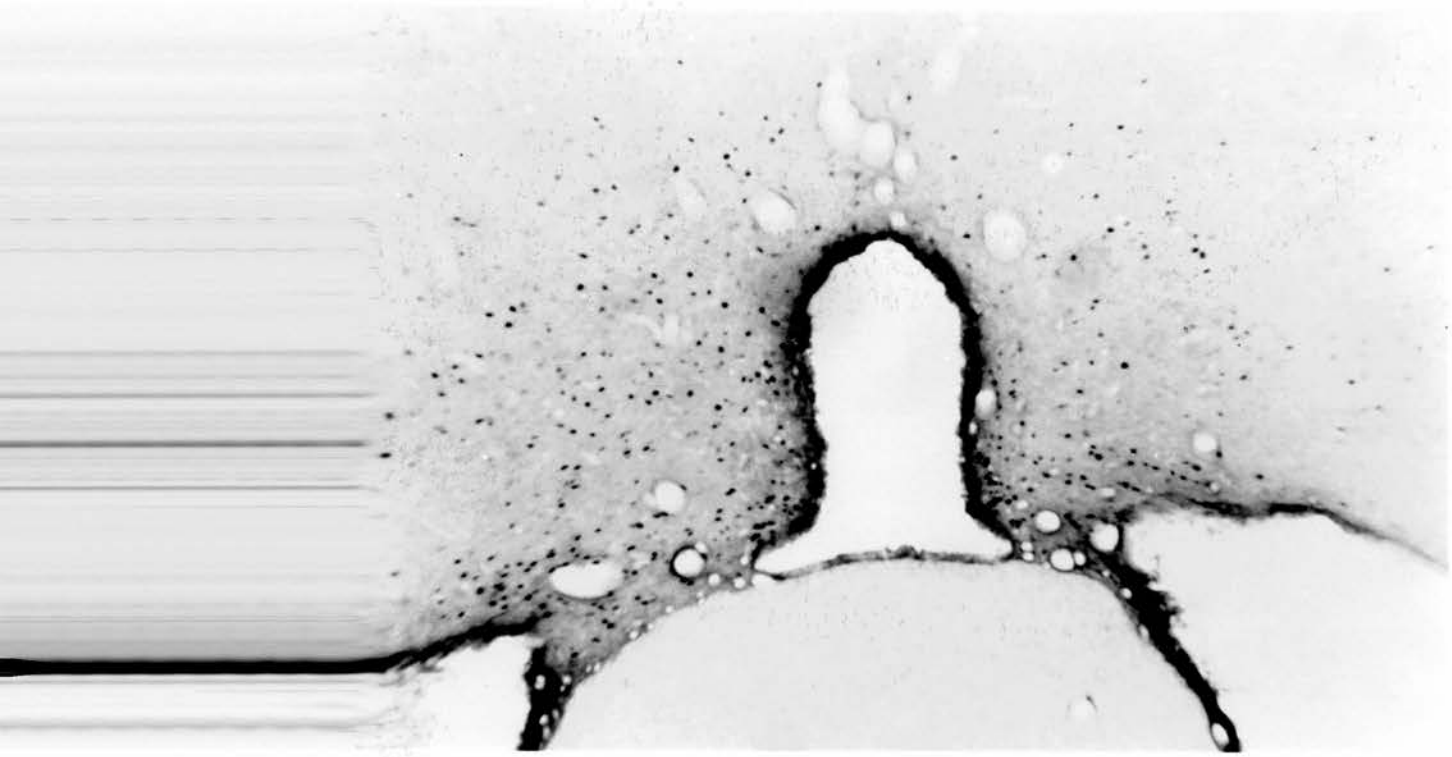
Fos- and oxytocin-immunoreactive neurones in the PVN of a parturient rat



100 μm

Fig. 1.7. Fos expression in the anterior hypothalamus of a parturient rat
Coronal section (30 μm) through the anterior hypothalamus, processed for standard Fos immunocytochemistry.
Fos expression was abundant in the anterior hypothalamus, at the rostral end of the optic chiasm, in parturient rats.
Scale bar 100 μm.

munoreactive nuclei in the anterior hypothalamus of a parturient rat



100 μm

Chapter 2

The involvement of cholecystokinin (CCK) receptor types in pathways controlling oxytocin secretion

As shown in the previous chapter, Fos expression is induced during parturition throughout the brain and allows the investigation of the pathways involved in the secretion of oxytocin during delivery of pups and thus in response to a physiological stimulus. In previous reports, intravenous administration of CCK has been shown to excite selectively magnocellular oxytocin neurones (Hamamura *et al.* 1991b, Luckman *et al.* 1993b), while inhibiting or having no effect on vasopressin neurones. Since this excitation of oxytocin neurones and the resulting increase in hormone release following an injection of CCK is associated with an increased expression of the immediate early gene c-*fos* in magnocellular neurones (Hamamura *et al.* 1991b, Verbalis *et al.* 1991b) and putative afferent neurones in the dorsal vagal complex of the brainstem (including the NTS and the dorsal motor nucleus of the vagus nerve, Luckman 1992), it is possible that CCK activates the same pathways mediating oxytocin secretion as parturition.

Using immunocytochemical detection of Fos combined with a second immunocytochemical procedure to identify hypothalamic neurones activated by CCK in terms of transmitter produced, it has been shown that in the SON Fos-immunoreactive nuclei are found exclusively in magnocellular oxytocin neurones, while in the PVN at least three neuronal populations express Fos: neurones producing corticotropin-releasing factor, and magnocellular and parvocellular oxytocin neurones (Verbalis *et al.* 1991b). Since parvocellular oxytocin neurones project to the dorsal vagal complex in the caudal medulla oblongata (Sawchenko & Swanson *et al.* 1982a), which in turn provides a direct afferent input to the hypothalamus, that is activated upon systemic administration of CCK (Luckman 1992, Onaka *et al.* 1995, Rinaman *et al.* 1994, Verbalis *et al.* 1991a), these neurones may form a neural circuit between the PVN and the brainstem as pointed out in the previous chapter. In the periphery, the activation of pituitary oxytocin release by systemic CCK is mediated by the vagus nerve (Fraser & Davison 1992, Van Dijk *et al.* 1984), including the gastric branches, that project to the dorsal vagal complex in the caudal medulla.

CCK and CCK receptors are present in neurones throughout this proposed pathway and it has therefore been suggested that CCK activates a chain of neurones, which may utilise CCK as a central neurotransmitter (Honda *et al.* 1993, Kawai *et al.* 1988, Meister *et al.* 1994, Miller *et al.* 1993b). There are two distinct CCK receptor types, the CCK_A and CCK_B receptor, which are both found in the central nervous system (Carlberg *et al.* 1992, Honda *et al.* 1993, Van Dijk *et al.* 1984), while in the periphery, the CCK_A receptor type seems to predominate (Miller *et al.* 1993b). In the CNS, the CCK_A receptor is primarily detected in the area postrema and the medial NTS of the brainstem, while the SON and PVN themselves possess only the CCK_B type receptor (Carlberg *et al.* 1992, Honda *et al.* 1993, Meister *et al.* 1994, Van Dijk *et al.* 1984). Since systemically administered CCK is unlikely to directly access CCK receptors in the hypothalamus, that is located within the blood-brain barrier (Ermisch *et al.* 1993), circulating CCK is likely to mediate oxytocin secretion by acting at one of the circumventricular sites, including the area postrema, which lack an efficient blood-brain barrier, or at the peripheral endings of the afferent vagus nerve (Miller *et al.* 1993b, Verbalis *et al.* 1986). Thus, it has been possible to show the induction of Fos immunoreactivity in the brainstem, including the area postrema and the dorsal vagal complex, in response to a systemic injection of CCK (Luckman 1992).

In the present study, we used two potent non-peptide CCK receptor antagonists, one selective for the CCK_A receptor (MK-329) and the other for the CCK_B receptor (L-365,260),

to establish which receptor type was involved in the neuroendocrine activation following systemic administration of CCK. Since the work presented here was my contribution to a larger study, involving in addition electrophysiological evidence, as well as evidence from plasma oxytocin concentrations and from *in situ* hybridisation (Luckman *et al.* 1993b), I will refer, whenever necessary, to the findings described in the complete study.

Recently, it has been shown that suckling-induced secretion of oxytocin is preceded by an increase in plasma CCK (Linden *et al.* 1990) and that both the increase in plasma CCK and oxytocin are impaired by abdominal vagotomy and by lesions in the lateral midbrain (Linden *et al.* 1990). These data suggest the involvement of the vagus nerve and possibly a facilitatory role of circulating CCK for the stimulation of suckling-induced pituitary oxytocin secretion. Furthermore, systemic CCK has been implicated in the initiation of maternal behaviour in ovariectomised, oestrogen-treated rats (Linden *et al.* 1989) and in the maintenance of maternal behaviour in lactating rats (Mann *et al.* 1994). Since the vagus nerve carries afferents from the uterus, which terminate in the dorsal vagal complex in the caudal medulla (Ortega-Villalobos *et al.* 1990), a region that is activated during parturition (see chapters one & three) and projects to the hypothalamus, including magnocellular neurones (Onaka *et al.* 1995, Raby & Renaud 1989a, 1989b, Sawchenko & Swanson 1982a), the vagus nerve could represent a common ascending pathway for the release oxytocin in response to a variety of stimuli. Thus, we examined in a second experiment whether the CCK receptor type involved in mediating the neuroendocrine response to systemic CCK, can, when administered during parturition, be effective at preventing oxytocin secretion (and Fos expression in the SON) and hence disrupt the progress of delivery and/or maternal behaviour.

Materials and Methods

Animals

We used rats from the Babraham colony, either male rats, which were anaesthetised with tribromoethanol (10 ml/kg intraperitoneally) and implanted with a jugular vein cannula two days prior to the experiment (experiment 1) or date mated pregnant Wistar rats, which were implanted with a jugular vein cannula under brief halothane anaesthesia on day 19 of pregnancy (experiment 2).

Drugs

CCK-8 (CCK26-33, sulphated; Sigma, UK) was dissolved in isotonic saline (1 mg/ml) and injected intravenously at a concentration of 20 µg/kg body weight, the injected volume being 100 µl/100 g body weight.

Selective non-peptide antagonists against the CCK_A and CCK_B receptor, MK-329 (3S(-)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-car-boxy-amide, also known as L-364,718 or devazepide) and L-365,260 (3R(±)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-N'-(3methlphenyl)urea) (both Merck Sharp & Dohme, UK), respectively, were dissolved in a solution containing 20% absolute ethanol and 80% propan-1,2-diol. Both antagonists were given intravenously to male rats at a concentration of 100 µg/kg. Pregnant rats were injected immediately after the birth of the first pup with the CCK_A receptor antagonist at a concentration of 1 mg/kg. The injected volume being in both experiments 10 µl/100 g body weight.

Experimental design

The effect of specific CCK receptor antagonists on Fos expression in the brain following intravenous administration of CCK (experiment 1)

On the day of the experiment the cannulae were connected to polyethylene tubings (of 0.1 ml volume). Twenty-two male rats were divided into four groups of five to six rats each:

- two groups were given an injection of vehicle followed after 20 min by either an injection of saline or CCK (20 µg/kg body weight, both n=5),
- one group was given the CCK_A receptor antagonist (100 µg/kg body weight) followed after 20 min by an injection of CCK (n=5)
- one group was given the CCK_B receptor antagonist (100 µg/kg body weight) followed after 20 min by an injection of CCK (n=5).

Rats were deeply anaesthetised with sodium pentobarbitone (50 mg/kg body weight iv) 90 min after the second injection and *in situ* perfusion-fixed with 4% paraformaldehyde in phosphate buffer.

The effect of a CCK_A receptor antagonist on the progress of parturition and Fos expression (experiment 2)

On the day of the experiment (= the day of expected parturition, day 21 of pregnancy) the cannulae were connected to polyethylene tubings as described above and rats were observed for delivery. Immediately after the birth of the first pup animals were given an intravenous injection of either vehicle (10 µl/100 g body weight, n=4) or the CCK_A receptor antagonist (1 mg/kg, n=4). 90 min after delivery of the second pup (by which time delivery is normally completed) rats were deeply anaesthetised and perfusion-fixed *in situ* as described above.

In both experiments the brains were removed, postfixed and stored at -80°C until cut on a microtome into 30 µm coronal sections through the hypothalamus and/or the brainstem and processed for standard Fos immunocytochemistry (see general materials and methods section).

Statistical analysis

Comparisons were made using non-parametric tests. In the first experiment the Mann-Whitney U-test was used to compare the number of Fos-immunoreactive nuclei after saline and CCK treatment, and the Kruskal-Wallis test to compare Fos immunoreactivity in rats treated with either CCK receptor antagonist to those treated with CCK only. In the second experiment the Mann-Whitney U-test was used to compare the cumulative time between delivery of pups 1-9 and to compare the number of Fos-immunoreactive nuclei in the SON of rats treated with the CCK_A receptor antagonist or vehicle.

Results

Fos expression in the hypothalamus and the brainstem in response to CCK, and CCK plus the CCK_A or CCK_B receptor antagonist

Systemic injection of vehicle followed by CCK (20 µg/kg) caused a significant increase in the number of Fos-immunoreactive nuclear profiles in the dorsal vagal complex and the area postrema of the brainstem and the SON and PVN of the hypothalamus compared to vehicle and saline-treated rats (U-test, $p<0.05$, Fig. 1, 2 & 3). In each of these areas the increase in Fos immunoreactivity in animals pretreated with the CCK_A receptor antagonist MK-329 (100 µg/kg) was significantly reduced compared to CCK-treated rats, while the CCK_B receptor antagonist L365,260 (100 µg/kg) significantly reduced the number of Fos-immunoreactive nuclei only in the SON (Kruskal-Wallis test, $p<0.05$). Fos expression in the

PVN in rats pretreated with L365,260 was reduced compared to CCK-treated rats, but this effect did not reach significance, while in the dorsal vagal complex and the area postrema L365,260 had no affect on Fos immunoreactivity.

The effect of an injection of the CCK_A receptor antagonist on the progress of delivery

In pregnant rats, the progress of delivery was not affected by an injection of the CCK_A receptor antagonist (1 mg/kg) after the birth of the first pup (cumulative time between pups 2-9: 57.5±6.8 min (n=4) vs 59.3±1.7 min (n=4) in vehicle-treated rats, Fig. 4 top). The first interbirth interval, that is normally excluded from statistical comparison because of its large intragroup variability, was also not significantly different between the two treatment groups (Fig. 4). Both groups of rats showed a similar number of pups per litter (11.3±1 pups/litter for controls and 11±0.4 pups/litter for CCK_A receptor antagonist treated rats, range 9-13 pups/litter) and in both groups mother rats displayed, once delivery started, normal maternal behaviour, including nest building activity, licking and retrieval of pups during and after delivery.

Fos expression in the SON in parturient rats injected with the CCK_A receptor antagonist or vehicle

The number of Fos-immunoreactive nuclei in the SON of parturient rats was higher than that in rats from experiment 1 given an injection of vehicle followed by saline (Fig. 4 bottom). However there was no significant difference for the incidence of Fos-immunoreactive in the SON between parturient rats given an injection of the CCK_A receptor antagonist MK-329 or vehicle.

Discussion

In male rats, a systemic injection of CCK induced a significant increase in the number of Fos-immunoreactive nuclear profiles in the dorsal vagal complex and the area postrema in the brainstem and in the SON and PVN in the hypothalamus. These results are in agreement with our data on the significant increase in oxytocin plasma concentrations following systemic administration of CCK (Luckman *et al.* 1993b) and the described activation of hypothalamic oxytocin (and corticotropin-releasing factor containing) neurones in the PVN in response to intravenous CCK (Verbalis *et al.* 1991b). Fos expression in the brainstem, including the area postrema and the dorsal vagal complex, suggests that neurones in these areas are part of the

afferent pathway involved in the release of oxytocin from the posterior pituitary in response to systemic CCK (Carter & Lightman 1987b). Alternatively, peripheral CCK, which does not penetrate the blood-brain barrier in significant amounts (Ermisch 1992), might stimulate magnocellular neurones by acting at circumventricular organs like the organum vasculosum of the lamina terminals (OVLT), a forebrain structure that has a strong neuronal input to the SON and PVN (Honda *et al.* 1990). However, lesions of the OVLT do not impair oxytocin release following an injection of CCK (Blackburn & Leng 1990a), while lesions of the area postrema severely attenuate the effects of CCK on oxytocin release (Carter & Lightman 1987b). The NTS and the area postrema are heavily interconnected and both areas are activated by intravenous CCK (Cunningham *et al.* 1994, Luckman 1992, Sawchenko & Swanson 1982a). Unlike the area postrema, the NTS is a source of a direct afferent input to magnocellular oxytocin neurones, part of which comprises noradrenergic neurones of the A2 cell group (Raby & Renaud 1989b, Sawchenko & Swanson 1982a). Peripheral administration of CCK has been shown to activate catecholaminergic neurones in the A2 cell group and to result in noradrenaline release in both the PVN and the dorsal part of the SON (Kendrick *et al.* 1991, Luckman 1992, Ueta *et al.* 1993).

Administration of the CCK_A receptor antagonist MK-329 before the injection of CCK abolished the increase in Fos expression in the NTS, the area postrema and the SON and PVN. Furthermore, MK-329, used at a dose range between 0.01 - 1 mg/kg body weight dose-dependently inhibited pituitary oxytocin release, while L365,260, used at the same dose range, was not effective (Luckman *et al.* 1993b). We cannot conclude from these data whether this effect is due to the blockade of CCK_A receptors in the NTS and the area postrema or in the periphery, e.g. at vagal afferents (Van Dijk *et al.* 1984), inhibiting the excitatory input from these brainstem areas or the periphery to the SON and PVN or whether MK-329 acted in addition on other, more rostral sites or on the hypothalamic oxytocin neurones themselves. However, apart from the NTS and the area postrema, that contain both types of CCK receptors (Hill & Woodruff 1990), most other areas in the brain possess mainly CCK_B receptor types and the SON in particular has been shown to express exclusively CCK_B receptors (Carlberg *et al.* 1992, Honda *et al.* 1993). Since it is also unlikely that MK-329 acted via the CCK_B receptor as despite their chemical similarity, binding studies indicate that the relative affinities of MK-329 and L365,260 for the CCKA and CCK_B receptor differ by more than one order of magnitude (Hill & Woodruff 1990), brainstem areas, and possibly peripheral afferents, are the most likely site of action of systemic MK-329. Furthermore, the

increase in electrical activity of supraoptic oxytocin neurones following intravenous CCK is completely abolished by the CCK_A receptor antagonist (1 mg/kg), but not the CCK_B receptor antagonist (Luckman *et al.* 1993b).

The attenuation of CCK-induced Fos expression in the SON and PVN, but not the brainstem, by the CCK_B receptor antagonist L-365,260 points either to an involvement of central CCK in mediating Fos expression in the hypothalamus after peripheral administration of CCK or to an inhibitory effect of L-365,260 on the afferent input from the NTS to the hypothalamus. Though we cannot conclude from our results which projection is involved in the activation of magnocellular oxytocin neurones, a likely source are noradrenergic neurones in the NTS, that co-localise CCK (Kawai *et al.* 1988) and project directly to the SON (Onaka *et al.* 1995b). Furthermore, a majority of neurones in the NTS that are retrogradely labelled from the SON and contain tyrosine hydroxylase, the rate limiting enzyme for catecholamine synthesis, express Fos following administration of CCK (Onaka *et al.* 1995b).

Since the CCK_B receptor antagonist did not affect secretory activity of oxytocin neurones (Luckman *et al.* 1993b), it remains possible that several neurotransmitters are involved in the regulation of pituitary oxytocin secretion, like the recently described peptidergic innervation from the NTS to hypothalamic oxytocin neurones (Sawchenko *et al.* 1985, 1988a, 1988b, 1990), and that Fos expression induced in the hypothalamus by intravenous CCK is more sensitive to antagonism than oxytocin secretion.

Unlike the inhibitory effect of MK-329 on hypothalamic activation after administration of CCK, the CCK_A receptor antagonist had no effect on the progress of delivery nor on Fos expression in SON neurones of parturient rats. Although we did not examine Fos expression in the NTS of parturient rats treated with MK-329, the lack of any effect of MK-329 on Fos expression in the SON and on the progress of delivery indicates that oxytocin release was not affected and hence an inhibitory effect of MK-329 on the afferent input from the brainstem to the SON in parturient rats is unlikely (Douglas *et al.* 1993a, Luckman *et al.* 1993a, Russell 1989b, 1991). Though we used a higher dose of the CCK_A receptor antagonist to try and block Fos expression in the SON of parturient rats than that used to prevent the release of oxytocin effects in response to CCK, we have shown in the complete study that MK-326 used at a dose range between 0.01 - 1 mg/kg body weight dose-dependently inhibits CCK-induced oxytocin secretion, without having an agonistic effect even at the highest dose (Luckman *et al.* 1993b).

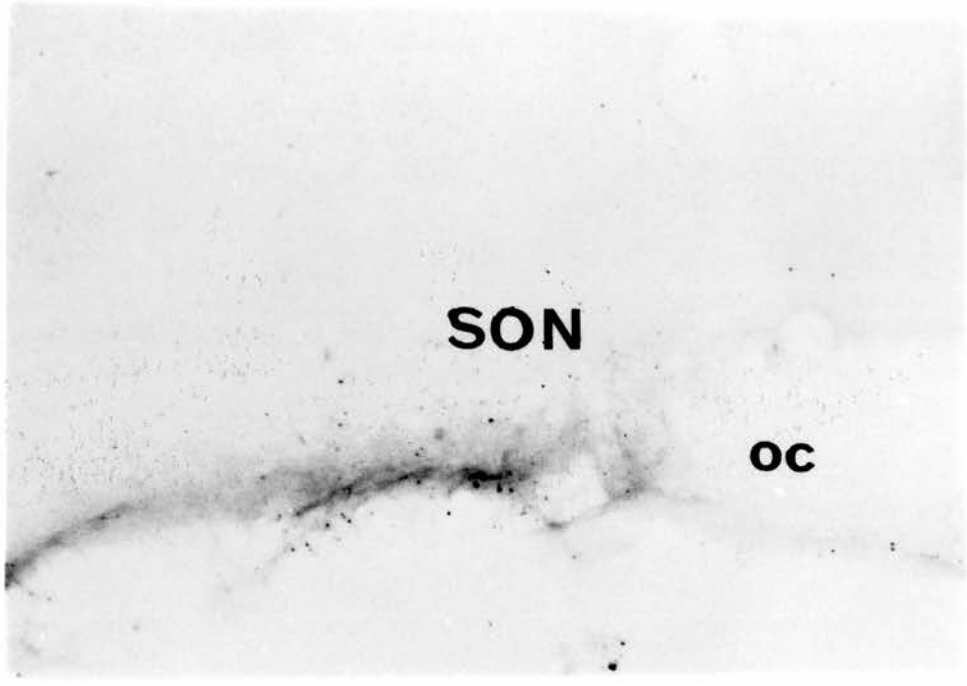
Thus, our results indicate a different sensitivity of Fos expression in SON neurones and pituitary oxytocin release to the inhibitory effects of a CCK_B receptor antagonist during

parturition and in response to CCK. Though parturition and an injection of CCK are both associated with an increase in Fos expression in the NTS, the proportion of Fos-immunoreactive nuclei contained in TH neurones in response to CCK is greater than that during parturition (c.f. Onaka *et al.* 1995b and chapter three), suggesting the existence of a common, yet not identical pathway. We and others have shown recently, that in response to CCK about 80% of Fos positive nuclei in the NTS that project to the SON or PVN contain TH (Onaka *et al.* 1995b, Rinaman *et al.* 1994), this indicates the physiological importance of the catecholaminergic pathway from the A2 region to the magnocellular hypothalamus.

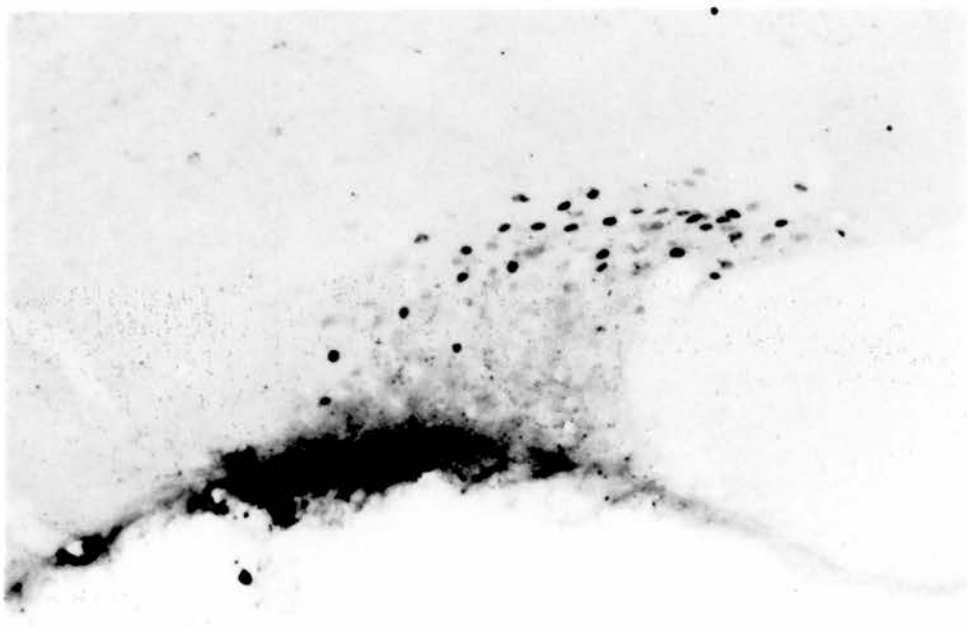
The recent finding that different peptides are co-localised in subsets of catecholaminergic NTS neurones (Kamai *et al.* 1988, Sawchenko *et al.* 1985), opens up the possibility that activation of different subpopulations of catecholaminergic NTS neurones could account for the observed differences with regard to the regulation of Fos expression in the SON in response to different stimuli. Furthermore, during parturition, but also in response to CCK, a substantial proportion of non-catecholaminergic neurones express Fos, that remain to be characterised in terms of the transmitter produced, and are likely to be involved in the control of Fos expression in the hypothalamus and oxytocin secretion from the posterior pituitary.

Fig. 2.1. Fos expression in the SON of rats injected with CCK
Rats were killed 90 min after an injection of isotonic saline (100 µl/100 g body weight iv, controls) or CCK (20 µg/kg body weight iv) and the brains cut into coronal sections (30 µm) through the SON and processed for standard Fos immunocytochemistry.
Control rats showed significantly fewer Fos-immunoreactive nuclei in the SON than rats killed after an injection of CCK.
oc = optic chiasm, scale bar 100 µm

control



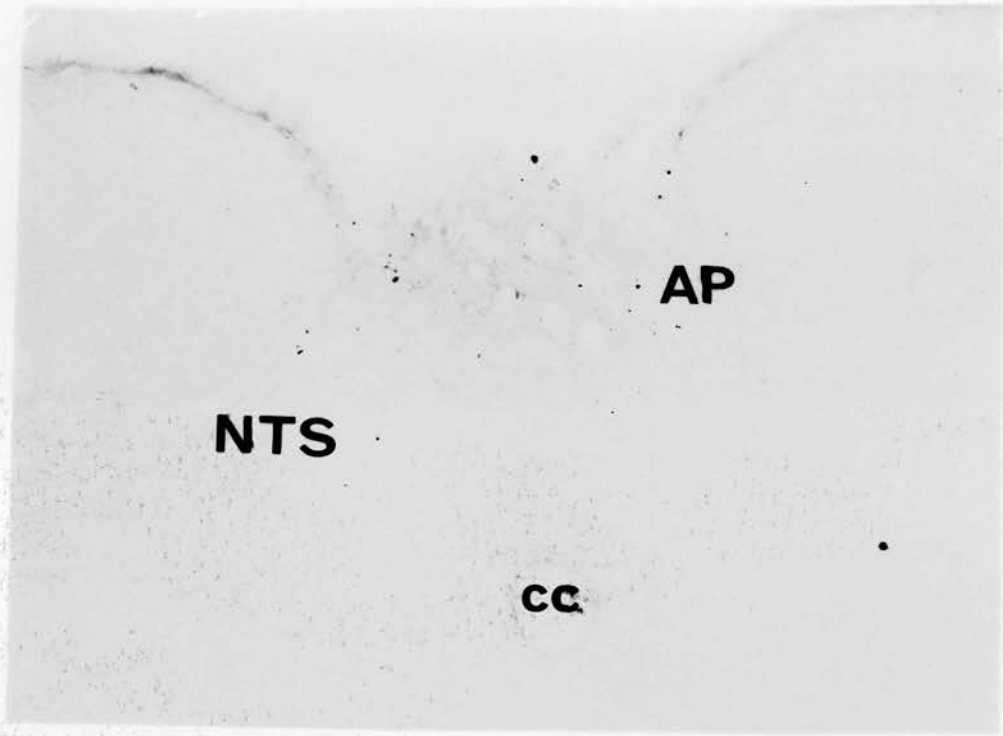
90 min after CCK (20 μg/kg, iv)



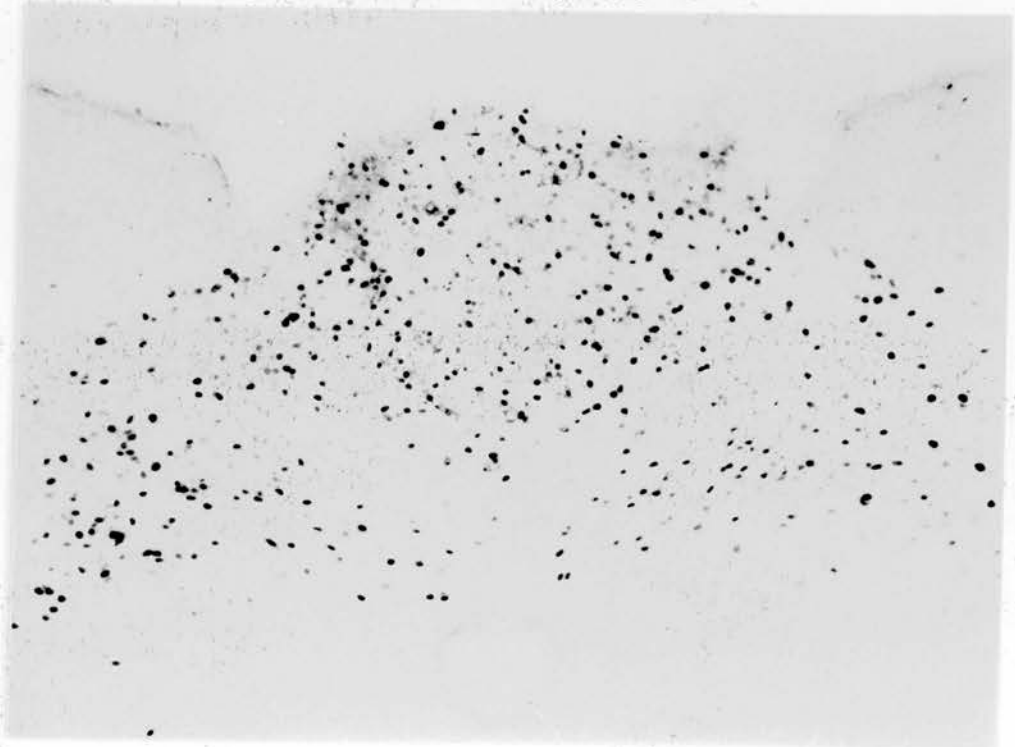
100 μm

Fig. 2.2. Fos expression in the brainstem of rats injected with CCK
Rats were killed 90 min after an injection of isotonic saline (100 µl/100 g body weight iv, controls) or CCK (20 µg/kg body weight iv) and the brains processed for Fos immunocytochemistry.
Control rats showed significantly fewer Fos-immunoreactive nuclei in the NTS and the area postrema than rats killed after an injection of CCK.
AP = area postrema, cc = central canal, scale bar 100 µm.

control



90 min after CCK (20 μg/kg, iv)



100 μm

Fig. 2.3. Fos expression in the hypothalamus and the brainstem of rats injected with CCK or CCK followed by an injection of a CCK_A (MK-329) or CCK_B (L365,260) receptor antagonist

The incidence of Fos-immunoreactive nuclei/section was counted in the SON and PVN and in the brainstem (the dorsal vagal complex (DVC) and area postrema) of rats injected intravenously with isotonic saline (open bars), CCK-8 alone (20 μg/kg body weight, filled bars), CCK with prior injection of MK-329 (CCK_A receptor antagonist, left-hatched bars) or L365,260 (CCK_B receptor antagonist, cross-hatched bars).

Animals (n=5-6 per group) treated with CCK alone showed significantly more Fos-immunoreactive nuclei compared to saline treated rats (†$p<0.05$, U-test). Pretreatment with either CCK receptor antagonist reduced this increase in Fos expression in the SON and PVN (*$p<0.05$, U-test), though in the PVN the difference between rats given CCK and those given CCK plus the CCK_B receptor antagonist was not significant. In the DVC and the area postrema, only the CCK_A receptor antagonist significantly reduced the expression of Fos compared to CCK alone treated rats, while in rats injected with the CCK_B receptor antagonist Fos expression in the brainstem was not significantly different from that seen in CCK-treated rats and significantly higher than that in saline treated rats (†$p<0.05$, U-test).

†, *$p<0.05$, U-test compared to isotonic saline and CCK alone, respectively

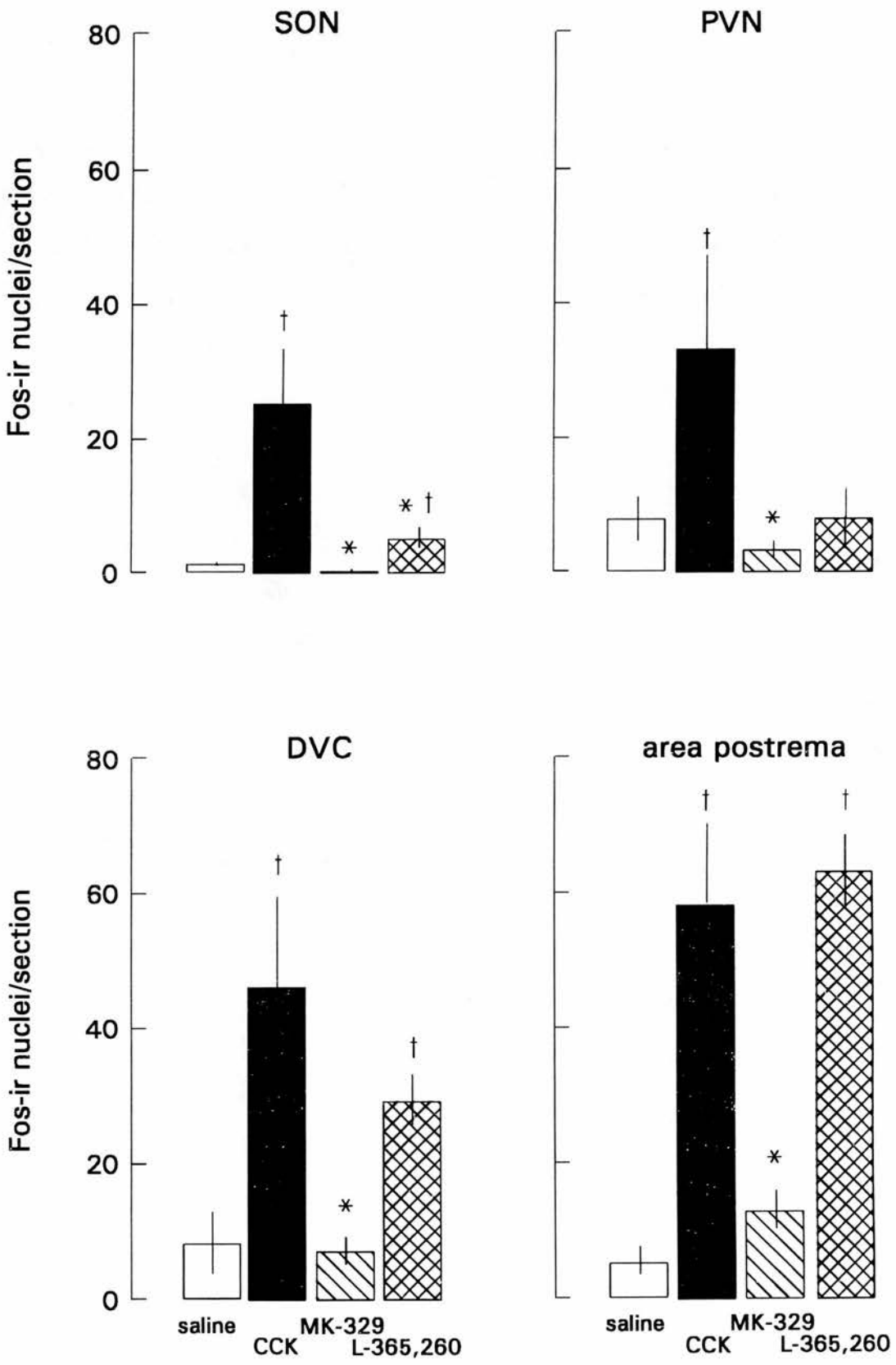
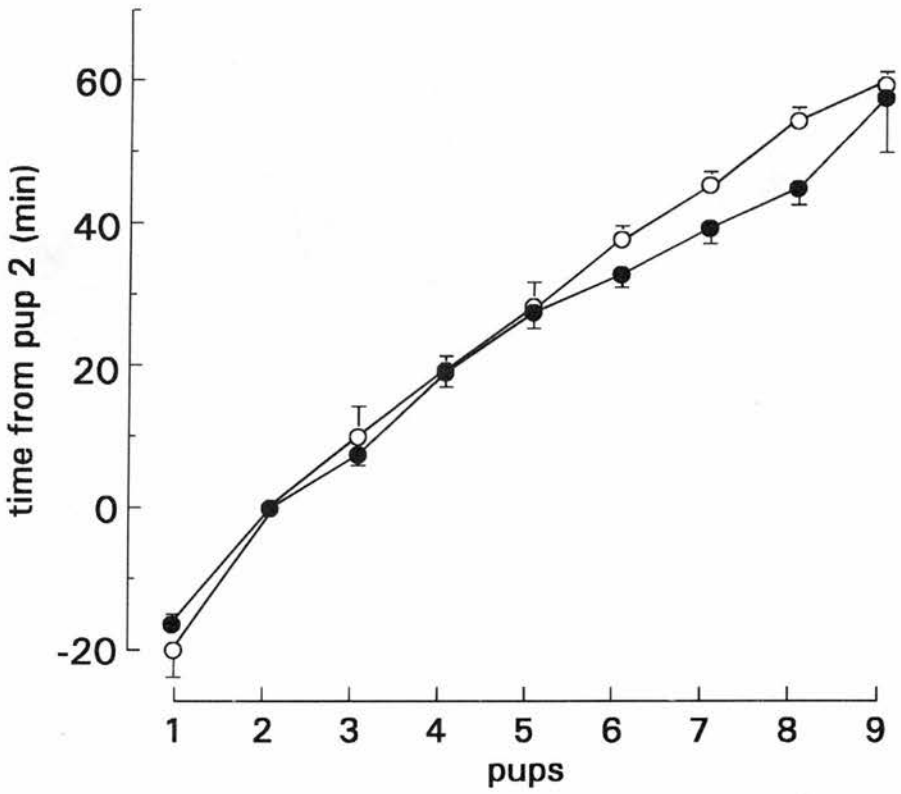
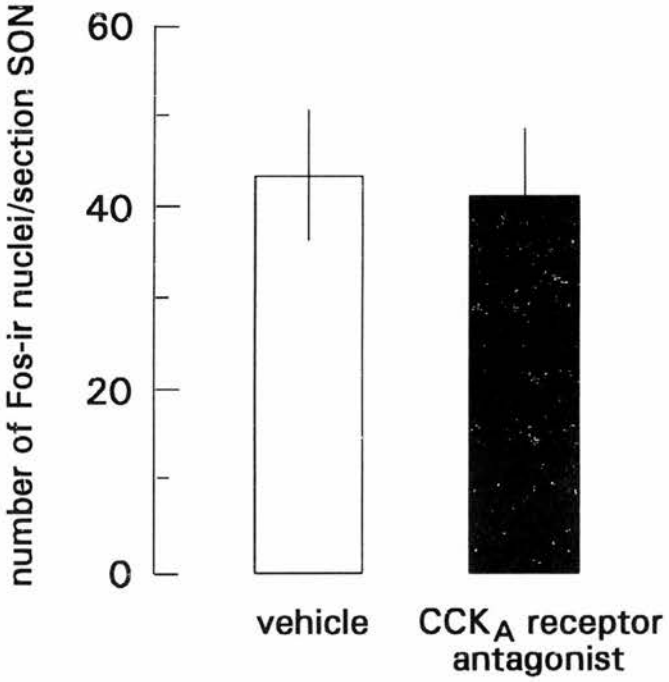

SON
PVN
DVC
area postrema
Fos-ir nuclei/section
0
20
40
60
80
saline
CCK
MK-329
L-365,260

Fig. 2.4. Effect of a CCK_A receptor antagonist on the progress of delivery and Fos expression in the SON

Top: The progress of delivery was assessed by the cumulative time between delivery of pups 1-9 in rats injected immediately after the birth of the first pup with the CCK_A receptor antagonist MK-329 (1 mg/kg, filled circles, n=4) or vehicle (10 µl/100 g body weight, open circles, n=4).
There was no significant difference for the time between delivery of pups 1-9 between the two groups.

Bottom: Fos-immunoreactive nuclei in the SON of parturient rats given MK-329
The incidence of Fos-immunoreactive nuclei/section SON was not significantly different between rats injected after the birth of the first pup with vehicle (open bar, n=4) or MK-329 (filled bar, n=4).


time from pup 2 (min)
60
40
20
0
-20
1
2
3
4
5
6
7
8
9
pups


number of Fos-ir nuclei/section SON
60
40
20
0
vehicle
CCK$_A$ receptor antagonist

Chapter 3

Induction of uterine activity with oxytocin in late pregnant rats replicates the expression of c-*fos* in neuroendocrine and brainstem neurones as seen during parturition

The oxytocin content of the rat neurohypophysis increases approximately two fold from the beginning of pregnancy to term and this accumulated excess is secreted into the circulation (Douglas *et al.* 1993b, Fuchs & Saito 1971, Kumaresan *et al.* 1979) between the births of the first and last pups of the litter. Findings in a variety of species, including the human, suggest that this release of oxytocin occurs in pulses (Fuchs *et al.* 1991, 1992), reaching peak plasma levels at the expulsion of each foetus (Higuchi *et al.* 1986b). Oxytocin pulses during delivery are superimposed on an elevated background secretion (Fuchs *et al.* 1991, Higuchi *et al.* 1986b) and in rats these pulses are preceded by a burst-like increase in activity in neurosecretory oxytocin neurones (Summerlee 1981).

In the first chapter, I have provided evidence for the physiological importance of pulsatile oxytocin secretion for the normal progress of parturition and have shown that at the time of

parturition, expression of Fos, the protein product of the immediate early gene *c-fos*, is increased in the SON and putative afferent neurones in the brainstem, including the NTS. Sensory afferents from the uterus and the vagina terminate in the NTS (Ortega-Villalobos *et al.* 1990), which projects directly to the hypothalamus, including supraoptic oxytocin neurones (Raby & Renaud 1989a, 1989b, Sawchenko & Swanson 1982a), and hence the NTS might be involved in the relay of sensory information from the uterus to the SON during delivery. Since a) neither magnocellular nor brainstem neurones appear to be activated to express Fos before the expulsion of pups and b) the continuous administration of oxytocin, although inducing delivery, apparently does not deplete pituitary oxytocin (Fuchs & Poblete 1970), I investigated firstly, whether the more physiological intermittent administration of oxytocin could induce delivery and activate magnocellular oxytocin neurones and putative afferent neurones in the ventrolateral medulla and the NTS and secondly, whether increased uterine activity in response to pulsatile oxytocin administration could play an important role for the activation of these afferent pathways.

Materials and Methods

Animals

Date mated Sprague-Dawley rats were implanted with a jugular vein cannula on day 20 of pregnancy under brief ether anaesthesia and in the morning of the next day the cannulae were connected to oxytocin- or saline-filled polyethylene tubings. The treatment (oxytocin or saline pulses) was started at 10.45 h and for four hours each 10 min a pulse of saline (0.03 ml) or oxytocin (10 mU oxytocin for 2 hours and then 20 mU oxytocin for 2 hours) was injected. All animals were assessed every 5-10 min for signs of labour (stretching, straining, vaginal bleeding), pup delivery and maternal behaviour (nest building, excessive grooming, licking of external genitals, licking of the pups during and after delivery, pup retrieval). Onset of delivery was defined as the time when the first pup was fully expelled and the time of birth for each following pup was recorded until at least the twelfth pup.

Experimental design

Dose-dependent effects of oxytocin on the onset of delivery

In order to induce delivery in late pregnant rats, I first injected pulses of oxytocin at a dose that was highly effective at restoring delivery that has been disrupted by morphine (see chapter one): animals were injected intravenously with 1 mU oxytocin every 10 min for four

hours (n=4), however since this dose proved not effective at inducing delivery, we used in separate rats increasing doses of oxytocin. Thus, rats were given 1 mU every 10 min for two hours and then 2 mU each 10 min for two hours (n=3), or 2 mU and then 4 mU (n=2) or 5 mU and 10 mU (n=2); however, none of these rats started to give birth during the treatment.

Another eighteen rats were given an even higher dose oxytocin treatment (10 mU oxytocin per pulse for two hours and then 20 mU oxytocin per pulse for two hours). Fourteen animals were injected at the same times with saline vehicle. Animals were killed either before birth of any pups (prepartum groups, n=4 in the oxytocin-treated group and n=5 in the control group) or 90 min after delivery of the second pup (parturient groups, n=14 in the oxytocin-treated group and n=9 in the control group). Four of the oxytocin-treated parturient rats were killed before the end of the treatment (during the last 30 min of the injections), while all remaining rats were killed after the treatment (30-60 min after the last injection for all prepartum and oxytocin-treated parturient rats). Control parturient rats were killed whenever delivery occurred (in most cases in the second half of the light phase on day 21). After 20.00 h, the normal start of the dark phase, the observation of rats was continued in red light.

Rats were killed by cervical dislocation, the brains were removed and immediately frozen under crushed dry ice and subsequently processed for standard Fos immunocytochemistry (see general materials and methods section) and the uteri were examined for foetuses and placentae.

To generate a larger group of rats which were injected with oxytocin but in which delivery was not induced during such treatment, I injected sixteen other rats with 10 mU oxytocin each 10 min for the entire four hour treatment period, rather than to increase the dose of oxytocin to 20 mU per pulse for the last two hours. Nine rats were given saline pulses at the same times (0.03 ml saline per pulse). 30-60 min after the treatment (and 90 min after delivery of the second pup, n=4, 2 in the oxytocin- and saline-treated group, respectively) animals were deeply anaesthetised with sodium pentobarbitone (50 mg/kg body weight iv) and *in situ* perfusion-fixed. The brains were postfixed and then kept frozen at -80°C until processed for immunocytochemistry. The *in situ* perfusion fixation was chosen to optimise conditions for double immunocytochemistry performed on brainstem sections of these rats.

The experiments, with and without *in situ* fixation, were performed over a few weeks, with about 6-10 rats treated on each experimental day and with equal numbers of term pregnant animals injected with either saline or oxytocin each day.

To assess whether oxytocin injections could have a direct effect on Fos expression in the brain, independent of uterine activity and parturition, 10 virgin rats were injected at a random stage of the oestrous cycle with the higher dose oxytocin regime (10 mU each 10 min for 2 hours and then 20 mU each 10 min for 2 hours) and were killed by cervical dislocation 30-90 min after the end of the treatment. The brains were frozen on crushed dry ice and processed for Fos immunocytochemistry (see general materials and methods section).

Fos immunocytochemistry

For immunocytochemistry on non-perfused brains, 15 μm coronal cryostat sections were cut and every fifth section was mounted on gelatinised slides. For Fos immunocytochemistry on *in situ* perfusion-fixed brains, coronal sections (30 μm) were cut on a microtome and every fourth section was collected in 0.1 M phosphate buffered solution and processed for immunocytochemistry (for details see general materials and methods section).

Sections were collected throughout the SON, from the anterior commissure to the median eminence, and through the brainstem, from the pyramidal decussation to the widening of the fourth ventricle rostral of the area postrema. We counted Fos-immunoreactive nuclei in every fifth section from the respective areas (on average 12 and 9 cryostat sections per SON and NTS, and 8 microtome SON sections), thus ensuring that sections from similar rostro-caudal positions between animals were analysed, so that a variation in cross section area and section volume is controlled for.

To test the specificity of the increase in Fos expression in the NTS and SON in response to oxytocin treatment (10/20 mU per pulse), Fos-immunoreactive nuclei were counted on cryostat sections containing the subfornical organ (SFO) or the area postrema. Every third section was collected of the respective area and a minimum of five sections per area were analysed.

Double immunocytochemistry

Double immunocytochemistry was performed for Fos and oxytocin or vasopressin, or Fos and tyrosine hydroxylase (TH, the rate limiting enzyme for the synthesis of catecholamines), on alternate free-floating coronal microtome sections (30 μm) through the SON or NTS, respectively (for details see general materials and methods section).

In the NTS, double- and single-labelled cells were counted in at least 42 alternate sections per animal to survey the catecholaminergic cell groups of the NTS and of the ventrolateral medulla (the A2/C2 and A1/C1 cell groups, respectively), while TH-immunoreactive cells located in the dorsal motor nucleus of the vagus were excluded, as the majority of these neurones at the level of the area postrema contain dopamine, unlike at the caudal level of the A2/C2 region, where the majority of TH-immunoreactive cells contain noradrenaline (Kalia *et al.* 1985). The counts were divided into seven groups of six sections each, so that each group extended rostro-caudally over a subdivision of 0.36 mm. The obex, just rostral of the area postrema (Paxinos & Watson 1986), was taken as reference point zero.

Statistical analysis

Comparisons between two groups were made using the non-parametric Mann-Whitney U-test and, in the case of multiple comparisons, the Bonferroni correction was applied as appropriate. Comparisons between more than two groups were made with the non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc test. Comparison of percentages was performed with the two-proportion t-test. For a value of $p<0.05$ a difference was considered significant.

Results

The effect of oxytocin pulses on the onset of delivery

Fourteen rats were injected with saline pulses for four hours in the morning of expected delivery as controls for pulsatile oxytocin administration. None of these rats started to give birth during the treatment, but gave birth within 17 hours after the last saline injection (median time of onset of delivery: 8.1 h after the start of the treatment = 18.51 clocktime, Fig. 1 & 2, top).

Eleven rats were injected with oxytocin pulses ranging from 1 mU per pulse up to 5 mU and 10 mU per pulse for four hours. Of these rats, none started to deliver during the treatment. Only two of these rats gave birth within the normal range (i.e. 17 hours after the last injection). The other nine animals gave birth between 20 and 41 hours after the end of the treatment. In comparison with the saline group, the onset of delivery occurred significantly later (median time 27.1 hours after the start of the treatment, test for medians and U-test, $p<0.05$, Fig. 1).

In contrast, fourteen out of eighteen rats (77%) injected with a higher dose regime of oxytocin (10 mU per pulse for 2 hours and then 20 mU for 2 hours) delivered during the four hour treatment (median time to first pup was 3.3 hours after the first injection, U-test, $p<0.01$ vs controls, Fig. 2, top).

All rats injected with oxytocin, regardless of the dose, demonstrated typical signs of labour (stretching and straining movements) promptly after beginning the oxytocin injections, while in saline-treated rats such behaviour was usually not observed during the treatment period, but only prior to delivery, a few hours after the end of the injections. Once the first pup was born, all rats, regardless of the treatment, showed normal maternal behaviour (licking of pups during and after delivery, retrieving and grouping of pups and nest building activity).

The litter size, which shows a weak inverse correlation with the time of onset of parturition (see chapter four), did not differ significantly between groups (13.4±0.5 pups per litter in prepartum and parturient controls, 11.5±1.5 pups per litter in lower dose and 14.3±0.6 pups per litter in higher dose oxytocin-treated prepartum and parturient rats). Neonatal pup survival was similar in all groups (98%, 100% and 100% of pups alive 6 hours after birth, respectively, for rats that were allowed to deliver).

The progress of delivery, as assessed by the time between delivery of the first and the twelfth pup, was prolonged in lower dose oxytocin-treated rats compared to controls (119.39±5.1 min, n=6, vs 100.2±5.8 min, n=7, U-test, $p<0.05$), but was similar for higher dose oxytocin-treated rats (99.4±2.9 min, n=10) and controls (Fig. 2, bottom). Comparison of the mean of all interbirth intervals in saline-treated rats (11±3 min, n=7) with the mean of the interbirth intervals during the first two hours and the last two hours of the higher dose oxytocin treatment, and after the end of the oxytocin treatment (11±2 min, n=3, 14±3 min, n=14, and 12±3 min, n=8, respectively) also showed no significant difference. Thus, although rats treated with the higher dose of oxytocin entered parturition early, the progress of parturition in these animals was not disturbed by the treatment and progressed normally even after the treatment was discontinued.

Fos expression in the SON of oxytocin-treated rats

Vehicle-treated rats that were killed before delivery of any pups showed a low incidence of Fos-immunoreactive nuclei in magnocellular neurones in the SON, compared with rats killed during spontaneous delivery, indicating activation of SON neurones during the birth of pups. Oxytocin-treated rats (10 mU or 10/20 mU per pulse) that were killed before delivery of

any pups showed a significantly higher incidence of Fos-immunoreactive nuclei in the SON than control prepartum rats (Fig. 3 & 5). All animals killed during parturition showed a significantly higher incidence of Fos-immunoreactive nuclei than control prepartum rats regardless of whether delivery had occurred spontaneously or had been induced with oxytocin (Fig. 3 & 5, Kruskal-Wallis test, $p<0.05$). This result was confirmed in another set of animals injected with 10 mU oxytocin or saline pulses for four hours and perfusion-fixed *in situ* at the end of the experiment but before delivery had started: as in the previous experiment, oxytocin-treated rats (n=12) showed significantly more Fos-immunoreactive nuclei per section SON than prepartum controls (n=7) (19.6±2.4 vs 7.6±2.5 Fos-immunoreactive nuclei/section U-test, $p<0.02$).

SON sections from spontaneously parturient and oxytocin-treated parturient rats (n=2, 2), processed for double immunocytochemistry (Fos and either oxytocin or vasopressin), showed Fos in both types of magnocellular neurones, however the dense cell packing in the SON made it difficult to reliably quantify the relative numbers of oxytocin and vasopressin neurones expressing Fos (Fig. 6).

Fos expression in the NTS of oxytocin-treated rats

Oxytocin-treated rats (10/20 mU per pulse) killed before the delivery of any pups showed significantly more Fos-immunoreactive nuclei in the NTS than saline-treated prepartum rats (Fig. 4 & 5, Kruskal-Wallis test, $p<0.05$), but a similar incidence of Fos-immunoreactive nuclei in the NTS to either parturient group (saline- or oxytocin-treated). In all parturient animals the incidence of Fos-immunoreactive nuclei was higher than in control prepartum rats (Kruskal-Wallis test, $p<0.05$).

Fos-immunoreactive cells in catecholaminergic brainstem neurones of oxytocin-treated prepartum rats (Fig. 7)

There were few Fos-immunoreactive nuclei in the NTS of saline-treated prepartum rats, while 10 mU pulses of oxytocin for four hours increased the incidence of Fos-immunoreactive nuclei in the NTS even before delivery (Fig. 8 & 9). The incidence of Fos-immunoreactive nuclei per NTS and ventrolateral medulla in parturient rats (treated either with 10 mU oxytocin pulses or saline, n=2, 2) and in oxytocin-treated prepartum rats was similar, but significantly greater than in saline-treated prepartum rats (Table 1). Although the incidence of Fos-immunoreactive nuclei was higher at every level of the NTS and the ventrolateral medulla

in oxytocin- compared to saline-treated prepartum rats, the difference was only significant at the caudal level of the NTS (U-test, $p<0.05$, Bonferroni correction applied as appropriate, Fig. 9, top).

The total number of TH-immunoreactive cells per NTS was also significantly greater in parturient and oxytocin-treated prepartum than saline-treated prepartum rats (Fig. 6, 8 & 9 and Tab. 1, Kruskal-Wallis test, $p<0.05$), while there was no significant difference in the number of TH-immunoreactive cells between groups in the A1/C1 region. The number of TH-immunoreactive cells at every individual level of the brainstem was greater in oxytocin- than saline-treated rats, however the difference reached significance only at the caudal levels of the NTS (U-test, $p<0.05$, Fig. 9, middle).

The total number of double-labelled cells (cells immunoreactive for both Fos and TH) per NTS and per ventrolateral medulla was significantly higher in parturient and oxytocin-treated prepartum than saline-treated prepartum rats (Table 1). The oxytocin-treated prepartum group showed consistently a greater incidence of double-labelled cells than the saline-treated group throughout the brainstem (Fig. 9, bottom), but the difference was only significant at the two most caudal levels of the NTS (U-test, $p<0.05$, Fig. 9, bottom).

Table 1

Incidence of Fos-, TH- and double-labelled (Fos- and TH) immunoreactive (ir) cells in the NTS and the ventrolateral medulla (VLM)

	Fos-ir nulcei/area		TH-ir cells/area		Fos+TH-ir cells/area	
groups	NTS	VLM	A2/C2	A1/C1	A2/C2	A1/C1
saline prepartum (n=7)	177±21	78±19	281±69	322±29	61±8	66±15
oxytocin prepartum (n=12)	475±74*	289±71*	660±51*	541±63	132±23*	165±41*
parturient (n=4)	709±188*	557±198*	920±124*	401±53	195±47*	242±75*

Oxytocin-treated prepartum and parturient rats showed a significantly higher incidence of Fos-, TH- and double-labelled (Fos- and TH-immunoreactive) cells in the A2/C2 region than saline-treated prepartum rats. In the A1/C1 cell group, only the incidence of Fos-immunoreactive nuclei and double-labelled cells was higher in oxytocin-treated prepartum and parturient rats compared to the saline-treated prepartum group (*$p<0.05$, Kruskal-Wallis test).

Both groups showed a similar distribution of Fos-immunoreactive nuclei, TH-immunoreactive cells and double-labelled cells (immunoreactive for both Fos and TH) in the brainstem with a peak incidence of labelled cells at the level of the area postrema in the NTS (Fig. 9). Although the absolute increment in the numbers of immunoreactive cells following pulsatile oxytocin compared to saline treatment was similar at all rostro-caudal levels, the percentage increase of labelled cells was greatest in the caudal NTS, as apparent from the significant differences between the two groups at the caudal levels of the NTS (Fig. 9 & 10).

The proportion of TH-stained cells that were also labelled for Fos in the NTS was not significantly different in saline- and oxytocin-treated prepartum rats and in the parturient group (Table 2). Likewise there were no significant differences between the groups for the proportion of TH-stained cells that were also labelled for Fos in the ventrolateral medulla (Table 2).

Table 2

Percentage of TH-immunoreactive (ir) cells expressing Fos and Fos-immunoreactive nuclei in TH-containing cells

groups	% TH-ir cells expressing Fos		% Fos-ir nuclei contained in TH-ir cells	
	NTS	VLM	A2/C2	A1/C1
saline prepartum (n=7)	21±5	32±2.6	40±6.2	96±5.5*
oxytocin prepartum (n=12)	31±7.2	20±2.9	26±2.7	58±5.1
parturient (n=4)	43±12	32±3.1	26±4.1	61±6.3

The percentage of TH-immunoreactive cells that expressed Fos before and during parturition, were, unlike the absolute numbers, not significantly different between groups in either the region of the NTS or the ventrolateral medulla. The number of Fos-immunoreactive nuclei that were detected in cells containing TH were also similar between groups, however in the A1/C1 cell group in saline-treated prepartum rats the percent of Fos-immunoreactive nuclei contained in TH-stained cells was nearly 100% and significantly higher than in oxytocin-treated prepartum and parturient rats (*$p<0.05$, Kruskal-Wallis test).

The proportion of Fos-immunoreactive cells that was also immunoreactive for TH was higher in the saline-treated prepartum group compared to the oxytocin-treated prepartum group and the parturient group, but the difference was only significant in the A1/C1 region (Table 2, Kruskal-Wallis test, $p<0.05$) and not in the NTS (Table 2). However the total number of double-labelled cells was very low in the saline-treated prepartum group, so that the proportional data have to be interpreted with caution.

Fos-immunoreactive nuclei in the SON and NTS of non-pregnant rats injected with oxytocin pulses

Ten non-pregnant rats injected with oxytocin pulses (10 mU every 10 min for 2 hours and then 20 mU for 2 hours) showed very few Fos-immunoreactive nuclei in either the SON (2.2±0.3 Fos-immunoreactive nuclei/section) or the NTS (4.6±0.8 Fos-immunoreactive nuclei/section). The incidence of Fos-immunoreactive nuclei in the SON or NTS of virgin rats treated with oxytocin was not significantly different from that in the respective regions in late pregnant rats injected with saline pulses (4.4±2.4 Fos-immunoreactive nuclei/section SON and 2.5±1.4 Fos-immunoreactive nuclei/section NTS), but was significantly less than that in late pregnant rats injected with oxytocin (c.f. Fig. 3 & 4: 17.7±6.1 Fos-immunoreactive nuclei/section SON and 12.0±1.9 Fos-immunoreactive nuclei/section NTS, Kruskal-Wallis test, $p<0.05$).

Fos-immunoreactive nuclei in the SFO and the area postrema of vehicle- and oxytocin-treated prepartum rats

Fos-immunoreactive nuclei in the area postrema and the SFO, two areas outside the blood-brain barrier which are sensitive to changes in blood pressure and plasma osmolality, were counted on cryostat sections from saline- (n=5) and oxytocin-treated prepartum rats (10/20 mU oxytocin for 4 hours, n=10). There was no significant difference in either area between the saline- and the oxytocin-treated groups (7.6±1.0 vs 6.3±1.1 Fos-immunoreactive nuclei/section in the area postrema and 1.2±0.4 vs 0.9±0.2 Fos-immunoreactive nuclei/section in the SFO).

Discussion

This study demonstrated that intermittent injections of oxytocin to late pregnant rats are highly effective at inducing delivery. In contrast, lower doses of oxytocin delayed rather than advanced parturition, indicating that stimulation of the uterus with doses of oxytocin which are ineffective at inducing delivery, might cause a down-regulation of oxytocin receptors (Engstroem *et al.* 1988) and hence a desensitisation of the uterus to endogenous oxytocin, resulting in a delayed onset of parturition.

Once parturition was induced during the oxytocin treatment, it progressed normally even after discontinuation of the treatment. As inhibition of pituitary oxytocin secretion during delivery disrupts the normal progress of delivery (Luckman *et al.* 1993a, Russell *et al.* 1989b, Zlatnik & Fuchs 1972), it appears that oxytocin treatment initiated endogenous oxytocin release and thus delivery, once induced by exogenous oxytocin, could be sustained. Although pulsatile oxytocin, when given before delivery, augments uterine contractions to a greater extent than a continuous oxytocin infusion (Randolph & Fuchs 1989), much higher doses of oxytocin were needed to consistently advance delivery in rats, than are needed to restore delivery interrupted by morphine. This observation can be explained by the dramatic increase in uterine oxytocin receptor density during the hours preceding delivery (Alexandrova & Soloff 1980, Fuchs *et al.* 1982, 1983), resulting in an enhanced sensitivity of the uterus to oxytocin during labour (Fuchs & Poblete 1970).

Strong uterine contractile activity, as seen during delivery, stimulates uterine afferent nerves, namely the hypogastric and pelvic nerves (Peters *et al.* 1987, Sato *et al.* 1989). The increased discharge frequency of the pelvic nerve during delivery of pups (Peters *et al.* 1987) and the lack of a reflex contraction of abdominal muscles and the diaphragm in response to vaginal distension (foetus-expulsion reflex) after lesioning of the pelvic nerves (Higuchi *et al.* 1986a, 1986b) together with a reduced increase in plasma oxytocin concentrations in these rats, indicate an involvement of uterine afferents for the reflex release of pituitary oxytocin and hence the normal progress of delivery in rats. However, the prolonged delivery in pelvic neurectomised rats can be only partly overcome by systemic administration of oxytocin, suggesting that factors other than the reduced release of oxytocin contribute to the prolongation of delivery in these rats (Higuchi *et al.* 1986a). It has been suggested that pelvic neurectomy abolishes reflex abdominal contractions that contribute to the expulsion of the foetuses (foetus-expulsion reflex, Higuchi *et al.* 1987). Alternatively, pelvic neurectomy might interfere with the pulsatile release of oxytocin from the posterior pituitary. Thus, pulsatile

rather than continuous infusion of oxytocin to pelvic neuroectomised rats might have provided a more physiological stimulation of uterine activity and might have been more effective at restoring delivery.

Although the afferent pathways between the uterus and the hypothalamus are not yet fully described, vaginal distension in pregnant and lactating rats facilitates the firing rate of neurones in the PVN (Negoro *et al.* 1973a) and the SON (Dreifuss *et al.* 1976) and vagal uterine afferents project to the dorsal vagal complex in the brainstem (Ortega-Villalobos *et al.* 1990), from where catecholaminergic neurones send excitatory projections, amongst others, to magnocellular SON and PVN neurones (Cunningham & Sawchenko 1991, Raby & Renaud 1989a, 1989b, Sawchenko & Swanson 1982a, 1991). Unlike the SON, which consists predominantly of magnocellular neurones, the PVN is a more heterogeneous nucleus, containing magnocellular and parvocellular neurones and increased Fos expression is apparent in the PVN before parturition (see chapter one, Luckman *et al.* 1993a, Luckman 1995). Therefore I restricted Fos counts in the present study to the SON, where the expression of the c-*fos* gene appears to be a reliable indicator of electrical activity and hormone secretion (Hamamura *et al.* 1991b, 1992, Hoffman *et al.* 1993). Thus, I can infer from the findings of similar numbers of Fos-immunoreactive nuclei in the SON of oxytocin-treated and spontaneously parturient rats and from the normal progress of delivery in the former that pulsatile oxytocin treatment in late pregnant rats stimulated secretory activity of SON neurones. Indeed, double immunocytochemistry for Fos and oxytocin or vasopressin performed on sections through the SON of oxytocin-treated and normal parturient rats confirmed Fos induction in a substantial number of supraoptic oxytocin neurones, while it also revealed activation of vasopressin neurones.

Similar to the SON, I observed an increase in Fos expression in the NTS of rats which delivered spontaneously and of rats in which delivery has been induced with oxytocin. Neurones in the NTS are known to project to the hypothalamus, including the magnocellular neurones (Raby & Renaud *et al.*1989a, 1989b). Recently, we have shown that a majority of NTS neurones, that project to the SON and express Fos in response to CCK are catecholaminergic (Onaka *et al.* 1995b), indicating the physiological importance of an afferent catchecholaminergic input from the NTS to the SON during CCK-induced oxytocin release. In this study, I found that the number of Fos-immunoreactive nuclei and the number of Fos and TH double-labelled cells in the NTS was significantly higher in oxytocin- compared to saline-treated prepartum rats, consistent with the hypothesis that an excitatory input from

catecholaminergic NTS neurones to the SON is activated during oxytocin-induced labour. Although in the NTS the proportion of activated TH-containing neurones was not significantly different between groups, the absolute number of TH-containing cells was significantly higher in the oxytocin- than the saline-treated prepartum group, particularly in the caudal and dorsal NTS, where predominantly noradrenergic neurones are located (Kalia *et al.* 1985). The increase in the number of NTS neurones containing TH might indicate an increased enzyme activity, possibly in response to increased catecholamine release stimulated through the pathway from the uterus proposed above. Various stimuli have been shown to increase the number of cells immunoreactive for TH in the locus coeruleus (Debure *et al.* 1992, Weiser *et al.* 1993) and the hypothalamus (Arbogast & Voogt 1991a), suggesting the existence of a population of "sleeping cells" in which TH expression is either inactive or at a low level, but increases in response to certain stimuli (Debure *et al.* 1992). Whether the observed change in the number of TH-containing cells in the NTS is reflected by a concomitant change in TH synthesis, as has been shown to occur under other circumstances (Debure *et al.* 1992, Arbogast & Voogt 1991a, Wang *et al.* 1993) remains to be investigated (see chapter six). As we did not observe an increase in the number of TH-containing cells in response to oxytocin-treatment in the A1/C1 region, uterine activity or delivery appear to induce TH immunoreactivity specifically in the A2/C2 region, consistent with the hypothesis of an important role for the excitatory noradrenergic projection from the NTS to oxytocin neurones in the SON. However a substantial number of NTS neurones that express Fos during parturition and following oxytocin treatment do not contain TH, indicating that other brainstem neurones, which remain to be identified in terms of transmitter produced, were also activated by the oxytocin treatment.

The activation of catecholaminergic neurones in the A1/C1 region, albeit less than that in catecholaminergic NTS neurones, might indicate that vasopressin neurones are also driven by afferents from the uterus, which is in agreement with reports on vasopressin release before and during spontaneous delivery (Fuchs & Saito 1971, Kumaresan *et al.* 1979).

The observation that Fos expression in the SON before delivery was higher in oxytocin-treated than control prepartum rats indicates that passage of the foetus through the lower birth canal is not essential for the reflex stimulation of oxytocin secretion at the end of pregnancy. The cervix, which has a higher density of oxytocin receptors and mechanoreceptors than the corpus and fundus uteri of the rat (Gorodeski *et al.* 1990), presents a firm barrier during pregnancy and softens only at the time of delivery. Oxytocin itself can reduce the rigidity of

the cervix (Khalifa *et al.* 1992), as well as relaxin (Downing & Sherwood 1985). Also, uterine contractions contribute to the softening of the cervix by stimulating prostaglandin release, which then induces collagen breakdown in the cervix (Fuchs 1987). Furthermore if labour is induced with oxytocin before softening of the cervix has occurred, the resulting uterine activity appears to be stronger (Olah *et al.* 1993) than when induced after dilatation of the cervix, suggesting that oxytocin-induced strong uterine contractions in late pregnant rats might be a more potent stimulus for SON oxytocin cell activation than spontaneous labour. The lack of an increase in Fos expression in the SON or brainstem in virgin rats in response to oxytocin supports the hypothesis that, if the uterus is not sensitive to oxytocin, the oxytocin treatment cannot excite brainstem and SON neurones.

The absence of an induction of the c-*fos* gene (and hence Fos protein) in the SFO and area postrema following oxytocin treatment, unlike following osmotic stimuli (Hamamura *et al.* 1991a, 1992) or changes in blood pressure (Badoer *et al.* 1993, Russ & Walker 1994), suggests that these two circumventricular organs do not mediate the stimulating actions of systemically administered oxytocin on SON neurones. Thus, we can exclude the possibility that a change in blood volume and/or plasma osmolality induced by the four hour oxytocin pulse treatment contributed significantly to the induction of Fos in the SON and NTS. In addition, increased Fos expression in the SON caused by a decrease in blood volume and hence blood pressure is only observed in response to severe hypovolemia (Badoer *et al.* 1993). Oxytocin itself has no effects on mean arterial blood pressure in male rats (Erickson & Millhorn 1991) and our own preliminary blood pressure measurements in term pregnant rats injected with oxytocin or saline pulses also showed no difference between groups. A direct effect of intravenous oxytocin on SON neurones is unlikely, as first, its plasma half-life is only about 1-2 min (Higuchi *et al.* 1986b), and second, the blood-brain barrier is rather impermeable for neurohypophysial peptides (Ermisch 1992, Van Bree *et al.* 1989). Thus, the most plausible explanation for the observed increase in Fos expression in SON and NTS neurones following oxytocin-induced labour and delivery without concomitant activation of neurones in the SFO or area postrema is that uterine contractions stimulated neurosecretory activity of SON neurones via catecholaminergic brainstem neurones.

In summary, I have shown that pulsatile oxytocin injections are very effective at inducing delivery in rats and that during such delivery SON magnocellular neurones and putative afferent neurones in the brainstem, of which a substantial proportion are catecholaminergic, are activated in a similar way as during spontaneous delivery. In addition, I have shown that

such pulsatile administration of oxytocin similarly activates neurones in the SON and brainstem even before the delivery of any pups. I suggest that uterine activity induced with pulsatile oxytocin administration is a potent stimulus for the excitation of magnocellular oxytocin neurones via afferent pathways, which are normally activated during the delivery of pups. Thus, the birth of pups appears not to be a prerequisite for the reflex excitation of SON neurones at the end of pregnancy in rats.

Fig. 3.1. Induction of delivery with an oxytocin pulse treatment
Time of onset of delivery in hours after 10.45 h (= start of a 4 hour oxytocin pulse treatment) on day 21 of pregnancy was compared for animals given each 10 min for 4 hours an injection of either isotonic saline (open bar, n=9), 1 mU oxytocin (wide hatched bar, n=4), 1 mU oxytocin for 2 hours and then 2 mU oxytocin for 2 hours (wide cross-hatched bar, n=3), 2 mU and then 4 mU oxytocin (narrow hatched bar, n=2), 5 mU and then 10 mU oxytocin (narrow cross-hatched bar, n=2) or 10 mU and then 20 mU oxytocin (filled bar, n=14). Since in the lower dose oxytocin-treated groups the numbers of rats per group were small (n=2-4), we indicated individual values by filled circles, rather than calculating the standard error.
Animals that were given 10 and 20 mU oxytocin for 4 hours delivered significantly earlier than saline treated rats (***$p<0.01$, U-test), while all animals given lower doses of oxytocin pulses taken together delivered significantly later than saline-treated rats ($p<0.05$, U-test).

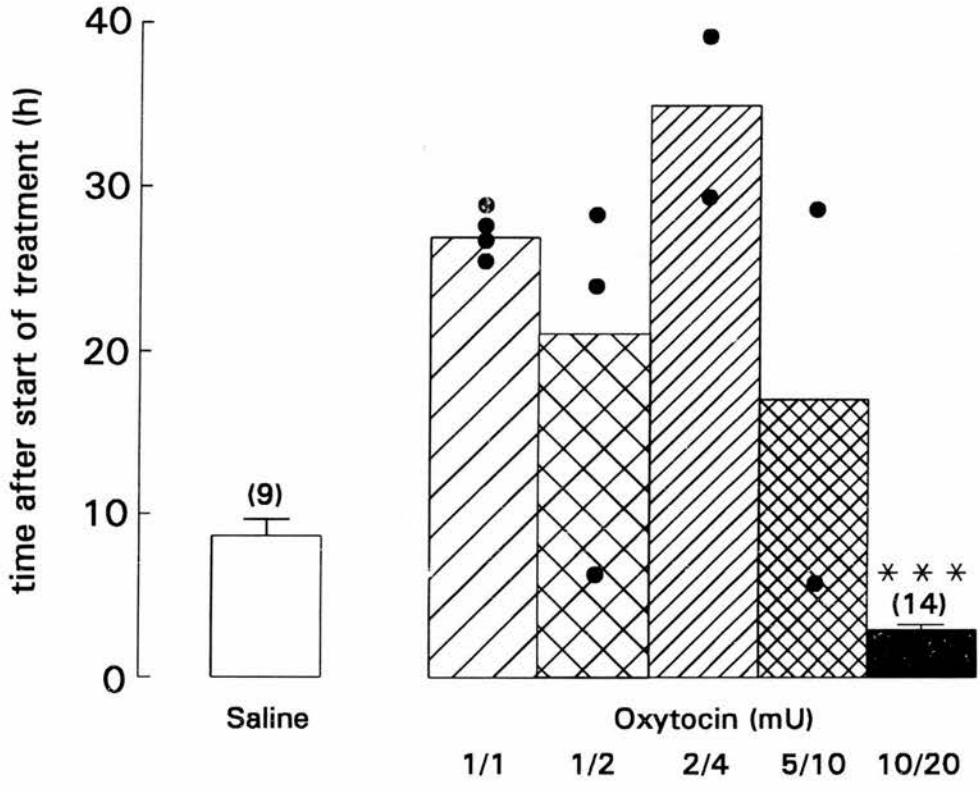

40
30
20
10
0
time after start of treatment (h)
(9)
Saline
* * *
(14)
Oxytocin (mU)
1/1
1/2
2/4
5/10
10/20

Fig. 3.2. Onset and progress of delivery during an oxytocin pulse treatment
Top: The time of onset of delivery in each rat injected with saline (0.03 ml each 10 min for 4 hours, open circles) or oxytocin pulses (10 mU each 10 min for 2 hours: wide hatched area and 20 mU oxytocin each 10 min for 2 hours: narrow hatched area, filled circles) on the day of expected term (day 21 of pregnancy) was assessed. Animals killed before delivery has occurred but after the end of the treatment are represented by a crossed circle.
While none of the control rats delivered during the saline-injections, 14/18 oxytocin-treated rats (77%) started to give birth during the 4 hour oxytocin treatment (median time of the onset of delivery: 3.3 h vs 8.1 h after start of the treatment in oxytocin- and saline-treated rats, ***$p<0.01$, U-test).
Bottom: Individual birth intervals were compared for rats injected with oxytocin or saline pulses for 4 hours.
There was no significant difference between oxytocin- (filled bars, n=14) and saline-treated rats (open bars, n=9) for any of the first eleven birth intervals (intervals between pups 1-12).

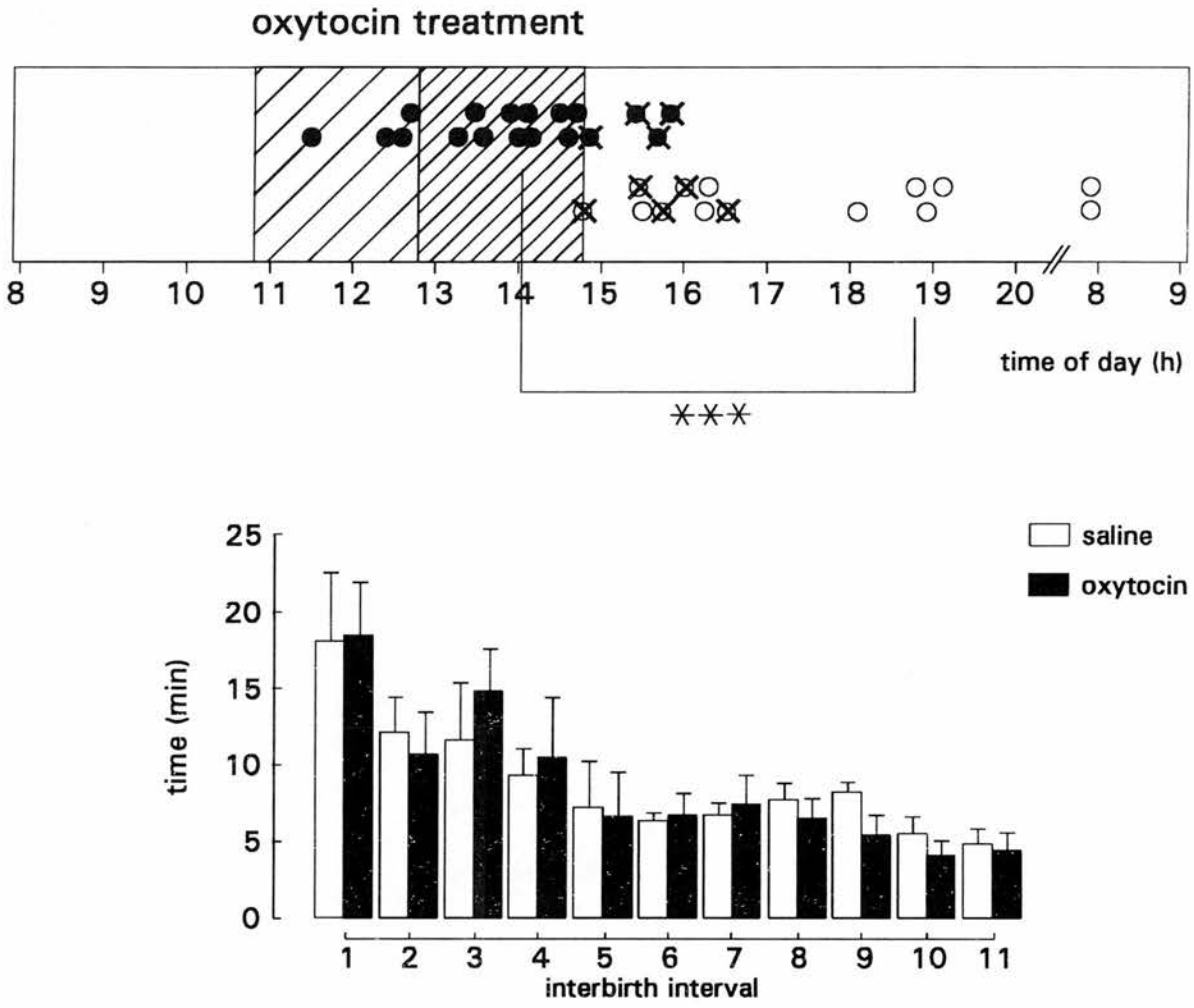

oxytocin treatment
8
9
10
11
12
13
14
15
16
17
18
19
20
8
9
time of day (h)
***
saline
oxytocin
time (min)
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
interbirth interval

Fig. 3.3. Fos expression in the SON of oxytocin-treated prepartum and parturient rats
Rats were given pulses of oxytocin (10/20 mU oxytocin for 4 hours) or saline and were killed by cervical dislocation after the treatment, before or during (90 min after delivery of the second pup) delivery. The brains were frozen, cut into coronal sections (15 μm) through the hypothalamus and processed for Fos standard immunocytochemistry.
In saline-treated prepartum rats there were few Fos-immunoreactive nuclei in the SON (A), while in the SON of oxytocin-treated rats (10 mU oxytocin each 10 min for 4 hours) killed before (B) and during parturition (C) we found a much greater incidence of Fos-immunoreactive nuclei, that was similar to the incidence in saline-treated parturient rats (D). oc = optic chiasm, scale bar 0.1 mm

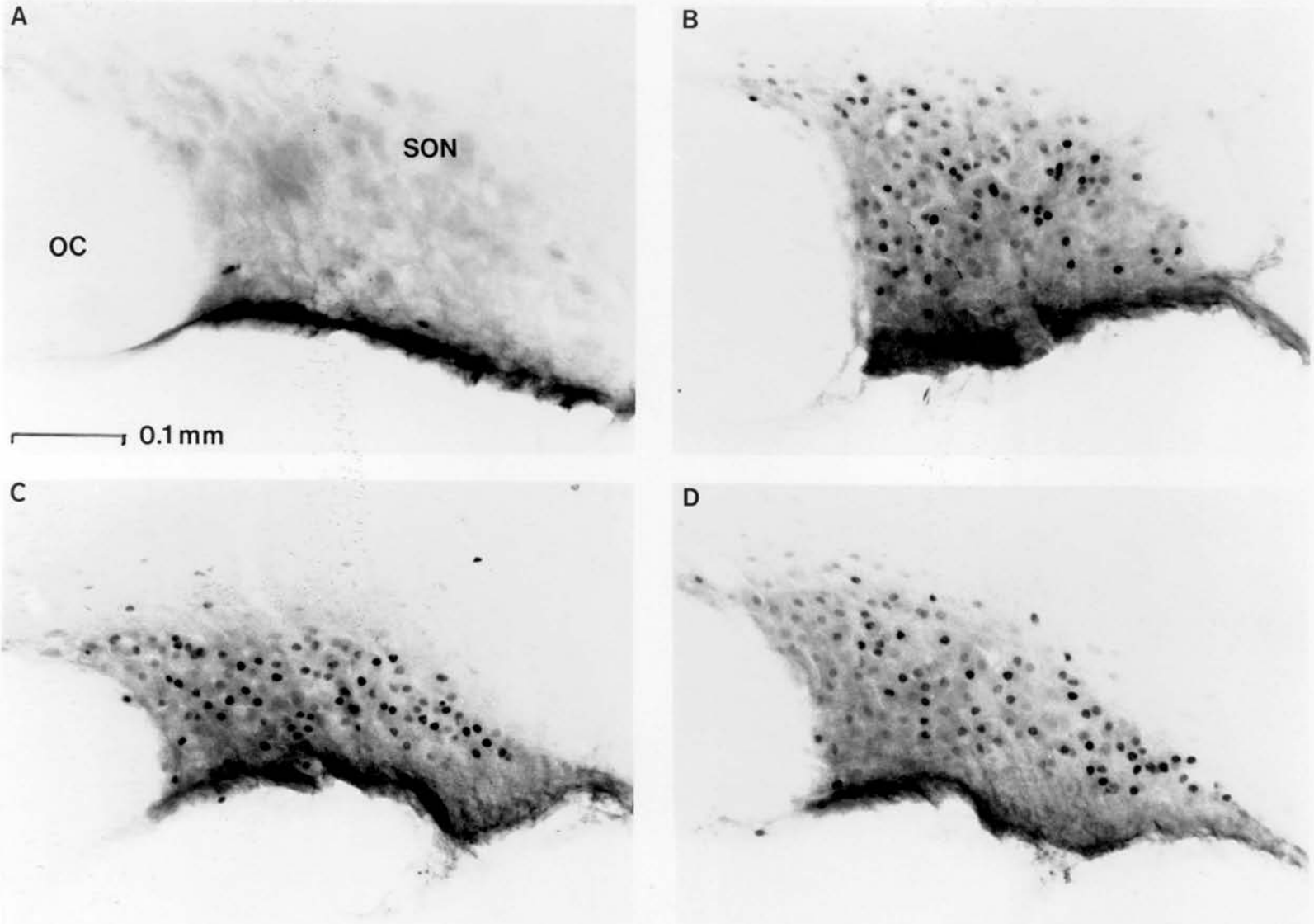

A
SON
OC
0.1 mm
B
C
D

Fig. 3.4. Fos expression in the NTS of oxytocin-treated prepartum and parturient rats
Similar to the SON, in saline-treated prepartum rats there were few Fos-immunoreactive nuclei in the NTS (A), while in the NTS of oxytocin-treated (10 mU oxytocin each 10 min for 4 hours) rats killed before (B) and during parturition (C) we found a much greater incidence of Fos-immunoreactive nuclei, that was similar to the incidence in saline-treated parturient rats (D).
AP = area postrema, cc = central canal, scale bar 0.1 mm

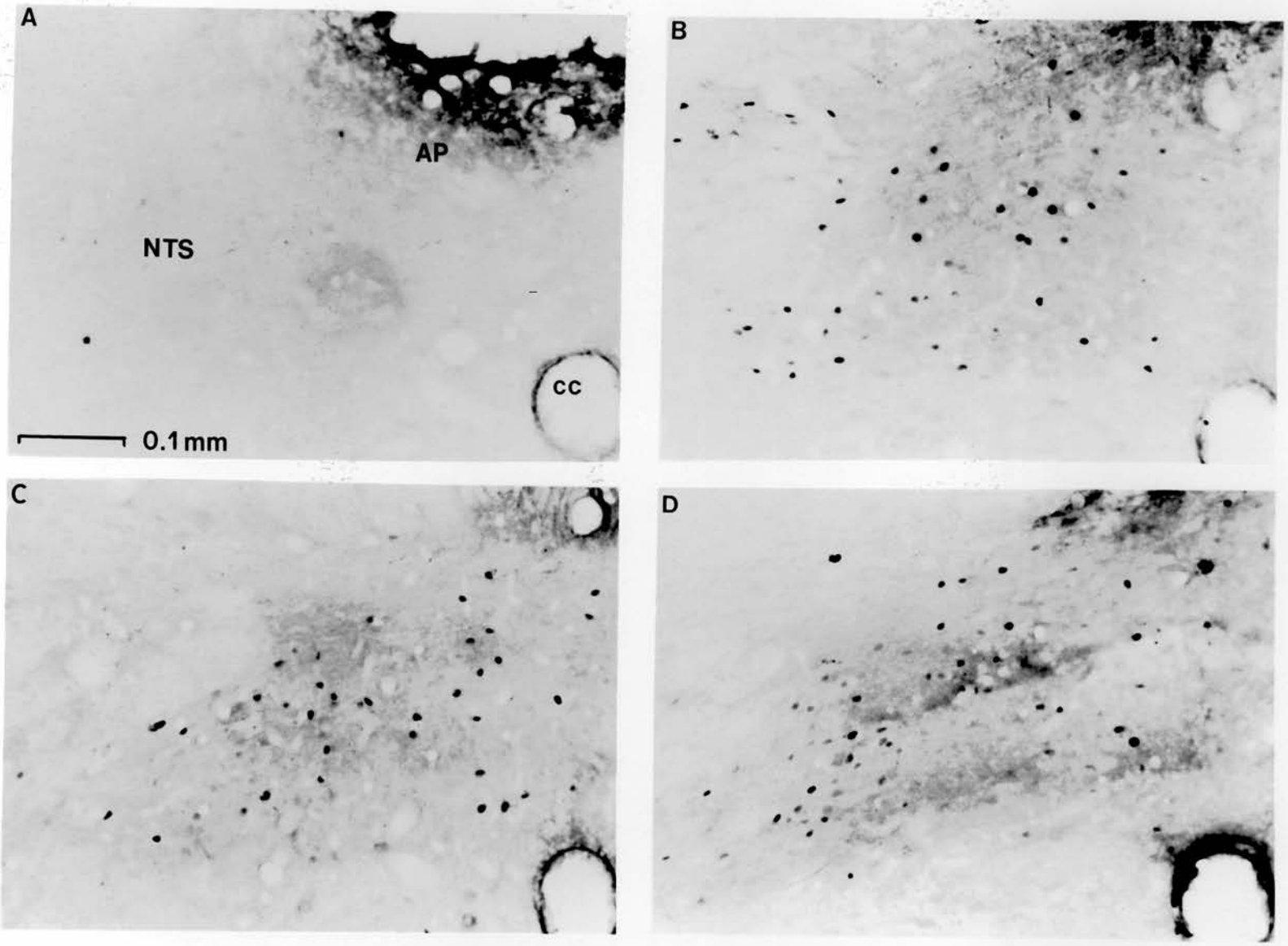

A
AP
NTS
cc
0.1 mm
B
C
D

Fig. 3.5. Fos expression in the SON and NTS of oxytocin-treated prepartum and parturient rats

Rats were injected with pulses of oxytocin (10/20 mU for 4 hours) or saline and killed before (prepartum) or during (parturient) delivery and the brains processed for Fos immunocytochemistry. The numbers of Fos-immunoreactive nuclei were counted on sections (on average 9-12 sections per area per rat) through the rostro-caudal extent of the SON and the NTS.

The number of Fos-immunoreactive nuclei per section SON and NTS (means± s.e.m.) was significantly higher in oxytocin-treated (filled bars, n=4) compared to saline-treated (open bars, n=5) prepartum rats (*$p<0.05$, Kruskal-Wallis test), but similar to that in the SON and NTS of parturient rats (oxytocin-treated rats, n=14 and saline-treated, n=9). In all parturient rats, the incidence of Fos-immunoreactive nuclei was significantly greater than that in saline-treated prepartum rats (*$p<0.05$, Kruskal-Wallis test).

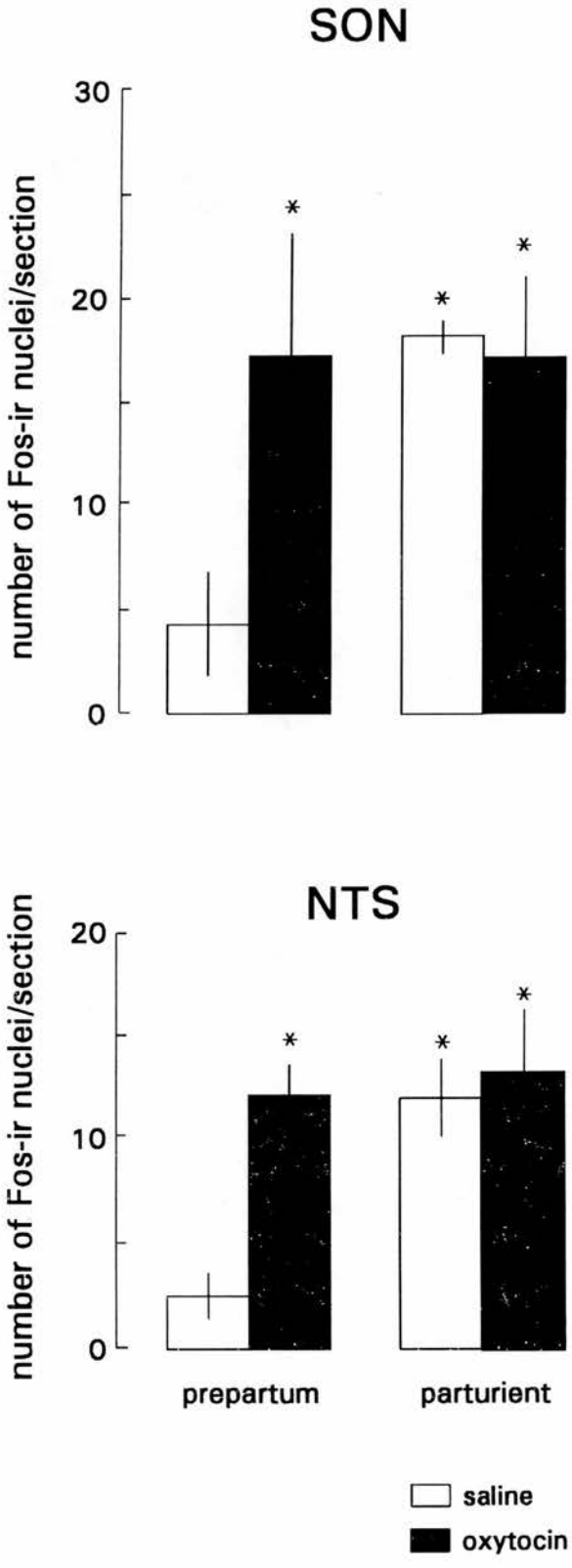
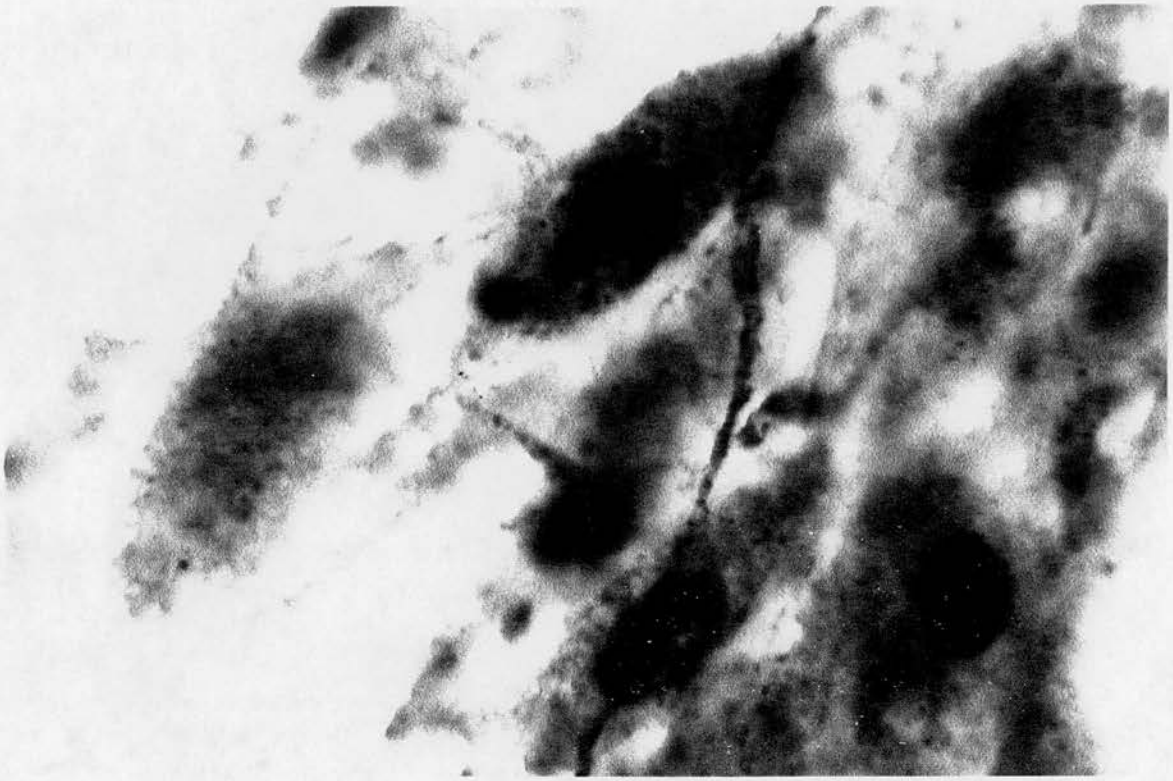

SON
30
20
10
0
number of Fos-ir nuclei/section
*
*
*
NTS
20
10
0
number of Fos-ir nuclei/section
*
*
*
prepartum
parturient
saline
oxytocin

Fig. 3.6. Fos expression in supraoptic oxytocin and vasopressin neurones during delivery that has been induced with pulses of oxytocin
High magnification view of a section through the SON processed for double immunocytochemistry for Fos (dark purple nuclear staining) and oxytocin (light brown cytoplasmic staining, **Top**) or vasopressin (light brown cytoplasmic staining, **Bottom**) in a rat in which delivery has been induced with pulses of oxytocin.

Oxytocin- and Fos-immunoreactive cells in the SON of a parturient rat



Vasopressin- and Fos-immunoreactive cells in the SON of a parturient rat

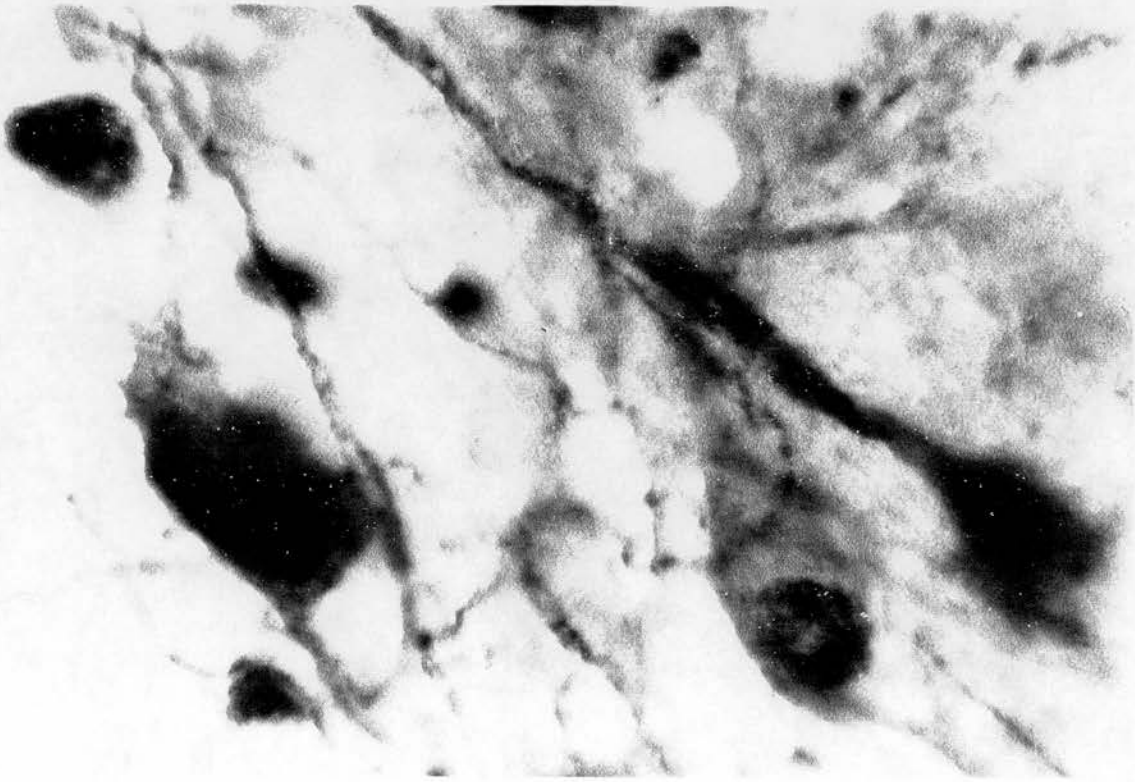
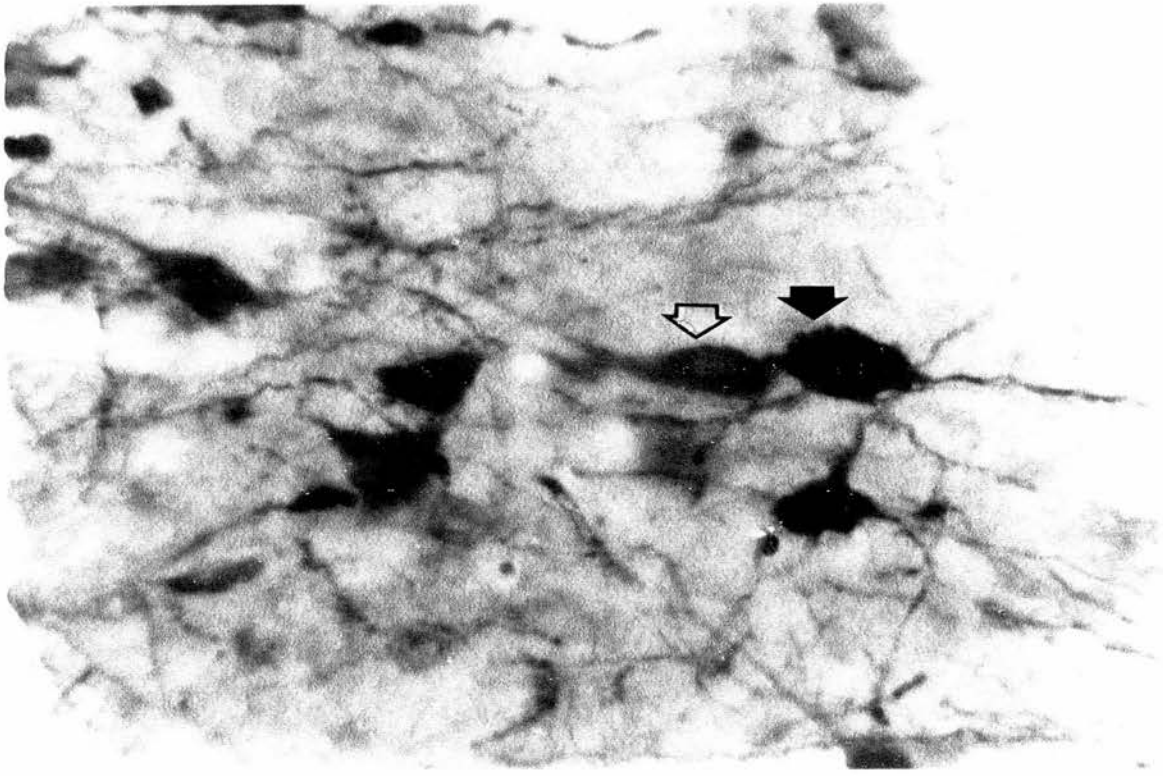


Fig. 3.7. Fos expression in catecholaminergic NTS neurones during delivery that has been induced with pulses of oxytocin
High magnification view of a section through the NTS (A2 region) at the level of the area postrema processed for double immunocytochemistry for Fos (dark purple nuclear staining) and tyrosine hydroxylase (TH, the rate limiting enzyme for catecholamine synthesis, light brown cytoplasmic staining) in a rat in which delivery has been induced with pulses of oxytocin.

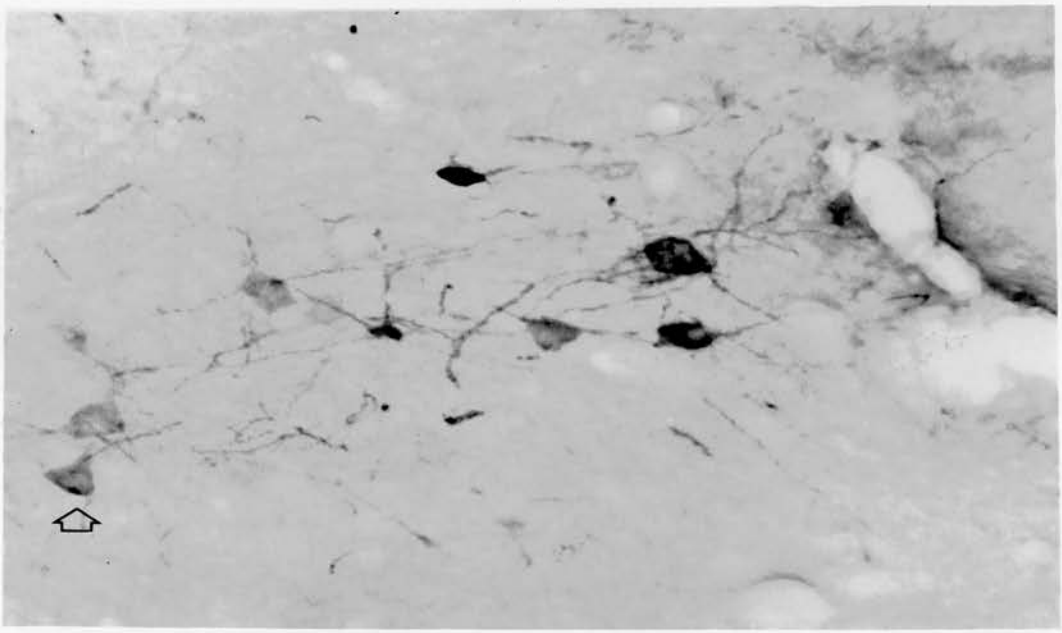
TH- and Fos-immunoreactive cells in the NTS



___ 10 μm

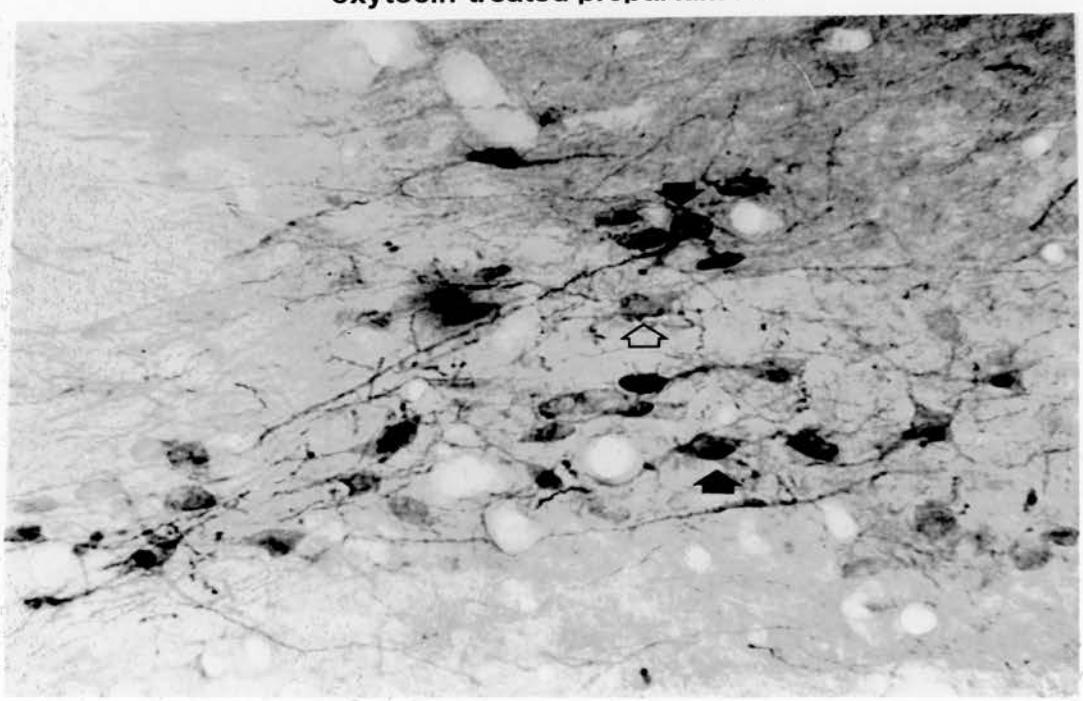
Fig. 3.8. Fos expression in catecholaminergic NTS neurones during delivery that has been induced with pulses of oxytocin
Double immunocytochemistry for Fos (dark nuclear staining) and TH (light cytoplasmic staining) in the A2 region at the level of the area postrema in rats injected with saline **(Top)** or oxytocin (10/20 mU, **Bottom)** for 4 hours and killed before delivery of any pups.
In the NTS of saline-treated rats, there were very few cells stained for either Fos or TH, while in oxytocin-treated rats we observed more cells stained for Fos, TH (open arrow) or both Fos and TH (filled arrow).
Scale bar 100 μm

saline-treated prepartum rat



NTS

oxytocin-treated prepartum rat



NTS

100 μm

Fig. 3.9. Incidence of cells stained for Fos, TH or both throughout the NTS and the ventrolateral medulla (VLM)

The incidence of cells immunoreactive for Fos (**Top**), TH (**Middle**) or both Fos and TH (double-labelled cells, **Bottom**) was assessed per six sections throughout the rostro-caudal extent, from 1.98 mm caudal to 0.18 mm rostral of the obex, of the NTS and the ventrolateral medulla (VLM) in oxytocin- (filled circles, n=12) and saline-treated (open circles, n=7) prepartum rats.

The incidence of cells (means±s.e.m.) immunoreactive for Fos (**Top**), TH (**Middle**) or both Fos and TH (double-labelled cells, **Bottom**) was always higher in oxytocin- compared to saline-treated prepartum rats in both the NTS (A2/C2 region) and the VLM (A1/C1 region). *$p<0.05$, comparing saline- and oxytocin treated rats at each level, U-test, Bonferroni correction applied as appropriate

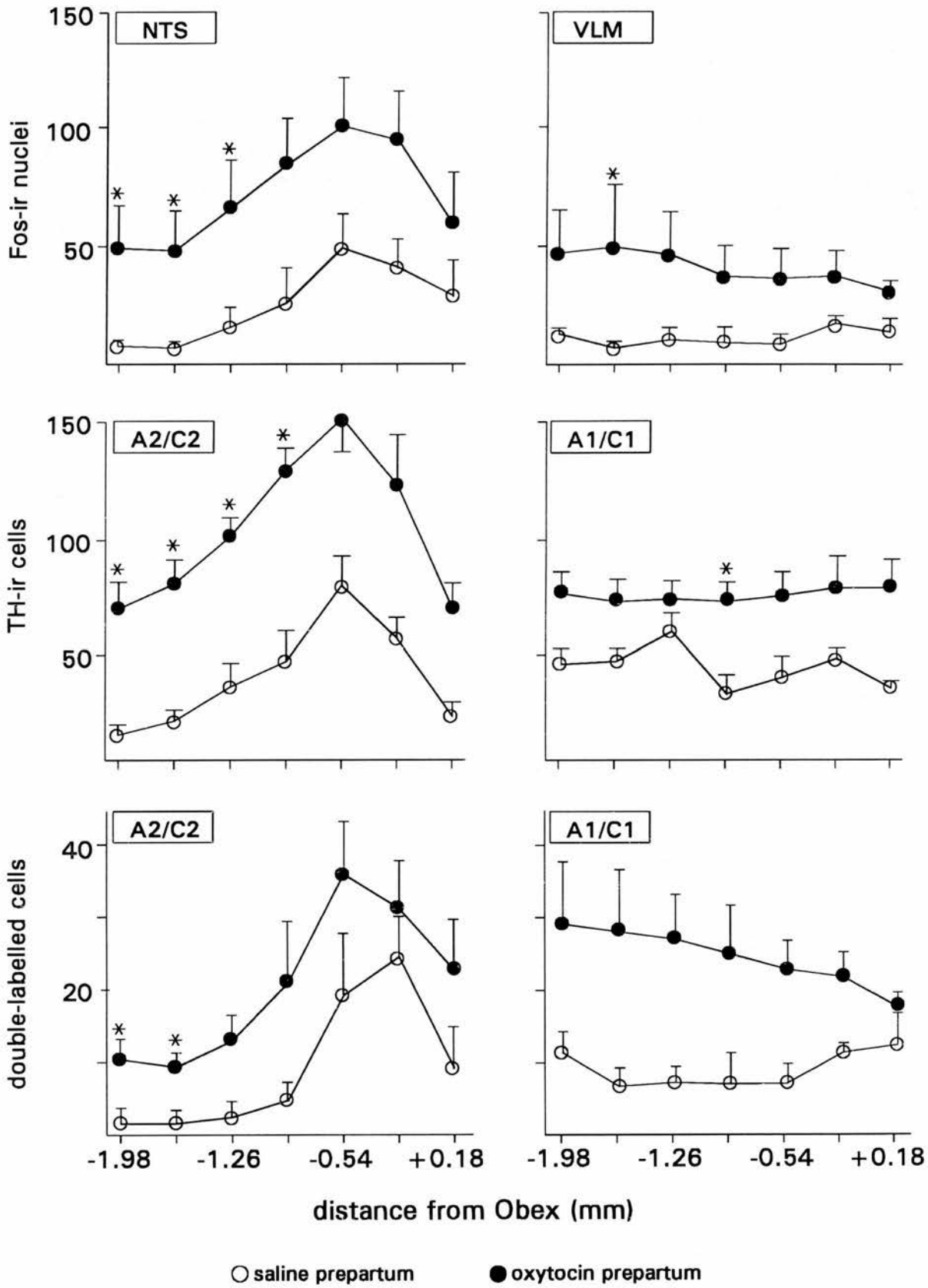
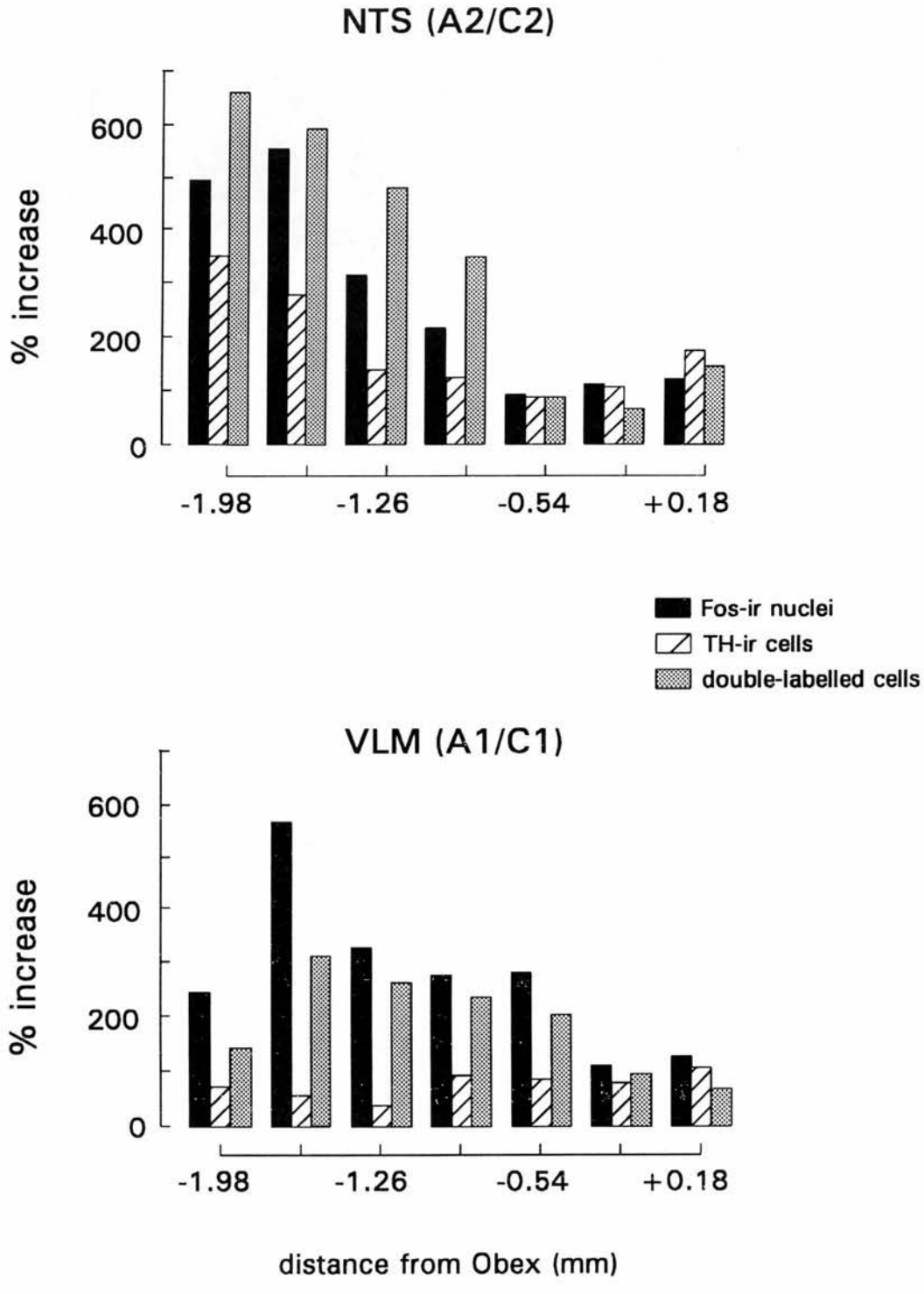

NTS
VLM
A2/C2
A1/C1
A2/C2
A1/C1
Fos-ir nuclei
TH-ir cells
double-labelled cells
150
100
50
40
20
-1.98
-1.26
-0.54
+0.18
distance from Obex (mm)
○ saline prepartum
● oxytocin prepartum

Fig. 3.10. Percentage increase in the number of cells immunoreactive for Fos, TH, or both throughout the brainstem of oxytocin-treated prepartum rats

The percentage increase in the number of cells immunoreactive for Fos (filled bars), TH (hatched bars) or both Fos and TH (double-labelled cells, shaded bars) was analysed throughout the rostro-caudal extent of the NTS (A2/C2 region) and the ventrolateral medulla (VLM, A1/C1) for saline- (n=7) and oxytocin-treated (n=12) prepartum rats. The difference in the number of cells stained for Fos, TH and Fos and TH is expressed as % increase in oxytocin- compared to saline-treated rats.

Oxytocin-treated rats showed always a higher number of labelled cells than saline-treated rats, particularly in the NTS, though the increase was more pronounced in the caudal compared to the rostral parts of the NTS.


NTS (A2/C2)
% increase
600
400
200
0
-1.98
-1.26
-0.54
+0.18
Fos-ir nuclei
TH-ir cells
double-labelled cells
VLM (A1/C1)
% increase
600
400
200
0
-1.98
-1.26
-0.54
+0.18
distance from Obex (mm)

Chapter 4

Oxytocin antagonists delay the initiation of parturition and prolong its active phase in rats

In the previous chapters, I have concluded from the effectiveness of pulsatile administration of oxytocin at inducing and maintaining delivery in rats, that endogenous oxytocin might play a critical role for parturition. Here, I administered two novel peptide oxytocin antagonists to late pregnant and parturient rats in order to further examine the importance of endogenous oxytocin in parturition. Although oxytocin is not the sole contractile agent at the uterus, since locally synthesised prostaglandins have also strong uterotonic effects amongst other local and systemic agents (Chan & Chen 1992, Chan *et al.* 1991, Fuchs 1987, Soloff 1989), oxytocin is one of the most potent substances at augmenting labour in humans and rats (Dawood 1989, Randolph & Fuchs 1989). However, the involvement of oxytocin in the initiation of parturition is less clear (Fuchs 1987), partly due to conflicting reports on gestation length after oxytocin antagonist (Chan *et al.* 1991) or oxytocin

antibody (Kumaresan *et al.* 1971) administration and a much smaller rise in plasma oxytocin concentrations before, than during parturition in many species (Fuchs *et al.* 1991, Thorburn & Challis 1979). Furthermore, the myometrial oxytocin receptor concentration in rats rises dramatically only shortly before delivery (Alexandrova & Soloff 1980, Fuchs *et. al.* 1982) and in parallel there is a dramatic increase in uterine sensitivity to oxytocin in the last hours before delivery (Fuchs 1969, Fuchs & Poblete 1970). Thus, the initiation of parturition by exogenous oxytocin has generally been found to require prolonged administration at high doses (see chapter three, Fuchs & Poblete 1970, Fuchs *et al.* 1983). Beside oxytocin, prostaglandins are known for their uterotonic action (Chan 1977), and α–adrenergic receptors in the rat myometrium are involved in uterine contractions at term (El Alj *et al.* 1988, Legrand *et al.* 1987), while stimulation of β–adrenergic receptors mediates uterine quiescence through most of pregnancy (El Alj *et al.* 1989) and hence β-adrenergic receptor agonists are commonly used for threatened preterm labour in humans (Higby *et al.* 1993, Kupferminc *et al.* 1993).

The objectives of the present study were first, to investigate the importance of oxytocin for the progress of delivery during the early and later stages by administering two novel peptide oxytocin antagonists, F382 (Ferring, Sweden) and OVT16 (Manning *et al.* 1989), systemically to rats during the delivery of pups, and second, to investigate the role of oxytocin in the initiation of parturition by giving repeated injections of an oxytocin antagonist to animals on the day of expected parturition but before the delivery of any pups. Furthermore, we thought to examine electrical activity of SON neurones during parturition, using Fos immunocytochemistry, to test the hypothesis that a reduction of uterine contractions in parturient rats following oxytocin antagonist administration alters the neurosecretory activity of oxytocin neurones and hence Fos expression.

Material and Methods

Animals

Date mated rats (Wistar rats in Babraham and Sprague-Dawley rats in Edinburgh) were implanted with a jugular vein cannula either on day 19 of pregnancy under halothane anaesthesia (Wistar rats) or on day 20 under brief ether anaesthesia (Sprague-Dawley rats). On the day of expected parturition (day 21 of pregnancy for both strains of rats) animals were continuously observed for signs of delivery. Once the first pup was born, animals were

carefully monitored and the time of each pup birth was recorded. During the dark period the observation was continued in red light.

Drugs

Oxytocin (Syntocinon, Sandoz) was diluted in isotonic saline. Two peptide oxytocin antagonists were used, desGly^9,d(CH$_2$)$_5$[Tyr(Me)^{2}Thr^4]OVT (OVT16) (Manning *et al.* 1989) and the compound F382® (des-Gly^9-[D-Trp^2,alloIle^4,Orn^8]dC$_6$OT, Ferring, Sweden). The effective dose (ED) was defined as the dose (in nmol/kg) that reduces the response to 2 units of agonist to equal the response to 1 unit; *in vivo* antioxytocic ED, measured by changes in the integrated uterine pressure in oestrogen-pretreated rats, is 1.7±0.3 (OVT16) and 2.9±0.2 (F382) (Manning *et al.* 1989 and P. Melin, personal communication). In contrast, both the *in vivo* antivasopressor and the antidiuretic ED of OVT16 are much greater (7.3±1.3 and ~100, respectively, Manning *et al.* 1989), thus clearly showing OVT16 to be a highly selective oxytocin antagonist. The *in vivo* duration of the antagonistic action in the uterine assay is more than 60 min for OVT16 (Manning *et al.* 1989) and 220±29 min for F382 (P. Melin, personal communication, calculated from the time at which there was an initial inhibition of the uterine response to oxytocin of at least 50% until recovery of 75% of the original response). Both peptide oxytocin analogues were dissolved in isotonic saline containing 0.03 mM HCl and were given in a volume of 0.05 ml/100 g body weight per injection, at a concentration of 30 or 60 µg/kg body weight. Since the pharmacological experiments performed by the supplier of the antagonists indicate a much longer duration of the antioxytocic effects of F382 compared to OVT16, we used both antagonists in the same experimental design to assess their efficacy on slowing the progress of delivery.

Morphine sulphate was dissolved in sterile isotonic saline solution (0.9%) and injected at a concentration of 1 mg/kg body weight in 0.05 ml/100 g rat.

Experimental design

Preliminary pharmacological experiments

Pilot pharmacological experiments were carried out to establish the activity and dose of the oxytocin antagonists needed to

1) prevent oxytocin-induced contractile activity of uterine strips *in vitro,*
2) to abolish milk-ejection in response to oxytocin *in vivo* in anaesthetised rats, and

3) to prevent exogenous oxytocin from restoring the progress of delivery following disruption by morphine treatment.

For that purpose

1) four untreated rats were killed by cervical dislocation 10-16 hours postpartum and the uteri were removed, cut into separate horns, each of which was then connected to a pressure transducer with a preload of 10 g, suspended in 50 ml of De Jalon solution (90 g NaCl, 42 ml 10% KCl, 5 g glucose, 5 g $NaHCO_3$, 2.7 ml 1 M $CaCl_2$, aerating gas 0_2 + 5% CO_2) and essayed in an *in vitro* uteri strip preparation (see detailed description in Russell *et al.* 1989b),
2) three lactating rats (two Sprague-Dawley rats and one Wistar rat, on day 9-13 of lactation, with all but one pup removed 12 hours prior to the experiment) were anaesthetised with urethane (1.25 g/kg body weight iv) and implanted with two venous cannulae each, one cannula in the femoral vein and the other in the jugular vein, to allow separate administration of oxytocin and the oxytocin receptor antagonist. One to three mammary glands were cannulated by G. Leng and J.A. Russell and the polyethylene tubings connected to a pressure transducer, attached to a calibrated pen writer, to allow continuos intramammary pressure recordings. After a minimum recovery time of 60 min, rats were injected with increasing doses of oxytocin (0.25-1 mU oxytocin = 0.5-2 ng/0.1 ml isotonic saline) into the jugular vein, with a minimum of 5 min between injections. Oxytocin was injected for 30-50 min before and 120 min following administration of either OVT16 or F382 (30 µg/kg body weight iv) into the femoral vein and
3) ten term pregnant Sprague-Dawley rats, implanted with a jugular vein cannula under ether anaesthesia on day 20 of pregnancy (for detailed description see above), were injected with morphine (1 mg/kg body weight iv, to prevent pituitary oxytocin secretion, see Russell *et al.* 1989b and chapter one), followed by a single dose of either OVT16 (30 µg/kg body weight iv, n=6) or vehicle (0.05 ml/100 g body weight iv, n=4) immediately after delivery of the second pup. Subsequently, all rats were given intravenous oxytocin injections (1 mU oxytocin (= 2 ng) each 10 min for 90 min), a treatment which I have shown in chapter two to completely restore normal parturition (Luckman *et al.* 1993a).

1) Effects of oxytocin receptor antagonists in vitro on oxytocin-induced uterine activity

Both oxytocin antagonists (OVT16 and F382, final concentration 0.45 μg/ml, within the range expected *in vivo* in extracellular fluid after intravenous injection of 30 μg/kg) added to the *in vitro* uterine strip preparation greatly reduced spontaneous and oxytocin-induced uterine contractile activity (oxytocin concentrations: 0.7-2.8 mU/ml = 1.4-5.6 ng/ml, equivalent to the intravenous administration of 10-40 mU oxytocin) and baseline pressure, while the antagonists did not affect the increase in uterine contractions in response to an injection of prostaglandin PGF2α (0.35μg), that was greater than that induced by the highest dose of oxytocin (5.6 ng = 2.8 mU). For OVT16, the effects of two lower doses (1/4 and 1/16 of the initial dose, final concentration 0.12 and 0.03 μg/ml) were also tested in the *in vitro* uterine strip preparation: while 1/4 of the original dose was similarly effective at reducing spontaneous and oxytocin-induced uterine contractions and decreasing baseline pressure as the initial dose, 1/16 of the dose was only effective at preventing the uterine response to the lowest dose of oxytocin and for a duration of less than 20 min, compared to more than 100 min following administration of the higher doses. Thus, all doses of the oxytocin antagonists that were effective at preventing the increase in uterine activity in response to 1 mU oxytocin were also effective at reducing baseline pressure of the uterus, and both effects showed a duration of more than 100 min for both antagonists.

2) Effects of oxytocin receptor antagonists on the intramammary pressure response to iv oxytocin in lactating rats

Both F382 and OVT16 given intravenously (at a concentration of 30 μg/kg body weight, respectively) to urethane-anaesthetised lactating rats (n=3) prevented the rise in intramammary pressure in response to intravenous oxytocin at increasing doses (0.5-2 ng equivalent to 0.25-1 mU) for at least 80 min (Fig. 1).

3) Effectiveness of OVT16 to block exogenous oxytocin actions during parturition

In rats injected with morphine, vehicle and oxytocin pulses delivery progressed normally as expected from previous experiments (see chapter one), while in rats given the oxytocin receptor antagonist OVT16 instead of vehicle, the effects of exogenous oxytocin to restore delivery were prevented (time between birth of pups 2-7: 78±7.9 min in antagonist- vs

43.8±7.6 min in vehicle-treated rats, U-test, $p<0.04$, Fig. 2), thus confirming the effectiveness of the oxytocin antagonist to block oxytocin actions during parturition.

Oxytocin antagonists (OVT16 and F382) and the progress of parturition

Nineteen rats (nine Wistar and ten Sprague-Dawley rats) were given a single injection of the vehicle immediately after birth of the second pup (0.05 ml/100 g body weight iv) as controls for seventeen oxytocin antagonist-treated rats (seven Wistar rats injected with F382 and ten Sprague-Dawley rats injected with OVT16, 30 μg/kg body weight). The nine Wistar control rats were given a second vehicle injection after birth of the fourth pup as controls for another group of Wistar rats injected with F382 (30 μg/kg body weight iv, n=7) after the birth of the fourth pup only. All animals were carefully monitored and each pup delivery was recorded. Pup survival was assessed 4-6 hours after the birth of the last pup (ratio of pups alive/all pups born) and the presence of milk in the pups' stomachs was noted. Mothers were given an overdose of sodium pentobarbitone (50 mg/kg body weight iv) and after laparotomy the uteri were examined for pups and placentae.

Oxytocin antagonist (F382) and the onset of parturition

Thirteen Wistar rats were injected with F382 (30 μg/kg body weight iv) before delivery of any pups, at 12.00, 14.00 and 16.00 h on the day of expected parturition. Twenty-five rats were given vehicle injections at the same times. Eleven other Wistar rats were injected with F382 (60 μg/kg body weight iv) starting at 10.00 h on day 21 and then every 2 hours until 22.00 h, while seven rats were given the vehicle at the corresponding times (0.05 ml/100 g body weight iv). When rats started to give birth within the treatment period, the injections, vehicle or oxytocin antagonist, were discontinued. As mentioned above, all animals were carefully monitored throughout delivery. Pup survival and the presence of milk in the pups' stomachs was assessed 4-6 hours after the birth of the last pup. Then, mothers were killed and after laparotomy the uteri were examined for pups and placentae.

Statistical analysis

The difference in the time of the onset of parturition between the three groups was calculated by assigning a rank to each rat according to the time of onset of delivery (the earlier the onset the lower the rank) and then comparing the rank sums using the non-parametric

Mann-Whitney U-test (one-tailed). For comparison of the progress of delivery, group means for the cumulative time between delivery of pups 2-4, 2-5, 2-6, 2-7, pups 4-7 and pups 6-10, respectively, were calculated and compared using the non-parametric Mann-Whitney U-test. A difference was considered significant for $p<0.05$. Group data are expressed as means±s.e.m.

Results

The effects of OVT16 and F382 on the progress of parturition

Rats injected with OVT16 or F382 (30 μg/kg) after delivery of the second pup gave birth to the following five pups more slowly than vehicle-injected rats (time from birth of pups 2-7: 100±8 and 83.3±11.7 min in F382 and OVT16 treated animals, respectively, and 51±6.3 and 48.9±6.4 min in the corresponding control groups (U-test, $p<0.05$, Fig. 3).

The cumulative time between pups 2-5 and 2-6 was also significantly longer in antagonist- compared to vehicle-treated rats, while the time between pups 2-4 was significantly longer in F382-treated rats only. When the course of delivery was divided into an early (time between delivery of pups 2-6) and a later stage (time between delivery of pups 6-10), the delay caused by the antagonist was only significant during the early, but not the later stage (Table 1).

Table 1

The effect of oxytocin antagonists (F382 or OVT16) injected after the birth of the second pup on the time to deliver four pups during an early and later stage of delivery

groups	time between pups 2-6	time between pups 6-10
controls (n=7)	38.7±7.9 min	32.3±3.9 min
F382-treated rats (n=9)	88±7.6* min	56.3±6.7 min
controls (n=10)	41±6.0 min	27.4±5.8 min
OVT16-treated rats (n=10)	75.2±12.5* min	32.7±6.2 min

* $p<0.05$, U-test compared to respective control group.

The effect of oxytocin antagonists (F382 or OVT16, 30 μg/kg body weight iv)) injected after the birth of the second pup on the time to deliver four pups during an early and later stage of delivery. Rats given either oxytocin antagonist delivered pups 2-6 significantly slower than control rats, while the time between delivery of pups 6-10 was not statistically different between groups.

Of seven rats injected with F382 (30 μg/kg) after delivery of the fourth pup, four rats showed no prolongation of the interbirth intervals following the injection (cumulative time from pups 4-7: 24.7±2.9 min (n=4) vs 27.5±3.1 min in controls, Fig. 4, shaded area A = means±s.e.m. of interbirth intervals in control rats). In the remaining three rats, delivery of pups 4-7 was much slower compared to controls and compared to the four unaffected animals (cumulative time from pups 4-7: 86±4.3 min) and similar to rats injected with an oxytocin antagonist after the second pup (shaded area B = means±s.e.m. of interbirth intervals in rats injected with F382 after the birth of the second pup). The delay induced by the antagonist in 3/7 rats was most pronounced for the first interbirth interval following the antagonist injection (time between birth of pups 4-5: 61.3±9.9 min, n=3, vs 9.2±2.5 min in controls), while the two following interbirth intervals were similar to control animals (time between delivery of pups 5-6 and 6-7, respectively: 9±2.5 and 19±4.1 min, n=3, vs 16±3.1 and 13±2.2 min in controls). We did not find any difference between rats affected and those unaffected by the antagonist injection after the birth of pup 4 which could account for this effect. The litter sizes were similar for both groups (11±1 pups/litter in the four not affected rats, range 8-14 pups, and 10±2 pups/litter in the three rats that showed a delay following the antagonist injection, range 8-14 pups) and all pups were suckled 4-6 hours after the completion of delivery.

Maternal behaviour, as assessed by vaginal licking, licking of pups during and after delivery, retrieval and grouping of pups and nest building, was normal in antagonist-treated animals. Pup survival 4-6 hours after delivery of the last pup was similar between all groups, regardless of treatment or strain of rats (79 - 85%) and all pups were suckled at the time of killing. Post mortem examination of the uteri revealed that none of the animals had pups or placentae left *in utero*. The average litter size was not different between groups (experimental Wistar rats had on average 10.5±0.5 pups per litter, and controls had 11.8±0.5 pups, both Sprague-Dawley groups had a mean of 12.3±0.4 pups per litter).

Of 31 Wistar rats not treated with F382 before delivery, 21 rats with litters of more than 11 pups all delivered between 12.20 h on day 21 and 09.00 h on day 22. The remaining 10 rats all had small litters (7 pups or less) and all delivered after 09.58 h on day 22, with the last one delivering after 9.35 h on day 23. In recognition of this effect of the litter size on the time of onset of parturition, all rats with litters of 7 pups or less were excluded from subsequent analysis.

The effect of F382 on the onset of parturition

As the times of onset of delivery in vehicle-treated rats from both control groups were similar, we have combined both control groups for statistical comparisons. Thus 53% (17/32) of animals injected with vehicle before the birth of any pup delivered in the light phase of day 21 of pregnancy and nine rats (28%) delivered in the first half of the next light phase (between 07.00 h and 13.00 h on day 22, Fig. 5), while during the dark phase only six rats (19%) started to give birth.

In rats given three repeated doses of 30 μg/kg F382 before the birth of any pups (at 12.00, 14.00 and 16.00 h), delivery occurred throughout the night, with no apparent preference for the light phase (4/13 rats started to deliver during the light phase on day 21, 4/13 during the dark phase and 5/13 during the day 22 light phase). The difference of the rank sums of the times of onset of delivery for the antagonist and control group with the Mann-Whitney U-test just failed to reach significance ($p<0.06$), although the median time of onset of delivery was later in antagonist-treated rats (04.08 h on day 22 vs 18.52 h on day 21, Fig. 5). Only one rat (that had been injected with F382 before delivery of any pups) had to be excluded from subsequent analysis on the basis that it had a small litter (5 pups, as revealed by post mortem examination in the morning of day 23 of pregnancy, by which time it had not yet given birth).

When seven injections of F382 at twice the previous dose (60 μg/kg) were given, starting at 10.00 h on day 21 and then every 2 hours until 22.00 h, the onset of delivery was significantly delayed compared to vehicle-treated animals (U-test, $p<0.01$) and the median time of onset of delivery was significantly later in the antagonist-treated group compared to control rats (10.23 h on day 22 vs 18.52 h on day 21 in controls, U-test, $p<0.01$, Fig. 5). Only two of eleven antagonist-treated animals started to deliver during the treatment (in the light phase of day 21), while the remaining nine rats gave birth in the light phase of the following day. The time between the birth of the second and the seventh pup was similar in all groups (63±11.5 min in control rats, 72.5±15.3 min and 61.8±5.2 min in rats previously injected with 30 or 60 μg/kg F382, respectively). At the end of the study (4-6 hours after completion of delivery) all pups were suckled and no pups or placentae were detected in the uteri in any rat.

Discussion

This study provided evidence for an involvement of oxytocin during both the initiation and the maintenance of parturition in rats. The role of oxytocin in initiating parturition has remained controversial due to the previous failure of oxytocin antagonists (Chan & Chen 1992, Chan *et al.* 1991) or oxytocin antibodies (Kumaresan *et al.* 1971) to prolong gestation significantly in rats, while being effective at prolonging the progress of delivery and disrupting maternal behaviour (Van Leengoed *et al.* 1986). I have now demonstrated that repeated administration of an oxytocin antagonist significantly prolongs gestation. The need for multiple injections could be related to the half-life of the antagonist, since a sustained action is obviously required to delay the onset of parturition for several hours compared with a relatively brief action to slow parturition once it has started. These data are in agreement with observations made in humans, indicating an acute decrease in uterine contraction frequency during a two hour infusion of a peptide oxytocin antagonist, but no long-term effect on the onset of delivery after such treatment (Goodwin *et al.* 1994). Here, rats treated with F382 before delivery of any pups started to give birth later, while the progress of the delayed delivery was normal, indicating that the antioxytocic effect of the antagonist was overcome. Since I have also demonstrated the effectiveness of OVT16 to prevent exogenous oxytocin from restoring delivery that has been disrupted by morphine, the prolongation of delivery seen in response to oxytocin antagonist administration during parturition can be attributed to the antioxytocic effects of the antagonists used.

The dose of oxytocin required as an agonist to initiate delivery is higher than that needed to augment labour and delivery that has started spontaneously (Fuchs & Poblete 1970, c.f. chapter one and three). This raises the possibility that the initiation of parturition may involve the activation of endometrial oxytocin receptors, leading to prostaglandin release (Chan *et al.* 1993). The endometrial oxytocin receptors might be more difficult to access from blood for both circulating oxytocin and oxytocin antagonists than myometrial receptors, since the vascularisation of the myometrial layer is greater than that of the endometrium, and particularly than that of the epithelial layer, into which only few vessels reach (Ramsey 1989). Thus, the lower dose of oxytocin agonist and antagonist required to affect the progress of parturition can be explained by a myometrial site of action, while the higher dose of either agonist or antagonist needed to affect the initiation of parturition is consistent with the hypothesis that such effects are mediated by endometrial oxytocin receptors, which are less accessible for blood-born substances. Stimulation of endometrial oxytocin receptors, e.g. by

local oxytocin, induces the release of prostaglandins (Chan & Chen 1992, Chan *et al.* 1993, Fuchs *et al.* 1982), that is important for the formation of gap junctions (Chan *et al.* 1991, Garfield & Beier 1989), for the induction of myometrial oxytocin receptors (Chan *et al.* 1991, Fuchs *et al.* 1984) and the effacement of the cervix (Huszar & Naftolin 1984), the latter of which can also be initiated by oxytocin itself (Khalifa *et al.* 1992). All of these changes are essential for the generation of strong contractions during labour and delivery and thus might be involved in the positive feedback onto hypothalamic neurosecretory oxytocin neurones (see chapter three). The physiological importance of endometrial oxytocin receptors in the uterus is supported by reports of oxytocin mRNA and peptide in the human (Miller *et al.* 1993a) and the rat endometrium at term (Lefebvre *et al.* 1992b), opening up the possibility that an increase in local oxytocin secretion, that is not necessarily reflected by a rise in circulating oxytocin concentrations, might contribute to the initiation of parturition.

In addition, there might be an involvement of vasopressin receptors, which are present in the uteri of rats (Chan *et al.* 1990), rabbits (Maggi *et al.* 1991a) and women (Maggi *et al.* 1990) and mediate uterine contractility. However, in contrast to oxytocin receptors, the vasopressin receptor concentration, at least in rabbits and humans, does not increase at term (Maggi *et al.* 1991a, 1992) and an infusion of vasopressin, unlike oxytocin, cannot induce labour in late pregnant rats (Fuchs & Poblete 1970). Also, both antagonists used in this study have been reported to bind with a much higher affinity to the rat oxytocin compared to the rat vasopressin receptor (Manning *et al.* 1989 and Melin, personal communication).

Once parturition has been initiated and delivery of pups has started, a single dose of an oxytocin antagonist prolonged delivery by about one hour, but did not prevent parturition, which is in agreement with the reported half-life of OVT16 (Manning *et al.* 1989). Our data showing that an oxytocin antagonist prolongs delivery more consistently when injected after the birth of the second rather than the fourth pup might indicate that delivery is more dependent on oxytocin during the early compared to the later stages of parturition. This could be due to factors other than oxytocin, e.g. prostaglandins, which are released during the process of parturition (Behrens *et al.* 1993) and can perhaps restore uterine activity that has been reduced by the antagonist treatment and thus ensure that delivery is completed even if the action of oxytocin is blocked. In addition, oxytocin plasma concentrations rise during the course of delivery and might diminish the effect of the antagonist treatment (Higuchi *et al.* 1986b). Due to the moderate effect on the progress of delivery following administration of peptide oxytocin antagonists, that was much less pronounced than the delay observed in

response to morphine (see chapter one), I have not assessed Fos expression in the SON of rats given an antagonist treatment during parturition. Repeated injections and/or administration of a non-peptide oxytocin antagonist, which is more resistant to enzymatic degradation, might result in a greater delay of pup birth and hence might be a suitable model to examine whether uterine activity is a critical factor for the induction of Fos expression in the SON and in putative afferent neurones during parturition.

Beside its classic uterotonic role, oxytocin has been shown to soften the cervix (Khalifa *et al.* 1992) and a high density of cervical oxytocin receptors (Gorodeski *et al.* 1990) supports an active role of cervical contractions (Olah *et al.* 1993) during the expulsive phase. These findings, together with the observation that preterm labour is accompanied by an increase in oxytocin receptor concentrations similar to that in term labour (Garfield *et al.* 1982, Garfield & Beier 1989) warrant further exploration of the effects of oxytocin antagonists in preterm labour, particularly as there seem to be no adverse effects on foetal or maternal well-being following treatment with oxytocin antagonists (Goodwin *et al.* 1994), while the current treatment of preterm labour with β-adrenergic agonists can have serious consequences for both the mother and the foetus (Kupferminc *et al.* 1993) and an uncertain efficacy (Higby *et al.* 1993).

In summary, I suggest that in the rat, oxytocin plays a key role both before and during the delivery of pups, as systemic administration of an oxytocin antagonist significantly delays the onset of delivery and prolongs its progress, the latter effect being more pronounced during the early than later stages of delivery.

Fig. 4.1. Effect of an oxytocin receptor antagonist on intramammary pressure
Intramammary pressure (in mmHg) was recorded from a cannulated mammary gland of a lactating rat (day 12 of lactation, after removal of all but one pup 12 hours prior to the experiment) in response to intravenous injections of oxytocin (0.25-1 mU iv, open arrows) before and after an injection of the oxytocin receptor antagonist F382 (30 μg/kg body weight iv).
Repeated injections of oxytocin resulted in a dose-dependent increase in intramammary pressure. After an intravenous injection of the F382 the intramammary response to oxytocin was completely abolished for 80 min, while 120 min after the antagonist injection the intramammary pressure increase to 1 mU oxytocin was fully restored.

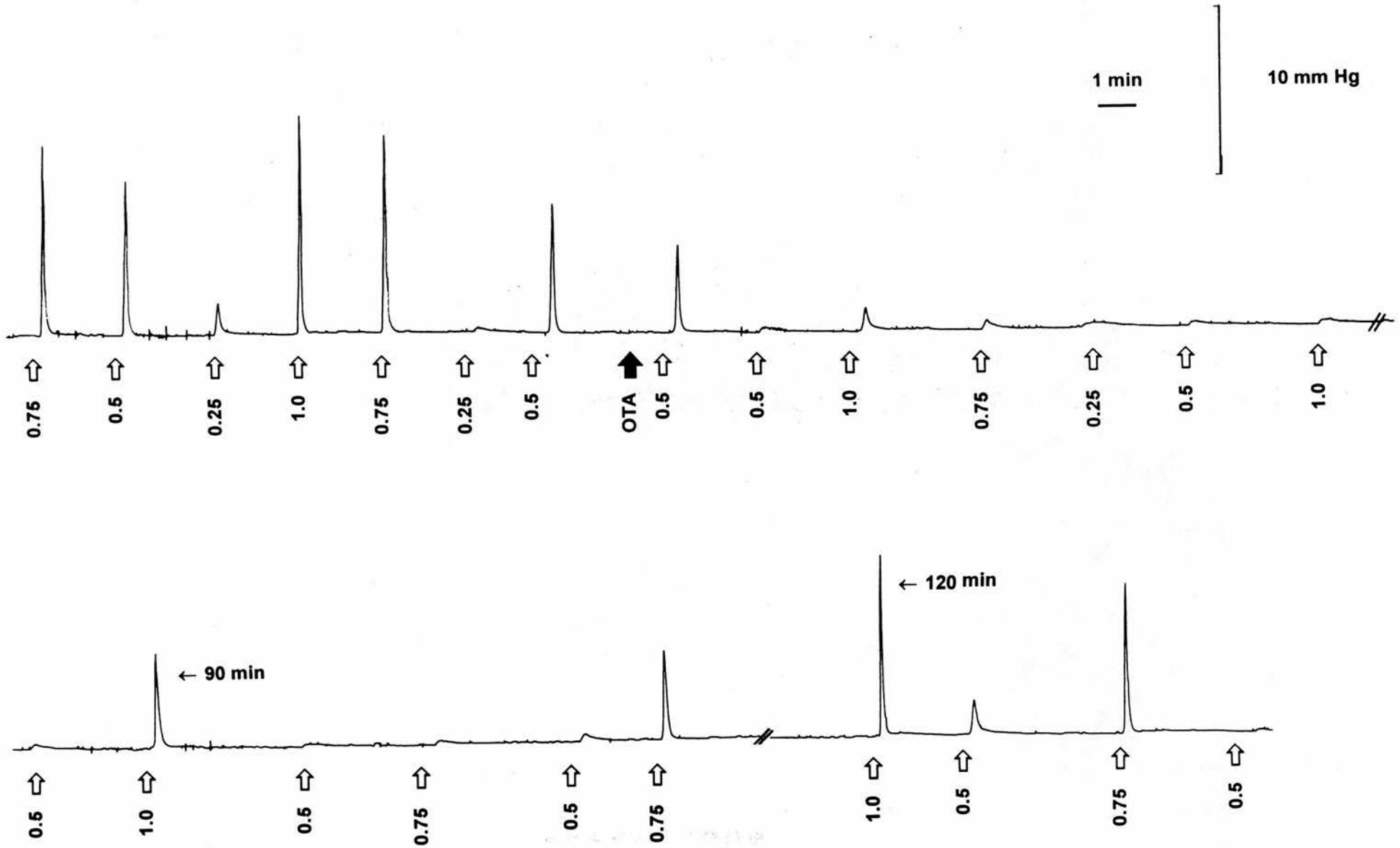

1 min
10 mm Hg
0.75
0.5
0.25
1.0
0.75
0.25
0.5
OTA
0.5
0.5
1.0
0.75
0.25
0.5
1.0
← 90 min
← 120 min
0.5
1.0
0.5
0.75
0.5
0.75
1.0
0.5
0.75
0.5

Fig. 4.2. Effectiveness of the oxytocin receptor antagonist OVT16 to block exogenous oxytocin actions during parturition

Animals were injected intravenously after the birth of the second pup with either a single injection of vehicle (controls, open bar, n=10), the oxytocin receptor antagonist OVT16 (30 μg/kg body weight iv, hatched bar, n=10), morphine (1 mg/kg body weight) plus vehicle (0.05 ml/100 g body weight iv) followed by pulses of oxytocin (1 mU oxytocin each 10 min iv, cross-hatched bar, n=4) or morphine plus OVT16 (30 μg/kg body weight iv, filled bar, n=6) followed by pulses of oxytocin and the cumulative time for delivery of pups 2-7 was compared between groups.

While rats given morphine, vehicle and oxytocin pulses showed no delay in delivering subsequent pups compared to vehicle treated rats, in rats treated with morphine, OVT16 and oxytocin delivery was significantly prolonged compared to controls and animals given morphine plus vehicle plus oxytocin pulses (*$p<0.05$, U-test). There was no significant difference between rats given only a single injection of OVT16 and rats given morphine, OVT16 and pulses of oxytocin.

These data suggest that OVT16 is highly effective at preventing exogenous oxytocin actions during parturition.

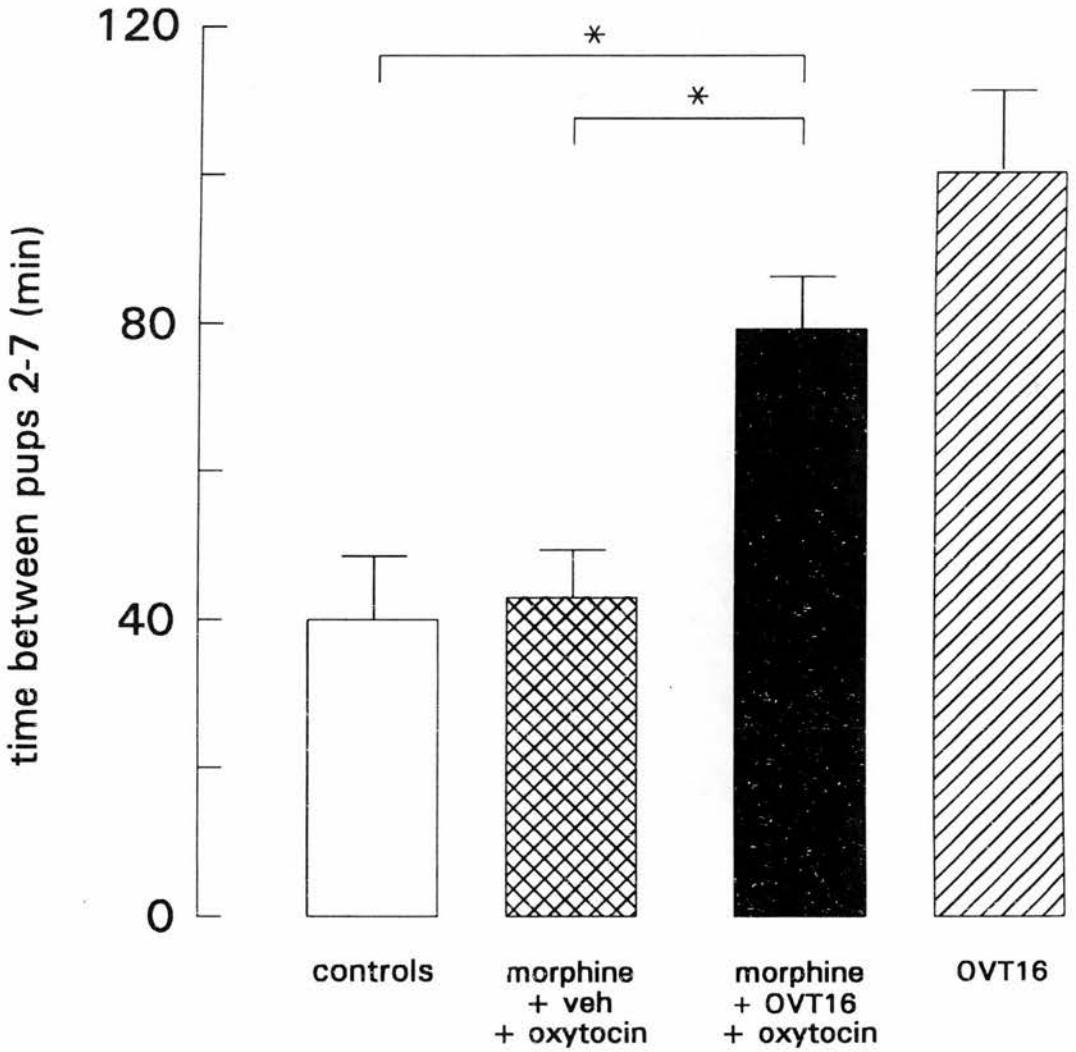

120
80
40
0
time between pups 2-7 (min)
*
*
controls
morphine
+ veh
+ oxytocin
morphine
+ OVT16
+ oxytocin
OVT16

Fig. 4.3. Effect of two oxytocin-antagonists (OVT16 and F382) on the progress of parturition

Wistar rats (open circles, n=9) and Sprague-Dawley rats (open squares, n=10) were given a single injection of the vehicle immediately after the birth of the second pup (0.05 ml/100 g body weight iv) as controls for rats injected intravenously with either F382 (filled circles, n=7) or OVT16 (filled squares, n=10) at a dose of 30 µg/kg body weight each (values are means±s.e.m.).

Antagonist-treated rats showed a delay in the delivery of the subsequent five pups, which was most pronounced during the first 80 min following the antagonist injection.

*$p<0.05$, U-test, compared to the respective controls

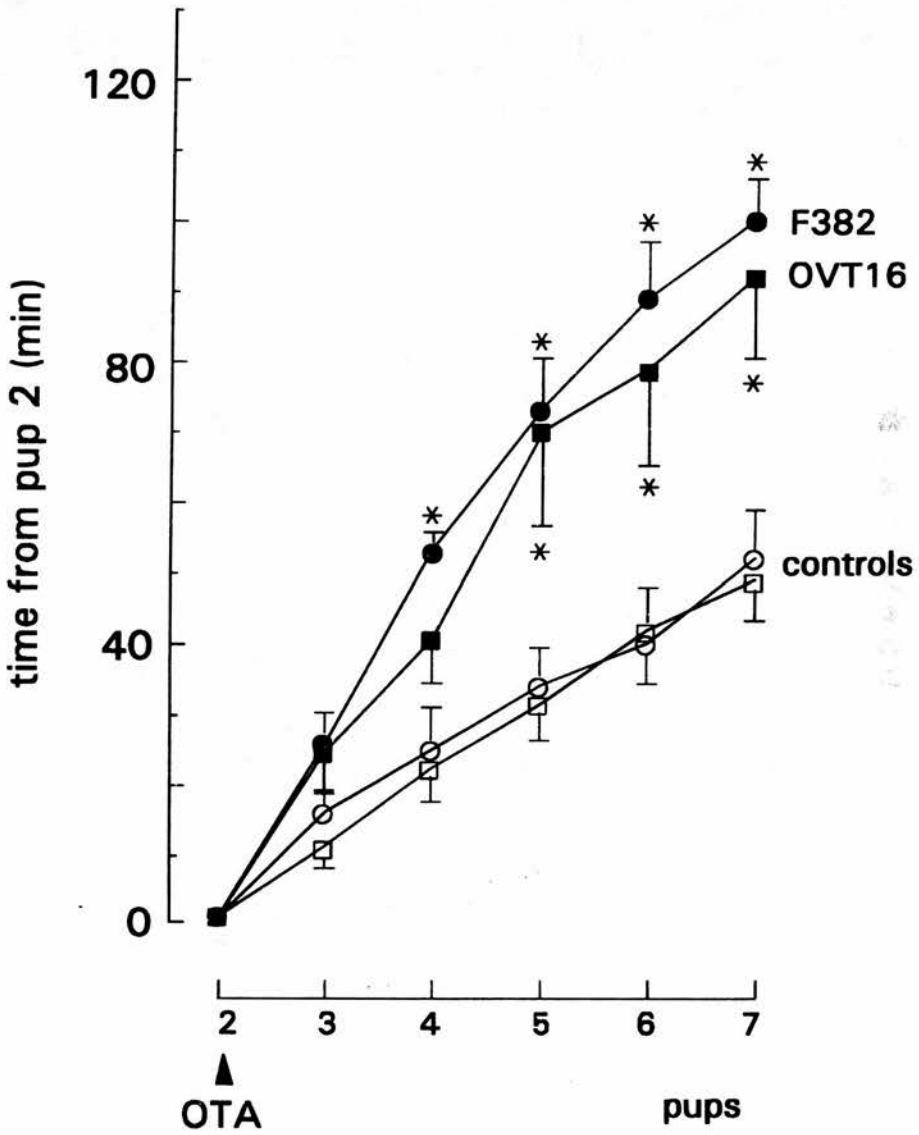

120
80
40
0
time from pup 2 (min)
F382
OVT16
controls
2
3
4
5
6
7
OTA
pups

Fig. 4.4. Effect of an injection of F382 after the birth of pup 4 on the progress of parturition

Seven Wistar rats were injected with the oxytocin antagonist F382 (30 μg/kg body weight iv) after the birth of the fourth pup and interbirth intervals were recorded. Four rats (filled circles) delivered the subsequent three pups within the control time range (shaded area A = means±s.e.m. of control animals). The remaining three rats (filled squares) delivered pups 4-7 in the time range of rats injected with F382 after the birth of the second pup (shaded area B = means±s.e.m. of rats injected with F382 after pup 2) and thus slower than control animals.

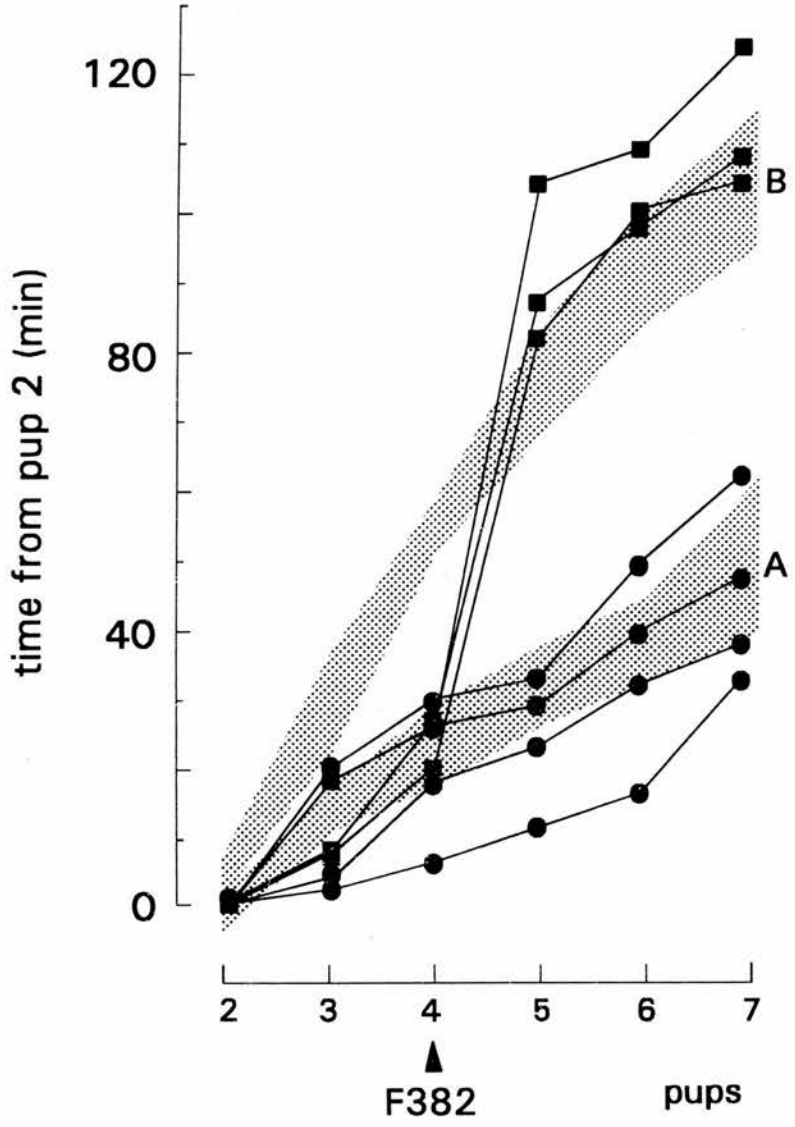
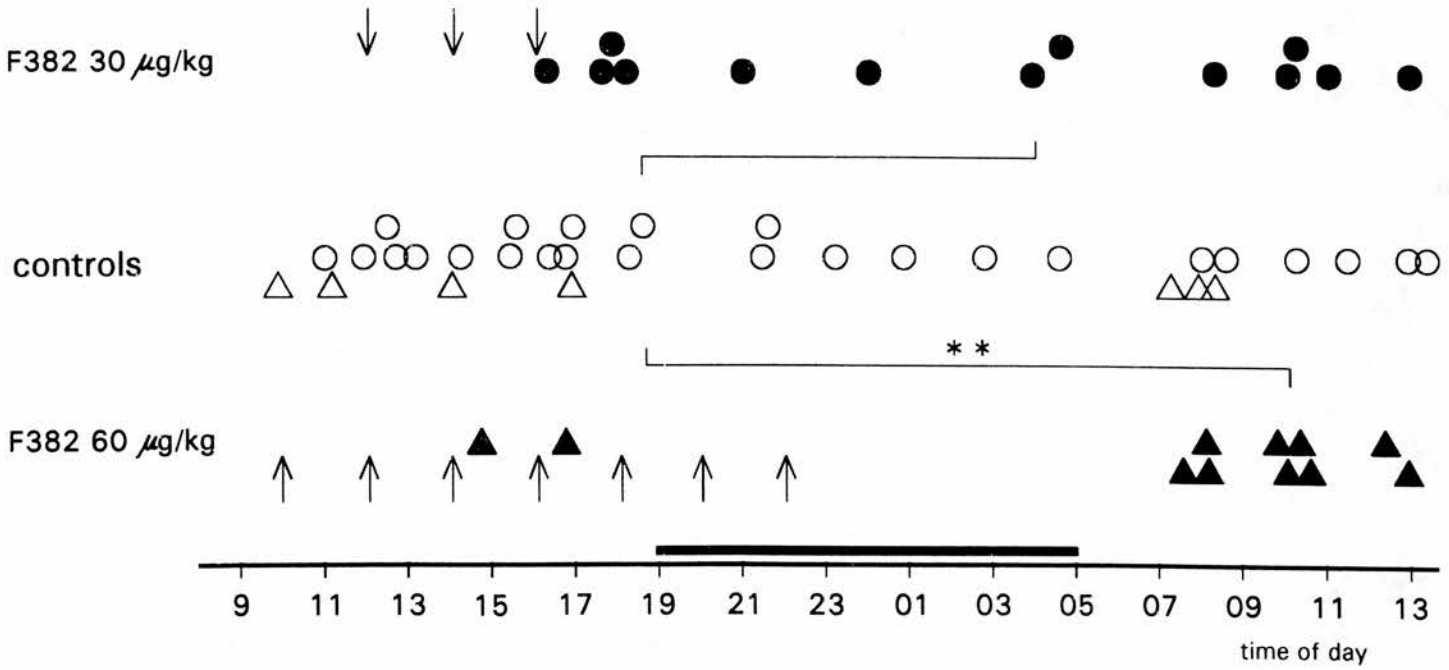

120
80
40
0
time from pup 2 (min)
B
A
2
3
4
5
6
7
F382
pups

Fig. 4.5. Dose-related effects of the oxytocin antagonist F382 on the onset of parturition

Rats were given either three intravenous injections of F382 at a concentration of 30 μg/kg body weight (filled circles represent the time of birth of the first pup in individual rats, n=13), or seven injections of F382 at a concentration of 60 μg/kg body weight (filled triangles, n=11) starting in the morning of the day 21 of pregnancy. Thirty-two animals were given three (open circles, n=25) or seven (open triangles, n=7) vehicle injections at the same time as experimental animals the antagonist. The x-axis indicates the time of day, bars represent the dark phase. The overall median of the time of onset of delivery for the two control groups combined (18.52 h on day 21) was earlier than that in rats injected with the oxytocin antagonist. Though the difference just failed to reach significance for rats treated with three injections of F382 (30 μg/kg, upper panel, median time: 04.08 h on day 22, $p<0.055$ vs controls), the delay was highly significant for the group given seven injections of F382 (60 μg/kg, lower panel, median time: 10.23 h on day 22, $^{**}p<0.01$, U-test, compared to controls).


F382 30 µg/kg
controls
F382 60 µg/kg
* *
9
11
13
15
17
19
21
23
01
03
05
07
09
11
13
time of day

Chapter 5

Progesterone treatment in late pregnancy delays the onset of delivery and prevents the normal activation of supraoptic neurones during such delivery

Delivery in rats is preceded by a decrease in the plasma progesterone concentration, resulting in an increased ratio of plasma oestrogen/progesterone (Csapo & Wiest 1969). This switch from a state of relative progesterone dominance during most of pregnancy to one of greater oestrogen dominance at the end of gestation is considered to be a crucial step towards the initiation of labour (Csapo & Wiest 1969, Csapo *et al.* 1980, Fuchs *et al.* 1983). Under the increasing influence of oestrogen, uterine changes, including the formation of gap junctions and the expression of oxytocin receptors, which are important for successful delivery, take place (Alexandrova & Soloff 1980, Chan *et al.* 1991, El Alj *et al.* 1993, Fuchs *et al.* 1983, Puri & Garfield & Beier 1982). If the normal fall in plasma progesterone is postponed, these changes are prevented and gestation is prolonged (Bosc *et al.* 1987, Garfield *et al.* 1982).

Furthermore, high levels of progesterone during pregnancy have been implicated in a functional deafferentation of the term pregnant uterus (Morizaki *et al.* 1989), that might contribute to a reduced transmission of sensory information from the uterus to the CNS during pregnancy and following progesterone administration (Frye & Duncan 1994). Beside a peripheral site of action, systemic progesterone also reduces the noradrenaline content of the hypothalamus and the medulla (Chaudhuri *et al.* 1992), possibly due to a reduced noradrenaline synthesis and release and oxytocin release, induced by vaginal distension, has been shown to be inhibited by an acute intracerebroventricular administration of progesterone (Roberts 1971).

In addition, oxytocin immunoreactivity in the hypothalamus increases at the end of pregnancy in rats (Jirikowski *et al.* 1989) and increasing concentrations of oestrogen have been implicated in contributing to these changes (Jirikowski *et al.* 1988). Since central oxytocin can induce maternal behaviour (Pedersen *et al.* 1982, McCarthy 1990) and maternal behaviour is initiated at the end of pregnancy (Numan 1988), when the plasma ratio of oestrogen/progesterone concentrations increases, a change in gonadal steroid concentrations in favour of oestrogen at term seems to affect both the uterus and the CNS, and thus could be critical for the normal initiation of parturition.

Although the factors involved in the initiation of parturition are not fully understood, we have shown in the previous chapters that oxytocin is of importance for both the initiation and the progress of delivery in rats. Thus, oxytocin might augment uterine contractions, which will then stimulate the reflex release of pituitary oxytocin via a catecholaminergic projection from the NTS to the SON (see chapter three, Onaka *et al.* 1995b). The recent finding of oxytocin mRNA and peptide in the endometrium of term pregnant rats (Lefebvre *et al.* 1992b) and humans (Miller *et al.* 1993a) opens up the possibility that local oxytocin initiates uterine activity which then feeds back onto hypothalamic magnocellular neurones to stimulate the release of oxytocin during parturition. The increased neurosecretory activity of magnocellular oxytocin neurones during delivery is reflected by increased expression of Fos, the protein product of the immediate early gene c-*fos*, in the SON and putative afferent neurones in the NTS (Fenelon *et al.* 1993, Luckman *et al.* 1993a, see chapter three). Using immunocytochemistry for Fos, a marker for neuronal activation (see previous chapters), we investigated the effects of a systemic injection of progesterone to late pregnant rats on the onset of delivery and the activation of supraoptic neurones and putative afferent neurones in the brainstem during delivery. Since recently, a dramatic increase in uterine oxytocin mRNA

content at term has been discussed as a mechanisms by which local synthesis and release of oxytocin could affect the timing of delivery (Chibbar *et al.* 1993, Lefebvre *et al.* 1992b), we also examined whether progesterone treatment would affect the increase in uterine oxytocin mRNA content in late pregnancy.

Material and Methods

Animals

On day 20 of pregnancy, at 09.30 h, date mated female Sprague-Dawley rats were given an injection of either progesterone (5 mg, Intervet, n=48) or vehicle (0.2 ml of 0.3vol% cresol, 89.7vol% arachis oil and 10vol% benzylalcohol, kindly provided by Intervet, n=23) subcutaneously into the right outer calf.

For intravenous oxytocin (Syntocinon, Sandoz, dissolved in sterile 0.9% saline) or saline administration twenty progesterone-treated rats were implanted with a jugular vein cannula one day prior to the experiment under brief ether anaesthesia. Eight progesterone-treated rats were implanted with a jugular vein cannula in the morning of day 20 of pregnancy and the other twelve progesterone-treated rats were implanted with a cannula in the morning of day 21. In the morning of the experiment, the cannulae were flushed with 0.1 ml heparinised saline (50 IU heparin/ml saline) and connected to oxytocin- or saline-filled polyethylene tubings.

All animals were assessed every 5-10 min from the morning of day 21 of pregnancy onwards for signs of labour (stretching, straining, vaginal bleeding), pup delivery and maternal behaviour (nest building, excessive grooming, licking of external genitals, licking of the pups during and after delivery, pup retrieval). Onset of delivery was defined as the time when the first pup was fully expelled and the time of birth of each pup was recorded until at least eleven pups were born. During the dark period the observation was continued in red light.

At the end of each experiment all rats were killed by cervical dislocation, laparotomised and the uteri removed under RNAse free conditions, stripped of foetuses and placentae and quickly frozen on dry ice and stored at -80°C until processed for *in situ* hybridisation (see below). Similarly, the brains were removed and quickly frozen on crushed dry ice and stored at -80°C until processed for immunocytochemistry (see general materials and methods section).

Assessment of weight of pups and foetuses

Three pups or foetuses from each litter were randomly selected (paying particular attention not to select obviously small or large pups/foetuses), and individually weighed. The average of the three measurements was calculated and considered representative for the respective litter. According to the treatment, the average weight of pups or foetuses was calculated for each treatment group.

Experimental design

Effects of progesterone on the onset and progress of delivery and Fos expression in the SON and NTS

Rats injected with either progesterone or vehicle as described above were killed in time-matched pairs, one of each group, either in the afternoon of day 21 of pregnancy but prior to delivery (between 12.00-15.00 h, both n=6, day 21 prepartum groups), during delivery, 90 min after the birth of the second pup (n=10, 11, parturient groups) or 6-12 hours after the birth of the first pup (both n=6, postpartum groups).

Since progesterone-treated rats delivered about one day later than vehicle-treated rats, one group of six progesterone-treated rats was killed in the afternoon of day 22 of pregnancy (between 12.00-15.00 h) prior to delivery (n=6, day 22 prepartum group).

All rats were killed by cervical dislocation and the brains and uteri were removed and frozen until processed for immunocytochemistry.

Effects of oxytocin administration to progesterone-treated rats on the onset and progress of delivery and Fos expression in the SON and NTS

Twelve progesterone-treated rats that had been implanted with a jugular vein cannula on day 20 of pregnancy were given pulses of oxytocin (10 mU oxytocin each 10 min for 2 hours and then 20 mU oxytocin each 10 min for 2 hours, n=8) or saline (0.03 ml each 10 min, n=4) on day 21 of pregnancy, from 11.00-15.00 h and were observed for delivery of pups.

Rats that had not given birth by the end of the treatment were killed between 15.30-16.30 h as described above and the brains were removed and frozen until processed for immunocytochemistry.

Eight other progesterone-treated rats that had been implanted with a jugular vein cannula on day 21 of pregnancy were given on day 22 of pregnancy from 09.00-13.00 h either oxytocin (n=5) or saline pulses (n=3) as described above. Animals were observed for delivery

of pups and were killed either 90 min after the birth of the second pup (parturient group) or time-matched within 90 min after the end of the treatment but before delivery of pups (prepartum groups). All rats were killed as described above and the brains and uteri were removed and frozen until processed for immunocytochemistry or *in situ* hybridisation.

Immunocytochemistry

For immunocytochemistry 15 μm coronal cryostat sections through the hypothalamus or the brainstem were cut and mounted on gelatinised slides. Sections containing the SON were collected throughout the hypothalamus from the anterior commissure to the median eminence. Brainstem sections were collected from the pyramidal decussation to the widening of the fourth ventricle rostral of the area postrema.

Sections through the hypothalamus were processed for Fos immunocytochemistry and sections through the brainstem were divided into two sets, one processed for immunocytochemical detection of Fos and the other for tyrosine hydroxylase (TH), the rate limiting enzyme in the catecholamine synthesis.

On average 9.0±0.4 sections per animal and area (SON or NTS) were counted for Fos-immunoreactive nuclei and 6.0±0.3 sections per animal per NTS for TH-immunoreactive cells, with the identity of the sections coded, using a microscope with a x10 objective and a brightfield condenser.

In situ hybridisation for oxytocin mRNA

Uterine transverse sections (15 μm) were cut on a cryostat under RNAse free conditions, mounted on gelatinised slides and stored at -80°C until processed for *in situ* hybridisation with a synthetic oligonucleotide probe complementary to the bases 912-939 (27 mer) of the rat oxytocin gene (Ivell & Richter 1984, see general materials and methods section).

For the quantitative analysis of oxytocin mRNA expression, an automated image analysis system (Joyce Loebl μMagiscan) was used and the optical density (silver grains/area) in the endo- and myometrium of the uterus was measured as determined by counterstained uterine sections. For each animal two slides and two sections/slide were analysed as follows: for each section the mean of three background measurements was subtracted from the mean of three measurements over each of the two areas of myometrium and the endometrium. Then, the mean for each area (from a total of four sections) per animal was established and finally

we calculated the group means±s.e.m. from individual measurements according to the treatment. To compare the grain density measurements from different autoradiographic films, optical densities measurements were transformed into dpm/mm^3 with a standard curve based on measurements of ten uterine standards (three measurement per standard minus three measurement of background density surrounding the standards) and assuming a 95% efficiency of the scintillation counter.

Statistical analysis

Comparisons between two groups were made with the non-parametric Mann-Whitney U-test. For comparisons between more than two groups the non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc test was used. Comparison of percentages were performed with the two-proportion t-test. A value for $p<0.05$ was considered significant.

Results

The effect of an injection of progesterone on the onset and progress of delivery

Animals injected with the vehicle one day prior to expected term delivered their pups between 11.00 and 20.00 h on day 21 of pregnancy (n=11). By the time the last vehicle-treated rat started to deliver, none of the ten progesterone-treated rats had given birth, but they delivered between 09.00 h on day 22 and 10.00 h on day 23, thus significantly later (28 hours) than controls (U-test, $p<0.001$, Fig. 1, top).

In progesterone-treated rats, delivery of pups was not only greatly delayed, but the progress of parturition was prolonged compared to vehicle-treated rats, particularly during the early stages of parturition (time between delivery of pups 2-6: 80.9±11 min vs 39.1±3 min in controls, Kruskal-Wallis test, $p<0.05$), while the time between delivery of pups 6-10 was not significantly different between groups (38.4±6 min vs 27.2±3.9 min in progesterone- and vehicle-treated rats, Fig. 1, bottom). Furthermore, the birth weight of pups born to progesterone-treated mothers was significantly higher than that of control pups (Fig. 2, top) and at the time of killing (90 min after delivery of pup 2) progesterone-treated rats still had on average 3±1 pups left in the uterus, while none of the vehicle-treated rats had any pups left *in utero* (Kruskal-Wallis test, $p<0.05$).

When neonatal survival was assessed 6-8 hours after the onset of delivery 99±1% of all pups born were alive in the control group compared to only 86±6% in the progesterone-treated group (U-test, $p<0.05$, Fig. 2, bottom). All vehicle-treated rats showed normal maternal

behaviour (licking pup pups, pup retrieval, nest building activity), while in most progesterone-treated rats no such behaviour was observed.

The litter size, which shows a weak negative correlation with the onset of delivery in rats (see chapter four) was not significantly different between groups (14.2±0.7 and 13.6±0.4 pups per litter in prepartum (vehicle- and progesterone-treated) rats and 12.8±1.2 and 12.7±0.9 pups per litter in parturient (vehicle- and progesterone-treated) rats, respectively).

The effect of pulsatile oxytocin treatment on day 21 or day 22 of pregnancy on the onset and progress of delivery in progesterone-treated rats

Animals that were given an injection of progesterone on day 20 and then on day 21 a four hour oxytocin pulse treatment (10 mU each 10 min for 2 hours and then 20 mU each 10 min for 2 hours, n=5) or saline (0.03 ml, n=3) starting at 11.00 h did not deliver during such treatment (Table 1).

Table 1

The effectiveness of pulses of oxytocin at inducing delivery in rats treated with progesterone

	number of rats in which delivery was induced	
groups	day 21	day 22
saline-treated rats	0/3	0/4
oxytocin-treated rats	0/5	6/8*

*$p<0.05$, U-test compared to respective control group.

Pulsatile administration of oxytocin (10 mU oxytocin each 10 min for 2 hours and then 20 mU each 10 min for 2 hours) on day 21 (11.00-15.00 h) was not effective at inducing delivery in rats that had been injected with progesterone (5 mg sc) on day 20 of pregnancy.

In contrast, 6/8 animals (75%) treated with progesterone and then given the same oxytocin pulse treatment as above on day 22 (09.00-13.00 h) started to deliver during the treatment and thus delivered significantly earlier than rats treated with progesterone alone, but later than vehicle-treated rats (Kruskal-Wallis test, $p<0.05$, time of first pup: 09.05, 10.05, 10.06, 12.05, 12.32 and 12.58 h, respectively, Table 1). None of the four saline-treated rats gave birth during the treatment and these rats were killed time-matched with an oxytocin-treated parturient rat after the end of the treatment.

Most rats that had been injected with progesterone and in which delivery had been induced with oxytocin pulses on day 22 showed normal maternal behaviour and the time between delivery of pups 2-6 (61.3±4 min vs 38.4±6 min in vehicle-treated rats, Kruskal-Wallis test, $p<0.05$) was shorter, and neonatal pup survival (99±1%) increased, compared to rats treated with progesterone only. However, delivery of pups 2-6 was still significantly slower than in vehicle-treated rats (Fig. 1, bottom). The time between pups 6-10 was not significantly longer than in vehicle rats (25.3±4.8 min in progesterone plus oxytocin-treated rats vs 27.2±3.9 min in controls). The average pup weight of progesterone-treated rats in which delivery was induced with oxytocin on day 22 was not significantly different from controls (Fig. 2, top).

The litter size was again not significantly different between groups (12.3±2.6 and 11.8±0.4 vs 12.3±1.8 and 12.8±0.3 pups per litter in rats given progesterone plus saline or oxytocin on day 21 and day 22, respectively).

The effect of an injection of progesterone on Fos expression in the SON and NTS of parturient rats

Vehicle-treated animals killed before or 6-12 hours after delivery of pups showed very few Fos-immunoreactive nuclei in the SON (1.5±0.3 Fos-immunoreactive nuclei/section, n=4), while the number of Fos-immunoreactive nuclei was significantly increased in rats killed during parturition (15.6±4.5 Fos-immunoreactive nuclei/section, n=6, Fig. 3). All rats given an injection of progesterone showed few Fos-immunoreactive nuclei in the SON whether they were killed before (day 21 and day 22, 1.7±1.0 Fos-immunoreactive nuclei/section, n=5, and 2.8±0.4, n=4), during (3.7±2.7 Fos-immunoreactive nuclei/section, n=7) or after delivery of pups (0.3±0.2 Fos-immunoreactive nuclei/section, n=3). Thus progesterone-treated parturient rats had significantly fewer Fos-immunoreactive nuclei per section SON than vehicle-treated parturient rats (Kruskal-Wallis test, $p<0.05$, Fig. 3).

In the NTS, vehicle-treated animals killed before or after delivery of pups showed very few Fos-immunoreactive nuclei in the NTS, while parturient rats showed a significantly higher number of Fos-immunoreactive nuclei (0.4±0.1 Fos-immunoreactive nuclei/section, n=4 and 0.8±0.2, n=3 vs 14.4±2.7, n=6, Kruskal-Wallis test, $p<0.05$). Likewise, rats given an injection of progesterone showed few Fos-immunoreactive nuclei in the NTS before (4.4±3.2, 0.3±0.2 Fos-immunoreactive nuclei/section, n=4, 4, on day 21 and day 22, respectively) and after delivery (2.3±1.9 Fos-immunoreactive nuclei/section, n=3), but an increased number of

Fos-immunoreactive nuclei during delivery (17.4±4.9 Fos-immunoreactive nuclei/section, n=6, Kruskal-Wallis test, $p<0.05$). Thus, in the NTS parturient rats regardless of whether they were given progesterone or vehicle, showed an increase in the number of Fos-immunoreactive nuclei compared to pre- and postpartum animals.

Table 2

Correlation between the incidence of Fos-immunoreactive (ir) nuclei in the SON and NTS, TH immunoreactivity in the NTS and the progress of delivery in progesterone- and vehicle-treated rats

correlation between	progesterone-treated rats (n=7)	vehicle-treated rats (n=6)	both groups combined (n=13)
time between pups 2-6 and Fos-ir nuclei in the SON	$r^2 = 0.07$	$r^2 = 0.01$	$r^2 = 0.14$
time between pups 2-6 and Fos-ir nuclei in the NTS	$r^2 = 0.02$	$r^2 = 0.27$	$r^2 = 0.02$
Fos-ir nuclei in the SON and the NTS	$r^2 = 0.02$	$r^2 = 0.35$	$r^2 = 0.03$
Fos-ir nuclei in the SON and TH-ir cells in the NTS	$r^2 = 0.78$*	$r^2 = 0.11$	$r^2 = 0.05$
Fos-ir nuclei in the NTS and TH-ir cells in the NTS	$r^2 = 0.001$	$r^2 = 0.01$	$r^2 = 0.001$

There was no correlation between the number of Fos-immunoreactive nuclei in the SON and the NTS and the time between delivery of pups 2-6 for either progesterone- or vehicle-treated rats, nor for both groups combined. Similarly, we did not observe a correlation between the number of TH-immunoreactive cells in the NTS and Fos expression in the SON in vehicle-treated rats, while in progesterone-treated rats, this correlation was highly significant (*$p<0.01$).

To examine whether the low level of Fos expression in the SON of progesterone-treated rats was the result of the slow progress of delivery, we calculated the correlation for the number of Fos-immunoreactive nuclei in the SON or NTS with the time between the births of pups 2-6 for progesterone- and vehicle-treated parturient rats and for both groups combined (Table 2).

There was no significant correlation between the progress of delivery (assessed by the time between delivery of pups 2-6) and the number of Fos-immunoreactive nuclei in the SON or NTS. Likewise, there was no correlation between the number of Fos-immunoreactive nuclei in the SON and NTS for neither group, nor all parturient rats combined. However, we observed a significant correlation between the number of TH-immunoreactive cells in the NTS and the number of Fos-immunoreactive nuclei in the SON for the progesterone-treated group, but not in the vehicle group, nor for the combined parturient group.

Effect of oxytocin injections on Fos expression in the SON and NTS of progesterone-treated rats

In rats that were given progesterone and then oxytocin or saline pulses on day 21 of pregnancy and killed after the treatment but before delivery of any pups, the number of Fos-immunoreactive nuclei in the SON and NTS was low and not significantly different from vehicle-treated prepartum rats (2.2±0.9 Fos-immunoreactive nuclei/section SON in oxytocin-treated rats, n=5, vs 0.7±0.3 in saline-treated rats, n=3, and 0.9±0.7 Fos-immunoreactive nuclei/section NTS, n=5 vs 3.8±2.9, n=3, Fig. 4).

In progesterone-treated rats, in which delivery was induced with the oxytocin pulse treatment on day 22 and which were killed 90 min after the birth of the second pup, the number of Fos-immunoreactive nuclei in the SON and NTS was higher than in saline-treated rats that did not deliver (6.9±1.7 Fos-immunoreactive nuclei/section SON, n=6 vs 2.2±0.2, n=4, U-test, $p<0.05$, and 2.5±1.3 Fos-immunoreactive nuclei/section NTS, n=6 vs 0.4±0.4, n=4), but lower than in vehicle-treated parturient rats (U-test, $p<0.05$). The two oxytocin-treated rats that did not start to deliver during the injections showed fewer Fos-immunoreactive nuclei per section SON (3.8 and 4.0 Fos-immunoreactive nuclei/section SON) and NTS (1.0 and 3.7 Fos-immunoreactive nuclei/section NTS) than parturient rats, however as there were only two rats in this groups we did not calculate the means±s.e.m.

Fos expression in progesterone-treated rats that were given pulses of oxytocin was lower in both the SON and the NTS when compared with rats injected with oxytocin without progesterone pretreatment (c.f. chapter three, Fig. 4).

The effect of an injection of progesterone on the number of TH-immunoreactive cells in the NTS

Vehicle-treated animals killed before delivery of pups showed very few TH-immunoreactive cells in the NTS, while the number of TH-immunoreactive cells was significantly increased in rats killed during parturition (1.9±0.6 TH-immunoreactive cells/section NTS, n=4 vs 7.3±0.8, n=5, U-test, $p<0.02$, Fig. 5).

In rats given an injection of progesterone the number of TH-immunoreactive cells/section NTS was low in rats killed on day 21 (1.7±0.6 TH-immunoreactive cells/section NTS, n=4) and similar to vehicle-injected rats before delivery. Progesterone-treated rats killed on day 22 but still before delivery had more TH-immunoreactive cells/section NTS than day 21 progesterone-treated rats (4.2±1.5 TH-immunoreactive cells/section NTS, n=4, U-test, $p<0.05$) and progesterone-treated parturient rats showed a small further increase in the number of TH-immunoreactive (6.3±1.2 TH-immunoreactive cells/section NTS, n=6). Thus, the incidence of TH-immunoreactive cells/section NTS in progesterone-treated parturient rats was significantly higher than in progesterone-treated day 21 prepartum rats (U-test, $p<0.01$, Fig. 5), but not significantly different from progesterone day 22 prepartum rats. Though progesterone-treated parturient rats had fewer TH-immunoreactive cells per section NTS than control parturient rats, this difference was not significant.

Rats injected with progesterone on day 20 of pregnancy and treated with oxytocin or saline pulses on day 21 showed a similar number of TH-immunoreactive cells/section NTS (4.6+1.7 and 3.9+1.9 TH-immunoreactive cells/section NTS, n=5, 3), that was not different from control prepartum animals. Progesterone-treated day 22 prepartum rats that were given oxytocin pulses and in which delivery was induced (n=6/8) showed a similar incidence of TH-immunoreactive cells in the NTS as progesterone plus saline treated rats (2.2±0.8 and 2.3±0.7 TH-immunoreactive cells/section NTS, respectively, n=6, 4) and as control prepartum rats (1.9±0.6 TH-immunoreactive cells/section NTS).

The effect of progesterone on the uterine OT mRNA content (Fig. 6 & 7)

The endometrial OT mRNA content was significantly higher in vehicle-treated rats on day 21 prepartum than in parturient and postpartum rats (1205±105 dpm/mm^3 vs 461±52 and 281±20 dpm/mm^3, respectively, Kruskal-Wallis test, $p<0.05$). In progesterone-treated rats, the endometrial oxytocin mRNA content in day 21 prepartum rats was similar to that in vehicle-treated prepartum rats and significantly higher than in day 22 prepartum, parturient or

postpartum progesterone-treated rats (1026±106 dpm/mm^3 vs 161±44 and 248±57 and 147±40 dpm/mm^3, Kruskal-Wallis test, $p<0.05$). The myometrial oxytocin mRNA content was not significantly different between groups.

Discussion

This study has shown that an intravenous injection of progesterone to late pregnant rats delays delivery by more than one day and markedly prolongs the progress of delivery, impairs neonatal survival and maternal behaviour.

Though the increased duration of delivery and the high neonatal mortality in progesterone-treated rats might be partly attributed to the increased foetal weight in rats in which gestation was prolonged (and thus a greater disproportion between the foetal size and the narrow birth canal), this is unlikely to be the only reason for the observed prolongation, since administration of pulsatile oxytocin on day 22 of pregnancy, that advanced delivery by about 6 hours, improved the progress of delivery and significantly reduced neonatal mortality, indicating that insufficient endogenous oxytocin release might have contributed to the slow delivery and increased mortality. Since oxytocin treatment was not fully effective at restoring the normal process of delivery, unlike in morphine-treated rats (see chapter one), progesterone treatment might have reduced uterine responsiveness to oxytocin (Chwalisz *et al.* 1991). Normally, the uterine sensitivity to oxytocin remains low until a few hours before delivery, when, parallel to the dramatic increase in uterine oxytocin receptor expression, the uterus becomes highly sensitive to oxytocin (Fuchs & Poblete 1970, Fuchs *et al.* 1983, Garfield *et al.* 1982). In humans, rats and sheep, uterine oxytocin receptor concentrations increase following oestrogen treatment, while progesterone administration can inhibit the effects of oestrogen (Burgess *et al.* 1992, Maggi *et al.* 1991a, Fuchs *et al.* 1983)

Thus, the lack of an effect of pulsatile oxytocin on delivery in progesterone-treated rats on the expected day of term (day 21 of pregnancy) is consistent with the hypothesis that oxytocin induces delivery by stimulating contractions of the uterus, which, due to the suppressive effects of progesterone on uterine oxytocin receptor expression, was not responsive to oxytocin on day 21 of pregnancy.

Since a) progesterone-treated rats that delivered spontaneously on day 22 showed normal Fos expression in the NTS and b) the NTS receives direct afferent projections from the uterus (Ortega-Villalobos *et al.* 1990), this is consistent with the hypothesis that uterine activity has been induced and has stimulated neurones in the brainstem as observed in response to normal

delivery and oxytocin-induced labour (see chapter one & three). However, the lack of concomitant Fos expression in the SON, but the improvement of delivery with oxytocin, suggests that oxytocin secretion during progesterone-delayed delivery was reduced.

Since in rats magnocellular neurones do not contain steroid hormone receptors (Fox *et al.* 1990, Rhodes *et al.* 1981a, Sar 1988), the inhibition of Fos expression in the SON during delivery following progesterone treatment is likely to involve interneurones and/or afferent neurones. Catecholaminergic neurones in the NTS represent a major excitatory input to the hypothalamus, including magnocellular oxytocin neurones (Cunningham & Sawchenko 1988, 1991, Onaka *et al.* 1994, Sawchenko & Swanson 1982a) and express oestrogen receptors (Heritage *et al.* 1977, 1980). As shown in chapter three, during normal and oxytocin-induced delivery there is not only an increase in the number of TH-immunoreactive NTS neurones that express Fos, but also an increase in the total number of TH-immunoreactive neurones. Since administration of oxytocin pulses to late pregnant rats leads to strong uterine activity (Randolph & Fuchs 1989), we have proposed that this might be an important stimulus for the initiation of the increased release of oxytocin during labour and delivery (Fuchs *et al.* 1991, Higuchi *et al.* 1986b), that is, at least partly, mediated by noradrenergic neurones in the NTS. Here, we described a more gradual increase in TH immunoreactivity in the NTS of progesterone- compared to vehicle-treated rats and a strong positive correlation between the incidence of TH-immunoreactive cells in the NTS and Fos expression in the SON in progesterone-treated rats, consistent with the hypothesis that progesterone affects the excitatory input provided by the catecholaminergic pathway from the NTS to the SON during delivery and thus reduces Fos expression in supraoptic neurones.

An alternative site at which progesterone treatment could act to inhibit electrical activity of supraoptic oxytocin neurones includes $GABA_A$ receptors, located on magnocellular supraoptic neurones (Buijs *et al.* 1987, Theodosis *et al.* 1986b). Progesterone, and particularly its metabolite allopregnanolone, are agonists at the $GABA_A$ receptor (Paul & Purdy 1992, Frye & Duncan 1994). Since prolonged exposure to high concentrations of progesterone reduces the binding affinity of GABA to the $GABA_A$ receptor, but not to allopregnanolone (Paul & Purdy 1992), progesterone treatment of late pregnant rats could inhibit electrical activity by a direct action on supraoptic neurones. Furthermore, $GABA_A$ receptors are located in the neural lobe on peptidergic axon terminals (Buijs *et al.* 1987, Zhang & Jackson 1993) and *in vitro* studies have shown a $GABA_A$ receptor mediated inhibition of hormone release from the neural lobe by progesterone and its metabolite

allopregnanolone (Zhang & Jackson 1994). Thus, progesterone could inhibit electrical activity of supraoptic neurones (and Fos expression) and hormone release from the posterior pituitary via $GABA_A$ receptors and hence impair the normal progress of delivery. Since progesterone can impair the induction of c-*fos* mRNA expression in peripheral organs (Kirkland *et al.* 1992, Subramaniam *et al.* 1993), a similar mechanism in the CNS could contribute to the reduced Fos expression in progesterone-treated rats observed in this study.

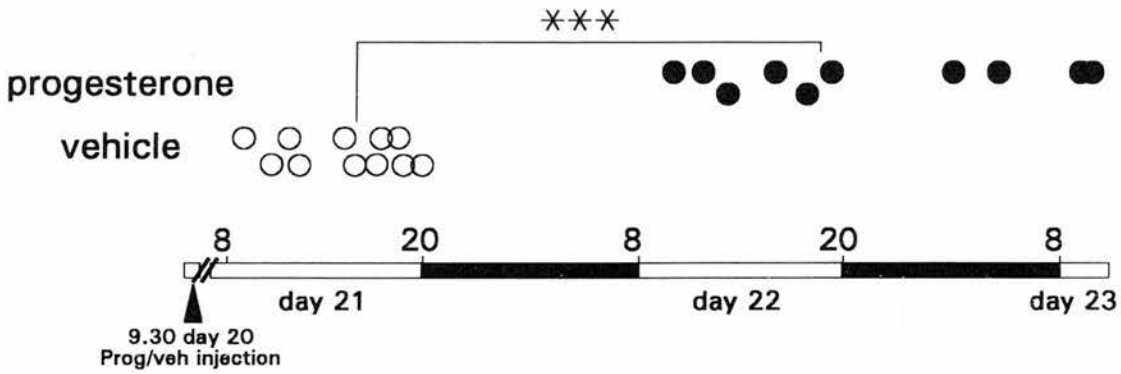
The recent finding of uterine oxytocin mRNA and peptide has opened up the possibility that local oxytocin could be involved in the initiation of uterine activity and hence the positive feedback stimulation of SON neurones (Chibbar *et al.* 1993, Lefebvre *et al.* 1992b). Though the uterine oxytocin mRNA content is much higher than that of the hypothalamus, the actual peptide content in the uterus at term is less than 1% of that in the posterior pituitary (5 mU = 10 ng compared to 700-800 mU = 1400-1600 ng, c.f. Lefebvre *et al.* 1992b, Fuchs & Saito 1971). This apparent mismatch seems to indicate a lack of storage capacity in the uterus, unlike in the posterior pituitary, in which a large amount of oxytocin peptide accumulates in the course of pregnancy (Kumaresan *et al.* 1979) and is eventually released in pulses during delivery (Higuchi *et al.* 1986b), while uterine oxytocin is most likely released as soon as it is synthesised. Similarly, a mismatch between the high oxytocin mRNA content but low peptide synthesis has also been described in the bovine and rat testis for both oxytocin and vasopressin (Foo *et al.* 1991, Ungefroren *et al.* 1994a). This phenomenon has been explained by a post-transcriptional block and it has been suggested that such a mechanism could be involved in the regulation of neurohypophysial peptide production in gonadal tissues, namely to restrain peptide synthesis and hence unwanted effects of the peptides (Ang *et al.* 1994, Ivell & Richter 1984b). Furthermore, the shorter poly(A) tail of the uterine compared to the hypothalamic transcript (Lefebvre *et al.* 1992b, Zingg & Lefebvre 1989) suggests that translation efficiency of the uterine transcript might be low and hence a major contribution of uterine oxytocin to plasma hormone concentrations seems unlikely. Unlike in sheep, in which oxytocin is transcribed and translated efficiently in the corpus luteum (Ivell *et al.* 1990a), the role of the oxytocin (and vasopressin) mRNA in gonadal tissues of rats, cows and humans (Chibbar *et al.* 1993, Miller *et al.* 1993a) remains unclear. However, uterine oxytocin might interact locally with endometrial oxytocin receptors, which in response to stimulation release prostaglandins (Chan *et al.* 1993) and thus could contribute to the initiation of uterine activity. Our finding that uterine oxytocin mRNA content decreases on day 21 of pregnancy regardless of whether delivery has occurred or not, indicates that the delayed delivery in progesterone-

treated rats was initiated despite a greatly reduced uterine oxytocin mRNA content. Thus, other factors, including prostaglandins synthesised in the uterine endometrium (Fuchs 1987) and/or the stretch of the uterine wall by the growing foetuses (Lye & Freitag 1990, Wathes *et al.* 1982), might have contributed to the initiation of labour in progesterone-treated rats. The lack of an effect of progesterone on uterine oxytocin mRNA also indicates that the uterine oxytocin gene is not inhibited by such treatment and is agreement with the observed increase in oxytocin mRNA in the rat uterus and placenta at a time during pregnancy when plasma progesterone concentrations are still elevated (Lefebvre *et al.* 1992a, 1992b).

In summary, we have shown that if the decline in plasma progesterone concentrations at the end of gestation is postponed, delivery is delayed and its progress disrupted, most likely due to a reduced secretion of endogenous oxytocin. Since during such delayed delivery NTS activation is normal, while SON activation is impaired and the increase in TH immunoreactivity in the NTS is altered, I suggest that a reduced catecholaminergic input from the NTS to the SON might have contributed to a reduced electrical stimulation of magnocellular neurones and hence impaired (pulsatile) hormone release during the delayed delivery following progesterone treatment.

Since progesterone did not completely prevent delivery, it might have impaired synchronised bursting activity of supraoptic oxytocin neurones (and hence Fos expression), possibly via an alteration of the excitatory noradrenergic input from the brainstem, while having no or less effect on background secretion. As shown in chapter one, pulsatile release of oxytocin is critical for the normal progress of delivery and thus, reduced pulsatile oxytocin release might explain the slower delivery in progesterone-treated rats.

Fig. 5.1. Effects of progesterone on the onset and progress of delivery
Top: Onset of delivery in rats injected with progesterone on day 20 of pregnancy
Each circle represents the time of birth of the first pup for individual rats injected subcutaneously with progesterone (5 mg, filled circles, n=10) or vehicle (0.2 ml, open circles, n=11) on day 20 of pregnancy (09.30 h). The x-axis indicates the time of day, bars represent the dark phase.
Progesterone-treated rats delivered significantly later (28 hours) than vehicle-treated controls (***$p<0.001$, U-test). While most control rats delivered in the light phase of day 21 of pregnancy, the times of onset of delivery were more spread out in the progesterone-treated group.
Bottom: Time between delivery of pups 2-6 and pups 6-10 in rats injected with progesterone or progesterone and oxytocin pulses
The cumulative time for delivery of pups 2-6 in rats injected with vehicle (0.2 ml, open bars, n=11) was significantly shorter compared to rats given progesterone (5 mg sc, filled bars, n=10, *$p<0.05$, Kruskal-Wallis test) on day 20 of pregnancy and to rats given progesterone on day 20 followed by oxytocin pulses (10/20 mU each 10 min for 4 hours) on day 22 (cross-hatched bars, n=6, *$p<0.05$, Kruskal-Wallis test). Though in rats given progesterone plus oxytocin, the time between pups 2-6 was shorter than in rats given only progesterone, this difference did not reach significance. There was no significant difference between groups for the time between delivery of pups 6-10.


***
progesterone
vehicle
8
20
8
20
8
day 21
day 22
day 23
9.30 day 20
Prog/veh injection

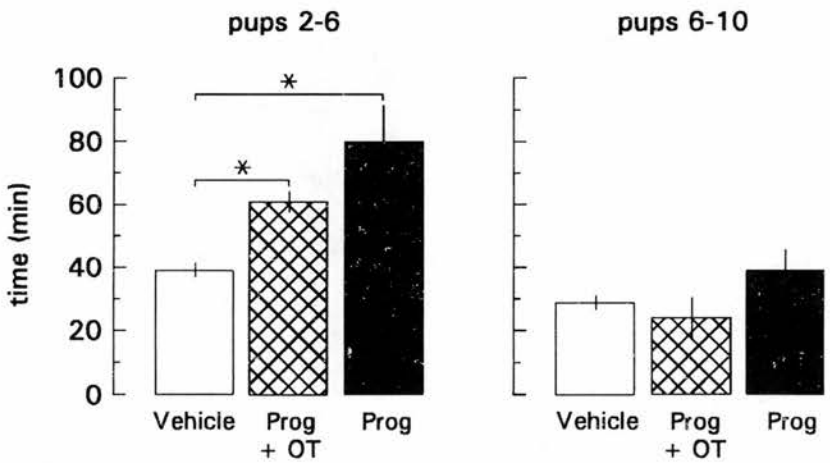

pups 2-6
pups 6-10
time (min)
100
80
60
40
20
0
*
*
Vehicle
Prog + OT
Prog
Vehicle
Prog + OT
Prog

Fig. 5.2. Effects of progesterone on neonatal weight and mortality rate

Top: Weight of pups born to mothers injected with vehicle, progesterone or progesterone and oxytocin pulses

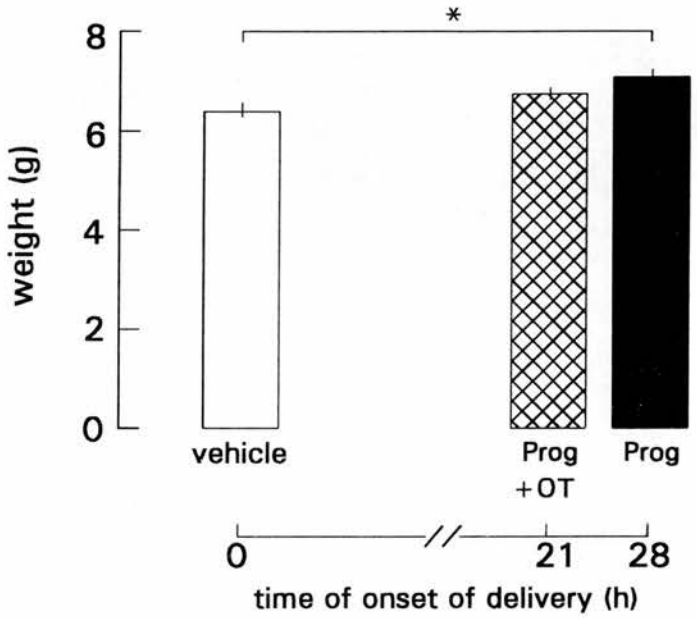
The average weight (means±s.e.m.) of three randomly selected pups per litter was compared for rats injected subcutaneously on day 20 of pregnancy with vehicle (0.2 ml, open bar, n=11), progesterone (5 mg, filled bar, n=10), or progesterone on day 20 followed by oxytocin pulses (10/20 mU each 10 min for 4 hours) on day 22 (cross-hatched bar, n=6).

Birth weight of pups born to mothers injected with vehicle was significantly lower than that of pups born to mothers injected with progesterone (*$p<0.05$, U-test). In contrast, the birth weight of pups born to rats given progesterone plus oxytocin was not significantly different from that of vehicle-treated rats.

On the y-axis is the time in hours of the onset of delivery in rats, with assigning time zero to the onset of delivery in vehicle-treated rats to indicate the positive correlation ($r^2=0.56$, data not shown) of the birth weight of rats with the length of gestation.

Bottom: Neonatal mortality of pups born to mothers injected with vehicle, progesterone or progesterone and oxytocin pulses

The neonatal mortality rate was calculated as the number of pups that died within 10 hours of delivery/number of all pups born, in % for rats injected subcutaneously on day 20 of pregnancy with vehicle (open bar, n=11), progesterone (filled bar, n=10), or progesterone and oxytocin pulses (cross-hatched bar, n=6). Neonatal mortality was significantly lower in vehicle and progesterone plus oxytocin treated rats than that in rats given only progesterone ($14\pm1.7\%$ vs $<1.5\%$ in the two other groups, *$p<0.05$, Kruskal-Wallis test).


*
8
6
4
2
0
weight (g)
vehicle
Prog
+OT
Prog
0
21
28
time of onset of delivery (h)

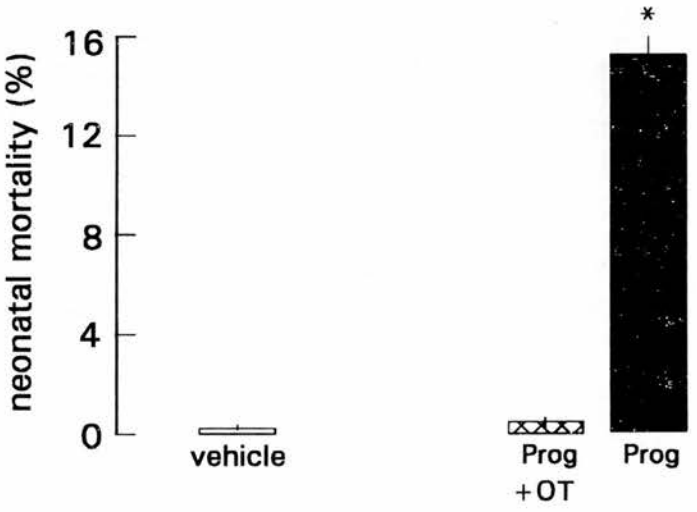

*
16
12
8
4
0
neonatal mortality (%)
vehicle
Prog
+OT
Prog

Fig. 5.3. Effect of progesterone on Fos expression in the SON and NTS during delivery
The number of Fos-immunoreactive nuclei on sections through the SON **(Top)** and the NTS **(Bottom)** was compared for of rats injected subcutaneously with vehicle (0.2 ml, open bars, n=4-6) or progesterone (5 mg, filled bars, n=4-7) on day 20 of pregnancy.
Rats were killed either on day 21 before delivery (or day 22, progesterone-treated rats only, prepartum rats), 90 min after the birth of the second pup (parturient rats), or 6-12 hours after the birth of the first pup (postpartum rats).
Vehicle-treated parturient rats showed a significant increase in Fos expression in the SON and NTS compared to prepartum and postpartum vehicle-treated rats (*$p<0.05$, Kruskal-Wallis test), while in progesterone-treated rats, an increase in Fos expression during parturition was only apparent in the NTS, but not the SON (*$p<0.05$, Kruskal-Wallis test, compared to prepartum and postpartum progesterone-treated rats).

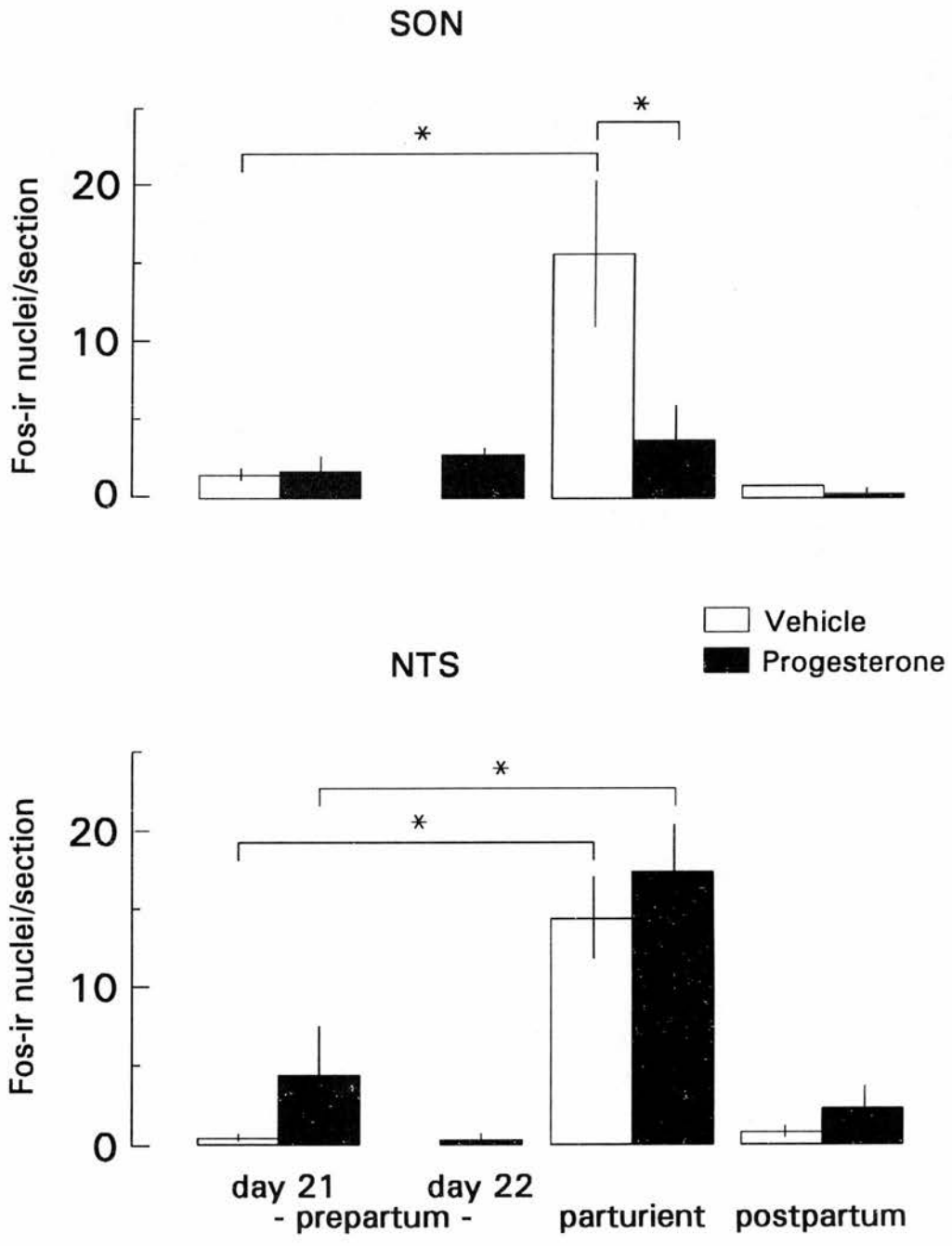

SON
Fos-ir nuclei/section
20
10
0
*
*
Vehicle
Progesterone
NTS
Fos-ir nuclei/section
20
10
0
*
*
day 21
day 22
- prepartum -
parturient
postpartum

Fig. 5.4. Effect of progesterone and oxytocin pulses on Fos expression in the SON and NTS during delivery

The number of Fos-immunoreactive nuclei on sections through the SON **(Top)** and the NTS **(Bottom)** was compared for of rats injected subcutaneously with progesterone (5 mg, filled bars, n=4-7) on day 20 of pregnancy followed by pulsatile administration of oxytocin (10/20 mU for 4 hours, hatched bars = prepartum rats, n=5, 2, cross-hatched bars = parturient rats, n=6) or saline (0.03 ml for 4 hours, shaded bars, n=3, 4) on day 21 or day 22 of pregnancy. Rats were killed either at the end of the treatment but before delivery of any pups (prepartum) or 90 min after the birth of the second pup (parturient).

Only two rats that were given oxytocin on day 22 did not deliver during the treatment (day 22, hatched bar) and thus the two individual values are indicated by filled circles.

The number of Fos-immunoreactive nuclei per section was not different between saline- and oxytocin-treated rats on day 21 of pregnancy, while there was a slight, but significant increase in the number of Fos-immunoreactive nuclei per section SON in rats in which delivery was induced with oxytocin pulses on day 22 compared to the respective saline group (*$p<0.05$, U-test).

However, all rats treated with progesterone plus oxytocin pulses had fewer Fos-immunoreactive nuclei in the SON and NTS than rats injected with pulses of oxytocin or saline on day 21 of pregnancy (Right, filled and open bars, respectively, c.f. chapter three) without progesterone pretreatment.

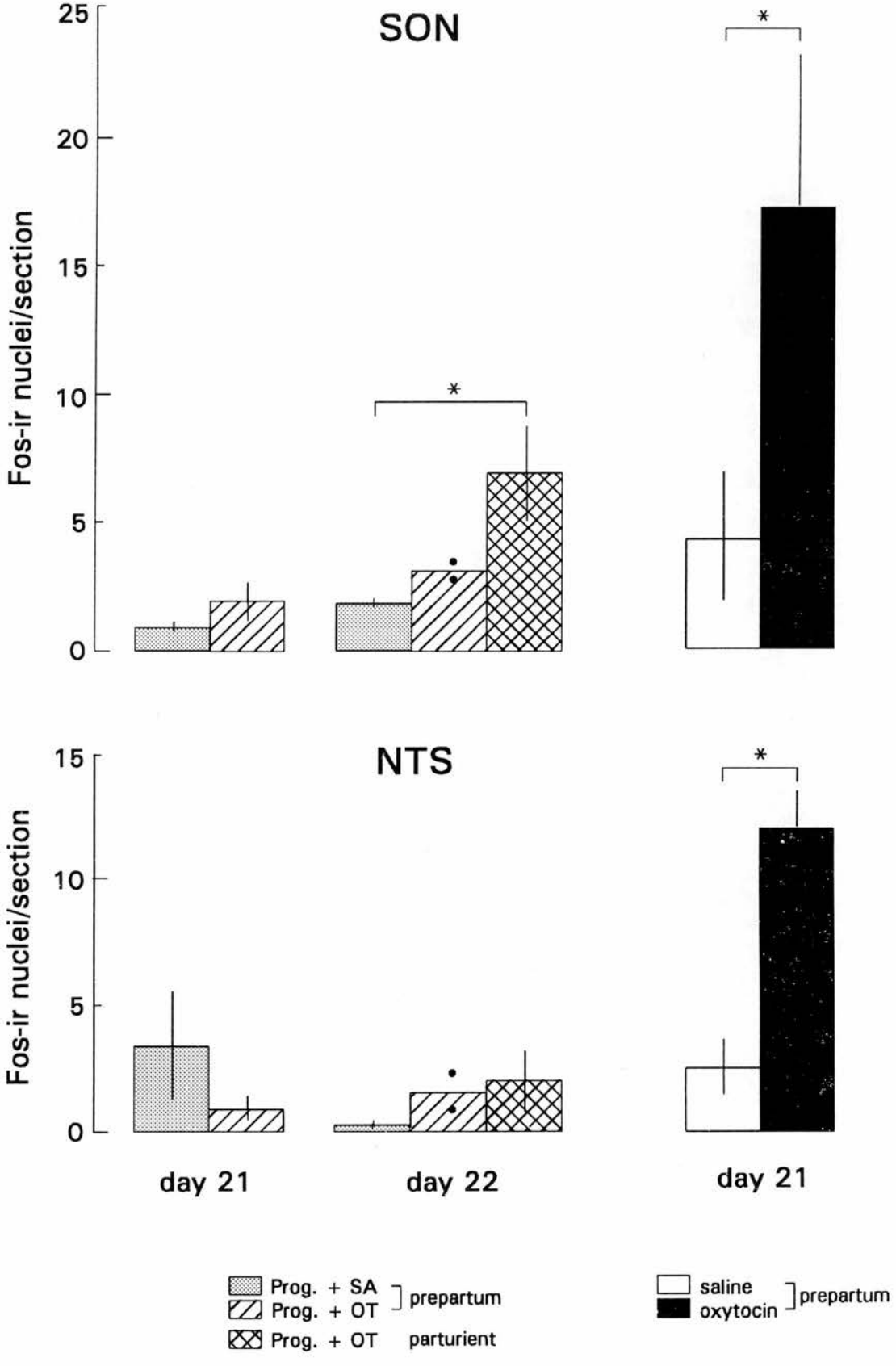

SON
NTS
Fos-ir nuclei/section
25
20
15
10
5
0
*
day 21
day 22
day 21
Prog. + SA
Prog. + OT
prepartum
Prog. + OT
parturient
saline
oxytocin
prepartum

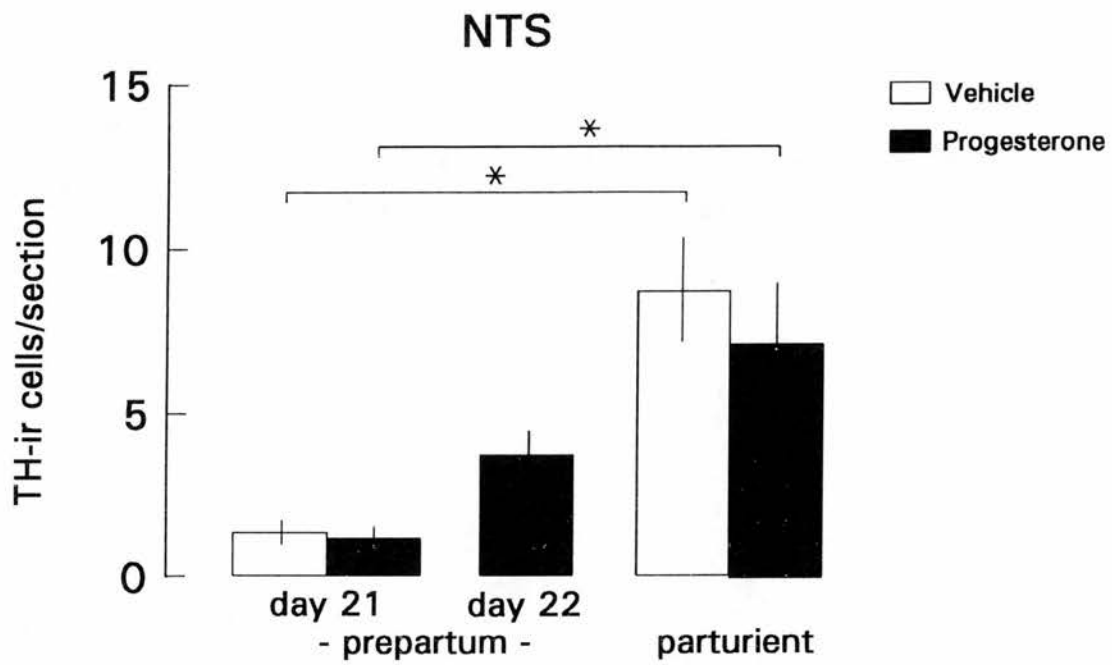
Fig. 5.5. Effect of progesterone on TH immunoreactivity in the NTS of late pregnant rats

Top: The number of TH-immunoreactive cells/section NTS was counted in rats injected subcutaneously with vehicle (0.2 ml, open bars, n=4-6) or progesterone (5 mg, filled bars, n=3-7) on day 20 and killed on day 21 (or day 22, progesterone-treated rats only) before delivery of any pups (prepartum) or 90 min after the birth of the second pup (parturient).

There was a significant increase in the number of TH-immunoreactive cells/section NTS in control parturient compared to control prepartum rats (*$p<0.05$, U-test), and similarly there was a significant increase in TH immunoreactivity in the NTS of progesterone-treated parturient compared to progesterone day 21 prepartum rats (*$p<0.05$, U-test). However, in progesterone-treated rats the increase in TH immunoreactivity was more gradual and less pronounced than in controls.

Bottom: The number of TH-immunoreactive cells/section NTS was counted in rats injected subcutaneously with progesterone followed by pulsatile administration of oxytocin (10/20 mU oxytocin for 4 hours, right hatched bars = prepartum rats, n=5, 2, cross-hatched bars = parturient rats, n=6) or saline (shaded bars, n=3, 4) on day 21 or day 22 of pregnancy and killed at the end of the treatment before (prepartum) or during delivery (parturient).

There was no significant difference between groups for the number of TH-immunoreactive cells/section NTS. Since only 2/8 oxytocin-treated rats did not deliver on day 22 during the oxytocin treatment (day 22, hatched bar), the individual values are indicated by filled circles.


NTS
15
10
5
0
TH-ir cells/section
Vehicle
Progesterone
*
*
day 21
day 22
- prepartum -
parturient

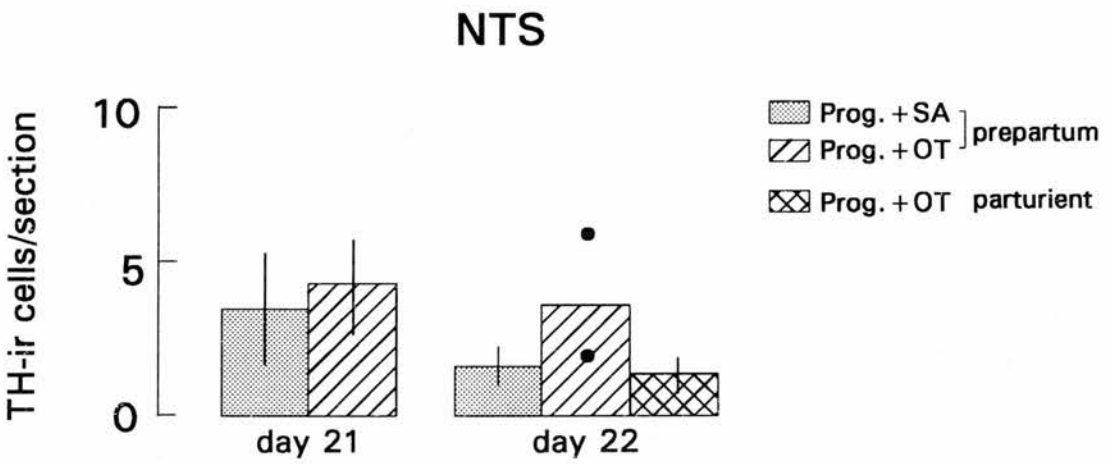

NTS
10
5
0
TH-ir cells/section
Prog. + SA
Prog. + OT
prepartum
Prog. + OT
parturient
day 21
day 22

Fig. 5.6. Effect of progesterone on uterine oxytocin mRNA content of late pregnant rats

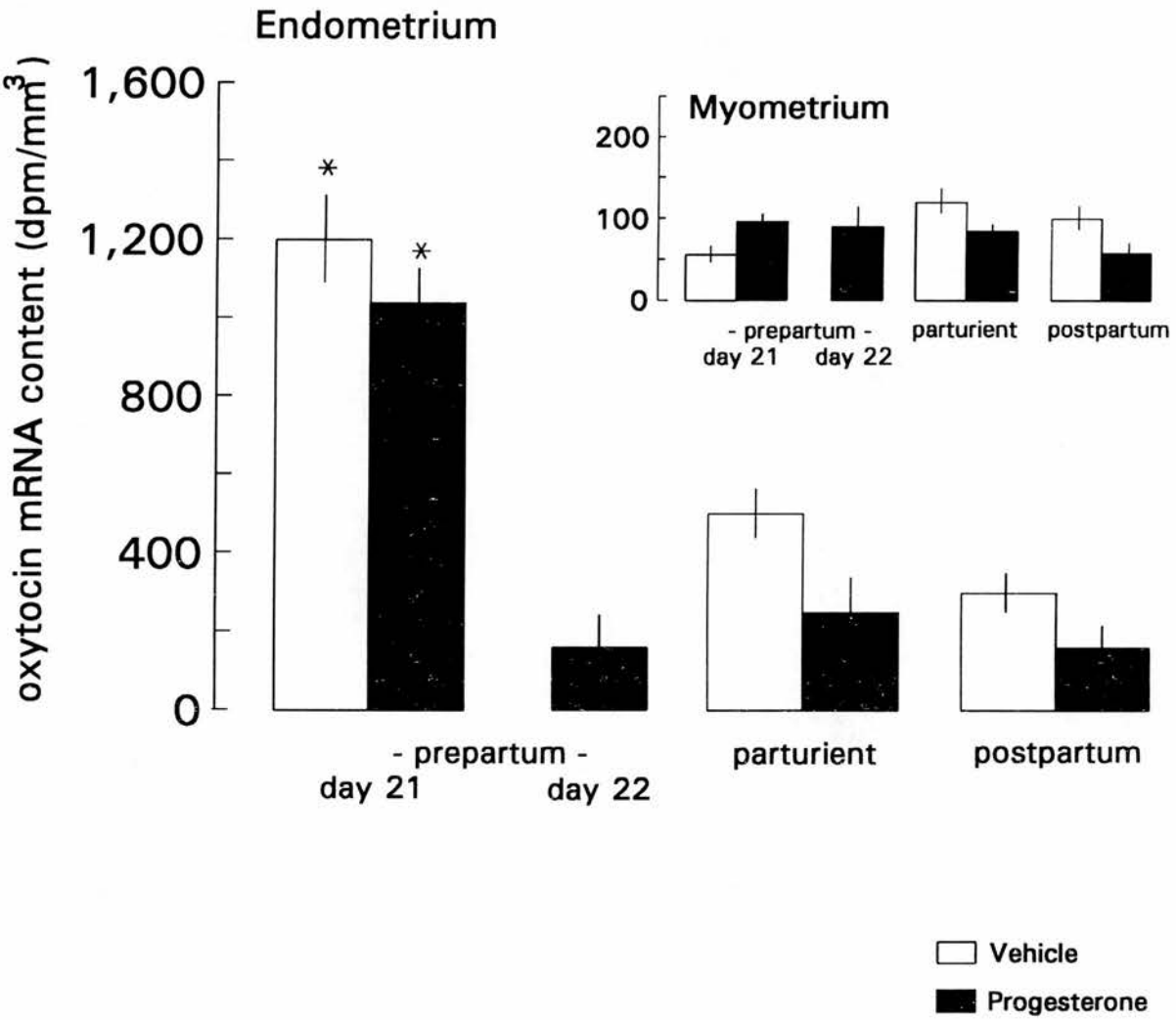
Oxytocin mRNA was counted using an automated image analysis system and the optical (= grain) density measurements were transformed using a standard curve into dpm/mm^3 endo- or myometrium. Rats were injected subcutaneously with vehicle (0.2 ml, open bars, n=4-5) or progesterone (5 mg, filled bars, n=4-5) on day 20 of pregnancy and killed on day 21 (or day 22, progesterone-treated rats only) before (prepartum), during (parturient) or after (postpartum) delivery of pups.

The myometrial oxytocin mRNA content was low and not significantly different between groups, while the endometrial oxytocin mRNA content of both vehicle- and progesterone-treated rats was significantly higher on day 21 than in all other groups (*$p<0.05$, Kruskal-Wallis test).

Fig. 5.6a. Radioactive *in situ* hybridisation for oxytocin mRNA in the uterus of late pregnant and parturient rats

Overleaf: Oxytocin mRNA expression (assessed by radioactive *in situ* hybridisation in transverse uterine sections) was significantly more abundant in the endometrium of term pregnant (**Left**) compared to a parturient (**Right**) rats.
Scale bar 100 μm

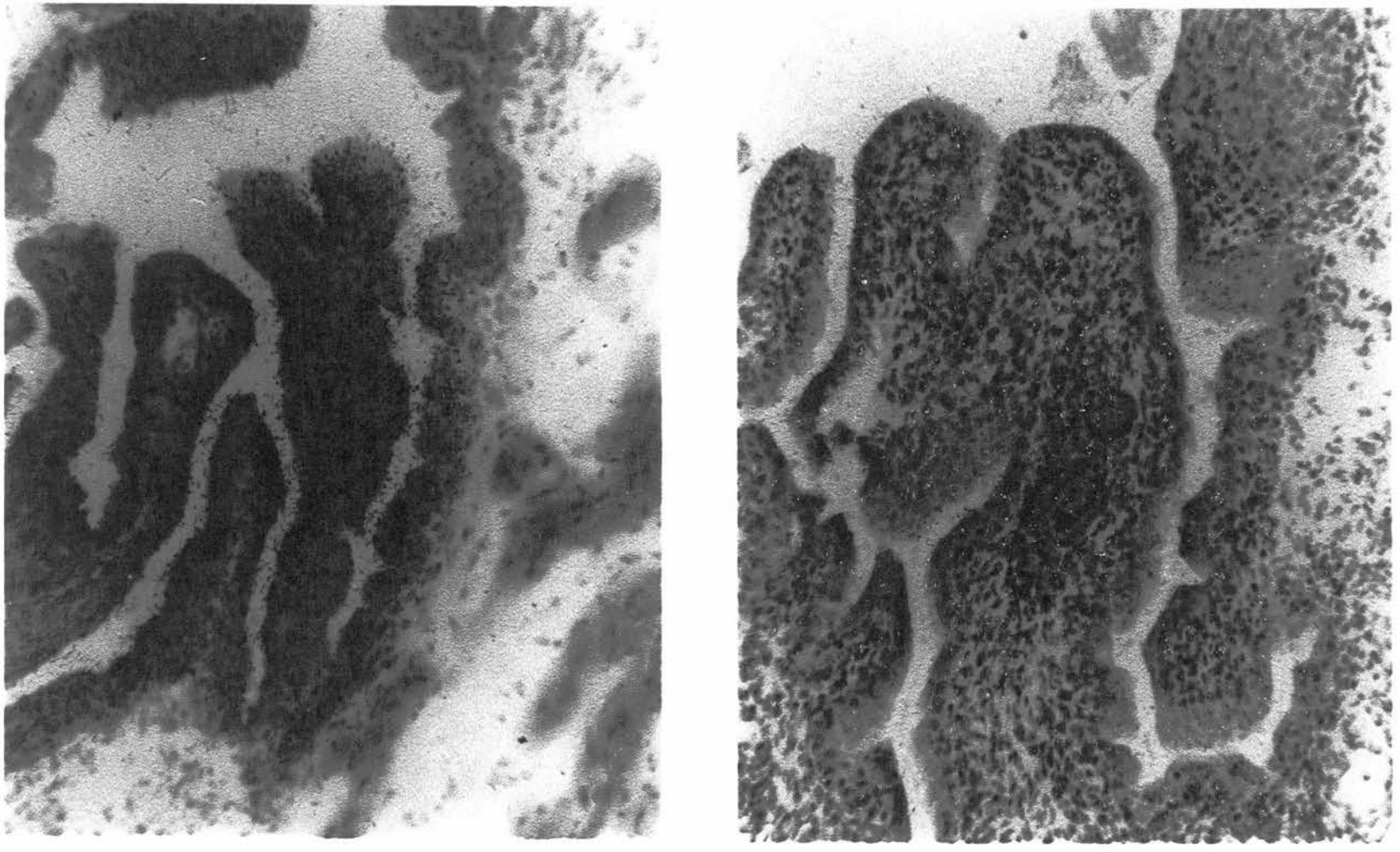
Fig. 5.6b. Overleaf: High magnification view of a uterine section processed for *in situ* hybridisation for oxytocin mRNA showed silver grains primarily in the uterine endometrium in term pregnant rats.


Endometrium
oxytocin mRNA content (dpm/mm3)
1,600
1,200
800
400
0
*
*
Myometrium
200
100
0
- prepartum -
day 21
day 22
parturient
postpartum
- prepartum -
day 21
day 22
parturient
postpartum
Vehicle
Progesterone

oxytocin mRNA in the rat uterus on day 21

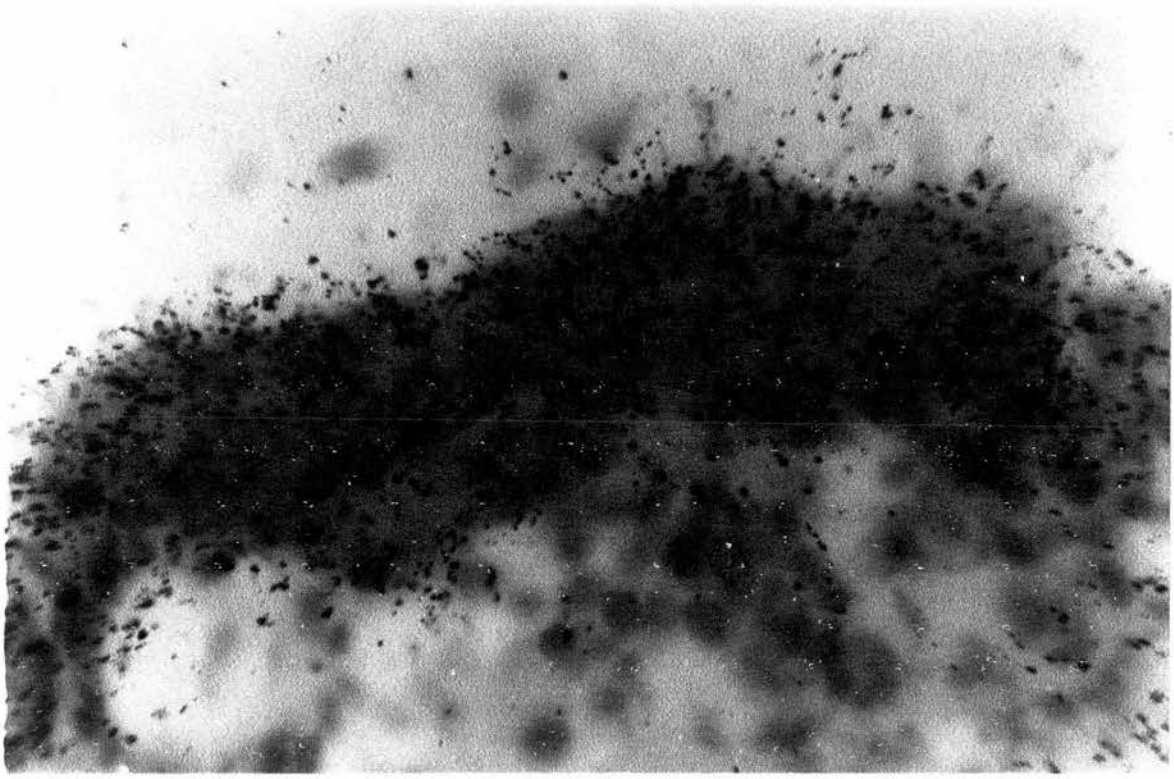
prepartum

during parturition



100 μm

Oxytocin mRNA in the endometrium



Chapter 6

Acute changes in TH mRNA expression in brainstem neurones of late pregnant rats

As shown in the previous chapters, circulating oxytocin is critical for delivery and occurs despite the restraint of endogenous opioid peptides on oxytocin neurones during pregnancy (Bicknell *et al.* 1988c, Neumann *et al.* 1992), suggesting the existence of an excitatory influence that can override the opioid inhibition on magnocellular neurones. Possible candidates involved in mediating the bursting activity of oxytocin neurones during delivery (Summerlee 1981) include the ascending noradrenergic pathways from the NTS to the SON, that provide a major excitatory input onto magnocellular neurones (Raby & Renaud 1989a, 1989b, Swanson & Sawchenko 1982a), and are involved in CCK-induced oxytocin release (Onaka *et al.* 1995, Rinaman *et al.* 1994). The involvement of catecholamines in the bursting activity of magnocellular oxytocin neurones is further supported by the observation that administration of an α_1-adrenergic agonist in an *in vitro* slice preparation to magnocellular

oxytocin neurones results in a burst-like activity similar to that seen during the milk-ejection reflex (Wakerley & Ingram 1993).

In the previous chapters, I have shown that in rats in which delivery is induced with oxytocin pulses the number of TH-immunoreactive cells in the NTS and Fos expression in the SON is increased (see chapter three), while a delay of delivery following an injection of progesterone alters the increase in TH immunoreactivity in the NTS and is accompanied by reduced Fos expression in SON neurones during such delivery (chapter five), pointing to an involvement of the noradrenergic projection from the NTS in the normal activation of supraoptic neurones during parturition.

Unlike magnocellular oxytocin neurones, that lack gonadal steroid receptors (Bethea *et al.* 1994, Fox *et al.* 1990, Rhodes et al 1981a), catecholaminergic brainstem neurones express nuclear oestrogen receptors and are hence a target for gonadal steroid regulation (Heritage *et al.* 1977, 1980). Thus, TH mRNA expression in the rat brainstem increases in catecholaminergic neurones in the ventrolateral medulla, that project to the medial preoptic area where LHRH neurones are located (Wright & Jennes 1993), at the time of the LH surge (Liaw *et al.* 1992c) and thus at the time of an increase in the plasma oestrogen/progesterone ratio (Freeman 1988). Similarly, *in vitro* studies have indicated a stimulatory effect of oestrogen and an inhibitory effect of progesterone on TH mRNA expression (Kedzierski *et al.* 1994, Kim *et al.* 1994, Wang & Porter 1986). Since an oestrogen or progesterone response element in the promotor region of the TH gene has not yet been detected, a change in the TH mRNA content of NTS neurones can be attributed to trans-synaptic or trans-membrane actions (Kohama & Bethea 1995). Most stimuli that affect TH expression have been reported to first activate TH mRNA expression, which in turn is followed by an increase in TH activity (Arbogast & Voogt 1993, McMahon *et al.* 1992, Nankova *et al.* 1994). However, gonadal steroids can modulate TH activity by protein phosphorylation (Wang & Porter 1986) and such changes in enzymatic activity are succeeded by a concomitant change in TH mRNA expression (Arbogast & Voogt 1991b, 1994).

The induction of TH mRNA expression *in vivo* can occur within 1-2 hours of a stimulus, including immobilisation stress, hypoxia and electrical stimulation (Czyzyk-Krzeska *et al.* 1994, Liaw *et al.* 1992b, Nankova *et al.* 1994) and this increase in TH mRNA is followed with some latency (2-6 hours) by an increase in TH protein and TH activity (McMahon *et al.* 1992, Nankova *et al.* 1994). Though no direct relationship between TH mRNA expression and catecholamine release has so far been demonstrated, at the time of the LH surge, an

increased release of catecholamines in the preoptic area (Demling *et al.* 1985, Rance *et al.* 1981) coincides with an increase in TH mRNA expression in neurones in the ventrolateral medulla (Liaw *et al.* 1992c). Similarly, noradrenaline release in the preoptic area is increased in response to A1 neurone stimulation (Fernandez-Galaz *et al.* 1994, Herbison *et al.* 1990), the latter of which is also associated with an increase in the TH mRNA content (Liaw *et al.* 1992b). Thus, an increase in TH mRNA expression can, under certain circumstances, reflect catecholamine release from the respective axon terminals.

In this chapter, we have investigated first, whether TH mRNA expression in the brainstem changes in untreated rats in relation to the reproductive stage and second, whether the observed change in TH immunoreactivity in oxytocin-treated and normal parturient rats and progesterone-treated late pregnant rats (see chapter three and five) is associated with a change in TH mRNA expression.

Materials and Methods

Animals

Vaginal smears from cycling female rats were taken daily and examined under the microscope until a minimum of two consecutive four day oestrous cycles were established.

For experiments on pregnant rats, date mated female Wistar rats from the Babraham colony were used. Twelve animals were either not treated and killed at different stages of the oestrous cycle (proestrous and metestrous) or at different stages of pregnancy (day 17 or day 21 of pregnancy, before delivery of pups) or on day 10 of lactation. Other date mated rats were either implanted with a jugular vein cannula under brief halothane anaesthesia on day 20 of pregnancy (n=12) or were given an injection of progesterone (Intervet, 5 mg sc, n=10) or vehicle (0.2 ml of 0.3vol% cresol, 89.7vol% arachis oil and 10vol% benzylalcohol, also kindly provided by Intervet, n=11) at 09.30 h on day 20 of pregnancy subcutaneously (we have shown in chapter five that such treatment delays delivery by about 28 hours). From the morning of day 21 onwards until the time of killing animals were observed for signs of labour (stretching, straining, vaginal bleeding), pup delivery and maternal behaviour (nest building, excessive grooming, licking of external genitals, licking of the pups during and after delivery, pup retrieval).

Experimental design

TH mRNA in the brainstem of untreated rats at different stages of the reproductive cycle

Eight rats, in which at least two consecutive oestrous cycles have been established, were killed by cervical dislocation in the afternoon of proestrous (n=4) or metestrous (= diestrous 1, n=4) between 15.00-15.30 h.

Twelve other rats were date mated and killed by cervical dislocation between 15.00-15.30 h on day 17 or day 21 of pregnancy (prepartum), respectively, or at the same time in the afternoon of day 10 of lactation. All pregnant rats were laparotomised and the uteri examined for pups and placentae and the brains removed under RNAse free conditions and immediately frozen on crushed dry ice and stored at -80°C until processed for *in situ* hybridisation for TH mRNA.

TH mRNA in the brainstem of rats treated with progesterone

Five progesterone- and five vehicle-treated rats were killed time-matched before the birth of any pups on day 21 of pregnancy between 11.00-13.00 h (day 21 prepartum groups). Six vehicle-treated rats were allowed to deliver and were killed 90 min after the birth of the second pup (between 11.00-15.00 h on day 21, parturient group). The remaining five progesterone-treated rats were killed on day 22 of pregnancy between 11.00-13.00 h but before the birth of any pups (day 22 prepartum group). All rats were killed by cervical dislocation, laparotomised and the uteri examined for pups and placentae and as described above the brains were removed and immediately frozen until processed for *in situ* hybridisation for TH mRNA.

TH mRNA in the brainstem of rats injected with oxytocin or saline pulses

Rats implanted with a jugular vein cannula were given 0.1 ml heparinised saline (50 IU heparin/ml saline) to flush the cannulae, which were then connected to polyethylene tubing filled with isotonic saline or oxytocin. From 11.00-15.00 h on day 21 of pregnancy animals were given each 10 min a pulse of saline (0.03 ml, n=6) or oxytocin (10 mU for 2 hours and then 20 mU for 2 hours, n=6). This treatment induces labour and Fos expression in SON neurones (see chapter three) and significantly increases the number of TH-immunoreactive cells in the NTS. At the end of the treatment (between 15.45-16.45 h) again all rats were killed in time-matched pairs (one animal of each group) by cervical dislocation, laparotomised

and the uteri examined for pups and placentae, the brains removed, frozen and eventually processed for *in situ* hybridisation for TH mRNA.

In situ hybridisation for tyrosine hydroxylase mRNA

For the quantitative analysis of tyrosine hydroxylase mRNA expression (see general materials and methods section) an automated image analysis system (Joyce Loebl μMagiscan) was used. The average optical density per cell (silver grains/cell) in the NTS and the ventrolateral medulla were measured on counterstained brainstem sections using a light microscope with x40 objective and a brightfield condenser. For each animal a minimum of 4 x 5 cells were measured throughout the brainstem in each the NTS and the ventrolateral medulla. On most sections, the area covered by the screen of the image analysis system (dorsal and lateral of the central canal and in the ventrolateral medulla), contained five or less cells, so that the cells included in the analysis were usually all or most of the cells containing silver grains per half section. Means±s.e.m. were calculated for each area per animal, which were then grouped according to the treatment and the group means±s.e.m. were established. Since for each five cells a different section was used, counts were taken throughout the rostro-caudal extent of the brainstem from the decussation of the pyramids to the rostral end of the area postrema (according to the rat brain atlas of Paxinos & Watson 1986). The majority of catecholaminergic neurones caudal to the area postrema synthesise noradrenaline, while adrenaline producing cells are found almost exclusively rostral to the area postrema and dopaminergic cells ventral of the NTS in the dorsal motor nucleus of the vagus (Kalia *et al.* 1985) and thus the TH mRNA containing cells that were included in the statistical analysis represent mainly noradrenergic neurones.

Only in oxytocin- and saline-treated rats, the brainstem was divided into a caudal and rostral part, with the former extending from the pyramidal decussation to the caudal end of the area postrema and the latter from the caudal to the rostral end of the area postrema. In progesterone- and vehicle-treated rats the caudal and ventral brainstem parts were grouped together since we did not observe a significant difference in the amount of silver grains/cell for the rostral and caudal divisions.

According to the number of silver grains per cell, we established a distribution histogram for all cells of one experimental group (range 63-123 cells, for 5-6 rats per group). For each experimental group, the total number of cells was assigned 100% and the number of cells

containing less than 100, 100-200, 200-300 silver grains etc. up to 900-1000 silver grains, was expressed as percent of the total number of cells analysed in each treatment group. Since the data for the distribution histogram were derived from a relatively small number of rats (n=5-6), we did not perform a statistical comparison of the groups medians, but confined ourselves to a qualitative representation of the data.

Furthermore, we counted the total number of cells containing silver grains for each animal (6-8 sections per area per animal) throughout the brainstem to assess whether a change in TH mRNA content reflected a recruitment of cells synthesising TH or a higher production rate of TH in the same number of cells.

Statistical analysis

Intergroup comparisons were made using non-parametric tests, either the Mann-Whitney U-test for comparison between two groups or, for comparisons between more than two groups, including subpopulations of groups, the Kruskal-Wallis test, followed by Dunn's post-hoc test. For a two-tailed value of $p<0.05$ a difference was considered significant.

Results

Neurones in the rat brainstem could be clearly identified on counterstained sections by the presence of silver grains overlying cells (Fig. 1), while there was hardly any background staining.

TH mRNA expression in the NTS and the ventrolateral medulla of untreated rats during the oestrous cycle, pregnancy and lactation (Fig. 2)

The content of TH mRNA per cell, expressed as an average number of silver grains/cell, was significantly elevated in the NTS of rats killed on day 21 of pregnancy before delivery of any pups compared to rats killed on day 17 of pregnancy and on day 10 of lactation (Kruskal-Wallis test, $p<0.05$). The lactating group showed among all groups the smallest intragroup variability (reflected by the small standard error). Though the TH mRNA content in NTS cells of rats killed on metestrous and proestrous was lower than in day 21 prepartum rats, the difference was not significant. In the ventrolateral medulla, the average amount of silver grains/cell was not significantly different between groups.

The numbers of cells per section expressing TH mRNA were not significantly different between groups in the NTS and the ventrolateral medulla, though rats killed on day 21 but

prior to delivery of any pups showed the highest number of TH containing cells in the NTS (Fig. 2). With the exception of the lactating group, the number of cells per section reflected, though at a lower, and hence not significant level, the changes in the TH mRNA content per cell.

TH mRNA expression in the NTS and ventrolateral medulla of late pregnant rats after a systemic injection of progesterone (Fig. 3)

The average number of silver grains/cell in the NTS was significantly higher in control day 21 prepartum rats compared to parturient rats and compared to progesterone-treated day 21 prepartum rats (Kruskal-Wallis test, $p<0.05$). In progesterone-treated day 22 prepartum rats, the average number of silver grains/cell was higher than in progesterone-treated day 21 prepartum rats, but lower than in the control prepartum group. However, these differences were not significant.

Similar to the first experiment, the numbers of cells per section expressing TH mRNA were not significantly different between groups (Fig. 3) for the NTS and the ventrolateral medulla. In comparison with the first experiment, we counted fewer cells containing silver grains/section in the NTS, while in the ventrolateral medulla the average number of cells containing silver grains per section was similar (c.f. Fig. 2 & 3).

TH mRNA expression in the NTS and the ventrolateral medulla of rats injected with pulses of oxytocin or saline (Fig. 4)

The content of TH mRNA per cell, expressed as an average number of silver grains/cell, was higher in the NTS of oxytocin- compared to saline-treated rats, however the difference was not significant in the Mann-Whitney U-test. Since I have shown in chapter three, that the percentage increase in the number of TH-immunoreactive was more pronounced in the caudal parts of the NTS, I divided the brainstem in a caudal (from the decussatio of the pyramids to caudal tip of the area postrema) and a rostral (throughout the area postrema) part and compared the TH mRNA content per cell in these subdivisions. The average number of silver grains/cell was significantly higher in oxytocin- compared to saline-treated rats in the rostral part of the NTS (Kruskal-Wallis test, $p<0.05$), while in the caudal parts the difference between the oxytocin- and the saline-treated groups was small and not significant. In both parts of the ventrolateral medulla the number of silver grains/cells was not significantly

different between groups. Oxytocin-treated rats showed in all comparisons a smaller intragroup variation than saline-treated rats (small standard error).

The numbers of cells per section expressing TH mRNA were not significantly different in the NTS and the ventrolateral medulla between groups and were similar to progesterone-treated rats (and hence lower than in the NTS of untreated rats).

The distribution histogram of cells in the NTS according to the number of silver grains per cell

To take a closer look at the TH containing cell population, we established a distribution histogram including all cells analysed in one group and these were subdivided according to the number of silver grains. In vehicle-treated parturient rats (n=6, number of cells n=83, median: 273 silver grains/cell, Fig. 5) the distribution histogram showed a shift to the left compared to vehicle-treated day 21 prepartum rats (n=5, number of cells analysed n=65, median: 410 silver grains/cell).

In progesterone-treated day 21 prepartum rats, the median of the amount of silver grains per cell was lower than in the vehicle-treated day 21 prepartum group (median: 246 vs 410 silver grains/cell, number of cells n=79, c.f. Fig. 5 & 6). In progesterone-treated rats killed on day 22 before delivery, there was a shift to the right compared to progesterone-treated day 21 prepartum rats (median: 346 vs 246 silver grains/cell, number of cells n=63, 79). Thus, in progesterone-treated day 21 prepartum rats there was a marked shift to the left compared to normal day 21 prepartum rats. There was a similar trend, albeit less pronounced, when progesterone-treated day 21 prepartum rats were compared to progesterone-treated day 22 prepartum rats (Fig. 6).

In oxytocin-treated (day 21 prepartum) rats the distribution of TH neurones in the NTS according to the amount of silver grains/cell was similar to that in saline-treated (prepartum) rats (463 and 419 silver grains/cell in oxytocin- and saline-treated rats, respectively, number of cells n=104, 123, Fig. 7) and the medians of both groups were not markedly different from control day 21 prepartum rats (c.f. Fig. 5 & 7). When the TH cell population in the NTS was subdivided into a caudal and a rostral group, there was a small shift to the right and hence to a higher amount of silver grains/cell in the rostral NTS of oxytocin-treated rats compared to the saline group (medians: rostral NTS: 516 vs 405 silver grains/cells, in oxytocin- and saline-treated rats, respectively, n=46, 54, Fig. 7, inset). In the caudal NTS, the medians were 407 and 341 silver grains/cell in oxytocin- and saline-treated rats (n=58, 69). Thus, in the saline-

treated group, the distribution of cells according to the silver grain content in the two divisions of the NTS was similar, while in oxytocin-treated rats, rostrally located cells in the NTS had a higher content of silver grains/cell.

Discussion

Here, we have shown that at the end of normal pregnancy, but before delivery of any pups, the TH mRNA content of NTS neurones is significantly higher than in rats during parturition. Since in day 21 pregnant rats, TH mRNA expression in the NTS was significantly higher than in day 17 prepartum rats, the observed up-regulation of TH mRNA occurs late in pregnancy. Furthermore, our data indicate that the TH mRNA content of the NTS decreases within a few hours and since TH mRNA expression in the brainstem does not show a diurnal variation (Liaw *et al.* 1992a), these acute changes in TH mRNA expression could be associated with labour and delivery.

The decrease in TH mRNA content during parturition was only seen in NTS neurones, that project preferentially to magnocellular oxytocin neurones and not in noradrenergic neurones in the ventrolateral medulla, that innervate almost exclusively vasopressin cells (Raby & Renaud 1989a, 1989b). Since some uterine afferents travel with the vagal nerve, that projects to the NTS (Ortega-Villalobos *et al.* 1990), this pathway could be involved in the regulation of TH mRNA expression in NTS neurones. Though our data suggest that TH mRNA expression declines prior to and/or during delivery and hence at a time of increased uterine activity, oxytocin treatment seems to be able to prevent the normal decline in TH mRNA expression and instead to induce TH mRNA expression in the NTS in term pregnant rats. Since oxytocin pulse treatment stimulates stronger uterine activity than that seen during spontaneous labour (Randolph & Fuchs 1989) and than that in response to saline treatment (Douglas *et al.* 1994), and is a more potent stimulus for the induction of Fos and TH expression in the NTS prior to delivery (see chapter three), the effects of oxytocin on TH mRNA expression, might, at least partly, be mediated by the stress and pain (McMahon *et al.* 1992, Nankova *et al.* 1994) that is likely to be associated with oxytocin-induced labour.

Though day 21 prepartum rats injected for four hours with saline pulses showed a lower TH mRNA content in both the NTS and the ventrolateral medulla than vehicle-treated and untreated day 21 prepartum rats, while rats given an oxytocin pulse treatment for four hours showed a TH mRNA content per NTS neurone similar to vehicle- and untreated prepartum rats. Whether saline treatment has a negative effect on TH mRNA expression in the

brainstem, which is compensated for by the oxytocin treatment, or whether other factors accounted for the general lower levels of TH mRNA expression in these two groups cannot be answered by this study. Possibly the time of killing, that was latest in saline- and oxytocin-treated rats (after 15.45 h compared to 15.00-15.30 h in untreated and 11.00-15.00 h in vehicle-treated rats) and thus closest to the time of parturition, might have contributed to the lower expression of TH mRNA in the brainstem and further supports the aforementioned hypothesis that labour is associated with a decrease in the TH mRNA content of NTS cells. Furthermore, saline injections might have had an effect on the general circulation, including blood pressure and volume, and thus might have influenced the TH mRNA content in the NTS. In progesterone-treated rats, the TH mRNA content in the NTS on day 21 of pregnancy was significantly lower than in vehicle-treated rats. Since in these rats delivery is delayed by more than one day and its progress impaired and not reflected by an increase in Fos expression in the NTS (see chapter five), our data support a physiological importance for the increase in TH mRNA expression in late pregnant rats. Thus, an impaired excitatory input from noradrenergic brainstem neurones to the SON might contribute to the lack of normal activation of supraoptic neurones during progesterone delayed delivery.

In the brainstem, TH mRNA expression has been reported to change during the oestrous cycle, namely to increase in A1 neurones at the time of the LH surge (Liaw *et al.* 1992c), when plasma oestrogen concentrations are high (Freeman 1988), pointing to the possible involvement of gonadal steroids, namely a stimulatory effect of plasma oestrogen, on TH mRNA expression. Indeed, in distinct areas of the rat hypothalamus, TH immunoreactivity is modulated by oestrogen treatment (Yuri & Kawata *et al.* 1994).

The decline in plasma and uterine progesterone concentrations is considered a critical step in the initiation of uterine activity and hence parturition (Bosc *et al.* 1987, Csapo & Wiest 1969, Csapo *et al.* 1980, Saito *et al.* 1985) and progesterone treatment of late pregnant rats prolongs the quiescent state of the uterus maintained during most of pregnancy (El Alj *et al.* 1989). Furthermore, progesterone, which has analgesic (Frye & Duncan 1994), sedative (Paul & Purdy 1992) and anxiolytic (Freeman *et al.* 1993) properties, could, when given to late pregnant rats, reduce the transmission of uterine sensations to the NTS and thus alter the induction of TH mRNA expression in these neurones.

Beside the effects of gonadal steroids on TH mRNA expression, there is evidence for the involvement of gonadal steroids in catecholamine synthesis and/or release (Chaudhuri *et al.* 1992, Demling *et al.* 1985) and hence a physiological implication of such regulation. Thus,

subcutaneous administration of progesterone to intact female rats can reduce the noradrenaline content of the hypothalamus and the medulla (Chaudhuri *et al.* 1992), while in oestrogen-primed ovariectomised rats, prior to the induced LH-surge, the release of noradrenaline in response to electrical stimulation of A1 neurones is higher than in animals not primed with oestrogen (Herbison *et al.* 1990).

An additional interpretation with regard to the observed changes in TH mRNA expression in neurones likely to mediate oxytocin release includes the involvement of catecholamines in the formation of synaptic contacts, as has been shown in the developing rat brain (Parnavelas & Blue 1982). Since at the end of pregnancy, morphological changes in the hypothalamus, including an increase in somatosomatic and somatodendritic appositions and an increase in "double synapses" (Theodosis & Poulain 1984, Theodosis *et al.* 1986a, 1986b, Tweedle & Hatton 1982), take place, the increase in TH mRNA expression in the NTS might indicate a role of the catecholaminergic projection from the NTS to the hypothalamus for the initiation of structural changes at term. Furthermore, the observation that in late pregnant rats, noradrenaline release in the SON is increased prior to rather than during parturition itself (Herbison, personal communication) suggests that the higher TH mRNA content in the NTS before compared to that during parturition might be the response to catecholamine release and could reflect synthetic activity. Though such presumed TH gene activation in the NTS prior to delivery is not associated with an increase in Fos expression (Luckman 1995), the induction of TH gene expression does not necessarily involve activation of the AP-1 site by Fos/Jun heterodimers (Ginzberg & Ziff 1994), but can also be mediated by a calcium responsive element (Kedzierski *et al.* 1994, Kim *et al.* 1994). Thus, the lack of Fos expression in catecholaminergic neurones in response to a stimulus does not exclude activation of TH gene expression.

However, such a presumed involvement of catecholamines prior to delivery does not explain the observed increase in TH mRNA expression and TH immunoreactivity in the NTS in response to oxytocin-induced labour. Since oxytocin-induced labour (and delivery) is a more potent stimulus of uterine activity (Randolph & Fuchs 1989), it might have activated TH gene expression by afferent pathways other than or in addition to those normally activated during labour and thus led to a concomitant expression of both TH mRNA and TH protein (Czyzyk-Krzeska *et al.* 1994, Kedzierski *et al.* 1994, Wang & Porter 1986). Indeed, a variety of stimuli, including stress and hypoxia, can rapidly induce TH gene expression and TH activity (Czyzyk-Krzeska *et al.* 1994, Nankova *et al.* 1994). Since repeated stimulation has

been shown to induce a more persistent and more pronounced increase in TH mRNA expression and a sustained rise in TH protein and activity (for 1-3 days), and it has been suggested that such sustained response can sensitise the system, so that it can display an augmented response in the case of repeated or prolonged activation (Nankova *et al.* 1994). Thus, oxytocin-induced labour in term pregnant rats might be an example for the stimulation of a highly sensitised system, namely the excitatory noradrenergic projections from the NTS onto the magnocellular hypothalamus, and thus might have induced the concomitant increase in TH mRNA and TH expression.

Unlike the number of TH-immunoreactive cells in the NTS, that was increased following oxytocin- and decreased following progesterone-treatment on the day of expected term, the number of NTS neurones expressing TH mRNA were not different between groups. This could be due to the greater sensitivity of *in situ* hybridisation compared with immunocytochemistry to detect positive cells and hence some cells faintly stained for TH protein might not have been included in the calculation. In addition, an increase in TH mRNA upon stimulation, that is further enhanced by an increased stability of the transcript, has been shown in some conditions to be more pronounced than the rise in TH protein and TH activity (Czyzyk-Krzeska *et al.* 1994, Kedzierski *et al.* 1994). The greater number of TH mRNA containing cells in the NTS of untreated rats in the first experiment compared to rats injected with progesterone, oxytocin or the respective vehicle seems to reflect experimental variability rather than a specific effect since in the ventrolateral medulla the number of TH mRNA containing cells was similar between experimental groups.

Finally, we have shown with the distribution histograms, made for each treatment group according to the amount of silver grains (and hence TH mRNA content) per cell for all cells counted, that in prepartum rats there were relative more cells containing a high amount of silver grains, while in parturient and progesterone-treated rats there was a shift to the left and hence to lower amounts of silver grains per cell. In saline- and oxytocin-treated rats, the distribution histograms were similar when the NTS was examined as an entity, while a tendency to a higher amount of silver grains/cell was apparent in the rostral part of the NTS in the oxytocin-treated group. Other workers have described a population of TH containing "sleeping cells", that normally contain very little TH mRNA, but can, in response to certain stimuli, increase their TH mRNA expression (Debure *et al.* 1992). Though we cannot rule out the possibility that the increase in TH mRNA expression in the NTS at the end of pregnancy is due to the existence of two catecholaminergic populations in the NTS, one which is only

activated in late pregnancy and shows a higher expression of TH mRNA before than during parturition, and another population, in which TH mRNA does not change during pregnancy, the similar shape of the distribution histograms in control and experimental groups indicates that the increased amount of silver grains per cell in the prepartum group is the result of an overall higher expression of TH mRNA compared to the parturient group.

Taken together, our data indicate that the catecholaminergic projection from the NTS to the SON might play a role for the excitation of hypothalamic oxytocin neurones at term and hence the increased release of oxytocin from the posterior pituitary during parturition. Whether noradrenaline is also involved in the initiation of morphological changes in the hypothalamo-neurohypophysial system of late pregnant rats and the facilitation of bursting activity during delivery and lactation remains to be investigated. However, recent data indicating a synergistic effect of catecholamines together with NPY and AMPA on oxytocin release in lactating rats (Parker & Crowley 1993a, 1993b) open up the possibility that the excitatory effects of catecholamines on magnocellular oxytocin neurones can be potentiated by other transmitters, some of which, as in the case of NPY, are co-localised within a subpopulation of noradrenergic NTS neurones that project to the hypothalamus (Sawchenko *et al.* 1985).

Fig. 6.1. ***In situ*** **hybridisation for TH mRNA in the brainstem**
Top: Coronal section through the hindbrain at the level of the area postrema with the region of the NTS dorsal and lateral of the central canal.
Bottom: Higher magnification view of cells in the NTS hybridised with an antisense oligonucleotide probe against the rat tyrosine hydroxylase gene (silver grains overlying cells counterstained with methylene blue).
cc = central canal, scale bar 100 μm

TH mRNA in the NTS

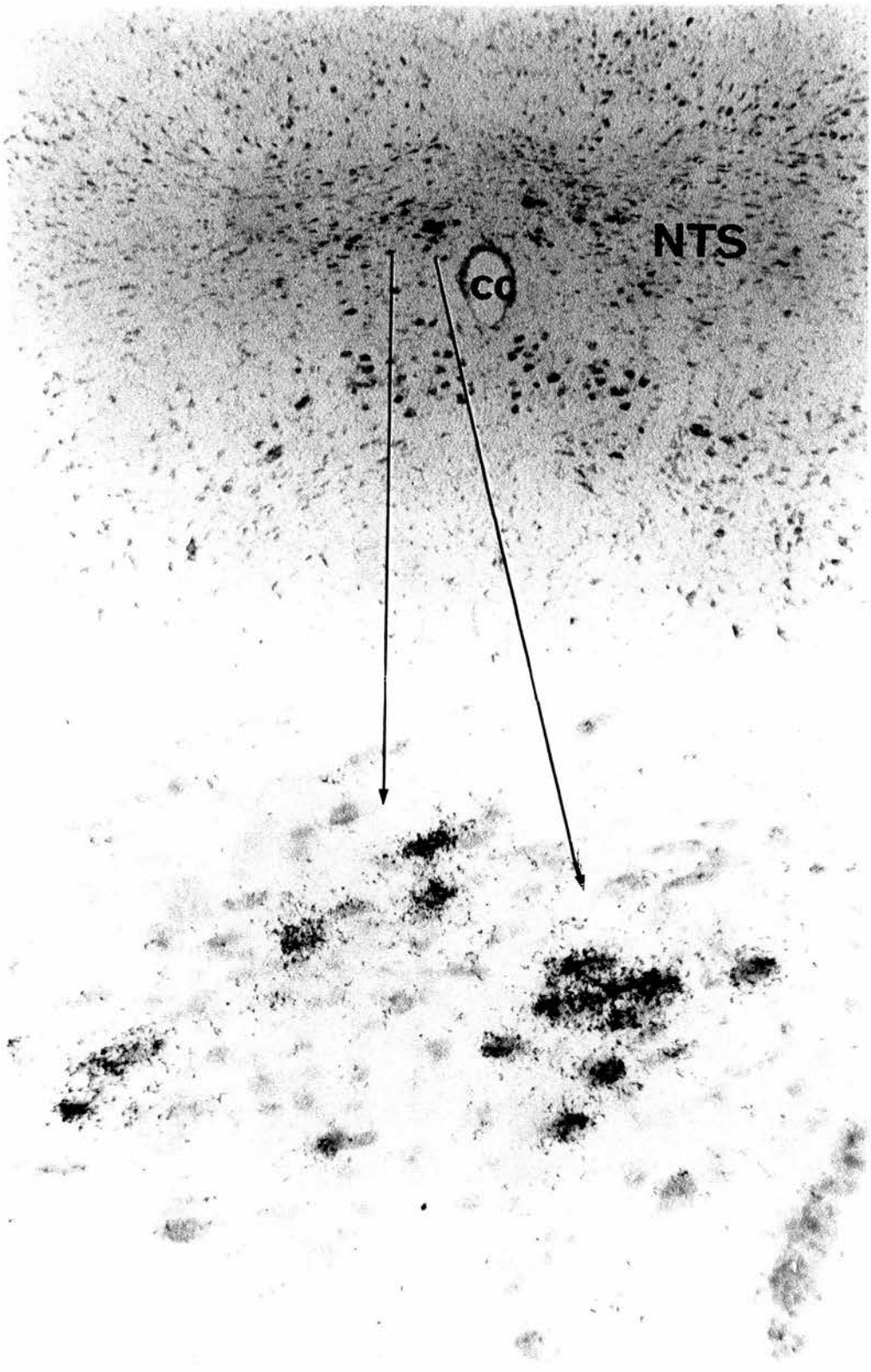


Fig. 6.2. TH mRNA expression in the brainstem of cycling, pregnant and lactating rats
Top: TH mRNA content was assessed using radioactive *in situ* hybridisation for TH mRNA in neurones of the NTS (A2 cells) and the ventrolateral medulla (VLM, A1 cells) of cycling, pregnant and lactating rats. The signal was counted using an automated image analysis system and expressed as number of silver grains/cell in A2 and A1 neurones of untreated cycling rats (killed in metestrous and proestrous, respectively, open and shaded bars, both n=4), pregnant rats (on day 17 and day 21 of pregnancy, respectively, killed before delivery of any pups, wide and narrow cross-hatched bars, both n=4) and lactating rats (day 10 of lactation, hatched bars, n=4). All rats were killed by cervical dislocation between 15.00-15.30 h.
The number of silver grains/cell was significantly higher in day 21 pregnant rats compared to day 17 pregnant and lactating rats (*$p<0.05$, Kruskal-Wallis test), while there was no significant difference between groups in the VLM.
Bottom: Means$\pm$s.e.m. number of cells containing silver grains in the NTS and the VLM of cycling, pregnant and lactating rats
The mean number of cells containing silver grains in the NTS and the VLM (6-8 sections per area per animal) was not significantly different between groups.

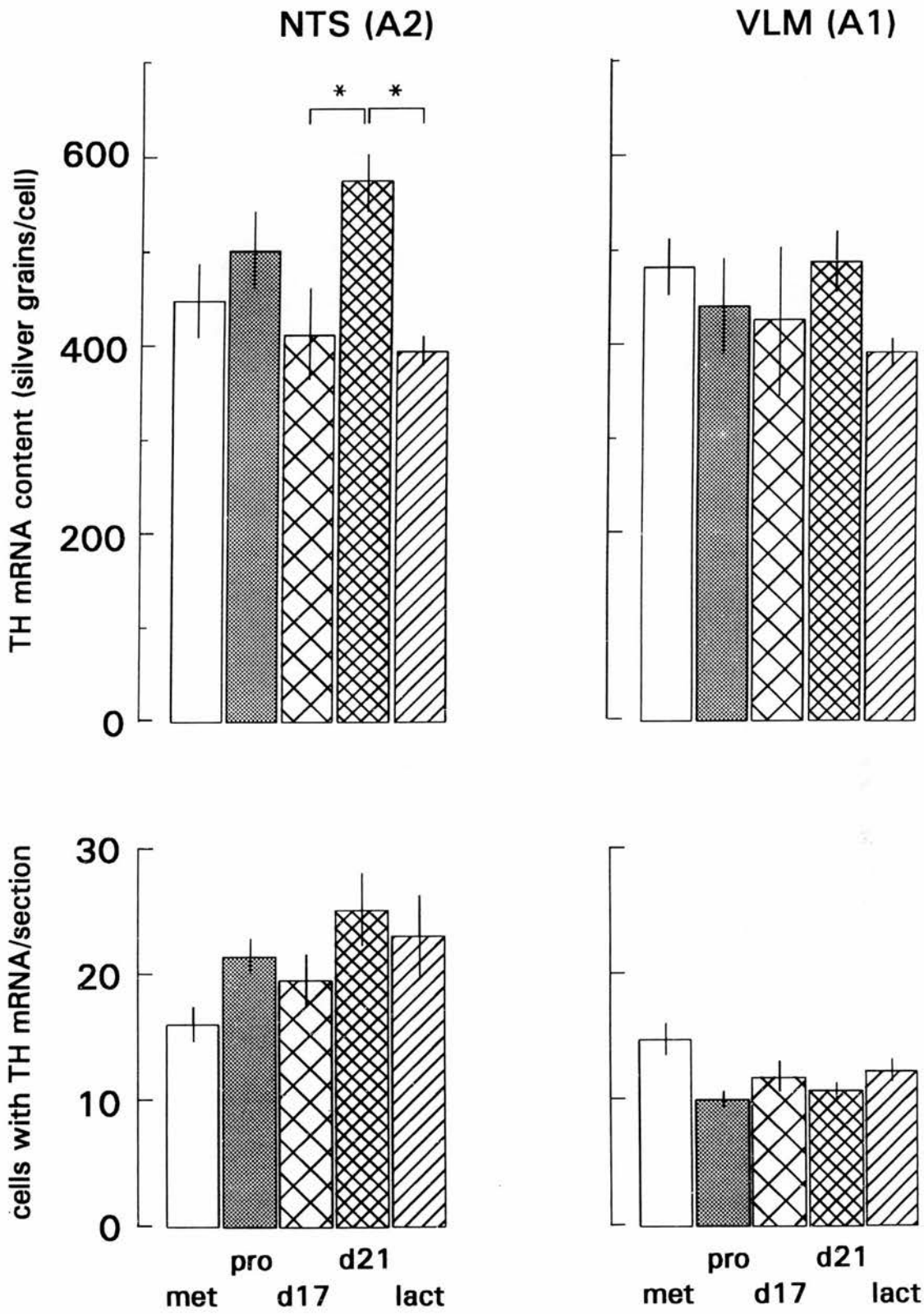

NTS (A2)
VLM (A1)
TH mRNA content (silver grains/cell)
600
400
200
0
*
*
cells with TH mRNA/section
30
20
10
0
met
pro
d17
d21
lact
met
pro
d17
d21
lact

Fig. 6.3. Effects of progesterone on TH mRNA expression in the brainstem of late pregnant rats

Top: TH mRNA expression (silver grains/cell) was counted in A2 and A1 neurones of rats injected subcutaneously with vehicle (0.2 ml, open bars, all n=5-6) or progesterone (5 mg, filled bars, all n=6) on day 20 of pregnancy and killed either on day 21 (or day 22, progesterone-treated rats only) before delivery of any pups (prepartum, between 11.00-13.00 h) or 90 min after the birth of the second pup (parturient, between 11.00-15.00 h on day 21).

We observed a significantly higher number of silver gains/cell in the NTS of vehicle-treated prepartum rats compared to vehicle-treated parturient and progesterone-treated day 21 prepartum rats (*$p<0.05$, Kruskal-Wallis test), while there was no significant difference between groups in the VLM.

Bottom: Means±s.e.m. number of cells containing silver grains in the NTS and the VLM of vehicle- and progesterone-treated rats

The mean number of cells containing silver grains in the NTS and the VLM (6-8 sections per area per animal) was not significantly different between groups.

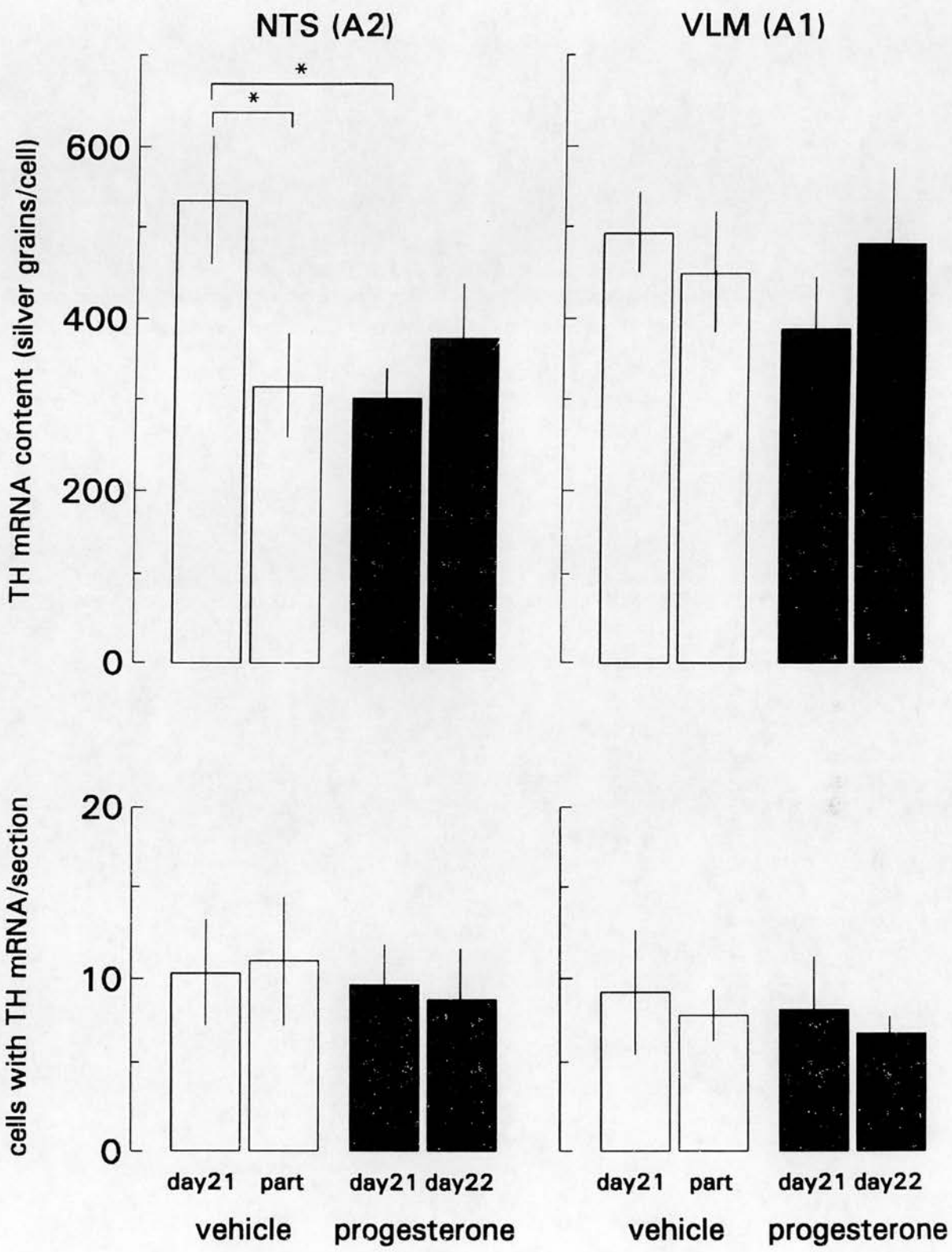

NTS (A2)
VLM (A1)
*
*
600
400
200
0
TH mRNA content (silver grains/cell)
20
10
0
cells with TH mRNA/section
day21
part
day21
day22
vehicle
progesterone
day21
part
day21
day22
vehicle
progesterone

Fig. 6.4. Effects of oxytocin on TH mRNA expression in the brainstem of late pregnant rats

Top: TH mRNA expression (silver grains/cell) was counted in A2 and A1 neurones of rats injected with pulses of saline (open bars, n=6) or oxytocin (10/20 mU each 10 min for 4 hours, filled bars, n=6) in the morning of day 21 of pregnancy and killed between 15.45-16.45 h on that day. In addition, the analysis was performed in the brainstem subdivided into a caudal part (extending from the pyramidal decussatio to the caudal end of the area postrema) and a rostral part (form the caudal to the rostral end of the area postrema) according to the rat brain atlas of Paxinos & Watson 1986 (see inset graphs).

When TH mRNA content was assessed in the entire NTS, there was no significant difference between the oxytocin- and the saline-treated groups, while there was a significant increase in the number of silver grains/cell in the rostral NTS of oxytocin- compared to saline-treated rats (*$p<0.05$, Kruskal-Wallis test). In the caudal NTS and the VLM, there was no significant difference between groups.

Bottom: Means$\pm$s.e.m. number of cells containing silver grains in the NTS and the VLM of saline- and oxytocin-treated rats

The mean number of cells containing silver grains in the NTS and the VLM (5-8 sections per area per animal) was not significantly different between groups, neither for the entire NTS and VLM, nor for the rostral and caudal subdivisions.

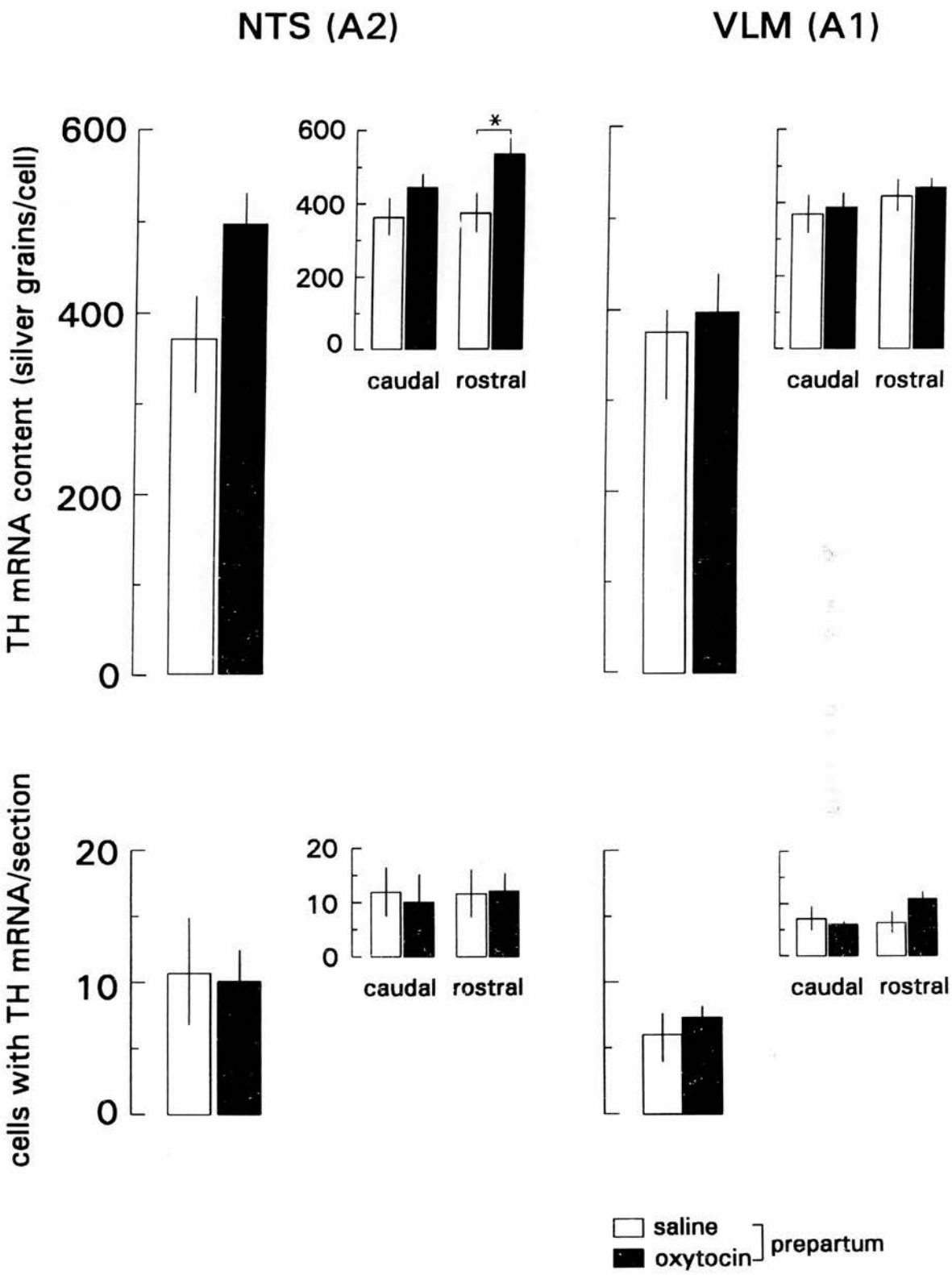

NTS (A2)
VLM (A1)
TH mRNA content (silver grains/cell)
600
400
200
0
*
caudal
rostral
cells with TH mRNA/section
20
10
0
saline
oxytocin
prepartum

Fig. 6.5. Distribution histogram of TH mRNA containing cells in the NTS of prepartum and parturient rats

Distribution histogram of cells containing TH mRNA in the NTS according to the amount of silver grains/cell for vehicle-treated prepartum and parturient rats were established. Cells (n=65, **TOP**) of five vehicle-injected rats killed before delivery were assigned 100% and the percentage was calculated of cells containing less than 100, 100-200 silver grains etc. up to 1000 silver grains. The same procedure was performed for cells (n=83, **Bottom**) of six vehicle-treated rats killed during parturition.

In parturient rats there was a shift to the left and hence to a lower level of TH mRNA content per cell compared with prepartum rats.

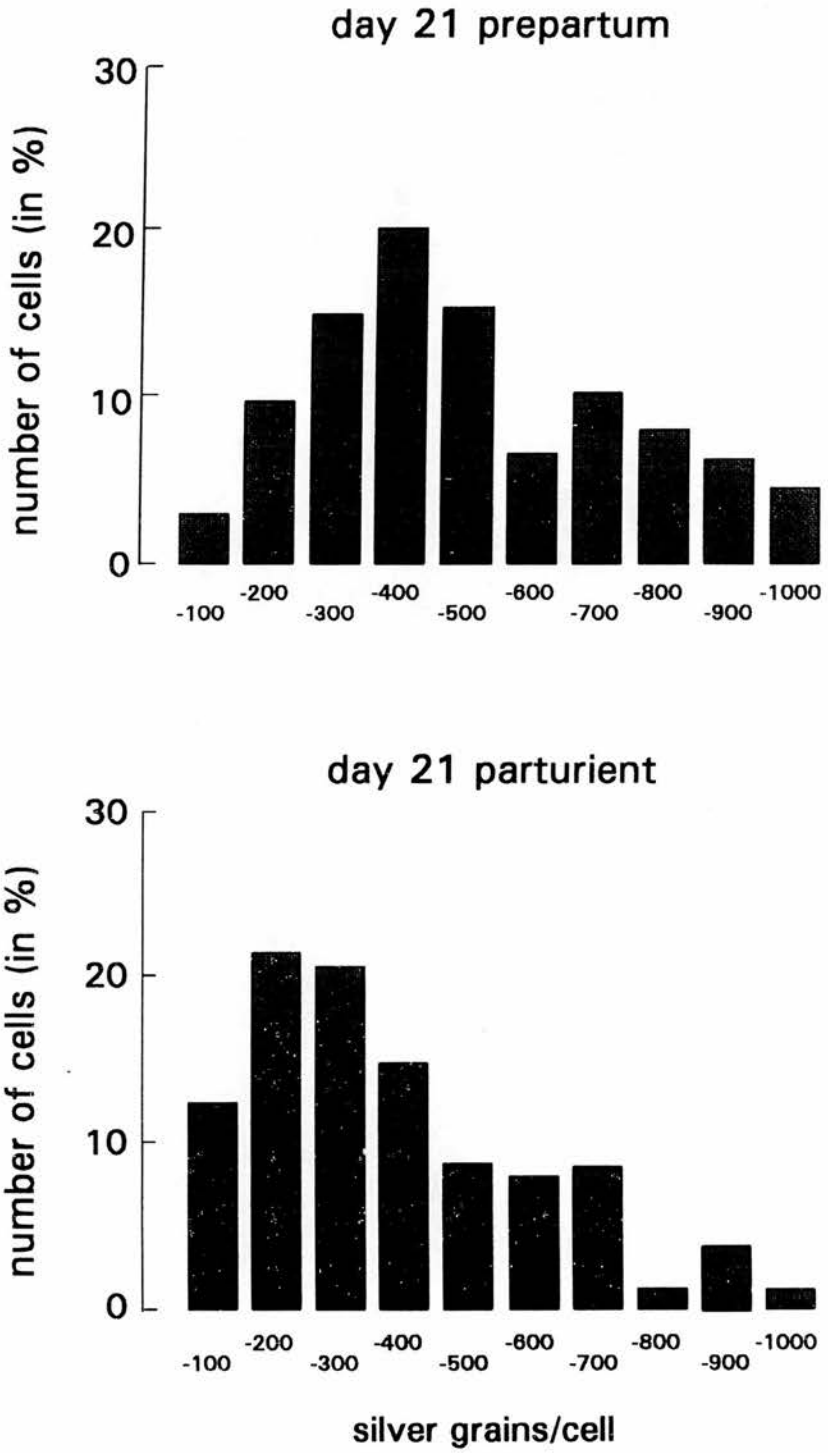

day 21 prepartum
number of cells (in %)
30
20
10
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
day 21 parturient
number of cells (in %)
30
20
10
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
silver grains/cell

Fig. 6.6. Effect of progesterone on the distribution histogram of TH mRNA containing cells in the NTS

Distribution histograms as described in Fig. 6.5. were established for cells of progesterone-injected rats (5 mg sc, on day 20 of pregnancy) killed before delivery either on day 21 (5 rats, number of cells =79, **TOP**) or day 22 (5 rats, number of cells n=63, **Bottom**).

Progesterone-treated rats killed on day 22 showed a marked shift to the right compared to progesterone-treated rats killed on day 21, while the latter showed a shift to the left compared to control prepartum rats (c.f. Fig. 6.5).

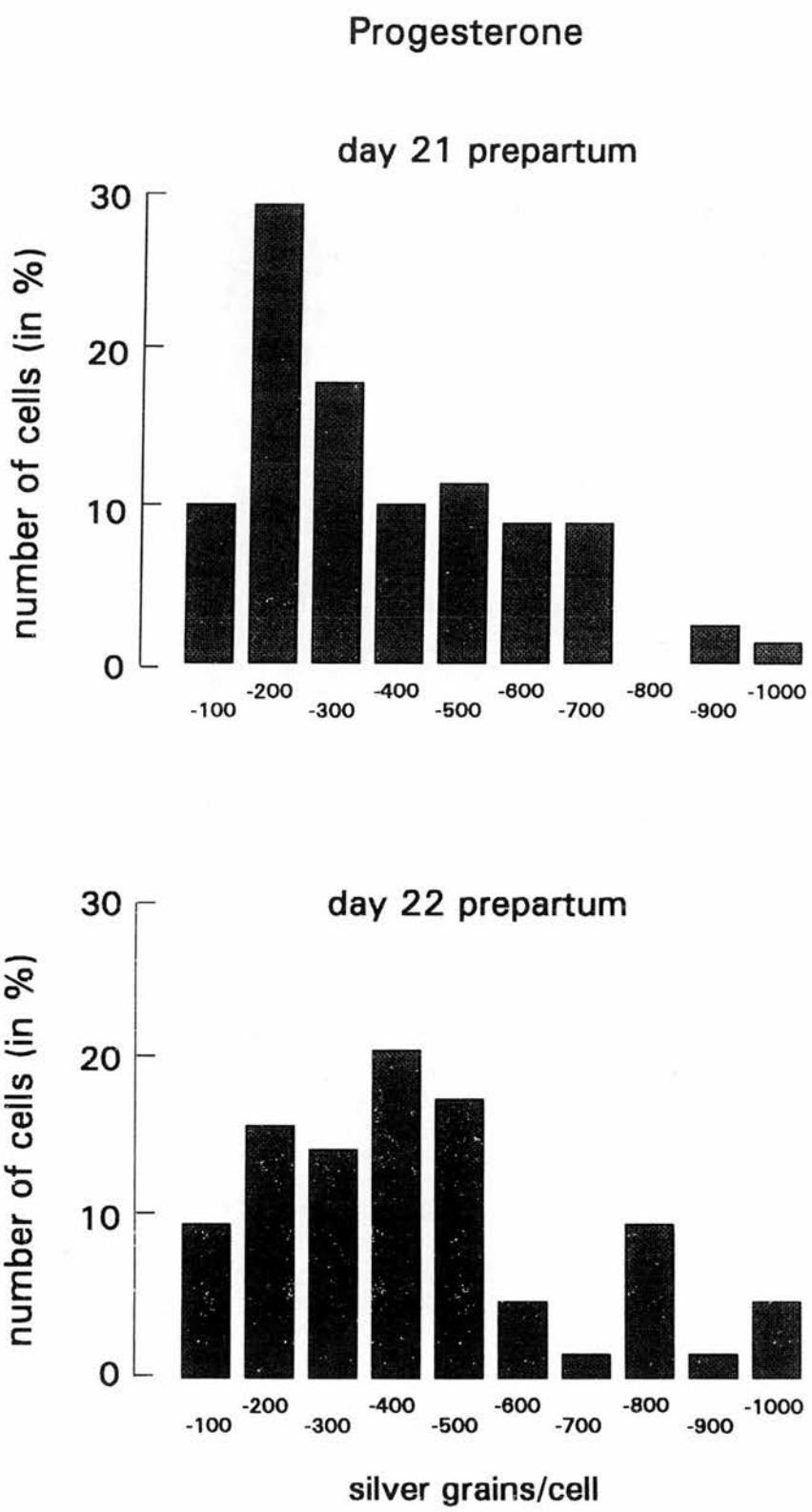
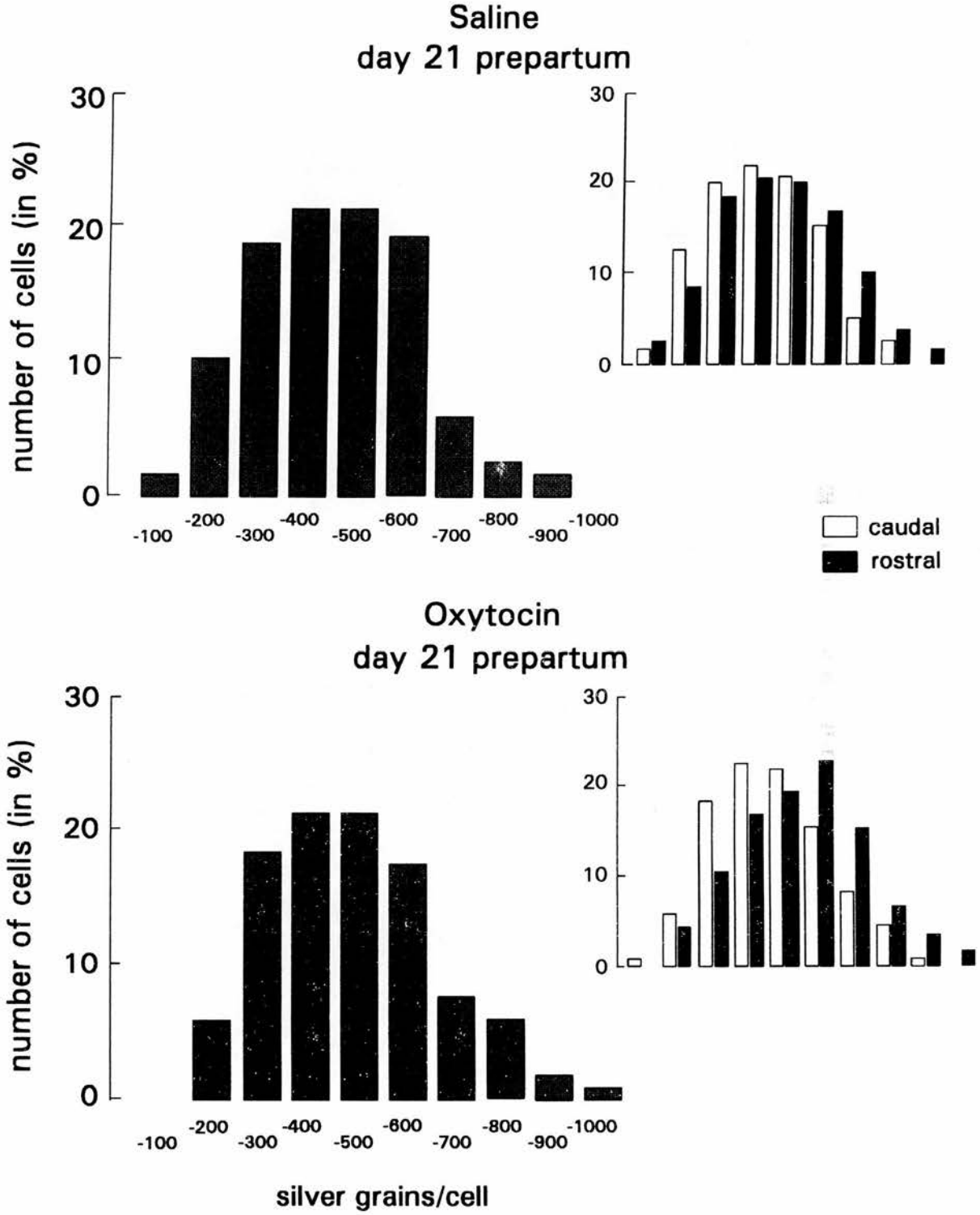

Progesterone
day 21 prepartum
number of cells (in %)
30
20
10
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
day 22 prepartum
number of cells (in %)
30
20
10
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
silver grains/cell

Fig. 6.7. Effect of oxytocin on the distribution histogram of TH mRNA containing NTS cells

Distribution histogram (see Fig. 6.5.) were established for cells of six saline- (number of cells=123, **TOP**) and six oxytocin-injected rats (number of cells=104, **Bottom**), all killed before delivery. In addition, separate distribution histogram were calculated for cells located in the caudal (open bars) and rostral (filled bars) NTS, respectively (insets: **Top** for saline-treated rats, number of cells n=69, 54, **Bottom** for oxytocin-treated rats, number of cell n=58, 46 in the caudal and rostral part, respectively).

When TH containing cells were analysed throughout the NTS, there was no significant difference in the distribution histograms for the two treatment groups. However, when cells in the rostral and caudal NTS were analysed separately, there was a small shift to the right and hence to a higher silver grain content per cell in the rostral NTS in oxytocin- compared to saline-treated rats (see inset graphs).


Saline
day 21 prepartum
number of cells (in %)
30
20
10
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
caudal
rostral
Oxytocin
day 21 prepartum
number of cells (in %)
silver grains/cell

Chapter 7

Effects of pulsatile oxytocin injections to late pregnant rats on uterine pressure and electrical activity of supraoptic neurones

In the previous chapters, I have put forward the hypothesis that at the end of pregnancy uterine activity, induced by exogenous oxytocin or spontaneously during labour, can exert a positive feed back onto magnocellular oxytocin neurones that will eventually initiate the increased release of oxytocin from the posterior pituitary.

It is known that at the end of pregnancy and during lactation (Dreifuss *et al.* 1976), but not in early and mid- pregnancy, vagino-cervical stimulation leads to an increase in firing rate of magnocellular neurones (Negoro *et al.* 1973b) and at the time of delivery, the birth of each pup is preceded by strong abdominal and uterine contractions (Higuchi *et al.* 1986a, 1987) and burst-like activity of putative oxytocin cells in the PVN (Summerlee 1981). The high frequency discharge of neurosecretory oxytocin cells is reflected by the pulsatile release of oxytocin during delivery, which is superimposed on an elevated background secretion (Higuchi *et al.* 1986b).

In the course of pregnancy, the neural lobe content of both oxytocin and vasopressin is increased compared to virgin rats (Kumaresan *et al.* 1979) and the accumulated excess of both peptides is released in the course of parturition (Fuchs & Saito 1971, Kumaresan *et al.* 1979). Though the role of vasopressin for labour and delivery remains unclear, there is evidence for a reflex activation of supraoptic vasopressin neurones, similar to oxytocin neurones, in response to vagino-cervical stimulation (Dreifuss *et al.* 1976).

An increase in neurosecretory activity of both types of magnocellular neurones during parturition, suggesting an involvement of vasopressin and oxytocin for labour and delivery, is in agreement with our observation that in parturient rats Fos expression is increased in both types of supraoptic neurones (see chapter one & three). Furthermore, I have shown that Fos expression can be induced in late pregnant rats in the SON, including oxytocin and vasopressin neurones, and in putative afferent neurones in the brainstem with a four hour oxytocin pulse treatment prior to delivery, indicating that vagino-cervical stimulation is not a prerequisite for the stimulation of neurosecretory activity in supraoptic neurones. Since we have shown previously that pulsatile administration of oxytocin can induce uterine contractions and delivery in late pregnant anaesthetised rats and that such induced labour is associated with an increase in Fos expression in the SON and putative afferent brainstem areas (Douglas *et al.* 1994), here we assessed electrical activity of supraoptic neurones and uterine pressure changes in late pregnant anaesthetised rats during pulsatile oxytocin administration.

Materials and Methods

Animals and Surgery

Date mated day 21 pregnant Wistar rats were anaesthetised with sodium pentobarbitone (initially 20-30 mg/kg intraperitoneally) and implanted with a jugular vein cannula (see general materials and methods section). Two rats were in addition implanted with a uterine balloon, one rat was implanted with two uterine balloons, one in each horn, and three rats were implanted with two balloons each, one in the uterus and the other in the cervix. Then the surgical placement of the electrodes was performed by Dr. G. Leng (see general materials and methods section).

After a minimum recovery time of 60 min the recording was started. Electrical activity of supraoptic neurones was recorded in 100 msec (10 Hz) bins, as spikes/sec, uterine and cervical pressure changes were recorded as analogue data in Volt and converted with a force

transducer into mmHg. Six of the seven rats, IG1-6, were injected with oxytocin pulses (10 mU oxytocin in 0.03 ml 0.9% saline, each 10 min for 2 hours and then 20 mU oxytocin each 10 min for 2 hours), while in one rat the cervix or uterine balloon was de- and inflated (by $\pm$1-2 and $\pm$1.5-3 ml, respectively) and cell activity in response to such acute pressure changes was recorded.

Cells were identified as projecting to the posterior pituitary by displaying a constant latency action potential in response to a biphasic stimulus (2 ms, 40 Volt) applied to the neural stalk. To classify antidromically activated cells as vasopressin or oxytocin neurones, the spontaneous firing pattern was taken into consideration (cells displaying phasic activity were presumed to be putative vasopressin neurones and cells showing continuous activity were classified as putative oxytocin neurones) and in addition most cells were characterised by their response to an intravenous injection of CCK (20 µg/kg body weight: cells displaying no change in activity or a transient decrease were classified as putative vasopressin neurones and cells showing a transient increase in firing rate were classified as putative oxytocin neurones). For cells that failed to display a clear response to CCK, we gave a injection of naloxone (5 mg/kg body weight iv) followed by a second injection of CCK, since in late pregnant rats electrical activity of supraoptic oxytocin neurones is under an inhibitory opioid tone (Bicknell *et al.* 1988c). For some cells, which were pharmacologically identified prior to the treatment, a minimum time of 10-20 min was allowed before the four hour oxytocin treatment was started.

Data analysis

The recordings were analysed using Spike 2 (Version 4.70, 1991, Cambridge Electronic Design Ltd., Cambridge) and a Genstat program written by David Brown. The latter was used to analyse correlations and long-term changes in cell activity, uterine and cervical pressure in the course of the four hour treatment. The recording time consisted of three periods: 1) the control period, 30-60 min, prior to the oxytocin treatment and 2) the treatment period that was subdivided either according to the dose of oxytocin into a first two hour period (during which animals were injected with 10 mU oxytocin each 10 min) and a second two hour period (during which 20 mU oxytocin were injected each 10 min) or into 60 min periods. For the statistical analysis using the Genstat program each period was subdivided into 200 sec bins and for each bin the following parameters were calculated:

- uterine and cervical pressure and contraction amplitude (in mmHg)
- frequency (in spikes/sec)
- activity quotient (in percent as the time in sec during which the cell was active divided by 200 sec, calculated for each 200 sec in 100 msec bins)

and finally the overall mean was calculated for each of the above variables during each period. In addition we calculated for each period:

- the correlation of each of the above parameters with time
- the correlation between uterine/cervical pressure and cell activity and
- the correlation between the two pressure recordings in the case of two balloons per rat (2x uterine pressure or uterine and cervical pressure)

Comparisons for means±s.e.m. of uterine and cervical pressure and amplitude were performed using the two-tailed non-parametric Mann-Whitney U-test. In addition we calculated the average spike activity for all analysed cells before and during each hour of the treatment using the rate program written on Spike 2. We compared electrical activity of magnocellular neurones during a period of at least 10 min prior to any treatment with 10 min periods during the end of each hour of the oxytocin treatment, or during 10 min periods after one or two hours of recording without any treatment. Spike activity during these periods was compared using the parametric t-test. A similar approach was used to assess statistically acute changes in neuronal activity, e.g. following an injection of CCK and each injection of oxytocin, and in response to vaginal probing, cervical and uterine distension. Thus, the average firing rate during 1-3 x 60 sec each before and after the stimulus was calculated and compared with each other using the parametric t-test. For all comparisons spike activity was calculated in 1 sec bins unless stated otherwise.

Results

Electrical activity was recorded in seven rats and of a total of 18 cells, of which 16 were analysed (the two recordings not analysed were excluded due to a duration of less than 2000 sec per recording). Of the 16 cells analysed, 11 displayed a clear response to CCK that allowed to identify them as either putative oxytocin (increase in firing rate) or putative vasopressin neurone (decrease in firing rate, Fig. 1 & 2), in addition to their classification according to the firing pattern (continuous and phasic activity for oxytocin and vasopressin neurones, respectively, Table 1).

All but one animal were implanted with a uterine balloon, so that uterine pressure could be recorded simultaneously with cell activity (Fig. 1). In one rat, uterine pressure was recorded from both horns and in three other rats uterine and cervical pressure were recorded in addition to neuronal activity.

Table 1

Individual recordings from supraoptic neurones without oxytocin treatment

cell	duration (sec)	firing pattern	response to CCK (spikes/sec) activity before	after	putative identification
C1	4500 sec	phasic	4.3±0.21	3.2±0.2*	vasopressin neurone
C2	2500 sec	phasic	-	-	vasopressin neurone
C3	7500 sec	continuous	2.5±0.1	3.4±0.1*	oxytocin neurone
C4	10000 sec	phasic	-	-	vasopressin neurone
C5	4300 sec	phasic	-	-	vasopressin neurone
C6	4000 sec	phasic	1.5±0.2	0.3±0.07*	vasopressin neurone
C7	4000 sec	phasic	6.7±0.5	3.0±0.5*	vasopressin neurone

*$p<0.05$, t-test compared to the value before the CCK injection

All but one animal were given a four hour oxytocin pulse treatment (10 mU oxytocin each 10 min for 2 hours and then 20 mU oxytocin each 10 min for 2 hours), while in one animal, implanted with two balloons, one in the uterine body and the other in the cervix, these balloons were alternately de- and inflated with saline (±1-3 ml) and cell activity in response to acute pressure changes was investigated.

Recordings from supraoptic neurones during periods without treatment

To assess spontaneous activity of supraoptic neurones in late pregnant rats, activity from seven cells was recorded for one hour or more without oxytocin treatment (Table 1). The average duration of these recordings, that served as a control period for recordings of neuronal activity during the four hour oxytocin treatment, was 5257±901 sec (= 88±15 min, range 2500-10000 sec = 42-167 min). Beside being identified by the firing pattern, 4/7 neurones were classified according to their response to an intravenous injection of CCK (20 µg/kg body weight) as putative oxytocin or vasopressin neurones (Table 1, Fig. 1 & 2).

The firing rate of cells during these control periods was calculated as average number of spikes during a period of 15-20 min at the beginning of the recording and then during 10 min periods at the end of each hour of the recording. Activity of vasopressin neurones significantly decreased in the course of 60 min without oxytocin treatment and without uterine pressure manipulations (C1 and C2, Table 2). However, when uterine pressure was changed manually by de- and inflation of a balloon, spike activity significantly increased in the course of one hour in 2/4 vasopressin neurones (C4 and C5, Table 2), while in the other two cells there was no significant change (C6 and C7, Table 2, Fig. 1). For one cell, in which electrical activity was recorded for a total of three hours, the spike activity was significantly decreased at the end of the recording compared to the beginning (C7, Table 2).

Table 2

Electrical activity of supraoptic neurones without oxytocin treatment

cell	type	neuronal activity (spikes/sec)			
		start of recording	after 1 hour	after 2 hours	after 3 hours
C1	vasopressin	3.3±0.2	2.5±0.2*		
C2	vasopressin	4.3±0.1	3.5±0.1*		
C3	oxytocin	2.5±0.1	1.8±0.1*	2.2±0.1	
C4	vasopressin	2.3±0.1	5.8±0.2*		
C5	vasopressin	1.5±0.8	2.2±0.1*		
C6	vasopressin	7.0±0.2	7.4±0.6		
C7	vasopressin	4.5±0.2	4.7±0.2	4.5±0.2	3.8±0.2*

*$p<0.05$, t-test compared to the control (first) value

Electrical activity without oxytocin treatment, but during uterine pressure manipulations, was recorded from only one putative oxytocin neurone (C3, Table 2), which showed a small but significant decline in spike activity in the course of the first hour (Table 2, Fig. 1).

Recordings from supraoptic neurones during the four hour oxytocin pulse treatment

Six animals were injected with pulses of oxytocin (10 mU oxytocin each 10 min for 2 hours and then 20 mU oxytocin each 10 min for 2 hours) and a total of nine cells were recorded during such treatment (Table 3).

The average recording time for all nine cells was 9100±1621 sec (152±27 min, range 3500-19000 sec = 58-317 min), with an average duration of 8268±1560 sec for six oxytocin neurones and 10767±3539 sec for three vasopressin neurones. Thus, for 3/9 cells recorded during the oxytocin treatment the recording spanned nearly the entire four hour treatment period (12000-19000 sec, Fig. 3 & 4). For three cells, the recording extended over more than half of the treatment time (2-3 hours) and for three other cells the recording covered at least one hour of the treatment period (3600-5500 sec). Apart from two cells, one putative oxytocin and one putative vasopressin neurone, which were identified according to their firing pattern only, all other cells we additionally identified by their response to an intravenous injection of CCK (20 µg/kg body weight)(Table 3, Fig. 2).

Table 3

Individual recordings from supraoptic neurones with oxytocin treatment

oxytocin treatment: 10 mU each 10 min for 2 hours and then 20 mU each 10 min for 2 hours					
cell	duration (sec)	firing pattern	response to CCK (spikes/sec)		putative identification
			before	after	
T1	12000 sec	continuous	3.8±0.2	6.6±1.3*	oxytocin neurone
T2	19000 sec	phasic	5.1±0.3	0.3±0.1*	vasopressin neurone
T3	14600 sec	continuous	1.3±0.1	1.8±0.2*	oxytocin neurone
T4	7000 sec	continuous	0.3±0.003	1.4±0.1*	oxytocin neurone
T5	7000 sec	continuous	-	-	oxytocin neurone
T6	3600 sec	continuous	4.4±0.2	5.3±0.3*	oxytocin neurone
T7	5500 sec	continuous	6.1±0.3	7.0±0.6	oxytocin neurone
T8	4300 sec	phasic	0.9±0.03	0.3±0.01*	vasopressin neurone
T9	9000 sec	phasic	-	-	vasopressin neurone

*$p<0.05$, t-test compared to the value before the CCK injection

Electrical activity during a minimum of 10 min prior to any treatment or at the beginning of a new recording served as the control value, to which all other values were compared (*$p<0.05$, t-test compared to the control value, Table 4a & b).

Thus, for six cells the control period was taken from before the start of the treatment, while for three cells the baseline was established from a 10 min period at the beginning of the

recording during the first hour of the oxytocin injections (Table 4). Activity was measured during the last 10 min of each hour of the treatment and these values were compared to the respective control value for each cell (Table 4a & b).

Table 4a

Change in electrical activity of oxytocin neurones during the oxytocin treatment

	oxytocin treatment 10 mU each 10 min for 2 hours and then 20 mU each 10 min for 2 hours				
cell	before treatment	1st hour	2nd hour	3rd hour	4th hour
T1	3.8±0.2	4.2±0.3	4.5±0.3	3.9±0.3	
T3	0.8±0.03	1.2±0.04*	1.6±0.04*	1.2±0.04*	
T5		0.3±0.003	0.7±0.04*	2.5±0.07*	2.4±0.7*
T7		4.7±0.1	5.0±0.1*	5.6±0.1*	5.8±0.1*
T4	0.3±0.003	0.5±0.04*			
T6	4.5±0.1	4.8±0.1*			

Table 4b

Change in electrical activity of vasopressin neurones during the oxytocin treatment

	oxytocin treatment 10 mU each 10 min for 2 hours and 20 mU each 10 min for 2 hours				
cell	before treatment	1st hour	2nd hour	3rd hour	4th hour
T2	1.2±0.1	1.7±0.1*	2.2±0.1*	3.1±0.2*	3.9±0.2*
T9		3.0±0.2	2.6±0.1	3.5±0.1*	3.3±0.1*
T8	0.2±0.03	0.5±0.03*			

*$p<0.05$, t-test compared to the control (first) value

Oxytocin neurones

In the first hour of the oxytocin treatment, electrical activity of all oxytocin neurones (n=4) increased. Likewise, in the course of the second hour electrical activity of putative oxytocin neurones showed a further increase, that was sustained in 2/4 cells, while in two cells

activity showed a slight decrease in the third hour. However, the firing rate remained higher than at the beginning of the treatment (Table 4a, Fig. 3 & 6).

Vasopressin neurones

Similar to oxytocin neurones, vasopressin neurones showed an increase in activity in response to the oxytocin treatment. For two cells, which were recorded through almost the entire four hour treatment, the firing rate increased progressively and was significantly higher at the end compared to the beginning of the recording (Table 4b, Fig. 4).

For those two vasopressin neurones, which showed a typical phasic pattern (T2 and T9), we calculated the activity quotient (the time in percent during which the cell is active, assessed for 0.1 sec bins throughout the recording, divided by the total recording time) that increased in both cells from the first to the third hour of the recording (46% and 45% in the first hours vs 56% and 74% in the third hour, respectively). The increase in the activity quotient was accounted for by a shortening of the silent intervals between periods of phasic activity (Fig. 4 & 11).

Uterine and cervical pressure and contraction amplitude

Uterine and cervical pressure and contraction amplitude were recorded in six animals, either prior to or during the oxytocin treatment and during acute pressure manipulations. The pressure changes during the oxytocin injections were compared to baseline values estimated from periods without such treatment (on average 3399$\pm$625 sec = 55$\pm$10.4 min).

Uterine pressure and contraction amplitude

In two animals, in which uterine pressure was recorded during a period without the oxytocin treatment and without pressure changes, uterine pressure and amplitude showed no dramatic change when the values at the beginning of the recording and after one hour were compared (an increase of uterine pressure by 0 and 1 mmHg, respectively), and no dramatic change in contraction amplitude ($\pm$1 mmHg and -6 mmHg, Table 5, Fig. 1). In the other two rats in which uterine and cervical pressure were manipulated uterine pressure decreased in the course of one hour (by 7.5 and 14.5 mmHg, respectively), while the amplitude showed either no significant change ($\pm$ 1 mmHg) or a decrease (by 7 mmHg, Table 5).

Table 5

Change in pressure and contraction amplitude during periods without oxytocin treatment

change in % (and mmHg, c.f. beginning/1 hour later)			
uterine pressure	uterine amplitude	cervical pressure	cervical amplitude
0% (0 mmHg)	+1% (1 mmHg)	200% (27 mmHg)	-71% (20 mmHg)
+1.1% (1 mmHg)	-11% (6 mmHg)	+45% (4 mmHg)	+50% (3 mmHg)
-50% (7.5 mmHg)	4% (1 mmHg)	-7% (3 mmHg)	+4% (1 mmHg)
-76% (14.5 mmHg)	-58% (7 mmHg)		

In contrast, during the four hour oxytocin pulse treatment uterine pressure showed a consistent and progressive increase in all animals examined, while the contraction amplitude decreased concomitantly (Fig. 3, 4, 5, 6 & 7). Thus, uterine pressure increased by 5.9±2.3 mmHg, 19.1±3.9* mmHg and 14.1±3.0 mmHg during the first and last two hours and after the end of the treatment, respectively (*$p<0.05$, U-test compared to baseline, Fig. 7). Unlike uterine pressure, the amplitude of uterine contractions decreased from a baseline value (= 100%) before the treatment by 0.6±0.02 mmHg, 4.1±0.2* mmHg and 0.6±0.1 mmHg during the first and last two hours and after the end of the treatment, respectively (*$p<0.05$, U-test compared to baseline, n=4, Fig. 4, 6 & 7). The steady increase in uterine pressure throughout the four hour treatment and the concomitant decline in contraction amplitude was most pronounced in two animals (Fig. 4 & 6). In one of these rats, in which uterine pressure was recorded from both uterine horns, the pressure in the two horns showed a synchronised increase when the oxytocin treatment was started, while there was no response of the uterus to an intravenous injection of CCK (Fig. 6). Though the synchronised activity between the two horns was apparent for most of the oxytocin treatment, the activity in both horns was most clearly correlated to each oxytocin injection during the last two hours of the oxytocin treatment, when both uterine horns displayed an increase in pressure and a decline in the contraction amplitude following each injection (Fig. 6, 7 & 8). However, the pressure in both horns showed a significant correlation at the beginning of the treatment and some further increase in the course of the treatment ($r^2= 0.75$ vs $r^2= 0.90$ at the beginning and the end of the treatment, respectively, $p<0.05$ for each period, Fig. 8).

The contraction frequency of the uterus showed no consistent change during the oxytocin injections, however, we did observe sometimes an increase in frequency following a pulse of oxytocin (Fig. 8), but not at other times (Fig. 1).

Cervical pressure and contraction amplitude

Cervical pressure and amplitude during periods without oxytocin treatment were recorded in three animals. Pressure and amplitude were measured during 10 min at the beginning of each recording and during 10 min after one hour of either no treatment or uterine and pressure manipulations. In the former case (n=1) there was very little change in cervical pressure and amplitude (a decrease in pressure by 3 mmHg and an increase in contraction amplitude by 1 mmHg). In the course of the uterine pressure manipulations (n=2), the cervical of one animal responded with a increase in both pressure and amplitude (by 4 and 3 mmHg, respectively), while the other animal showed a decrease in pressure and amplitude (by 27 and 20 mmHg, respectively, Table 5). Thus, the cervix showed no consistent change in pressure or contraction amplitude without any treatment or following uterine pressure changes.

In contrast, in both animals, in which cervical pressure and contraction amplitude were measured during the oxytocin treatment, we observed a decrease in cervical pressure and a concomitant increase in contraction amplitude and frequency during the oxytocin injections (Fig. 9, 10 & 11). These changes were directly opposite to those seen in the uterus during the oxytocin pulse treatment (Fig. 7). Thus, the cervical pressure decreased during the treatment compared to the baseline value by 13 and 4 mmHg during the first two hours and by 14 and 15 mmHg during the last two hours, respectively, and continued to decrease in the 60 min after the end of the treatment (by 10 and 20 mmHg from baseline), while the contraction amplitude increased (by 4 and 22 mmHg during the first two hours and by 12 and 31 mmHg during the last two hours, respectively, and remained at that level during the 60 min after the end of the treatment (Fig. 7).

Uterine pressure and cervical pressure in relation to each other

Unlike the significant correlation between the pressure in the two uterine horns of the same animal, apparent prior to the oxytocin treatment, there was no significant correlation between uterine and cervical pressure of the same animal before we started the treatment (r^2= 0.48 and r^2= -0.50, respectively, Fig. 10 & 11), but a significant positive correlation during the last two hours of the treatment (r^2= 0.96 and r^2= 0.84, $p<0.001$). Unlike the case for the

two uterine horns, we did not consistently observe a period of increased synchrony between cervical and uterine activity in response to an injection of oxytocin (Fig. 10 & 11). Thus, in one animal an injection of oxytocin, but not CCK, induced a series of synchronised and increased uterine and cervical contractions (Fig. 10 & 12), while in another animal CCK, but not oxytocin triggered a period of synchronised activity between the uterus and the cervix (Fig. 13).

In addition, we observed peak pressures which occurred simultaneously in the cervix and the uterus, though they were of higher amplitude in the former (Fig. 10). These cervical peak pressure increments showed no obvious relation to oxytocin injections, however they occurred more often in the second half of the treatment (Fig. 9 & 10), superimposed on the rhythmic contractions of both the uterus and the cervix and were not associated with an acute change in neuronal activity (Fig. 10).

The effect of oxytocin pulses on neuronal activity

Since the oxytocin pulse treatment did not only augment uterine activity, but also induced an increase in electrical activity of both types of supraoptic neurones, we investigated the effect of each oxytocin injection on neuronal activity. Thus, the mean firing rate of each neurone (n=6) was calculated for 1-2 x 60 sec bins immediately after each oxytocin injection and at mid-time between two injections.

Oxytocin neurones

In 2/6 oxytocin cells we observed a consistent excitation of the firing rate immediately following an injection of oxytocin, while at mid-time between two injections neuronal activity was relatively lower (Fig. 8, 14 & 15, left). The difference between the pre- and post-injection values, though often not very marked, reached significance more often in the second, compared to the first half of the treatment (Fig. 15). In two other neurones, we observed for a thin majority of oxytocin injections an excitation of neuronal activity, while in the remaining two neurones no consistent association of firing rate with the oxytocin injections was apparent (Fig. 15, right).

One of the two neurones displaying an increase in electrical activity following an injection of oxytocin fired at a rather slow rate and showed a small, though significant overall increase in activity during the oxytocin treatment (0.8 ± 0.03 and 1.6 ± 0.04 spikes/sec before and during the second hour of the treatment), while the other neurone displayed a higher

discharge rate (4.7±0.1 and 5.8±0.1 spikes/sec in the first and the fourth hour of the treatment), indicating that the neuronal responsiveness to systemic oxytocin does not dependent on the firing rate of the cell. The two neurones that did not show a consistent change in activity in response to oxytocin both fired at a relatively high rate (3.8±0.2 and 4.5±0.1 spikes/sec before and 4.2±0.3 and 4.8±0.1 spikes/sec during the first hour of the treatment, respectively). The two remaining neurones, that showed more often an increase than a decrease in response to oxytocin pulses, both displayed an activity of less than one spike/sec before the treatment (0.3±0.1 and 0.3±0.003, respectively) and a slight increase during the injections (0.5±0.04 spikes/sec in the first hour in the case of one neurone and 2.5±0.07 spikes/sec in the third hour in the case of the other).

Vasopressin neurones

In contrast to oxytocin neurones, three vasopressin neurones showed a decrease in spike activity following each oxytocin pulse but an increase at mid-time between the first and the second oxytocin injection (Fig. 11, 14, 15 & 16). In two cells, for which activity was recorded through all or most of the four hour treatment the firing rate increased progressively in the course of the treatment and was higher at mid-time between two oxytocin injections than immediately following an injection (Fig. 15, left). Though a similar pattern was observed in the third cell, the duration of the recording was only one hour and thus the response to only 5/25 oxytocin injections could be analysed (Fig. 15, right).

Correlation of uterine and cervical pressure with neuronal activity

Thus, it appears that the electrical activity of both supraoptic oxytocin and vasopressin neurones is influenced by stimuli which induce changes in uterine pressure. Therefore, we investigated whether there was any correlation between uterine and cervical pressure and neuronal activity.

Oxytocin neurones

A correlation between electrical activity and uterine or cervical pressure was calculated for five cells, of which three were recorded for more than one hour. In these three cells the correlation between the uterine pressure and the cell firing increased and was positive for the last 1-2 hours of the treatment (Table 6, Fig. 6, 9 & 10). Since in one animal uterine pressure was recorded from both uterine horns, we calculated the correlation between neuronal activity

and the pressure for each horn separately (Table 6). The results indicate that at the beginning of the treatment there was no correlation for either horn with oxytocin neurone activity, while at a later stage of the treatment, the correlation between cell activity and uterine pressure was highly significant for both horns (Table 6). In contrast, two cells, which were only recorded during the first hour of the oxytocin treatment, showed a very small increase in electrical activity during that time and we failed to observe a correlation between uterine pressure and cell activity (r^2= 0.25 and r^2= 0.1 at the beginning of the treatment and r^2= -0.3 and r^2= 0.4 after one hour of the oxytocin injections, respectively, data not shown).

Table 6

Correlation of neuronal activity and uterine pressure

oxytocin treatment 10 mU oxytocin each 10 min for 2 hours and then 20 mU oxytocin each 10 min for 2 hours					
oxytocin neurones			vasopressin neurones		
cell	first 1-2 hours	last 1-2 hours	cell	first 1-2 hours	last 1-2 hours
T3	r^2= 0.12	r^2= 0.45	T2	r^2= -0.3	r^2= 0.68*
T5	r^2= 0.1	r^2= 0.83*	T9	r^2= -0.03	r^2= 0.66*
	r^2= 0.01	r^2= 0.80*			
T7	r^2= 0.3	r^2= 0.55			

*$p<0.05$, t-test

Since cervical pressure was only recorded in two animals treated with oxytocin, a correlation between cervical pressure and oxytocin cell activity in relation to the treatment could only be calculated for one oxytocin neurone. For this neurone we observed no correlation with cervical pressure before the treatment (r^2= 0.03), and though in the course of the treatment the correlation coefficient increased to r^2= 0.4, this correlation was not statistically significant (Fig. 9).

Vasopressin neurones

For two vasopressin neurones recorded through most of the four hour oxytocin pulse treatment, no correlation was found between cell activity and uterine pressure at the beginning of the recording (r^2= -0.3 and r^2= -0.03, respectively), while during the last two hours of the oxytocin injections there was a significant positive correlation between uterine pressure and neuronal activity (r^2= 0.68 and r^2= 0.66, respectively, $p<0.05$, Fig. 4 & 16). When analysed in greater detail, one vasopressin cells showed a marked correlation between uterine contractions and phasic cell activity during most of the four hour treatment (Fig. 16, 17 & 17a). Statistical analysis performed for each of the 200 sec intervals, into which the four hour treatment was subdivided, revealed a positive correlation between uterine pressure and cell activity for almost every interval (r^2= 0.35-0.6), and thus a highly significant correlation for the entire treatment period ($p<0.01$, Fig. 17 & 17a).

Cervical pressure and vasopressin cell activity, analysed for one cell, showed no significant correlation at the beginning of the recording (r^2= -0.03), but a significant positive correlation during the last two hours of the treatment (r^2= 0.66, $p<0.05$, Fig. 11).

Effect of cervical and uterine pressure changes on neuronal activity in term pregnant rats

Since the findings so far suggest that uterine and cervical activity can modulate supraoptic neurone activity in late pregnant rats, we thought to investigate in a last experiment whether acute changes in uterine pressure, induced by de- and inflation ($\pm$1-3 ml) of balloons located in the uterine corpus or the cervical end, would affect neuronal activity. Thus, in one animal implanted with two balloons, electrical activity of one oxytocin and one vasopressin cell in response to manipulations of the balloon volume was recorded. For comparison of firing rate, the number of spikes were counted in 1-3x60 sec bins just prior to and immediately after a change in cervical or uterine pressure and compared using the parametric t-test.

Oxytocin neurone

In the case of the oxytocin neurone, an acute increase in cervical pressure induced a gradual, but significant decline in cell activity, while a subsequent decrease in cervical pressure induced a gradual increase in spike activity and when this procedure was repeated, it yielded the same result (Fig. 18 & 18a). However, the firing rate in response to an acute pressure change seemed, after an initial response, to move towards its baseline value despite a

continuation of the induced pressure change. Uterine pressure, recorded simultaneously, was not affected by such cervical pressure manipulation (Fig. 18a).

Vasopressin neurone

Changes in vasopressin cell activity in response to cervical distension, though displaying a similar trend to those of the oxytocin cell (namely a decrease in activity following inflation of the balloon in 4/5 times and an increase in activity in response to deflation in 3/6 times), were less consistently observed (Fig. 19 & 19a). Again cervical pressure changes had no effect on uterine activity (Fig. 19a).

De- and inflation of the balloon located in the uterine corpus (±2 ml), resulted in only minor pressure changes within the uterus, with no change in contraction amplitude and frequency, and no alteration of the phasic activity of a vasopressin cell nor in a change in cervical contractions (Fig. 20). Also cervical pressure, recorded simultaneously, was not affected by uterine pressure changes.

However, a more pronounced decrease in uterine pressure (3-3.5 ml) induced a gradual decline in the frequency of rhythmic cervical contractions (Fig. 21a) and a significant increase in vasopressin cell activity (during the 10 min following the pressure change compared to the same period before the change, Fig. 21). Similarly, following an intravenous injection of the oxytocin antagonist F382 (30 μg/kg body weight, Fig. 22a), uterine pressure declined, while the amplitude and frequency of the contractions remained unchanged. The phasic activity of the putative vasopressin neurone became more continuous and firing rate showed an significant increase during a 15 min period after the antagonist administration (compared to a period of the same duration prior to the antagonist injection (Fig. 22). An injection of CCK (20 μg/kg body weight iv) to the same animal clearly inhibited cell activity (Fig. 22 & 22a).

The effect of vaginal probing on supraoptic neurone activity in term pregnant rats

Finally, we examined the effects of vaginal probing, a stimulus that can activate or inhibit magnocellular oxytocin neurones depending on the reproductive state (Negoro *et al.* 1973a, 1973b), on electrical activity of oxytocin and vasopressin cells. The oxytocin neurone had been exposed to cervical pressure manipulation prior to vaginal probing, while the vasopressin neurone had been recorded through the four hour oxytocin pulse treatment.

Both neurones showed a significant decrease in firing rate in response to vaginal probing (Fig. 23 & 23 a). This rapid and significant decline in firing rate was observed for each of the three probings performed during the recording of the vasopressin cell (Fig. 23 & 23a).

Discussion

In this study, we have shown that electrical activity of supraoptic neurones increases in the course of a four hour oxytocin pulse treatment in term pregnant rats, while during periods without any treatment, a decrease in firing rate was observed. Since the SON is located within the blood-brain barrier, circulating oxytocin, which does not readily cross this barrier (Ermisch 1992, Weber *et al.* 1993), is unlikely to have a direct effect on supraoptic neurones. The increase in electrical activity of supraoptic neurones was accompanied by an increase in uterine pressure and cervical contractions, which were positively correlated with the firing rate at the end of the oxytocin treatment. Thus, our data are consistent with the hypothesis that uterine activity can modulate supraoptic neurone activity. Though in the present study, we have not examined the effects of saline injections on uterine and cell activity, in a previous chapter (see chapter three) we have shown that only pulsatile oxytocin administration induces Fos expression in supraoptic (oxytocin and vasopressin) neurones, while saline injections were not effective. Furthermore, we have shown that in term pregnant anaesthetised rats oxytocin injections augment uterine activity to a greater extent than saline injections (Douglas *et al.* 1994). Thus, a likely explanation for the observed increase in firing rate is the increase in intrauterine pressure in response to oxytocin.

Previous work has shown that uterine activity during labour that has been induced with an infusion of oxytocin bears a close resemblance to that observed during spontaneous labour (Fuchs & Poblete 1970), namely a progressive increase in contraction frequency and amplitude (Higuchi *et al.* 1986b). However, labour induced with pulsatile administration of oxytocin is characterised by a greater than normal increase in intrauterine pressure (Randolph & Fuchs 1989). This is in agreement with our observations firstly that unlike during normal labour, uterine activity in response to intermittent oxytocin injections resulted in Fos expression in the SON and brainstem neurones even before delivery of pups and secondly that oxytocin treatment augmented primarily intrauterine pressure, while decreasing contraction amplitude. Though we have not systematically analysed the contraction frequency, we did observe an increase in contraction frequency following an injection of oxytocin or CCK in the cervix and, less consistently, in the uterus. The high amplitude rhythmic contractions of the

cervix, which were not always accompanied by uterine contractions in response to both oxytocin and CCK, are in agreement with reports on the ability of the cervix to contract independently of the uterus (Olah *et al.* 1993) and with reports on a higher density of oxytocin receptors at the cervical compared to the ovarian end of the rat uterus (Gorodeski *et al.* 1990). In addition, oxytocin has been implicated in the effacement of the cervix (Khalifa *et al.* 1992) and thus, the effects of pulsatile oxytocin treatment on delivery might have a dual site of action: one to augment uterine activity important for the expulsion of foetuses and the other to contribute to the softening of the cervix, which is a prerequisite for normal delivery.

In the course of spontaneous labour, cervical and uterine activity becomes more coordinated and this is reflected by an increase in distally directed contractions (Crane & Martin 1991a, Fuchs & Poblete 1970). Here, we found a significant correlation between uterine and cervical contractions at the end, but not at the beginning of the treatment. In addition, in the course of the treatment, we observed an increasing incidence of high amplitude peaks in cervical pressure, that appeared superimposed on normal contractions and were, in most cases, associated with a concomitant peak of smaller magnitude in the uterus. In contrast, alterations of uterine pressure in rats not pretreated with oxytocin were not accompanied by a concomitant change in cervical pressure, unless a sustained decrease in uterine pressure was induced, which resulted in a decline of high amplitude cervical contractions. Similarly, manipulations of cervical pressure without oxytocin treatment did not affect uterine activity. Thus, oxytocin injections seem able to initiate synchronised and coordinated uterine activity, as seen prior to spontaneous delivery (Fuchs & Poblete 1970, Crane & Martin 1990a).

Since the induction of Fos in the NTS and SON during labour but before delivery is only observed following oxytocin treatment, we cannot be certain whether physiologically uterine and/or cervical contractions represent the key stimulus for supraoptic neurone excitation (or whether an increase in firing rate normally precedes the augmentation of uterine activity). However, our data indicate that a gradual but sustained increase in uterine pressure and cervical activity, induced by pulsatile oxytocin, has at least the potential to increase the electrical discharge of supraoptic neurones at term.

In addition, vasopressin cell activity decreased immediately following an oxytocin injection, concomitantly with a transient reduction in uterine pressure and contraction amplitude, while neuronal activity and uterine pressure both showed a maximum at mid-time between two injections, suggesting that oxytocin injections play a modulatory role for the

synchronisation of uterine and neuronal activity in late pregnancy. Beside such significant correlation between vasopressin cell activity and uterine pressure observed throughout the oxytocin treatment, we also observed a positive correlation between the progressive increase in cervical contractions and vasopressin cell activity. Though we also found a significant positive correlation between uterine pressure and oxytocin cell activity, we did not see a synchronisation between uterine and oxytocin cell activity, but rather a higher firing rate immediately following a pulse of oxytocin, when uterine pressure showed a transient decreased.

A relationship between supraoptic neurone activity and uterine contractions during induced labour suggests a physiological significance of oxytocin and vasopressin release during parturition in the rat, which in the case of vasopressin, remains to be clarified.

Shortly before delivery of pups, background activity of both types of magnocellular neurones increases (Richard *et al.* 1988) and during labour and delivery the release of a substantial amount of vasopressin has been reported in rats (Fuchs & Saito 1971, Kumaresan *et al.* 1979). Since vasopressin V_1-receptors are present in the uterus and mediate uterine contractions (Chan *et al.* 1990, Maggi *et al.* 1991a, 1992), vasopressin could, in addition to oxytocin, play a role for the augmentation of intrauterine pressure.

Unlike the excitatory effect of the gradual rise in uterine pressure and cervical contractions during the oxytocin pulse treatment, moderate inflation of a balloon placed in the body of the uterus had no effect on vasopressin cell activity. Since the pregnant rat uterus is already greatly expanded, it might offer little resistance to further distension and this might explain the rather small pressure change induced by such manipulation, which proved ineffective at modulating neuronal activity. In contrast, when uterine pressure was reduced by a systemic injection of an oxytocin receptor antagonist or by an extensive deflation of the uterine balloon, we observed a moderate increase in firing rate of a putative vasopressin neurone over a period of 5-15 min. Since the latter experiments were performed after the four hour oxytocin treatment, SON neurones might have become more sensitive to uterine pressure changes. Alternatively, the decrease in uterine pressure following either the oxytocin antagonist injection or the extensive deflation, might represent a more potent stimulus than moderate inflation of the balloon. The excitation of vasopressin neurones following a decrease in uterine pressure is not consistent with the excitation of supraoptic neurones observed during the gradual increase in uterine activity by oxytocin pulses. Thus, it could be that in late pregnancy, all sensations from the uterus are predominantly excitatory to supraoptic

vasopressin neurones. At this point of the discussion it seems important to emphasise that the effects of uterine pressure changes on supraoptic neurone activity presented here are based on two cells only, one supraoptic oxytocin and one vasopressin cell, and hence any interpretation of our observations should keep the preliminary nature of the data in mind.

Since neurosecretory vasopressin neurones co-express dynorphin (Watson *et al.* 1982), a kappa opioid receptor ligand, and endogenous opioids restrain oxytocin neurones at the hypothalamic level (Bicknell *et al.* 1988c, 1993) and oxytocin release from axon terminals in the posterior pituitary (Bicknell & Leng 1982, Van de Heijning 1991a), the stimulation of vasopressin cells in late pregnancy might contribute, via the release of dynorphin either from terminals in the posterior pituitary or from dendrites within the SON, to the inhibition of premature oxytocin secretion. Thus, the decrease in electrical activity of oxytocin neurones towards the end of the four hour oxytocin treatment, could reflect an inhibition of oxytocin neurones by dynorphin released from increasingly activated vasopressin neurones. Since dynorphin has also an autoinhibitory effect on vasopressin neurones themselves (Bicknell 1993), this might serve to control premature release of both vasopressin and oxytocin release at term.

A tight control of neurohypophysial hormone release would be expected to occur particularly in term pregnant animals, when the uterus is already highly sensitive oxytocin (Fuchs & Poblete 1970, Fuchs *et al.* 1983), but the cervix not yet fully effaced. At that time a sudden increase in oxytocin neurone activity (and hormone release), e.g. in response to acute vagino-cervical stimulation, could, by augmenting intrauterine pressure against a tight cervix, endanger the foetus(es). Similarly, it would seem sensible to restrain the acute release of vasopressin, since vasopressin can also induce uterine contractions (Maggi *et al.* 1991a).

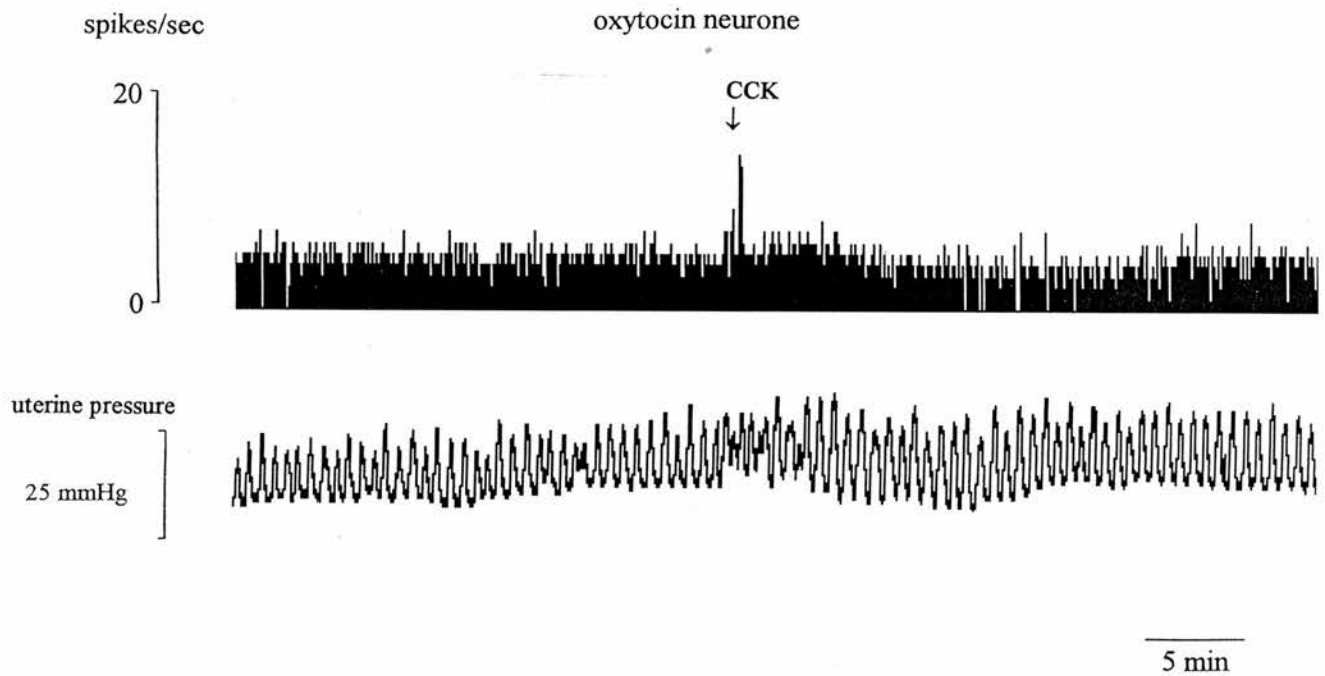
While we observed excitation of SON neurones during a gradual increase in uterine pressure and cervical contractions, acute cervical distension and vaginal probing resulted in an inhibition of oxytocin and vasopressin cell activity. Since others have reported increased neuronal activity in response to vaginal distension in term pregnant rats (Negoro *et al.* 1973b), it might be that our rats were closer to parturition than those described in previous studies (Negoro *et al.* 1973a, 1973b) and hence at this late stage of pregnancy acute cervical distension might inhibit rather than excite supraoptic neurones.

The reported increase in supraoptic neurone activity and hormone release following vaginal distension in lactating rats (Dreifuss *et al.* 1976) might reflect a decreased inhibitory tone on SON neurones in these rats, in which neurohypophysial hormone release would not

any deleterious effects. Thus, our observations and previous reports can both be accommodated by the postulation of an inhibitory influence on supraoptic neurones that is predominantly present at term, when the control of neurohypophysial hormone release is of critical importance. Likely candidates involved in such regulation are endogenous opioids, which restrain supraoptic neurones at term (Bicknell *et al.* 1988c) and whose inhibitory control is augmented in late pregnancy, when plasma progesterone concentrations decline (Soaje & Deis 1994).

In summary, we have shown that pulsatile administration of oxytocin to late pregnant rats initiates synchronised activity in the uterus and the cervix, which could represent a potent stimulus for activation of afferent pathways and the increased electrical activity of supraoptic neurones necessary for the release of large amounts of neurohypophysial hormones during parturition. Together with the reported observation that delivery can be induced in anaesthetised rats with such oxytocin treatment (Douglas *et al.* 1994), we have provided a model which allows to use electrophysiological recording to investigate the regulation of the reflex release of neurohypophysial hormones in term pregnant rats.

Fig. 7.1. Electrical activity of supraoptic neurones in term pregnant anaesthetised rats
Neuronal activity (upper trace, spikes/sec) and uterine pressure (lower trace, in mmHg) of two supraoptic neurones recorded in term pregnant rats under sodium pentobarbitone anaesthesia (30-40 mg/kg body weight). Both cells, a putative oxytocin (**Top**) and vasopressin (**Bottom**) cell were identified by their response to an intravenous injection of CCK (20 μg/kg body weight).
There was no significant change in electrical activity for either cell or uterine activity in the course of the 1 hour recordings.


spikes/sec
oxytocin neurone
20
CCK
0
uterine pressure
25 mmHg
5 min

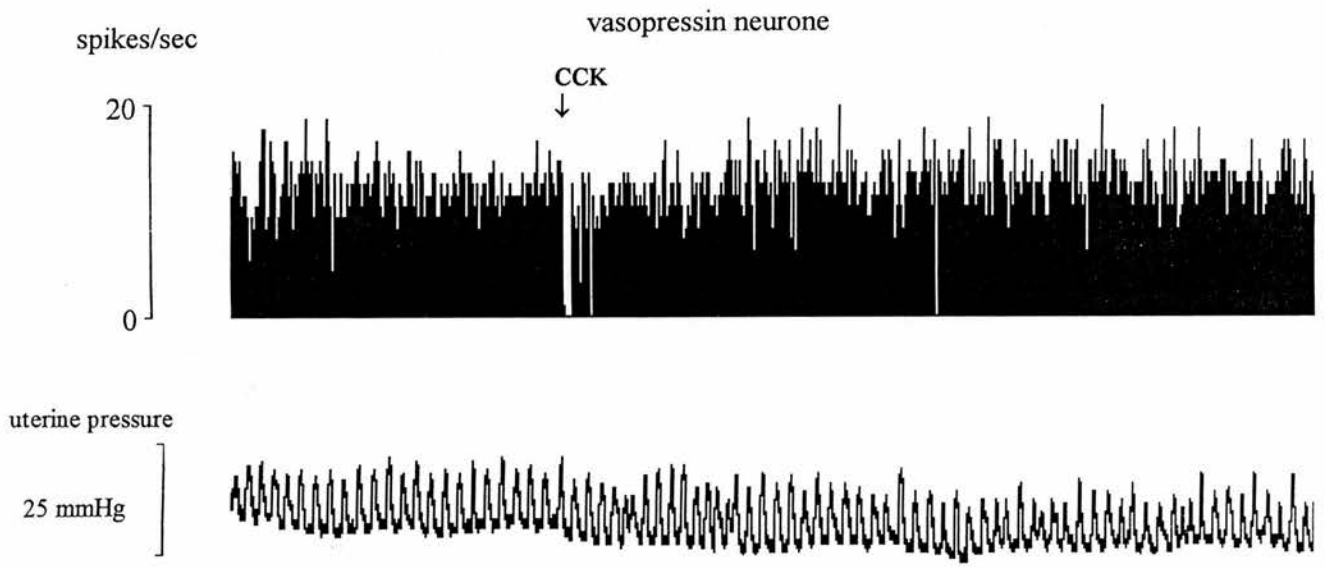

spikes/sec
vasopressin neurone
CCK
20
0
uterine pressure
25 mmHg

Fig. 7.2. Effects of CCK on electrical activity of supraoptic neurones and uterine and cervical pressure in term pregnant anaesthetised rats
Neuronal activity (middle trace, spikes/sec), uterine pressure and cervical pressure (in mmHg) were recorded in two anaesthetised animals injected intravenously with CCK (20 μg/kg body weight).
The putative oxytocin neurone responded to an injection of CCK with an increase in firing rate (**Top**), while the putative vasopressin neurone showed a decrease in firing rate (**Bottom**). In contrast, uterine and cervical activity were not affected by the injections of CCK.

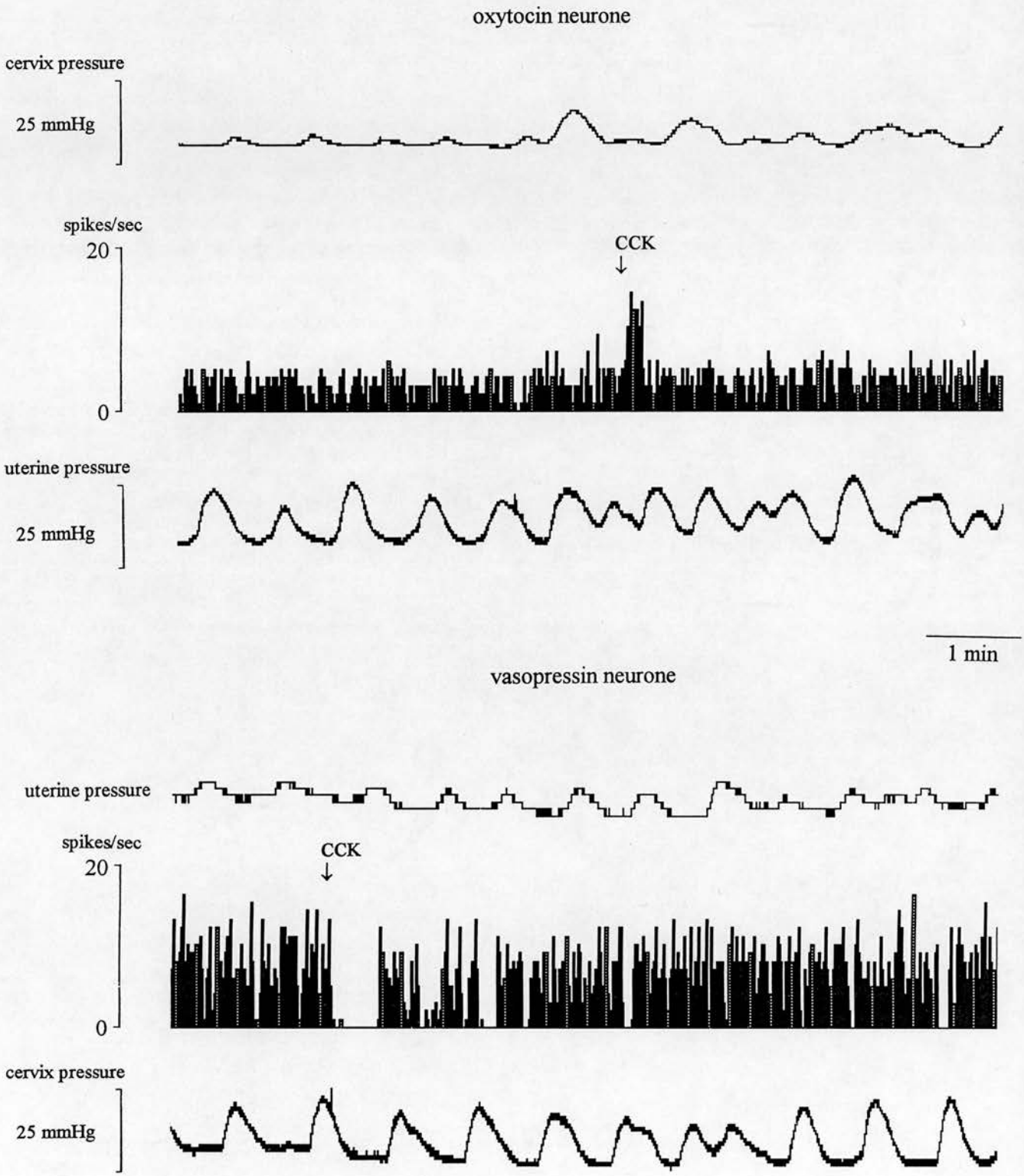

oxytocin neurone
cervix pressure
25 mmHg
spikes/sec
20
0
CCK
uterine pressure
25 mmHg
1 min
vasopressin neurone
uterine pressure
spikes/sec
20
0
CCK
cervix pressure
25 mmHg

Fig. 7.3. Electrical activity of a supraoptic oxytocin neurone and uterine pressure in a term pregnant anaesthetised rat during a 4 hour oxytocin treatment
Neuronal activity (upper trace, spikes/sec) and uterine pressure (lower trace, in mmHg) of a supraoptic oxytocin neurone were recorded throughout the 4 hour oxytocin pulse treatment (arrow = pulse of oxytocin: 10 mU each 10 min for 2 hours and then 20 mU each 10 min for 2 hours).
Electrical activity as well as uterine pressure increased gradually in the course of the treatment.

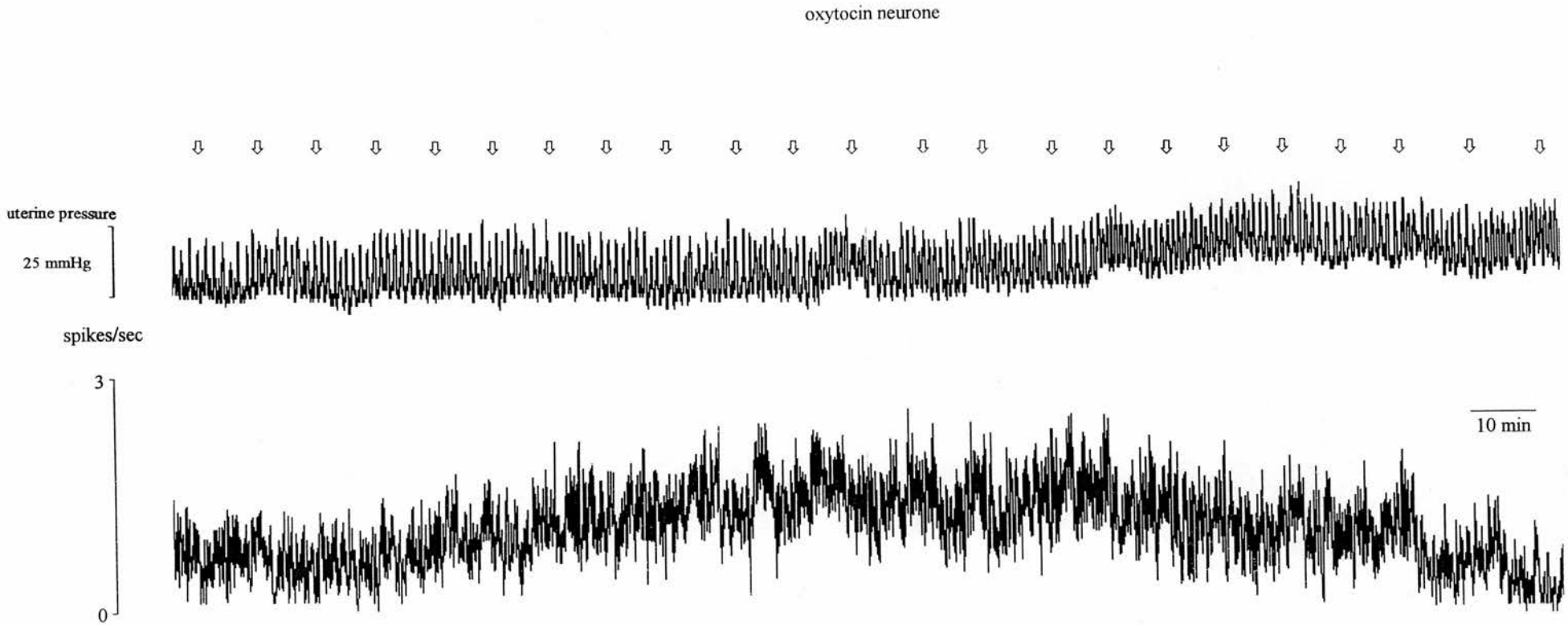

oxytocin neurone
uterine pressure
25 mmHg
spikes/sec
3
0
10 min

Fig. 7.4. Electrical activity of a supraoptic vasopressin neurone and uterine pressure in a term pregnant anaesthetised rat during a 4 hour oxytocin treatment
Neuronal activity (upper trace, spikes/sec) and uterine pressure (lower trace, in mmHg) of a supraoptic vasopressin neurone was recorded throughout the 4 hour oxytocin pulse treatment (arrow = pulse of oxytocin, details see Fig. 7.3.).
Electrical activity as well as uterine pressure increased in the course of the treatment.

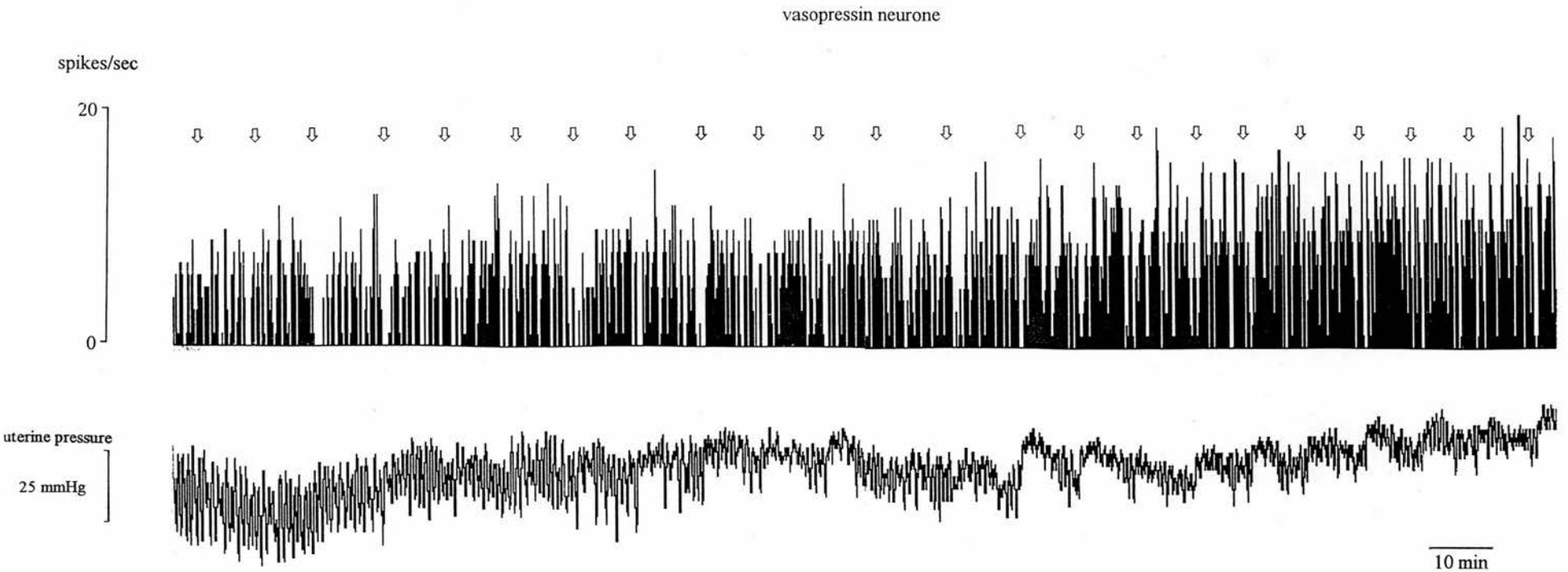

vasopressin neurone
spikes/sec
20
0
uterine pressure
25 mmHg
10 min

Fig. 7.5. Uterine pressure and supraoptic neurone activity in response to an injection of CCK and oxytocin

Neuronal activity (middle trace, spikes/sec) of a supraoptic oxytocin neurone (as identified by the increase in firing rate to CCK) and uterine pressure were recorded from both uterine horns (upper and lower trace, in mmHg) of a term pregnant anaesthetised rat during the first 2 hours of the oxytocin pulse treatment (arrow = a pulse of 10 mU oxytocin).

While there was no significant increase in uterine pressure in response to an injection of CCK, uterine pressure in both horns increased with the first injection of oxytocin.

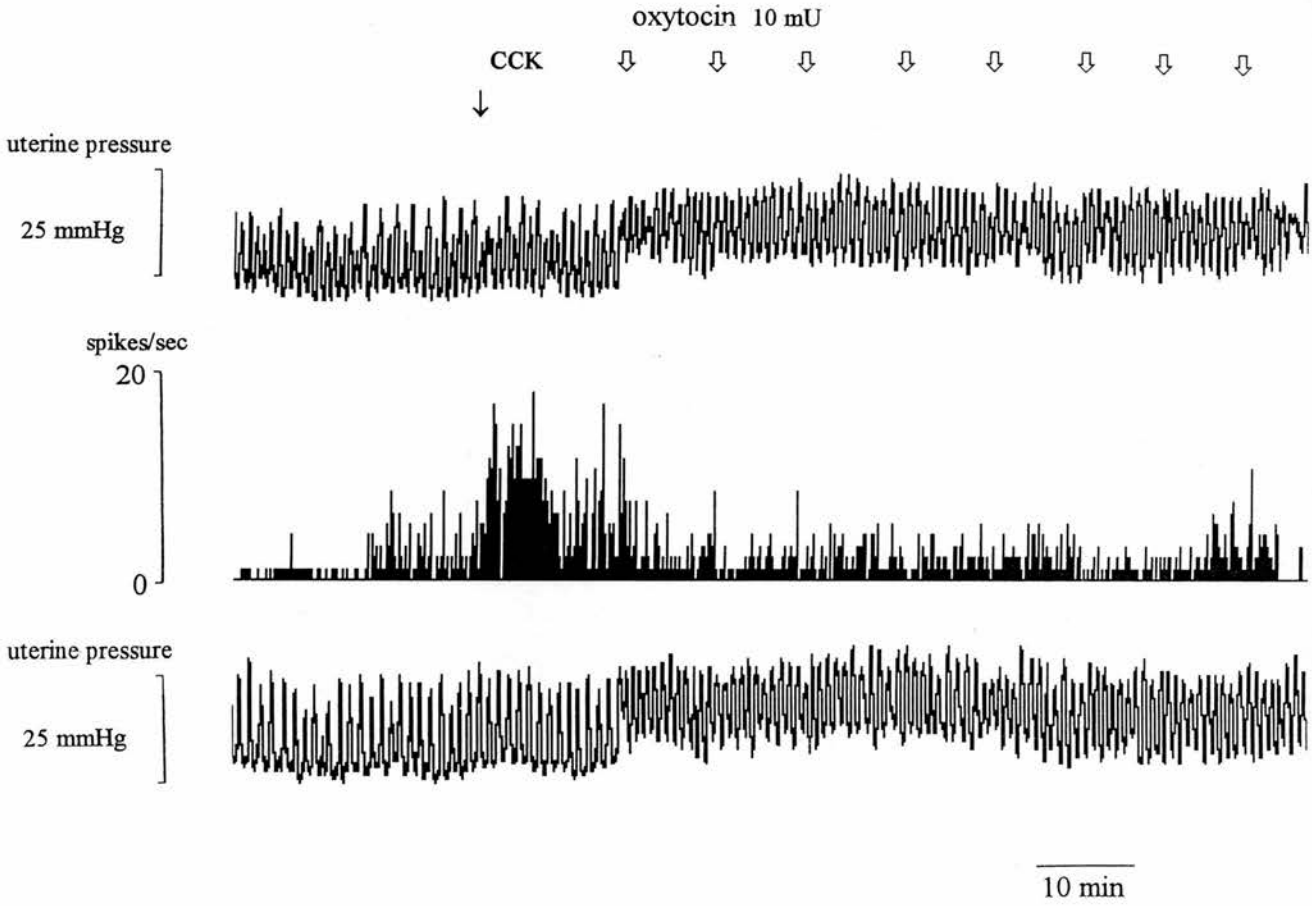

oxytocin 10 mU
CCK
uterine pressure
25 mmHg
spikes/sec
20
0
uterine pressure
25 mmHg
10 min

Fig. 7.6. Uterine pressure and supraoptic oxytocin neurone activity during the oxytocin treatment

Neuronal activity (middle trace, spikes/sec) of a putative oxytocin neurone and uterine pressure recorded from both uterine horns (upper and lower trace, in mmHg) of a term pregnant anaesthetised rat were recorded during the 3rd hour of the 4 hour oxytocin pulse treatment (arrow = a pulse of 20 mU oxytocin).

There was a significant increase in firing rate of the putative oxytocin neurone in the course of the recording and concomitantly an increase in uterine pressure that was synchronised between the two horns.

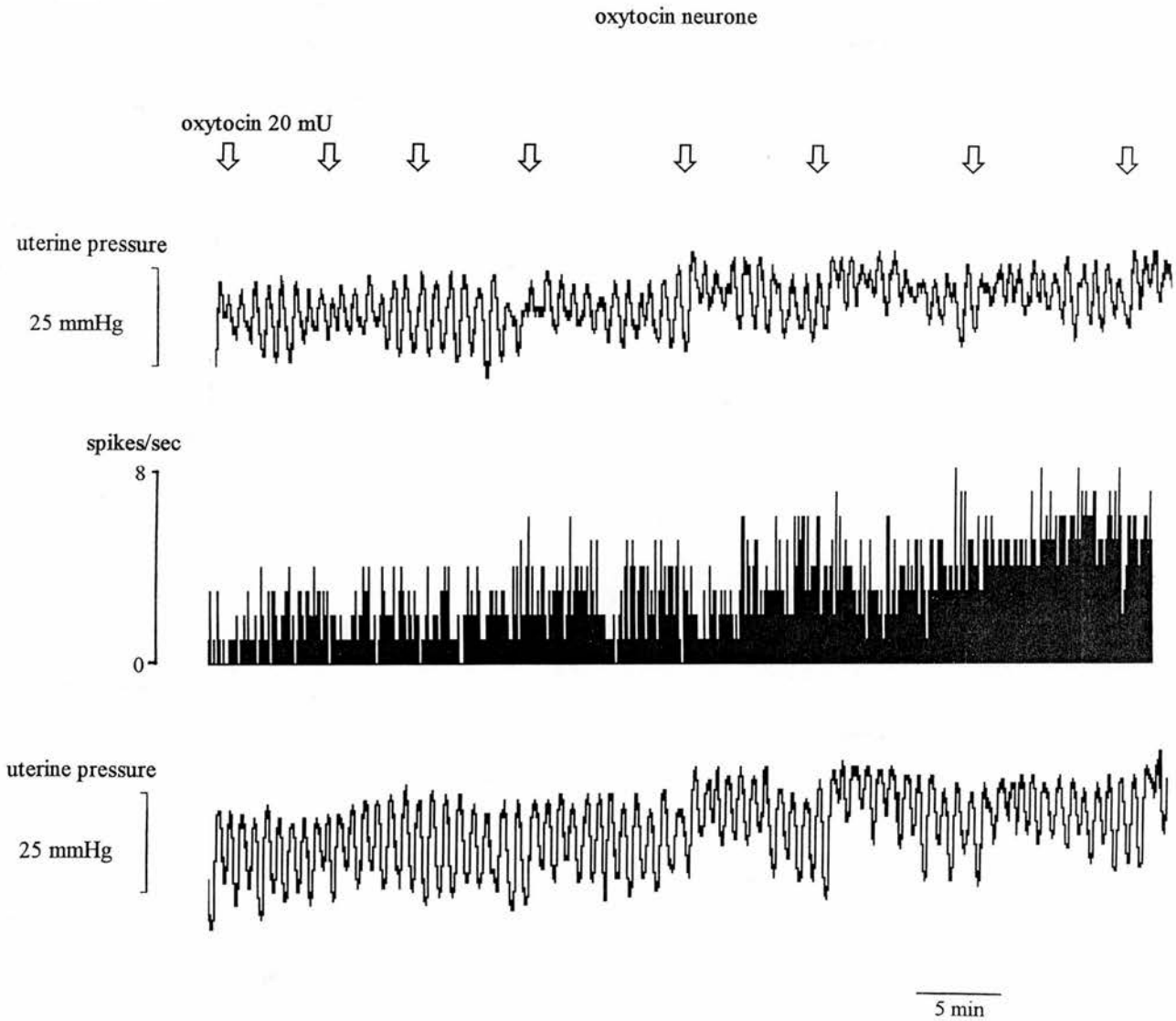

oxytocin neurone
oxytocin 20 mU
uterine pressure
25 mmHg
spikes/sec
8
0
uterine pressure
25 mmHg
5 min

Fig. 7.7. Uterine and cervical pressure and contraction amplitude during the oxytocin treatment

Top: The change in uterine pressure and contraction amplitude (n=4) during and after the 4 hour oxytocin pulse treatment was expressed as % change from baseline (= 100%).

Uterine pressure significantly increased and contraction amplitude decreased in the course of the treatment compared to the baseline value (= assessed during a period before any treatment, *$p<0.05$, U-test).

Bottom: Likewise, the change in cervical pressure and contraction amplitude (n=2) was expressed as % change form baseline (= 100%) during and after the 4 hour oxytocin pulse treatment. Because of n=2 for all cervical measurements, the two individual values are indicated as filled circles.

In both animals analysed, there was a decrease in cervical pressure and an increase in the contraction amplitude during the oxytocin treatment.

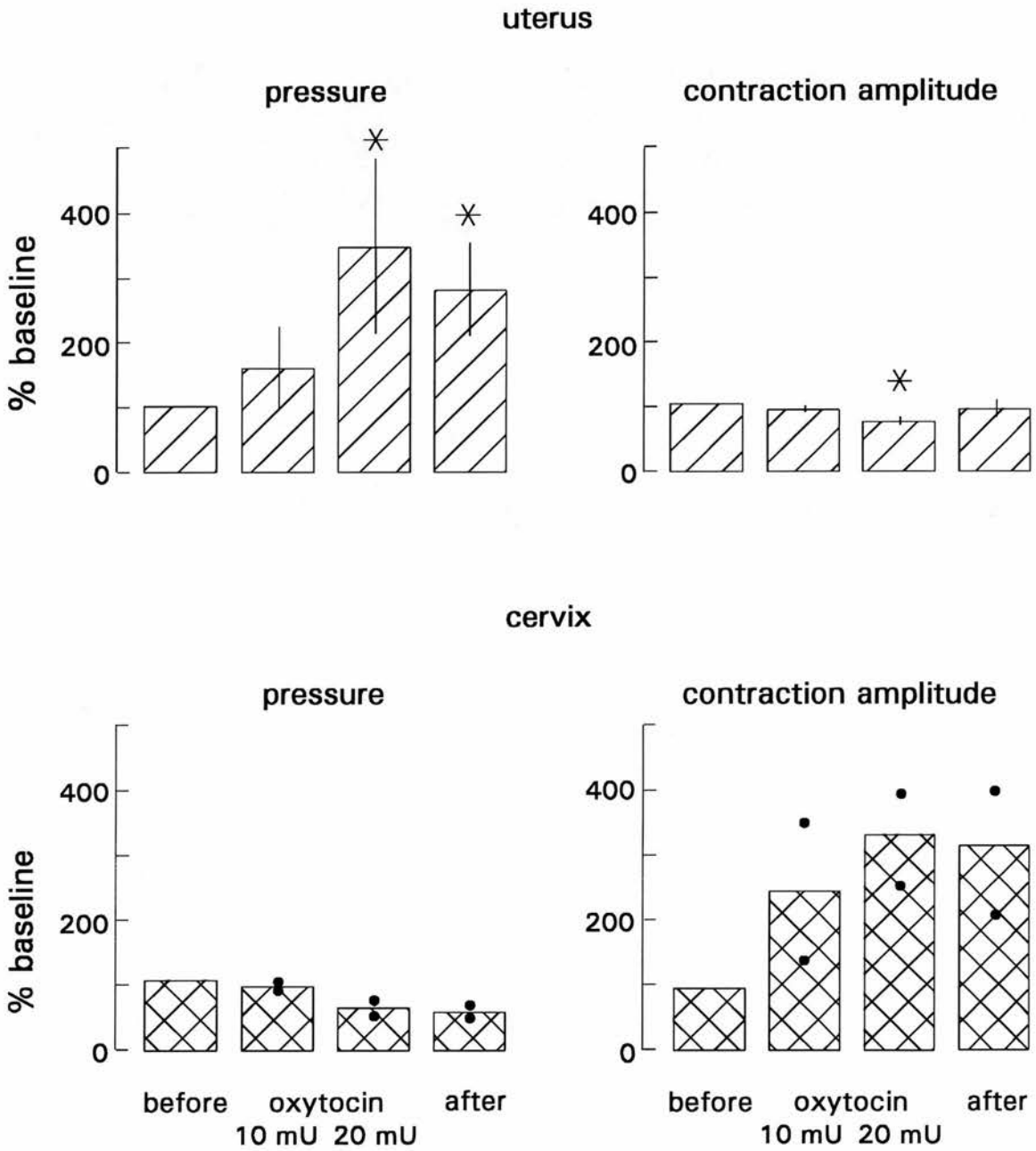
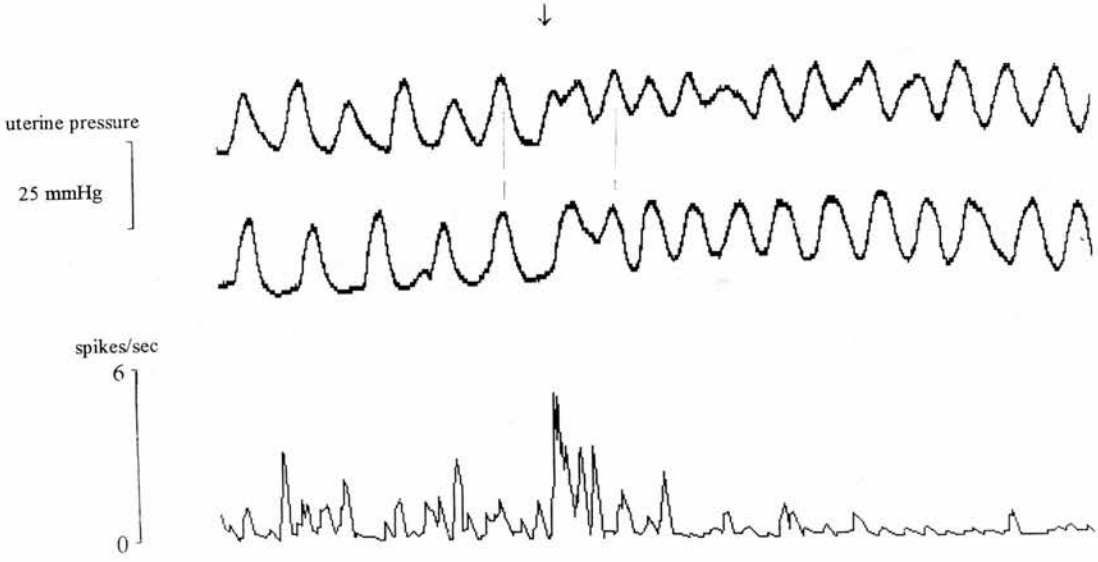
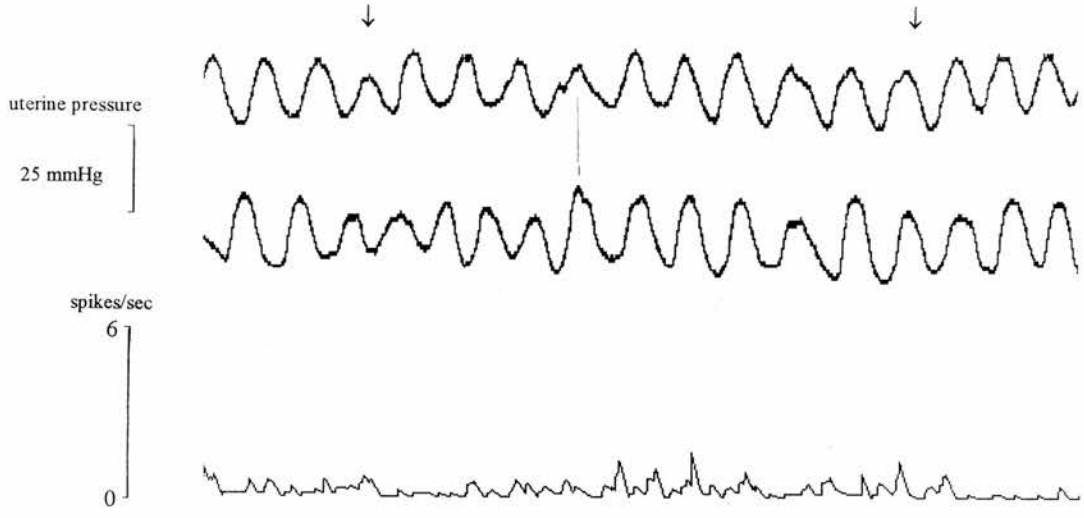

uterus
pressure
contraction amplitude
% baseline
400
200
0
cervix
pressure
contraction amplitude
% baseline
400
200
0
before
oxytocin
10 mU
20 mU
after

Fig. 7.8. Concomitant recording of uterine activity from both horns and electrical activity of an oxytocin neurone during the oxytocin pulse treatment
Electrical activity of a putative oxytocin neurone (spikes/sec) and uterine pressure (in mmHg) recorded simultaneously from both uterine horns are shown at various times during the 4 hour oxytocin pulse treatment (arrow = a pulse of oxytocin). **Top:** 2nd hour of the treatment, **Middle:** 3rd hour of the treatment, **Bottom:** 4th hour of the treatment.
In the course of the treatment, uterine contractions became more synchronised between the two horns. The firing rate showed, albeit not consistently, an increase in spike activity following an injection of oxytocin.

oxytocin neurone





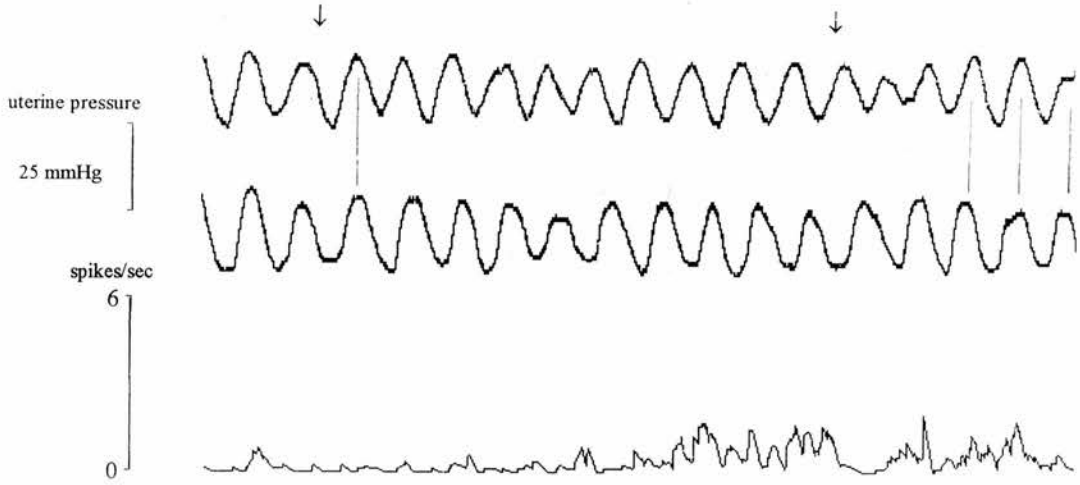


Fig. 7.9. Oxytocin neurone activity and uterine and cervical pressure during the oxytocin treatment

Electrical activity of a putative oxytocin neurone (middle trace, spikes/sec), uterine pressure (upper trace, in mmHg) and cervical pressure (lower trace, in mmHg) were recorded from a term pregnant anaesthetised rat during the last 2 hours of the oxytocin pulse treatment (arrow = a pulse of 20 mU oxytocin). At the end of the recording the cell was identified by an increase in firing rate to CCK as a putative oxytocin neurone.

The contraction amplitude of the cervix increased in the course of the treatment, while neither uterine nor cell activity exhibited a significant change during the oxytocin treatment.

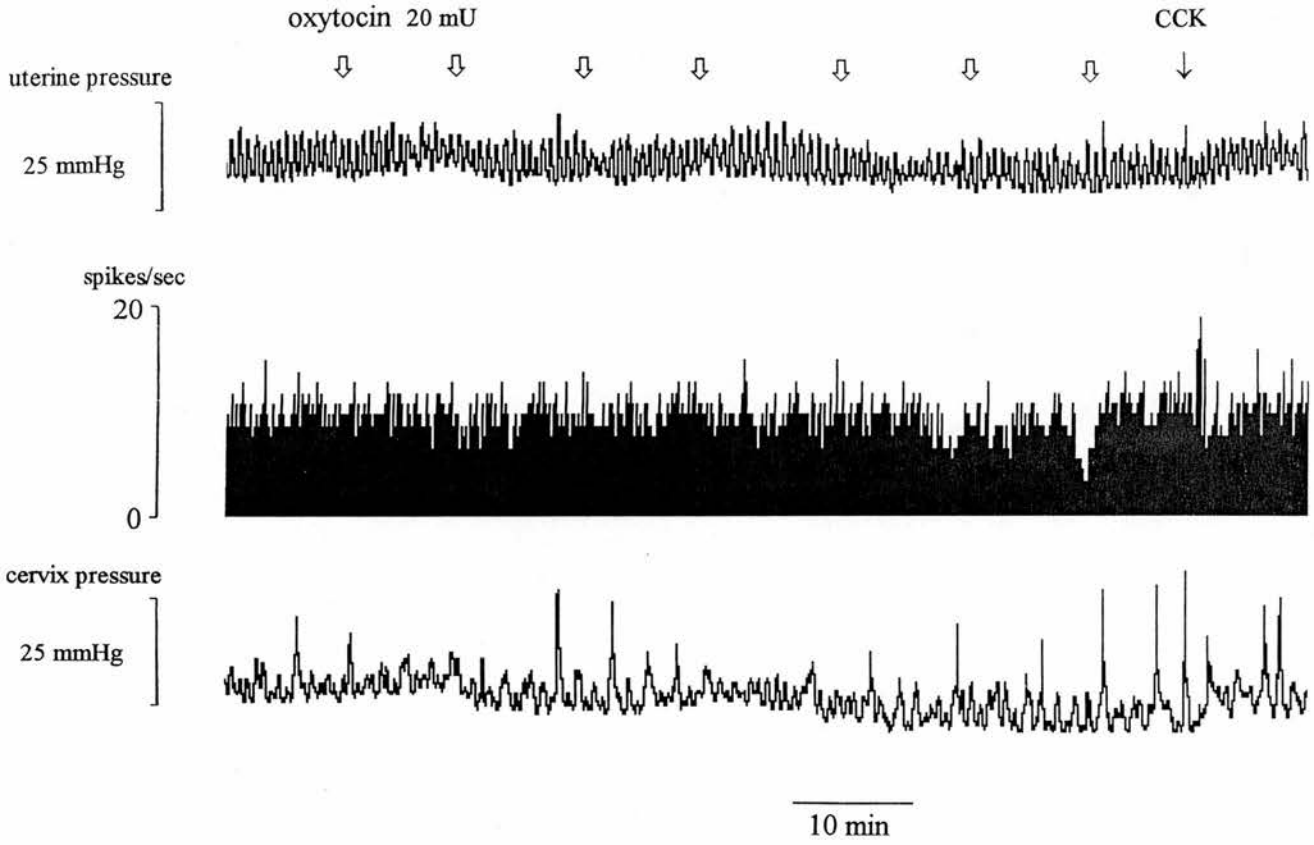

oxytocin 20 mU
CCK
uterine pressure
25 mmHg
spikes/sec
20
0
cervix pressure
25 mmHg
10 min

Fig. 7.10. Oxytocin neurone activity and uterine and cervical pressure during the oxytocin treatment

Electrical activity of a putative oxytocin neurone (lower trace, spikes/sec), uterine pressure (top trace, in mmHg) and cervical pressure (middle trace, in mmHg) are shown at various times during the 4 hour oxytocin pulse treatment (arrow = a pulse of oxytocin). **Top:** 2nd hour of the treatment, **Middle:** 3rd hour of the treatment, **Bottom:** 4th hour of the treatment. The cell was identified by an increase in firing rate in response to an injection of CCK as a putative oxytocin neurone.

Unlike the cell, neither the uterus nor the cervix were affected by the injection of CCK. However, we observed an increasing number of pressure peaks in the cervix in the course of the treatment that were usually accompanied by peaks of smaller magnitude in the uterus. These peaks were not related to injections of oxytocin and could reflect abdominal straining.

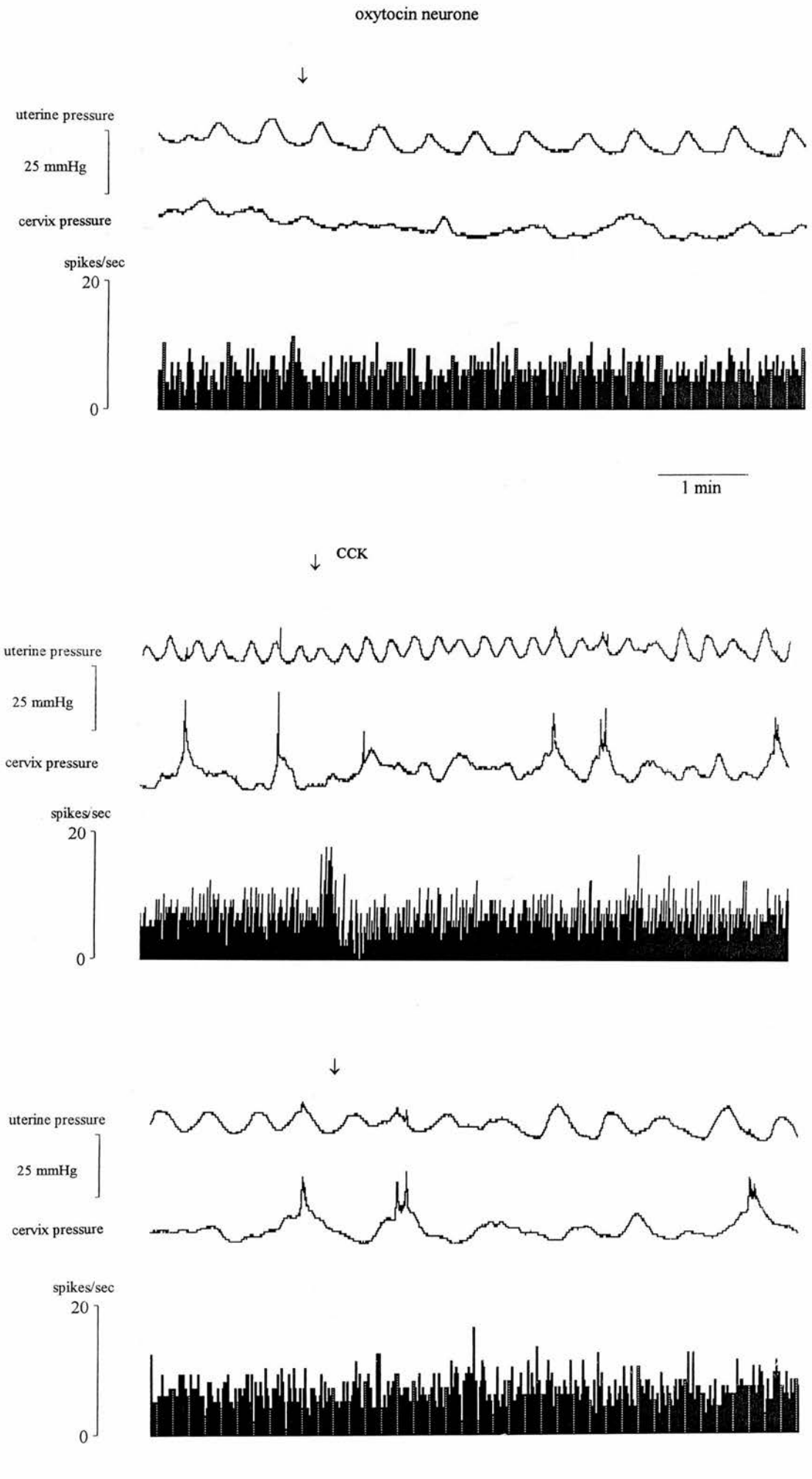

oxytocin neurone
uterine pressure
25 mmHg
cervix pressure
spikes/sec
20
0
1 min
CCK
uterine pressure
25 mmHg
cervix pressure
spikes/sec
20
0
uterine pressure
25 mmHg
cervix pressure
spikes/sec
20
0

Fig. 7.11. Vasopressin neurone activity and uterine and cervical contractions in relation to oxytocin pulses

Electrical activity of a putative vasopressin neurone (middle trace, spikes/sec), uterine pressure (top trace, in mmHg) and cervical pressure (bottom trace, in mmHg) recorded at various times during the 4 hour oxytocin pulse treatment (arrow = a pulse of oxytocin) are shown. **Top:** 1st hour of the treatment, **Middle:** 2nd hour of the treatment, **Bottom:** 4th hour of the treatment.

The phasic activity of the putative vasopressin neurone showed a progressive increase in spike frequency and a shortening of the silent periods in the course of the treatment. Concomitantly, uterine contractions became more regular and cervical contractions increased in amplitude and frequency. During the last hour of the treatment we observed more often a synchronised activity between the uterus and the cervix than at the beginning of the treatment. In response to some of the oxytocin injections, the cell showed a silent period.

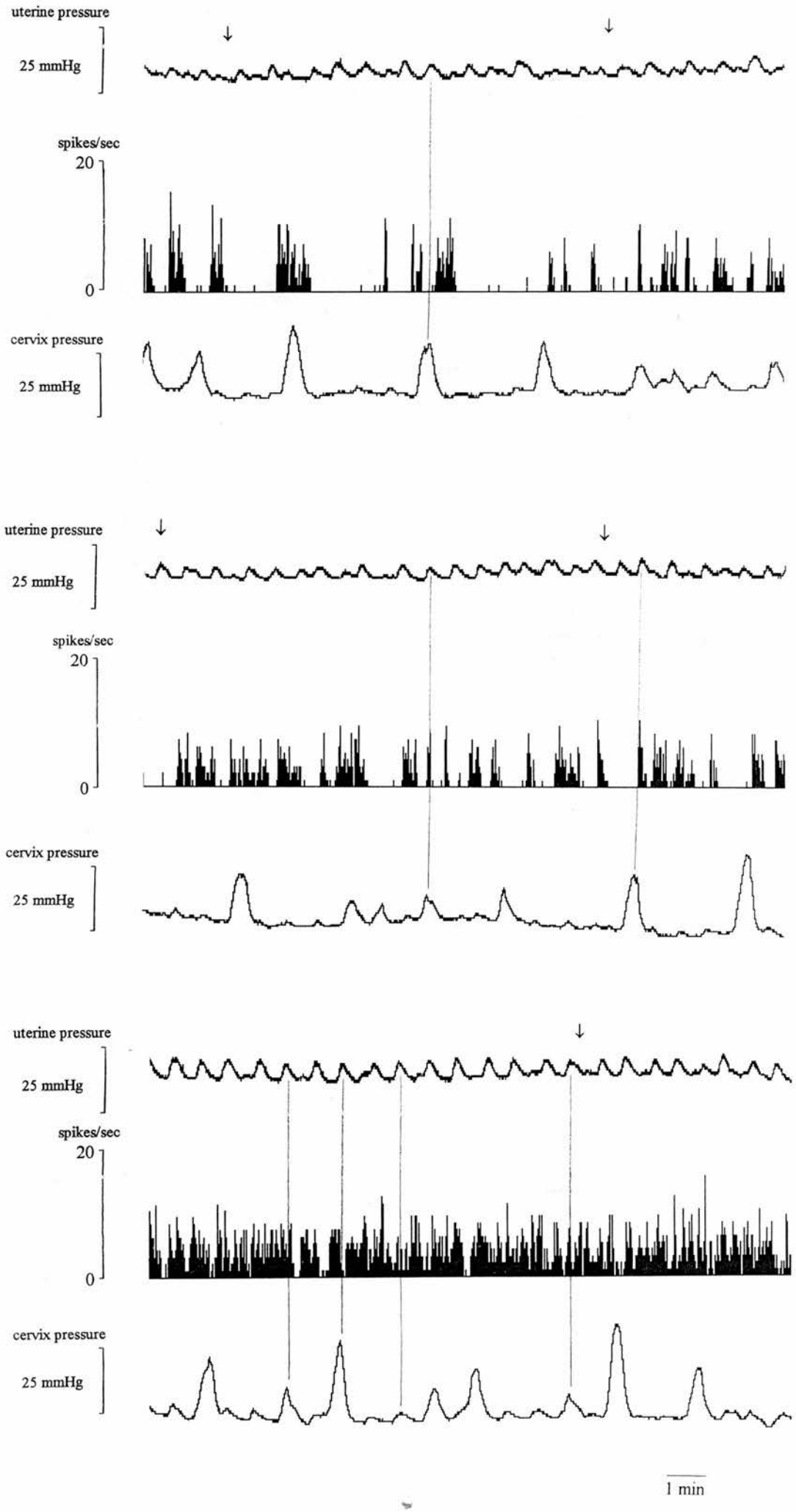

uterine pressure
25 mmHg
spikes/sec
20
0
cervix pressure
25 mmHg
uterine pressure
25 mmHg
spikes/sec
20
0
cervix pressure
25 mmHg
uterine pressure
25 mmHg
spikes/sec
20
0
cervix pressure
25 mmHg
1 min

Fig. 7.12. Effects of an injection of CCK on uterine and cervical activity
Uterine pressure (top trace, in mmHg) and cervical pressure (bottom trace, in mmHg) at the end of the 4 hour oxytocin pulse treatment (arrow = 20 mU oxytocin) and in response to an injection of CCK are shown.
In response to the last injection of oxytocin, intrauterine pressure and cervical contraction amplitude increased, while a subsequent injection of CCK had no additional effect on either the uterus or the cervix.

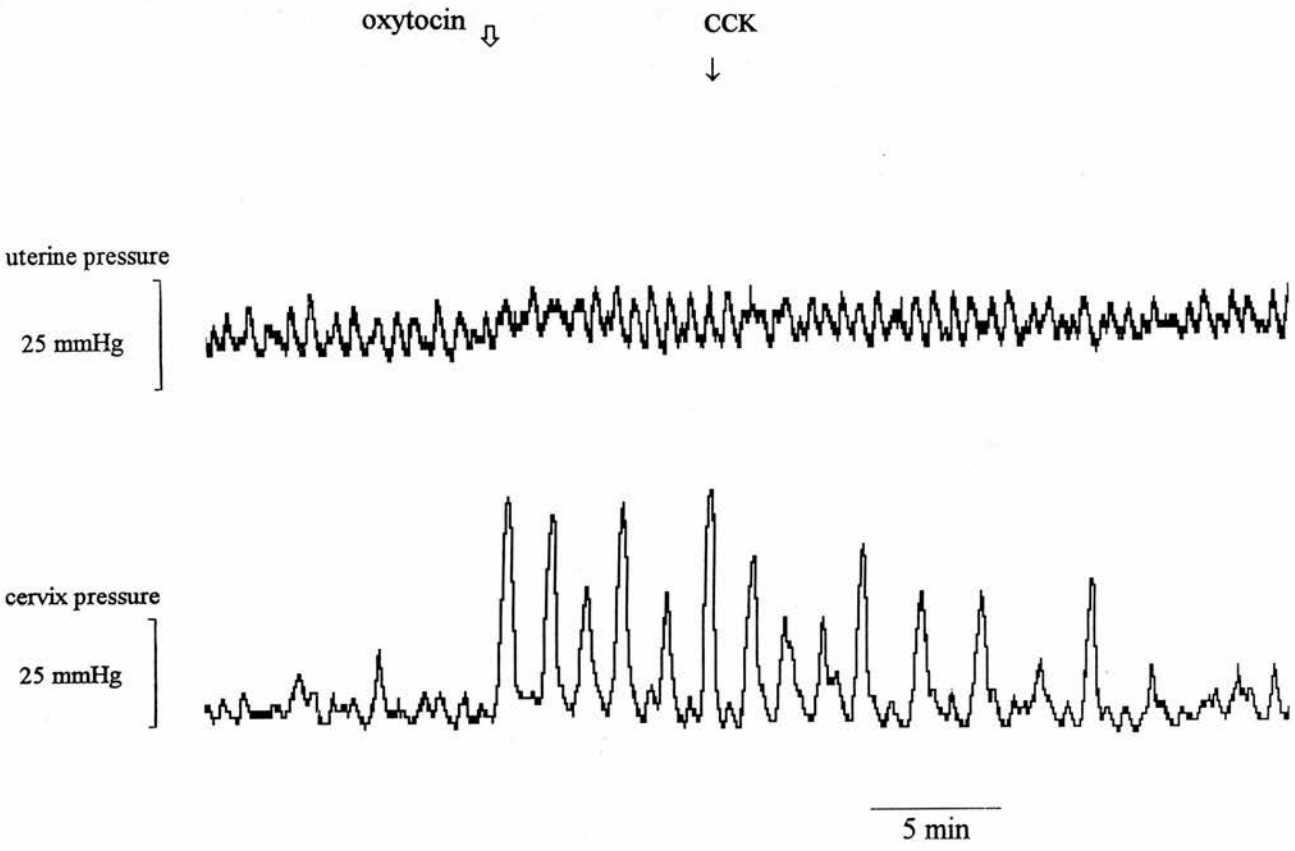

oxytocin
CCK
uterine pressure
25 mmHg
cervix pressure
25 mmHg
5 min

Fig. 7.13. Effect of an injection of CCK on uterine and cervical activity
Electrical activity of a putative vasopressin neurone (middle trace, spikes/sec, identified by the decrease in firing rate in response to CCK), uterine pressure (top trace, in mmHg) and cervical pressure (bottom trace, in mmHg) recorded after discontinuation of the 4 hour oxytocin pulse treatment are shown. **Top:** 10 min after the last oxytocin injection, **Bottom:** 20 min after the last oxytocin injection. An injection of CCK triggered a series of high amplitude contractions that were synchronised between the uterus and the cervix.

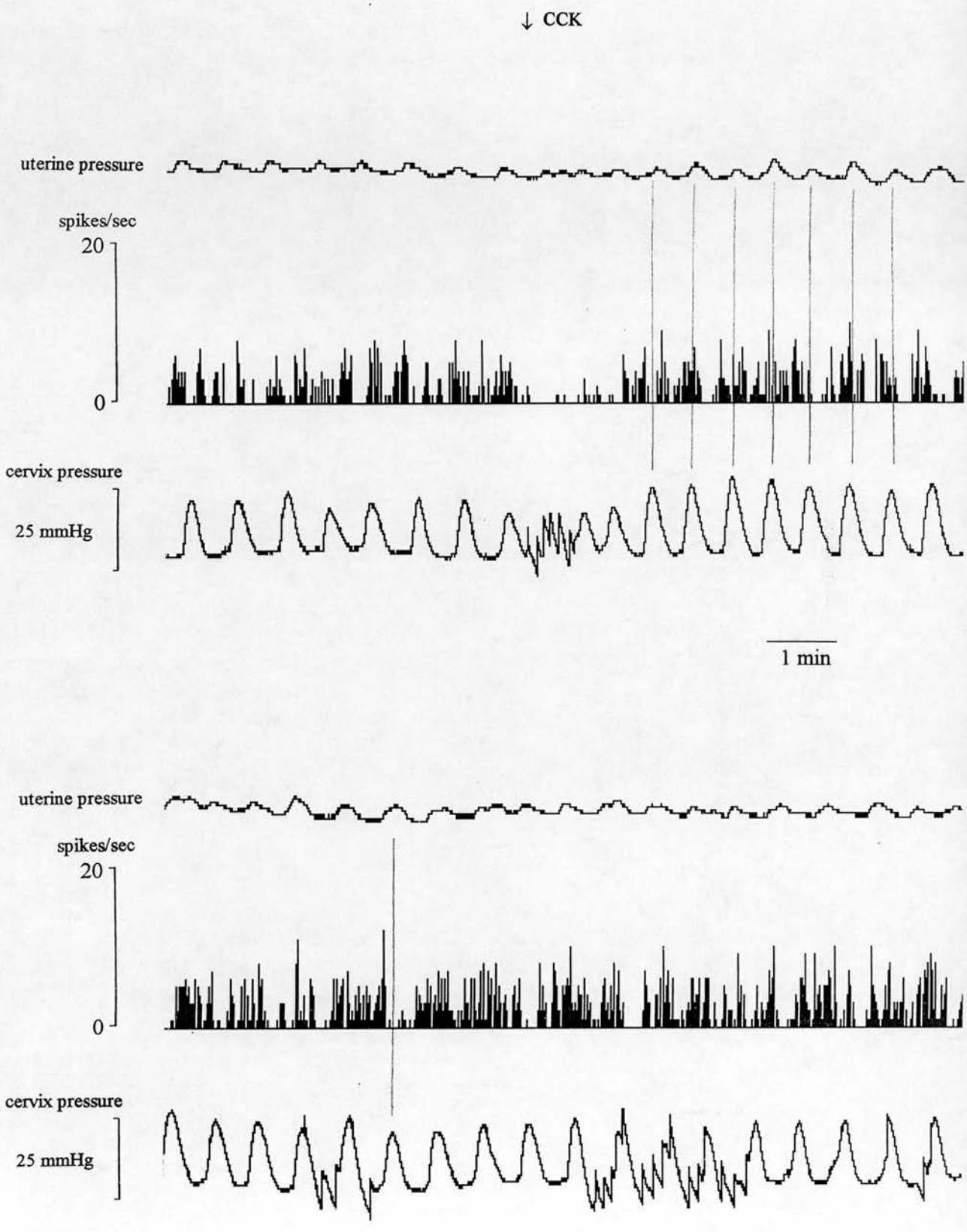

↓ CCK
uterine pressure
spikes/sec
20
0
cervix pressure
25 mmHg
1 min
uterine pressure
spikes/sec
20
0
cervix pressure
25 mmHg

Fig. 7.14. Effects of intermittent oxytocin injections on oxytocin and vasopressin neurone activity

Neuronal activity (upper trace, spikes/sec) of two supraoptic neurones (**Top:** a putative oxytocin neurone, **Bottom:** a putative vasopressin neurone) and uterine pressure (lower trace, in mmHg) were recorded during the oxytocin treatment.

The oxytocin neurone displayed more often an increase in firing rate following an injection of oxytocin, while the vasopressin neurone displayed more often a transient decrease in firing rate after the injections.

The uterus in one animal showed an inconsistent and small response to the oxytocin injections (**Top**), while in the other animal uterine pressure increased in response to each oxytocin injection (**Bottom**).

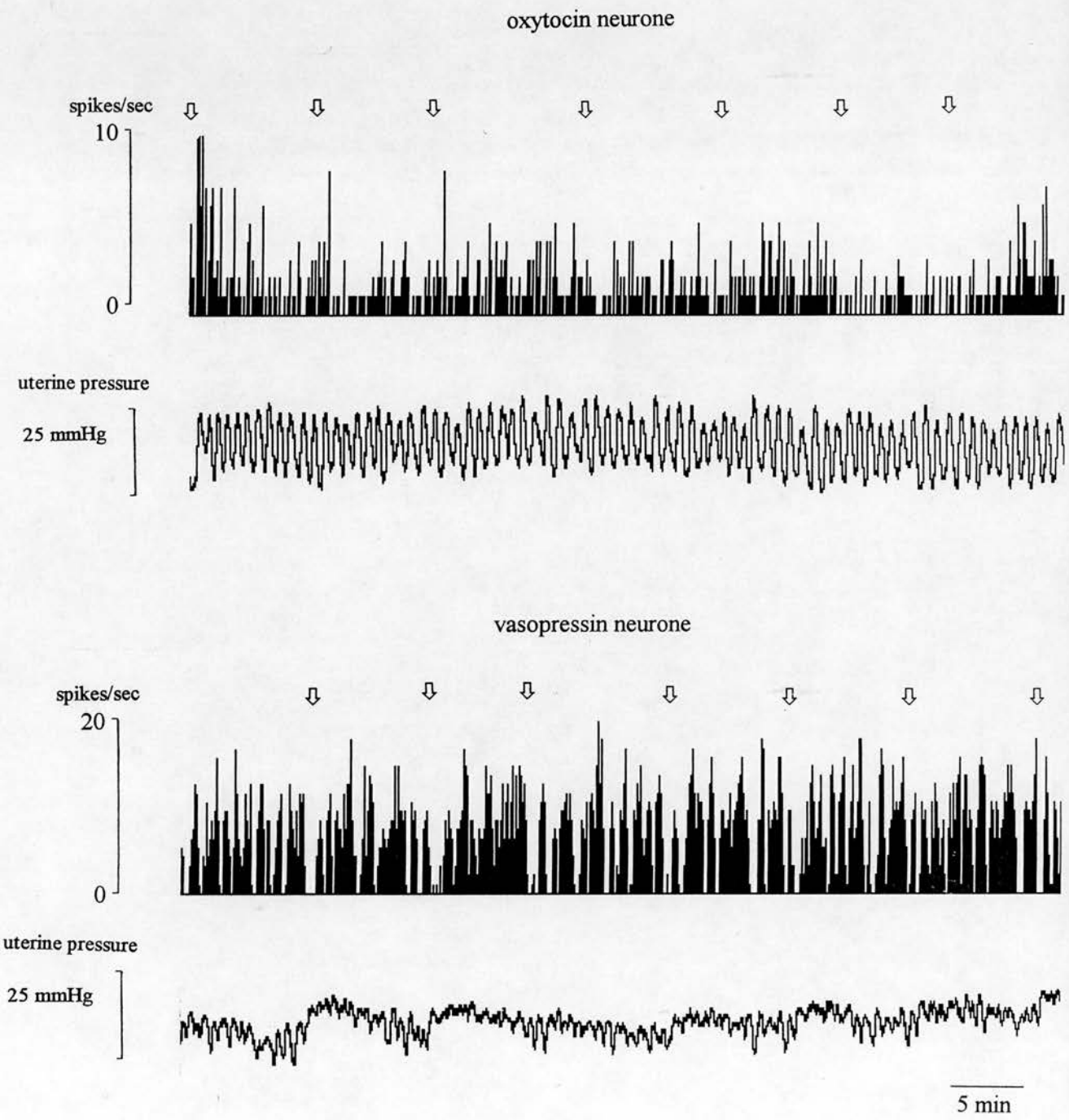

oxytocin neurone
spikes/sec
10
0
uterine pressure
25 mmHg
vasopressin neurone
spikes/sec
20
0
uterine pressure
25 mmHg
5 min

Fig. 7.15. Oxytocin and vasopressin neurone activity in relation to oxytocin pulses
Mean spike activity (spikes/sec) of four supraoptic cells during 2x60 sec bins immediately after each oxytocin pulse (filled bars) and at mid-time between two oxytocin pulses (shaded bars) was calculated.
Top: Two putative oxytocin neurones.
Left: The firing rate immediately after an injection of oxytocin (filled bars) was more often higher than at mid-time between two injections (shaded bars). Though this pattern was apparent throughout the treatment, the difference between the two values was more often significant in the second half of the treatment (*$p<0.05$, t-test for the two respective values). Furthermore, there was a gradual increase in spike activity in the course of the first 2-3 hours of the treatment, but a decline in spike activity thereafter.
Right: Here, the firing rate immediately following a pulse of oxytocin (filled bars) was more often lower than at mid-time between two pulses (shaded bars), however the difference between the two values was not significant for any of the injections.
Bottom: Two putative vasopressin neurones.
Left: Unlike the oxytocin neurone, the vasopressin neurone showed always a higher firing rate at mid-time between two oxytocin injections (shaded bars) compared to that immediately following an injection (filled bars). However the difference between the two values was significant only during the first half of the treatment (*$p<0.05$, t-test for the two respective values). The overall firing rate showed a progressive increase in the course of the treatment.
Right: Similar to the vasopressin cell described above, this cell showed more often a higher firing rate at mid-time between two injections (shaded bars) than immediately following an injection of oxytocin (shaded bars), though the difference between the two values was in most cases not significant (*$p<0.05$, t-test for the two respective values). The overall firing rate showed, similar to the previously described vasopressin cell, a progressive increase in the course of the treatment.

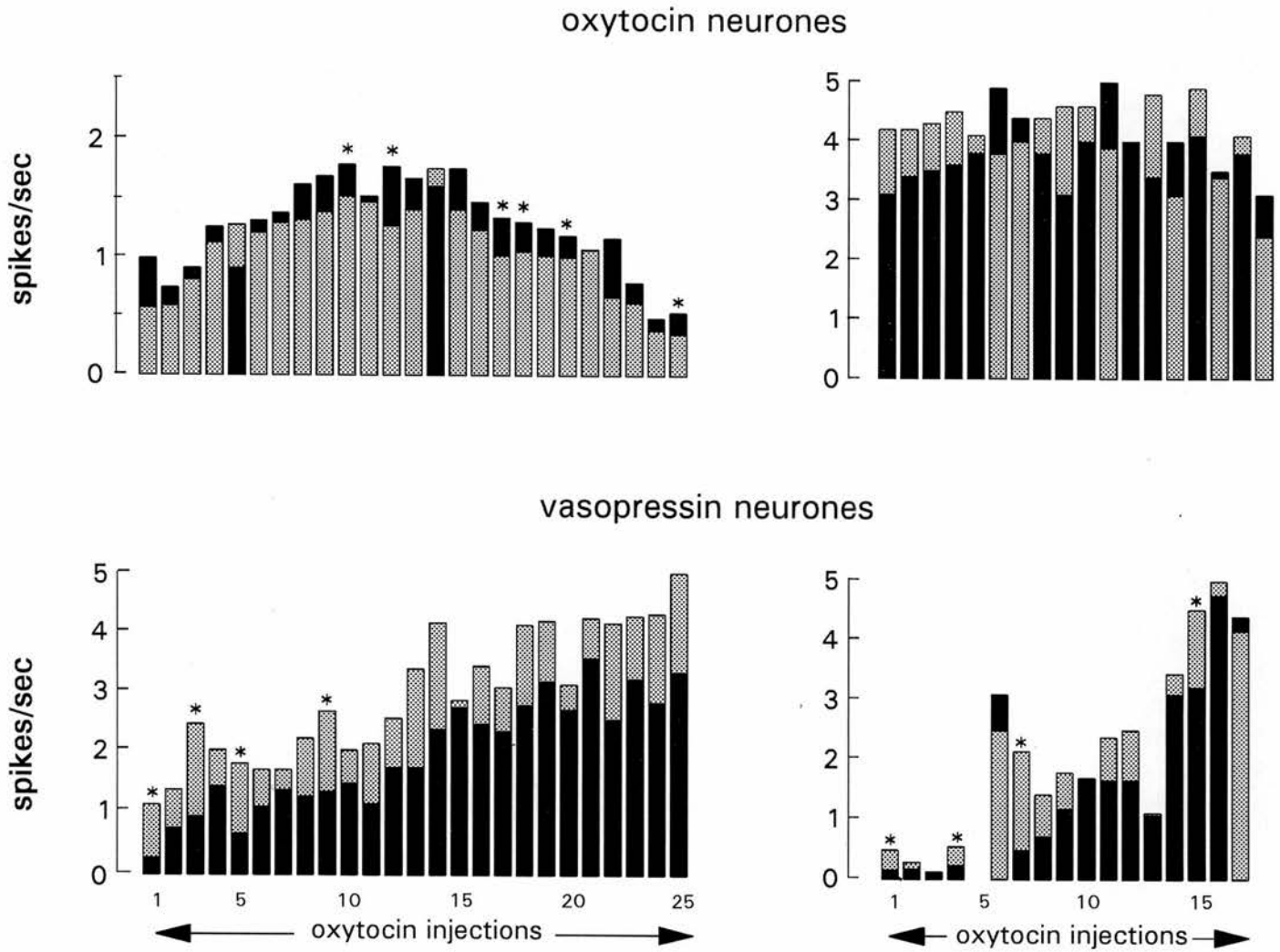
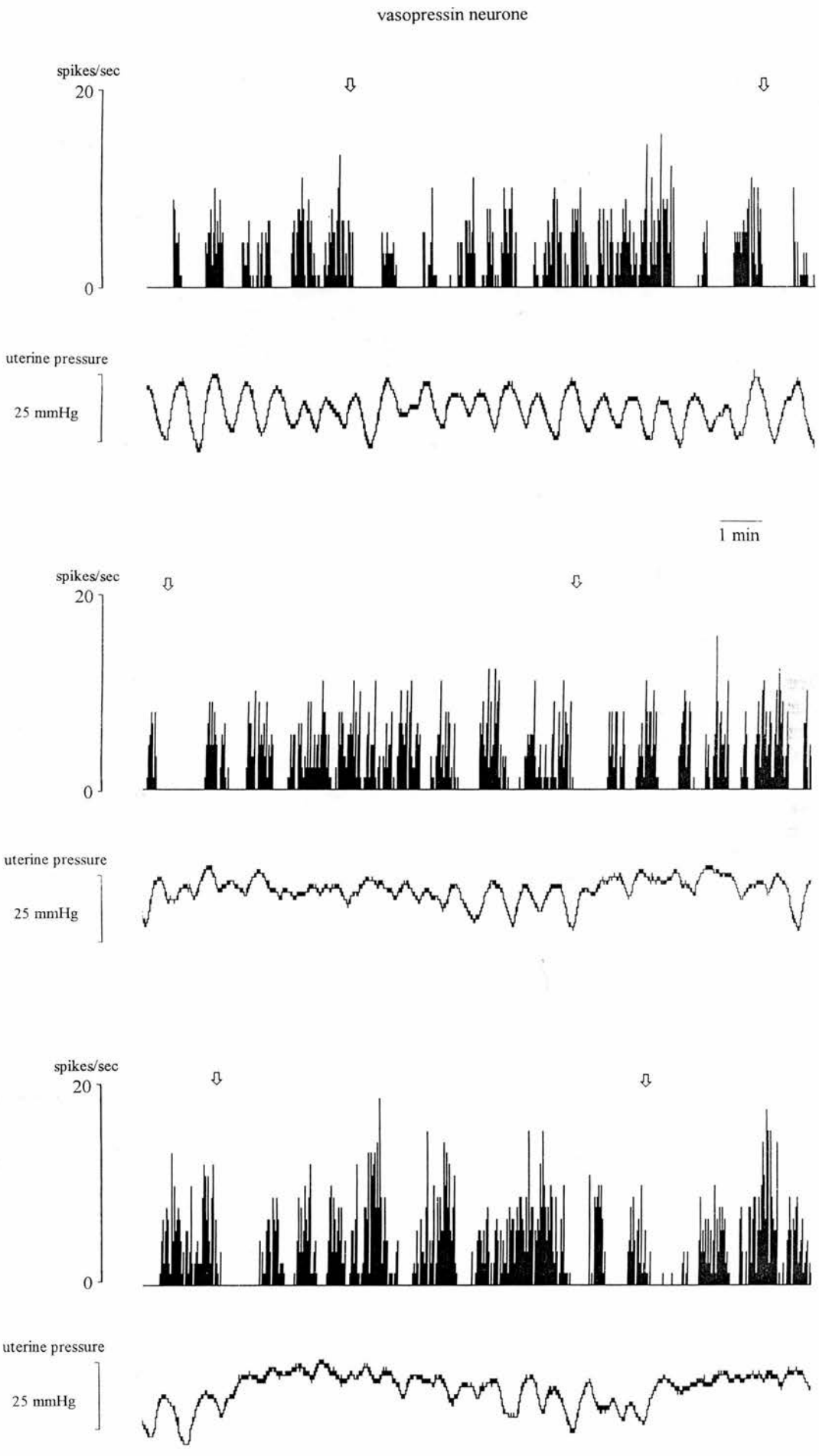

oxytocin neurones
spikes/sec
0
1
2
*
*
* *
*
*
0
1
2
3
4
5
vasopressin neurones
spikes/sec
0
1
2
3
4
5
*
*
*
1
5
10
15
20
25
oxytocin injections
0
1
2
3
4
5
*
*
*
1
5
10
15
oxytocin injections

Fig. 7.16. Synchronisation between neuronal activity and uterine pressure
Electrical activity of a putative vasopressin neurone (upper trace, spikes/sec) and uterine pressure (lower trace, in mmHg) during three 20 min periods throughout the oxytocin treatment (arrow = a pulse of oxytocin) are shown. **Top:** 1st hour. **Middle:** 2nd hour, **Bottom:** 3rd hour.
There was a marked synchronisation between the phasic activity of the putative vasopressin neurone and uterine contractions. Furthermore, the cell displayed in response to most of the oxytocin injections a silent period.


vasopressin neurone
spikes/sec
20
0
uterine pressure
25 mmHg
1 min
spikes/sec
20
0
uterine pressure
25 mmHg
spikes/sec
20
0
uterine pressure
25 mmHg

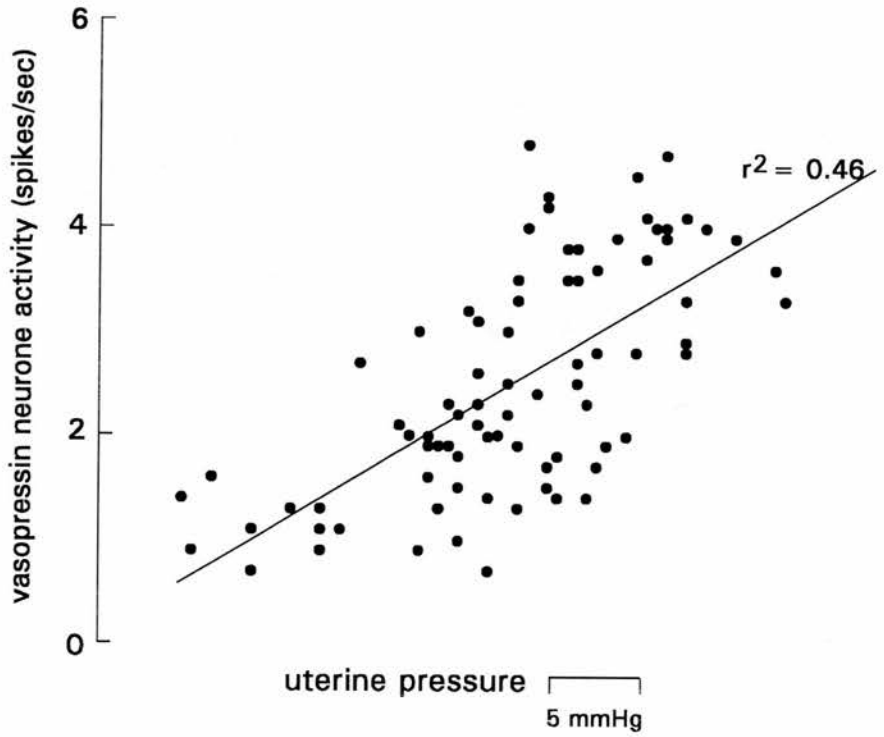
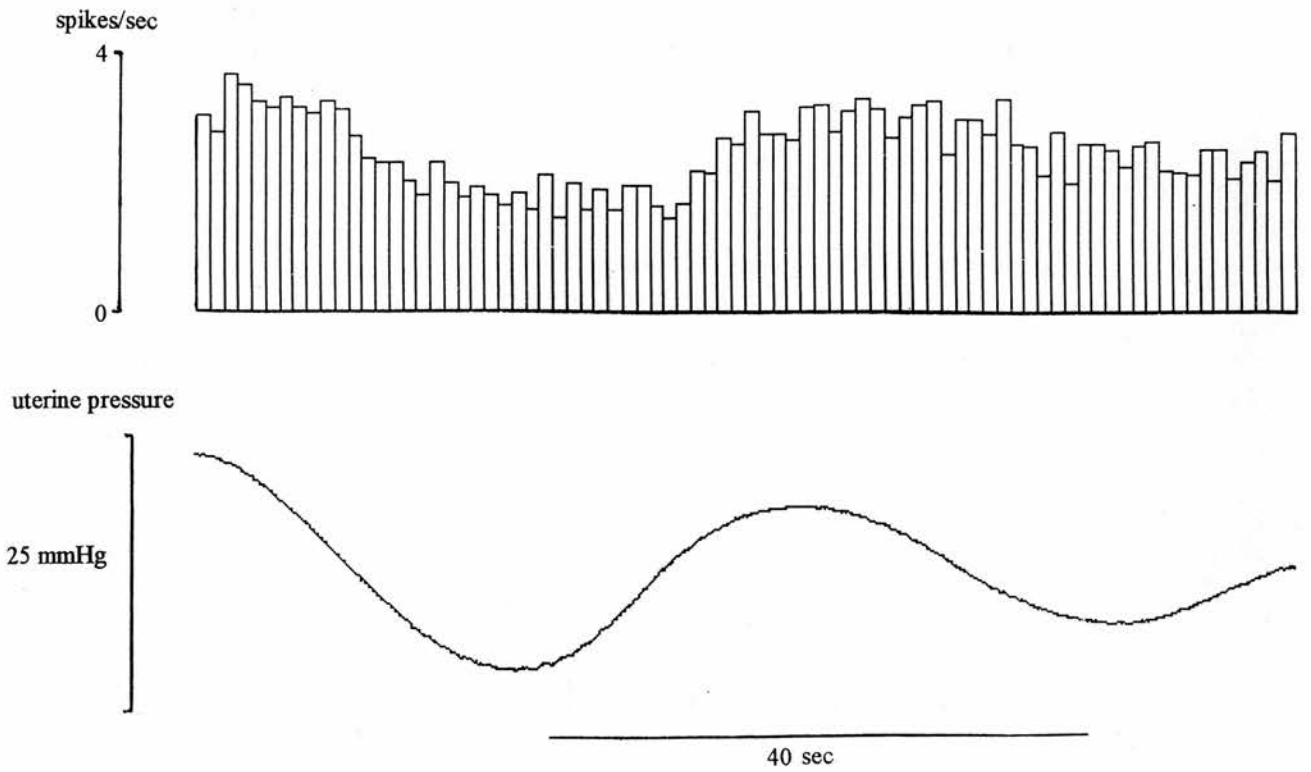


Fig. 7.17. Correlation between vasopressin neurone activity and uterine pressure
Correlation analysis of electrical activity of a supraoptic vasopressin neurone and uterine pressure was performed for each 200 s interval of the entire 4 hour oxytocin pulse treatment.
There was a significant correlation for most of the 200 s intervals and thus an overall highly significant correlation between the increase in electrical activity and uterine pressure (r^2=0.46, $p<0.01$, Genstat analysis).

Fig. 7.17a. Vasopressin neurone activity in relation to uterine contractions
Top: Correlation of electrical activity (spikes/sec) of a putative vasopressin neurone (same as in Fig. 7.16.) with uterine activity in relation to the oxytocin injections was calculated for each 80 sec (in 1 sec bins) during the last 2 hours of the oxytocin pulse treatment.
Bottom: Uterine activity was correlated with itself for the same period.
There was a striking synchronisation of electrical activity and uterine pressure, following each pulse of oxytocin.


spikes/sec
4
0
uterine pressure
25 mmHg
40 sec

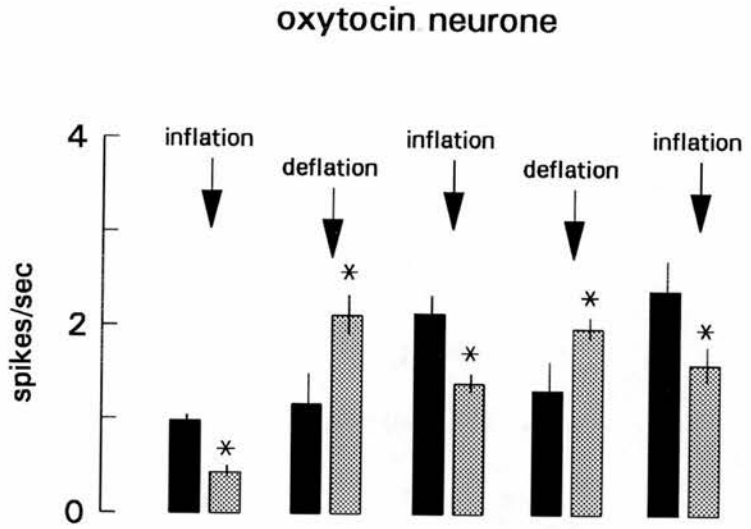
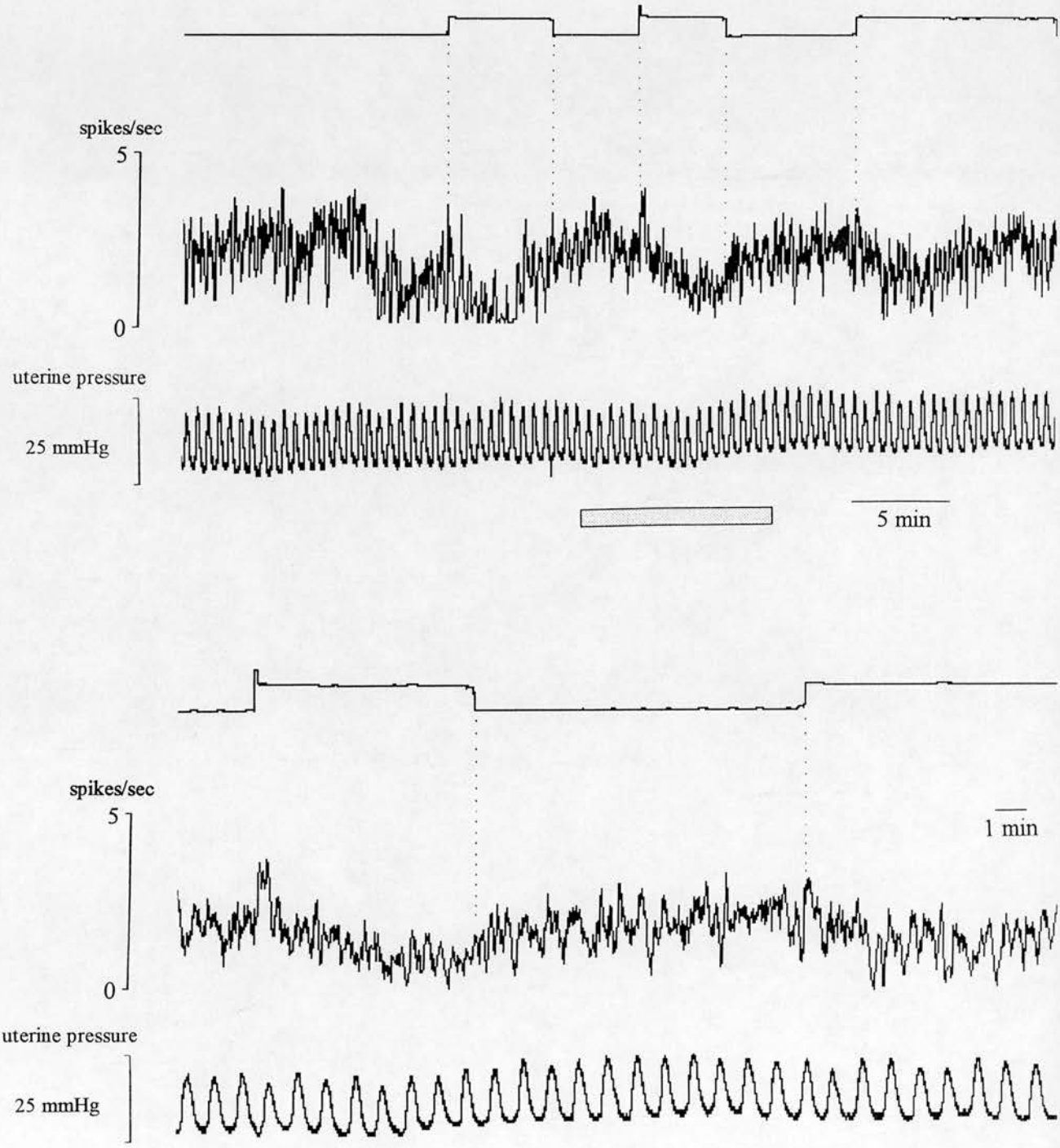


Fig. 7.18. Effects of cervical pressure changes on oxytocin neurone activity
Electrical activity of a putative oxytocin neurone (spikes/sec) was recorded during 180 sec prior to (black bars) and immediately after (shaded bars) de- and inflation (±1.5-3 ml saline) of a balloon placed in the cervix.
Electrical activity decreased consistently and significantly in response to an acute increment in uterine pressure, while reduction of uterine pressure resulted in an increase in spike activity (*$p<0.05$, t-test, for comparison of the respective pre- and post-stimulus value).

Fig. 7.18a. Effects of cervical pressure changes on oxytocin neurone activity and uterine pressure
Electrical activity of a putative oxytocin neurone (middle trace, spikes/sec) and uterine pressure (lower trace, in mmHg) were recorded in response to de- and inflation of a balloon placed in the cervix (±1.5-3 ml saline, upper trace). **Top:** Entire 50 min recording. **Bottom:** High magnification view of the time period indicated in the top trace by a shaded bar.
There was a decrease in neuronal activity in response to an increase in cervical pressure and an increase in firing rate following subsequent decrease in cervical pressure, while uterine pressure was not affected by cervical pressure manipulations.


spikes/sec
5
0
uterine pressure
25 mmHg
5 min
spikes/sec
5
0
1 min
uterine pressure
25 mmHg

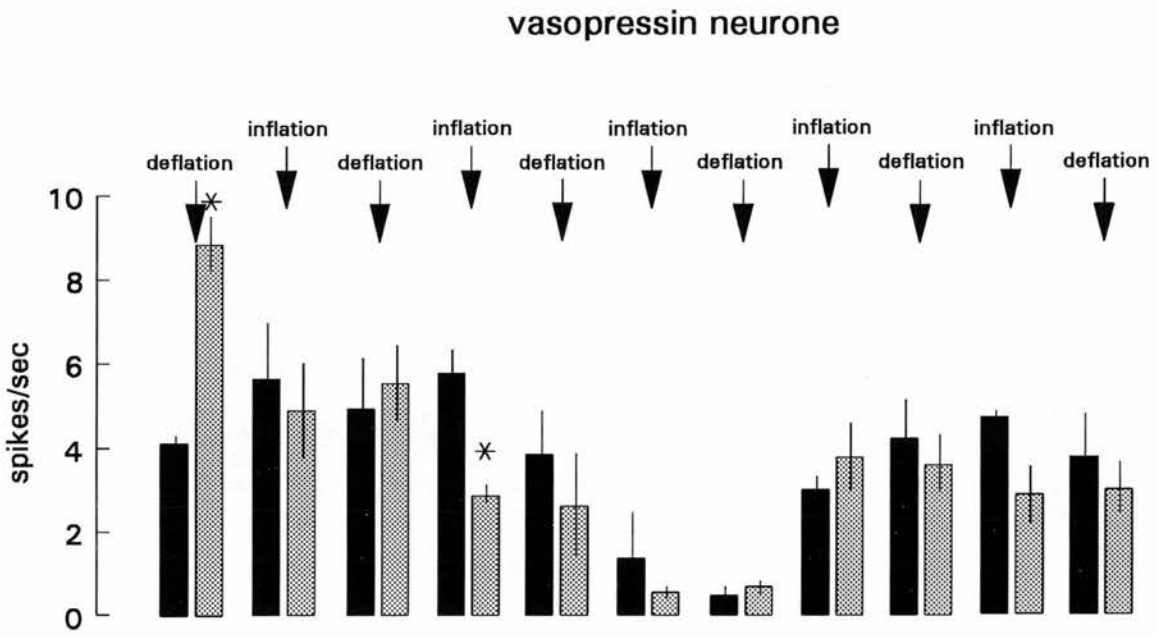


Fig. 7.19. Effects of cervical pressure changes on vasopressin neurone activity
Electrical activity of a putative vasopressin neurone (spikes/sec) was calculated during 180 sec prior to (black bars) and immediately after (shaded bars) de- and inflation (±1.5-3 ml saline) of a balloon placed in the cervix was assessed.
Electrical activity increased in response to deflation of the uterine balloon in 3/6 times and decreased following inflation in 4/5 times, however these changes in electrical activity in response to uterine pressure manipulations were less consistently observed than in the case of the oxytocin cell (see Fig. 7.18, *$p<0.05$, t-test, for comparison of the pre- and post-stimulus value).

Fig. 7.19a. Effects of cervical pressure changes on vasopressin neurone activity and uterine pressure
Electrical activity of a putative vasopressin neurone (middle trace, spikes/sec) and uterine pressure (lower trace, in mmHg) in response to de- and inflation of a balloon placed in the cervix (±1.5-3 ml saline, upper trace). **Top** & **Bottom** are two subsequent 50 min periods during the same recording.
Though we observed mostly decrease in neuronal activity in response to an increase in cervical pressure and an increase in firing rate following subsequent decrease in cervical pressure in half of the cases, the effects of cervical pressure manipulations on vasopressin neurone activity were less marked than those observed for the oxytocin cell (see Fig. 7.18a).

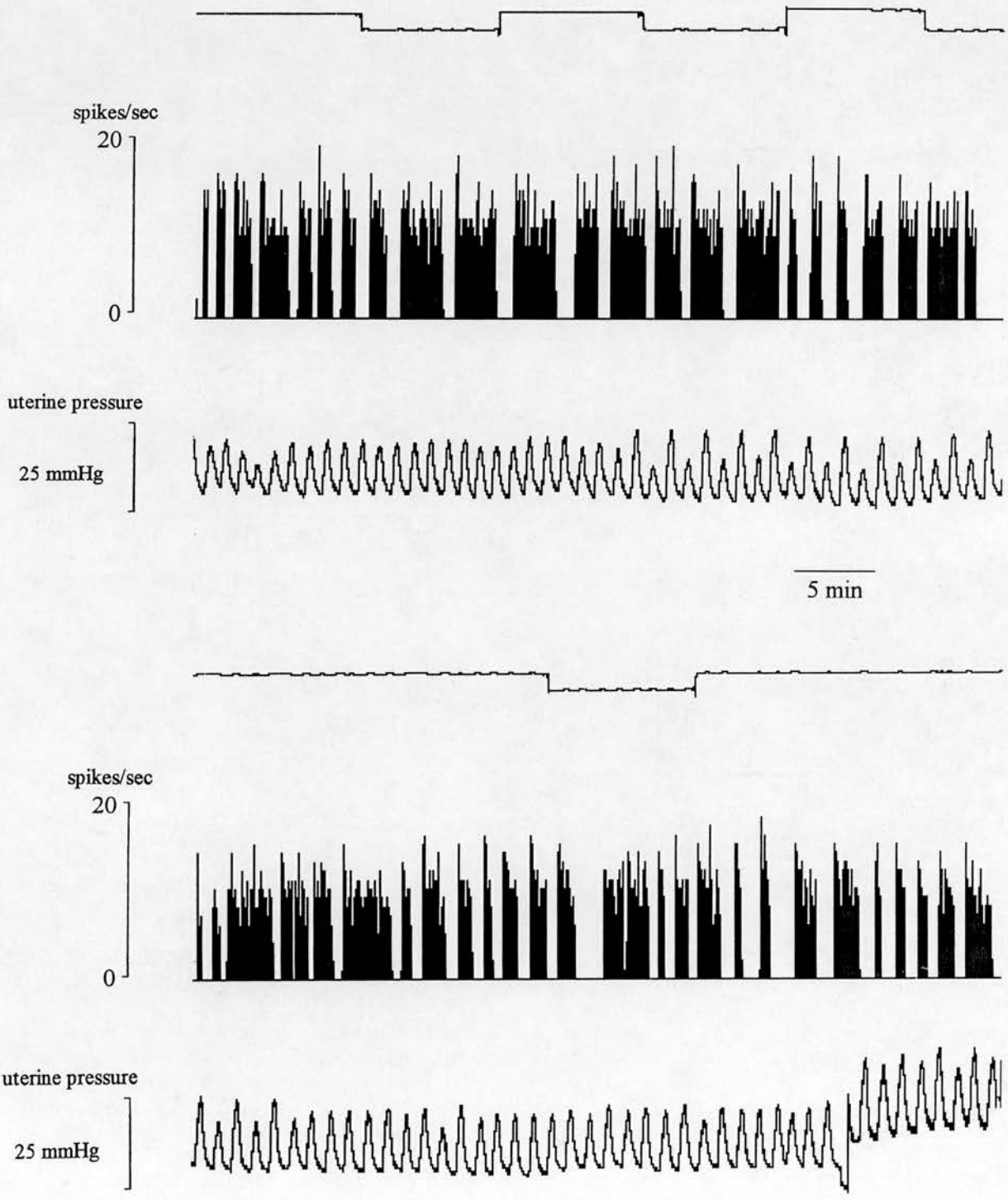
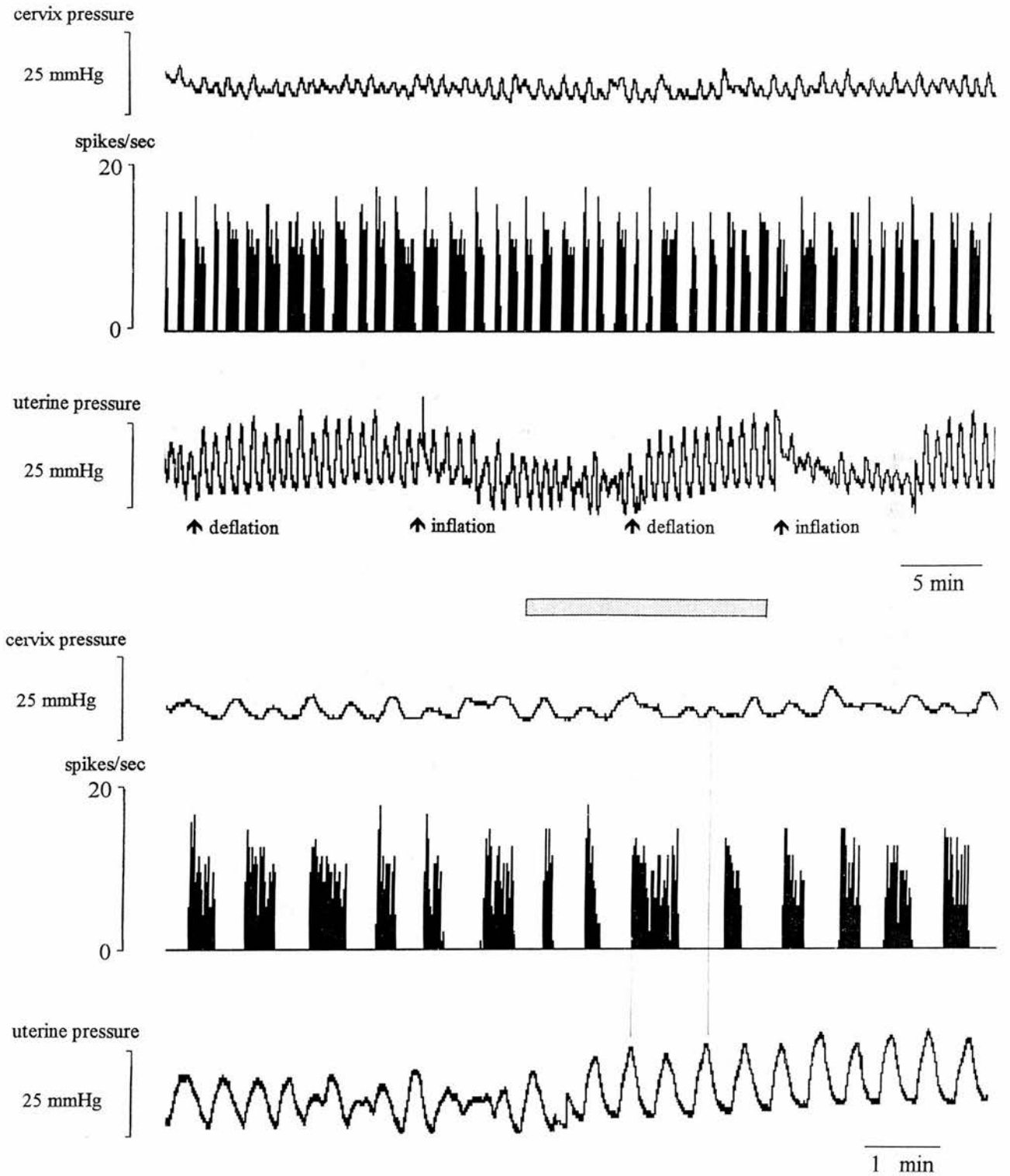

spikes/sec
20
0
uterine pressure
25 mmHg
5 min
spikes/sec
20
0
uterine pressure
25 mmHg

Fig. 7.20. Effects of uterine distension on vasopressin neurone activity and cervical contractions

Electrical activity of a putative vasopressin neurone (middle trace, spikes/sec) and cervical pressure (upper trace, in mmHg) in response to uterine distension (1.5-3 ml) via a balloon placed in one uterine horn (uterine pressure: lower trace, in mmHg) were assessed. **Top:** Entire 50 min recording. **Bottom:** High magnification view of the time period indicated in the top trace by a shaded bar. We observed no significant change in firing rate or in cervical pressure in response to uterine distension via a balloon, possibly because the resulting pressure changes in the uterus were rather small.


cervix pressure
25 mmHg
spikes/sec
20
0
uterine pressure
25 mmHg
deflation
inflation
deflation
inflation
5 min
cervix pressure
25 mmHg
spikes/sec
20
0
uterine pressure
25 mmHg
1 min

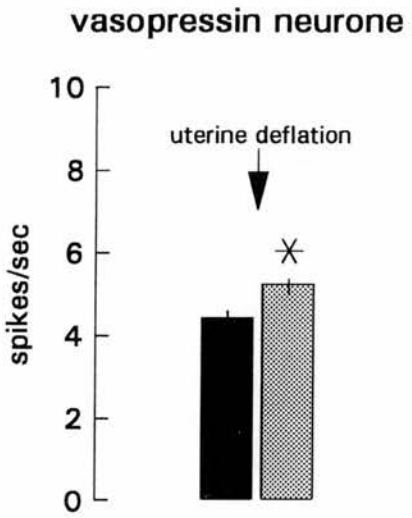
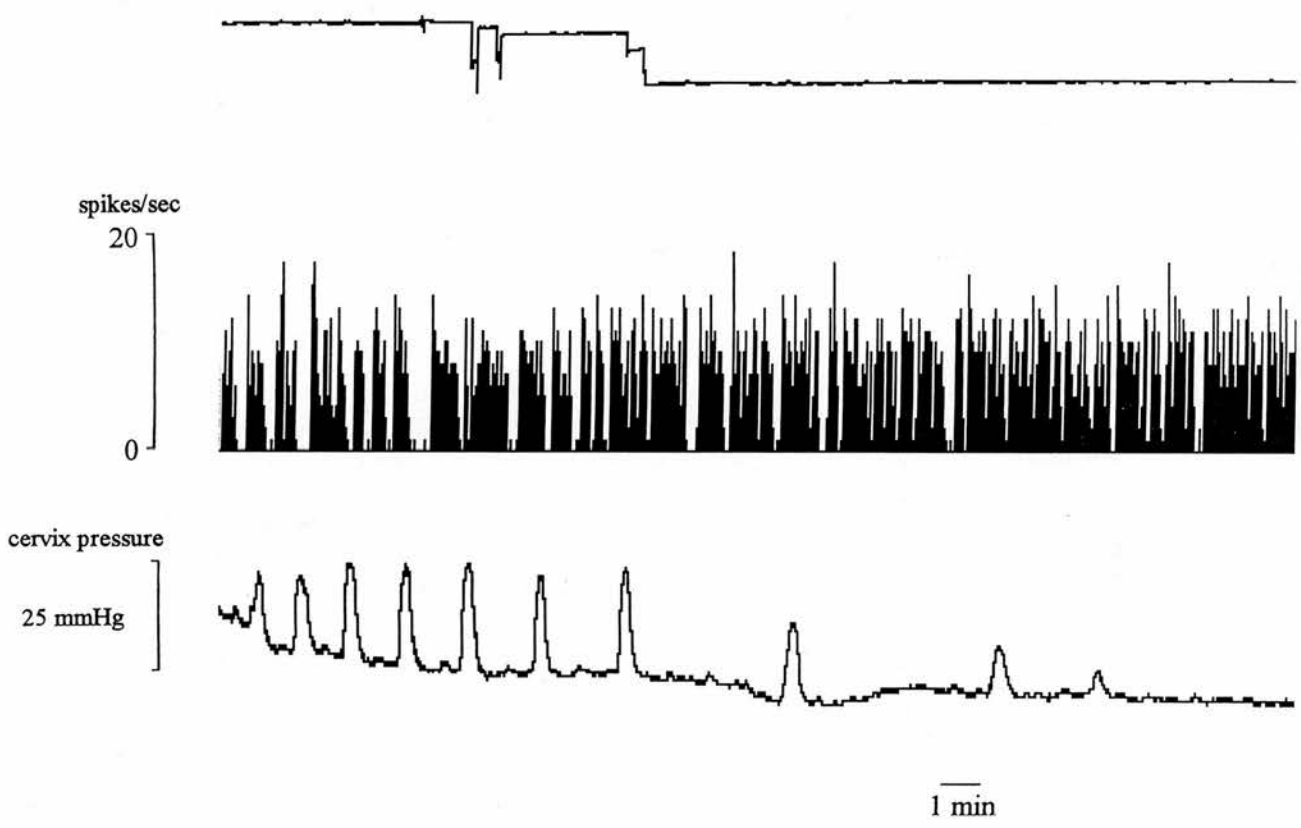


Fig. 7.21. Effects of uterine pressure changes on vasopressin neurone activity
Electrical activity of a putative vasopressin neurone (spikes/sec) was assessed during 10 min before (black bar) and after (shaded bar) a large decrease in uterine pressure induced by deflation of a balloon (3-4 ml) placed in one uterine horn. Electrical activity increased significantly in response to the decrement in uterine pressure (*$p<0.05$, t-test, for comparison of the pre- and post-stimulus value).

Fig. 7.21a. Effects of uterine pressure changes on vasopressin neurone activity and cervical contractions
Electrical activity of a putative vasopressin neurone (middle trace, spikes/sec) and cervical pressure (lower trace, in mmHg) were assessed in response to a large decrease in uterine pressure (upper trace, decrease by 150-200 mmHg).
Concomitantly with the decrease in uterine pressure, the firing rate of the putative vasopressin neurone increased in the course of 10-15 min (compared to a period of the same duration before the pressure change) and at the same time the frequency of high amplitude cervical contractions decreased.


spikes/sec
20
0
cervix pressure
25 mmHg
1 min

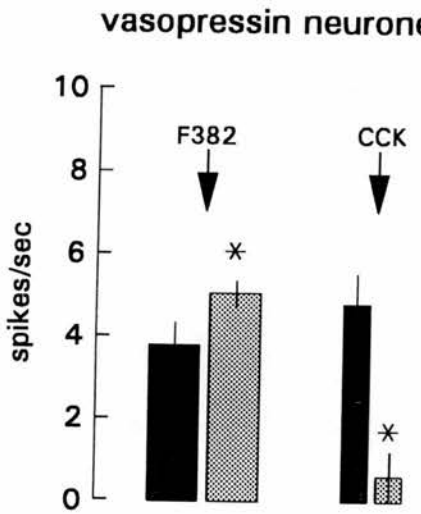
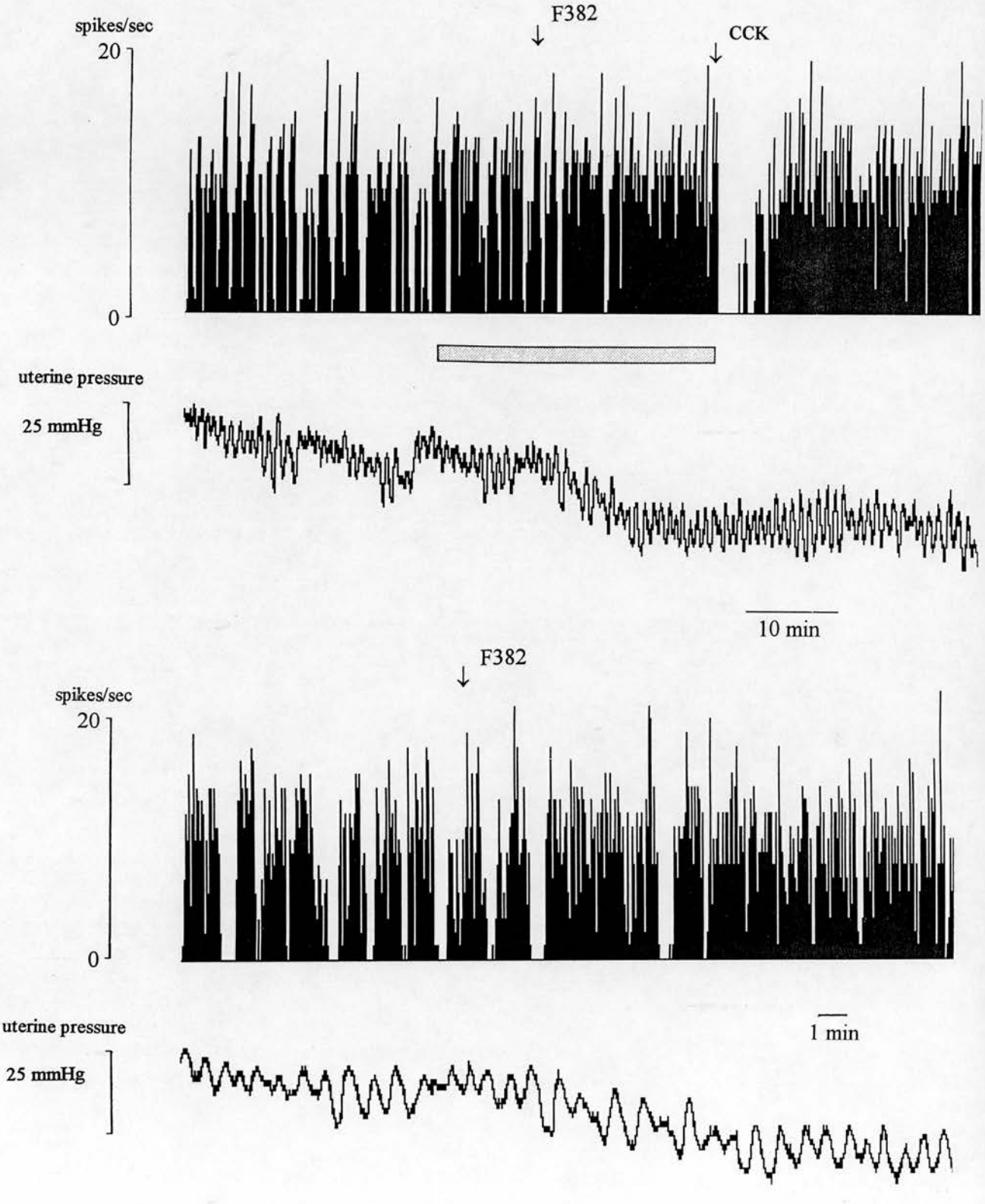


Fig. 7.22. Effects of F382 and CCK on vasopressin neurone activity
Electrical activity of a putative vasopressin neurone (spikes/sec) was assessed during 15 min before (black bar) and after (shaded bar) an injection of the oxytocin receptor antagonist F382 (30 μg/kg body weight iv) and 120 sec before (black bar) and after (shaded bar) an injection of CCK (20 μg/kg body weight iv). There was a significant increase in spike activity following an injection of F382 and a significant decrease in spike activity in response to an injection of CCK (*$p<0.05$, t-test, comparing the respective pre- and post-stimulus values).

Fig. 7.22a. Effects of F382 and CCK on vasopressin neurone activity and uterine pressure
Electrical activity of a putative vasopressin neurone (upper trace, spikes/sec) and uterine pressure (lower trace, in mmHg) were recorded in response to F382 (30 μg/kg, **Top** & **Bottom**) and CCK (20 μg/kg, **Top**). **Bottom:** Higher magnification view of the period indicted in the top trace by a shaded bar.
Uterine pressure showed a rapid decrease in response to an injection of F382, while the contraction frequency and amplitude were not affected. Concomitantly with the decrease in uterine pressure, the firing rate of the vasopressin neurone increased significantly in the course of 15 min following the injection of F382. In contrast, in response to an injection of CCK, neuronal firing rate declined rapidly and significantly (see Fig. 22), whereas uterine pressure showed no marked change.


spikes/sec
20
0
F382
CCK
uterine pressure
25 mmHg
10 min
F382
spikes/sec
20
0
uterine pressure
25 mmHg
1 min

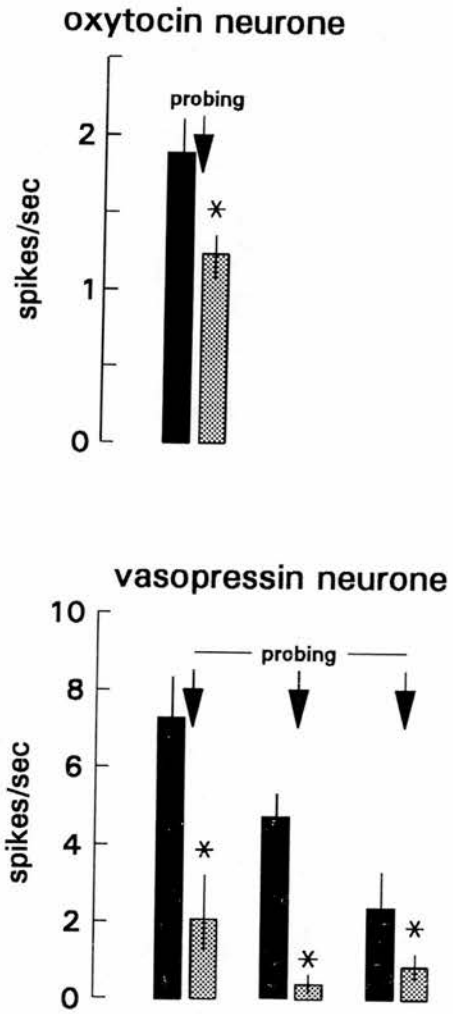
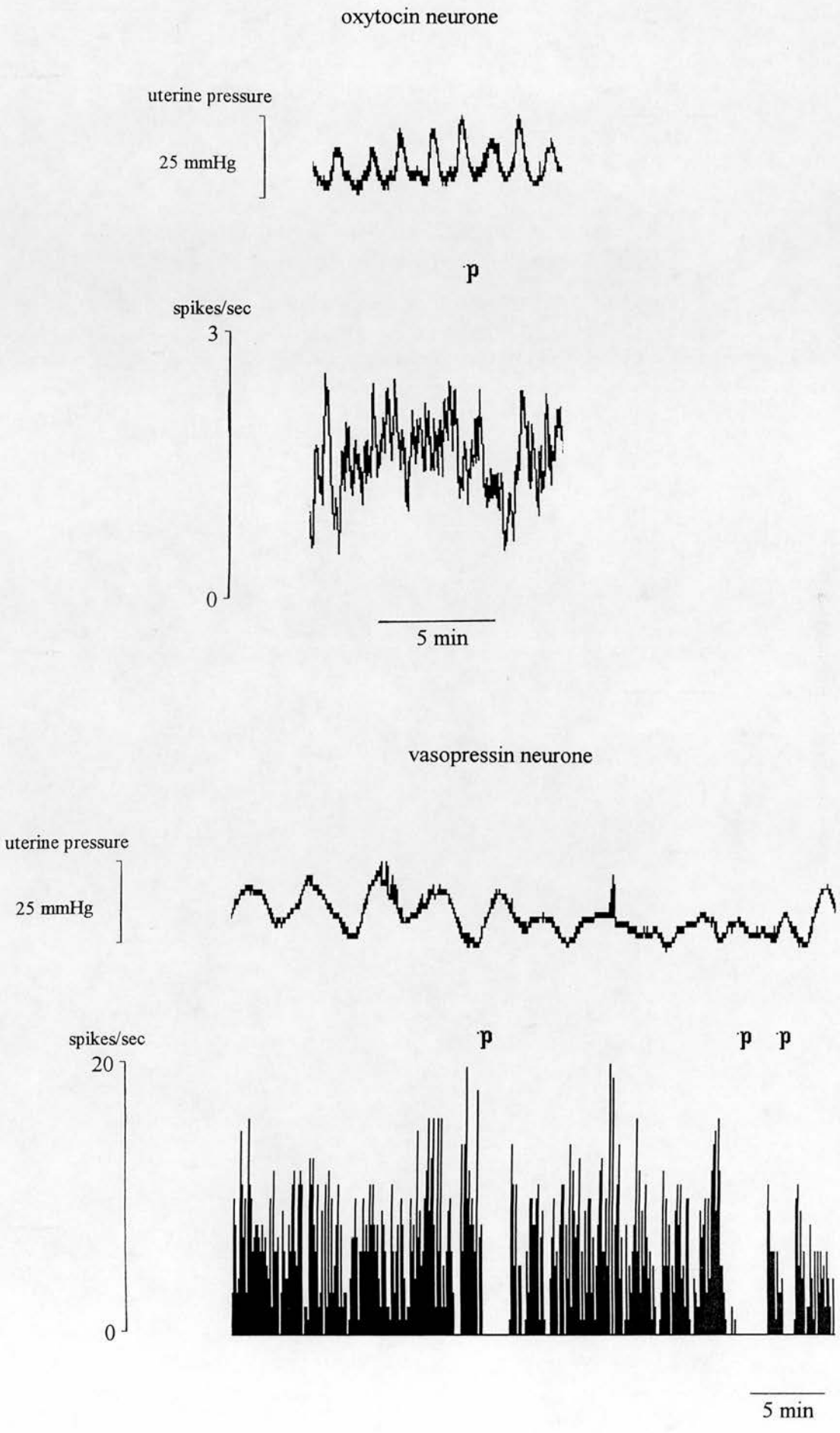


Fig. 7.23. Effects of cervical probing on oxytocin and vasopressin neurone activity
Electrical activity of a putative oxytocin (**Top**) and vasopressin (**Bottom**) neurone was assessed during 60 sec before (black bars) and after (shaded bars) vaginal probing.
Both neurones responded to vaginal probing with a significant decrease in electrical activity (*$p<0.05$, t-test for the two respective values).

Fig. 7.23a. Effects of cervical probing on oxytocin and vasopressin neurone activity and uterine pressure
Electrical activity of a putative oxytocin neurone (**Top**, lower trace, spikes/sec) and a putative vasopressin neurone (**Bottom**, lower trace, spikes/sec) and uterine pressure (upper traces, in mmHg) were recorded in response to vaginal probing (p = vaginal probing).
Both neurones were transiently inhibited following vaginal probing. In the case of the vasopressin neurone, for which electrical activity was recorded during repeated probings, the observed decrease in firing rate was reproducible. In contrast, uterine pressure was not affected by vaginal probing.


oxytocin neurone
uterine pressure
25 mmHg
p
spikes/sec
3
0
5 min
vasopressin neurone
uterine pressure
25 mmHg
spikes/sec
20
0
p
p
p
5 min

General Discussion

I started the presented studies with two main objectives: firstly, to assess the importance of endogenous oxytocin for parturition in the rat and secondly, to investigate afferent pathways involved in oxytocin secretion during late pregnancy and parturition.

The role for oxytocin during labour and delivery has remained controversial due to the apparent lack of a dramatic increase in plasma oxytocin concentrations until delivery. Here, we have demonstrated the importance of oxytocin for normal delivery in rats and we have also provided evidence for an intermittent release of oxytocin, that is critical for the normal progress of parturition, since pulsatile, but not continuous, administration of physiological doses of oxytocin was effective at sustaining the normal progress of delivery in rats in which pituitary oxytocin secretion had been inhibited by an injection of morphine. Similarly, in humans, pulsatile release of oxytocin has been described during spontaneous delivery (Dawood 1989, Fuchs *et al.* 1991) and pulsatile administration of oxytocin has been shown to be more effective at inducing labour and delivery than a continuous infusion (Dawood 1989).

The apparent importance of the pattern of oxytocin release supports the hypothesis that the hypothalamus plays a key role in controlling parturition. Though the finding of large quantities of oxytocin mRNA in the uterus of term pregnant rats has led to the suggestion that the uterus might be a major source of oxytocin during parturition, the amount of oxytocin peptide present in the term pregnant rat uterus is less than 1/100 of that in the posterior pituitary (c.f. Lefebvre *et al.* 1992b and Fuchs & Saito 1971), indicating that, unlike in the posterior pituitary, oxytocin is not stored in the uterus, but is constitutively released. Such a mismatch between the amount of the oxytocin transcript and peptide has also been observed in the rat testis and a post-transcriptional block has been postulated with regard to oxytocin in gonadal tissues of the rat (Foo *et al.* 1991). Since a common feature of oxytocin mRNA found in gonadal tissues is a shorter poly(A) tail compared to the hypothalamic transcript (Lefebvre *et al.* 1992b, Foo *et al.* 1991), this might indicate a less efficient translation of the transcript (Carter & Murphy 1991, Zingg & Lefebvre 1989) and might hence represent a mechanism by which unnecessary translation is prevented in peripheral tissues. However, even small amounts of uterine oxytocin could have paracrine effects, mediated via endometrial oxytocin receptors, including the stimulation of prostaglandin synthesis and release, the latter of which contributes to the induction of myometrial oxytocin receptors and enhances uterine activity (Chan *et al.* 1988, 1993, Fuchs 1987). Increased uterine activity could then lead to the positive feedback onto supraoptic neurones and oxytocin release. The physiological importance of this positive feedback loop is strengthened by the delay of the onset of delivery following administration of an oxytocin receptor antagonist, which reduces uterine activity, to late pregnant rats (see chapter four).

Furthermore, the critical role of uterine activity in stimulating the release of pituitary oxytocin is supported by the observation that when labour and delivery are initiated by pulsatile oxytocin treatment, the progress of delivery is maintained even after the discontinuation of the treatment. In contrast, a continuous infusion of oxytocin, that augments uterine activity to a lesser extent than pulses of oxytocin (Randolph & Fuchs 1989), has been reported previously to be unable to induce pituitary oxytocin release, albeit that such infusion results in preterm labour and delivery (Fuchs & Poblete 1970). Here, we have shown, using Fos immunoreactivity as a marker for neuronal activation, that in response to pulsatile oxytocin treatment in late pregnancy, Fos expression is increased in supraoptic neurones and in putative afferent neurones in the brainstem. This increase in Fos expression, that was observed regardless of whether rats started to deliver during such treatment or not, mimicked

Fos expression seen during spontaneous delivery, suggesting that delivery of pups itself is not necessarily the critical stimulus for the initiation of the reflex release of oxytocin. Indeed, in the last chapter, we consolidated our hypothesis that uterine activity is a critical stimulus for magnocellular neurones by showing that electrical activity of supraoptic neurones increases concomitantly with the gradual augmentation of uterine pressure induced by intermittent oxytocin administration to late pregnant rats. Thus, we have established a model, using pulsatile oxytocin treatment of late pregnant rats, which makes it possible to study the afferent projections from the uterus to the SON in anaesthetised rats and thus to investigate the relevance of afferent projections for the initiation of neurosecretory activity in term pregnant rats.

In summary, we have been able to establish the importance of pituitary oxytocin release, induced, at least partly, by uterine activity in late pregnant rats, for the initiation and maintenance of delivery. In addition, we have made some observations which opened up new questions and which require further investigation before the physiological relevance of these results can be fully understood. In the last chapter, I described a significant correlation between uterine contractions and electrical activity of magnocellular neurones in the course of the oxytocin pulse treatment, indicating the existence of an afferent pathway from the uterus to the SON, which might serve to synchronise neuronal activity and thus possibly to improve the efficiency of hormone release. Since the most striking synchronisation was observed for uterine contractions and vasopressin cell activity, the release of vasopressin, in addition to that of oxytocin, during labour and delivery seems to play a physiological role, which at present remains unclear. Since vasopressin is a potent vasoconstrictor, it could help to maintain blood pressure during delivery (that is accompanied by a substantial blood loss). Furthermore, vasoconstriction has been shown to augment intrauterine pressure, at least following electrical nerve stimulation (Hutchison *et al.* 1994) and thus vasopressin might contribute to the induction of uterine activity at term. Vasopressin receptors are present in the rat uterus (Chan *et al.* 1990) and in humans and rabbits vasopressin receptors mediate uterine activity (Maggi *et al.* 1991a, 1992) and hence both oxytocin and vasopressin might act synergistically to initiate uterine activity, although only the oxytocin receptor density increases dramatically at term (Maggi *et al.* 1991a). Alternatively, I would like to suggest that vasopressin release at term could help to prevent premature release of oxytocin via the postulated co-release of dynorphin (Bondy *et al.* 1989b), an endogenous kappa opioid receptor agonist, that is co-expressed with vasopressin in secretory granules of magnocellular neurones (Watson *et al.*

1982) and could, upon secretion, inhibit via kappa opioid receptors, oxytocin release either at the hypothalamic (Sumner *et al.* 1992) or the neurohypophysial level (Bondy *et al.* 1989b, Herkenham *et al.* 1986). Since an opioid inhibition of prolactin release in term pregnant rats is enhanced by the normal decline of plasma progesterone concentrations (Soaje & Deis 1994), changes in gonadal steroid concentrations could be critical for the control of preterm release of oxytocin.

An inhibition of oxytocin cell activity might be particularly important at a time when the cervix is not fully softened, since at that time release of oxytocin would result in an increase in intrauterine pressure, which would endanger the foetus(es) that cannot yet be expelled. This hypothesis is in agreement with our observation that vaginal probing inhibited oxytocin cell activity (and hence hormone release). However, in response to such an acute stimulus as vaginal probing vasopressin neurone activity was similarly reduced, indicating that both types of magnocellular neurones are under an inhibitory control at term, possibly because both types of neurohypophysial hormones can induce uterine activity. By contrast, in the course of induced or spontaneous labour, the cervix is softened (Olah *et al.* 1991) and this is reflected by the increase in amplitude of cervical contractions (see chapter seven), which would seem to be the appropriate stimulus for oxytocin (and vasopressin) release.

The activation of both supraoptic vasopressin and oxytocin neurones during labour, and particularly in response to oxytocin-induced labour, is supported by the detection of Fos protein in both types of neurones (see chapter three). Though Fos expression is an indirect measurement of neuronal activity and in magnocellular neurones of hormone release, a large body of evidence in addition to that provided in this work supports the association of Fos expression with neurosecretory activity in the magnocellular hypothalamus (Hamamura *et al.* 1991a, 1991b, Luckman *et al.* 1993a, Verbalis *et al.* 1991b). In contrast, the absence of Fos expression in the SON does not necessarily indicate a lack of oxytocin secretion, as seen in rats injected with CCK following pretreatment with a CCK_B receptor antagonist (see chapter two and Luckman *et al.* 1993b). However, the conclusion drawn from our studies in parturient rats treated with morphine and in rats treated with progesterone on day 20 of pregnancy, that reduced expression of Fos in the SON indeed reflects reduced secretion, seems warranted for two reasons: first, an injection of morphine to parturient rats has been shown previously to inhibit oxytocin secretion (Russell *et al.* 1989b, 1991) and second, the impaired progress of delivery in both morphine- and progesterone-treated rats is improved by administration of exogenous oxytocin.

The role of the immediate early gene c-*fos* is generally considered to involve induction of late genes (Sheng & Greenberg 1990), through binding of Fos heterodimers to so-called AP-1 sites contained within the respective late genes. However, the vasopressin and the oxytocin gene possess only a site which differs from the canonical AP-1 site by one base (Leng *et al.* 1993a) and to which binding of Fos has not yet been demonstrated. Thus, the exact role of Fos expression during parturition in supraoptic neurones awaits further investigation. Since in the SON, Fos expression in response to increased neurosecretory activity is dependent on trans-synaptic activation and not on spike activity *per se* (Luckman *et al.* 1994), this observation opens up the possibility that neurotransmitter(s) themselves modulate Fos expression in postsynaptic elements. Such stimulus specificity, which could explain the lack of a simple relationship between immediate early gene induction and the amount of hormone secretion, might involve activation of distinct subpopulations of NTS neurones that project to the hypothalamus and, as shown recently, co-localise different neurotransmitters (Kawai *et al.* 1988, Sawchenko *et al.* 1985, 1988a, 1988b, 1990). Thus, depending on the stimulus, a specific subpopulation of afferent neurones could be activated, resulting in the release of a certain combination of neurotransmitters within the hypothalamus and hence differential stimulation of second messenger systems in the postsynaptic neurone, which might account for the observed differences in immediate early gene expression. Since labour is associated with pain and stress (Bonica & McDonald 1990), this might lead to activation of afferent pathways in addition to those stimulated by uterine contractions and hence contribute to the induction of Fos expression and oxytocin release during parturition (Jezova *et al.* 1993, Lightman & Young 1989, Smith & Day 1994, Walker *et al.* 1992). This might be particularly the case during oxytocin-induced labour, which is characterised by stronger uterine activity than spontaneous labour (Randolph & Fuchs 1989) and activates, unlike normal labour, supraoptic and putative afferent NTS neurones prior to delivery of pups (see chapter three). Though in rats, noxious stimuli not involved in reproduction, e.g. hindpaw pinches, also stimulate magnocellular oxytocin neurones and oxytocin release (Akaishi *et al.* 1988, Onaka & Yagi 1991), in the NTS (Bailey & Wakerley 1994) and the PVN (Akaishi *et al.* 1988) there is a degree of specificity with regard to neuronal subpopulations activated in response to footpinch and vaginal distension. Hence some of the Fos-immunoreactive neurones in the hypothalamus and the NTS during parturition are likely to reflect specific activation of afferent neurones and magnocellular neurones in response to labour, while others might be activated due to the accompanying pain and stress.

Since vaginal distension seems to be a less potent stimulus for oxytocin release than suckling (Poulain & Wakerley 1982), uterine activity might have to reach a certain threshold before magnocellular neurone activation can occur. At the end of pregnancy, gonadal steroid concentrations show a typical change, namely an increased ratio of plasma oestrogen/progesterone concentrations (Csapo *et al.* 1980), that in turn seems to modulate the excitability of hypothalamic neurones *in vitro* (Kow *et al.* 1991) and the sensitivity of magnocellular oxytocin neurones to vaginal-cervical stimulation *in vivo* (Negoro *et al.* 1973a, 1973b). Thus, progesterone treatment of late pregnant rats might reduce the responsiveness of magnocellular oxytocin neurones to the uterine activity during spontaneous labour.

In humans, uterine activity in the last trimester of pregnancy is increased at night, coinciding with high plasma oxytocin concentrations (Fuchs *et al.* 1992) and the latter are strongly correlated with the ratio of plasma oestrogen/progesterone concentrations. In baboons, it has been shown that plasma oestrogen and progesterone concentrations both exhibit a nocturnal surge during the third trimester of pregnancy (Wilson *et al.* 1991) and that in the last 10 days prior to delivery, the oestrogen surge is shifted forward, resulting in an increased ratio of plasma oestrogen/progesterone, that is associated with increased uterine activity (Wilson *et al.* 1991). This indicates that an alteration in the circadian rhythm of gonadal steroid plasma concentrations might be involved in the initiation of labour. An interference with these circadian rhythms, e.g. by an injection of progesterone (see chapter five), might have contributed to the observed delay in the onset of parturition in our experiments.

During parturition, Fos is also expressed in putative afferent neurones in the brainstem and I have shown, using double immunocytochemistry, that in parturient and late pregnant rats a significant proportion of Fos immunoreactive nuclei is found in catecholaminergic neurones in the NTS. Since firstly, the catecholaminergic cell group in the NTS represents a major excitatory pathway to magnocellular oxytocin neurones in the SON and PVN (Onaka *et al.* 1995, Raby & Renaud 1989a, 1989b, Rinaman *et al.* 1994, Sawchenko & Swanson 1982a) and secondly, the NTS receives sensory information from the pelvic reproductive organs, including the uterus (Hubscher & Berkley 1994, Ortega-Villalobos 1990), this nucleus is likely to be involved in the mediation of oxytocin release during parturition.

Further evidence for the importance of an afferent input from the uterus for oxytocin release during delivery is provided by the observation that following progesterone treatment of late pregnant rats, pulsatile oxytocin could not induce delivery on the day of expected term.

This is consistent with the hypothesis that when the expression of functional uterine oxytocin receptors is delayed by progesterone treatment (Fuchs *et al.* 1983, Maggi *et al.* 1991a), the uterus remains relatively insensitive to oxytocin and thus the positive feedback of uterine activity onto magnocellular oxytocin neurones is prevented. However, at the time of spontaneous parturition progesterone-treated rats still fail to show a normal increase in Fos expression in the SON, while Fos expression in the NTS is not affected. These data imply that during such delayed delivery the afferent input from the uterus to the NTS is intact, but the excitatory projection from the NTS to the SON is impaired. This hypothesis is supported by our data showing altered TH immunoreactivity and TH mRNA expression, namely a reduced number of TH containing cells at the time of parturition and lower TH mRNA content on day 21 of pregnancy in NTS neurones of progesterone-treated rats and suggests that progesterone might act at the level of catecholaminergic NTS neurones, possibly by affecting TH gene transcription and translation and thus the excitatory input from the NTS to the SON. Up till now, there is little data on the relationship between TH mRNA expression and catecholamine release from axon terminals. However, electrical stimulation of catecholaminergic A1 neurones leads to increased noradrenaline release in the preoptic area (Herbison *et al.* 1990) and is followed within less than one hour by an increase in TH mRNA expression in the same cell group (Liaw *et al.* 1992b), pointing to the possibility that TH gene expression and transmitter release or electrical activation are somehow correlated. Furthermore, the α_2-adrenoreceptor mediated autoinhibition of catecholamine release in the hypothalamus is attenuated by oestrogen (Karkanias & Etgen 1993) and hence at term, when the ratio of oestrogen/progesterone plasma concentrations is elevated, even small amounts of catecholamines would be expected to lead to a more pronounced excitation compared to mid-pregnancy, when plasma progesterone levels are high.

Since magnocellular neurones in rats and monkeys do not express nuclear gonadal steroid receptors (Bethea *et al.* 1994, Fox *et al.* 1990, Rhodes *et al.* 1991a, Sar 1988), while catecholaminergic neurones in the NTS contain oestrogen receptors, the latter are targets for gonadal steroid modulation (Heritage *et al.* 1977, 1980). Other workers have described an inhibitory effect of progesterone on TH mRNA expression and a negative correlation of TH activity, measured by the accumulated metabolite concentration, and plasma progesterone concentrations *in vivo* (Arbogast & Voogt 1993, Liaw *et al.* 1992a, 1992b, Wang & Porter 1986), which is in agreement with our observations. The observation that in normal pregnant

rats there is an increase in TH mRNA expression in the NTS prior to delivery could indicate an involvement of this pathway in priming neurosecretory oxytocin neurones for the coordinated burst-like increase in electrical activity seen during delivery and lactation (Belin & Moos 1986, Lambert *et al.* 1993, Moos & Richard 1989), which is critical for the release of large amounts of oxytocin.

In addition, morphological changes in the magnocellular hypothalamus and the neural lobe of late pregnant rats, including an increased apposition between oxytocin neurones in the former and a greater access of axon terminals to the capillaries in the latter (Theodosis *et al.* 1984, 1986a, Tweedle & Hatton 1982), have been implicated in the facilitation of bursting activity and hormone release. *In vitro,* morphological changes of posterior pituitary glial cells can be induced within hours by catecholamine administration (Bicknell *et al.* 1989, Luckman & Bicknell 1990). Though a similar involvement of catecholamines for the induction of structural plasticity *in vivo* has not yet been shown, the neural lobe receives a direct noradrenergic projection from NTS neurones (Bicknell *et al.* 1988a, Garten *et al.* 1989, Saavedra 1985). Furthermore, in the developing rat brain, ascending catecholaminergic pathways have been implicated in the organisation of synaptic contacts (Parnavelas & Blue 1982). Thus, the observed acute changes in TH mRNA expression and TH immunoreactivity in brainstem neurones of late pregnant rats could indicate a contribution of a noradrenergic projection from the NTS to the initiation of structural plasticity in the neural lobe and possibly in the hypothalamus at the end of gestation.

However, the significance of morphological changes in suckled rats for the bursting activity and increased hormone release seems to be permissive rather than causal for the synchronised high frequency discharge of the oxytocin neurone population, since similar morphological changes are also observed during chronic dehydration (Chapman *et al.* 1986, Theodosis *et al.* 1986a), a condition that is not accompanied by a burst-like discharge of oxytocin neurones (Leng *et al.* 1993b). Thus, once the morphological changes have taken place, additional factors seem necessary to generate the bursting activity of oxytocin neurones, possibly including the intranuclear release of oxytocin, that is more pronounced during parturition and lactation (Moos & Richard 1989, Moos *et al.* 1992, Neumann *et al.* 1993) than following hyperosmotic stimulation (Moos *et al.* 1992) and enhances the burst amplitude of oxytocin neurones (Lambert *et al.* 1993, Moos & Richard 1989, Neumann *et al.* 1995) and hence the amount of hormone released from the posterior pituitary. The release of intranuclear oxytocin release in turn, might be dependent on a gating mechanism that involves the

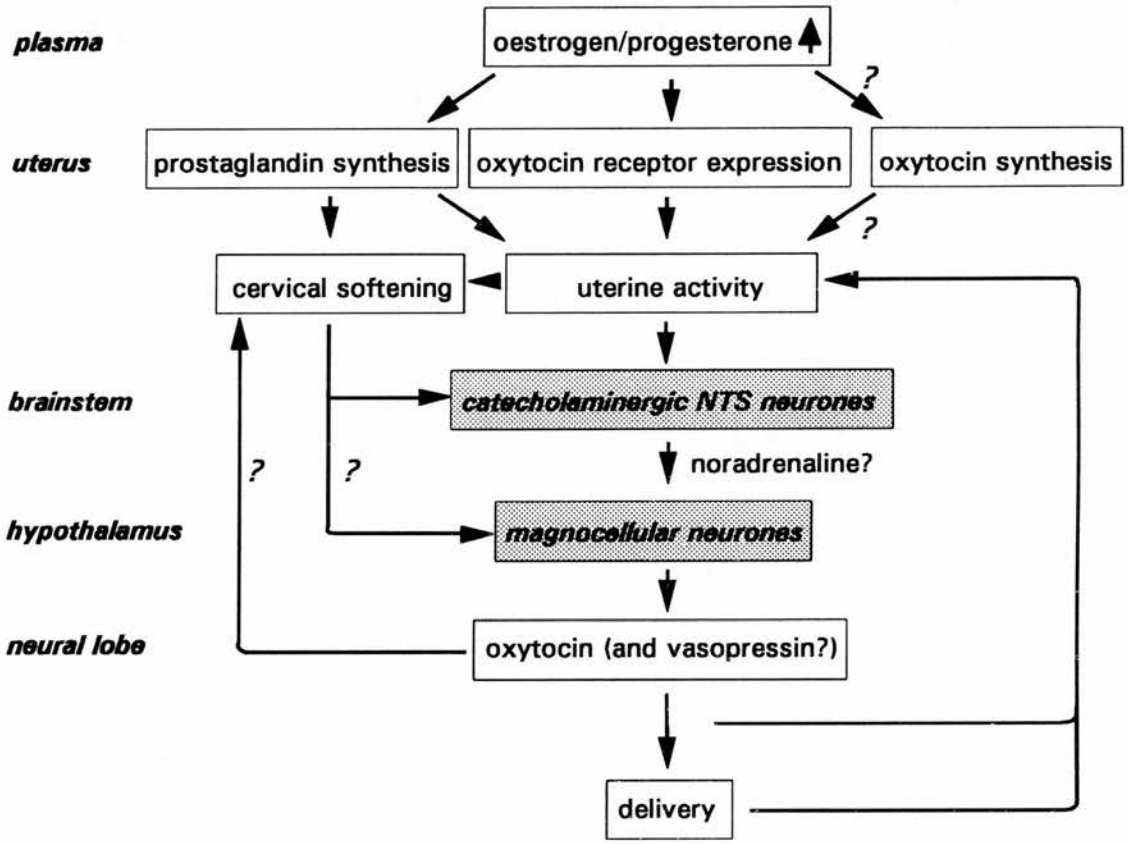
synergistic actions of transmitter co-release. In this respect, glutamate, an excitatory amino acid widely distributed in the brain, including the hypothalamus, might play an important role: 1) glutamate has been implicated in the release of most pituitary hormones, including oxytocin and vasopressin (Brann 1995), 2) a high density of glutamate-containing nerve terminals has been described in the SON (Meeker *et al.* 1989), 3) patch-clamp analysis has shown that glutamate could account for almost all fast excitatory postsynaptic potentials (EPSP) in the rat SON (Wuarin & Dudek 1993) and finally electrophysiological recordings form supraoptic neurones suggest that activation of non-NMDA receptors might be critical for the induction of clustered spike activity (Hu & Bourque 1992). A physiological significance of glutamate for bursting activity of supraoptic neurones is further supported by the observation that central co-administration of AMPA and α_1-adrenergic agonists greatly potentiates the excitatory effects of the adrenergic agonist on oxytocin release in lactating rats (Parker & Crowley 1993b).

Likewise, NPY has been demonstrated to have an enhancing effect on α_1-adrenergic agonist-induced oxytocin release in lactating rats (Parker & Crowley 1993a), indicating that synergistic actions of neurotransmitters might represent a mechanism by activation of magnocellular neurones can be greatly enhanced without being reflected by a dramatic increase in any one transmitter release. This hypothesis also supports a physiological relevance of the described co-expression of NPY in a subset of catecholaminergic NTS neurones that project to the hypothalamus (Sawchenko *et al.* 1985) and the up-regulation, via the Y_1-receptor, of TH mRNA expression in the rat brain following central administration of NPY (Hong *et al.* 1994).

In this respect it is intriguing firstly, that α_1-adrenergic agonist administration *in vitro* using brain slice preparations can induce bursting activity of oxytocin neurones that bears a close resemblance to the activity observed during the milk-ejection reflex *in vivo* (Wakerley & Ingram 1993) and secondly, that noradrenaline release within the SON is increased in the hours prior to delivery (Herbison, personal communication).

In summary, while we have not fully elucidated the complex regulation of the initiation of parturition, our studies provide evidence for an involvement of oxytocin prior to as well as during delivery. The role of oxytocin prior to delivery might involve a gradual augmentation of uterine activity that will eventually feed back to magnocellular neurones (via the NTS) and initiate the burst-like reflex release of oxytocin, crucial for the normal progress of delivery in

rats and humans (see Fig. D). The importance of uterine contractions for the activation of magncocellular neurones at term is emphasised by the increased electrical activity of supraoptic neurones during oxytocin-induced labour and delivery. Thus, pulsatile oxytocin treatment offers the chance to investigate the regulation of neurosecretory activity in the SON at the end of pregnancy, since such treatment activates, most likely via the stimulation of uterine activity, putative catecholaminergic cells in the NTS and leads to an increase in TH immunoreactivity in these neurones. Furthermore, the observation that TH mRNA expression in the NTS is significantly higher before than during parturition, points to an involvement of catecholaminergic NTS neurones in the reflex release of oxytocin, possibly by priming magnocellular neurones for the burst-like release of oxytocin during normal parturition.


plasma
oestrogen/progesterone
?
uterus
prostaglandin synthesis
oxytocin receptor expression
oxytocin synthesis
?
cervical softening
uterine activity
brainstem
catecholaminergic NTS neurones
noradrenaline?
? ?
hypothalamus
magnocellular neurones
neural lobe
oxytocin (and vasopressin?)
delivery

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