Central vasopressin signalling and aggressive behaviour

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Declaration

This thesis and the work described within it are, except where specified, my own work. This work has not been submitted for any other degree or professional qualification.

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Acknowledgements

Abstract

Although many signalling molecules appear relevant to the production of complex behaviours, those that are important to the physiological regulation of behaviour, and so those that characterise individual styles of behaviour, are unknown. Vasopressin is the strongest candidate regulator of social behaviour. Experiments were carried out in consideration that vasopressin may directly regulate aggressive behaviour in lactating rats. Patterns of immediate early gene expression during/subsequent to aggressive behaviour suggested specific neural circuits may have significant direct regulatory influence over particular behaviours, and that activation of the V1b vasopressin receptor, in these circuits, may contribute to this putative regulatory signalling. *In situ* hybridisation studies indicated that patterns of vasopressin release, rather than receptor expression, might be important for any peripartum changes in behaviour driven by vasopressin. Although their relative importance is unknown, central actions of vasopressin may exert a strong regulatory influence over a range of behaviours, across a range of species.

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Abbreviations

3V	Third ventricle
5-HT	Serotonin
ABC	Avidin-biotin complex
AC	Anterior commissure
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
AH	Anterior hypothalamic area
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BDNF	Brain-derived neurotrophic factor
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
CaMKII	α-Ca^{2+}-calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CC	Central canal
CeA	Central amygdala
COMT	Catechol-O-methyltransferase
c.p.m.	Counts per minute
CRF	Corticotropin releasing factor
c.s.f.	Cerebrospinal fluid
cst	Commissural stria terminalis
CTP	Cytidine triphosphate
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DMH	Dorsomedial hypothalamus
dPAG	Dorsal periaqueductal gray
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetic acid
eGFP	Enhanced green fluorescent protein

ERα	Estrogen receptor-α
GABA	Gamma-amino-butyric acid
GD	Day of gestation
Gl	Main olfactory bulb, glomerular layer
GTP	Guanosine triphosphate
HPA	Hypothalamo-pituitay-adrenocortical
i.c.v.	Intracerebroventricular
IgG	Immunoglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
i.p.	Intraperitoneal
LAL	Long attack latency
LBNST	Bed nucleus of the stria terminalis, lateral division
LC	Locus coeruleus
LDCV	Large dense core vesicle
LHb	Lateral habenular nucleus
lPAG	Lateral/ventrolateral periaqueductal gray
LS	Lateral septum
LV	Lateral ventricle
MAO-A	Monoamine oxidase-A
MBNST	Bed nucleus of the stria terminalis, medial division
MeA	Medial amygdala
MiOB	Mitral cell layer of the main olfactory bulb
MPOA	Medial preoptic area
mPVN	Paraventricular nucleus, magnocellular division
Ni-DAB	Nickel-intensified diaminobenzidine
NOS	Nitric oxide synthase
OB	Olfactory bulb (main and accessory)
OC	Optic chiasm
OFC	Orbitofrontal cortex
OT	Optic tract
PAG	Periaqueductal gray

PB	Phosphate buffer
PBS	Phosphate buffered saline
PB-T	Phosphate buffer/0.2% Triton X-100
PCR	Polymerase chain reaction
PD	Postnatal day
pPVN	Paraventricular nucleus, parvocellular division
PVN	Paraventricular nucleus
RGS2	Regulator of G-protein signalling-2
r.p.m.	Revolutions per minute
SAL	Short attack latency
s.c.	Subcutaneous
SCN	Suprachiasmatic nucleus
SD	Standard deviation
s.e.m.	Standard error of the mean
SON	Supraoptic nucleus
SSC	Saline sodium citrate
st	Stria terminalis
STWS	Scott's tap water substitute
TGF-α	Transforming growth factor α
tRNA	Transfer ribonucleic acid
trpc2	Transient receptor potential cation channel (subfamily C, member 2)
UTP	Uridine triphosphate
UV	Ultraviolet
VMH	Ventromedial hypothalamus
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

General introduction

Aggressive behaviour

Aggressive behaviours are traditionally defined as those with the intention of harming others. They have time and energy costs, and carry a risk of injury, which limits their use, but they are adaptive if used acutely and successfully when conflicts of interest arise. Forms of aggressive behaviour have been variously classified according to species/context, although the most pronounced interspecific differences are between humans and other species. Aggressive behaviour in humans has been subject to heavy sociological constraints and intensely politicized. Thus most of the aggressive behaviours with putative parallels in other species are unfashionable (except in staged contexts), which has consequences for their expression and patterns of expression. Nevertheless, the classification by context common to the animal literatures is reasonably easily reconcilable with human patterns of behaviour. The different classes have different stimuli and are associated with different, but stereotyped and predictable, patterns of aggressive behaviour. In non-human mammals aggressive behaviour is commonly designated as intermale (territorial or otherwise competitive), maternal, non-maternal female territorial/competitive, fear-induced, shock-induced, pain-induced, isolation-induced, predatory, infanticidal or as a displacement activity. The common classification can be made as comparable patterns of behaviour vary with context reasonably predictably. The frequency of aggressive behaviours also vary similarly – within and between species – according to age, natural history stage and experience, in developmental changes that are easily related to supposed function.

In intraspecific aggressive behaviour the risk associated with the behaviour applies to all participants, and this presumably underlies the existence of additional ritualized interactions between individuals in conflict, which do not involve physical contact; so-called agonistic displays. These imply that there is a contrived interpretation – as well as display – of the behaviours expressed during conflicts. The common patterns of display, interpretation and development of behaviour – across numerous species – strongly suggest that aggressive behaviours have some conserved biological determinants.

The dependence of aggressive behaviour on both extrinsic factors (such as the environment, the size and hormonal status of the opponent) and intrinsic factors (such as size, life history stage and experience) are suggestive of reasonably complex neural control. Neuroscientific evidence describing how such a system might function – how many factors combine to produce behaviour, to favour aggressive over other behaviours or favour one aggressive behaviour over another – is minimal. As the neural substrates that contribute to regulation of ostensibly the same behaviour may be distributed across various systems and are not necessarily fixed across context/time there is some difficulty in determining the nature of the contributions made by the neurochemical systems that have been implicated. The converse problem also applies. The signalling molecules and brain regions that have been implicated in aggressive behaviour are listed below. The list includes factors linked to any of the described classes of aggressive behaviour (in any of a variety of rodent and primate species), but in most cases, the only model that has been tested is intermale aggressive behaviour in rodents – commonly the intermale aggressive

behaviour observed in a resident intruder test. A resident-intruder test usually involves a dyadic encounter wherein one individual (the intruder) is introduced into the home cage of another (the resident) for a short test period. In most cases, any offensive aggressive behaviour used by the resident is considered territorial aggressive behaviour. It is not clear how much consistency in regulation of behaviour should be expected across contexts. Some differences are presumed because the stimuli and contexts are specific to each and there are some differences in behavioural pattern and form. Some consistencies might be expected because there appear to be consistencies in behavioural pattern and form, and because signals of internal state would presumably influence all forms of behaviour.

Signalling molecules implicated in aggressive behaviour

Of the many molecules suggested to be involved in regulation of aggressive behaviour, some − acetylcholine (ACh), interleukin-1β (IL-1β) and transforming growth factor α (TGF-α); thus far linked to aggressive behaviour only via artificially potentiated activity − may be physiologically irrelevant (ACh: Pucilowski *et al*, 1991; TGF-α: Hilakivi-Clarke *et al*, 1992; IL-1β: Cirulli *et al*, 1998). Others (brain-derived neurotrophic factor (BDNF), α-Ca^{2+}-calmodulin-dependent kinase II (CaMKII), regulator of G-protein signalling-2 (RGS2), breakpoint cluster region (BCR), interleukin-6 (IL-6), catechol-O-methyltransferase (COMT), transient receptor potential cation channel (subfamily C, member 2; trpc2), substance P and adenosine) are implicated only by such potentially extra-physiological assessment and/or through global, unconditional knockout models (BDNF: Lyons *et al*, 1999; CaMKII: Chen *et al*, 1994; RGS2: Oliveira-Dos-Santos *et al*, 2000; BCR: Voncken

et al, 1998; IL-6: Alleva *et al*, 1998; COMT: Gogos *et al*, 1998; trpc2: Leypold *et al*, 2002; Stowers *et al*, 2002; Kimchi *et al*, 2007; substance P: De Felipe *et al*, 1998; adenosine: Ledent *et al*, 1997; Navarro *et al*, 2000; Giménez-Llort *et al*, 2002). As unconditional knockout models include the potential for developmental effects on additional systems (and in some cases this has been described) the substances are again only tenuously linked to the behaviour. It is of course very likely that some of the widely-used signalling molecules are involved, but the approaches used do not indicate that they are strong determinants of behaviour. Other molecules that have been more convincingly implicated include nitric oxide synthase (NOS), gamma-amino-butyric acid (GABA), glutamate, histamine, serotonin (5-HT), noradrenaline, dopamine, monoamine oxidase-A (MAO-A), opioids, oxytocin, and corticotrophin releasing factor (CRF). Modulation of behaviour by their acute antagonism suggests that they have at least a facilitatory effect in some forms of aggressive behaviour (NOS: Nelson *et al*, 1995; Demas *et al*, 1997; Demas *et al*, 1999a; Gammie and Nelson, 1999; Gammie *et al*, 2000; Chiavegatto *et al*, 2001; GABA: Stork *et al*, 2000; Uhlírová *et al*, 2004; Gourley *et al*, 2005; glutamate: Vekovischeva *et al*, 2004; Navarro *et al*, 2006; Vekovischeva *et al*, 2007; histamine: Nath *et al*, 1982; Yanai *et al*, 1998; 5-HT: see Olivier *et al*, 1995 for review; Sakaue *et al*, 2002; noradrenaline: see Haller *et al*, 1998 for review; Sallinen *et al*, 1998; Marino *et al*, 2005; dopamine: Vukhac *et al*, 2001; Rodríguez-Arias *et al*, 1999; MAO-A: Florvall *et al*, 1978; Datla and Bhattacharya, 1990; Brunner *et al*, 1993; Cases *et al*, 1995; opioids: Benton, 1985; Haney and Miczek, 1989; Teskey and Kavaliers, 1988; Konig *et al*, 1996; oxytocin: Ferris *et al*, 1992a; DeVries *et al*, 1997; Winslow *et al*, 2000;

Harmon *et al*, 2002; Lubin *et al*, 2003; Consiglio *et al*, 2005; Ragnauth *et al*, 2005; CRF: Tazi *et al*, 1987; Elkabir *et al*, 1990; Farrokhi *et al*, 2004).

Androgens have also been observed to facilitate aggressive behaviour, although they are not required in all contexts (see Davidson and Levine, 1972; Bouissou, 1983 for reviews; Demas *et al*, 1999b; Trainor *et al*, 2006). It is not clear that they have any direct regulatory influence, but they seem to have organizational effects with important regulatory consequences. The perinatal period seems to be particularly sensitive to such effects, but ongoing organization is possible (de Ruiter *et al*, 1993; Motelica-Heino *et al*, 1993; Oyegbile and Marler, 2005). Other steroid hormones may have similar organizational effects: glucocorticoids appear to be important in the timing of the changes in patterns of aggressive behaviour at puberty (Wommack *et al*, 2005).

Brain regions implicated in aggressive behaviour

Attempts to determine neural sites with regulatory influences over aggressive behaviour are associated with difficulties similar to those encountered in investigation of applicable neurochemical substances. Studies using electrical stimulation may be supra-physiological, lesion and pharmacological manipulations are generally not designed to allow demonstration of more than facilitatory effects and studies involving immediate early gene expression, as a measure of nuclear activation, tend to identify signalling that is difficult to link to behaviour, as opposed to, for example, processing of coincidental contextual signals or regulation of additional – even co-regulated – facets of the response to the context. Nevertheless,

there are nuclei quite convincingly (repeatedly) associated with aggressive behaviour: parts of the main and accessory olfactory bulbs (OB), lateral septum (LS), bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), anterior hypothalamic area (AH), medial amygdala (MeA), ventromedial hypothalamus (VMH), periaqueductal gray (PAG) and orbitofrontal cortex (OFC). Very generally, loss of activity in the OB, LS, BNST, MPOA, AH, MeA, VMH or PAG is associated with lower measures of aggressive behaviour, and lesion of the OFC with higher levels, although there are examples of non-conformation with these generalizations (see Lonstein and Gammie, 2002 and Nelson and Trainor, 2007 for reviews).

Although the studies referred to above suggest many factors may be required to allow aggressive behaviour to occur, they do not give any convincing indication that these factors are involved in the physiological regulation of aggressive behaviour. There are data from two human studies that suggest that some aspects of 5-HT signalling may be relatively important, as correlations could be made between indirect measures of 5-HT$_{1A}$ receptor availability/activity and individual scores of 'aggression' measured on the Brown-Goodwin Aggression History Scale, Spielberger State-Trait Anger Scale, Aggression Rating Scale and Buss-Durkee Hostility Inventory. However, the 'aggression' measured in these scales incorporates much more than behaviour, thus no link with behaviour *per se* is clear (Cleare and Bond, 2000; Parsey *et al*, 2002).

It might be presumed that all of the mentioned signalling molecules contribute to regulation of behaviour because it is expected that behaviour would select anything

that provides information regarding the internal environment to contribute to its regulation, and it is difficult to imagine that any particular signal would not contain any such information. Thus, a wide range of signals may influence a behaviour, and presumably in somewhat varied combinations, or at least with different relative degrees of influence, depending on context. As there may, then, be many, and changeable, regulators of behaviour – which may influence behaviour such that it is the output of a balance of activity across various sites and chemical systems, fully integrated only at the level of motor pattern generation - it may be that individual strong central regulators of aggressive behaviour do not exist and it could be very difficult to assign a regulatory role to a particular signalling molecule or a particular anatomical location. The prediction of levels of behaviour using a neurological measure might require the computation of a complex composite score.

There are, however, three recent studies of two systems not yet mentioned, which suggest that strong regulators of behaviour may exist and may be identifiable. These studies also allow for the possibility – in contrast to the situation described above, although that situation remains possible – that there is some circuitry in the brain dedicated to behaviour.

The first study demonstrated that individual measures of aggressive behaviour displayed by male mice in a resident-intruder test were positively correlated with their individual numbers of oestrogen receptor-α (ERα) immuno-positive cells in the LS, BNST and AH (Trainor *et al*, 2006). Although these positive correlations were found, such relationships may be context-dependent as there are context (age, sex

and experience)-dependent differences in the effects of ERα/oestrogen receptor-β knockout on aggressive behaviour (although these differences may only arise as a result of developmental changes consequent to gene knockout; Ogawa *et al*, 1997; Ogawa *et al*, 1998; Ogawa *et al*, 1999; Nomura *et al*, 2002; Scordalakes and Rissman, 2003; Nomura *et al*, 2006). Also, because the only evidence that oestrogen is required for normal levels of some aggressive behaviour comes from knockout studies and exogenous hormone application (which also has inconsistent effect: Mayer *et al*, 1990 cf. Svare and Gandelman, 1975; van de Poll, 1986), it remains possible the correlations between receptor expression and behaviour are artefactual, that the receptor expression is coincidental, even co-regulated, but causally linked to another output.

The second and third studies together provide a more convincing indication that strong, identifiable regulators of behaviour may exist. The levels of vasopressin V1a receptor binding in particular brain regions of male prairie voles were found to be correlated with individual scores of four behaviours (paternal behaviour, partner preference, interactions with juvenile voles and aggressive behaviour in the resident intruder test; Hammock *et al*, 2005), and LS vasopressin content (by radioimmunoassay) and vasopressin-immunoreactivity of male rats were correlated with individual latencies to attack a conspecific intruder in a resident-intruder test (Everts *et al*, 1997). A relatively large body of data – detailed below – indicates that vasopressin is important for expression of these behaviours. With this background, it is considered that the correlations are indicative of association with behaviour rather than coincidental signalling. The number of behaviours found to be associated with

the vasopressin system, in the receptor study, also suggests that the correlations are associated with behaviour rather than any of the other functions of the peptide: correlation between these other outputs and four behaviours is less likely than their correlation with one. Being correlated with so many different behaviours, the vasopressin system appears a surprisingly powerful (and the most powerful identified) putative regulator of behaviour.

Vasopressin

Arginine vasopressin is 9 amino acids arranged thus: Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH$_2$, with a disulphide bond linking the two cysteine residues. It is found in most mammals. Mammals in which arginine vasopressin does not occur (some marsupials), contain other vasopressins (lysine vasopressin and phenylalanine vasopressin; Chauvert *et al*, 1980), and members of the family Suidae appear to express both arginine vasopressin (in the corpus luteum; Pitzel *et al*, 1984; Choy and Watkins, 1988) and lysine vasopressin (in the central nervous system; Popenoe *et al*, 1952). Single amino acid substitutions distinguish arginine vasopressin from these other vasopressins, and from vasotocin, which occurs in all non-mammalian vertebrates. As vasotocin occurs in all non-mammalian vertebrates – including Agnatha species (Suzuki *et al*, 1995) – it is considered to have existed for around 500 million years. It is the presumed evolutionary precursor of all vertebrate vasopressins and is a possible precursor of the vertebrate isotocin/oxytocin peptide lineage (Acher *et al*, 1995). Vasopressin/oxytocin-like peptides, and associated receptors, have also been identified in various invertebrate species (Van Kesteren *et al*, 1995; Van Kesteren *et al*, 1996; Fujino *et al*, 1999).

Arginine vasopressin (vasopressin), or anti-diuretic hormone, is named in part for its arginine at position 8, which distinguishes it from the otherwise similar (in structure and function) lysine vasopressin common to pigs and some marsupials, and partly for two of its functions; its pressor and anti-diuretic effects. It acts on arterioles, increasing vascular resistance (Oliver and Schafer, 1895), and in the collecting ducts of the kidney to promote water reabsorption (Farini, 1913; Vongraven, 1913; cited in e.g. Zimmerman and Robinson, 1976). It has a third major peripheral function in the anterior pituitary where it contributes to stimulation of adrenocorticotropic hormone (ACTH) secretion from corticotrophs as part of the hypothalamo-pituitary-adrenocortical (HPA) axis (De Wied et al, 1961; Doepfner et al, 1963).

Each of these three effects relies on signalling via one of the three identified vasopressin receptors. All three receptors are membrane bound G-protein coupled receptors with seven transmembrane domains, an extracellular N-terminal peptide-binding domain and an intracellular C-terminal. The V2 receptor – mediator of vasopressin's anti-diuretic effects – is coupled to the G_s protein subtype and cyclic adenosine monophosphate (cAMP) signalling (see Jard, 1983 for review; Orloff and Handler, 1967; Chase and Aurbach, 1968). The V_{1a} and V_{1b} receptors – respectively mediators of vasopressin signalling in the vasculature and anterior pituitary – both signal through $G_{q/11}$/inositol triphosphate pathways (see Jard et al, 1987 for review; Michell et al, 1979).

The vasopressin active in the periphery is derived from the hypothalamus. The vasopressin utilised in the HPA axis is produced, at least in most part, in the

parvocellular division of the paraventricular nucleus (pPVN). The neurones involved project to the median eminence, where they release vasopressin into the portal circulation that travels to the anterior pituitary (Vandesande *et al*, 1977; Carlson *et al*, 1982). Vasopressin released by magnocellular neurones of the paraventricular nucleus (PVN) and supraoptic nucleus (SON), into the systemic (and perhaps occasionally portal) circulation, may also contribute to the levels of vasopressin in the portal system (Oliver *et al*, 1977 cf. Recht *et al*, 1981). Release of vasopressin into the portal circulation from pPVN neurones is regulated by signals from the circadian rhythm generator, circulating levels of cortisol and many – potentially all – stressors (Makara 1992; Watts *et al*, 2004). In rats and primates, the actions of vasopressin in the anterior pituitary generally supplement and potentiate those of CRF, which is the principal secretagogue of ACTH, is produced in the same nucleus (probably the same cells) as the implicated vasopressin, and is responsive to many, if not all, of the same stimuli (see Owens and Nemeroff, 1991 for review; Vale *et al*, 1981; Makara, 1992). The production of vasopressin in the pPVN and pituitary sensitivity to vasopressin are elevated during chronic exposure to some stressors, following adrenalectomy and in lactation (Whitnall *et al*, 1987; de Goeij *et al*, 1991; de Goeij *et al*, 1992; Bartanusz *et al*, 1993; Aguilera *et al*, 1994; Chowdrey *et al*, 1995; Rabadan-Diehl *et al*, 1997; Rabadan-Diehl and Aguilera, 1998; Walker *et al*, 2001). At these times the expression and effects of pPVN vasopressin are also elevated relative to the expression and effects of pPVN CRF – which can be lower relative to the control situation – and vasopressin assumes *a*, sometimes *the*, greater role in maintaining the activity within the HPA axis.

The vasopressin released into the systemic circulation to act at the kidneys and on blood vessels is produced in the magnocellular neurones of the SON and PVN. Each of these neurones has one axon which projects to the neurohypophysis (Armstrong, 1995) where it terminates at the perivascular basement membrane (e.g. Nagasawa *et al*, 1970). The neurones release vasopressin in response to osmotic stimuli, contributing to regulation of blood volume and blood pressure (see Share, 1988 for review; Bankir, 2001; Koshimizu *et al*, 2006). The circulating vasopressin may also act at the liver, heart, adrenal, skeletal muscle, lung, small intestine, pancreas, mesentery, bladder, reproductive organs, spleen, breast, white adipose tissue and various types of blood cell, which each express vasopressin receptors (of relatively uncharacterised effect; Lolait *et al*, 1995; Saito *et al*, 1995; Fay *et al*, 1996; Thibonnier *et al*, 1996; Fujiwara *et al*, 2007), may promote water absorption via actions in the colon (Bridges *et* al, 1984; Cristia *et al*, 2007) and may act at circumventricular organs as a feedback signal (Jurzak and Schmid, 1998). Centrally acting vasopressin is otherwise derived from within the blood brain barrier.

Seven populations of vasopressin neurones have been characterized in the rat brain and six are understood to have central effects. The one population without known central effect is that of the pPVN which produces the vasopressin for use in the HPA axis and is probably distinct from an additional pPVN vasopressin population that projects centrally to various brainstem and spinal cord centres involved in regulation of the autonomic nervous system (Swanson *et* al, 1980; Sawchenko and Swanson, 1982; Kc *et al*, 2002). The remaining five populations include one in each of the

suprachiasmatic nucleus (SCN), MeA and BNST, and the magnocellular populations of the SON and PVN already mentioned.

Central signalling by SON/mPVN vasopressin

That there are central effects of vasopressin derived from the magnocellular division of the paraventricular nucleus (mPVN) is inferred from the information known about the functionally similar SON. The central effects of magnocellular vasopressin are interesting as the axonal projections of the magnocellular neurones terminate in the pituitary, around 4mm distant from the cell bodies (Norströ and Sjöstrand, 1971), and evidence for central collaterals is sparse (Silverman *et al*, 1981; Mason *et al*, 1984; Alonso *et al*, 1986). All of the characterized central activity of magnocellular vasopressin involves somatodendritically released peptide. The dendrites of the SON vasopressin neurones form a dense plexus on the ventral surface of the nucleus. They contain large numbers of large dense core vesicles (LDCVs) filled with vasopressin and vasopressin receptors, such that upon release the vasopressin has autoregulatory and paracrine effects. This is probably important for physiological regulation of the electrical activity of these neurones and, in turn, neurohypophyseal vasopressin release (see Ludwig and Pittman, 2003 for review). It is difficult to demonstrate that the dendritically released vasopressin has additional physiological functions, but the local concentrations of peptide found in the SON following magnocellular stimulation can be high, and sufficiently high to allow for the possibility that the peptide not only acts in the SON, but also travels and acts at additional sites. The structure of neuropeptide systems is supportive of such putative hormonal activity (see Ludwig and Leng, 2006 for review). Unlike conventional

neurotransmitters, preferentially targeted to synapses, peptides are contained in LDCVs localised to, and released from, all parts of the plasma membrane (Morris and Pow, 1991). They do not appear to have the reuptake mechanisms of the small neurotransmitters and so are presumed to rely on a slower enzymatic degradation to quench their activity, and their receptors have relatively high ligand binding affinities, facilitating signalling at lower concentrations, which may equate to signalling over greater distances (e.g. the Kd for vasopressin at vasopressin receptors is in the high pM/very low nM range (e.g. Swank and Dorsa, 1991; Ventura *et al*, 1999; Hawtin *et al*, 2005) and for glutamate at glutamate receptors is in the nM/µM range (see Briley *et al*, 1981 for review)). The frequently observed inconsistencies between sites, or abundance, of peptide receptor expression and peptidergic fibre input, suggest these features of peptide systems have a functional relevance (Herkenham, 1987). In such a system the expression of vasopressin receptors, rather than presence of vasopressin fibres, would be more indicative of a site susceptible to vasopressinergic regulation. There is evidence for central expression of the V2 receptor only in the PAG (Yang *et al*, 2006; Yang *et al*, 2007), but the central distribution of the V1a and V1b receptors is extensive (V1a: Ostrowski *et al*, 1994; Szot *et al*, 1994; Hurbin *et al*, 1998; Pietranera *et al*, 2004; V1b: Lolait *et al*, 1995; Vaccari *et al*, 1998; Hernando *et al*, 2001). With peptide systems constructed in this way it is difficult to imagine that there would not be at least some overspill from one vasopressin system into another, but the relative importance of hormonal activity could be far greater.

Data from studies of dendritically released magnocellular oxytocin demonstrate that there is differential regulation of peptide release from magnocellular axons versus

soma/dendrites: dendritic peptide release (from isolated SON) occurs in response to application of melanocortin 4 receptor agonists, which, following intracerebroventricular (i.c.v.) administration, inhibits the electrical activity of the cells *in vivo*, decreasing neurohypophyseal peptide secretion (Sabatier *et al*, 2003). Additional data demonstrate that some stimuli induce intranuclear (intra-SON) peptide release and neurohypophyseal peptide release with different latencies (>1 hour apart) and over different time scales (Moos *et al*, 1989; Ludwig *et al*, 1994); potentially some environments lead to intranuclear release in the absence of associated neurohypophyseal release (Wotjak *et al*, 1998). Although the intranuclear peptide release (very probably dendritic peptide release) temporally dissociated from axonal release may have undetected latent autoregulatory effects and no distant activity, it is also possible that its main effects are exerted at distant sites. It is interesting that the dendritic release that seems to occur without synchronous axonal release occurs at times when peptide activity in the brain may be important for behavioural responses to the particular situation. Potentially, the SON is a source of the peptide signalling implicated in these behaviours.

Central signalling by SCN vasopressin

In the SCN there are circadian fluctuations in vasopressin mRNA expression, which is translated into circadian fluctuations in vasopressin release, and is believed to give rise to the circadian rhythmicity of vasopressin concentrations in the cerebrospinal fluid (c.s.f.) (Reppert *et al*, 1981; Uhl and Reppert, 1986; Burbach *et* al, 1988). The projection sites of the vasopressin neurones of the SCN are centred largely in the medial hypothalamus (in the dorsomedial hypothalamus (DMH), the

subparaventricular zone and ventral PVN), but they may also include the ventral LS, BNST, MPOA, organum vasculosum of the lamina terminalis, parts of the thalamus and the PAG (Hoorneman and Buijs, 1982; Watts *et* al, 1987; Watts and Swanson, 1987; Leak and Moore, 2001; Abrahamson and Moore, 2001). Little is known about the functions of the vasopressin produced by the SCN. It is understood to contribute to the circadian pattern of HPA axis activity; probably via indirect (interneurone–mediated) modulation of activity in pPVN HPA axis neurones following release in the DMH/PVN (Kalsbeek *et al*, 1992; Buijs *et al*, 1993; Vrang *et al*, 1995; Hermes *et al*, 2000). This input to the pPVN may also impact on the pPVN output to autonomic centres in the spinal cord, which influence adrenal sensitivity to ACTH (Buijs *et al*, 1999; Ishida *et al*, 2005; Ulrich-Lai *et al*, 2006). Indirect evidence suggests the rhythmic patterns of gonadotropin-releasing hormone and luteinising hormone secretion are influenced by SCN vasopressin (De La Iglesia *et al*, 1995; Van Der Beek *et al*, 1997; Mahoney and Smale, 2005; Miller *et al*, 2006), and correlative studies indicate that it may be relevant to the daily rhythm in general activity/locomotor behaviour (Gerkema *et al*, 1994; Delville *et al*, 1998; Jansen *et al*, 1999; Jansen *et al*, 2000; Hut *et al*, 2002; Van der Veen *et al*, 2005).

Central signalling by BNST and MeA vasopressin

The parvocellular vasopressin neurone populations in the BNST and MeA are sexually dimorphic (generally more pronounced in males than females) and seem to be similarly controlled by gonadal steroid hormones (De Vries *et al*, 1984; De Vries *et al*, 1985; Miller *et al*, 1989a; Miller *et al*, 1989b; Miller *et al*, 1992; Wang and DeVries, 1995). Correlative data suggest they may be important for some forms of

social behaviour, but their functions are otherwise unknown. Although the evidence linking them with social behaviour is only correlative (Bester-Meredith *et al*, 1999; Bester-Meredith and Marler, 2001), one study has demonstrated co-variation between not only BNST vasopressin content and behaviour, but also between behaviour and vasopressin-immunoreactivity in the LS (Compaan *et al*, 1993), a known efferent target of both the BNST and MeA vasopressin populations and a site where vasopressin contributes to regulation of social behaviour (De Vries and Buijs, 1983; Caffe *et al*, 1987; Lui *et al*, 2001). Other projection sites of the BNST and/or MeA vasopressin populations include the PAG, dorsal raphe nucleus, locus coeruleus (LC) and hippocampus (De Vries and Buijs, 1983; De Vries *et al*, 1985; Caffe *et al*, 1987), which have also been implicated in social behaviour (Pucilowski *et al*, 1986; Hennessey *et al*, 1992; Kask *et al*, 2000; de Oca and Fanselow, 2004; Machado and Bachevalier, 2006; Bannai *et al*, 2007; Carvalho-Netto *et al*, 2007; Cooper and Huhman, 2007). However the actions of vasopressin in these projection sites have been found to produce effects which may be responsible for indirect effects on behaviour: vasopressin has osmoregulatory effects in the LS (Abrão Saad *et al*, 2004), antinociceptive effects in the PAG (Yang *et al*, 2007) and may contribute to regulation of blood pressure, heart rate, posture and vestibulospinal reflexes in the LC (Berecek *et al*, 1984; Berecek, 1986; Andre *et al*, 1992). Thus it is not known that these are sites where these populations affect behaviour directly. It is not known that these populations directly affect behaviour at all. It is not really known that vasopressin has any direct effect on behaviour. The data that seem to indicate this – when combined with the mentioned finding that V1a receptor binding in particular

brain regions is correlated with individual levels of behaviour in prairie voles – are detailed below.

Vasopressin and social behaviour

Central actions of vasopressin appear to be important for behavioural responses in various tests of anxiety and depression, various forms of aggressive behaviour and the behaviours that characterize social bonding between sexual partners and between parents and their infants (see Caldwell *et al*, 2008 for review). Of these behaviours, those that constitute long-term pair-bonding have been most convincingly suggested to be regulated by central actions of vasopressin. Such stable pair bonding involves long-term cohabitation with, and a preference for, one partner, direction of reproductive behaviours towards this partner alone, aggressive responses to unfamiliar conspecifics and biparental care. These are characteristic features of monogamous species. Their association with vasopressin is largely based on a series of studies in vole species.

The initial association was based on interspecific differences in the structure of the vasopressin system: vasopressin receptor binding in many brain regions, other than the LS, was found to be higher in the monogamous prairie vole than the non-monogamous montane vole and in the LS vasopressin receptor binding was higher in the montane vole. LS vasopressin receptor binding was also higher in the non-monogamous meadow vole compared to the monogamous pine vole (Insel *et al*, 1994). Intraspecific differences associated with reproductive stage have also been found. In prairie voles, vasopressin-immunoreactivity in the LS and lateral

habenular nucleus (LHb) has been observed to be higher in males that have spent 3 days in cohabitation with a female, and males at postpartum day 6, than in sexually naïve controls (Bamshad *et al*, 1993; Bamshad *et al*, 1994), and PVN/SON vasopressin mRNA is higher in postpartum males and females than in sexually naive controls (Wang *et al*, 2000). In montane voles the PVN/SON vasopressin mRNA and LS vasopressin-immunoreactivity were the same in all groups (Bamshad *et* al, 1993; Wang *et al*, 2000). A complementary set of studies suggest these inter- and intra-specific differences impact on behaviour. I.c.v. administration of vasopressin or a V1a receptor antagonist prior to mating of prairie voles leads to higher or lower scores, respectively, in a post-mating partner preference test (Winslow *et al*, 1993), whilst artificially enhancing V1a receptor expression in the ventral pallidum of meadow voles leads to higher levels of partner preference than observed in controls (Lim *et al*, 2004). Similarly, intra-septal administration of vasopressin induces paternal behaviour in sexually naïve prairie voles, V1a receptor antagonists have the opposite effect (and inhibit the effect of exogenous vasopressin), and paternal behaviour can be produced in non-paternal meadow voles by i.c.v. injection of vasopressin (Wang *et al*, 1994; Parker and Lee, 2001).

Some correlative data from experiments in mice and marmosets suggest that vasopressin is relevant to pair-bonding in species other than voles. The monogamous California mouse has higher vasopressin-immunoreactivity in the BNST and V1a receptor binding in the LS than the polygamous white-footed mouse (Bester-Meredith *et al*, 1999) and the male marmoset (a paternal male) has a higher density

of pre-frontal cortical V1a receptors when paternal than when non-paternal (Kozorovitskiy *et al*, 2006).

Although less than 5% of mammalian species are monogamous, a much higher proportion pair bond over the short-term and/or use components of pair-bonding in different contexts. The use of maternal behaviours and aggressive displays aimed towards conspecifics is particularly widespread. Data suggest that vasopressin is required for some forms of aggressive behaviour in non-monogamous as well as monogamous species and it is thus a good candidate regulator of these forms of aggressive behaviour. Association with the intermale aggressive behaviour observed in resident intruder tests has been most intensively investigated.

Vasopressinergic signalling and aggressive behaviour

Activational effects of vasopressin on intermale aggressive behaviour expressed during a resident-intruder test have been observed in Syrian hamsters, California mice and prairie voles. In the socially inexperienced prairie vole, i.c.v. administration of vasopressin heightens scores of aggressive behaviour (Winslow *et al*, 1993) and prairie voles with high levels of post-mating aggressive behaviour show higher levels of activation (inferred from immediate early gene expression) of their anterior hypothalamic vasopressin population during an aggressive encounter than those with lower aggression measures (Gobrogge *et al*, 2007). In the Syrian hamster, microinjection of vasopressin into the anterior hypothalamus has a facilitatory effect on aggressive behaviour and produces a dose-dependent facilitation of flank marking (Ferris *et al*, 1984; Ferris *et al*, 1997). Central (i.c.v.) injection of a

V1a receptor antagonist inhibits aggressive behaviour in prairie voles and California mice (Winslow *et al*, 1993; Bester-Meredith *et al*, 2005), and in the hamster, targeted injection of a V1a receptor antagonist to the AH prevents the facilitation of aggressive behaviour and flank-marking by local vasopressin administration (Ferris *et al*, 1988; Ferris and Potegal, 1988). A knockout mouse model and an antagonist study in hamsters suggest the V1b receptor may also be important (Wersinger *et al*, 2002; Blanchard *et al*, 2005), and both the density of vasopressin-immunoreactivity in specific central locations of mice, rats and hamsters, and the abundance of V1a receptor expression in particular brain regions of the hamster, have been correlated with intraspecific variation in aggressive behaviour (Compaan *et al*, 1993; Everts *et al*, 1997; Cooper *et al*, 2005; Albers *et al*, 2006; Frazier *et al*, 2006; Grimes *et al*, 2006; Veenema *et al*, 2006). These receptor studies, together with the correlations between individual measures of receptor expression and particular aspects of pair bonding found in prairie voles, suggest receptor expression may be an important aspect of vasopressinergic regulation of behaviour.

Some instances of male aggressive behaviour that do not appear to strongly depend on vasopressin include the aggressive displays of the California mouse in a neutral arena aggression test, which are unaffected by i.c.v. administration of a V1a receptor antagonist (Bester-Meredith *et al*, 2005), and predatory aggression in mice, which is unaffected by knockout of the V1b receptor, although it produces deficits in aggressive behaviour in the resident-intruder paradigm (Wersinger *et al*, 2002). Also, the aggressive behaviour displayed by male Syrian hamsters in the resident-intruder

test becomes independent of vasopressin under 'winter-like' photoperiods (during short days, the non-breeding season; Caldwell and Albers, 2004).

Compared to aggressive behaviour in males, aggressive behaviour in females has been little studied. Studies of aggressive behaviour in females are commonly studies of maternal aggression, the aggressive behaviour expressed during the peripartum period. In rodents, it is directed against conspecific intruders of the nest site and is presumed to protect the young offspring from the potentially infanticidal behaviour of these conspecifics. Measures of maternal aggression are lower in V1b receptor knockout mice than in wildtype controls (Wersinger *et al*, 2007) and scores of maternal aggression are altered by i.c.v. administration of a V1a receptor antagonist (Nephew and Bridges, 2007). In view of the supposed function of maternal aggression it is interesting that vasopressin may be implicated in its production as vasopressin's involvement in behaviour seems to be important when the behaviour is ostensibly directly linked to reproductive success.

To further evaluate the contributions of vasopressin to maternal aggression a model of maternal aggression was developed in the lactating rat.

Experimental model

Female rats isolate themselves from conspecifics during pregnancy and display aggressive behaviours towards intruders of their nest site from day 16 of gestation (GD16) until weaning (in the laboratory until around postnatal day 21 (PD21)). The levels of aggressive behaviour displayed peak at around PD2/3, remain high to the

end of the first lactational week and thereafter steadily decline (Flannelly and Flannelly, 1987; Mayer *et al*, 1987). To evoke this intruder-induced aggressive behaviour, a resident-intruder test was used and the tests were conducted between PD4 and PD6.

Various attributes of an intruder, including its age, sex, size and hormonal status, can alter the pattern of aggressive behaviour displayed by a resident peripartum rat. Weight-matched, cycling female intruders were used in all behavioural experiments. Although they can be infanticidal (Fleming *et al*, 1980; Sheehan *et al*, 2000), such behaviour is much less prevalent than in males, allowing the pups to remain with the dam during the test (potentially allowing a more accurate maternal response) and providing a lower risk of physical injury to the maternal resident.

The behaviours defined as aggressive were classified as such after observing numerous examples of maternal aggression. They are, therefore, personally defined, but they correspond well with those identified in other studies. The behaviours measured and descriptions of their characteristics are presented in Table 1.1. The measures used in analysis of aggressive behaviour were composite measures of the duration of all so-designated aggressive behaviours and the number of attacks. Again these are standard measures. Latency to attack is another common measure of aggressive behaviour. It is reported in the results to conform to convention, but was not used in analysis as observation suggested it could easily vary with, for example, where the nest site was in relation to the intruder's entry site and the nature of the dam's activity at the time of intruder entry.

This model was used to investigate the following:

1. that central actions of vasopressin are relevant to maternal aggression, and

2. the brain regions and receptor subtypes involved in vasopressinergic regulation of maternal aggression.

Additional investigations were carried out to consider:

1. that the peripartum period may be accompanied by a change in the magnitude and/or distribution of expression of central vasopressin receptors, and

2. that the magnocellular neurones of the PVN and SON have axonal connections to brain regions relevant to behaviour.

Table 1.1 Behaviour analysis

Descriptions of the behaviours recorded and their classification as aggressive, maternal or other

Behaviour	Description/notes	Classification
Attack	Fighting in a closed ball; frequently occurred following sniffing, lateral threat and chasing behaviours; episodes often interspersed with clawing/punching and pinning down	Aggressive
Biting tail	Attempt to pull conspecific by tail	Aggressive
Bite	Bite on any part of body	Aggressive
Clawing/ punching	Clawing/punching with forelimbs (usually aimed at head/chest following rearing) or pushing/kicking with hindlimbs (usually associated with pinning down)	Aggressive
Chasing	Pursuit which generally ends in flight of conspecific, sniffing or attack	Aggressive
Lateral threat	Threatening advance from the side, often resolved without additional aggressive behaviour, sometimes preceded attack	Aggressive
Pinning down	Standing over supine conspecific, preventing its movement, usually occurred following attack	Aggressive
Sniffing/clawing	Intense sniffing accompanied by clawing at fur in the same area and occasionally biting, usually around the neck region	Aggressive
Nesting	Moving bedding, usually around pups, sometimes covering them	Maternal
Nursing		Maternal
Pup related	Pup investigation/sniffing, licking/grooming, retrieval; often appeared disorganised	Maternal
Drinking		Other
Eating		Other
Environmental exploration	Any exploratory behaviour (generally sniffing) not aimed directly at conspecific	Other
Escape	Jump made to exit fighting ball, or flight from conspecific	Other
Exposed underside	Exposure of underside to conspecific following conspecific's aggressive behaviour; possibly submissive	Other
Freezing	Freezing after attack	Other
Grooming self	Occurred with high frequency following initiation of encounters with conspecific	Other
Rearing	Often accompanied by clawing/punching	Other
Sniffing	Sniffing of conspecific	Other
Other	Any other behaviour, usually inactivity	Other

Notes on materials and methods

1. Animals were used under the conditions of personal and project licences granted by the UK Home Office. All experiments were performed according to the regulations and guidelines issued by the Home Office and the University of Edinburgh Biological Services

2. Statistical tests were performed by SigmaStat (Version 3.10 (2004); Systat Software, Inc.)

3. Suppliers of commercially sourced materials are indicated in the text (details of each supplier are provided in Appendix 11)

4. Details, including constituents, of the following solutions are noted in Appendix 10: acid alcohol, Buffer P1, Buffer P2, Buffer P3, Buffer QBT, Buffer QC, Buffer QF, cryoprotectant, 0.1 % diethylpyrocarbonate-treated water (DEPC-H_2O), dithiothreitol (DTT), gelatin subbing solution, 4 % paraformaldehyde/1× phosphate buffer (PB), 4 % paraformaldehyde/ phosphate buffered saline (PBS), PB, PB/0.2 % Triton X-100 (PB-T), PBS, saline sodium citrate (SSC), Scott's tap water substitute (STWS), potassium alum, TE buffer, triethanolamine/acetic anhydride, TY culture medium

Chapter 2

Vasopressinergic regulation of maternal aggression

Introduction

Two studies implicate vasopressin in the regulation of maternal aggression. Rats treated with a V1a receptor antagonist (i.c.v.) show a higher number and higher duration of attacks than saline-treated controls (Nephew and Bridges, 2008) and global, unconditional knockout of the V1b receptor gene (*v1br*) is associated with lower levels of attack behaviour in mice (Wersinger *et al*, 2007).

The V1b receptor has also been linked to regulation of intermale aggression. *v1br* knockout mice typically show less aggressive behaviour than wildtype littermates in resident-intruder/neutral arena tests (Wersinger *et al*, 2002; Wersinger *et al*, 2007) and male Syrian hamsters exhibit lower levels of some components of aggressive behaviour ('offensive sideways' and 'chase' behaviours) when pre-treated with an orally administered V1b receptor antagonist (Blanchard *et al*, 2005). It is not actually clear in either of these experiments, or in the study of knockout females, that the V1b receptor is directly involved in modulation of aggressive behaviour: knockout of the V1b receptor appears to affect development of the testosterone system (Wersinger *et al*, 2002) and the V1b receptor antagonist may be effective at oxytocin receptors (Serradeil-Le Gal *et al*, 2002 cf. Griffante *et al*, 2005; Hodgson *et al*, 2007). If the V1b receptor is directly involved in the behaviour the nature of its involvement is unclear. Study of the male *v1br* knockout mice suggests their behavioural phenotype does not result from deficits in the activity of pituitary corticotrophs, as both basal and intruder exposure-associated levels of corticosterone are similar to those of wild-type mice. Conspecific exposure-induced activation of

potentially relevant brain regions (the OB, BNST, preoptic area, PVN, MeA and PAG) in naturally low-aggressive individuals is also equivalent between genotypes (Wersinger *et al*, 2002). The V1b receptor is expressed in various additional peripheral organs and brain regions (Lolait *et al*, 1995; Vaccari *et al*, 1998; Hernando *et al*, 2001), and its actions at many of these sites may be important.

The disruption of maternal aggression by central (i.c.v.) administration of a V1a receptor antagonist more convincingly links vasopressin with the behaviour and suggests central actions of vasopressin are relevant. It is unclear if the central V1a receptor antagonism influences behaviour by direct effects, via effects on peripheral actions of vasopressin (e.g. via disruption of the autoregulation of vasopressin cells of the mPVN and SON that secrete vasopressin from the neurohypophysis), by otherwise modulating activity of peripheral systems, or through a combination of these effects.

Studies of intermale aggression support the idea that central actions of vasopressin at the V1a receptor are linked to aggressive behaviour and have identified candidate regions for involvement. Mentioned in the introduction, the level of V1a receptor binding in the LS has been found to correlate with mating system (polygamous or monogamous), which predicts levels of aggressive behaviour directed against conspecifics, in the males of four vole species (Insel *et al*, 1994). Mating systems in two mouse species, the monogamous California mouse and polygamous white-footed mouse, are also predictive of levels of both aggressive behaviour in resident-intruder/neutral aggression tests and V1a receptor binding in the LS (Bester-

Meredith *et al*, 1999). Experiments in mice bred for long- and short- attack latency (LAL and SAL mice) again implicate LS vasopressin: following exposure to a conspecific, which SAL mice attack with shorter latency than LAL mice, the density of vasopressin-immunoreactivity in the LS and BNST is higher in the LAL group (Compaan *et al*, 1993). Similarly, in rats, both the vasopressin content of the LS (by radioimmunoassay) and density of vasopressin-immunoreactivity in lateral septal fibres measured following a resident-intruder test have been positively correlated with individual differences in latency to attack a conspecific intruder (Everts *et al*, 1997). Additional regions where vasopressin may regulate aggressive behaviour include the mitral cell and internal plexiform layers of the main olfactory bulb, where measures of receptor binding were found to correlate with individual scores of aggressive behaviour in the prairie vole (Hammock *et al*, 2005); the AH, PVN, LH and central amygdala (CeA), where levels of V1a receptor binding are higher in Syrian hamsters that have been subject to relative social isolation, and have higher levels of aggressive behaviour, than more socially experienced, less aggressive, controls (Albers *et al*, 2006); and the lateral VMH, where levels of V1a receptor binding are higher in Syrian hamsters that have repeatedly won as residents in the resident-intruder test, compared to those that have been repeatedly submissive and non-tested controls (Cooper *et al*, 2005).

To test that central actions of vasopressin may be involved in regulation of maternal aggression, the activity of central vasopressin-responsive neurones associated with expression of maternal aggression was considered. The OB, LS, PVN, CeA and VMH have been mentioned as regions where vasopressin may act to contribute to

production of aggressive behaviour, and as such were appropriate for investigation. The BNST and MeA, which provide at least some of the vasopressinergic input to the LS, were also examined. Studies of immediate early gene expression commonly implicate these nuclei (or at least some of their sub-divisions) in various forms of aggressive behaviour: maternal aggression in mice (Gammie and Nelson, 2001; Gammie *et al*, 2004; Hasen and Gammie, 2005; Hasen and Gammie, 2006), aggressive behaviour in cycling female California mice (Davis and Marler, 2004), intermale aggressive behaviour in Syrian hamsters (Kollack-Walker and Newman, 1995; Delville *et al*, 2000), intermale aggressive behaviour in rats (Veening *et al*, 2005) and intermale aggressive behaviour in LAL/SAL mice (Haller *et al*, 2006). The PAG is also frequently implicated by these studies and so was also investigated. Finally, the level of activation of vasopressin receptor-expressing neurones in the SON and mPVN was studied as the V1a- and V1b-receptors in the SON and mPVN appear to colocalise with expression of vasopressin rather than oxytocin (Berlove and Piekut, 1990; Hurbin *et al*, 1998; Hurbin *et al*, 2002) and it was thus considered that the activation of these neurones would indicate the possibility of activation of vasopressin secretion from these cells (whether into the brain or periphery). To test for aggressive behaviour-associated activation of the other major source of centrally-derived, peripherally-active vasopressin - the vasopressin population of the pPVN that projects to the median eminence as part of the HPA axis – an additional experiment was required. Thus, two experiments were carried out:

Experiment 1

Tissue from lactating rats that had been subject to a resident-intruder test was stained for Fos and CRF. CRF is a 41 amino acid peptide widely synthesized in the rat brain, with major populations in the pPVN, BNST and CeA (Merchenthaler *et al*, 1982; Swanson *et al*, 1983). Acting centrally and peripherally, it is the iconic peptide of stress and anxiety: the primary secretagogue at corticotrophs (Vale *et al*, 1981) and a major regulator of behavioural, autonomic and immune responses to stressors (see Owens and Nemeroff, 1991 for review). The CRF neurones of the pPVN are the only CRF population integral to the HPA axis. Their CRF provides, together with vasopressin, the stimulus for secretion of ACTH from pituitary corticotrophs, following release at the median eminence. However, only a sub-population of pPVN neurones project to the median eminence. Additional, distinct neurone populations appear to have only central projections (Swanson and Kuypers, 1980; Swanson *et al*, 1980). Vasopressin is expressed in neurones that project centrally (Hallbeck and Blomqvist, 1999) and neurones that project to the median eminence (e.g. de Goeij *et al*, 1991), which are likely to be different neurone populations (Swanson *et al*, 1980) and to be differentially regulated. By contrast, there is only minimal evidence that the pPVN CRF population contains a centrally-projecting sub-population (Rodaros *et al*, 2007).

In (at least) one vasopressin population, vasopressin is colocalised with CRF. It is presumed that this vasopressin is involved in the HPA axis because potentially all CRF cells project to the median eminence and because the enhanced production of vasopressin, and its enhanced colocalisation with CRF in fibres at the median

eminence, which is induced by chronic stress/adrenalectomy/lactation, occurs as a result of enhanced vasopressin expression in 'CRF' cells (Whitnall *et al*, 1987; de Goeij *et al*, 1991; Walker *et al*, 2001). As there is a relatively high likelihood of there being multiple vasopressin-containing neuronal phenotypes in the pPVN (under differential regulation), and a lower likelihood of there being multiple CRF pPVN neurone populations, CRF, rather than vasopressin, was used as a neural marker here to allow the vasopressin neurones of the pPVN that project to the median eminence to be considered in relative isolation. The activation of CRF neurones was inferred from cellular colocalisation of CRF and Fos protein expression, as described below. This experiment also considered Fos/CRF expression in the BNST and CeA.

Experiment 2

Tissue from lactating rats that had been subject to a resident-intruder test was stained for Fos and either the V1a- or V1b-receptor. It has previously been established that each of the regions to be investigated (the mitral cell layer of the main olfactory bulb (MiOB), LS, BNST, pPVN, mPVN, SON, CeA, MeA, VMH and PAG) expresses at least one of the V1 receptor subtypes. The V1a receptor is expressed in the LS, BNST, CeA, pPVN, mPVN, VMH and PAG (Ostrowski *et al*, 1994; Szot *et al*, 1994; Hurbin *et al*, 1998; Pietranera *et al*, 2004), and was found by *in situ* hybridisation to be expressed in the MeA (see Chapter 3). The V1b receptor is expressed in the MiOB, septum, PVN and SON (Lolait *et al*, 1995; Vaccari *et al*, 1998; Hernando *et al*, 2001).

Fos, as a product of an immediate early gene, is a marker of neural activation. In this context 'neural activation' is generally synonymous with electrophysiological activity. This is probably because voltage-activated Ca^{2+} entry is a feature of neuronal excitation and Ca^{2+} (via regulation of adenylate cyclases and calmodulin) can stimulate the calcium/cAMP response elements of the *c-fos* gene, promoting *c-fos* gene transcription. However not all stimuli that increase intracellular Ca^{2+} concentrations and Fos expression produce neuronal excitation (Sabatier *et al*, 2003), and neuronal excitation is not always associated with *c-fos* induction (Luckman *et al*, 1994; Sgambato *et al*, 1997). Fos expression is not, therefore, necessarily indicative of electrical activity and it is not the case that a lack of Fos expression indicates a lack of electrical activity in a particular brain region. Neither is it the case that a lack of change in activity (including a lack of change in Fos expression) induced by a new context would deny neuronal signalling in a particular region a role in responding to the context. The existing activity may play a permissive role or there may be functional changes in circuitry at a resolution higher than investigated. As the stimuli that induce Fos expression are variable its expression does not identify the nature of cellular input or output. Thus although in the experiments here it may be found to be colocalised with CRF/vasopressin receptor, this can only lead to speculation of their involvement in cell activity.

Fos, rather than another immediate early gene product, was chosen for use, as previous studies of maternal aggression in mice have shown it to be (of those tested) the most comprehensive indicator of changes in regional activity (Gammie and Nelson, 2001; Hasen and Gammie, 2006).

Materials and methods

Experiment 1

Animals

Female rats (280-340 g; Hsd:Sprague Dawley®™SD®™ strain, Harlan UK Ltd.) were acclimatised for one week before pairing with a male. During acclimatisation, and from impregnation to GD18, they were group housed. They were individually housed from GD18 until the end of the experiment. Polycarbonate cages were used and food and water were freely available. During the week of acclimatisation the rats were fed a standard Teklad Global 14 % Protein Rodent Maintenance Diet (diet 2014, Harlan UK Ltd.). Thereafter this was supplemented with a high protein (19 %), high fat (9 %) diet (Teklad Global 19 % Protein Extruded Rodent Diet; diet 2019, Harlan UK Ltd.). The rooms used for housing were maintained on a 12:12 light:dark cycle (lights on at 07:00 h) with an ambient temperature of 20 ± 2 ˚C and relative humidity of 50 ± 10 %. At least 24 h before the start of behavioural testing the rats were moved to the experimental room. At the same time the rats to be used in the behaviour test were transferred (with pups) to polycarbonate cages ($480 \times 265 \times 210$ mm, floor area 940 cm^2), with stainless steel flat lids and external feed hoppers (Techniplast UK Limited). Control rats were maintained in cages of similar size ($425 \times 266 \times 200$ mm; with feed hoppers integrated into the lids). The day of parturition was PD1. All behavioural tests were carried out between PD4 and PD6.

Behaviour test

Tests were carried out between 08.30 and 12.30 h. A size matched virgin rat was placed in the cage of each test (lactating) rat at time 0. The interaction was carefully, remotely monitored and recorded as a black and white digital file for later analysis. The virgin rat was removed at +30 min and at least 24 h would lapse before it was used in another test. The tested rats were kept in the experimental room until + 90 min, when they were removed for tissue collection. Tissue was collected at this time in accordance with previous studies suggesting peak levels of Fos protein expression occur 1-2 h following stimulus presentation (e.g. Verbalis *et al*, 1991; Miyata *et al*, 1995; Matsunaga *et al*, 2000; Spencer and Houpt, 2001). The control rats experienced similar cage movement and disturbances at the same times as their corresponding experimental subjects, but no conspecifics were introduced into their cages.

Tissue collection

At +90 min the test and corresponding control rats were killed by sodium pentobarbitone overdose (1.5 ml intraperitoneal (i.p); 54.7 g/l) and transcardially perfused with heparinsed (129 mg/l, 151 units/mg) saline (0.9 % weight/volume (w/v)) followed by 4 % paraformaldehyde/1× PB (methods detailed in Appendix 1). The brains were removed and post-fixed in 4 % paraformaldehyde/15 % sucrose/1× PB, at 4 ˚C (24 h), cryoprotected in 30 % sucrose/1× PB (4 ºC, 24-48 h), snap frozen on dry ice and stored at -70 ˚C.

Tissue preparation

52 μm coronal sections of the collected brains were cut using a freezing microtome (Leica; -20 to -22 ˚C). Sections were collected into 1× PB and transferred into cryoprotectant for storage at -20 ˚C.

Immunocytochemistry

The prepared tissue sections were brought to room temperature for immunocytochemical processing through the stages detailed in Appendix 2. Briefly, sections were incubated (48 h) in a rabbit anti-Fos immunoglobulin G (IgG) solution (polyclonal antibody raised against amino acids 4-17 of human c-Fos; Calbiochem, UK) and the resulting binding was developed via an indirect, avidin-biotin complex (ABC) amplified, biotin-horseradish peroxide conjugate method, using nickel-intensified diaminobenzidine (Ni-DAB) as the chromogen. The second primary antibody solution was anti-CRF rabbit antiserum (polyclonal antibody raised against human/rat CRF; Peninsula Labs, Inc., USA) diluted 1:15000 in 3 % normal sheep serum/1× PB-T. After a 72-h exposure binding was visualised as above, but with diaminobenzidine (DAB) alone as the chromogen. With omission of this second primary antibody, no additional staining developed during the second visualisation reaction. Omission of both primary antibodies resulted in absence of specific staining.

Analysis of immunocytochemistry

The slides were coded such that the identity of each sample was unknown during analysis. Total numbers of Fos-positive, CRF-positive and Fos/CRF doubled-

labelled cells were counted in the lateral division of the BNST (LBNST), pPVN and CeA under a 20×/0.5 or 40×/0.7 objective lens. Classification of positive cells was subjective during counting. *Post-hoc* analysis suggested positive cells had a minimum density of staining > 40 × mean staining + (3 × standard deviation (SD)) of unlabelled cells (Fos) and > 20 × mean staining + 3SD of unlabelled cells (CRF). Unlabelled cells were those within regions adjacent to those investigated wherein staining was comparable to that obtained with omission of the primary antibody. For each nucleus, at least 8 sections were analysed per animal. Two-way analysis of variance (ANOVA), followed by pairwise Holm-Sidak tests, was used to compare measures of staining between test and control groups. In the comparisons of Fos labelling and CRF labelling, $p < 0.05$ was considered to reflect a difference. Strictly, the Fos/CRF double-labelling data demanded non-parametric analysis. Two-way ANOVA was therefore an inappropriate test, but nevertheless carried out as there is no non-parametric equivalent, hence results should be treated with caution. However, the reported 'significant' p-values were 0.001 and 0.015, low enough to give reasonable confidence in the significance of the differences. Unpaired t-tests were additionally used to analyse the double-labelling data.

Analysis of behaviour

For each tested maternal rat, the duration of each occurrence of each behaviour listed in Table 1.1, and its time of its onset in the 30-min test, was recorded using The Observer software (Noldus IT Ltd., The Netherlands). The sum of the durations of all aggressive behaviours (as defined in Table 1.1) was used as the 'aggressive behaviour score' for each rat.

Experiment 2

Animals

Female rats bred in the Biological Research Facility, University of Edinburgh as part of the transgenic (vasopressin-enhanced green fluorescent protein (eGFP)) colony described in Appendix 6 were used. Animals were 17-20 weeks old, 250-350 g at the time of experiment.

The housing of animals, behaviour test and methods of tissue collection were as described for Experiment 1.

Tissue preparation

52 μm coronal sections of the collected brains were cut using a freezing microtome (Leica; -20 to -22 ºC). Alternate sections were collected separately, in 1× PB, for either V1a- or V1b-receptor labelling. Sections were transferred to cryoprotectant for storage at -20 ºC.

Immunocytochemistry

Immunocytochemical processing for Fos and vasopressin receptor was as detailed in Appendix 2. Briefly, sections were first stained for Fos protein as in Experiment 1: tissue was incubated for 48 hours in a rabbit anti-c-Fos antibody solution (polyclonal antibody raised against amino acids 4-17 of human c-Fos; Calbiochem, UK) and immunoreactivity visualised using an ABC-peroxidase method with Ni-DAB as chromogen. The tissue was then exposed for 72 h to either:

1. V1a receptor antibody (rabbit polyclonal antibody raised against a 19 amino acid N-terminal (extracellular) fragment of the rat V1a receptor; Alpha Diagnostic International, San Antonio, USA) diluted 1:250 in 3 % normal sheep serum/1× PB-T; or

2. V1b receptor antibody (rabbit polyclonal antibody raised against an 18 amino acid N-terminal (extracellular) fragment of the rat V1b receptor; Alpha Diagnostic International, San Antonio, USA) diluted 1:750 in 3 % normal sheep serum/1× PB-T.

For the V1a receptor labelled tissue, Vector-VIP (Vector Labs., UK) was used as the chromogen. V1b receptor labelling was detected with DAB. Specificity of each antibody for its V1 receptor subtype has been previously described (Hurbin *et al*, 2002; Folny *et al*, 2003). No specific staining was apparent following the second developing reaction when the second primary antibody was omitted, nor when the second primary antibody solution was pre-incubated with the specific antigen (Alpha Diagnostic International, San Antonio, USA). Omission of both primary antibodies resulted in the absence of any specific staining.

Analysis of immunocytochemistry

The slides were coded such that the identity of each sample was unknown during analysis. In both sets of tissue total numbers of Fos-positive cells and double-labelled cells (cells co-labelled for either Fos/V1a receptor or Fos/V1b receptor) were counted in the MiOB (half of each mitral cell layer, along dorsal-ventral axis, counted per section), LS, BNST, SON, pPVN, mPVN, MeA, CeA, VMH, dorsal division of the periaqueductal gray (dPAG) and lateral/ventrolateral division of the

periaqueductal gray (lPAG), under a 20×/0.5 or 40×/0.7 objective lens. Definition of positive cells was subjective during counting, but *post-hoc* sampling suggested positive cells had a minimum intensity of staining > 12× mean staining + 3SD (Fos), > 10× mean staining + 3SD (V1a receptor) or > 8× mean staining + 3SD (V1b receptor) of unlabelled cells. Unlabelled cells were those in a region wherein staining was comparable to that obtained with omission of the primary antibody. For each nucleus, at least 6 sections were analysed per animal. Two-way ANOVA, followed by pairwise Holm-Sidak comparisons, was used to compare measures of staining between groups. In all cases, assumptions of normality and homoscedasticity were violated. Nevertheless, in all but one 'significant' outcome, p ≤ 0.008. Given the nature of the data (see Results), this seems sufficiently low to allow for the possibility that the likelihoods of differences between the groups are real. The comparison of Fos/V1b receptor double labelling in the SON yielded a p-value of 0.041.

Analysis of behaviour

Behaviour was analysed as in Experiment 1. For each rat subject to the behaviour test, the duration of each occurrence of each behaviour listed in Table 1.1 and the time of its onset in the 30 minute test was recorded using The Observer software (Noldus IT Ltd., The Netherlands). The sum duration of all aggressive behaviours (as defined in Table 1.1) was used as the 'aggressive behaviour score' for each rat.

Results

Behaviour

Outwith the 30 min of the test, the behaviour of the dams of the tested group was observed, but not recorded nor quantified. In both experiments they were observed, before testing, to be almost wholly maternal (as defined in Table 1.1), generally nursing. There were occasional, brief periods of self-grooming and environmental exploration. Environmental exploration tended to coincide with a local disturbance. The rats reverted to this pattern of behaviour shortly after the end of the test (within 1-2 min) and it was generally observed in animals of the control group throughout. Each rat, tested and control, showed some acknowledgement of the cage disruption just prior to the test, but the disturbance was short-lived.

Tested behaviour

A summary of the behaviour of the tested groups from both experiments is displayed in Table 2.1. Some results are also described below.

Excepting one rat in Experiment 2, all lactating dams exposed to an intruder displayed aggressive behaviours directed towards this conspecific. In Experiment 1 the latency to attack was 54 ± 13 s (mean $\pm$ standard error of the mean (s.e.m.)), with a range of 21 to 123 s. In Experiment 2 the latency was 116 ± 25 s (mean $\pm$ s.e.m.), with a range of 26 to 250 s (excluding the one rat that failed to show aggressive behaviour). From this point until the end of the test the lactating rats alternated between aggressive and other (generally exploratory and maternal) behaviours in an

oscillatory pattern, a change occurring every few minutes. The nature and perceived intensity of the aggressive behaviour was variable. The number of attacks in Experiment 1 ranged from 7 to 44 per test (mean ± s.e.m.: 22 ± 5). In Experiment 2 this ranged from 6 to 33 per test (mean ± s.e.m.: 17 ± 3; again excluding the rat that showed no aggressive behaviour). In both experiments sniffing (of the intruder) occupied the longest duration for any single behaviour (mean ± s.e.m.: Experiment 1, 450 ± 79 s; Experiment 2, 452 ± 44 s). Self-grooming and environmental exploration were also consistently dominant behaviours (mean ± s.e.m.: self grooming: Experiment 1, 268 ± 62 s; Experiment 2, 173 ± 31 s; environmental exploration: Experiment 1, 218 ± 25 s; Experiment 2, 379 ± 38 s). These behaviours occurred in what were considered 'aggressive' and 'non-aggressive' periods. Whilst exploratory and maternal behaviours tended to characterise the 'non-aggressive' periods, they appeared disorganised and maladaptive.

Aggressive behaviour scores

In Experiment 1, aggressive behaviour scores (the total duration of all behaviours classed as aggressive) ranged from 197 to 430 s per 30-min test (mean ± s.e.m.: 332 ± 34 s). This measure was dominated by pinning down (mean ± s.e.m.: 166 ± 24 s). The time spent engaged in attack (mean ± s.e.m.: 45 ± 12 s), lateral threat (mean ± s.e.m.: 42 ± 16 s), clawing/punching (mean ± s.e.m.: 30 ± 9 s) and sniffing/clawing (mean ± s.e.m.: 26 ± 18 s) was moderate. Biting (mean ± s.e.m.: 6 ± 4 s), biting tail (mean ± s.e.m.: 8 ± 6 s) and chasing (mean ± s.e.m.: 2 ± 1 s) were each of very short duration.

In Experiment 2, aggressive behaviour scores ranged from 0-500 s (mean ± s.e.m.: 175 ± 51 s). The behavioural profile underlying this was reasonably different from that in Experiment 1. The largest component was clawing/punching (mean ± s.e.m.: 58 ± 26 s), followed by attack (mean ± s.e.m.: 43 ± 12 s), lateral threat (mean ± s.e.m.: 38 ± 14 s), and pinning down (mean ± s.e.m.: 24 ± 7 s). Biting (mean ± s.e.m.: 7 ± 3 s) and chasing (mean ± s.e.m.: 2 ± 1 s) again occupied very little time.

As a response to an indication in the raw data, Spearman correlation and linear regression analysis were used to test for a negative association between the time engaged in maternal, versus aggressive, behaviour. A significant correlation was found (p < 0.001; Spearman's r = -0.795; Figure 2.1). This is not considered to be an artefactual consequence of aggressive behaviours limiting the time available for maternal behaviours as the average duration of maternal + aggressive behaviours was only 419 ± 30 s (mean ± s.e.m.) out of the 1800 s available per test. An average of 352 ± 42 s (mean ± s.e.m.) was spent in pursuits classified as 'other' and these were generally characterised by inactivity. To check for specificity in the correlation between aggressive and maternal behaviours, Spearman correlation and linear regression analysis were used to test for an association between the duration of aggressive behaviours and the duration of grooming behaviour. Grooming was, of the various dominant behaviours, the least ostensibly connected to aggressive or maternal behaviours. No correlation between grooming and aggressive behaviours was found (p = 0.450; Figure 2.1).

Immunocytochemistry

Experiment 1

Staining

Intense immunocytochemical staining for CRF was localised to very few discrete, compact nuclei. Cell bodies were easily and clearly discerned. Somatic staining within the pPVN was generally of lower density than that in the LBNST and CeA. In keeping, the fibres leading towards the median eminence displayed the highest observed concentrations of label. Staining of fibres was otherwise generally of lower density and more punctate than that of cell bodies, but it was also clear and commonly multiple stained fibres could be associated with a single cell. Some fibres could be seen to extend over reasonably long distances.

The staining threshold for a Fos- or CRF-labelled (counted) cell was subjectively defined at the time of analysis, with effort to maintain consistency across all sections. *Post-hoc* analysis (sampling of at least 6 cells/region/rat) suggested the minimum density of CRF-linked staining in a positive cell to be at least 20× greater than the mean background staining + 3SD (background staining = the staining of cells in an adjacent region comparable to that observed with omission of the CRF antibody). The staining density of an accepted Fos-positive cell was similarly estimated, by sampling, to be at least 40× greater than the mean background staining + 3SD.

Measurements

In the test rats, there was significant activation of Fos protein expression in the LBNST, pPVN and CeA ($p < 0.05$ versus controls; Figure 2.2). The difference between means was most significant and largest in the pPVN (mean ± s.e.m.: 29.8 ± 5.1 versus 13.2 ± 2.1; $p = 0.000$), followed by the LBNST (36.0 ± 2.2 versus 23.9 ± 3.4; $p = 0.008$), and CeA (24.1 ± 2.2 versus 15.2 ± 2.3; $p = 0.048$). In all three regions, the numbers of CRF-immunoreactive neurones were similar between groups (mean ± s.e.m.: LBNST, 39.7 ± 1.4 (tested group) and 37.9 ± 1.5 (control group); pPVN, 51.1 ± 1.9 (tested group) and 48.9 ± 1.2 (control group); CeA, 38.5 ± 2.4 (tested group) and 37.6 ± 2.8 (control group); Figure 2.2). Although there was no area effect ($p = 0.068$) and no interaction ($p = 0.543$) in the Fos/CRF double-labelling data, pairwise *post hoc* tests were performed. These suggested no significant difference between the pPVN of test and control rats ($p = 0.069$). In the LBNST and CeA there appeared to be significant activation of CRF neurones in the test animals ($p = 0.001$ (BNST) and $p = 0.015$ (CeA); Figure 2.2). These latter reported likelihoods of differences are inaccurate because the data (which failed tests of normality) did not meet the requirements of the two-way ANOVA or *post hoc* tests, but the data are fitting with the possible differences (mean ± s.e.m.: BNST, 6.5 ± 1.4 (tested group) versus 2.3 ± 0.6 (control group); CeA, 4.1 ± 0.7 (tested group) versus 1.0 ± 0.2 (control group)). In the pPVN the lack of difference is associated with a high estimated error in the tested group rather than a lack of difference between group means (mean ± s.e.m.: 3.8 ± 1.2 (tested group) versus 1.5 ± 0.3 (control group)). Analysis of the double-labelling data by t-tests produced similar

results (uncorrected p-values = 0.001 (CeA), 0.018 (BNST) and 0.081 (pPVN)). The

Bonferroni-corrected p-value for the BNST is 0.054.

Experiment 2

Staining

Immunocytochemical staining for both the V1a and V1b receptors was weaker than for CRF (Figure 2.3). Cell bodies were readily identifiable. Fibres were generally only faintly stained and more difficult to define clearly on a single focal plane. Intensity of V1b receptor staining was consistently greater than for the V1a receptor and the punctate pattern of staining more dramatic. The distribution of stained cells was widespread: throughout large extents of the hypothalamus, limbic regions and brainstem, the hippocampus and layer V of the neocortex.

The distribution of labelling for the V1a receptor was very similar to that for the V1b receptor. Although this is unlikely to be a result of cross-reactivity between the receptors (Hurbin *et al*, 2002; Folny *et al*, 2003) the extent of cross reactivity with other proteins is unknown and this may be the reason for such widespread labelling and the apparent similarities between the distributions of the two receptors. It is not clear whether the observed distribution of the V1a receptor is consistent with the distribution of labelling found using *in situ* hybridisation (see Part 3). Although very similar, the distribution of staining for mRNA was not universally matched to that for protein. Moreover, in the areas in which mRNA expression was quantified (by cell counts and density measurements; see Part 3) the number of cells in each region expressing mRNA does not correlate with the number of cells in each region expressing the protein. The numbers of cells expressing the protein appear to positively correlate with the size of the region, but this is not the case for the number

of cells expressing the mRNA, the regional densities of mRNA expression, or composite measures of mRNA cell counts and density measurements (see results in Part 3). The disparity between protein and mRNA expression in some regions is to the extent that there is no mRNA expression despite protein expression. However, in some of these regions (e.g. the SON and mPVN) functional evidence attests to presence of the V1a receptor. Thus it is possible variance in regulation of mRNA across brain regions may account for the mismatch between particular regional extents of labelling for protein and mRNA.

As for the analysis of labelling in Experiment 1, identification of labelled cells was subjective during analysis, with care taken to maintain consistency across all tissue. Subsequent sampling (at least 6 cells/region/animal) demonstrated the density of labelling in a 'positive' (counted) cell to be at least 10 times (V1a receptor), 8 times (V1b receptor) or 12 times (Fos) greater than mean background staining + 3SD. Background measurements were made in areas without receptor labelling (where staining levels were as in control tissue not exposed to the particular primary antibody).

Measurements

Data sets consistently failed tests of normality and inappropriate tests (two-way ANOVA) were used in analysis. In most comparisons the differences supported by the analysis were sufficiently strong to allow for the possibility that they could be real.

Fos labelling

In both the V1a receptor- and V1b receptor-labelled tissue, test rats showed significant activation of Fos expression in the MiOB, LS, BNST, pPVN, MeA, VMH, dPAG and lPAG ($p \leq 0.008$ versus controls; Figure 2.4). No significant differences in expression levels were found in the SON, mPVN or CeA (Figure 2.4). The concordance between the data from the V1a receptor- and V1b receptor-labelled tissue is strong, both in areas where differences were found and areas where they were not (see summary in Figure 2.4 and values in Table 2.2). The data are characterised by large differences where they exist and low standard deviation. The error associated with the means is low even where differences are not found, indicative of the similarities in group mean values.

Fos/V1a receptor labelling

The regions wherein Fos/V1a receptor double-labelling was higher in tested than control rats were similar to those in which the test was associated with higher Fos expression generally. The numbers of double-labelled cells were higher in the MiOB, LS, pPVN, MeA, VMH, dPAG and lPAG of the test, compared to control, rats ($p \leq 0.002$; Figure 2.5; Table 2.2), with no differences observed in the BNST, SON, mPVN or CeA (Figure 2.5; Table 2.2). Again the differences, where apparent, were large. The lack of difference in the BNST reported by the analysis does not fit well with the data for this area (mean ± s.e.m. for tested group: 3.5 ± 0.5; and for control group: 1.3 ± 0.3). This may be a result of the relatively low error values associated with these means as compared to those in other regions and thus an overestimation of

the error in this region. Using an unpaired t-test to test for differences between groups in this area yields a p-value of 0.001.

Fos/V1b receptor labelling

Analysis of the Fos/V1b receptor double-labelling data again leads to differences being reported in the areas in which Fos expression was found to be activated, in this case including the BNST (p ≤ 0.002; Figure 2.6; Table 2.2). Additionally, a 'significant' difference (p = 0.041) was found in the SON. The raw data suggests this difference, although the result of an invalid method of comparison, to be fair. A univariate comparison of groups by t-test provides a p-value of 0.014. However, there is a suggestion the V1b receptor-expressing cells in the SON match, almost exactly, those that express the V1a receptor (Hurbin *et al*, 1998). The lack of activation of V1a receptor-labelled SON cells is therefore surprising.

Vasopressin receptor labelling

The regions wherein measures of cell activation are higher in tested rats are the same regardless of whether Fos expression, V1a receptor/Fos double-labelling or V1b receptor/Fos double-labelling is considered. Given the widespread receptor distribution it is possible that the double-labelling data are simply a reflection of general Fos activation. Using (again invalid) two-way ANOVA, followed by pairwise Holm-Sidak comparisons, to test for differences, between groups, in the proportion of Fos-positive nuclei co-localised with the V1a receptor or V1b receptor labelling suggests this may be the case (Figure 2.7; Table 2.3). The only 'significant' difference was found in the mPVN of V1b receptor labelled tissue (p < 0.0001),

where the higher measure occurred in the control group and the difference was probably overestimated by the test. Even use of (inappropriate) t-tests/Mann Whitney rank sum tests to test for differences in each region produced 'significant' outcomes in only 4 regions: the pPVN (p = 0.012, t-test) and MeA (p = 0.039, t-test) in V1a receptor stained tissue, and mPVN (p = 0.011, t-test) and pPVN (p = 0.018, Mann Whitney rank sum test) in V1b receptor stained tissue. In two of these regions (those in the V1b receptor stained tissue) higher measures occurred in the control group.

Analysis of neural activation and behaviour

Fos expression and aggressive behaviour

The BNST, pPVN and CeA were analysed for Fos expression in both experiments and the two analyses gave rise to similar cell counts for each region. The mean values for each animal were plotted against their aggressive behaviour (sum duration) by linear regression. This involved a re-analysis of the BNST in tissue from Experiment 1 to include both the medial and lateral subregions such that the data were consistent with those obtained in Experiment 2 (see Table 2.4). Data from the V1b receptor- rather than V1a receptor-labelled tissue were used as this group had a larger sample size. Spearman correlation and regression analyses were used to determine the nature and strength of any associations.

Significant positive correlations (BNST: Spearman's r = 0.657; pPVN: Spearman's r = 0.550; CeA: Spearman's r = 0.688) were found between the behaviour and Fos expression in all three areas (Figure 2.8). The strongest result occurred in the CeA (p = 0.002), followed by the BNST (p = 0.012) and then pPVN (p = 0.031). To test whether these results were reflective of a specific or more generalised neural activation the data from additional brain regions were similarly analysed. The regions analysed were those investigated in Experiment 2 present in the tissue stained for Experiment 1. These regions were the MiOB, LS, SON, MeA and VMH (see data in Table 2.4). Analyses were made using the Fos counts compiled from the two experiments, as for the BNST, pPVN and CeA. No additional significant

relationships were found (Figure 2.9). Significant correlations were restricted to the BNST, pPVN and CeA.

To test that a relationship between the levels of Fos expression in these three areas may have been associated with their common link to the measure of aggressive behaviour, Pearson correlation and linear regression analyses were used to test for associations between the levels of Fos expression in the different regions. None was found (pPVN versus BNST: $p = 0.534$; BNST versus CeA: $p = 0.169$; CeA versus pPVN: $p = 0.809$; Figure 2.10). The levels of Fos expression in each of the three areas thus appears to be characterised by independent regulation and so in each case the possibility that the region has an independent link with the behaviour is (at least) as likely as the possibility of an indirect or artefactual link supported by activity in another of the regions. In keeping, the link between Fos expression in these regions and aggressive behaviour is strongest when a combined Fos count from all three regions is regressed against the behaviour ($p < 0.001$; Spearman's $r = 0.762$; Figure 2.11), rather than the Fos expressed in any one region.

The aggressive behaviour understood to correlate with Fos expression in the BNST, pPVN and CeA included attack, biting, clawing/punching, chasing, lateral threat, pinning down, and sniffing/clawing. Each of these components was separately tested (Spearman correlation and linear regression analysis) for correlation with Fos expression in the CeA, the area found to be the most likely to correlate with aggressive behaviour. Pinning down was the only measure to produce a more reliable association with Fos expression then the total aggressive behaviour

(Spearman's r = 0.669; p = 0.001; Figure 2.12). In fact, it was the only component to produce a significant outcome. Pinning down was an important element of the aggressive behaviour score. Taking both experiments into account, it occupied, on average, 84 s of the average 241 s aggressive behaviour score. This was almost twice the duration of the next highest scoring behaviour. Pinning down was therefore tested (Spearman correlation and linear regression analysis) for association with Fos expression in the BNST or pPVN, but no correlations were found (BNST: p = 0.062; pPVN p = 0.391). Links between the additional aspects of the aggressive behaviour and Fos expression in the pPVN and BNST were therefore sought. The durations of both attack and lateral threat were found to correlate with Fos expression in the pPVN (attack: p = 0.003, Spearman's r = 0.561; lateral threat: p = 0.023, Spearman's r = 0.507; Figure 2.12). The combined duration of both behaviours was also strongly correlated with the pPVN Fos measures (p = 0.003, r = 0.569; Figure 2.13). Fos expression in the BNST was not significantly related to any of the measured components of aggressive behaviour by linear regression.

Measures of double-labelling and aggressive behaviour

To determine if the neural populations linked with aggressive behaviour could be characterised by any of the labelled proteins (CRF, V1a receptor or V1b receptor), the measures of double-labelling in the BNST, pPVN and CeA were tested for correlation with the aggressive behaviour scores. Fos/CRF double-labelling in the BNST was negatively correlated with the duration of aggressive behaviour (Spearman's r = -0.738; p = 0.041; Figure 2.14). In the BNST and CeA, Fos/V1b receptor double-labelling was positively correlated with the aggressive behaviour

scores (BNST: Spearman's r = 0.418; p = 0.032; CeA: Spearman's r = 0.717; p = 0.001; Figure 2.15). As these correlations between Fos/V1b receptor labelling and aggressive behaviour were found, the measures of Fos/V1b receptor double-labelling in the CeA and pPVN were tested for correlation with the duration of pinning down and attack/lateral threat, respectively. The Fos/V1b receptor double-labelling in the CeA was correlated with the duration of pinning down (Spearman's r = 0.524; p = 0.025; Figure 2.16).

Fos/V1a receptor and Fos/V1b receptor double-labelling in the SON were also tested for correlation with the aggressive behaviour scores. These tests were carried out in consideration that the vasopressin derived from these populations may be an important source of the putative regulatory vasopressin. No correlations were found (V1a: p = 0.292; V1b: p = 0.959; Figure 2.17).

Fos expression and maternal behaviour

As the duration of maternal behaviour was negatively correlated with aggressive behaviour scores, Spearman correlation and linear regression analyses were performed to consider that the neural populations associated with aggressive behaviour may also be associated with maternal behaviour. Firstly, the Fos expression in the BNST, pPVN and CeA was tested for correlation with the sum duration of all maternal behaviours. Fos expression in the BNST was negatively correlated with maternal behaviour (Spearman's r = -0.532; p = 0.017; Figure 2.18). The linear regression analysis using CeA Fos data yielded a p-value of 0.051 (Spearman's r = -0.363; Figure 2.19). No correlation was found between maternal

behaviour and Fos expression in the pPVN (p = 0.140; Figure 2.19). The remaining analysed brain regions were then tested for correlation with maternal behaviour. None was so linked (Figure 2.19).

Each measured component of maternal behaviour (nursing, pup-related behaviour and nesting behaviour) was separately tested (Spearman correlation and linear regression analysis) for correlation with Fos expression in the BNST and Fos expression in the CeA. Nesting behaviour and Fos expression in the BNST were the only variables to significantly correlate (Spearman's r = -0.509; p = 0.044; Figure 2.20).

Measures of double-labelling and maternal behaviour

Finally, the levels of Fos double-labelling with the V1a receptor, V1b receptor or CRF, in the BNST, pPVN and CeA, were tested for correlation with the sum duration of all maternal behaviours. Fos/V1b receptor double-labelling in the CeA produced the only significant outcome (Spearman's r = -0.684; p = 0.045; Figure 2.21). Each of the components of maternal behaviour (nesting, nursing and pup-related) was tested for correlation with Fos/V1b receptor double-labelling in the BNST and CeA. No significant correlations were found.

Discussion

In Experiment 1, the BNST, pPVN and CeA were activated in connection with the resident-intruder test. The test was also associated with activation of the CRF populations in the BNST and CeA, but Fos labelling in pPVN CRF neurones was not different between the tested and control groups.

In Experiment 2, the MiOB, LS, BNST, pPVN, MeA, VMH and PAG were activated in association with the test, whereas there were no differences in the numbers of Fos-positive cells in the CeA, mPVN and SON between tested and control groups. The test-induced changes in Fos expression in vasopressin-responsive neurones had the same distribution as the changes in general Fos expression, except that Fos/V1b receptor double-labelling in the SON was higher in the tested, compared to control, group. This latter result – and the apparent activation of CeA CRF neurones during the test in Experiment 1 – suggests that although there was no difference in the numbers of Fos-positive nuclei in the CeA and SON between the tested and control conditions in Experiment 2, this may not be reflective of a lack of difference in activity.

The results suggest there was limited – if any – test-associated activation of the vasopressin systems that release vasopressin into the periphery. It appears that the vasopressin (and CRF) population integral to the HPA axis was not significantly activated during the resident-intruder test and there was only a possible - certainly not clear - activation of SON magnocellular vasopressin neurones. It is unclear as

the activation appeared to be restricted to within the V1b receptor-expressing population and it is not obvious what the V1b receptor population in the SON represents. The V1b receptor is reported to colocalise with the V1a receptor in the vasopressin cells of the SON (Hurbin *et al*, 1998), but no significant activation of the V1a receptor population (= V1b receptor population) was observed. It may be that a small elevation of Fos expression in SON vasopressin neurones has occurred during the test. These results are surprising. The lack of activation of the pPVN CRF population is surprising as a stress (ACTH, corticosterone) response to (male) intruder-exposure has been observed in lactating rats (Deschamps *et al*, 2003). Potentially, the inclusion of CRF neurones that project centrally (rather than to the median eminence), in the population studied, has obscured an activation within the HPA axis neurones alone. It may have been useful to additionally label for vasopressin such that the CRF/vasopressin double-labelled population could have been studied in isolation from any additional, potentially differentially regulated, CRF population. That there is not an obvious activation of magnocellular neurones is surprising as peripheral actions of vasopressin (water retention) would seem appropriate to the changes in behaviour. However, the result is in keeping with a previous experiment that suggests the SON is not activated during the expression of maternal aggression in mice (Gammie and Nelson, 2001). In addition to there being no obvious activation of these vasopressin populations, there were no correlations between the Fos expression in these populations and scores of aggressive behaviour. Although Fos is not a reliable indicator of basal levels of neuronal activity and it may be that basal activity of peripheral vasopressin is related to behaviour, the results provide no indication that this is the case.

In contrast to the apparently little activation of peripheral vasopressin systems associated with the resident-intruder test, there appeared to be extensive test-associated activation of the vasopressin responsive neurones in the brain. Most interestingly, levels of Fos expression in the BNST, pPVN and CeA were positively correlated with individual scores of aggressive behaviour, and in the BNST and CeA, levels of Fos expression in V1b receptor-labelled neurones were also positively correlated with these scores, suggesting vasopressin may contribute to the nuclear activation that appears to be closely associated with aggressive behaviour. Although the output from the BNST and CeA can modulate the activity of peripheral systems, their best described such output is the regulation of stress and autonomic activity, predicted to occur at least in part via their CRF populations (Gray, 1993; Herman and Cullinan, 1997; Champagne *et al*, 1998), and one of these populations (that of the BNST) appears negatively associated with the aggressive behaviour. The lack of apparent activation of the magnocellular vasopressin system – and lack of correlation between Fos expression in magnocellular nuclei and behaviour - additionally suggest that osmoregulatory effects of vasopressin activity within the brain would not (alone) account for the activation of vasopressin-responsive neurones correlated with behaviour. It is therefore considered that the vasopressin signalling found to be correlated with behaviour may be directly connected with the behaviour.

That the measures of Fos expression in the CeA and pPVN were more tightly correlated with the durations of pinning down and attack/lateral threat behaviours, respectively, than with the sum duration of all so-designated aggressive behaviours, is again suggestive that the signalling correlated with behaviour is associated with

behaviour rather than, or in addition to, a co-regulated response to the test. It is difficult to envisage that these different parts of a coherent behavioural response, produced in the same general context, would be individually associated with circuits other than those directly involved in behaviour.

The Fos expression correlated with behaviour may be representative of strong regulatory signals or may represent a mechanism for gating behaviour within the brain. These are not necessarily different things.

Table 2.1 Behavioural profiles of tested rats in Experiment 1 and Experiment 2

For each of the behaviours recorded during the 30 minute test the mean (± s.e.m.) duration (s) of its expression by rats of Experiment 1 and Experiment 2, and the mean duration of its expression by all rats (Experiments 1 and 2) is displayed. The numbers of rats observed to exhibit each behaviour are displayed in parentheses after each duration.

Behaviour	Classification	Mean duration (s; ± s.e.m.) of behaviour – Experiment 1	Mean duration (s; ± s.e.m.) of behaviour – Experiment 2	Mean duration (s; ± s.e.m.) of behaviour – Experiments 1 and 2
Attack	Aggressive	45 ± 12 s (8)	43 ± 12 s (10)	44 ± 8 s (18)
Biting tail	Aggressive	8 ± 6 s (2)	0 ± 0 s (0)	3 ± 2 s (2)
Bite	Aggressive	6 ± 4 s (5)	7 ± 3 s (9)	7 ± 2 s (14)
Chasing	Aggressive	2 ± 1 s (6)	2 ± 1 s (9)	2 ± 0 s (15)
Clawing/punching	Aggressive	30 ± 9 s (7)	58 ± 26 s (10)	46 ± 16 s (17)
Lateral threat	Aggressive	42 ± 16 s (7)	38 ± 14 s (8)	40 ± 10 s (15)
Pinning down	Aggressive	166 ± 24 s (8)	24 ± 7 s (8)	84 ± 20 s (16)
Sniffing/clawing	Aggressive	26 ± 18 s (3)	2 ± 1 s (5)	12 ± 8 s (8)
Nesting	Maternal	34 ± 21 s (4)	104 ± 43 s (9)	74 ± 27 s (13)
Nursing	Maternal	1.5 ± 1 s (3)	25 ± 23 s (2)	15 ± 13 s (5)
Pup related	Maternal	77 ± 24 s (8)	97 ± 27 s (10)	89 ± 18 s (18)
Drinking	Other	4 ± 4 s (1)	13 ± 7 s (6)	9 ± 4 s (7)
Eating	Other	8 ± 8 s (1)	9 ± 6 s (4)	9 ± 5 s (5)
Environmental exploration	Other	218 ± 25 s (8)	379 ± 38 s (11)	311 ± 30 s (19)
Escape	Other	1 ± 1 s (1)	0 ± 0 s (1)	0 ± 0 s (2)
Exposed underside	Other	0 ± 0 s (0)	0 ± 0 s (1)	0 ± 0 s (1)
Freezing	Other	28 ± 16 s (6)	0 ± 0 s (0)	12 ± 7 s (6)
Grooming self	Other	268 ± 62 s (8)	173 ± 31 s (11)	213 ± 33 s (19)
Rearing	Other	37 ± 15 s (8)	25 ± 11 s (9)	30 ± 9 s (17)
Sniffing	Other	450 ± 79 s (8)	452 ± 44 s (11)	451 ± 40 s (19)
Other	Other	356 ± 86 s (8)	349 ± 41 s (11)	352 ± 42 s (19)

Table 2.2 Aggressive behaviour, Fos expression and Fos/vasopressin receptor co-expression

The mean (± s.e.m.) numbers of labelled cells per section are displayed. *p = 0.008, **p = 0.002, ***p < 0.001, ^{o}p = 0.041 (two-way ANOVA, Holm-Sidak method); ^{o}p = 0.014, $^{\#}$p = 0.001 (unpaired t-tests) versus control group

Area	Fos-positive cells per section in V1a receptor stained tissue (mean ± s.e.m.)		Fos-positive cells per section in V1b receptor stained tissue (mean ± s.e.m.)		Fos/V1a receptor double-labelled cells per section (mean ± s.e.m.)		Fos/V1b receptor double-labelled cells per section (mean ± s.e.m.)	
	Control group	Tested group	Control group	Tested group	Control group	Tested group	Control group	Tested group
MiOB	36.9 ± 5.8	128 ± 14.7***	48.6 ± 7.6	158.8 ± 10.9***	4.1 ± 0.8	12.9 ± 2.9***	4.5 ± 0.8	10.8 ± 1.3***
LS	51.1 ± 9.0	166.5 ± 4.8***	36.3 ± 4.7	127.7 ± 9.1***	3.8 ± 0.5	17.4 ± 0.7***	7.8 ± 1.2	25.3 ± 2.2***
BNST	27.5 ± 1.6	58.6 ± 3.0***	21.0 ± 2.5	42.6 ± 2.6*	1.3 ± 0.3	3.5 ± 0.5#	2.5 ± 0.3	6.0 ± 0.5***
SON	39.0 ± 6.3	43.2 ± 5.3	34.8 ± 7.2	48.4 ± 7.3	8.5 ± 1.0	10.4 ± 1.3	3.6 ± 0.5	5.7 ± 0.6⁰
pPVN	18.1 ± 3.0	61.5 ± 3.3***	12.9 ± 2.2	54.1 ± 4.3***	1.4 ± 0.3	8.2 ± 1.0***	2.4 ± 0.4	6.4 ± 0.4***
mPVN	4.3 ± 0.9	6.7 ± 1.1	3.5 ± 1.0	5.8 ± 0.8	0.6 ± 0.2	0.8 ± 0.1	1.4 ± 0.3	1.1 ± 0.3
MeA	41.5 ± 6.2	157.3 ± 8.6***	23.6 ± 2.8	115.4 ± 7.3***	1.1 ± 0.3	7.0 ± 1.1***	2.8 ± 0.3	11.0 ± 0.9***
CeA	23.1 ± 2.5	33.8 ± 5.3	14.5 ± 2.3	16.2 ± 2.4	3.7 ± 0.9	4.8 ± 0.9	2.0 ± 0.2	2.4 ± 0.3
VMH	23.9 ± 1.7	62.3 ± 6.1***	30.7 ± 2.8	76.6 ± 7.9***	1.8 ± 0.3	6.8 ± 0.9***	2.0 ± 0.1	5.9 ± 0.6***
dPAG	26.1 ± 3.3	85.8 ± 6.4***	26.2 ± 3.7	75.8 ± 7.9***	1.5 ± 0.3	7.1 ± 0.8***	2.0 ± 0.2	5.4 ± 0.6**
lPAG	42.3 ± 5.5	102.6 ± 3.4***	34.7 ± 2.7	95.8 ± 6.1***	3.5 ± 0.5	7.5 ± 1.0**	3.7 ± 0.4	7.4 ± 0.8***

| Area	Proportion of Fos-positive cells per section in V1a receptor labelled cells (mean ± s.e.m.)		Proportion of Fos-positive cells per section in V1b receptor labelled cells (mean ± s.e.m.)	
	Control group	Tested group	Control group	Tested group
MiOB	0.11 ± 0.010	0.10 ± 0.015	0.106 ± 0.017	0.068 ± 0.007
LS	0.081 ± 0.011	0.105 ± 0.006	0.233 ± 0.043	0.205 ± 0.023
BNST	0.048 ± 0.010	0.060 ± 0.008	0.135 ± 0.026	0.139 ± 0.007
SON	0.245 ± 0.034	0.257 ± 0.030	0.186 ± 0.055	0.169 ± 0.042
pPVN	0.074 ± 0.015	0.133 ± 0.013[##]	0.192 ± 0.020	0.123 ± 0.009[o]
mPVN	0.147 ± 0.022	0.131 ± 0.023	0.552 ± 0.112	0.191 ± 0.030[*,##]
MeA	0.027 ± 0.005	0.044 ± 0.052[#]	0.142 ± 0.024	0.099 ± 0.011
CeA	0.153 ± 0.027	0.143 ± 0.018	0.164 ± 0.022	0.160 ± 0.021
VMH	0.078 ± 0.012	0.111 ± 0.012	0.068 ± 0.007	0.078 ± 0.005
dPAG	0.057 ± 0.009	0.085 ± 0.013	0.091 ± 0.014	0.075 ± 0.009
lPAG	0.083 ± 0.004	0.074 ± 0.011	0.113 ± 0.015	0.081 ± 0.010

Table 2.3 Aggressive behaviour and Fos expression in vasopressin-responsive cells

The mean (± s.e.m.) proportions of the total number of Fos-labelled cells per section found in V1a receptor- or V1b receptor-labelled cells. *$p < 0.0001$ (two-way ANOVA, Holm-Sidak method); #$p = 0.039$, ##$p \leq 0.012$ (unpaired t-tests); o$p = 0.018$ (Mann Whitney rank sum test) versus control group

Area	Number of Fos-positive cells per section (mean ± s.e.m.)
MiOB	131 ± 18
LS	115 ± 11
BNST (total)	56 ± 3
SON	46 ± 7
MeA	77 ± 8
VMH	32 ± 4

Table 2.4 Fos-positive cell counts in the MiOB, LS, BNST, SON, MeA and VMH from Experiment 1 tissue

The mean (± s.e.m.) numbers of labelled cells per section are displayed
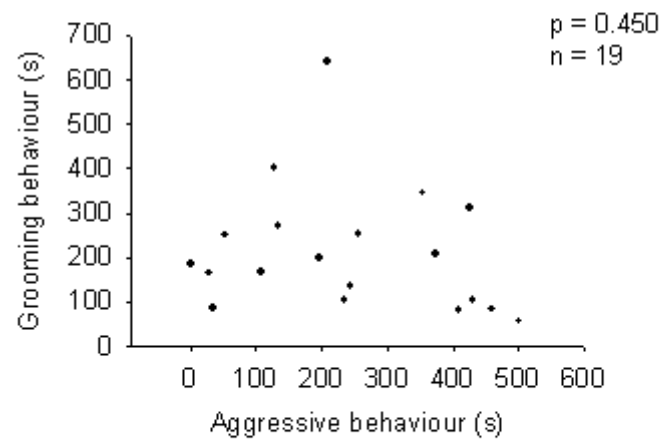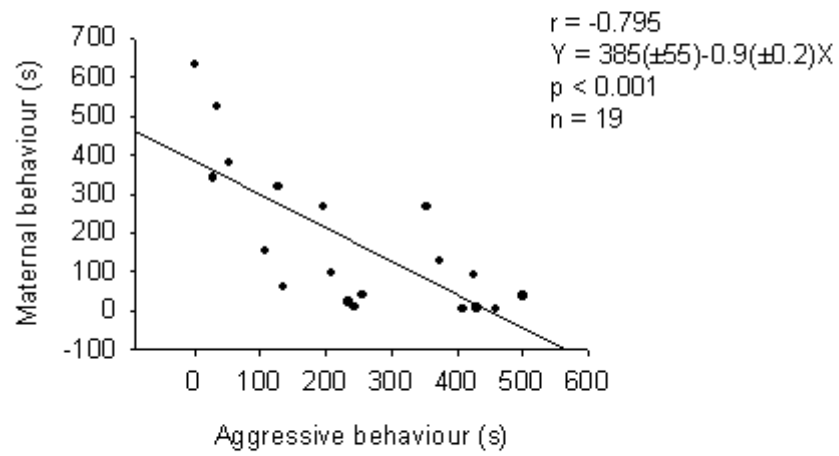
Figure 2.1 Correlation between the durations of aggressive and maternal behaviours, and aggressive and grooming behaviours
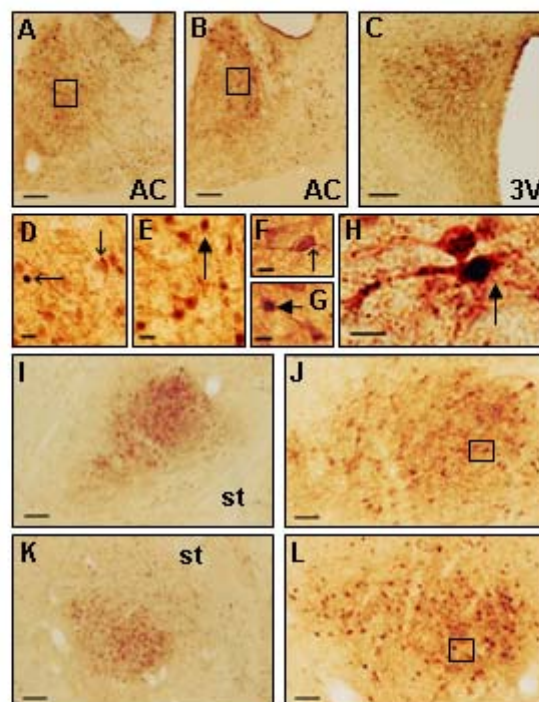
Figure 2.2 Aggressive behaviour and Fos/CRF co-expression. Mean (+ s.e.m.) counts of Fos- (M), CRF- (N) and Fos/CRF- (O) labelled cells in control (grey bars; n = 7) and test (black bars; n = 8) groups. *p < 0.05, **p ≤ 0.015, or ***p ≤ 0.001 versus control group (two-way ANOVA, Holm-Sidak method). Photomicrographs, A-L, were taken through a 10x/0.3 (A-C, I, K; scale bar = 100 μm), 20x/0.5 (D, E, F, G; scale bar = 10 μm, and J, L; scale bar = 50 μm), or 100x/1.3 (H; scale bar = 10 μm) objective lens. A, B (LBNST), C (pPVN), I and K (CeA), indicate the levels at which counts were made. D, E, F and G are expansions of the areas indicated in A, B, J and L, respectively. A, D (LBNST) F, I and J (CeA) are examples of staining from a control rat. B, E (LBNST), and G, K, L (CeA) are examples from a test rat. Filled arrows indicate examples of double-labelled cells. H shows an example at higher resolution. Open arrows indicate cells labelled only for Fos (dark, nuclear stain), or CRF (brown, cytoplasmic stain). AC, anterior commissure; 3V third ventricle; st, stria terminalis
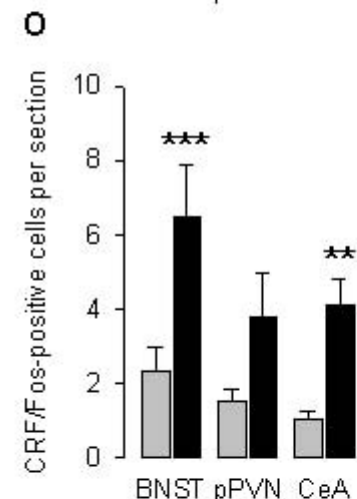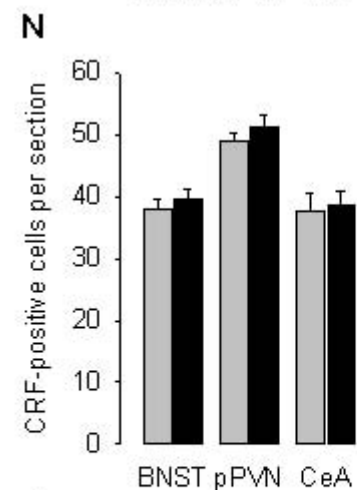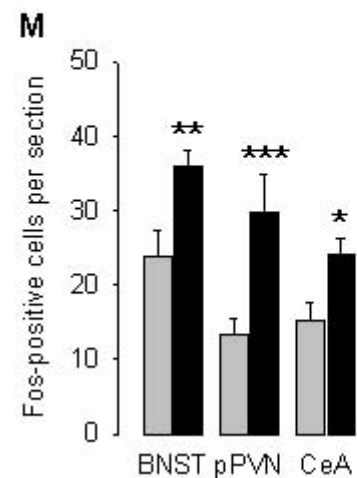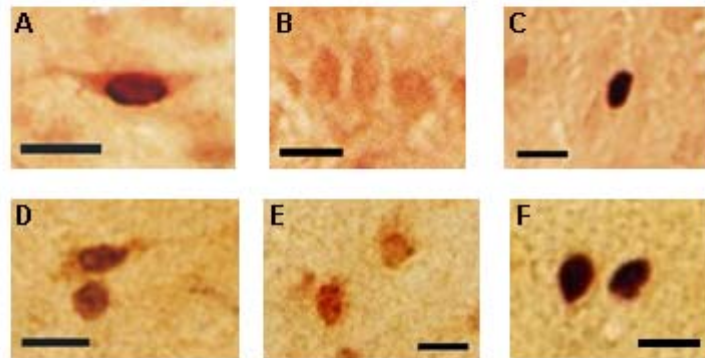
Figure 2.3 Immunostaining analysis. Examples of double-labelled cells (A, D; 40x/0.7 objective lens; scale bar = 10 μm), cells labelled only for receptor (B, E; 20x/0.5 objective lens; scale bar = 10 μm), or only for Fos (C, F; 20x/0.5 objective lens; scale bar = 10 μm), in tissue stained for Fos and V1a receptor (A-C), or Fos and V1b receptor (D-F).
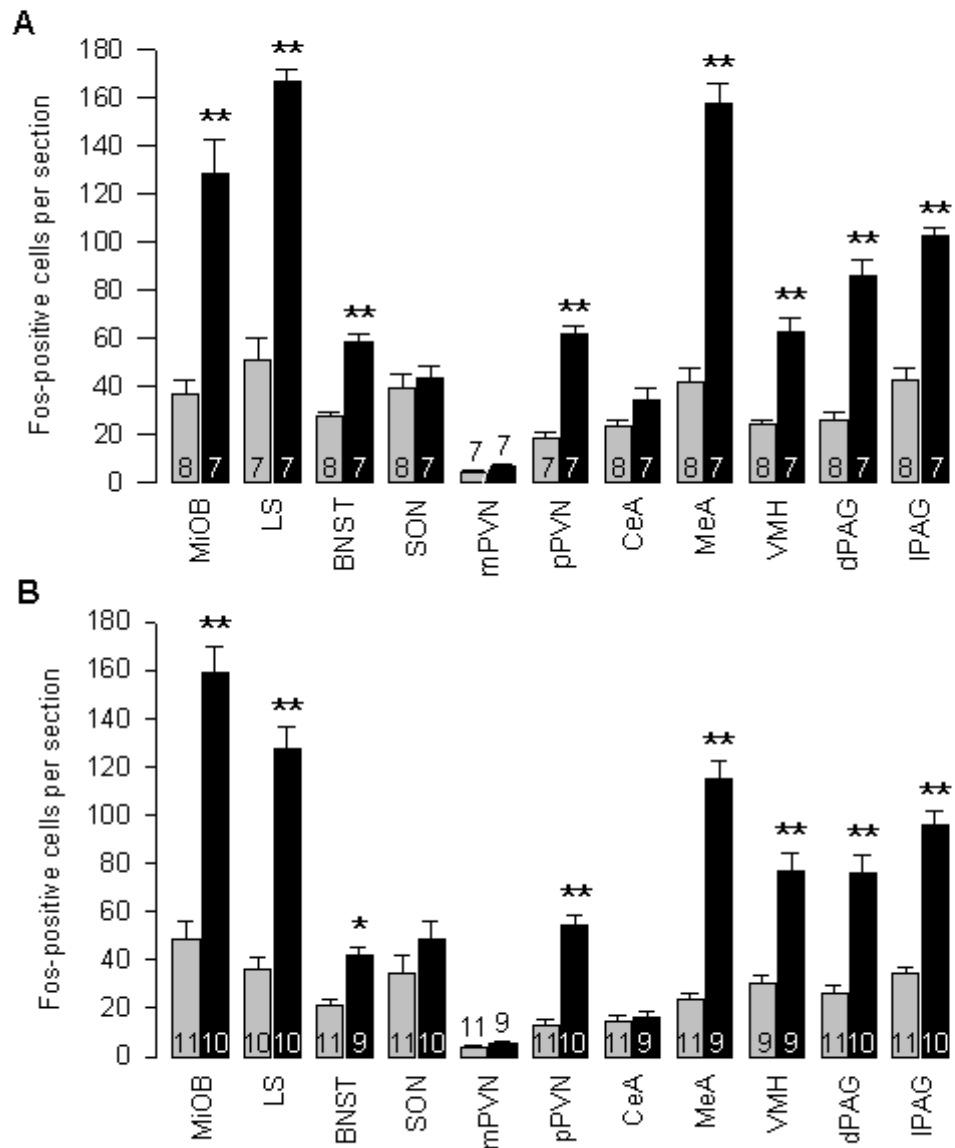
Figure 2.4 Aggressive behaviour and Fos expression. Mean (+ s.e.m.) counts of Fos-labelled cells in tissue sections additionally immuno-labelled for the V1a receptor (A) or V1b receptor (B) protein. Counts are displayed for control (grey bars) and test (black bars) groups. n, for each group, is indicated on (or above) each bar. *p = 0.008, **p < 0.001 versus control group (two-way ANOVA, Holm-Sidak method). Figures 2.5 and 2.6 include photomicrographs that indicate the levels at which the counts were made, and demonstrate the different levels of Fos expression between groups.
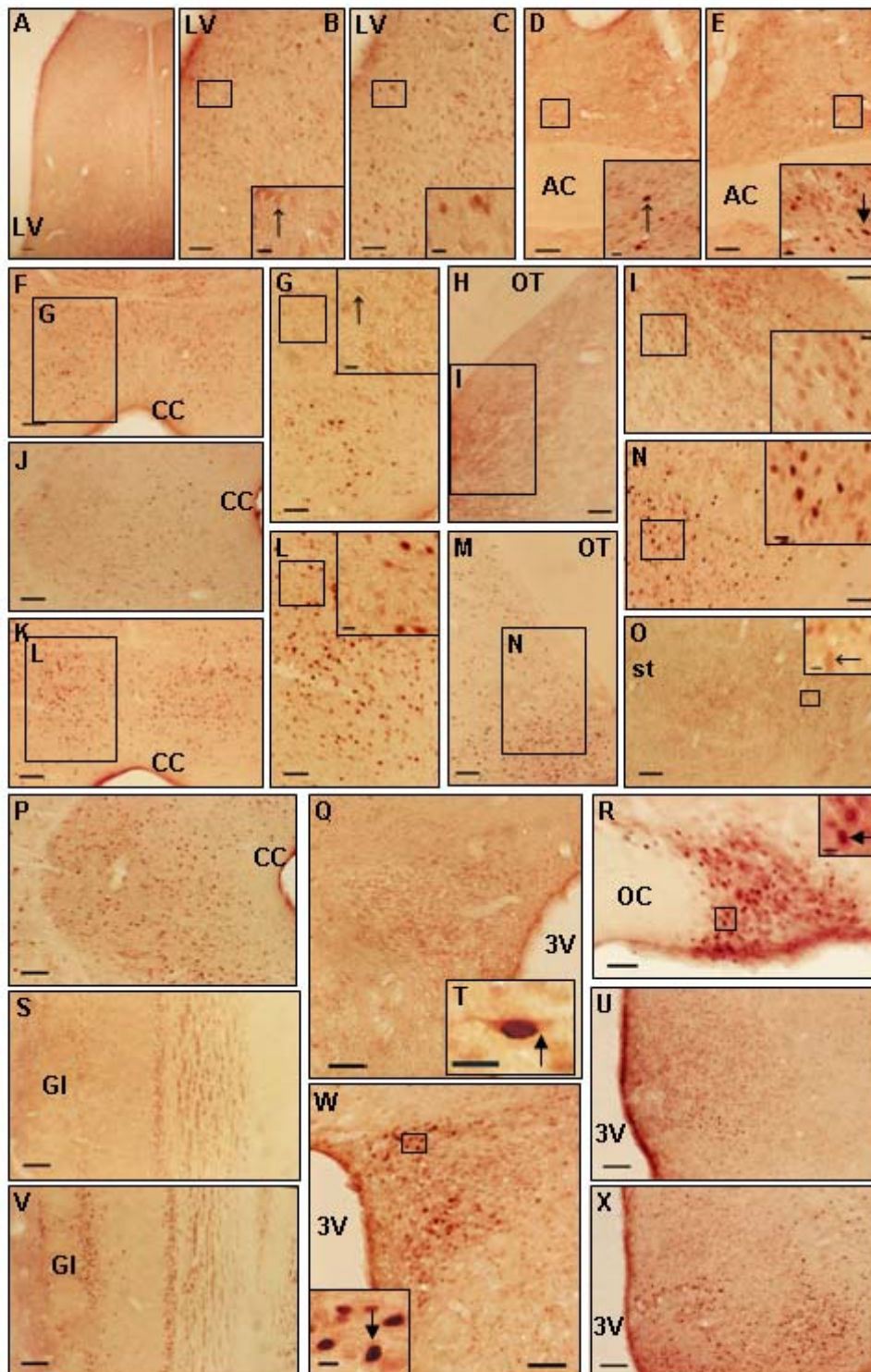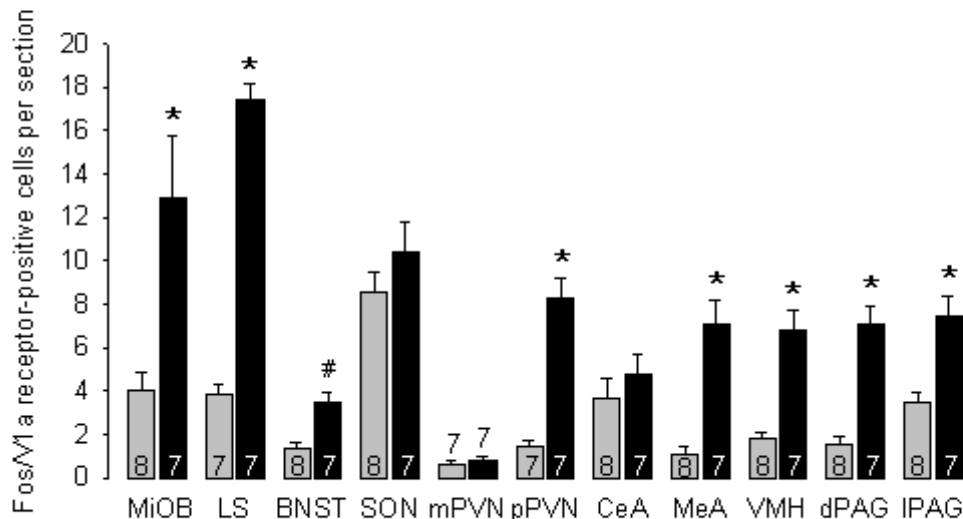
Figure 2.5 Aggressive behaviour and Fos/V1a receptor co-expression.

Above: Mean (+ s.e.m.) counts of Fos/V1a receptor-labelled cells in control (grey bars) and test (black bars) groups. n, for each group, is indicated on (or above) each bar. *p ≤ 0.002 (two-way ANOVA, Holm-Sidak method); #p = 0.001 (unpaired t-test) versus control group

Opposite: Photomicrographs (A-X) were taken through a 5x/0.15 (A; scale bar = 100 μm), 10x/0.3 (D-F, H, J, K, M, O-Q, S, U-X; scale bar = 100 μm (inserted expansions, scale bar = 10 μm)), x20/0.5 (B, C, G, I, L, N, R; scale bar = 50 μm (inserted expansions, scale bar = 10 μm)), or x40/0.7 (T; scale bar = 10 μm) objective lens. Expansions are from the boxed region in the same figure, except where otherwise indicated. S (OB), A (LS), D (BNST), F (dPAG), H (MeA), J (IPAG), O (CeA), Q (PVN), R (SON), and U (VMH) indicate the levels at which counts were made. B (LS), D (BNST), F, G (dPAG), H, I (MeA), J (IPAG), Q (pPVN), S (OB) and U (VMH) are examples of the suggested areas from a control rat. C (LS), E (BNST), K, L (dPAG), M, N (MeA), P (IPAG), V (OB), W (PVN) and X (VMH) are examples from a test rat. Filled arrows indicate examples of double labelled cells. T shows an example at higher resolution. Open arrows indicate cells labelled only for Fos (dark, nuclear stain), or V1a receptor (pink, cytoplasmic stain). LV, lateral ventricle; AC, anterior commissure; 3V third ventricle; OT, optic tract; CC, central canal; Gl, glomerular layer, olfactory bulb; OC, optic chiasm; st, stria terminalis
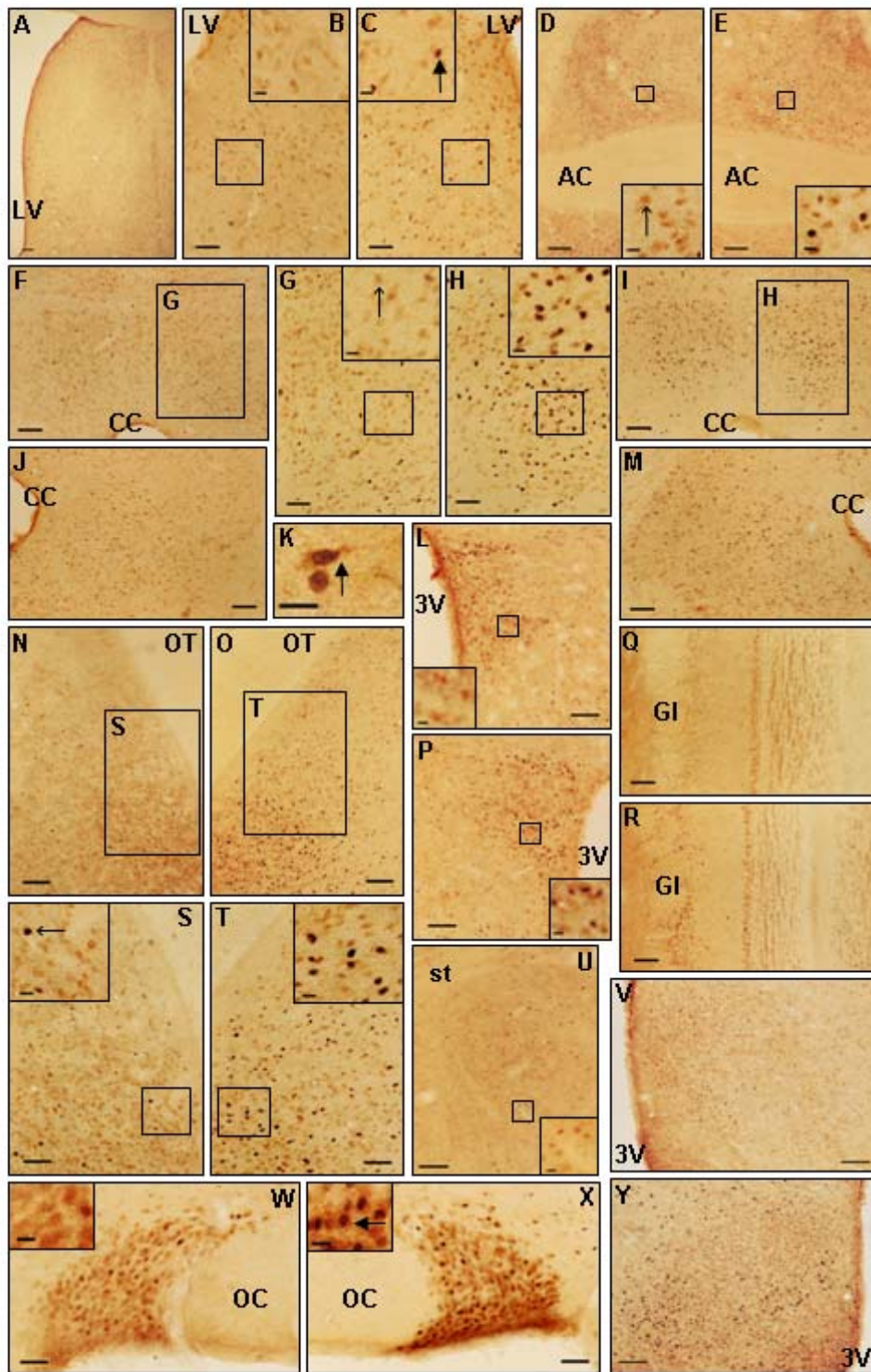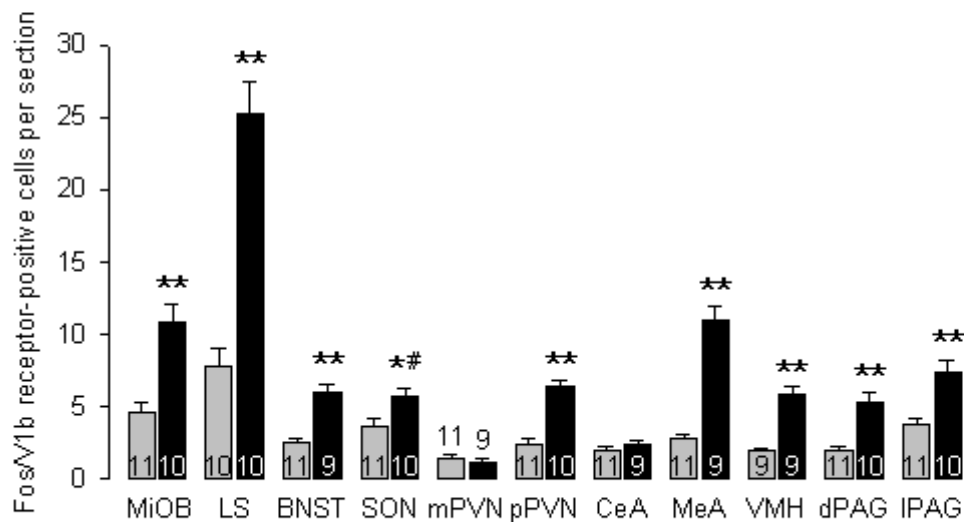
Figure 2.6 Aggressive behaviour and Fos/V1b receptor co-expression.

Above: Mean (+ s.e.m.) counts of Fos/V1b receptor-labelled cells in control (grey bars) and test (black bars) groups. n, for each group, is indicated on (or above) each bar. *p = 0.041, **p ≤ 0.002 (two-way ANOVA, Holm-Sidak method); #p = 0.014 (unpaired t-test) versus control group

Opposite: Photomicrographs (A-Y) were taken through a 5x/0.15 (A; scale bar = 100 μm), 10x/0.3 (D-F, I, J, L-R, U, V, Y; scale bar = 100 μm (inserted expansions, scale bar = 10 μm)), x20/0.5 (B, C, G, H, S, T, W, X; scale bar = 50 μm (inserted expansions, scale bar = 10 μm)), or x40/0.7 (K; scale bar = 10 μm) objective lens. Expansions are from the boxed region in the same figure, except where otherwise indicated. A (LS), D (BNST), F (dPAG), J (lPAG), L (PVN), N (MeA), Q (OB), U (CeA), V (VMH) and W (SON) indicate the levels at which counts were made. B (LS), D (BNST), F, G (dPAG), J (lPAG), L (pPVN), N, S (MeA), Q (OB), V (VMH) and W (SON) are examples of the suggested areas from a control rat. C (LS), E (BNST), H,I (dPAG), M (lPAG), P (pPVN), O,T (MeA), R (OB), Y (VMH) and X (SON) are examples from a test rat. Filled arrows indicate examples of double labelled cells. K shows an example at higher resolution. Open arrows indicate cells labelled only for Fos (dark, nuclear stain), or V1b receptor (brown, cytoplasmic stain). LV, lateral ventricle; AC, anterior commissure; 3V third ventricle; OT, optic tract; CC, central canal; Gl, glomerular layer, olfactory bulb; OC, optic chiasm; st, stria terminalis
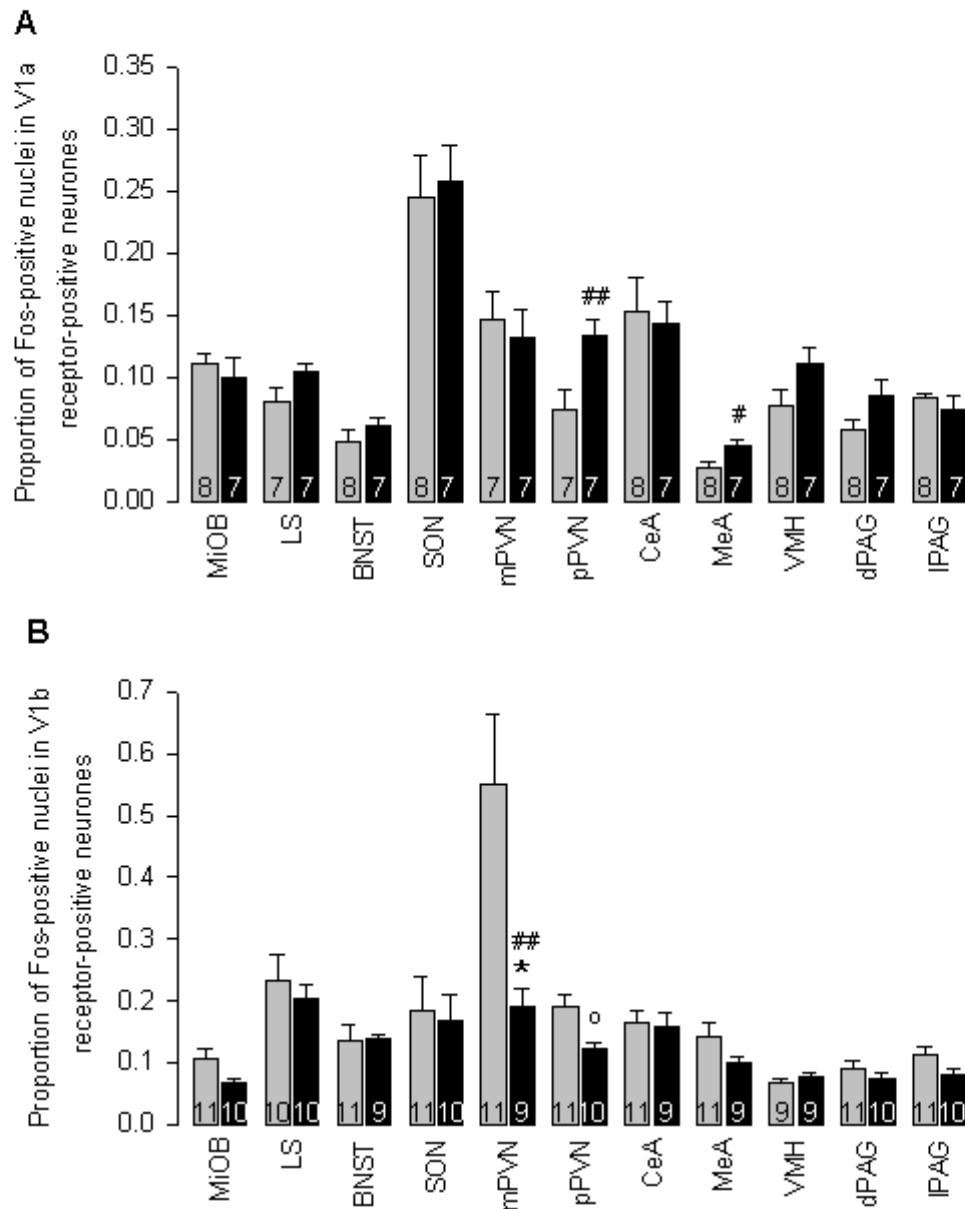
Figure 2.7 Aggressive behaviour and Fos expression in vasopressin receptor-expressing cells. Mean (+ s.e.m.) proportions of the total number of Fos-labelled cells per section found in cells immuno-labelled for the V1a receptor (A) or V1b receptor (B) protein. Results are displayed for control (grey bars) and test (black bars) groups. n, for each group, is indicated on (or above) each bar. *p < 0.0001 (two-way ANOVA, Holm-Sidak method); #p = 0.039, ##p ≤ 0.012 (unpaired t-tests); °p = 0.018 (Mann Whitney rank sum test) versus control group

A
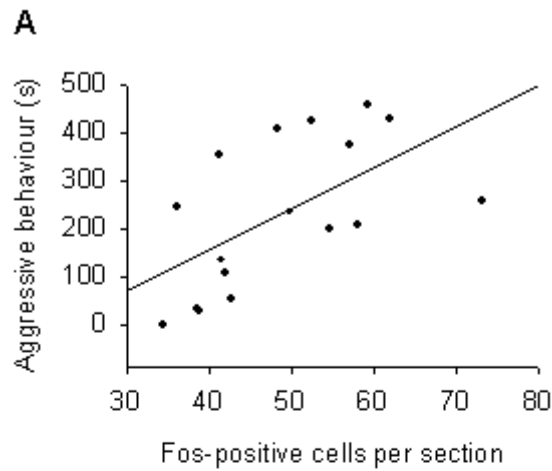
BNST
r = 0.657
Y = -189(±151) + 8.6(±3.0)X
p = 0.012
n = 17

B
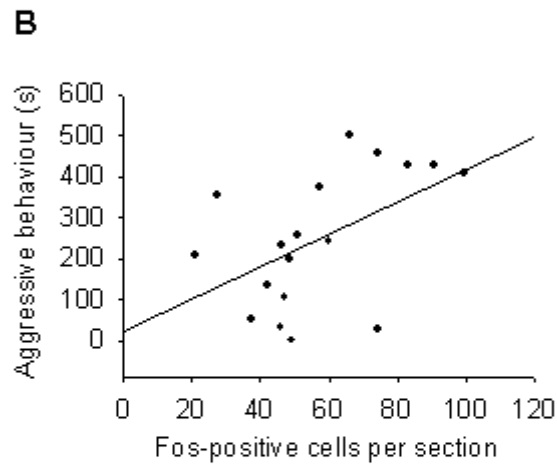
pPVN
r = 0.550
Y = 24(±101)+3.9(±1.7)X
p = 0.031
n = 18

C
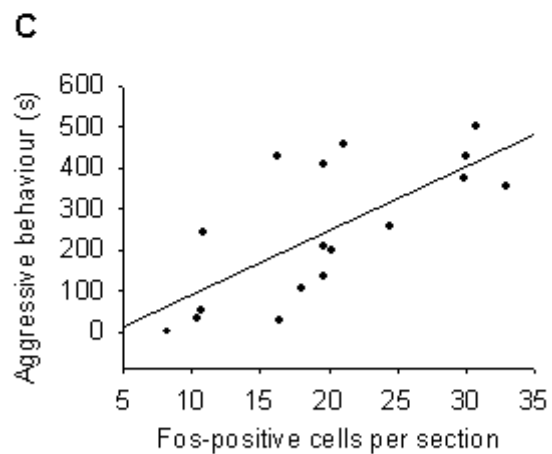
CeA
r = 0.688
Y = -64(±86)+15.6(±4.0)X
p = 0.002
n = 17

Figure 2.8 Correlation between the duration of aggressive behaviour and the number of Fos-positive cells in the BNST (A), pPVN (B), or CeA (C)

A
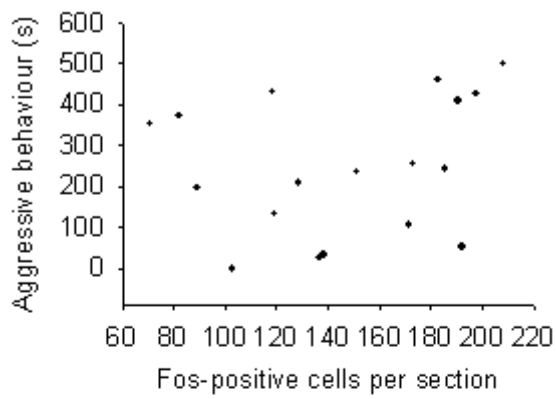
MiOB
p = 0.368
n = 18

B
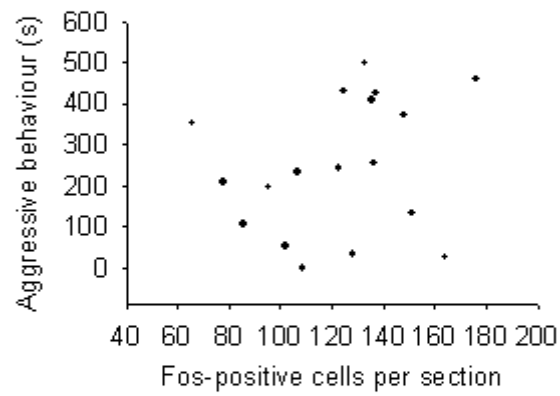
LS
p = 0.368
n = 18

C

SON
p = 0.149
n = 17

D

MeA
p = 0.342
n = 17

E

VMH
p = 0.590
n = 17
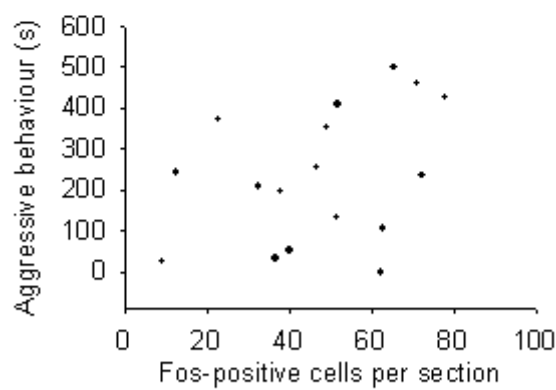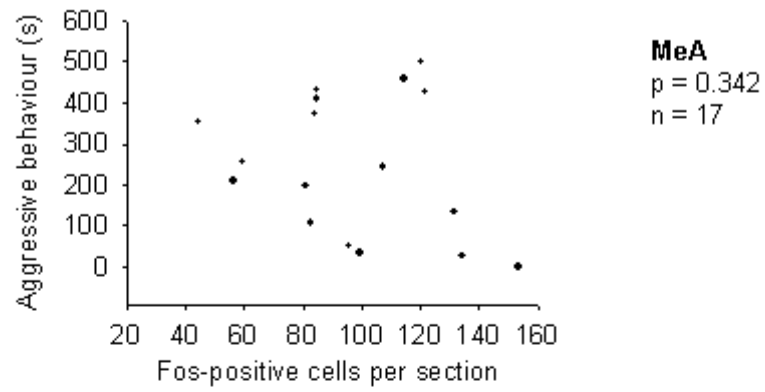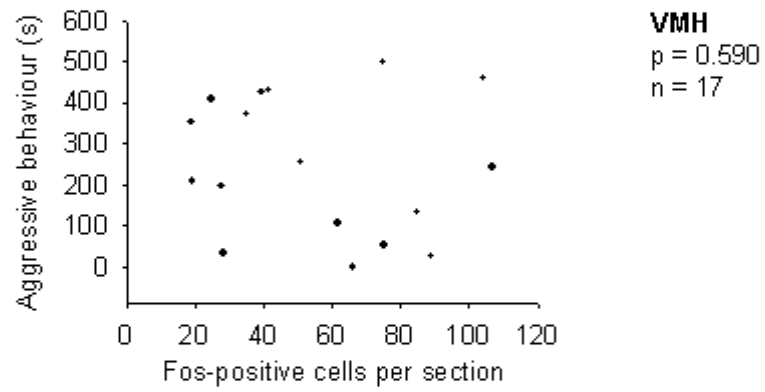
Figure 2.9 Linear regression analysis of the duration of aggressive behaviour and the number of Fos-positive cells in the MiOB (A), LS (B), SON (C), MeA (D) or VMH (E)

A
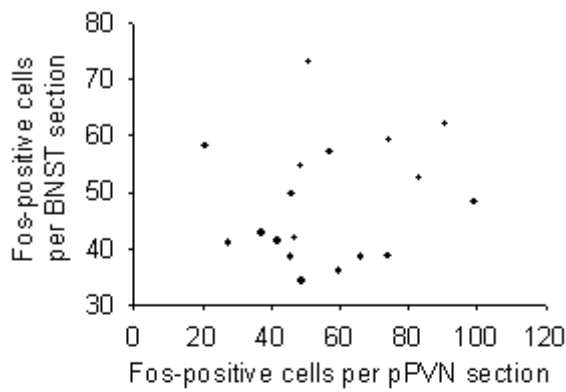
BNST vs. pPVN
p = 0.534
n = 18

B
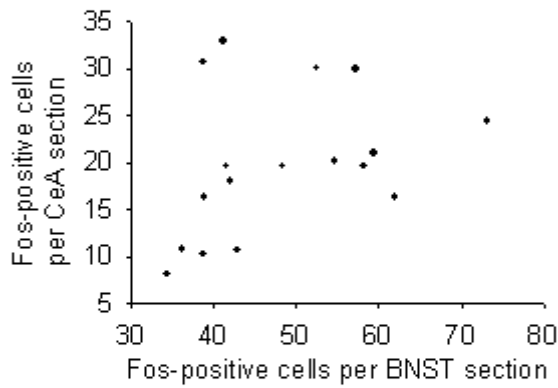
CeA vs. BNST
p = 0.169
n = 17
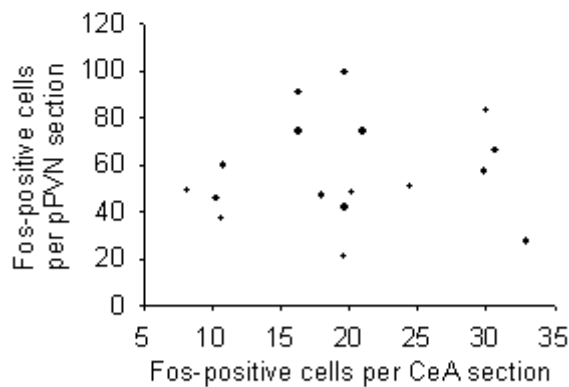
pPVN vs. CeA
p = 0.809
n = 17

Figure 2.10 Linear regression analysis of the numbers of Fos-positive cells in the BNST and pPVN (A), CeA and BNST (B), and pPVN and CeA (C)
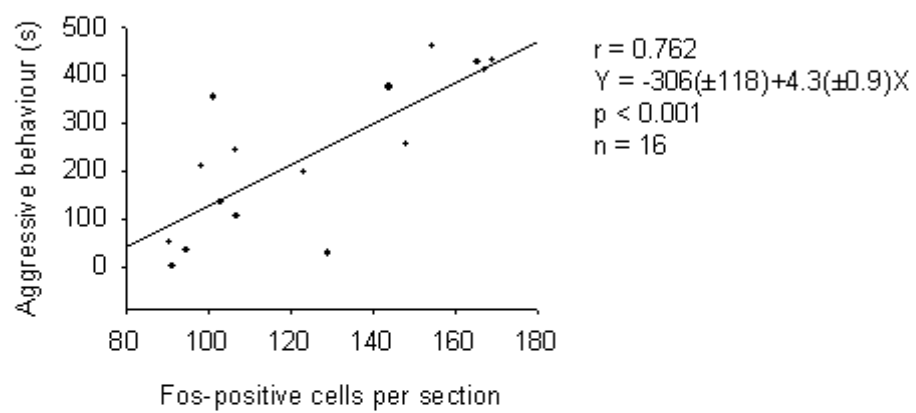
Figure 2.11 Correlation between the duration of aggressive behaviour and the sum of the numbers of Fos-positive cells per section in the BNST, pPVN and CeA

A

CeA
r = 0.669
Y = -75(±44)+8.3(±2.1)X
p = 0.001
n = 17

B

pPVN
r = 0.507
Y = -22(±27)+1.1(±0.5)X
p = 0.023
n = 18

C

pPVN
r = 0.561
Y = -21(±20)+1.1(±0.3)X
p = 0.003
n = 18

Figure 2.12 Correlations between the duration of pinning down and Fos expression in the CeA (A), the duration of lateral threat and Fos expression in the pPVN (B) and the duration of attack and Fos expression in the pPVN (C)

r = 0.569
Y = -43(±39)+2.3(±0.7)X
p = 0.003
n = 18
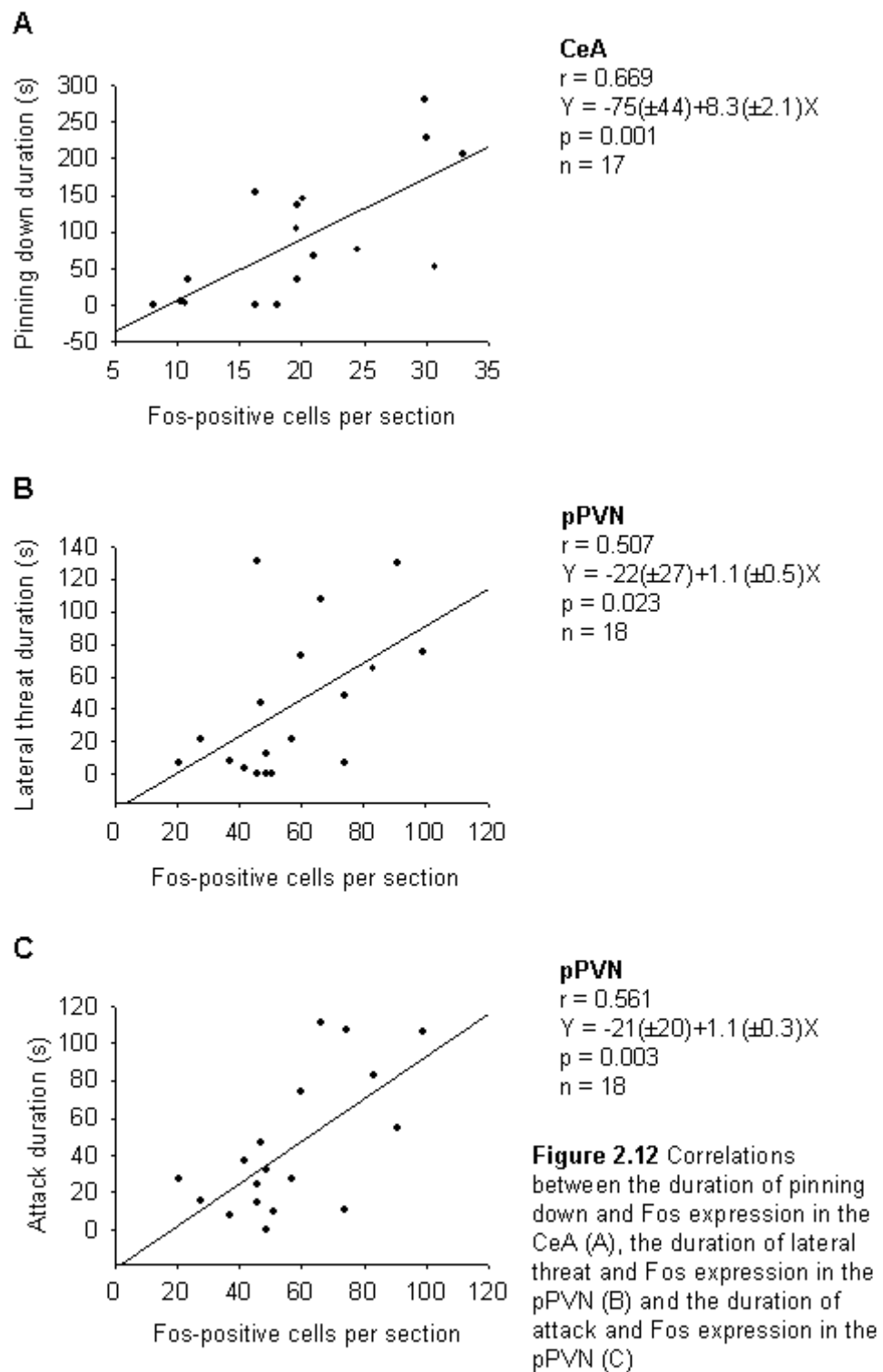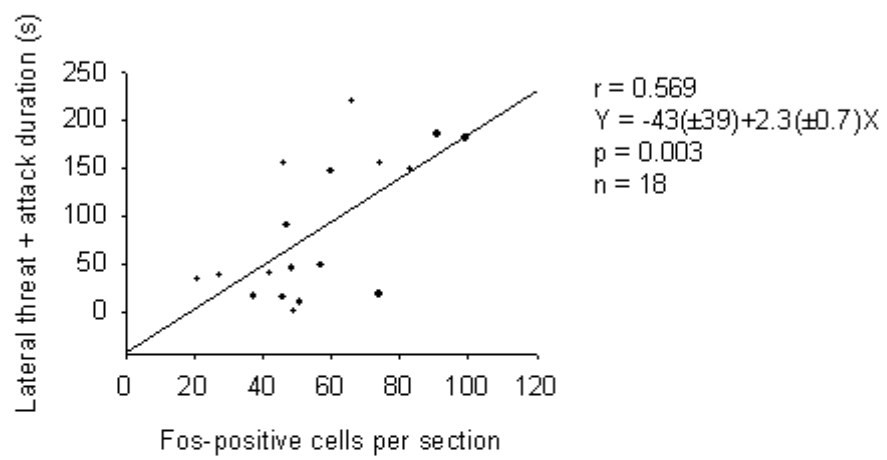
Figure 2.13 Correlation between the duration of lateral threat plus attack behaviours and the number of Fos-positive cells in pPVN

BNST
r = -0.738
Y = 446(±51) − 17.7(±6.8)X
p = 0.041
n = 8
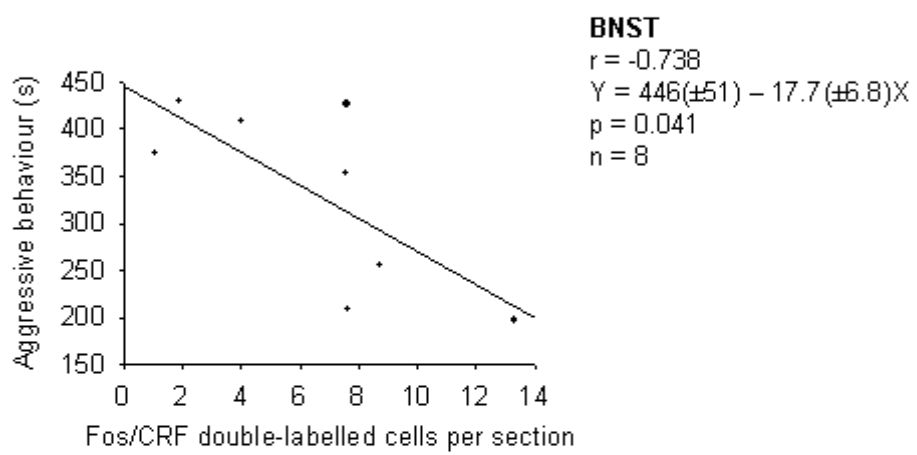
Figure 2.14 Correlation between the duration of aggressive behaviour and the number of Fos/CRF double-labelled cells in the BNST

A

BNST
r = 0.418
Y = -277(±162) + 70(±26)X
p = 0.032
n = 9

B

CeA
r = 0.717
Y = -299(±95) + 201(±38)X
p = 0.001
n = 9
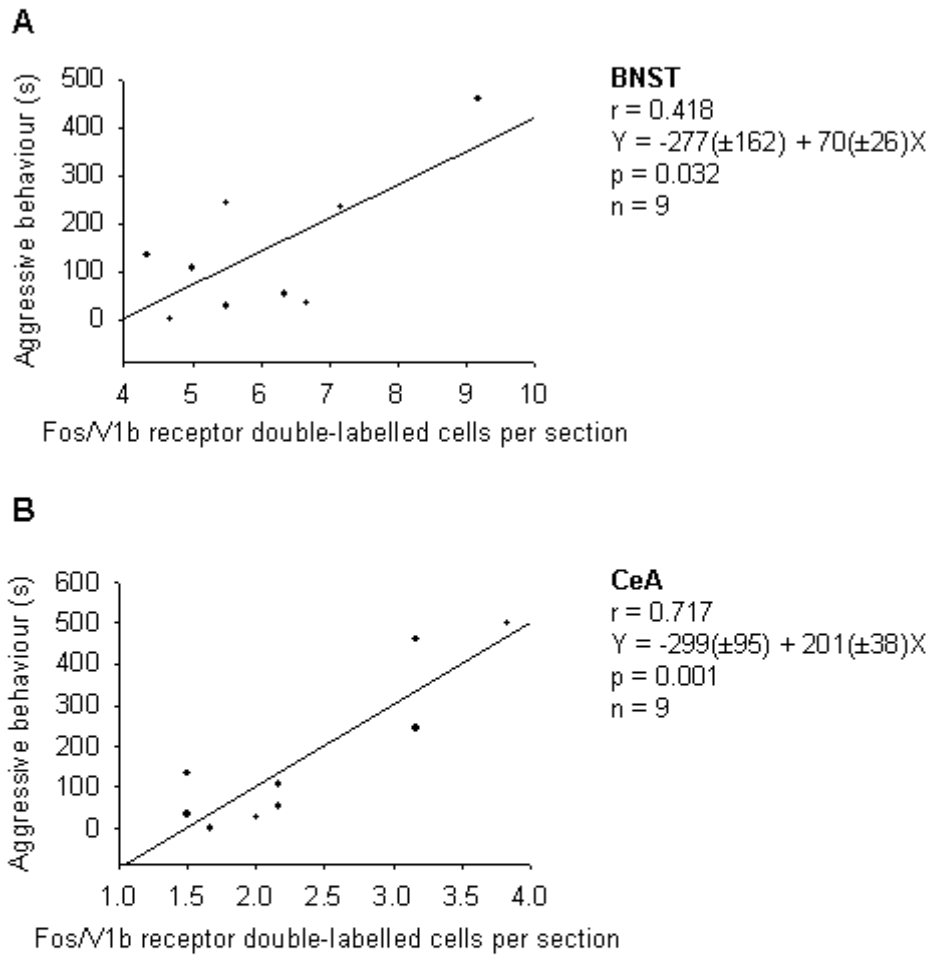
Figure 2.15 Correlation between the duration of aggressive behaviour and the number of Fos/V1b receptor double-labelled cells in the BNST (A) or CeA (B)

CeA
r = 0.524
Y = -31(±20) – 22.6(±8.0)X
p = 0.025
n = 9
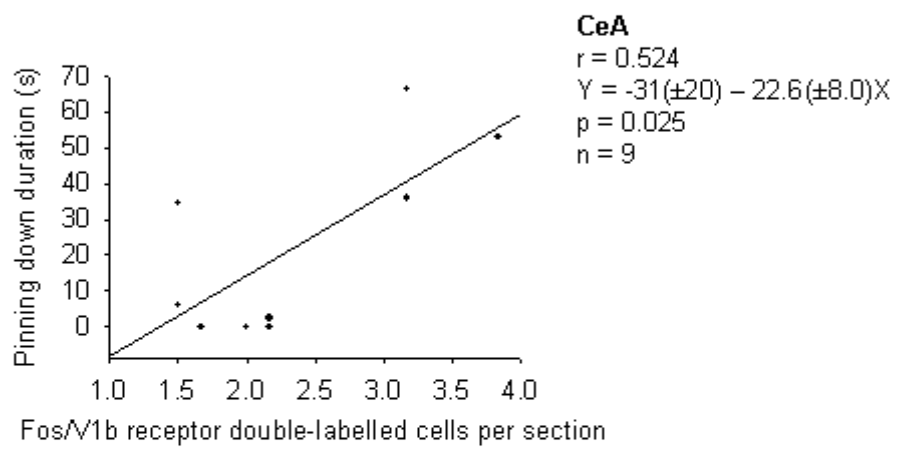
Figure 2.16 Correlation between the duration of pinning down and the number of Fos/V1b receptor double-labelled cells in the CeA

A

p = 0.292
n = 7

B

p = 0.959
n = 10
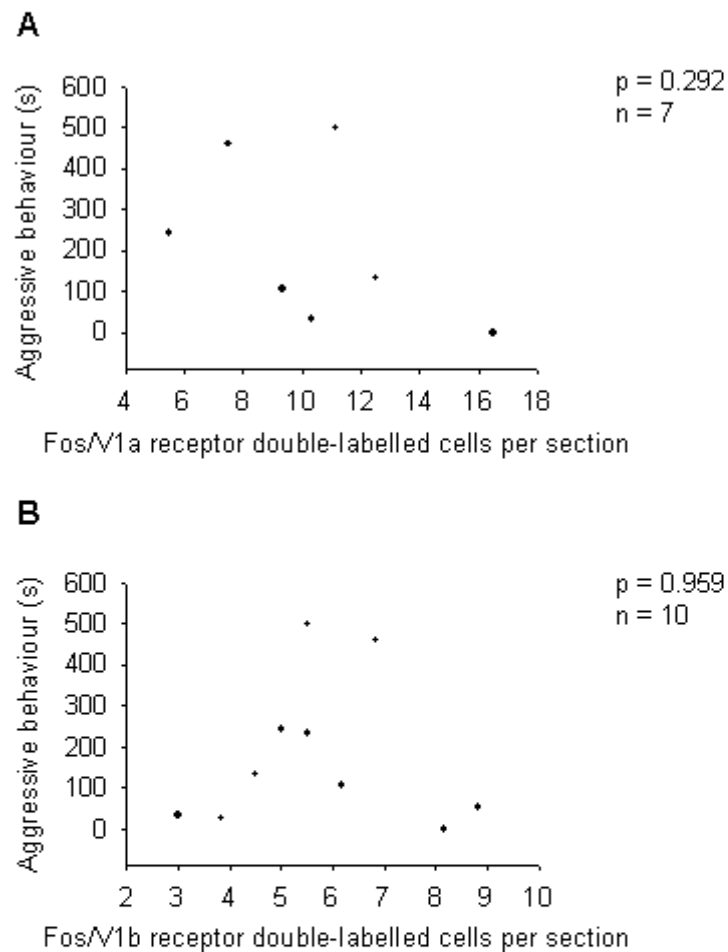
Figure 2.17 Correlation between the duration of aggressive behaviour and the number of Fos/V1a receptor double-labelled cells (A) and Fos/V1b receptor double-labelled cells (B) in the SON

BNST
r = -0.532
$Y = 679(\pm191) - 10.2(\pm3.8)X$
p = 0.017
n = 17
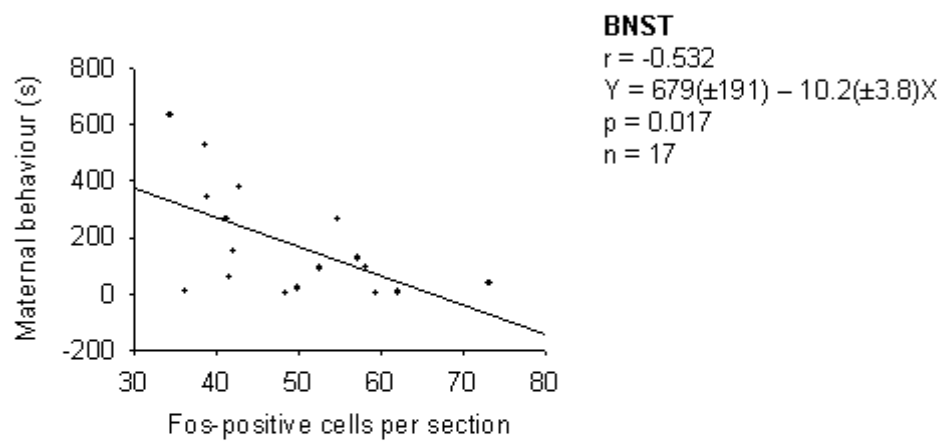
Figure 2.18 Correlation between the duration of maternal behaviour and the number of Fos-positive cells in the BNST

A

Maternal behaviour (s) vs Fos-positive cells per section

CeA
p = 0.051
n = 17

B

Maternal behaviour (s) vs Fos-positive cells per section

pPVN
p = 0.140
n = 18

C

Maternal behaviour (s) vs Fos-positive cells per section

MiOB
p = 0.125
n = 18

D

Maternal behaviour (s) vs Fos-positive cells per section

LS
p = 0.327
n = 18
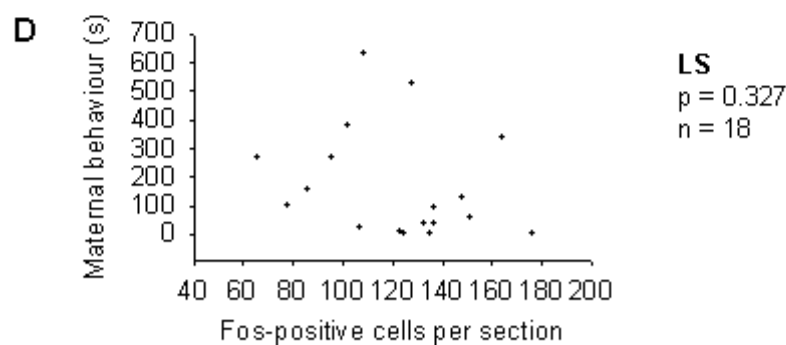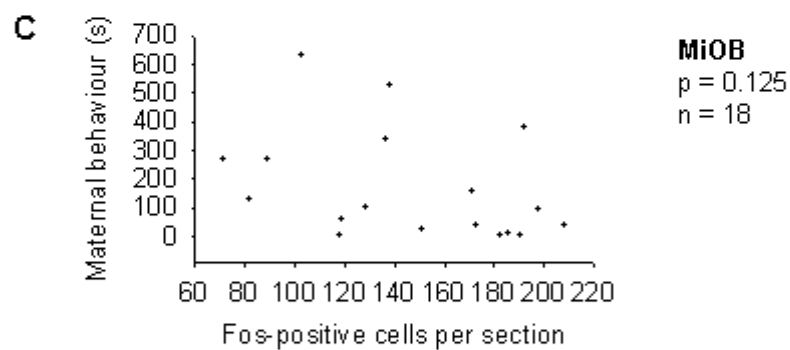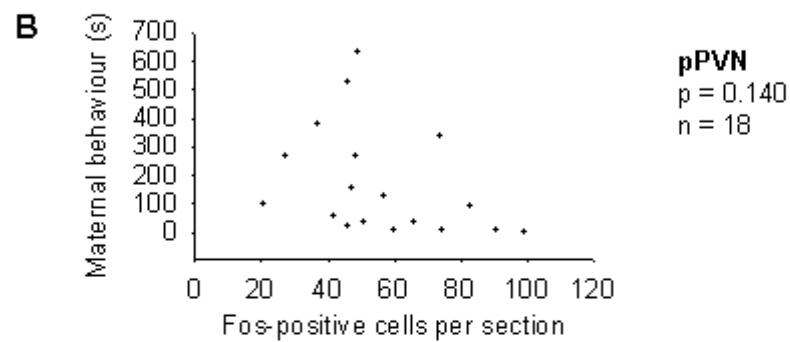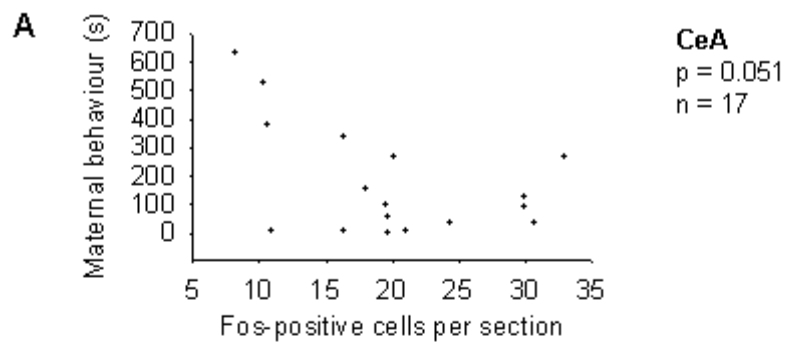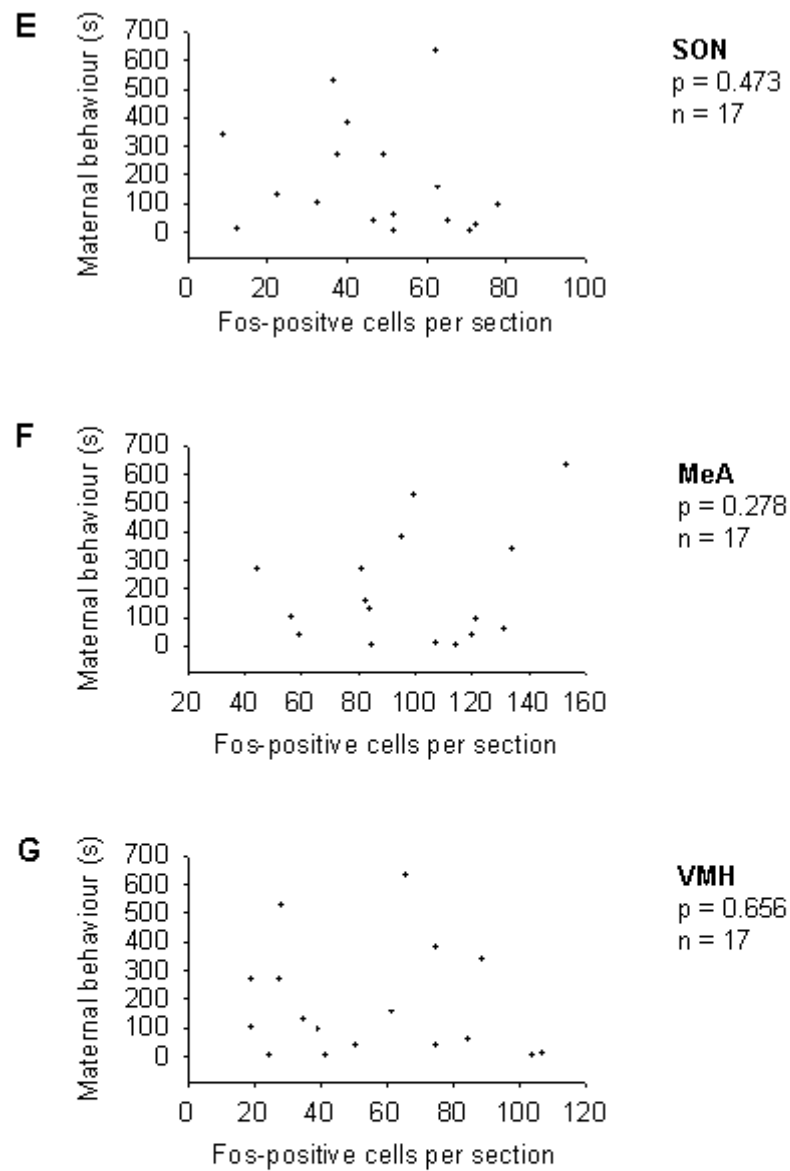
Figure 2.19 Linear regression analysis of the duration of maternal behaviour and the number of Fos-positive cells in the CeA (A), pPVN (B), MiOB (C), LS (D), SON (E), MeA (F) or VMH (G)

BNST
r = -0.509
Y = 355(±130) − 5.7(±2.6)X
p = 0.044
n = 17
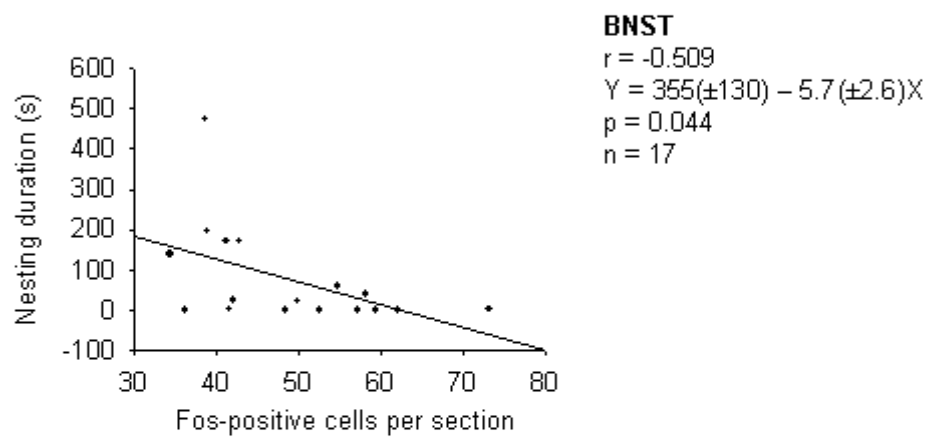
Figure 2.20 Correlation between the duration of nesting behaviour and the number of Fos-positive cells in the BNST

CeA
r = -0.684
Y = 690(±196) − 192(±79)X
p = 0.045
n = 9
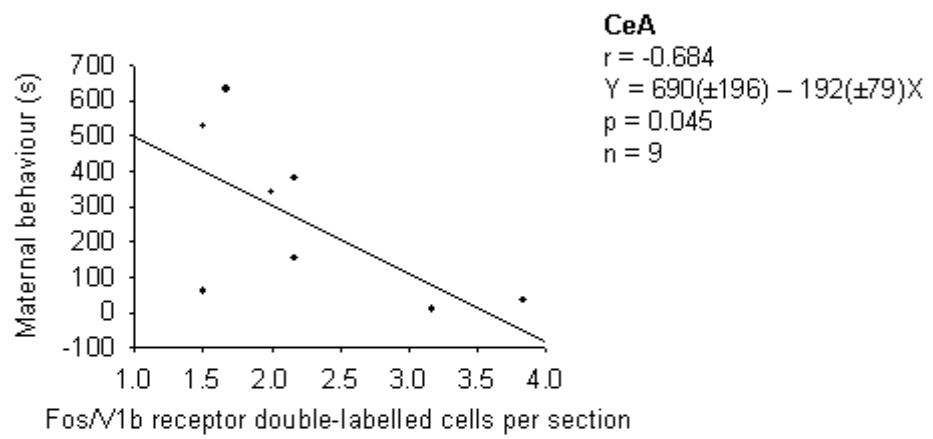
Figure 2.21 Correlation between the duration of maternal behaviour and the number of Fos/V1b receptor double-labelled cells in the CeA

Chapter 3

**Central expression of vasopressin
receptors during the peripartum**

Introduction

Central actions of vasopressin appear to be important for various forms of aggressive behaviour, including maternal aggression. The distribution and extent of V1a receptor expression in particular brain regions has been correlated with levels of some of these forms of aggressive behaviour, both within and between species, as described in Chapters 1 and 2 (Compaan *et al*, 1993; Insel *et al*, 1994; Everts *et al*, 1997; Bester-Meredith *et al*, 1999; Hammock and Young, 2005; Hammock *et al*, 2005). Artificial manipulation of V1a receptor expression can induce dramatic changes in the expression of social behaviours (Landgraf *et al*, 1995; Pitkow *et al*, 2001; Landgraf *et al*, 2003; Lim *et al*, 2004). Effects on aggressive behaviour have not been examined, but naturally occurring, social history-dependent differences in V1a receptor binding in the AH, PVN, lateral hypothalamus, CeA and lateral VMH of Syrian hamsters may be linked to coincidental differences in expression of aggressive behaviour (Cooper *et al*, 2005; Albers *et al*, 2006).

Plasticity in vasopressin receptor expression may be an important aspect of the putative regulation of aggressive behaviour associated with the vasopressin system. Thus, it was considered that changes in vasopressin receptor expression may be associated with the changes in frequency of expression of aggressive behaviour evident in peripartum rats. *In situ* hybridisation was used to attempt to determine levels of V1a- and V1b-receptor mRNA expression in the LS, BNST, PVN, SON, CeA and MeA in groups of rats that would, or would not, be expected to display aggressive behaviour in a resident-intruder test, based on their reproductive stage.

Although the expression of the V1b receptor has not been linked to expression of aggressive behaviour, the results obtained in Part 1 suggest actions of vasopressin via this receptor to be at least as relevant as those via the V1a receptor.

Methods

Animals

Virgin and late pregnant (GD16-GD17) rats were purchased from Harlan UK Ltd. (250-350 g; Hsd:Sprague Dawley®™SD®™ strain). Following a week of acclimatisation some of the virgin rats were impregnated. The rats were group housed until GD18; late pregnant and lactating rats were housed alone. The rats were housed in polycarbonate cages with food and water freely available. All rats were fed the standard Teklad Global 14 % Protein Rodent Maintenance Diet (diet 2014, Harlan UK Ltd.). For pregnant and lactating rats this was supplemented with a high protein (19 %), high fat (9 %) diet (Teklad Global 19 % Protein Extruded Rodent Diet; diet 2019, Harlan UK Ltd.). Housing was under a 12:12 light:dark cycle (lights on 07:00 h), ambient temperature of 20 ± 2 ˚C and relative humidity 50 ± 10 %.

Tissue collection

Brains were collected from virgin rats (n = 4 – 8) and rats at GD10 (n = 6 – 8), GD21 (n = 5 – 7), GD22 (n = 5 – 6), 2 h postpartum (n = 1 – 6), PD2 (n = 4 – 7), PD7 (n = 6), PD13 (n = 4 – 8) and PD21 (n = 6 – 8). n is dependant on both area and method of quantification (see below). Group 1 included all rats at GD21, GD22, 2 h postpartum, PD2, PD7 and PD13. Group 2 consisted of virgin rats, rats at GD10, and rats at PD21. Group 1 contained rats that would have been expected, under the conditions of the experiment, to give an aggressive response to an intruder. Rats of Group 2 would not have been expected to show aggressive behaviour. Rats were

killed by conscious decapitation, the brains immediately removed, snap frozen on dry ice and stored at -70 ˚C.

Probe synthesis

V1a receptor

A 396 base pair clone extending from the start of the fifth to the mid-seventh transmembrane domain of the rat V1a receptor (kindly provided by S.J. Lolait, University of Bristol, UK) was used for preparation of ^{35}S-labelled sense- and antisense-strand probes by *in vitro* transcription. The template, subcloned in pGEM3z, was linearised by EcoRI or HindIII. Radiolabelled riboprobes were generated using ^{35}S-uridine triphosphate (UTP) in RNA polymerization catalyzed by SP6 (following EcoRI digestion) or T7 (following HindIII digestion) polymerases, to produce antisense or sense probes, respectively.

V1b receptor

The ^{35}S-labelled sense and antisense probes for the V1b receptor were prepared by *in vitro* transcription of the 464 base pairs immediately upstream of the translation start site for the rat V1b receptor (clone again provided by S.J. Lolait, University of Bristol, UK). The template, subcloned in pGEM4z, was linearised by Hind III or EcoRI for production of antisense or sense probes, respectively. SP6 polymerase (for antisense strands) or T7 polymerase (for sense strands) was used to catalyse the polymerisation of probes incorporating ^{35}S-UTP.

Labelled probes were purified using Sephadex columns.

Newly linearised templates and labelled probes were used in each hybridisation. In total, three hybridisations were carried out using each probe: one to process all PVN/SON tissue, one to process all LS/BNST tissue and one to process all amygdala tissue.

Appendices 3 and 4 contain details of the methods for probe amplification and labelling, respectively.

Tissue preparation

Coronal cryostat sections (15 μm) of the collected brains were thaw mounted onto poly-L-lysine slides, fixed (4 % paraformaldehyde/PBS), acetylated (0.1 M triethanolamine/acetic anhydride) and dehydrated in increasing concentrations of ethanol (70 %, 80 % and 95 %, in DEPC-H_2O).

Hybridisation

The brain sections were incubated in a pre-hybridisation solution (2 h; 50 ºC) followed by the hybridisation solution containing the labelled probe ($2x10^6$ counts per min (c.p.m.)/slide; 16-18 h; 55 ºC for V1a receptor, 50 ºC for V1b receptor). Single stranded or loosely bound RNA was removed by incubation in 30 μg/ml RNase (1 h, 37 ºC), washes in 2× SSC (room temperature), and 0.1× SCC (65 ºC for V1a receptor, 60 ºC for V1b receptor).

Visualisation

The slides were exposed to autoradiographic emulsion for 7 weeks (all LS/BNST sections and V1b receptor PVN/SON sections), or 12 weeks (all amygdala and V1a receptor PVN/SON sections), developed, fixed and counterstained for analysis.

Details of the tissue preparation, hybridisation and visualisation are available in Appendix 5.

Analysis

V1a receptor

No V1a receptor mRNA expression was evident in the SON or mPVN. The autoradiograms were analysed for V1a receptor mRNA expression in the LS, BNST, pPVN, CeA and MeA. The slides were coded such that the identity of each sample was unknown during analysis. Identification of labelled cells was subjective during counting, but *post-hoc* sampling suggested selected cells possessed a minimum density of staining $> 5 \times$ the mean staining $+ 3SD$ of unlabelled cells. The number of positive cells per nucleus was counted in the LS, LBNST, medial division of the BNST (MBNST), CeA and MeA. 4-8 sections of each nucleus were analysed per rat. In each section the density of silver grains was analysed (using NIH Image, version 1.62) in three different 292397 μm^2 areas (LS) or three different 6463 μm^2 areas (LBNST, MBNST, CeA and MeA). As it was possible to measure the density of staining within the entire pPVN, no complementary cell counts were made.

Two methods of statistical analysis were used to compare the two measures of mRNA expression between groups:

(i) Two-way ANOVA, followed by pairwise Holm-Sidak tests, was used to compare cell counts and density measurements between groups, but in both cases the data were heteroscedastic and failed tests of normality

(ii) Unpaired t-tests and Mann Whitney rank sum tests (with critical p-values of 0.05) were used to consider differences between groups, for both measures in all areas

V1b receptor

In the tissue labelled for the V1b receptor there was no indication of specific, cellular labelling at 7 (LS/BNST and PVN tissue) or 12 weeks (amygdala tissue) of exposure to autoradiographic emulsion. At these time points the background labelling was increasing without any indication of increasing specific hybridisation and analysis would have been inappropriate.

Results (V1a receptor)

Hybridisation signal

In all regions studied the hybridisation signal achieved was strong and convincingly localised to cells. Single labelled cells were thus easy to identify, unless tightly clustered, in which case the number of cells labelled was estimated by the density and extent of staining.

Distribution of labelling

There was evidence of hybridisation in many regions. The intensity of labelling varied by region. In the SON no labelling was apparent despite that V1a receptor mRNA has previously been found in the rat SON (Hurbin *et al*, 1998) and that immunocytochemical detection of the protein and functional evidence attest to presence of the receptor in this area (Gouzenes *et al*, 1999; Hurbin *et al*, 2002). The apparent lack of mRNA transcription may be due to low levels of *de novo* receptor synthesis, or transcription may have been obscured by rapid rates of mRNA translation/degradation.

Measurements

The data was unsuitable for a rigorous statistical analysis. The data did not conform to the assumptions built into the two-way ANOVA. The results of the (invalid) ANOVA suggested only one potential (p = 0.001) difference between groups: the number of positive cells in the LS was higher in Group 1 (mean ± s.e.m.: Group 1, 97.5 ± 2.8; Group 2, 90.5 ± 3.3; Figure 3.1). This 'difference' is a result of *post hoc*

tests carried out despite no interaction (p = 0.267) and the result was not borne out in a t-test (p = 0.113). Using (invalid) t-tests/Mann Whitney rank sum tests to compare density measurements and cell counts between groups in all areas - interpreting each result using a critical value of p = 0.05 - resulted in 'differences' being reported between groups for the number of labelled cells in the MeA (mean ± s.e.m.: Group 1, 2.9 ± 0.3; Group 2, 1.8 ± 0.3; p = 0.012 (t-test); Figure 3.1) and CeA (mean ± s.e.m.: Group 1, 5.5 ± 0.4; Group 2, 3.9 ± 0.5; p = 0.006 (Mann Whitney rank sum test); Figure 3.1), and the density of labelling in the MeA (mean ± s.e.m.: Group 1, 0.0064 ± 0.0004 m^2/m^2; Group 2, 0.0050 ± 0005 m^2/m^2; p = 0.031 (t-test); Figure 3.1) and LS (mean ± s.e.m.: Group 1, 0.044 ± 0.002 m^2/m^2; Group 2, 0.037 ± 002 m^2/m^2; p = 0.048 (t-test); Figure 3.1). Potentially the lack of difference in amygdala nuclei by ANOVA and the appearance, through ANOVA, of an unexpected indication that the number of positive cells in the LS may be the measure most likely to differ between groups, can be similarly explained. The LS and amygdala constitute the upper and lower extremes of both measurements (positive cells and density), respectively. The standard error associated with each mean is generally low, but again highest for the LS measures and lowest for the amygdala measures. Thus it is possible both that the higher error associated with the other regions conceals differences in the amygdala, and that that the lower error associated with the other areas reveals a false difference in the LS.

If any real difference between groups does exist, it is subtle. In consideration of both sets of results (positive cells and density), which are complementary, and the results of the various tests, a small difference in LS V1a receptor expression (higher in

Group 2) seems plausible. It is probably correct to suggest that no differences were found in either measure between the different BNST and PVN groups.

To allow testing for more subtle temporal changes in receptor expression across the peripartum, the GD22 and 2 h postpartum data were combined to produce a 'peripartum' group of reasonable size for analysis. One-way ANOVA was used to compare time points in each area, for each type of measurement, and two-way ANOVA was used to compare time points for each type of measurement.

Use of one-way ANOVA to compare density measurements across time points did not demonstrate any differences in any brain region. Two-way ANOVA (again invalid; Holm-Sidak method) reported PD2 versus virgin, GD21 and PD21 (all p-values < 0.0001), and PD13 versus virgin (p = 0.001) differences in the LS (Figure 3.2). In the pPVN, there were PD13 versus GD10 and 'peripartum' (p-values < 0.0001), and 'peripartum' versus GD21 (p = 0.001) differences (Figure 3.2).

Use of one-way ANOVA to compare positive cell counts across time points indicated a PD2 versus virgin difference in the CeA (p < 0.05, ANOVA on ranks, Dunn's method; Figure 3.2). In the MeA, one-way ANOVA (Holm-Sidak method) suggested differences between PD13 and virgin (p = 0.000149), PD13 and GD21 (p = 0.000201), PD2 and virgin (p = 0.000941), and PD2 and GD21 (p = 0.00123; Figure 3.2).

Comparisons of positive cell counts by two-way ANOVA (invalid; Holm-Sidak method) reported general differences between the following peripartum stages: PD7 and virgin (p < 0.0001), PD7 and GD21 (p < 0.0001), PD7 and PD21 (p < 0.0001), PD2 and virgin (p = 0.000194), PD2 and GD21 (p = 0.000782), PD2 and PD21 (p = 0.000584), PD13 and virgin (p = 0.00167). In the LS there were PD7 versus virgin, GD10, GD21, 'peripartum', PD13 and PD21 differences (all p-values < 0.0001), PD2 versus virgin (p < 0.0001), GD21 (p = 0.001) and PD21 (p = 0.001) differences, 'peripartum' versus virgin (p = 0.001) and GD10 versus virgin (p = 0.002) differences (Figure 3.2).

Discussion

There was no clear difference in the levels of V1a receptor mRNA expression between rats at typically aggressive or non-aggressive reproductive stages. Previously, levels of V1a receptor binding in the rat BNST were found to be consistent across various reproductive stages (ovariectomy, pregnancy and lactation; Kremarik *et al*, 1991), and in both male and female, prairie and montane voles, the postpartum (PD1 or PD6) levels of V1a receptor binding in the LS, BNST, VMH and amygdala were equivalent to those found in sexually naïve controls (Wang *et al*, 2000). The Syrian hamster provides the only example of altered V1a receptor expression during the peripartum: receptor binding in the DMH was higher, and in the VMH was lower, in a group containing hamsters at PD7 and PD14, compared to cycling virgins (Delville *et al*, 1995).

The suggestion that any relevant central expression of V1a receptors is unchanged in rats, peripartum, is tentative as the data obtained could not be subject to a rigorous statistical analysis, not all conceivably relevant areas were tested and the approach used may have been too crude to detect changes in the relevant circuits. The outcomes of the statistical analysis allowed for the possibility of a small change in receptor mRNA expression in at least the LS. However, understanding the potential relative difference in mRNA expression to be small does not indicate that any associated phenotypic effects would be small. A more useful result may have been obtained had regions with dissimilar levels of receptor expression been considered in separate experiments, and had comparisons between only virgin rats and rats at

PD2/3 (when the highest levels of aggressive behaviour are observed) been made. This suggestion is broadly supported by the analysis of receptor expression across the various peripartum stages. It may also have been preferable to measure receptor binding rather than mRNA as a low but sustained change in mRNA expression levels could accumulate at this level. Also, receptor binding would probably provide a better indication of the abundance of functional receptors, and as such could be more forgivably expected to vary in temporal coincidence with behaviour. It is also likely that visualisation of V1b receptor expression would be possible, using an available specific ligand (Serradeil-Le Gal *et al*, 2003).

In the absence of any peripartum change in vasopressin receptor expression, any change in vasopressin signalling important for the peripartum changes in behaviour would be likely to be associated with the pattern of vasopressin release. The frequency and/or scale of release may be altered. There is the capacity for this: the accumulation of hypothalamic vasopressin mRNA is 2-3 times greater in late pregnant and lactating rats than in cycling controls (Van Tol *et al*, 1988; Zingg and Lefebvre, 1988). If changes in vasopressin release were fully accountable for any peripartum changes in behaviour associated with vasopressin signalling, receptor levels might still be important for individual levels of behaviour. Comparing individual scores of aggressive behaviour with subsequent levels of V1a receptor mRNA expression or V1a receptor binding may indicate this.
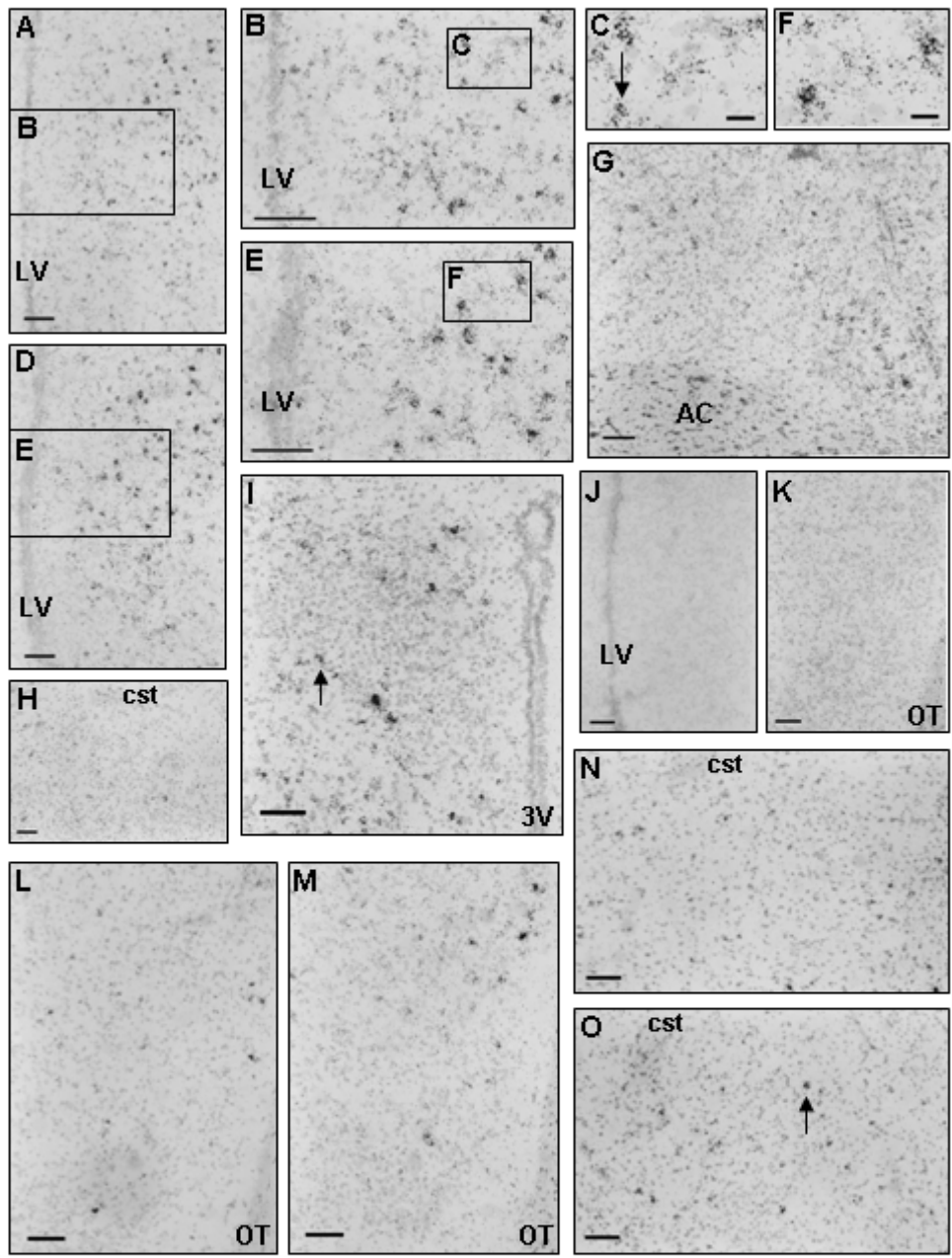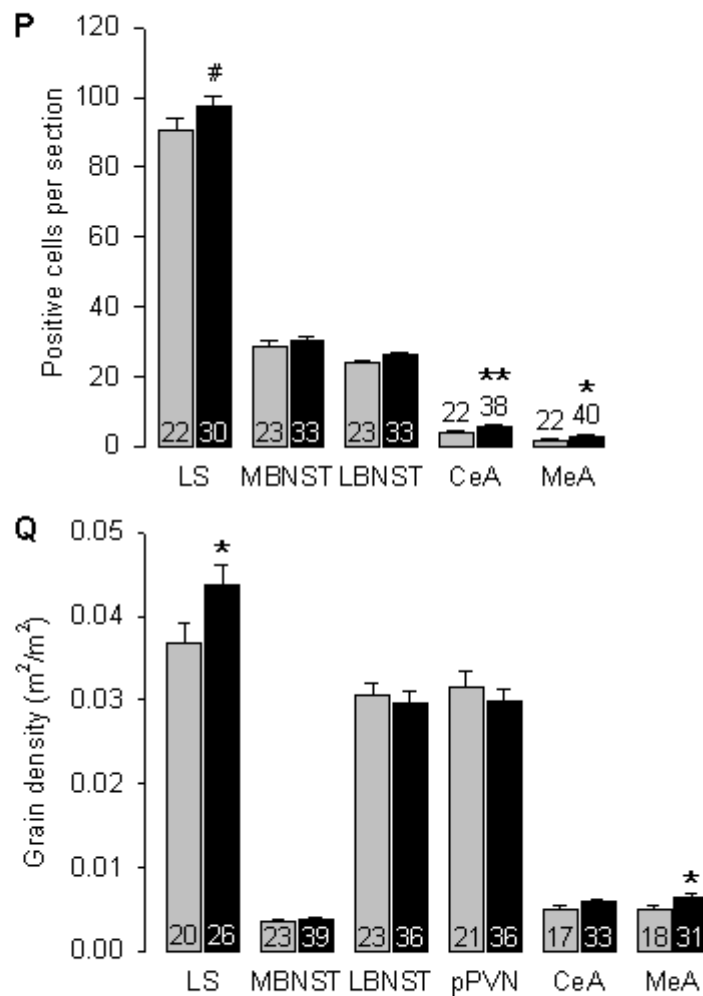
Figure 3.1 V1a receptor mRNA expression and reproductive stage. Mean (+ s.e.m.) counts of silver grain-labelled cells (P), and mean density measurements (+ s.e.m.; Q) in groups 1 (grey bars) and 2 (black bars). Group 1 includes rats at GD21, GD22, 2 h postpartum, PD2, PD7 and PD13. Group 2 includes virgin rats, rats at GD10, and rats at PD21. n, for each group, is indicated on (or above) each bar. #p = 0.001 (two-way ANOVA, Holm-Sidak method), *p < 0.05 (unpaired t-test), **p = 0.006 (Mann Whitney rank sum test) versus control group. Photomicrographs, A-O, were taken through either a 10x/0.3 objective lens (A, D and G-O; scale bar =100 μm) or 20x/0.5 objective lens (B, C, E and F; scale bar = 100 μm (B and E) or 20 μm (C and F)). Examples of the levels at which measurements were made for the LS, BNST, pPVN, MeA and CeA are indicated in A, G, I, L and N, respectively. A, L and N are examples of the LS, MeA and CeA in a control rat. D, M and O are examples from a test rat. Arrows indicate examples of silver grain labelling of single cells. H, J and K show examples of the CeA, LS and MeA, respectively, from sense probe labelled sections. LV, lateral ventricle; AC, anterior commissure; 3V, third ventricle; OT, optic tract; cst, commissural stria terminalis
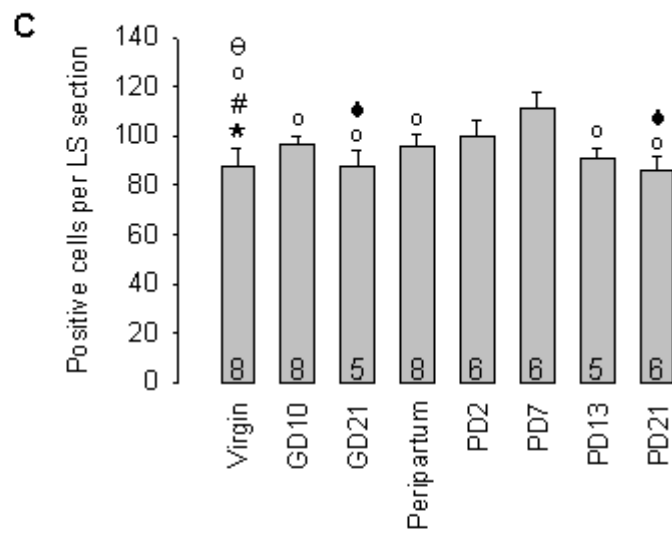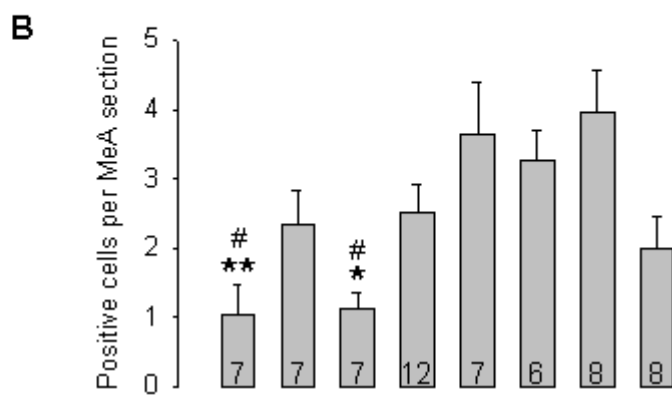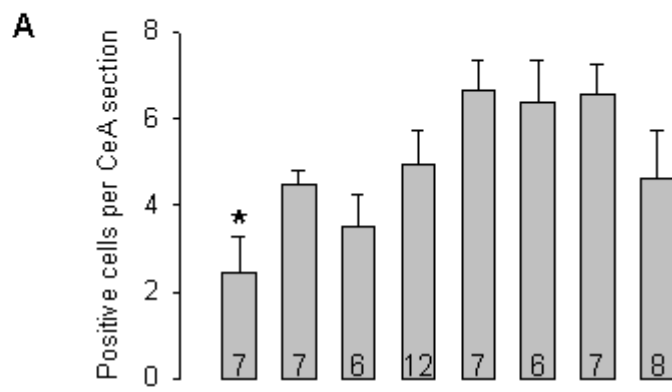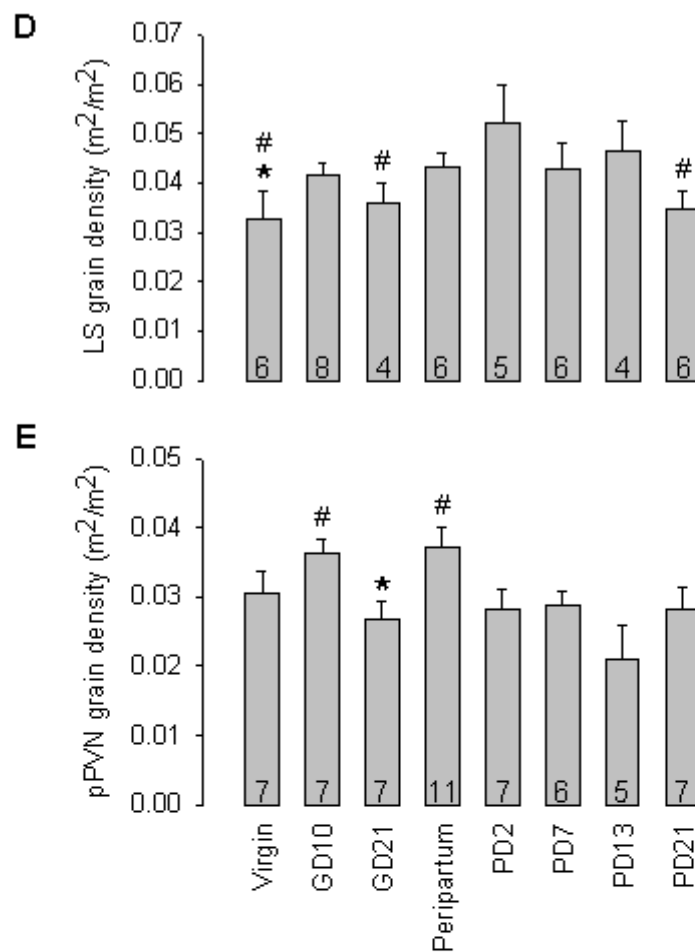
Figure 3.2 V1a receptor mRNA expression across the peripartum.
A Mean (+ s.e.m.) numbers of labelled cells in the CeA of virgin rats, rats at GD10, GD21, 'peripartum', PD2, PD7, PD13 and PD21. *p < 0.05 vs PD2 (one-way ANOVA on ranks, Dunn's method)
B Mean (+s.e.m.) numbers of labelled cells in the MeA of virgin rats, rats at GD10, GD21, 'peripartum', PD2, PD7, PD13 and PD21. *p = 0.00123 vs PD2; **p < 0.001 vs PD2; #p < 0.001 vs PD13 (one-way ANOVA, Holm-Sidak method)
C Mean (+s.e.m.) numbers of labelled cells in the LS of virgin rats, rats at GD10, GD21, 'peripartum', PD2, PD7, PD13 and PD21. *p = 0.002 vs GD10; #p = 0.001 vs 'peripartum', °p < 0.0001 vs PD7; ⁺p = 0.001 vs PD2; ᵉp < 0.0001 vs PD2 (two-way ANOVA, Holm-Sidak method)
D Mean (+ s.e.m.) density measurements from the LS of virgin rats, rats at GD10, GD21, 'peripartum', PD2, PD7, PD13 and PD21. *p = 0.001 vs PD13; #p < 0.0001 vs PD2 (two-way ANOVA, Holm-Sidak method)
E Mean (+ s.e.m.) density measurements from the pPVN of virgin rats, rats at GD10, GD21, 'peripartum', PD2, PD7, PD13 and PD21. *p = 0.001 vs 'peripartum'; #p < 0.0001 vs PD13 (two-way ANOVA, Holm-Sidak method)
♦ n, for each group, is indicated on each bar.

Chapter 4

Central connectivity of magnocellular neurones

Introduction

The source of the vasopressin that appears to act centrally in association with maternal aggression is unknown. More than one vasopressin system has been implicated in other forms of aggressive behaviour. In mice, vasopressin-immunoreactivity in the BNST following exposure to a conspecific is lower in the more aggressive, compared to less aggressive, individuals (Compaan *et al*, 1993). Thus, the parvocellular vasopressin population here – and perhaps the similarly regulated population in the MeA – may provide some of the peptide implicated in the behaviour. However Syrian hamsters, in which exogenous vasopressin facilitates aggressive and flank-marking behaviours (Ferris *et al*, 1988; Ferris and Potegal, 1988), do not appear to have these populations. They appear to have very few parvocellular vasopressin cells outwith the SCN (Ferris *et al*, 1995) and their SCN is not required for flank marking behaviour (Delville *et al*, 1998). Their additional major source of vasopressin is the magnocellular populations of the hypothalamus. 75% of the cells in these populations project to the neurohypophysis and can be eliminated by injection of suicide lectins into the neurohypophysis. The remaining 25% of hypothalamic magnocellular neurones appear sufficient to support flank-marking behaviour (Ferris *et al*, 1992b). Magnocellular vasopressin populations may also be relevant to aggressive behaviour in species that do have parvocellular populations additional to the SCN. Male rats subject to maternal separation as pups are more aggressive as adults, and have higher levels of vasopressin-immunoreactivity and vasopressin mRNA in their SON and PVN, in comparison with untreated controls (Veenema *et al*, 2006). California mice cross-fostered with

the less aggressive while-footed mouse produce lower scores of aggressive behaviour than control California mice and have lower vasopressin-immunoreactivity in both the BNST and SON (Bester-Meredith and Marler, 2001). It is also possible that the activation of V1b receptor labelled SON cells observed to occur during display of maternal aggression (in Chapter 2) is indicative of involvement of SON vasopressinergic neurones. They may be involved even if this indication is misleading as it is not clear that more than basal vasopressin signalling is important.

Unlike the vasopressin cells of the BNST and other parvocellular populations, the magnocellular vasopressin neurones of most species (again the exception is the Syrian hamster) do not have well-described central projections. In the rat, each magnocellular neurone is understood to have a single axon that projects to the neurohypophysis and there is only minimal evidence for some central collaterals that potentially project to the lateral hypothalamus, OB, cerebral cortex, LHb, subcommisural organ, MeA and LC (Mason *et al*, 1984; Alonso *et al*, 1986). Magnocellular neurones of the SON may also project to the PVN (Silverman *et al*, 1981). It is therefore possible that the dendrites of magnocellular neurones, rather than the axons, are their main source of centrally-acting vasopressin. To consider that potential behavioural effects of magnocellular neurone-derived vasopressin may occur following axonal release of peptide into the brain, projections from the magnocellular vasopressin neurones of the SON and PVN to regions that appear important for behaviour (the LS, BNST, CeA, MeA and PAG) were sought.

Methods

Animals

17 to 36 week old female rats (240 to 300 g) were selected from the vasopressin-eGFP transgenic colony described in Appendix 6. Each rat was housed alone during the three days immediately post-operative and from GD18 onward. At other times the rats were group housed. Polycarbonate cages were used for housing, under a 12:12 light:dark cycle (lights on 07:00 h), an ambient temperature of 20 ± 2 °C and relative humidity 50 ± 10 %. Food and water was always available. Throughout the study, rats were provided with the Teklad Global 14 % Protein Rodent Maintenance Diet (diet 2014, Harlan UK Ltd.). During pregnancy and lactation this was supplemented with a high protein (19 %), high fat (9 %) diet (Teklad Global 19 % Protein Extruded Rodent Diet; diet 2019, Harlan UK Ltd.).

Surgery

Under Hypnorm/Hypnovel anaesthesia, single, unilateral stereotaxic injections of 1μl undiluted fluorescent latex microspheres (Red Retrobeads[TM], Lumafluor Inc., USA) were made in the LS (co-ordinates: anteroposterior, -0.2 mm from bregma; mediolateral, ±2.3 mm from midline; dorsoventral, -4.0 mm from dura; angle, 20º from vertical; n = 6), BNST (co-ordinates: anteroposterior, -0.3 mm from bregma; mediolateral, ±3.0 mm from midline; dorsoventral, -5.7 mm from dura; angle, 17º from vertical; n = 8), CeA (co-ordinates: anteroposterior, -2.5 mm from bregma; mediolateral, ±4.0 mm from midline; dorsoventral, -7.0 mm from dura; n = 6), MeA (co-ordinates: anteroposterior, -2.6 mm from bregma; mediolateral, ±3.2 mm from

midline; dorsoventral, -8.0 mm from dura; n = 7) or PAG (co-ordinates: anteroposterior, -7.0 mm from bregma; mediolateral, ±2.0 mm from midline; dorsoventral, -4.7 mm from dura; angle, 21º from vertical, or: anteroposterior, -7.0 mm from bregma; mediolateral, ±2.4 mm from midline; dorsoventral, -4.9 mm from dura; angle, 21º from vertical ; n = 6). Control injections were made in the PVN (co-ordinates: anteroposterior, -2.1 mm from bregma; mediolateral, ±1.7 mm from midline; dorsoventral, -7.5 mm from dura; angle, 10º from vertical; n = 4), which has well described vasopressinergic input from the SCN. Surgical methods are detailed in Appendix 7.

Breeding and tissue collection

After a post-operative recovery period of at least 7 days, the rats were paired with a (wildtype) male. Once pregnant they were returned to their home cages for 18 days and thereafter housed alone. The day of parturition was PD1. Between PD4 and PD6 the rats were injected with a lethal dose of sodium pentobarbitone (1.5 ml i.p.; 54.7 g/l) and the tissue was immediately fixed by transcardial perfusion of 4 % paraformaldehyde (methods detailed in Appendix 1). The brains were quickly removed, stored in post-fix (4 % paraformaldehyde/15 % sucrose/1× PB) for approximately 24 h, cryoprotected in 30 % sucrose/1× PB (24-48 h), snap frozen on dry ice and stored at -70 ºC.

Tissue preparation

52 μm coronal microtome sections of the collected brains were prepared (Leica microtome; -20 to -22 ºC) and temporarily stored in 0.1× PB (room temperature). At

this point epifluorescent illumination (Leica light microscope; N 2.1 filter block (Leica); 10×/0.3 objective lens) was used to verify the site of each injection and the absence of tracer back-flow into the injection track. Brains with unsuitable injections were eliminated from further processing. The sections prepared from brains with acceptable injections were transferred to cryoprotectant and stored in light-proof containers at -20 ºC.

Immunofluorescent staining

The tissue was brought to room temperature and immunolabelled for oxytocin as described in Appendix 8. Briefly, the tissue was exposed to a polyclonal, rabbit anti-oxytocin antibody (anti-oxytocin rabbit polyclonal antibody, raised against rat oxytocin; Calbiochem, UK) for 20 h. Binding was visualised using an indirect, ABC method, with a streptavidin-Alexa Fluor® 633 conjugate (Invitrogen, UK). Omission of the primary antibody resulted in absence of specific labelling.

Imaging

SCN sections were imaged in PVN-injected brains. SON and PVN sections were imaged in all other brains. The BNST was imaged in brains with injections in the LS, CeA and MeA, the MeA in brains injected in the BNST and the CeA in brains with PAG injections. Confocal images were produced using a multi-track configuration with excitation wavelengths of 488, 543 and 633 nm, and detection between 500 and 550 nm (green colour applied), 565-615 nm (red colour applied) and > 650 nm (blue colour applied). Images were acquired as z-series and combined using a maximum intensity projection (Zeiss LSM Image Browser Version 3,2,0,115, Carl Zeiss Ltd.).

Sample SCN, BNST, CeA and MeA sections (at least 4 per brain) were imaged under 20×/0.5 and 63×/1.4 objective lenses. All available SON and PVN sections (at least 11 SON sections and 7 PVN sections per brain) were imaged under a 20×/0.5 objective lens and 2/3 sections per brain under a 63×/1.4 lens. With illumination by incandescent light through the 488 nm laser line and excitation at 543 nm (detection 565-615 nm), under a 10×/0.3 lens, sample injection site images were constructed. Imaging methods are detailed in Appendix 9.

Results

Labelling for vasopressin and oxytocin

The reporter GFP for vasopressin and immunocytochemical staining for oxytocin produced clear and photo-stable labelling of both cells and fibres. In both the SON and PVN the labelling for oxytocin or vasopressin tended to be restricted to particular subregions of the containing nucleus and cellular colocalisation of the two signals was limited.

Tracer

Where concentrated the signal produced by the tracer was strong. Where more diffuse (as when retrogradely transported) the occurrence of tracer in a particular cell, or in some cases nucleus, could have been overlooked without active searching. The nature of the punctate signal (Figure 4.1, U-Y) was strongly indicative that it could be ascribed to tracer rather than autofluorescence or error in imaging parameters. Bleaching did not obviously occur with the typical number of scans (three or four). The bleaching evident with more repeated high resolution scanning was not to the extent of being problematic.

Injections

Three successful injections were made in the LS and the BNST, four in the PAG and two in each of the CeA, MeA and PVN (sites shown in Figure 4.1, A-J and Figure 4.2).

CeA: A reconstruction of an injection in the CeA, using widefield light microscope images superimposed on sequential atlas diagrams (Figure 4.3, A) gives an indication of the volume occupied by tracer at each injection site. In the CeA a relatively large proportion of the nucleus was stained.

PVN: The tracer injected into the PVN covered a similar proportion of the nucleus as that targeted to the CeA. Both PVN injections were centred dorsal to the middle of the nucleus and excluded the ventral part of the pPVN and more lateral mPVN.

BNST: The dorsal BNST (dorsal to the anterior commissure) was covered to a similar extent as the CeA in the dorsal-ventral and medial-lateral planes, but not along the anterior-posterior axis: approximately half of this area was covered by each injection. The injections were placed at different levels such that the majority of the nucleus was covered by the three injections.

LS: The injections in the LS were all similarly placed (centred as in Figure 4.1, A) and occupied an estimated 1/3 of the area of a coronal section at the mid-point of the injection. None of the injections reached the more anterior and posterior parts of the nucleus. The extension of the injections was from around Bregma + 0.7mm to Bregma - 0.4mm (as in Paxinos and Watson, 1998).

MeA: Injections in the MeA were aimed towards the mid-to-ventral aspect of the nucleus to avoid labelling in the optic tract. Thus they both excluded the postero-dorsal part of the nucleus. The ventral edge (posteroventral) was also incompletely

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covered and tracer spilled out of the medial nucleus, into more lateral areas, at the injection mid-point. Generally the basomedial amygdala was excluded.

PAG: In the PAG all injections were centred close to the central canal. Two were located in the dPAG: one very central, one towards the lateral edge. The central injection covered a large extent of the dorsal subregion (unilaterally) extending almost to its dorsal edge. The more lateral injection did not reach the dorsal nor medial edge and crossed into the lateral subregion at some points. A further two injections were located lateral to the central canal. These extended to occupy the dorsal-ventral plane of the lPAG in the medial half of the nuclear subdivision, but did not extend so fully in the lateral portion, nor did either reach the lateral edge. Each PAG injection occupied approximately 1/2 of the nucleus along its anterior-posterior axis, all centred around its mid-point (roughly Bregma - 7.04mm; Paxinos and Watson, 1998).

Retrograde labelling

Despite the incomplete labelling of each nucleus, connections could be found between the injection sites and established afferent nuclei. The following projections were sought and found (Figure 4.1, U-Y and Figure 4.2):

(i) from the BNST to the LS, CeA and MeA,

(ii) from the MeA to the BNST,

(iii) from the CeA to the PAG, and

(iv) from the SCN to PVN

A comprehensive screening for the afferent projections to each injection site was not carried out. It was nevertheless apparent that retrograde labelling occurred in only very discretely localised areas. Each PVN and SON was particularly thoroughly searched and there was no evidence of tracer in the vasopressin- or oxytocin- labelled cells of either the SON or mPVN, regardless of injection site (obviously disregarding the cases where injections were targeted to the PVN; Figure 4.1, K-T). Figure 4.3 (B and C) contains a larger version of one of each of the example SON and PVN images from Figure 4.1. In a few cases (Figure 4.1, M; expanded view in Figure 4.4) a very small extent of retrograde labelling was apparent in the pPVN, but it was not colocalised with an additional marker.

In the SCN colocalisation of signal for vasopressin with signal for tracer was apparent, but only observed in 1-2 SCN cells per section. The tracer labelling in these cells was generally low such that this alone would not have identified the area of staining as a cell body.

The extent of retrograde labelling in the SCN (and elsewhere) was not quantified. Figure 4.2 (C) shows a typical distribution and density of retrograde labelling in a single slice. The extent of labelling of the 'control' nuclei from rats with injections in the LS, BNST, amygdala or PAG was similar. Generally, retrograde labelling appeared not to be distributed across the whole nucleus, but restricted to (variously sized) subregions. Images U-Y in Figure 4.1, higher resolution than Figure 4.2 (C), demonstrate the irregularity of labelling and the difficulty with identifying labelled

cells in the absence of a somatic marker. The areas highlighted in Figures U-Y

provide some of the best examples of putative somatic labelling.

Discussion

No connections were found between the magnocellular neurones of the PVN/SON and the LS, BNST, CeA, MeA or PAG. This is in keeping with previous observations: True Blue/Granular Blue/horseradish peroxidase-wheat germ agglutinin tracer injected into the LS is not transported to either the PVN or SON, and lesion of the PVN does not affect the vasopressin fibres in the LS, MeA or PAG (De Vries and Buijs, 1983). However, following tracer injection in the LS, BNST, CeA, MeA or PAG, only minimal retrograde labelling was evident in the pPVN, which electrophysiological data suggest has efferent connection with the BNST, LS, PAG and possibly MeA (Pittman *et al*, 1981; Ingram and Moos, 1992). Thus, although no connections between magnocellular neurones and the LS, BNST, CeA, MeA or PAG were found, this may be due to limitations in the methodology. The incomplete labelling of each tracer-injected nucleus is a major limitation. The incomplete labelling is a result of the relatively limited diffusion of the latex beads, which often makes them preferable to other tracers: although the limited diffusion does not prevent inadvertent labelling of neighbouring areas, it greatly reduces it. As the cost of this – a bias of labelling to particular nuclear sub-regions – may produce different problems in attempts to describe nuclear interconnections between gross anatomical sites, tracer studies, regardless of the nature of the tracer, seem inferior to electrophysiological techniques in such investigation (although it is possible the electrophysiological results (which are only few) arise from recording of fibres passing through the areas concerned). Some difficultly in visualisation of the retrogradely transported latex beads may also limit their value. Very small somatic

deposits of tracer were observed to occur, requiring that extensive and thorough observation be carried out before proposing a negative result. In addition, such sparse labelling may have been particularly vulnerable to bleaching.

Despite the lack of expected connections from the pPVN to BNST, LS and PAG, all of the other connections designed as controls were found. Bi-directional connection between the MeA and BNST (Krettek and Price, 1978; Coolen and Wood, 1998), direct CeA input to the PAG (Rizvi *et al*, 1991) and BNST input to the CeA and LS (De Vries and Buijs, 1983; Dong *et al*, 2000) have been found in previous tracing studies. Each of these links was apparent. The retrograde tracing also tracked the established projection from the SCN to PVN (Hoorneman and Buijs, 1983; Leak and Moore, 2001). This perhaps indicates that the projections from the pPVN to BNST, LS and PAG are fewer than between these areas. Similarly, although it cannot be suggested that the apparent complete absence of connection from the mPVN/SON to LS, BNST, CeA, MeA or PAG is truly the case, it may be predicted that such projections are relatively few.

Without centrally-projecting axons, contribution of magnocellular vasopressin to central vasopressin signalling in the LS, BNST, CeA, MeA or PAG would depend on peptide release from the somatodendritic compartment and subsequent hormonal activity. Putative central hormonal signalling is characterised by a lack of spatial and temporal constraint, which peptide systems are designed to facilitate. The very widespread activation of vasopressin-responsive cells suggested by the immunocytochemisry experiments to occur during the resident-intruder test is in

keeping with the idea that hormonal activity of vasopressin in the brain may contribute to behaviour, and so would allow that magnocellular neurones, despite their apparent lack of centrally-projecting axons, to contribute vasopressin to regulation of the observed behaviour.

Figure 4.1 Tracer injections in the LS, BNST, MeA, CeA and PAG: retrograde labelling in the SON, PVN and control regions

A-E (10x/0.3; scale bar = 100 µm) show example injection sites (pink, 565-615 nm collected light) in the LS, BNST, MeA, CeA and dPAG, respectively. The location of injections in additional brains included in analysis are indicated by black circles in F-J. K-T (20x/0.5 lens; scale bar = 50 µm) are example confocal images of the PVN (K-O), or SON (P-T), paired with the injection site (A-E) directly above (green, 500-550 nm collected light; blue, > 650 nm collected light). U-Y (63x/1.4 lens; scale bar = 10 µm), also associated with the injection sites A-E, are confocal images taken in the region indicated in the upper, right corners (red, 565-615 nm collected light). The boxed areas are expanded directly below (scale bars = 10 µm). LV, lateral ventricle; AC, anterior commissure; OT, optic tract; CC, central canal.
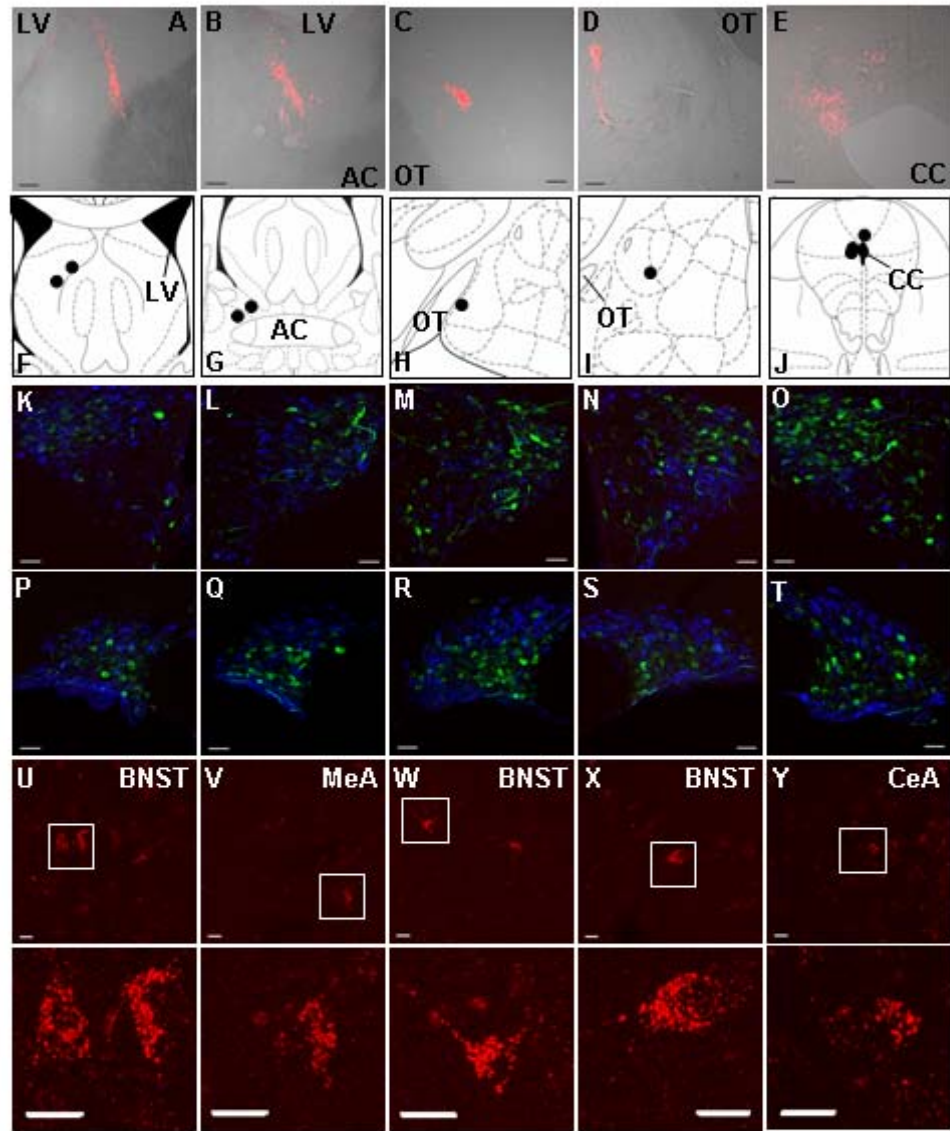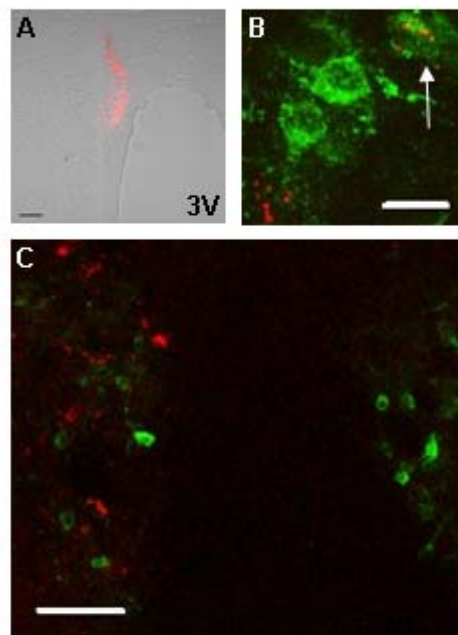
Figure 4.2 Tracer injection in the PVN: retrograde labelling in the SCN

False transmission image, A (10x/0.3; scale bar = 100 µm) displays position of tracer injection site (pink, 565-615 nm collected light). Red staining (565-615 nm collected light) in B (63x/1.4; scale bar = 10 µm) and C (20x/0.5; scale bar = 50 µm) demonstrates retrograde labelling in the SCN. Arrow indicates a GFP-positive cell (green, 500-550 nm collected light) retrogradely-labelled with tracer. 3V, third ventricle
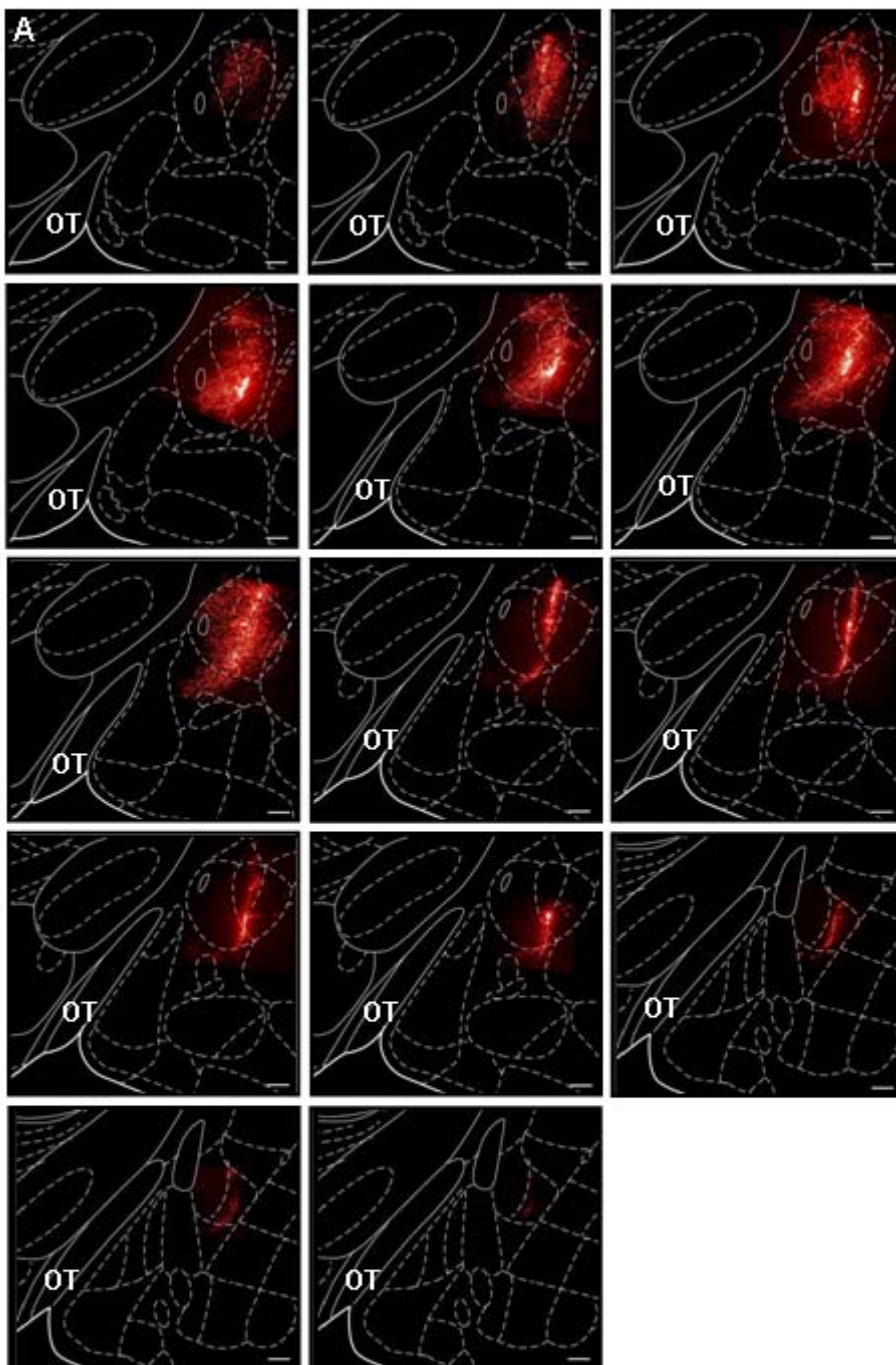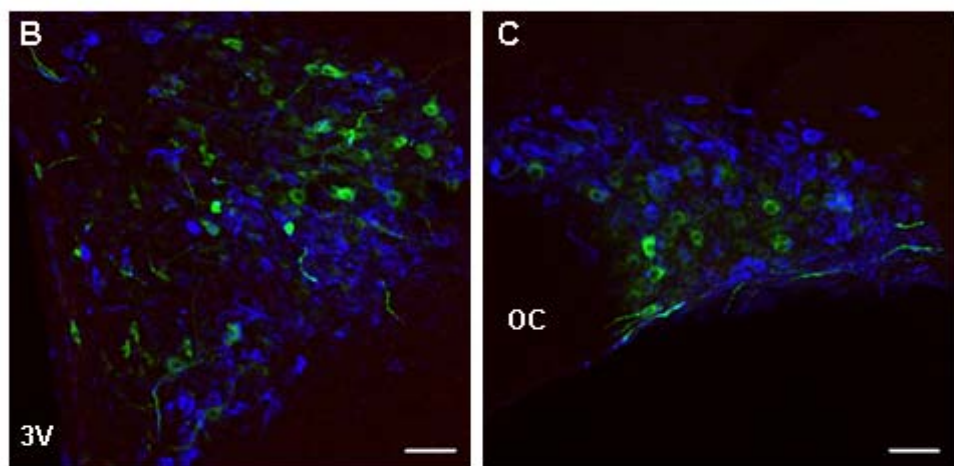
Figure 4.3 Example of volume occupied by injection and corresponding retrograde labelling in the PVN/SON

A Digital photomicrographs of a single tracer injection (red) in the CeA, taken from serial tissue sections (anterior, top left, to posterior, bottom right; 10x/0.3 objective lens; N2.1 filter cube (Leica)), superimposed on diagrams of coronal brain sections (bregma – 2.30 to bregma – 3.14), from the atlas of Paxinos and Watson (1998). Scale bars (200 μm) correspond to the injection images. OT, optic tract.

B and C Confocal images of the PVN (B), and SON (C), paired with the injection in A (20x/0.5 objective lens; scale bar = 50 μm; green, 500-550 nm collected light; red, 565-615 nm collected light; blue, > 650 nm collected light). OC, optic chiasm; 3V, third ventricle
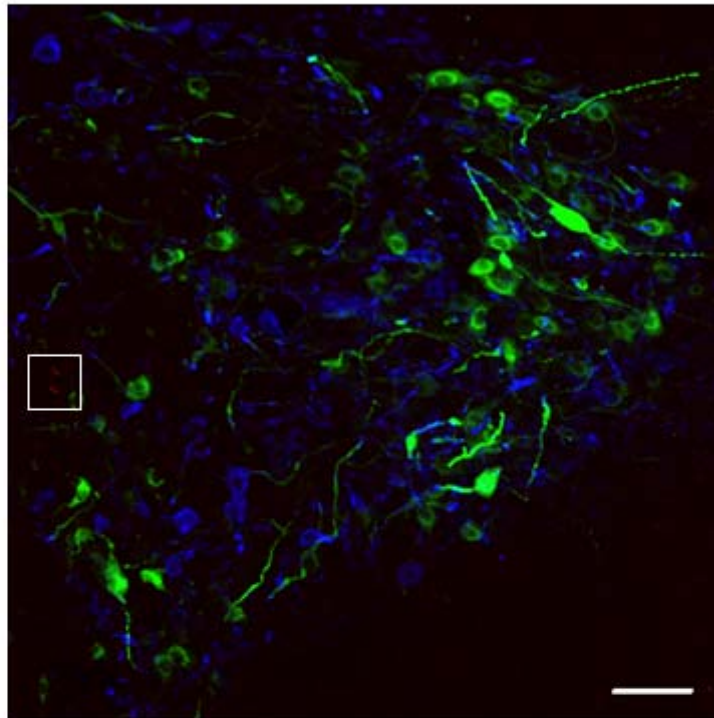
Figure 4.4 Retrograde labelling in the PVN

Confocal image of a PVN section from a rat with tracer injected in the MeA (20x/0.5 objective lens; scale bar = 50 µm; green, 500-550 nm collected light; red, 565-615 nm collected light; blue, > 650 nm collected light). The boxed area indicates an example of red signal.

Chapter 5

General discussion

In the first presented experiments immunocytochemical labelling for Fos was used to identify brain regions activated in association with maternal aggression. This was combined with immunocytochemical labelling for the V1a receptor, V1b receptor or CRF. These proteins were used as markers of particular central vasopressinergic or vasopressin-responsive populations.

In the vasopressin neurones of the pPVN that project to the median eminence and the vasopressin populations of the mPVN and SON – the sources of vasopressin for peripheral use – there was limited, if any, difference in levels of Fos expression between lactating rats subject to a resident intruder test (that displayed aggressive behaviour) and non-tested controls. The basal activity of these populations may have been relevant to the aggressive behaviour displayed during the test. This was not found, but as Fos is an unreliable guide to basal levels of neuronal activity a link cannot be convincingly ruled out.

Fos was, however, surprisingly useful in connecting vasopressin signalling within the brain with aggressive behaviour. Initial consideration of general Fos expression within various brain regions revealed widespread induction of Fos during the test. The levels of Fos expression in the BNST, pPVN and CeA were positively correlated with scores of aggressive behaviour. The Fos expression in the CeA and the pPVN was more tightly correlated with particular components of aggressive behaviour than the general aggressive behaviour score: activity in the CeA was associated with pinning down and activity in the pPVN with attack and lateral threat. As Fos is only one of many immediate early genes, is linked only to particular changes in cellular

activity, to changes in cellular activity that are widely used, and was measured in large/complex areas that are relevant to a range of functions, the correlations between Fos expression in the BNST/pPVN/CeA and aggressive behaviour are surprising. The correlations between Fos expression in different regions and quite specific behaviours (pinning down, lateral threat and attack) are useful because they indicate that the signalling is linked to behaviour, rather than coincident – possibly co-regulated – output, as it is difficult to conceive that this would vary so specifically. The activation of these sites may be reflective of aspects of signalling that have strong regulatory influences over the observed behaviour, may be regions where the behaviour is gated, or both.

Vasopressin is expected to provide a contribution to this potentially regulatory signalling as positive correlations between activation of neurones expressing the V1b receptor and scores of aggressive behaviour were found in the BNST and CeA, and in the CeA the activation of V1b receptor-expressing neurones was positively correlated with pinning down. The previous findings linking vasopressin receptor binding in particular brain regions to particular behaviours in prairie voles are helpful because they suggest the correlations here are not just a consequence of the observed extensive distribution of vasopressin receptor expression and that the putative vasopressin signalling is not a passive facilitation of a regulatory input characterised upstream. In turn, the results here suggest the receptor correlations with behaviour in prairie voles may be linked to behaviour rather than/in addition to coincident function. But despite these correlations, and the putative implication of vasopressin in the regulation of a strikingly high number of behaviours, the results are unable to

provide an indication of the relative importance of vasopressin signalling to behaviour.

In a previous study investigating isolation potentiated startle – which suggests vasopressin signalling may be relevant to regulation of another class of behaviour - V1a receptor binding in the LBNST was positively correlated with behavioural measures, but CRF and oxytocin receptor binding – in the shell of the nucleus accumbens and intermediate zone of the LS, respectively – also shared such relationships with the behaviour and linear combination of the three measures provided a better predictor of behaviour than any measure in isolation (Nair *et al*, 2005). Thus, the three systems (of three investigated) all have an identifiable association with the behaviour and investigation of only one here should not allow the suggestion that vasopressin is a major regulator of the behaviour observed without sufficient investigation of other systems. One might predict the number of relevant systems to be high, as it would seem appropriate for anything that signals something of state to be selected to contribute to regulation of behaviour.

Such signals may affect behaviour as a direct result of their actions as required for their additional physiological functions. Vasopressin, for example, may contribute to the peripartum changes in behaviour only as a result of its additional roles in maintenance of peripartum physiology. As such it would be an efficient and very reliable indicator of some aspects of internal state and optimal behavioural functioning. The changes in blood volume and associated re-setting of the threshold for neurohypophyseal vasopressin secretion that occur peripartum may, in particular,

provide alterations in vasopressin signalling that contribute to the changes from non-peripartum to peripartum behaviour. Release of vasopressin from the neurohypophysis is maintained near maximum efficiency by the firing patterns of magnocellular vasopressin neurones, which may depend on autoregulatory communication by vasopressin released into the magnocellular nuclei in response to neuronal stimulation. Application of vasopressin or specific vasopressin antagonists impacts on the electrical activity of magnocellular vasopressin neurones (Moos and Desarménien, 2004). In rats, the relative abundance of vasopressin and vasopressin receptors, colocalised in the LDCVs found in SON magnocellular dendrites, is varied according to the hydration state of the animal, suggestive that the vasopressin has an autoregulatory role (Hurbin *et al*, 2002). Vasopressin affects the firing pattern of magnocellular neurones according to their existing pattern of activity: vasopressin induces or enhances phasic firing in neurones with relatively low existing activity, it decreases the intraburst frequency of relatively continuously active neurones, leading them to acquire a phasic activity pattern, and has little effect on the activity of neurones with existing phasic patterns of activity (Gouzenes *et al*, 1998). Thus, autoregulatory actions of vasopressin may contribute to regularisation of activity within magnocellular neurones, this being a method of ensuring efficacy of neurohypophyseal peptide secretion, such that maintaining a higher blood volume associated with changes in stimulation of magnocellular neurones would be associated with changes in intranuclear peptide release. If the locations and density of central vasopressin receptors are unchanged in the peripartum (as found, in the areas investigated, for the V1a receptor by *in situ* hybridisation), such changes in magnitude/frequency of vasopressin signalling would be important for vasopressin to

impact on the changes in behavioural pattern. Also, although it is not understood that magnocellular vasopressin has any direct association with behaviour, the observed lack of centrally-projecting magnocellular neurones by retrograde tracing suggests that somatodendritic sources would supply any magnocellular vasopressin that may be associated with behaviour. It is presumed that at least the pPVN – being so proximate to the mPVN – would see such a peripartum change in basal intranuclear vasopressin release, but the parameters of neuropeptide signalling, as they are currently understood, suggest the impact might be much more widespread (see Ludwig and Leng, 2006 for review). This does suggest that blood volume, and in turn body weight, would be somewhat predictive of behaviour, but body weight should be an important influence on behaviour and the suggestion may not be inappropriate. A study that has demonstrated higher plasma vasopressin concentrations in a group of patients with autistic spectrum disorders compared to controls (without differences in body mass index or blood pressure) is further indicative that this osmoregulatory system may be associated with behaviour (Boso *et al*, 2007).

One problem with the suggestions that changes in magnocellular stimulation may be associated with changes in behaviour via actions of somatodendritically released magnocellular vasopressin at distant sites is that they are based on the assumption that the vasopressin is able to reach these sites. Although current ideas about the characteristics of neuropeptide systems suggest neuropeptide activity to be relatively unrestricted in time and space, it is not really clear to what extent they are (un)restricted in either dimension. The dynamics of their movement and metabolism

in the extracellular fluid is not well described and is very likely to vary across the brain. What is expected, is that vasopressin, once released, must reach the c.s.f. or be metabolised by neuropeptidases and neuropeptidases do not act sufficiently quickly to metabolise all vasopressin before it reaches the c.s.f.. There is therefore sufficient scope for the long-distance signalling proposed to occur and it seems unlikely that intranuclear release of vasopressin in a particular system would not at least contaminate other vasopressin systems. It may not be the case, but current understanding of central peptide systems would at least allow that the vasopressin involved in behaviour is not necessarily different to that with additional functions and regulated according to these functions.

At the other extreme of potential mechanisms through which vasopressin may be considered to signal to facilitate peripartum changes in behaviour, the implicated vasopressin would be incorporated into circuits designated for production of behaviour (which are not known to exist) and its synthesis/secretion would be regulated by intermediary signals of the peripartum. Activated, rather than basal, release of peptide would be important and it would be released at synapses with post-synaptic effects relating to behaviour. It is very difficult to conceive that it would not affect neighbouring cells, but it may only signal locally over a reasonably short time-scale.

Between these two extremes, various combinations are possible, but a system more towards the first proposal is preferred for a number of reasons. This system may be more efficient: it does not require duplication of potentially many neurochemical

systems, it leaves less room for error in co-ordination of behaviour and internal state, and it does not require activation of the vasopressin system in association with behaviour-eliciting stimuli. The system would also provide a relatively high potential for flexibility in alternating between behaviours as the degree of peptidergic effects would be relatively balanced across all behaviours. In the second proposal it would be possible to potentiate one behaviour without affecting others: without rapid loss of the peptide signal, this may leave the particular behaviour with a relatively increased likelihood of occurrence despite the immediate context.

If, as proposed, signalling necessarily associated with the peripartum is implicated in behaviour, then many additional systems that have altered regulation at this time (including additional vasopressin systems such as those involved in peripartum changes in thermoregulation and nociception) should equally be expected to contribute. The various signals, including vasopressin, would also be expected to influence peripartum changes in behaviour additional to aggressive behaviours. The results from the first experiments described may indicate this to be the case. Vasopressin signalling was found to be associated with maternal behaviour in addition to aggressive behaviour. However, the association with maternal behaviour was negative. If vasopressin is a signal of the peripartum state, it is not intuitively obvious that it would negatively regulate maternal behaviour. The negative association is also a slightly unexpected result in view of the previous observation that vasopressin has – if anything – a positive effect on maternal behaviour when administered to an isolated lactating rat, with pups (Pedersen *et al*, 1992). Potentially, the negative correlation is observed as a reflection of a system

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constructed to prevent concurrent activation of more than one of the generally mutually exclusive forms/styles of behaviour. In response to a particular environmental stimulus, it is preferable to exhibit the most appropriate behaviour, but equally important to inhibit those that would be inappropriate. Such a system may exist for co-ordinated regulation of reproductive and defensive behaviours in mice as neurones activated by stimuli that elicit defensive behaviours have been observed to provide excitatory input to regions that receive inhibitory input from neurones responsive to stimuli that elicit reproductive behaviours (Choi *et al*, 2005). Potentially, vasopressin signalling in a peripartum context without a stimulus promoting aggressive behaviour would be positively associated with maternal behaviour and negatively associated with aggressive behaviour. The results obtained are therefore in keeping with the consideration that vasopressin potentially impacts a range of behaviours and are indicative of context-dependent effects on the frequency of expression of these behaviours.

Such putative effect on the frequency of expression of behaviour is interesting because the frequency of expression of particular behaviours in particular contexts characterises their physiological versus pathological dimensions. The possibility that vasopressin may contribute to regulation of multiple behaviours – those identified here and those identified in the prairie vole studies, including isolation potentiated startle – is interesting because it allows that vasopressin may be a strong regulator of behaviour in breadth as well as depth, which may be suggestive of co-regulation and covariance between multiple behavioural traits. Such a phenotypic connection has been found to exist between various cognitive traits (Jensen, 1998) and is expected to

be the result of conservation of regulatory factors across traits (Petrill, 1997). That particular factors may influence a range of behaviours is also interesting in view of behavioural pathologies as these are generally characterised by problems across a wide spectrum of contexts and behaviours. With regard to physiology it is intriguing as such putative general regulation of behaviour would presumably be important for the production of a trans-context behavioural style, a lifestyle, or personality.

However, the implication of vasopressin in the basis of both physiological and pathological dimensions of behaviour and the suggestion that multiple traits may have some common sources of variability cannot reasonably be made without first demonstrating that vasopressin is relatively influential in regulation of behaviour. Although behaviour is considered to result from interactions between multiple genomic loci and environmental stimuli, small effects of single locus genotype appear to be identifiable with sufficiently large samples (e.g. Butcher *et al*, 2005). Thus, use of candidate gene association studies and whole genome analysis might be successful in identification of loci with relatively strong effect on phenotype – typically the only loci identified using such approaches. They may also provide a rough indication of the degree of influence exerted by each locus/implicated system. It is presumed that the vasopressin system (and therefore any system with greater effect) would be identified as a regulator of behaviour using such techniques as the extent of expression of the V1a receptors found to be correlated with particular behaviours in prairie voles was – in some areas – found to correlate with a polymorphism in the promoter region of the *v1ar* gene (Hammock *et a*l, 2005). Also, polymorphisms in the human *v1ar* gene have been linked to incidence of autistic

traits (Kim e*t* al, 2002; Wassink *et al*, 2004; Yirmiya *et al*, 2006). The prevalence of autistic spectrum conditions indicates that individual components of their multigenic basis are beneficial and contribute to 'normal' variation in behaviour.

Following identification of loci associated with the structure of the vasopressin system and behaviour, and determination of other loci associated with the structure of additional systems and behaviour, there would exist a background on which their relationships with behaviour could begin to be characterised, from where potentially practical questions could be addressed. Estimation of the extent and distribution of effects of individual systems on behaviour would require investigation of interactions and interdependence of multiple locus genotypes, gene × environment effects and developmental effects. Relationships between 'normal' variability in behaviour and pathological behaviour could be assessed. It might be possible to predict sub-classes of behavioural disorders – based on behavioural phenotypes – according to different potential aetiologies. The information may aid in the ability to detect genotypes at risk of pathology, genotypes for which particular environments/experiences may pose a relatively high health risk and could provide an opportunity for development of prophylactic and rehabilitation strategies.

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Appendices

Appendix 1

Perfusion fixation

Anaesthesia was induced by injection of sodium pentobarbitone (1.5 ml i.p.; 54.7 g/l; Ceva Sante Animale, France). The heart was exposed on appearance of apnoea, and a blunt needle inserted through the left ventricle into the ascending aorta. The right atrium was pierced to allow loss of fluid, and the animal perfused with 50-100 ml, ice-cold, heparinised (129 mg/l; 151 units/mg; Sigma-Aldrich, UK) saline (0.9 % w/v), delivered through the inserted needle, using a peristaltic pump (MO45; Autoclude Ltd., UK). This was followed by perfusion with 300-400 ml, ice-cold 4 % paraformaldehyde/$1\times$ PB.

Appendix 2

Immunocytochemical staining of free-floating rat brain sections

52 μm tissue sections were processed through the stages below. Except where otherwise stated, all steps were carried out with agitation, at room temperature.

First primary antibody application

1. 4×15 min washes in $1\times$ PB-T

2. 5 min wash in $1\times$ PB

3. 15 min wash in 0.3 % H_2O_2 (Sigma-Aldrich, UK; volume/volume (v/v)), prepared in 80 % $1\times$ PB/20 % methanol (Fisher Scientific, UK; v/v)

4. 3×10 min washes in $1\times$ PB-T

5. 20 min wash in 3 % skim milk powder/$1\times$ PB

6. 3×10 min washes in $1\times$ PB-T

7. 30 min wash in 3 % normal sheep serum (Sigma-Aldrich, UK)/$1\times$ PB-T

8. Incubation in Fos antibody (anti-c-Fos (Ab-2) rabbit polyclonal antibody, raised against amino acids 4-17 of human c-Fos; Calbiochem, UK; diluted 1:1000 in 3 % normal sheep serum/$1\times$ PB-T; 48 h; 4 ºC).

Visualisation 1

1. 3×10 min washes in $1\times$ PB-T

2. Incubation in biotinylated goat anti-rabbit secondary antibody solution (10 μl biotinylated goat anti-rabbit antibody : 30 μl normal goat serum : 1 ml $1\times$ PB-T)

for 1 hour (antibody and serum components of Vectastain Elite ABC Kit; Vector Laboratories, UK).

3. 3 × 10 min washes in 1× PB-T

4. Incubation in pre-prepared (30-60 min before use) ABC solution (20 μl streptavidin : 20 μl biotinylated horseradish peroxidase : 1 ml 1× PB-T) for 1 h (streptavidin and biotinylated peroxidase components of Vectastain Elite ABC Kit; Vector Laboratories, UK).

5. 2 × 10 min washes in 1× PB-T

6. 5 min wash in 0.1 M sodium acetate (Fisher Scientific, UK; pH 6.0)

7. Incubation in visualisation solution (25 mg DAB (Sigma-Aldrich, UK) in 50 ml distilled water added to 50 ml 0.2 M sodium acetate (pH 6.0) containing 2.5 g ammonium nickel (II) sulfate (Fisher Scientific, UK) and 0.08 g ammonium chloride (Sigma-Aldrich, UK), with 100 μl H_2O_2 added just before use) for approximately 2.5 min (sections monitored to determine optimal incubation time)

8. 5 min wash in 0.1 M sodium acetate (pH 6.0)

9. 2 × 5 min washes in 1× PB

Second primary antibody application

1. 2 × 5 min washes in 1× PB-T

2. 15 min wash in 0.3 % H_2O_2 (v/v: prepared in 80 % 1× PB/20 % methanol (v/v))

3. 2 × 10 min washes in 1× PB-T

4. 20 min wash in 3 % normal sheep serum/1× PB-T

5. Incubation in second primary antibody solution (detailed in main text) for 72 h, at 4 ºC.

Visualisation 2

1. 3×10 min washes in $1\times$ PB-T

2. Incubation in biotinylated anti-rabbit secondary antibody solution (10 µl biotinylated goat anti-rabbit antibody : 30 µl normal goat serum : 1 ml $1\times$ PB-T) for 1 h.

3. 3×10 min washes in $1\times$ PB-T

4. Incubation in pre-prepared (30-60 min before use) ABC solution (20 µl streptavidin : 20 µl biotinylated horseradish peroxidase : 1 ml $1\times$ PB-T) for 1 h.

5. 2×10 min washes in $1\times$ PB-T

6. 5 min wash in $1\times$ PB

7. Visualisation with either:

   • 99 ml $1\times$ PB : 25 mg DAB (in 1 ml ddH$_2$O) : 100 µl H$_2$O$_2$, or:

   • 94 ml $1\times$ PB : 2.28 ml Reagent 1 : 2.28 ml Reagent 2 : 2.28 ml Reagent 3 : 100 µl H$_2$O$_2$ (Reagents 1-3 from Vector VIP Substrate Kit, Vector Labs, UK)

   Choice of chromogen is indicated in the main text. Sections were monitored during reaction to detect optimal incubation time.

8. 4×5 min washes in $1\times$ PB

Tissue mounting and coverslipping

Sections were mounted onto gelatine (5 %) subbed, glass microscope slides, dehydrated through washes (each 5 min) in increasing concentrations of ethanol (70 %, 80 %, 95 %, 2×100 %; Fisher Scientific, UK), cleared in zylene (2×5 min washes), and coverslipped (VWR, UK) using DePeX (VWR, UK).

Appendix 3

Probe amplification and linearisation for *in situ* hybridisation

Plasmid replication

Clones of (i) a 396 base pair fragment of the rat V1a receptor (extending from the start of the fifth to the mid-seventh transmembrane domain), in pGEM3z, and (ii) a 464 base pair fragment immediately upstream of the translation start site for the rat V1b receptor, in pGEM4z, were generously provided by S.J. Lolait, University of Bristol, as approximately 7.6 μg/μl solutions in TE buffer (pH 8.0). Both plasmid solutions were processed as follows.

1 μl ice cool plasmid solution, in 100 μl ice cool HB101 cell solution (> 18^8 colony forming units/μg; Promega, UK), was chilled on ice (in 4 ml Simport Cultubes^{TM} Sterile Culture Tubes; Jencons (Scientific) Ltd., UK; 1 h), heat-shocked in a 42 ºC waterbath (75 s), and returned to ice (2-3 min).

400 μl TY medium was added, and the solution placed in a shaking incubator (Unitron, Infors AG, Switzerland; 200 revolutions per minute (r.p.m.); 37 ºC). After a 30-min incubation, a 200 μl sample of the solution was spread over an agar/ampicillin plate (3.5 g Agar (Sigma-Aldrich, UK) : 5 μg ampicillin (Sigma-Aldrich, UK) : 100 ml ddH$_2$O). The plates were incubated at 37 ºC (15 h), and chilled at 4 ºC (2.5 h). A colony from each plate, in 1.5 ml TY (in 4 ml Simport Cultubes^{TM} Sterile Culture Tubes) was incubated at 37 ºC (shaking incubator; 4

hours). The solution was transferred to 50 ml TY (in 250 ml flask, sealed with parafilm), and returned to 37 ºC (shaking incubator, 16 h).

Plasmid purification

The cultures were centrifuged (in 50 ml Falcon tubes; 15 min; 5000 r.p.m.; 4 ºC), supernatant removed, and pellet kept on ice. Plasmid DNA was purified using the Qiagen HiSpeed Plasmid Midi Kit (Qiagen, UK), following the supplied instruction:

1. 4 ml ice cool Buffer P1 was used to resuspend the pellet

2. 4 ml Buffer P2 was added, the solution mixed, and incubated for 5 min (room temperature)

3. 4 ml Buffer P3 was added, the solution mixed, and transferred to a QIAfilter Cartridge, for a further incubation (10 min, room temperature)

4. The lysate was filtered (with pressure) into a HiSpeed Tip (pre-equilibrated with 4 ml gravity filtered Buffer QBT).

5. The HiSpeed Tip was washed with 20 ml Buffer QC, and the DNA eluted in 5 ml Buffer QF

6. 3.5 ml isopropanol was added to the eluate, the solution mixed and incubated (5 min, room temperature), and transferred to a 20 ml syringe with attached QIAprecipitator

7. Using pressure, the solution was filtered through the QIAprecipitator

8. 2 ml 70 % ethanol was added to the syringe, and used to wash the QIAprecipitator

9. The membrane was twice air-dried by applying pressure to the air in the syringe, and the outlet dried with absorbent paper

10. The QIAprecipitator was attached to a 5 ml syringe, and 1 ml Buffer TE applied to elute the DNA

11. The eluate was transferred into the 5 ml syringe, and used to elute remaining DNA into the same collection tube

12. The optical density/DNA concentration was measured (eluate diluted 1:5 in TE Buffer; BioMate 3 UV-Vis Spectrophotometer, Thermo Scientific, USA.), before storage at -20 ºC.

Plasmid linearisation

Plasmids were digested to produce templates for sense and antisense probe generation. A double digest preparation was used as a control. For linearisation of the V1a receptor probe-containing plasmids, the following components were added to three separate 0.5 ml eppendorfs (on ice), before incubation in a 37 ºC waterbath (2 h):

1. For antisense template: 20 μl purified plasmid DNA, 4 μl EcoRI (Promega, UK), 10 μl Buffer H (Promega, UK), 10 μl bovine serum albumin (BSA; Promega, UK), 56 μl DEPC-H_2O

2. For sense template: 20 μl purified plasmid DNA, 4 μl HindIII (Promega, UK), 10 μl Buffer E (Promega, UK), 10 μl BSA, 56 μl DEPC-H_2O

3. Double digest: 5 μl purified plasmid DNA, 1 μl EcoRI, 1 μl HindIII, 2.5 μl Buffer M, 2.5 μl BSA, 13 μl DEPC-H_2O

For linearisation of the V1b receptor probe-containing plasmids, the following components were added to three separate 0.5 ml eppendorfs (on ice), before incubation in a 37 ºC waterbath (2 h):

1. For antisense template: 20 µl purified plasmid DNA, 4 µl HindIII, 10 µl Buffer E, 10 µl BSA, 56 µl DEPC-H_2O

2. For sense template: 20 µl purified plasmid DNA, 4 µl EcoRI, 10 µl Buffer H, 10 µl BSA, 56 µl DEPC-H_2O

3. Double digest: 5 µl purified plasmid DNA, 1 µl EcoRI, 1 µl HindIII, 2.5 µl Buffer M, 2.5 µl BSA, 13 µl DEPC-H_2O

The antisense and sense template preparations were processed through the following steps:

1. Addition of 100 µl phenol:chloroform isoamyl alcohol (Fisher Scientific, UK), vortexing and centrifugation (13000 r.p.m., 5 min)

2. Removal of top layer, addition of an equal volume of chloroform:isoamyl alcohol (Sigma-Aldrich, UK), vortexing and centrifugation (13000 r.p.m., 5 min)

3. Removal of top layer and addition of 5 M NaCl (volume of NaCl added = 1/10 volume of layer collected)

4. Addition of 100 % ethanol (volume equal to total volume of collected layer + NaCl)

5. Solution mixed, incubated on dry ice (5 min), thawed, and returned to dry ice (5 min)

6. Centrifugation (13000 r.p.m., 15 min)

7. Removal of supernatant, and drying of remaining DNA pellet (air-drying, approximately 1 h)

8. Pellet re-suspended in 15 μl DEPC-H$_2$O and stored at -20 ºC

Electrophoresis of plasmid fragments

1 μl of each digested plasmid solution (sense, antisense or double digest), was combined with 3 μl ddH$_2$O and 1 μl BlueJuice^{TM} (Invitrogen, UK). The 3 solutions were run on an agarose gel (1.5 g agarose (Promega, UK), 100 ml 1× TBE buffer and 1 μl ethidium bromide (Sigma-Aldrich, UK)), in 1× TBE buffer (Promega, UK), with 100 base pair (Invitrogen, UK), and 200-10000 base pair (Hyperladder I, Bioline, UK) ladders (140 V, 50 min). An ultraviolet (UV)-light transilluminator and imaging software (Syngene Gene Genius Bioimaging System, Synoptics Ltd., UK) were used to detect DNA migration.

Appendix 4

Probe labelling for *in situ* hybridisation

Labelling

For each probe, the following components were added to two separate 0.5 ml eppendorfs:

1. To produce labelled antisense probes: 4 μl 5× transcription buffer (Promega, UK), 2 μl adenosine triphosphate (ATP; Promega, UK), 2 μl cytidine triphosphate (CTP; Promega, UK), 2 μl guanosine triphosphate (GTP; Promega, UK), 2 μl 100 mM DTT, 1 μl linearised antisense template, 1 μl RNaseIn (Promega, UK), 8 μl ^{35}S-UTP (PerkinElmer, UK), 2 μl SP6 (Promega, UK)

2. To produce labelled sense probes: 4 μl 5x transcription buffer, 2 μl ATP, 2 μl CTP, 2 μl GTP, 2 μl 100 mM DTT, 1 μl linearised sense template, 1 μl RNaseIn, 2 μl ^{35}S-UTP, 2 μl T7 (Promega, UK)

The solutions were vortexed and incubated for 2 h (37 ºC waterbath (T7), or 40 ºC waterbath (SP6)). 2 μl DNase was added and the solutions further incubated (15 min).

Purification

Separate Nick Columns (GE Healthcare, UK) were used for purification of sense and antisense probes. The water was poured out of each column, 1 ml TE buffer added, and this poured out. 3 ml TE buffer was added and eluted, and one labelling solution applied to each Column gel bed. 400 μl TE buffer was added and eluted. A second

400µl TE buffer was added, and the eluate collected. The specific activity of the eluate (2 µl in 3.5 ml scintillation fluid), was counted (over 4 min, Beckman LS1801 scintillation counter; Beckman Coulter (UK) Limited). Counts ranged from 233552 c.p.m./µl to 491920 c.p.m./µl (antisense), and 118220 c.p.m./µl to 203216 c.p.m./µl (sense) for the V1a receptor probe, and from 159510 c.p.m./µl to 432496 c.p.m./µl (antisense) and 159510 c.p.m./µl to 374780 c.p.m./µl (sense) for the V1b receptor probe. The antisense probes were used up to 7 days after labelling, the sense probes up to 17 days later (decay accounted for).

Appendix 5

***In situ* hybridisation: tissue preparation, hybridisation and visualisation**

Tissue preparation

The collected brains were stored at -70 ˚C. 15 μm coronal cryostat sections were prepared (Bright lnstrument Co. Ltd., UK), at -16 ˚C, and thaw-mounted onto polysine slides (VWR, UK). Sections were mounted across sets of 5 slides, such that on each slide, the second section was 75 μm posterior to the first, the third 150 μm posterior, and the fourth 225 μm posterior. After preparation, slides were returned to -70 ˚C until use. 1-2 slides per animal were used in each hybridisation.

Immediately prior to hybridisation the selected slides were removed from -70 ˚C and kept on dry ice, until processing through the following stages:

1. Transfer to ice cool 4 % paraformaldehyde/$1\times$ PBS (prepared in DEPC-H_2O), for 10 min

2. 2×5 min washes in 1x PBS (prepared in DEPC-H_2O)

3. 10 min immersion in 0.1 M triethanolamine/0.027 M acetic anhydride

4. 2×3 min washes in $1\times$ PBS

5. Dehydration through consecutive washes in 70 %, 80 % and 95 % ethanol (prepared with sterile ddH_2O; each 2 min)

6. Air-drying for 30-60 min

Prehybridisation

Prehybridisation solution was prepared as: 5.86 ml DEPC-H_2O : 2.4 ml 5 M NaCl : 200 µl 1 M Tris : 400 µl Denhardt's solution (50×; Sigma-Aldrich, UK) : 80 µl 250 mM ethylene-diamine-tetra-acetic acid (EDTA) : 1 ml salmon testes DNA (9.1 mg/ml; Sigma-Aldrich, UK) : 50 µl yeast transfer RNA (tRNA; 50 mg/ml; Invitrogen, UK). Deionised formamide was prepared by mixing with Amberlite® (10 g Amberlite (VWR, UK) per 100 ml formamide (VWR, UK)), for at least 1 h (room temperature) and filtering through a double layer of paper (Whatman No. 1, Whatman, UK). Prehybridisation solution and deionised formamide were combined (1:1 ratio, by volume) and 200 µl of the resulting solution applied to each slide. Slides were placed in humidified boxes – prepared by addition of a 10 ml deionised formamide + 6 ml DEPC-H_2O + 4 ml 20× SSC solution, to a box (volume = 4 L) fitted with two layers of Whatman No. 1 filter paper - and left to incubate at 50 ˚C (2 h).

Hybridisation

Hybridisation solution was prepared as: 6.66 ml DEPC-H_2O : 2.4 ml 5 M NaCl : 200 µl 1 M Tris : 400 µl Denhardt's solution : 80 µl 250 mM EDTA : 200 µl (9.1 mg/ml) salmon sperm DNA : 2 g dextran sulphate (Sigma-Aldrich, UK) : 50 µl 50 mg/ml yeast tRNA (Sigma-Aldrich, UK). The volume of labelled probe solution required to provide a final concentration of 10^7 c.p.m./ml, and volume of 1 M DTT required to provide a final concentration of 10^{-2} M, were added to a combination of deionised formamide and hybridisation solution, in a volume ratio of 1 (deionised formamide) : 1 (hybridisation solution + DTT + probe solution). The probe solution was added to

the combined deionised formamide and hybridisation solution before a 10 min waterbath incubation (70 ˚C), cooling on ice (1 min), and addition of DTT.

The prehybridisation solution was drained from each slide, and 200 µl of the final hybridisation solution applied to each. The slides were returned to the humidified boxes for incubation at 55 ˚C (for V1a receptor) or 50 ˚C (for V1b receptor) (16-18 h).

Posthybridisation

The solution was drained from the slides and the slides washed in 2× SSC (3 × 5 min, room temperature). An RNase solution was prepared as: 1 ml 5 M NaCl : 100 µl 1 M Tris : 20 µl 500 mM EDTA : 8.88 ml ddH$_2$O : 5 µl RNase A (61 mg protein/ml, 5400 Kunitz units/ml; Sigma-Aldrich, UK), and 200 µl applied to each slide. The slides were placed in humidified boxes – prepared by addition of a 2 ml 5 M NaCl + 200 µl 1 M Tris + 40 µl 500 mM EDTA + 17.75 ml ddH$_2$O solution to boxes (volume = 4 L) fitted with one layer of Whatman No. 1 filter paper – and incubated at 37 ˚C (60 min).

The RNase solution was then drained from the slides, and the slides washed in SSC:

1. 2× SSC, room temperature, 30 min

2. 0.1× SSC, 65 ˚C (for V1a receptor)/60 ˚C (for V1b receptor), 60 min

3. 65 ˚C/60 ˚C 0.1× SSC, left at room temperature to cool, 60 min

4. 65 ˚C/60 ˚C 0.1× SSC, left at room temperature to cool at the beginning of wash 3, 60 min

Slides were dehydrated through consecutive washes in 50 %, 70 %, and 90 % ethanol/0.003 M ammonium acetate (Sigma-Aldrich, UK), each for 2 min, and left to air-dry.

Autoradiogram production

Each slide was coated in 42 ˚C emulsion (Ilford Photo K.5 emulsion in gel form, Calumet, UK), air-dried overnight in a light-proof box, and transferred to light-proof storage at 4 ˚C. After 7 (LS/BNST tissue), or 12 (PVN and amygdala tissue), weeks, the slides were brought to room temperature and processed through the following washes:

1. developer (Kodak Professional D-19 Developer; Sigma-Aldrich, UK; 80 g/l in ddH$_2$O; 5 min)

2. ddH$_2$O (5 s)

3. fixer (Ilford Hypam rapid fixer (Calumet, UK); diluted 1:4 in ddH$_2$O; 2 × 5 min)

4. ddH$_2$O (2 × 5 min)

Counterstaining

Following developing, the slides were immediately processed as follows:

1. staining with filtered hematoxylin (Shandon Harris Hematoxylin, Thermo Scientific, USA; 5 min)

2. rinse in tap water (~10 s)

3. differentiation in acid alcohol (~10 s)

4. rinse in tap water (~10 s)

5. wash in STWS (3 min)

6. wash in tap water (3 min)

7. staining with filtered 1 % Eosin Y (VWR, UK; 2 min)

8. rinse with tap water (~5 s)

9. wash in potassium alum (3 min)

10. rinse in tap water (5-15 min)

Slides were finally dehydrated through consecutive washes in 70 %, 80 %, 95 % (each 4 min), and 100 % (2 × 4 min) ethanol, cleared in xylene (Genta Medical, UK; 2 × 4-10 min), and coverslipped with DePeX (VWR, UK).

Appendix 6

Generation of vasopressin-eGFP $^{+/+}$ rat colony

Male, Wistar rats heterozygous for an inserted construct encoding vasopressin precursor conjugated to eGFP were provided by Dr. Y. Ueta (Ueta *et al.*, 2005).

Breeding

Systematic genotyping of offspring from transgenic-transgenic pairings and transgenic-wildtype backcrosses was used, with an appropriate breeding programme, to generate five homozygous breeding pairs. Genotyping of F_1 and F_2 generations arising from these animals confirmed eGFP expression. Animals from the F_2, and subsequent, generations were used in studies.

Genotyping

Tissue preparation

0.2 cm diameter ear notches or 0.2 cm tail tips were collected and stored at -20 ºC. Prior to amplification by polymerase chain reaction (PCR), DNA was purified from tail tissue samples. Ear tissue samples were processed as crude tissue lysates.

DNA extraction and purification from tail tissue samples

DNA from each tissue sample was extracted and purified using the Qiagen Dneasy Tissue Kit (Qiagen Limited, UK), according to the supplied manufacturers' protocol. Samples were thus processed through the following stages:

1. Overnight incubation in 180 μl buffer ATL + 20 μl proteinase K (waterbath, 55 ºC)

2. Addition of 200 μl buffer AL/200 μl ethanol mixture

3. Washing through a Dneasy Mini Spin Column by centrifugation at 8000 r.p.m. for 1 min

4. Washing of the same Dneasy Mini Spin Column following addition of 500 μl buffer AW1 (8000 r.p.m., 1 min) and then 500 μl buffer AW2 (13000 r.p.m., 3 min)

5. Elution of purified DNA using 100 μl buffer AE (8000 r.p.m., 1 min)

6. Storage of purified sample at 4 ºC.

The concentration of DNA within each purified sample was measured by spectrophotometry (BioMate 3 UV-Vis Spectrophotometer, Thermo Scientific, USA) after dilution (1:29) in ddH_2O. The volume required to provide the optimal concentration of template for PCR (10 ng/μl) in a 20 μl reaction preparation was determined and added to a 0.2 ml, thin walled PCR tube. ddH_2O was added to make each sample up to 11.7 μl.

Preparation of ear tissue lysates

Ear notches, 150 μl DirectPCR Lysis Reagent (Ear) (Euromedex, France) and 7.5 μl proteinase K (Qiagen Limited, UK) were added to 0.6 ml tubes. These were incubated on a rocking plate (Shake'n'Stack hybridization oven, Thermo Scientific, USA; 55 ºC) for 6 h. This was followed by a 45-min incubation in a waterbath at 85

ºC. 1 µl of the resulting lysate from each sample was added to 10.7 µl ddH$_2$O in a 0.2 ml, thin walled PCR tube.

PCR

To complete the 20 µl PCR reaction preparation, the following components were added to each 11.7 µl sample, after their combination:

1. 2 µl sense primer (sequence: 5'-C CAT CTT CTT CAA GGA CGA C-3'; 10 pM; Invitrogen, UK)

2. 2 µl antisense primer (sequence: 5'-CG GCC ATG ATA TAG ACG TTG TG-3'; 10 pM; Invitrogen, UK)

3. 2 µl deoxyribose-nucleotide triphosphate mix (100 mM, Fermentas, UK)

4. 2 µl 10× PCR buffer (TaqPlus Precision DNA Polymerase Buffer, Stratagene, The Netherlands)

5. 0.3 µl *Taq* polymerase (TaqPlus Precision DNA Polymerase (5 U/µl), Stratagene, The Netherlands)

The following PCR programme (Techne Genius Thermocycler, Jencons PLS, UK) was used for amplification:

1. 94 ºC (5 min)

2. 35 × (94 ºC (40 s), 55 ºC (45 s), 72 ºC (45 s))

3. 72 ºC (7 min)

4. 4 ºC (indefinite)

Samples were stored at 4 ºC until subject to gel electrophoresis (maximum overnight).

Gel electrophoresis of PCR product

9 µl of each PCR sample, with 1 µl 10× BlueJuice^TM gel loading buffer, was run on an agarose gel (1.5 g agarose + 1 µl ethidium bromide per 100 ml 1× TBE buffer; 160 V, 25 min) next to 5 µl of a 100 base pair DNA ladder solution (Invitrogen, UK). A UV-light transilluminator and imaging software (Syngene Gene Genius Bioimaging System, Synoptics Ltd., UK) were used to detect DNA migration. Samples at the position corresponding to 171 base pairs were presumed eGFP positive.

Appendix 7

Stereotaxic tracer injection

Drugs

Anaesthetic: Fentanyl/fluanisone (Hypnorm; Janssen-Cilag, UK) and midazolam (Hypnovel; Roche Pharmaceuticals, UK) in sterile ddH$_2$O (1 Hypnorm : 1 Hypnovel : 2 water); 2.7 ml/kg (i.p.); 5 min before start of surgery

Analgesic: Rimadyl (Pfizer, UK; 5 mg/kg, subcutaneous (s.c.)); at the time of induction of anaesthesia and again the following morning, or: buprenorphine (Reckitt Benckiser Healthcare, UK; 0.05 mg/kg, s.c.); at end of surgery

Reversal: Naloxone (Sigma-Aldrich, UK; 0.1 mg in 1 ml saline (0.9 % w/v), i.p.); at end of surgery, or: buprenorphine (0.05 mg/kg, s.c.); at end of surgery

Surgery

Hair was removed from around the surgical site, and rats were fitted in a stereotaxic frame (Digital Dual Manipulator Lab Standard; World Precision Instruments, UK; over a heat pad). Vetasept Povidone Iodine (Animalcare, UK), and ethanol (90 %), were applied to the surgical site, and the skull exposed by an approximately 1cm anterior-posterior incision. Bregma and lambda were levelled. A burr hole (approximately 1 mm diameter) was drilled at the anteroposterior and mediolateral co-ordinate cross. 1 μl tracer (undiluted red fluorescent RetroBeads™; Lumafluor Inc., USA), was introduced into a MicroFil^{TM} needle (34 gauge; World Precision Instruments, UK) attached to a 10 μl Hamilton syringe (Sigma-Aldrich, UK). The

needle tip was lowered to 0.2 mm below the dorsoventral co-ordinate, and retracted to the dorsoventral co-ordinate, where 1 µl tracer was injected. The needle was retracted a further 0.2 mm, and maintained at this point for 15-20 min, before removal over 10 min. 2 or 3 sutures were used to close the incision, iodine applied, and chosen reversal agent administered. Rats were given 5 ml warmed (27-28 ºC) saline (0.9 %; s.c.), placed in a heat box (27-28 ºC) for approximately 1 h (until active), and then transferred to a cage (warmed by heat mat), with provision of soft diet.

Appendix 8

Immunofluorescent staining of free-floating rat brain sections

52 μm tissue sections were processed as below. Exposure to light was prevented where possible.

Primary antibody application

Tissue was removed from -20 ºC/cryoprotectant, and washed in 0.1 M PB-T (4 × 15 min), 20 % methanol/80 % 0.1 M PB (15 min), and PB-T (3 × 10 min). Washes in 3 % skim milk powder/0.1 M PB (30 minutes), 0.1 M PB-T (3 × 10 minutes), and 3 % normal goat serum/0.1 M PB-T (45 min), were used to inhibit non-specific labelling. Sections were then transferred to the antibody solution (60 ml 0.1 M PB-T : 2 ml normal goat serum : 1.24 ml anti-oxytocin rabbit polyclonal antibody (raised against rat oxytocin; Calbiochem, UK; final dilution 1:500) and left to incubate at 4 ºC (20 h).

Visualisation

Tissue was removed from 4 ºC, washed in 0.1 M PB-T (3 × 10 min), and exposed to a solution of biotinylated goat anti-rabbit IgG (10 μl biotinylated goat anti-rabbit antibody : 30 μl normal goat serum : 1 ml 0.1 M PB-T). After incubation for 1 h (room temperature), sections were washed in 0.1 M PB-T (3 × 10 min), and incubated (1 h; room temperature) in ABC solution (1 ml 0.1 M PB-T : 20 μl biotin (Vector Laboratories, UK) : 1.5 μl streptavidin-Alexa Fluor® 633 conjugate

(Invitrogen, UK)), prepared approximately 60 min before use. After 3×10 min washes in 0.1 M PB-T and 3×5 min washes in 0.1 M PB, sections were mounted, air-dried and coverslipped with Mowiol.

Mowiol preparation

2.4 g Mowiol (Calbiochem, UK), 4.76 ml glycerol (density 1.26 g/l; Sigma-Aldrich, UK) and 12 ml ddH$_2$O were mixed and left at room temperature, on shaker, for 15-16 h. 12 ml 0.2 M Tris (pH 8.5) was added, and the solution heated to 50 ºC (waterbath; 2 h). After centrifugation (2000 r.p.m.; 15 min), 0.72 g 1,4-diazabicyclooctane (Sigma-Aldrick, UK) was added, before storage at -20 ºC (moved to 4 ºC on day of use).

Appendix 9

Confocal imaging

Tissue was imaged using a Zeiss LSM510 upright microscope (LSM510 scanning head combined with Axioplan research microscope), with argon-krypton (488 nm laser; tube current = 6.1 A; laser power < 8 %) and helium-neon (543 and 633 nm lasers; laser power 100 %) laser types, and a 10×/0.3 plan neofluar, 20×/0.5 plan neofluar or 63×/1.4 (OIL) plan apochromat lens.

For detection of the three fluorescent labels, multi-track configuration (tracks switched after each line) was used with the following tracks:

1. excitation at 488 nm, detection between 500 and 550 nm

2. excitation at 543 nm, detection between 565 and 615 nm

3. excitation at 633 nm, detection above 650 nm

The second track, used in multi-track configuration with incandescent light through the 488nm laser line, was used to produce the injection site images.

All frame sizes were 1024 × 1024 pixels, with zoom factor = 1 and 8 bit data depth. Lines were averaged using the mean (of 2). The pinhole was set to 1 Airy Unit for the shortest wavelength laser and adjusted to equal diameter for the additional laser lines.

The fluorescent images were collected as z-series (slice interval = 2.64 μm with 20×/0.5 lens, 1.00 μm with 63×/1.4 lens) and combined as a maximum intensity projection.

Appendix 10

Solutions

Acid alcohol

70 % (v/v) ethanol (Fisher Scientific, UK)

1 % (v/v) concentrated HCl (VWR, UK)

Prepared in distilled, deionised water

Buffer P1 (Qiagen, UK)

50 mM Tris-Cl, pH 8.0

10 mM EDTA

100 µg/ml RNase A

Buffer P2 (Qiagen, UK)

200 mM sodium hydroxide

1 % SDS (w/v)

Buffer P3 (Qiagen, UK)

3 M potassium acetate

pH 5.5

Buffer QBT (Qiagen, UK)

750 mM sodium chloride

50 mM 3-(N-morpholino) propanesulfonic acid, pH 7.0

15 % isopropanol (v/v)

0.15 % Triton® X-100 (v/v)

Buffer QC (Qiagen, UK)

1 M sodium chloride

50 mM 3-(N-morpholino) propanesulfonic acid, pH 7.0

15 % isopropanol (v/v)

Buffer QF (Qiagen, UK)

1.25 M sodium chloride

50 mM Tris-Cl, pH 8.5

15 % isopropanol (v/v)

Cryoprotectant

100 ml phosphate buffered saline (0.081 M disodium hydrogen orthophosphate

(BDH, UK), 0.0227 M sodium dihydrogen orthophosphate (Fisher Scientific, UK)

and 0.15 M sodium chloride (Sigma-Aldrich, UK), pH 7.4)

60 ml ethylene glycol (Acros Organics, UK)

40 ml glycerol (Fisher Scientific, UK)

pH 5.5

0.1 % Diethylpyrocarbonate-treated water (DEPC-H_2O)

0.1 % diethylpyrocarbonate (v/v; Sigma-Aldrich, UK) in distilled, deionised water

Autoclaved 2 hours after combined

1 M Dithiothreitol (DTT)

0.001 M dithiothreitol (Sigma-Aldrich, UK) in autoclaved, distilled, deionised water

Filtered through a 0.2 μm acrodisc® (Pall Life Sciences, Inc., USA)

Gelatin subbing solution

5 % (w/v) gelatine (VWR, UK)

0.001 M chromium potassium sulphate (VWR, UK)

Prepared in distilled water

4 % Paraformaldehyde/1× PB

4 % (w/v) paraformaldehyde (Sigma-Aldrich, UK)

0.081 M disodium hydrogen orthophosphate (VWR, UK)

0.0227 M sodium dihydrogen orthophosphate (Fisher Scientific, UK)

Prepared in distilled, deionised water

pH 7.4

4 % paraformaldehyde/PBS

4 % (w/v) paraformaldehyde (Sigma-Aldrich, UK)

0.137 M sodium chloride (Sigma-Aldrich, UK)

0.0027 M potassium chloride (Sigma-Aldrich, UK)

0.01 M disodium hydrogen orthophosphate (VWR, UK)

Prepared in autoclaved PBS made using DEPC-H_2O

pH 7.4

1× Phosphate buffer (PB)

0.081 M disodium hydrogen orthophosphate (VWR, UK)

0.0227 M sodium dihydrogen orthophosphate (Fisher Scientific, UK)

Prepared in distilled water

pH 7.4

1× Phosphate buffer/0.2 % Triton X-100 (PB-T)

0.2 % (v/v) Triton X-100 (VWR, UK) in 1× PB

pH 7.4

10× Phosphate buffered saline (PBS)

0.163 M disodium hydrogen orthophosphate (VWR, UK)

0.015 M potassium dihydrogen orthophosphate (Fisher Scientific, UK)

0.027 M potassium chloride (Sigma-Aldrich, UK)

Prepared in 0.1 % DEPC-H_2O and autoclaved

pH 7.4

20× Saline sodium citrate (SSC)

3 M sodium chloride (Sigma-Aldrich, UK)

0.3 M Tri-sodium citrate (Sigma-Aldrich, UK)

Prepared in DEPC-H_2O and autoclaved

pH 7.0

Scott's tap water substitute (STWS)

2 % (w/v) Magnesium sulphate (Sigma-Aldrich, UK)

0.35 % (w/v) Sodium hydrogen carbonate (Fisher Scientific, UK)

Prepared in distilled, deionised water

Potassium alum

5 % (w/v) Aluminium potassium suphate (VWR, UK)

Prepared in distilled, deionised water

TE buffer

10 mM Tris-Cl (Sigma-Aldrich, UK)

1 mM EDTA (Sigma-Aldrich, UK)

Prepared in distilled, deionised water and autoclaved

pH 7.4

Triethanolamine/acetic anhydride

0.1 M triethanolamine (Sigma-Aldrich, UK)

0.027 M acetic anhydride (Sigma-Aldrich, UK)

Prepared in autoclaved, distilled, deionised water

TY culture medium

1.6 % (w/v) bacto-tryptone (Sigma-Aldrich, UK)

1 % (w/v) bacto-yeast extract (Sigma-Aldrich, UK)

0.5 % (w/v) sodium chloride (Sigma-Aldrich, UK)

Prepared in distilled, deionised water

Appendix 11

Suppliers

Alpha Diagnostic Intl. Inc.

6203 Woodlake Center

San Antonio, Texas 78244 USA

Animalcare Limited

Common Road, Dunnington

York, YO19 5RU

Autoclude Ltd.

Unit 7, Carnival Park

Carnival Court, Basildon

Essex, SS14 3WN

Beckman Coulter (U.K.) Limited

Oakley Court, Kingsmead Business Park

London Road, High Wycombe

Buckinghamshire HP11 1JU

Bioline Ltd.

16 The Edge Business Centre, Humber Road

London, NW2 6EW

Bright lnstrument Co Ltd,

St Margarets Way, Huntingdon

Cambridgeshire, PE29 6EU

Calbiochem UK

Merck Chemicals Ltd., Boulevard Industrial Park,

Padge Road, Beeston

Nottingham NG9 2JR

Calumet UK

Promandis House, Bradbourne Drive

Tilbrook, Milton Keynes, MK7 8AJ

Carl Zeiss Ltd.

15-20 Woodfield Road, Welwyn Garden City

Hertfordshire, AL7 1JQ

Ceva Sante Animale

La Ballastière - BP 126

33501 Libourne Cedex

France

Euromedex

24 rue des Tuileries, BP 684

F-67460 Souffelweyersheim

France

Fermentas UK

Sheriff House, Sheriff Hutton Industrial Park

York, YO6 0RZ

Fisher Scientific UK Ltd

Bishop Meadow Road, Loughborough,

Leicestershire, LE11 5RG

Genta Medical

Unit 17D, Marston Business Park, Rudgate

Tockwith, York, YO26 7QF

GE Healthcare Life Sciences

The Grove Centre, White Lion Road

Amersham, Bucks, HP7 9LL

Harlan UK Limited/Harlan Teklad

Shaw's Farm, Blackthorn, Bicester

Oxon, OX25 1TP

Infors-AG

Rittergasse 27

CH-4103 Bottmingen

Switzerland

Invitrogen Ltd

3 Fountain Drive, Inchinnan Business Park

Paisley

Janssen-Cilag Ltd.

P.O. Box 79, Saunderton

High Wycombe, Bucks

HP14 4HJ

Jencons-PLS

Unit 15, The Birches, Willard Way

Imberhorne Industrial Estate, East Grinstead

West Sussex, RH19 1XZ

Leica Microsystems GmbH

Ernst-Leitz-Strasse 17-37

35578 Wetzlar

Germany

Lumafluor Inc.

1213 Silverstrand Drive

Naples, FL 34110, USA

Pall Life Sciences,

Europa House, Havant Street

Portsmouth, Hampshire, PO1 3PD

Peninsula Labs

611 Taylor Way, Belmont

CA 94002, USA

PerkinElmer LAS (UK) Ltd

Chalfont Road, Seer Green

Beaconsfield, Bucks, HP9 2FX

Pfizer Limited

Ramsgate Road, Sandwich

Kent, CT13 9NJ

Promega

Delta House, Southampton Science Park

Southampton, SO16 7NS

Qiagen

Qiagen House, Fleming Way

Crawley, West Sussex

RH10 9NQ

Reckitt Benckiser Healthcare

103-105 Bath Road, Slough

Berkshire, SL1 3UH

Roche Products Ltd

Hexagon Place, 6 Falcon Way

Welwyn Garden City

Hertfordshire, AL7 1TW

Sigma-Aldrich Company Ltd.

The Old Brickyard, New Road

Gillingham, Dorset, SP8 4XT

Stratagene Europe

Gebouw California, Hogehilweg 15

1101 CB Amsterdam Zuidoost

The Netherlands

Synoptics Ltd

Beacon House, Nuffield Road

Cambridge, CB4 1TF

Systat Software Inc.

24 Vista Centre, 50 Salisbury Road

Hounslow, London, TW4 6JQ

Tecniplast UK Ltd

Kettering Venture Park, Kettering

Northamptonshire, NN15 6XR

Thermo Fisher Scientific, Inc.

81 Wyman Street, Waltham

MA 02454, USA

Vector Laboratories, Ltd.

3 Accent Park, Bakewell Road

Orton Southgate, Peterborough

PE2 6XS

VWR International Ltd

Hunter Boulevard, Magna Park

Lutterworth, Leicestershire

LE17 4XN

Whatman plc

Springfield Mill, James Whatman Way

Maidstone, Kent, ME14 2LE

World Precision Instruments, LTD

Astonbury Farm Business Centre

Aston, Stevenage

Hertfordshire, SG2 7EG