Neurosteroids and Maternal Behaviour

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DECLARATION

I hereby declare that this thesis is of my own composition, that the experiments of which it is recorded have been done by me, or under my supervision, unless acknowledged in the text. That it has not been accepted in any previous application for a higher degree, and that all sources of information have been specifically acknowledged in the reference.

David Ishaya

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ABSTRACT

Maternal behaviour is exhibited by female rats post partum. This includes, nest building, nursing, pup retrieval, licking and grooming. Lactating mothers also show maternal defense behaviour with aggression directed against conspecifics. This behaviour has been attributed, in part, to the dramatic changes in steroid and neuropeptide hormones that accompany pregnancy and parturition.

Peripheral steroid release is required for normal parturition and lactation. The brain is also a target for steroid hormones and evidence suggests that central release of steroid and peptides can induce many maternal behaviour. Neurosteroids are known to modulate a wide range of neurotransmitter and receptor systems in the central nervous system. They affect primary excitatory and inhibitory systems (glutamate and gamma amino butyric acid (GABA), and so regulate a range of behaviours including learning, anxiety, cognition, sexual behaviour and aggression. 5α-reductase type 1 (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD) are the two enzymes involved in the synthesis of neurosteroids; allopregnenolone (Allop) and tetrahydrodeoxycorticosterone (THDOC) in the brain from progesterone and deoxycorticosterone, respectively. In this study, brain regions such as the supraoptic nucleus (SON), medial amygdala (mAmyg), medial preoptic nucleus (MPO), bed nucleus of stria terminalis (BNST), and paraventricular nucleus (PVN) were activated in lactating mothers following the exhibition of maternal aggressive behaviour. Using immunocytochemistry for the immediate early gene (Fos-IR) as a marker of neuronal activation, there were approximately two to three folds more Fos-immunoreactive (Fos-IR) cells, in each brain region analysed in the aggressive lactating dams compared with non-aggressive controls.

In brain regions such as the SON, mAmyg, MPO, BNST and PVN the Fos-IR cells were also double labelled for 5α-R and 3α-HSD. Glutamic acid decarboxylase (GAD) labelled cells were also observed in these brain regions but there were no Fos-IR cells labelled for GAD in any of the brain regions analysed. Together these results implicate neurosteroids in the exhibition of maternal aggression. Activation of cells containing the enzymes 5α-R and 3α-HSD suggest that neurosteroid synthesis in specific brain regions may play a role in regulating aggressive behaviour perhaps through the GABAergic mechanism, but further work is warranted.

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LIST OF ABBREVIATIONS

3α-HSD	3 alpha-hydroxysteroid dehydrogenase
3α, 5α- THPROG	3 alpha, 5 alpha-tetrahydroprogesterone
5-HT	5-hydroxy tryptamine
5α-R	5 alpha-reductase type 1
17β-HSD	17 beta-hydroxysteroid dehydrogenase
AAS	Anabolic androgenic steroid
ACTH	Adrenocorticotropin hormone
Allop	Allopregnanolone
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOB	Accessory olfactory bulb
BNST	Bed nucleus of stria terminalis
CeA	Central amygdala
CNS	Central Nervous System
cPAG	Caudal periqueductal gray
CRH	Corticotropin releasing hormone
DAB	Diaminobenzidine
DD3	Dihydrodiol dehydrogenase
DHEA	Dehydroepiandrostenedione
DHEAS	Dehydroepiandrostenedione sulphate
DHP	Dihydroprogesterone
DHT	Dihydrotestosterone
E_2	Oestradiol
Fos-IR	Fos-immunoreactivity

FSH	Follicle stimulating hormone
GABA	Gamma amino butyric acid
GAD	Glutamic acid decarboxylase
GH	Growth hormone
GH-RH	Growth hormone releasing hormone
GH-RIH	Growth hormone releasing inhibiting hormone
GnRH	Gonadotropin releasing hormone
ICC	Immunocytochemistry
IEG	Immediate early gene
IGRs	Ionotropic glutamate receptors
LH	Luteinizing hormone
mAmyg	Medial amygdala
MPO	Medial preoptic nucleus
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
MSH-RH	Melanocyte-stimulating hormone releasing hormone
MSH-RIH	Melanocyte-stimulating hormone release inhibiting hormone
MVs	Microvesicles
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NHS	Normal horse serum
NMDA	N-methyl D-aspartate
NSS	Normal sheep serum
P	Prolactin
PB	Phosphate buffer

PBS	Phosphate buffered saline
PB-T	Phosphate buffered triton
PREG	Pregnanolone
PREGS	Pregnenolone sulphate
P-RH	Prolactin releasing hormone
P-RIH	Prolactin releasing inhibiting hormone
PROG	Progesterone
PVN	Paraventricular nucleus
S.E.M	Standard error of the mean
SON	Supraoptic nucleus
StAR	Steroidogenesis acute regulatory protein
THP	Tetrahydroprogesterone
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone

CHAPTER 1

INTRODUCTION

MATERNAL BEHAVIOUR AND AGGRESSION

Female rats are generally social animals and are not normally aggressive. Following parturition, they exhibit maternal behaviour to the pups which includes: lactation, retrieving, licking, grooming, nest building, and crouching. Previous studies have reported increased aggressive behaviour directed against conspecific intruder rats at this time (E.g. Bosch, et al. 2005). These aggressive behaviours include: sniffing, biting, clawing/kicking, rearing, tail rattles directed against the intruder during the peripartum and postpartum periods (Pinna, et al. 2003). This behavioural pattern relies partly on the hormonal interplay that characterises late gestation, parturition and lactation (Bitran, et al. 1991 & Fleming, et al. 1981). Maternal aggression in rats begins during the last trimester, reaches it peaks around days 1 to 7 of lactation, and declines during the course of lactation. As the pups mature, changes in pup stimuli such as smell, size, body hair, mobility, vocalisation, and milk consumption may contribute to the observed decline in maternal aggression (Albert, et al. 1994).

MATERNAL BEHAVIOUR AND ANXIETY

Maternal behaviour can be divided into sensory-motor and emotional components in rats. The former include pup retrieval, nursing, licking and grooming, building a nest, and exhibiting maternal aggression to protect the pups from intruders. The emotional components include reduced anxiety in the presence of anxiety-induced situations such as being placed in the open field, or in the open arm of a plus-maze, being startled by a loud noise or presented with a threatening male intruder (Ferreira, et al. 1989, Ferreira, et al. 2002, Fleming, et al. 1981, Hansen, et al. 1986, Hard, et al. 1986 and Leng & Russell, 2006). In males, aggressive behaviour was found to be inversely related to the anxiety-linked behaviour (Nyberg, et al. 2003, Veenema, et al. 2004 & Bosch, et al. 2005). Less is known about the relationship between anxiety and maternal aggression in lactating females (Bosch, et al. 2005).

Reproductive hormones have a major effect on social behaviour, and dramatic changes in steroid hormone and neuropeptide levels within the mother's brain may underlie emotional liability following parturition (Belelli, et al. 2005). Connection of physiology to reproductive behaviour is important to the understanding of proximate mechanisms involved in the control of reproductive success. Socioendocrine studies can help in understanding the origin of individual differences in reproductive behaviour (Bercovitch, 1999, Bercovitch, 2002 & Ziegler, 2002).

EMBRYOGENESIS OF MATERNAL BEHAVIOUR

Research into neural mechanisms related to aggressive behaviour has focused on males. Sex differences in regions subserving aggressive behaviour are evident from embryogenesis. So it is therefore useful to study this behaviour in females. During embryogenesis, there is sexual differentiation in which the brain is programmed into either male or female. Brain development becomes feminised under the influence of extremely minute quantities of oestradiol. Exposure of the fetal brain to the higher concentrations of oestradiol present in the maternal blood is prevented by the production of fetal alpha-fetoprotein production. This prevents the maternal oestradiol from crossing the blood-brain barrier, and the embryo becomes fully feminised (Senger, 1999). Alpha-fetoprotein does not bind to testosterone which has the tendency of entering the brain and being converted to oestradiol. In the male developing embryo this high concentration of oestradiol leads to masculinisation of the brain. Defeminisation abolishes the likelihood of female reproductive behaviours at post puberty and masculinisation results into potential male-like behaviour post pubertally (Senger, 1999)

NEUROENDOCRINE INTERACTIONS

There is a constant interaction between the endocrine organs and the central nervous system (CNS). Hormones have profound influence on the function of the CNS. The CNS in turn has an important role in the control of the endocrine function (Schmidt-Nielsen, et al. 1997). Studies have shown that the hypothalamus plays a dominant role in this control.

THE HYPOTHALAMIC CONTROL SYSTEM

The hypothalamus is located below the thalamus, just at the base of the brain immediately above the pituitary gland, posterior to the optic chiasm, where it forms the floor of the third ventricle (Schmidt-Nielsen, et al. 1997). The hypothalamus regulates many systems: for example, temperature regulation and the regulation of food and water intake (Schmidt-Nielsen, et al. 1997). The hypothalamus regulates the endocrine system via the pituitary gland. Information is transferred to the anterior pituitary (adenohypophysis) through a portal circulation, and to the posterior pituitary (neurohypophysis) through neural connections (Schmidt-Nielsen, et al. 1997). The supraoptic (SON) and paraventricular (PVN) nuclei are part of this system (Schmidt-Nielsen, et al. 1997).

The neuropeptides oxytocin and vasopressin are secreted from the neurohypophysis from the axon terminals of magnocellular neurones whose cell bodies are situated in SON and PVN of the hypothalamus. The PVN is divided, but almost all SON neurones project to the posterior pituitary and synthesize either vasopressin or oxytocin e.g (Russell, et al. 2003). Usually many neurones synthesize both, but in an unequal amounts, and it seems unlikely that the little amount of vasopressin in a cell that predominantly produces oxytocin and vice versa, is of any functional importance

(Mezey, et al. 1989, Xi, et al. 1999). The oxytocin and vasopressin cells in the magnocellular neurones must make enough peptides to satisfy effective concentrations required in the circulatory system for a long period. This is reflected in the high rate of vasopressin and oxytocin gene expression (Bowers, 1994).

In contrast, the adenohypophysis itself produces hormones, and their release into the blood stream is also regulated by the hypothalamus. It is now well established that there are 9 hypothalamic regulating hormones involved.

Hormone	**Abbreviation**
1. Growth hormone releasing hormone	GH-RH
2. Growth hormone releasing inhibiting hormone	GH-RIH
3. Prolactin releasing hormone	P-RH
4. Prolactin releasing inhibiting hormone	P-RIH
5. Melanocyte- stimulating hormone releasing hormone	MSH-RH
6. Melanocyte - stimulating hormone release inhibiting hormone.	MSH-RIH
7. Corticotropin (ACTH) releasing hormone	CRH
8. Thyrotropin releasing hormone	TRH
9. Gonadotropin hormone releasing hormone	GnRH

Three of these hormones (GH, P, and MSH) are under dual hypothalamic control, one stimulatory and the other inhibitory. Thus, their release is not regulated by simple feedback signals, although feedback signals are involved in their control.

Four of these hormones (CRH, TSH, and GnRH) depend strongly on a negative feedback system. They regulate the release of ACTH, TSH, LH and FSH from the pituitary gland. Their targets are the adrenal cortex, the thyroid and the gonads respectively, which when stimulated, produce corticosteroids, thyroxine and sex steroids. These feedback and inhibit the primary hormone secretion (Schmidt-Nielsen, et al. 1997).

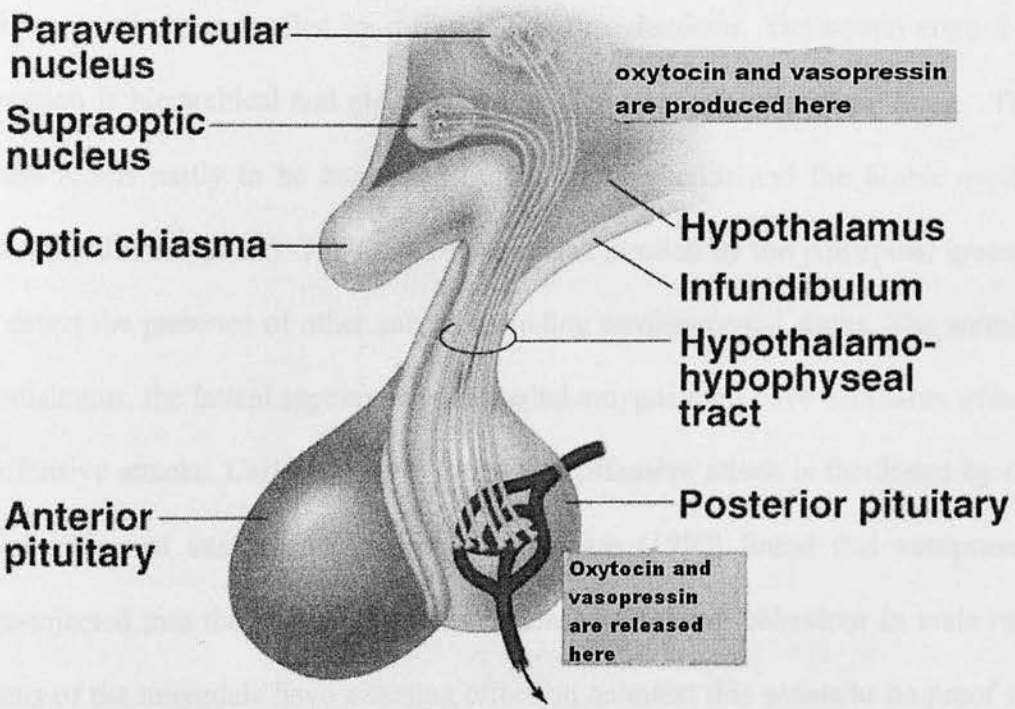


Figure 1.1 Pituitary gland: showing anterior and posterior pituitary
http://www.biosbcc.net/barron/physiology/endo/hypopit.htm

NEUROENDOCRINE BASIS OF AGGRESSION

Aggressive behaviours are species specific; so that, the patterns of movements (for example, posturing, biting, striking) are organised by neural circuits whose development in largely programmed by genes. Most aggressive behaviours are related to reproduction, for example, aggressive behaviour that is exhibited by some males to gain access to mates, defending territory, which is required to attract mates or build nest, or defend offspring against intruders are all related to reproduction.

The three major types of aggressive behaviours are offensive, defensive, and predatory, and are controlled by different brain mechanisms. The neural control of aggression is hierarchical and programmed by the neural circuits in the brain. This process seems partly to be controlled by the hypothalamus and the limbic system (especially the amygdala). The limbic system is controlled by the perceptual systems that detect the presence of other animal including environmental status. The anterior hypothalamus, the lateral septum and the medial amygdala all have excitatory effects on offensive attacks. Carlson, (1998) found that offensive attack is facilitated by the central action of vasopressin, a peptide. Koolhaas (1990) found that vasopressin micro-injected into the medial amygdala increased offensive behaviour in male rats. Lesions of the amygdala have a taming effect on animals; this seems to be proof for the amygdala's involvement in defensive attack of a particularly wild rhesus monkey against human handlers (Carlson, 1998).

Androgens (especially testosterone) have been known to increase aggression in both males and females. Males tend to exhibit aggression in competition for sexual partners during breeding seasons, but this can even extend into the non-breeding season. Females exhibit defensive aggression to defend their offspring and to protect

their nest or territory. Androgens have an organisational effect on the aggressiveness of females, and a certain amount of prenatal androgenisation appears to occur naturally (Carlson, 1998).

VASOPRESSIN AND OXYTOCIN

The vasopressin system has been implicated in the aggressive behaviour of male rats and mice and female prairie voles (Stribley, et al. 1999). In male rats and mice injection of vasopressin into the amygdala or lateral septum has been found to increase aggressive behavioural scores (Koolhas, et al. 1998). In the rat, centrally administered vasopressin can, like oxytocin, induce maternal behaviour in appropriately primed virgin rats (Pederson, et al. 1982, Leng, et al. 2006).

In both rats and mice, postpartum aggressive behaviour has been demonstrated to be suckling-dependent (Garland, et al. 1988), but not dependent on prolactin secretion (Mann, et al. 1980). The established role of oxytocin is in the milk ejection reflex during lactation: acting via oxytocin receptors on the myo-epithelial cells in the mammary gland causing them to contract. Moreover, oxytocin also plays a vital role in the contraction of the myometrium during the final stages of delivery (Russell, et al. 2003).

Oxytocin is one of the key neuropeptides regulating initiation and maintenance of maternal behaviour (Pedersen, et al. 2003). However, the neuropeptidergic regulation of maternal aggression is less clear and even partly controversial (Oliver, et al. 2005). Underlying many studies on oxytocin neurones and their roles in parturition and lactation is an assumption that, because these cells then exhibit exceptional modes of

behaviour, the animals only become competent to display maternal behaviour in pregnancy (Russell, et al. 2003).

Microinjection of oxytocin into the amygdala in the hamster has been demonstrated to increase aggressiveness of lactating females towards intruding males (Ferris, et al. 1992). It has been reported by Giovenardi and co-workers (1998), that pharmacological manipulation of the oxytocin system within the central nucleus of the amygdala (CeA) or the parvocellular division of the PVN elicits changes in maternal aggression (Giovenardi, et al. 1998, Johns, et al. 1998, Elliott, et al. 2001, Lubin, et al. 2003 & Bosch, et al. 2005). In rats, maternal aggression has been reported to be both dependent and independent of suckling (Mayer, et al. 1987 & Leng & Russell, 2006) and independent of prolactin secretion (Erskine, et al. 1980) and probably oxytocin secretion (Factor, et al. 1982). However, aggressive behaviour is evidently influenced by the somatosensory inputs to the snout and ventral body surface influenced by the volatile odours acting through the olfactory bulb (Ferreira, et al. 1987, Stern & Kolunie, 1993 & Kolunie & Stern, 1995).

The vasopressin and oxytocin systems are often studied by observation of expression of immediate early genes: most importantly and commonly the expression of Fos-IR cells, the protein product of *c-fos*, in the populations of hypothalamic neurones. Oxytocin cells strongly express Fos-IR cells and other immediate early genes (Fos B, Erg-1) within ninety minutes after the birth of the first to second pup (Russell, et al. 2003, Meddle, et al. 2000, Lin, et al. 1995 & Lin, et al. 1998).

NEUROSTEROIDS

The term neurosteroid was first termed in 1981 by Etuenne-Emile Baulieu and colleagues. The concept that steroids may be synthesized in the brain was initiated by Baulieu and co-workers (Baulieu, 1997). They discovered that steroids such as pregnanolone, and their sulphate and lipoid esters, were found in higher concentrations in nervous system tissue than in the plasma. They also found that steroids remain in the nervous system long after surgical removal of gonads and adrenal glands. To indicate the origin and to differentiate them from the classical steroid producing organs such as the gonads, adrenal glands and placenta, they have been named neurosteroids (Wagner, 2001).

Neurosteroids, by definition, are steroids found within the CNS synthesised *de novo*. These are molecules structurally similar to cortisone, progesterone and the gonadal hormones. They include metabolites of progesterone and androgens which are formed in the nervous system, and may have a physiological role (Belelli, et al. 2005). In the nervous system, neurosteroids are synthesised within the myelinated glial cells, from cholesterol or steroidal precursors imported from the peripheral sources (Mellon, et al. 1993), these include 3-beta-hydroxy-delta 5-compounds, such as pregnanolone (PREG) and dehydroepiandrostenedione (DHEA), their sulfates (pregnanolone sulfate and dehydroepiandrostenedione sulfate (DHEAS)), and reduced metabolites such as the tetrahydroderivative of progesterone-3-alpha-hydroxy-5 alpha-pregnane-20-one (3 alpha, 5 alpha-THPROG) and allopregnanolone. These compounds can act as allosteric modulators of neurotransmitter receptors, such as $GABA_A$, N-Methyl D-aspertate (NMDA), and sigma receptors. Progesterone (PROG) is also a neurosteroid, and a progesterone receptor has been identified in peripheral and central glial cells

(Baulieu, 1997). In rodents, the neurosteroid pregnanolone sulphate (PREG-S) has been shown to produce antidepressant-like effects (Barrot, et al. 1999). At different regions of the brain, neurosteroid concentration varies according to behavioural and environmental conditions, such as stress, sex recognition or aggressiveness. Neurosteroid concentrations in the circulation have been reported to vary according to the time of the day, extent of light exposure, food consumption, the presence of another animal, and handling frequency (Baulieu, 1997).

STEROIDOGENESIS

Steroidogenesis refers to the biosynthesis of compounds from simple precursor molecules such as glucose or acetate. Steroids are generally synthesised from cholesterol by a series of enzymatic actions. Understanding of the principal pathways of steroid hormone biosynthesis was achieved over two decades ago through advances in steroid radioisotopic and chromatographic techniques (Sutcliff, et al. 1996). When the enzymes of individual pathways could be examined in more detail, the dissection of the complex pattern of enzyme activities began. Separate pathways may employ the same enzymes such as some cytochrome P450s, aromatase, and several dehydrogenases (Orth, et al. 1992). 17 alpha-hydroxylase was later cloned and expressed in tissue culture cells, revealing that contrary to the enzyme in rat, human and cattle 17 alpha-hydroxylase cannot convert 17 alpha-hydroxyprogesterone to androstenedione (Bradshaw, et al. 1987, Fevolt, et al. 1989)

CHOLESTEROL SYNTHESIS

Cholesterol is produced from Acetyl-CoA in four stages:

1. Condensation of three acetate units to form a six-carbon intermediate mevalonate. This is the major point of regulation of cholesterol synthesis.

2. The conversion of mevalonate to activated isoprene units is the next step. Isomerization of this compound will yield the second activated isoprene, dimethylallylpirophosphate.

3. Polymerization of six 5-carbon linear squalene molecules.

4. Cyclisation of squalene to form the four rings of the steroid nucleus, with further changes which include oxidations, removal or migration of methyl groups, produces cholesterol (Nelson, et al. 1982).

FORMATION OF STEROID HORMONES

All steroid hormones have in common a four-ring structure (designated rings A, B, C, and D) called a cyclopentanoperhydrophenantrene nucleus with numbered carbon atoms 1 to 9. The steroid group may be a planar (flat) structure with subtituents such as a methyl group at C18 or C19 and the ethyl group side chain at C 17 projecting above the plane. It has been proposed that the basic 17 carbon nucleus minus the methyl groups and side chain carbon carbon double bonds be called gonane. Slight modifications to the gonane give rise to the various biologically active steroid hormones. Addition of an angular methyl to represent C18 leads to the formation of

oestrane, the parent structure of the oestrogens. On the other hand, addition of both methyl groups and projection of hydrogen at carbon 5 from above the plane of the nucleus to below the plane gives rise to androstane the parent structure of the androgens. The third alternative is to add carbons, 20 and 21 at position 17 to give rise to pregnane, which is a precursor for progestins and adrenal steroids. This simply leads to the point that C18 steroids are estrogens, C19 compounds are androgens and C21 compounds are either adrenal corticoids or progestins (McDonald, 1980).

OESTROGEN AND TESTOSTERONE BIOSYNTHESIS

Oestrogens are hormones that are synthesised in various tissues such as the ovaries, placenta, testes, and brain. 17 beta-oestradiol is the primary oestrogen of ovarian origin. Oestrogen is more abundant in pregnancy due to its placental synthesis. The enzymes involved in oestrogen synthesis are same as those involved in androgen synthesis. Oestrogen is formed by a complex process that involve three hydroxylation steps each requiring oxygen and NADPH. The aromatase enzyme complex is thought to include a P450 mixed function oxidase. The oestradiol formed is the substrate involved in the formation of testosterone, whereas oestrane is produced from the aromatisation of androstenedione (Murray, et al. 2000).

Significant amounts of oestrogens are produced by peripheral aromatisation of androgen to oestradiol (E2). In humans, the peripheral aromatisation of testosterone to oestradiol accounts for 80% of the production of oestradiol. In females, adrenal androgens are important substrates as 50% of total oestrogen in pregnancy is produced here (Murray, et al. 2000).

Aromatase was described more than thirty years ago as an enzyme involved in steroidogenesis - responsible for the conversion of testosterone to oestradiol. This same enzyme is expressed in the brain (Naftolin, et al. 1971). It is now accepted that aromatase is found in the hippocampus, both in glial cells and neurones, as shown at the mRNA level (Abdelgadir, et al. 1994). The main site of aromatase expression in premenopausal women is the ovarian follicle, where FSH induces aromatase followed by oestradiol production in a cyclic fashion (Simpson, et al. 1994). Aromatase has also been expressed in the brains of fish, birds, and lower mammals (rodents) through highly conserved promoters named I.f and II respectively (Simpson, et al. 1994)

The initial entry of cytoplasmic cholesterol into the mitochondrion, which is enhanced by steroidogenesis acute regulatory protein (StAR), represents a major step in neurosteroidogenesis. Six enzymes encoded by at least five specific genes then catalyses the conversion of cholesterol to the biologically active oestrogen, oestradiol. The aromatase enzymes catalyses the final step, i.e., the conversion of C19 steroids to oestrogens. Metabolites of progesterone particularly allopregnanolone, formed in the brain can potentiate GABA actions by acting on $GABA_A$ receptors. This depends upon the receptor components and the post-receptor signaling mechanism. (Sedar, et al. 2005).

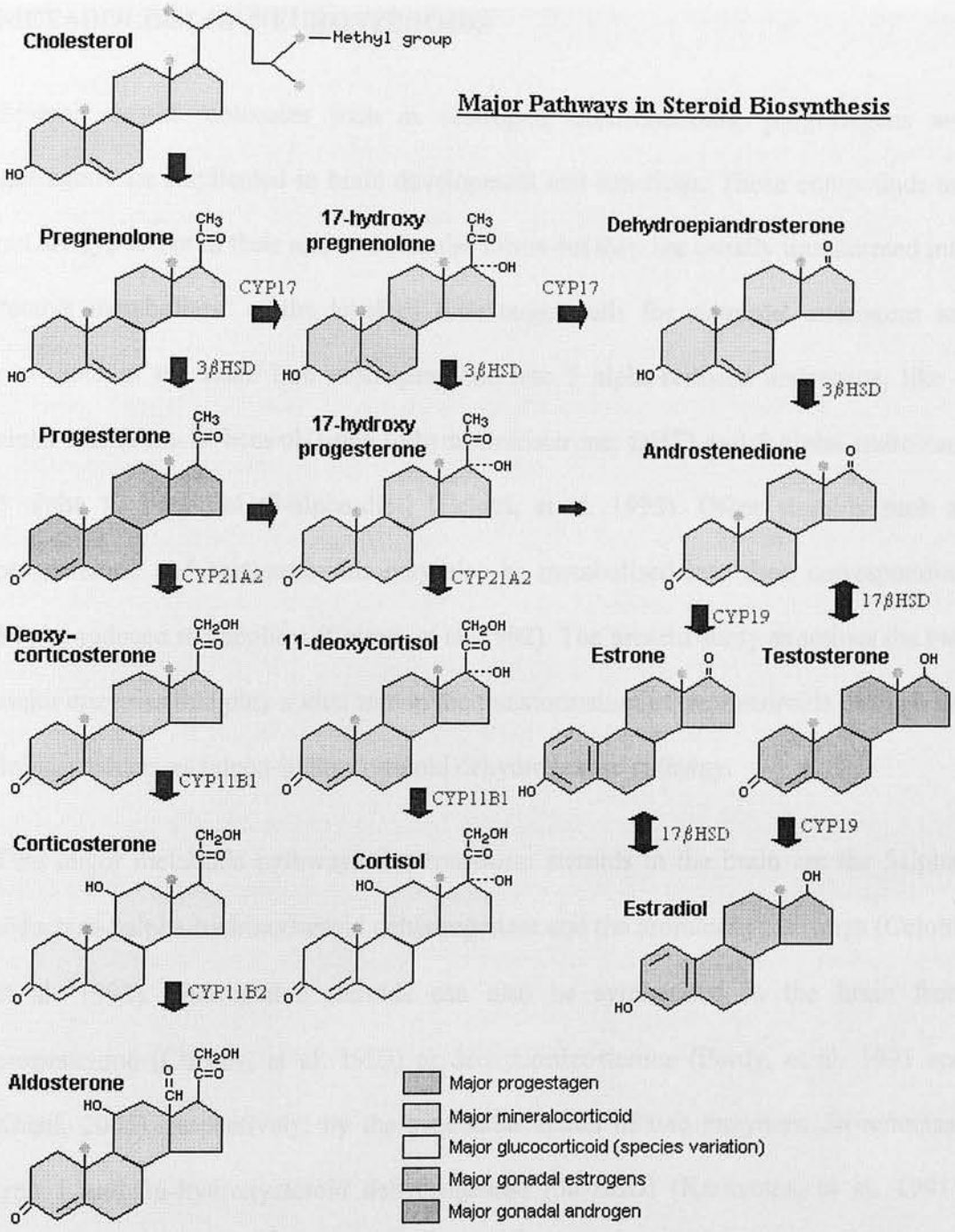


Figure 1.2 The major pathways in steroidogenesis

From:
http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/basics/steroidogenesis.html

METABOLISM OF NEUROSTEROIDS

Several steroid molecules such as oestrogen, corticosteroids, progestagens and androgens are implicated in brain development and functions. These compounds are not always active in their native molecular forms but they are usually transformed into "active metabolites" at the level of their target cells for example, androgens are converted in the brain into oestrogens and into 5 alpha-reduced androgens, like 5 alpha-androstan-17 beta-ol-3-one (dihydrotestosterone; DHT) and 5 alpha-androstan-3 alpha 17 beta-diol (3-alpha-diol) (Celotti, et al. 1998). Other steroids such as progesterone and corticosteroids may also be metabolised into their corresponding 5alpha-reduced metabolites (Celotti, et al. 1992). The present study examines the two major enzymes that play a vital role in the transformation of neurosteroids through the 5alpha-reductase-3alpha-hydroxysteroid dehydrogenase pathway.

Two major metabolic pathways that transform steroids in the brain are the 5alpha-reductase-3alpha-hydroxysteroid dehydrogenase and the aromatase pathways (Celotti, et al. 1998). Neuroactive steroids can also be synthesised in the brain from progesterone (Cheney, et al. 1995) or deoxycorticosterone (Purdy, et al. 1991 and Khisti, 2005), respectively, by the sequential action of two enzymes, 5α-reductase type 1 and 3α-hydroxysteroid dehydrogenase (3α-HSD) (Karavolas, et al. 1991). Other related enzymes include cytochrome P450, aromatase and 17 beta-hydroxysteroid dehydrogenase (17β-HSD) (Stoffel-Wagner Birgit, 2003). These processes give rise to the formation of neuroactive steroids in the brain which modulates $GABA_A$, N-methyl-D-aspertate (NMDA), muscarinic, serotonin (5-HT), kainite, glycine and sigma receptors, neuroprotection in or outgrowth of dentritic spines and synaptogenesis (Stofell-Wagner, 2003). These modulate brain functions to

affect behaviours such as aggression, anxiety and cognition (Pinna, et al. 2005). The involvement of neuroactive steroids in clinical conditions such as fatigue, premenstrual syndrome, postpartum depression, catamenial epilepsy, and depressive disorders has also been reported (Stoffel-Wagner, 2003).

5α-REDUCTASE

5α-reductase type 1 (5α-R) is one of the enzymes involved in the 5α-reductase-3α-hydroxysteroid dehydrogenase steroid formation enzyme system. The 5α-R isoenzymes are coded by 2 genes, referred to as the 5α-R type 1 and type 2 (Lephart, et al. 2001) and both proteins catalyse the same biochemical reaction (Russell and Wilson., 1994). The type 1 gene is located on the short arm of chromosome 5, while the type 2 gene on the short arm of chromosome 2 (Lephart, et al. 2001).

Anderson and Russell (1993) reported that in humans the 2 isoenzymes of 5α-R, which differ in biochemical characteristics and tissue distribution as well as in their responsiveness to specific inhibitors of their enzymatic activity, have been identified.

5α-R metabolises progesterone to 5α-dihydroprogesterone (5α-DHP) which is then further reduced to a potent neurosteroid 3α, 5α-tetra hydroprogesterone (3α, 5α THP) (Mellon, 1994) this is displayed by a mechanism that involves the irreversible transfer of two hydrogen atoms from NADPH that reduces the 4-5 double bond of the substrates such as androgens (testosterone) or progesterone (Celotti, et al. 1992 & Russell, et al. 1994). Progesterone is suggested to be a better substrate to testosterone due to its abundance as substrate to 5α-R, higher affinity to the enzyme and it's concentrating biochemical properties within the brain (Lephart, 1993). Once the conversion of progesterone to 5α-dihydroprogesterone or testosterone to 5α-dihydrotestosterone has taken place these reduced metabolites becomes better

substrates for 3α reduction through 3α-dihydroxysteroid dehydrogenase (Celotti, et al. 1992 & Lephart, 1993).

DISTRIBUTION OF 5α-REDUCTASE IN THE BRAIN

Brain distribution of 5α-R is more uniform and widespread than P450 aromatase in both cell type and brain region (Celotti, et al. 1992, Lephart, et al. 1993, Martini, et al. 1982, Guennoun, et al. 1995). 5α-R has been demonstrated in both neurone and glial cells, neurones show higher rates of enzymatic activity than glial cells and white matter structures contain very high levels of 5α-R.

Lephart and co-wokers (1993), demonstrated 5α-R type 1 enzyme in the medial basal hypothalamic and preoptic area, amygdala and frontal cortical tissue of male and female rats. Other tissues found to demonstrate 5α-R are the skin expressing type 1 isoenzyme and the prostate expressing type 2 isoenzyme. The olfactory bulb is characterised by expression of intense 5α-R type 1 mRNA, likewise the basolateral anterior amygdaloid nucleus and sparse expression in the thalamus, striatum and hippocampus (Roberto, et al. 2006).

3α-HYDROXYSTEROID DEHYDROGENASE (3α-HSD)

The 3α-HSD also referred to as dihydrodiol dehydrogenase (DD3), has 3 functional isoenzymes in human, based on their affinity for 5α-dihydrotestosterone (Khanna, et al. 1995, & Penning, 1997). The human brain expresses 3α-HSD, which either catalyse the reduction of 5α-dihydroxytestosterone (5α-DHT) into allopregnanolone or reverse this reaction depending on the optimal condition (Roberto, et al. 2006 & Penning, et al. 2004). So far only one isoform of 3α-HSD has been identified in rodent brain (Hara, et al. 1988 & Penning, et al. 2003). 3α-HSD type 2 is thought to

be responsible for the production of neurosteroids (Khanna, et al. 1995), whereas the type 1 isoenzyme is expressed only in the liver. It plays a role in the physiological inactivation of steroid hormones, metabolism of xenobiotics, such as polycyclic aromatic hydrocarbons, pesticides and the reduction of keto-containing drugs by using NADPH or NADH as a co-factor (Brinstock,et al. 1992). The fact that the mRNA expression levels of 3α-HSD are greater than those of 5α-R type 1 mRNA isoforms this suggest that 5α-R is the rate-limiting step in the formation of 3α, 5α-reduced metabolites of progesterone (Russell & Wilson, 1994). The rat 3α-HSD enzyme shares 84% sequence similarity with human liver dihydrodiol dehydrogenase (DD2) which is considered the human counterpart of the rat 3α-HSD (Penning, et al. 1996).

DISTRIBUTION OF 3α-HSD IN THE BRAIN

Previous studies (Khanna, et al. 1995, Cheng, et al. 1994, Penning, et al. 1996 & Krieger & Scott, 1984) have described the distribution of 3α-HSD in the rat brain. Results have consistently shown that the olfactory bulb and the olfactory tubercle have the highest brain concentration of the 3α-HSD enzyme, while moderate concentrations are evident in other brain regions such as the amygdala and the striatum. They also report the utilisation of both dihydrotestosterone and dihydroprogsterone as substrates.

NEUROSTEROIDS AND BEHAVIOUR

Neurosteroids affect the most widely distributed neurotransmitter and receptor systems in the central nervous system: glutamate and gamma-amino butyric acid (GABA) mechanisms. By affecting primary excitatory and inhibitory systems, neurosteriods modulate a wide array of behaviours including aggression, anxiety and cognition. Generally neurosteriods that positively modulate $GABA_A$ receptors

decrease anxiety, and exhibit a bimodal effect on aggression (Belellis, et al. 2005). Several studies in mice that are not related to anabolic androgenic steroids (AAS) have unequivocally revealed that impairment of GABAergic transmission plays a vital role in the expression of aggressive behaviour (Pinna, et al. 2005, Miczek, et al. 2003, Pinna, et al. 2003 & Guidotti, et al. 2001).

Neurosteroidal control of some behavioural activities may apply to all mammalian species including humans (Baulieu & Robel, 1990). Neurosteroids may serve physiological functions not mediated through the classical steroid hormone receptor (Majewska, et al. 1987): releasing GnRH from the hypothalamus (progesterone and its metabolites) (Flood, et al. 1992), attenuating excitatory amino acid responses of cerebella purkinje fibre via NMDA receptors; modulating behavioural responses (corticosterone, pregnenolone, dehydroepiandrosterone and progesterone) (Orchinik, et al. 1991) and enhancing memory (pregnanolone and its metabolites) (Flood, et al. 1992). The neurosteroid allopregnanolone (Allop) is a potent endogenous $GABA_A$ receptor complex modulator, and thus consequently exerts sedation, hypnosis, anxiety, and anticonvulsant effects (Gacior, et al. 1997). Male mice socially isolated showed increased aggressiveness due to reduced responsiveness to $GABA_A$ receptor acting drugs, and a deregulation of brain levels of 3alpha, 5alpha-tetrahydroprogesterone (allopregnenolone: 3 alpha, 5 alpha-THP), a neurosteroid endowed with potent positive allosteric modulator of $GABA_A$ (Pinna, et al. 2005). Allop administration can significantly increase aggression in male rodents at moderate doses, implicating the involvement of $GABA_A$ receptor in the aggression-heightening effects (Zitzman, et al. 2005).

NEUROSTEROIDS AND THE POSTPARTUM PERIOD

It is possible that neurosteroids play a role in postpartum psychiatric conditions particularly since there is a rapid drop in oestrogen and progesterone following delivery. It may be hypothesised that this dramatic decrease in oestrogen in the brain causes a disturbance in the brain neurosteroid-sulphate balance. This may lead to anxiety and depression through GABAergic activity (Buckerwater, et al. 1999).

NEUROTRANSMITTERS

The two principal neurotransmitters found in the brain are gama aminobutyric acid (GABA) and glutamate which are inhibitory and excitatory in action respectively, both are synthesised in the brain from the Krebs citric acid molecule α-ketoglutarate from glutamic acid with vitamin B6 pyridoxal phosphate as a co-factor. The release of glutamate by synaptic terminals opens ionic channels in the postsynaptic membrane via three classes of the ionotropic glutamate receptors (IGRs) named after the agonists that activate them: NMDA, AMPA, and kainate receptors (Chun-rong, et al. 2005). However, IGRs can also be expressed presynaptically throughout the brain and the spinal cord (Engelman & MacDermott, 2004). Both decreased and enhanced release of GABA by activation of presynaptic kainate receptors were reported in the hippocampus, basolateral amygdala, and ventrobasal thalamus (Chun-rong, et al. 2005; Braga, et al. 2003). The magnocellular cells of the SON primarily function in parturition and lactation but they also have clear microvesicles (MVs) that contain glutamate (Ponzio, et al. 2006). Glutamate has also been reported to cause cell death in the event of excessive release. Fernandez, et al. (2005), found that exposure of cultured cells to glutamate and other excitatory amino acids acting via specific receptors (i.e. ionotrophic and/or metabotrophic glutamate receptors, caused massive

nuclear alteration leading to damage of cell components including mitochondria, finally causing cell death. Excitotoxicity mediated by N-methyl-D-aspartate (NMDA) receptors also induces expression and activation of the apoptotic protein caspases (Fernandez, et al. 2005 & Zeron, et al. 2004). IGR subunits are expressed not only in primary afferent terminals; some IGR-immunoreactive terminals also establish symmetric synapses, suggesting that they may be GABAergic (Lu, et al. 2002).

GLUTAMIC ACID DECARBOXYLASE (GAD)

Glutamic acid decarboxylase (GAD) is the enzyme that catalyses the rate-limiting stage in the formation of gamma-amino butyric acid (GABA), which is considered to be the major inhibitory neurotransmitter in the central nervous system, it is also found in several tissues outside the central nervous system. Biological function of glutamic acid decarboxylase and gamma-amino butyric acid extend beyond regulation of neurotransmission to include immunological effects as well as modulation of cell proliferation, protein synthesis and metabolism. Two isoforms of GAD derived from a single separate gene are known to be present in the rat brain, GAD65 and GAD67, based on their relative molecular weight in kDa. Both isoforms are known to have significant levels of homology in the catalytic portion of the molecule, but differ greatly in the first 95 amino acids in the N-terminal region of the protein.

GAMMA AMINO BUTYRIC ACID (GABA)

GABA is the major inhibitory neurotransmitter of the brain, occurring in 30-40 percent of all synapses and second only to glutamate as a major neurotransmitter. The GABA concentration in the brain is 200-1000 times greater than that of the monoamines or acetylcholine (Fernandez, et al. 2005). GABA is also synthesised in the brain the same way glutamate is synthesised from the Krebs citric acid molecule α-ketoglutarate a reaction known as GABA shunt.

During maternal behaviour, neurones in the medial preoptic area and bed nucleus of stria terminalis are active and show an increase in the labelling of early gene products Fos-IR cells and fosB (Arrati, et al. 2005 & Numan, et al. 2003). $GABA_A$ receptors are located on postsynaptic neurones and $GABA_B$ receptors are located mainly on presynaptic neurones. Activation of $GABA_A$ receptors produces postsynaptic hyperpolarisation whereas activation of $GABA_B$ receptors inhibits presynaptic neurotransmitter release and produces postsynaptic hyperpolarisation via a G-protein mechanism (Durkin, et al. 1999). GABAergic neurotransmission involves ionotrophic GABA and metabotrophic $GABA_B$ receptor subtypes charecterised by their pharmacological (Bormann, et al. 1988), and electrophysiological (Hillet, et al. 1981) properties. Although fast inhibitory transmission through $GABA_A$ receptor activation is commonly found in the basal ganglia, the function as well as the cellular and subcellular localisation of $GABA_B$ receptors is still poorly known (Charara, et al. 2000). The output pathways as well as the intrinsic synaptic circuits of the basal ganglia use mainly GABA as transmitter (Smith, et al. 1998, & Parent, et al. 1995). The GABA receptor is a hetero-oligometric protein which gates a chloride channel and process modulatory sites for benzodiazepines and a variety of other substances (Olsen, et al. 1990)

The $GABA_A$ receptor belongs to the family of seven transmembrane domain metabotrophic receptors that use G-proteins to regulate ion channels and intracellular second messengers. Whereas stimulation of $GABA_A$ receptors produces fast postsynaptic increase in chloride ion conductance which can be blocked by bicuculline, activation of $GABA_B$ receptors induces either slow postsynaptic increase in potassium ion conductance or presynaptic decrease in calcium ion conductance (Hill, et al. 1981).

The GABA innervation appears to play a role in patterning the pulsatile discharge of oxytocin cells that is observed both during parturition and during suckling-induced reflects milk ejection (Leng & Russell, 1999 & Moos, 1995) and also implicated in maternal behaviour (Arrati, et al. 2005). The classic account of parturition is that in late pregnancy, the high circulating concentrations of progesterone induce uterine quiescence while the uterus acquires contractile ability. During catabolism, metabolites of progesterone can have membrane actions, especially the Allop can act at $GABA_A$ receptors to potentiate the actions of GABA, depending upon the particular subunit of the receptors. GABA is also an important neurotransmitter in oxytocin cells and about 45 percent of all synapses onto the oxytocin cells contain GABA and the total numbers of GABA synapses are more in lactating rats than in virgins (Leng & Russell, 1999 & El Majdoubi, et al. 1997). GABA receptors are expressed not only on the cell bodies and dendrites of supraoptic neurones, but also on the nerve endings, where they may modulate stimulus secretion coupling (Jackson & Zhang, 1995). Some mechanisms are responsible for the switch from $\alpha 1$ to $\alpha 2$ $GABA_A$ receptors subunit expression at the end of pregnancy. This may play a role in maternal aggression and the pattern is reversed and altered at the end of lactation (Leng & Russell, 1999). IRGs can also act as heteroceptors when expressed in

gamma amino butyric acid (GABA)-ergic terminals and has been reported by Chun-Rong, et al. (2005) to modulate the release of GABA in various brain regions such as the activation of the presynaptic NMDA receptors which enhances release of GABA from inter-neurones onto Purkinje cells (Smart & Duguid, 2004).

Classical neurotransmitters are synthesised from dietary amino acid glutamate by removal of a carboxylic group (COOH) from glutamate catalysed by glutamic acid decarboxylase (GAD). The effect is relatively short lasting and after being released from the synapses they can readily be replenished quickly after use. There is no one-to-one relationship between transmitters and functions; any one transmitter can have many relations to behaviour. Transmitter system also appears to influence one another in that if the level of activity of one transmitter is changed, it in turn changes the activity of other transmitters.

There are some underlining processes that lead to aggression; one of the obvious principles is that there are multiple systems controlling virtually every behaviour. Visual information following the introduction of an intruder follows a ventral route through the temporal lobe and dorsal route through the parietal lobe. The former plays a role in object recognition and the later plays a role in spartial location. Considering the general principle of brain organisation, suggests that there are multiple systems both cortical and subcortical that contribute to maternal aggression (Carlson, 1998).

THE AIMS OF THIS STUDY

Despite the recent work on maternal aggression and the neurotransmitters, there is still uncertainty about the role neurosteroids play in maternal behaviour, particularly aggression, and lack of attribution of a specific neurotransmitter either inhibitory (GABA) or excitatory (glutamate) to maternal aggression. This necessitated the study of maternal aggression exhibited by lactating female rats during the peripartum and lactating period. Lactating mothers exhibit maternal behaviour and also show defense behaviour with aggression directed against conspecific intruder rats.

The aim of the study was to examine the role neurosteroids play in the exhibition of maternal aggression by combining immediate early gene immunocytochemistry with detection of the enzymes GAD, 5α-reductase and DD3 in the rat brain.

Activation of neuronal circuits containing these enzymes during maternal aggression would provide evidence to suggest a role for neurosteroids in peripartum aggression in female rats.

CHAPTER 2

MATERIALS AND METHODS

SELECTION AND HOUSING OF ANIMALS:

Female Sprague-Dawley rats (body weight between 250 and 350g supplied by Harlan) were housed in environmentally enriched polypropylene cages with wood shaving bedding, under standard laboratory conditions (12 hours light/dark cycle; lights on at 08:00 A.M, 22°C, 60% humidity; and *ad libitum* access to water and standard rat chow (Harlan), as recommended by the Home Office Animal (Scientific) Procedure Act, 1986. Rats were mated and pregnancy was confirmed the next day by the presence of seminal plug in the cage. This was taken as pregnancy day 1. Pregnant rats were housed in groups of four in standard rat cages (40 x 60 x 20 cm); from day 18 of pregnancy rats were housed individually. On days 22 and 23 of pregnancy the rats delivered, with a litter size of 6-16 pups, and the day of parturition was marked as lactation day 1 the experiment was conducted between 5th-7th days of lactation. It was ensured that rats from both the experimental group (n=16) and control group (n=16) exhibited maternal behaviour such as lactation, pup retrieval, hovering over pups, nest building, pup licking and grooming, to ensure they were good mothers. Rats were moved to the experimental room at least 2-3 days before the start of the experiment. This allowed time for the rats to adapt to the new environment before the experiment began.

EXPERIMENTAL PROCEDURES:

1. TEST FOR MATERNAL AGGRESSION

All rats were assessed for maternal aggression using a resident intruder maternal defense test. A conspecific virgin intruder of approximately same weight and age to the lactating dam was marked using a marker pen for easy identification and was introduced into the cage of the resident dam (with pups), to quantify the dam's defensive aggressive behaviour. The virgin intruder was left in the dams' cage for 30 minutes and video footage was recorded with a digital camera during this period. Time was recorded as zero when the intruder was placed into the dam's cage. At the end of the resident-intruder test the intruder rat was removed from the cage. Behavioural analysis of the dam was performed for each individual, using "Observer 5.0" software (Noldus IT., Tracksys LTD. United Kingdom). The behaviours of both the intruder and the resident were analyzed during the 30 minutes period and scored for: attacks, eating, biting, drinking, rearing, pup related behaviour, nest building, freezing, escape, avoidance, and clawing. Any behaviour exhibited by the rats that was not among these categories, was scored as other behaviour. Latency to first attack, frequency of attacks, total duration of attacks and other various forms of aggressive behaviours such as pinning down were also analysed using the behavioural analysis data.

2. PERFUSION-FIXATION

Perfusion-fixation was performed at 1 hour following the end of the maternal aggression test. Rats were transferred to the perfusion room immediately prior to perfusion. Rats were weighed and euthanised with an overdose of anesthetic agent; pentobarbital sodium (40-50mg/kg body weight adjusted for body size), and then placed back into the in home cage. When moribund the rats' chest was opened with scissors to expose the still beating heart. Transcardial perfusion was performed by inserting a blunted needle into the heart chamber that was attached to silastic tubing through to a perfusion pump. The tube end was inserted into the bottle containing heparinized physiological saline packed in ice (1000 ml 0.9% physiological saline and heparin 5000 iu, Multiparin.). This heparinised/saline solution was pumped into the circulation to flush out all blood. As soon as the liver appeared pale, the tube was immediately transferred from the heparinised saline bottle into the bottle containing 4% paraformaldehyde (40g paraformaldehyde in 1000 ml 0.1M phosphate buffer). The pump was stopped once 300ml of fixative had been pumped through the body and the carcass became rigid. The brain was immediately removed and post fixed overnight at 4°C in small labelled vials filled with post fixative (15% sucrose in 4% paraformaldehyde see later). 24 hour later the brain was transferred to 30% sucrose in PB over night until the brain had sunk. Brains were then removed and frozen on powdered dry ice. Brains were then stored at -70°C.

3. BRAIN SECTIONING

The brain was sectioned into 52μm sections on a freezing microtome. Before sectioning, the microtome was turned on and the temperature adjusted to between -20 to -22°C. The embedding medium tissue tek was used to fix the brain onto the microtome and the brain was covered with foil and left for 20 minutes to equilibrate to cutting temperature. The 52μm sections were collected into a Petri dish containing 0.1M PB and later transferred to a small labelled vials containing section cryoprotectant (see later) and stored in the freezer at -20°C.

Fos-IR/GAD DOUBLE IMMUNOCYTOCHEMISTRY

BACKGROUND: Glutamic acid decarboxylase (GAD) is the enzyme that catalyses the rate-limiting stage in the formation of gamma-amino butyric acid (GABA), which is considered to be the major inhibitory neurotransmitter in the central nervous system (Negron, et al.2002), c- fos is an immediate early gene IEG), one of a family of such genes; immediate, refers to its rapid activation after a particular stimulus (aggression) and early, refers to the relatively short duration of it's activity (about 2-3 hours) after the stimulus. In conjunction with other IEGs, it begins a cascade of neural events. Although it is not yet known what role it plays in maternal aggression, it is used as a marker of neural excitation. The protein product, Fos, can be visualised with immunocytochemisrty (ICC) (Negron, et al. 2002).

AIMS:

1. To map the brain for immediate early gene expression (c-Fos) in aggressive lactating rats; to identify the brain regions involved in the exhibition of female aggressive behaviour.

2. To investigate whether any of these activated cells in the aggressive lactating rat brain are GABAergic by examining whether they contain the enzyme GAD.

HYPOTHESIS: Neurones involved in maternal aggression, as mapped by Fos expression, are GABAergic and are thus inhibitory.

IMMUNOCYTOCHEMISTRY

Double labelling immunocytochemistry for Fos-IR and GAD was performed on brain sections from control (n=6) and experimental (n=6) rats. Sections from the olfactory bulb, fore brain, and hind brain were washed in 0.1M Phosphate buffer with 2% triton X-100 (BDH 306324N) (PB-T) to remove fixative, endogenous peroxidase was blocked by washing in 30% hydrogen peroxidase and unspecific staining was blocked by normal sheep serum. Immunocytochemistry procedure was run using c-fos polyclonal antibody raised in rabbit (Ab-2 Rabbit polyclonal; Oncogene Science) diluted at 1:1000 in PB-T and incubated for 2 days at 4°C. The antibody-antigen complex was visualized by the ABC method with a vector elite kit (Vector, Bucks, UK). Diaminobenzidine (DAB, Sigma D5637) with nickle ammonium sulphate (BDH 101674A) were used as a black chromogen. Sections were further double labelled using glutamic acid decarboxylase (GAD) antibody (Rabbit anti-GAD 65/67

polyclonal: Chemicon International), diluted at 1:2000 Concentration in PB-T and incubated for 2 days at 4°C. GAD immunoreactivity was also visualized by the ABC method. Final complex was visualised using 25mg (1ml) DAB, as a brown chromogen and 3% hydrogen peroxide (Sigma H1009) (99ml PB + 100µl 3% hydrogen peroxide + 1ml DAB). Sections were stored at 4°C in PB until they were mounted. Specific details of immunocytochemistry are shown below.

BRAIN TISSUE FIXATIVES

Heparinised Saline

9g of Sodium Chloride in 1000 ml distilled water stirred to dissolve the salt completely. 5000 iu/ml (Multiparin) heparin was added and was stored in the fridge at 4°C overnight. 30 ml of heparinised saline was infused intracardially and pumped to the tissues at the rate of 15 - 25ml per minute.

4% Paraformaldehyde in PB (pH 7.3 - 7.4)

To prepare 1 litre of 4% paraformaldehyde in PB solution:

Solution A: 40g of parafomaldehyde (BDH294474L) was added to 500 ml of warmed distilled water. Drops of sodium hydroxide were added.

Solution B: 11.5g of disodium hydrogen orthophosphate (BDH103834G) and 2.72g Sodium di-hydrogen orthophosphate (BDH102455S) was dissolved in 500 ml distilled water.

Solutions A and B were combined, mixed and cooled on ice and pH was adjusted to 7.3. The solution was kept in the fridge at 4°C overnight. During perfusion 400 ml of 4% paraformaldehyde in PB was pumped into each animal.

POST-FIXATIVE SOLUTIONS

15% Sucrose in 4% Paraformaldehyde.

15g sucrose (BDH102745C) was added to 100 ml of 4% paraformaldehyde. The brains were stored in small labelled vials filled with this solution at 4°C overnight.

30% Sucrose in PB solution

30g sucrose was added to 100 ml 0.1M phosphate buffer, and stirred thoroughly. The brains were removed from the 15% sucrose in paraformaldehyde solution and placed in the 30% sucrose overnight or until they sank to the bottom of the vial.

FREEZING OF THE BRAIN

The brains were removed from their vials containing 30% sucrose and were cut into three parts; the olfactory bulb, the mid-brain and the brain stem. Each portion was wrapped in foil and frozen on dry ice and stored at -70°C once the brains are frozen they can never be thawed.

CRYOPROTECTANT FOR THE CUT SECTIONS

Cut brain sections that were not processed for immunocytochemisrty the same day were stored at -20°C in phosphate buffered saline (PBS) with ethylene glycol and glycerol.

Preparation:

To prepare 0.2M PB, 400 ml distilled water was added to 100ml 1M PB

0.9g NaCl was added to 100 ml 0.2M PB to make the PBS

To 100 ml PBS; 60ml ethylene glycol and 40ml glycerol were added.

PREPARATION OF SOLUTIONS

1. 1M Phosphate Buffer (PB)

1M PB stock solution was first prepared as follows:

1 Litre of double distilled water was heated in microwave for 5 minutes

115g disodium-hydrogen-orthophosphate ($Na_2HPO_4.2H_2O$, BDH 103834G)

27.2g sodium-dihydrogen-orthophosphate ($NaH_2PO_4.2H_2O$, BDH102455S)

Salts were completely dissolved on a heated stirrer and the solution allowed to cool in the fridge.

To prepare 0.1M PB from the stock solution, 900 ml double distilled water was added to 100 ml of stock solution.The pH was adjusted to 7.3-7.4 by adding concentrated NaOH or HCl.

PB-T

2 ml triton X-100 (BDH 306324N) was added to 1000 ml 0.1M PB and mixed until the triton was completely dissolved.

Sodium Acetate Buffer (0.2M pH 6.0)

16.4g of sodium acetate (BDH102364Q) dissolved in 1000 ml double distilled water.

pH was adjusted to 6.0 using acetic acid.

Normal sheep serum (NSS) in 0.1M PB-T

Three aliquots of normal sheep serum (Sapona) were dissolved in 97ml of 0.1M PB-T, to produce 3% normal sheep serum.

Normal horse serum (NHS) in 0.1 M PB-T

To prepare 3% normal horse serum in PB-T, 3 ml of horse serum (Sigma) were dissolved in 97 ml of PB-T.

0. 3% hydrogen peroxidase in PB solution

1ml of 30% hydrogen peroxide (Sigma H1009) was added to 99ml 0.1M PB and stirred thoroughly.

Diaminobenzidine Tetrachloride (DAB) 25mg/ml

1g of Diaminobenzidine tetrachloride (DAB, Sigma D5637) was dissolved in 40 ml of warmed double distilled water. The solution was then aliquot into 1ml vials and stored at -20°C.

VISUALIZING SOLUTIONS

Nickle-DAB Solution

Solution A: 25g of Nickle ammonium sulphate (BDH 101674A) and 0.08g Ammonium chloride was dissolved in 50 ml 0.2M Sodium acetate (BDH 102364Q)

Solution B: 1ml of aliquot DAB (Sigma D5637) was dissolved in 50 ml double distilled water.

Solution A and B were added on a stirrer and 100µl of 30% hydrogen peroxide (Sigma H1009) was added prior to use. This solution was used in visualization of c-fos.

PB-DAB solution

1ml of DAB (sigma D5637) was dissolved in 99 ml 0.1M PB and 100µl of 30% hydrogen peroxide (sigma H1009) was added prior to use on stirrer. This solution was used in the visualization of GAD immunoreactivity.

Gelatine for subbing slides

5g gelatine in 1000 ml of distilled water was heated in microwave for 5 minutes and 0.5g chromic potassium sulphate was added on a stirrer until completely dissolved. The solution was cooled in the fridge and filtered before subbing slides. Slides were loaded onto a slide rack and then immersed in the solution and allowed to dry overnight before mounting sections.

IMMUNOCYTOCHEMISTRY

The aim of immunocytochemistry is to detect labelled antigen in a biological specimen. Successful immunolabelling depends on optimal preservation of antigen sites and on the target tissue antigen being rendered insoluble at its original site in the cytoplasm. Most sensitive methods lead to production of a labelled antigen of clear contrast against the back ground of the tissue.

IMMUNOLABELLING

Avidin-biotin complex is frequently used with the enzyme system to yield satisfactory result. Avidin, a glycoprotein, has a high affinity for the small water-soluble vitamin biotin, and biotin can be conjugated to a variety of biological molecules including antibodies. Biotinylated antibody can thus bind to more than one molecule of avidin to form a large molecule. Though avidin may bind non-specifically to negatively charged structures in the specimen due to its high isoelectric point at neutral pH, and may also react with molecules such as lecithin through the carbohydrate moiety (Jackson, et al. 1993). These effects were minimised by incubation of sections in normal sheep serum.

Avidin (bottle "A") and biotinylated horseradish peroxides (bottle "B") (Vector, Bucks, UK), were mixed at 20µl per ml PB-T, and were allowed to stand for at least 30 minutes at room temperature for the complex to form before incubating sections. This preformed complex is attached to the biotinylated antibody that sections were incubated in.

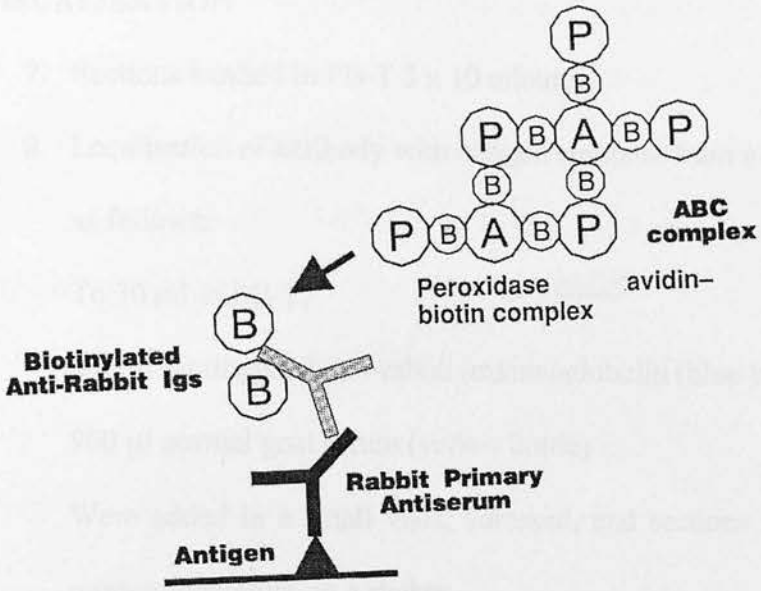


Figure 2.1 Avidin-biotin immunolabelling
Modified from; Jackson & Blythe, (1995)

DETAILED PROTOCOL

1. Sections washed in PB-T 4 x 15 minutes at room temperature to remove fixative.
2. Sections washed in 0.1M PB for 5 minutes
3. Sections washed in hydrogen peroxide (99ml PB + 1ml 3% hydrogen peroxide) for 15 minutes to block endogenous peroxidase.
4. Sections washed in PB-T for 3 x 10 minutes
5. Sections washed in 3% normal sheep serum (1 aliquot of NSS + 33 ml PB-T) for 30 minutes to block non-specific staining.
6. Sections incubated in rabbit polyclonal Fos antibody (rabbit polyclonal; Oncogene Science) at 1:1000 diluted in PB-T. Vortexed in a small labelled vial and incubated for two days at 4°C

VISUALISATION

7. Sections washed in PB-T 3 x 10 minutes
8. Localisation of antibody with antigen complex from a vectastain elite ABC kit as follows;

   To 30 ml of PB-T,

   300 µl biotinylated anti-rabbit immunoglobulin (blue bottle)

   900 µl normal goat serum (yellow bottle)

   Were added in a small vials, vortexed, and sections incubated for 1 hour at room temperature on a shaker.
9. Sections washed in PB-T 3 x 10 minutes
10. Sections incubated in ABC solution for 1 hour

    To 30 ml of PB-T,

    600 µl avidin DH (bottle A)

    600 µl biotinylated horseradish peroxidase (bottle B)

    were added, vortexed, and allowed to incubate for at least 30 minutes before sections were incubated for 1 hour at room temperature on a shaker.
11. Sections washed in PB-T 2 x 10 minutes
12. Sections rinsed in 0.1M acetate buffer for 5 minute (0.2M acetate buffer 50:50 with double distilled water, pH adjusted to 6.0) by adding drops of acetic acid.
13. Visualisation solution:

    5g nickel Ammonium sulphate and 0.16g Ammonium chloride were dissolved in 100 ml 0.2M Sodium acetate.

    50 mg DAB was dissolved in 100 ml double distilled water.

    The nickel and DAB solutions were added and 200µl of 30% hydrogen peroxide was added prior to use. This result in a black reaction product.

After 1 minute of adding the solution to the sections, the Petri dishes were placed on a white background and one section was then picked out with a brush placed on a microscope slide and examined under a microscope (Leitz) to monitor the reaction. This process was repeated until the reaction product was clearly visible. The reaction was then terminated with a wash of sodium acetate.

- Sections washed in 0.1M acetate buffer for 5 minutes to terminate the reaction.
- Sections were rinsed in PB for 4 x 5 minutes
- Sections washed in PB-T for 2 x 10 minutes.
- Sections washed in hydrogen peroxide (1ml 30% Hydrogen peroxide + 99 ml PB) for 15 minutes to block unreacted peroxidase.
- Sections washed in PB-T 2 x 10 minutes.
- Sections washed with 3% normal sheep serum (3 ml NSS + 97 ml PB-T) for 30 minutes.
- Sections incubated in GAD antibody (Rabbit anti-GAD 65/67 polyclonal: Chemicon International) 1:2000 for 2 days at 4°C.
- Sections washed in PB-T 3 x 10 minutes to remove excess antibody
- Incubation of the sections in anti-rabbit immunoglobulin to localize the antigen-antibody complex, for 1 hour at room temperature.
- To 30 ml PB-T;
- 300 µl biotinylated anti-rabbit immunoglobulin (blue bottle), and
- 900 µl normal goat serum (yellow bottle), were added.
- Sections washed in PB-T for 3 x 10 minutes
- Sections incubated in ABC solution for 1 hour at room temperature.
- To 30 ml PB-T;
- 600µl Avidin (bottle A), and

- 600µl Biotinylated Horse Radish Peroxidase (bottle B) were added, vortexed, and allowed to incubate for 40 minutes before adding the section.
- Sections washed in PB-T 2 x 10 minutes.
- Sections rinsed in 0.1M PB for 5 minutes
- Visualisation with 99 ml PB + 1 ml DAB + 100µl 30% hydrogen peroxide for 5 minutes.
- Sections washed in PB 3 x 10 minutes
- Sections were mounted in serial order on gelatine coated slides (Menzel-Glaser, Ca 76 x 26 mm), and allowed to air dry.
- Slides were placed in a slide rack and taken through ascending concentrations of alcohol and xylene for 5 minutes each. (70%, 80%, 95%, 100%, 100% alcohol, xylene 1, and xylene 2)
- Slides were coverslipped (VWR 631-0137) using a DPX mountant and allowed to air dry in a fume hood.

DAB-NICKLE AMMONIUM SULPHATE VISUALISATION

Immunocytochemical techniques depend on the enzyme-substrate reactions which convert colourless chromogens into coloured, visible end products. Sensitive hydrogen peroxide-diaminobenzidine (DAB) reaction, with horseradish peroxidase antibody label has been used to produce a brown end product insoluble in alcohol, xylene and other inorganic solvents and visible in light microscopy (Jackson, et al. 1993).

Safety precautions were taken against the possible carcinogenic property of DAB by the use of gloves, laboratory coat, and the use of bleach solution to neutralize all equipments in contact with DAB.

5α-REDUCTASE AND 3α-HSD IMMUNOCYTOCHEMISTRY

BACKGROUND

The neurosteroids (allopregnanolone and tetrahydrodeoxycorticosterone) are potent positive modulators of $GABA_A$ receptors, and are synthesised in the brain through the 5 alpha-reductase-3 alpha-hydroxysteroid dehydrogenase pathway from progesterone or deoxycorticosterone, respectively, by the sequential action of 5α-R and 3α-HSD (DD3) enzymes in the brain.

In this study 5α-R 1 (R-17): sc-20399 and DD3 (3α-HSD) (R-17): sc-20424 (Santa Cruz biotechnology) antibodies raised in goat were used for single and double Fos-IR immunocytochemistry to detect 5α-R type 1 (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD) enzymes, in the brains of aggressive and non-aggressive lactating rats.

AIM:

1. To immunolabel 5α-R and 3α-HSD (DD3) enzymes, in the rat brain.
2. To investigate whether neurons expressing Fos protein in aggressive lactating rats contain 5α-R or 3α-HSD enzymes.

HYPOTHESIS: Neurones in the brain activated following maternal aggression (identified by Fos-IR) contain 5α-R and 5α-HSD enzymes thus implicating a role of neurosteroids in this behaviour.

DOUBLE IMMUNOCYTOCHEMISTRY FOR FOS-IR AND 5α-REDUCTASE

PROCEDURE:

Double labelling Immunocytochemistry for Fos and 5α-R was performed on the brain sections from control (n=5) and aggressive (n=5) rats.

1. Sections were immunolabelled for Fos protein as previously described.
2. Sections washed in PB-T 2 x 10 minutes.
3. Sections washed with 3% normal horse serum (7 ml NHS + 227 ml PB-T) for 30 minutes.
4. Sections incubated in 5α-reductase 1 (R-17): sc-20399 goat polyclonal (Santa Cruz biotechnology), (60 µl + 30 ml 3% NHS in PB-T) 1: 500 dilution for 2 days at 4°C.
5. Sections washed in PB-T 3 x 10 minutes to remove excess antibody.
6. Incubation of the sections in anti-goat immunoglobulin to localise the antigen-antibody complex, for 1 hour at room temperature.

   To 30 ml PB-T;

   300 µl biotinylated anti-goat immunoglobulin (blue bottle), and

   900 µl normal horse serum (yellow bottle), were added.
7. Sections washed in PB-T for 3 x 10 minutes
8. Sections incubated in ABC solution for 1 hour at room temperature.

   To 30 ml PB-T;

600µl avidin (bottle A), and

600µl biotinylated horse radish peroxidase (bottle B) were added, vortexed, and allowed to incubate for 40 minutes before adding the section.

9. Sections washed in PB-T 2 x 10 minutes.
10. Sections rinsed in 0.1M PB for 5 minutes
11. Visualisation with 198 ml PB + 2 ml DAB + 200 µl 30% hydrogen peroxide for 5 minutes.
12. Sections washed in PB 3 x 10 minutes
13. Sections were mounted and coverslipped as previously described.

DOUBLE LABEL IMMUNOCYTOCHEMISTRY FOR FOS-IR AND 3α-HYDROXYSTEROID DEHYDROGENASE

Double labelling Immunocytochemistry for Fos-IR and 3α-HSD was performed on brain sections from control (n=5) and aggressive (n=5) rats.

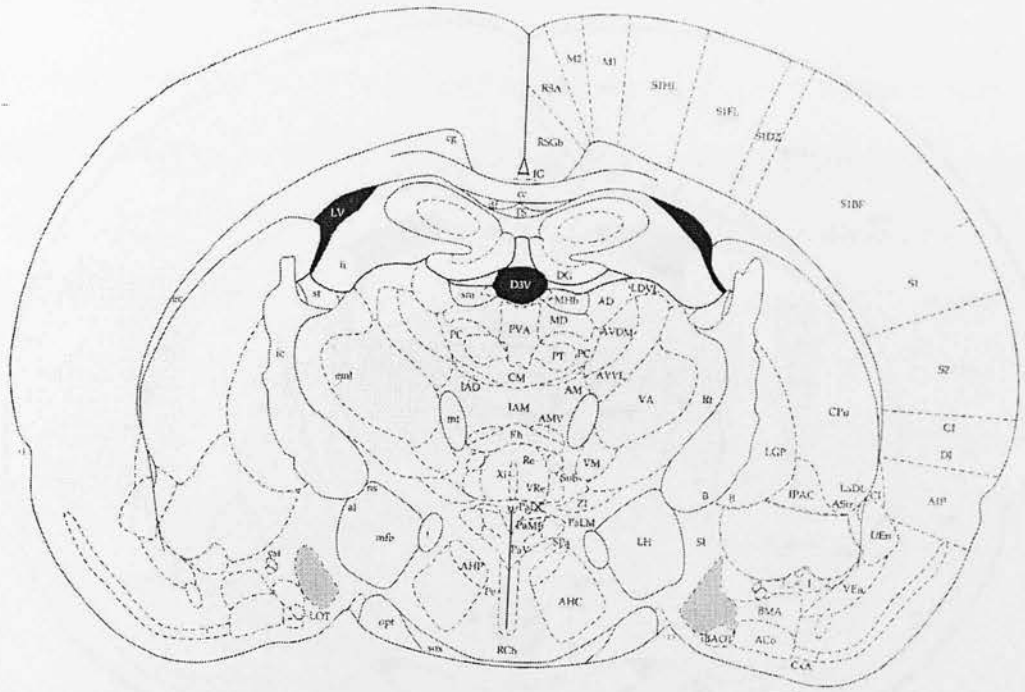
The same procedure was followed as for Fos-IR and 5α-reductase. The antibody used was a goat polyclonal DD3(R-17) sc-20424 Santa Cruz Biotechnology at a concentration of 1:500 incubated for 2 days at 4°C.

SINGLE IMMUNOCYTOCHEMISTRY LABELLING

In single immunocytochemistry labelling the Fos-IR was omitted and the sections were incubated directly into the 5α-reductase or 3α-HSD antibodies, for 48 hours. Sections were visualised using anti-goat vectastain ABC kit and normal horse serum to localise the antigen, using DAB as the chromogen.

IMMUNOREACTIVITY ANALYSIS

Brain sections were examined for Fos-IR using a Leitz light microscope. Single Fos-IR immunolabeling was counted in each brain region from 5 sections. For double-labelling brains were analysed for Fos/GAD double labelled cells, Fos/5α- reductase double labelled cells, Fos/3α-HSD double labelled cells included the supraoptic nucleus (SON), medial amygdala (mAmyg), medial preoptic nucleus (MPO), basal nucleus of stria terminalis (BNST) and the paraventricular nucleus (PVN) as shown in fig 2.2 below.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S1HL
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S2
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LDVL
PVA
MD
AVDM
PT
PC
CM
IAD
AVVL
IAM
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VA
AMV
Rh
Re
VM
VRe
Sub
ZI
PaLM
PaMP
SPa
LH
SI
AHP
AHC
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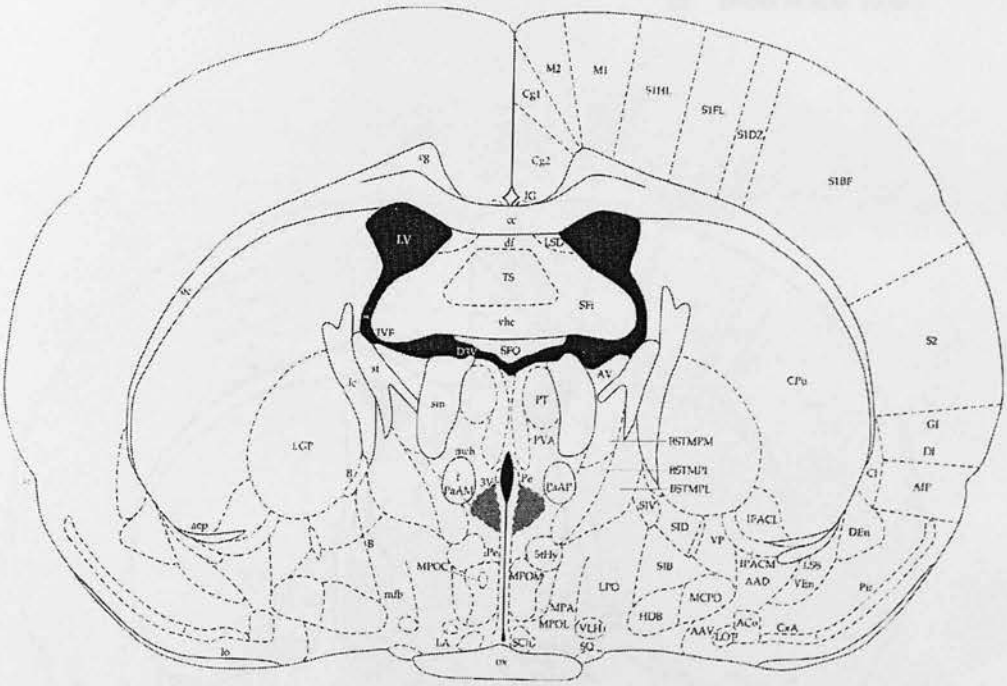
Medial amygdala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JC
cc
LV
df
LSD
TS
SFi
vhc
IVF
DIV
SFO
AV
PT
PVA
sm
LGP
BSTMPM
BSTMPL
BSTMDL
PaAM
CeAP
SIV
SID
VP
IPACL
IPACM
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HDB
AAV
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Paraventricular nucleus

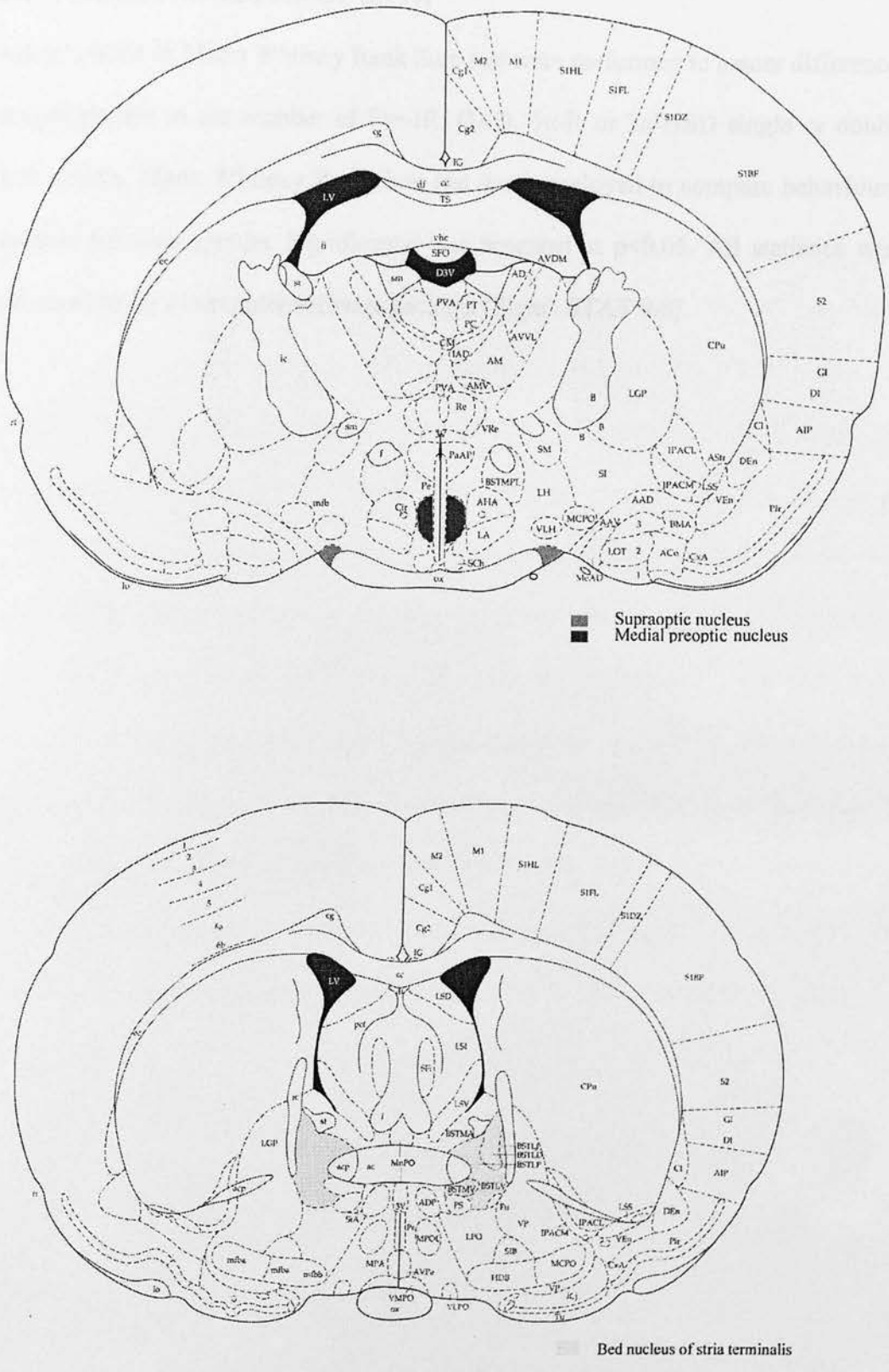


Figure 2.2 Brain areas of the lactating rat analysed in this study
Modified from; Paxinos & Watson, (1998).

STATISTICAL ANALYSIS OF DATA

Student's t-test or Mann Whitney Rank Sum test were performed to assess differences between groups in the number of Fos-IR, GAD, 5α-R or 3α-HSD single or double labelled cells. Mann Whitney Rank Sum test were employed to compare behavioural measures between groups. Significance was accepted at $p<0.05$. All statistics were performed using a computer software package (Sigma STAT 9.0).

CHAPTER 3

RESULTS

BEHAVIOUR

All lactating females exposed to the virgin intruders demonstrated aggressive behaviour during the 30 minutes of the maternal defense test (Fig 3.1, Fig 3.2). The average attack time was 108 ± 86s (range: 36-378s). The mean latency to attack was 292s. The time the resident spent on pinning down the intruder was 72 ± 18s (range: 0-270s). The time spent by the resident dam showing behaviour relating to the pups (e.g pup retrieval, hovering over the pups, lactating) was 360 ± 120 (range: 0-18s). Residents groomed themselves 283 ± 36s (range: 108-378s) and the time spent drinking and eating was 54s (range: 0-216s). Residents sniffed the intruder 216s ± 185s (range: 108-288s) and the time spent by the intruder freezing was 900s (range: 0-1386s). Both intruders and residents spent 414 ± 54s and 414 ± 90s respectively, exploring the environment. No statistical comparisons were made. These behavioural results are consistent with the findings of Hansen, et al. (2005) and Nelson, (2000).

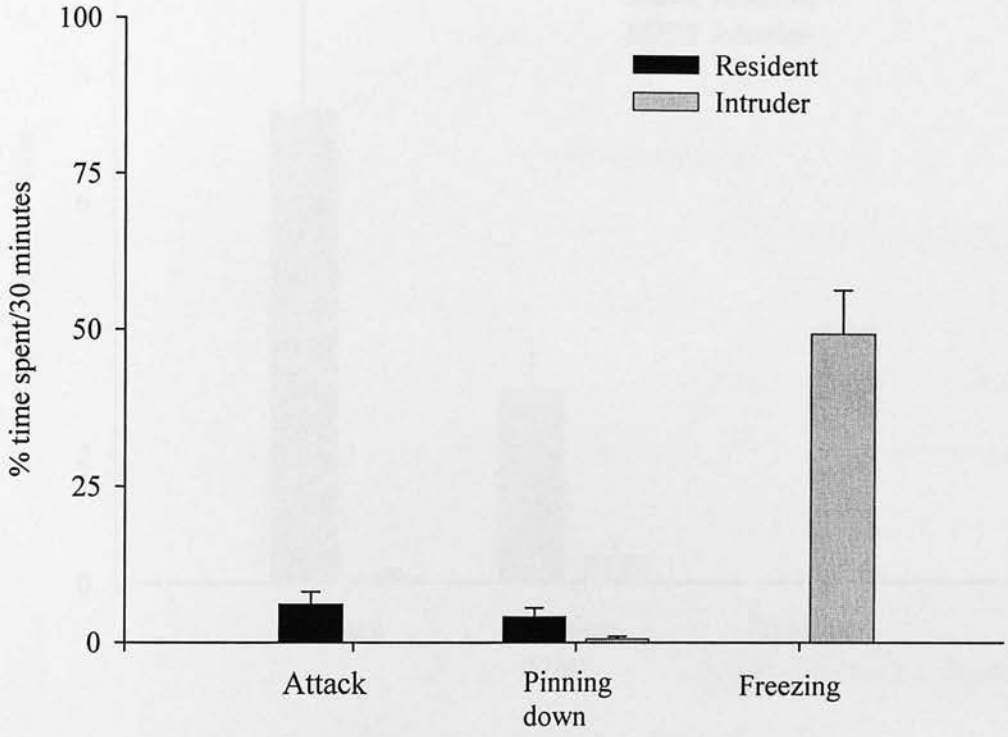


Figure 3.1 Percentage of time spent by the resident and intruder attacking pinning down and freezing during maternal defense test. Data are expressed as mean + S.E.M.

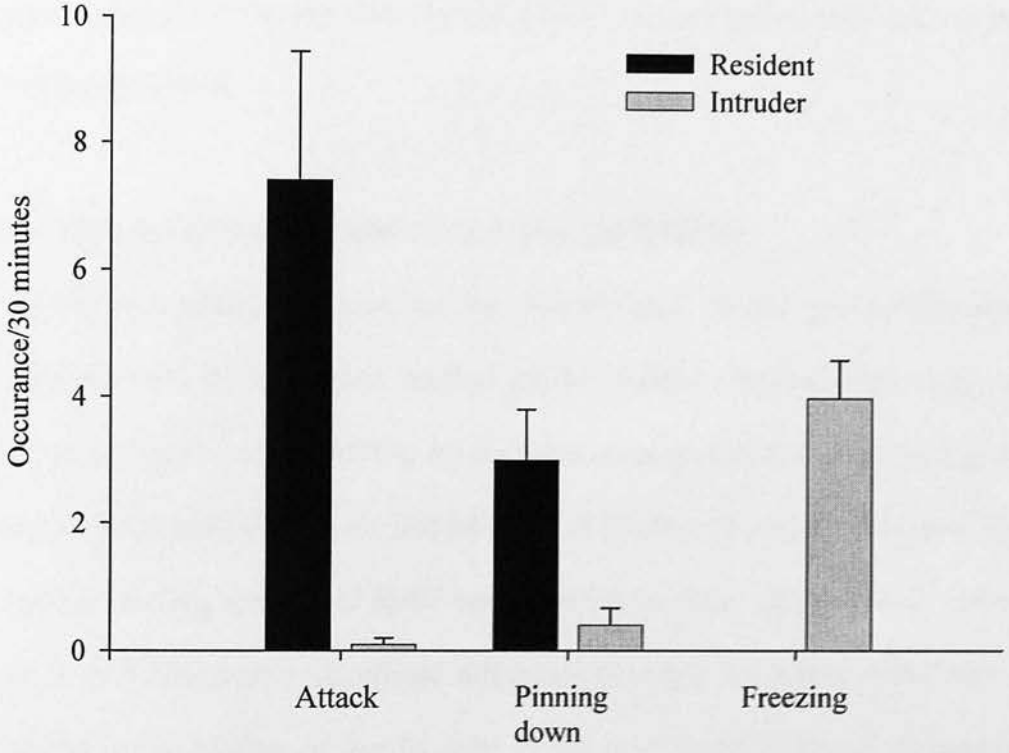


Figure 3.2 Number attacks, pinning down and freezing during maternal defense test by the resident and intruder. Data are expressed as mean + S.E.M.

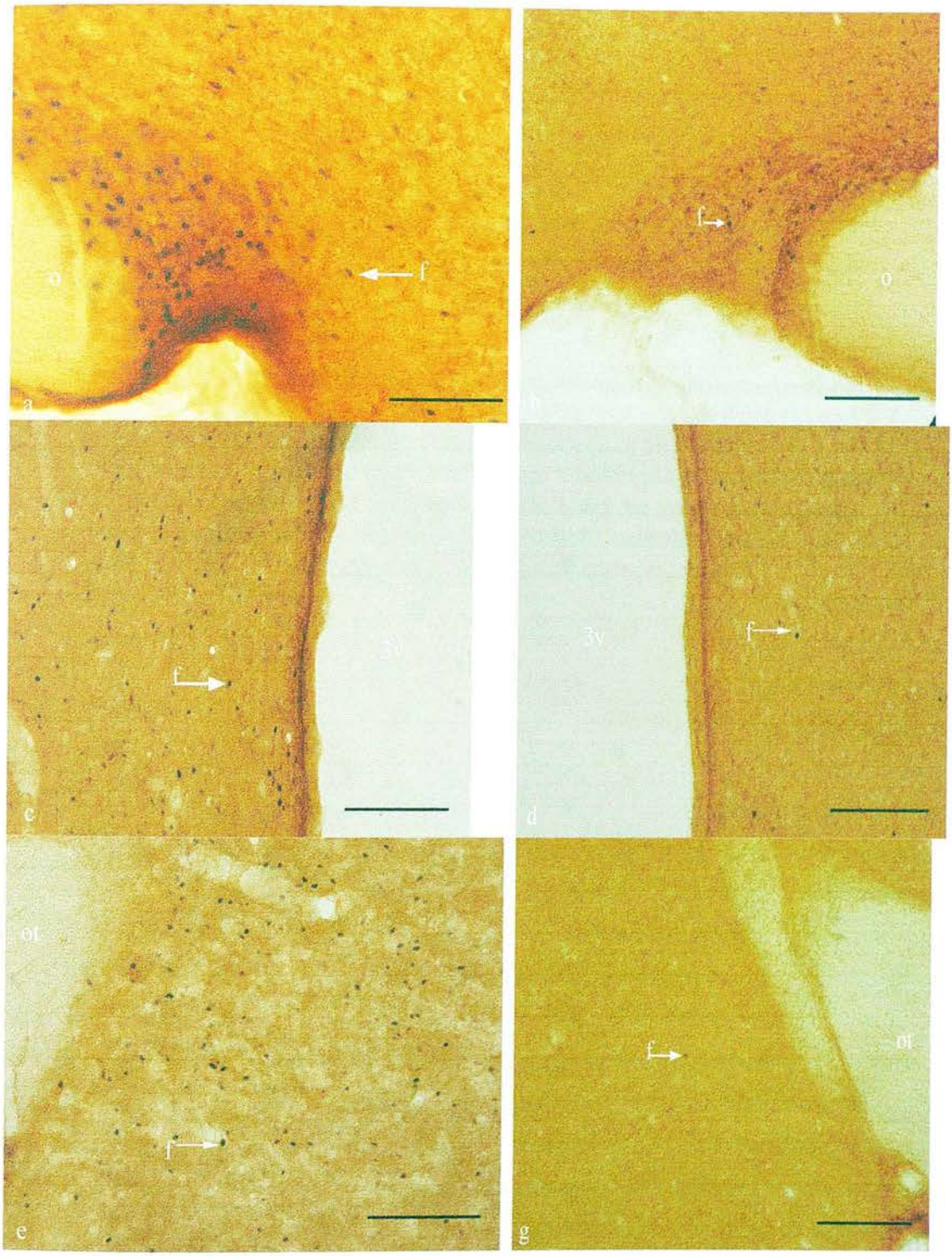
DISTRIBUTION OF GAD IN THE BRAIN

GAD single labelled cells were expressed in the SON, mAmyg, MPO, BNST, PVN, the cerebral cortex, especially in the somatosensory and piriform cortices (Fig 3.3 (g)). Other brain regions also expressed GAD single labelled cells such as the CeA and hippocampus.

Fos-IR/GAD DOUBLE IMMUNOCYTOCHEMISTRY

The brain regions examined for the Fos-IR/GAD double immunocytochemistry labelling were the supraoptic nucleus (SON), medial amygdala (mAmyg), and the medial preoptic nucleus (MPO). All the three areas analysed contained many neurons that showed dark or black nuclear labelling of the Fos-IR protein, but no evidence of double labelling with GAD 65/67 was observed in either aggressive or control rats. There was however, a significant difference between the experimental and control groups in the number of Fos-IR cells in the brain areas analysed. Aggressive rats expressed greater number of Fos-IR cells than controls in the SON ($p< 0.002$) ($t_{4.023}$), (mAmyg) ($p<0.0001$) ($t_{14.913}$), MPO ($p<0.00001$) ($t_{17.825}$), (Fig 3.4).

Figure 3.3



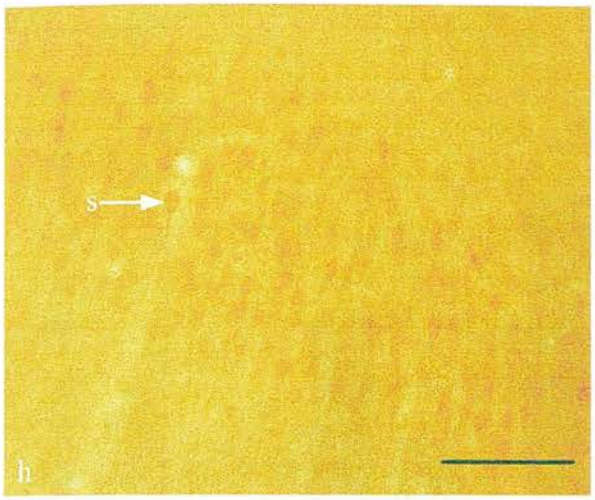


Figure 3.3 Fos-IR/GAD double immunocytochemistry photomicrographs from the SON of a lactating aggressive rat following maternal defense (a) and a control rat (b). Fos-IR and GAD in aggressive (c,e) and control (d,g) rats in the MPO (c,d) and mAmygd (e,g).GAD single labelling is shown in (h) from the cortex. Arrows with *f* point to Fos-IRcell and the arrow with *s* in (h) point to single labelled GAD cell. The *o* in (a,b) indicates optic chiasm, *3v* in (c,d) indicates 3rd ventricle and *ot* in (e,f) indicates optic tracts. Scale bars = 100μm.

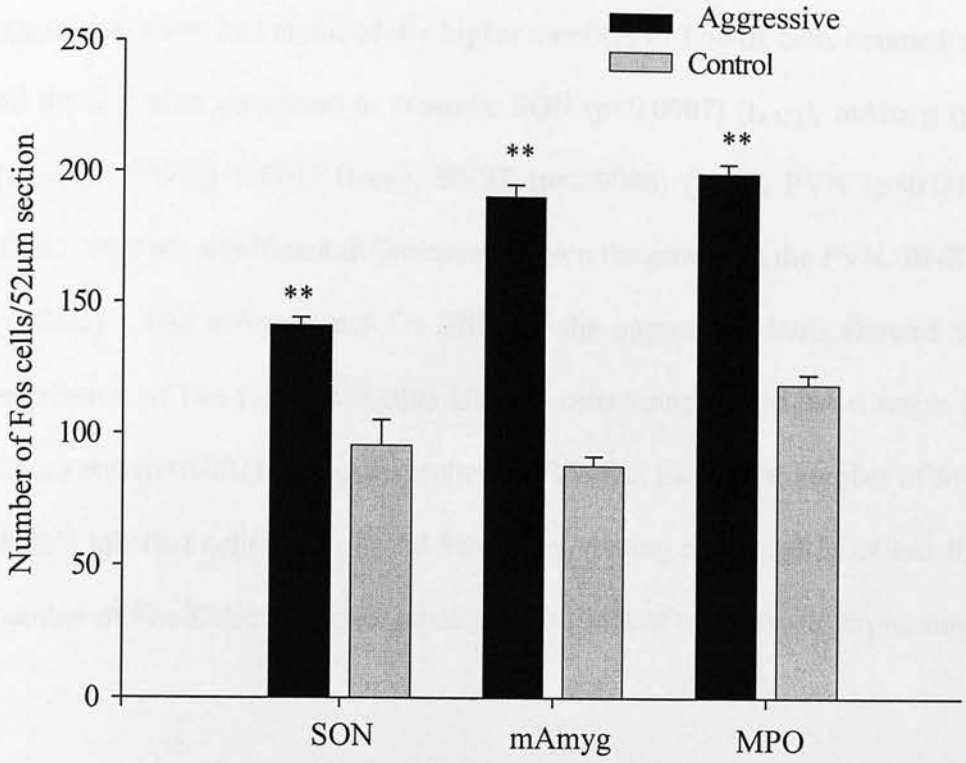


Figure 3.4 Fos-IR cells in the SON, mAmyg, and MPO of lactating rats tested for maternal defense test (n=10) and control (n=10). Data are expressed as mean + S.E.M.. **p<0.01, t-test.

5α-REDUCTASE TYPE 1 BRAIN DISTRIBUTION

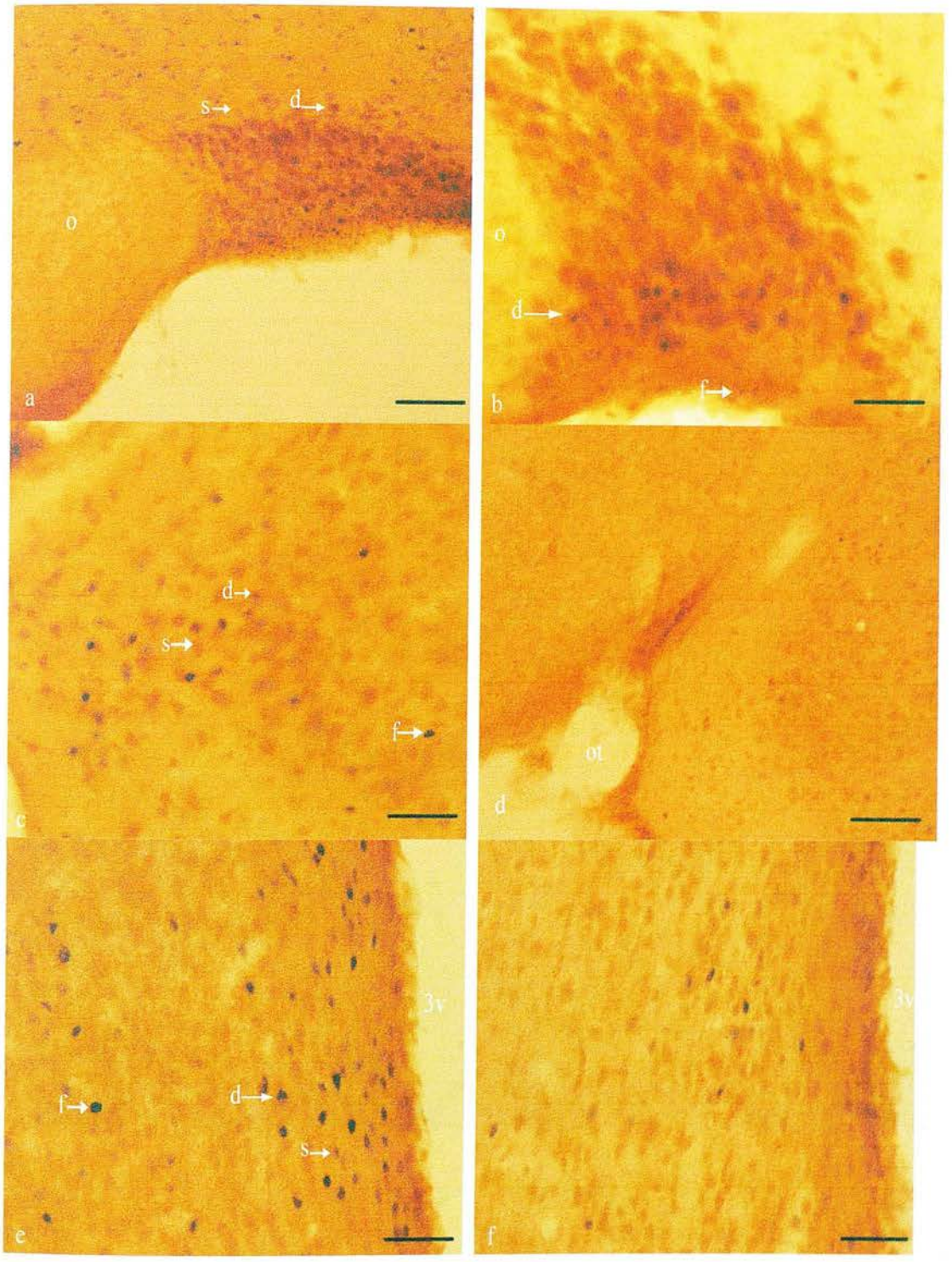
The 5α-R immunoreactivity was observed throughout the brain including the Cortex, SON, PVN (all sub divisions), MPO, mAmyg and BNST (see Fig 3.5).

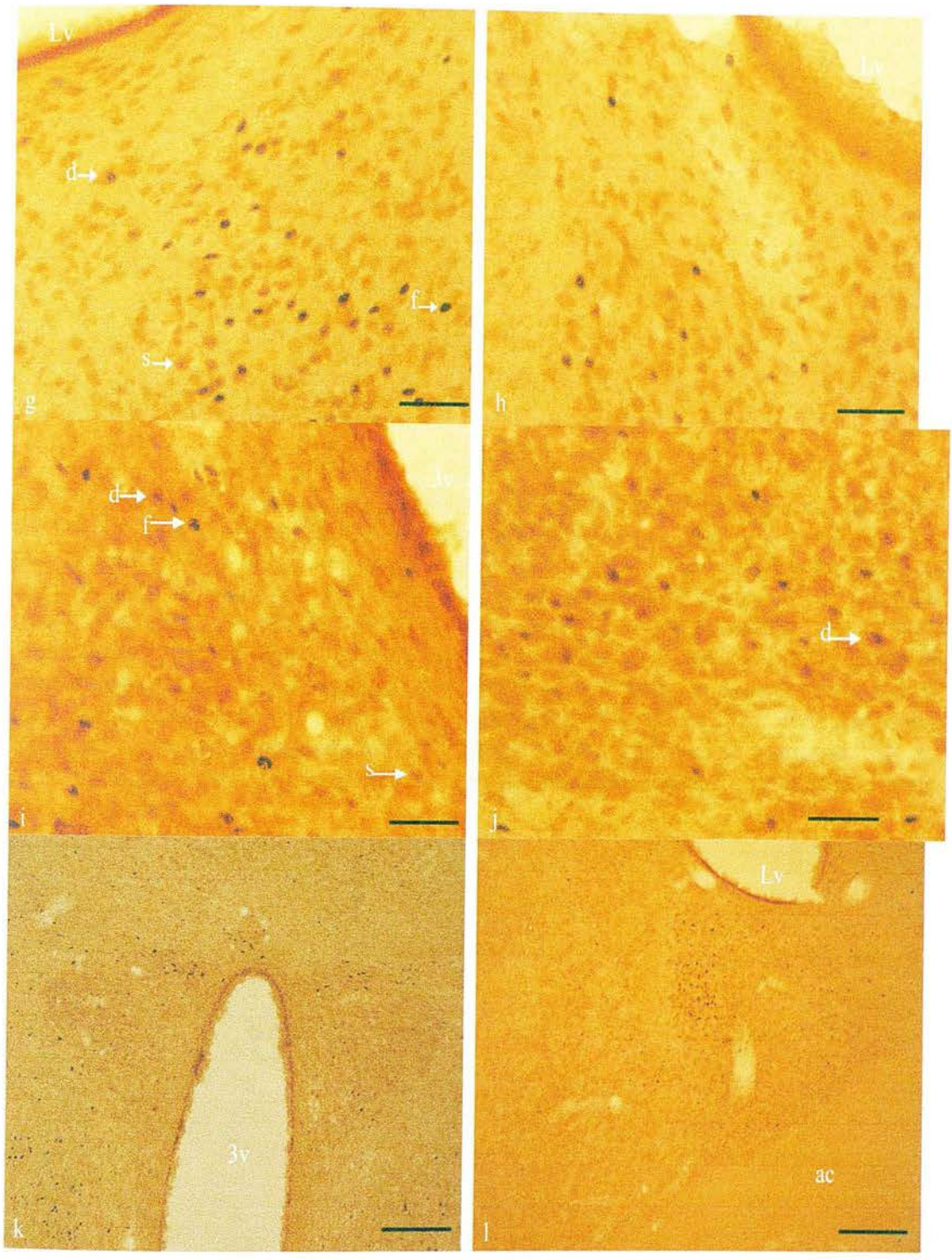
Fos-IR/5α-REDUCTASE TYPE 1 DOUBLE IMMUNOCYTOCHEMISTRY

Brain regions examined for Fos-IR/5α-R immunolabelling were the SON, mAmyg, MPO, BNST and PVN (parvocellular and magnocellular divisions). All the sites analysed contained many cells that showed light brown cytoplasmic labelling of 5α-R and dark or black nuclear labelling of the Fos-IR protein. Double labelled neurons were identifiable by the presence of a darkly (black) stained Fos immunoreactive nucleus surrounded by a light brown cytoplasmic stain (Fig 3.5). Experimental

aggressive dams had significantly higher numbers of Fos-IR cells counted/sections in all the five sites compared to controls; SON ($p<0.0007$) ($t_{9.153}$), mAmyg ($p<0.0003$) ($t_{11.352}$), MPO ($p<0.001$) ($t_{7.975}$), BNST ($p<0.0008$) ($t_{9.007}$), PVN ($p<0.004$) ($t_{5.861}$). There were not significant differences between the groups in the PVN, BNST or SON ($p>0.05$). The mAmyg and the MPO of the aggressive dams showed significant expression of Fos-IR/5α-R double labelled cells compared to the controls ($p<0.001$) ($t_{8.090}$) and ($p<0.002$) ($t_{7.060}$), respectively. PVN has the lowest number of fos-IR/5α-R double labelled cells (6% of total Fos-IR expressing cells) and SON has the highest number of Fos-IR/5α-R double labelled cells (54% of total Fos-IR expressing cells).

Figure 3.5

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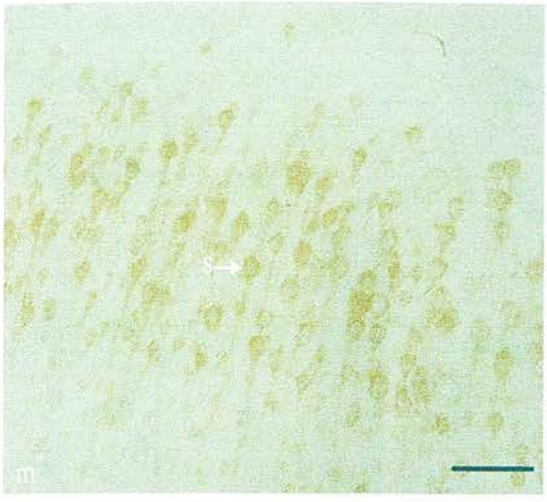
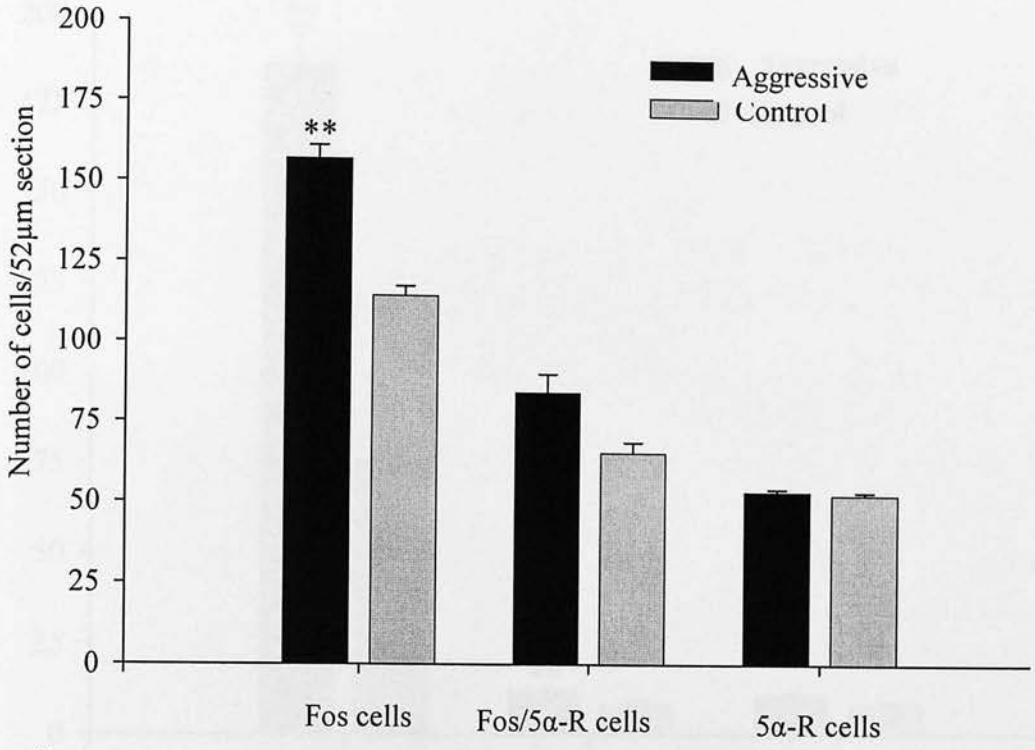


Figure 3.5 Fos-IR/5α-R double immunocytochemistry photomicrographs from the SON of a lactating rat following maternal defense (a) and a control rat (b). Fos-IR and 5α-R in aggressive (c,e,g,i) and control (d,f,h,j) rats in the mAmyg (b,c) MPO (d,e) BNST (f,g) and PVN (i,j). 5α-R single labelling is shown in (m) from the cortex. Photomicrographs (k) and (l) are lower magnifications of the PVN and BNST respectively. Arrows with's' point to 5α-R single label cells, arrows with 'f' point to Fos-IR cells (black stained nucleus), and arrows with'd' point to double labelled cells (cells immunolabelled for both Fos-IR and 5α-R). The '*o*' in (a,b) indicates optic chiasim, '*ot*' in (d) indicates optic tract, '*3v*' in (e,f,j,k) indicates 3rd ventricle, '*LV*' in (g,h) indicates lateral ventricle and '*ac*' in (l) indicates anterior commissure. Scale bars = 100μm)

a. SON


200
175
150
125
100
75
50
25
0
Number of cells/52µm section
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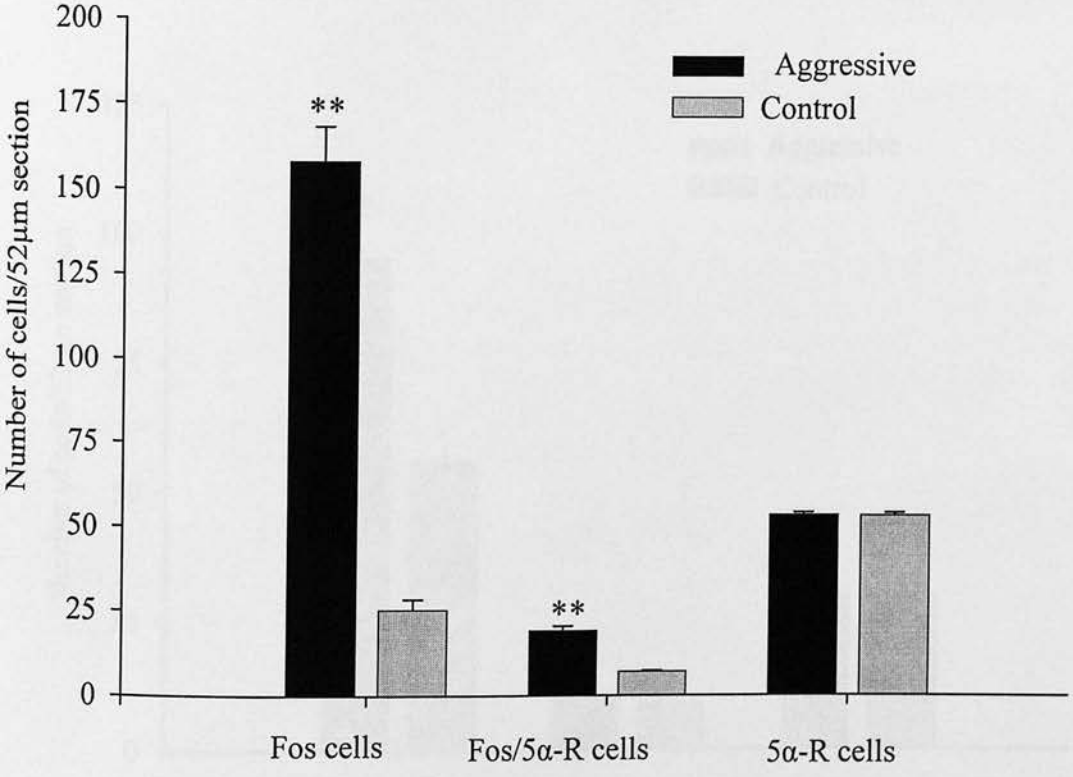
Aggressive
Control
Fos cells
Fos/5α-R cells
5α-R cells

b. mAmyg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Number of cells/52µm section
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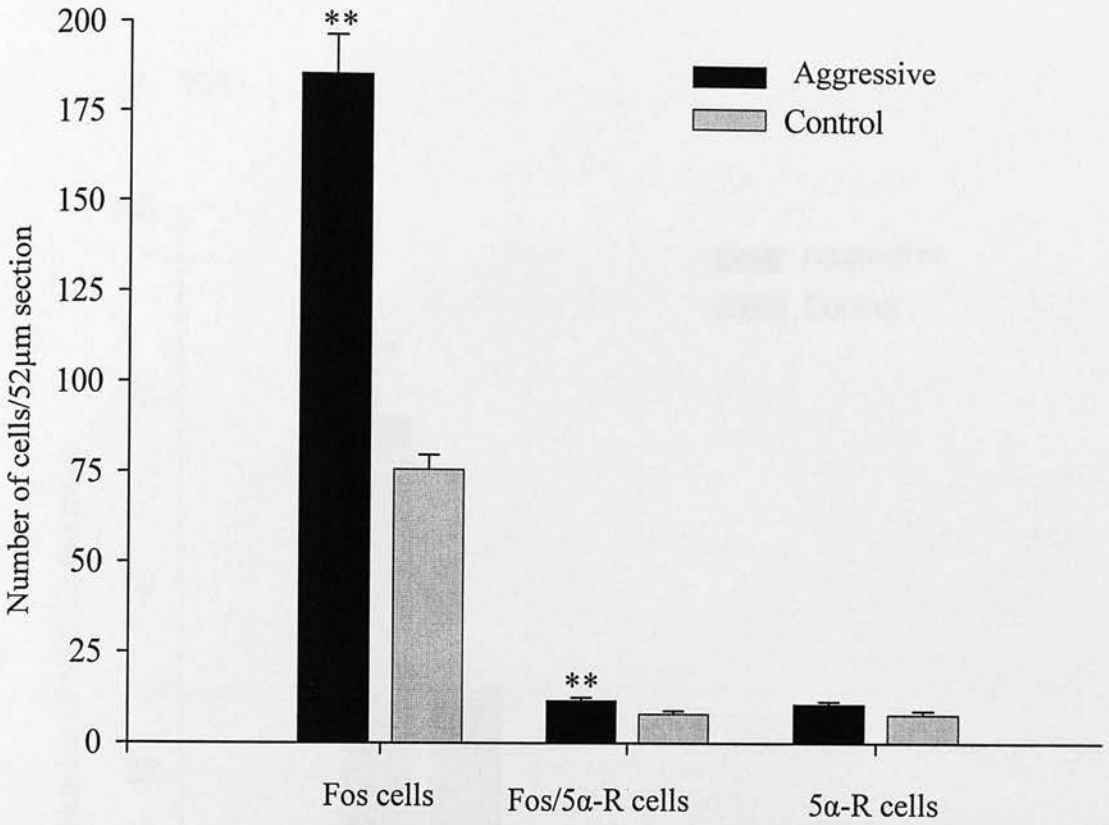
**
Aggressive
Control
Fos cells
Fos/5α-R cells
5α-R cells

c. MPO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150
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Number of cells/52µm section
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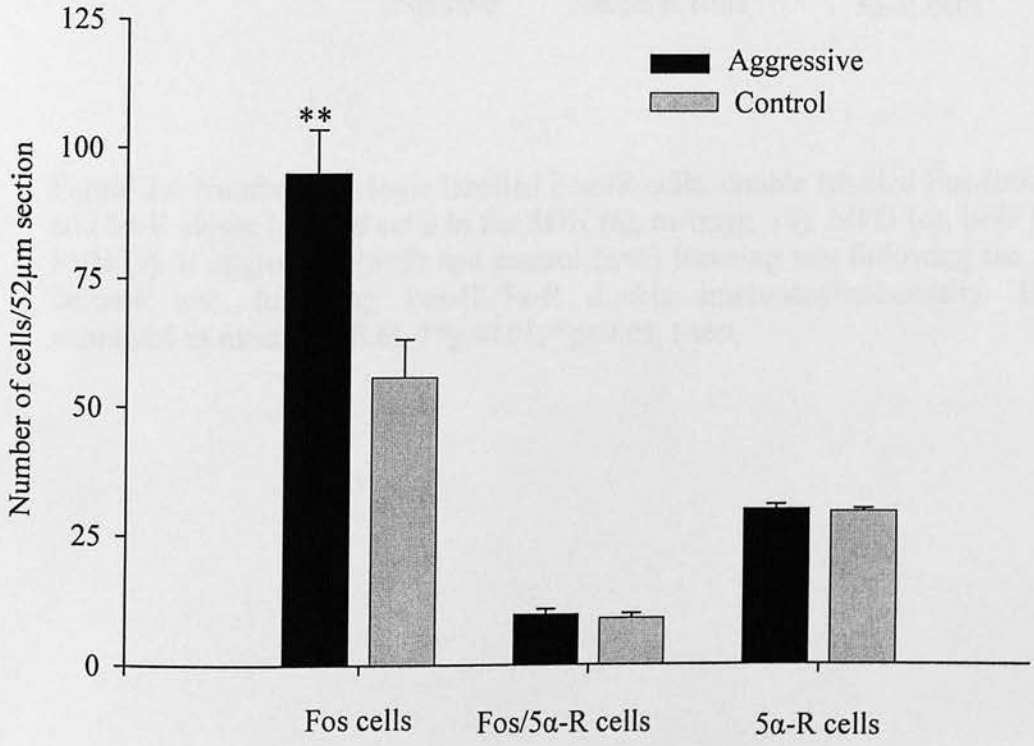
Aggressive
Control
Fos cells
Fos/5α-R cells
5α-R cells

d. BNST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50
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0
Number of cells/52µm section
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Aggressive
Control
Fos cells
Fos/5α-R cells
5α-R cells

e. PVN

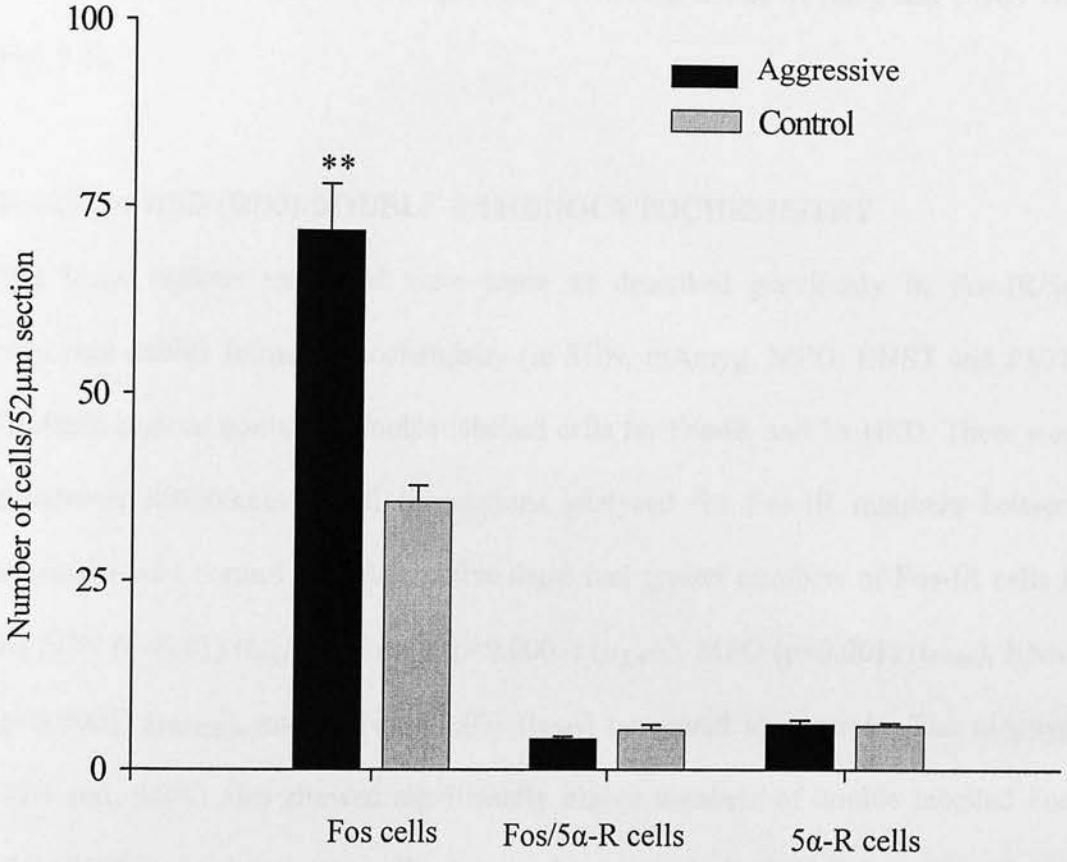


Figure 3.6 Numbers of single labelled Fos-IR cells, double labelled Fos-R/5α-R cells and 5α-R single labelled cells in the SON (a), mAmyg (b), MPO (c), BNST (d) and PVN (e) of aggressive (n=5) and control (n=5) lactating rats following the maternal defense test, following Fos-IR/5α-R double immunocytochemisrty. Data are expressed as mean + S.E.M. **p<0.01, *p<0.05, t-test.

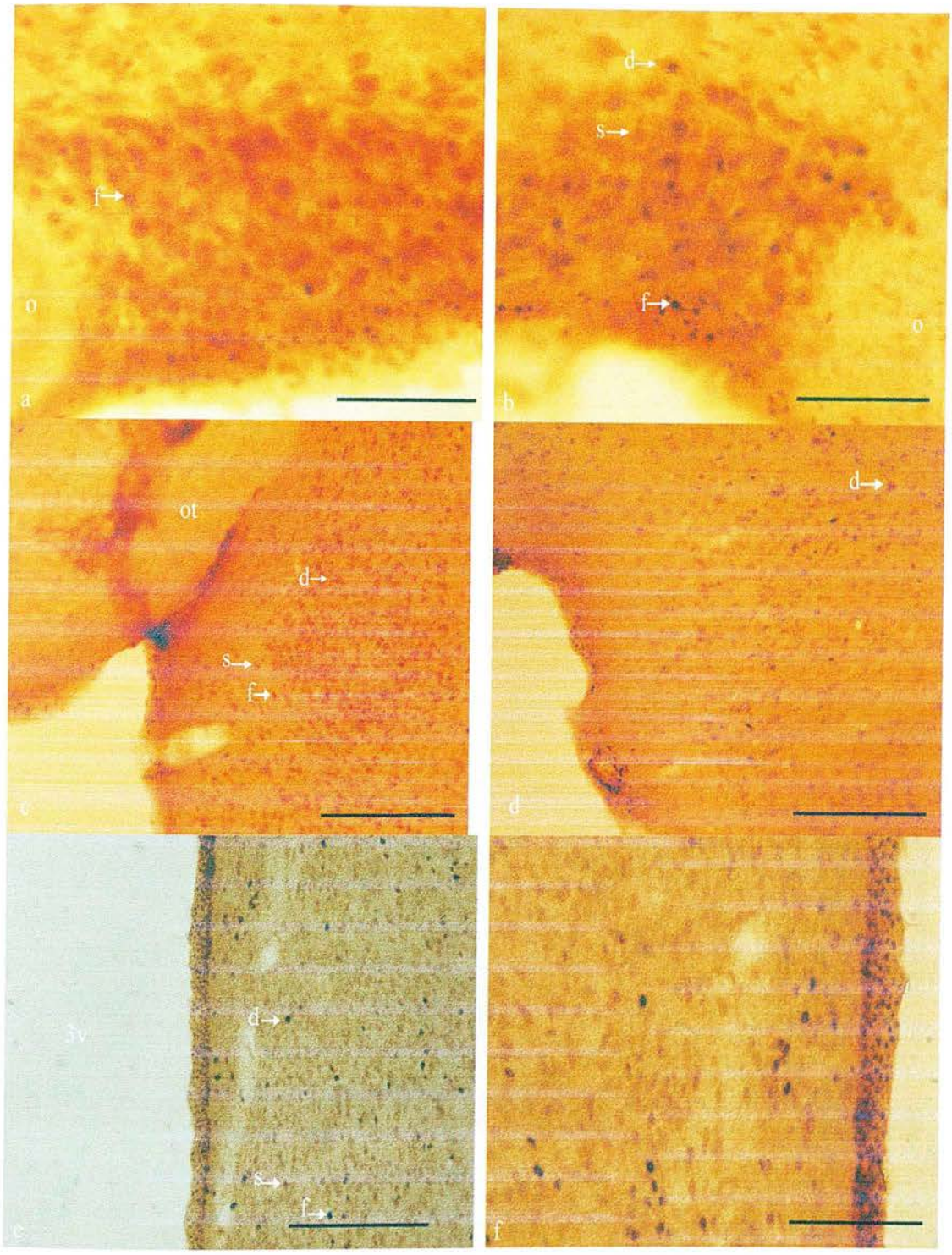
3α-HYDROXYSTEROID DEHYDROGENASE (DD3) BRAIN DISTRIBUTION

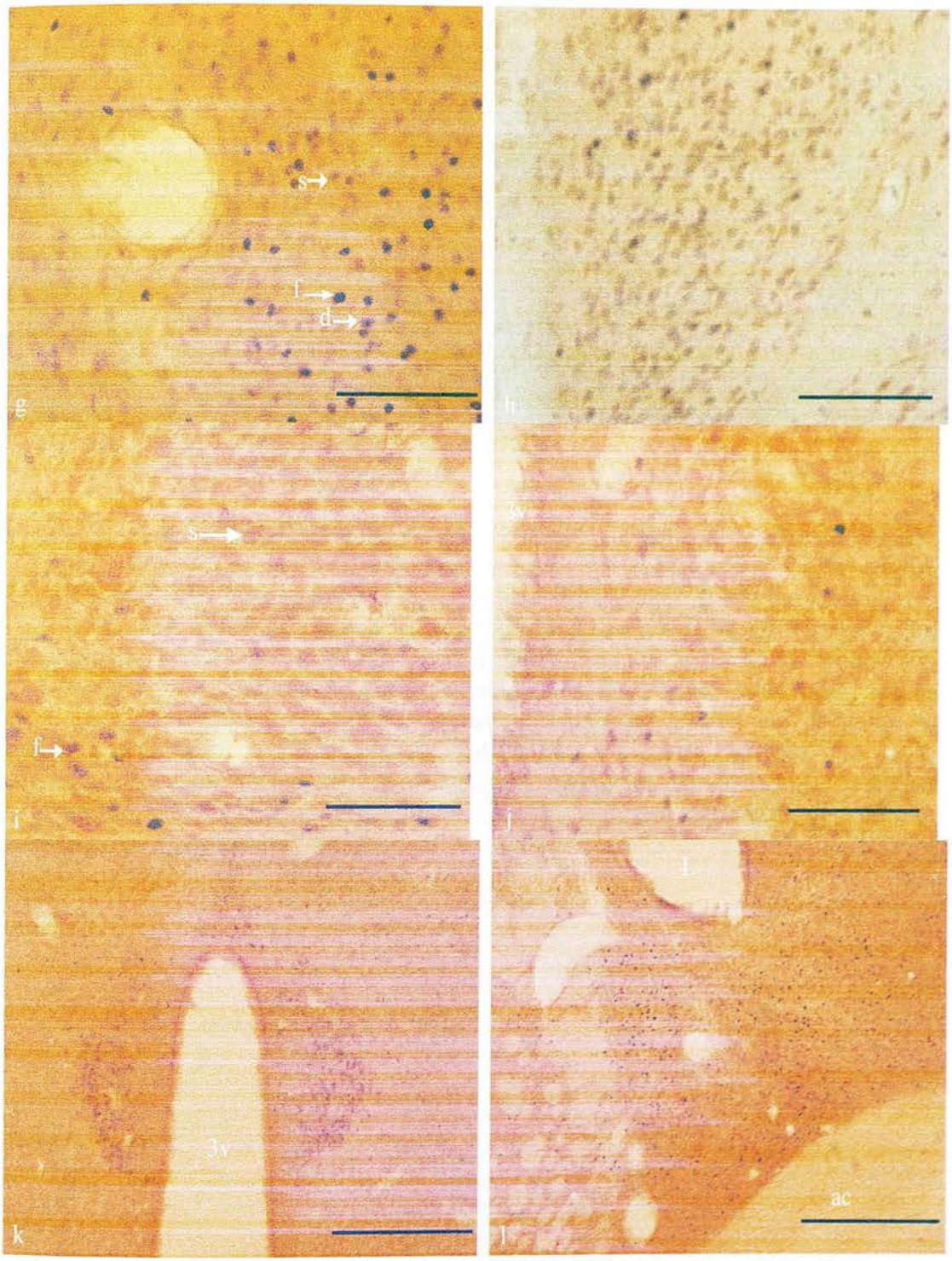
3α-HSD immunoreactivity was observed throughout the brain including the cortex, SON, PVN (parvocellular and magcellular divisions), MPO, mAmyg and BNST (see Fig. 3.7).

Fos-IR/3α-HSD (DD3) DOUBLE IMMUNOCYTOCHEMISTRY

The brain regions examined were same as described previously in Fos-IR/5α-reductase double immunocytochemistry (ie SON, mAmyg, MPO, BNST and PVN). All brain regions contained double labelled cells for Fos-IR and 3α-HSD. There were significant differences in all the regions analysed for Fos-IR numbers between aggressive and control rats. Aggressive dams had greater numbers of Fos-IR cells in the SON ($p<0.01$) ($t_{4.517}$), mAmyg ($p<0.0002$) ($t_{12.453}$), MPO ($p<0.001$) ($t_{7.495}$), BNST ($p<0.0001$) ($t_{13.108}$), and PVN ($p<0.02$) ($t_{3.747}$) compared to controls. The mAmyg, SON and MPO also showed significantly higher numbers of double labelled Fos-IR/3α-HSD immunoreactive cells compared to controls ($p<0.004$) ($t_{5.687}$), ($p<0.005$) ($t_{5.375}$) and ($p<0.001$) ($t_{8.232}$), respectively. There were no significant differences in the BNST and PVN. There were also no significant differences in the number of 3α-HSD cells in any of the regions analysed between the aggressive and control groups $p>0.05$). The PVN has the least number of Fos-IR/3α-HSD expressed (6.4% of total Fos-IR expressed) and SON has the highest expression (13% of total Fos-IR expressed).

Figure 3.7

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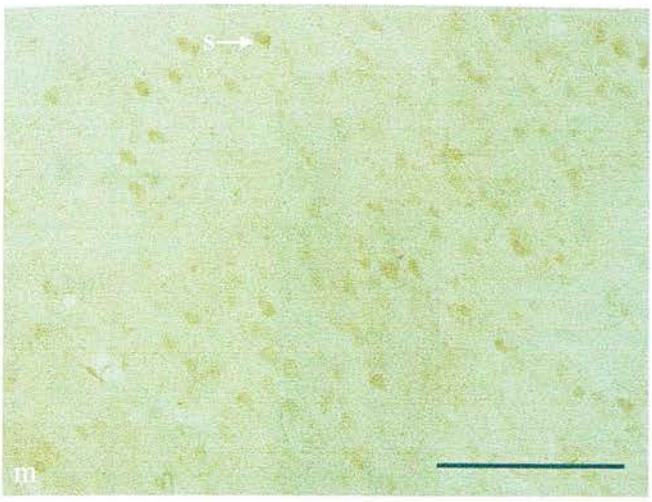
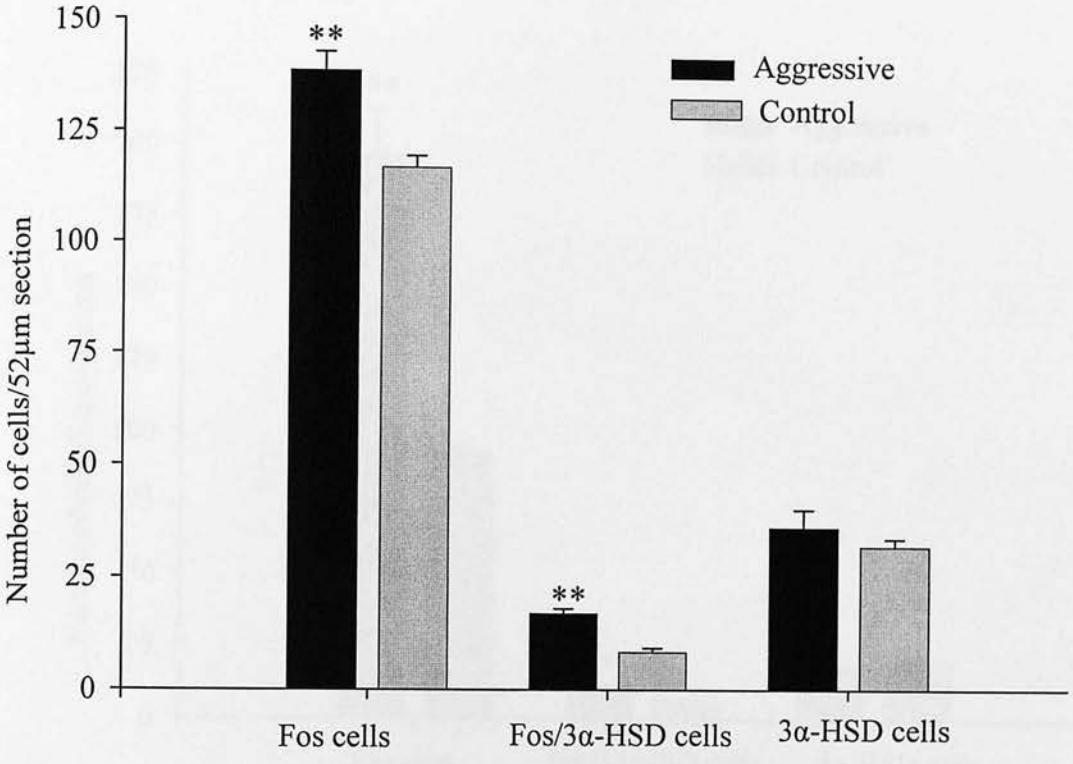


Figure 3.7 Fos-IR/dd3 (3α-HSD) double immunocytochemistry photomicrographs from the SON of a lactating rat following maternal defense (a) and a control rat (b). Fos-IR and 3α-HSD in aggressive (c,e,g,i) and control (d,f,h,j) rats in the mAmyg (c,d) MPO (e,f) BNST (g,h) and PVN (i,j). 3α- HSD single labelling is shown in (m) from the cortex. Microphotographs (k) and (l) are lower magnifications of the PVN and BNST respectively. Arrows with's' point to 3α-HSD single label cells, arrows with 'f' point to Fos-IR cells (black stained nucleus), and arrows with'd' points to double labelled cells (cells immunolabelled for both c-fos and 3α-HSD). The '*o*' in (a,b) indicates optic chiasm, '*ot*' in (d) indicates optic tract, '*3v*' in 'e,f,j,k' indicates 3rd ventricle, '*L*' in (g,l) indicates lateral ventricle and '*ac*' in (l) indicates anterior commissure. Scale bars = 100μm.

a. SON


150
125
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Number of cells/52µm section
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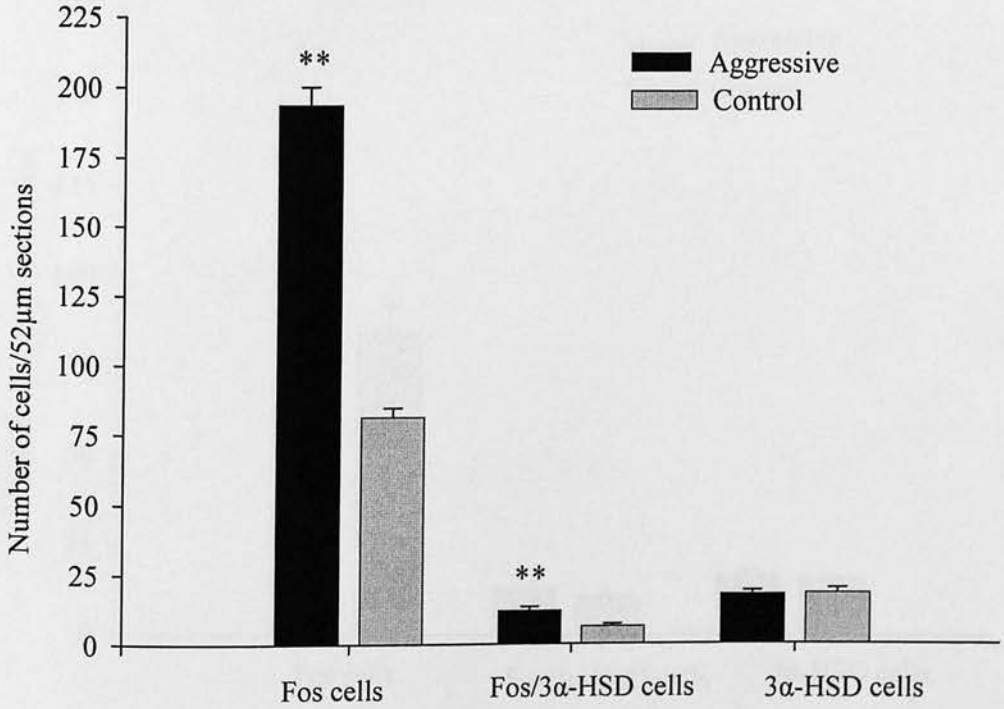
Aggressive
Control
Fos cells
Fos/3α-HSD cells
3α-HSD cells

b. mAmyg


225
200
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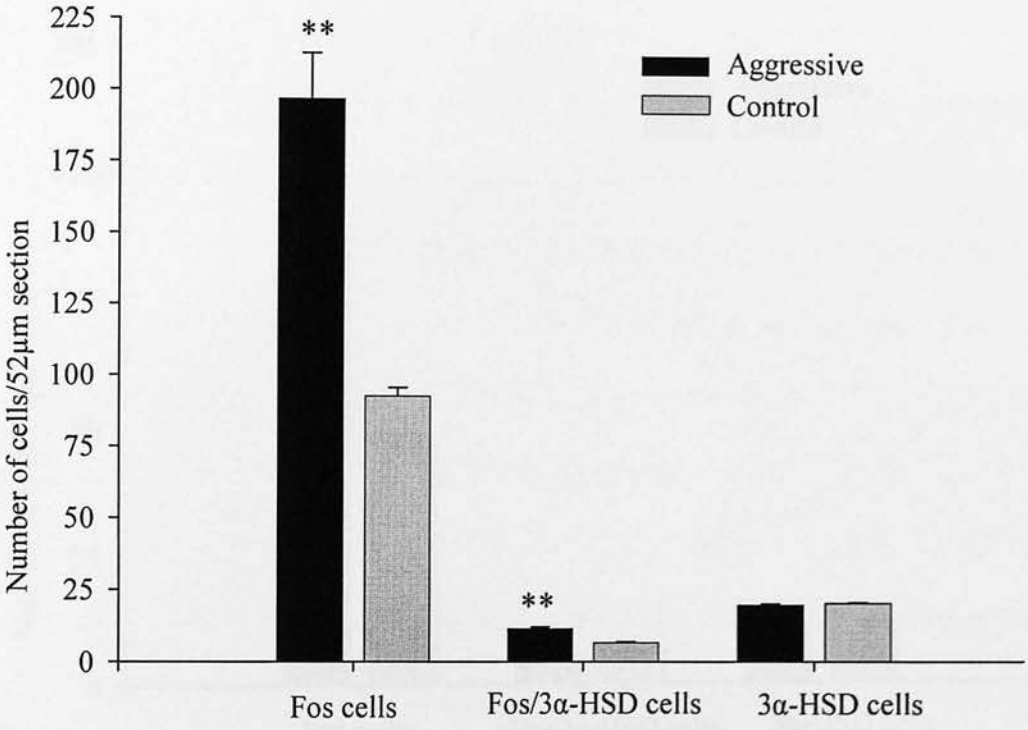
75
50
25
0
Number of cells/52µm sections
**
**
Aggressive
Control
Fos cells
Fos/3α-HSD cells
3α-HSD cells

c. MPO


225
200
175
150
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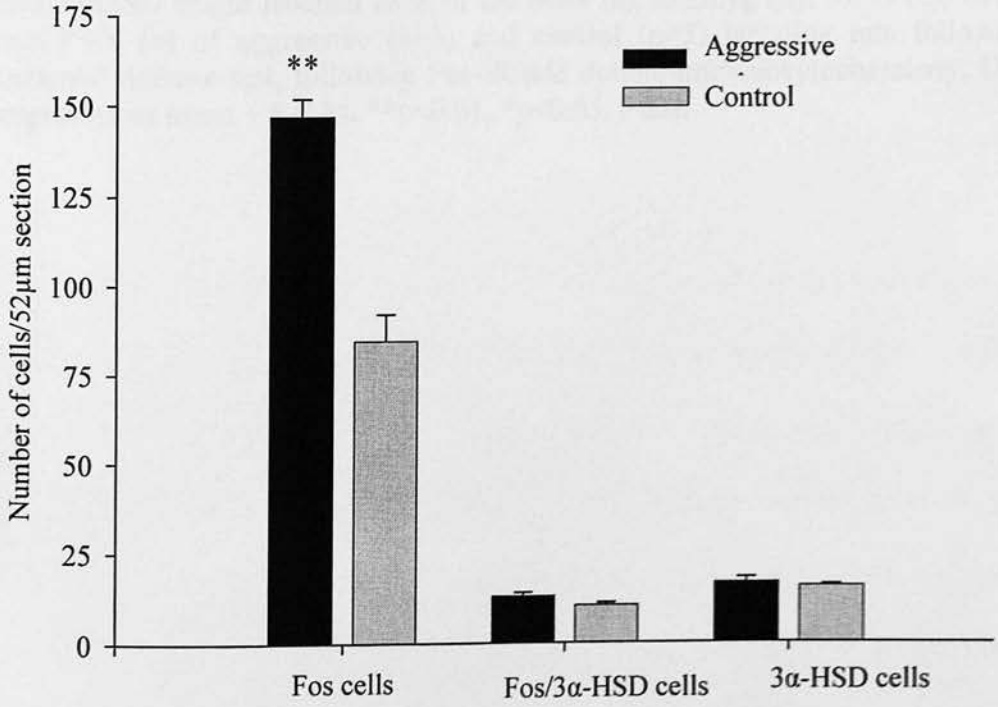
100
75
50
25
0
Number of cells/52µm section
**
**
Aggressive
Control
Fos cells
Fos/3α-HSD cells
3α-HSD cells

d. BNST


175
150
125
100
75
50
25
0
Number of cells/52µm section
**
Aggressive
Control
Fos cells
Fos/3α-HSD cells
3α-HSD cells

e. PVN

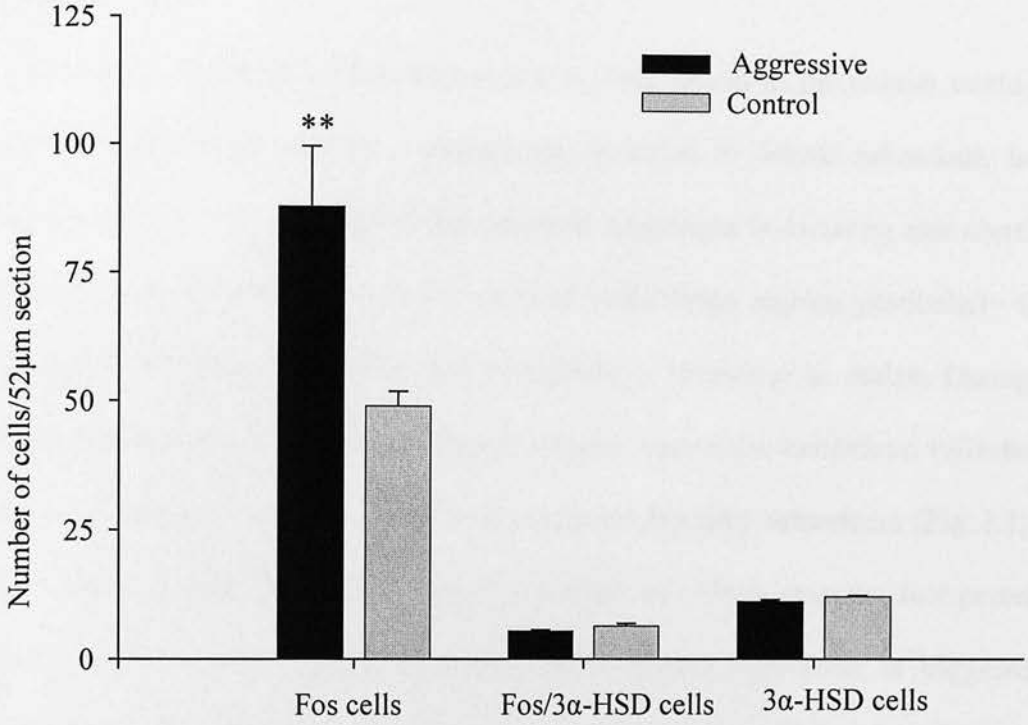


Figure 3.8 Numbers of single labelled Fos-IR cells, double labelled, Fos-IR/3α-HSD and 3α-HSD single labelled cells in the SON (a), mAmyg (b), MPO (c), BNST (d) and PVN (e) of aggressive (n=5) and control (n=5) lactating rats following the maternal defense test, following Fos-IR/dd3 double immunocytochemisrty. Data are expressed as mean + S.E.M. **$p<0.01$, *$p<0.05$, t-test.

CHAPTER 4

DISCUSSION

Maternal aggression is a phenomenon that is wide spread in the animal world, and represents for many species, a remarkable deviation in female behaviour. In the present study it has been shown that maternal aggression in lactating rats correlates with the high expression of Fos-IR cells in many brain regions particularly those previously implicated in the control of aggressive behaviour in males. During the maternal defense test, lactating residents showed aggressive behaviour, reflected by attacks, pinning down, and the intruders frequent freezing behaviours (Fig 3:1, Fig 3:2). These changes make a lactating dam capable of a fierce response to a perceived threat. Mayer, et al. (1993), considered that maternal aggression is triggered by changes in the endocrine pattern characteristics of late gestation, parturition and lactation (Ferreira, et al. 2002, Mayer, et al. 1990 & Mayer, et al. 1990), Prolactin (Svare, et al. 1983), and oxytocin (Cosiglio, et al. 1996, Giovenardi, et al. 1998, Hansen, et al. 1986 & Mayer, et al. 1987).

FOS-IMMUNOREACTIVITY

In this study the Fos-IR was used as a marker for neuronal activation to identify brain circuits involved in aggression. In addition, enzyme systems important for the production of neurosteroids in the brain and are thought to be responsible for mediating maternal aggression were investigated. Previous reports demonstrated the GABAergic neuronal pathways (Lonstein & De Vries, 2000) and noradrenergic neurones (Meddle, et al. 2000, Antonijevic, et al. 1995 & Lin, et al. 1998) are important in regulating the suite of maternal behaviours following parturition. These

brain regions (SON, mAmyg, BNST, MPO and PVN) may be important for the performances of maternal aggression and the high level of Fos-IR cells expressed during aggression may reflect the influence these brain regions have in regulating maternal behaviours. The medial amygdala showed significantly higher levels of Fos-IR cells in aggressive rats compared to controls. Activation of this site may not be unconnected to the role this brain region plays in maternal aggression and other forms of aggression as reported by Gammie, et al. (2001), Joppa, et al. (1995), Bester-Meredith & Miller (2001) and Davis & Marler (2004) as well as in maternal behaviours (Numan & Insel, 2003). Amygdala has also been reported by Carlson (1989), to be involved in the control of the limbic system, which can not be separated from aggressive behaviours. Medial amygdala projects reciprocally to the accessory olfactory bulb (AOB) and plays a role in mediating responses such as pheromonal and chemosensory signals (Hansen, et al. 2005, Wood & Swann, 1999 & Meredith & Westberry, 2004). This may be the underlining reason why most attacks by the resident are longer after persistent sniffing of the intruder.

The medial preoptic nucleus (MPO) also showed significantly higher levels of Fos-IR in aggressive dams. This result is consistent with a large body of research on this region of the brain, which defines a role for the MPO with wide range of sexual, social and aggressive behaviours (Hansen & Gammie, 2005). The MPO has also been reported to show increasing Fos-IR cells during pregnancy through to lactation (Lin, et al. 1998). Moreover Fos-IR increases in the MPO following pup exposure (Numan & Numan, 1994) and during prolonged mother-young interactions in rats (Stack, et al. 2000) and after reunion of pups with mother in mice (Mathieson, et al. 2002). The results obtained in this study is consistent with Hansen & Gammie, (2005) who

reported that MPO showed higher Fos-IR cells in aggressive lactating mice compared to their non-aggressive counterparts (Gammie & Hansen, 2001 and Gammie, et al. 2004). Thus the present findings support the hypothesis that MPO plays a role in mediating maternal aggression perhaps through their connections with the medial amygdala (Numan & Insel, 2003). The MPO has also been shown to play a role in intermale aggression (Edwards, et al. 1993 & Albert, et al. 1986) and in other social behaviours (Joppa, et al. 1995, Sato, et al. 1998 & Wang, et al. 1997).

The aggressive dams also showed robust maternal behaviour such as hovering over the pups, lactation and retrieval of pups during the resident intruder test which may also be responsible for eliciting fos-IR cells. This finding is consistent with previous reports by Lonstein & De Vries, (2000), Fleming, et al. (1994), Lonstein, et al. (1998) & Walsh, et al. (1996). The number of Fos-IR cells obtained was highly expressed in all the sites analysed in aggressive dams, about twice the number of Fos-IR cells expressed by the control. Lonstein & DeVries (2000) showed that population of neurones that express Fos-IR cells after peripheral sensory stimulation or the performance of a particular behaviour may be especially important for the process, and this hypothesis is supported by the ability of central infusion of Fos-IR intense oligonucleotides to alter the display of numerous behaviours including maternal behaviour in sheep (Hebb, et al. (1999), Heilig, et al. (1993), Hooper, et al. (1994) & Hou, et al. (1997). The Fos-IR expression showed by the SON and the PVN might be due to the involvement of these sites in the synthesis and release of oxytocin, vasopressin and corticotropin releasing hormone (CRH) system and their role in maternal aggression and anxiety. This is supported by Bosch, et al. (2005) & Windle, et al. (2004) that oxytocin is likely to promote maternal aggression indirectly through

the regulation of fear or anxiety. This is probably through regulation of the brain CRH system in the PVN and amygdala or by another possible mechanism of inhibition of GABA release by oxytocin within the amygdala (Neumann and Bosch, unpublished observation). Thus there seems to be a link between local c-fos expression, the availability of inhibitory or excitatory amino acids within the amygdala (Ebner, et al. 2005, Hubber, et al. 2005) the activity of the brain CRH system (Liebsch, et al. 1995) the state of anxiety and the level of maternal aggression (Gammie, et al. 2004). Negron (1993), showed that CRH is one of the peptides which have tremendous effect on the reduction of anxiety and fearfulness in lactating dams associated with heightened maternal aggression.

GAD AND FOS-IMMUNOREACTIVITY

There was a large distribution of single labelled pyramidal GABAergic neurones in the granular cortex (fig 3.3(g) but Fos-IR/GAD double labelled cells were not evident in any of the five regions analysed. Since GABAergic neurones can be either short inhibitory interneurones or longer projection neurones (Fisher, et al. 1988, Millhorn, et al. 1987, Roland, et al. 1993, Schued, et al. 1989, Sun, et al. 1993 & Tredici, et al. 1983). GABAergic influences from those sites analysed may be found both in close proximity to these areas. GAD cells in the cerebral cortex suggest inhibitory output from this sub-region may be especially important sources of GABAergic output. GABAergic neurones in the cerebral cortex may provide inhibitory mechanism that is necessary for the normal display of maternal aggression during the maternal defense test. Neurones from the brain regions responsible for maternal behaviour may be tonically inhibited by GABAergic neurones from another region probably the mAmyg to prevent the display of maternal behaviour during intermittent aggressive encounters

with the intruder. This hypothesis is supported by the inability of infusion of $GABA_A$ receptor antagonist bicuculline into $cPAG_{vl}$ to produce kyphosis in dams interacting with non suckling pups a stimulus that normally does not elicit this posture (Lonstein & De Vries, 2000). It is probable that maternal aggression provides an excitatory input to GABAergic mAmyg neurones which, when stimulated disinhibits premotor neurones in the medulla to allow for the display of aggression. It is also likely that GABAergic neurones act in consonance with the hormonal milieu accompanying pregnancy and lactation to influence aggression in the lactating dams perhaps through the oxytocin system. This hypothesis is supported by Moos (1995) & Voi sin, et al. (1995) that GABA innervations appears to play a role in patterning the pulsatile discharge of oxytocin cells that is observed during peripartum periods. It is also probable that GABAergic neurones could be influenced by progesterone, considering the fall in its ovarian production 24hrs preceding parturition (Leng & Russell, 2006). This hypothesis is supported by Flugge, et al. (1986), Herbison, et al. (1997) & Lonstein, et al. (2000) that GAD activity can be influenced by the ovarian hormones since dramatic changes in ovarian activity occurs throughout pregnancy and lactation, it is possible that this hormonal interplay influence onset of maternal behaviour and maternal aggression by changes in GABAergic transmissions in cells that are important for these behaviours.

There was no evidence of Fos-IR/GAD double labelled cells but this does not completely exclude the role of GABA in maternal aggression. It is probable that a mechanism different from a direct involvement of GABAergic signal transduction may be operative in this actions. The SON of the aggressive dams showed significantly higher Fos-IR cells than non-aggressive dams. Oxytocin is a crucial

hormone for the initiation of parturition and milk let-down and both has been implicated in maternal aggression. These hormones are released by the axon terminals of neurone in the neurohypophysis whose cell bodies are situated in the PVN and SON. Hansen, et al. (1986), associate oxytocin to the initiation of maternal aggression while Ferris, et al. (1997) reported vasopressin microinjections into the anterior hypothalamus to significantly shorten the latency to bite and increase biting attacks during aggression (Meddle, et al unpublished studies).

5α-REDUCTASE AND FOS-IMMUNOREACTIVITY

Progesterone, which is also termed a pregnancy hormone decreases in the circulation during the peripartum period before the onset of parturition. Due to its high levels through pregnancy it may form the bulk of neurosteroid precursors including other androgens and glucocorticoids. Lephart, et al. (2001), reported that out of all the steroids, progesterone has the highest affinity for the 5α-R enzyme and non-polar biochemical nature that enhances its concentrating ability in the brain lipophilic environment. Lephart, et al. (2001), has shown that the major pathways in the brain that steroids are metabolised are the aromatisation of the androgens and the 5α-reduction of progesterone and androgens. The 5α-R enzyme converts a number of androgens, progestagens and glucocorticoids to their 5α-reduced metabolites throughout the brain (Lephart, 1993, Celotti, et al. 1997, Celotti, et al. 1997, Celotti, et al. 1992). This study conforms to Lephart, et al. (1993) by the detection of 5α-R in the brain regions analysed. It may be hypothesised that this enzyme contributes to the neuroendocrine dynamics that accompany parturition and underlie maternal aggression during lactation. Once the irreversible conversion of testosterone to 5α-DHT or 5α-DHP has taken place these reduced metabolites becomes a better substrate

for 3α-reduction via 3α-HSD (DD3) (Celotti, et al. 1992 & Lephart, 1993). The reaction is reversible between 3α-5α- reduced metabolites and the 3α-, 5α-reduced metabolites of progesterone represents the most potent natural neurosteroids (Allop, and THDOC) and these are potentiators of $GABA_A$ receptor activation (Celotti, et al. 1992 & Lephart, et al. 1993). Agis-B albio, et al. (2006), reported that synthesis of Allop and THDOC in the brain from progesterone or deoxycorticosterone, respectively is catalysed by the sequential action of 5α-R type 1 and 3α-HSD. 3α-HSD catalyse the reduction of 5α-DHT or 5α-DHP to allopregnenolone or tetrahydrodeoxycorticosterone.

3α-HYDROXYSTEROID DEHYDROGENASE AND FOS-IMMUNOREACTIVITY

All the brain regions analysed (SON, PVN, mAmyg, MPO, BNST) contained 3α-HSD immunoreactive cells. This finding is consistent with Negri-Cesi, et al. (1996) and Celotti & Martini (1992) who reported that brain contains an active 5α-R-3α-HSD system that appears to be widely distributed through the brain. 3α-HSD is crucial in the formation of neuroactive steroids (Allop and THDOC) (Paul & Purdy, 1992). Fos-IR/3α-HSD expression was higher in the magnocellular division of the PVN compared to the parvocellular portion, perhaps due to the fact that neuroactive steroids may serve functions mediated through the oxytocin system. Since oxytocin is thought to originate from centrally projecting PVN neurones, and may be released centrally from oxytocin neurone dendrites is one of the key neuropeptides regulating the initiation and maintenance of maternal behaviour (Pedersen & Boccia, 2002, Numann & Insel, 2003) and supported by Consiglio & Lucion (1996), that electrolytic lesions of the PVN reduced maternal aggression, and enhanced oxytocin release was

recently reported within the PVN of lactating Wistar rats during the maternal defense test Bosch, et al. (2004). Also findings by Giovenardi, et al. (1998), Johns, et al. (1998), Elliott, et al. (2001), Lubia, et al. 2003) supported this hypothesis by reporting that pharmacological manipulation of oxytocin system within the central nucleus of amygdala or the parvocellular division of the PVN elicits changes in maternal aggression. Neuroactive steroids (Allop and THDOC) may act to modulate maternal aggression via the GABAergic responses via $GABA_A$ receptor system to exert its physiological effects of oxytocin in modulating maternal behaviour and aggression (Orchinik & Murray 1991).

FEAR AND ANXIETY

Most studies show that sensitised females treated with sex steroids, develop aggression (Mayer, et al. 1990 & Mayer, et al. 1990) and demonstrate less fear (Hansen, et al. 1986), behavioural features of lactating animals. Ferreira, et al. (2000) suggests that both endocrine factors and maternal care contribute to the expression of aggression and fear reduction. It is probable that reduced anxiety and fear, increased aggression observed in lactating rats could be related to the expression of maternal care. Lactating females show decreased anxiety in the plus maze test in contrast to the levels exhibited both by the sensitised and ovariectomised rats, though could display attacks on the intruders the aggression level is usually lower than that of the lactating dams (Ferreira, et al. 2002). A decrease of brain neurosteroids availability has been associated with psychiatric conditions, including anxiety, aggression, premenstrual dysphoria and cognitive and mood disorders (Pinna, et al. 2006, Guidotti, et al. 1998 & Barbaccia, 2004). The selective reduction in anxiety in lactating but not in the sensitised rats suggest that maternal behaviour per se is not the essential cue to

produce anxiolysis in the elevated plus maze, but other factors, possibly of endocrine origin. Supporting this idea it has been demonstrated that progesterone and its metabolites exert an anxiolytic effect in females in different animal models (Bitran, et al. 1991, Bitran, et al. 1993 & Fenandez-Guasti & Picazo, 1992). Interestingly, in non human primates, dams who show frequent behavioural signs of anxiety achieve higher score in maternal protectiveness, suggesting a link between protective parenting style and emotionality (Maestripieri, 1999). However, in rats manipulations that resulted in enhanced emotionality consequently reduced maternal aggressive behaviour (Lonstein, et al. 1998, Boccia & Pedersen, 2001). In males it has been shown that exposure to an aggressive male resident result in enhanced anxiety (Heinrichs, et al. 1992, Liebsch, et al. 1995, Haller, et al. 2003). Oxytocin also regulates anxiety-related behaviour (McCarthy, et al. 1996 & Bosch, et al. 2005) an effect that could be localized within the amygdala (Bale, et al. 2001, Neumann, 2002). Thus oxytocin may promote maternal aggressive behaviour indirectly through transient regulation of fear and anxiety possibly by regulation of the activity of the brain CRH System (Windle, et al. 2004). As another possible mechanism oxytocin inhibits the release of GABA within the CeA, but not PVN during maternal aggression (Neumann & O. J. Bosch, unpublished observation). Thus, it is probable that there is a link between local oxytocin release, the availability of inhibitory or excitatory amino acids with the CeA (Ebner, et al. 2005 & Huber, et al. 2005) the activity of the brain CRH System (Liebsch, et al. 1995), the state of anxiety, and the level of maternal aggression (Gammie, et al. 2004).

NEUROACTIVE STEROIDS AND MATERNAL AGGRESSION

Neuroactive steroids (Allop and THDOC) which are synthesised by neurones (Mellon, et al. 2001) are positive allosteric modulator of $GABA_A$ receptor. At different regions of the brain, neurosteroid concentration varies according to behavioural and environmental conditions, such as stress, sex recognition or aggressiveness (Barrot, et al. 1999). Akk, et al. (2005), reported that neurosteroids can reach the site(s) involved in potentiation of the $GABA_A$ receptor function by diffusion through the plasma membrane rather than by direct binding to a site on the receptor from the external medium. It is probable that this process underlie the aggressive behaviour shown by the lactating dams during maternal defense test. Studies by Pelletier, et al. (2004) showed that in addition to 5α-R and 3α-HSD, cortical and hippocampal neurones express 20α-HSD, the enzyme that converts progesterone into the active 20α-hydroxyprogesterone or converts Allop into 20α-hydroxyAllop. Cortical and hippocampal neurones also express 3β-HSD, the enzyme that converts pregnenolone into progesterone (Guennoun, et al. 1995). Thus, glutamatergic cortical hippocampal neurones and, also, very likely, the olfactory mitral neurones, may synthesise both progesterone and 5α-DHP, which may be involved in the regulation of intracellular progesterone-receptor function (Roberto, et al. 2006). Grobin, et al. (2006) showed that neurosteroids have been reported to influence prefrontal cortex structure and thalamocortical connectivity and increase the proliferation of human embryonic neural stem cells expressed in the cerebral cortex Wang, et al. (2005). It is probable that a receptor mechanism different from a direct modulation of $GABA_A$ receptor signal transduction may be operative in these actions. Although the neurosteroid that act at $GABA_A$ receptors to positively modulate GABA

action are indiscriminately synthesised and secreted from glial cells and neurones in all brain regions (Roberto, et al. 2006). Perhaps this is one of the mechanisms of influencing maternal aggression through the adreno-pituitary endocrine system. In this study the expression of 5α-R and 3α-HSD in the brain has not been evenly distributed, the high density of expression was observed in the SON with MPO having the lowest density of expression, this is confirmed by Roberto, et al. (2006) that 5α-R and 3α-HSD expression matches the non uniform distribution of Allop and 5α-DHP (Cheney, et al. 1995 & Dong, et al. 2001). Because neurosteroids act through GABA receptors and $GABA_A$ receptors are expressed in neuronal populations of several brain regions, it is imperative to establish whether Allop, which acts positively on $GABA_A$ receptors reaches the $GABA_A$ receptor by diffusion from contiguous synapse, or perhaps via a local paracrine mechanism (Roberto, et al. 2006).

During late pregnancy oxytocin cells express $GABA_A$ receptors with a subunit composition that confer sensitivity to the progesterone metabolite Allop (Brusaards, et al. 1997). The type of $GABA_A$ receptors changes 1-2 hours after the birth of the first pup and the type of $GABA_A$ also changes and become insensitive to Allop (Brussaard, et al. 1997, Brussaard, et al. 2000). As plasma and brain concentration of Allop fall to pre-pregnancy levels in the last two days of pregnancy (Concas, et al. 1998), the sensitivity of oxytocin cells to GABA also falls (Brussaard, et al. 1997). At this time $GABA_A$ receptors binding in the SON also decreases (Amico, et al. 2000), consequently the oxytocin cells should increase their activity. It may be hypothesised that the influence of neurosteroid through the oxytocin mechanism act in this manner to mediate fast synaptic inhibition by activating ligand-gated chloride channels. Binding of 3α-reduced neurosteriods to GABA receptors leads to either inhibition or

potentiation of the inhibitory effects of GABA. Hence, anticonvulsive, anaesthetic and anxiolytic effects of neuroactive steroids are mediated by their capacity to positively modulate $GABA_A$ receptor function, ie these substances act to increase GABAergic effects by increasing the frequency and duration of chloride channel opening (Majewska, 1992). On the other hand, inhibition of $GABA_A$ receptor function which is mostly documented for the neurosteroids PREG-S and DHEAS producing effects ranging from anxiety and excitability to seizure susceptibility (Paul & Purdy, 1992). There is also evidence that neurosteroids may regulate gene expression by activating progesterone receptors (Grazzini, et al. 1998).

NEURONAL CIRCUITRY IN MATERNAL AGGRESSION

The medial amygdala plays an important role in the control of the limbic system as examplified by the violent organised involuntary movements of the collateral limbs during aggressive attack (Carlson, 1998). The amygdala also occupies a central position within the brain with regards to aggression, a position somewhat analogous to that of the striatum: It receives a major input from the cerebral cortex and in turn exerts GABA-mediated inhibitory effects upon the output structure of the amygdala to influence movements involved in aggressive attacks (Levesque & parent, 2005, Levy, et al. 1997, Obeso, et al. 2000 & Cesaro, et al.2003). Our results showed GABAergic neuronal cells in the cerebral cortex which may be responsible for the cortical input into the medial amygdala during maternal aggression. This hypothesis conforms to the findings by Levesque & parent (2005) that the cortical inputs imposes upon the subthalamic nucleus a functional organisation so that the nucleus is commonly subdivided into sensorimotor territory, associative territory and the limbic territory. Neurones in the sensorimotor territory are believed to be associated with the

execution of movements, those in the associative territory with the planning of movements and those in the limbic territory with the motivational and emotional aspects of movements. This neuronal circuitry may apply to the aggressive lactating rats during the maternal defense test, since significantly high levels of Fos-IR cells were expressed in the mAmyg and other brain regions examined in this study coupled with the demonstration of the GABAergic neurones in the cortex. Amygdala has specifically been linked to the control of the limbic system and involvement in the control and organisation of the musculoskeletal system during all forms of aggressive attacks (Carlson, 1998), figure 4.1 illustrates the neural circuits that may be involved in maternal aggression.

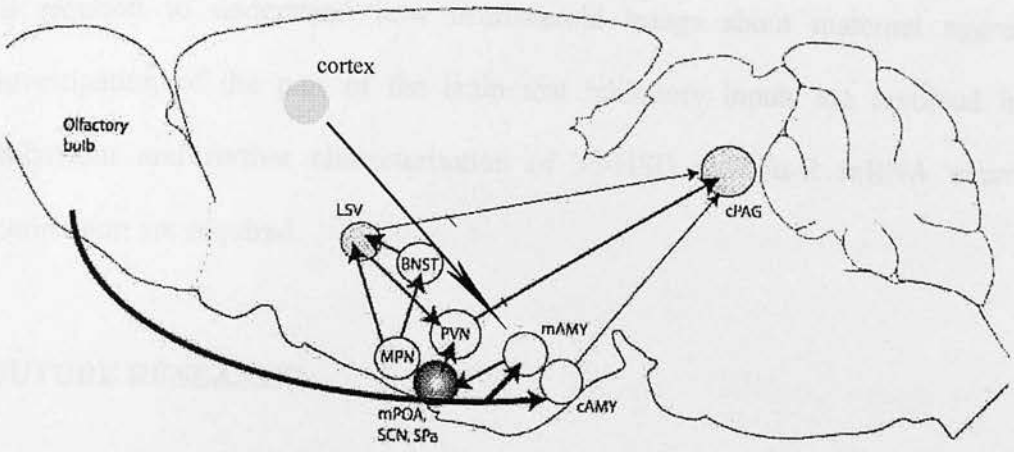


Figure 4.1 Neural Circuits involved in maternal aggression.
Modified from; Negron, (2004).

The above diagram is based on the immediate early gene expression (Fos-IR) and the GABAergic neuronal input in aggressive lactating rats.

GENERAL CONCLUSION

The present results show that 5α-R is expressed in the neurones and glial cells in the SON, Amyg (M), MPO, BNST and the PVN. There were no significant differences in the number of cells expressing 5α-R between the aggressive and non aggressive rats. The medial amygdala and MPO showed significantly higher levels of the 5α-R/Fos-IR double labelled cells in aggressive compared to non-aggressive rats. 5α-R cells did not vary between aggressive and non aggressive dams, perhaps due to the fact that no changes in enzyme synthesis could be detected in the short time from the behaviour to sampling. Neurosteroids are synthesised by the 3α-HSD-5α-R Type 1 enzyme system and play important role in maternal aggression and are also important in influencing

other behaviours such as mood, memory, reproductive behaviours, cognition and learning (Lephart, et al. 2001, Blomqvist, 2000, & Fink, et al. 1998). Further research is required to understand how neurosteroid brings about maternal aggression, investigation of the part of the brain that inhibitory inputs are involved in this behaviour and further characterisation of 3α-HSD and 5α-R mRNA expression peripartum are required.

FUTURE RESEARCH

1. Neurosteroids (Allop, THDOC, alphaxalone) may be blocked by fenasteride during the peripartum period. Any effects on maternal aggression and anxiety in lactating dams will be monitored.
2. $GABA_A$ receptor antagonist could be administered ICV to the lactating rats before being exposed to a maternal aggression test. Any effects on maternal aggression performance would indicate involvement of the GABAergic system.
3. Blockade of the 3α-HSD and 5α-R enzymes in lactating rats before anxiety and maternal defense test would investigate the involvement of these enzymes in aggressive behaviour.
4. Intracerebral injection of neurosteroids (Allop, Alphaxalone) to the virgin rats followed by test for aggression and anxiety could also provide some clues to neurosteroids involvement in initiation of maternal aggression since virgin rats normally do not exhibit aggressive behaviour.
5. Intracerebral injection of neurosteroids (Allop, Alphaxalone) in lactating rats before maternal defense and anxiety tests may change aggression levels,

(anxiety, latency to attack and maternal behaviour) which I would predict to be increased with elevated neurosteroid levels.

Hypothesis to be tested would include:

- Increased level of central neurosteroids heightens aggressive performance, anxiety and shortens latency to attack.
- Neurosteroids are critical for the exhibition of maternal aggression.
- Activation of the GABAergic system forms part of the neural circuit involved in displays of aggressive behaviour in lactating dams.

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