THE EFFECTS OF PERINATAL EXCITATORY AMINO ACIDS ON NEUROENDOCRINE FUNCTION IN THE RAT

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ABSTRACT

Glutamate is important in neuroendocrine regulation. Recent studies in our laboratory have found that low doses of domoic acid (DOM) and kainic acid (KA) given to rats over postnatal days (PND) 8 to 14, produce changes suggestive of permanent endocrine dysfunction. These changes include early eye opening, enlarged pituitary glands, and a novelty- or stress-induced seizure-like syndrome. The objectives of this study were to characterize effects on serum corticosterone (CORT), adrenocorticotropic hormone (ACTH), oxytocin, and prolactin concentrations in neonatal rats following sequential injections of DOM or KA over PND 8-14, and to determine whether these drug treatments correlate with long-term changes in behavioural and neuroendocrine responses to stress.

In the first experiment, eighty Sprague-Dawley (SD) rat pups were randomly assigned to one of five treatment groups: 5 or 20μg/kg DOM, 25 or 100μg/kg KA, or saline. Drugs were administered by subcutaneous injection and physical assessments were performed daily from PND 8-14. On PND 14, trunk blood was collected 30 minutes following drug injection and assayed for serum CORT concentrations by radioimmunoassay (RIA). Adrenal and thymus wet weights were recorded. There were significant litter effects in serum CORT concentrations, adrenal and thymus gland weights (CORT: $F(7, 71) = 7.87, p<.05$; adrenal weights: $F(7, 71) = 8.36, p<.05$; thymus weights: $F(7, 71) = 3.53, p<.05$). When analysed with litter as the co-variate, no significant difference between the drug groups was found. These results rule out early increases in serum CORT as an explanation for later effects. The significant differences in serum CORT concentrations, adrenal and thymus gland weights of the litters support the important methodological consideration of having all drug groups represented in each litter.

In the second experiment, thirty-four 16-month-old SD rats treated with either 20μg/kg DOM or saline from PND 8-14 were used. Blood was collected prior to and immediately after exposure to a mild stressor. Serum CORT, tail flick latency, and appearance of a previously observed novelty-induced seizure-like syndrome (NIS-L) were recorded. Also, trunk blood was collected 1 month later and assayed for baseline serum ACTH. No significant drug treatment differences were found in CORT, ACTH, or tail flick latencies. However, a significant number of male and female drug-treated rats exhibited NIS-L following mild stress (males: $X^2(1) = 5.89, p<.05$; females: $X^2(1) = 5.89, p<.05$). The presence of NIS-L was not correlated with CORT concentrations. The results suggest that this neonatal drug treatment does not permanently alter neuroendocrine function with regard to CORT and ACTH release. Tail flick latencies were not altered, also suggesting that there was no change in antinociceptive response to mild stress. Interestingly, while serum CORT concentrations were not altered, aged rats still manifested the NIS-L syndrome, further indicating a dissociation between increased

CORT and NIS-L.

In the third study, 2 separate cohorts of 75-day-old rats (N=80) received one of the five drug treatments, described in study 1, from PND 8-14. Blood was collected prior to, immediately following, or two hours following exposure to a moderate stressor (the Novel Water Maze, NWM) and assayed for either serum ACTH or CORT by RIA. Appearance of the NIS-L syndrome was also recorded. No significant treatment differences were found in CORT or ACTH, but a significant number of male and female drug-treated rats exhibited NIS-L following NWM testing. The presence of NIS-L was not correlated with either CORT or ACTH concentrations. The results of this study provide further evidence that daily injections of low doses of DOM and KA during the second postnatal week do not alter neuroendocrine function at the level of the adrenal gland and its release of CORT and also do not alter the secretion of ACTH from the pituitary. While serum ACTH and CORT levels were not altered, adult rats still manifested the NIS-L syndrome, further strengthening the idea of a dissociation between the release or concentration of these stress hormones and NIS-L.

The results of each of the three studies indicate that perinatal administration of low doses of DOM and KA from PND 8-14 does not cause either an immediate or longterm change in neuroendocrine function with regard to either circulating CORT or ACTH, but does produce permanent behavioural change that manifests as the NIS-L syndrome. We conclude that the NIS-L syndrome does not appear to reflect an exaggerated neuroendocrine response to stress.

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ABBREVIATIONS

Abbreviation	Term
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AMPH	Amphetamine
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APV	D,L-2-amino-5 phosphonovaleric acid (NMDA receptor antagonist)
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BDNF	Brain derived neurotropic factor
CNS	Central nervous system
CNQX	6-cyano-7-nitroquinoxalone-2,3-dione (AMPA receptor antagonist)
CORT	Corticosterone
CPM	Counts per minute
CPP	(±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid
CRH	Corticotropin-releasing hormone
DA	Dopamine

Abbreviation	Term
df	Degrees of freedom
DMN	Dorsomedial nuclei
DOM	Domoic acid
E	Embryonic day
EAA	Excitatory amino acid
EPSP	Excitatory postsynaptic potential
FSH	Follicle-stimulating hormone
GABA	γ-amino butyric acid
GAS	General adaptation syndrome
GH	Growth hormone
GH-RH	Growth hormone-releasing hormone
GluR	Glutamate receptor subunit
GnRH	Gonadotropin releasing hormone
G-proteins	GTP-binding proteins
GR	Glucocorticoid receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
HSD	Honestly significant difference
IC_{50}	Inhibitory concentration 50
IGF-1	Insulin-like growth factor-1
iGluR	Ionotropic glutamate receptor

Abbreviation	Term
ip	Intraperitoneal
KA	Kainic acid
LH	Lutenizing hormone
MBH	Medial basal hypothalamus
mGluR	Metabotropic glutamate receptor
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MSG	Monosodium glutamate
MSH	Melanocyte stimulating hormone
MSH-RF	Melanocyte stimulating hormone-releasing factor
MSH-RIF	Melanocyte stimulating hormone releasing and inhibiting factor
MWM	Morris Water Maze
ng	Nanograms
NIS-L	Novelty-induced seizure-like syndrome
nM	Nanomolar
NMA	N-methyl-D,L- aspartate
NMDA	*N*-methyl-D-aspartate
non-NMDA	non-*N*-methyl-D-aspartate
NPY	Neuropeptide Y

Abbreviation	Term
NSB	Nonspecific binding
NT	Neurotransmitter
NWM	Novel Water Maze
pg	Picograms
PIF	Prolactin inhibiting factor
PND	Postnatal day
POA	Preoptic area
POMC	Proopiomelanocortin
PRF	Prolactin releasing factor
PRL	Prolactin
PVa	Periventricular nuclei
PVN	Paraventricular nuclei
RIA	Radioimmunoassay
rpm	Rotations per minute
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SHRP	Stress hyporesponsive period
SON	Supraoptic nuclei
SS	Somatostatin
T3	Triiodothyronine

Abbreviation	Term
T4	Thyroxine
TB	Total bound
TC	Total counts
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VIP	Vasoactive intestinal peptide
VMN	Ventromedial nuclei

1.0. INTRODUCTION

1.1. Neurobiology of Glutamate

Glutamate is the most abundant excitatory amino acid (EAA) transmitter in the mammalian central nervous system (CNS) (Brann & Mahesh, 1992; Jorgensen et al., 1995; Ozawa et al., 1998). The majority of neurons can be excited by glutamate (McDonald & Johnston, 1990). Glutamate neurotransmission underlies many normal physiological processes that occur in the brain. It plays a role in the formation of neural networks during development, in mediating synaptic transmission, and is the primary neurotransmitter (NT) in a molecular model of learning and memory processes (long-term potentiation) (for review see Brann & Mahesh, 1994). However, high concentrations of glutamate or glutamate-like compounds can produce neuronal damage and have been implicated in a variety of pathological and neurodegenerative disorders, including global and focal ischemia, Huntington's disease, Parkinson's disease, Alzheimer's disease, and cell death following epilepsy (for review see Bettler & Mulle, 1995). Maintenance of optimal glutamate concentrations in the CNS is, therefore, important in both the developing and adult brain.

1.1.1. Synthesis, catabolism, and release

Glutamate is a "nonessential" amino acid that is synthesized from glucose via the

Krebs cycle and transamination of α-ketoglutarate (Cooper et al., 1996). Glutamate can also be produced from glutamine that is synthesized in glial cells, transported into nerve terminals, and converted to glutamate by glutaminase (Gonzales & Jaworski, 1997). Neuronal glutamate is stored in synaptic vesicles until its release into the synapse by a calcium-dependent exocytotic process. Plasma membrane glutamate transporter proteins terminate the synaptic actions of glutamate by transporting it back to the presynaptic neuron and to surrounding glial cells (Gonzales & Jaworski, 1997). Glutamate molecules taken back up into the presynaptic neuron are directly stored in synaptic vesicles, while molecules transported to glial cells are converted to glutamine by glutamine synthetase (Cooper et al., 1996). This glutamine is then recycled to nearby nerve terminals and serves as a precursor for glutamate.

1.1.2. Classification of glutamate receptors

Glutamate elicits responses on target cells by binding to receptors and depolarizing neuronal membranes (Feldman et al., 1997). Glutamate receptors are categorized as metabotropic or ionotropic. The current classification scheme for glutamate receptors is presented in Figure 1.1.

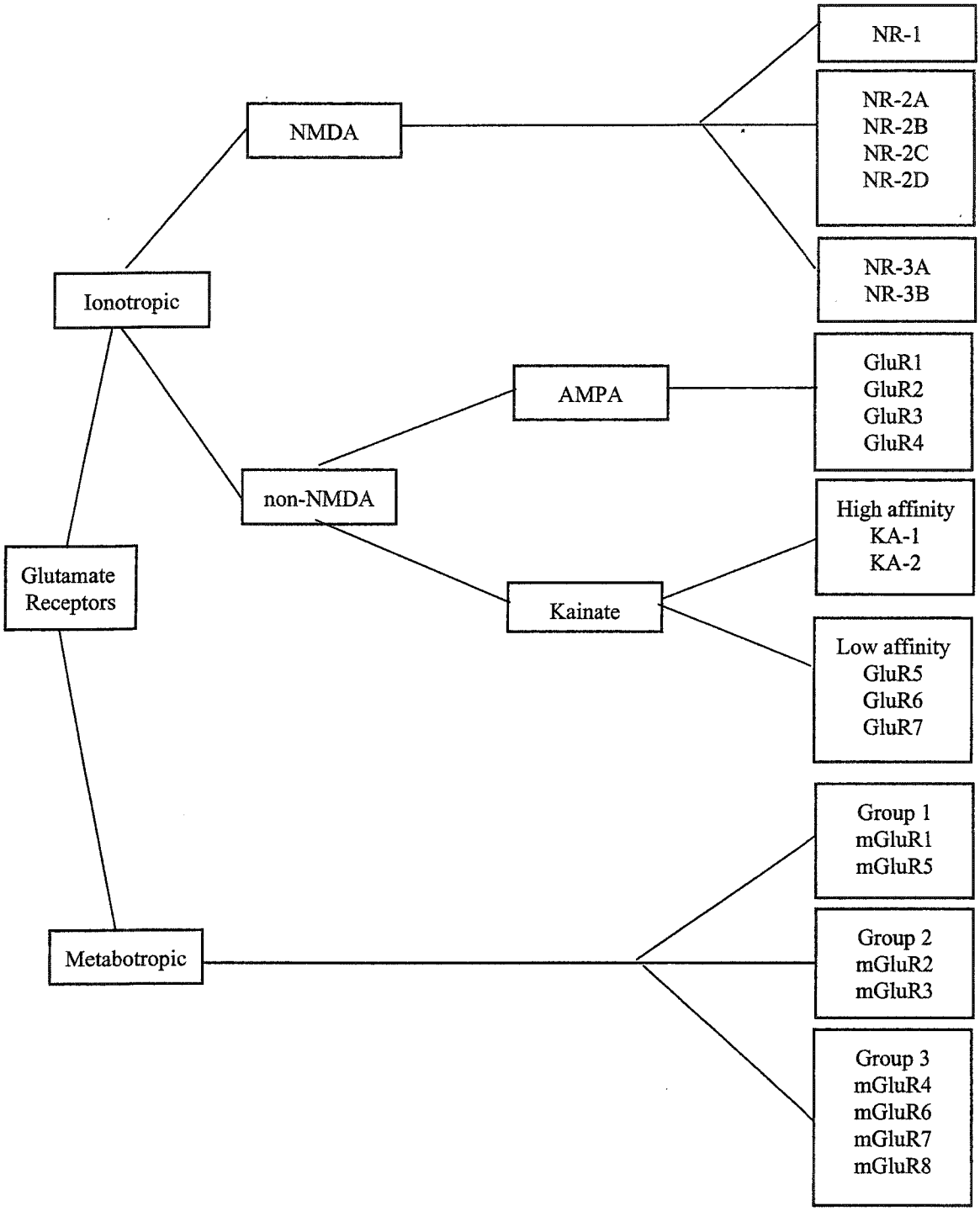


Figure 1.1. Glutamate receptor classification. Adapted from Jorgensen, M., et al. (1995). *Pharmacology & Toxicology, 76*, 312-319; and Ozawa, S., et al. (1998). *Progress in Neurobiology, 54*, 581-618.

1.1.2.1. Metabotropic receptors

Metabotropic glutamate receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins) and modulate the production of intracellular second messengers, such as adenylate cyclase, via the release of calcium from intracellular stores (Schoepp & Conn, 1993). Currently, eight receptor subtypes (mGluR1-mGluR8) have been identified (for review see Ozawa et al., 1998). These subtypes can be further classified into group 1 (mGluR1 and mGluR5), group 2 (mGluR2 and mGluR3), and group 3 (mGluR4, mGluR6, mGluR7, and mGluR8) (Tanabe et al., 1992).

1.1.2.2. Ionotropic receptors

Ionotropic glutamate receptors (iGluRs) form cation-specific ion channels (Ozawa et al., 1998) and are divided into two classes based on agonist specificities: *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors. The non-NMDA receptors are further subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors (Figure 1.1).

1.1.2.2.1. NMDA receptors

The NMDA receptors are ligand-gated cation channels that are blocked by a magnesium ion and are highly permeable to calcium (McDonald & Johnston, 1990).

Depolarization of the cell membrane by the influx of monovalent cations (usually via non-NMDA channels) causes the magnesium block to be relieved. There are seven known subunits (NR-1, NR-2A, NR-2B, NR-2C, NR-2D, NR-3A, and NR-3B) that can come together to form tetrameric NMDA receptor complexes (Ritter et al., 2002) (Figure 1.1). The NR-1 subunit must be present in order to form a functional NMDA receptor channel (Monyer et al., 1992). The NMDA receptors display different properties depending on which of the NR-2 and NR-3 subunits are assembled with NR-1. These receptors are distributed throughout the brain with the highest density in the CA1 region of the hippocampus (Ozawa et al., 1998). The NMDA receptor subtypes are differentially expressed early in development and are all present by postnatal day (PND) 7 (Al-Hallaq et al., 2002; Ritter et al., 2002; Takai et al., 2003).

1.1.2.2.2. Non-NMDA Receptors

The non-NMDA receptors, AMPA and kainate, are distinct receptor complexes although they can be activated by many of the same agonists (Bettler & Mulle, 1995). The AMPA receptors mediate fast excitatory neurotransmission and are distributed throughout the CNS (Bleakman & Lodge, 1998; Ozawa et al., 1998). They are tetrameric assemblies derived from combinations of four protein subunits designated glutamate receptor subunit (GluR) 1, GluR2, GluR3, and GluR4.

Kainate receptors are also widely distributed in the CNS. Five kainate receptor

subunits have been identified as either low-affinity (GluR5, GluR6, and GluR7) or high-affinity (KA-1 and KA-2) (Jorgensen et al., 1995). At low concentrations, kainic acid (KA) has a greater affinity for the high affinity KA-1 and KA-2 subunits, while domoic acid (DOM), at low concentrations, is selective for low affinity subunits, with the greatest selectivity for the GluR6 subunit (Verdoorn et al., 1994).

Expression of non-NMDA receptor subunits is detectable early in embryonic development (Bettler & Mulle, 1995). There is a transient surge in the expression of glutamate receptors during early postnatal development followed by a sharp decline of expression with the refinement of synaptic connections (Bettler & Mulle, 1995). In the developing rat hippocampus, there are three patterns of AMPA receptor subunit expression: (1) transiently increased expression around PND 7, followed by a decrease to lower levels than birth by PND 35 for GluR2 and GluR4 subunits; (2) transiently increased expression occurring around PND 18 for the GluR3 subunit; (3) no change in expression from birth to PND 35 for the GluR1 subunit (Ritter et al., 2002). Kainate receptor subunits have more complex patterns of expression. By PND 35, expression of all five receptor subunits was lower than birth levels. Also, each receptor subunit had a specific period of increased expression (Ritter et al., 2002). For example, GluR6 expression increased at PND5, while an elevation in KA2 was not seen until PND 14 (Ritter et al., 2002).

1.1.3. Domoic acid and kainic acid

Both DOM and KA are glutamate analogues and EAAs that are similar in structure and function to one another, but show differences in pharmacological and behavioural action (Tasker & Strain, 1992; Tasker et al., 1996; Doucette et al., 2000). Domoic acid is a naturally occurring excitotoxin found in both seaweed (*Nitzschia diatomee* in Prince Edward Island) and phytoplankton. It was responsible for an outbreak of gastrointestinal distress and neurotoxicity in approximately 150 people in Eastern Canada following consumption of contaminated mussels in the late 1980's. Kainic acid is derived from both seaweeds and marine algae (*Digenia simplex*) (Tasker et al., 1991). Although non-NMDA receptors are involved in the actions of DOM and KA, there is evidence that NMDA receptors are also involved in the actions of DOM, possibly through the release of endogenous glutamate (Novelli et al., 1992; Berman & Murray, 1997; Tasker & Strain, 1998). Furthermore, antagonists that interact selectively with low affinity KA receptor binding sites have been shown to reduce DOM toxicity while not affecting KA toxicity in vivo (Tasker et al., 1996). Domoic acid has also been shown to be 8-10 times more potent than KA in adult rodents (Tasker et al., 1991) and 6 times more potent in neonatal rodents (Doucette et al., 2000). Administration of DOM and KA can cause behavioural and pathological effects. High doses of KA result in seizures (Holmes & Thompson, 1988). Even low doses of DOM and KA, which do not produce any overt signs of toxicity, can cause physiological and behavioural effects including earlier eye opening, conditioned place preference, and altered activity levels (Doucette et

al., 2003).

1.2. The hypothalamic-pituitary-adrenal axis

The hypothalamus, pituitary, and adrenal cortex are the major neuroendocrine structures that control the hormonal regulation of the stress response. These structures collectively form the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus activates the sympathetic nervous system and controls secretions from the pituitary. The pituitary regulates secretions from the adrenal cortex. The adrenal glands, in turn, secrete glucocorticoids from the cortex and catecholamines from the medulla.

1.2.1. Anatomy and function

1.2.1.1. The hypothalamus

The hypothalamus is a relatively complex structure located directly beneath the thalamus at the base of the brain and posterior to the optic chiasm (Nelson, 2000). It is divided in two by the third ventricle and is composed of several collections of nerve cell bodies. These nuclei are paired, with one on either side of the ventricle (Brown, 1994) (Figure 1.2).

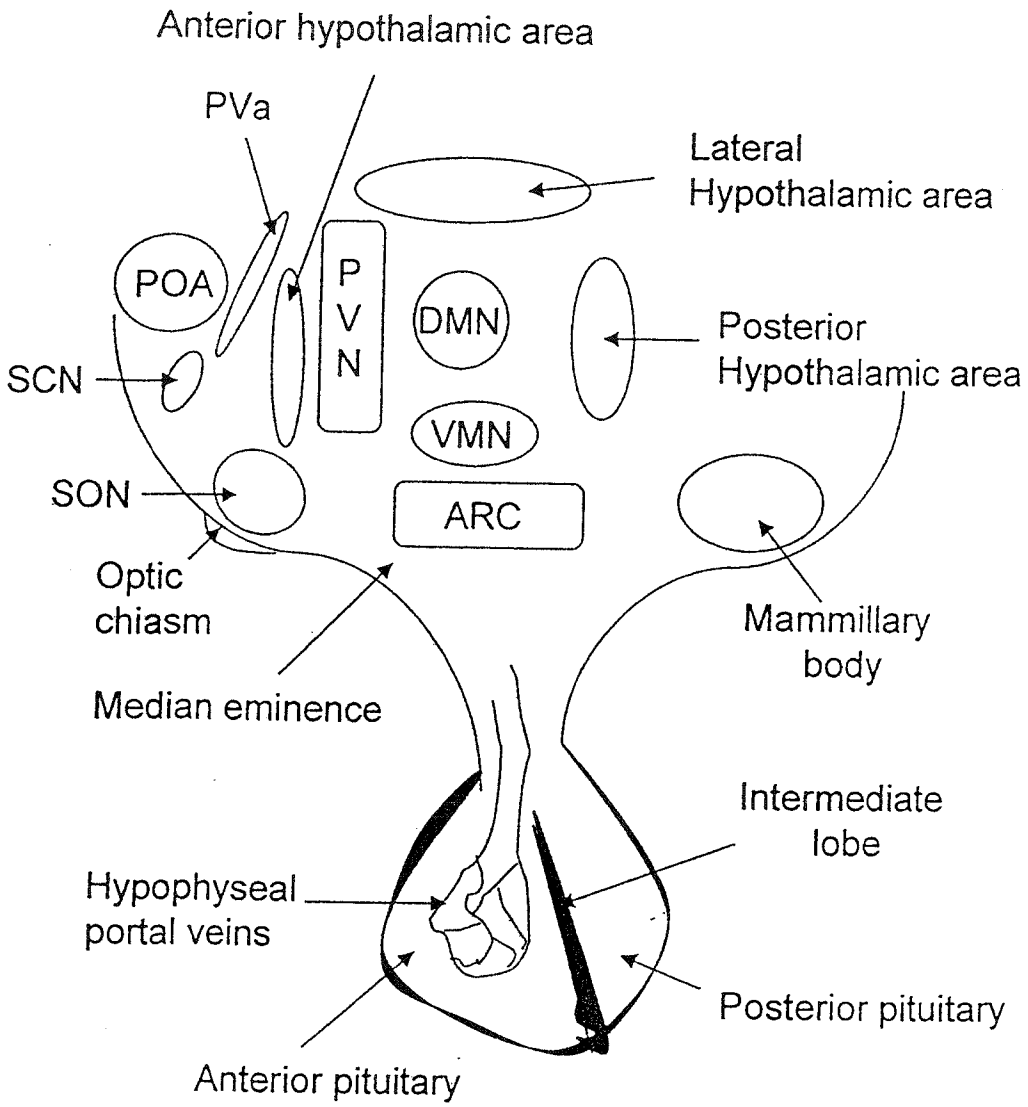


Figure 1.2. Sagittal view of nuclei of the hypothalamus. The hypothalamus is a collection of these small nuclei that lie just below the thalamus. Adapted from Brown, R. E. (1994). *An Introduction to Neuroendocrinology*. Cambridge: Cambridge University Press.

The main role of the hypothalamus is to maintain internal homeostasis. Therefore, it has many functions, including synthesizing hormones, regulating the sympathetic and parasympathetic autonomic nervous system, controlling the temperature regulation of the body, regulating electrolyte balance, controlling emotional behaviour such as anger, fear, or euphoria, and mediating motivational arousal such as hunger, thirst, and aggression (Pinel, 2003). Also, it contains a "biological clock" which determines and regulates biological rhythms. Each of these functions can be localized to particular hypothalamic nuclei. The basal hypothalamus is not surrounded by the blood brain barrier. This is adaptive because it must be able to detect hormonal changes in the blood.

Two groups of hypothalamic neurosecretory cells release hormones into the circulation for neuroendocrine communication. The magnocellular neurosecretory system is composed of large neurons located in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) and extending to the posterior pituitary. The parvicellular system is composed of relatively small cells found primarily in the preoptic area (POA), ventromedial nucleus (VMN), and arcuate nucleus (ARC). It projects to the median eminence where the cells release their hormones into the primary capillary plexus of the pituitary blood supply. These hormones stimulate or inhibit anterior pituitary secretions (Brown, 1994).

The majority of the blood supplied to the anterior lobe of the pituitary is indirectly

derived from the hypophyseal arteries through a capillary portal plexus. The superior hypophyseal arteries send branches into the median eminence of the hypothalamus where they form a network of capillaries called the primary plexus. Hypothalamic hormones released into the primary plexus travel through the hypophyseal portal veins of the infundibulum to the secondary plexus in the adenohypophysis where they stimulate pituitary cells to release their hormones (Brown, 1994). The capillaries of the portal system first appear in the median eminence of the rat on PND 5 (Watkins & Choy, 1979). They increase in number until they reach adult levels around PND 14-18. Prior to this, corticotropin-releasing hormone (CRH) passes from the hypothalamus to the pituitary via diffusion (Watkins & Choy, 1979).

1.2.1.1.1. Hypothalamic hormones

Hypothalamic Releasing Hormones

Thyrotropin releasing hormone (TRH) is synthesized primarily in the PVN and the anterior periventricular nuclei (PVa). Secretion of TRH is regulated by catecholaminergic neurotransmitters, somatostatin, and the opioids. It stimulates thyrotropes to produce and release thyroid-stimulating hormone (TSH) (Pinel, 2003).

Corticotropin-releasing hormone (CRH) stimulates the release of adrenocorticotropic hormone (ACTH) from corticotropes (de Kloet, 2000). It is

synthesized primarily in the PVa and PVN (refer to section 1.2.2.1. for more discussion).

Gonadotropin-releasing hormone (GnRH) is synthesized in the POA, suprachiasmatic nucleus (SCN), and ARC and acts on gonadotropes to stimulate the release of lutenizing hormone (LH) and follicle-stimulating hormone (FSH) (Brown, 1994). A host of neurotransmitters and neuropeptides regulate the release of GnRH, many of which interact with each other and the gonadal steroids to modulate its release. Glutamate regulates GnRH secretion, as activation of the NMDA receptors stimulates GnRH release (Dhandapani & Brann, 2000).

Paired Hypothalamic Releasing and Inhibiting Hormones

Growth hormone-releasing and inhibiting hormones (GH-RH/somatostatin (SS)) regulate the secretion of growth hormone (GH) from somatotropes (Brown, 1994). GH-RH is synthesized primarily in the ARC and also the VMN. Its release is controlled by catecholaminergic and serotonergic neurotransmitters, as well as by the opioids and TRH. Somatostatin is synthesized primarily in the PVa and POA and released into the hypophyseal portal system. Somatostatin is also produced in the VMN and dorsomedial nuclei (DMN), but is released in other areas of the brain where it acts as a neuromodulator (Brown, 1994).

Prolactin releasing and inhibiting factors (PRF/PIF) control the secretion of

prolactin (PRL) from lactotropes. Vasoactive intestinal peptide (VIP), TRH, oxytocin, and β-endorphin have been shown to increase prolactin release. The neurosecretory cells that synthesize PRFs are located in the PVN and POA. Dopamine (DA), a neurotransmitter released from the ARC, is the major PIF (Brown, 1994). In females, PRL is released in a cyclic pattern with periodic surges while in males, prolactin is secreted in a tonic acyclic pattern (Neill, 1972).

Melanocyte stimulating hormone releasing and inhibiting factors (MSH-RF/MSH-RIF) regulate the release of melanocyte stimulating hormone (MSH) from melanotropes (Brown, 1994). Dopamine has been reported to act as a MSH-RIF, inhibiting MSH release in the rat (Tiligada & Wilson, 1989).

1.2.1.2. The pituitary gland

The pituitary (hypophysis) is located between the brain and peripheral endocrine organs in a depression (sella turcica) in the sphenoid bone at the base of the skull. It plays a crucial role as a transducer of nervous system stimuli into hormonal information (Yoshimura & Gorbman, 1986). Both structurally and functionally, the pituitary gland is the most complex of the organs of internal secretion (Holmes & Ball, 1974).

The pituitary gland is made up of the neurohypophysis and the adenohypophysis. The neurohypophysis, also called pars nervosa or posterior lobe, is an extension of the

hypothalamus (Brown, 1994). It includes the posterior lobe and the infundibulum and accounts for approximately 20% of the pituitary gland. The adenohypophysis, also called the pars distalis or anterior lobe, accounts for approximately 80% of the pituitary gland and is composed of the pars distalis, pars intermedia, and pars tuberalis.

1.2.1.2.1. Pituitary hormones

Hormones of the adenohypophysis

Seven hormones are produced and released from the adenohypophysis. Adrenocorticotropic hormone is produced in corticotropes by proteolytic cleavage of a large precursor protein, proopiomelanocortin (POMC) (Carrasco & Van der Kar, 2003) (refer to section 1.2.2.1. for more discussion).

Thyroid-stimulating hormone, produced in thyrotropes, stimulates the synthesis and release of triiodothyronine (T3) and thyroxine (T4) from thyroid cells (Brown, 1994).

Somatotropin (GH), produced in somatotropes, is a major participant in control of growth and metabolism (Brown, 1994). It has direct effects that result from growth hormone binding to its receptor on target cells. Growth hormone also has indirect effects mediated primarily by insulin-like growth factor-1 (IGF-1), a hormone secreted from tissues such as the liver in response to GH. The majority of the growth-promoting actions

of GH are due to IGF-1 acting on its target cells (Woelfle et al., 2003).

Prolactin, produced in lactotropes, is essential for initiating milk synthesis in the mammary glands and has many other functions related to growth, osmoregulation, fat and carbohydrate metabolism, reproduction, and parental behaviour (Schulkin, 1999). It also plays a role in the stress response as both PRL mRNA and plasma PRL have been reported to increase following stressors (Gala, 1990; Dave et al., 2000) (refer to section 1.3.1.2). The hypothalamus suppresses PRL secretion and DA is the major PIF (Shaar & Clemens, 1974). Glutamate is a modulator of DA release in the CNS and all of the GluR subtypes are involved in DA regulation (Whitton, 1997). Therefore, drugs acting at GluRs may stimulate DA release, which, in turn, would inhibit PRL secretion. Prolactin secretion is positively regulated by TRH and GnRH (Brown, 1994).

The gonadotropins FSH and LH are produced and secreted by gonadotropes. They stimulate the gonads and are essential for reproduction (Brown, 1994). Gonadotropins are also produced in gonadotropes of the pars tuberalis (Stevens & Lowe, 1997).

Melanocyte-stimulating hormone is synthesized in melanotropes of the pars intermedia of most vertebrates. It is a pigmentation regulator, causing darkening of the skin of fish, amphibians, and reptiles. It also regulates skin colour in humans (Norman & Litwack, 1987). Several forms of MSH are similar in structure to ACTH as they are

cleaved from the same precursor molecule, POMC (Brown, 1994). Melanotropes also synthesize the opioid peptide β-endorphin. Like ACTH and MSH, β-endorphin is derived from POMC. β-endorphin has morphine-like activity and is secreted in response to CRH (Brown, 1994).

Hormones of the neurohypophysis

Oxytocin and vasopressin (antidiuretic hormone) are synthesized in neurosecretory cells of the PVN and SON and transported down axons through the infundibulum to the pars nervosa where they are stored in nerve terminals (Pinel, 2003). The pars nervosa also stores neurophysins, large carrier proteins for oxytocin and vasopressin (Brown, 1994).

In addition to stimulating milk ejection during lactation, stimulating uterine smooth muscle contractions at birth, and the establishment of parental behaviour (Nelson, 2000), oxytocin also plays a role in the stress response (Lang et al., 1983; Gibbs, 1984; Neumann, 2002) (refer to section 1.3.1.2).

Kanic acid and NMDA differentially modulate oxytocin release from the hypothalamus and posterior pituitary (Pampillo et al., 2001). Administration of KA increases oxytocin release from the hypothalamus and this effect is blocked by selective antagonists. It has no effect on oxytocin release from the pituitary. In contrast,

administration of NMDA had no effect on hypothalamic oxytocin release, however, it decreased oxytocin release from the pituitary (Pampillo et al., 2001).

Vasopressin binds to receptors in the kidney and promotes reabsorption of water back into circulation. It also causes widespread constriction of arterioles, which leads to raised blood pressure (Nelson, 2000).

1.2.1.3. Hypothalamic-pituitary interaction

Hypothalamic-hypophyseal interactions are complex, and one hypothalamic hormone may control the secretion of several pituitary hormones (Brown, 1994). For example, TRH stimulates both PRL and thyrotropin release. Also, pituitary hormones may be transported back to the hypothalamus to act as neuromodulators. Neurohypophyseal hormones can travel by retrograde axonal transport and adenohypophyseal hormones may be carried by efferent portal vessels (Bergland & Page, 1979). Pituitary hormones may be released into the cerebrospinal fluid and stimulate hypothalamic nuclei around the third ventricle. Some 'hypothalamic hormones' may be secreted from cells in other brain regions, such as the limbic system, and act as neuropeptides (Brown, 1994). In addition to being regulated by the hypothalamic hormones, the pituitary hormones are also controlled by many neurotransmitters and neuropeptides. For example, DA and γ-amino butyric acid (GABA) may be released into the portal veins to regulate pituitary hormone release (Shaar & Clemens, 1974; Ben-

Jonathan et al., 1989). Finally, hypothalamic hormones interact with other hormones, such as the gonadal steroids, to control the secretion of pituitary hormones (Brown, 1994).

1.2.1.4. The adrenal glands

The adrenal glands in mammals are located at the craniomedial poles of both kidneys. They consist of an outer cortex, which surrounds the inner medulla. Each of these parts has a separate and specific function (Nelson, 2000).

1.2.1.4.1. The adrenal medulla

The adrenal medulla is composed of chromaffin cells which synthesize and store adrenaline (epinephrine) and noradrenaline (norepinephrine) (Nelson, 2000). Chromaffin cells function as part of the autonomic nervous system. Hormonal secretion from the chromaffin cells is controlled by the neural signals from the sympathetic nervous system. Adrenaline and noradrenaline are released following stress as part of the "fight or flight" stress response (Brown, 1994). Adrenaline acts to increase heart rate and blood glucose levels while noradrenaline increases blood pressure and constricts blood vessels (Carrasco & Van der Kar, 2003).

1.2.1.4.2. The adrenal cortex

The adrenal cortex is made up of three distinct regions in mammals. The outer zona glomerulosa is characterized by whorls of cells and makes up about 10-15% of the adrenal cortex (Nelson, 2000). The middle zona fasciculata consists of cells arranged in vertical columns and represents 75-80% of the gland. The innermost zona reticularis, is composed of an irregular network of cells.

All zones of the adrenal cortex produce steroid hormones (Nelson, 2000). Mineralocorticoids, particularly aldosterone, are produced by the zona glomerulosa. Sodium deprivation stimulates the secretion of aldosterone and it acts to increase the reabsorption of sodium in the kidneys, salivary glands, and sweat glands (Brown, 1994).

Glucocorticoids are produced in the zona fasciculata and reticularis. The zona reticularis also produces small amounts of gonadal steroids (ie. androgen, estrogen, and progesterone). The glucocorticoids (eg. cortisol and corticosterone (CORT)) are released in response to ACTH, and act to modulate carbohydrate metabolism. They are also involved in regulating immune cells during infection and have been reported to both enhance and suppress immune expression in the brain and periphery (Schulkin, 1999). In the mammalian CNS, there are two types of glucocorticoid receptors called Type I and Type II. (Carrasco & van der Kar, 2003) (refer to section 1.3.1.1).

1.2.2. Regulation of the HPA axis

1.2.2.1. Hormones

There are three hormones intricately involved in the regulation of the HPA axis and its response to stress. Corticotropin-releasing hormone, synthesized in response to internal and external stimuli, is released in a distinct day-night rhythm and regulated by many NTs and neuropeptides such as acetylcholine (Ohmori et al. 1995), serotonin (Jones et al., 1976), histamine (Grossman & Costa, 1993), opioids (Grossman & Costa, 1993), and glutamate (Joanny et al., 1997). Adrenocorticotropic hormone is secreted in response to CRH. In turn, ACTH stimulates the synthesis and release of glucocorticoids (Carrasco & van der Kar, 2003). Glucocorticoids signal back to both CRH and ACTH to inhibit their secretion (Verderame, 1986).

1.2.2.2. Feedback mechanisms

Hormonal feedback, particularly to the hypothalamus, is a method of monitoring hormone levels in general circulation. Hormones form integral feedback systems (loops) at all three levels of the HPA axis. This feedback can be either positive or negative to stimulate or reduce the secretion of hormones, respectively (Miller & O'Callaghan, 2002) (Figure 1.3).

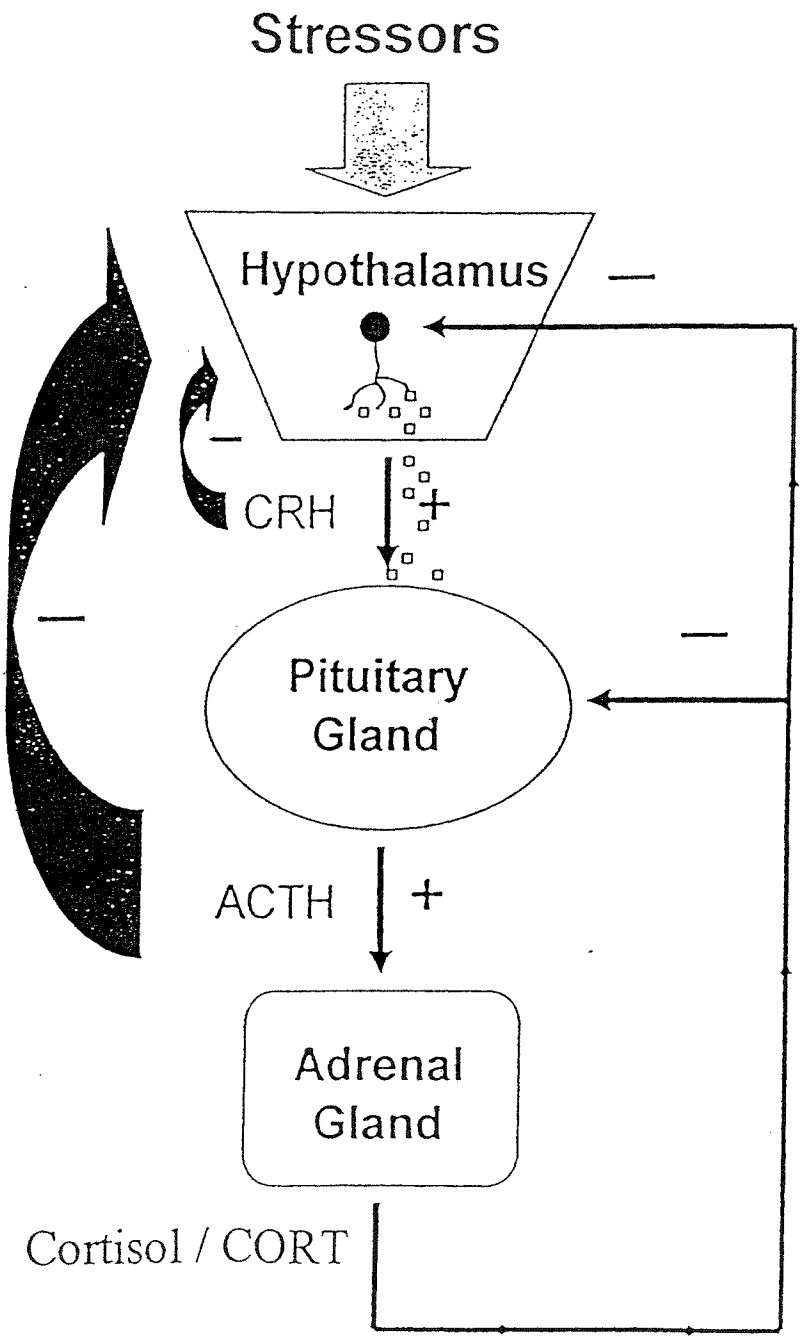


Figure 1.3. Feedback mechanisms of the HPA axis. Adapted from Brown, R. E. (1994). *An Introduction to Neuroendocrinology*. Cambridge: Cambridge University Press; and Nelson, R. J. (2000). *An Introduction to Behavioural Neuroendocrinology*. Massachusetts: Sinauer Associates, Inc.

First order feedback loops are self-regulatory. Hormone levels in the blood are monitored only by hormone-sensitive nerve cells in the brain. There is only one endocrine gland and one hormone involved in the feedback mechanism. For example, oxytocin and vasopressin each feed back to inhibit their own release from the hypothalamus (Nelson, 2000).

Second order feedback loops involve both hypothalamic hormones and adenohypophyseal hormones, such as GH, MSH, and PRL, whose target cells are not an endocrine gland. These pituitary hormones regulate their own secretion through negative feedback to the hypothalamic releasing hormones, inhibiting their secretion (Nelson, 2000). The hypophyseal hormones also provide positive feedback to the hypothalamic release-inhibiting hormones, which further inhibits the release of the hypophyseal hormones (Brown, 1994).

Third order feedback loops involve the hypothalamus, the pituitary gland, and either the adrenals, gonads, or thyroid (Brown, 1994). There are three levels of feedback within this system. In the hypothalamic-pituitary-adrenal axis system (Figure 1.3), corticosteroids provide long-loop negative feedback to inhibit CRH release and inhibit the secretion of ACTH (Miller & O'Callaghan, 2002). Release of ACTH provides negative feedback to the hypothalamus, as a short-loop feedback. Ultra-short loop feedback is provided by CRH to help regulate itself (Ono et al., 1985). Altering the level of one hormone in a feedback loop affects the levels of other hormones within that system

(Brown, 1994).

Neurotransmitters also play a role in the function of the feedback system and can alter the neuroendocrine system. An increase in NTs stimulating the release of CRH activates the entire HPA system (Jones et al., 1976; Ohmori et al., 1995; Joanny et al., 1997).

1.2.2.3. Circadian rhythms

Many hormonal systems are regulated by circadian rhythms (Dunn et al., 1972). In the nocturnally active rat, plasma CORT and ACTH peak around the beginning of the dark phase (Dunn et al., 1972; de Boer and van der Gugten,1987). Circadian patterns of hormone release need to be considered such that all test animals are sampled during the time when hormone concentrations are relatively stable to eliminate the possibility of a difference in concentrations due to time of day.

1.2.2.4. Role of glutamate in neuroendocrine regulation

The EAAs have been found to play an important role in neuroendocrine regulation in the hypothalamus (Brann & Mahesh, 1994). Studies have shown that endogenous and exogenous EAAs are able to excite hypothalamic neurons, including those in the ARC and the median eminence (Arnauld at al., 1983; van den Pol et al., 1990). Kainate was

found to be the more potent excitant. High concentrations of glutamate occur in presynaptic axons in the SCN, VMN, ARC, and parvocellular and magnocellular paraventricular nuclei in the rat hypothalamus. Almost all medial hypothalamic neurons respond to endogenous glutamate or to the glutamate agonists, quisqualate and kainate (van den Pol et al., 1990) and spontaneous excitatory postsynaptic potentials (EPSPs) in hypothalamic nuclei can be blocked by glutamate receptor antagonists (van den Pol et al. 1990; Brann & Mahesh, 1992). Excitatory postsynaptic potentials in the ARC and the PVN of the rat were almost completely blocked by the non-NMDA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (van den Pol et al., 1990). The NMDA receptor antagonists only slightly suppressed EPSPs in the PVN (Waurin & Dudek, 1991). These studies give evidence that glutamate neurotransmission, particularly acting via non-NMDA receptors, is important in neuroendocrine regulation.

1.2.2.4.1. EAA receptors in the hypothalamus

There are many glutamate receptors in the hypothalamus and the pituitary gland. Functional NMDA and non-NMDA receptors have been identified early in development of the rat hypothalamus, although they are present in lower concentrations than in other brain regions (van den Pol et al., 1995). The presence of functional glutamate receptors expressed early in hypothalamic development suggest that glutamate may influence many factors in neuronal development such as synaptogenesis and enzyme regulation. Also, the hypothalamus and pituitary in the rat exhibit moderate levels of NMDA receptor binding

(Kus et al., 1995). Compared to NMDA receptor sites, kainate receptor sites are reported to be higher in the hypothalamus. Moderate levels of AMPA-binding sites are found in the hypothalamus (Brann, 1995).

1.2.2.4.2. EAAs and regulation of hormone secretion

Excitatory amino acids play a role in regulating the secretion of GnRH, LH, FSH, GH, PRL, ACTH, oxytocin, and vasopressin (Brann & Mahesh, 1994). Administration of NMDA stimulates PRL secretion in intact and castrated male rats and cycling female rats, and administration of an NMDA antagonist blocks this effect (Login, 1990). Kainate administration stimulates PRL secretion in cycling females (Abbud & Smith, 1991). Although the mechanism(s) of stimulation of PRL secretion by EAAs is unclear, EAAs may act through the regulation of PRF and PIF or by regulating DA (the major PIF) release in the hypothalamus. Makara and Stark (1975) first demonstrated that administration of glutamate through the third ventricle potently stimulates ACTH secretion. Kainate, NMDA, and quisqualate have been subsequently shown to increase ACTH secretion (Brann & Mahesh, 1994).

1.3. Neuroendocrine and behavioural responses to stress

1.3.1. The stress response

Stress can be defined as an adaptive response of the body to any challenge (stressor) causing a change from internal homeostasis. Stressors are a diverse classification of stimuli, such as activities, events, or changes, that cause a deviation from homeostasis. There are physical, physiological, and psychological sources of stressors. Physical stressors are those related to the environment such as extreme temperatures, noise, pollution, or air quality. Physiological stressors are those that affect the physical body such as illness, lack of sleep, or food and water deprivation. Psychological stressors are those that relate to emotionality, fighting, social subordination, or lack of control in a situation. The stress response has been defined as nonspecific, meaning that various stimuli will elicit a similar response of the body (Seyle, 1976). The degree of the response may vary and no two individuals will necessarily respond to stress in exactly the same way.

A stress response occurs when a stressor disrupts homeostasis (Nelson, 2000). It is comprised of various physiological and behavioural responses that attempt to return the body to a state of homeostasis. Physiological changes that occur include activation of energy resources to maintain brain and muscle function, sharpened attention on the perceived threat, increased local cerebral glucose use, redistribution of blood flow toward

vital organs such as the brain, heart, and lungs, and inhibition of appetite, growth, immune function, reproduction, and pain perception (Carrasco & Van der Kar, 2003). These responses are aimed at maintaining survival. Within seconds of exposure to a threat or a perceived threat, epinephrine and norepinephrine are secreted from the adrenal medulla. Minutes later, the adrenal cortex secretes glucocorticoids and pituitary hormones such as prolactin, oxytocin, and vasopressin, are released (Carrasco & Van der Kar, 2003). These hormones facilitate coping with stress and behavioural adaptation, and prepare the organism for the next encounter with a stressor (de Kloet, 2000).

In 1936, Hans Seyle proposed a theory called the general adaptation syndrome (GAS) as the mechanism by which organisms cope with stress (Seyle, 1976). The alarm reaction is the first response to a stressor. This stage is initiated by the hypothalamus and includes elevated sympathetic activity, the release of CRH followed by the release of ACTH (Carlson et al., 2000). The release of glucocorticoids marks the beginning of the stage of resistance. Blood sugar and blood pressure increase and they increase metabolic activity throughout the body. With prolonged exposure to the stressor and inability to cope, an organism enters the third phase of the GAS, exhaustion. If the stressor continues, death can occur due to tissue destruction resulting from a prolonged overall increase in ACTH, glucocorticoids, and sympathetic nervous system activity (Carlson et al., 2000).

1.3.1.1. Glucocorticoid receptors

The effects of CORT are mediated by two glucocorticoid receptors. Type I, or mineralocorticoid receptors (MRs) bind both mineralocorticoids and glucocorticoids and have a higher affinity for CORT than do the Type II receptors. Type II, or glucocorticoid receptors (GRs) bind only glucocorticoids and have a lower affinity for CORT, thus require larger concentrations of CORT for activation (de Kloet 1995; Vazquez 1998; Carrasco & van der Kar 2003). Glucocorticoid receptors are more widely expressed than MRs (Kellendonk et al., 2002). Unlike MRs, which are found only in the brain and in epithelial cells of the kidney and colon, GRs are expressed in most cell types of the body. Mineralocorticoid receptors are present in areas of the limbic system, such as the septum and amygdala, and in the brain stem but not in the hypothalamus or pituitary. While GRs are expressed in these areas as well, there is also a high concentration of GRs in the hypothalamic PVN and pituitary (Brown 1994; de Kloet 1995). The highest expression of both Type I and Type II receptors in the brain is in the hippocampus and the localization of these receptors has been shown to overlap (Kellendonk et al., 2002).

During the first postnatal week, GR and MR concentration declines to about 20% of levels found in adults. After this initial decrease, receptor expression slowly begins to increase. Approximately 65-80% of the adult hippocampal GR and MR expression is reached by postnatal week 3 and there is also an increase of GR and MR binding capacity in the developing hippocampus (Vazquez, 1998).

Both GRs and MRs act as ligand-dependent transcription factors. There is a dual action of MRs and GRs in the hippocampus that is crucial for basal modulation and stress regulation of the HPA axis. Mineralocorticoid receptors mediate the effects of low basal circadian levels of circulating glucocorticoids and the tonic inhibitory influence of corticosteroids on HPA axis activity. Cells in the hippocampus control HPA axis activity indirectly by stimulating GABAergic neurons in the ventrolateral septal region. These neurons project to CRH-containing neurons in the PVN (Gesing et al., 2001). Binding to GRs, on the other hand, mediates the effects of high stress levels of glucocorticoids through negative feedback to terminate stress-induced HPA axis activation and regulates behavioural responses to stress, thus protecting the organism from its own stress response (Brown, 1994). This dual system allows the organism to cope with a wide range of glucocorticoid levels from 0.5 nM to 100 nM. Maximum stability and optimal viability for the organism occurs under conditions of predominant MR and little GR occupation (de Kloet, 2000).

Both of these receptors are down-regulated during chronic stress and aging (Brown 1994; Vazquez, 1998). Glucocorticoid receptors are down-regulated in response to high levels of glucocorticoids whereas MRs show a diurnal rhythm, with highest receptor concentrations at the end of the light period during the time of peak pituitary-adrenal activity (Brown 1994).

Glucocorticoid receptors and behaviour

Both types of glucocorticoid receptors play a role in a wide variety of specific and generalized functions depending on the given cell type (Kellendonk et al., 2002). For example, MRs are important in sodium and water reabsorption. Glucocorticoid receptors are involved in many physiological processes including energy homeostasis, adaptation to stress, modulation of the immune system, and modulation of brain function. The high concentration of MRs and GRs in the hippocampus support a role for these receptors in learning and memory, the primary functions of the hippocampus. Experiments using the Morris Water Maze have shown that MR and GR activation contribute to the efficiency of the various stages of processing spatial information (Oitzl & de Kloet, 1992).

Mineralocorticoid receptor-mediated effects are critical to processes of exploration, interpretation, and development of a behavioural repertoire to cope with the situation when an organism is exposed to novel environments (de Kloet, 2000). For example, one study (Gesing et al., 2001) examined the effect of acute stressors on anatomical and functional properties of the hippocampal MRs. Three different types of stressors were used. Cold stress, a physical stressor, was induced by placing animals in individual cages maintained at 4^0C for 4 hours without access to food or water. Novelty stress, a psychological stressor, was induced by placing the animals singly in a new cage for 30 minutes. Swim stress, a combined physical and psychological stressor, was induced by placing the animals in water maintained at 25^0C for 15 minutes. Animals that

experienced acute stressors with a psychological component (ie. novelty or swimming) had significantly higher hippocampal MR density 24 hours following the stressor that was associated with changes in the regulatory control of the HPA axis. Cold stress, which may have been both a physical and physiological stressor due to food and water deprivation in conjunction with cold exposure, was found to be ineffective. The stressor specificity demonstrates the importance of the limbic structures in the control of HPA axis activity. Activation of MRs in limbic structures is also important in behavioural reactivity and response selection during retrieval of stored information (de Kloet, 2000). Hippocampal MRs have been shown to modulate anxiety-like behaviour in rats because MR blockade 10 minutes prior to testing, but not GR antagonism, led to an anxiolytic effect in rats tested in the Black-White box test (Smythe et al., 1997). The MR system is incredibly sensitive to changes in CORT concentrations in both young and adult animals (Vazquez, 1998). Therefore, any alteration in CORT levels could significantly alter MR mRNA and potentially affect an organism's behavioural reactivity.

Glucocorticoid receptor-mediated effects that can be induced by the stress of a novel situation promote memory consolidation and information storage (Oitzl & de Kloet, 1992). Administration of a specific GR agonist to the basolateral amygdala enhances retention performance in a dose-dependent manner immediately following inhibitory avoidance training. Administration of GR antagonists into the same area produces memory impairment, including impairing retention performance in a water maze spatial task (Roozendaal, 2003). Post-training infusions of the specific GR agonist RU 28362

into the hippocampus have been shown to enhance memory consolidation for both appetitive and aversive tasks (Roozendaal, 2003). Therefore, GRs affect cognitive aspects of HPA axis regulation and their activation is important in memory consolidation and retention processes. If CORT is chronically elevated, ongoing behaviour can change to behaviour that would be of greater benefit to the organism. Therefore, the concentration of circulating CORT can have a considerable impact on an organism's behaviour. Also, GRs and MRs appear to have opposing effects on anxiety. While MR activation has been shown to be anxiolytic, GR activation is anxiogenic (de Kloet, 2000; Kellendonk et al., 2002). Sustained increases of glucocorticoids increases anxiety levels. Blockade of GR activity, inactivation of the GR gene in the brain, and downregulation of GR protein levels have all been shown to reduce anxiety in animals tested on the elevated plus and elevated zero mazes (Kellendonk et al., 2002). This provides evidence of a direct function of GRs in emotional behaviour.

The open field test is commonly used in developmental toxicology studies, but is also widely used as a novel, mildly stressful environment to evaluate psychological stress and curiosity and to study behaviours expressed in mildly stressful situations (Oitzl et al., 1994; van den Buuse et al., 2002). The test consists of an open arena in the shape of a circle or square divided into evenly spaced grids. The animal is placed in the arena and left to freely explore for a period of 2 to 10 minutes. Dependent measures typically include activity levels (number of grids entered), which reflect both emotional reactivity and exploratory behaviour, and amount of defecation, which reflects emotional reactivity

(Vorhees, 1986). Low activity scores can occur as a result of increased duration of freezing, a species-specific defensive reaction of rats that is often displayed when they are placed in fearful situations (Vorhees, 1986).

1.3.1.2. Neuroendocrine responses to stressors

The neuroendocrine response to stress depends on both the nature and intensity of the stress stimulus involved (Donohoe et al., 1987; Zelena et al., 1999). Many factors, including species differences, age, gender, health, motivation, and past experiences, also aid in determining the response of an individual to a particular stressor.

The primary neuroendocrine response to a stressor involves the activation of the HPA axis. Approximately 50-90% of the PVN neurons projecting to the median eminence are responsible for the release of CRH to initiate the stress response (Miller & O'Callaghan, 2002). Administration of CRH will produce most of the signs associated with exposure to a stressor. This demonstrates its importance in the initiation of the stress response. Glucocorticoids, GABA, and norepinephrine all have the ability to terminate CRH production. On the other hand, glutamate has been reported to stimulate CRH release in a dose dependent manner (Joanny et al., 1997). In addition, there are many CRH receptors in various brain areas, including the amygdala, as well as in organs such as the heart and gastrointestinal tract, suggesting that CRH has many functions, including mediating the behavioural response to stress (Miller & O'Callaghan, 2002).

Caldeira & Franci (2000) have shown that CORT secretion is altered in response to acute stress after PVN lesion has been induced. Lesioning the parvocellular neurons of the PVN by administering ibotenic acid (a glutamate analogue) and exposing the animals to a stressor (ether) 3 weeks later, resulted in a significantly higher increase in CORT concentrations of lesioned animals compared to controls. No significant differences were seen in basal CORT levels of control and lesioned animals. These findings reinforce the plasticity of the system as it was able to adjust for, and even overcompensate for, the lack of CRH from the PVN.

Hormone levels and activity of the HPA axis can persist for extended periods of time following exposure to stressors (Servatius et al., 2000). Plasma CORT concentrations in rats are elevated for at least 24 hours following single or repeated exposures to mild restraint and inescapable footshock (Servatius et al., 2001). Responses to stressors do not always end within hours after the termination of the stressor, but can persist for an undetermined period of time, which can lead to disease, and, ultimately, death.

There is much evidence to indicate that stress and PRL levels are related, and that both acute and chronic stress can stimulate PRL release (Freeman et al., 2000). Acute restraint stress has been shown to significantly increase plasma PRL levels in rats (Klenerova et al., 2001). In addition, compared to control rats, male and female rats exposed to chronic stress (footshock) showed an increase in PRL mRNA levels after only

one day of stress. After 3 days of stress exposure, PRL mRNA levels were even more elevated. However, after 14 days of stress, there were no significant differences in PRL mRNA levels in control and stressed groups. This suggests that chronic stress increases the synthesis of PRL mRNA during the first days of the stress and that the levels return to normal between the third and fourteenth day of stress in rats (Dave et al., 2000). Early treatment with monosodium glutamate (MSG) is able to completely block stress-induced release of PRL following two minutes of ether stress (Mizunuma et al., 1983).

In addition to the increase in many classic stress hormones in the plasma, there is also an increase in oxytocin during the stress response (Neumann, 2002). Oxytocin release can be induced by a variety of stressors (Lang et al., 1983). Both physical immobilization and swimming produced significant elevations in plasma oxytocin concentrations of both male and female rats but did not change plasma vasopressin levels (Lang et al., 1983). A later study by Gibbs (1984) also found that plasma oxytocin levels were significantly elevated following five minutes of either restraint or ether stress.

The release of oxytocin in response to stress depends on the nature of the stressor (Gibbs, 1986; Kasting, 1988; Wotjak et al., 1998). Oxytocin and vasopressin were simultaneously released, though in different proportions, during restraint stress, hemorrhage, endotoxin, and hypertonic saline administration (Kasting, 1988). Vasopressin alone was released following diethylstilbestrol administration, and oxytocin release occurred without the release of vasopressin following forced running,

hyperthermia, and hypothermia (Kasting, 1988). Also, several stimuli involve more than one type of stress. For example, swimming is both a physical and emotional stressor, combining cold stress, physical exercise, and fear of drowning. It has been shown to be a robust stressor in activating the oxytocin system (Neumann, 2002).

Oxytocin is also produced in the PVN and SON (Jezova et al., 1993). However, there is evidence to suggest that there are differential effects of stress on oxytocin in the PVN and SON. When the SON-neurohypophysial tracts are intact while those from the PVN are severed, no stress-induced release of oxytocin is observed following immobilization (Jezova et al., 1993). Therefore, the PVN is essential for immobilization-induced oxytocin release. A significant increase in oxytocin levels in the PVN following shaker stress provides further evidence for the importance of the PVN in mediating stress-induced oxytocin release (Nishioka et al., 1998). Even though the evidence clearly shows a relationship between oxytocin and stress, the physiological functions of oxytocin released in response to stress are still unclear, although there is some evidence of hypophysiotropic action (Jezova et al., 1995).

1.3.1.3. Sex differences in the stress response

Many sex differences in neuroendocrine and behavioural responses to stressors have been reported (Jezova et al., 1996). Kant et al. (1983) compared plasma CORT, PRL, and GH concentrations in response to three stressors (forced running,

immobilization, and footshock) in male and female Sprague-Dawley rats and found that plasma CORT rose more rapidly in females following all three stress conditions. There were no significant sex differences found with respect to PRL concentrations, but all stressors were found to decrease GH levels in males while not affecting female GH concentrations. A rise in plasma vasopressin levels following immobilization was found in females but not in males and stress-induced oxytocin release was greater in females than in males (Jezova et al., 1996). Studies examining behavioural differences have often found conflicting results. A decrease in feeding and body weight was found in male rats, but not in female rats following chronic immobilization stress of 20 minutes/day for 3 weeks suggesting that males may be more sensitive to moderate stress (Faraday, 2002). In contrast, Donohoe et al. (1987) found that two hours of immobilization stress decreased feeding and body weight similarly in male and female rats. In another aspect of a recent study, both males and females exposed to the chronic immobilization stress paradigm showed decreased open field activity levels on the first day of exposure compared with their non-stressed counterparts, however, while activity levels of the females remained low on subsequent testing days, those of males returned to control levels after the first day (Faraday, 2002). Variation in the results of studies looking at male versus female stress responses may be due to methodology, including which stressor was used, whether the stressor caused pain, the duration and intensity of the stressor, and the number of exposures to the stressor (Faraday, 2002). Gender differences in the stress response of humans are less well studied. However, a significant rise in plasma ACTH and PRL release in females following heat exposure for 20 minutes at 80^{0}C in a sauna

bath has been reported, although cortisol levels were similar in both females and males (Jezova et al., 1994). Cortisol levels are reported to be higher in men than women following psychological stress (Traustadottir et al., 2003).

1.3.1.4. Pathophysiological consequences of stress

It has been well established that exposure to chronic stress can lead to a variety of illnesses and psychological disorders such as inflammatory diseases, asthma, heart disease, depression, and many others (Sharpley, 1998; Miller & O'Callaghan, 2002). However, the mechanisms by which stress leads to disease are not well understood. It is likely that a combination of factors are involved, including environmental and genetic factors, individual differences, as well as the strength of the stressor and the length of time it is presented. Depression and cardiovascular disease are believed to be specifically related to a dysfunction of the HPA axis (Miller & O'Callaghan, 2002). In addition to leading to various diseases, stress can also induce thymus atrophy and suppress immune function (Morishita et al., 1998). The thymus is the primary site of T-lymphocyte production and maturation (Engler & Stefanski, 2003). The thymus provides a link between the neuroendocrine and immune systems as glucocorticoids have been shown to induce thymocyte apoptosis, which contributes to the atrophy of the thymus under stress (Tarcic et al., 1998). Therefore, an increase in CORT concentrations would augment thymus atrophy.

Physiological responses to stressors include alterations in the response to pain (da Silva Torres et al., 2003). In addition to glucocorticoid release, endogenous opioids are also secreted following a stressor (Mooring et al., 1985). These opioids are involved in analgesia. Behaviourally, measures such as the tail flick test can be used to determine stress-induced analgesic response. In the tail flick test, a third of the rat's tail is dipped in a hot water bath maintained at a particular temperature, typically 50^0C. The latency to perform the reflexive response of flicking the tail in response to the hot water is measured. An animal experiencing stress should have an increased tail flick latency because the subsequent secretion of β-endorphins will suppress the discomfort of having the tail dipped in a hot water bath. The tail flick test is a commonly used tool to examine different effects of exposure to stress on the nociceptive response (Castilho et al., 1999; Hawranko et al., 1999; da Silva Torres et al., 2003).

1.3.2. Developmental issues in the stress response

During development, the HPA axis is different from that of the adult. Mechanisms underlying normal HPA development and the mechanisms of adaptation are unlike those that a mature system would employ given the same challenges (Vazquez, 1998). During the final week of gestation, basal CORT levels in the fetal rat are within or slightly above the adult range (Sapolsky & Meaney, 1986). After birth, CORT levels decrease dramatically. Although plasma CORT levels decrease immediately following birth, they are higher than at any time during the subsequent two weeks (Sapolsky &

Meaney, 1986).

Both heat and electric shock induce a significant increase in plasma CORT of both 2 and 9 day old rats (Zarrow et al., 1966). The time for maximum response was 30 and 60 minutes, respectively. Although the concentration of CORT in the adrenal glands of 9 day old rats increased following both heat and electric shock, only heat induced an increase in CORT levels of the 2-day-old animals. However, the increased adrenal response was obtained in 5 minutes in both 2- and 9-day-old animals. These results show that an adrenal response can be made during the stress hypo-responsive period (SHRP). In a second similar study, Zarrow et al. (1967) found that there was no sex difference in the response of the 2 day old female rat pups to heat stress and electric shock.

Butte et al. (1973) showed that there is a small increase in circulating CORT levels in response to histamine stress in 1 and 2 day old rat pups. Between day three and fifteen, however, they found no increase in CORT in response to histamine stress. The neonatal rat is less sensitive to the stimulatory effects of stress and the inhibitory effects of circulating CORT during this period. Therefore, a stressor of higher intensity is required in order to stimulate an HPA axis response (Butte et al., 1973). In the rat, this period (SHRP) is a time from approximately PND 2 to PND 14 during which basal and stress-induced ACTH and CORT release are low compared to adults (Walker et al., 1986). This period is defined by the relative inactivity of the adrenocortical axis upon exposure to stress (Levine et al., 1992). The pattern of response is that of

hyporesponsiveness at all levels of the HPA axis. There is blunted ACTH secretion, resulting from immaturity of neural inputs to the CRH neurons, decreased pituitary peptide content, or decreased sensitivity to the CRH stimulus (Walter et al., 1986). The adrenal glands are also hyporesponsive to circulating ACTH levels (Levine, 2001). The pups fail to respond, or respond only weakly to a variety of stressors, such as surgery, handling, ether, or changes in temperature (Levine, 2001). These stressors reliably elicit an increase in ACTH and CORT secretion in rats at other ages. Immediately following the SHRP period, rats enter a new phase of stress responsiveness. They fail to terminate glucocorticoid secretion, thereby exhibiting high and sustained levels of circulating ACTH and corticosteroids (Vazquez, 1998).

Although there are many theories, the mechanism(s) underlying SHRP are still uncertain. Walker et al. (1986) examined the possibility that enhanced sensitivity of the hypothalamus or the pituitary to the inhibitory feedback effects of circulating glucocorticoids may contribute to SHRP. They found that 5 day old adrenalectomized rats increased ACTH secretion following exposure to ether or ovine CRH, demonstrating that negative feedback does play a role in mediating SHRP. In a similar study, Walker & Vrana (1993) characterized pituitary responsiveness to synthetic CRH using cultured pituitary cells of rats at PND 1, 6, 11, 16, and 20. There was significant ACTH release at all ages with the greatest release occurring at PND 6 and 11, during SHRP when CORT concentrations were lowest. The pituitary glands are still functional during this period, although they might require increased stimulation for activation.

1.3.2.1. Ethological relevance

The SHRP is a protective mechanism that ensures stable low levels of glucocorticoids throughout early development (Bakker et al., 2001). One adaptive function of the SHRP is related to the influence of glucocorticoids on the growth and development of the central nervous system. During the SHRP, catabolic processes are limited and postnatal central nervous system development can occur in the absence of high levels of glucocorticoids (Sapolsky & Meaney, 1986). Glucocorticoids are generally catabolic, inhibiting cell division, protein synthesis, and the uptake of amino acids and glucose in target tissues (Sapolsky & Meaney, 1986). This has been shown to have serious negative consequences for the developing animal. Rats treated with glucocorticoids during the first week of life have permanently reduced brain weights and DNA content, which appears to be due to the postnatal suppression of cell division (Sapolsky & Meaney, 1986). Also, neuronal myelination, which is dependent on postnatal cell division, is affected by glucocorticoid treatment during the first week of life. Low levels of glucocorticoids appear to facilitate myelination while high levels inhibit this process (Sapolsky & Meaney, 1986).

During the SHRP, when CORT levels are typically low, high levels of CORT can enhance central nervous system maturation (van der Helm-Hylkema & de Wied, 1976). This would not be beneficial for the animal, as each process must progress at a certain rate. Accelerating the rate of development may cause other problems for the animal.

Rats exposed to high levels of glucocorticoids show altered social behaviour and performance in a variety of learning paradigms. In order to ensure normal physical growth and development of the pups, a battery of assessments is generally employed (Vorhees, 1986). These assessments included daily weight gain, eye opening, and auditory startle. The development of the auditory startle reflex is assessed by observation of its presence or absence followed by the delivery of a click (Vorhees, 1986). This reflex typically develops around PND 11 in the rat. Eye opening occurs when the sutures of the eyelids break, slowly revealing increasing amounts of the eyes until they are fully open. This occurs around approximately PND 13-14 in the rat. The time of eye opening has been accelerated by one to two days by a single injection of ACTH or ACTH fragment on PND 3 (van der Helm-Hylkema & de Wied, 1976).

Activation of the HPA axis by external stimuli in the adult animal activates physiological processes which facilitate the behavioural fight or flight response (Carlson et al., 2000). This effect may have no specific adaptive function in the young animal. Rat pups (PND 2-14) have such a limited behavioural repertoire that they are virtually incapable of the fight or flight response, therefore, this response is not advantageous. However, rat pups are capable of producing ultrasonic vocalizations in response to stressors, which in turn activates the HPA axis in the dam, who is capable of making an appropriate adaptive response (Allin & Banks, 1972; Smotherman, 1974; Bell, 1979).

1.3.2.2. Effect of early experiences

The HPA response to stressful stimuli can be altered by many environmental events during development (Francis et al., 1996). Animals that have experienced handling show decreased HPA responsivity to stress. In contrast, physical trauma or exposure to an endotoxin enhanced HPA responsivity to stress (Francis et al., 1996). Levine (1994) has shown that maternal behaviour can influence SHRP as prolonged maternal deprivation (a 24-hour separation) results in a disinhibition of HPA axis function. Pups that were separated from their mother showed significant increases in ACTH and CORT following exposure to a mild stressor, such as novelty or saline injection. Normally reared pups do not manifest an ACTH response to these stimuli (Levine, 1994). Also, ACTH remains elevated for at least two hours following an acute saline injection, suggesting a possible deficiency in the negative feedback mechanism.

Dam-pup interaction contributes to the regulation of the HPA axis during development and the dam plays a role in regulating offspring physiology until weaning. Maternal separation during this time can alter development (Kuhn et al., 1990). Pups isolated from their mothers for a single 24 hour period show elevated plasma levels of ACTH and CORT and attenuation of SHRP following exposure to stressors such as novelty, saline injection, or exogenous administration of ACTH (Cirulli et al., 1992). Lehmann et al. (2002) found that the effects of early maternal separation are long-lasting. Maternal separation for a single 24 h period at any of PND 4, 9, or 18 did not affect

baseline CORT levels at 5 and 20 months of age. However, it did result in a significant increase in the CORT response to a 30 minute restraint stress in the 5 month old rats only. In another study, adult rats that had undergone maternal deprivation for 180 minutes as neonates (PND 3 -12) showed increased anxiety-related behaviour compared to their control counter-parts (Wigger & Neumann, 1999).

1.3.2.3. Role of glutamate in development

NMDA receptors have been shown to be involved in diverse processes including learning and memory (Aguado et al., 1997; Mickley et al., 2000). Ketamine, a NMDA receptor antagonist, has been used to investigate the role of NMDA receptors in learning. It has been shown to act as a mildly aversive unconditioned stimulus when injected following the consumption of a flavored solution (Aguado et al., 1997). Administration of ketamine to pups in utero on E 19 thirty minutes prior to pairing a conditioned stimulus (saccharin) with an unconditioned stimulus (lithium chloride) blocked the taste mediated conditioned response normally seen up to 2.5 weeks postnatally (Mickley et al., 2000). These findings clearly implicate the NMDA receptors in taste aversion learning processes.

Domoic acid has previously been shown to manifest some of its effects indirectly via NMDA receptor activation (Tasker et al., 2004). Blockade of NMDA receptors by (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), administered

concomitantly with DOM, attenuated the conditioning effect of DOM on PND 13 (Doucette et al., 2004). There are two ways by which DOM could activate NMDA receptors. Firstly, DOM can bind to KA receptors on the postsynaptic membrane, resulting in the opening of ion channels and the entry of sodium into the cell causing depolarization of the cell membrane and relieve of the magnesium block in the NMDA receptor channel (Tasker et al., 1996). Calcium is then free to enter the cell and exert its effects. Secondly, DOM may bind to KA receptors on the presynaptic membrane of the neuron and stimulate the release of endogenous glutamate into the synapse (Tasker et al., 1996). Glutamate is then free to bind to both AMPA/KA and NMDA receptors on the postsynaptic membrane.

Activation of non-NMDA receptors and subsequent effects have been less well studied. High doses of KA administered to young rats have been shown to produce seizures that are similar to human temporal lobe epilepsy (Holmes et al., 1988; Sarkisian, et al., 1997). Male Wistar rats (PND 22-26) treated with 8-11 mg/kg KA were observed for behavioural changes for 4 hours following injection and tested in a variety of behavioural paradigms up to PND 220 (Holmes et al., 1988). Animals treated with KA developed spontaneous seizures. Long-term detrimental effects on learning, activity levels, and social interaction were also observed. Serial administration of convulsant doses of KA to neonatal rats beginning on PND 20 resulted in seizures with progressively longer onset latency and decreased severity as measured by electroencephalogram changes. In contrast, adults that had received this KA treatment exhibited severe seizures

after each of the 4 KA injections (Sarkisian et al., 1997).

Xi et al. (1997) were the first to examine non-NMDA receptor activation via DOM and its subsequent effects on neonatal rats. Neonatal rats (PND 2 and 10) were found to be highly sensitive to the neuroexcitatory effects of DOM as it induced behavioural effects at the lowest dose given (0.05 mg/kg) and produced seizures at 0.2 mg/kg. These changes were similar to those of adults, but were produced at much lower doses. One criticism of these findings is that the rating scale used to assess behavioural toxicity was designed for adult mice. A 2- or 10-day-old rat pup is quite limited in the behavioural responses it is capable of making and certainly does not have the same behavioural repertoire as an adult mouse, which was the model used to develop the rating scale. Despite the use of this rating scale, this study still demonstrated that DOM is a potent neurotoxin to neonatal rats.

There has been some research on the effects of non-NMDA receptor agonists on the HPA axis in both neonatal and adult rats. Chautard et al. (1993) administered N-methyl-D, L-aspartic acid (NMA), the racemic form of NMDA (1.25-10 μg/g), KA (0.75-5 μg/g), and quisqualic acid (2.5-30 μg/g) to 7-day-old rats during SHRP (see section 1.3.2) and found that all three EAAs induced a dose-related increase in plasma ACTH and CORT concentrations. However, KA was the most effective and potent at eliciting ACTH and CORT responses followed by NMA and lastly quisqualic acid. These results suggested that during SHRP, the HPA axis can be stimulated by EAAs

acting at either the hypothalamic or suprahypothalamic level.

Kent et al. (1996) further pursued the findings of Chautard et al. (1993) by examining the development of the HPA response to an acute injection of NMDA (5 mg/kg) and KA (2.5 mg/kg) in pups administered at one of PND 6, 12, or 18 (ie. during or immediately after the SHRP). Similar to Chautard et al. (1993), both NMDA and KA induced age- and time-dependent elevations of ACTH and CORT. Also, administration of a KA receptor antagonist (CNQX at 0.69 mg/kg) attenuated the response, suggesting that KA exerts its effects via the KA receptor at both PND 12 and 18, while the NMDA receptor antagonist (D,L-2-amino-5 phosphonovaleric acid (APV) at 7.5 mg/kg) was only effective at PND 18, indicating that the NMDA receptor is not mature until this time. Unlike Chautard et al. (1993), they found that some pups did exhibit overt behavioural changes such as scratching, convulsions, and hyperlocomotion. When the data was analysed without the animals demonstrating behavioural abnormalities, ACTH and CORT levels were still elevated over baseline.

Both Chautard et al. (1993) and Kent, et al. (1996) demonstrated that acute, high doses of KA can activate the HPA axis of pups during SHRP. It is possible that lower doses of the compound chronically administered may also produce comparable effects during this period.

Endogenous glutamate has been shown to trigger plasma ACTH release in adult

rats in response to footshock, immobilization, and ether stress (Zelena et al., 1999). Simultaneous blockade of NMDA and AMPA glutamate receptor subtypes for 4-5 days, followed by exposure to the stressful stimuli resulted in a small inhibition of ACTH release in response to immobilization stress. This simultaneous blockade did not affect ACTH response to footshock or ether stress, suggesting that involvement of glutamatergic pathways in neuroendocrine response during stress is selective for discrete stress stimuli (Zelena et al., 1999).

Few studies have investigated non-NMDA receptor activation using low doses of non-NMDA agonists. Doucette et al. (2003) reported that low doses of the selective KA receptor agonists, DOM and KA, administered during the second postnatal week produce changes in eye opening, conditioned place preference, and activity levels of neonatal rats. Furthermore, Tasker et al. (2004) showed that the acquisition of the conditioned place preference may be in part due to NMDA receptor involvement, because CPP, a competitive NMDA receptor antagonist, was able to block the effect. Rats given this neonatal drug treatment of low doses of DOM and KA also exhibit long-lasting behavioural effects. When placed in novel environments as adults, drug-treated rats displayed a collection of behaviours similar to low-level seizures (refer to section 1.4.2) (Doucette et al., 2004).

The development of an organism includes critical periods for its normal maturation during which it is particularly vulnerable to insult or stress (Vorhees, 1986;

Eriksson, 1997). The brain growth spurt period occurs from the day of birth until the third postnatal week of life in the rat. Many biochemical changes occur as well as axonogenesis, development of neurotransmitter systems, synapse formation and refinement, and myelination. Exposure to even low doses of neurotoxins during this period can cause irreversible changes in brain function of the resulting adult rat (Vorhees, 1986; Eriksson, 1997).

An organism's susceptibility to neurotoxins differs dramatically depending on its stage of development. This principle of critical periods is one of Wilson's 6 principles of teratology (Wilson as cited in Vorhees, 1986). The time of exposure to a toxin greatly impacts the nature and extent of the neurotoxic effect (Wilson as cited in Vorhees, 1986). There are four periods of susceptibility to damage during development which differ in their vulnerability to insult. They are termed preimplantation, organogenesis, histogenesis, and functional organization. Neurotoxic effects on the preimplantation stage are not graded. The organism either dies from the insult or survives with no major malformations. Exposure to a toxin during organogenesis may result in malformations. Insults during histogenesis are detectable as cellular abnormalities, growth changes, or functional impairments. The period of development known as functional organization may be the period of maximum vulnerability to damage that affects postnatal behaviour (Wilson as cited in Vorhees, 1986). During this period, fine and ultrafine changes are occurring, including the establishment of receptor and secretory sites and activation of biochemical pathways. Also, the effects of neurotoxin exposure may not be manifested

until later in development (Wilson as cited in Vorhees, 1986).

The glutamate system of the rat appears to develop around the third week of postnatal life (Bettler & Mulle, 1995). There is a rapid increase in the expression of glutamate receptors during early postnatal development, particularly during a stage of intense synaptogenesis. This is followed by a sharp decline of expression dependent on refinement of synaptic connections (Bettler & Mulle, 1995). During such a period of significant refinement of the system, disruption of this process by some kind of stress (physiological or chemical) leads to abnormal brain development and permanent changes (Vorhees, 1986).

1.4. Pertinent recent results from our laboratory

In a series of recent studies in our laboratory, we have used the non-NMDA agonists, DOM and KA, to stimulate the glutamate system of rats during a critical period of brain development. Rat pups were injected daily with either 5 or 20μg/kg of DOM, 25 or 100μg/kg KA, or saline from PND8-14. These doses of DOM and KA were chosen because they have been shown previously to be equally efficacious and non-toxic with regard to overt behaviour and are well below those that would produce overt signs of toxicity (Doucette et al., 2000). However, drug group differences in eye opening, activity levels, and conditioned place preference indicate that the doses of both of these compounds were behaviourally and physiologically relevant.

1.4.1. Effects on neonatal rats

A number of measures of physical development were assessed, including eye opening, which is a manifestation of the rate of central nervous system maturation (Binns & Salt, 1997). Both male and female 20μg/kg DOM-treated and male 25μg/kg KA-treated animals attained eye opening criterion prior to the saline-treated pups (Doucette et al., 2003). An earlier study by van der Helm-Hylkema & de Wied (1976) found earlier eye opening in rats that had been given a single injection of either ACTH or an ACTH fragment on PND3. Increased levels of corticosterone will enhance the rate of central nervous system maturation, therefore, a corticosterone response stimulated by DOM or KA given over PND 8-14 may be the mechanism responsible for the earlier eye-opening seen in the drug-treated pups. Although acute injections of high doses of KA increased CORT and ACTH concentrations in neonatal animals (Chautard et al., 1993; Kent et al., 1996), it is not known if chronic injections of low doses of DOM and KA may stimulate a CORT response or not.

Low level KA receptor stimulation also produced other changes in neurobehavioural development. KA-treated female rats showed a decrease in activity levels on PND 13 during olfactory testing (Doucette et al., 2003). DOM-treated animals were also better able to form an association from a single pairing of an odour and the drug in an olfactory conditioning paradigm. During testing on PND 13, they demonstrated a conditioned place preference for the odour. Animals were forced to choose between the

odour that had been previously paired with the drug and a novel odour, therefore it could not be concluded if the DOM-treated rats preferred the conditioning odour or were avoiding the novel odour. A subsequent study was aimed at clarifying the role of glutamate in simple associative learning processes during early development by removing this confound and testing whether DOM had a specific effect on learning versus a general alteration in odour responsiveness (Tasker et al., 2004). Pups were injected with 20μg/kg DOM either alone, or in combination with CPP, or saline from PND 8-14. They were conditioned to a peppermint odour on PND 8, re-exposed to peppermint or to a novel odour, almond, on PND 9, and tested for a conditioned place preference using a standard 3-choice paradigm on PND 13. Results indicated that DOM-treated pups spent significantly more time over the conditioning odour than did saline controls. Injections of a low dose of DOM appetitively reinforces a previous association with a conditioned stimulus in a single learning trial. This effect was antagonized by CPP, suggesting involvement of NMDA receptors in this paradigm (Tasker et al., 2004).

1.4.2. Effects on the resulting adult rats

Beginning at 120 days of age, rats subjected to perinatal drug injections were tested for various forms of learning and memory using different paradigms. Drug-treated rats placed in novel, mildly stressful environments, such as the open field, the Morris Water Maze (MWM), and the Novel Water Maze (NWM), were noted to manifest many abnormal behaviours not seen in any of the saline rats tested (Doucette et al., 2004). The

abnormal behaviours were characterized by a combination of hunched body posture, facial clonus, mastication with tongue-protrusion, repetitive head bobbing, repetitive eyeblinking or squinting, vibrissae and ear twitching, and tonic clonic forepaw movements. Collectively, these behaviours, termed the novelty-induced seizure-like syndrome (NIS-L), were suggestive of a stage 2 seizure. Drug-treated rats showed significant increases in hippocampal brain-derived neurotropic factor (BDNF) gene transcription with no significant increase in neuropeptide Y (NPY) mRNA (Doucette et al. 2004). These neurochemical changes are also observed in human patients with epilepsy (Takahashi et al., 1999). The glutamate system can be altered by many mechanisms during development (Ikonomidou et al., 2000). Therefore, it is probable that this early drug treatment has altered brain development, resulting in an animal that responds differently to novel, mildly stressful environments. The novelty of each test is a crucial component of precipitation of the NIS-L syndrome. Once a situation is no longer novel, the animals do not exhibit the syndrome.

1.4.3. Possible explanations for the NIS-L syndrome

Seizure behaviours in rats are grouped into 5 stages, ranging from mild (stage 1: facial clonus) to severe (stage 5: rearing and falling) (Racine, 1972). The NIS-L syndrome may be a low level seizure because the collection of behaviours by which it is defined are similar to those expressed by animals in a stage 2 seizure. It can be hypothesized that because the development of the glutamate system is easily altered, early

manipulation of this system may stabilize synaptic connections normally destined for removal, resulting in an intrinsically hyper-excitable brain in adulthood. This hyper-excitable brain may be more apt to experience seizures or it may also result in an animal that is hyper-responsive to novelty.

An alternative (or perhaps complimentary) explanation for the NIS-L syndrome is that the animals may be exhibiting exaggerated stress responses to the testing environments because their coping strategies for mildly stressful situations may have been altered. In addition to the collection of behaviours termed NIS-L, the drug-treated rats also displayed crouching, failure to groom, and porphyrin secretion while on the platform between trials. These are typical behaviours of the stress response in animals (Blanchard et al., 1998). The drug-treated rats also showed signs of altered spatial memory. However, manifesting these stress-related behaviours did not result in a decreased learning/working memory. These results are indicative of a heightened stress responsivity.

Necropsy data on these rats, at about one year of age showed that many DOM-treated animals had enlarged pituitary glands and that 6/11 of the DOM-treated females showed evidence of mammary tumours and had milk in their mammary glands (Doucette, 2003). This may indicate that non-NMDA receptors play an important role in the development of the limbic-hypothalamic circuitry. It may be that early activation of the glutamate system using low doses of DOM or KA may have permanently affected the

development of the HPA axis, resulting in an axis that is hyper-responsive to moderate stressors. The significance of these findings is that the data suggests that the limbic and HPA neuronal circuits may have been permanently "re-wired" using very low doses of a low affinity KA agonist.

1.4.4. Rationale, hypothesis, and objectives

The previous findings of early eye opening and NIS-L in DOM- and KA-treated animals suggest changes in HPA axis development. Mammary tumours and lactation in DOM-treated animals suggest an alteration in pituitary function. There is no reason to believe that only the pituitary and mammary glands have been altered by this neonatal drug treatment regime. The adrenal cortex or the pineal gland could also have been affected. The low level of glutamate system stimulation by DOM and KA could be contributing to a generalized neuroendocrine dysfunction that may affect other endocrine glands. However, there is evidence of enlarged pituitary glands, mammary tumours, milk production, enhanced central nervous system maturation, and an exaggerated behavioural response in a stressful paradigm (Doucette, 2003). Early activation of the HPA axis can produce a hyper-responsive HPA axis in the resulting adult (Sapolsky & Meaney, 1986). All of these taken together implicate an altered HPA axis as a possible consequence of early glutamate receptor activation. Therefore, further exploration of this phenomenon using hormonal measures of stress responsivity (CORT and ACTH) and behavioural indices (NIS-L and stress-induced analgesia (tail flick)) was warranted.

The overall hypothesis directing this thesis is that low doses of kainate receptor selective ligands, given perinatally during critical periods of CNS development, will cause immediate and long-term changes in HPA axis function as evidenced by both altered hormone secretions (CORT, ACTH, oxytocin, and PRL) and changes in stress-related behaviours.

The specific objectives of this study were:

(1) to determine whether early activation of the glutamate system caused alterations in HPA axis development by assessment of serum CORT concentrations in neonates following sequential injections of DOM or KA over PND 8-14, and

(2) to study baseline hormone (CORT, ACTH, oxytocin, and PRL) levels during development and determine whether this DOM and KA drug treatment correlates with long-term changes in behavioural (NIS-L and tail flick) and neuroendocrine (CORT, ACTH, oxytocin, and PRL) response to stress.

Study 1 will focus on neonatal rats at 14 days of age. Results from this study will indicate immediate effects of treatment that might account for long-term changes in stress responsiveness. Study 2 will focus on aged rats approximately 16 months old (available from a previous study) and will examine baseline concentrations of CORT, ACTH, oxytocin, and PRL as markers of pituitary and adrenal function, and CORT concentrations immediately following a novel stressor. Study 3 will focus on baseline ACTH, CORT, and oxytocin concentrations and ACTH, CORT and oxytocin

concentrations immediately and 2 hours following a novel stressor in adult rats at 75 days of age. Results of studies 2 and 3 will confirm the presence of the NIS-L syndrome and determine if there is a later or sustained change in neuroendocrine regulation as a result of neonatal drug treatment.

Collectively, these studies will investigate whether chronic perinatal drug treatment during a critical period of brain development affects later neuroendocrine regulation and response to stress. This is important for understanding how an outwardly mild perinatal event can produce long-lasting permanent changes in the brain.

2.0. MATERIALS AND METHODS

2.1. Experimental animals

All experiments used Sprague-Dawley rats obtained from Charles River Laboratories (St. Constant, PQ). The animal colony room was kept at 22^{0}C and maintained on a 12-hour light : dark schedule with lights on at 0700 hours. Purina rat chow and water were available ad libitum. Husbandry practices and all experimental procedures were approved in advance by the UPEI Animal Care Committee and were according to the guidelines of the Canadian Council on Animal Care (1993).

Neonatal experiments

Subjects were one hundred and ten male and female offspring of eleven untimed Sprague-Dawley dams. The day of birth was designated as PND 0. The litters were culled to 10 pups (5 male and 5 female when possible) on PND 0.

Adult experiments

Subjects were two cohorts of eighty male and female offspring of eight untimed pregnant Sprague-Dawley dams. The day of birth was designated as PND 0. The litters were culled to 10 pups (5 male and 5 female when possible) on PND 0. On PND 8,

animals in each litter were identified using a nontoxic, permanent marker to place an identification number on the rump. Animals were randomly selected and placed in one of five treatment conditions: 5μg/kg DOM, 20μg/kg DOM, 25μg/kg KA, 100μg/kg KA, or saline. There were 8 pups in each treatment condition. Animals were weighed and each was given the appropriate subcutaneous injection (in the nape of the neck) daily from PND 8 - 14. All injections were given in the morning starting at 0830 hours. A number of measures of physical development were assessed, including daily weight gain, auditory startle (from PND 10-13), and eye opening (from PND 12-14). On PND 23, animals were weaned, housed in pairs, and left until they were 75 days old.

Aged experiments

Subjects were thirty-two male and female Sprague-Dawley rats (approximately sixteen months old) that had been born in-house and been subjected to non-invasive testing as part of another experiment. Rats had been randomly divided into groups on PND 8 and injected subcutaneously in the nape of the neck with either 20μg/kg domoic acid or saline (10 ml/kg) from PND 8-14. On PND 8, each pup had been removed from the dam, weighed, given the appropriate subcutaneous injection and placed in a conditioning chamber containing either peppermint or no odour for 30 minutes according to the condition. They were tested for a conditioned place preference on PND 9 and 13 (Tasker et al. 2004). On PND 60, animals were run in the MWM as a test for spatial memory. Rats were housed in pairs and then left until they were sixteen months old.

2.2. Equipment

2.2.1. Cannulas

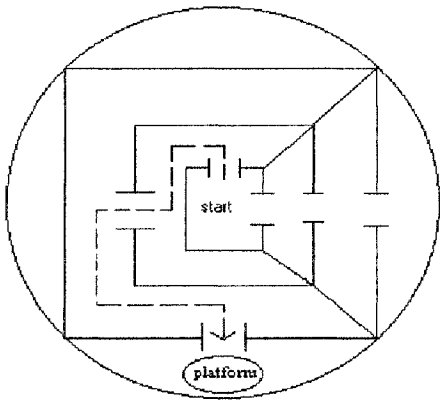
Where required, whole blood was collected via cannulas constructed "in house". For the intravascular end, 25 gauge needles (Becton Dickinson, Franklin Lakes, New Jersey) were removed from their hubs and attached to 10cm of sterile Silastic tubing (0.64mm ID x 1.19mm OD) obtained from Dow Corning (Midland, MI). At the distal end, 23 gauge needles (Becton Dickinson, Franklin Lakes, New Jersey) were attached to 1 ml tuberculin syringes (Becton Dickinson, Franklin Lakes, New Jersey).

2.2.2. Novel water maze and open field apparatus

The NWM was constructed similar to that described by Kant et al. (1988). It consisted of black plexiglas walls (30.5 cm high) arranged as 3 concentric squares placed inside a 150 cm diameter fiberglas pool. There were openings in some of the walls such that the animals had a series of choices between 2 alternative pathways. Choosing one set of three openings led to an escape platform located 2 cm above the water surface (Figure 2.1.).

The open field apparatus consisted of a rectangular plastic arena (80 cm long x 54 cm wide x 25 cm high) sectioned into 8 grids (27cm x 20cm) (Figure 2.1). Rats were

A



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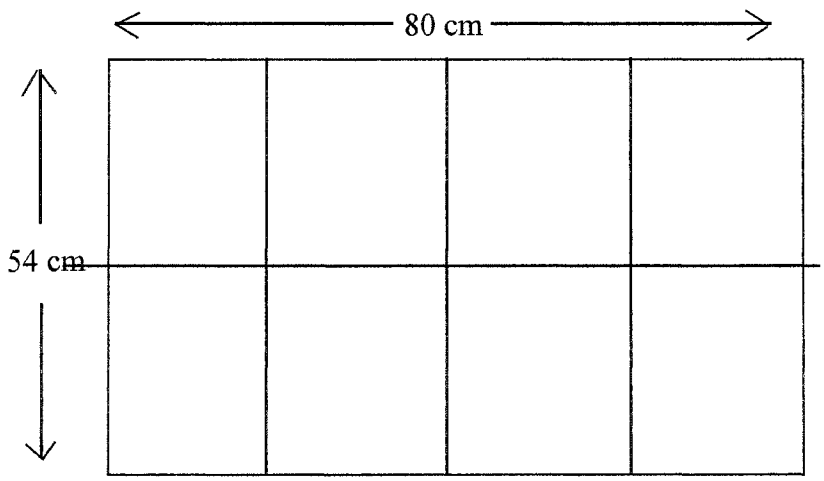

Figure 2.1. Schematic diagrams of the behavioural testing arenas. A. Overhead view of the Novel Water Maze. The configuration used for each of the 6 trials is indicated by the dashed line. B. Overhead view of the open field.

given 10 minutes to explore the open field.

2.3. Drugs and reagents

Domoic acid (Diagnostic Chemicals Ltd., Charlottetown, PEI) was injected subcutaneously at a dose of either 5μg/kg or 20μg/kg in a volume of 10 mL/kg. Kainic acid (Diagnostic Chemicals Ltd., Charlottetown, PEI) was injected subcutaneously at a dose of either 25μg/kg or 100μg/kg in a volume of 10 ml/kg. Stock solutions of DOM and KA (1 mg/mL of sterile physiological saline) were prepared. A 2 μg/ml solution of DOM was prepared by removing 100μl from 50mL of saline and replacing it with 100μl of stock DOM solution (1 mg/ml). A dose of 20μg/kg was given by injecting 0.2 ml/20g of rat. All other solutions were similarly prepared.

2.4. General procedures

On PND 8, animals in each litter were identified using a nontoxic, permanent marker to place an identification number on the rump. Animals were randomly selected and placed in one of five previously described treatment conditions (see section 2.1). These doses were chosen as they have previously been shown to be non-toxic, but behaviourally and physiologically relevant (Doucette et al., 2003). There were 8 pups in each treatment condition. Animals were weighed and each was given the appropriate subcutaneous injection daily from PND 8 - 14. All injections were given in the morning.

2.5. Physical assessments

2.5.1. Auditory startle

Development of the auditory startle reflex was assessed on PND 10 - 13. Each pup was removed from the dam and tested using a "clicker" held approximately 2 cm above the subject's head. Whether or not the pup startled to the sound was recorded.

2.5.2. Eye opening

On PND 12 -14, subjects were inspected twice daily (once in the morning and again in the late afternoon) for eye opening. Eye opening was operationally defined as a break in the sutures of the eyelids of both eyes. Whether or not the eyes were open was recorded and which eye opened first was recorded when possible.

2.6. Blood collection procedures

Rats were acclimatized to being in a Turkish towel and having their tails stroked. Once a day for a week, each rat was placed in the towel and its tail was stroked for 5 minutes. This was done in order to habituate the rat so that any differences in stress levels would not be due to handling or procedural variables.

Baseline

On the day of blood collection, each rat was placed in a Turkish towel. Its tail was washed with warm soapy water to both clean the tail and dilate the blood vessels. An elastic band was used as a tourniquet and tied at the base of the tail. Blood was collected from a point approximately 10 cm distal from the base of the tail. The needle was inserted into the vein with the bevel facing upward. Approximately 0.45 ml of blood was slowly drawn up into the syringe. The needle was then removed from the tail and hydrogen peroxide was rubbed on the tail to prevent infection. Gauze was applied to stop the bleeding. Blood was collected from one animal, followed by its cage mate so that each animal would not be able to smell blood and thus become stressed prior to blood collection. The blood was placed in a microcentrifuge tube and was left to cool and clot for 3 minutes. It was spun down in a microcentrifuge at 16,000 x g for 10 minutes at room temperature. The serum was pipetted using glass pipettes and plastic bulbs and was placed in labelled cryovials. The samples were stored at -20^{0}C until the CORT, ACTH, or oxytocin radioimmunoassay (RIA) was performed. All blood samples were collected between 0800 hours and 1300 hours because levels of CORT are relatively stable for approximately six hours after the lights come on (de Boer & van der Gugten, 1987).

Novel Water Maze

A rat was placed in the centre of the NWM maze facing a wall. A clear plexiglass cover was placed over the maze to prevent the animal from climbing the walls. The rat was given 60 seconds to find the platform. If it failed to find the platform after this time, a maximum score of 60 was assigned and the rat was guided to the platform by holding its tail and/or shoulders. It was then given 60 seconds on the platform. Behaviours such as grooming, squinting, head bobbing, mastication, rearing, and scratching were recorded by an observer blind to the experimental treatment of the animals (collectively termed "NIS-L"). Any animal that exhibited the NIS-L syndrome while on the platform was noted. A rat was given 6 consecutive trials in the pool. After its final 60 seconds on the platform, the rat was immediately placed in a turquish towel and blood (~0.5 ml) was collected in the same manner as for the baseline blood collection. The rat was then placed in a drying cage, consisting of a cage lined with paper towel, for 15 minutes before being returned to its home cage. Animals in the 75-day-old group also underwent a third blood collection (~0.5 ml) two hours following the final trial in the maze. All blood samples were stored at -20^{0}C until the CORT, ACTH, or oxytocin RIA was performed. All testing and blood collection was done between 0800 hours and 1300 hours.

2.7. Hormone assays

2.7.1. Corticosterone

Serum CORT was measured in baseline samples (PND14, PND 75, and PND 480 rats), in samples taken immediately following NWM exposure (PND 75 and PND 480 animals), and in samples taken 2 hours following NWM exposure (PND 75 rats). All serum samples were measured in duplicate by RIA using a Coat-A-Count Rat Corticosterone kit purchased from Intermedico (Markham, Ontario). Uncoated tubes were labelled for total counts (TC) and non-specific binding (NSB). Rat corticosterone antibody-coated tubes were labelled for standards and samples. Fifty microlitres of all standards and samples were pipetted into the appropriate tubes. One millilitre of ^{125}I rat corticosterone was added to every tube. The tubes were vortex mixed and incubated at room temperature for 2 hours. All visible moisture was then decanted from all tubes except the two TC tubes. The tubes were counted for 1 minute in a gamma counter (Riastar, Canberra Packard, Canada). The sensitivity of the assay, as determined by the manufacturer, was 5.7 ng/ml with 100% cross-reactivity for corticosterone and very low to no cross-reactivity to other compounds that may be present in rat serum. All samples were done in duplicate in a single run, the values were averaged and the concentration was expressed in nanograms (ng) of CORT per ml.

2.7.2. ACTH

Baseline serum samples (75 and 480 day old rats) and samples taken immediately and 2 hours following NWM exposure (75 day old rats) were analysed for ACTH. All serum samples were measured in duplicate by radioimmunoassay using kits purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA). These kits required 2 overnight incubations. All reagents were reconstituted with the RIA buffer. TC tubes, NSB tubes, total bound (TB) tubes, standards, and samples tubes were labelled in duplicate. Two hundred microlitres of RIA buffer was pipetted into each NSB tube and 100μl of buffer was pipetted into each TB tube. One hundred microlitres of each standard and sample were pipetted into the appropriate duplicate tubes. Following this, 100μl of primary antibody (rabbit anti-ACTH serum) was pipetted into the TB tubes, standards, and samples. The contents of each tube were vortex mixed and incubated overnight at 4^0C. One hundred microlitres of the ^{125}I-ACTH tracer solution was added to each tube. The contents of each tube were vortex mixed and again incubated overnight at 4^0C. One hundred microlitres of goat anti-rabbit IgG serum and normal rabbit serum were added to each tube except the TC tubes. The tubes were incubated at room temperature for 90 minutes. Following this incubation, 500μl of RIA buffer was added to each tube except the TC tubes. All of the tubes were centrifuged at 3000 rotations per minute (rpm), or 1700 x g, for 20 minutes at 4^0C. All of the supernatant was aspirated immediately following centrifugation. The TC tubes were not aspirated. A gamma counter (Riastar, Canberra Packard, Canada) was used to count the counts per minute (CPM) of the pellets.

The inhibitory concentration50 (IC_{50}) of this assay was 13.2 pg/tube with 100% cross-reactivity for ACTH and no cross-reactivity with any other peptide. All samples were done in duplicate, the values were averaged and the concentration was expressed in picograms (pg) of ACTH per ml.

2.7.2. Oxytocin

Baseline serum samples (75 and 480 day old rats) were analysed for oxytocin. Oxytocin concentrations were measured in duplicate by RIA using kits provided by Phoenix Pharmaceuticals Inc. (Belmont, CA). This procedure was the same as for ACTH analysis (see section 2.7.2). The IC_{50} of this assay was 5.89 pg/tube with 100% cross-reactivity for oxytocin. All samples were done in duplicate, the values were averaged and the concentration was expressed in pg oxytocin per ml.

2.7.4. Prolactin

Aliquots of the serum samples were sent to the National Hormone & Peptide Program at the Harbor-UCLA Medical Center (Torrance, CA) for prolactin analysis.

2.8. Other methods

2.8.1. Neonatal corticosterone experiment

2.8.1.1. Blood collection and endocrine gland dissections

Blood was collected between 1200 hours and 1530 hours one half hour following the appropriate drug injection on PND14. One litter at a time was injected and brought into a separate room 30 minutes later. Pups were immediately decapitated sequentially. Trunk blood was collected in microcentrifuge tubes. The blood was allowed to cool, clot, and coagulate at room temperature for approximately 10 minutes prior to centrifugation. It was then spun at 3000 rpm for 10 minutes in a microcentrifuge. Serum was pipetted into labelled cryovials. The remaining solids were discarded. The serum samples were stored in a freezer (-20^{0}C) until radioimmunoassay.

The thymus and adrenal glands were dissected out and the wet weights recorded in order to determine if there were differences between groups.

2.8.2. Aged rats experiment

2.8.2.1. Tail flick test and open field

The tail flick test was done in the 16-month-old group as a measure of pain sensitivity and stress-induced analgesia. A third of the rat's tail was dipped into a water bath maintained at $50^0C \pm 0.5^0C$. The latency of the rat to flick its tail was recorded. The maximum time allowed was 30 seconds. This was repeated three times with one minute between trials and the latencies from the second and third trials were averaged and designated as baseline latency. After the baseline tail flick latencies were recorded, the rat was immediately placed in an open field for 10 minutes. Any animal that exhibited the NIS-L syndrome was noted. Following the open field, the tail flick test was repeated.

2.8.2.2. Other experimental procedures

On the day of euthanasia, animals were weighed one at time and deeply anaesthetized with isoflurane. They were then rapidly decapitated.

The brains were rapidly removed and each dipped in liquid nitrogen for 15 seconds then wrapped in a small piece of plastic wrap along with an identification number and stored in a labelled plastic scintillation vial. The vials were kept temporarily on ice until they were stored in the -80^0C freezer. The pituitary glands were dissected out

of the skulls and the wet weights were recorded. They were individually stored in small labelled plastic cryovials containing 10% formalin. The adrenal glands were dissected out and the wet weights were recorded.

Approximately 3ml of trunk blood was collected from each animal in 3 microcentrifuge tubes. Blood was left to cool and clot for 3 minutes at room temperature prior to centrifugation. The blood was spun for 10 minutes in a microcentrifuge and the serum was pipetted off the top and placed in two labelled cryovials. The cryovials were placed on ice prior to storage in the -40^{0}C freezer until they were analysed for oxytocin and PRL concentrations.

2.9. Statistical analysis

All analyses were performed using SPSS 10.0 for Windows. Unless otherwise stated, all analyses for differences between groups were conducted using one-way (Treatment) or two-way (Treatment x Sex) analyses of variance (ANOVAs). Repeated measures ANOVAs were employed for analysis of tail flick latencies and serum CORT and ACTH baseline, immediate, and 2 hour analyses. Alpha was set at .05. Tukey's honestly significant difference (HSD) test was employed following a significant F value. Exploration of relationships between variables were conducted using Pearson r or Biserial correlations. Frequency data for occurrence of NIS-L was analysed using Chi square tests.

3.0. RESULTS

All means and appropriate statistical values are listed in Appendices A and B.

3.1. Neonatal experiment

3.1.1. Physical assessments

Analysis of the day on which the auditory startle reflex first appeared revealed no significant difference between groups. The first day to startle ranged from 12.6 to 12.8 days.

Weight gain was analysed on PND 9, after an acute injection, and again on PND 14, following serial injections. A two-way (Treatment x Sex) ANOVA with Repeated Measures failed to reveal a significant Treatment effect ($F(4, 99) = 0.72, p > .05$). There was, however, a significant Sex effect ($F(1,99) = 5.02, p < .05$) on weight gain. Males gained more weight than females during these two time periods.

3.1.2. Serum corticosterone and gland weights

Mean serum CORT concentrations for the different treatment conditions are presented in Figure 3.1. There were no significant Treatment or Sex effects on serum

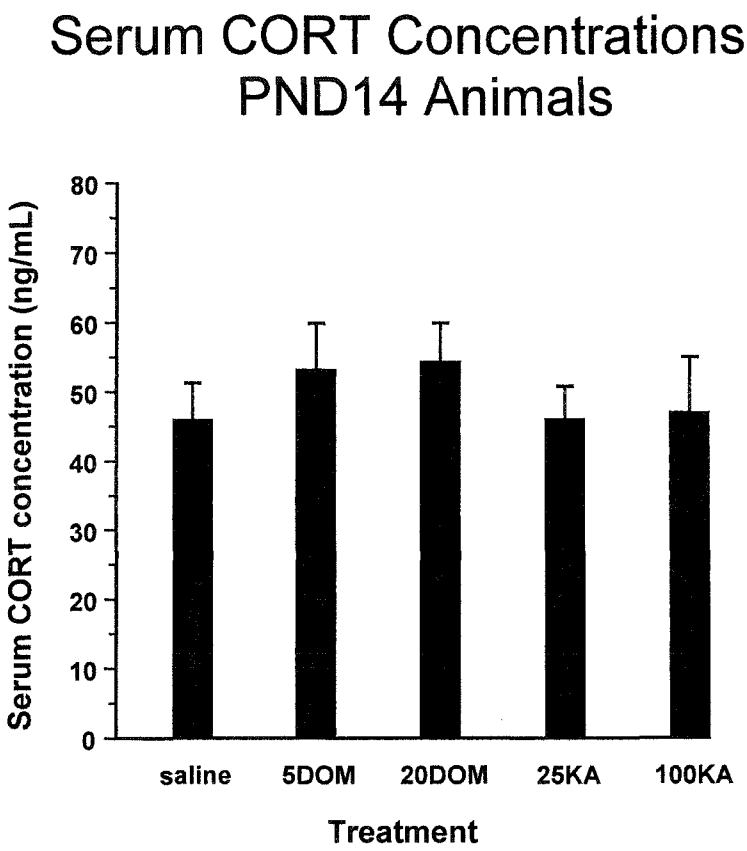

Figure 3.1. Mean (± SEM) baseline serum corticosterone concentrations of PND14 rat pups (N=8 males and 8 females/treatment condition).

corticosterone concentrations (Treatment: $F(4, 69) = 0.43, p > .05$; Sex: $F(1, 69) = 1.78, p > .05$). Baseline serum CORT concentrations ranged from 45.91 ± 5.37 ng/mL to 54.34 ± 5.57 ng/mL. However, as there appeared to be large litter variations, a Treatment x Sex analysis of covariance (ANCOVA) was employed with litter as the covariate in an attempt to minimize a possible confound to the Treatment effect on CORT concentrations and gland weights. Both absolute gland weights and the ratio of gland weights to body weights were analysed. There were no significant Treatment, Sex, or Treatment x Sex effects on CORT concentrations, adrenal gland weights or thymus gland weights both with and without the covariance factor included, except for a significant Sex effect on the ratio of thymus weights to body weights ($F(1, 68) = 5.80, p < .05$). Females had a significantly higher ratio of thymus weight to body weight than did males.

3.1.2.1. Litter effects

In order to determine the impact of litter on CORT concentrations, adrenal gland weights, and thymus gland weights, one-way ANOVAs with Litter as the grouping variable were conducted. Mean CORT concentrations, adrenal gland weights, and thymus gland weights of pups in each litter are presented in Figures 3.2 and 3.3. There was a significant difference in serum CORT concentrations between the different litters ($F(7, 71) = 7.87, p < .05$). For example, litter 5 had significantly lower serum CORT concentrations than litters 1, 2, 4, 8, and 11 (Figure 3.2). There was also a significant difference in the adrenal gland weights and thymus gland weights of pups of different

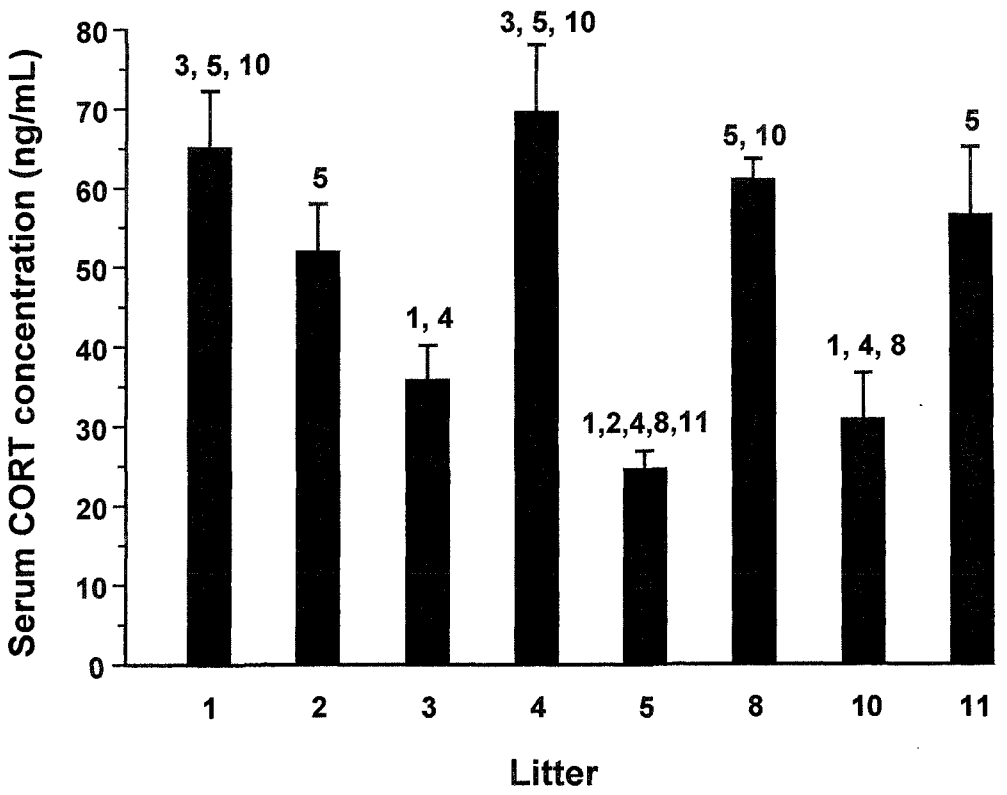

Figure 3.2. Mean (± SEM) baseline serum corticosterone concentrations of PND14 rat pups of the different litters (N=10 pups/litter). Numbers represent litters that are significantly different from the reference litter.

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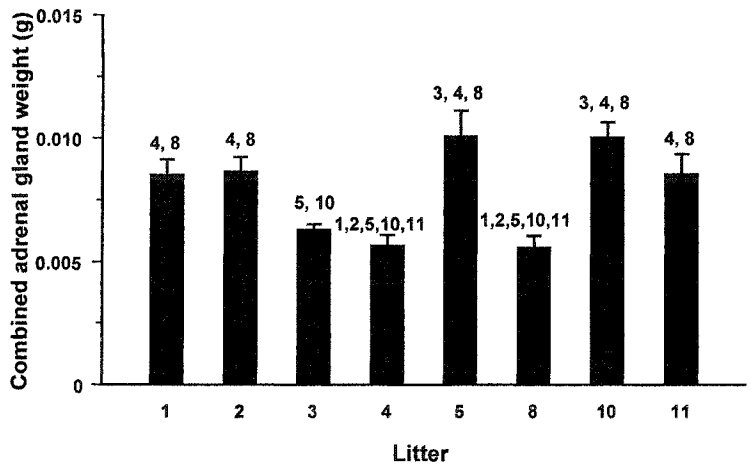

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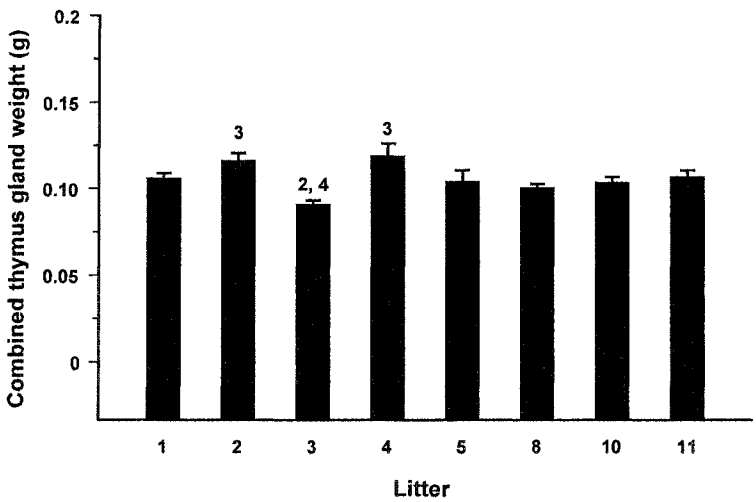

Figure 3.3. Litter effects on gland weights. A. Mean (± SEM) adrenal gland weights of PND14 rat pups of the different litters (N=10 pups/litter). B. Mean (± SEM) thymus gland weights of PND14 rat pups of the different litters (N=10 pups/litter). Numbers represent litters that are significantly different from the reference litter.

litters (adrenal gland weights: $F(7, 71) = 8.36, p < .05$; thymus gland weights: $F(7,71) = 3.53, p = 0.003$). For example, pups in litter 3 had lower adrenal gland weights than pups in litters 5 and 10 and lower thymus gland weights than pups in litters 2 and 4 (Figure 3.3). Also, significant differences in the ratio of gland weights to body weights were found for both the adrenals and the thymus (adrenals: $F(7, 71) = 12.14, p < .05$; thymus: $F(7, 71) = 5.28, p < .05$) (Table I). There was a significant correlation whereby litters with high serum CORT concentrations had lower adrenal gland weights ($r(77) = -.239, p = 0.034$).

3.2. Adult rats experiment

3.2.1. Neonatal physical assessments

There were no significant Treatment, Sex, or Treatment x Sex effects with respect to the day of eye opening (Treatment: $F(4, 70) = 0.49, p > .05$; Sex: $F(1,70) = 0.67, p > .05$) or auditory startle (Treatment: $F(4, 70) = 0.39, p > .05$; Sex: $F(1, 70) = 2.77, p > .05$).

3.2.2. Adult serum corticosterone and behavioural observations

Baseline serum CORT concentrations, serum CORT concentrations immediately following NWM testing, and serum CORT concentrations two hours following NWM

Table I.

Litter Effects on the Ratio of Organ to Body Weight

Litter	Adrenal weight/body weight Mean	SE	Thymus weight/body weight Mean	SE
1	$0.000254^{5,10}$	0.000017	$0.00317^{2,5}$	0.0001
2	$0.000297^{4,8}$	0.000020	$0.00398^{1,3,8}$	0.0001
3	$0.000221^{5,10}$	0.000008	0.00318^{2}	0.0001
4	$0.000178^{2,5,10,11}$	0.000013	0.00372	0.0002
5	$0.000361^{1,3,4,8}$	0.000031	0.00376^{1}	0.0002
8	$0.000183^{2,5,10,11}$	0.000017	0.00328^{2}	0.0001
10	$0.000361^{1,3,4,8}$	0.000022	0.00371	0.0001
11	$0.000285^{4,8}$	0.000003	0.00355	0.0002

Note. N = 9-10 rats/group, superscript numbers represent litters that are significantly different from the reference litter.

testing are shown in Figure 3.4. Because preliminary analysis revealed a significant Sex effect, with a marked elevation in circulating CORT in female rats relative to males in all three cases (baseline: $F(1, 70) = 59.97, p < .05$; immediate: $F(1, 70) = 205.60, p < .05$; 2 hours: $F(1, 70) = 65.48, p < .05$), separate Repeated Measures analyses with Treatment as the grouping variable for each sex were performed. Repeated Measures analysis with baseline, immediate, and 2 hour time points as the Repeated Measures variable failed to reveal significant Treatment effects (males: $F(4, 35) = 0.26, p > .05$; females: ($F(4, 35) = 1.47, p > .05$). However, there was a Repeated Measures Time effect (males: $F(1, 35) = 3.91, p = 0.056$; females: $F(1, 35) = 6.05, p = 0.02$). Subsequent analysis using paired samples t-tests revealed significant elevations in immediate serum CORT compared to baseline and 2 hour CORT concentrations in both males and females (males: $t(39) = -11.42, p < .05$ and $t(39) = 10.63, p < .05$, respectively; females: $t(39) = -11.69, p < .05$ and $t(39) = 12.76, p < .05$, respectively).

During NWM testing, the NIS-L syndrome was found to occur. Separate Chi Square analyses (males and females, individually) for each drug (DOM and KA) were performed on the incidence of the NIS-L syndrome during the NWM testing. A significant number of DOM-treated male and female rats and KA-treated male rats exhibited the NIS-L syndrome compared to saline-treated animals (DOM males: $X^2(24) = 10.15, p < .05$; DOM females: $X^2(24) = 7.20, p < .05$; KA males: $X^2(24) = 4.94, p < .05$ [saline: 0%; DOM males: 68.75%; DOM females: 56.25%; KA males: 43.75%; KA females: 18.75%]). Subsequent Chi Square analysis revealed that the incidence of NIS-L

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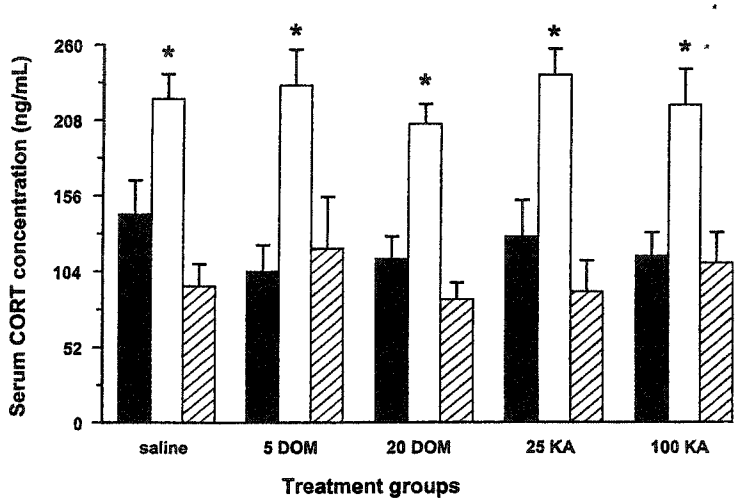

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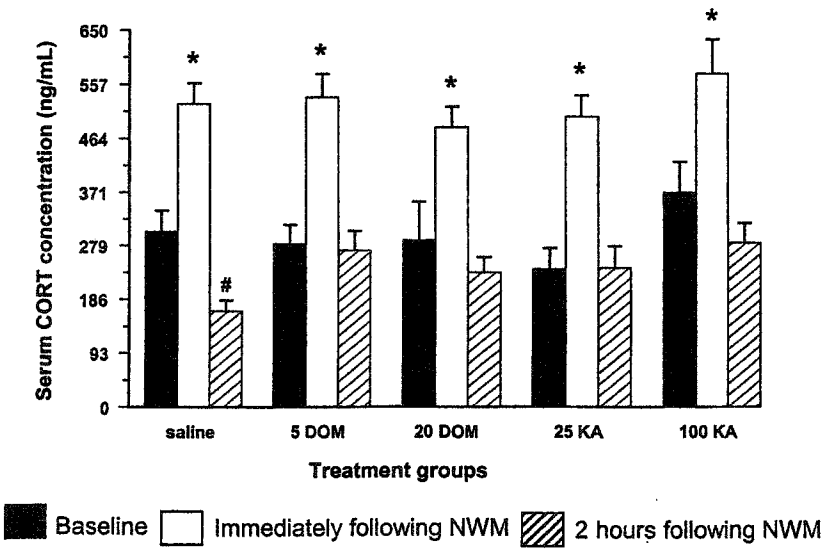

Figure 3.4. Serum CORT concentrations in PND 75 rats. A. Mean (± SEM) serum CORT concentrations in adult male rats (N = 8 rats/test condition). B. Mean (± SEM) serum CORT concentrations in adult female rats (N = 8 rats/test condition). Baseline concentrations and concentrations immediately and 2 hours following NWM testing are represented. * indicates a significant difference from baseline and 2 hours and # denotes a significant difference from baseline only.

in both male and female DOM animals, regardless of dose, was significantly greater than saline while for KA, the significance was found for only the 25μg/kg KA males (Table II and Figure 3.5).

To explore whether the occurrence of the NIS-L syndrome was correlated with CORT concentrations, Point Biserial Correlations were performed. Expression of the NIS-L syndrome was given a score of 1. Failure to manifest this syndrome was given a score of 2. There was a significant correlation revealed for KA-treated females. In this group, as baseline CORT concentrations increased, the expression of the NIS-L syndrome increased. This negative correlation was significant both with and without the inclusion of the saline-treated controls (including saline: $r_{pb}(24) = -0.44$, $p < .05$; excluding saline: $r_{pb}(16) = -0.51, p < .05$).

Serum CORT concentrations of animals expressing the NIS-L syndrome were also compared with both their saline controls and with those rats in the same Treatment condition that did not express the NIS-L syndrome. Repeated Measures analysis failed to reveal significant Treatment effects in male rats. Therefore, males that displayed NIS-L (regardless of Treatment condition) had comparable CORT concentrations to saline controls at all three time points (DOM NIS-L males: $F(1, 17) = 0.08, p > .05$; KA NIS-L males: $F(1, 13) = 0.13, p > .05$). Repeated Measures analysis failed to reveal a significant Treatment effect in DOM-treated female animals that displayed the NIS-L syndrome ($F(1, 15) = 0.14, p > .05$), but there was a significant Treatment effect in KA-treated

Table II.

NIS-L Incidence Following NWM Testing of the First Cohort of Animals Tested for CORT Concentrations

Groups	X^2	% rats exhibiting NIS-L
Males		
5 DOM	5.33*	50
20 DOM	12.44*	87.5
25 KA	7.27*	62.5
100 KA	2.29	25
saline		0
Females		
5 DOM	7.27*	62.5
20 DOM	5.33*	50
25 KA	1.07	12.5
100 KA	2.29	25
saline		0

Note. degrees of freedom (df) = 16 for all groups (*$p < .05$)

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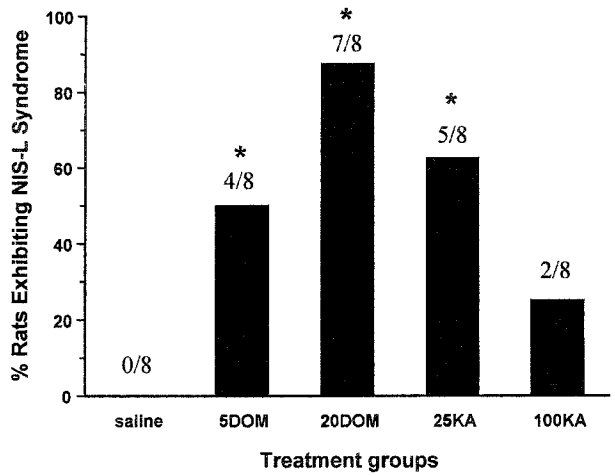

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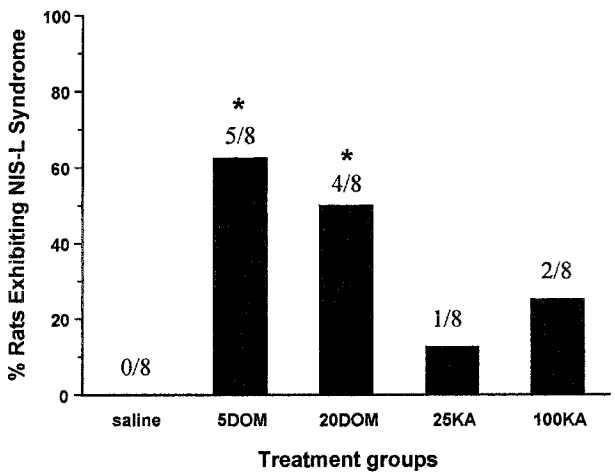

Figure 3.5. Incidence of NIS-L in the first cohort of PND 75 rats. A. Percentage of adult male rats exhibiting NIS-L on the platform of the NWM prior to blood collection for CORT analysis. B. Percentage of adult female rats exhibiting the NIS-L syndrome on the platform of the NWM prior to blood collection for CORT analysis. * denotes a significant difference from saline.

females that displayed the NIS-L syndrome ($F(1, 9) = 10.85, p < .05$). There was a significant elevation of serum CORT concentrations in KA-treated female rats that expressed the NIS-L syndrome compared to saline controls. Repeated Measures analysis (males and females, individually) for each drug (DOM and KA) of animals that displayed the NIS-L syndrome versus their non-NISL expressing counterparts failed to reveal significant Treatment effects (DOM males: $F(1, 14) = 0.26, p > .05$; DOM females: $F(1, 14) = 2.76, p > .05$; KA males: $F(1, 14) = 0.03, p > .05$; KA females: $F(1, 14) = 3.07, p > .05$).

Paired samples t-tests were conducted for each treatment condition to compare CORT levels at all 3 experimental time points. Although CORT concentrations were significantly elevated immediately following NWM testing, levels had returned to baseline values at 2 hours post-testing in both males and females (males: $t(39) = 10.63, p < .05$; females: $t(39) = 12.76, p < .05$) (Figure 3.6). In addition, in most cases, CORT levels at 2 hours dropped slightly below baseline levels. There were no significant differences in any of the male drug treatment groups, however, CORT levels of the saline controls at two hours were notably lower than their baseline levels and this difference approached statistical significance ($t(7) = 2.24, p = 0.06$). CORT concentrations in all groups of female animals were similar with no significant Treatment differences between baseline and 2 h concentrations in any of the treatment groups except for the saline controls, in which two hour CORT concentrations were significantly lower than baseline ($t(7) = 4.33, p < .05$) (Figure 3.6).

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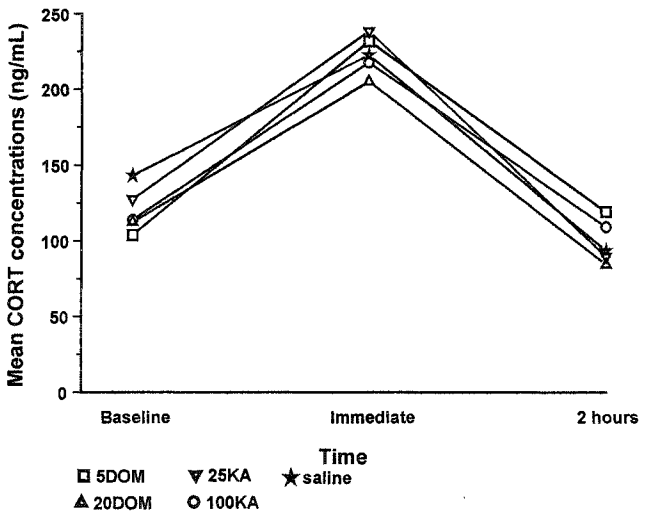

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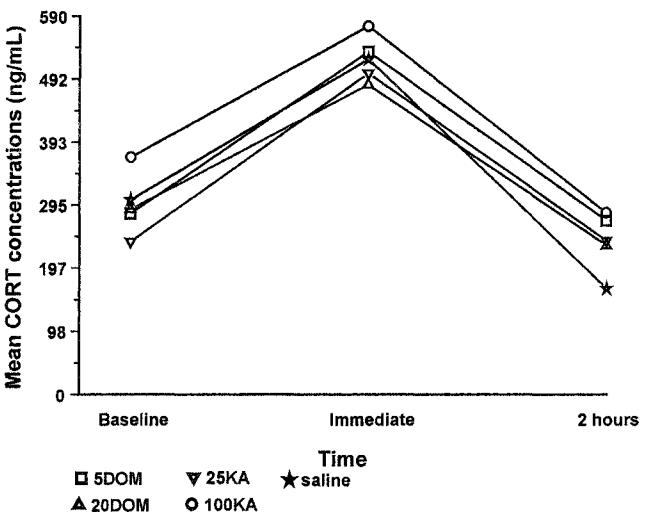

Figure 3.6. Serum CORT concentrations in PND 75 rats over time. A. Mean serum CORT concentrations in adult male rats over the 3 blood collection times (N = 8 rats/test condition). B. Mean serum CORT concentrations in adult female rats over the 3 blood collection times (N = 8 rats/test condition). Baseline concentrations and concentrations immediately and 2 hours following NWM testing are represented.

3.2.3. Adult serum ACTH and behavioural observations

Both baseline serum ACTH concentrations and serum ACTH concentrations immediately following and two hours following NWM testing are shown in Figure 3.7. Because preliminary analysis revealed a significant Sex effect such that there was an elevation in circulating ACTH in female rats relative to males immediately following and two hours following NWM testing (immediate: $F(1, 69) = 16.27, p < .05$; 2 hours: $F(1, 69) = 3.97, p = 0.05$), separate Repeated Measures analyses for each sex with Treatment as the grouping variable were performed. Repeated Measures analysis with baseline, immediate, and 2 hour time points as the Repeated Measures Time variable failed to reveal a significant Treatment effect (males: $F(4, 34) = 1.26, p > .05$; females: $F(4, 35) = 1.04, p > .05$). Analysis using paired samples t-tests revealed significant elevations in serum ACTH concentrations immediately following NWM testing in both males and females (males: $t(38) = -10.35, p < .05$; females: $t(39) = -11.51, p < .05$). When compared with the baseline concentrations of 40.06 ± 2.36 pg/mL in males and 42.02 ± 2.48 pg/mL in females, serum ACTH markedly increased to 65.59 ± 2.30 pg/mL in males and 81.20 ± 3.14 pg/mL in females immediately following NWM testing. There were no Treatment x Sex interactions at any of the 3 time points.

During NWM testing, the NIS-L syndrome was again observed. Separate Chi Square analyses for males and females, individually, and for each drug (DOM and KA) were performed on the incidence of the NIS-L syndrome during NWM testing. A

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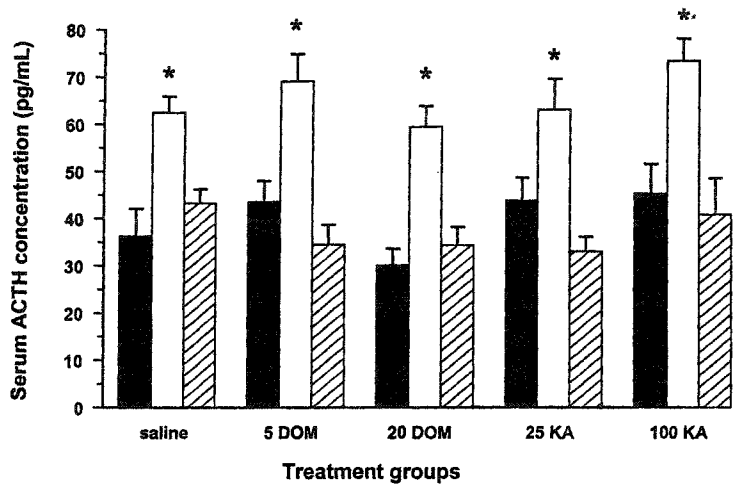

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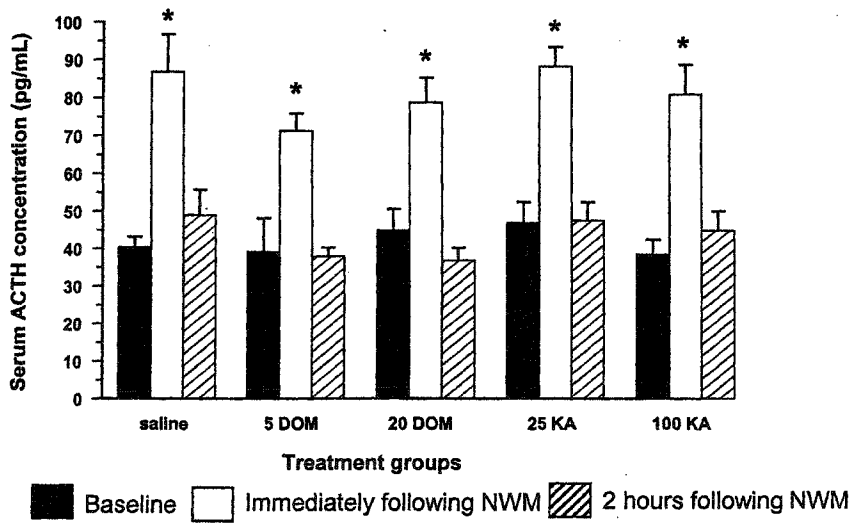

Figure 3.7. Serum ACTH concentrations in PND 75 rats. A. Mean (± SEM) serum ACTH concentrations in adult male rats (N = 7-8 rats/test condition). B. Mean (± SEM) serum ACTH concentrations in adult female rats (N = 8 rats/test condition). Baseline concentrations and concentrations immediately and 2 hours following NWM testing are represented. * indicates a significant difference from baseline and 2 hours.

significant number of DOM- and KA-treated male and female rats exhibited the NIS-L syndrome compared to their saline-treated controls (DOM males: $X^2(23) = 13.38, p < .05$; DOM females: $X^2(24) = 10.15, p < .05$; KA males: $X^2(24) = 10.15, p < .05$; KA females: $X^2(24) = 6.00, p < .05$ [saline: 0%; DOM males: 80%; DOM females: 68.75%; KA males: 68.75%; KA females: 50%]). Subsequent Chi Square analysis revealed the incidence of NIS-L in DOM- and KA-treated male and DOM-treated female animals regardless of dose was significantly greater than saline. NIS-L incidence of the 25μg/kg KA females was also significantly greater than saline (Table III and Figure 3.8).

To explore whether the occurrence of the NIS-L syndrome was correlated with ACTH concentrations, Point Biserial Correlations were performed. Expression of the NIS-L syndrome was given a score of 1 and failure to manifest this syndrome was given a score of 2. No significant correlations were found at any of the three time points tested in any of the Treatment groups (refer to Appendix A for all r_{pb} values).

Serum ACTH concentrations of animals expressing the NIS-L syndrome were also compared with both their saline controls and with those rats in the same Treatment condition that did not express the NIS-L syndrome. Repeated Measures analysis failed to reveal significant Treatment effects in either male or female rats. Therefore, males and females that displayed NIS-L (regardless of Treatment condition) had comparable ACTH concentrations to saline controls at all three time points (DOM NIS-L males: $F(1, 18) =$

Table III.

NIS-L Incidence Following NWM Testing of the Second Cohort of Animals Tested for ACTH Concentrations

Groups	X^2	% rats exhibiting NIS-L
Males		
5 DOM	9.60*	75
20 DOM**	10.50*	86
25 KA	7.27*	62.5
100 KA	9.60*	75
saline		0
Females		
5 DOM	7.27*	62.5
20 DOM	9.60*	75
25 KA	7.27*	62.5
100 KA	3.69	37.5
saline		0

Note. df = 16 for all groups, except for **, where df = 14 (*$p < .05$)

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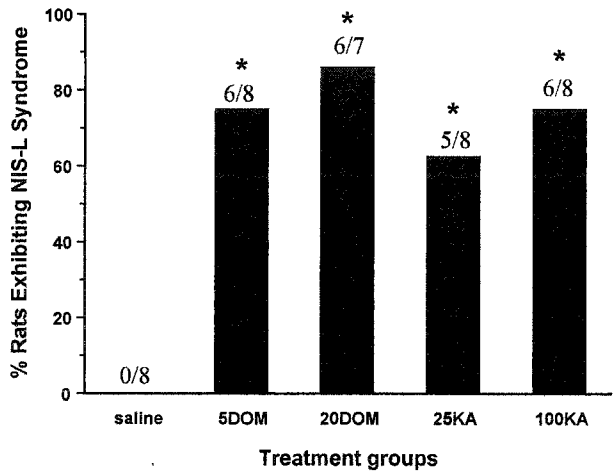

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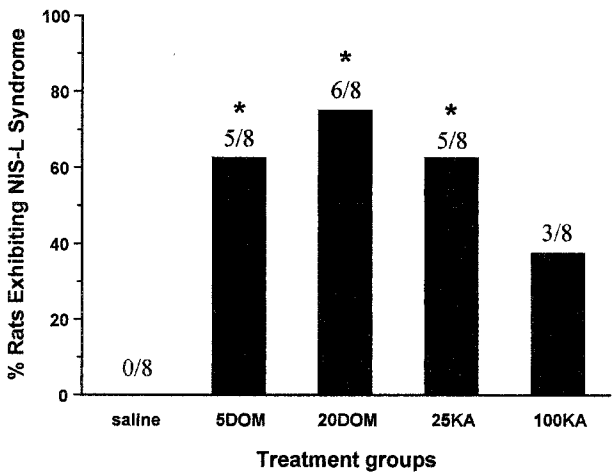

Figure 3.8. Incidence of NIS-L in the second cohort of PND 75 rats. A. Percentage of adult male rats exhibiting NIS-L on the platform of the NWM prior to blood collection for ACTH analysis. B. Percentage of adult female rats exhibiting the NIS-L syndrome on the platform of the NWM prior to blood collection for ACTH analysis. * denotes a significant difference from saline.

0.17, $p > .05$; DOM NIS-L females: $F(1, 17) = 1.30$, $p > .05$; KA NIS-L males: $F(1, 17) = 0.06$, $p > .05$; KA NIS-L females: $F(1, 14) = 0.12$, $p > .05$). Also, Repeated Measures analysis (males and females, individually) for each drug (DOM and KA) of animals that displayed the NIS-L syndrome versus their non-NISL expressing counterparts failed to reveal significant Treatment effects (DOM males: $F(1, 13) = 0.07$, $p > .05$; DOM females: $F(1, 14) = 0.02$, $p > .05$; KA males: $F(1, 14) = 0.24$, $p > .05$; KA females: $F(1, 14) = 1.05$, $p > .05$).

Paired samples t-tests were conducted for each treatment condition to compare ACTH levels at all 3 experimental time points. Although ACTH concentrations were markedly elevated immediately following NWM testing, levels had returned to baseline values at 2 hours post-testing in both males and females (males: $t(38) = 10.96$, $p < .05$; females: $t(39) = 13.50$, $p < .05$) (Figure 3.9). Serum ACTH concentrations at 2 hours post-NWM testing were not significantly different from baseline levels in either males or females (males: $t(38) = 1.02$, $p > .05$; females: $t(39) = -0.41$, $p > .05$).

3.2.4. Adult serum oxytocin

Oxytocin concentrations of the adult rats group were unable to be determined as 32.5% of the samples were below the level of detection of the assay. Therefore, it was supposed that either a methodological error or problem with transportation of the assay kits had occurred. As a result, oxytocin concentrations immediately and 2 hours

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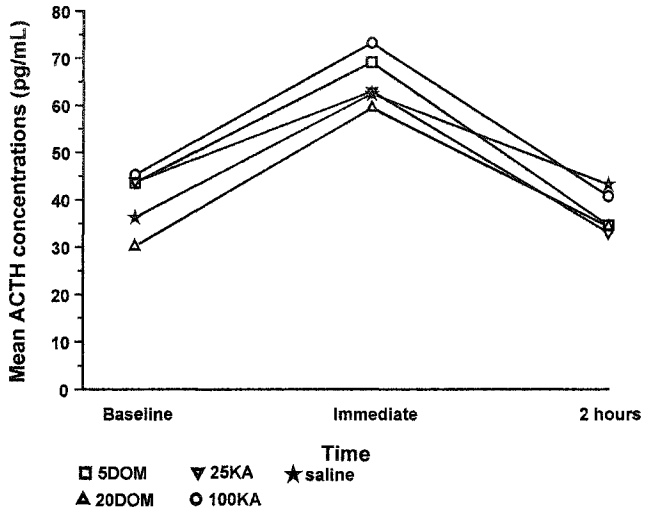

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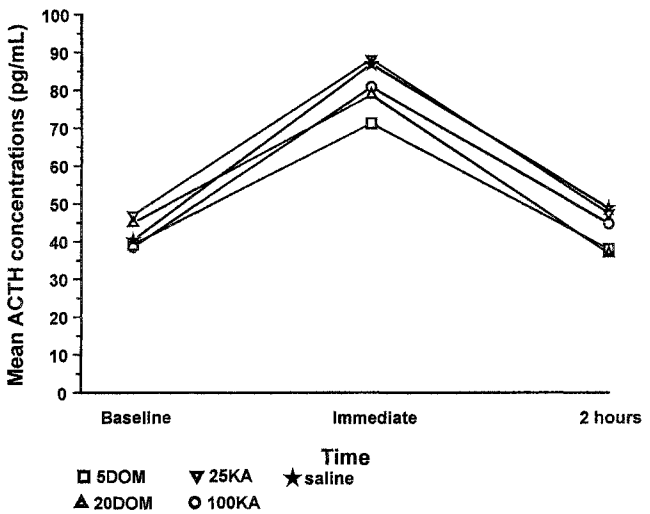

Figure 3.9. Serum ACTH concentrations in PND 75 rats over time. A. Mean serum ACTH concentrations in adult male rats over the 3 blood collection times (N = 7-8 rats/test condition). B. Mean serum ACTH concentrations in adult female rats over the 3 blood collection times (N = 8 rats/test condition). Baseline concentrations and concentrations immediately and 2 hours following NWM testing are represented.

following NWM exposure were not analysed. Refer to Appendix C for the raw data.

3.3. Aged rats experiment

3.3.1. Serum corticosterone and behavioural observations

Both baseline serum CORT concentrations and serum CORT concentrations immediately following NWM testing are shown in Figure 3.10. Preliminary analysis revealed a significant Sex effect, such that there was a marked elevation in both baseline and post-NWM circulating CORT concentrations in female rats, relative to male rats (baseline: $F(1, 30) = 30.56, p < .05$; immediate: $F(1, 29) = 87.06, p < .05$). Therefore, separate Repeated Measures analyses for each sex, with Treatment as the grouping variable, were performed. Repeated Measures analysis with baseline, immediate, and 2 hour time points as the Repeated Measures variable failed to reveal significant Treatment effects (males: $F(1, 15) = 0.71, p > .05$; females: $F(1, 14) = 0.16, p > .05$). However, there was a significant Repeated Measures Time effect (males: $F(1, 15) = 11.39, p < .05$; females: $F(1, 14) = 7.58, p < .05$). Subsequent analysis using paired samples t-tests revealed significant elevations in post-NWM serum CORT concentrations compared to baseline in both males and females (males: $t(16) = -3.26, p < .05$; females: $t(15) = -2.85$, $p < .05$). When compared with the baseline concentrations of 137.41 ± 13.59 ng/mL in males and 352.41 ± 35.32 ng/mL in females, serum corticosterone increased significantly to 195.12 ± 9.62 ng/mL in males and 463.56 ± 26.87 ng/mL in females immediately

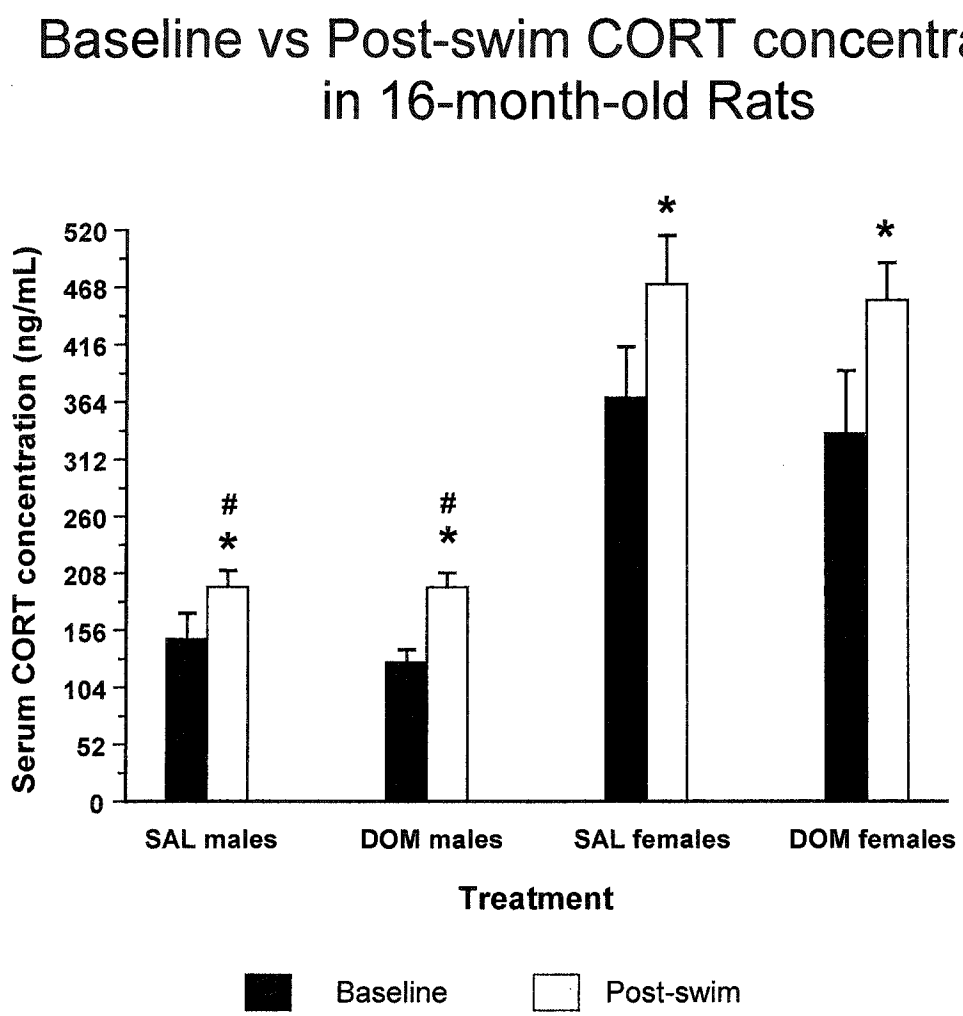

Figure 3.10. Mean (± SEM) serum corticosterone concentrations in aged rats prior to and immediately following NWM testing (N = 8-9 rats/test condition). * indicates a significant difference from baseline and # denotes a significant sex difference.

following testing in the NWM. There were no significant Treatment x Sex interactions.

During NWM testing, the NIS-L syndrome was observed. Separate Chi Square analyses for males and females were performed on the incidence of the NIS-L syndrome during NWM testing. A significant number of both male and female drug-treated animals exhibited the NIS-L syndrome in the NWM compared to saline-treated animals (males: $X^2(1) = 5.89, p < .05$ [saline: 0%; 20 DOM: 50%], females: $X^2(1) = 5.89, p < .05$ [saline: 0%; 20 DOM: 50%]) (Figure 3.11). However, subsequent Repeated Measures comparisons of CORT concentrations of male and female "NIS-L syndrome positive" rats compared with saline controls were performed to determine if including the rats that did not express the NIS-L syndrome in the analysis may have masked any Treatment effect on CORT concentrations. No significant Treatment differences for either male or female "NIS-L syndrome positive" rats were revealed (males: $F(1, 11) = 1.17, p > .05$; females: $F(1, 10) = 0.62, p > .05$). Repeated Measures analysis also failed to reveal a significant Treatment effect of serum CORT concentrations of male and female "NIS-L syndrome positive" rats and their non-NIS-L expressing counterparts (males: $F(1, 6) = 1.28, p > .05$; females: $F(1, 6) = 0.60, p > .05$). CORT concentrations of either sex were not significantly correlated with the expression of the NIS-L syndrome (males: $r_{pb}(17) = 0.18, p > .05$; females: $r_{pb}(16) = 0.31, p > .05$).

There was also no relationship between the latency to find the platform on the 6th trial in the maze and CORT concentrations for either males or females, indicating a

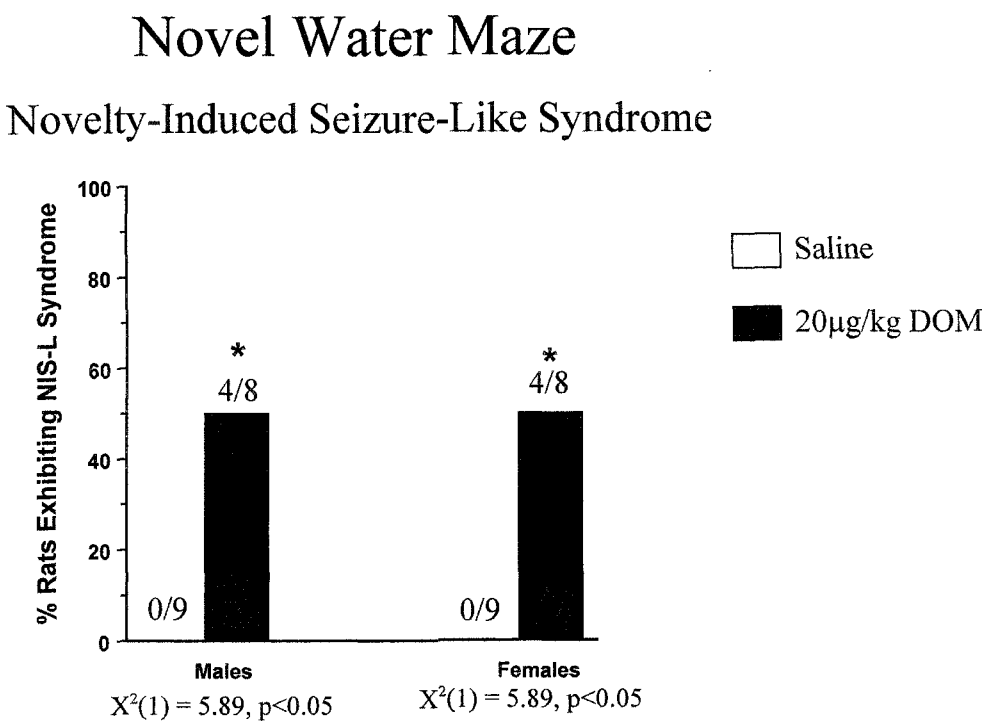

Figure 3.11. Percentage of aged male and female rats exhibiting the novelty-induced seizure-like syndrome on the platform of the NWM. * denotes a significant difference from saline.

dissociation between alterations in CORT concentration and spacial mapping ability.

3.3.2. Serum ACTH

Serum ACTH concentrations in trunk blood obtained from 16-month-old rats are presented in Figure 3.12. There were no significant Treatment or Sex differences found in serum ACTH concentrations (Treatment: $F(1, 27) = 0.73, p > .05$; Sex: $F(1, 27) = 3.14, p > .05$). Also, ACTH concentrations were not found to be significantly correlated with the expression of the NIS-L syndrome.

3.3.3. Serum oxytocin and prolactin

Baseline serum oxytocin concentrations of the aged rats are presented in Figure 3.13. There was a significant main effect of Treatment on serum oxytocin concentrations ($F(1, 28) = 15.41, p < .05$). A significant elevation in circulating oxytocin was found in DOM-treated rats relative to saline controls. However, oxytocin concentrations of both males and females were found to be comparable and there was no significant Treatment x Sex interaction. In addition, Point Biserial Correlation revealed a significant correlation between the expression of NIS-L and oxytocin levels ($r_{pb}(30) = 0.341, p < .05$). As oxytocin levels increased, incidence of NIS-L increased. There was, however, no significant correlation between oxytocin concentration and the expression of NIS-L when saline rats were excluded.

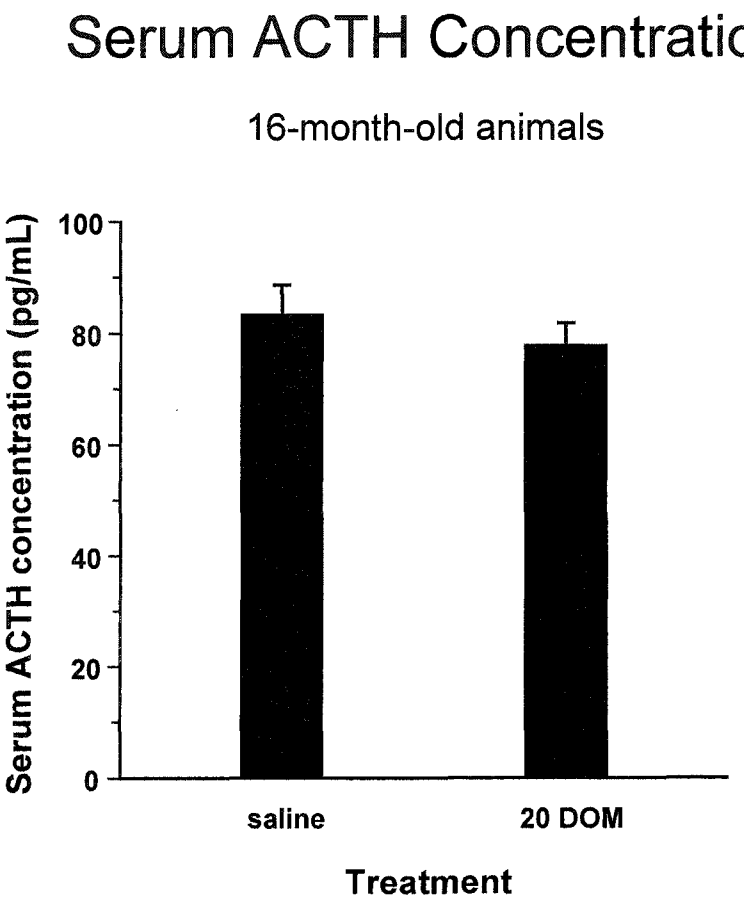

Figure 3.12. Mean (± SEM) baseline serum ACTH concentrations in aged rats (N = 8-9 rats/test condition).

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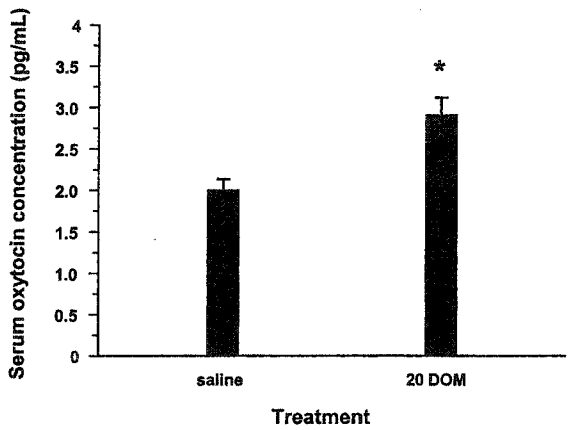

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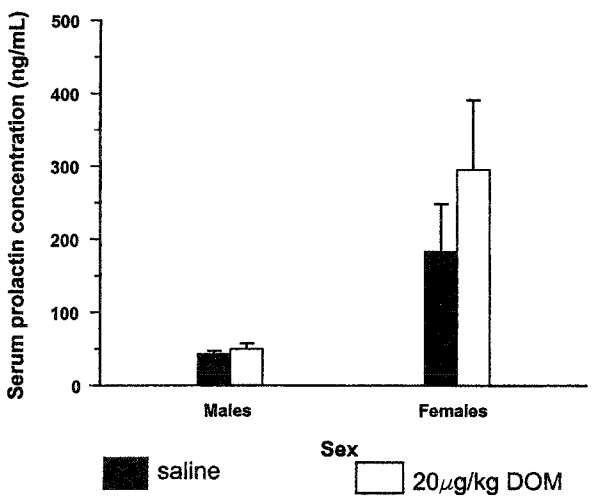

Figure 3.13. Baseline serum oxytocin and prolactin concentrations in PND 480 rats. A. Mean (± SEM) baseline serum oxytocin concentrations in aged rats (N = 8-9 rats/test condition). B. Mean (± SEM) baseline serum prolactin concentrations in aged rats (N = 8-9 rats/test condition). * indicates a significant difference from saline.

Baseline serum prolactin concentrations of the aged rats are presented in Figure 3.13. There was no significant Treatment effect on serum prolactin concentrations ($F(1, 28) = 0.96, p > .05$). However, a significant Sex effect was revealed ($F(1, 28) = 10.09, p < .05$). Females had significantly higher serum prolactin concentrations than did males. There was no Treatment x Sex interaction. Because of the substantial differences in prolactin levels, separate Point Biserial Correlations were conducted for males and females to determine whether prolactin levels were correlated with the NIS-L syndrome. There were no significant correlations found for either sex.

3.3.4. Tail flick test and open field test

As shown in Figure 3.14, Repeated Measures analysis failed to reveal a significant Treatment effect on tail flick latencies ($F(1, 29) = 0.40, p > .05$). However, there was a significant Sex effect ($F(1, 29) = 6.93, p < .05$). Males had significantly longer tail flick latencies that females. Also, there were no significant Treatment x Sex interactions. The NIS-L syndrome was observed during open field testing. Separate Chi Square analyses for males and females were performed on the incidence of the NIS-L syndrome during open field testing. Fifty percent of DOM-treated males exhibited the NIS-L syndrome while in the open field ($X^2(1) = 5.89), p < .05$), while none of the saline-treated males displayed the syndrome (Figure 3.15). No female rats in either group exhibited the NIS-L syndrome in the open field (Figure 3.15).

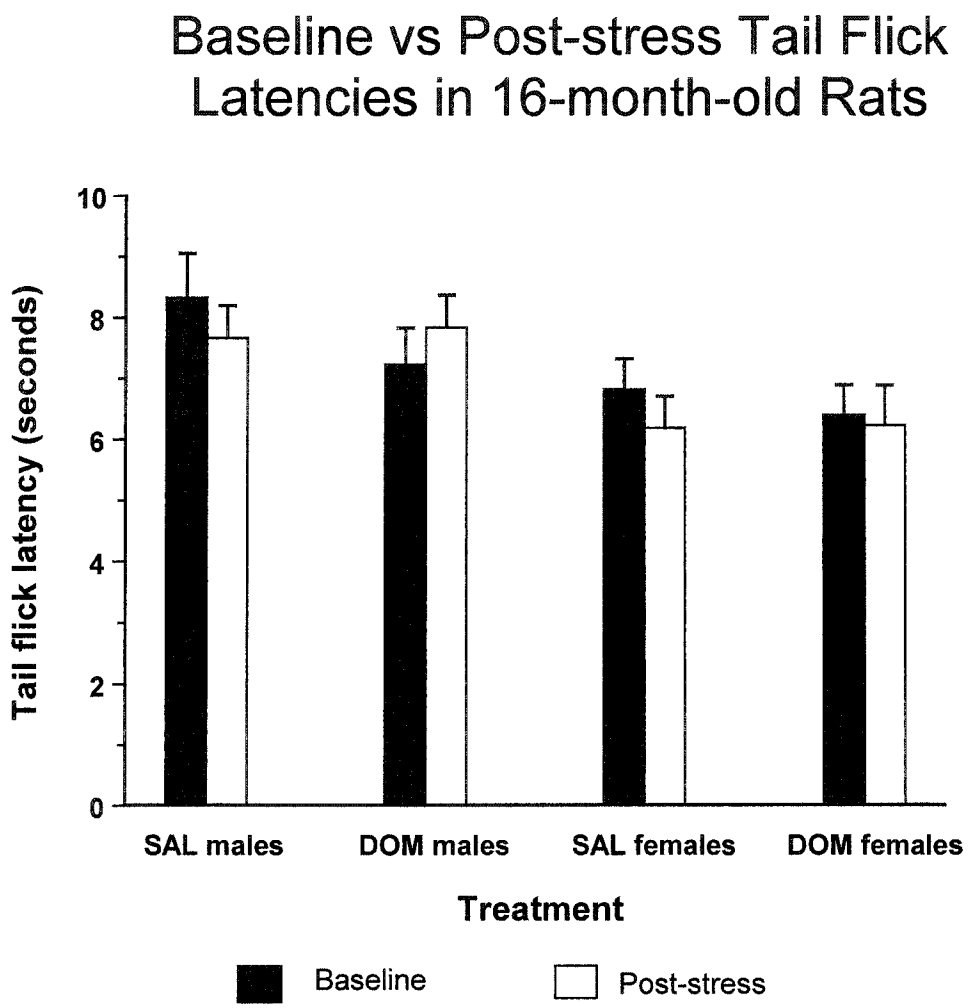

Figure 3.14. Mean (± SEM) latencies of aged rats to tail flick in a water bath maintained at 50°C before and after a 10 minute trial in the open field (N = 8-9 animals/treatment condition).

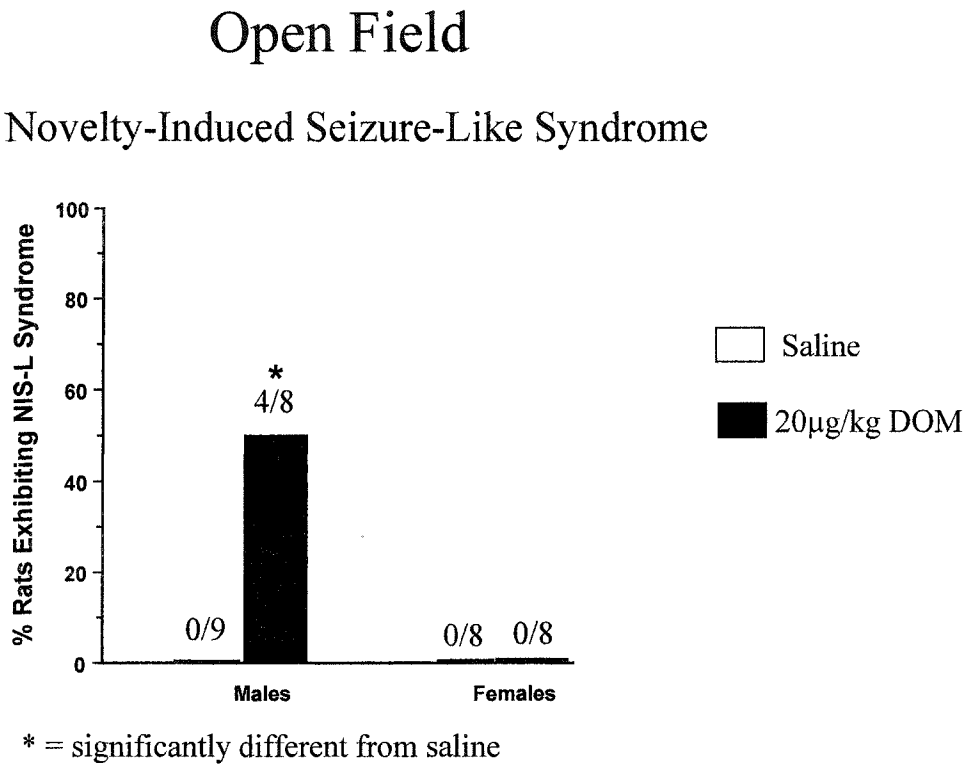

Figure 3.15. Percentage of aged male and female rats exhibiting the novelty-induced seizure-like syndrome in the open field. * denotes a significant difference from saline.

3.3.5. Gland weights

There were no significant differences between Treatment groups on either absolute or gland to body ratio pituitary and adrenal weights in 16-month-old rats (absolute pituitary: ($F(1, 28) = 0.32, p > .05$; absolute adrenal: $F(1, 28) = 1.92, p > .05$; ratio pituitary: ($F(1, 28) = 0.33, p > .05$; ratio adrenal: $F(1, 28) = 1.43, p > .05$). However, females had significantly larger pituitary glands and adrenal glands than did male rats (absolute pituitary: $F(1, 28) = 4.47, p = 0.04$; absolute adrenals: $F(1, 28) = 10.69, p = 0.003$; ratio pituitary: $F(1, 28) = 8.40, p = 0.007$; ratio adrenals: $F(1, 28) = 16.93, p = 0.000$). There were no significant Treatment x Sex interactions for any of the glands.

4.0. DISCUSSION

The primary aim of this thesis was to determine if perinatal administration of low levels of DOM and KA caused changes in neuroendocrine function as measured by serum CORT and ACTH and to determine if this was an explanation for NIS-L. The results obtained confirm that administration of the drug during this critical period of development results in a change that manifests as the NIS-L syndrome. However, the findings also clearly show no evidence that low levels of these compounds caused an alteration in neuroendocrine function as measured by serum CORT and ACTH.

4.1. Methodological considerations

Although drug treatment did not significantly alter CORT concentrations, absolute gland weight, or ratio of gland to body weight of the adrenal and thymus glands of PND14 rats, these measures were significantly different for rats in different litters. Several studies have shown that variations in maternal care, particularly with regard to frequency of licking and grooming, affect the development of play and open field behaviour as well as behavioural and neuroendocrine responses to stress (Moore & Power, 1992; Liu et al., 1997; Champagne et al., 2003). There are substantial naturally occurring differences in maternal licking and grooming in rat dams (Caldji et al., 2000). Adult offspring of dams that exhibited more licking and grooming of pups showed reduced ACTH and CORT responses to acute restraint stress, enhanced glucocorticoid

negative feedback sensitivity, and decreased levels of hypothalamic CRH messenger mRNA (Liu et al., 1997). The results of different CORT levels and adrenal and thymus weights of the individual litters shown in the current study, as well as findings in other studies (Wigger & Neumann, 1999; Lehmann et al., 2002), clearly show that maternal factors other than nutrition can influence development. These significant litter effects indicate the importance of having all drug groups represented within a litter, where possible, rather than designating one particular litter as one particular drug treatment. If each litter was composed of only one drug group, a confound of maternal regulation would be introduced into the study. By having all drug groups represented in all of the litters, variations in maternal care will be evenly distributed across all treatment conditions.

4.2. Neonatal rats experiment

Earlier eye opening was not seen in DOM- and KA-treated neonatal rats as had been reported in earlier work in our lab (Doucette et al., 2003). On PND 14, the day of sacrifice, not all rats had their eyes open, therefore, the eye opening measure was truncated. This might have been the reason that earlier eye opening in these rats was not found.

Serum CORT concentrations of the different litters were correlated with both thymus and adrenal gland weights in order to determine if there was a relationship

between CORT levels and organ weights. A significant negative correlation was found, indicating that there was an inverse relationship between serum CORT levels and adrenal gland weight, i.e. litters with high serum CORT concentrations had lower adrenal gland weights. One possible explanation for this finding is that the medulla and zona glomerulosa may be developing more slowly (or atrophied), accounting for lower adrenal weights. Conversly, the zonas fasciculata and reticularis (which secrete CORT) may be more fully developed. If this occurred, CORT could still be secreted even though the overall weight of the glands decreased.

Daily injections from PND8-14 of low doses of DOM and KA did not produce an alteration in serum CORT concentrations on PND14. Blood samples were collected half an hour following the final drug administration, therefore, any short or long-term effects of the drugs should still have been apparent. Neonatal CORT concentrations in this study were comparable to those found in other studies (see Chautard et al., 1993; Kent et al., 1996). However, both Chautard et al. (1993) and Kent et al. (1996) showed a significant increase in CORT following an acute injection of KA (2.5mg/kg or 0.75μg/g) during the stress hypo-responsive period. Administration of lower doses of KA did not elicit an HPA response in their study. The doses used in the present study were presumably not high enough to evoke an acute CORT response, and repetitive injections from PND 8-14 did not result in a treatment effect. This effectively rules out early increases in serum CORT as the mechanism responsible for later effects in drug-treated animals.

4.3. Adult rats experiment

In contrast to an earlier study by Doucette et al. (2003), the current study did not find earlier eye opening in the DOM- and KA-treated rat pups. One possible explanation for this discrepancy is that in the earlier study, the animals experienced increased handling. It was a neurotoxicological study involving extensive daily testing and handling. In the current study, the animals were not handled as frequently. In rats, handling can increase CORT levels and elevated CORT concentrations can enhance CNS maturation (Vazquez, 1998). Therefore, a possible handling by treatment interaction might have been the reason for the earlier eye opening seen in drug-treated rat pups in the earlier study.

The results of the experiments on 75-day-old rats indicate that daily injections of low doses of DOM and KA from PND 8-14 did not alter neuroendocrine function with regard to serum ACTH and CORT secretion of animals that had just entered adulthood. While serum ACTH and CORT levels were not altered, adult rats still manifested the NIS-L syndrome, suggesting a dissociation between both ACTH and NIS-L and CORT and NIS-L. Importantly, there were no significant Treatment effects on physical development. Therefore, although the drugs did not produce any overt toxic effects on the rats, these compounds were behaviourally active as there was evidence of later effects.

Corticosterone is able to bind to both GRs and MRs (Brown, 1994). These

receptors have different, and sometimes opposing, roles in a wide variety of functions. For example, optimal activation of hippocampal GRs facilitate memory consolidation whereas MR activation is involved in the modulation of attentional processes and behavioural reactivity (Kellendonk et al., 2002). There is a possibility that both GRs and MRs could be increased in the drug-treated rats. However, there is no evidence of any impact on memory in the drug-treated rats as all treatment groups performed similarly in the NWM test. There is evidence of changes in behavioural reactivity in the form of the NIS-L syndrome, which suggests a change in MRs. Animals exposed to an acute novelty stress exhibit higher hippocampal MR expression and this alteration in MR concentration is involved in changes in coping strategies and behavioural reactivity to a novel situation (Gesing et al., 2001). If receptor expression was changed in these drug-treated animals that express the NIS-L syndrome, altered sensitivity to stress hormones would result. This hypothesis could be tested by administering a MR antagonist to determine whether the NIS-L syndrome would still be exhibited by drug-treated rats. A change in MR expression may not be the cause of the behaviours, but may, in part, explain why novelty is a trigger of the NIS-L syndrome.

Blood samples were collected both immediately and two hours following NWM testing to determine whether there was an alteration in the hormonal peak following a water maze stressor. Also, two hour samples were collected to determine whether there was a difference in the rate of return of hormone concentrations to baseline in drug-treated rats. This was considered likely because of previous reports that early activation

of the HPA axis can produce a HPA axis in the resulting adult that exhibits higher and prolonged ACTH and CORT concentrations in response to stressors (Sapolsky & Meaney, 1986). Both serum ACTH and CORT concentrations of all the treatment groups were restored to baseline levels by two hours following NWM testing. This is consistent with literature reports that ACTH is elevated 3-5 minutes after the onset of the stress and returns to basal concentrations 30 minutes following a stressor while CORT concentrations peak at 15-30 minutes and return to baseline within 60-90 minutes (de Souza & van Loon, 1982; Kant et al., 1987; Rittenhouse et al., 2002). Interestingly, in most cases, hormone levels at two hours were slightly below basal concentrations. It is unlikely that this is due to circadian fluctuation as all blood samples were collected within a period of relative hormone stability. This finding does suggest that baseline hormone concentrations might not have actually been "baseline" but a weak stress response to handling. Although animals were habituated to handling and the blood collection procedure prior to any experimental manipulation, they might still have been somewhat affected by the stress of handling. Habituation may involve decreased sensitivity to the stressor, a decreased affective response to the same degree of stimulation, or an altered biochemical response to a repeated stimulus (Kant et al., 1987). Unlike for the baseline blood sampling, at the time of the two hour blood sample collection, animals had been previously handled several times and may have habituated to the handling procedure, thereby experiencing decreased anxiety to a familiar stimulus, and this could account for lower hormone concentrations than baseline samples. An alternate explanation for lower CORT and ACTH concentrations at 2 hours post-stressor than baseline concentrations is

that the system might be exhausted. However, this is unlikely because exposure to the NWM did not elicit the greatest possible hormonal response. Maximal levels of CORT and ACTH were not reached following the stressor as several studies using various stressors have reported levels of CORT 30-45% higher and levels of ACTH 50-60% higher than those in the current study (Dunn et al., 1972; Kant et al., 1987; Pardon et al., 2002). Therefore, it is improbable that the system was exhausted and not capable of producing normal baseline hormone concentrations.

The 75-day-old (adult rats) group was significantly stressed due to NWM testing. Both male and female rats had significantly elevated serum CORT and ACTH concentrations when sampled immediately after testing. Therefore, this was verification of the NWM as a paradigm that was able to elicit a stress response. There were also robust sex differences. Females had significantly higher ACTH and CORT concentrations than males both immediately and two hours following NWM testing and significantly higher baseline CORT concentrations. Baseline ACTH concentrations appeared higher in females than males, but this difference was not significant. Females also showed a greater ACTH response than males immediately following NWM testing. This is consistent with past findings in the literature which also report a higher release of ACTH in females than males following stress (Le Mevel et al., 1979; Jezova et al., 1996). Sex differences in HPA axis functioning have been shown to be due to the effect of sex steroids (particularly estrogen) (Lesniewska et al., 1990). Vamvakopoulos & Chrousos (1993) found that estrogen has direct stimulatory effects on CRH gene expression because

of estrogen-responsive elements in the promoter area of the CRH gene. Therefore, CRH neurons are a target of ovarian steroids and this may, in part, mediate sex differences in the stress response.

Hormone concentrations of animals expressing the NIS-L syndrome were compared to saline controls to determine whether experiencing the behavioural manifestations of the syndrome was associated with an alteration in hormone secretion. While hormone concentrations of male rats that displayed NIS-L were found to be comparable to those of saline rats, CORT concentrations of female rats that displayed the NIS-L syndrome and had received either 25μg/kg KA or 100μg/kg KA were significantly higher than those of saline controls. Also, baseline CORT concentrations of KA-treated females were significantly correlated with the expression of NIS-L such that the higher the CORT concentration, the higher the probability of expressing the NIS-L syndrome. However, neither CORT nor ACTH concentrations of any other treatment group were correlated with NIS-L. NIS-L is scored as an "all-or-none" phenomenon. Various degrees of the NIS-L syndrome are not scored because the rat either demonstrates the behaviours or does not. There was a small number of females that expressed the syndrome in these two drug conditions (25μg/kg KA females: N=1; 100μg/kg KA females: N=2). It is difficult to draw conclusions from data of only one or two animals. If more animals in these two drug treatments had shown NIS-L, the results may have been different.

Fewer animals that received the higher of the "low" doses of KA (100μg/kg) expressed the NIS-L syndrome than animals in any other treatment condition. Past findings have shown that responses to DOM and KA are not always identical at equitoxic doses (Strain & Tasker, 1991). Also, at low doses, DOM has a higher affinity for "low affinity" KA receptors composed of receptor subunits GluR5-7 while KA is more selective for "high affinity" KA receptors composed of receptor subunits KA1 and KA2 (Verdoorn et al., 1994; Hampson & Manalo, 1998). Therefore, DOM and KA may be targeting different receptor subtypes and may not have had identical actions in these animals, resulting in the differential expression of the NIS-L syndrome.

4.4. Aged rats experiment

The results obtained in 16-month-old rats indicate that neonatal drug treatment with low doses of DOM and KA from PND 8-14 does not permanently alter standard indicators of the stress response. This is true both at the levels of the pituitary gland and adrenal gland regardless of either absolute or proportion of organ weight and their hormone release. Interestingly, while serum CORT and ACTH concentrations were not different between saline and drug-treated rats, DOM-treated rats still manifested the NIS-L syndrome, indicating a dissociation between increased CORT and ACTH and the NIS-L syndrome. In addition, baseline PRL concentrations were not altered, however, there was a significant elevation in oxytocin of the DOM-treated rats compared to saline controls one month following NWM testing. This oxytocin elevation was positively

correlated with NIS-L incidence.

While not correlated with the production of the NIS-L syndrome, both male and female rats had significant elevations of CORT after testing in the NWM maze, clearly indicating that the animals were significantly stressed. This was important since had the animals not been stressed, any differences in hormone concentrations of animals expressing NIS-L could not be attributed to an exaggerated stress response. However, there were no significant differences between the saline animals and the drug-treated animals both in baseline CORT levels or concentrations following the moderate stress of the NWM. Females had significantly higher CORT concentrations than males, which is consistent with findings in the literature (Beiko et al., 2004).

The Novel Water Maze was used as the novel stressor because past studies using this maze have shown that drug-treated rats exhibit the NIS-L syndrome while on the platform between trials (Doucette et al., 2004). The open field was used as another novel stressor that was presumed to be less intense than the NWM and did not include the physical stress of swimming. A higher percentage of drug-treated rats displayed the NIS-L syndrome on the platform of the NWM than in the open field. Interestingly, it was only the male DOM-treated rats that exhibited NIS-L in the open field. All of the animals identified as exhibiting the NIS-L syndrome in both the NWM and the open field were drug-treated rats, that is to say, there were no false positives. This is consistent with our previous data (Doucette et al., 2004) and confirms that perinatal DOM causes NIS-L.

The tail flick test was used in these studies to determine if there was evidence of a stress-induced analgesic response. Rationale for the use of this test stems from the physiological mechanisms involved. When an animal is stressed, beta-endorphins are released from the pituitary. Beta-endorphins are involved in analgesia (Mooring et al., 1985). Therefore, a difference in tail flick latencies may indicate that a pituitary dysfunction in beta-endorphin secretion has occurred. If the animal is stressed, it should show an increased tail flick latency since there is an increase in secretion of beta-endorphins, thus suppressing the discomfort experienced by having the tail dipped in the hot water bath. Control of the noxious stimuli is important in stress-induced analgesia. Rats subjected to inescapable shocks show long-term analgesia whereas when they are able to escape the shocks, analgesia does not develop (Maier, 1986). Also, the stressors typically used to produce stress-induced analgesia are quite severe (Maier, 1986; Vidal & Jacob, 1986), therefore, it was not expected to be found in this study unless there was some permanent change in HPA axis sensitivity. Indeed, there were no significant differences in baseline tail flick latencies as compared to latencies following a 10 minute open field exposure and there were no significant drug treatment effects on tail flick latencies. This finding suggests that there was no change in analgesic response.

4.5. Alternative explanations for the NIS-L syndrome

Expression of the NIS-L syndrome does not appear to be due to an enhanced stress response of the animals to the novelty of the mazes as measured by serum ACTH

and CORT. One alternative explanation for the behavioural manifestations of the NIS-L syndrome is that it may be a low-level seizure. The behaviours observed in this syndrome are similar to those seen in an animal experiencing a Stage 2 seizure (Doucette et al., 2004). Facial clonus, head nodding, and mild forelimb clonus, all part of the NIS-L syndrome, are typical manifestations of Stage 2 seizures (Racine, 1972). In addition, in a recent study in our laboratory, in situ hybridization revealed marked elevations in brain-derived neurotropic factor (BDNF) mRNA in the CA1 and dentate gyrus regions of the hippocampus without corresponding increases in NPY mRNA in rats treated with 20μg/kg DOM (Doucette et al., 2004). These markers are also observed in human patients with epilepsy (Takahashi et al., 1999). In particular, BDNF is highly expressed in brain areas thought to be involved in limbic seizures and has been shown to have neuroprotective effects (Zhu & Roper, 2001). These drug-treated rats also displayed a significant elevation of baseline oxytocin (Doucette et al., 2004). It has been shown that following generalized seizures induced by kainic acid administration, there is a selective activation of oxytocin-containing neurons in the PVN which results in a significant and long-lasting increase in oxytocin mRNA (Peikut et al., 1996; Sun et al., 1996). Rats treated neonatally with DOM and KA showed an increase in serum oxytocin one month following NWM testing. That neurochemical correlate, taken together with increased BDNF mRNA expression in the absence of an increase in NPY, provide some evidence that the NIS-L syndrome may be a low-level seizure. To further strengthen this hypothesis, drug-treated rats are currently being tested for alterations in mossy fiber sprouting. Abnormal mossy fiber sprouting is one of the best anatomical correlates of

animal models of epilepsy (Holmes, 1991). Therefore, any differences found will give further insight into whether or not the collection of behaviours that make up the NIS-L syndrome is truly a seizure.

Other studies have found increases in plasma ACTH and CORT five minutes following the induction of seizures and elevation of these hormone levels for a minimum of 60 minutes (Young et al., 1990; Daniels et al., 1990). Serum ACTH and CORT elevation relating to drug treatment was not found in the current study, however, there are several explanations to account for these findings. First, the stressor used to elicit NIS-L was milder and of a different nature than the methods used to elicit seizures in other studies. Young et al. (1990) administered electrically-induced seizures to animals via an ear clip, resulting in a 30 second seizure. Daniels et al. (1990) used a single dose of 1mg/mL KA injected into the CA1 region of the left dorsal hippocampus to induce a seizure. Rats exhibited seizures within 10 minutes of receiving the KA injection. The novel environments that precipitated the NIS-L syndrome are more representative of a situation that humans and animals are likely to encounter on a daily basis. This type of stimulus may also induce seizures of a different nature than those produced by electrical or chemical stimuli.

Secondly, the NIS-L syndrome is more characteristic of a low-level seizure than status epilepticus. In the study by Daniels et al. (1990), animals experienced behavioural changes typical of grand mal seizures including hyperactivity, wet dog shakes, and

rotatory movement. These behaviours were not seen in NIS-L animals, however, they did demonstrate chewing, head bobbing, and forelimb clonus which are typical Stage 2 seizure behaviours in rats (Racine, 1972). Therefore, the possibility exists that NIS-L is indeed a low-level seizure and may not be causing elevations in ACTH and CORT. Also, NIS-L may be used as a model of developmental seizure disorders.

In people, the prevalence of epilepsy is higher in males (Mejias-Aponte et al., 2002). Male rats have been found to be more susceptible to the convulsant effects of agents that produce temporal lobe-like seizures and present a higher amount of full limbic convulsions than females (Mejias-Aponte et al., 2002). If NIS-L is truly a low-level seizure, this may, in part, explain why more males than females exhibited the NIS-L syndrome in the NWM and open field.

A second possible explanation for the behavioural manifestations of the NIS-L syndrome is that the animals may be hyper-responsive to novelty. Dopamine pathways may have been affected as a consequence of neonatal exposure to DOM and KA. Evidence for this stems from the fact that expression of the NIS-L syndrome includes a large motor component. Many reports suggest that dopamine neurotransmission is involved in behavioural responses to novelty in that reward is related to increased dopamine activity and dopamine modulates exploratory behaviour in animals (Legault & Wise, 2001). Therefore, the novelty-seeking behaviour of the drug-treated animals may be altered. Evidence of this comes from a preliminary study which found that drug-

treated rats exhibited markedly increased locomotor behaviour when tested in a conditioned place preference task (Ryan, unpublished observations). Further, when the situation is no longer novel, animals fail to express the NIS-L syndrome. Currently, a study is being conducted in the UPEI Behavioural Science Laboratory to investigate response to novelty and novelty-seeking behaviour in both a hole board test and the elevated plus maze. Information gained from that study will provide further insight into the etiology of the NIS-L syndrome.

4.6. Conclusions

The aim of this study was to examine the interaction between the glutamate system and the neuroendocrine system, particularly with regard to early glutamate receptor activation. Four groups of animals at three different developmental time points were investigated, from the neonatal rat at PND14, to when the animal has just entered adulthood at PND 75, to old age at PND 480. The results of each of these studies indicate that this neonatal drug treatment does not cause either an immediate or longterm change in neuroendocrine function with regard to early CORT elevation and does not cause a longterm change in neuroendocrine function with regard to ACTH secretion. However, present findings confirmed the results of a previous study (Doucette et al. 2004), whereby stimulation of KA receptors with low doses of selective agonists during a critical period of brain development produced a permanent and long-lasting change (seen as late as 15 months) in the rat CNS. This change presented itself as the collection of behaviours

termed "NIS-L". At this point, the NIS-L syndrome does not appear to be an exaggerated response to stress as measured by neuroendocrine release of ACTH and CORT.

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APPENDIX A: STATISTICAL VALUES FOR THE NEONATAL RATS EXPERIMENT, ADULT RATS EXPERIMENT, AND AGED RATS EXPERIMENT

Neonatal (PND 14) Animals (* denotes significance)

Analysis	Source	Statistical Values	P values
2-way ANOVA (auditory startle)	Treatment Sex Treatment x Sex	0.29 2.73 1.13	0.881 0.102 0.348
2-way ANOVA with Repeated Measures (weight gain)	Treatment Sex Treatment x Sex	0.72 5.02 0.40	0.581 0.027* 0.806
2-way ANOVA (CORT)	Treatment Sex Treatment x Sex	0.43 1.78 0.41	0.785 0.186 0.802
2-way ANCOVA with litter as covariate (CORT)	Treatment Sex Treatment x Sex	0.45 1.87 0.41	0.776 0.176 0.802
2-way ANOVA (thymus gland weights)	Treatment Sex Treatment x Sex	0.65 0.88 0.10	0.629 0.351 0.983
2-way ANCOVA with litter as covariate (thymus gland weights)	Treatment Sex Treatment x Sex	0.66 0.91 0.11	0.622 0.343 0.980
2-way ANOVA (ratio of thymus to body weights)	Treatment Sex Treatment x Sex	0.51 5.93 0.16	0.730 0.018* 0.958
2-way ANCOVA with litter as covariate (ratio of thymus to body weights)	Treatment Sex Treatment x Sex	0.49 5.80 0.16	0.740 0.019* 0.960
2-way ANOVA (adrenal gland weights)	Treatment Sex Treatment x Sex	0.70 0.92 1.43	0.597 0.403 0.233

2-way ANCOVA with litter as covariate (adrenal gland weights)	Treatment Sex Treatment x Sex	0.68 1.83 1.29	0.621 0.181 0.281
2-way ANOVA (ratio of adrenal to body weights)	Treatment Sex Treatment x Sex	0.39 3.49 1.14	0.816 0.066 0.343
2-way ANCOVA with litter as covariate (ratio of adrenal to body weights)	Treatment Sex Treatment x Sex	0.40 3.41 1.08	0.810 0.069 0.375
1-ANOVA (litter effects on CORT)	Litter	7.87	0.000*
1-ANOVA (litter effects on thymus gland weights)	Litter	3.53	0.003*
1-ANOVA (litter effects on ratio of thymus to body weights)	Litter	5.28	0.000*
1-ANOVA (litter effects on adrenal gland weights)	Litter	8.36	0.000*
1-ANOVA (litter effects on ratio of adrenal to body weights)	Litter	12.14	0.000*

Adult (PND 75) Animals (* denotes significance)

Analysis	Source	Statistical Values	P values
2-way ANOVA (auditory startle) - CORT cohort rats	Treatment Sex Treatment x Sex	0.39 2.77 0.72	0.813 0.100 0.580
2-way ANOVA (eye opening)-CORT cohort rats	Treatment Sex Treatment x Sex	0.49 0.67 0.86	0.745 0.415 0.492
2-way ANOVA (baseline CORT)	Treatment Sex Treatment x Sex	0.90 59.97 1.07	0.469 0.000* 0.380
2-way ANOVA (immediate CORT)	Treatment Sex Treatment x Sex	0.68 205.60 0.59	0.608 0.000* 0.671
2-way ANOVA (2 hour CORT)	Treatment Sex Treatment x Sex	2.12 65.48 1.04	0.087 0.000* 0.391
ANOVA with Repeated Measures (all males & CORT)	Treatment Time	0.26 3.91	0.904 0.056
ANOVA with Repeated Measures (all females & CORT	Treatment Time	1.47 6.05	0.234 0.019*
Paired samples t-test (to determine if rats were sig stressed by NWM) - CORT males	Time	-11.42	0.000*
Paired samples t-test (to determine if rats were sig stressed by NWM) - CORT females	Time	-11.69	0.000*
Paired samples t-test (to determine if CORT immediately after NWM was sig different from 2hrs) - males	Time	10.63	0.000*

Paired samples t-test (to determine if CORT immediately after NWM was sig different from 2hrs) - females	Time	12.76	0.000*
Chi Square (to determine if a sig number of 5 & 20 DOM rats exhibited NISL in NWM) - CORT males	Expression of NISL	10.15	0.001*
Chi Square (to determine if a sig number of 5 & 20 DOM rats exhibited NISL in NWM) - CORT females	Expression of NISL	7.20	0.007*
Chi Square (to determine if a sig number of 25 & 100 KA rats exhibited NISL in NWM) - CORT males	Expression of NISL	4.94	0.026*
Chi Square (to determine if a sig number of 25 & 100 KA rats exhibited NISL in NWM) - CORT females	Expression of NISL	1.71	0.190
Chi Square (to determine if a sig number of 5 DOM rats exhibited NISL in NWM) - CORT males	Expression of NISL	5.33	0.021*
Chi Square (to determine if a sig number of 5 DOM rats exhibited NISL in NWM) - CORT females	Expression of NISL	7.27	0.007*
Chi Square (to determine if a sig number of 20DOM rats exhibited NISL in NWM) - CORT males	Expression of NISL	12.44	0.000*
Chi Square (to determine if a sig number of 20 DOM rats exhibited NISL in NWM) - CORT females	Expression of NISL	5.33	0.021*

Chi Square (to determine if a sig number of 25 KA rats exhibited NISL in NWM) - CORT males	Expression of NISL	7.27	0.007*
Chi Square (to determine if a sig number of 25 KA rats exhibited NISL in NWM) - CORT females	Expression of NISL	1.07	0.302
Chi Square (to determine if a sig number of 100 KA rats exhibited NISL in NWM) - CORT males	Expression of NISL	2.29	0.131
Chi Square (to determine if a sig number of 100 KA rats exhibited NISL in NWM) - CORT females	Expression of NISL	2.29	0.131
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - all males	NISL & baseline CORT	0.18	0.277
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - all males	NISL & immediate CORT	-0.05	0.773
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - all males	NISL & 2 hour CORT	-0.10	0.541
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - DOM & SAL males	NISL & baseline CORT	0.18	0.397
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - DOM & SAL males	NISL & immediate CORT	-0.07	0.757

Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - DOM & SAL males	NISL & 2 hour CORT	-0.14	0.526
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - DOM males	NISL & baseline CORT	-0.04	0.879
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - DOM males	NISL & immediate CORT	-0.14	0.607
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - DOM males	NISL & 2 hour CORT	-0.14	0.612
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - KA & SAL males	NISL & baseline CORT	0.20	0.353
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - KA & SAL males	NISL & immediate CORT	-0.04	0.848
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - KA & SAL males	NISL & 2 hour CORT	-0.05	0.801
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - KA males	NISL & baseline CORT	0.17	0.533
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - KA males	NISL & immediate CORT	-0.03	0.927

Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - KA males	NISL & 2 hour CORT	-0.04	0.888
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - all females	NISL & baseline CORT	-0.02	0.891
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - all females	NISL & immediate CORT	0.06	0.717
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - all females	NISL & 2 hour CORT	-0.004	0.980
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - DOM & SAL females	NISL & baseline CORT	0.25	0.250
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - DOM & SAL females	NISL & immediate CORT	0.26	0.221
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - DOM & SAL females	NISL & 2 hour CORT	-0.02	0.918
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - DOM females	NISL & baseline CORT	0.28	0.299
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - DOM females	NISL & immediate CORT	0.32	0.230

Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - DOM females	NISL & 2 hour CORT	0.37	0.165
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - KA & SAL females	NISL & baseline CORT	-0.44	0.031*
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - KA & SAL females	NISL & immediate CORT	-0.24	0.257
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - KA & SAL females	NISL & 2 hour CORT	-0.20	0.357
Point Biserial Correlation (to determine whether NISL was related to baseline CORT) - KA females	NISL & baseline CORT	-0.51	0.044*
Point Biserial Correlation (to determine whether NISL was related to immediate CORT) - KA females	NISL & immediate CORT	-0.26	0.328
Point Biserial Correlation (to determine whether NISL was related to 2 hr CORT) - KA females	NISL & 2 hour CORT	-0.09	0.742
ANOVA with Repeated Measures (DOM NISL & saline males & CORT)	NISL Time	0.08 2.70	0.787 0.119
ANOVA with Repeated Measures (DOM NISL & saline females & CORT)	NISL Time	0.14 9.57	0.717 0.007*
ANOVA with Repeated Measures (KA NISL & saline males & CORT)	NISL Time	0.13 3.06	0.724 0.104

ANOVA with Repeated Measures (KA NISL & saline females & CORT)	NISL Time	10.85 10.46	0.009* 0.010*
ANOVA with Repeated Measures (DOM NISL vs non-NISL males & CORT)	NISL Time	0.26 0.29	0.616 0.600
ANOVA with Repeated Measures (DOM NISL vs non-NISL females & CORT)	NISL Time	2.76 0.86	0.119 0.368
ANOVA with Repeated Measures (KA NISL vs non-NISL males & CORT)	NISL Time	0.03 1.13	0.866 0.306
ANOVA with Repeated Measures (KA NISL vs non-NISL females & CORT)	NISL Time	3.07 2.88	0.101 0.112
Paired samples t-test (to determine if baseline CORT was sig different from 2hrs) - males	Time	1.96	0.058
Paired samples t-test (to determine if baseline CORT was sig different from 2hrs) - females	Time	2.44	0.019*
2-way ANOVA (baseline ACTH)	Treatment Sex Treatment x Sex	0.66 0.41 1.19	0.622 0.523 0.323
2-way ANOVA (immediate ACTH)	Treatment Sex Treatment x Sex	0.62 16.27 1.42	0.649 0.000* 1.42
2-way ANOVA (2 hour ACTH)	Treatment Sex Treatment x Sex	1.71 3.97 0.52	0.158 0.050* 0.719
ANOVA with Repeated Measures (all males and ACTH)	Treatment Time	1.26 0.99	0.304 0.326
ANOVA with Repeated Measures (all females and ACTH)	Treatment Time	1.04 0.17	0.401 0.680

Paired samples t-test (to determine if rats were sig stressed by NWM) - ACTH males	Time	-10.35	0.000*
Paired samples t-test (to determine if rats were sig stressed by NWM) - ACTH females	Time	-11.51	0.000*
Chi Square (to determine if a sig number of 5 & 20 DOM rats exhibited NISL in NWM) - ACTH males	Expression of NISL	13.38	0.000*
Chi Square (to determine if a sig number of 5 & 20 DOM rats exhibited NISL in NWM) - ACTH females	Expression of NISL	10.15	0.001*
Chi Square (to determine if a sig number of 25 & 100 KA rats exhibited NISL in NWM) - ACTH males	Expression of NISL	10.15	0.001*
Chi Square (to determine if a sig number of 25 & 100 KA rats exhibited NISL in NWM) - ACTH females	Expression of NISL	6.00	0.014*
Chi Square (to determine if a sig number of 5 DOM rats exhibited NISL in NWM) - ACTH males	Expression of NISL	9.60	0.002*
Chi Square (to determine if a sig number of 5 DOM rats exhibited NISL in NWM) - ACTH females	Expression of NISL	7.27	0.007*
Chi Square (to determine if a sig number of 20DOM rats exhibited NISL in NWM) - ACTH males	Expression of NISL	10.50	0.001*

Chi Square (to determine if a sig number of 20 DOM rats exhibited NISL in NWM) - ACTH females	Expression of NISL	9.60	0.002*
Chi Square (to determine if a sig number of 25 KA rats exhibited NISL in NWM) - ACTH males	Expression of NISL	7.27	0.007*
Chi Square (to determine if a sig number of 25 KA rats exhibited NISL in NWM) - ACTH females	Expression of NISL	7.27	0.007*
Chi Square (to determine if a sig number of 100 KA rats exhibited NISL in NWM) - ACTH males	Expression of NISL	9.60	0.002*
Chi Square (to determine if a sig number of 100 KA rats exhibited NISL in NWM) - ACTH females	Expression of NISL	3.69	0.055
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - all males	NISL & baseline ACTH	-0.03	0.872
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - all males	NISL & immediate ACTH	0.09	0.602
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - all males	NISL & 2 hour ACTH	0.07	0.690
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - DOM & SAL males	NISL & baseline ACTH	-0.002	0.993

Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - DOM & SAL males	NISL & immediate ACTH	-0.05	0.827
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - DOM & SAL males	NISL & 2 hour ACTH	0.18	0.424
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - DOM males	NISL & baseline ACTH	0.06	0.841
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - DOM males	NISL & immediate ACTH	0.02	0.935
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - DOM males	NISL & 2 hour ACTH	-0.26	0.360
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - KA & SAL males	NISL & baseline ACTH	-0.14	0.516
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - KA & SAL males	NISL & immediate ACTH	0.10	0.652
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - KA & SAL males	NISL & 2 hour ACTH	0.11	0.623
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - KA males	NISL & baseline ACTH	0.04	0.873

Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - KA males	NISL & immediate ACTH	0.32	0.233
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - KA males	NISL & 2 hour ACTH	-0.05	0.864
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - all females	NISL & baseline ACTH	-0.09	0.583
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - all females	NISL & immediate ACTH	0.08	0.607
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - all females	NISL & 2 hour ACTH	0.02	0.923
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - DOM & SAL females	NISL & baseline ACTH	-0.16	0.459
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - DOM & SAL females	NISL & immediate ACTH	0.31	0.140
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - DOM & SAL females	NISL & 2 hour ACTH	0.28	0.189
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - DOM females	NISL & baseline ACTH	-0.17	0.519

Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - DOM females	NISL & immediate ACTH	0.28	0.288
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - DOM females	NISL & 2 hour ACTH	0.03	0.902
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - KA & SAL females	NISL & baseline ACTH	-0.01	0.958
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - KA & SAL females	NISL & immediate ACTH	-0.15	0.500
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - KA & SAL females	NISL & 2 hour ACTH	-0.18	0.407
Point Biserial Correlation (to determine whether NISL was related to baseline ACTH) - KA females	NISL & baseline ACTH	0.04	0.871
Point Biserial Correlation (to determine whether NISL was related to immediate ACTH) - KA females	NISL & immediate ACTH	-0.28	0.286
Point Biserial Correlation (to determine whether NISL was related to 2 hr ACTH) - KA females	NISL & 2 hour ACTH	-0.35	0.184
ANOVA with Repeated Measures (DOM NISL & saline males & ACTH)	NISL Time	0.17 0.78	0.682 0.388

ANOVA with Repeated Measures (DOM NISL & saline females & ACTH)	NISL Time	1.30 0.02	0.270 0.890
ANOVA with Repeated Measures (KA NISL & saline males & ACTH)	NISL Time	0.06 0.001	0.807 0.973
ANOVA with Repeated Measures (KA NISL & saline females & ACTH)	NISL Time	0.12 4.16	0.733 0.061
ANOVA with Repeated Measures (DOM NISL vs non-NISL males & ACTH)	NISL Time	0.07 0.87	0.798 0.369
ANOVA with Repeated Measures (DOM NISL vs non-NISL females & ACTH)	NISL Time	0.02 0.34	0.891 0.568
ANOVA with Repeated Measures (KA NISL vs non-NISL males & ACTH)	NISL Time	0.24 2.61	0.634 0.129
ANOVA with Repeated Measures (KA NISL vs non-NISL females & ACTH)	NISL Time	1.05 0.75	0.322 0.403
Paired samples t-test (to determine if ACTH immediately after NWM was sig different from 2hrs) - males	Time	10.96	0.000*
Paired samples t-test (to determine if ACTH immediately after NWM was sig different from 2hrs) - females	Time	13.50	0.000*
Paired samples t-test (to determine if baseline ACTH was sig different from 2hrs) - males	Time	1.02	0.313
Paired samples t-test (to determine if baseline ACTH was sig different from 2hrs) - females	Time	-0.41	0.681

Aged (PND 480) Animals (* denotes significance)

Analysis	Source	Statistical Values	P values
2-way ANOVA (baseline CORT)	Treatment Sex Treatment x Sex	0.47 30.56 0.02	0.498 0.000* 0.887
2-way ANOVA (immediate CORT)	Treatment Sex Treatment x Sex	0.07 87.06 0.06	0.795 0.000* 0.814
ANOVA with Repeated Measures (males & CORT)	Treatment Time	0.74 11.39	0.411 0.004*
ANOVA with Repeated Measures (females & CORT)	Treatment Time	0.16 7.58	0.700 0.016*
Paired samples t-test (to determine if rats were sig stressed by NWM) - males	Time	-3.26	0.005*
Paired samples t-test (to determine if rats were sig stressed by NWM) - females	Time	-2.85	0.012*
Chi Square (to determine if a sig number of DOM rats exhibited NISL in NWM) - males	Expression of NISL	5.89	0.015*
Chi Square (to determine if a sig number of DOM rats exhibited NISL in NWM) - females	Expression of NISL	5.89	0.015*
ANOVA with Repeated Measures (saline & NISL males)	Treatment Time	1.17 5.40	0.302 0.040*
ANOVA with Repeated Measures (saline & NISL females)	Treatment Time	0.62 3.35	0.450 0.097
ANOVA with Repeated Measures (NISL & non-NISL males)	Treatment Time	1.28 21.50	0.301 0.004*

ANOVA with Repeated Measures (NISL & non-NISL females)	Treatment Time	0.60 4.27	0.468 0.084
Point Biserial Correlation (to determine whether NISL was related to CORT) - males	Expression of NISL & CORT	0.18	0.502
Point Biserial Correlation (to determine whether NISL was related to CORT) - females	Expression of NISL & CORT	0.31	0.250
Pearson Correlation (relationship b/ween finding the platform on the 6th trial & CORT) - males	Finding the platform & CORT	-0.05	0.851
Pearson Correlation (relationship b/ween finding the platform on the 6th trial & CORT) - females	Finding the platform & CORT	-0.11	0.692
2-way ANOVA (baseline ACTH)	Treatment Sex Treatment x Sex	0.73 3.14 0.08	0.400 0.088 0.787
Point Biserial Correlation (to determine whether NISL was related to ACTH)	Expression of NISL & ACTH	-0.09	0.645
2-way ANOVA with Repeated Measures (tail flick latencies)	Treatment Sex Treatment x Sex Time	0.40 6.93 0.07 0.72	0.534 0.013* 0.793 0.402
Chi Square (to determine if a sig number of DOM rats exhibited NISL in open field) - males	Expression of NISL	5.89	0.015*
Chi Square (to determine if a sig number of DOM rats exhibited NISL in open field) - females	Expression of NISL	0.06	0.808
2-way ANOVA (pituitary gland weights)	Treatment Sex Treatment x Sex	0.32 4.47 0.89	0.575 0.040* 0.354

2-way ANOVA (ratio of pituitary to body weights)	Treatment Sex Treatment x Sex	0.33 8.40 0.58	0.573 0.007* 0.453
2-way ANOVA (adrenal gland weights)	Treatment Sex Treatment x Sex	1.92 10.69 1.95	0.177 0.003* 0.174
2-way ANOVA (ratio of adrenal to body weights)	Treatment Sex Treatment x Sex	1.43 16.93 1.48	0.241 0.000* 0.235
2-way ANOVA (body weights)	Treatment Sex Treatment x Sex	0.02 167.30 0.29	0.889 0.000* 0.594
2-way ANOVA (baseline oxytocin)	Treatment Sex Treatment x Sex	15.41 1.63 1.73	0.001* 0.212 0.200
Point Biserial Correlation (to determine if oxytocin was correlated with NISL)	Expression of NISL & oxytocin	0.34	<0.05*
Point Biserial Correlation (to determine if oxytocin was correlated with NISL) - NISL vs non-NISL rats only	Expression of NISL & oxytocin	0.03	0.961
2-way ANOVA (baseline prolactin)	Treatment Sex Treatment x Sex	0.96 10.09 0.75	0.335 0.004 0.393
Point Biserial Correlation (to determine if prolactin was correlated with NISL) - males	Expression of NISL & prolactin	-0.19	0.493
Point Biserial Correlation (to determine if prolactin was correlated with NISL) - females	Expression of NISL & prolactin	-0.11	0.665

APPENDIX B: ALL MEANS AND STANDARD ERRORS FOR THE NEONATAL RATS EXPERIMENT, ADULT RATS EXPERIMENT, AND AGED RATS EXPERIMENT

Neonatal (PND 14) rats

Test	Treatment	Mean (± SEM)
Auditory startle (mean first day to startle)	5 DOM animals 20 DOM animals 25 KA animals 100 KA animals saline animals	12.77 (0.13) 12.64 (0.15) 12.64 (0.14) 12.67 (0.14) 12.82 (0.16)
Adrenal gland weights (mg)	5 DOM animals 20 DOM animals 25 KA animals 100 KA animals saline animals	8.74 (0.69) 7.38 (0.55) 8.12 (0.75) 7.61 (0.67) 7.71 (0.60)
Ratio of adrenal to body weight	5 DOM animals 20 DOM animals 25 KA animals 100 KA animals saline animals	0.00029 (0.000025) 0.00025 (0.000021) 0.00027 (0.000023) 0.00026 (0.000026) 0.00026 (0.000023)
Thymus gland weights (mg)	5 DOM animals 20 DOM animals 25 KA animals 100 KA animals saline animals	109 (4.97) 104 (3.93) 101 (3.97) 108 (4.03) 108 (3.64)
Ratio of thymus to body weight	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	0.00342 (0.000125) 0.00351 (0.000143) 0.00324 (0.000150) 0.00345 (0.000166) 0.00344 (0.000182)
Ratio of thymus to body weight	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	0.00371 (0.000228) 0.00361 (0.000246) 0.00355 (0.000136) 0.00372 (0.000169) 0.00380 (0.000160)

Weight gain on PND 9 (g)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	2.27 (0.120) 2.21 (0.101) 2.21 (0.109) 2.20 (0.087) 2.23 (0.128)
Weight gain on PND 9 (g)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	2.34 (0.075) 2.20 (0.123) 2.07 (0.098) 2.14 (0.097) 2.21 (0.095)
Weight gain on PND 14 (g)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	2.48 (0.108) 2.20 (0.173) 2.48 (0.169) 2.29 (0.142) 2.55 (0.142)
Weight gain on PND 14 (g)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	2.21 (0.171) 2.09 (0.175) 2.09 (0.188) 2.22 (0.149) 2.15 (0.140)
CORT concentrations (ng/ml)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	45.54 (11.19) 52.00 (6.64) 42.05 (7.03) 40.53 (6.69) 48.16 (5.22)
CORT concentrations (ng/ml)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	60.86 (6.99) 56.69 (9.36) 49.99 (6.79) 54.33 (15.81) 43.66 (9.73)
CORT concentrations (ng/ml)	5 DOM animals 20 DOM animals 25 KA animals 100 KA animals saline animals	53.20 (6.67) 54.34 (5.57) 46.02 (4.83) 46.97 (8.08) 45.91 (5.37)

CORT concentrations (ng/ml)	Litter 1	65.12 (7.16)
	Litter 2	51.90 (6.08)
	Litter 3	35.77 (4.40)
	Litter 4	69.59 (8.45)
	Litter 5	24.59 (2.31)
	Litter 8	60.97 (2.62)
	Litter 10	30.81 (5.83)
	Litter 11	56.51 (8.61)
Adrenal gland weights (mg)	Litter 1	8.52 (0.62)
	Litter 2	8.64 (0.60)
	Litter 3	6.31 (0.21)
	Litter 4	5.66 (0.43)
	Litter 5	10.08 (1.04)
	Litter 8	5.58 (0.49)
	Litter 10	10.04 (0.61)
	Litter 11	8.56 (0.81)
Ratio of adrenal to body weights (mg)	Litter 1	0.254 (0.017)
	Litter 2	0.297 (0.020)
	Litter 3	0.221 (0.008)
	Litter 4	0.178 (0.013)
	Litter 5	0.361 (0.031)
	Litter 8	0.183 (0.017)
	Litter 10	0.361 (0.022)
	Litter 11	0.285 (0.003)
Thymus gland weights (mg)	Litter 1	106 (3.29)
	Litter 2	116 (4.57)
	Litter 3	91.0 (2.60)
	Litter 4	119 (7.57)
	Litter 5	104 (6.73)
	Litter 8	100 (2.36)
	Litter 10	103 (3.42)
	Litter 11	107 (3.99)
Ratio of thymus to body weights (mg)	Litter 1	3.17 (0.10)
	Litter 2	3.98 (0.13)
	Litter 3	3.18 (0.09)
	Litter 4	3.72 (0.20)
	Litter 5	3.76 (0.18)
	Litter 8	3.28 (0.08)
	Litter 10	3.71 (0.12)
	Litter 11	3.55 (0.16)

Adult (PND 75) Animals

Test	Treatment	Mean (± SEM)
Auditory startle (mean first day to startle)	5 DOM animals	12.81 (0.26)
	20 DOM animals	12.69 (0.22)
	25 KA animals	12.81 (0.19)
	100 KA animals	12.81 (0.19)
	saline animals	13.06 (0.25)
Eye opening (mean first day to open eyes)	5 DOM animals	14.75 (0.21)
	20 DOM animals	14.75 (0.19)
	25 KA animals	14.50 (0.18)
	100 KA animals	14.63 (0.22)
	saline animals	14.88 (0.20)
Baseline CORT concentrations (ng/ml)	5 DOM males	103.86 (17.88)
	20 DOM males	112.49 (15.17)
	25 KA males	127.58 (24.85)
	100 KA males	114.05 (16.10)
	saline males	143.23 (23.17)
Baseline CORT concentrations (ng/ml)	5 DOM females	281.46 (33.48)
	20 DOM females	288.26 (66.05)
	25 KA females	237.63 (36.62)
	100 KA females	369.84 (52.75)
	saline females	302.84 (36.17)
Immediate CORT concentrations (ng/ml)	5 DOM males	231.81 (24.69)
	20 DOM males	205.08 (13.56)
	25 KA males	238.52 (17.85)
	100 KA males	217.67 (24.69)
	saline males	222.57 (16.98)
Immediate CORT concentrations (ng/ml)	5 DOM females	534.24 (40.22)
	20 DOM females	482.75 (34.95)
	25 KA females	500.46 (36.47)
	100 KA females	574.28 (58.96)
	saline females	522.66 (35.63)

2 hour CORT concentrations (ng/ml)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	119.24 (35.71) 84.48 (29.87) 89.71 (21.45) 109.22 (20.82) 93.70 (15.22)
2 hour CORT concentrations (ng/ml)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	270.54 (33.23) 231.75 (26.50) 239.24 (38.28) 282.89 (34.34) 164.97 (18.70)
Baseline CORT concentrations (ng/ml)	5 DOM NISL males 20 DOM NISL males 25 KA NISL males 100 KA NISL males	106.65 (37.54) 110.99 (17.43) 104.38 (16.86) 124.56 (44.22)
Baseline CORT concentrations (ng/ml)	5 DOM NISL females 20 DOM NISL females 25 KA NISL females 100 KA NISL females	293.65 (51.96) 197.73 (56.66) 339.24 (N=1) 503.59 (103.68)
Immediate CORT concentrations (ng/ml)	5 DOM NISL males 20 DOM NISL males 25 KA NISL males 100 KA NISL males	245.31 (45.92) 211.12 (14.01) 221.53 (21.38) 250.24 (70.38)
Immediate CORT concentrations (ng/ml)	5 DOM NISL females 20 DOM NISL females 25 KA NISL females 100 KA NISL females	508.55 (36.97) 443.44 (72.38) 570.46 (N=1) 631.00 (78.34)
2 hour CORT concentrations (ng/ml)	5 DOM NISL males 20 DOM NISL males 25 KA NISL males 100 KA NISL males	148.20 (63.26) 85.90 (13.26) 86.80 (25.69) 139.79 (3.17)
2 hour CORT concentrations (ng/ml)	5 DOM NISL females 20 DOM NISL females 25 KA NISL females 100 KA NISL females	248.50 (49.36) 195.20 (31.78) 376.37 (N=1) 230.92 (65.59)
Baseline CORT concentrations (ng/ml)	5 DOM non-NISL males 20 DOM non-NISL males 25 KA non-NISL males 100 KA non-NISL males	101.08 (8.78) 122.98 (N=1) 166.23 (60.17) 110.55 (18.54)

Baseline CORT concentrations (ng/ml)	5 DOM non-NISL females 20DOM non-NISL females 25 KA non-NISL females 100 KA non-NISL females	261.16 (33.63) 378.79 (108.11) 223.12 (38.82) 325.26 (53.73)
Immediate CORT concentrations (ng/ml)	5 DOM non-NISL males 20 DOM non-NISL males 25 KA non-NISL males 100 KA non-NISL males	218.31 (24.79) 162.83 (N=1) 266.83 (28.44) 206.81 (26.71)
Immediate CORT concentrations (ng/ml)	5 DOM non-NISL females 20DOM non-NISL females 25 KA non-NISL females 100 KA non-NISL females	577.06 (95.26) 522.07 (57.98) 490.46 (40.50) 555.37 (76.11)
2 hour CORT concentrations (ng/ml)	5 DOM non-NISL males 20 DOM non-NISL males 25 KA non-NISL males 100 KA non-NISL males	90.29 (37.28) 774.57 (N =1) 94.57 (45.56) 99.03 (26.93)
2 hour CORT concentrations (ng/ml)	5 DOM non-NISL females 20DOM non-NISL females 25 KA non-NISL females 100 KA non-NISL females	307.29 (33.28) 268.29 (37.11) 219.65 (37.98) 300.22 (41.02)
Baseline CORT concentrations of NISL rats only (collapsed across drug) (ng/ml)	DOM NISL males DOM NISL females KA NISL males KA NISL females	109.41 (16.43) 251.02 (39.61) 110.15 (15.57) 448.80 (81.14)
Immediate CORT concentrations of NISL rats only (collapsed across drug)	DOM NISL males DOM NISL females KA NISL males KA NISL females	223.55 (18.22) 479.61 (27.00) 229.73 (21.94) 610.82 (49.53)
2 hour CORT concentrations of NISL rats only (collapsed across drug) (ng/ml)	DOM NISL males DOM NISL females KA NISL males KA NISL females	108.55 (24.36) 224.81 (30.54) 101.94 (20.25) 279.40 (61.17)
Baseline CORT concentrations of NISL rats only (collapsed across drug) (ng/ml)	DOM non-NISL males DOM non-NISL females KA non-NISL males KA non-NISL females	105.46 (8.09) 328.38 (63.76) 129.11 (23.05) 270.26 (34.30)

Immediate CORT concentrations of NISL rats only (collapsed across drug) (ng/ml)	DOM non-NISL males DOM non-NISL females KA non-NISL males KA non-NISL females	207.22 (22.18) 545.64 (48.78) 226.82 (21.56) 520.42 (40.53)
2 hour CORT concentrations of NISL rats only (collapsed across drug) (ng/ml)	DOM non-NISL males DOM non-NISL females KA non-NISL males KA non-NISL females	87.14 (29.05) 285.00 (24.77) 97.54 (21.81) 256.84 (29.09)
Baseline CORT vs. Immediate CORT - all males (ng/ml)	Baseline males Immediate males	120.24 (8.68) 223.13 (8.69)
Baseline CORT vs. Immediate CORT - all females (ng/ml)	Baseline females Immediate females	296.01 (20.95) 522.88 (18.56)
Immediate CORT vs. 2 hour CORT - all males (ng/ml)	Immediate males 2 hour males	223.13 (8.69) 99.27 (9.76)
Immediate CORT vs. 2 hour CORT - all females (ng/ml)	Immediate females 2 hour females	522.88 (18.58) 237.88 (14.69)
Baseline CORT vs. 2 hour CORT - all males (ng/ml)	Baseline males 2 hour males	120.24 (8.68) 99.27 (9.76)
Baseline CORT vs. 2 hour CORT - all females (ng/ml)	Baseline females 2 hour females	296.01 (20.95) 237.88 (14.69)
Baseline ACTH concentrations (pg/ml)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	43.62 (4.47) 30.13 (3.50) 43.79 (4.84) 45.31 (6.15) 36.24 (5.86)
Baseline ACTH concentrations (pg/ml)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	39.23 (8.88) 44.96 (5.68) 47.00 (5.48) 38.50 (3.88) 40.43 (2.71)

Immediate ACTH concentrations (pg/ml)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	69.10 (5.79) 59.46 (4.35) 62.96 (6.49) 73.21 (4.67) 62.45 (3.40)
Immediate ACTH concentrations (pg/ml)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	71.28 (4.60) 78.80 (6.51) 88.17 (5.16) 80.87 (7.78) 86.86 (9.85)
2 hour ACTH concentrations (pg/ml)	5 DOM males 20 DOM males 25 KA males 100 KA males saline males	34.54 (4.18) 34.32 (3.86) 32.97 (3.14) 40.77 (7.70) 43.27 (2.99)
2 hour ACTH concentrations (pg/ml)	5 DOM females 20 DOM females 25 KA females 100 KA females saline females	37.97 (2.36) 36.83 (3.39) 47.46 (5.01) 44.80 (5.17) 48.89 (6.77)
Baseline ACTH concentrations (pg/ml)	5 DOM NISL males 20 DOM NISL males 25 KA NISL males 100 KA NISL males	41.65 (3.61) 32.29 (3.27) 42.58 (5.38) 45.40 (8.39)
Baseline ACTH concentrations (pg/ml)	5 DOM NISL females 20 DOM NISL females 25 KA NISL females 100 KA NISL females	41.63 (14.70) 46.77 (7.04) 44.27 (4.91) 38.65 (5.79)
Immediate ACTH concentrations (pg/ml)	5 DOM NISL males 20 DOM NISL males 25 KA NISL males 100 KA NISL males	69.70 (7.84) 59.18 (5.14) 55.79 (4.26) 72.15 (6.06)
Immediate ACTH concentrations (pg/ml)	5 DOM NISL females 20 DOM NISL females 25 KA NISL females 100 KA NISL females	70.78 (6.95) 73.22 (6.81) 87.18 (4.84) 93.62 (13.90)

2 hour ACTH concentrations (pg/ml)	5 DOM NISL males 20 DOM NISL males 25 KA NISL males 100 KA NISL males	36.23 (5.49) 35.28 (4.43) 31.29 (4.17) 42.45 (10.40)
2 hour ACTH concentrations (pg/ml)	5 DOM NISL females 20 DOM NISL females 25 KA NISL females 100 KA NISL females	35.37 (2.28) 38.77 (4.43) 49.64 (6.36) 52.90 (3.01)
Baseline ACTH concentrations (pg/ml)	5 DOM non-NISL males 20 DOM non-NISL males 25 KA non-NISL males 100 KA non-NISL males	49.51 (17.84) 17.17 (N=1) 45.79 (10.92) 45.05 (1.25)
Baseline ACTH concentrations (pg/ml)	5 DOM non-NISL females 20DOM non-NISL females 25 KA non-NISL females 100 KA non-NISL females	35.23 (1.39) 39.53 (11.01) 51.55 (13.53) 38.41 (5.66)
Immediate ACTH concentrations (pg/ml)	5 DOM non-NISL males 20 DOM non-NISL males 25 KA non-NISL males 100 KA non-NISL males	67.33 (3.62) 61.13 (N=1) 74.90 (14.79) 76.39 (6.66)
Immediate ACTH concentrations (pg/ml)	5 DOM non-NISL females 20DOM non-NISL females 25 KA non-NISL females 100 KA non-NISL females	72.12 (6.03) 95.56 (10.88) 89.82 (12.97) 73.23 (8.52)
2 hour ACTH concentrations (pg/ml)	5 DOM non-NISL males 20 DOM non-NISL males 25 KA non-NISL males 100 KA non-NISL males	29.47 (2.01) 28.54 (N=1) 35.78 (5.26) 35.75 (1.89)
2 hour ACTH concentrations (pg/ml)	5 DOM non-NISL females 20DOM non-NISL females 25 KA non-NISL females 100 KA non-NISL females	42.30 (4.42) 31.02 (3.34) 43.83 (9.42) 39.94 (7.50)
Baseline ACTH concentrations of NISL rats only (collapsed across drug) (pg/ml)	DOM NISL males DOM NISL females KA NISL males KA NISL females	36.97 (2.72) 44.44 (7.31) 44.12 (4.96) 42.16 (3.64)

Immediate ACTH concentrations of NISL rats only (collapsed across drug) (pg/ml)	DOM NISL males DOM NISL females KA NISL males KA NISL females	64.44 (4.74) 72.11 (4.64) 64.71 (4.47) 89.59 (5.52)
2 hour ACTH concentrations of NISL rats only (collapsed across drug) (pg/ml)	DOM NISL males DOM NISL females KA NISL males KA NISL females	35.75 (3.36) 37.22 (2.46) 37.37 (5.98) 50.86 (3.97)
Baseline ACTH concentrations of NISL rats only (collapsed across drug) (pg/ml)	DOM non-NISL males DOM non-NISL females KA non-NISL males KA non-NISL females	38.73 (14.91) 36.95 (3.72) 45.49 (6.00) 43.34 (6.07)
Immediate ACTH concentrations of NISL rats only (collapsed across drug) (pg/ml)	DOM non-NISL males DOM non-NISL females KA non-NISL males KA non-NISL females	65.26 (2.94) 81.50 (7.46) 75.50 (8.38) 79.45 (7.29)
2 hour ACTH concentrations of NISL rats only (collapsed across drug) (pg/ml)	DOM non-NISL males DOM non-NISL females KA non-NISL males KA non-NISL females	29.16 (1.20) 37.78 (3.82) 35.77 (2.94) 41.40 (5.49)
Baseline ACTH vs. Immediate ACTH - all males (pg/ml)	Baseline males Immediate males	40.06 (2.36) 65.59 (2.30)
Baseline ACTH vs. Immediate ACTH - all females (pg/ml)	Baseline females Immediate females	42.02 (2.48) 81.20 (3.14)
Immediate ACTH vs. 2 hour ACTH - all males (pg/ml)	Immediate males 2 hour males	65.59 (2.30) 37.25 (2.12)
Immediate ACTH vs. 2 hour ACTH - all females (pg/ml)	Immediate females 2 hour females	81.20 (3.14) 43.19 (2.18)
Baseline ACTH vs. 2 hour ACTH - all males (pg/ml)	Baseline males 2 hour males	40.06 (2.36) 37.25 (2.12)
Baseline ACTH vs. 2 hour ACTH - all females (pg/ml)	Baseline females 2 hour females	42.02 (2.48) 43.19 (2.18)

Aged (PND 480) Animals

Test	Treatment	Mean (± SEM)
Baseline CORT concentrations (ng/ml)	20 DOM males saline males	126.25 (11.85) 147.33 (23.72)
Baseline CORT concentrations (ng/ml)	20 DOM females saline females	335.38 (56.77) 367.56 (46.31)
Immediate CORT concentrations (ng/ml)	20 DOM males saline males	194.75 (13.14) 195.44 (14.67)
Immediate CORT concentrations (ng/ml)	20 DOM females saline females	456.38 (33.88) 470.75 (43.94)
Baseline CORT (ng/ml)	20 DOM NISL males	114.75 (17.68)
Baseline CORT (ng/ml)	20 DOM NISL females	323.50 (78.40)
Immediate CORT (ng/ml)	20 DOM NISL males	183.00 (19.86)
Immediate CORT (ng/ml)	20 DOM NISL females	408.50 (54.66)
Baseline CORT (ng/ml)	20 DOM non-NISL males	137.75 (15.95)
Baseline CORT (ng/ml)	20DOM non-NISL females	347.25 (93.79)
Immediate CORT (ng/ml)	20 DOM non-NISL males	206.50 (17.85)
Immediate CORT	20DOM non-NISL females	504.25 (28.99)
Baseline CORT vs. Immediate CORT - all males (ng/ml)	Baseline males Immediate males	137.41 (13.59) 195.12 (9.62)
Baseline CORT vs. Immediate CORT - all females (ng/ml)	Baseline females Immediate females	348.94 (37.42) 463.56 (26.87)
Baseline Tail Flick Latencies (seconds)	20 DOM males saline males	7.23 (0.60) 8.33 (0.73)
Baseline Tail Flick Latencies (seconds)	20 DOM females saline females	6.39 (0.49) 6.81 (0.50)
Immediate Tail Flick Latencies (seconds)	20 DOM males saline males	7.84 (0.53) 7.66 (0.53)

Immediate Tail Flick Latencies (seconds)	20 DOM females saline females	6.23 (0.65) 6.18 (0.52)
Baseline ACTH concentrations (pg/ml)	all 20 DOM animals all saline animals	77.79 (3.94) 83.21 (5.41)
Baseline ACTH concentrations (pg/ml)	20 DOM males saline males	82.90 (5.44) 90.59 (7.67)
Baseline ACTH concentrations (pg/ml)	20 DOM females saline females	72.69 (5.40) 76.65 (7.32)
Pituitary gland weights (mg)	20 DOM males saline males	14 (1.5) 16 (1.5)
Pituitary gland weights (mg)	20 DOM females saline females	34 (10) 24 (3.2)
Ratio of pituitary to body weights	20 DOM males saline males	0.000017 (0.000002) 0.000020 (0.000002)
Ratio of pituitary to body weights	20 DOM females saline females0	0.000066 (0.000026) 0.000048 (0.000005)
Adrenal gland weights (mg)	20 DOM males saline males	54 (6.2) 54 (5.5)
Adrenal gland weights (mg)	20 DOM females saline females	142 (330) 89 (14)
Ratio of adrenal to body weights	20 DOM males saline males	0.000066 (0.000006) 0.000067 (0.000008)
Ratio of adrenal to body weights	20 DOM females saline females	0.000284 (0.000073) 0.000185 (0.000032)
Baseline oxytocin concentrations (pg/ml)	all 20 DOM animals all saline animals	2.91 (0.21) 2.00 (0.13)
Baseline oxytocin concentrations (pg/ml)	20 DOM males saline males	2.91 (0.28) 1.68 (0.14)
Baseline oxytocin concentrations (pg/ml)	20 DOM females saline females	2.90 (0.32) 2.29 (0.16)
Baseline oxytocin concentrations (pg/ml)	20 DOM NISL animals	2.88 (0.26)

Baseline oxytocin concentrations (pg/ml)	20 DOM non-NISL animals	2.93 (0.35)
Baseline prolactin concentrations (ng/ml)	20 DOM males saline males	50.00 (7.35) 43.13 (4.15)
Baseline prolactin concentrations (ng/ml)	20 DOM females saline females	295.25 (95.34) 183.11 (65.08)

APPENDIX C: BASELINE SERUM OXYTOCIN CONCENTRATIONS OF ADULT RATS

Raw data of serum oxytocin concentrations of PND 75 rats

ID	Sex	Drug	Oxytocin (pg/ml)
1	female	saline	1.85
2	female	saline	2.54
3	female	saline	2.39
4	female	25μg/kg KA	1.51
5	female	25μg/kg KA	1.49
6	male	saline	2.07
7	male	saline	1.29
8	male	25μg/kg KA	2.21
9	male	25μg/kg KA	1.31
10	male	25μg/kg KA	2.80
11	male	25μg/kg KA	1.72
12	male	25μg/kg KA	1.12
13	male	25μg/kg KA	0.97
14	female	5μg/kg DOM	2.82
15	female	5μg/kg DOM	1.13
16	male	saline	1.12
17	male	saline	0.91
18	male	saline	1.56
19	female	5μg/kg DOM	2.37
20	female	5μg/kg DOM	2.17
21	male	20μg/kg DOM	2.15
22	male	20μg/kg DOM	1.06
23	female	25μg/kg KA	1.86

24	female	25μg/kg KA	2.22
25	female	100μg/kg KA	2.14
26	female	100μg/kg KA	3.27
27	female	100μg/kg KA	1.85
28	female	100μg/kg KA	1.31
29	female	100μg/kg KA	1.72
30	male	5μg/kg DOM	1.44
31	male	5μg/kg DOM	1.46
32	male	5μg/kg DOM	1.51
33	female	20μg/kg DOM	1.61
34	female	20μg/kg DOM	1.77
35	female	saline	1.56
36	female	saline	1.43
37	male	5μg/kg DOM	1.32
38	male	5μg/kg DOM	2.11
39	male	5μg/kg DOM	2.83
40	male	100μg/kg KA	1.80
41	male	100μg/kg KA	1.54
42	male	100μg/kg KA	1.19
43	female	20μg/kg DOM	2.82
44	female	20μg/kg DOM	0.51
45	male	20μg/kg DOM	0.61
46	male	20μg/kg DOM	0.00
47	male	20μg/kg DOM	0.00
48	male	100μg/kg KA	0.00
49	male	100μg/kg KA	0.51

50	female	25μg/kg KA	0.59
51	female	25μg/kg KA	0.00
52	male	100μg/kg KA	0.00
53	male	100μg/kg KA	0.00
54	male	100μg/kg KA	0.00
55	female	25μg/kg KA	0.00
56	female	25μg/kg KA	0.74
57	male	saline	0.00
58	male	saline	0.00
59	male	saline	0.00
60	female	20μg/kg DOM	0.00
61	female	20μg/kg DOM	0.00
62	male	20μg/kg DOM	0.00
63	male	20μg/kg DOM	0.00
64	male	20μg/kg DOM	0.00
65	male	25μg/kg KA	0.00
66	male	25μg/kg KA	0.00
67	female	saline	0.00
68	female	saline	3.16
69	female	saline	0.53
70	male	5μg/kg DOM	0.00
71	male	5μg/kg DOM	0.59
72	female	100μg/kg KA	0.00
73	female	100μg/kg KA	0.00
74	female	100μg/kg KA	0.58
75	female	20μg/kg DOM	0.00

76	female	20μg/kg DOM	0.00
77	female	5μg/kg DOM	0.87
78	female	5μg/kg DOM	0.00
79	female	5μg/kg DOM	0.00
80	female	5μg/kg DOM	0.76