INFLUENCES OF NEONATAL ENVIRONMENTS ON STRESS-INDUCED BEHAVIOURS, MAMMARY GLAND MORPHOLOGY, AND ESTROGEN RECEPTOR ALPHA AND P53 EXPRESSION IN BALB/C MICE

A Thesis Submitted to the Committee on Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of Master of Science
in the Faculty of Arts and Science

TRENT UNIVERSITY

Peterborough, Ontario, Canada

Watershed Ecosystems Graduate Program

January 2008

Your file Votre référence
ISBN: 978-0-494-31715-0
Our file Notre référence
ISBN: 978-0-494-31715-0

ABSTRACT

Influences of Neonatal Environments on Stress-Induced Behaviours, Mammary Gland Morphology, and Estrogen Receptor Alpha and p53 Expression in Balb/c Mice

Ayesha Salleh

Early-life experiences influence HPA axis development, affecting hormone levels, stress-induced behaviours and possibly normal and malignant mammary gland development. Influences of neonatal experiences on stress-induced behaviours and mammary gland development were examined in pubertal (PND 30-35) and adult (PND 55-65) mice. Similar to other studies, immobility in the forced swim test was higher in maternally separated (MS; 4 hrs/day; PND2-22) compared to handled (H; 15 mins/day; PND2-22) mice (p's<0.05), but this difference was age- and gender-dependent. Additionally, neonatal manipulations influenced mammary gland growth, whereby H compared to MS and typically reared (TR; control) adult mammary glands were significantly more differentiated (p's<0.05). Examination of mRNA and protein expression of p53 and estrogen receptor alpha (ERα), found that only ERα protein expression was higher in adult mammary glands of both H and MS, compared to TR mice (p's<0.05). These results suggest that neonatal manipulations influence mammary gland development, but the interaction is complex.

Keywords: Neonatal manipulation, depressive behaviour, mammary gland development, estrogen receptor alpha, tumour suppressor gene p53.

Acknowledgements

I would like to extend my deepest appreciation to Dr. Leslie Kerr for agreeing to be my thesis supervisor and for your never-ending guidance, patience and unwavering support for the past four years. Your faith in me helped me to get to this point! To my supervisory committee; Dr. Carolyn Kapron and Dr. Craig Brunetti, thank you for all your constructive and helpful comments and ideas during the course of my research. To Dr. Janet Yee and the members of her lab (especially Brad Moore and Melissa Page) thank you for all your guidance and patience with me when I was learning the intricacies of real-time PCR. Thank you also to Dr. Brad White and members of the DNA profiling centre (Sylvia Coulson, Kristyne Wozney, Gaby Szanto) for help with real-time PCR. Thank you to Debbie Lietz and Tina Fridgen for your guidance in histology. Thank you to Jason Allen and the animal care staff for your help with mice rearing. Thank you to Linda Cardwell for all the wonderful advice you have given me. To Dr. Neil Emery and the staff of the office of graduate studies, thank you for your help with course work and thesis preparation. To the past and present members of the Kerr lab; Josh McGrath, Tina Trevelyan, Tricia Ralph, Valerie Taylor, Kori St. Cyr, Yogindra Persaud, Mautin Hundeyin and Meagan Munro, my research experience was very memorable because of you! Finally, I will be forever grateful to my family and friends for all their patience and support throughout the course of my research.

The forced swim test data used in the present study was jointly collected by Ayesha Salleh, Tricia Ralph, Tina Trevelyan and Josh McGrath. The estrogen receptor alpha western blot data was initially collected by Tricia Ralph, and replicated and expanded by Yogindra Persaud and Ayesha Salleh.

Table of contents

List of figures

List of tables

List of abbreviations

ACTH	Adrenocorticotropic hormone
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
cDNA	Copy deoxyribonucleic acid
CHEK2	Checkpoint homologue gene 2
CORT	Corticosterone
CRH	Corticotropin releasing hormone
Ct	Cycle threshold
DMBA	Dimethylbenzene anthracene
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ERE	Estrogen responsive elements
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GC	Glucocorticoid
GnRH	Gonadotropin releasing hormone
H	Handled; dams and pups separated for 15 minutes /day
HCl	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2 gene
HPA	Hypothalamic pituitary adrenal
HPG	Hypothalamic pituitary gonadal
Hsp90	Heat shock protein 90
LAU	Lobular alveolar unit
LHPA	Limbic hypothalamic pituitary adrenal
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MDM2	Mouse double minute 2 gene
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
MS	Maternally separated; dams and pups separated for 4 hours/day
NH	Non-handled; dams and pups not exposed to any form of outside stimulation
p53	Tumour suppressor gene p53
PBS	Phosphate buffered saline
PI3	Phosphatidylinositol 3
PMSF	Phenylmethylsulphonyl fluoride
PND	Post-natal day
PTEN	Phosphatase and tensin homologue gene

PVN	Paraventricular nucleus (of the hypothalamus)
RNA	Ribonucleic acid
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SDS	Sodium dodecyl sulphate
TBST	Tris-buffered saline with Tween
TDLU	Terminal ductal lobular unit
TEB	Terminal end bud
TR	Typically Reared; dams and pups are not separated

Chapter 1: Introduction

1.1 General introduction

Breast cancer is currently the most common cancer affecting Canadian women, where one in every nine women will develop this disease (Canadian Cancer Society). In men, breast cancer cases are much rarer, and it is expected that 170 men in Canada will develop this disease in 2007 (Canadian Cancer Society). Breast cancer is a late onset disease, such that the majority of cases are diagnosed after the age of 50 (Canadian Cancer Society). To date, inherited mutations in a handful of genes have been identified to increase the risk of heritable breast cancer, such as the high penetrance genes breast cancer 1 and 2 (BRCA1, BRCA2), and the low penetrance genes phosphatase and tensin homolog (PTEN), checkpoint homolog (CHEK2) and tumour suppressor gene p53 (p53) (Kenemans et al., 2004). However, heritable breast cancer is estimated to account for five to ten percent of all cases (Radford and Zehnbauer, 1996). Thus, approximately 90% of breast cancer cases do not have known inherited genetically linked causal factors, and it is estimated that 40% of these sporadic breast cancer cases may be attributed to environmental and lifestyle factors (Lacey Jr. et al., 2002). To date, some of the generally accepted breast cancer risk factors include age at menarche and menopause, and number and timing of pregnancies throughout a woman's lifetime (Dumitrescu and Cotarla, 2005; Hilakivi-Clarke, 1997a). Other factors include diet, exposure to exogenous estrogens, and early-life and/or chronic stress exposure (Hilakivi-Clarke, 1997a; Jacobs and Bovasso, 2000; Lacey Jr. et al. 2002). All of these risk factors directly or indirectly alter patterns of gene expression and subsequent protein translation (via genetic/epigenetic changes), as well as result in post-transcriptional protein modifications, which can then affect ligand-receptor sensitivity and receptor-gene binding capabilities (Borek, 2004). These

alterations further influence either levels of circulating mitogenic hormones (e.g., estrogen) or de-regulate cell-cycle control (via signal transduction mechanisms related to the cell-cycle), thereby increasing breast cancer risk (Dumitrescu and Cotarla, 2005; Lacroix et al., 2006; Russo and Russo, 2006).

Early-life or chronic stress, are risk factors that remain to be better elucidated. Several studies link exposure to differing life experiences, both in childhood and throughout life, and subsequent coping strategies, with changes in breast cancer risk (Butow et al., 2000; Jacobs and Bovasso, 2000; Price et al., 2001). In humans, studies have suggested that women who are able to cope better in stressful situations, have a reduced risk of developing breast cancer, respond better to chemotherapy and have a higher chance of survival compared to women who are less able to cope with stress (Hilakivi-Clarke, 1997a; Temoshok 1987). Coping abilities are attributed to differences in personality, which can arise from both genetic and environmental influences (Bleiker et al., 1996). Since the brain and mammary glands are structures that continue to develop post-natally, physiological influences exerted by compounds such as neurotransmitters and hormones, have the potential to affect the development of these structures. Differential exposure to environments early in life and throughout life, have been shown to significantly alter brain development and subsequently influence mammary gland development, through, for example, alterations in circulating hormone levels. Thus, and not surprisingly, studies have shown that exposure to various types of stressful life events early in life or throughout life is associated with increased risk of developing anxiety and depressive disorders, and breast cancer risk (Butow et al., 2000; Heim and Nemeroff, 2000; Kruk and Aboul-Enein, 2004; Temoshok, 1987). For example, in humans, positive

experiences (e.g., high level of maternal nurturing) have been shown to increase active coping behaviours and decrease stress-induced anxiety of the adult offspring, whereas negative experiences (e.g., parental neglect, childhood abuse and trauma) can have the opposite effect (Heim and Nemeroff, 2001). Furthermore, the effects of these negative experiences may be augmented when coupled with heritable predisposition such as familial history of depression (Heim and Nemeroff, 2001). Instances of childhood trauma (physical, sexual and/or psychological), parental lack of attention and warmth, and inconsistencies in parenting styles are associated with an increased vulnerability to developing anxiety disorders and symptoms, such as major depressive disorder (MDD) (Heim and Nemeroff, 2001). In terms of breast cancer risk, day-to-day stressors, major life events and depression are associated with increased breast cancer risk (Butow et al., 2000; Kruk and Aboul-Enein, 2004). However, due to the number of uncontrollable variables in these types of associative studies such as recall capability, mood and personality, a number of other studies have not found any significant associations between differing life events and breast cancer risk (Duijts et al., 2003; Nielsen and Gronbaek, 2006).

Since human data exploring the complex interaction between physiological and psychological changes over time in response to life experiences is virtually unattainable, the mouse model is used to examine the developmental changes that may occur in the brains of mice subjected to neonatal environment manipulations, by analyzing stress-induced anxiety behaviours in pubertal and adult mice. The neonatal manipulations used in the present study involve separating the dam and her pups for a certain amount of time each day (brief or protracted), for the first three weeks of life. These neonatal

manipulations are widely used in both rats and mice to examine the effects of dam-pup interactions on a variety of normal and stress-induced behaviours of the offspring. Furthermore, in contrast to what is known about changes in hormonal control and cell-cycle regulation that occur following tumourigenesis, the developmental environment of the normal mammary gland that influences its susceptibility to tumour development remains to be better elucidated (Anderson and Clarke, 2004). We propose that physiological changes resulting from exposure to different life experiences/environments can influence brain functioning, via the body's main stress axis, the hypothalamic-pituitary-adrenal (HPA) axis. In turn, this leads to changes in stress-induced behaviours, and additionally, impacts the development of the mammary gland. As a result, changes in the mammary gland environment during development may increase or decrease breast cancer susceptibility.

Thus, the present study examined the effects of manipulating the neonatal environment of Balb/c mice on stress-induced mouse behaviours, gross morphology of the mammary gland and the expression of genes related to mammary gland growth, to further support the observations that neonatal manipulations significantly affect anxiety and coping behaviours, and elucidate the influence of differing early-environments on mammary gland development.

1.2 HPA axis activation

The HPA axis is responsible for mediating the body's stress responses in order to maintain homeostasis. Under non-stressful situations, activity of the HPA axis fluctuates throughout the day, in accordance with ultradian and circadian rhythms (Tsigos and Chrousos, 2002). In response to a stressful situation, the limbic system (e.g., amygdala,

hippocampus, frontal cortex) stimulates the paraventricular nucleus (PVN) of the hypothalamus to release corticotropin releasing hormone (CRH), resulting in the stimulation of the anterior pituitary which causes the release of hormones such as adrenocorticotropin hormone (ACTH), prolactin, and β-endorphin (Hilakivi-Clarke, 1997a; Anisman et al., 1998, Tsigos and Chrousos, 2002). In the classical stress response, the ACTH in turn stimulates the adrenal cortex to release glucocorticoids (GC). Glucocorticoids (referred to as cortisol in humans and corticosterone in rodents) are able to exert their metabolic effects by binding to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) present in a diverse array of organs including the brain and mammary glands (Tsigos and Chrousos, 2002). Upon ligand-receptor binding, glucocorticoids translocate into the nucleus and interact with glucocorticoid responsive elements (GREs) on responsive genes and affect gene transcription. Alternatively, the activated receptors can affect other transcription regulators thus indirectly influencing gene transcription, or affect protein translation by influencing the stability of messenger RNA (mRNA) of hormone- responsive proteins (Tsigos and Chrousos, 2002). In both humans and rodents, the HPA axis is regulated by negative feedback loops (Levine, 2000), whereby increased production of GC can inhibit CRH and ACTH production and release at the level of the hypothalamic PVN and the anterior pituitary.

The activation of the HPA axis and subsequent increase in GC release can influence physiological processes such as development of the immune system, production of gonadal and growth hormones and regulation of receptor densities in various organs (Swaab et al., 2005; Tsigos and Chrousos, 2002). Apart from the influence of GC on the hypothalamus and anterior pituitary, GC is also known to affect various neural structures

responsible for emotional memory and cognitive appraisal (e.g., prefrontal cortex, hippocampus and amygdala; Swaab et al., 2005). For example, in the rodent brain, differential levels of stress-induced GC as a result of early life stressor exposure, have been shown to impact GR densities through up-regulation in the amygdala and prefrontal cortex, but down-regulation in the hippocampus, which potentially affects anxiety behaviour (Swaab et al., 2005). In terms of mammary gland development, increased CRH and GC production via HPA axis activation can suppress the hypothalamic-pituitary-gonadal (HPG) axis by reducing release of gonadotropin releasing hormone (GnRH), or via direct suppression of the gonads themselves, which reduces circulating levels of reproductive hormones (Swaab et al., 2005; Tsigos and Chrousos, 2002). Reduction of these reproductive hormones, including estrogen and progesterone, can influence mammary gland development (Silberstein, 2001). Alternatively, GC also interacts with its receptors in the mammary gland and directly influences mammary gland development (Silberstein, 2001).

1.3 Neonatal manipulations and anxiety behaviours

In non-human primates and rodents, neonatal environment manipulation models are utilized to investigate the effects of the parent-offspring relationship on behaviour. The mouse model of early-life experiences that will be employed in this study has been shown to differentially affect HPA axis development (Anisman et al., 1998, Levine, 2000). These neonatal manipulations involve separating dams and pups for short (e.g., 15 minutes) and longer (3 to 6 hours, up to 24 hours) periods of time typically during the first three weeks of life. The shorter separation time (i.e., 15 minutes), mimics behaviour of wild dams when foraging for food (Champagne et al., 2003; Fleming and Rosenblatt,

1974; Jans and Woodside, 1990; Levine, 2000). These early life manipulations have been shown to significantly alter stress related behaviours and stress-induced hormone levels in juvenile and adult, male and female mice (Levine, 2000; Meerlo et al., 1999; Parfitt et al., 2004; Papaioannou et al., 2002; Romeo et al., 2003). It has been shown that short periods of separation (typically referred to as handled rodents; H) result in pups that have an attenuated stress response as adults, exemplified by increased exploratory behaviour and lower anxiety (eg; open-field and elevated plus- maze tests), as well as lower levels of stress-induced hormones such as ACTH and corticosterone (Anisman et al., 1998; Meerlo et al., 1999). In contrast, longer periods of separation (referred to as maternally separated rodents; MS) result in animals that have a prolonged stress response, are more anxious, have decreased exploratory behaviour and exhibit less effective coping strategies as adults (Anisman et al., 1998; Pryce and Feldon, 2003; Wigger and Neumann, 1999).

The specific mechanisms causing behavioural and physiological differences between neonatal manipulation groups are not well-understood, but the differential effects of the two manipulations are widely-accepted as being mediated by differential HPA axis activation. The differences in HPA reactivity between the rodents subjected to H and MS manipulations are proposed to be associated with developmental changes in brain activity, including HPA hormone receptor expression in the hypothalamus, amygdala and hippocampus, which may subsequently alter rodent anxiety behaviours (Anisman et al., 1998; Caldji et al., 2000; Liu et al., 2000; Pryce and Feldon, 2003). It has been suggested that the differences in maternal care (instances of arched-back feeding and licking/grooming behaviour) between various neonatal manipulation groups may be partly responsible for the observed differences in HPA reactivity (Anisman et al., 1998;

Francis and Meaney, 1999). Although HPA axis activity is genetically programmed, it is thought that developmental influences such as negative early life experiences (long dam-pup separation period) may be able to sensitize and hence increase HPA reactivity, while positive life experiences (short dam-pup separation period) may blunt the HPA axis post-natally (Anisman et al, 1998; Francis and Meaney, 1999; Swaab et al., 2005). Thus, these differences in HPA axis development and reactivity between neonatal manipulation groups may additionally influence the growth and development of other HPA axis reactive organs, such as the mammary gland.

1.4 Mammary gland development

Mouse and human mammary glands are morphologically similar and undergo comparable growth and proliferation at different stages of development (Cardiff and Wellings, 1999; Haslam, 2006). During the foetal stage, the mammary gland forms from the epidermal placodes in both mice and humans. In mice, five pairs of epidermal placodes are present; which become the future site of the nipples. These placodes develop into the mammary mesenchyme, forming the primary mammary rudiments (termed anlage). By birth, these rudiments have developed into a minimally branched structure that has just begun to penetrate into the mammary fat pad (Parmar and Cunha, 2004; Sternlicht et al., 2006). In the human mammary glands, similar foetal developmental processes are observed, albeit with two differences; only one pair of mammary placodes are formed, and the human anlage develops into several branched mammary structures that group together around the nipple (Hovey et al., 2002; Sternlicht et al., 2006). In both species, the rudimentary ducts are composed primarily of epithelial and myoepithelial cells during the foetal stage (Hovey et al., 2002; Parmar and Cunha, 2004).

In human and rodent mammary glands, growth is minimal from birth until pre-puberty, but undergoes rapid development during puberty, pregnancy and lactation (Ball, 1998; Cardiff and Wellings, 1999; Silberstein, 2001). Due to the surge of estrogen, progesterone and other mammogenic hormones at puberty in both humans and mice, the mammary glands rapidly differentiate and proliferate into an organized network of ducts and lobules with an "open" structure (referring to the considerable space between ductal branches) (Ball, 1998; Cardiff and Wellings, 1999; Sternlicht et al., 2006). This "open" type of structure allows for continued growth and differentiation at later stages of development (i.e., pregnancy, lactation) (Sternlich et al., 2006). Also at puberty, the appearance of immature club-shaped terminal end buds (TEBs) is first detected. TEBs, composed of body cells and a layer of cap cells at the apical end of the bud, form at the leading edge of the proliferating ducts, allowing the ducts to penetrate into the adipose tissue of the mammary gland (Cardiff and Wellings, 1999; Silberstein, 1999; Parma and Cunha, 2004). TEBs are considered immature structures as they contain undifferentiated and hence, highly pluripotent cap cells. In both mice and humans, the body and cap cells of the TEBs continue to develop, into epithelial and myoepithelial cells, respectively, as proliferation occurs (Henninghausen and Robinson, 1998; Dunbar and Wysolmerski, 2001). Throughout puberty and into adulthood, and with each menstrual/estrous cycle (in human and rodent, respectively), TEBs develop dichotomously, via bifurcation and lateral branching into more differentiated ducts and lobules (Silberstein, 2001). This process involves high levels of both mitotic and apoptotic activity (Hovey et al., 2002). The highest level of cell differentiation occurs at the luteal (human) and proestrus (mouse) phases of the menstrual/estrous cycle, which coincide with a peak in plasma

estrogen levels (Hovey et al., 2002; Russo et al., 1999). In the human mammary gland, ductal branching occurs in a radial pattern, extending out from the nipple and forming distinct lobes that have separate ductal systems. In this respect, the branching pattern of the human mammary gland is more complex than that of the mouse, such that in the mouse, ductal branching penetrates the mammary fat pad in a linear fashion, to form one main, albeit intricate, ductal system (Cardiff and Wellings, 1999). In humans, TEBs develop into a network of ductal branches that end in terminal ducts, which are surrounded by alveolar buds. This terminal duct and its associated alveolar buds form the terminal ductal lobular unit (TDLU), the functionally active unit of the human mammary gland. The TDLU structure can be likened to a small grape-like unit, at the end of a duct (Cardiff and Wellings, 1999; Russo and Russo, 2004, Richert et al., 2000). With continued growth of the mammary glands, the TDLUs differentiate into more mature structures, termed lobules, dependent on the stage of mammary gland development. For example, with each menstrual cycle lobule 1 (lob1) structures continue to differentiate and grow progressively into lob2, whereas differentiation from lob2 to lob3 only occurs during pregnancy (Russo and Russo, 2004). As the lobules differentiate from lob1 to lob3, the number of associated alveolar buds (termed ductules in lob2 and lob3) increase, accompanied by a concomitant decrease in the size of each alveolar bud (Russo and Russo, 2004). In the mouse, TEBs develop into the lobular alveolar unit (LAU), which forms the functional unit of the mouse mammary gland (Ball, 1998, Cardiff and Wellings, 1999). Unlike in human TDLUs, the mouse LAU is composed of a single bud, typically with a globular end (Cardiff and Wellings, 1999; Richert et al., 2000). In addition, ductal proliferation from TEBs to more mature ducts and lobules is less

extensive than in humans, as there is considerable space between ductal branches (Cardiff and Wellings, 1999; Parmar and Cunha, 2004;). By early adulthood (approximately postnatal day 55 in the mouse), ductal branching in the mouse mammary gland has typically completely filled the mammary fat pad (Cardiff and Wellings, 1999; Parmar and Cunha, 2004). There is some discrepancy in the literature on the nomenclature used for naming various mammary gland structures, and analogies that are drawn between mammary structures in both species. Some researchers suggest that the TDLUs are analogous to the LAUs in mice (Cardiff and Wellings, 1999; Parmar and Cunha, 2004), whereas others argue that TDLUs are more closely related with TEBs in mice (Hilakivi-Clarke, 2006). Regardless of the analogous structures, it is evident that in both mice and humans, tumourigenesis most commonly originates in the least differentiated structures (i.e. those with the most pluripotent cells) of the mammary gland (i.e., TEBs in mice, TDLUs or lob1 in humans) (Fenton, 2006, Hilakivi-Clarke, 2006) .

Importantly, as related to development and risk of tumourigenesis, the cellular composition of both human and mouse ductal structures are similar, such that in both types of mammary glands, a continuous layer of luminal epithelia lines the entire ductal system, which is then surrounded by a layer of myoepithelial cells and a basement membrane (Anderson and Clarke, 2004; Hennighausen and Robinson, 1998; Dunbar and Wysolmerski, 2001). In the human breast, the ducts are surrounded by fibroblasts and embedded in an intra-lobular stroma (Anderson and Clarke, 2004). In the mouse mammary gland, myoepithelial cells surround both ducts and TEBs, albeit discontinuously around the TEBs and secondary/tertiary ducts (Henninghausen and Robinson, 1998; Dunbar and Wysolmerski, 2001). In both mice and humans, mature

mammary glands are comprised of highly branched ductal structures ending with clusters of alveoli (humans) or blunt and globular terminal ducts (mice). In addition, these ductal structures are organized within the mammary stroma, which is made up of many cell types, including adipocytes, fibroblasts, endothelial cells and the extra cellular matrix (Parma and Cunha, 2004; Rizki and Bissell, 2004). However, compared to the human stroma, the mouse stroma contains significantly more adipose tissue (Parmar and Cunha, 2004). Hence in the human breast, the TDLUs are more closely associated with the fibrous connective tissue of the stroma, whereas in the mouse, the LAUs are closely associated with the adipocytes (Parmar and Cunha, 2004). Regardless of the species however, mammary gland proliferation and differentiation is a continuous process and is influenced by hormonal fluctuations from the menstrual/estrous cycle, and during pregnancy and lactation (Cardiff and Wellings, 1999).

Indeed, in both species, ductal proliferation and elongation depend on the intimate interaction between the stromal and epithelial compartments of the mammary gland (Parma and Cunha, 2004). For example, in order for new ducts to penetrate the mouse mammary fat pad, the mammary epithelium emits chemical signals (such as from growth factors, prolactin) to the stroma to allow further ductal and alveolar development (Hennighausen and Robinson, 1998). In humans, xeno-transplantation studies suggest that proper ductal and alveolar development depend on a competent stroma (Kuperwasser et al., 2004; Parmar and Cunha, 2004). In mice, growth factors in the stroma rather than in the epithelia are responsible for differences in lateral branching patterns between mouse strains (Sternlicht et al., 2006). In addition, the action of estrogen, progesterone and other growth factors (prolactin, growth hormone) on the mammary gland depend on

specific receptor expression patterns in the stroma and epithelia (Clarke, 1996). For example, in the mouse, ERα expression in the stroma but not the epithelia is essential for development of ductal structures (Hennighausen and Robinson, 1998).

Apart from puberty, pregnancy and lactation are two other stages where rapid mammary development is observed in both rodents and humans, marked by a significant change in mammary gland morphology. At pregnancy, an increase in ductal proliferation and differentiation is observed especially in luminal epithelial cells, in order to facilitate milk secretion during lactation (Anderson and Clarke, 2004). Post-lactation, the mammary gland again undergoes significant ductal reorganization via apoptosis (Cardiff and Wellings, 1999; Richert et al., 2000; Russo et al., 2001). In humans, a high level of proliferation and differentiation of mammary lobules are observed, such that lob2 and lob3 differentiate into lob4, a type of lobule that is only found in the parous mammary gland. Lob4 is characterized by increased number and size of ductules (called acini at this stage) (Russo and Russo, 2004). As pregnancy progresses, proliferation decreases, and the newly formed Lob4 acini acquire secretory capability which allow for the secretion of colustrum and eventually milk during lactation (Russo and Russo, 2004). Although during lactation, no significant changes in mammary gland morphology are observed, the post-lactational mammary gland undergoes major structural regression, resulting in the degeneration of secretory acini (via apoptosis), and the re-development and proliferation of terminal ducts/tubules and connective tissue (Russo and Russo, 2004). Due to the significant morphological changes during pregnancy and lactation, the parous mammary gland contains significantly more of the differentiated lob3 and virtually no lob1 compared to the nulliparous gland (Russo and Russo, 2004). This is true until the age of

40 years, where the parous mammary gland begins to regress into lob1 and lob2 structures, resulting in a decrease of lob3. However, the parous mammary gland never fully regresses to the structure of a nulliparous gland. Subsequently, the mammary glands of nulliparous women consist mainly of lob1 (up to 80% of total lobules) and occasionally lob2 and lob3, while the parous mammary gland is predominantly made up of lob3 (up to 90% of total lobules) (Russo and Russo, 2004).

Similar to humans, the mouse mammary gland undergoes rapid ductal proliferation during pregnancy and lactation, giving rise to more differentiated terminal ductules and alveolar structures that allow for milk production. Hence, the ratio of epithelial cells to adipocytes is significantly increased at this time. By the third week of pregnancy (last stage), the production of milk protein in the alveolar epithelial cells is observed (Hennighausen and Robinson, 1998; Dunbar and Wysolmerski, 2001; Richert et al., 2000). During lactation, the luminal compartment is filled with milk, while the secreting alveolar epithelial cells continue to expand into the mammary fat pad causing a further decrease in adipocytes (Richert et al., 2000). As in humans, the parous mouse mammary gland undergoes dramatic reorganization of ductal structures post-weaning. At this time, the secretory alveolar cells involute and undergo apoptosis, while adipocytes begin to infiltrate back into the mammary gland. The alveolar buds are surrounded by a layer of myoepithelium until involution is complete. The epithelia to adipocyte ratio increases and there is significant rearrangement of the epithelia and stroma (Hennighausen and Robinson, 1998, Richert et al., 2000). Subsequently, at 21 days post-weaning, the mammary gland resembles that of the nulliparous mouse, albeit with a more

arborized ductal structure, and the presence of a few alveolar buds that are not found in the nulliparous gland (Richert et al., 2000).

The next stage of significant morphological change in the human mammary gland occurs at menopause, where the atresia of the female ovarian follicles results in the termination of ovarian estrogen production, and subsequent cessation of the menstrual cycle (Russo and Russo, 2004). Since estrogen is the primary mitogen in mammary gland development, the cessation of endogenous ovarian estrogen production causes regression of the mammary structures in both parous and nulliparous mammary glands, to the extent that after the age of 60 years, the morphology of both types of mammary glands appear similar (Russo et al., 2001; Russo and Russo, 2004). This is primarily due to the regression of lob3 to lob1 in parous mammary glands. Unlike humans, rodents maintain reproductive capability throughout the adult life span and do not undergo menopause (Haslam, 2006). Instead, mammary gland growth is influenced by each bout of pregnancy and lactation.

The development of the mammary gland is influenced by the fluctuations in mammogenic hormones, and other growth hormones involved in its development. One of these mechanisms includes the interaction of the HPA and HPG axes. Aside from the role of the HPA axis in affecting anxiety behaviours, it also can significantly influence the mammary gland, both directly via CORT receptors in the gland, and indirectly, through the influence of the HPA axis on the HPG axis (Silberstein, 2001, Tsigos and Chrousos, 2002). Since H and MS neonatal manipulations differentially influence HPA axis development, influencing the level of circulating CORT, which can then affect circulating levels of mammotropic hormones and growth factors, mice subjected to these neonatal

manipulations may exhibit morphological differences in growth and differentiation of the mammary gland attributable to differential hormonal changes that occur, during development. These alterations in the growth and differentiation of the mammary gland may be in the form of gross morphological changes and/or differences in protein and RNA expression levels and profiles of genes important for mammary gland growth. Consequently, morphological differences in the mammary glands of mice between neonatal manipulation groups may indicate altered risk of tumour initiation via changes in gross morphology, protein and/or RNA expression profiles. Since the human and rodent mammary glands are structurally similar and contain analogous structures (Cardiff and Wellings, 1999; Russo and Russo, 2001), some aspects of mouse mammary gland development can be paralleled to that of the human condition.

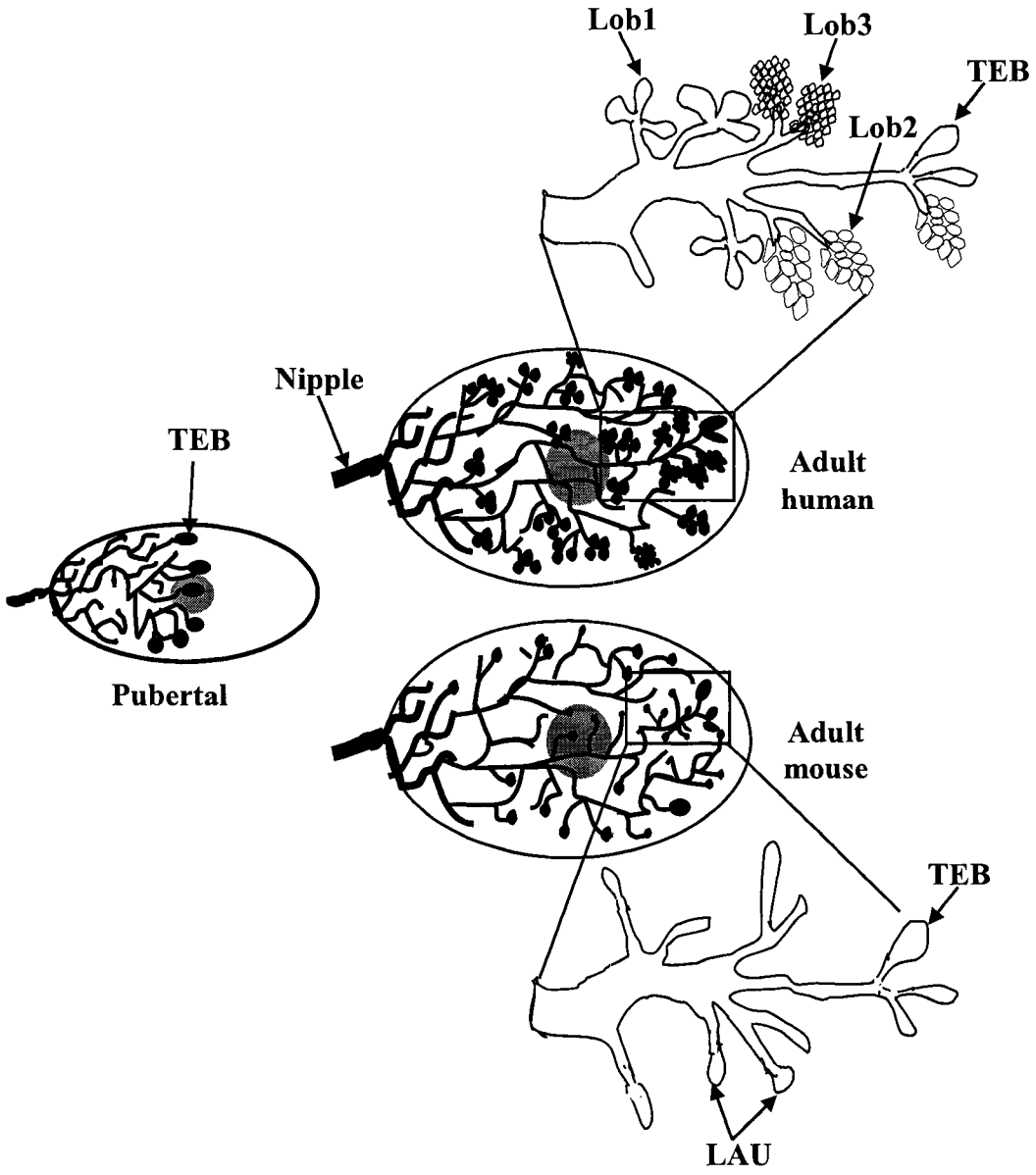


Figure 1.Comparative development of mouse and human mammary glands. At puberty, the surge of mammogenic hormones initiates the differentiation of highly proliferative terminal end buds (TEB) in both humans and mice. TEBs differentiate into lobules types 1,2,3 in humans, and lobular alveolar units (LAUs) in the nulliparous mouse. Adapted from Russo and Russo (2004) and Wiseman and Werb (2002).

1.5 Estrogen receptor alpha (ERα)

Aside from examining gross morphological differences (e.g. TEB numbers and extent of branching patterns) in the mammary glands of neonatally manipulated mice, analyzing protein and mRNA expression of genes related to mammary gland development is essential as protein and mRNA expression profiles provide important clues to the differences that may exist in mammary gland growth among neonatal manipulation groups. Of particular importance are the estrogen receptors, responsible for mediating the effects of estrogen on mammary gland development. The estrogen receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are highly homologous, although it is thought that ERα is responsible for estrogen-induced proliferation of both human and rodent mammary glands (Anderson and Clarke, 2004). In the mammary gland, estrogen interacts with its receptors in the epithelial (mouse and humans) and stromal (mouse only) compartments to regulate growth (Anderson and Clarke, 2004; Parmar and Cunha, 2004). The interaction between estrogen and ERα also changes depending on the stage of mammary gland development (Shyamala et al., 2002). For example, in the pubertal mouse mammary gland, there is an inverse relationship between estrogen levels and ERα protein expression, whereas at adulthood, ERα protein expression is increased with higher levels of estrogen (Shyamala et al., 2002).

Due to the importance of ERα in regulating not only normal but also malignant mammary gland growth, ERα protein and mRNA levels are consistently assessed in breast cancer cases, such that breast tumours are classified based on their estrogen receptor levels. In humans, ERα mRNA levels are higher in malignant tumours compared to normal surrounding tissue (Lofgren et al., 2006), whereas ERα protein levels are

significantly different between various breast tumour types (Tang et al., 2006). Similarly in the rodent, ERα expression is significantly different in mammary tumours compared to normal tissue (Yoshidome et al., 2000). Moreover, in both humans and mice, ERα protein levels are detected at higher levels in the less differentiated structures of the mammary glands (i.e., TEBs) (Russo et al., 1999). These findings suggest that ERα expression levels are linked to different stages of normal growth and tumourigenesis, and thus any alterations in ERα expression may be indicative of changes in the developmental environment of the mammary gland, and possibly related to tumourigenesis susceptibility. Thus, examining the levels of ERα mRNA and protein, along with the gross morphology of H and MS mammary glands will provide insight as to the differences that may exist in the developmental environment of the mammary gland, which may increase or decrease its susceptibility to tumour development.

1.6 Tumour suppressor gene p53 (p53)

Similar to ERα, which is involved in mammary gland growth and development, the tumour suppressor gene p53 is responsible for cell growth and differentiation, its role is to control DNA repair and when necessary to induce cell growth arrest or apoptosis, regardless of the cell type (Jerry et al., 2002; Meek, 2004). Known as the "guardian of the genome", and rightly so, stimulation of p53 from sources such as DNA damage and activation of oncogenes, cause enhanced binding of p53 proteins to DNA along with accumulation of p53 in the nuclei (Jerry et al., 2002; Hoogervorst et al., 2005). As a result, post-translational modifications of p53 allow it to act on target genes that can induce senescence, cell cycle arrest and DNA repair (Jerry et al., 2002).

Point mutations of the tumour suppressor gene p53 are the most commonly found mutation in sporadic breast cancers. These mutations lead to the loss of function of the p53 protein, causing defects in the DNA binding capability of p53 and the downstream activation of p53 responsive genes (Kenemans et al., 2004). Thus, damaged cells may escape DNA repair and apoptosis induction and subsequently propagate mutation accumulation (Almog and Rotter, 1997). In support of these observations, p53 mutations and post-transcriptional modifications are most commonly found in a large percentage of breast cancer cases (Lacroix et al., 2006; Lerebours and Lidereau, 2002). Furthermore, aside from loss of function, p53 protein expression levels in breast tumours have been significantly associated with tumour proliferation rate (Lacroix et al., 2006), short term breast cancer survival rate (Railo et al., 2007) and failure of endocrine therapy for cancer treatment (Kai et al., 2006). Moreover, p53 over-expression has been detected in breast tumours, compared to normal surrounding tissue (Hussein and Ismael, 2004). Thus, p53 mRNA and protein expression status, coupled with ERα expression status and morphological examination of the mouse mammary gland may further elucidate the effects of neonatal manipulations on mammary gland development and risk of tumour development.

Differences in stress-induced behaviours, mammary gland morphology, and ERα and p53 expression may not only be influenced by various neonatal manipulations, but are possibly dependent on stage of development (Colorado et al., 2006; Hovey et al., 2002; Parfitt et al., 2004; Shyamala et al., 2002). It is expected that morphological or protein and mRNA expression differences observed in adult mice will not be completely similar to pubertal mice because of the differences in stage of growth in brain, HPA axis

and mammary gland development. In terms of stress-induced behaviours, gender and stage of development significantly influence behaviours of rodents subjected to neonatal manipulations (Colorado et al., 2006; Papaioannou et al., 2002; Parfitt et al., 2004; Romeo et al., 2003). For example, gender difference may be attributed to estrogen action on the HPA axis that occurs post-puberty in females, which may modulate differences in anxiety behaviours seen at various developmental stages (Hiroi and Neumaier, 2006; Morgan et al., 2004). In terms of mammary gland development, significant variations in pubertal and adult mouse mammary glands are observed, as a result of differences in the hormonal milieu between pubertal and adult mice (Hovey et al., 2002). Therefore it is expected that anxiety behaviours, gross morphology and expression of various proteins in the mammary gland will differ between pubertal and adult mice between and within treatment groups.

1.7 Objectives of the study

The present study aimed to examine the influence of H and MS manipulations on:

1) Stress-induced behaviours in response to acute stressor exposure (forced swim test) in pubertal and adult mice.

2) Gross morphological differences in branching patterns and TEB and LAU numbers that may be present in the mammary glands of H and MS mice, and to examine developmental changes that occur in these structures from puberty to adulthood.

3) mRNA and protein expression profiles of ERα and p53 via reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), immunohistochemical staining and western blot in pubertal and adult mouse mammary glands to analyze the influence of neonatal manipulations on these growth-related genes.

1.8 Hypotheses

It is expected that mice exposed to the neonatal manipulations of H and MS will exhibit differences in stress-induced behaviours in the forced swim test, and changes in mammary gland development compared to the TR control group. It is expected that H compared to MS mice will show more active coping and less depressive-like behaviours. In terms of mammary gland morphology, it is hypothesized that mammary glands of H and MS mice will be significantly different, characterized by variations in branching patterns and TEB and LAU counts, where H compared to MS mouse mammary glands may contain more differentiated structures. In addition, ERα and p53 mRNA and protein expression profiles are hypothesized to be significantly different in H compared to MS mouse mammary glands due to differences in the cellular composition, and density of various mammary gland structures (e.g., TEBs and LAUs). Furthermore, differences in stress-induced behaviours, mammary gland morphology and ERα and p53 expression in pubertal compared to adult mice both within and between groups are also expected to be found.

Chapter 2: General methodology

2.1 Animals

A total of 36 female and 16 male Balb/c mice (Charles River Laboratories, Saint-Constant, Quebec), approximately 65 days old were used for breeding in this study. Upon arrival, females and males were housed separately in cages of 3-4 mice/cage. Following 7-10 days of acclimatization in a temperature controlled room (22 $\pm$ 2 °C) under artificial illumination (12:12 h light/dark; lights on at 7a.m.), 2-4 females were housed with 1 male in a polypropylene shoebox cage (19 x 29 x12.5 cm.). Once females were pregnant, they were separated into individual shoebox cages and provided with nesting material. One day following birth, litters were randomly cross-fostered to reduce the possibility of litter effects and assigned to handled (H), maternally separated (MS) or typically reared (TR) treatment groups. Since some studies have suggested that rodent mothers tend to spend more time caring for male than female pups and that in mixed litters, neonatal handling is more advantageous to males (Moore and Morelli, 1979) all litters were culled to a ratio of 3:2 females to males. Neonatal manipulations were performed between PND 2-22. On PND 23-25, pups were ear-punched, weaned and housed in same-sex, same-litter groups with 1-4 mice per cage. Previously, we demonstrated that housing mice either individually or in same-sex groups of up to four mice per cage did not influence forced swim test behaviours (unpublished data). Approximately half the cages from each neonatal environment were randomly designated to be examined at puberty, while the remainder were examined in early adulthood. Cages and water bottles were cleaned weekly; food (Lab Diet 5015) and water were provided *ad libitum*.

Starting at PND 29, puberty onset was assessed in females. Puberty onset was determined by the onset of estrus (microscopic evaluation of cells in vaginal smears). The

stage of estrous cycle was determined by examining the abundance of leukocytes and cornified and non-cornified epithelial cells in the vaginal smears (Inderdeo et al., 1996). Estrus was determined when the majority of cells observed were cornified epithelial cells. Male mice were handled in a similar manner to compensate for estrus testing in females. At puberty (PND 30-35), the predetermined subset of mice from each neonatal manipulation group was subjected to the forced swim test. Since some studies have suggested that the stage of estrous cycle can affect female mouse behaviours (Morgan and Pfaff, 2001), females were subjected to the forced swim test only when they were determined to be in estrus in order to reduce variability in forced swim test behaviours. Males were also subjected to the swim test at the same time as their female counterparts, between PND 30-35. The procedure was repeated at young adulthood (PND 55-65) with a different subset of cages from each treatment group. Data in this study are a union of replicated studies carried out over two sequential years, and meet the statistical requirement of homogeneity of variance. All experiments were performed following protocols approved by the Trent University Animal Care Committee (Canadian Council of Animal Care approved facility).

2.2 Neonatal manipulations

Neonatal manipulations were conducted once daily (between 1130h and 1700h) from PND 2-22 (procedures adapted from Plotsky and Meaney, 1993; Pryce and Feldon, 2003). The H treatment involved separating dams from their pups for 15 min/day. Dams were first removed from the home cage and placed in a clean paper towel lined cage. Pups were then placed individually in paper towel lined plastic cups. After 15 minutes,

dams and pups were reunited in the home cage. MS involved a 4 hour separation between dams and pups. Pups were removed from the home cage and the entire litter was placed in a clean, paper towel lined cage, while dams remained in the home cage. After 4 hours, pups were reunited with the dam in the home cage. TR groups were exposed to standard laboratory housing procedures (weekly cage and water bottle changes) and did not undergo any other manipulation.

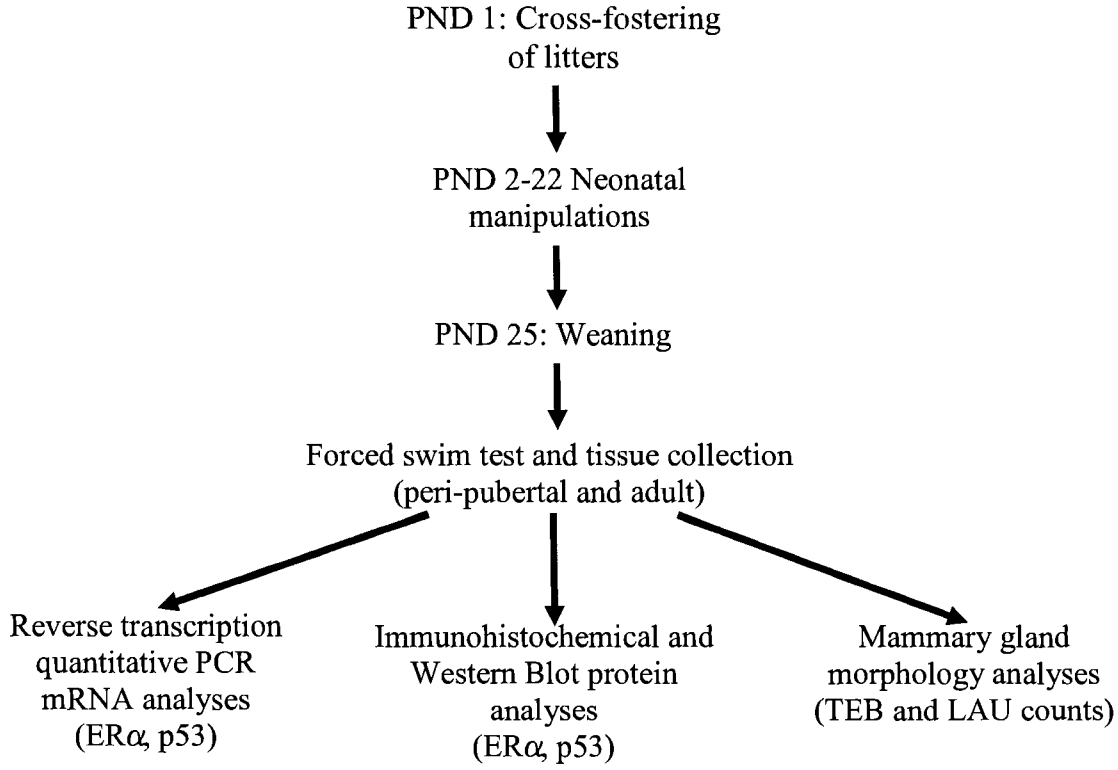


Figure 2. Flowchart of general experimental methodology. Balb/c mice were subjected to neonatal manipulations and then exposed to a forced swim test at either peri-puberty (PND 30-35) or adulthood (PND 55-65). Mammary glands were then immediately harvested and processed for mRNA, protein and gross morphological analyses.

Chapter 3: Neonatal Environment, Gender, and Age at Testing Differentially Influence Forced Swim Test Behaviours in Balb/c Mice

3. 1 Introduction

The parent-offspring relationship is important in shaping physiological and behavioural responses to stressors in the adult offspring. In humans, a high, consistent level of maternal nurturing in childhood increases active coping behaviours and decreases stress-induced anxiety in the adult offspring, whereas inconsistent parental care can lead to increased stress-induced anxiety in adults (Penza et al., 2003). Both human and animal studies have shown that negative early-life environments (e.g., abuse, protracted maternal separation in rodents, variable foraging demand in non-human primates) affect limbic-hypothalamic-pituitary-adrenal (LHPA) axis development, and result in adult offspring with elevated stress-induced secretions of hormones such as corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and glucocorticoids (cortisol in humans and corticosterone in rodents; CORT); levels of which are directly correlated with increased stress-induced anxiety behaviours (Coplan et al., 2001;Levine, 2000; Morgan and Pfaff, 2001; Pryce and Feldon, 2005; Rosenblum et al., 2001). Importantly, alterations in or dysfunctions of the LHPA axis play an important role not only in stress reactivity, but also in affective states such as depression and anxiety (Feijo de Mello et al., 2003; Piccinelli and Wilkinson, 2000). Aside from the influence of early life environments on LHPA axis development and subsequent function and reactivity, gender may also differentially influence LHPA axis development. For example, compared to men, women have a two-fold increase in lifetime risk of developing depression and more often than men report concurrent symptoms such as those consistent with anxiety disorders (Marcus et al., 2005). Since controlling for potentially confounding and

interactive variables in human studies is challenging, an established rodent model that manipulates mother-neonate interaction is widely used to analyze the effects of early-life experiences on brain development and subsequent behavioural repertoires, including stress-induced behaviours of the adult offspring. However, most rodent neonatal manipulation studies to date have focused on examining these behaviours only in adult males (Anisman et al., 2001; Caldji et al., 2000; Ladd et al., 2000; Meerlo et al., 1999; Pryce and Feldon, 2003), with little work done on female and adolescent populations. Also until recently, the effects of neonatal environments were typically examined in rats, but emerging data indicate that manipulation of neonatal environments also produce robust behavioural effects in mice; in for example, the elevated plus maze (Renard et al., 2005; Romeo et al., 2003) and modified forced swim tests (MacQueen et al., 2003; Papaioannou et al., 2002) with results comparable to those in the rat (Anisman et al., 2001; Gariepy et al., 2002; Schmidt et al., 2002; Zaharia et al., 1996). In particular, the forced swim test and the more recently described and validated modified forced swim test is favorable due to its ability to detect behaviours associated with anxiety and coping in the rodent (Cryan et al., 2005).

Neonatal handling (H) and maternal separation (MS) are two well established rodent models of early-life environmental manipulations that differentially influence the development and life-time activity of several neural systems and can have differing and enduring effects on LHPA axis activity and related hormone levels. In this rodent model, offspring are subjected to brief (e.g., 1-15minutes/day; H) or protracted (e.g., 2-6 hours/day; MS) periods of maternal separation in the first few weeks of life. The combination of brief hypothermia and changes in the mother's feeding and nesting

behaviours that result due to the H and MS conditions have been suggested to differentially alter LHPA axis reactivity, anxiety behaviours, stress hormone levels, and brain peptide expression of the adult male H and MS offspring in response to chronic or acute stressors (Levine, 2000; Liu et al., 1997, Pryce and Feldon, 2003). Importantly however, the interpretation of the influences of H and MS neonatal conditions on behavioural and hormonal profiles in the adult offspring differ depending on the comparison group (e.g., non-handled; NH or animal facility (typically) reared; TR conditions). Whereas the NH condition represents a total absence of experimenter manipulations, TR animals are exposed to typical animal facility rearing conditions including cage and water bottle changes throughout the experimental period. Research has shown that compared to NH rats, adult H males show decreased stress-induced anxiety behaviours as exhibited by increased exploratory behaviours in the open field (Caldji et al., 2000), increased time spent in the open arms of an elevated plus maze (Meerlo et al., 1999; Severino et al., 2004) and decreased immobility in a forced swim test (Papaioannou et al., 2002). Conversely, MS compared to NH adult male rats exhibited higher anxiety and stressor reactivity in an acoustic startle test (Caldji et al., 2000) and elevated plus maze test (Kalinichev et al., 2002) but not in open field or neophagia (propensity to try new food) tests (Caldji et al., 2000). When compared to TR adult males, MS adult rats also showed increased anxiety in the elevated plus maze test (Wigger and Neumann, 1999), whereas in other studies, H and TR adult male rats showed similar behavioural profiles in both the acoustic startle and elevated plus maze tests (Pryce et al., 2001). Taken together, and considering only the neonatally manipulated groups, MS rodents typically are more anxious compared to H rodents. The differences

in anxiety behaviours of H and MS animals are further correlated with changes in HPA axis reactivity, such that the HPA hormone profiles in the H adult rodent are in contrast to those of MS. That is, relative to both NH and MS male rats, H adults exhibit decreased basal CRH levels in the hypothalamus and CRH mRNA expression in the median eminence, and decreased stress-induced levels of ACTH and CORT (Anisman et al., 1998; Plotsky and Meaney, 1993). The decreased hormonal reactivity to stressors in H males is further correlated to an increased sensitivity to HPA negative feedback, evidenced by faster return to baseline CORT levels in response to a stressor (Anisman et al., 1998; Meaney et al., 1996). Importantly, the magnitude of the hormonal and behavioural changes in neonatal manipulated rodents is dependent on the separation schedule used (e.g., MS condition from post natal days 1-14 or 1-21). Overall however, H animals compared to MS are behaviourally and physiologically less reactive when exposed to a stressor.

Although there is extensive literature on anxiety behaviours of neonatally manipulated male adult rodents, there currently is limited research examining the differential effects that gender or age at testing may have on behavioural and physiological outcomes of neonatal manipulations. Recently, some studies indicate that there is a general shift towards more gender-specific behaviours as rodents mature, although it is unclear when these behavioural differences actually begin to emerge (Laviola et al., 2003). The few studies examining gender differences and neonatal environment manipulations have found gender dependent effects on anxiety behaviours in both H and MS conditions, and as may be expected, the extent of behavioural differences observed are dependent on the comparison group as well as the stressor

paradigm used. For example, compared to TR rats, anxiety behaviours of adult MS male rats were unchanged but were decreased in MS females in a defensive withdrawal test (Eklund and Arborelius, 2006). In contrast, when examined in an elevated plus maze test and compared to the TR condition, MS male rats were slightly more anxious, while MS females showed decreased anxiety (Renard et al., 2005), but in comparison to the NH condition, both MS males and females showed decreased exploratory behaviours (Kalinichev et al., 2002). Conversely, compared to NH rats, the H condition had an adverse effect in females, but not males, as shown by increased immobility behaviour in a chronic forced swim paradigm (Papaioannou et al., 2002). Overall, it is presently unclear at this stage whether one gender is more susceptible than the other to the effects of neonatal manipulations.

In addition to the differential influence of gender on the effects of neonatal environment on anxiety behaviours, the age at which these behavioural differences occur may also differ between genders. Research comparing anxiety behaviours of pubertal and adult rodents is limited. Overall, studies have demonstrated that pubertal rats showed more exploratory, novelty seeking, and play behaviours than adults, and thus may display higher activity and perhaps less anxiety-like behaviours in all novel testing situations (Laviola et al., 2003; Ray and Hansen, 2005). The few studies that have examined the interaction between neonatal conditions and age on anxiety behaviours have demonstrated that for pubertal (post natal day 28; PND28) males, MS rats exhibited increased activity (low anxiety) in a novel open field test compared to both H and TR rats, which showed similar levels of activity (Colorado et al., 2006). Moreover, when compared to NH rats, both pre-pubertal MS males and females (PND15) showed

increased activity in an open field test (Arnold and Siviy, 2002). In contrast, H peri-pubertal males (PND25-29) exhibited decreased anxiety/fear in a defensive-withdrawal test compared to MS and NH males, while no differences were observed between MS and NH conditions. At adulthood however, the researchers found no differences in anxiety behaviours among neonatal manipulation groups (Parfitt et al., 2004).

The interactive effects of neonatal manipulations, gender, and age at testing on behavioural outcomes are unclear. The present study explored the effects of early dam-pup environments on coping and anxiety behaviours (measured by the modified forced swim test) of both male and female mice at puberty and at early adulthood. In the present study, Balb/c mice were subjected to H (15min/day from PND 2-22) and MS (4 hr/day from PND 2-22) manipulations, and the behavioural effects of these two neonatal manipulations were examined using a modified forced swim test, performed at two distinct development stages; puberty (PND 30-35) and young adulthood (PND 55-65) in both male and female mice.

3.2 Materials and Methods

Forced swim test

The forced swim test was administered between 0800h – 1100h and at two distinct stages, puberty (PND 30-35) or young adulthood (PND 55-65). All females were in behavioural estrus at the time of forced swim testing. Mice were individually placed in a clean, opaque cylindrical plastic container (22cm in diameter) containing approximately 1250ml of tap water at 28-30°C. After 1 minute of acclimatization to the water, animal behaviour was recorded at 20 second intervals, for 5 minutes in pubertal mice and 11 minutes in young adult mice; based on shorter test times for adolescent mice (Hansen-Trench and Barron, 2005). 'Immobility' was recorded if the mouse was not upright (on

its side) and made only small movements of the forepaws and/or tail in order to keep afloat. The amount of time spent immobile is a measure of the level of anxiety in the rodent, a high amount of time spent immobile is indicative of depressive behaviour and poor coping response (Hall et al., 2001; Porsolt et al., 1977). 'Swimming' was recorded if the mouse swam freely in the container with active use of forepaws and tail to propel the mouse forward either around the sides of the cylinder or in the centre (Hall et al., 2001). 'Struggling' was recorded if the mouse was actively pawing the walls of the container or lifting the forepaws above the surface of the water. Both swimming and struggling are considered adaptive (coping) behaviours, of which high levels indicate adaptation/coping to the novel environment (Cryan et al., 2005). Finally, 'active floating' was recorded when the mouse actively maintained a level floating position (as opposed to the immobile position where the mouse floated more on one side of its body, in a tilted position), typically in the center of the container, while making small forepaw and/or tail movements. During active floating, whisker movement and visual environmental surveying were typically observed. The forced swim test was performed with the experimenters blind to the neonatal condition experienced by each mouse. At the completion of the test, each mouse was removed from the water and dried with a clean towel.

Statistical analyses

Data from the forced swim test were normalized according to the number of observations recorded during the test period and were thus expressed as ratios of time a mouse spent exhibiting each behaviour. This allowed comparison of behaviours between

adult and pubertal mice, as they were subjected to the forced swim test for different durations of time (i.e., 6 minutes for pubertal mice and 12 minutes for adult mice).

Effects of age, treatment, and gender. To analyze the effects of neonatal manipulations, age at testing and gender on forced swim test behaviours, three-way analyses of variance (ANOVAs) for the factors of age (pubertal, adult), treatment (H, MS, TR), and gender were performed for immobility, swimming, active floating, and struggling behaviours.

Significance was set at $p<0.05$. Significant main or interaction effects were further analyzed using Newman-Keuls post-hoc comparisons. All statistical analyses were conducted using the statistical package STATISTICA 6 (Statsoft, USA). Data are presented as means $\pm$ S.E.M.

3.3 Results

Effects of Age (pubertal, adult), Treatment (H, MS, TR) and Gender on forced swim test behaviours.

The present study examined the effects of short (15 minutes/day; H) and long (4 hours/day; MS) periods of daily maternal separation (between PND2-22) on behaviours of Balb/c mice in a forced swim paradigm. The forced swim test was conducted at two developmental stages (i.e., puberty and adulthood) in both male and female mice to determine if age and gender differentially influenced the forced swim test behaviours of immobility, swimming, active floating and struggling among mice exposed to the H, MS, and TR neonatal manipulations.

Forced swim test behaviour:

Immobility. The significant main effects of Age ($F(1,100)= 48.86$, $p<0.0001$), Treatment ($F(2,100)= 7.61$, $p<0.001$), Gender ($F(1,100)= 5.31$, $p<0.05$) and significant interaction effects of Age x Gender ($F(2,100)=10.14$, $p<0.01$) and Age x Treatment x

Gender ($F(2,100)= 3.48$, $p<0.05$) indicated that from puberty to adulthood, immobility behaviour significantly decreased in males, in all neonatal manipulation groups ($p's<0.05$) (Figure 1). At puberty, immobility behaviour was moderately decreased in H males compared to both MS ($p=0.073$) and TR ($p=0.085$) males (Figure 1). However, by adulthood, immobility behaviours in males were similar among neonatal manipulation groups.

In females, immobility behaviour significantly decreased from puberty to adulthood in H females ($p=0.05$) and moderately decreased in TR females ($p=0.108$), but unlike other manipulation groups, immobility behaviour in MS females remained elevated from puberty to adulthood (Figure 1). Unlike in males, at puberty, immobility behaviours were similar among neonatally manipulated females, whereas at adulthood, MS compared to H females, displayed significantly more immobility behaviour ($p=0.006$) (Figure 1). This effect was primarily due to the elevated levels of immobility in MS female adults. Furthermore, gender differences were apparent only in pubertal MS mice, where MS males compared to females, displayed significantly more immobility ($p=0.02$).

Swimming. Significant main effects of Age ($F(1,100)=8.36$, $p<0.01$), Gender ($F(1,100)= 7.37$, $p<0.05$), Treatment ($F(2,100)=4.90$, $p<0.05$) and a significant interaction effect of Gender x Treatment ($F(2,100)=4.74$, $p=0.01$) were observed for swimming behaviour in neonatally manipulated mice. Overall, females displayed significantly more swimming behaviour than males, while adult compared to pubertal mice, exhibited significantly more swimming behaviour (Figure 2). Additionally, neonatal conditions significantly affected swimming behaviour, but only in males, such

that regardless of age, TR males displayed significantly more swimming than both H ($p=0.027$) and MS ($p=0.0002$) males (Figure 2). In females, swimming behaviour was similar among all neonatal manipulation groups. Similar to what was observed in immobility, gender differences were only apparent in the MS condition, such that MS females compared to MS males, displayed significantly more swimming behaviour ($p=0.004$) (Figure 2). This was primarily due to the difference in swimming behaviour between male and female MS pubertal mice.

Active floating. Significant main effects of Age ($F(1,100)=34.10$, $p<0.0001$) and Treatment ($F(2,100)=6.10$, $p<0.005$) and significant interaction effects of Age x Gender ($F(2,100)=4,39$, $p<0.05$) and trend towards Gender x Treatment ($F(2,100)=3.07$, $p=0.05$) effect indicated that overall, adult compared to pubertal mice exhibited significantly more active floating, (Figure 3). *Post hoc* analyses of Gender X Treatment interactions show that in male mice, neonatal manipulations significantly affected active floating behaviour such that regardless of age, TR males exhibited significantly less active floating compared to H ($p=0.003$) and MS ($p=0.0001$) males (Figure 3). In female mice, H compared to both MS ($p=0.044$) and TR ($p=0.022$) mice, displayed more active floating behaviour, regardless of age (Figure 3). Similar to observations in immobility and swimming behaviours, gender difference was only observed in the MS condition, such that MS males exhibited significantly more active floating than their female counterparts ($p=0.0003$) (Figure 3).

Struggling. A significant main effect of Age ($F(1,100)=8.77$, $p<0.01$) revealed that overall, struggling behaviour was significantly decreased from puberty to adulthood. Additionally, a significant main effect of Treatment ($F(1,100)=5.06$, $p<0.01$) indicated

that, regardless of age or gender, H compared to both MS ($p=0.044$) and TR ($p=0.044$) mice exhibited significantly more struggling behaviours (Figure 4).

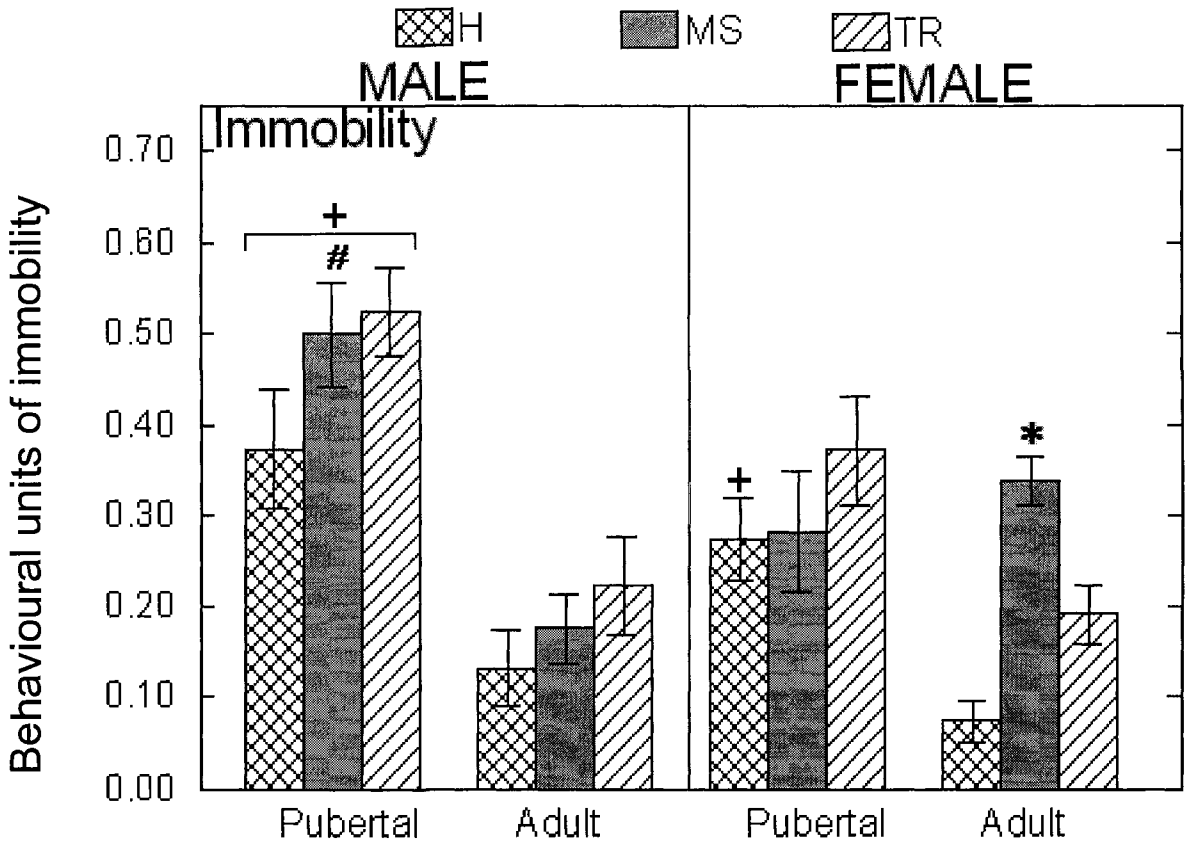


Figure 3.The influences of gender and developmental stage on mean (± S.E.M.) immobility behaviour among H, MS, and TR Balb/c mice. Y-axis units represent the ratio of time a mouse spent exhibiting a forced swim test behaviour. Immobility is a validated behaviour within the forced swim test that is reduced by administration of anti-depressants. Increased immobility is related to increased depressive-like behavioural tendencies in the forced swim test. Significance symbols: Age (+); Treatment (*); Gender (#); p's<0.05; *n* per group: 3- 17 mice.

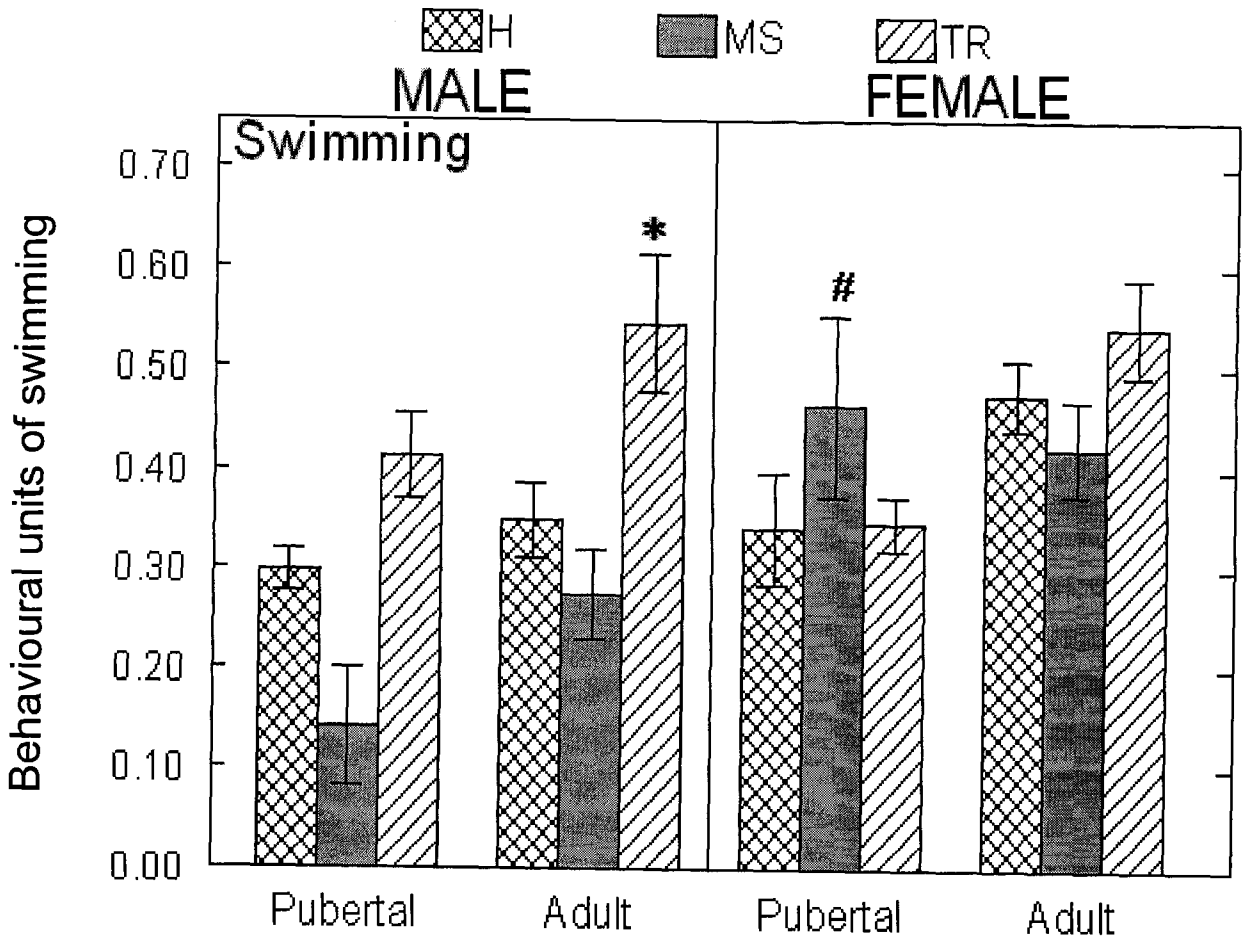


Figure 4.The influences of gender and developmental stage on mean (± S.E.M.) swimming behaviour in male and female H, MS and TR Balb/c mice. Significance symbols: Age (+); Treatment (*); Gender (#); p's<0.05. *n* per group: 3-17 mice.

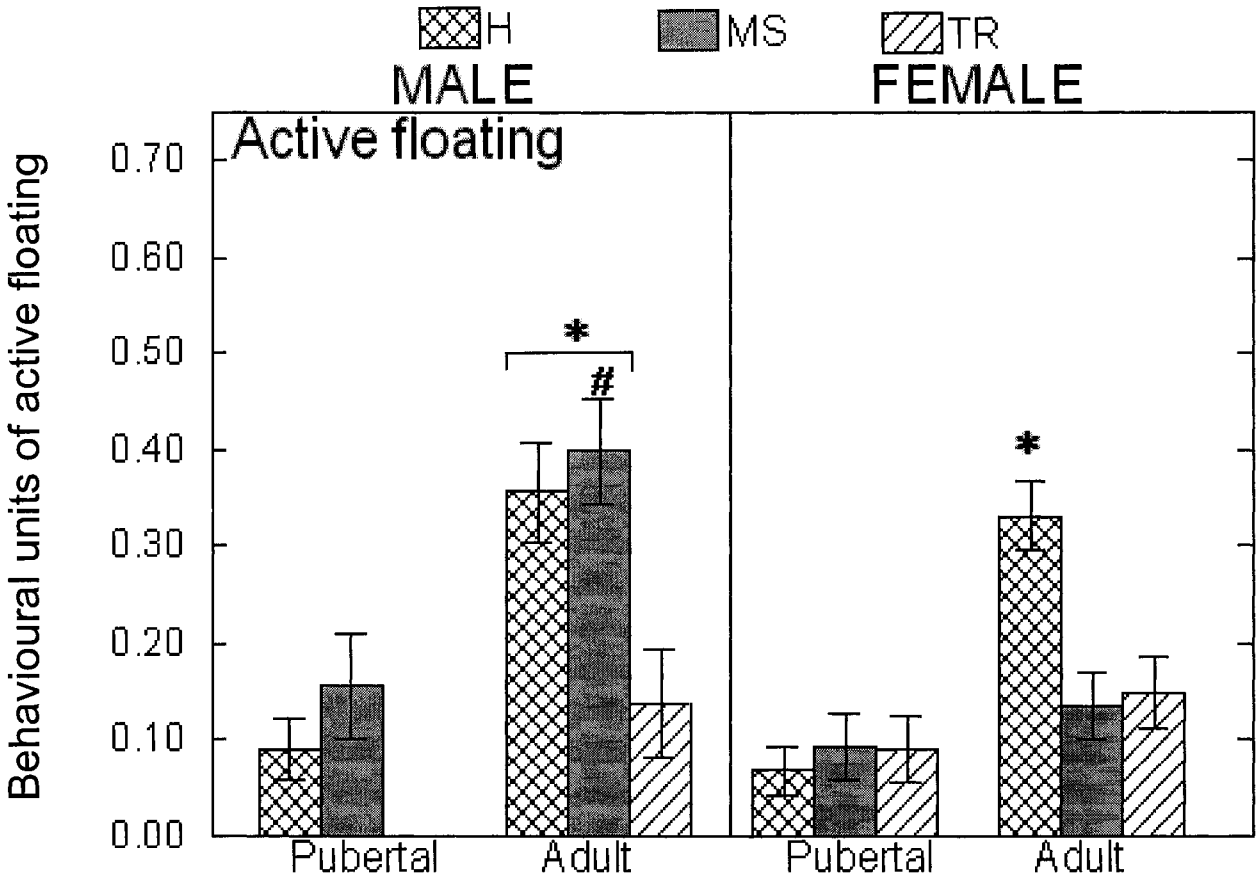


Figure 5.The influences of gender and developmental stage on mean (± S.E.M.) active floating behaviour in male and female H, MS and TR Balb/c mice. Significance symbols: Age (+); Treatment (*); Gender (#); p's<0.05. *n* per group: 3-17 mice.

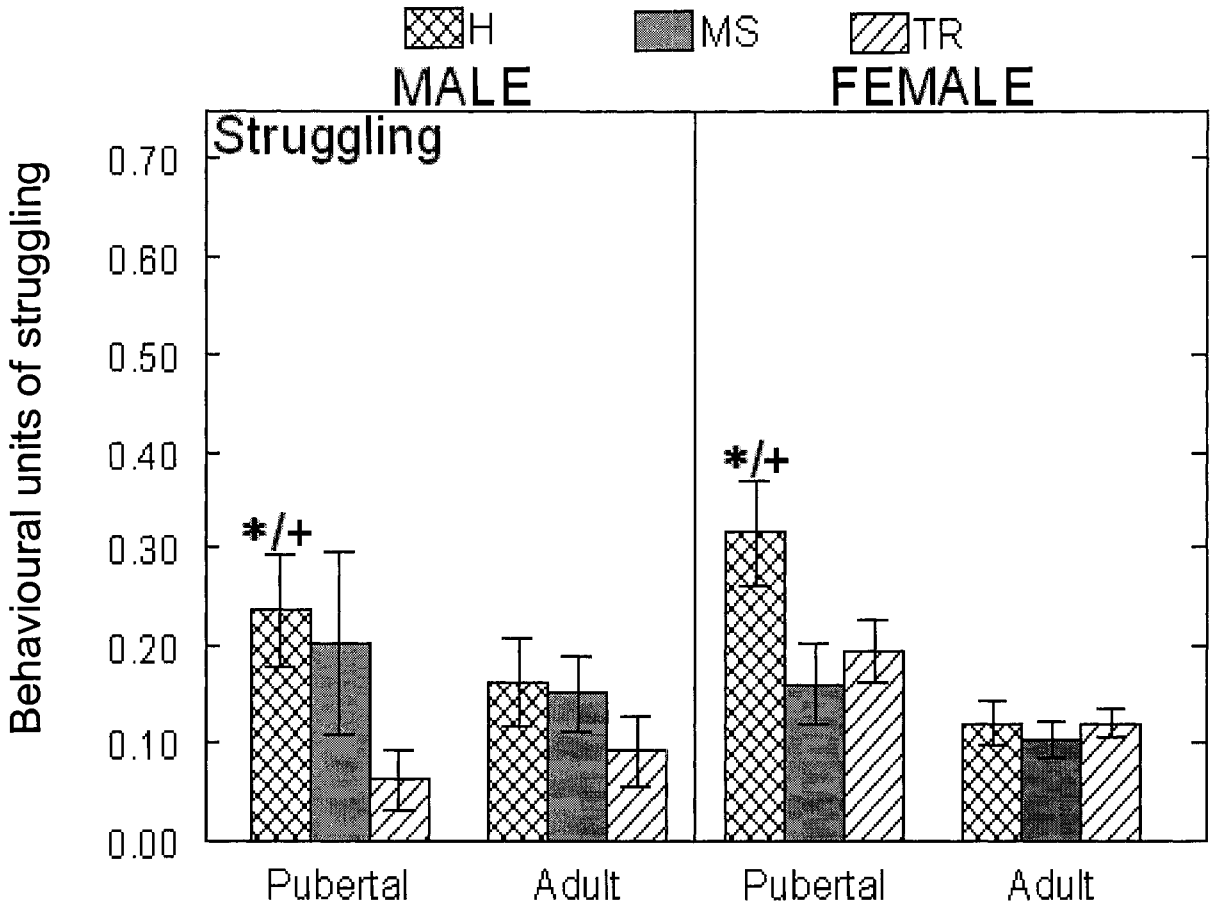


Figure 6.The influences of gender and developmental stage on mean (± S.E.M.) struggling behaviour in male and female H, MS and TR Balb/c mice. Significance symbols: Age (+); Treatment (*); Gender (#); p's<0.05. *n* per group: 3 to 17 mice.

3.4 Discussion

The present study demonstrates that gender and age at testing (pubertal or adult) differentially influence forced swim test behaviours among neonatal manipulated mice. Similar to other studies (Boccia and Pedersen, 2001; Caldji et al., 2000; Kalinichev et al., 2002), moderately higher levels of immobility (an indication of depressive behaviour) were observed in MS compared to H male mice. Unlike other studies, however, this difference was observed only at puberty. In females, immobility behaviour was significantly higher in MS compared to H females, but unlike in males, this difference was only observed in adulthood. The active coping behaviours of swimming, active floating and struggling were also significantly influenced by neonatal manipulations, and these differences were dependent on gender and the active coping behaviour being observed.

The present study also demonstrates that gender differences in forced swim test behaviours were only observed in the MS condition, such that females exhibited significantly more swimming and significantly less active floating behaviours compared to their MS male counterparts. In terms of depressive behaviours (i.e., immobility), gender differences were observed only at puberty, such that MS males compared to MS females displayed significantly higher levels of immobility.

Overall, the data of the present study demonstrate that neonatal environmental conditions and gender interact to differentially influence force swim test behaviours in Balb/c mice and that similar to the human condition, these behavioural effects are also dependent on the age at which the animal was tested.

In addition to typically reported forced swim test behaviours, we documented a novel behaviour which we termed "active floating". This behaviour is characterized by small forepaw and tail movements by the mouse in order to maintain an upright and stable floating position (typically in the center of the container; i.e., thigmotaxis was rarely observed in this behaviour), which was also accompanied by whisker movements and visual surveying of the environment. Although limited movement by the mouse is observed in both active floating and immobility, there are significant behavioural differences between the two actions. For immobility, the mouse is considered immobile when it makes only enough movements to stay afloat, usually in a body position that is not upright (i.e., floating on one side of its body), which is typically accompanied by thigmotaxis. Immobility behaviour is suggested to be an indication of behavioural depression, whereas on the other hand, we believe that active floating does not reflect increased depressive-like behaviours as it involves higher energy expenditure and active surveying of the environment; behaviours not observed in immobility. Instead, active floating, like struggling and swimming, may be a form of active coping behaviour that increases the chances of escape from a stressful and novel situation (Cryan et al., 2005). More importantly, immobility typically was not observed directly after active floating (or vice versa) during the forced swim test, suggesting that the two behaviours are indeed distinct. To the best of our knowledge, only one other paper (Belozertseva et al., 2006) has characterized a similar behaviour, which the authors refer to as an active/mobile behaviour in rats subjected to a classical forced swim test. Future research will focus on further characterizing this behaviour in mice to distinguish it from immobility, and to examine if active floating is reproducible in different mouse strains.

The significant differences in active coping and depressive behaviours from puberty to adulthood was expected as studies have shown that pubertal mice generally exhibit increased stress-induced anxiety, novelty-seeking, and risk-taking behaviours compared to their adult counterparts (Laviola et al., 2003; Ray and Hansen, 2005; Romeo et al., 2004; Slawecki et al., 2005). Moreover, one study showed that even across various stages of adolescence (i.e., early, late), differences in depressive behaviours and fear conditioning were observed in mice (Hefner and Holmes, 2007). The higher levels of depressive (immobility) behaviour in pubertal compared to adult mice can be related to changes in HPA activity from puberty to adulthood. It has been observed that both male and female peri-pubertal (DOA28) rats had elevated HPA stress-induced reactivity and thus extended stress-induced CORT response compared to adult rats (Romeo et al., 2005; Romeo et al., 2006). In pubertal rodents, the developing HPA axis is exposed to increased levels of gonadal hormones which influence HPA activity and neural development (Severino et al., 2004). This highly changing hormonal environment in addition to the known effects of neonatal manipulations on neural development may interact to influence brain/HPA activity in pubertal mice, thereby increasing reactivity to stressors in pubertal rodents (Kellog et al., 1998).

The present study demonstrates that H and MS manipulations produce gender dependent effects in males and females. The finding that immobility is significantly higher in MS than in H adult female mice is in agreement with results of previous studies in female rodents, using other tests for depressive or anxiety-related behaviours (Kalinichev et al., 2002; Wigger and Neumann, 1999). However, this effect was not observed in pubertal females. In females rodents, studies have shown that after puberty, the surge of ovarian hormones stimulate HPA function (Romeo et al., 2004) and can exert

either anxiolytic or anxiogenic effects depending on the perceived threat of the situation (Hiroi and Neumaier, 2006; Morgan et al., 2004). For example, if the animal is in a situation it deems as safe (such as in the closed arms of an elevated plus maze), estrogen can stimulate HPA function and reduce both anxiety and depressive-related behaviours (Morgan et al., 2004). Similarly, estradiol administration has been shown to decrease immobility times in rodents subjected to a forced swim test, suggesting that estrogen is capable of inducing anti-depressant effects (Estrada-Camarena et al., 2006).However, in a more threatening scenario such as the forced swim test, the stimulation of the stress axis by estrogen may increase anxiety, fear and depressive behaviours (Hiroi and Neumaier, 2006; Morgan et al., 2004). In the present study, females were tested only during behavioural estrus, where the levels of circulating estrogen are high, but decreasing (Hiroi and Neumaier, 2006). Hence, changes in HPA reactivity due to the effects of the H and MS manipulations (Anisman et al. 1998; Plotsky and Meaney, 1993) may interact with the stimulatory effects of estrogen to influence stress-induced behaviours in female mice. The stimulatory effects of estrogen may also be more apparent in adult than in pubertal mice, since compared to adult females, pubertal females have just begun to experience estrous cycles and thus have not been exposed to high levels of estrogen for a long period of time. Interestingly, the finding that immobility behaviour was significantly different in H and MS adult, but not pubertal female mice is similar to what is observed in humans such that in women, depression and anxiety-related disorders are diagnosed more frequently in young adults compared to adolescent females (Piccinelli and Wilkinson, 2000). Other researchers have observed different behavioural effects of neonatal manipulations compared to what was found in the present study, which may have been due to differences in species, separation schedules, and the stage of estrous cycle at

testing. For instance, one study using mice, found that compared to TR mice, MS (3 hours/day; PND1-14) mice in diestrus (at a time was estrogen levels are low) showed reduced anxiety in the open field test but not in the elevated plus maze, whereas MS mice in behavioural estrus exhibited similar levels of anxiety in both the open field test and elevated plus maze compared to TR controls, suggesting an influence of estrous cycle on anxiety (Romeo et al., 2003). In addition, unlike other mouse strains, the Balb/c mice used in the present study are known to show high levels of anxiety and neophobia (Anisman et al., 1998; Caldji et al., 2000; Priebe et al., 2005). Therefore, it is possible the genetic predisposition of Balb/c mice to anxious behaviour could have augmented the behavioural effects of MS in females thus increasing anxiety behaviours, not seen in other rodent species or strains in previous studies.

Unlike observations in female mice, differences in depressive-like behaviours in male mice between H and MS conditions were apparent at puberty, such that MS compared to H male mice displayed moderately increased immobility. At adulthood however, and in support of some studies (Millstein and Holmes, 2006; Parfitt et al., 2004), immobility behaviour was similar between H and MS males. These findings can be paralleled to the human condition as a higher prevalence of depression and anxiety are detected in adolescent than in adult men (Sisk and Zehr, 2005). However, studies of H and MS manipulations on adult male rodents have regularly found that the H condition reduces stress-induced anxiety and depressive behaviours with a concomitant decrease in HPA reactivity (Anisman et al., 1998; Plotsky and Meaney, 1993). That is, H adult male rodents typically exhibit decreased stress-induced reactivity in the elevated plus maze, open field test and chronic forced swim test (Caldji et al., 2000; Meerlo et al., 1999; Papaioannou et al., 2002; Severino et al., 2004), accompanied by lower stress-induced

levels of ACTH and CORT relative to NH animals (Anisman et al., 1998; Pryce and Feldon, 2003). The differential behavioural observations in male mice in our study compared to previous research may have been a result of the nature of the behavioural test used, both in terms of the severity of the stressor and the 'type' of stress-induced behaviour being measured. It has been suggested that more aggressive tests are able to bring out greater behavioural differences between treatment groups (Meerlo et al., 1999).

Active coping behaviours were also differentially influenced by neonatal manipulation and gender. Regardless of age, active floating was higher in H compared to MS and TR females, whereas in males active floating was higher in H and MS compared to TR males. In contrast, swimming behaviour was significantly different only in male mice such that regardless of age, swimming was lower in H and MS compared to TR males. The differences observed in behavioural profiles among male and female H and MS mice may be due to the fact that behavioural tests do not always elicit similar behavioural repertoires in males and females. This is expected as males and females have different social and reproductive roles and hence may show differences in adaptation to stressful situations (Palanza, 2001), suggesting that similar to humans (Piccinelli and Wilkinson, 2000), male and female mice employ different coping styles when exposed to the same stressor (Palanza, 2001). Moreover, the single exposure to the swimming pool in the modified forced swim test employed in the present study may have elicited a different set of behavioural outcomes due to the novelty of the test situation, compared to the double exposure in a traditional forced swim test used by other researchers (MacQueen et al., 2003; Papaioannou et al., 2002). In addition, comparison between male and female behavioural profiles demonstrate that gender-dependent forced swim test behaviours are only observed in MS mice such that at puberty, MS males had significantly higher levels

of depressive-like behaviour (immobility) than MS females. In terms of active coping, and regardless of age, MS females compared to males, displayed significantly more swimming, but significantly less active floating. These finding suggest that differences in depressive behaviour and coping styles between genders become more apparent when the paradigm used involves a prolonged period of maternal separation.

For the mouse pup, maternal interactions serve as the majority, if not the only stimuli during the neonatal period (Pryce et al., 2005) and hence, any disruption or change to the dam-pup relationship has a strong impact on the developing pup. Not only is the total amount or quality of maternal care received by the pup important, but the consistency of the care throughout the day as well as during the entire neonatal development period may play a part in the development of stress-induced coping behaviours. In the present study, all litters were culled to a ratio of 60% females to 40% males since it has been suggested that dams spend more time caring for male than female pups (Moore and Morelli, 1979). However, the effects of litter culling on maternal behaviours are not well understood. Furthermore, some evidence suggests that the H and MS manipulations influence amount, quality, and timing of dam-pup interactions (Boccia and Perdersen, 2001; Liu et al., 1997), which can alter the development of neurobiological systems (such as the HPA axis) in the maturing offspring and also subsequently influence behaviour (Liu et al., 1997; Pryce et al., 2005). In our study, unlike the H pups that experience individual, brief (15 minute) separation from the dam (Caldji et al., 1999; Meerlo et al., 1999, Millstein and Holmes, 2007) the 4 hour daily absence of maternal care experienced by MS pups disrupts the physical (warmth, food) and possibly the emotional (nurturing, grooming) needs of the pups (Pryce et al., 2005). Thus, it may be and some researchers suggest that the increased level or type of maternal

care received by H pups may result in offspring that are less reactive, both physiologically and behaviourally, to stressful situations (Anisman et al., 1998; Plotsky, et al., 1993; Pryce et al., 2005). These findings may explain why in the present study, gender dependent effects were mainly observed in MS and not H mice. It should also be noted that some researchers have found no differences between maternal behaviours of H and MS dams (Millstein and Holmes, 2007; Romeo et al., 2003), which suggest that other influences such as species and separation paradigms, may be responsible for the observed behavioural outcomes of the neonatally manipulated offspring (Anisman et al., 1998; Priebe et al., 2005; Shoji and Kato, 2006; Zaharia et al, 1996).

The findings of the present study lend further support to the influences of neonatal environments on rodent behaviour, and suggest that the effects of H and MS neonatal conditions on modified forced swim test behaviours are dependent on gender and stage of development in Balb/c mice. The present study further elaborated on the effects of H and MS neonatal manipulations and demonstrated that regardless of the form of neonatal manipulation, coping abilities were more developed in adult than in pubertal mice (reflected by differences in active coping behaviours), suggesting that these skills develop over puberty and into adulthood. Furthermore, gender dependent effects distinctly emerge when mice are subjected to neonatal conditions that involve protracted removal from maternal care, as was the case for mice in the MS condition, evident from differences in depressive and active coping behaviours between MS male and female mice.

Chapter 4: Neonatal Conditions Differentially Influence Adult Mammary Gland Morphology in Female Balb/c Mice

4.1 Introduction

Psychosocial factors have long been linked to breast cancer risk, such that personality type, various life experiences and depression have been associated with the development of the disease (Hilakivi-Clarke, 1997a, Jacobs and Bovasso, 2000). In humans, individuals who are more prone to breast cancer may have poor coping abilities (sense of hopelessness/ depressive symptoms), are anti-emotional, and experience difficulty in expressing hostility (Hilakivi-Clarke, 1997a; Temoshok, 1987). In addition, severe life events and stressful daily activities have also been associated with increased breast cancer risk (Butow et al., 2000; Jacobs and Bovasso, 2000 Kruk and Aboul-Enein, 2004). However, the relationship between psychological and behavioural characteristics and breast cancer risk are not well developed as it is unclear whether the impact on breast cancer risk is indirect, such as via changes in other behaviours (e.g., sleep patterns, exercise habits, diet) or direct, such as through changes in the neuroendocrine or immune systems (Butow et al., 2004). Furthermore, human studies typically assess stressful life events using questionnaires (e.g., Life Events and Difficulties Schedule; LEDS), which may be biased as they are dependent on the mood or changes in personality of the individual at the time of survey completion (Butow et al., 2004), in addition to being retrospective and thus increasing the possibilities of recall error or bias (Butow et al., 2004; Ollonen et al., 2005). Consequently, it is not surprising that studies examining the relationship between breast cancer risk and coping styles or stressful life events yield inconsistent results (Duijts et al., 2003; Nielsen and Gronbaek, 2006).

One possible mechanism through which psychosocial factors may affect breast cancer risk is by affecting mammary gland growth and differentiation. The mammalian mammary gland is one of a few organs that undergoes the majority of its development after birth; specifically at puberty, pregnancy, lactation, and menopause (Clarke, 2006; Russo and Russo, 2004). Hence, its growth is highly influenced by fluctuations of hormones and other growth factors that affect its development, including prolactin, glucocorticoids, and the classical mammogens: progesterone and estrogen, all of which are influenced by activity of the hypothalamic-pituitary-gonadal (HPG) axis (Cardiff and Wellings, 1999; Russo and Russo, 2004; Tsigos and Chrousos, 2002). Thus, many physiological risk factors that are associated with alterations in hormone levels and/or prolonged exposure to hormones have been identified to influence breast cancer risk, including age at menarche, menopause, or first pregnancy, as well as timing and number of pregnancies (de Waard and Thijssen, 2005; Dumitrescu and Cotarla, 2005; Hilakivi-Clarke et al, 2002). For example, past research found that mammary gland morphology and cellular activity are different between women with high breast cancer risk (e.g., nulliparous women) compared to lower risk groups (e.g., early first pregnancy, multi-parity) (Russo and Russo, 2004). Aside from these physiological factors, psychosocial factors may also influence breast cancer risk in a similar manner by influencing hormone levels and subsequent mammary gland proliferation via action of the HPA axis; the body's main stress axis (Tsigos and Chrousos, 2002). For example, increased corticotrophin releasing hormone (CRH) and glucocorticoid production via HPA axis activation can suppress HPG functioning by reducing gonadotropin releasing hormone (GnRH) release, or via direct suppression of the gonads themselves, which reduces circulating levels of reproductive hormones (Tsigos and Chrousos, 2002). Alternatively,

glucocorticoid interaction with its receptors in the mammary gland can also directly influence mammary gland development (Silberstein, 2001).

Since many extraneous and confounding variables are present in human studies examining the link between breast cancer risk and life experiences, an animal model is desirable due to the ease in controlling experimental environments and examining physiological changes (e.g., mammary gland morphology, protein/RNA expression, hormone levels). In these studies, the animals are typically manipulated (holding/stroking the animal, injection with a vehicle (Hilakivi-Clarke, 1997a) to simulate differences in life experiences and the resulting impact of these manipulations on physiological variables (hormone levels, induced-tumour growth) are measured. In one such study, female rat pups handled (daily injection with a drug-vehicle) for the 2nd and 3rd post natal week had a decrease in DMBA-induced tumour initiation and tumour growth rate compared to non-handled rats, suggesting that experiential differences, related to handling at an early age, could profoundly influence mammary tumour growth (Hilakivi-Clarke, 1997b). However, in contrast to what is known about changes in hormonal control and cell-cycle regulation that occur once tumour initiation has begun, the developmental environment of the normal mammary gland that influences its susceptibility to tumour development is less known (Anderson and Clarke, 2004). Therefore, an examination of the normal developmental environment of the mammary gland is necessary in order to understand the mechanisms involved in any cellular changes that occur in the gland, which may predispose it to tumourigenesis. In the present study, the early life environments of mice are manipulated to assess the impact of these differing environments on normal mammary gland growth and subsequent cancer risk.

As discussed in Chapters 1 and 3, well-established models of rodent H and MS neonatal manipulations are utilized in the present study to investigate the effects of differing neonatal environments on mammary gland morphology. These neonatal manipulations affect stress-induced behaviours (mediated by alterations in the development of the HPA axis) in rodents. Aside from the role of the HPA axis in affecting stress-induced behaviours, the activation of the HPA axis and subsequent increase in hormones, primarily corticosterone (CORT), can influence physiological processes such as immune system development, regulation of gonadal and growth hormones and regulation of receptor densities in various organs (Tsigos and Chrousos, 2002). These physiological processes in turn, affect the development of organs, especially those that develop post-natally.

Since H and MS neonatal manipulations differentially influence HPA axis development and subsequent reactivity to stressors, influencing the level of circulating CORT, mice subjected to these neonatal manipulations may exhibit morphological differences in growth and differentiation of the mammary gland attributable to hormonal changes that occur, during development. Alterations in the growth and differentiation of the mammary gland can be examined through gross morphological changes (such as terminal end bud counts and branching patterns) as well as through examination of protein and RNA expression levels and profiles, factors that directly influence the overt morphological changes in the mammary glands. Consequently, morphological differences in the mammary glands of mice between neonatal manipulation groups may indicate altered risk of tumour initiation via changes in protein and/or RNA expression profiles. Since the human and rodent mammary glands are structurally similar and contain analogous structures (Cardiff and Wellings, 1999) some aspects of the mouse mammary

gland development can be paralleled to that of the human condition. Thus, the aim of the present study was to identify morphological differences in mammary gland development between the H and MS neonatal manipulations groups (two groups that are known to show distinct differences in HPA development and anxiety behaviours), and to investigate whether differences in mammary gland development can be related to subsequent risk of tumour initiation.

Mammary gland morphology and breast cancer susceptibility

Several studies have indicated that differences in mammary gland morphology, (primarily differences in lobular composition and structure) between women may be a factor affecting susceptibility to breast cancer (Russo and Russo, 2004; Russo et al., 2005). However, how mammary gland lobular composition influences tumour development risk is not well-understood. The Russo group have suggested that the risk of tumourigenesis in the mammary gland is related to the degree of differentiation of the mammary structures, which is most evident in the lobular composition between parous and nulliparous women. Since the most frequent site of tumourigenesis is in less differentiated TDLUs or lob1 structures (Cardiff and Wellings, 1999; Fenton, 2006; Hilakivi-Clarke, 2006; Russo and Russo, 1998), the mammary glands of nulliparous women, which contain significantly more undifferentiated and more proliferative lob1 structures, may be more susceptible to tumourigenesis compared to their parous counterparts. In addition to the level of differentiation, proliferation activity between lobular structures may influence susceptibility to tumourigenesis. For example, immunohistochemical studies show that lob1 compared to lob2 or lob3, have significantly higher proliferation indices in both parous and nulliparous women, and that the same structure (e.g., lob1) between nulliparous and parous women can have different

proliferative indices (Russo and Russo, 2004). Another factor related to lobular composition that may influence mammary gland tumourigenesis risk is the cellular activity of various lobular structures. For example, proliferative capability of lob1 have been demonstrated *in vitro* such that when exposed to chemical carcinogens (e.g., DMBA), the cells in lob1structures have a shorter cell division time and faster growth rate, increased survival and multi-nucleation compared to lob3 structures (Russo et al., 2001; Russo et al., 1989). Similarly, mouse TEBs, are more sensitive to the effect of carcinogens than LAUs (Russo and Russo, 1987). For example, higher TEB counts are associated with an increase in the number of tumours in rodents exposed to chemical carcinogens (Fenton, 2006; Russo and Russo, 1987). These findings of how differences in mammary gland morphology may be related to cancer risk point to the importance of examining the processes that are involved in the transition from normal mammary gland development to neoplasia, and that differences in mammary gland morphology can be used to infer the cellular activity, proliferation and differentiation status of the mammary gland.

The majority of breast cancer cases are spontaneous and not associated with specific genetic mutations (Lacey et al., 2002; Radford and Zehnbauer, 1996). Instead, they are attributed to epigenetic changes arising from differences in a number of factors including physiological (e.g., parity status, age at puberty and pregnancy) and lifestyle factors (i.e., psychosocial factors, diet, exercise), which may induce subtle changes in the developmental environment of the mammary gland, subsequently influencing breast cancer risk. The present study examines one of the factors that may induce these subtle changes, namely the influence of early-life events/experiences on mammary gland growth. In the present study, mammary gland growth and development were assessed in

mice subjected to the H and MS neonatal conditions, two manipulations known to affect HPA reactivity (and stress-induced CORT levels) (Pryce and Feldon, 2003). Since CORT directly interacts with the HPG axis as well as the mammary gland itself via glucocorticoid receptors (Tsigos and Chrousos, 2002), it was expected that the mammary glands of H compared to MS mice would be morphologically different due to changes in circulating levels of gonadal hormones. The increased HPA reactivity in MS mice may cause growth suppression of the mammary gland due to CORT-induced HPG axis inhibition and subsequent decrease in estrogen production (Tsigos and Chrousos, 2002). Since differences in mammary gland morphology are associated with differentiation and proliferation activity of mammary gland structures, assessment consisted of examination of TEBs, LAUs and branching, which was conducted at two developmentally important stages in the mouse, puberty (PND30-35) and young adulthood (PND55-65).

4.2 Materials and Methods

4.2.1 Tissue preparation and carmine alum staining

Immediately following the forced swim test, mice were decapitated and the 4th and 5th left inguinal glands of pubertal and adult female mice from each of the H, MS and TR treatment groups were dissected, stretched out onto a clean glass slide and immersed in a 25% glacial acetic acid solution in 95% ethanol overnight. Following this step, mammary glands were immersed in xylene for 1 to 3 days to de-lipidize the adipose rich glands. When glands appeared translucent (indicating that most adipose tissue had dissolved), they were re-hydrated in serial ethanol solutions in the following order: 95% ethanol for 1 hour, 90% ethanol for 1-2 hours followed by 70% ethanol immersion for overnight. Mammary glands were then ready for carmine alum staining. The carmine stain was

prepared by boiling 1g of carmine and 2.5g of aluminium potassium sulphate in 0.5 litres of water. The mixture was then cooled, and filtered into a clean glass bottle. Alum thymol (0.05g) was then added as a preservative. Mammary glands were immersed in carmine alum for between 6-18 hours, until consistent colour development of all structures within the mammary gland was evident. Following staining, mammary glands were de-stained and serially dehydrated in 3 ethanol washes (70% ethanol for 2 hours, 90% ethanol for 2 hours and 95% ethanol for 2 hours) and immersed in xylene for 1-3 days until mammary glands again appeared translucent. The mammary glands were then mounted using Permount mounting medium (Fisher Biotechnology, Canada) and coverslipped.

4.2.2 Terminal end bud (TEB) and lobular alveolar unit (LAU) counts

TEB and LAU counts were performed under a dissecting microscope (Wild, Switzerland). TEBs are large club-shaped structures, composed of body cells and cap cells that have high differentiation capability, and are the site of ductal proliferation (Russo and Russo, 2004). Structures that corresponded to this club-like description were classified as TEBs. TEBs in each mammary gland were counted blind to the treatment group, three times using a mechanical counter, and the average number of TEBs across counts was then recorded.

Due to the high numbers of LAUs in the mouse mammary gland, a procedure adapted from Olivo and Hilakivi-Clarke (2005) was used to determine the density of LAUs. A scale of 0-5 (0, absent; 1, low; 2, low–moderate; 3, moderate; 4, moderate–high; 5, high) was used to indicate the relative density of LAUs in each mammary gland. Similar to TEB counts, LAU counts were also performed three times and the average value recorded. In order to minimize observational bias when performing these counts, the researcher was blind to the identity of each mammary gland whole mount.

4.2.3 Determination of LAU/TEB ratio as a marker of differentiation rate

In the mouse mammary gland, immature TEB structures, consisting of undifferentiated cells are most abundant during puberty. As the mammary gland matures, TEBs proliferate and differentiate into a network of more mature ducts and lobules, of which the functional unit is termed the lobular alveolar unit (LAU). As a result, adult mouse mammary glands consist primarily of LAUs, and less TEBs (Russo and Russo, 2004, Silberstein, 2001). A ratio of the LAU density to the number of TEBs was determined for each mammary gland, as an indication of the rate of differentiation for the particular mammary gland. Since the number of TEBs should decrease from puberty to adulthood in the mouse mammary gland (Anderson and Clarke, 2004), a higher TEB count would result in a low LAU/TEB ratio, and may point to a lower rate of proliferation in the mammary gland. Conversely, a high LAU/TEB ratio, due to a lower number of TEBs, would signify increased proliferation in the mammary gland.

4.2.4 Branch point analysis

Images of pubertal and adult inguinal mouse mammary glands from each treatment condition were captured under a dissecting microscope (Wild, Switzerland) using a digital camera (Nikon, USA). These images were then analyzed for the number of branch points using image software (Adobe Photoshop 9.0, USA). By using a 5cm x 5cm grid available in the image software, the number of branch points in 10 representative quadrants of each mammary gland was quantified. The total number of branch points in all 10 quadrants per mammary gland, as well as the average number of branch points per quadrant was recorded for each pubertal and adult mammary gland.

4.2.5 Statistical analyses

Preliminary descriptive analysis of distribution, skewness and kurtosis indicated that the data set did not violate the assumptions of normality.

Effects of Treatment and Age. To determine the effects of neonatal manipulations and age on mouse mammary gland morphology, a two-way analysis of variance (ANOVA) for the effects of Treatment (H, MS, TR) and Age (pubertal, adult) was performed for each marker of mammary gland growth; TEB, branch point counts and LAU/TEB ratio. Analyses of TEB data are shown in Appendix B.

Significance was set at $p<0.05$. Significant main or interaction effects were further analyzed using Newman-Keuls post-hoc comparisons. All statistical analyses were conducted using the statistical package STATISTICA 6 (Statsoft, USA). Data are presented as means $\pm$ S.E.M.

4.3 Results

4.3.1 LAU/TEB ratio

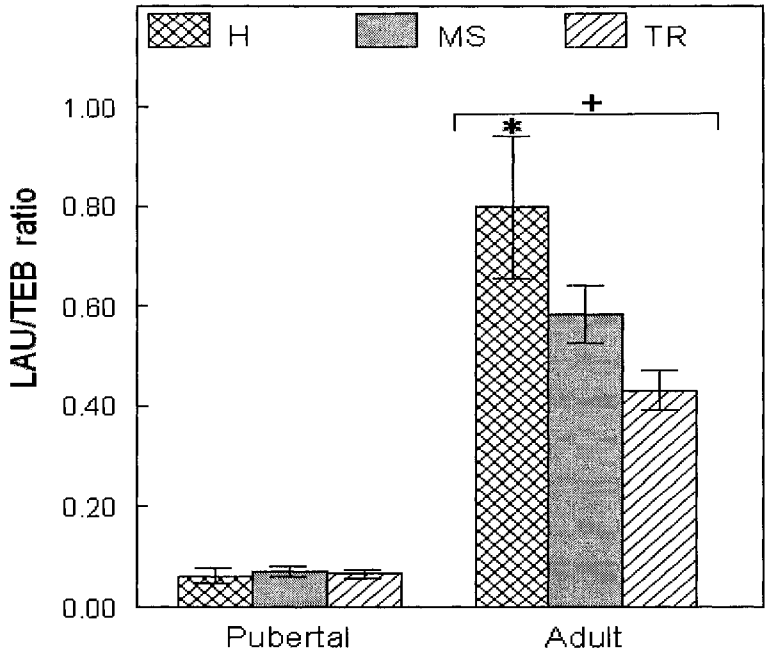
The LAU/TEB ratio was calculated as an indication of the rate of mammary gland differentiation from less differentiated TEB structures, to the more differentiated LAUs in each mammary gland. Hence, a higher LAU/TEB ratio is indicative of a more differentiated mammary gland. A Treatment x Age ANOVA revealed significant main effects of Age ($F(1,76)= 83.14$, $p<0.001$), a moderate effect of Treatment ($F(2,76)=3.02$, $p=0.05$), and a significant Treatment x Age interaction effect ($F(2,76)=3.17$, $p<0.05$). Posthoc analysis of the interaction revealed that as expected, adult mice had significantly higher LAU/TEB ratios compared to pubertal mice, regardless of neonatal manipulation group, and that neonatal conditions only significantly influenced adult, but not pubertal LAU/TEB ratios (Figure 5A). That is, mammary glands of H adult females had

significantly higher LAU/TEB ratios than both TR ($p=0.002$) and MS ($p=0.041$) mice (Figure 5A). This indicated that the mammary glands of H mice were significantly more differentiated than those of MS and TR mice.

4.3.2 Branch point analysis

A branch point analysis was conducted to determine the number of branching bifurcations in each mammary gland sample, as an approximation of mammary gland growth. A Treatment x Age ANOVA on the number of branch points in mouse mammary glands indicated that age significantly influenced branching in the mouse mammary gland ($F(1,53)=210.45$, $p<0.0001$), regardless of neonatal condition. That is, adult mammary glands contained significantly more branch points than pubertal glands (Figure 6A). However, neonatal conditions did not significantly affect the number of branch points in either pubertal or adult mammary glands (Figure 6A, 6B).

A.



B.

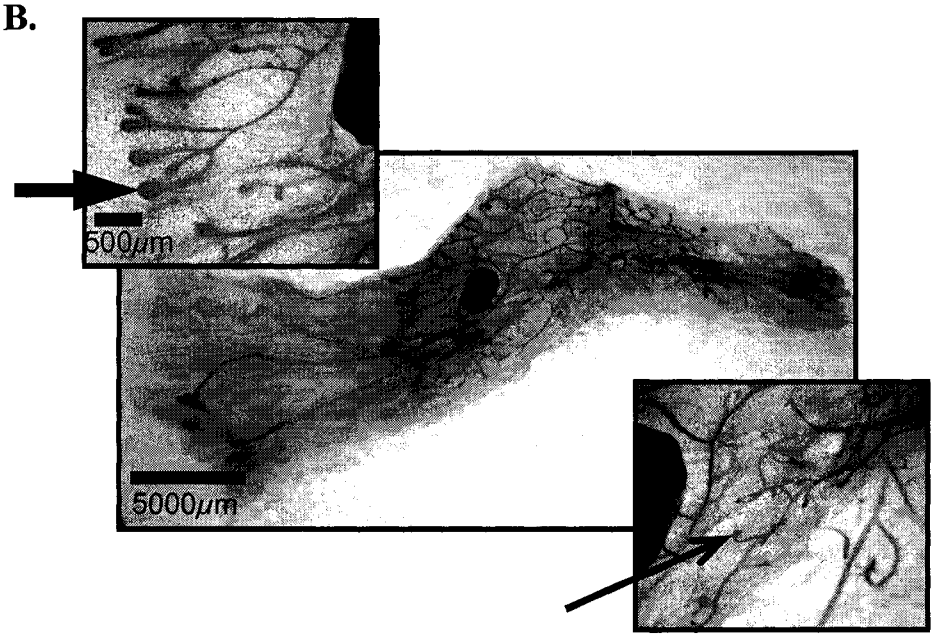
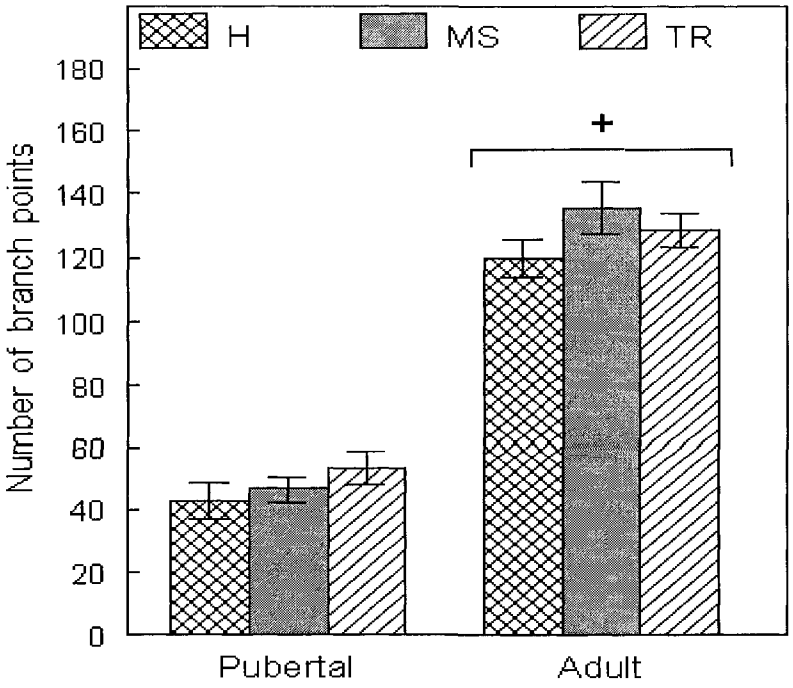


Figure 7.A: Mean (± S.E.M) LAU/TEB ratio for pubertal and adult H, MS and TR female Balb/c mice. The LAU/TEB ratio was calculated to approximate the degree of mammary gland differentiation. Significance symbols: *, $p<0.05$; effect of neonatal manipulation; +,$p<0.01$, effect of age.
n **per group: 11-19 mice. B: Representative pictures of TEB and LAU in a pubertal mouse mammary gland. Thick arrow points to a TEB; thin arrow indicates an LAU. Whole mammary gland photo taken at 6X magnification; inset pictures are at 10X magnification.**

A.



B.

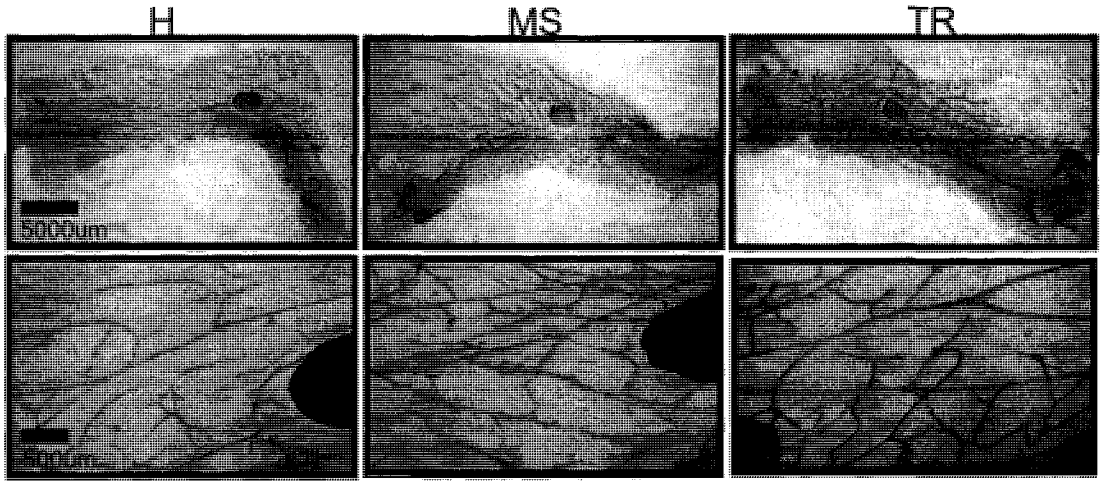


Figure 8.A: Mean (± S.E.M) branch points in mammary glands of pubertal and adult H, MS and TR female Balb/c mice. Branch points in 10 random sections were counted for each mammary gland. The number of branch points was significantly higher in adult than pubertal mammary glands, regardless of neonatal manipulation group (+, p <0.001). *n* per group: 11-19 mice. B: Representative pictures of branching patterns in the mammary glands of H, MS and TR adult female mice, at 6X magnification (top row) and 10X magnification (bottom row).

4.4 Discussion

The present study demonstrates that manipulating neonatal conditions in mice influences mammary gland development resulting in significant changes in mammary gland morphology, especially during young adulthood. It was observed that the mammary glands of mice subjected to neonatal manipulations, specifically those that involved short (H) and protracted (MS) periods of maternal separation for the first three weeks of life exhibited differences in differentiation rates, exemplified by a ratio of LAU to TEB number (representing the rate of glandular differentiation). It was found that adult mammary glands of H mice had significantly higher LAU:TEB ratios and thus greater mammary gland differentiation compared to both MS and TR mice. However, pubertal mammary glands had similar LAU/TEB ratios, regardless of manipulation condition. These findings suggest that differing neonatal environments can influence the morphological development of mammary glands, possibly due to changes in HPA development (influencing circulating hormone levels), as evidenced indirectly by changes in stress-induced behaviours, and the resulting impact of these alterations to the activity of the HPA axis on other organ systems, including mammary glands.

With the onset of the estrous cycle in rodents, undifferentiated TEBs first appear in the mouse mammary gland, and with the significant increase in circulating hormones including estrogen, prolactin and progesterone, these structures rapidly extend into the mammary fat pad as puberty progresses. As ductal extension/elongation continues, TEBs differentiate into more mature ducts and lobules, and form LAUs, the functional unit of the mouse mammary gland (Silberstein, 2001). Thus as expected in the present study, pubertal mice had significantly more TEBs, but significantly less LAUs and branch points compared to their adult counterparts (data shown in Appendix B). However, no effects of

neonatal manipulation on mammary gland morphology were observed between treatment conditions in pubertal mice. This may not indicate the absence of developmental differences in the HPA axis and mammary glands among mice in the different neonatal manipulation conditions, but rather that at this early (and rapid) stage of development, those differences are not yet observable, at least at the level of the mammary gland. Future studies will examine mammary glands at various stages of puberty (e.g., pre-puberty, late puberty) for a more complete analysis of mammary gland growth in response to neonatal manipulations at this allometric stage of development. Interestingly, as discussed in Chapter 3, H and MS conditions significantly affected forced swim test behaviours in only adult but not pubertal female mice, which support the idea that changes in HPA reactivity between groups are not yet apparent at this stage.

In contrast to the lack of morphological differences among the pubertal mammary glands, the significant differences in TEB counts between the normal adult mammary gland (i.e., TR) and those from mice that have been manipulated (i.e., MS and H) (data shown in Appendix B) demonstrate that these subtle neonatal manipulations, both brief and protracted, significantly impact development in a manner different from the normal, unmanipulated neonatal environment. Previous research has shown that TEBs compared to other mammary structures, are at an increased risk of carcinogen-induced tumourigenesis due to their highly proliferative nature and that a higher TEB number at time of carcinogen exposure is related to an increased number of mammary tumours (Fenton, 2006; Russo and Russo, 1987). The proliferative nature of cells in the TEBs (and thus high mitotic activity) increases the likelihood of mutation accumulation (Fenton, 2006; Russo and Russo, 1989). Since 5-10 genetic and epigenetic changes are believed to be required for mammary tumourigenesis to occur (Wazer and Band, 1999), an increase

in TEB numbers may increase tumourigenesis risk. Thus, it would appear, at least at this stage of development that the mammary glands of both H and MS mice may be less susceptible to tumourigenesis, due to the decreased numbers of hormonally sensitive TEBs compared to the mammary glands of TR mice. However, the development of the mammary gland is governed by a variety of signalling pathways, involving a large number of genes including the mammogenic hormone receptors, epidermal growth factor, hepatocyte growth factor and transforming growth factor-β (Silberstein, 2001), that regulate among others, TEB development/growth. Our calculations of differentiation rates (ratio of LAU/TEB) revealed that H adult mammary glands were the most differentiated, followed by MS and TR mammary glands. The high degree of differentiation (an indication of lower proportion of TEBs and possibly lower numbers of highly proliferative TEB cap and body cells) in H mammary glands suggests that these glands may be at a lower risk for mammary tumourigenesis. Thus, the differences in observations between LAU/TEB ratio and TEB counts indicate that the development of the mammary gland depends on the subtle interplay of various mammogenic factors that regulate mammary gland development from puberty to adulthood, suggesting that other morphological factors aside from TEB numbers alone may also be associated with tumourigenesis susceptibility.

The development of the mouse mammary gland is influenced by external factors during various developmental stages, affecting its growth. This includes the neonatal period, which is especially sensitive to changes in the environment. Previous studies have indicated that exposure to differing external environments during the neonatal period, both in terms of exposure to various chemicals (e.g., estrogenic compounds, polychlorinated biphenyls) as well as experimental manipulations (e.g. vehicle injections)

influence mammary gland growth (Fenton, 2006; Hilakivi-Clarke, 1997; Padilla Banks et al., 2006). For example, neonatal exposure to genistein caused stunted mammary branching and decreased TEBs in pubertal CD-1 mice (Padilla-Banks et al., 2006), whereas exposure to diethylstilbestrol (DES) and tamoxifen during this same period resulted in significant dose-dependent differences in postpubertal mammary gland growth (alveolar and branching development) of Balb/c mice (DES increased ductal growth whereas tamoxifen decreased ductal growth) (Hovey et al., 2005). These changes in mammary gland morphology were suggested to be due to disruptions of the hypothalamic pituitary gonadal (HPG) axis (Padilla-Banks et al., 2006), such that genistein potentially interrupts HPG signalling and secretion of pituitary hormones (Padilla-Banks et al., 2006). Furthermore, the significantly altered expression of estrogen and progesterone receptor mRNA (Padilla-Banks et al., 2006) and prolactin mRNA (Hovey et al., 2005) in the mammary glands of rodents exposed to estrogenic chemicals (e.g., genistein, diethylstilbestrol) compared to unexposed controls, suggest a disruption in endocrine system functioning, ultimately impacting on normal mammary gland growth and differentiation (Hovey et al., 2005; Padilla-Banks et al., 2006).

Similarly, manipulations of the dam-pup environment during the neonatal period, such as those used in the present study, have been found to affect behavioural attributes including exploration activity, and associated HPA axis activity (Anisman et al., 1998. Plotsky and Meaney, 1993; Pryce and Feldon, 2001), along with the development of the mammary gland. Previous research found that neonatal handling (injection of a vehicle substance) between post natal days 5 and 20 delayed the onset of puberty in handled compared to non-handled rats (Hilakivi-Clarke, 1997), whereas another study showed that handling (tactile stimulation for 1 minute/day) from post natal days 1 through 10

decreased ovulation rate in rats one year later (Gomes et al., 2005), suggesting that the regulation of the HPG axis is influenced by various forms of neonatal manipulations. Additionally, one of these studies also found that handled females compared to their non-handled counterparts had significantly lower rates of DMBA-induced tumour incidence (Hilakivi-Clarke, 1997), possibly due to differences in mammary gland growth/morphology as a result of changes to HPG axis regulation. The present study adds to these findings by demonstrating that dam-pup manipulations during the neonatal period also influence mammary gland growth, potentially via the interaction of the HPA and HPG axes. In support of the present findings, one study found that ACTH administration in neonatal rats (which could be considered as mimicking one part of the HPA response observed in MS rodents) delayed puberty and decreased plasma estrogen in adults, which can potentially decrease mammary gland differentiation (Alves et al., 1997).

Over time, as differences in HPA reactivity between manipulation groups interact with various organ and tissue systems, changes in mammary gland development between groups may be more dramatic. Apart from puberty and young adulthood, it would be interesting to analyze the mammary glands of more mature adult mice that have been subjected to neonatal manipulations, since in humans, the majority of breast cancer cases are diagnosed after 50 years of age (Haslam, 2005, 2006). Additionally, it would be interesting to see how parity status may interact with neonatal conditions in affecting mammary gland morphology. In humans and mice, parity significantly changes the morphology of the mammary gland (increased differentiation) such that tumourigenesis risk is decreased, both with early- and multi-parity (Cardiff and Wellings, 1999; Russo and Russo, 2004). Whether parity may abolish differences in mammary gland morphology as a result of neonatal manipulations remains to be elucidated.

Although it has been shown that the TEBs in mice are more susceptible to neoplastic growth when exposed to chemical carcinogens than other structures in the mammary gland (Fenton, 2006; Hilakivi-Clarke et al., 2006), and that neonatal manipulations can affect carcinogen-induced tumour growth rates (Hilakivi-Clarke, 1997b) not much is known about how the microenvironment of the normal mammary gland must change to cause alterations in glandular development thereby increasing the risk of tumourigenesis. One possible factor involved in regulating normal and malignant mammary gland growth is the interaction between the mammary stroma and epithelia. For example, estrogen interaction with its receptors in the stroma (but not in the epithelia) is crucial for ductal proliferation (Mueller et al., 2002). Whereas other researchers find that the interaction of estrogen with its receptors in the epithelia and stroma are needed for normal mouse mammary gland growth (Henninghausen and Robinson, 1998; Mallepell et al., 2006; Parmar and Cunha, 2004). Additionally, the stage of mammary gland development also influences hormone-receptor interactions. For example, one study looking at the interaction of administration of physiological doses of estrogen with its receptors in ovariectomized (induced menopause) mouse mammary glands found a heightened sensitivity to estrogen, but similar ERα levels in late versus early menopausal glands (Haslam, 2005, 2006), suggesting differences in hormone-receptor interaction between these two stages. Thus, aside from the circulating levels of various mammogenic hormones, the interaction between each hormone and its respective receptors potentially impacts on mammary gland growth. Apart from gross morphological analysis, future studies will look at the influence of neonatal manipulations on the levels and distribution of gonadal receptors (e.g., estrogen and progesterone receptors) in various mammary gland structures, in addition to plasma levels of gonadal hormones.

Differences in mammary gland growth may be assessed by examining not only morphological but also cellular differences that may exist between different structures of the mammary glands (e.g., TEBs, LAUs). Recently, stem cell populations have been characterized in both human and mouse mammary epithelia, which are proposed to be the site of origin of mammary tumours. In mice, mammary stem cells compared to normal cells are more prone to MMTV-induced tumourigenesis (Smith, 2005), whereas in humans, stem cells have the ability to continuously divide and self-renew, thus reducing the efficacy of currently available cancer treatments (Kalirai and Clarke, 2006). Therefore, identification of stem cell populations in mammary glands may be an important marker for cancer susceptibility. Additionally, in post-menopausal human mammary glands, the TDLU or lob1 structures are the most frequent site of mammary gland neoplasia (Clarke, 2006; Russo and Russo, 2004). However, aside from the number of each of these structures present in the gland, there are also significant differences in the cellular activity within these structures between high and low breast cancer risk groups (Russo and Russo, 2004), which may account for differences in breast cancer susceptibility. For example, *in vitro* studies have shown that lob1 and lob3 cells have significantly different cell division times and growth rates when exposed to a chemical carcinogen (DMBA) (Russo and Russo, 2004). Furthermore, lob1 structures have significantly different proliferation rates in nulliparous versus parous mammary glands (Russo and Russo, 2004). Therefore, future studies will examine differences at the cellular level that may exist between mammary glands of neonatally manipulated mice. For example, via immunohistochemical staining of proliferating cells (using PCNA or Ki-67 antibodies) and by assessing mitotic activity of mammary epithelial cells.

4.5 Conclusion

In humans, severe life experiences such as loss of a significant person, depression, and personality traits such as poor coping abilities have been associated with an increased risk of breast cancer development (Butow et al., 2000; Hilakivi-Clarke, 1997; Price et al., 2001; Temoshok, 1987). Similar observations have been found in rodents such that neonatal personality type (i.e., neophobic or neophilic) is associated with timing of spontaneous mammary tumour development and mortality rate (Cavigelli et al., 2006). However, the physiological mechanisms involved in these observations are not well understood as the developmental regulation of the normal mammary gland remains to be elucidated. Some researchers have suggested that differences in mammary gland growth and morphology, caused by differences in exposure to estrogen can be attributed to this difference in breast cancer risk (Fenton, 2006; Hilakivi-Clarke et al., 2006; Russo and Russo, 1999). Hence, in the present study a rodent neonatal manipulation model was used to examine differences in mammary gland morphology, as this model has widely been used to elicit differences in anxiety behaviours and stress hormone levels. It was found that in mice, brief maternal separation resulted in adult mammary glands that were more differentiated than the mammary glands from mice exposed to protracted maternal separation and normal (control) mammary glands, indicating that early-life experiences, aside from affecting regulation of stress-induced behaviours, also influence the development of the normal mammary gland environment.

Chapter 5: Influences of neonatal environments on mRNA and protein expression of estrogen receptor alpha (ERα) and tumour suppressor gene p53 (p53) in Balb/c female mammary glands

5.1 Introduction

The progression of cells from the normal to the malignant phenotype is associated with aberrant gene expression, functioning, and regulation that typically result in a dysregulation of the cell cycle and uncontrolled cell proliferation. In the case of breast cancer, a number of genes have been identified as biomarkers of breast cancer development/progression, such that mutations and loss of function, or increased/decreased expression of certain genes are associated with an increased risk of developing the disease. For example, germ line mutations in genes such as BRCA1, BRCA2, p53, and PTEN are associated with an increased risk of breast cancer (Kenemans et al., 2004), whereas significantly different levels of protein expression of genes such as estrogen receptor alpha (ERα), c-Myc, human epidermal growth factor receptor 2 (HER-2), and cyclin D1 have been found in cancerous compared to normal breast tissue (Leong and Leong, 2006; Lofgren et al., 2006; McNeil et al., 2006; Roy and Thompson, 2006). Since these genes are responsible for controlling normal cell growth and development, and cell-cycle regulation, it is important to examine their expression and regulation in the normal cellular microenvironment to determine the changes that may occur, initiating tumourigenesis. Two such genes are ERα and the tumour suppressor gene p53. ERα is important in mediating the mitogenic effects of estrogen, important for mammary gland growth. For example, ERα knockout mouse studies show that ERα is essential for normal mammary gland development (Hewitt and Korach, 2003). Additionally, the interaction between estrogen and ERα has been found to be different

depending on stage of growth (pubertal, adult), whereas ERα protein expression is significantly different between cancerous and non-cancerous tissues (Lofgren et al., 2006; Mueller et al., 2002; Shyamala et al., 2002). On the other hand, the p53 gene is responsible for cell growth and differentiation, DNA repair, and apoptotic regulation. Importantly, mutations in the p53 gene are the most common mutation (20-30%) seen in sporadic breast cancers, the most prevalent form of breast cancer (Borresen-Dale, 2003; Kenemans et al., 2004). These mutations result in the loss of function of the p53 protein, resulting in uncontrolled cell proliferation.

Since cancer is a disease that is characterized by uncontrolled cell proliferation and growth, the present study examines the expression of both p53 and ERα, in the mammary glands of mice, due to the involvement of these genes in cell growth and proliferation and the significant association of these genes with tumourigenesis. Thus, apart from examining the influences of neonatal manipulations on gross mammary gland morphology (as discussed in chapter 4), changes in the expression levels of these two genes between manipulation groups may indicate differences in cell-cycle regulation and perhaps, mammary gland growth, thus potentially influencing risk of tumourigenesis. We examined the expression levels (mRNA and protein) of these two genes in the mouse mammary gland, in order to elucidate any differences that may exist between neonatal manipulation groups.

Estrogen receptor alpha (ERα)

The responsiveness of mammary glands to changes in estrogen levels is mediated by ERα, and the more recently characterized, estrogen receptor beta (ERβ). Although the two estrogen receptors are highly homologous, they exhibit differences in expression in the mammary gland and other estrogen sensitive tissues (Hewitt and Korach, 2003). It is

thought that ERα alone is responsible for estrogen-induced proliferation of the mammary glands as indicated by studies examining mammary gland development in ERα and ERβ knockout mice (Anderson and Clarke, 2004; Hewitt and Korach, 2003). ERβ is suggested to be more active in the brain and cardiovascular system, responsible for functions such as sexual behaviour, and can also interact with and modulate the actions of ERα (Anderson and Clarke, 2004; Harris, 2007).

ERα is part of the nuclear receptor superfamily and consists of six functional domains including a DNA binding domain, and ligand-dependent and ligand-independent domains (Toran-Allerand, 2004, Weihua et al., 2003), although more recently, it was shown that ERα is no longer confined to the nucleus, and that cell surface ERα has also been identified in both rodents and humans (Speirs and Walker, 2007; Weihua et al., 2003). The classical pathway of estrogen receptor function is as a transcriptional regulator, whereby the binding of estrogen to its receptors causes activation by dissociation from chaperone proteins such as a heat-shock protein (hsp90), receptor dimerization and subsequent interaction with estrogen-responsive elements (EREs) in the promoter region of target genes (Speirs and Walker, 2007; Toran-Allerand, 2004, Weihua et al., 2003). The net result of this activation is the suppression or enhancement of target gene transcription (Toran-Allerand, 2004; Dickson and Stancel, 2005). Moreover, activated ERα can bind to EREs of other transcriptional factors, and indirectly affect gene transcription (Toran-Allerand, 2004). However, more recently, other co-repressors/activators have been identified that influence ERα mediated transcriptional activity, such that these factors are able to increase/decrease the responsiveness of ERα to estrogen (Dickson and Stancel, 2005). Furthermore, ligand and DNA-independent transcriptional pathways have also been elucidated; for example, the phosphorylation of

the estrogen receptor and its co-activators by receptors such as epidermal growth factor receptor (EGFR) via the activation of mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3 (PI3) pathways (Anderson et al., 1998; Clarke, 2006). Thus, the expression and activation of ERα, and its influence on mammary gland development can be influenced by a host of different factors involved in ERα signalling.

Previous research has shown that ERα expression in the mammary gland is closely linked to stage of development, cellular differentiation and fate and is related to risk of tumourigenesis. In both human and mouse mammary glands, ERα expression is detected in approximately 4-15% of total cells in the mature gland (Anderson and Clarke, 2004, Shyamala et al., 2002). In humans, ERα is only expressed in the mammary epithelia (15-30% of epithelial cells), with no staining observed in the myoepithelia, fibroblasts, stroma or adipose tissue (Anderson et al., 1998). In the mouse, ERα is present in the mammary epithelia (10-60% expression in epithelial cells) and certain sections of the stroma (e.g., surrounding the primary ducts), as well as in adipose tissue (Anderson and Clarke, 2004; Hazlam and Woodward, 2003; Shyamala et al., 2002). Studies have shown that in mice, stromal and epithelial ERα are necessary (albeit at different stages) for mammary gland development, as ERα knockout mice show stunted ductal branching (Hazlam and Woodward, 2003; Mueller et al., 2002). Moreover, ERα expression in the mammary gland is closely tied to the stage of mammary gland development in both humans and rodents. In human and mouse mammary glands, ERα expression has been detected as early as in the foetal stage (Shyamala et al., 2002), and it is believed that ERα action is independent of estrogen at this stage of development, as the ovaries are not yet fully functional (Shyamala et al., 2002). By puberty, ovariectomy followed by exogenous estrogen administration studies in mice show that an increase in estrogen is associated

with a decrease of ERα, whereas at adulthood, increased estrogen is related to increased ERα the mammary glands (Mueller et al., 2002; Shyamala et al., 2002). Post-menopause however, ERα expression is higher than in the pre-menopausal gland (Anderson and Clarke, 2004). Interestingly, in both humans and mice, ERα is always detected adjacent to, but not in, proliferating mammary epithelial cells, suggesting a paracrine effect of estrogen in ductal proliferation (Anderson and Clarke, 2004; Russo et al., 1999). Additionally, it has been suggested that the developmental fate of the cell is related to ERα expression, where in mice, ERα is detected in epithelial cells that have the potential to differentiate into secretory luminal cells, but not in the myoepithelia that do not have this capability (Shyamala et al., 2002).

ERα and breast cancer risk

ERα is a popular target for therapeutic agents against breast cancer due to the interaction between ERα and estrogen in controlling cell proliferation. It is proposed that an increase in cell proliferation will increase the chance of spontaneous errors in DNA replication, or will further propagate the replication of cells that already contain such mutations (Dickson and Stancel, 2005). In both humans and rodents, studies have shown that ERα expression is significantly different in normal compared to cancerous tissue and among tissues with various differentiation capabilities. In post menopausal women, ERα protein expression was found to be higher in tissue surrounding malignant breast tumours compared to benign tumours (Saceda et al., 1988), whereas another study showed that the ERα mRNA was increased, while the ratio of ERβ to ERα protein expression was reduced in normal compared to malignant breast tissue (Lofgren et al., 2006). ERα protein expression is also significantly different between tumour types, such that high (aggressive) compared to low grade ductal carcinomas are associated with increased ERα

expression (Tang et al., 2006). Similarly in mice, ERα protein expression has been found to become significantly altered with tumour progression, where loss of ERα expression is observed with mammary tumour progression (Yoshidome et al., 2000).

In normal adult human mammary glands, immunohistochemical studies indicate that ERα protein expression is highest in the least differentiated lobules (lob1), and decreased in more differentiated lobules (lob2 and lob3) (Russo et al., 1999, Russo et al., 2002). Similarly in rats, highest ERα expression was found in the epithelial cells of undifferentiated TEBs, with decreased staining observed in the more differentiated LAUs (Russo et al., 1999). Thus, since least differentiated mammary structures are related to increased ERα expression, it is suggested that mammary glands that contain more undifferentiated structures will also show increased expression of ERα.

Tumour suppressor gene p53 (p53)

The loss of function of the p53 gene, commonly through point mutations and post-translational modifications is an important event in the development of breast cancer (Almog and Rotter, 1997; Kenemans et al., 2004). The expression and activity of p53 in the normal cell is relatively low. However, p53 protein functioning is essential when cells are exposed to a stressor, such irradiation, carcinogen exposure and oxidative stress (Lacroix et al., 2006). Due to its function as a transcription regulator, p53 is able to induce cell cycle arrest and apoptosis in damaged cells, minimizing aberrant cell growth. Three functional domains have been characterized in p53, which include a DNA-binding domain, an N-terminal domain involved in interactions with mouse double minute 2 (MDM2) and apoptosis proteins, and a C-terminal domain involved in p53 activity regulation (Lacroix et al., 2006). Stimulation of p53 from sources such as DNA damage and activation of oncogenes cause enhanced binding of p53 proteins to DNA along with

accumulation of p53 in the nuclei (Hoogervorst et al., 2005; Jerry et al., 2002). For example, DNA strand breaks initiate phosphorylation that disturbs the p53 - MDM2 (a p53 transcription inhibitor) pathway, inhibiting p53 degradation and causing accumulation of p53 (Meek, 2004). On the other hand, oncogene activation induces the expression of another protein ($p14^{ARF}$) which blocks the ability of MDM2 to degrade p53 (Meek, 2004). Moreover, post-translational modifications (e.g., methylation, phosphorylation) of p53 allow it to act on target genes that can induce senescence, cell cycle arrest and DNA repair (Jerry et al., 2002; Lacroix et al., 2006; Meek, 2004). In addition to the direct influence of p53 on cell-cycle regulation, other genes and interaction factors associated with p53, such as regulatory proteins, can affect the expression and function of p53, where the disruption or changes in activation/expression in these interactive factors may also affect p53 functioning (Lacroix et al. 2006). Numerous target genes are p53-responsive, including those that are involved in angiogenesis, mitochondrial apoptosis and those controlling the progression from G1 to S phase in mitosis (Lacroix et al., 2006; Meek, 2004).

p53 and breast cancer risk

The loss of function of p53 via mutation of the gene, deletion and post-transcriptional modification increases the risk of breast tumourigenesis. In humans, mutations and expression patterns of p53 are linked to breast cancer prognosis, efficacy of treatment, and breast tumour subtypes (Kai et al., 2006; Lacroix et al., 2006; Railo et al., 2007). Furthermore, significant differences in p53 protein expression have been found in tumour compared to normal breast tissue (Hussein and Ismael, 2004). In the mouse, evidence indicates that mitogen exposure to mouse mammary cells with both homozygous and heterozygous p53 deletions can cause strong carcinogenic effects

(Medina et al., 2002), whereas mice with p53 mutations compared to controls, show an increased frequency of sporadic tumour growth in the mammary gland (Wijnhoven et al., 2005).

In terms of protective functioning, loss of p53 functioning abolishes the protective effects of hormone elevation caused by early pregnancy in mice and delays mammary epithelia involution after pregnancy (Kuperwasser et al., 2000; Jerry et al., 2002). For example, p53 homozygous knockout mice treated with three weeks of estrogen/progesterone (mimicking the effects of pregnancy) showed an increase in mammary tumourigenesis due to carcinogenic exposure compared to similarly treated mice with functional p53 (Medina, 2004). Moreover, due to its hormonally responsive nature, research has shown that p53 may be a potential mediator of the protective effects of pregnancy, such that estrogen/progesterone treated (pregnancy-mimicking) rodent mammary glands showed a sustained increase in transcriptionally-active p53 protein expression, which may hinder carcinogen-induced cell proliferation (Sivaraman et al., 2001). This indicates the importance of p53 in conferring the cancer protective effects of early pregnancy in mice. Therefore, defects in p53 functioning can not only cause the accumulation of genetic mutation, but also abrogate established cancer protective factors (i.e, pregnancy), increasing the likelihood of tumourigenesis. Thus, when coupled with increased TEB numbers and subsequent ERα over-expression, status of p53 expression and functioning may further add to the risk of tumour development in the mammary gland.

Aside from examining mutations in the p53 gene that lead to disruption in p53 function, it is equally important to analyze the expression patterns of p53. Studies have shown that the frequency of p53 mutations is significantly lower in breast cancers

compared to other common cancers (Gasco et al., 2002), and suggest other mechanisms such as upstream and downstream targets responsible for disruption of p53 activity. For example, p53 protein expression was detected in the cytoplasm, but not the nuclei of cells in certain breast tumours (Gasco et al., 2002), indicating the presence of factors that inhibit p53 migration into the nucleus of these cancerous cells, and ultimately affecting its function. Thus, differences in p53 expression between neonatal manipulation groups in the present study may indicate differences in the mechanisms that regulate p53 expression between groups, possibly influencing cell growth, differentiation, and risk of tumourigenesis.

Although the deregulation and subsequent changes in expression of ERα and p53 may result in separate reaction cascades that can influence the risk of mammary tumour development, studies have also shown direct interaction of these gene products with one another. Frequently, the interaction dynamics between different genes are differentially altered between normal and cancerous cells. For example, *in vitro* studies have shown direct binding of estrogen receptors to the p53 gene (Yu et al., 1997). Interestingly, in normal endometrial cell lines, p53 protein expression was shown to repress the effects of estrogen receptor-mediated transcriptional activation by estrogen (Yu et al, 1997). However, in breast cancer cell lines, p53 has been shown to both up-regulate and down-regulate ERα expression (Angelone et al., 2004; Lewandowski et al., 2005), perhaps an indication of deregulation in gene expression. Therefore, interactions of these two genes with one another may also influence the mammary gland environment.

Since differing neonatal environments have been shown to influence the growth and differentiation of mouse mammary glands possibly due to alterations in the HPA and

HPG axes between treatment groups, it is anticipated that ERα and p53 expression may also be differentially expressed in the mammary glands of neonatally manipulated mice. Increased ERα expression has been shown to be directly related to a higher number of undifferentiated structures in the mouse mammary glands (Russo et al., 1999), whereas both ERα and p53 are differentially expressed in various mammary tumour types (Hussein and Ismael, 2004; Lofgren et al., 2006), compared to normal tissues. Thus in the present study, ERα and p53 mRNA and protein expression were examined in the mammary glands of neonatally manipulated adult mice to assess the influence of neonatal manipulations on these two regulators of mammary gland growth.

5.2 Materials and methods

5.2.1 Estrogen receptor alpha (ERα) immunohistochemical staining

5.2.1.1 Tissue preparation

Immunohistochemical staining for estrogen receptor alpha (ERα) was performed on paraffin embedded inguinal mammary gland sections from neonatally manipulated pubertal and adult mice. The 4^{th} and 5^{th} right inguinal glands of each mouse were dissected, placed in a cassette and fixed in a 4% neutral buffered formalin solution overnight. Mammary glands were then washed in distilled water (3 x 15 minutes each), followed by a 30 minute wash in 50% ethanol and finally immersed in 70% ethanol at 4° C for at least 2 days before tissues were processed. Tissues were then paraffin embedded. Embedded mammary glands were sectioned using a microtome to a thickness of 5-6μm and two serial sections were placed on each slide that was pre-coated with Mayer's

albumin. Slides were baked at 65° C for 2 to 3 hours to facilitate the adhesion of tissue sections to the slide surface.

5.2.1.2 Immunohistochemical staining

Sections were de-paraffinized in xylene (3 x 10 minutes), followed by re-hydration in 95% ethanol (2 x 3 minutes), and 1 wash each in 75% ethanol and 50% ethanol before a distilled water rinse. Sections were then washed for 10 minutes with a 3% hydrogen peroxide solution, and rinsed with distilled water. Following this re-hydration process, tissue sections were boiled for 6 minutes at 95°-100° C in 0.05M sodium citrate buffer (pH 6.0), cooled to room temperature by immersion in a distilled water bath, and rinsed in 1X phosphate buffered saline (PBS) (pH 7.0-7.4). To reduce non-specific binding, sections were incubated for 30 minutes at 25° C with either 6% goat or rabbit serum (prepared in 1% bovine serum albumin (BSA)), depending on the primary antibody to be used. Tissue sections were then incubated with either a rabbit polyclonal ERα (MC-20) or goat polyclonal β-actin (I-19) primary antibody (both from Santa Cruz Biotechnology, California) at 4° C overnight. Primary antibody concentrations of 1:60 and 1:50 for ERα and β-actin, respectively, diluted in 1% BSA were used. Following three washes with PBS, appropriate biotinylated secondary IgG antibodies were applied at a concentration of 1:150 for 1 hour at 25° C. As a negative control, primary antibodies were omitted for one mammary gland section per slide. ERα and β-actin immunoreactivity were visualized using an avidin-biotin complex/horseradish peroxidase (DakoCytomation, Denmark), followed by application of diaminobenzidine. Tissue sections were then counterstained with a 20% solution of Harris' Haematoxylin (Sigma-Aldrich, Canada). Following staining, slides were de-hydrated in 4 serial ethanol

solutions followed by 3 washes of xylene. After processing, slides were mounted with Permount (Fisher Biotechnology, Canada) and coverslipped.

5.2.1.3 Quantification of ERα protein expression

Tissue sections were examined under a light microscope (Weiss) attached to a digital camera (Microleaf Lumina) to determine positive staining for ERα protein. ERα positive cells are indicated by the presence of brown stained nuclei in the mammary tissue. Five areas, representative of the entire organ from each tissue section and its corresponding negative control were photographed at 20X and the digital images were analyzed using image software (Adobe Photoshop 9.0, USA). Positive and negative stained nuclei in mammary stroma and epithelia were counted and expressed as the percentage of positively stained nuclei relative to the total number of nuclei in each of 5 random areas. For each neonatal manipulation group, tissue sections from 4 or 5 mice were used, and approximately 100-250 nuclei were counted per tissue section, with a total of 600-1500 nuclei for each mouse.

5.2.1.4 Statistical analyses

Effects of Treatment and Age. To determine the effects of neonatal manipulations (treatment) and age on ERα protein expression, a two-way analysis of variance (ANOVA) for the effects of Treatment (H, MS, TR) and Age (pubertal, adult) was performed on epithelial, stromal and total expression of ERα protein.

Significance was set at $p<0.05$. Significant main or interaction effects were further analyzed using Newman-Keuls post-hoc comparisons. All statistical analyses were conducted using the statistical package STATISTICA 6 (Statsoft, USA). Data are presented as means $\pm$ S.E.M.

5.2.2 Total protein analysis of ERα and p53 using Western Blot

5.2.2.1 Homogenization of mammary gland samples

Mammary glands from pubertal and adult H, MS and TR mice were frozen in liquid nitrogen, wrapped in aluminium foil, and pulverized using a hammer. Pulverized glands were then transferred to a microcentrifuge tube, 2 to 3 equal volumes of homogenization buffer were added and the glands were homogenized using a stainless steel sonicator. The homogenization buffer contained: 25mM Tris hydrochloric acid, 2mM magnesium chloride, 1mM ethylenediamine tetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 1mM phenylmethylsulphonyl fluoride (PMSF), 1μM leupeptin, 1μM pepstatin and 1μM aprotinin in distilled water. Homogenization was performed for 15 seconds, after which samples were immediately cooled on ice. This process was repeated until tissue samples were completely homogenized. Homogenized glands were then centrifuged at 15,000g for 1 minute. Following centrifugation, the supernatant from each sample (containing the total protein of the mammary gland) was transferred into a new microcentrifuge tube.

5.2.2.2 Total protein quantification

A direct contact (DC™) protein assay (Bio-Rad, USA) adapted from a microplate assay protocol from the manufacturer was run to determine the protein concentration of homogenized mammary glands from each neonatal manipulation group. Briefly, 5 μL each of prepared BSA standards ranging from 100-2000mg/ml and diluted protein samples were pipetted into a 96-well flat bottom plate. 25μL of reagent A' (alkaline copper tartrate solution) was then added into each well, followed by 200μL of reagent B

(Folin reagent). Samples were gently mixed by tapping the microplate. After 15 minutes, the absorbance of the samples at 750nm was read using a microplate reader (Bio-Rad, USA). Standards and samples were run in duplicates or triplicates to improve the accuracy of the absorbance measurements. "Blank" measurements were also taken by substituting protein samples with distilled water to correct for instrumentation error.

5.2.2.3 Sample preparation and gel electrophoresis

Mammary gland protein samples were mixed 1:1 with 2X sample buffer to facilitate separation of protein bands during electrophoresis. The sample buffer contained: 0.5 mol Tris hydrochloric acid (pH 7.8) , 10% sodium dodecyl sulphate (SDS), 0.1% bromophenol blue, 2ml of glycerol and 20% β-mercaptoethanol made in distilled water to a quantity of 10ml. Effective detection of protein bands required the use of 10μg of total protein for ERα detection and 23μg of total protein for p53 detection. Four adult mammary gland samples and three pubertal samples from each manipulation group were used for each Western blot analysis, and gels were run in duplicate or triplicate to improve accuracy. Samples were loaded onto 8-16% prepared tris-glycine gels (Invitrogen, Canada) along with a molecular weight marker, and positive controls for ERα (recombinant ERα protein, Sigma-Aldrich) and p53 (BW5147 cells, Santa Cruz Biotechnologies, California). Electrophoresis was conducted at a constant voltage of 90-100V for 2-3 hours. This was followed by a transfer onto nitrocellulose membrane. A transfer "sandwich" was made by stacking blotting sponges, blotting paper, the tris-glycine gel, a sheet of nitrocellulose membrane followed by blotting paper and more blotting sponges. This "sandwich" was inserted into the electrophoresis apparatus and ran

at a constant amperage of 150mA for 90 minutes. Successful transfer of protein bands onto the nitrocellulose membrane was verified by Ponceau S staining.

5.2.2.4 Protein detection/visualisation

Nitrocellulose membranes were blocked with a 5% low-fat milk solution in 1X tris buffered saline with added Tween (TBST) for 30 minutes to reduce non-specific binding. Following 3 washes with 1X TBST, membranes were then incubated at 4°C overnight with primary antibodies. The concentrations of primary antibodies used were as follows: 1:300 for ERα (MC-20, Santa Cruz Biotechnologies, California), 1:300 for β-actin (I-19, Santa Cruz Biotechnologies), (Santa Cruz Biotechnologies), and 1:750 for p53 (CM-5, NovaCastra Laboratories, UK) and diluted in a 1% low-fat milk in 1X TBST solution. Membranes were then washed 3 times with 1X TBST and incubated with appropriate horseradish peroxidase conjugated secondary IgG antibodies for 1 hour. Visualisation of nitrocellulose membranes was performed using an electrochemiluminescence kit (containing luminol as the chemiluminescent agent) as per directions of the manufacturer, (Amersham Biosciences, Canada), followed by exposure onto Kodak™ Biomax® light photographic film (Sigma-Aldrich, Canada). Film exposure times were 15 minutes, 6 minutes and 1 minute for ERα, p53 and β-actin, respectively.

5.2.2.5 Western blot analysis

Analyses of western blot films were conducted using Scion Image Software (developed at the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/). Briefly, each blot was analyzed by measuring the optical density of ERα or p53 protein bands for each sample. Optical density values were

then corrected for background density. The optical density values of protein bands were also normalized using β-actin bands for each sample to compensate for sample leakage and other inaccuracies that may have occurred during loading.

5.2.2.6 Statistical analyses

Effects of Treatment and Age. A one-way analysis of variance (ANOVA) for the effect of replicate gel runs on optical density of protein bands indicated that replicate runs significantly affected relative optical density of these bands. Hence, to determine the effects of neonatal manipulation on ERα and p53 total protein expression, one-way analyses of covariance (ANCOVAs) for the effect of Treatment (H, MS, TR) were performed on pubertal and adult normalized relative optical density measurements of protein bands. Additionally, adult ERα protein expression data collected by Ayesha Salleh were combined with previously collected data from the Kerr laboratory to increase sample size. These data were generated by honours students Tricia Ralph (from September 2004 to April 2005) and Yogindra Persaud (from September 2005 to April 2006). Significance was set at $p<0.05$. Significant main or interaction effects or *a priori* assumptions were further analyzed using Newman-Keuls post-hoc comparisons. All statistical analyses were conducted using the statistical package STATISTICA 6 (Statsoft, USA). Data are presented as means $\pm$ S.E.M

5.2.3 Reverse transcriptase real-time quantitative polymerase chain reaction (RT-qPCR)
5.2.3.1 Primer design

Gene specific primers for real-time PCR were designed using Primer3 software (Whitehead Institute, USA). Primers were designed according to the following parameters:

- primers were approximately 15-25 nucleotides in length
- small amplicon size (90-100 nucleotides)
- approximately 50% guanidine and cytosine content
- minimum number of primer dimers to avoid secondary structure formation
- annealing temperature (Tm) of approximately 60°C
- annealing sites less than 2 kilobases upstream from the 3' end of gene sequence

Gene specific mRNA sequences for *Mus musculus* were obtained from the National Centre for Biotechnology Information (NCBI) and the European Bioinformatics Institute -Wellcome Trust Sanger Institute (EBI-WTSI) gene databases to facilitate primer design. The accession numbers associated with each gene are as follows:

Table 1.NCBI accession numbers for *Mus musculus* genes used in real-time qPCR analysis

Gene	Accession number
Estrogen receptor alpha (ERα)	NM_007956.2
Transformation related protein p53 (Trp53)	NM_0011640.1
β-Actin	NM_007393.1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001001303

Primer-dimer formations and annealing temperatures were determined using a free oligonucleotide tool kit (Operon Biotechnologies, USA). Designed primers were checked against the *Mus musculus* genome using NCBI's Basic Local Alignment Search Tool (BLAST) to ensure primer pairs only amplified one specific gene. The primers designed for each gene are listed in Table 2.

Table 2.Primer pairs for genes of *Mus musculus* used in qPCR experiments

Gene	Primer sequence(5'-3')	Length (base pairs)	Size (base pairs)
p53	F:GAAGACTCCAGTGGGAACC R:CAGTTCAGGGCAAAGGACT	19 19	126
GAPDH	F:CCTGGAGAAACCTGCCAAG R:GGTCCTCAGTGTAGCCCAAG	19 20	95
ERα	F:GGCACATGAGTAACAAAGGC R:GGTGGGCATCCAACATCT	20 18	99
β-actin	F: AGCCTTCCTTCTTGGGTATG R: GGTCTTTACGGATGTCAACG	20 20	90

5.2.3.2 Sample preparation for RNA isolation

Thoracic mammary glands of H, MS and TR pubertal and adult mice for RNA isolation were dissected and immediately immersed in RNA later (Sigma-Aldrich, Canada) to protect against RNA degradation. Mammary gland samples were then incubated at 4°C overnight. Following overnight incubation, the RNA later solution was discarded and mammary gland samples were kept frozen at -80°C. Prior to RNA isolation, frozen mammary glands were pulverized by immersing aluminium foil wrapped glands in liquid nitrogen, and smashing the glands into powder with a hammer. RNA isolation was conducted using two different methods:

1) RNA isolation-Trizol (Invitrogen) method

1 mL of Trizol (Invitrogen, Canada) was added for every 100mg of mammary gland tissue into clean microcentrifuge tubes. Mammary gland tissues were homogenized using a plastic homogenizing pestle. Following incubation with Trizol, samples were incubated for 5 minutes at room temperature. Subsequently, 200μl of chloroform was added to each tube, and tubes were shaken vigorously for 2 minutes. Samples were then

centrifuged at 12,000g at 4°C for 19 minutes. Following centrifugation, the resulting aqueous phase was removed from each sample and transferred into a clean microcentrifuge tube. 500μL of isopropyl alcohol was added to each tube and incubated at room temperature for 10 minutes. Following incubation, samples were centrifuged at 12,000g for 15 minutes. The resulting supernatant was removed and the remaining RNA pellet was washed with 75% ethanol and air dried. RNA pellets were diluted with an appropriate volume of RNase free water (Sigma-Aldrich, Canada) in order to obtain RNA concentrations of 1μg/ml.

2) RNA isolation using RNAeasy isolation kit (Qiagen, USA)

Similar to the Trizol method of RNA isolation, mammary gland tissue was smashed, and homogenized in a phenol solution (Qiazol). 200 ul of chloroform was added to each sample, shaken vigorously and incubated for 2-3 minutes at room temperature. Samples were then centrifuged at 12,000 *g* at 4°C for 15 minutes. The resulting aqueous phase was removed and transferred into a clean microcentrifuge tube. 600μL of 70% ethanol was added to each sample and mixed thoroughly using a vortex machine. This solution was immediately transferred into an RNAeasy spin column attached to a collection tube and centrifuged at 12,000g for 15 seconds. Each sample was then washed with 700μL of RW1 buffer followed by 2 washes with 500μL RPE buffer following manufacturer's protocols. Between each wash step, samples were centrifuged briefly at 12,000g at room temperature. Following these washing steps, RNaeasy spin columns were dried by centrifugation at 12,000g for 1 minute. The resulting isolated RNA was eluted from each spin column by adding 30-50ul RNAse free water.

The concentration and purity of isolated RNA was determined using a spectrophotometer (Nanodrop, Stratagene, USA). Uncontaminated and undegraded RNA has a 260nm/280nm absorbance ratio between 1.9 and 2.1 (Qiagen, USA). Only RNA samples with a ratio value greater than 1.85 were used for subsequent PCR experiments.

5.2.3.3 First-strand cDNA synthesis

Prior to cDNA synthesis, RNA samples were pre-treated with DNase I (Invitrogen, Canada) according to the manufacturer's instructions, to reduce DNA contamination that may be present in the samples. First strand cDNA synthesis was then carried out following manufacturer's instructions using reverse transcriptase (Superscript III, Invitrogen, Canada) and a non-specific poly (T) primer. This primer anneals to the 3' end of any RNA sequences with a poly (A) 'tail'. For each cDNA reaction, a sample without the addition of reverse transcriptase (no RT sample) was run alongside those with reverse transcriptase to check for the presence of genomic DNA. cDNA synthesis parameters for the cDNA reactions were 52°C for 60 minutes followed by 70°C for 15 minutes.

5.2.3.4 Real-time polymerase chain reaction (qPCR)

To analyze the mRNA expression levels of each gene of interest, qPCR reactions were performed using a protocol for SYBR green fluorescence adapted from Stratagene (USA). Fresh master mix was prepared before each run to increase consistency of the run. Briefly, the master mix contained: RNase free water, 10X PCR buffer, 50mM magnesium chloride, 5mM dNTPs, 25μM each of forward and reverse primers, dimethyl sulphoxide (DMSO), 50% glycerol, SYBR green and ROX reference dye. 24μl of this master mix was transferred into each well on a 96-well PCR plate. Subsequently, 1μl of cDNA was

added to each well. Three mammary gland cDNA samples per neonatal manipulation group (i.e., three biological replicates) were used; each sample was loaded in triplicate along with a no reverse transcriptase (no RT) sample to check for genomic DNA contamination. GAPDH was used as a control gene, to normalize the expression data for both ERα and p53. A standard curve, made from a set of cDNA serial dilutions (typically serially diluted from cDNA made from 100ng of starting RNA) from one control mouse mammary gland sample, was also loaded on each PCR plate. The qPCR reactions were carried out using Applied Biosystems (USA) 7900HT real-time PCR machine.

Cycling parameters for all qPCR reactions were as follows:

95°C: 10 minutes

95°C: 30seconds
60°C: 1 minute — 40 cycles
72°C: 32 seconds

95°C: 15 seconds
60°C: 15 seconds — Dissociation curve
95°C: 15 seconds

A dissociation curve analysis was done to check for specificity of PCR product amplification, and to check for primer dimers. PCR products were then subjected to gel electrophoresis to ensure correct amplicon size.

5.2.3.5 mRNA expression quantification using $2^{-\Delta\Delta Ct}$ method

The $2^{-\Delta\Delta Ct}$ mRNA expression quantification method allows for the relative quantification of the fold-change in expression of the target gene in a treatment group,

relative to a control group (Livak and Schmittgen, 2001). The relative change in mRNA expression for each gene (i.e., ERα, p53, and GAPDH) in mammary glands of mice between neonatal manipulation groups was calculated using the equations outlined in Livak and Schmittgen (2001). The first step involved normalizing the target gene expression cycle threshold (Ct) with the chosen internal control gene; in this case GAPDH. Hence, the Ct of GAPDH was subtracted from the Ct value of the target gene (i.e., p53 or ERα) for each replicate sample (known as the ΔCt value), and the mean values were calculated. Following this, the average ΔCt for each treatment group of H, MS and TR was calculated. The changes in gene expression between treatment groups were then calculated by subtracting the ΔCt values of H or MS from the TR control group, hence obtaining a ΔΔCt value. The change in expression between treatment groups were then calculated using the formula: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

5.2.3.6 Statistical analyses

t-tests were used to calculate the difference in p53 mRNA expression in H and MS groups, compared to TR adult mammary glands. ERα mRNA expression could not be calculated at the time of publication due to challenges faced with the optimization of the assay.

5.3 Results

The influence of various neonatal conditions on ERα, an important receptor related to mammary gland growth, and the cell-cycle regulator p53 were assessed using RT-qPCR to determine the mRNA expression levels of these genes, and immunohistochemical staining and western blotting to determine protein expression patterns.

5.3.1 Expression of ERα protein by immunohistochemical staining

A Treatment X Age ANOVA for total ERα protein expression (combined stromal and epithelial ERα) in the mammary glands of neonatally manipulated mice revealed a significant main effect of Age, such that regardless of neonatal conditions, total ERα expression was significantly higher in pubertal compared to adult mammary glands ($F(1,125)=6.82$, $p=0.01$) (Figure 7). A significant Treatment X Age interaction ($F(2, 125)=3.50$, $p<0.05$) further revealed that this Age effect was primarily due to the significant decrease in ERα expression from puberty to adulthood in TR mice ($p=0.039$). Thus at adulthood, MS compared to TR adult mammary glands expressed significantly more ERα protein ($p=0.04$), whereas H compared TR adult mammary glands expressed moderately more ERα protein ($p=0.103$) (Figure 7, 8B). Neonatal manipulations did not significantly affect total ERα expression in pubertal glands (Figure 7, 8A).

Additionally, Treatment X Age ANOVAs for ERα expression in either the mammary epithelial or the stromal compartments revealed that the significant difference in total ERα expression between pubertal and adult mammary glands was mainly due to the significant main effect of Age in stromal ERα expression ($F(1,25)=16.01$, $p<0.001$). In contrast, ERα protein in the mammary epithelia alone was not significantly affected by neonatal manipulations.

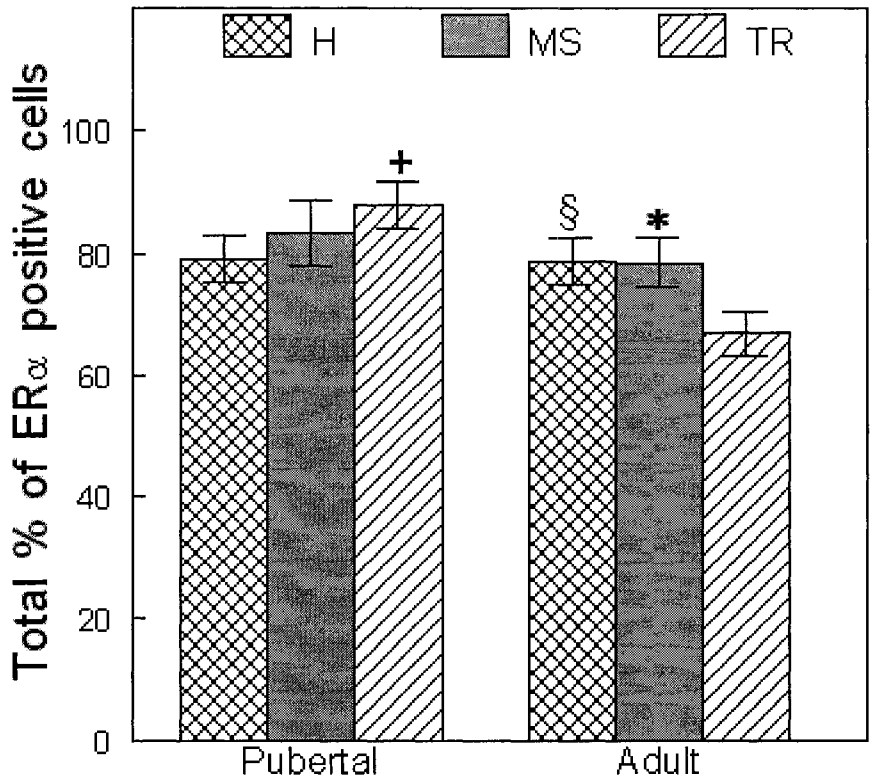


Figure 9.Mean (±S.E.M) percentage of cells positively stained for ERα in the mammary glands of neonatally manipulated pubertal and adult mice. ERα positively stained cells were counted in the mammary epithelia and stroma, and the percentage of positive cells was calculated for all random tissue sections of samples examined. Neonatal manipulations significantly influenced mammary gland ERα expression only at adulthood. Significance and trend symbols: *, $p<0.01$; +, $p<0.05$; §, $p=0.10$. *n* per group= 5.

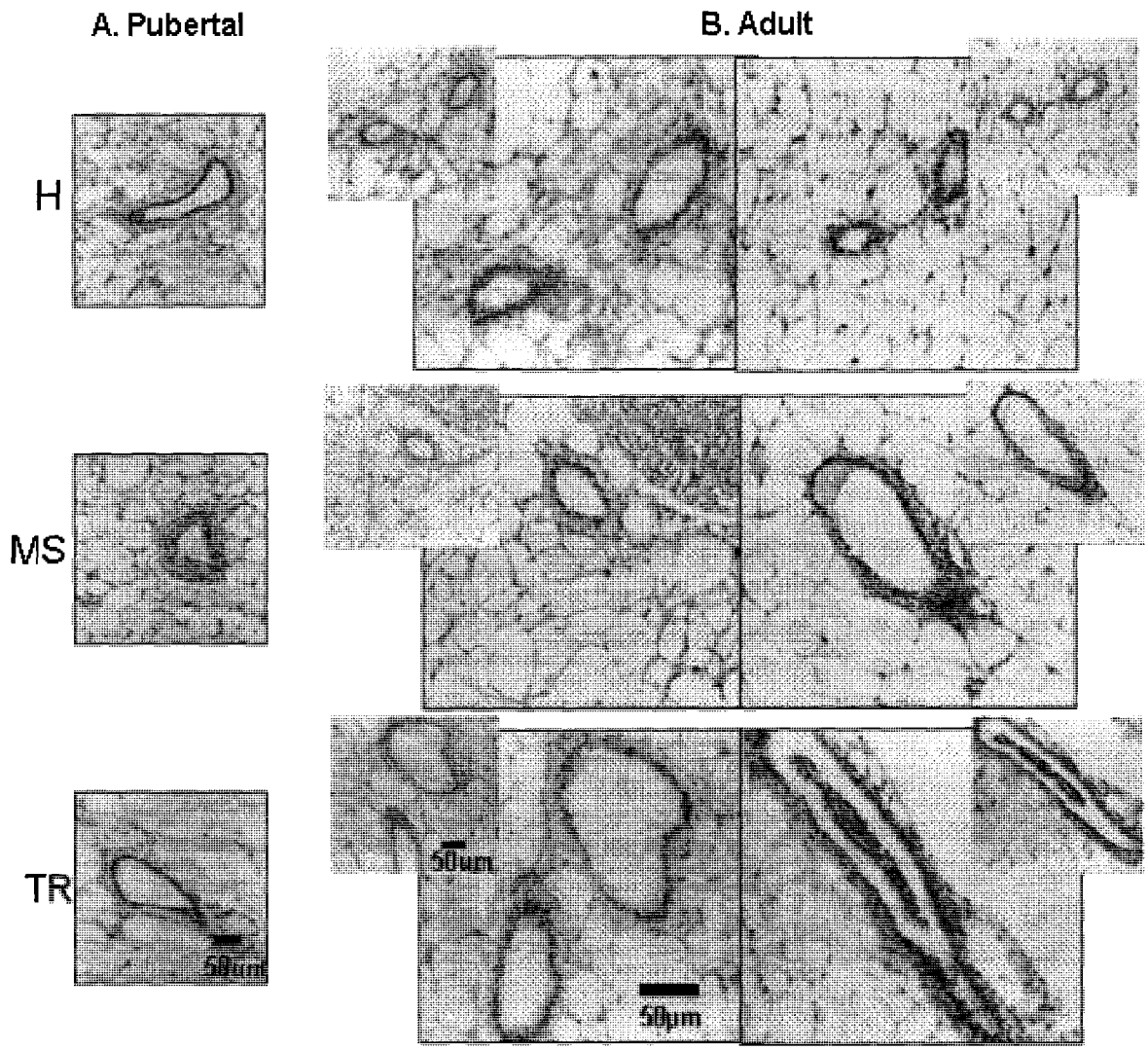


Figure 10.Representative sections of mammary glands positively stained for ERα protein (brown nuclei) in neonatally manipulated female mice. A. ERα protein expression was similar across neonatal manipulation groups in pubertal mice. B. In adult mice, TR compared to H and MS mammary glands, expressed lowest levels of ERα protein. Inset pictures show negative controls for each mammary gland section.

5.3.2 Expression of ERα and p53 protein in Western Blot analyses

A one-way ANCOVA, covarying out the effect of replicate runs for the effect of Treatment on ERα expression in pubertal mammary glands indicated that neonatal manipulations did not significantly affect ERα protein levels (Figure 9A). However, in adult mammary glands, a significant effect of Treatment ($F(2, 56)=7.34$, $p<0.01$) revealed that neonatal manipulations significantly affected ERα protein expression. Specifically, TR compared to both H ($p=0.012$) and MS ($p=0.0002$) adult mammary glands, had significantly lower ERα protein expression (Figure 9B).

For p53 protein expression, a one-way ANCOVA for the effect of Treatment on pubertal mammary glands revealed a trend towards an effect of Treatment ($F(2,22)=2.63$, $p=0.0947$), such that H compared to MS ($p=0.1004$) pubertal mammary glands, showed moderately higher p53 protein expression (Figure 10A). No differences in p53 expression were observed between MS and TR glands. p53 protein expression was not significantly influenced by neonatal environments in adult mouse mammary glands (Figure 10B).

5.3.3 p53 mRNA expression in adult mammary glands

RT-qPCR assays were successfully optimized for only p53 and GAPDH mRNA expression. One-way t-tests revealed that p53 mRNA expression was similar in adult mammary glands across neonatal manipulation groups (Figure 11).

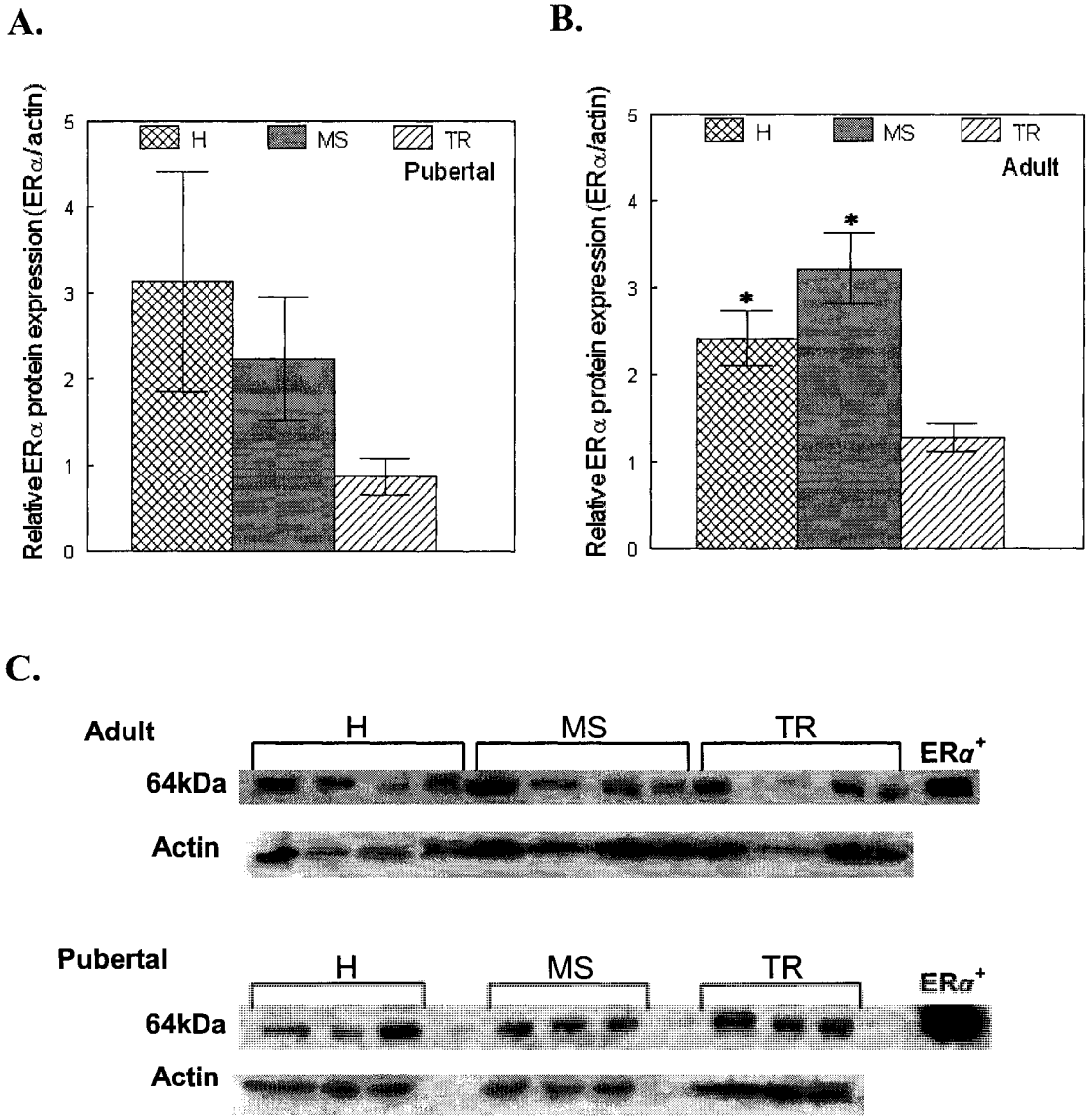


Figure 11.Mean (±S.E.M) relative ERα expression from Western Blot analyses of ERα protein expression in A: pubertal and B: adult mammary glands of neonatally manipulated mice. *n* per group: 10 - 12 mammary glands. C: Representative western blot images of ERα expression in adult and pubertal mammary glands. Expression of ERα protein among neonatal manipulation conditions were normalized using the corresponding actin expression level for each sample.
(Significance symbols: *, p's < 0.05).

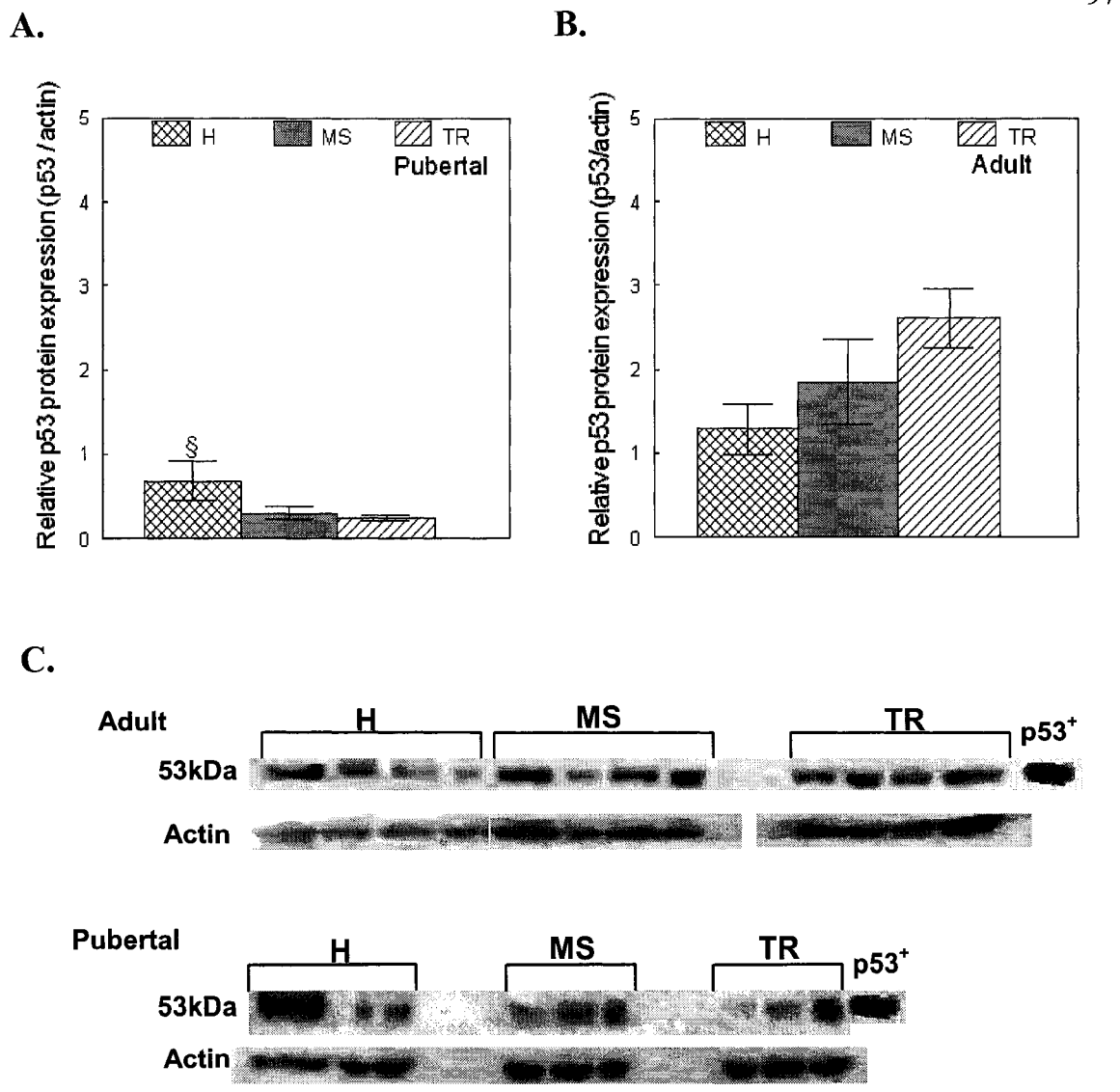


Figure 12.Mean (±S.E.M) relative p53 expression from Western Blot analyses of p53 protein expression in mammary glands of A: pubertal and B: adult neonatally manipulated mice. *n* per group: 3- 4 mammary glands. C: Representative Western Blot images of p53 expression in adult and pubertal mammary glands. Expression of p53 protein among neonatal manipulation conditions were normalized using the corresponding actin expression level for each sample. (Trend towards significance symbols: §, p=0.10).

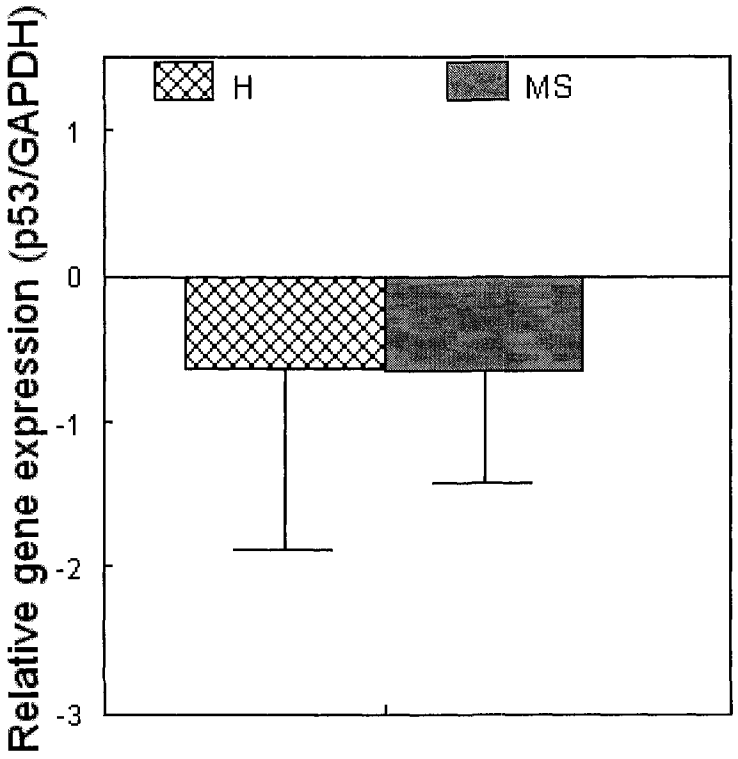


Figure 13.Expression (+S.E.M) of p53 mRNA in H and MS adult mammary glands relative to TR (control). No significant differences in mRNA expression were observed among mammary glands of neonatally manipulated adult mice. *n* per group= 3 mammary glands.

5.4 Discussion

The results of the present study reveal that ERα and p53 expression were differentially expressed in the mammary glands of neonatally manipulated mice. In mammary glands of pubertal mice, no significant differences in ERα protein expression (as measured by both western and by immunohistochemical analyses) were detected, whereas contrary to our predictions, the mammary glands of both adult H and MS mice had significantly increased ERα expression compared to TR adult mice. In contrast, p53 protein expression was moderately different between neonatal manipulation groups in pubertal, but not in adult mammary glands. That is, at puberty, H compared to MS mammary glands showed a moderate increase in p53 protein expression. In terms of mRNA expression, p53 levels in the mammary glands of adult mice were similar among neonatal manipulation groups.

Overall, the data demonstrate a differential, age-related ERα (adulthood) and p53 (pubertal) protein expression in mice and also suggest that p53 but not ERα protein levels are differentially influenced by the H and MS neonatal manipulations. The effects of neonatal manipulation on protein expression levels in pubertal and adult mice may be a result of the interaction between the physiological (e.g. hormonal) effects induced by the manipulations and the stage of mammary gland development. Indeed, at PND 30-35, pubertal mammary glands are going through rapid cell proliferation and development (Silberstein, 2001). At this developmental stage, surges of estrogen and other mammary-related mitogens interact with their respective receptors, orchestrating the complex interactions required for mammary gland development (Fendrick et al., 1998; Silberstein, 2001). In general, with increasing age, ERα staining intensity increases throughout the

mammary gland (Fendrick et al., 1998). Interestingly however, estrogen treatment of the pre-pubertal mammary gland significantly increases ERα levels, whereas estrogen treatment of the pubertal gland results in a down-regulation of ERα (Shyamala et al., 2002). These contrasting effects of estrogen suggest that there may be differences in the functional activation of ERα in the mammary gland during estrogen sensitive and insensitive stages. Importantly, in our studies, neonatal manipulations are suggested to differentially influence not only corticosterone levels but also circulating estrogen levels. Although hormone levels among H, MS, and TR mice in our study remain to be examined, this may be one possible factor influencing the differential expression of protein levels in the mammary glands.

However, as discussed in previous chapters, and as observed from results of the morphological analysis of the mammary gland (chapter 4), differences in HPA axis activity among pubertal female mice in various neonatal manipulation groups may not yet be noticeable at this early stage of development (recall that mammary glands were taken at the onset of the first estrous cycle experienced by the mouse). Additionally, ERα expression is associated with different mammary gland structures, such that TEBs are associated with a high level of ERα, whereas more differentiated structures have been shown to have decreased ERα protein expression (Russo et al., 1999). To support this, our mammary gland morphology results (discussed in chapter 4) found similar TEB numbers among pubertal mice. Thus, it is not surprising that ERα expression is also similar among pubertal mice.

In young adult mice, ERα protein expression analyses indicated that H and MS mice had generally higher levels of ERα protein compared to TR mice. In adult mice (approximately PND 77-80), levels of estrogen were found to be related to mammary

gland ERα expression, such that increased estrogen administration was associated with increased ERα expression (Shyamala et al., 2002). Consequently, our results may indicate that estrogen levels were higher in both H and MS compared to TR adult mice. Moreover, increased levels of estrogen in the mammary gland may be an indication of increased levels of cell proliferation, as ERα influences expression of progesterone receptors, promoting the proliferation of epithelial cells (Shyamala et al., 2002). Additionally, a significant effect of age was only apparent in the TR (control) group, such that ERα protein expression decreased from puberty to adulthood. This finding is in support of the observations that ERα levels have been found to be generally higher in pubertal compared to adult mice, and may be expected as increased cell proliferation is observed in pubertal compared to adult mammary glands (Cardiff and Wellings, 1999; Clarke, 2003; Richert et al., 2000; Shyamala et al., 2002). Interestingly, this age effect was not apparent in the two neonatally manipulated groups, indicating that ERα levels remained high in H and MS from puberty to adulthood. The elevated levels of ERα in H and MS compared to TR mice, could potentially indicate high levels of proliferation in these mammary glands from puberty to adulthood, and perhaps lower levels of proliferation in TR mice during the same time period. Thus, the results of the present study support our gross morphological analysis of mammary glands (chapter 4) which demonstrate that the mammary glands of H and MS adult mice contain significantly less TEBs compared to TR (data shown in Appendix B). However, LAU/TEB ratio calculations indicated that H compared to both MS and TR adult mammary glands were significantly more differentiated (chapter 4). Perhaps, given that the mammary glands were taken at an early stage of adulthood (i.e., PND 55-65, an approximate equivalent to 18-20 years of age in humans), the alterations in hormone levels, and hence level of

proliferation may not have yet reached their peak nor might they have had sufficient opportunity to significantly influence ERα levels in the mammary glands, resulting in the similar levels of ERα protein expression (and proliferation) between H and MS mice. These possibilities are currently being examined in our lab.

Aside from total expression of ERα protein, studies have shown that in both rodent and human mammary glands, the pattern of stromal and epithelial ERα is significant in mediating the effects of estrogen on mammary gland growth (Fendrick et al., 1998; Shyamala et al., 2002; Woodward, Xie and Haslam, 1999). Whereas other researchers show that the ERα in the epithelia (Mallepell et al., 2006) or stroma alone (Mueller et al., 2002) is sufficient to orchestrate growth, others contend that a complex interaction between stromal and epithelial ERα is needed for proper development of the mammary gland (Mallepell et al., 2006). Additionally, these functional differences are proposed to be age-dependent (Shyamala et al., 2002). Stromal ERα has been implicated in the maintenance of ductal structures, and its expression may be independent of ovarian steroid action (Shyamala et al., 2002). Moreover, stromal ERα is detected more often in the stroma surrounding primary mammary ducts, rather than more distal branches, suggesting the stromal ERα is more important in the growth of the primary duct, than secondary or tertiary branches (Shyamala et al., 2002). On the other hand, the role of epithelial ERα was shown to be essential in ductal morphogenesis, side branching and alveolar development, as shown by mammary gland transplant studies (Mallepell et al., 2006). In the present study however, the differences in ERα expression observed among young adult mammary glands exposed to various neonatal conditions were observed only on total ERα levels (i.e., the summation of epithelial and stromal ERα counts), and not stromal or epithelial ERα expression alone, and that generally, higher percentage of ERα

expression was found in the epithelia compared to the stromal compartment of the mammary gland. How this pattern of stromal/epithelial ERα expression found in the present study relates to mammary gland growth is not yet known. Moreover, researchers have also shown that estrogen action on mammary gland proliferation occurs in a paracrine manner, such that cells positive for ERα are non-proliferating, but are typically adjacent to proliferating cells (Daniel et al., 1987; Mallepell et al., 2006). Thus, the co-localization patterns of ERα positive and proliferating cells, and how they may be affected by different neonatal conditions still need to be addressed in order to explore why significant differences in ERα protein expression, were observed among neonatal manipulation groups.

The similar ERα expression profiles and gross mammary gland morphology in H and MS mice may potentially indicate a decreased risk of tumourigenesis susceptibility, due to increased proliferation (as indicated by ERα expression) and possibly, subsequent decrease in numbers of highly proliferative mammary structures (TEBs). Contrary to one study however (Russo et al., 1999), we found that high levels of ERα (assessed through immunohistochemical staining and western blots) are related to a lower number of undifferentiated structures. These researchers counted the number of ERα positive cells in specific TEBS and LAUs in 55 day old rats, whereas quantification of our immunohistochemical results involved counting the number of ERα positive cells in a whole field of view, regardless of the mammary structure (i.e., ductal branch, TEB or LAU) in mice. Thus, this difference in quantification method may account for differences in results. Additionally, Shyamala et al. (2002) found that in rodents, estrogen only modulated the level (intensity) of ERα in the mammary epithelial cells, but not the percent of cells expressing ERα. Future studies will need to elucidate on ERα protein

expression and intensity in specific structures (i.e., identification of various mammary structures, before quantification), in order to more accurately assess the differences in mammary gland ERα expression present among neonatal conditions.

In terms of p53 expression, the moderate increase in p53 expression in H compared to MS pubertal mammary glands, indicate that p53 regulation may be different between these two manipulation groups. p53 expression is relatively low in the normal cell, and usually increases in response to cellular stressors, including intrinsic changes such as replication errors, and external changes such as exposure to carcinogens (Lacroix et al., 2006). An increase in p53 in H mammary glands may indicate that these cells are being exposed to a higher level of cellular stressors, compared to those in the MS pubertal gland. Alternatively, higher levels of p53 protein expression may be an indication of increased protection from tumourigenesis. In support of this, upregulation of p53 protein expression and increased nuclear accumulation were found in mouse mammary glands treated with an estrogen/progesterone course of treatment (to mimic pregnancy) and by pregnancy itself, compared to virgin mouse glands (Tu et al., 2005), which the authors suggest to be one of the mechanisms involved in the protective effects of pregnancy on tumour development. Some researchers suggest a model whereby pregnancy sensitizes the p53 pathway to changes in the hormonal environment, exerting a protective effect on the mammary gland (Jerry et al., 2002; Minter et al., 2002). Moreover, another study found that 8 week old rat offspring whose mothers were exposed to a whole wheat diet during pregnancy exhibited an increase in p53 protein expression in the mammary gland, along with lower DMBA-induced tumour incidence and lower number of TEBs, which the authors believe are indicative of reduced tumourigenesis risk (Yu et al., 2006). Although the mechanisms involved in the modulation of p53 expression is complex, the

increased expression of p53 in the H pubertal mammary gland may be an indication of some form of protection against tumourigenesis, and needs to be further explored.

In contrast to ERα expression, p53 mRNA and protein expression in adult mammary glands were not affected by neonatal manipulations. These observations suggest that, changes, if any, which may lead to loss of function of the p53 protein, is most probably not at the transcriptional level, but may instead be at the post-translational level. Indeed, one study showed that in response to radiation, p53-induced transcription of a downstream protein important in the p53 apoptosis pathway ($p21^{wafl}$) in virgin mammary epithelial cells were only significantly up-regulated if preceded by estrogen-progesterone pre-treatment, but not when the virgin mammary epithelia was exposed to radiation alone (Jerry et al., 1998; Kuperwasser et al., 2000). Moreover, virgin mouse mammary glands typically show high levels of p53 mRNA, which are not indicative of p53 protein function, as radiation increases p53 mRNA but not p53 activity in virgin glands (Blackburn and Jerry, 2002). Thus, although no significant differences in p53 were found among adult mammary glands exposed to differing neonatal conditions, gross morphological analyses of these glands (discussed in chapter 4) suggest that cellular proliferation (a process associated with p53 functioning) may be different among adult mammary glands, which may be related to differences in p53 activity among neonatal manipulations groups. Future studies will aim at clarifying the association between p53 mRNA and protein expression, and its association with p53 activity, such as via analysis of the apoptotic index in the mammary glands of neonatally manipulated mice. Additionally, since the p53 antibody used for immunohistochemical staining was designed to detect full length p53 protein, and not mutated variant p53 proteins, the presence of other non-functional p53 variants was not verified in the present study.

Although the present study did not examine the interaction between the ERα and p53 proteins, other studies have shown direct interaction of the two, such that wild-type p53 can suppress estrogen-mediated transcription in endometrial cell lines, whereas mutant p53 proteins did not have the same effect (Yu et al., 1997). In mice, a specific p53 mutation (similar to a mutation found in human breast cancer) increased development of spontaneous tumours, that were frequently ERα positive (Wijnhoven et al., 2005). In humans, studies show that breast tumours with high p53 protein expression have been found to be frequently negative for estrogen receptor, but associated with a high proliferation rate (Lacroix et al., 2006). Thus, whether the protein expression patterns of ERα and p53 in the mammary glands of neonatally manipulated mice can be directly associated with each other and how they may interact to influence mammary gland development still needs to be explored.

The present study revealed that ERα and p53 protein expression patterns can be altered by changes in neonatal conditions. These differences in protein expression, along with gross morphological differences in the mammary gland, apparent only at young adulthood suggest that alterations in the neonatal environment (i.e., dam-pup interactions, thermoregulation) due to neonatal manipulation conditions, significantly influences the mammary gland developmental environment, and over time, may influence the susceptibility to mammary tumour development.

Chapter 6: General discussion and conclusion

The present study investigated the influence of the pre-pubertal environment on depressive-like behaviour, and on mammary gland growth and development in Balb/c mice. Alterations of the pre-pubertal (neonatal) environment used in the present study involved separating mouse dams from their pups for brief (15 minutes/day; handled; H) and prolonged (4 hours/day; maternally separated; MS) periods of daily maternal separation for the first three weeks of life. These maternal separation paradigms are well-established and are typically employed to examine the effects of dam-pup interactions, differences in neonatal environments on brain development, and resulting changes in behavioural and hormonal reactivity of rodents exposed to different stressful conditions (Pryce and Feldon, 2003). Since these neonatal manipulations have been found to differentially affect mouse stress-induced behaviours and stress hormone levels (e.g., ACTH, CORT) (Anisman et al., 1998; Meerlo et al., 1999; Pryce and Feldon, 2003; Wigger and Neumann, 1999), we hypothesized that mammary gland development (which like the brain, completes development after birth) would be influenced by neonatal manipulation-induced alterations in the body's main stress axis, the HPA axis. Thus, the present study examined the influence of neonatal manipulations on mammary gland morphology and mRNA and protein expression of two genes associated with cell and mammary gland growth.

The present study showed that:

1) Stress-induced behavioural differences (examined using the forced swim test) were influenced by neonatal manipulations, gender and age. Immobility behaviour (a depressive-like behaviour, Cryan et al., 2005) was higher in MS compared to H

mice, but this difference was dependent on gender and age of testing. The active coping behaviours of swimming, struggling and active floating were also influenced by neonatal manipulations, but dependent on gender, and the behaviour being observed. Active floating was higher in H compared to MS and TR females, whereas in males active floating was higher in H and MS compared to TR males, in both age groups. In contrast, swimming behaviour was different only in male mice such that regardless of age, swimming was lower in H and MS compared to TR males. Struggling behaviour was only influenced by neonatal manipulation, such that overall, H compared to MS and TR mice displayed higher levels of struggling. Interestingly, gender differences were apparent only in the MS manipulation group.

2) Differences in mammary gland morphology were observed among neonatal manipulation groups, but only at adulthood, such that a ratio of LAU/TEB, used as an index of mammary gland differentiation rate, was higher in H compared to both MS and TR (control) adult mammary glands.
3) ERα protein expression differences were only apparent at adulthood such that ERα protein expression was higher in H and MS compared to TR mammary glands. In contrast, p53 mRNA and protein expression was similar among the adult mammary glands of neonatally manipulated mice.

Similar to other studies (Boccia and Pedersen, 2001; Caldji et al., 2000; Kalinichev et al., 2002), and in support of our hypothesis, stress-induced behavioural differences among neonatally manipulated mice were observed, such that MS compared to H rodents displayed higher levels of immobility. This difference was age- and gender-dependent,

such that in male mice, higher levels of immobility in MS than H mice were observed only at puberty, whereas in females, this difference was only observed in adulthood. Active coping behaviours were also gender-dependent, and as discussed in chapter 3, suggest that male and female rodents, like humans, employ different coping strategies (Picinelli and Wilkinson, 2000) when exposed to the same stressor (Palanza, 2001). In addition, gender differences in forced swim test behaviours were only observed in MS mice, suggesting that a prolonged period of maternal separation results in more apparent gender-specific coping styles. A prolonged period of maternal separation (such as the MS manipulation) may interrupt the physiological/emotional needs of the pups (Pryce et al., 2005), and may influence dam-pup interactions, as has been found by some researchers (Anisman et al., 1998; Plotsky, et al., 1993; Pryce et al., 2005). As discussed in chapter 3, differences in dam-pup interactions may influence the development of neurobiological systems (such as the HPA axis) in the maturing offspring (Liu et al., 1997; Pryce et al., 2005), and subsequently influence behaviour.

In females, H compared to MS adults showed less depressive-like behaviours (immobility), whereas active coping behaviours of active floating and struggling were generally higher in adult H compared to MS females. These findings may indicate that HPA axis regulation may indeed be different between H and MS females in our study, as they are in male rodents (Anisman et al., 1998; Plotsky and Meaney, 1993). For example, H compared to MS rodents generally exhibit an attenuated CORT response to a stressor (Anisman et al., 1998; Pryce and Feldon, 2003). Reduced levels of circulating CORT, as well as attenuated stress-induced CORT levels may decrease the inhibition of HPG axis activity and can lead to an increase in estrogen production and accelerate mammary gland development (Tsigos and Chrousos, 2002). This would explain why, and in support of our

hypothesis, an increased level of differentiation in H adult mammary glands was observed. Conversely in MS mice, increased CORT levels can suppress the HPG axis leading to decreased estrogen production, which may explain the lower rate of differentiation in MS mammary glands. TR female mice however, did not show a distinct behavioural pattern, and had significantly lower mammary gland differentiation rates compared to H adult females. At this juncture, it is difficult to speculate how TR mouse behaviours can be related to HPA regulation and thus, basal and stress-induced levels of CORT and estrogen among H, MS and TR mice are currently being analyzed in our laboratory in order to determine whether CORT levels may be directly associated with the mammary gland development patterns observed among neonatally manipulated groups. Furthermore, it is important to note that CORT and estrogen levels are some of the many factors that can be associated with mammary gland growth. Moreover, as discussed in chapter 5, the interaction between these mammary gland growth hormones and their corresponding receptors, and regulation of the receptors themselves, may be differentially influenced by neonatal manipulations.

In terms of ERα expression, significant differences in ERα protein expression were only observed in adult mammary glands, such that H and MS mammary glands showed similarly high levels of ERα protein expression compared to TR mammary glands. As discussed in chapter 5, ERα expression may be used as an indication of circulating estrogen levels, such that in adult rodents, ERα expression has been shown to be positively related to estrogen levels (Shyamala et al., 2002). Thus, our ERα expression results may indicate that estrogen levels are significantly higher in H and MS than TR mammary glands, suggesting higher proliferation rates in H and MS adult mammary glands, which is in agreement with differences in TEB numbers among adult mammary

glands (data shown in Appendix B). However, LAU/TEB calculations indicated that H compared to both MS and TR adult mammary glands were significantly more differentiated (chapter 4). It is possible that since these mammary glands were observed at young adulthood (PND 55-65), changes in hormone levels, and proliferation rates may not have reached their peak, resulting in similar ERα protein levels between H and MS mice. Additionally, it may be that the regulation of estrogen on ERα and mammary gland growth in MS mammary glands may be different from H mammary glands, and remains to be elucidated. Moreover, aside from ERα/estrogen levels, the development of the mammary gland is governed by a variety of signalling pathways, involving a large number of genes including the other mammogenic hormone receptors, epidermal growth factor, and transforming growth factor-β (Silberstein, 2001). The influence of neonatal manipulations on protein expression of these genes, and how they may be associated with mammary gland growth in neonatally manipulated mice need to be determined.

In contrast to observations in adult mice, the effects of H and MS manipulations on pubertal mice were not observed in mammary gland development such that gross mammary gland morphology and ERα protein expression were similar among neonatal conditions. Similarly, forced swim test behaviours among neonatally manipulated female mice were generally similar at this stage of development (chapter 3). This may indicate that at this early time point in mouse development (PND30-35), differences in HPA axis development (if any) among neonatally manipulated mice may not have exerted an observable effect on behaviour or mammary gland growth, which only became apparent at young adulthood.

In terms of p53 expression, similar levels of p53 mRNA and protein expression among adult mammary glands could indicate that p53 is not influenced by neonatal

manipulation in adult mice. Alternatively, these observations suggest that differences in p53 (if any), which may lead to loss of function of the p53 protein may lie at the post-translational level. As discussed in chapter 5, p53 mRNA expression is not always indicative of protein expression/function (Blackburn and Jerry, 2002; Jerry et al., 1998; Kuperwasser et al., 2000). Since gross morphological analyses of neonatally manipulated mammary glands suggest that in adult glands, cellular proliferation (associated with p53 functioning) may be different among mammary glands, future studies will need to measure p53 activity, such as by examination of the apoptotic index in the mammary glands. Additionally, although the interaction of p53 and ERα were not examined in the present study, research has shown a direct interaction of the two proteins (Yu et al, 1997), and that an association between ERα and p53 levels are found in mammary tumours in both mice and humans (Lacroix et al., 2006; Wijnhoven et al., 2005). Thus, whether the protein expression patterns of ERα and p53 in the mammary glands of neonatally manipulated mice can be directly associated, and how their interaction may influence mammary gland development need to be explored.

In conclusion, subtle manipulations of the rodent neonatal environment result in significant changes in stress-induced behaviours, and mammary gland development, especially in the young adult female Balb/c mouse. Although future studies are still needed to determine whether these changes are stable over time (e.g., in mature or aged mice), and the specific mechanisms involved in altering mammary gland development among different neonatal experiences, this line of research is shedding some insight into the link between life experiences, stressors and the risk of breast cancer development.

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Appendices

Appendix A: Descriptive statistics and ANOVAs of forced swim test behaviours in pubertal and adult Balb/c mice

Gen -der	Age (pubertal/ adult)	Treatm ent	Swim Mean	Swim Std. Dev.	Imm Mean	Imm Std. Dev.	Actf Mean	Actf Std. Dev.	Strgl Mean	Strgl Std. Dev.
F	P	H	0.34	0.17	0.27	0.13	0.07	0.08	0.32	0.17
F	P	MS	0.46	0.23	0.28	0.16	0.09	0.10	0.16	0.11
F	P	TR	0.35	0.07	0.37	0.19	0.09	0.10	0.19	0.09
F	A	H	0.4	0.14	0.07	0.09	0.33	0.14	0.12	0.09
F	A	MS	0.42	0.18	0.34	0.11	0.14	0.14	0.10	0.07
F	A	TR	0.54	0.19	0.19	0.14	0.15	0.13	0.12	0.06
M	P	H	0.30	0.05	0.37	0.15	0.09	0.07	0.24	0.13
M	P	MS	0.14	0.12	0.50	0.11	0.16	0.11	0.20	0.19
M	P	TR	0.41	0.07	0.52	0.08	0.00	0.00	0.06	0.05
M	A	H	0.35	0.12	0.13	0.13	0.36	0.16	0.16	0.15
M	A	MS	0.27	0.15	0.17	0.13	0.40	0.18	0.15	0.13
M	A	TR	0.55	0.17	0.22	0.13	0.14	0.14	0.09	0.09

Immobility ANOVA and post hoc tests

Effect	Univariate Tests of Significance for RIMM (Swim_M_F_02to05_tr Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	7.027585	1	7.027585	410.9102	0.000000
SEX	0.090783	1	0.090783	5.3082	0.023294
AGE	0.835667	1	0.835667	48.8624	0.000000
TRT	0.260294	2	0.130147	7.6098	0.000839
SEX*AGE	0.173442	1	0.173442	10.1413	0.001933
SEX*TRT	0.016406	2	0.008203	0.4796	0.620422
AGE*TRT	0.045002	2	0.022501	1.3157	0.272898
SEX*AGE*TRT	0.118911	2	0.059455	3.4764	0.034704
Error	1.710248	100	0.017102		

Cell No.	Newman-Keuls test; variable immobility Probabilities for Post Hoc Tests Error: Between MS = .01710, df = 100.00			
	TRT	{1} .17083	{2} .29818	{3} .26870
1	H		0.000266	0.001842
2	MS	0.000266		0.333737
3	TR	0.001842	0.333737	

Newman-Keuls test; variable RIMM (Swim_M_F_02tc
Probabilities for Post Hoc Tests
Error: Between MS = .01710, df = 100.00

Cell No.	SEX	AGE	{1} .30935	{2} .20123	{3} .45335	{4} .16944
1	F	P		0.007448	0.000532	0.001829
2	F	A	0.007448		0.000105	0.422857
3	M	P	0.000532	0.000105		0.000139
4	M	A	0.001829	0.422857	0.000139	

Newman-Keuls test; variable RIMM (Swim_M_F_02to05_truncated)
Probabilities for Post Hoc Tests
Error: Between MS = .01710, df = 100.00

Cell No.	AGE	TRT	{1} .30981	{2} .36183	{3} .41338	{4} .09599	{5} .27225	{6} .19951
1	P	H		0.261565	0.068216	0.000192	0.416889	0.048253
2	P	MS	0.261565		0.265917	0.000117	0.131697	0.003644
3	P	TR	0.068216	0.265917		0.000121	0.014785	0.000207
4	A	H	0.000192	0.000117	0.000121		0.000758	0.026907
5	A	MS	0.416889	0.131697	0.014785	0.000758		0.117621
6	A	TR	0.048253	0.003644	0.000207	0.026907	0.117621	

Newman-Keuls test; variable RIMM (Swim_M_F_02to05_truncated)
Probabilities for Post Hoc Tests
Error: Between MS = .01710, df = 100.00

Cell No.	SEX	AGE	TRT	{1} .27429	{2} .28288	{3} .37196	{4} .07415	{5} .33896	{6} .19121	{7} .37376	{8} .50000	{9} .52381	{10} .13093	{11} .17521	{12} .22303
1	F	P	H		0.902234	0.501369	0.054733	0.623987	0.460713	0.611678	0.019785	0.009387	0.247076	0.488958	0.463864
2	F	P	MS	0.902234		0.410742	0.051995	0.423007	0.555446	0.562600	0.019911	0.010243	0.256197	0.536059	0.667470
3	F	P	TR	0.501369	0.410742		0.001503	0.636926	0.108441	0.979643	0.162884	0.136284	0.017620	0.080918	0.212804
4	F	A	H	**0.054733**	0.051995	0.001503		0.005954	0.339804	0.001691	0.000171	0.000118	0.417321	0.319502	0.213139
5	F	A	MS	0.623987	0.423007	0.636926	0.005954		0.219818	0.871859	0.102472	0.068744	0.053385	0.184511	0.348446
6	F	A	TR	0.460713	0.555446	**0.108441**	0.339804	0.219818		0.131287	0.000716	0.000321	0.663538	0.818952	0.649095
7	M	P	H	0.611678	0.562600	0.979643	0.001691	0.871859	0.131287		0.073148	0.084566	0.020230	0.094767	0.264558
8	M	P	MS	0.019785	0.019911	0.162884	0.000171	0.102472	0.000716	**0.073148**		0.733454	0.000181	0.000432	0.002570
9	M	P	TR	0.009387	0.010243	0.136284	0.000118	0.068744	0.000321	**0.084566**	0.733454		0.000176	0.000246	0.001046
10	M	A	H	0.247076	0.256197	0.017620	0.417321	0.053385	0.663538	0.020230	0.000181	0.000176		0.526692	0.551478
11	M	A	MS	0.488958	0.536059	0.080918	0.319502	0.184511	0.818952	0.094767	0.000432	0.000246	0.526692		0.772155
12	M	A	TR	0.463864	0.667470	0.212804	0.213139	0.348446	0.649095	0.264558	0.002570	0.001046	0.551478	0.772155	

Swimming ANOVA and post hoc tests

Effect	Univariate Tests of Significance for RSWIM (Swim_M Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	12.46954	1	12.46954	488.9160	0.000000
SEX	0.18789	1	0.18789	7.3670	0.007825
AGE	0.21315	1	0.21315	8.3573	0.004712
TRT	0.25016	2	0.12508	4.9043	0.009294
SEX*AGE	0.00055	1	0.00055	0.0217	0.883137
SEX*TRT	0.24191	2	0.12095	4.7425	0.010772
AGE*TRT	0.04495	2	0.02248	0.8813	0.417454
SEX*AGE*TRT	0.07526	2	0.03763	1.4755	0.233605
Error	2.55045	100	0.02550		

Cell No.	Newman-Keuls test; variable RSWIM Probabilities for Post Hoc Tests Error: Between MS = .02550, df = 10			
	TRT	{1} .39120	{2} .35704	{3} .48468
1	H		0.358807	0.013311
2	MS	0.358807		0.002487
3	TR	0.013311	0.002487	

Cell No.	Newman-Keuls test; variable RSWIM (Swim_M_F_02to05_truncated) Probabilities for Post Hoc Tests Error: Between MS = .02550, df = 100.00							
	SEX	TRT	{1} .42637	{2} .43415	{3} .47875	{4} .33259	{5} .23881	{6} .50115
1	F	H		0.889813	0.618673	0.096798	0.003300	0.541785
2	F	MS	0.889813		0.427218	0.169682	0.004017	0.457202
3	F	TR	0.618673	0.427218		0.050003	0.000502	0.689717
4	M	H	0.096798	0.169682	0.050003		0.096823	0.026568
5	M	MS	0.003300	0.004017	0.000502	0.096823		0.000240
6	M	TR	0.541785	0.457202	0.689717	0.026568	0.000240	

Struggling ANOVA and post hoc tests

Effect	Univariate Tests of Significance for RSTRGL (Swim_ Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	2.180048	1	2.180048	182.9939	0.000000
SEX	0.006589	1	0.006589	0.5531	0.458790
AGE	0.104511	1	0.104511	8.7727	0.003820
TRT	0.120473	2	0.060236	5.0563	0.008094
SEX*AGE	0.031344	1	0.031344	2.6310	0.107944
SEX*TRT	0.050716	2	0.025358	2.1286	0.124368
AGE*TRT	0.048810	2	0.024405	2.0486	0.134297
SEX*AGE*TRT	0.014727	2	0.007363	0.6181	0.541023
Error	1.191323	100	0.011913		

Cell No.	Newman-Keuls test; variable RSTRGL (Swim_M_F_C Probabilities for Post Hoc Tests Error: Between MS = .01191, df = 100.00					
	SEX	AGE	{1} .23031	{2} .11513	{3} .18209	{4} .14276
1	F	P		0.004001	0.146728	0.024842
2	F	A	0.004001		0.110165	0.404199
3	M	P	0.146728	0.110165		0.235711
4	M	A	0.024842	0.404199	0.235711	

Active floating ANOVA and post hoc tests

Effect	Univariate Tests of Significance for RACTF (Swim_M Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	2.355330	1	2.355330	133.5411	0.000000
SEX	0.045593	1	0.045593	2.5850	0.111032
AGE	0.601497	1	0.601497	34.1033	0.000000
TRT	0.215270	2	0.107635	6.1026	0.003157
SEX*AGE	0.046689	1	0.046689	2.6471	0.106884
SEX*TRT	0.154826	2	0.077413	4.3891	0.014890
AGE*TRT	0.108362	2	0.054181	3.0719	0.050729
SEX*AGE*TRT	0.038630	2	0.019315	1.0951	0.338483

Cell No.	TRT	{1} .24806	{2} .20654	{3} .11829
1	H		0.180951	0.000257
2	MS	0.180951		0.005212
3	TR	0.000257	0.005212	

Newman-Keuls test; variable RACTF
Probabilities for Post Hoc Tests
Error: Between MS = .01764, df = 10

Cell No.	SEX	TRT	{1} .23624	{2} .12311	{3} .12764	{4} .26776
1	F	H		0.043979	0.021642	0.499566
2	F	MS	**0.043979**		0.922727	0.012911
3	F	TR	**0.021642**	0.922727		0.009216

Newman-Keuls test; variable RACTF (Swim_M_F_02
Probabilities for Post Hoc Tests
Error: Between MS = .01764, df = 100.00

Appendix B: Descriptive statistics and ANOVAs of LAU/TEB ratios and branch point counts in pubertal and adult female mouse mammary glands

Trt	Age (pubertal/ adult)	TEB mean	TEB Std. dev.	LAU mean	LAU Std. dev.	LAU/ TEB ratio	LAU/TEB ratio Std. dev.	Valid N
H	Pubertal	19.68	7.88	1.18	0.75	0.06	0.05	11
H	Adult	7.46	4.36	4.14	0.66	0.80	0.53	14
MS	Pubertal	18.33	4.39	1.25	0.45	0.07	0.03	12
MS	Adult	7.03	2.56	3.68	0.75	0.59	0.25	19
TR	Pubertal	19.32	7.60	1.27	0.65	0.07	0.03	11
TR	Adult	10.87	4.96	4.13	0.74	0.43	0.15	15

Treatment	Age (pubertal/adult)	Branch point mean	Branch point Std. dev.	Valid N
H	Pubertal	42.75	16.46	8
H	Adult	119.8	19.06	10
MS	Pubertal	46.5	11.55	8
MS	Adult	135.57	30.42	14
TR	Pubertal	53.37	15.86	8
TR	Adult	128.45	17.59	11

ANOVA and post hoc tests for TEB counts in adult mammary glands

Effect	Univariate Tests of Significance for TEBMEAN (TEBS Sigma-restricted parameterization Type III decomposition SS	Degr. of Freedom	MS	F	p
Intercept	3371.267	1	3371.267	214.0631	0.000000
TRT	139.277	2	69.638	4.4218	0.017651
Error	708.702	45	15.749		

Cell No.	Newman-Keuls test; variable TEB MEAN (TEBS_03-05_with Dif Probabilities for Post Hoc Tests Error: Between MS = 15.749, df = 45.000 TRT	{1} 7.4643	{2} 7.0263	{3} 10.867
1	H		0.758500	
2	MS	0.758500		
3	TR	0.020495	0.025097	

ANOVA and post hoc tests for LAU/TEB ratios

Effect	Univariate Tests of Significance for LAU/TEB (TEBS_ Sigma-restricted parameterization Type III decomposition SS	Degr. of Freedom	MS	F	p
Intercept	8.926662	1	8.926662	130.2925	0.000000
TRT	0.414227	2	0.207114	3.0230	0.054542
AGE	5.696208	1	5.696208	83.1412	0.000000
TRT*AGE	0.434547	2	0.217273	3.1713	0.047554
Error	5.206950	76	0.068512		

Cell No.	Newman-Keuls test; variable LAU/TEB (TEBS_03-05) Probabilities for Post Hoc Tests Error: Between MS = .06851, df = 76.000 TRT	AGE	{1} .06330	{2} .79798	{3} .07270	{4} .58532	{5} .06692	{6} .43106
1	H	P		0.000126	0.995406	0.000140	0.971894	0.003166
2	H	A	0.000126		0.000148	0.040591	0.000123	0.001734
3	MS	P	0.995406	0.000148		0.000117	0.955047	0.000878
4	MS	A	0.000140	0.040591	0.000117		0.000160	0.134832
5	TR	P	0.971894	0.000123	0.955047	0.000160		0.001883
6	TR	A	0.003166	0.001734	0.000878	0.134832	0.001883	

ANOVA for branch point counts

Effect	Univariate Tests of Significance for branch point (BRAN Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	434856.8	1	434856.8	1002.535	0.000000
TRT	1148.8	2	574.4	1.324	0.274653
AGE	91282.7	1	91282.7	210.447	0.000000
TRT*AGE	562.3	2	281.1	0.648	0.527104
Error	22989.1	53	433.8		

Appendix C: Western blot ERα and p53 protein expression analyses using Scion Image Software.

Optical density of adult ERα mammary gland samples			
Sample	Replicate 1	Replicate2	Replicate 3
H1	1.97	2.37	2.13
H2	1.08	1.74	4.35
H3	0.21	1.00	1.24
H4	1.29	2.55	10.70
MS1	4.15	1.25	36.33
MS2	1.12	0.94	4.32
MS3	14.70	1.33	5.28
MS4	3.00	2.24	3.80
TR1	0.44	1.64	8.47
TR2	1.38	5.89	0.85
TR3	0.21	7.08	3.30
TR4	1.40	1.77	2.31

Optical density of pubertal ERα mammary gland samples			
Sample	Replicate 1	Replicate2	Replicate 3
H1	5.87	0.53	0.33
H2	0.99	0.43	1.17
H3	11.86	1.94	4.96
MS1	0.68	1.19	1.68
MS2	5.94	0.40	1.21
MS3	3.01	5.54	0.37
TR1	1.30	0.50	1.51
TR2	15.02	0.23	2.97
TR3	0.70	0.39	1.46

Optical density of adult p53 mammary gland samples			
Sample	Replicate 1	Replicate2	Replicate 3
H1	0.48	0.87	2.49
H2	0.78	1.48	3.55
H3	0.25	0.69	1.93
H4	3.15	0.73	10.44
MS1	0.93	2.90	1.48
MS2	0.37	5.18	1.08
MS3	1.01	1.08	1.78
MS4	0.36	0.57	5.36
TR1	0.68	4.32	3.39
TR2	2.71	1.94	0.07
TR3	2.38	1.85	2.87
TR4	6.59	0.09	3.30

Optical density of pubertal p53 mammary gland samples				
Sample	Replicate 1	Replicate2	Replicate 3	Replicate 4
H1	2.00	0.14	0.05	0.10
H2	0.70	0.09	1.45	1.06
H3	0.74	0.09	0.14	1.75
MS1	1.02	0.36	0.18	0.32
MS2	1.99	0.05	0.56	0.77
MS3	0.24	0.09	0.21	0.27
TR1	0.24	0.11	0.34	0.33
TR2	1.37	0.19	0.11	0.29
TR3	0.92	1.48	0.19	0.34

ANOVA and post hoc tests for ERα protein expression in pubertal and adult mammary glands

Adult mammary glands

	Univariate Tests of Significance for MOD_ERA (WES Sigma-restricted parameterization Type III decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	31.6266	1	31.62661	17.18945	0.000116
RUNNER	29.8732	1	29.87318	16.23643	0.000170
TRT	27.0068	2	13.50342	7.33927	0.001476
Error	103.0336	56	1.83989		

	Newman-Keuls test; variable MOD_E Probabilities for Post Hoc Tests Error: Between MS = 1.8399, df = 56			
Cell No.	TRT	{1} 2.4041	{2} 3.2097	{3} 1.2660
1	H		0.070588	0.011854
2	MS	0.070588		0.000231
3	TR	0.011854	0.000231	

Pubertal mammary glands

Effect	Univariate Tests of Significance for Era (stat_ERA_p Sigma-restricted parameterization Type III decomposition SS	Degr. of Freedom	MS	F	p
Intercept	63.0102	1	63.01024	9.591392	0.005461
run	19.4953	1	19.49525	2.967559	0.099646
trt	19.9799	2	9.98994	1.520665	0.241679
Error	137.9586	21	6.56946		

ANOVA and post hoc tests for p53 protein expression in pubertal and adult mammary glands

Adult mammary glands

Effect	Univariate Tests of Significance for p53 OD (STAT_p53 Sigma-restricted parameterization Type III decomposition SS	Degr. of Freedom	MS	F	p
Intercept	1.67930	1	1.679300	1.086527	0.306486
RUN	8.83465	1	8.834645	5.716122	0.024047
TRT	6.77356	2	3.386778	2.191287	0.131255
Error	41.73029	27	1.545566		

Pubertal mammary glands

Effect	Univariate Tests of Significance for p53 OD (STAT_p53 Sigma-restricted parameterization Type III decomposition SS	Degr. of Freedom	MS	F	p
Intercept	0.001379	1	0.001379	0.011472	0.915675
RUN	0.364865	1	0.364865	3.034690	0.095468
TRT	0.632221	2	0.316111	2.629185	0.094657
Error	2.645091	22	0.120231		

Cell No.	Newman-Keuls test; variable p53 OD (Probabilities for Post Hoc Tests Error: Between MS = .12023, df = 2 TRT	{1} .52832	{2} .24213	{3} .23823
1	H		0.100449	0.213682
2	MS	0.100449		0.981680
3	TR	0.213682	0.981680	

Appendix D: ERα expression analysis of mammary glands using immunohistochemical staining.

Treat-ment	Age (pubertal/adult)	TEB/ Branches Mean	TEB/ Branches Std. Dev.	Stroma Mean	Strom a Std. Dev.	Total Mean	Total Std. Dev.	Valid N
H	Pubertal	51.85	10.97	27.34	12.64	79.19	16.58	19
H	Adult	55.36	10.80	23.47	11.91	78.83	18.97	24
MS	Pubertal	51.18	12.91	32.41	12.93	83.44	23.09	20
MS	Adult	53.05	11.32	18.64	12.09	78.64	19.44	24
TR	Pubertal	55.53	11.78	32.37	9.30	87.93	16.52	19
TR	Adult	48.46	13.67	25.59	9.84	67.10	17.66	25

ANOVA and post hoc tests for ERα expression in the TEB/Branches of mouse mammary glands

	Univariate Tests of Significance for ER_tebbr% (ER_ Sigma-restricted parameterization Type III decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	357138.4	1	357138.4	2484.180	0.000000
TRT	69.1	2	34.5	0.240	0.786833
AGE	10.2	1	10.2	0.071	0.789915
TRT*AGE	697.2	2	348.6	2.425	0.092641
Error	17970.6	125	143.8		

	Newman-Keuls test; variable **ER_tebbr%** (ER_ALPHA_COUNTS) Probabilities for Post Hoc Tests Error: Between MS = 143.77, df = 125.00							
Cell No.	TRT	AGE	{1} 51.853	{2} 55.362	{3} 55.527	{4} 48.458	{5} 51.181	{6} 53.051
1	H	P		0.601976	0.746262	0.621886	0.854153	0.743111
2	H	A	0.601976		0.964123	0.322945	0.662046	0.526965
3	TR	P	0.746262	0.964123		0.380939	0.757640	0.776530
4	TR	A	0.621886	0.322945	0.380939		0.456152	0.590567
5	MS	P	0.854153	0.662046	0.757640	0.456152		0.865702
6	MS	A	0.743111	0.526965	0.776530	0.590567	0.865702	

ANOVA for ERα expression in the stroma of mouse mammary glands

Effect	Univariate Tests of Significance for ER_strma% (ER_ Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	91565.31	1	91565.31	691.0436	0.000000
TRT	346.39	2	173.20	1.3071	0.274272
AGE	2121.71	1	2121.71	16.0126	0.000107
TRT*AGE	558.22	2	279.11	2.1064	0.125970
Error	16562.87	125	132.50		

ANOVA and post hoc tests of total ERα expression in mouse mammary glands

Effect	Univariate Tests of Significance for ER_ALL% (ER_A Sigma-restricted parameterization Type III decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	810374.7	1	810374.7	2278.055	0.000000
TRT	271.9	2	136.0	0.382	0.683142
AGE	2426.9	1	2426.9	6.822	0.010106
TRT*AGE	2492.0	2	1246.0	3.503	0.033105
Error	44466.4	125	355.7		

Cell No.	Newman-Keuls test; variable **ER_ALL%**(ER_ALPHA_COUNTS) Probabilities for Post Hoc Tests Error: Between MS = 355.73, df = 125.00							
	TRT	AGE	{1} 79.197	{2} 78.832	{3} 87.935	{4} 67.100	{5} 83.444	{6} 78.642
1	H	P		0.949370	0.281407	0.151515	0.459982	0.994860
2	H	A	0.949370		0.387964	0.102490	0.701539	0.973514
3	TR	P	0.281407	0.387964		0.003928	0.434669	0.486488
4	TR	A	0.151515	0.102490	0.003928		0.036095	0.044641
5	MS	P	0.459982	0.701539	0.434669	0.036095		0.837527
6	MS	A	0.994860	0.973514	0.486488	0.044641	0.837527	

Appendix E: Sample of p53 mRNA expression results using RT-qPCR results using the $2^{-\Delta\Delta CT}$ method.

Sample	Detector	Ct	Quantity	Qty Mean	Qty SD	Ct Mean	Ct SD
H1	trp53	27.79	28.65	26.33	3.07	27.91	0.16
H2	trp53	27.36	39.30	38.47	5.86	27.40	0.21
H3	trp53	25.55	149.37	144.00	6.65	25.60	0.06
MS1	trp53	28.25	20.36	14.58	5.03	28.75	0.44
MS2	trp53	29.18	10.29	10.81	0.98	29.11	0.12
MS3	trp53	25.71	132.92	123.01	29.21	25.84	0.35
TR1	trp53	27.94	25.63	25.39	1.51	27.95	0.08
TR2	trp53	28.95	12.18	13.25	1.80	28.84	0.18
TR3	trp53	28.66	15.08	14.19	0.80	28.74	0.08

Sample	Detector	Ct	Quantity	Qty Mean	Qty SD	Ct Mean	Ct SD
H1	gapdh	28.60	0.32	0.30	0.03	28.72	0.13
H2	gapdh	27.05	0.93	0.80	0.15	27.29	0.29
H3	gapdh	20.39	88.49	88.73	6.09	20.39	0.10
MS1	gapdh	27.45	0.71	0.79	0.09	27.30	0.16
MS2	gapdh	28.52	0.34	0.41	0.06	28.27	0.23
MS3	gapdh	24.20	6.56	7.14	0.65	24.08	0.13
TR1	gapdh	28.37	0.38	0.41	0.03	28.26	0.11
TR2	gapdh	26.74	1.15	1.38	0.20	26.49	0.22
TR3	gapdh	28.54	0.34	0.40	0.06	28.30	0.22