SOCIAL STRESS ALTERS GENE EXPRESSION IN A TRANSGENERATIONAL RODENT MODEL OF POSTNATAL DEPRESSION

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List of abbreviations

Actb	Actin beta.
AON	Autonomic nervous system.
ACTH	Adrenocorticotropic Hormone.
ANS	Autonomic nervous system.
AVP	Arginine Vasopressin.
AvpR1a	Arginine vasopressin receptor 1A.
cDNA	Complementary DNA.
CeA	Central amygdala.
Crh	Corticosteroid Releasing Hormone.
CSS	Chronic Social Stress.
CON	Control.
CNS	Central nervous system.
DEPC	Diethylpyrocarbonate.
DNase	Deoxyribonuclease.
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase.
Gr	Glucocorticoid Receptor.
GC	Glucocorticoid.
Hcrt	Hypocretin (Orexin) Neuropeptide Precursor.
Hcrtr1	Orexin receptor type 1.
Hcrtr2	Orexin receptor type 2.
HPLC	High-pressue liquid chromatography.
HPA	Hypothalamic pituitary adrenocortical.
HPRT	Hypoxanthine-guanine phosphoribosyltransferase.
MeA	Medial amygdala.
Mr	Mineralocorticoid Receptor.
Mrfap1	Morf4 Family Associated Protein 1.
mRNA	Messenger ribonucleic acid.
OxR1	Orexin receptor 1.
Oxr2	Orexin receptor 2.
Oxt	Oxytocin.
OxtR	Oxytocin Receptor.
PCR	Polymerase chain reaction.
Pgk1	phosphoglycerate kinase 1.
PPD/A	Postnatal depression and anxiety.
PrlR	Prolactin Receptor (Long form).
PVN	Paraventricular nucleus of hypothalamus.
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR.
RNase	Ribonuclease.
RT	Reverse Transcription.
Sdha	Succinate dehydrogenase complex, subunit A.
SDS	Sodium dodecyl sulfate.
SNP	Single Nucleotide Polymorphism.
SON	Supraoptic nucleus.
TBE	Tris-Borate-EDTA.
T_m	Melting Temperature.
β-actin	Beta Actin.

Conference Proceedings

Stella Spears, Mohammad Taliefar, Benjamin Nephew, and Christopher Murgatroyd. Multi-Generational Effects of Chronic Social Stress: Initial PCR Examination of the PVN. Tufts Cummings School of Veterinary Medicine, North Grafton, MA. 2013.

M. Taliefar, G. Podda, B. Nephew, C. Murgatroyd. Social stress alters gene expression in a transgenerational rodent model of postnatal mood disorders. European College of Neuropsychopharmacology (ECNP). Nice, France, 2014.

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Abstract

Maternal mood disorders such as depression and chronic anxiety can be severely detrimental to the health and wellbeing of both the affected mother and her offspring. Despite a prevalence rate of 12-15% in the UK (Segre et al. 2007), there are few animal models of postnatal depression. In this rodent model of postnatal depression and anxiety, chronic social stress exposure during lactation induces deficits in maternal care, and this stress exposure also alters the behaviour of subsequent generations. It was hypothesised that chronic social stress is an ethologically relevant form of early life stress for the developing female offspring and may have effects on subsequent adult stress related gene expression. In this study, I extend the previous findings (Nephew and Bridges, 2011) by examining several neural systems within amygdala, hypothalamic and supraoptic nucleus regions involved in the control of the stress response and expression of maternal care that may be mediating the behavioural changes in the stressed model. This research was done in collaboration with Tuft University (USA), by providing brain tissue samples from a rodent model exposed to chronic social stress during lactation and a control model (F0 generation) and their female pups were then grown (F1 generation). Using mRNA extracted from a specific region for the expression of key genes involved in regulating maternal behaviours, I analysed my research findings through Reverse transcription polymerase chain reaction. I discovered that the behavioural changes were correlated with increased corticosteroid, releasing hormone mRNA expression in the F0 central nucleus of the amygdala, increases in the expression of mineralocorticoid receptor mRNA in the F1 paraventricular nucleus, and in oxytocin receptor mRNA expression in the F1 central nucleus of the amygdala of stressed compared to control dams. In conclusion, my data supports the hypothesis that social stress during lactation has long-term effects on the maternally relevant neuropeptide systems.

Chapter 1 Introduction

Maternal disorders such as postnatal depression and anxiety (PPD/A) affect women within four weeks of childbirth, which is caused by exposure to stress after childbirth. Prevalence rates are around 12-15% of new mothers in the UK population (Segre et al. 2007), although are higher than 30% in some populations, such as women living in rural areas of developing countries (Villegas et al, 2011), and in low-income, ethnic minority women (Gress-Smith et al, 2012).

The two key risk factors for PPD/A include depression symptoms during the pregnancy and a family history of psychiatric disorders (O'hara and Swain, 2009). Currently, doctors use the EPDS (Edinburgh Postnatal Depression Scale) as a screening scale for recognising women with depressive indications throughout pregnancy (Cox et al., 1987). When identified with major depression, the patient needs to be monitored closely for up to a year after delivery. PPD/A is responsible for causing dysphoric mood, fatigue, anorexia, sleep disturbances, anxiety, lactation difficulties, impaired concentration and decision making (Association, 2000). PPD/A engenders disabling and longer-lasting disorders, which often impairs women's ability to care for their babies (O'Hara and Swain 1996). A recent study has established that post-maternal behaviour has reflective properties on both the physiological and psychological health of offspring (Nephew and Bridges, 2011). Other studies have shown the long-term effect of PPD/A on offspring, causing an increased risk of developing a variety of psychiatric problems, such as depression and behavioural disturbances. Social and accomplishment shortfall disorders also develop which continue into adulthood (Anderson & Hammen 1993, Billings & Moos 1983, Goodman et al. 1993 and Hammen et al. 1987b, Champagne and Meaney, 2006; Champagne, 2008).

Mothers who have previously suffered from PPD/A have a 50% risk for upcoming depression-like symptoms (Llewellyn et al., 1997). However, regardless of such evidence, too little attention has been paid to PPD/A as causing a negative effect on maternal care and psychological development in the offspring; even though it has also been suggested that mood disorders during lactation are associated with impaired child growth and development (Patel et al. 2003; Surkan et al. 2008).

Currently, little is known about the consequence of PPD/A as an early life stress on adult behaviour; there is an imminent need for preclinical studies to investigate the mechanisms underlying PPD/A in order to develop improved preventative measures and treatments for these disorders. Improving mental health in mothers also represents a preventative measure to ensure the health of their offspring.

Recent rodent studies have been concentrating on the use of ethologically relevant stressors, such as Chronic Social Stress (CSS) and the effects of stress on mood disorders (Herzog et al., 2009; Brunton and Russell, 2010; Nephew and Bridges, 2011). In addition, previous results from rodent studies demonstrate that exposure to social stress impairs maternal care and lactation, increases anxiety, and alters basal levels of hormones and stress response and maternal care. In support of my study, previous elementary studies have suggested further correlation with tissue-specific alterations in the neurohormones, oxytocin (*Oxt*), oxytocin receptor (*OxtR*), vasopressin (*Avp*), and prolactin receptor (*PrlR*) in hypothalamic regions (Murgatroyd and Nephew, 2013). However, the mechanisms of CSS transgenerational effects on maternal care are unknown.

Corticotrophin Releasing Hormone (*CRH*), the central effector of the hypothalamicpituitary-adrenal (HPA) axis and key mediator of fear and anxiety states, has been implicated in the adverse effects of psychological stress in both animals and humans. Furthermore, the role of the glucocorticoid receptor (*GR*) in the transgenerational transmission of maternal care has been investigated in rodents (Zhang et al, 2013). However, most of this work has focused on the effects of altered maternal care on the offspring behaviour and neuroendocrinology, not the effects of stress on the mother. In addition, the hypocretin system, such as the expression of orexin and its receptors (*OxR1* and *OxR2*), has been implicated in the expression of both maternal care (D'Anna and Gammie, 2006) and depressive behaviour and pathophysiology (Arendt et al, 2013; Nollet and Leman, 2013). However, whether this system is involved in the decreased expression of maternal care observed in my rodent model of CSS is unknown. It is suggested that increased focus on the mother may reveal novel safe and effective preventative interventions aimed at

ameliorating adverse effects of postpartum depression and anxiety on offspring (Murgatroyd and Nephew, 2013).

In this study, I investigate whether these neural systems are altered in key amygdalar and hypothalamic regions within the brains of dams that were exposed to CSS during lactation compared to control dams, and whether the observed changes in these systems are correlated with related behavioural measures. I will present evidence that CSS in lactating dams is accompanied by alterations in several brain systems relevant to the observations of decreased maternal care, impaired lactation, and increased maternal anxiety. Alternative hypothesis may be, automatic maintenance of a steady state or state of balance of neural systems within genrations by stress adaptaion and regulation of homeostasis.

1.1 CSS

An active focus of maternal depression and anxiety research has been the role of CSS in the development of postnatal depression. As a recent study suggests, CSS is becoming more common in humans, even more so in lactating mothers. It has been shown that postnatal depression is correlated with a high level of social conflict and a low level of social support (Nephew and Bridges, 2011). Previous studies have reported an effective and ethologically related CSS based rodent model for postnatal depression (Carini et al., 2013; Murgatroyd and Nephew, 2013; Nephew and Murgatroyd, 2013). CSS attenuates maternal behaviour and effects on both dam and offspring has been previously hypothesised, which is ideal for early life stress for the developing female offspring and may have an effect on neuroendocrinology (Murgatroyd and Nephew, 2013).

1.2 Hypothalamic pituitary adrenocortical axis (HPA axis)

Stress can be defined as a perturbation (stress response) of an organism's physiological and/or behavioural homeostasis as a result of exposure to certain events or situations (termed stressors). Perturbations can occur in response to rewarding events (called eustress); however, researchers have concentrated much more intensively on perturbations resulting from aversive events (called distress) (Novak et al., 2013). In many cases, the stress response is brief and homeostasis are reversed, however it may be chronic where the homeostasis are not restored, releasing physiological dysregulation such as stress response (Novak et al., 2013). Postnatal depression is considered to have a neurochemical origin in numerous signalling pathways in different brain areas, and indeed various regionally selective impairments of structural plasticity have been reported (Manji and Duman, 2001).

The HPA axis is one of the many systems that are triggered throughout the introduction of stressful measures (Novak et al., 2013), which also releases cortisol and the primary Glucocorticoid (GC) of human and nonhuman primates alike; by measuring the concentration of the hormone released, the index of the stress response can be produced (O'Connor et al., 2000). The two-step hormonal mechanism of HPA induction is much slower than an AON system to drive sympathy adrenomedullary activation. Disturbances

in HPA axis regulation seem to play a reflective role in the development, course and remission of major depression and affective disorder. The major endocrine response to stress occurs via activation of the HPA axis, leading eventually to increases in circulating GC, which are essential for the metabolic adaptation to stress. The major players in the HPA axis are the hypothalamic neuropeptides, CRH, the pituitary hormone, adrenocorticotropic hormone, and the negative feedback effects of adrenal GC. In addition, a number of other neuropeptides, including AVP, Oxt, pituitary adenylate cyclase activating peptide and orexin can affect HPA axis activity by influencing the expression and secretion of CRH, and also by modulating pituitary corticotroph function or adrenal steroidogenesis. Of these peptides, AVP co-secreted with CRH from axonal terminals in the external zone of the median plays a prominent role by potentiating the stimulatory effect of CRH and by increasing the number of pituitary corticotrophs during the chronic challenge (Aguilera, 2011).

1.3 Neuroanatomy and Stress

Stress can be defined as an alarm of an organism's physiological and/or behavioural homeostasis as a result of introduction to convinced events or circumstances which are termed stressors (Novak et al., 2013). The brain has a major role in the body's acuity and response to stress, which is essential for survival and well-being of all species by providing appropriate physiological responses to environmental and homeostatic challenges. The brain triggers a stress feedback that is corresponding with the nature of the stimulus. In regards to non-physical stresses or psychogenic stressors based on prior experience or innate programs (Herman et al., 2003), these responses require processing in the prosencephalon and can occur in anticipation of or in reaction to stressful events. The restoration and conservation of homeostasis entails the coordinated activation and control of neuroendocrine and autonomic stress systems. Most stress responses are mediated largely by circuits in the hypothalamus and the brainstem, so that the particular influences of the neuroendocrine and autonomic systems are adjusted in accordance with stressor modality and intensity. Adaptation is the major objective under conditions of stress for all organisms, which involves an effective and highly preserved set of interlocking systems

and aims to preserve physiologic reliability even in the most challenging of conditions (Ulrich-Lai and Herman, 2009). If the brain continues to perceive something as dangerous, the hypothalamus releases CRH, which travels to the pituitary gland, triggering the release of adrenocorticotropic hormone (ACTH). This hormone travels to the adrenal glands, prompting them to release cortisol, which causes the body to stay at high alert and when the threat passes, cortisol levels fall, which is produce by the parasympathetic nervous system.

1.3.1 Amygdala and Hypothalamus

The mammalian amygdala and hippocampus are electrical regions involved in emotional processes, learning and remembering. They have been widely studied in humans using functional imaging (fMRI), positron emission tomography (PET) and in vivo brain morphometry using structural Magnetic resonance imaging (MRI) (Amunts et al., 2005). The amygdala represents a complex of many subnuclei, which have been described using different parcellation schemes (Heimer et al. 1999). The amygdala plays an important role in Species-specific defence reactions (SSDR) that are learned through interaction with the environment and others of the same species. Emotional response is created only after the signals have been relayed between the different regions of the brain, and activating the sympathetic nervous systems; which controls the physiological response (Bracha, 2004) In another study it has been reported that damaged amygdala can cause impairment in the recognition of fear (Adolphs et al., 2005).

The hypothalamus is extremely important, which is involved in the mediation of endocrine, autonomic and behavioural functions. The hypothalamus: (1) controls the release of eight major hormones by the hypophysis, which governs body temperature, thirst, hunger, sleep, circadian rhythm, moods and the release of other hormones in the body (Amunts et al., 2005). (2) Temperature regulation, (3) control of food and water intake, (4) sexual behaviour and reproduction, (5) control of daily cycles in physiological state and behaviour and (6) mediation of emotional responses (Aguilera, 2011).

1.4 Maternal care and CNS function

Although there are plenty of studies on the role of maternal care influencing the HPA axis, however in this study, I focused on main neurohormones, such as *Oxt, OxtR, Crh* and *GC*, which have a pivotal role in maternal care in influencing the CNS and both the physiological and behavioural changes associated with maternal care; they are also involved in mediators of the stress response (Nephew and Murgatroyd, 2013). *Crh* secretion by the PVN is a major modulator of the HPA axis through its actions on ACTH release into the transmission, and is also involved in gestation, parturition, and maternal care (Pedersen et al., 1991; Klampfl et al., 2013). It has been hypothesised that low Crh receptor activity is required for the expression of characteristic maternal behaviour. Early studies exposed that *Crh* reduced maternal care and increased infanticide (killing of an infant child by its mother during the early months of life) in an induced virgin rodent model of maternal care (Pedersen et al., 1991), and intracerebroventricular (administration of drugs or chemicals into the ventricular system of the brain) of Crh also inhibits maternal aggression in mice (D'Anna and Gammie, 2009; Gammie et al., 2004; Gammie et al., 2008). In recent studies it has been shown that the behavioural alterations in the maternal care of rat and mouse strains bred for differing levels of anxiety, has been associated with differences in central *Crh* and *Avp* (Bosch et al., 2006; Kessler et al., 2011; Klampfl et al., 2013), which have found higher level of *Crh* within PVN in high anxiety behaviour against low anxiety behaviour rats, plus central Crh was found to be involved in maternal anxiety in several strains of rats (Klampfl et al., 2013). The social behaviour functions of *Oxt* have been recognised in several species (Donaldson and Young, 2008), and Oxt is a mainly powerful mediator of maternal care and this was established initially in the late 70's through intracerebroventicular injection (Pedersen and Prange., 1979; Pedersen et al., 1982; Van Leengoed et al., 1987; Pedersen at al., 1994; Chapagne et al., 2001). Also the actions of *OxtR* have been associated in impulsive maternal care in prairie voles (Olazabal and Young, 2005). Although *OxtR* knockout mice display shortages in maternal care (Takayanagi et al., 2005), central *Oxt* activity may not be a factor in all phases of maternal care after it is commenced. Study in sheep have reinforced the theory that *Oxt* specifically mediates the induction of maternal care (DaCosta et al., 1996).

Chapter 2 Method

There are several technologies for performing gene expression analysis such as real time quantitative qRT-PCR (using SYBR green chemistry or Taqman chemistry), digital PCR, In situ hybridization, microarrays, and massively parallel signature sequencing (MPSS).

In my study I used qRT-PCR using SYBR green chemistry, which is a technique to amplify a specific region of DNA using changes in temperature to control the activity of the polymerase and the binding of primers: At the first stage (95°C) all double stranded DNA are "melted" to a single strand. At the second stage (around 60°C) the primers bind to the gene of interest. At the third stage the polymerase have the opportunity to bind and can begin to copy the DNA strand; the optimal temperature is 72°C, which allows the enzymes to perform more efficiently. This temperature change is repeated, though around 40-50 cycles. Once the SYBR Green binds to the double-stranded DNA of the PCR products, it will produce fluorescence light when excited. The strength of the fluorescence increases as the PCR products rises generating the cycle threshold (Ct) value (defined as the number of cycles required for the fluorescent signal to cross the threshold).

2.1 Chemical, Molecular kits and Equipment

Sodium dodecyl sulfate (SDS)	SIGMA-ALDRICH
Phenol/Chloform/Isoamyl Alcohal, 25:24:1	Fisher BioReagents
Trisure™	Bioline
Isopropyl alcohol	SIGMA-ALDRICH
Chloroform	Fisher Scientific
Ethanol, Absolute	Fisher Scientific
Certified™ Molecular Biology Agarose	Bio-Rad Laboratories
DEPC-Treated Water	Bioline
Trizma®Base, Tris Base	SIGMA-ALDRICH
Boric Acid	Fisher Scientific
Ethylenediaminetetraacetic Acid	Fisher Scientific
Lithium Bromide	Sigma-Aldrich
TRIzol®Reagent	Ambion®
Diethyl pyrocarbonate	Sigma-Aldrich
Cresyl Violet acetate	Sigma-Aldrich
Methylated spirit industrial	Fisher Scientific
Xylene	Fisher Scientific

	Eosin	Fisher Scientific
	Rabbit Anti-oxytocin Polyclonal Antibody	Millipore
	Phosphate buffered saline	Fisher Scientific
	Bovine serum albumin	Fisher Scientific
	4',6-diamidino-2-phenylindole (DAPI)	Molecular Probes®
	Tetro cDNA Synthesis Kit	Bioline
	SensiFAST™ SYBR Hi-ROX kit	Bioline
	EasyLadder I, 100-2000 bp	Bioline
	HyperLadder™ 25bp, 25-500 bp	Bioline
	Oligonucleotide Primers	Invitrogen™
	MyTaq™ DNA Polymerase	Bioline
	Crystal 5x DNA Loading Buffer	Bioline
	RNaseZap® RNase Decontamination	Ambion®
	AllPrep DNA/RNA Mini Kit	QIAGEN
	ISOLATE II RNA Mini Kit	Bioline
	ISOLATE II Genomic DNA Kit	Bioline
	RNA 6000 Nano total assay	Agilent Technologies
	Agilent DNA 1000 Kit	Agilent Technologies

TBE Buffer (10X):	121.1g Trizma®Base, Tris Base (1M), 61.8g Boric Acid (1M) and 7.4g EDTA (0.02M) pH 8.0. Dilute in 1000 mL with RNase-free H_2O.
Crystal Violet Staining Reagent:	2g *Crystal Violet*, 20ml 95% Ethanol, 0.8g Ammonium oxalate and 80ml Distilled water. Mix for 24 hour and filter.
GTC buffer:	4.5 M guanidinium thiocyanate, 2% N-lauroylsarcosine, 50 mM EDTA pH 8, 25 mM Tris-HCl pH 7.5, 0.1 M beta-mercaptoethanol,0.2% antifoam.

	Multipurpose Centrifuge	Eppendorf
	Vortex Mixers	Fisher Scientific
	Autoclave, Touchclave	Fisher Scientific
	Microbalance	Fisher Scientific
	Analytical Balance	Fisher Scientific
	Ultrasonic Bath SW3H	Fisher Scientific
	Heating Modules	Fisher Scientific
	Stirring Modules	Fisher Scientific
	pH meter	Fisher Scientific
	Hotplate	Fisher Scientific
	Multichannel pipette	Eppendorf
	Biological Class II safety cabinet	Fisher Scientific
RNA&DNA analyses	DNA LoBind 1.5mL Tubes, PCR clean	Eppendorf
	ART® self-sealing barrier pipette tips	Sigma-Aldrich
	Eppendorf Mastercycler® PCR Cycler	Eppendorf
	StepOnePlus™ Real-Time PCR	Applied Biosystems®
	MicroAmp® Fast Optical 96-Well Reaction Plate	Applied Biosystems®
	MicroAmp® Optical Adhesive Film	Applied Biosystems®

	NanoDrop 2000c spectrophotometer	Thermo Fisher Scientific
	2100 bioanalyzer	Agilent Technologies
	Gel Doc™ XR+	Bio-Rad Laboratories
	-20°C Freezer	Labcold
	Laminar flow safety cabinet PCR Prep station	Labcaire Fisher Scientific
Tissue	1ml syringe & Needle 29g	Becton Dickinson
	-80°C Freezer	Fisher Scientific
	Cryostat (-19°C)	Leica
	Sample Corer, 1mm, 0.8mm and 5mm diameter	Fine Science Tools GmbH
	1mm Microscope Slide	Fisher Scientific
	Multipurpose Centrifuge	Eppendorf
	Vortex Mixers	Fisher Scientific
	Autoclave, Touchclave	Fisher Scientific
	Microbalance	Fisher Scientific
	Analytical Balance	Fisher Scientific
	Ultrasonic Bath SW3H	Fisher Scientific
	Heating Modules	Fisher Scientific
	Stirring Modules	Fisher Scientific
	pH meter	Fisher Scientific
	Hotplate	Fisher Scientific
	Multichannel pipette	Eppendorf
	Biological Class II safety cabinet	Fisher Scientific
RNA&DNA analyses	DNA LoBind 1.5mL Tubes, PCR clean	Eppendorf
	ART® self-sealing barrier pipette tips	Sigma-Aldrich
	Eppendorf Mastercycler® PCR Cycler	Eppendorf
	StepOnePlus™ Real-Time PCR	Applied Biosystems®
	MicroAmp® Fast Optical 96-Well Reaction Plate	Applied Biosystems®
	MicroAmp® Optical Adhesive Film	Applied Biosystems®
	NanoDrop 2000c spectrophotometer	Thermo Fisher Scientific
	2100 bioanalyzer	Agilent Technologies
	Gel Doc™ XR+	Bio-Rad Laboratories
	-20°C Freezer	Labcold
	Laminar flow safety cabinet PCR Prep station	Labcaire Fisher Scientific
Tissue	1ml syringe & Needle 29g	Becton Dickinson
	-80°C Freezer	Fisher Scientific
	Cryostat (-19°C)	Leica
	Sample Corer, 1mm, 0.8mm and 5mm diameter	Fine Science Tools GmbH
	1mm Microscope Slide	Fisher Scientific

2.2 Animals

Animals used in this study were of the Sprague Dawley rat model, developed and maintained at Tufts University (USA). CSS models were subjected to chronic social stress protocol (appendix 1), and this was done by placing a novel male intruder into the lactating female home cage for 1 hour from day 2 to 16 of lactation. Control (CON) models were not exposed to chronic social stress after behavioural testing, such as total maternal care and maternal anxiety, during day 2, 9 and 16 (early, mid and late lactation). On day 23, animals were euthanized. Brains were collected and frozen at -80°C, then micropounched to obtain samples from PVN, CeA, MeA and SON, after which, they were shipped to us at Manchester Metropolitan University. The rats used in this study were sustained in accordance with the guidelines of the Committee on the care and use of laboratory animal resources, National Research Council, and the research protocol was approved by the Tufts Institutional Animal Care and Use Committee (USA).

2.3 Simultaneous DNA and RNA isolation

One of the major class of RNA in cells are messenger RNA (mRNA) which plays an important role in this experiment. Since I measured the gene expression using mRNA, due to synthesization on a DNA template by transcription, the base paring was used to synthesize RNA complementary to the template strand of DNA. The mRNA molecules are at present recognized to account for 3-5% of the total cellular RNA, they are quite heterogeneous with respect to size, reflecting on the variety of the gene product which they encode (P Jones et al. 1994). Extracted DNA will be used in a later Epigenetic study.

Using 0.8 mm brain punches shipped samples from Tufts University, which had been frozen at -80°C until extraction, I used the following method to perform simultaneous DNA and RNA extraction.

1. Brain punches were homogenize in the 400 µl of GTC buffer (see buffer recipe) using 1 ml micro fine syringe with 29G needle, then mixture incubated on ice for 15 minute.

2. 200 µl of the mixture were transferred to new tube for DNA extraction and remaining 200 µl for RNA extraction.

   Note: *RNA extraction is performed first, since RNA is less stable than DNA (DNA can be left at room temperature for several hours, to be completed after RNA extraction).*

RNA EXTRACTION

1. Adding 20µl of 3M sodium acetate (NaoAc), 200 µl of acidic phenol and 100 µl of Chloroform: Isoamyl alcohol then short vortexed and incubated on ice for 15 minute, followed by centrifuge for 20 minute at 13,000 RPM on 4°C temperature.
2. ~200 µl aqueous phase transferred to new tube to mix with 200 µl of 70% Ethanol (EtOH) to precipitate RNA, then vortexed and incubated on ice for 15 minute.
3. The mixture is then transferred to RNA column from RNeasy Mini Kit (Qiagen) then centrifuged for 2 min at 11,000 RPM on 4°C temperature after which the flow-through is discarded.
4. 500 µl of wash buffer added to the column, centrifuged for 2 minute at 11,000 RPM on 4°C temperature and then flow-through is discarded.

   Note: *This step is repeated twice.*
5. Without adding any solution to the column, the column is centrifuged for 2 minute at 11,000 RPM on 4°C temperature and then flow-through is discarded.
6. 20µl of DEPC treated water were added to the column and then incubated at room temperature for 2 minute. After centrifuged for 2 minute at 11,000 RPM on 4°C temperature. Then flow-through were collected and stored on ice for measurement such as RNA concentration, purities and integrity using the NanoDrop 2000C (Thermo Fisher Scientific).

DNA EXTRACTION

Using the 200 µl of the remaining lysate, I conducted the following procedure for DNA extraction:

1. 200µl of lysis Buffer from DNeasy Blood & Tissue Kit (Qiagen) and 200 µl of EtOH (75%) were added to the mixture and then short vortexed. The mixture was incubated at room temperature for 10 minutes.
2. Mixture was then transferred to DNA spin column from the Kit and then centrifuged for 1 min at 11,000 RPM, then flow-through was discarded.
3. 500 µl of wash buffer were added to the column, then Centrifuged for 1 minute at 10,000 RPM and the flow-through was discarded.
   Note: *This step is repeated twice.*
4. Without adding any solution the column, the column were centrifuged for 2 min at 11,000 RPM, then flow-through was discarded.
5. 50 µl of DEPC treated water were added directly on to the membrane and left the column for 2 minutes at room temperature, and then centrifuged for 1 minute at 11,000 RPM, flow-through were collected and stored on ice for measurement.

2.3.1 Simultaneous DNA and RNA isolation using TRI Reagent

As shown in table 5, when the previous method was used for simultaneous RNA and DNA extraction, I noticed a very low RNA concentration, which were affecting my qRT-PCR performance; as a consequence I developed the following improved method which allowed a several fold increase in the concentration of total RNA and DNA from~0.8 mm brain samples.

1. 400 µl of TRIzol solution (Life Technologies) was added to tube with 0.8 mm brain samples, which causes complete dissociation of the nucleoprotein complex. Then using 1ml micro fine syringe with 29G needle the samples were homogenized, then the mixture was incubated on ice for 15 minutes.

2. 80 µl of chloroform ($CHCl_3$) was added to the mixture, then vortex and incubated at room temperature for 15 minutes, after which the mixture was centrifuged for 15 min at 13,000 RPM on 4°C temperature.

   Note: *After centrifuge the mixture will be carved up into lower red phenol-chloroform phase (Protein), an Interphase (DNA) and colourless upper aqueous phase (RNA).*

3. Using pipette, ~200 µl colourless upper aqueous phase (RNA phase) were removed into a new tube by angling the tube at 45°, avoiding any contamination by other layers.

   Note*: The lower red phenol-chloroform phase, which are the Protein and the Interphase, are the DNA left on the side for DNA extraction using DNeasy Blood & Tissue Kit, as shown above "DNA extraction using DNeasy Blood & Tissue Kit".*

4. 200 µl of Isopropyl alcohol (C_3H_8O or C_3H_7OH) was added to the mixture (1:1 ratio) than vortexed. Mixture incubated at the room temperature for 15 minutes, followed by centrifuge for 10 minute at 11,000 RPM on 4°C temperature.
5. Without disturbing the RNA pellet, the precipitated isopropanol was removed, leaving the RNA pellet at the bottom of the tube.
6. 1 ml of EtOH (75%) added to the mixture then vortexed, then mixture incubated on the ice for 15 minutes, then centrifuged for 5 minutes at 9,000 RPM on 4°C temperature.

   Note: *(This step can be repeated if needed)*

7. Remaining EtOH were removed and 20 µl DEPC treated water added to RNA pellet at the bottom of the tube.
8. With open lid, RNA samples were incubated on heating block at 55-59°C for 15 minutes (heating cover used to block up any contamination) which caused any remaining ethanol to evaporate.
9. Purities and integrity were measured using the Nano-Drop 2000C (Thermo Fisher Scientific) for equalization prosses.

2.3.2 Equalizing total RNA concentration for RT input

In order to match the total RNA concentration in all samples used for reverse transcription reaction, a specific amount of DEPC treated water was added to each sample to give an equal amount of concentration (between 2.5-5 ng/µl). Using UV absorbance results, the following formula was used:

$$\left(\frac{Total\ RNA\ Conc\ (ng/\mu l)\ \times\ Total\ volume\ (\mu l)}{Lowest\ Total\ RNA\ Conc\ (ng/\mu l)}\right) - Total\ Volume\ \alpha\ = DEPC\ trated\ water\ to\ add\ (\mu l)$$

2.3.3 Reverse Transcription

The RT reaction was performed using the Tetro-cDNA reverse transcription kit (Bioline). As shown in table 1, the following manufactures protocol was followed in order to make a master mix. After which the master-mix was incubated using PCR cycler (Eppendorf) at 45°C for 30min and 85°C for 5mins, then chilled on ice. Before storage the cDNA were diluted by 1:5 (minimum cDNA concentration of 2.5 ng/µl) using DEPC treated water. The master-mix procedure was used, since the use of a master mix reduces the number of pipetting steps, which consequently reduces the chances of cross-well contamination and other pipetting errors.

	1 Reaction	Master Mix (20 Reactions with 10%)
Total RNA or mRNA	10µl	NA
Primer: Oligo dT or Random Hexamers	1µl	22µl
10 mM dNTP mix	1µl	22µl
5X RT Buffer	4µl	88µl
Ribosafe RNase Inhibitor (10 u/µl)	1µl	22µl
Tetro Reverse Transcriptase (200u/µl)	1µl	22µl
DEPC H2O	2µl	44µl
Total volume	20µl	220µl

Table 1. RT master-mix reaction setup for 20 reactions.

2.3.4 qRT-PCR

A Plate layout plan was made for each of the samples and target genes, including the no-template control (NTC) for each target gene. The following SYBR green Hi-Rox (Bioline) manufactures protocol was used to make a master-mix for each primer.

	1 Reaction	Master Mix (20 Reactions with 10%)
SYBR Green Mix (2x)	10μl	NA
Forward Primer (10μM)	1μl	22μl
Reverse Primer (10μM)	1μl	22μl
Template	4μl	88μl
DEPC H2O	2μl	44μl
Total volume	20μl	220μl

Table 2. Real-time PCR master-mix reaction setup for 20 reactions.

Using the StepOneplus (ABI Technology) qRT-PCR machine, the following manufacturer SYBR Green (Bioline) suggestion used for real-time PCR condition was followed:

Cycles	Temp	Time	Cycles	Data Collection	Notes
1	95°C	2min	1	off	Polymerase activation
40	95°C	5s	1	OFF	Denaturation*
	60-65°C	10s	40-45	ON	Annealing**
	72-72°C	5-20s	1	OFF	Extension***
Melt Curve	95°C	15s	1	OFF	
	60°C	1min		OFF	
	60°C +			ON	
	3°C	15s		ON	
	95°C				

Table 3. Real-time PCR condition used in this study.

Using StepOne software (ABI Technology), I was able check to see if there was any multiple peak curve or abnormal amplification plot after each PCR. The PCR specificity was examined by 3% agarose gel using 5 μl from each reaction. Ct Value data were exported from StepOne™ software (ABI Technology) to digital spreadsheet (excel format) for analysis.

2.3.5 Statistical Analysis

Two strategies are commonly employed to quantify the results obtained by qRT-PCR: the standard curve method and the comparative threshold method. The Standard Curve Method involves constructing a standard curve from an RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. However RNA standards can be used, their stability can be a source of variability in the final analyses. In addition, using RNA standards would involve the construction of cDNA plasmids that have to be in vitro transcribed into the RNA standards and accurately quantitated; a time-consuming process.

The Comparative Ct Method involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or cDNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate housekeeping gene (Figure 5). The comparative Ct method is also known as the 2-ΔΔCT method:

$$2\text{-}\Delta\Delta C_t = (C_{t\ \text{Sample}} - C_{t\ \text{Sample housekeeping}}) - (C_{t\ \text{Reference}} - C_{t\ \text{Reference housekeeping}})$$

ΔCT sample is the Ct value for any sample normalized to the housekeeping gene and ΔCT reference is the Ct value for the calibrator also normalized to the housekeeping gene. To obtain tight data from qRT-PCR, the experiments were repeated twice. Before each analysis the data were tested for normal distribution using the Kolmogorov-Smirnov test. Data are presented as Mean ± SD and were analysed using an independent t-test. All statistical analysis was carried out using IBM SPSS statistics version 20.0.

Chapter 3 Result

3.1 RNA Quantitation

Quantitation is the most important and necessary step prior to reverse transcription by averaging the RNA concentration mixture for optimum performance. In this experiment I used Nano-Drop 2000C (Therno Scientific) UV spectrometer to assess RNA concentartrion and purity, after which the concentration from each region was averaged to the lowest concentration.

RNA plus DNA concentration and including quality information from PVN, MeA, CeA and SON regions was measured using A260/A280 ratio represents quality (table 4); A260/A230 ratio represents the purity. Looking through my data (table 4), I noticed an improvement in concentration by ten-fold when I compared the Simultaneous RNA/DNA column based method to the novel method using tri-reagent.

		Average concentration (ng/µl)	260/280	260/230
RNA	F0 CeA	172.54**	1.55	0.29
	F0 MeA	98.9**	1.70	0.55
	F0 PVN	22.12*	1.85	0.54
	F0 SON	5.7*	2.27	0.15
	F1 PVN	95.38**	1.75	1.4
	F1 CeA	785.45**	1.7	1.3
DNA	*F0 PVN*	*21.96**	*2.04*	*0.3*
	F0 SON	*9.2**	*1.49*	*0.61*
	F1 PVN	*120.22***	*2.04*	*0.3*

Table 4. RNA and DNA concentration measurement obtained from Nano-Drop 2000C (Therno Scientific) total RNA and DNA concentration, plus purity information (A260/A280) and contamination (A260/A230) obtained. *Simultaneous RNA/DNA column based method, ** Improved method with TRIzol (Life Technologies).

For sensitive and reliable quantification of low abundant RNA gene expression, qRT-PCR is the method of choice and for high sensitivity and good reproducibility of results, it is important to use intact RNA (Bar et al., 2003; Wang and Brown, 1999). Many factors present in samples as well as exogenous contaminants can inhibit the RT as well as the PCR. Also, using high quality RNA have been shown to decrease low cycle threshold (Ct)

value of the PCR runs compare to less-quality (Fleige and Pfaff., 2006). Also, with increasing RNA quality, the variability of the qRT-PCR result was decreased (Huch et al., 2005). For this reason, I noticed a need to improve the RNA extraction protocol to improve the RNA quality.

3.2 Brain region-specific housekeeping gene selection and relative gene expression analysis

In my study, I used six housekeeping genes *β-actin, Hprt, Gapdh, Pgk1, sdha,* and *Mrfap1,* which are typically constitutive genes (Sikand et al., 2012, de Jonge et al., 2007), transcribed at a relatively constant level, and usually needed for cell maintenance. It is generally assumed that their expression is unaffected by experimental conditions. Normalization by an internal reference gene is widely used in gene expression analysis, which reduces tissue derived effects on qRT-PCR (Wittwer et al., 1997), plus sample-to-sample variations in qRT-PCR efficiency and errors in sample quantification (Pfaffl, 2001).

When performing qRT-PCR experiments, variation in the amount of starting material, sample collection, RNA preparation and quality, and reverse transcription (RT) efficiency can contribute to quantification errors. Normalization to endogenous control genes is currently the most accurate method to correct for potential RNA input or RT efficiency biases (de Kok et al., 2005). Careful selection of an appropriate control or set of controls is extremely important, as significant variation has been observed between samples, even for the most commonly used housekeeping genes, including *β-Actin* and *GAPDH* (de Kok et al., 2005). An ideal endogenous control generally demonstrates gene expression that is relatively constant and highly abundant across tissues and cell types. However, one must still validate the chosen endogenous control or set of controls for the target cell, tissue, or treatment, as no single control can serve as a universal endogenous control for all experimental condition (Suzuki et al., 2000). The chart below (Figure 1) shows one of the differences in Ct value box-plot of different housekeeping genes used within different brain regions. Lower Ct value and smaller box-plot represents a better housekeeping expression between CON and CSS within the specific region. In addition, one can notice that housekeeping expression within F1 PVN, F0 PVN, F0 MeA, F0 CeA were more constricted

than F1 CeA and F0 SON. Generally, I noticed a different level of housekeeping expression within different brain regions. In my experiments, I found the following genes best; *Pgk1, Mrfap1* and *Sdha* due to lower variation and similar Ct values between CON and CSS samples.

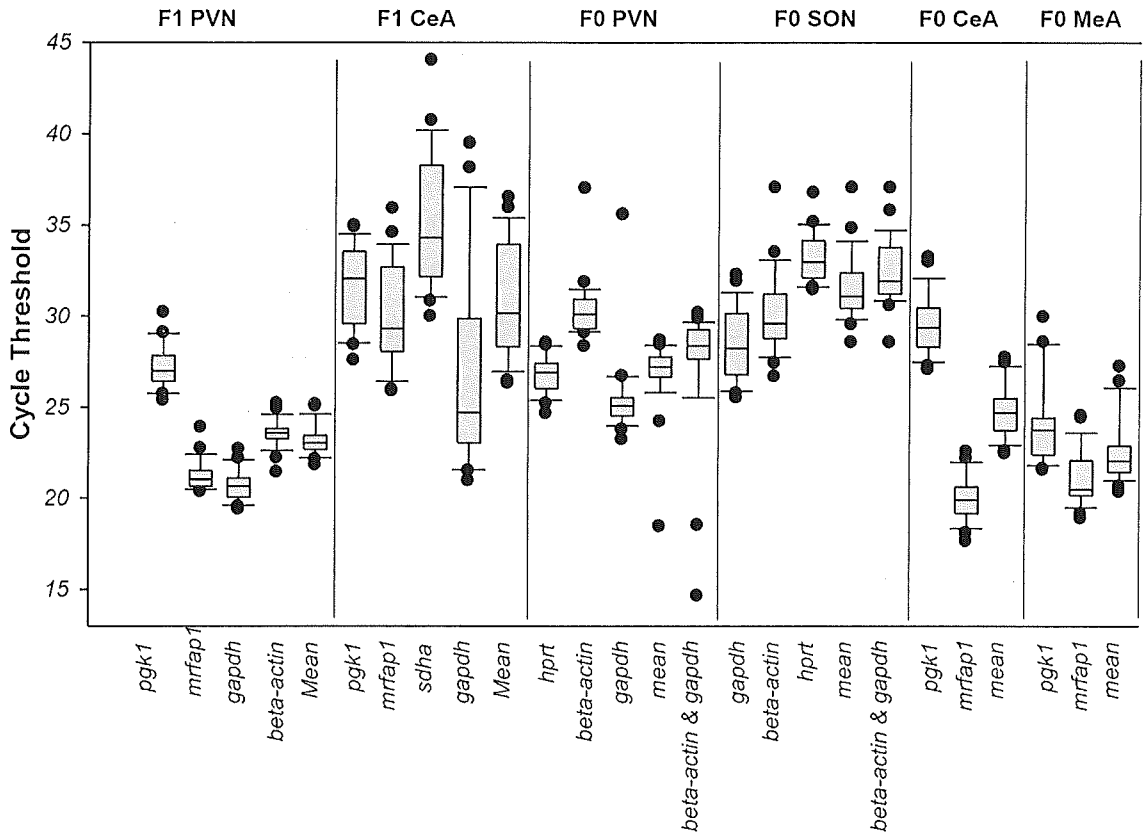

Figure 1. Cycle threshold value for housekeeping gene, quick Box-plot analysis of comparison of different housekeeping genes within different brain regions.

By normalizing, the Ct value against each of the housekeeping genes, examining the results using SPSS (IBM) version 20.0, comparing the mean performing independent-sample T-test for each housekeeping gene plus the mean value of housekeeping gens.

3.2.1 RNA Expression within CeA and MeA

There is extensive evidence that the amygdala is crucially involved in regulating stress-effecting memory (McGaugh & Roozendaal., 2002). My findings indicate stress activated neurotransmitters enhance the consolidation of memory for emotionally arousing experiences through actions involving the amygdala, such amygdala activation strengthens the storage of different kinds of information through the amygdala. qRT-PCR was performed using primers (Appendix 2) against the 27 samples from F0 CeA, F1 CeA and F0 MeA cDNA. An agaorse gel was used to confirm the correct size of base pairs (bp) from each primer. After each qPCR, the melting curve was checked for a clear peak, demonstrating the absence of any non-specific binding in any of the samples and the lack of primer dimers.

To assess the specific gene expression within F0 and F1 CeA, comparisons between the two groups CON and CSS were made using independent-sample T-test analysis. As can be seen from the chart below (figure 2) Mean + SEM relative gene expression of *Gr, OxtR, Avpr1r, Oxt, Avp, Prl* and Crh within the F0 plus *Gr, OxtR, Oxt, Prl, Avp, Crh* and *Mr* within F1. Relative expression values were determined from the combined relative values of the two housekeeping genes *Mrfap1* and *Pgk1*.

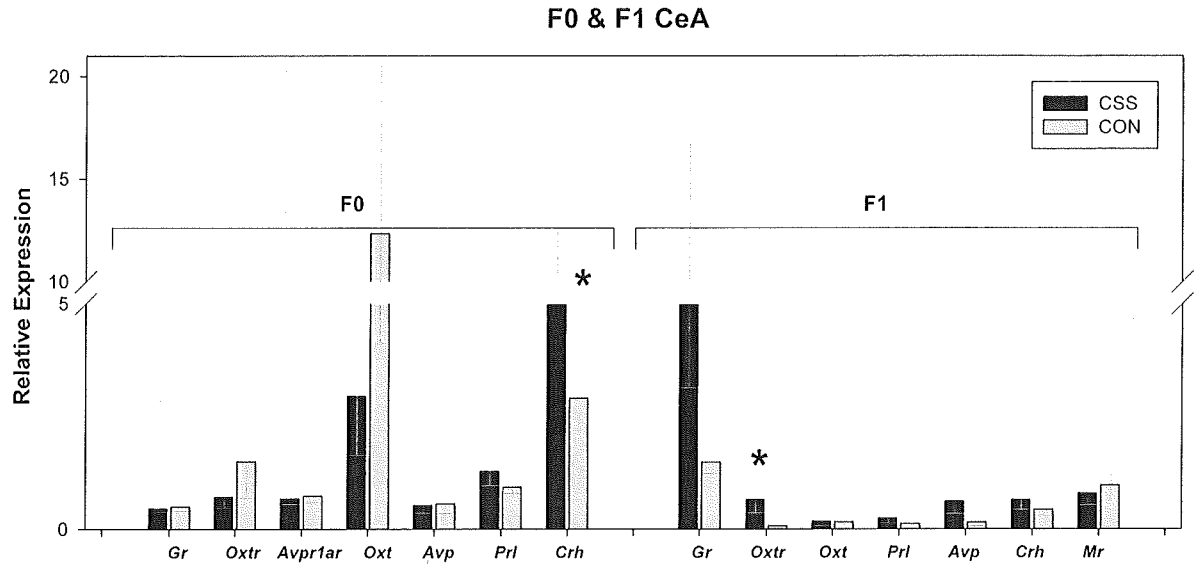

Figure 2. Chart representing an overview of relative gene expression changes of *Gr, OxtR, Avpr1r, Oxt, Avp, Prl, Crh* and *Mr* (Mean + SEM. n=13-15 rats per group) of F0 and F1 CeA between two groups of CON and CSS normalized against mean value of housekeeping genes (*Mrfap1* and *Pgk1*).*Indicates a significant effect of treatment (t-Test, p≤0.05). **Indicates a marginally significant (t-Test, p=0.07-0.051).

The data revealed (Figure 2) significant increased expression of *Crh* within F0 CSS (p=0.045) and F1 CSS (p=0.206). However, there were no significant differences of *Crh* within F1 genration, which may suggest a decrease in stimulation of pituitary synthesis of ACTH, as part of the HPA Axis, from F0 to F1 generation. Furthermore, the data (Figure 4) revals significant increase expression of *OxtR* (p=0.025) within F1 CSS and reverse within F0 genration; this might suggest a neuroendocrine adjustment period through increased receptors for neurotransmitter *Oxt.* This theory is supported by the same data since the level of *Oxt* within F1 generation is similar (p= 0.917) is similar to F0 (p=0.252). There was an additional increase of neurotransmitter within F1 generation; a noticible increase of *Gr* within F1 CSS (p=0.146) from F0 CSS (p=0.635) and elevated *Avp* from F0 to F1, however data were not significant. Surprisingly, the *Prl* remained non-significantly upregulated within F0 and F1 CSS compare to CON, which may be due to physiologic hypersecretion disturbances in lactation, sleep and stress which is possibly a sign for

Pituitary hypersecretion, such as Prolactinoma and Acromegaly. There were no significant differences in *Avpr1ar* and *Mr* expression within CSS and CON. From the overall data (Figure 2), one can notice a reduced difference in gene expression within F1 compare to F0 CeA, which may be due to homeostasis by neuroendocrine adjustment.

I assessed gene expression within F0 MeA, by comparisons between the two groups CON and CSS, using independent-sample T-test analysis. The data below (figure 3) display an overview of Mean + SEM relative gene expression of *Avpr1a, Gr, PrlR* and *Oxt*. Relative expression values were determined from the combined relative values of the two housekeeping genes *Mrfap1* and *Pgk1*.

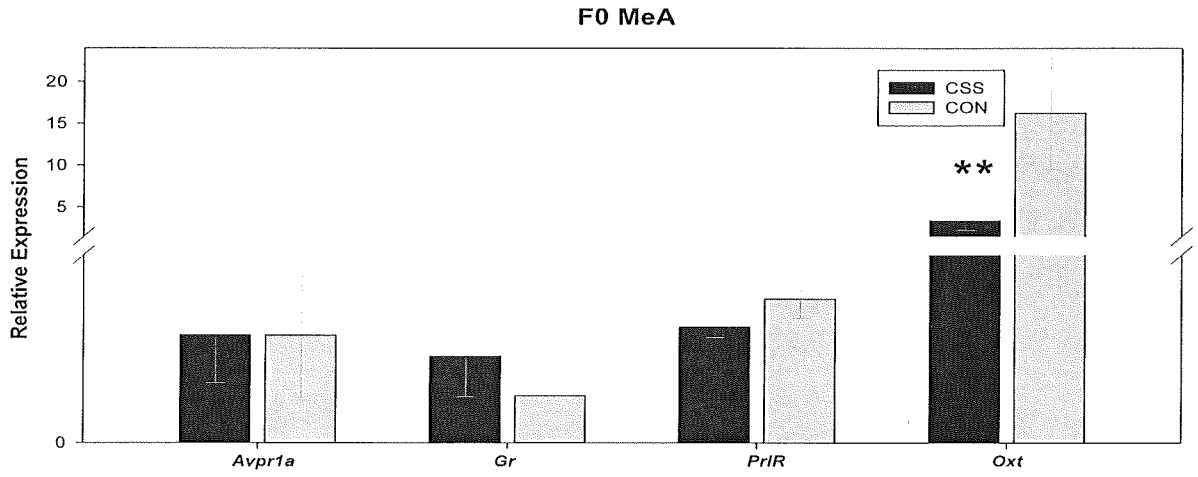

Figure 3. Chart repsenting an overview of relative gene expression changes of *Gr, Avpr1a, Gr, PrlR* and *Oxt* (Mean + SEM. n=13-15 rats per group) of F0 MeA between two groups of CON and CSS normalized against mean value of housekeeping genes (*Mrfap1* and *Pgk1*).*Indicates a significant effect of treatment (t-Test, p≤0.05). **Indicates a marginally significant (t-Test, p=0.07-0.051).

Looking through my data (Figure 3), I noticed a marginally significant decrease in *Oxt* (p=0.68) within CSS, which may be due to stress mood disorders and lack of maternal care. These results endorse my hypothesis that social stress has an effect on gene expression by decreasing *Oxt* expression; however this is not a significant finding. Additional data (Figure 3) show no significant differences between CSS and CON gene expressions of *Avpr1a, Gr* and *PrlR* within F0 MeA.

3.2.2 RNA Expression within PVN

Stress responses are controlled in large part by the PVN of the hypothalamus, which contains three functionally distinct neural populations that modulate multiple stress effectors such as HPA axis. In addition, the PVN region appears to participate in chronic stress responses (Herman et al., 2008).

Assessing gene expression within F0 and F1 PVN, via comparisons between the two groups CON and CSS, using independent-sample T-test analysis. Data below (figure 4) display an overview of Mean + SEM relative gene expression of *Oxt, OxtR, Avp, Prl, Crh, Mr, Gr* and *Avp1ar* within F0 and *Oxt, OxtR, Prl, Crh, Avp, Avpr1ar, Hcrtr1, Hcrtr2, Hcrt, Gr* and *Mr* within F1 generation. Relative expression values were determined from the combined relative values of the two housekeeping genes *Mrfap1, Pgk1, Gapdh* and *β-actin*.

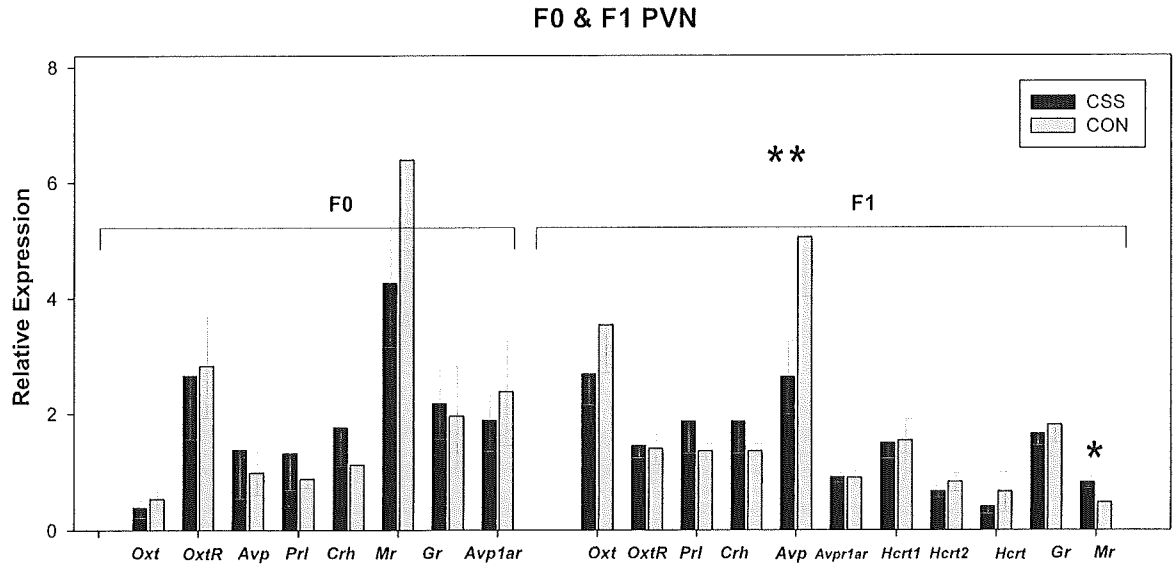

Figure 4. Chart repsenting an overview of relative gene expression changes of *Oxt, OxtR, Avp, Prl, Crh, Mr, Gr* and *Avp1ar* within F0 and *Oxt, OxtR, Prl, Crh, Avp, Avpr1ar, Hcrtr1, Hcrtr2, Hcrt, Gr* and *Mr* in F1 genration (Mean + SEM. n=13-15 rats per group), within PVN reagion between two groups of CON and CSS. Normalized against mean value of housekeeping genes (*Mrfap1, Pgk1, Gapdh* and *β-actin*).*Indicates a significant effect of treatment (t-Test, p≤0.05). **Indicates a marginally significant (t-Test, p=0.07-0.051).

From our data (Figure 4), marginally significant decrease in *Avp* (p=0.051) within F1 CSS, where as *Avp* (p= 0.848) in F0 CSS were not significantly different except a marginally upregulation within CSS. *Avp* mediates rodent maternal care and maternal aggression (Bosch and Neumann, 2008) and when elevated in humans suffering from depression and animal model of depression (Goekoop et al., 2006; Surget and Belzung, 2008; Rotzinger et al., 2010). In addition, a sturdy significant upregulation of *Mr (0.006)* within F1 CSS, while a non-significant lower *Mr* (p=0.249) Expression within F0 CSS can observed. Greater Mr levels within PVN may suggest increased psychological stress (Gesing et al., 2001). *Crh* has been identified as an important reulator of *Mr*, however a non-significant upregulation has been obsevered within F1 CSS (p=0.178). Similarly, unexpected *Gr* (p=0.660) expression were not significant within F1 since Glycoctricoid hormones represent the end product of HPA axis.

From prior studies, Orexin were determined to play an important role as motivation behaviours within the hypothalamus (Johnson et al., 2012; Sakurai, 2014). For this reason, I exclusively chose PVN and SON region to assess the differences in gene expression of *Hcrt, Hcrtr1* and *Hcrtr2* in CSS and CON. From my data (Figure 4) I noticed a slight down-requlation within F1 PVN CSS which was not significant (p=0.469, p=0.937, p=0.391). I was not able to peform the same test on F0 genration due to low quantity of cDNA.

A non-significant down regulation of *oxt, Oxtr* and *Avp1ar* within F0 CSS and *Oxt, Avp* and *Avp1ar* within F1 CSS can be observed, also a non-significant upregulation of *Avp, Prl* and *Crh* within F0 CSS and *OxtR, Prl* and *Crh* within F1 CSS can be perceived.

3.2.3 RNA Expression within SON

Assessing gene expression within F0 SON, via comparisons between the two groups CON and CSS, using independent-sample T-test analysis. Data below (figure 5) display an overview of Mean + SEM relative gene expression of *Oxt, OxtR, Avp, Prlr, Crh, Hcrt1, Hcrt2, Hcrt, Avp1ar* and *Gr* within F0 generation. Relative expression values were determined from the combined relative values of the two housekeeping genes *Gapdh* and *β-actin*.

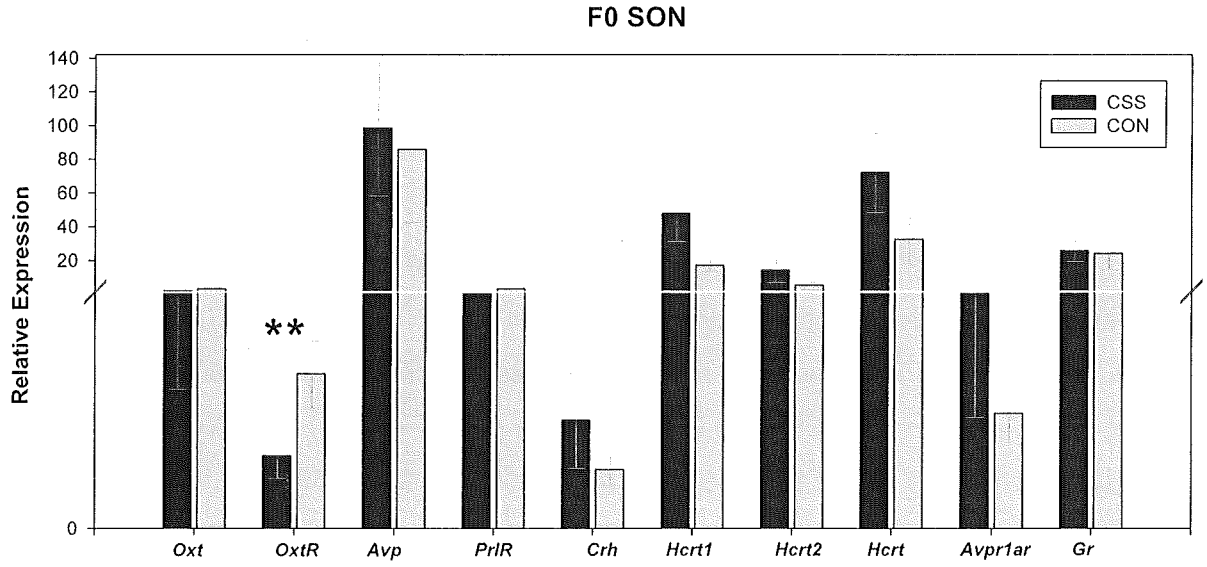

Figure 5. Chart representing an overview of relative gene expression changes of *Oxt, OxtR, Avp, Prlr, Crh, Hcrt1, Hcrt2, Hcrt, Avp1ar* and *Gr* (Mean + SEM. n=13-15 rats per group) of F0 SON between two groups of CON and CSS normalized against mean value of housekeeping genes (*Gapdh* and *β-actin*).*Indicates a significant effect of treatment (t-Test, p≤0.05). **Indicates a marginally significant (t-Test, p=0.07-0.051).

Looking through my data (Figure 5), there were no significant differences between the two groups, however marginally significant downregulation of *OxtR* (p=0.057) within F0 CSS can be observed. This dowregulation is similar to other regions such as F0 CeA and F0 PVN, nonetheless they are not significant or marginally significant, which suggests changes in endocrine response by reducing the OxtR for neurotransmitter Oxt. Even though there are reduced amounts of *OxtR*, the expression of *Oxt* (p=0.290) remains alike

within CSS and CON. Expression of *Gr* and *PrlR* within CSS and CON remains similar. Looking through my data (Figure 5), a non-significant upregulation of *Avp*, *Avpr1ar* and *Crh* within CSS ($p=0.348$, $p=0.390$, $p=0.481$) can be observed, which may be due to lack of maternal care.

Simillar to PVN region, Orexin have also played an important role as motivation behaviours within SON (Wang et al., 2003; Yukari et al., 1999). However, looking through the data, no significant differences of *Hcrt, Hcrtr1* and *Hcrtr2* ($p=0.863$, $p=0.434$, $p=0.110$) expression within CSS and CON can be observed, though a general upregulation within CSS can be observed.

3.3 Gene-Behaviour Association

In collaboration with Tufts University, I obtained F0 behavioural result, such as maternal care and maternal anxiety, which were tested using individual t-test on each day of lactation to independently test for diffrences on each day. Maternal care and maternal aggression were assessed on days 2, 9, and 16 of lactation (early,mid, and late lactation) between 0900 and 1200 h in all dams to assess the effects of early life CSS at different time points during lactation (Carini et al, 2013a; Murgatroyd et al, 2013). Frequencies and durations of pup retrieval, pup grooming, nursing, nesting, self-grooming, and general locomotor activity were scored by an observer who was blind to the treatment using ODLog behavioural analysis software (Macropod Inc., USA). Total maternal care included the combined durations of pup grooming and nursing and the combined durations of self-grooming, nesting, and locomotor activity during maternal care testing, referred to as maternal anxiety.

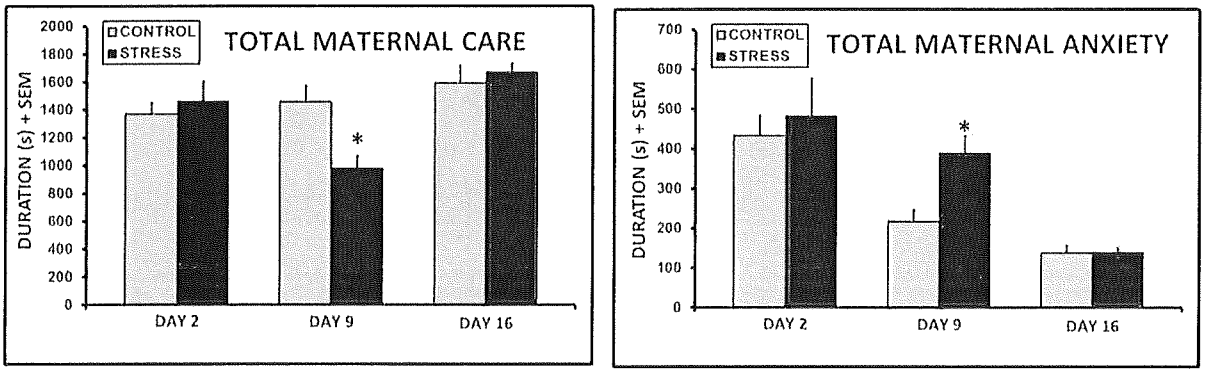

Figure 6. Mean durations + SEM of F0 maternal care during 30-minute maternal care observations on lactation days 2, 9, and 16. * Indicates a significant effect of CSS on that lactation day, $p<0.05$.

Relative mRNA expression levels were compared with individual t-test for each brain region plus for normal distribution using the kolmogrove test. Pearson correlation were used to test for significant gene-behaviour associations (table 5). All graphical results are presented as mean + SEM, and the level of statistical significance was $p<0.05$.

Gene	Nucleus	Generation	Day 9 Behavior	r	R2	P value
CRH	CeA	F0	Maternal Anxiety	0.48	0.23	**0.04**
OxtR	CeA	F1	Maternal Anxiety	0.51	0.26	**0.01**
MR	PVN	F1	Maternal Anxiety	0.51	0.26	**0.01**

Table 5. *Pearson correlation (Mean + SEM) results within F0 and F1 generation for gene-behaviour (day 9) associations in both control and early life CSS dams. All gene-behaviour correlation use the mean duration of the behaviour.*

As shown in table 5, there were significant gene behaviour correlations between CeA *Crh*, PVN *Mr*, CeA *OxtR* and day 9 maternal anxiety, which support my hypothesis that CSS induces stress related nuropetide, corticotropin releasing hormone and changes from F1 generation suggests long-term effects on maternal neuroendocrinology, oxytocin receptor, and mineralocorticoid receptor.

Chapter 4 Discussion

The present investigation has compared several neural systems within amygdala, hypothalamic and supraoptic nucleus regions involved in the control of the stress response and expression of maternal care that may be mediating the behavioural changes in the stress model within two generations (F0 and F1) in terms of model of PPD/A.

In this study, the aim was to assess the affect of CSS on mRNA expression. Returning to the hypothesis posed at the beginning of this study, my study supports and expands on previous data (Nephew et al, 2011); it is now possible to state that the ethologically relevant CSS model of postnatal depression has long-term effects on maternal neuroendocrinology, which are associated with impaired maternal care in both F0 and F1 dams. Exposure to social stress depresses maternal care, impairs lactation and increases maternal anxiety. These effects were associated with significant changes in gene expression of the behaviourally important neuropeptides *OxtR* and *Crh*, as well as changes in gene expression of *Mr*, in which the neuroendocrine effects involve primary mediators of the stress response and maternal care. The identification of oxytocin receptor, corticotropin releasing hormone, and HPA axis factors as preventative and treatment targets for social stress induced impairments in maternal care and lactation in the current study supports clinical interest in these factors. It is concluded that CSS induces behavioural, physiological and neural changes often observed in depressed and/or anxious dams exposed to social stress; this and similar models are valuable resources for both mechanistic studies of postnatal depression and anxiety and lactation difficulties, as well as translationally relevant testing of preventative interventions and treatments in humans.

In contrast to the maternal care and anxiety data, typical rodent maternal care involves retrieving all pups back to a well-formed nest, selfgrooming by the dam, grooming of the pups, and then a substantial nursing bout. The lack of differences in maternal care on day 2 indicates that the two groups were behaviourally similar at the start of the experiment. After one week of CSS on day 9 of lactation, dams spent more time gathering their pups to the nest and less time grooming and nursing the pups, supporting initial work on CSS (Nephew et al, 2011). The increase in pup retrieval duration often involved circling the

cage while carrying the pups and/or moving the nest location, and these actions were likely to affect other maternal care behaviours such as grooming and nursing which are usually expressed after the pups are gathered in the nest. One potential ethological explanation for the increase in pup retrieval on day 9 is that the pup carrying and change in nest location is an attempt to minimize the ongoing threat of the male intruder to the safety of the litter. In addition to this theory, maternal care is known to be very rewarding to the dam, and has been found to be even more rewarding than drugs of abuse on day 8 of lactation (Mattson et al, 2001). It is postulated that CSS disrupts the normal reward system in the brains of CSS dams, rendering the care of pups as less rewarding in CSS dams, which is supported by the decrease in overall maternal care on day 9; a naturally occurring, ethologically relevant measure of behavioural anhedonia that is a primary treatment target in clinical PPD/A research.

The most obvious finding to emerge from this study is that I found three significant gene expression changes within CeA and PVN. Within CeA I found two significant up-regulations in the expression of *Crh* in F0 and *OxtR* in F1 of those mothers exposed to CSS. Within the PVN I found one significant upregulations in the expression of *Mr* in F1 of those mothers exposed the CSS. The second major finding was that, using F0 (day 9) and F1 (day 2) behaviour results from Tuffs University (USA), multiple pearsons correlation analysis revealed significant gene behaviour correlations. As shown in table 7, Crh within F0 CeA, OxtR within F1 CeA and Mr within F1 PVN correlated with maternal care. These maternal care data further support the hypothesis that CSS alters the neuroendocrine control of maternal behaviour during lactation, as related work indicates that the expression of maternal aggression is strongly mediated by central changes in Crh activity in rodents (D'Anna and Gammie, 2009; Gammie et al, 2004; Gammie et al, 2008). My study supports prior work in highly aggressive multiparous females that have elevated central *Crh* compared to less aggressive primiparous dams (Nephew et al, 2009). Another potential mediator of the increase in maternal aggression in the CSS dams is the non-significant decrease *Oxt* expression within CSS. Early life maternal separation stress accentuates the later display of maternal aggression and this is associated with decreased CeA *OxtR*

expression (Veenema et al, 2007). Furthermore, CeA *OxtR* mRNA levels are decreased in multiparous rat dams that display much higher levels of aggression when compared to primiparous dams (Nephew et al, 2009), which supported earlier reports of an inverse relationship between CeA *OxtR* and maternal aggression (Giovenardi et al, 1997, 1998). While the regions where OxtR levels are decreased differ between these studies, this may be due to significant differences between animal models. Given that the normal progression from maternal aggression towards the expression of sexual receptivity following weaning involves the decrease in maternal aggression at the end of lactation, it is hypothesized that CSS could impact post-lactational sexual receptivity. This hypothesis is supported by the changes in *OxtR* and *Mr* and *Crh*: neuroendocrine factors known to mediate sexual behavior and depression and anxiety (Carter, 1992; Pariante and Lightman, 2008). In contrast to the maternal care and anxiety data, while this test is used as a standard measure of anhedonia in numerous animal models of depression, it is postulated that more ethologically and clinically relevant reward mediated behaviours, such as maternal care and social behaviour, will be more valuable in translational research and more likely to identify safe and effective preventative measures and treatments.

The decrease in maternal care on lactation day 9 was accompanied by an increase in maternal anxiety, and depression and anxiety are often comorbid in human mothers (Reck et al, 2008; Stuart et al, 1998; Sutter-Dallay et al, 2004). Instead of caring for the pups at a level similar to the control dams on day 9, the CSS dams spent more time nesting, self-grooming, and moving around the cage: evidence of an overall increase in maternal anxiety. These results support findings in both dams exposed to CSS and their F1 offspring (Carini et al, 2013b; Nephew et al, 2011). As with maternal care, there were only significant differences between the control and stressed dams in maternal anxiety on day 9; levels were similar on day 16. Further specific cognitive testing is needed to confirm adverse effects of CSS on decision-making.

The results of this investigation show that overall down-regulation of *Oxt* within CSS but not as significant as previously reported (Murgatroyd and Nephew, 2013). Nevertheless, I obtained marginally significant down-regulation within F0 MeA (P=0.06). Reduction of *Oxt*

have been associated with stress-related mood disorders and the control of maternal behaviour in both rodents and humans (Murgatroyd and Nephew, 2012). A recent study has suggested that low level of plasma Oxt in humans can increase the risk for postnatal depression (Skrundz et al., 2011) which suggests that plasma *Oxt* during pregnancy is negatively associated with postnatal depression. *Oxt* expression within amygdala acts as a strong mediator of maternal care in a variety of mammals (Bosch and Neumann 2012; Nephew 2012). As indicated in figure 5e, my marginally significant down-regulation of *Oxt* within MeA ($p=0.068$) plus significant positive correlation between MeA Oxt and maternal care ($p=0.04$), supports my hypnosis of novel model of postnatal depression. However, the lack of significant differences within other regions such as PVN, which has previously shown the decrease in PVN Oxt expression, is associated with maternal care (Veenema, Bredewold et al.2007). My data suggests a lack of difference in maternal care between CON and CSS, and I thereby propose an improved social stress protocol needed at Tufts University (USA) to improve my animal model of postnatal depression.

The results of this investigation show a significant up-regulation within F1 CeA CSS ($P=0.02$), which supports my results. The latest research has shown variant in *OxtR* is associated with amygdala which has a role in modulating fear and aggression (Huber et al., 2005; Terenzi and Ingram., 2005; McCarthy et al., 1996; Bosch et al., 2005; Furman et al., 2011). Previous studies have suggested that variances in OxtR distribution may be related to the species-typical pattern of social behaviour, in particular maternal behaviour (Insel and Shapiro, 1992). However, in a recent study in prairie vole model it has been shown that there are no significant differences of *OxtR* within hypothalamus region between the mothers that show good maternal care and control (Olazábal and Young, 2006). This supports my results from F0 generation where I did not obtain any significant differences. Nevertheless, a significant difference was found in second generation F1 CeA, which may suggest trans-generational effects caused by epigenetics influences, which is supported by new studies where epigenetics have played an important role in *OxtR* expression (Ebstein et al. 2012, Meyer-Lindenberg et al. 2010, Tost et al. 2010).

The results of this investigation show no overall trend of *Prlr* or any significant differences. However, the findings of the current study do not support the previous research, where significant down-regulation of *Prlr* within early life CSS was noticed (Murgatroyd and Nephew, 2012). My *Prlr* result does not meet my initial hypothesis since *Prl* plays an important role in stress-related mood disorders and/or the control of maternal behaviour in both rodents and humans. Moreover, previous studies have implicated *Prl* in the control of rodent maternal behaviour (Bridges et al., 1990; Bridges and Ronsheim, 1990; Bridges and Mann, 1994; Bridges et al., 2001). Additionally, low plasma levels of Prl are associated with postnatal depression in humans (Abou-Saleh et al., 1998).

The results of this investigation show a significant up-regulation of Crh in F0 CeA CSS (P=0.04) which support the previous findings (Bosch et al. 2006, Kessler et al. 2011, Klampfl et al, 2013). This study produced results which corroborated the finding of a great deal of the previous work in this field, since Crh is associated in the etiology of stress-related mood disorders and/or the control of maternal behaviour in both rodents and humans (Murgatroyd and Nephew, 2012). This increases in response to stress, and reduces or stops the maternal care in rats and primates (Saltzman et al., 2011; Pedersen et al., 1991), and suppresses maternal aggression in mice (Gammie et al., 2004). *Crh* also plays an important role in the development of treatments for stress-induced mood disorders (Heim et al., 1997). Up-regulation of *Crh* causes an increase in sympathetic excitation. The findings from my data are supported by previous studies, which suggest that an increase of *Crh* and *Mr* indicates an increased exposure to mineralocorticoid during lactation, mediating the changes in maternal anxiety (Makino, Gold et al. 1994; Herman and Cullinan 1997; Shepard, Barron et al. 2000). This, therefore, causes a negative feedback on elevated plasma mineralocorticoid, mediating behavioural adaptation. Previous studies have shown association with activation of *Crh*, which after long-term antidepressant administration reduced the *Crh* expression within hypothalamus (Brady et al., 1991). From table 7, I would suggest that the increase in Mr and Crh may have allowed for maternal care parallel to the control animals on day 16 either through a direct neuroendocrine effect and/or through a decrease in peripheral mineralocorticoid

(Brummelte and Galea 2010). The correlations between Mr and Crh expression and maternal anxiety support the suggestion that CSS induced elevated mineralocorticoid, as exogenous mineralocorticoid increases anxiety related behaviours in nulliparous and maternal rats, and CeA Crh has an anxiogenic role (Swiergiel, Takahashi et al. 1993; Makino, Gold et al. 1994).

The results of this investigation show non-significant changes of *Hcrt* and *Hcrt receptor 1&2 (Hcrtr1 and Hcrtr2)* within CSS, however I have noticed a significant correlation between SON *Hcrtr2* and PVN *Gr* expression. A possible explanation for these results may be due to the association of Hcrt and glucocorticoid, which has been reported in previous studies; they suggest enhanced glucocorticoid production in rats and humans caused by *Hcrt*. (Lin et al., 1999; Spinazzi et al., 2006). It has also been suggested that *Hcrt* receptors may mediate the fasting-induced surge in glucocorticoid blood levels in rats (Dallman et al., 1999). Moreover, *Hcrtr2* expression within F0 SON were positively correlated with maternal anxiety (results from tufts university (USA)). The *Hcrt* is known to regulate encouragement, wakefulness and feeding behaviour (Preedy et al., 2011) when intracerebroventricular administration of Hcrt increases food intake (Sakurai et al., 1998). From my data, I would suggest that an increase of *Hcrtr2* mediates the stress induced changes in CeA *Crh* and maternal anxiety (Sakamoto, Yamada et al. 2004).

The results of this investigation show no significant differences in *Gr* within CSS. Nevertheless I obtained a very strong significant up-regulation of *Mr* within F1 PVN CSS (P=0.006). This study produced results which corroborate the findings from previous studies where *Mr* expression were up-regulated in CSS adult tree shrews (Meyer et al., 2001), humans (Baes et al., 2014) and in rats (Gesing et al., 2001). This supports my hypothesis that during postnatal depression *Mr* are involved in HPA axis impairment causing stress response in adult life that leads to depression.

The data must be interpreted with caution because my result does not support my initial hypothesis since I did not obtain any significant differences in most of the specific genes, especially *Oxt, Avp* and *PrlR*. This could be due to my study limitations (see Appendix 3),

suggesting further investigations needed. However, the hypothesis that CSS alters the neuroendocrine control of maternal behaviour during lactation is almost certain, since related work indicates that the expression of maternal aggression is strongly mediated by central changes in CRH activity in rodents (Gammie, Negron et al. 2004; Gammie, Seasholtz et al. 2008; D'Anna and Gammie 2009). After reviewing the behavioural study facts supplied by Tufts University (USA) the lack of differences in peripheral hormones was not surprising given the timing of the sample collection on lactation day 23, the presentation of the stressor for 1hr/day, and the fact that the stressor was not applied until both populations had fully developed HPA axes. It is possible that there were differences during mid-lactation that were not present by day 23, and it is postulated that the increases in *Mr* and *Crh* are suggestive of adaptive neural responses to the social stressor. Early life represents a critical phase in brain development as many regions are not fully formed at birth or undergo extended postnatal maturation. Previous studies have reported that possible stress, mainly during the critical phase of early development, can have significant lasting consequences for brain structure and function later in life (Figure 7) (Loi et al., 2014).

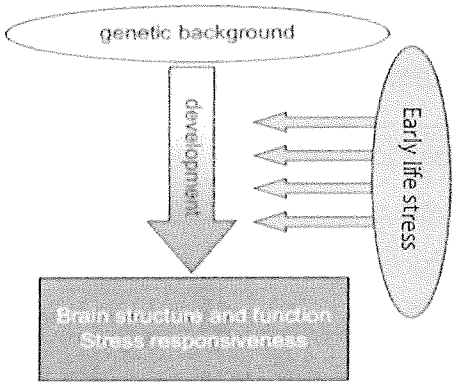

Figure 7. General scheme highlighting that life events, especially when experienced during the early development stage, may strongly impact the development of the brain, especially in genetically susceptible individuals (Loi et al., 2014).

It is also very important to take into account the neurological development of animal models to humans. Since rats and humans age at different phases of their lives, the precise correlation between the ages of laboratory rats and humans is still a subject of debate. The complete findings indicate that rats grow quickly throughout their juvenile and become sexually mature at about the sixth week, nevertheless reach social adulthood 5-6 months later. In adulthood, every day of the animal is approximately equivalent to 34.8 human days (i.e., One rat month is comparable to three human years) (Sengupta, 2013). In this study animals were exterminated on day 23, which according to a recent study represents weanling (around six month's age) (Sengupta, 2013). These results, therefore, need to be interpreted with caution, since the associations between maternal depressions, maternal behaviour and child outcomes are complex, and not all studies have found a relationship between maternal depression and indicators of poor parenting. Variations in the type, severity, chronicity and timing of maternal depression (Campbell et al., 1995), heterogeneity in sampling (community versus high-risk multiproblem samples), and potentiating risk factors, such as family adversity, low social support and financial stress (Sameroff et al., 1993), all contribute to differences in outcomes in children. On the other hand, stress factors can be responsible for adverse child outcomes in the absence of maternal depression.

Chapter 5 Appendix

5.1 Appendix 1 : CON and CSS Animal Models

Sprague Dawley rats are the most widely used outbred rat models for biomedical research. This model is ideal for applications requiring accurate estimation of gestational age and the superb reproductive act of the Sprague Dawley rat makes it an ideal model for producing timed-pregnant females; the age at paring (mating) for male and female is 8-10 weeks (Suckow et al., 2005). At Tufts University (USA) twenty seven females rats (F0) were sustained at a temperature (21–25°C) and light (14h:10h light: dark cycle; lights on at 05:00 h) controlled rooms and food and water were available ad libitum throughout the studies. F0 females were mated by placing two females with one male. Maternal behaviour was noted in all rats on the day of birth (retrieval of all puppies, cleaning and grooming of pups, and nursing), and dams were then randomly assigned to the CON or the CSS group. The CSS F0 dams were subjected to a chronic social stress protocol from days 2 to 16 of lactation (Nephew and Bridges, 2011). This process consisted of placing a similarly sized (220–300 g) novel male intruder into a lactating female's home cage for 1 h from days 2to 16 of lactation. Control dams were not divulged to the CSS protocol; they were only tested for maternal care and maternal aggression on days 2, 9, and 16 of lactation. The pups were left in the cage during the intruder presentation, and the CSS exposure results in depressed maternal care and increased anxiety-related behaviour and maternal aggression (Nephew and Bridges, 2011). In the following generation (F1), the control and early life CSS F1 females were the progeny of the F0 control and CSS dams; the deviations between the discourses of the control and early life CSS F1 females were limited to the exposure of the early life CSS F1 females to depressed maternal care and engagement between their F0 mothers and the male intruders during age 2 to 16 days. The F1 control and early life CSS animals were treated identically after the age of 16 days (Carini and NEPHEW, 2013).

5.1.1 Behavioural testing

At Tufts University (USA) maternal care and maternal aggression were assessed on days 2, 9, and 16 (early, mid and late lactation), of early life CSS on mean pup body weight across lactation, behaviours, milk intake, at veterinary medicine Tufts University (USA) (Carini and NEPHEW, 2013). For the maternal care and maternal aggression study a digital camera was used for behavioural observation without human intervention. Maternal care testing consisted of the re-entry of all eight pups to the home cage after a 30-minute removal, and behaviour was then video recorded for 30 minutes. Subsequently, the rat brain samples were shipped to me at Manchester Metropolitan University (UK) for gene expression analysis.

5.1.2 Microdissection

Rats used in this study were euthanized at Tufts University (USA) by cervical dislocation. Since this study involved a stress paradigm, no anesthesia were given, to avoid interfering with the normal physiological regulation of stress hormones. The brain was cryosectioned at thickness of 20 µm at -20°C temperature. Segments were mounted on super-frost glass slides and held in -20°C for referencing to a standard rat stereotaxic atlas using Paxinos 6 (Paxinos and Watson, 2007). In order to verify anatomical precision and to ensure inclusion of the region of interest, the neurons within the specific region were collected using a 0.8 mm internal diameter sample corer.

5.2 Appendix 2: Primer sequences used for cDNA

Primer-BLAST programme is used to design primers that are specific to the intended PCR target. Publicly available, Primer-Blast is developed at NCBI and it uses "primer3" to design PCR primers. This tool combines BLAST with a global alignment algorithm to ensure a full primer-target alignment and is sensitive enough to detect targets that have a significant number of mismatches to primers. Primer-BLAST allows users to design new target-specific primers in one step as well as to check the specificity of pre-existing primers. Primer-BLAST also supports placing primers based on exon/intron locations and excluding single nucleotide polymorphism (SNP) sites in primers. (Ye et al., 2012). Primer sequences and

conditions for qRT-PCR reactions are listed in Table. 1, some of the primers were obtained from a previous study (Murgatroyd and Nephew, 2013).

Gene	Sequence (forward, reverse)	Length	Start	Stop	T_m	Gc%	Self comp	Self 3'comp	Location (intron spanning)	Product length
Oxt	F TCTGACCTCCGCCTGCTACATC	(Murgatroyd and Nephew, 2013)								
	R AAGCAGCGCCTTTGCCGCC									
OxtR	F GTACTGGCCTTCATCGTGTGC	21	838	858	61.60	57.14	4.00	2.00	Exon1-2	133
	R TGCAGCAGCTGTTGAGGCTG	20	970	951	63.63	60.00	8.00	2.00		
PrlR	F GTAGATGGAGCCAGGAGAGTTC	(Murgatroyd and Nephew, 2013)								
	R ACCAGAGTCACTGTCGGGATCT									
Avp	F CAGATGCTCGGCCCGAAG	(Murgatroyd and Nephew, 2013)								
	R TTCCAGAACTGCCCAAGAG									
Avpr1a	F CAGCAGCGTGAAGAGCATTT	20	1087	1106	59.48	50.00	3.00	2.00	Exon 1-2	175
	R CGCCGTGATTGTGATGGAAG	20	1261	1242	59.63	55.00	3.00	0.00		
Crh	F CAGAACAACAGTGCGGGCTCA	21	121	141	63.43	57.14	3.00	2.00	Exon 1-2	119
	R AAGGCAGACAGGGCGACAGAG	21	239	219	64.42	61.90	2.00	0.00		
Hcrtr1	F CAGGGAGGGCCTATAATTGA	(Jöhren et al., 2001) (Sakurai et al., 1998)							AF041244 a	260
	R CCCTCAACTCCAGTCCTAGC									
Hcrtr2	F TCCCCCTCTCATAAACTTGG	(Jöhren et al., 2001) (Sakurai et al., 1998)							AF041246 a	314
	R CAATGTTGTTGGGGTGCTTA									
Hcrt	F CCACTGCACCGAAGATACCA	20	31	50	59.75	55.00	4.00	0.00	Exon 1-2	208
	R AGTTCGTAGAGACGGCAGGA	20	238	219	60.32	55.00	3.00	0.00		
nucb2	F GGCCCAGAAGCAGGAGTATC	20	1362	1381	59.89	60.00	4.00	2.00	Exon 13-14	79
	R CCCTGATGGAGCAATCCCTT	20	1440	1421	59.45	55.00	4.00	0.00		
Gr	F AGGGGAGGGGGAGCGTAATGG	21	78	98	65.53	66.67	2.00	0.00		119
	R CCTCTGCTGCTTGGAATCTGC	21	196	176	61.62	57.14	4.00	4.00		
Mr	F TACGACAATTCCAAGCCCGACACC	24	2724	2747	65.18	54.17	4.00	0.00		99
	R TACCTTGGCCCACTTCACGACCTG	24	2822	2799	66.45	58.33	5.00	3.00		
β-actin (Actb)	F TTGCTGACAGGATGCAGAA	19	1004	1022	56.96	47.37	5.00	3.00	Exon 5-6	101
	R ACCAATCCACACAGAGTACTT	21	1104	1084	56.57	42.86	6.00	4.00		
Hprt	F TGGTCAAGCAGTACAGCCCC	20	512	531	62.12	60.00	6.00	0.00	Exon 6-9	261
	R TACTGGCCACATCAACAGGA	20	772	753	58.64	50.00	6.00	0.00		
Gapdh	F CATCACCATCTTCCAGGAGC	" sqrdl" (Murgatroyd and Nephew, 2013)								
	R TAAGCAGTTGGTGGTGCAGG									
Pgk1	F GAAGGGAAGGGAAAAGATG	19	389	407	52.87	47.37	2.00	0.00	Exon 4-6	180
	R AAATCCACCAGCCTTCTGTG	20	568	549	58.08	50.00	3.00	2.00		
sdha	F AGACGTTTGACAGGGGAATG	20	1666	1685	57.81	50.00	4.00	0.00	Exon 13-16	160
	R TCATCAATCCGCACCTTGTA	20	1825	1806	56.93	45.00	3.00	2.00		
Mrfap1	F CATCTGGGACTGTGGATGGG	20	1028	1047	59.82	60.00	4.00	0.00	Exon 2	190
	R CCAACAAACTCATCAGGCAGG	21	1217	1197	59.46	52.38	4.00	0.00		

Table 6. Primer Sequence used in this study.

Appendix 3: Limitations of the Study

5.3 Gene analysis

Due to low mRNA concentration and low cDNA volume, I was not able to analyse all the genes mentioned in the method. I mainly focused on genes that had previously been reported to have an effect on CSS (Nephew et al, 2009). In terms of housekeeping genes, careful selection of an appropriate control or set of controls have been selected, as significant variation has been observed between brain regions, even for the most commonly used housekeeping genes, including *β-Actin* and *GAPDH* (de Kok et al., 2005).

5.4 qRT-PCR results

A possible explanation for lack of significant differences results may be due to lack of adequate guidelines set during these experiments. In this study I have failed to meet the minimum information for publication of quantitative real-time PCR experiments (MIQE) which are the guidelines that target the reliability of qRT-PCR results (Bustin et al., 2009). MIQE is a set of guidelines that define the minimum data required for evaluating qRT-PCR investigation. This investigation met most of the guidelines such as sample achievement, handling, preparation and control. However, I failed to meet the minimum requirements in Primer specificity and PCR efficiency. Checking the primer specificity using BLAST, one third of primer designs contain products on potentially unintended templates, mostly primers designed in previous experiments. The following primers *OxtR* primer contains fifty nine, *Avpr1a* contain two, *Gr* contain three, *β-actin* contain twenty five, *Hprt1* contain three, *Mr* contain twenty two products on potentially unintended templates. Additionally, in regards to qRT-PCR technique used in this study, it has a lack of specificity of its binding when using SYBR green reagent dye which does not discriminate the double-stranded DNA from the PCR products and those from the primer-dimer; overestimation of the target concentration is a common problem. Where accurate quantification is an absolute necessity, further assay for the validation of results must be performed.

After double checking my primers, and primers from the previous study, from table below (table 9), I noticed "Products on potentially unintended templates" for some primers which will have an impact on my qRT-PCR by binding to non-specific template. For this reason I have designed a new primers sequence for future studies, as shown in table 10.

	Problem with current Sequence (5'->3')	Length	Start	Stop	Tm	Gc%	Self comp	Self 3'comp	*Location (intron spanning)*	*Product length*
OxtR NM_012 871.2	***F*** GTACTGGCCTTCATCGTGTGC	21	838	858	61.60	57.14	4.00	2.00	Exon1/2	133
	R TGCAGCAGCTGTTGAGGCTG	20	970	951	63.63	60.00	8.00	2.00		
	Products on potentially unintended templates									
	NM_001106737.1 Rattus norvegicus ninein (GSK3B interacting protein) (Nin), mRNA	product length = 3658 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 6137 A.....T.....A.... 6118 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2480 A.G.....AC....C... 2499 product length = 2197 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 6137 A.....T.....A.... 6118 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 3941 .C...G..GA.....C 3960								
	NM_001191812.1 Rattus norvegicus espin-like (Espnl), mRNA	product length = 2874 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2926 ..TCC.C.....A.... 2907 Reverse primer 1 TGCAGCAGCTG-TTGAGGCTG 20 Template 53 ..G...G....T..... 73 product length = 486 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2926 ..TCC.C.....A.... 2907 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2441 CC.G.....CG..... 2460								
	NM_019249.1 Rattus norvegicus protein tyrosine phosphatase, receptor type, F (Ptprf), mRNA	product length = 1627 Forward primer 1 GTACTGGCCTTCATCGTGTGC 21 Template 162 .GGG.....C.T..... 182 Forward primer 1 GTACTGGCCTTCATCGTGTGC 21 Template 1688 C.G.....C.C... 1668								
	NM_001270972.1 Rattus norvegicus zinc finger RNA binding protein (Zfr), mRNA	product length = 610 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1130 A.....C..CT.... 1111 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 521 .C...TT.....CA.... 540 product length = 22 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1133 .....A.C..CT.... 1114 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20								

		Template 1112 A.....A.C..CT.... 1131 product length = 613 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1133 .....A.C..CT.... 1114 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 521 .C...TT.....CA.... 540
	XM_001056210.3 PREDICTED: Rattus norvegicus zinc finger RNA binding protein (Zfr), mRNA	product length = 610 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1130 A.....C..CT.... 1111 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 521 .C...TT.....CA.... 540 product length = 22 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1133 .....A.C..CT.... 1114 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1112 A.....A.C..CT.... 1131 product length = 613 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1133 .....A.C..CT.... 1114 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 521 .C...TT.....CA.... 540
	NM_001170484.1 Rattus norvegicus RNA binding motif protein 4 (Rbm4), mRNA	product length = 22 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 969 A.....A.C..CT.... 950 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 948 .....A.C..CT.... 967
	XM_001062568.3 PREDICTED: Rattus norvegicus myeloid/lymphoid or mixed-lineage leukemia 2 (Mll2), mRNA	product length = 733 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11927 ..TT.....TT.... 11908 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11195 A.....C..AG...A. 11214 product length = 676 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11870 ...T..T.....TT.... 11851 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11195 A.....C..AG...A. 11214 product length = 604 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11798 ...T.T.....TT.... 11779 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11195 A.....C..AG...A. 11214 product length = 691 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11885 A..T..T.....CT.... 11866 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20

		Template 11195 A.....C..AG...A. 11214 product length = 544 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11738 A..T..T.....TT.... 11719 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11195 A.....C..AG...A. 11214
	XM_002729854.2 PREDICTED: Rattus norvegicus myeloid/lymphoid or mixed-lineage leukemia 2 (LOC100362634), mRNA	product length = 733 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 12149 ..TT.....TT.... 12130 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11417 A.....C..AG...A. 11436 product length = 676 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 12092 ...T..T.....TT.... 12073 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11417 A.....C..AG...A. 11436 product length = 604 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 12020 ...T.T.....TT.... 12001 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11417 A.....C..AG...A. 11436 product length = 691 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 12107 A..T..T.....CT.... 12088 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11417 A.....C..AG...A. 11436 product length = 544 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11960 A..T..T.....TT.... 11941 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 11417 A.....C..AG...A. 11436
	NM_001108486.1 Rattus norvegicus leucine rich repeat containing 28 (Lrrc28), mRNA	product length = 2182 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2475 G.....G..G..T.A... 2456 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 294 .....C...GA.....C 313
	NM_001191666.1 Rattus norvegicus muscleblind-like splicing regulator 1 (Mbnl1), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 516 .....CA.CA.... 497 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 492 GC.....C..CA.... 511
	NM_001105734.1 Rattus norvegicus dual specificity phosphatase 10 (Dusp10), mRNA	product length = 22 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 400 .....AG..CT.... 381 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 379 .....AC.A.CT.... 398

	XM_003750732.1 PREDICTED: Rattus norvegicus procollagen, type VI, alpha 3 (Col6a3), mRNA	product length = 3887 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 4610 ..GG..C.A.....T... 4591 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 724 .....TT.GA.....A.. 743
	XM_003754548.1 PREDICTED: Rattus norvegicus procollagen, type VI, alpha 3 (Col6a3), mRNA	product length = 3887 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 4610 ..GG..C.A.....T... 4591 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 724 .....TT.GA.....A.. 743
	XM_574368.3 PREDICTED: Rattus norvegicus multiple inositol polyphosphate phosphatase 1-like (RGD1664801), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 329 .....CCC..CA.... 310 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 305 ..AG.....CA.G..... 324
	NM_001106379.1 Rattus norvegicus autoimmune regulator (Aire), mRNA	product length = 1145 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1225 .....T...AGCC..... 1206 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 81 ...GC.GC...C..... 100
	NM_019263.1 Rattus norvegicus multiple inositol-polyphosphate phosphatase 1 (Minpp1), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 329 .....CCC..CA.... 310 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 305 ..AG.....CA.G..... 324
	NM_001009708.1 Rattus norvegicus LIM domain only 4 (Lmo4), mRNA	product length = 514 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 520 A....G...AC.....A. 501 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 7 .....TG.C..CC.... 26
	XM_003754166.1 PREDICTED: Rattus norvegicus additional sex combs like 2 (Drosophila), transcript variant 2 (Asxl2), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1914 G..T.....C..CA.... 1895 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1890 A.....CA.CA.... 1909
	XM_003754165.1 PREDICTED: Rattus norvegicus additional sex combs like 2 (Drosophila), transcript variant 1 (Asxl2), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2058 G..T.....C..CA.... 2039 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2034 A.....CA.CA.... 2053
	NM_001271495.1 Rattus norvegicus similar to Msx2-interacting protein (SPEN homolog) (SMART/HDAC1-associated repressor protein) (LOC690911), mRNA	product length = 541 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 5484 ...G.....CC.G.A... 5465 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 4944 A.....A.AGA..... 4963
	NM_001033888.1 Rattus norvegicus numb homolog (Drosophila)-like (Numbl), mRNA	product length = 133 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 864 G.....A..G.T.C... 845 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 732 A.....C.CCT.... 751
	XM_002729562.2 PREDICTED: Rattus norvegicus similar to Msx2-interacting protein	product length = 541 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 5574 ...G.....CC.G.A... 5555

	(SPEN homolog) (SMART/HDAC1-associated repressor protein) (LOC690911), mRNA	Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 5034 A.....A.AGA..... 5053
	XM_001080881.3 PREDICTED: Rattus norvegicus spectrin, beta, non-erythrocytic 5 (Sptbn5), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 5747 .T.....CC..CA.... 5728 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 5723 A.....CA.GA.... 5742
	XM_003751283.1 PREDICTED: Rattus norvegicus formin 2-like (LOC100360457), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 644 A.....TC..CT.... 625 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 620 A.....A.CA..A.... 639
	XM_003752690.1 PREDICTED: Rattus norvegicus formin 2-like (LOC100360457), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 644 A.....TC..CT.... 625 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 620 A.....A.CA..A.... 639
	XM_003750119.1 PREDICTED: Rattus norvegicus additional sex combs like 2 (Drosophila), transcript variant 1 (Asxl2), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1920 G..T.....C..CA.... 1901 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1896 A.....CA.CA.... 1916
	XM_003750120.1 PREDICTED: Rattus norvegicus additional sex combs like 2 (Drosophila), transcript variant 2 (Asxl2), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2064 G..T.....C..CA.... 2045 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2040 A.....CA.CA.... 2059
	XM_003750121.1 PREDICTED: Rattus norvegicus additional sex combs like 2 (Drosophila), transcript variant 3 (Asxl2), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2043 G..T.....C..CA.... 2024 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 2019 A.....CA.CA.... 2038
	NM_001173433.1 Rattus norvegicus transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila) (Tle1), mRNA	product length = 25 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1334 A.....CA.CA.... 1316 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1310 C..T.....C..CA.... 1329
	NM_001106064.1 Rattus norvegicus calcium homeostasis endoplasmic reticulum protein (Cherp), mRNA	product length = 710 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1271 A.GG....G..C..... 1252 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 562 A.....A..CA..A.... 581 product length = 604 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1165 G..T..T.....CT.... 1146 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 562 A.....A..CA..A.... 581
	XM_003753401.1 PREDICTED: Rattus norvegicus keratin-associated protein 10-7-like (LOC100912367), mRNA	product length = 544 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 616 .....GACT...A. 597 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20

		Template 73 G..TC.....G.G..... 92 product length = 586 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 616 .....GACT...A. 597 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 31 G..TC.....G.G..... 50 product length = 475 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 616 .....GACT...A. 597 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 142 ...TC.....G.G...A. 161 product length = 499 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 571 .....GACT...A. 552 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 73 G..TC.....G.G..... 92 product length = 541 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 571 .....GACT...A. 552 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 31 G..TC.....G.G..... 50 product length = 430 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 571 .....GACT...A. 552 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 142 ...TC.....G.G...A. 161 product length = 409 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 481 .....GACT...A. 462 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 73 G..TC.....G.G..... 92 product length = 451 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 481 .....GACT...A. 462 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 31 G..TC.....G.G..... 50 product length = 340 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 481 .....GACT...A. 462 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 142 ...TC.....G.G...A. 161

	XM_001056210.3 PREDICTED: Rattus norvegicus zinc finger RNA binding protein (Zfr), mRNA	product length = 610 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1130 A.....C..CT.... 1111 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 521 .C...TT.....CA.... 540 product length = 22 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1133 .....A.C..CT.... 1114 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1112 A.....A.C..CT.... 1131 product length = 613 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 1133 .....A.C..CT.... 1114 Reverse primer 1 TGCAGCAGCTGTTGAGGCTG 20 Template 521 .C...TT.....CA.... 540								
Avpr1a NM_012871.2	***F*** CAGCAGCGTGAAGAGCATTT	20	1087	1106	59.48	50.00	3.00	2.00	Exon 1/2	175
	R CGCCGTGATTGTGATGGAAG	20	1261	1242	59.63	55.00	3.00	0.00		
	Products on potentially unintended templates									
	XM_002730026.2 PREDICTED: Rattus norvegicus dynein, axonemal, heavy polypeptide 7 (Dnah7), mRNA	product length = 3659 Forward primer 1 CAGCAGCGTGAAGAGCATTT 20 Template 10762 ..C..T.C.C.G..... 10743 Reverse primer 1 CGCCGTGATTGTGATGGAAG 20 Template 7104 ..T.AA.C....C..... 7123								
	XM_001065965.3 PREDICTED: Rattus norvegicus dynein, axonemal, heavy polypeptide 7 (Dnah7), mRNA	product length = 3659 Forward primer 1 CAGCAGCGTGAAGAGCATTT 20 Template 12007 ..C..T.C.C.G..... 11988 Reverse primer 1 CGCCGTGATTGTGATGGAAG 20 Template 8349 ..T.AA.C....C..... 8368								
Mr NM_012871.2	***F*** GGCCCAGAAGCAGGAGTATC	20	1362	1381	59.89	60.00	4.00	2.00	Exon 13-14	79
	R CCCTGATGGAGCAATCCCTT	20	1440	1421	59.45	55.00	4.00	0.00		
	Products on potentially unintended templates									
	NM_017230.2 Rattus norvegicus peptidyl arginine deiminase, type III (Padi3), mRNA	product length = 1680 Forward primer 1 GGCCCAGAAGCAGGAGTATC 20 Template 1292 ....A.TGG.A..... 1311 Reverse primer 1 CCCTGATGGAGCAATCCCTT 20 Template 2871 AG.....G..C..... 2852								
	XM_575165.5 PREDICTED: Rattus norvegicus titin (Ttn), mRNA	product length = 408 Forward primer 1 GGCCCAGAAGCAGGAGTATC 20 Template 61224 ..A.G....C.....G 61243 Forward primer 1 GGCCCAGAAGCAGGAGTATC 20 Template 61631 .A.A...GTC..... 61612								
	XM_001065955.4 PREDICTED: Rattus norvegicus titin (Ttn), mRNA	product length = 408 Forward primer 1 GGCCCAGAAGCAGGAGTATC 20 Template 61023 ..A.G....C.....G 61042 Forward primer 1 GGCCCAGAAGCAGGAGTATC 20 Template 61430 .A.A...GTC..... 61411								
Actb (β-actin) NM_012871.2	***F*** TTGCTGACAGGATGCAGAA	19	1004	1022	56.96	47.37	5.00	3.00	Exon 5-6	101
	R ACCAATCCACACAGAGTACTT	21	1104	1084	56.57	42.86	6.00	4.00		
	Products on potentially unintended templates									
	XM_002728532.2 PREDICTED: Rattus norvegicus actin, cytoplasmic 2-like (LOC684969), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 960 978								

		Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1060 G.....G..T..... 1040
	XM_002729196.2 PREDICTED: Rattus norvegicus actin, gamma 1 propeptide-like (LOC100361457), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 994 ..... 1012 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1094 G.....G..T..... 1074
	XM_002725322.2 PREDICTED: Rattus norvegicus actin, cytoplasmic 2-like (LOC684969), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 964 ..... 982 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1064 G.....G..T..... 1044
	NM_001127449.1 Rattus norvegicus actin, gamma 1 (Actg1), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1000 ..... 1018 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1100 G.....G..T..... 1080
	NM_012893.1 Rattus norvegicus actin, gamma 2, smooth muscle, enteric (Actg2), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 944 ..... 962 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1044 T.....G..T..... 1024
	XM_003752139.1 PREDICTED: Rattus norvegicus actin, cytoplasmic 1-like (RGD1660119), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1007 ..... 1025 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1107 G.....G..TA..... 1087
	XM_003754851.1 PREDICTED: Rattus norvegicus actin, cytoplasmic 1-like (RGD1660119), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 980 ..... 998 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1080 G.....G..TA..... 1060
	XM_003749740.1 PREDICTED: Rattus norvegicus uncharacterized LOC100911020 (LOC100911020), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 557 .....C..... 575 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 657 G.....G..T.....C. 637
	XM_003753892.1 PREDICTED: Rattus norvegicus actin, cytoplasmic A3a-like (LOC100912821), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 113 .....C..... 131 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 213 G.....G..T.....C. 193
	NM_001106409.1 Rattus norvegicus actin, beta-like 2 (Actbl2), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1050 ....C..... 1068 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1160 C.....A..T..A..... 1130
	NM_019212.2 Rattus norvegicus actin, alpha 1, skeletal muscle (Acta1), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 995 .C.....C.C..... 1013 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1095 G..G.....T..... 1075
	NM_019183.1 Rattus norvegicus actin, alpha, cardiac muscle 1 (Actc1), mRNA	product length = 101 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1124 .....TC.T.....A.. 1142

		Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1224 C.....G.....T.. 1204
	NM_001106020.2 Rattus norvegicus ATP-binding cassette, subfamily A (ABC1), member 13 (Abca13), mRNA	product length = 88 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 13276 .....AT.A.GA..... 13294 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 13363 A.....A....G.... 1334
	NM_001108775.1 Rattus norvegicus RAB6B, member RAS oncogene family (Rab6b), mRNA	product length = 3387 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 320 .G.....A..G.....T 338 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 3706 C...CC.....T... 3688
	NM_001105733.1 Rattus norvegicus calcium channel, voltage-dependent, beta 4 subunit (Cacnb4), mRNA	product length = 1726 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 861 .AA.A....T..... 879 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 2586 G...A...T.AC..... 2568
	XM_003751686.1 PREDICTED: Rattus norvegicus probable ATP-dependent RNA helicase DDX60-like (LOC100911190), partial mRNA	product length = 1432 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 519 ..T..TCA.....T... 537 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 1950 C.....CA..T.....T.. 1930
	XM_002728402.3 PREDICTED: Rattus norvegicus mCG11385-like (LOC100360801), mRNA	product length = 1912 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 186 ..T..TCA.....T... 204 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 2097 C.....CA..T.....T.. 2077
	XM_001077365.3 PREDICTED: Rattus norvegicus deleted in lung and esophageal cancer 1 isoform DLEC1-N1, transcript variant 1 (Dlec1), mRNA	product length = 597 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 952 .GCTC.C..... 970 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1648 ..TGG.C..T..... 1630
	XM_003754612.1 PREDICTED: Rattus norvegicus mCG11385-like (LOC100360801), mRNA	product length = 1945 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 219 ..T..TCA.....T... 237 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 2163 C.....CA..T.....T.. 2143
	XM_001062041.3 PREDICTED: Rattus norvegicus deleted in lung and esophageal cancer 1 isoform DLEC1-N1 (Dlec1), mRNA	product length = 597 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 952 .GCTC.C..... 970 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1648 ..TGG.C..T..... 1630
	XM_003754612.1 PREDICTED: Rattus norvegicus mCG11385-like (LOC100360801), mRNA	product length = 1945 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 219 ..T..TCA.....T... 237 Reverse primer 1 ACCAATCCACACAGAGTACTT 21 Template 2163 C.....CA..T.....T.. 2143
	XM_001062041.3 PREDICTED: Rattus norvegicus deleted in lung and esophageal cancer 1 isoform DLEC1-N1 (Dlec1), mRNA	product length = 597 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 952 .GCTC.C..... 970 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 1648 ..TGG.C..T..... 163
	NM_001106943.1 Rattus norvegicus forkhead box O4 (Foxo4), mRNA	product length = 313 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 2198 C.TT...A.....T 2216

		Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 2510 .A.TG.....A.....G 2492 product length = 2484 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 27 .GAG...G...G..... 45 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 2510 .A.TG.....A.....G 2492								
	NM_012671.2 Rattus norvegicus transforming growth factor alpha (Tgfa), mRNA	product length = 1827 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 381 .C....TG..C.....T 399 Forward primer 1 TTGCTGACAGGATGCAGAA 19 Template 2207 CA...CT.....T... 2189								
Hprt1 NM_012583.2	***F*** GGCCCAGAAGCAGGAGTATC	20	512	531	62.12	60.00	6.00	0.00	Exon 6-9	261
	R CCCTGATGGAGCAATCCCTT	20	772	753	58.64	50.00	6.00	0.00		
	Products on potentially unintended templates									
	XM_003752165.1 PREDICTED: Rattus norvegicus hypoxanthine phosphoribosyltransferase 1 (Hprt1), mRNA	product length = 261 Forward primer 1 TGGTCAAGCAGTACAGCCCC 20 Template 588 ..... 607 Reverse primer 1 TACTGGCCACATCAACAGGA 20 Template 848 829								
	NM_001270981.1 Rattus norvegicus fibronectin type III domain containing 5 (Fndc5), mRNA	product length = 918 Forward primer 1 TGGTCAAGCAGTACAGCCCC 20 Template 87 ....GC..GC.G..... 106 Forward primer 1 TGGTCAAGCAGTACAGCCCC 20 Template 1004 C.....T.A.....A 985								
	NM_001099481.1 Rattus norvegicus vomeronasal 2 receptor, 68 (Vom2r68), mRNA	product length = 1604 Reverse primer 1 TACTGGCCACATCAACAGGA 20 Template 1855 ..T.....TTCA..... 1836 Reverse primer 1 TACTGGCCACATCAACAGGA 20 Template 252 .....ATG.G.....A.. 271								

Table 7, Products on potentially unintended templates.

Controls in real-time PCR reactions prove that signals obtained from experimental samples represent the amplicon of interest, thereby validating specificity. All experiments should include a no-template control (NTC), and qRT-PCR reactions should also include a no-reverse transcriptase control. NTC controls should contain all reaction components except the DNA or cDNA sample. Amplification detected in these wells is due to either primer-dimers or contamination with completed PCR reaction product. This type of contamination can make expression levels look higher than they actually are. No-RT reactions should contain all reaction components except the reverse transcriptase. If amplification products are seen in no-RT control reactions, it indicates that DNA was amplified rather than cDNA. This can also artificially inflate apparent expression levels in experimental samples.

	NEW Sequence (5'->3')	Length	Start	Stop	Tm	Gc%	Self comp	Self 3'comp	*Location (intron spanning)*	*Product length*
OxtR NM_012871.2	***F*** ACGGAGTCTTCGATTGCTGG	20	545	564	60.11	55.00	5.00	0.00	Exon 1	92
	R CGGTACAATGTAGACGGCGA	20	636	617	59.90	55.00	7.00	2.00		
	Products on potentially unintended templates									
	NA									
Prl NM_012629.1	***F*** TGAGGAACAAAACAAGCGGC	20	357	376	59.62	50.00	3.00	3.00	N/A	116
	R CTTGCAGGGATGGGAGTTGT	20	472	453	59.96	55.00	4.00	0.00		
	Products on potentially unintended templates									
	NA									
Avp NM_016992.2	***F*** ATGCTCGCCATGATGCTCAA	20	21	40	60.46	50.00	5.00	2.00	Exon 2/3	89
	R GGGCAGTTCTGGAAGTAGCA	20	109	90	59.68	55.00	4.00	2.00		
	Products on potentially unintended templates									
	NA									
Oxt NM_012996.3	***F*** CGGTGGATCTCGGACTGAAC	20	8	27	60.18	60.00	4.00	0.00	Exon 1-3	424
	R ATCATCACAAAGCGGGCTCA	20	431	412	60.04	50.00	4.00	1.00		
	Products on potentially unintended templates									
	NA									
Hcrtr1 NM_013064.1	***F*** AGGTGGATGGAAGCGTGAAG	20	57	76	60.04	55.00	2.00	1.00	Exon 1-3	630
	R AGAGATAATCGCGCCACAGG	20	686	667	59.97	55.00	5.00	1.00		
	Products on potentially unintended templates									
	NA									
Hcrtr2 NM_013064.1	***F*** CGCAACTGGTCATCTGCTTC	20	96	116	59.55	55.00	4.00	1.00	Exon 1-5	862
	R TTCGTGCTCGGATCTGCTTT	20	957	938	60.04	50.00	4.00	1.00		
	Products on potentially unintended templates									
	NA									
nucb2 NM_013064.1	***F*** CGCCAGACACGGGACTTTAT	20	353	372	60.11	55.00	4.00	2.00	Exon 4-10	714
	R CCTCTGCATTCTGCGGGTTA	20	1066	1047	60.11	55.00	4.00	2.00		
	Products on potentially unintended templates									
	NA									
Actb NM_031144.3	***F*** CGCCAGACACGGGACTTTAT	20	17	36	60.04	55.00	4.00	0.00	Exon 1-4	481
	R CCTCTGCATTCTGCGGGTTA	20	497	478	60.04	55.00	5.00	0.00		
	Products on potentially unintended templates									
	NA									
Pgk1 NM_053291.3	***F*** AGCCAAGTCGGTTGTGCTTA	20	166	185	59.89	50.00	4.00	2.00	Exon 3-6	484
	R AGCTCCTCCCAAGATAGCCA	20	649	630	60.03	55.00	4.00	2.00		
	Products on potentially unintended templates									
	NA									

Table 8. New primer sequences.

Chapter 6 : References

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