ABSTRACT

CHARACTERIZATION OF THE POTENTIAL ROLE OF LUMAN AS A NOVEL REGULATOR OF ANIMAL STRESS RESPONSES

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This thesis is an investigation of a knockout (KO) mouse line of Luman/CREB3. Luman KO mice are viable and survive into adulthood, however, the Mendelian distribution is skewed, in that less than 5% of pups born from heterozygous (het) breeding pairs expressed the KO genotype. The Luman-deficient mice are smaller in size, have less visceral fat, low fertility and exhibit a maternal defect, characterized by a low rate of pup survival. Through further characterization of the mice I determined that when Luman is deficient, the mice exhibit a blunted stress response, characterized by low levels of both anxiety and depressive-like behaviour, in addition to low glucocorticoid (GC) levels and increased glucocorticoid receptor (GR) expression. Resulting from the change in GC/GR levels, LUMAN alters the GR response leading to an increase in activity and dysregulation in the central stress response system, the hypothalamic pituitary adrenal (HPA) axis. These observations lead me to two hypotheses: (1) deficiency of LUMAN increases GR expression, resulting in increased negative feedback in the HPA axis, leading to low GC secretion, and (2) when LUMAN is deficient the secretion of GCs is insufficient leading to a compensatory increase in GR during development which persists through life. While these possibilities are not mutually exclusive I designed experiments to dissect the mechanism of action. I have shown that LUMAN binds to GR through the LxxLL motif, and through this interaction activates GR in the absence of ligand. Once activated, LUMAN binds to the promoter region of genes containing glucocorticoid...
response elements (GREs) through the DNA binding domain (DBD), increasing the activity of GR. These results suggest that LUMAN alters GR expression, through acting as a transcription factor and a cofactor. On the other hand, in the absence of LUMAN basal secretion levels are not altered, however, the cells are more sensitive to cellular stress when LUMAN is absent. These data suggest that both mechanisms may play a part in how LUMAN regulates GR. I postulate that LUMAN alters HPA axis reactivity through increasing GR expression and decreasing GC secretion, leading to a blunted response to stress.
Acknowledgements

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Declaration of work performed

All work reported in this thesis was performed by me under the supervision of Dr. Ray Lu and my advisory committee with the following exceptions: The growth curve and mammary gland slides and scoring were completed by Drs. Minghua Zeng and Pat Turner. Sholl analysis was performed by Ari Mendell, co-immunoprecipitation experiments were performed by Tiegh Taylor and behavioural assays were completed by myself as well as Khoa Tran with the help of Jennifer Lymer for training and analysis.
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List of abbreviations

ACSS2    Acyl-CoA Synthetase Short-Chain Family Member 2
ACTH     Adenocorticotropic Releasing Hormone
AR       Androgen Receptor
ARE      Androgen Response Element
ARF      Adenosine Diphosphate-ribosylation factor
ATF4     Activating Transcription Factor 4
ATF6     Activating Transcription Factor 6
BAD      Bipolar Affective Disorder
BCL2     B-cell lymphoma 2
BDNF     Brain Derived Neurotrophic Factor
BFA      Brefeldin A
BiP      Binding Protein
bZIP     Basic Leucine Zipper Domain
CAG      Trinucleotide Repeats
CA       Catecholamine
cAMP     Cyclic Adenosine Monophosphate
CGN      Cis Golgi Network
CHOP     C/EBP homologous protein
CNS      Central Nervous System
COP      Coat protein complex
CORT     Corticosterone
CRE      Creb Response Element
CREB3    CAMP Responsive Element Binding Protein 3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Cytochrome P450 Family 3 Subfamily A Member 4</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>DDC</td>
<td>Dopamine Decarboxylase</td>
</tr>
<tr>
<td>DEX</td>
<td>DEXamethasone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA Methyl Transferase</td>
</tr>
<tr>
<td>ER (α and β)</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum Associated Degradation</td>
</tr>
<tr>
<td>ERGIC</td>
<td>Endoplasmic Reticulum Golgi Intermediate Complex</td>
</tr>
<tr>
<td>ERES</td>
<td>Endoplasmic Reticulum Exit Sites</td>
</tr>
<tr>
<td>ERSE</td>
<td>ER stress response element</td>
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<td>ERO-1α</td>
<td>ER oxidoreductin 1</td>
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<td>Tarcrolimus</td>
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<td>FKBP5</td>
<td>FK506 Binding Protein 5</td>
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<td>FOXO1</td>
<td>Forkhead Box O1</td>
</tr>
<tr>
<td>GADD34</td>
<td>growth arrest and DNA damage-inducible protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GCs</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GLuc</td>
<td>Gaussia Luciferase</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid Response Element</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GRP94</td>
<td>Glucose Regulated Protein 94</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyl Transferase</td>
</tr>
<tr>
<td>HCF-1</td>
<td>Host Cell Factor 1</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Acetylase</td>
</tr>
<tr>
<td>Herp</td>
<td>Homocysteine-induced endoplasmic reticulum protein</td>
</tr>
<tr>
<td>Het</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic Pituitary Adrenal Axis</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>I.P</td>
<td>intraperitoneal injection</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Inositol-requiring Enzyme 1α</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun amino-terminal kinases</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand Binding Domain</td>
</tr>
<tr>
<td>LRF</td>
<td>Luman Recruiting Factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid Receptor</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCOA</td>
<td>Nuclear Receptor Coactivator</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear Receptor</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear factor like 2</td>
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</table>
NTD  N-Terminal Transactivation Domain
ORF  Open Reading Frame
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
Per 1  Period Circadian Clock 1
PERK  protein kinase RNA-like endoplasmic reticulum kinase
PFA  Paraformaldehyde
PNMT  Phenylethanolamine N-Methyltransferase
PR (PRA and PRB)  Progesterone Receptor
PTSD  Post-Traumatic Stress Disorder
RIP  Regulated Intramembrane Proteolysis
RIP140  Nuclear Receptor Interacting Protein 1
RNA  Ribonucleic acid
RT PCR  Reverse Transcription Polymerase Chain Reaction
S1P/S2P  Sphingosine-1/2-phosphate
SDS  Sodium dodecyl sulfate
SEC  Secretory Protein
SIRT1  Sirtuin 1
SLC2A1  Solute Carrier Family 2 Member 1
SNS  Sympathetic Nervous System
SPGCs  Secretory Protein Gene Components
SRC  Steroid Receptor Coactivator
SRP  Signal Recognition Particle
SQLE  Squalene epoxidase
TA  Transactivation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Tg</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>Tm</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-Box Binding Protein 1</td>
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Chapter 1: Literature Review

1.1 Hypothalamic Pituitary Axis and Stress

Survival of an organism is determined by maintaining a dynamic equilibrium with its internal and external environment, referred to as homeostasis. This balance exists at all levels of organization including molecular, cellular, physiological and behavioural levels. Stress is a threat to this balance. However, there are mechanisms in place in order to maintain homeostasis through altering physiological processes and behavior (O'Connor et al., 2000). The primary endocrine component of the stress response involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis. This process involves a neuroendocrine cascade leading to the synthesis and secretion of glucocorticoids (GCs), which bind to, and activate the glucocorticoid receptor (GR). Within this cascade, one of the key effectors is corticotropin releasing hormone (CRH), which acts on the pituitary to elicit the release of adenocorticotropin releasing hormone (ACTH), which subsequently acts on the adrenal glands to cause the release of GCs (McEwen and Magarinos, 1997; Pariante and Lightman, 2008; Rivier et al., 1983). GCs then exert negative feedback on this cascade reducing CRH and ACTH secretion to bring the system back to a homeostatic equilibrium. GCs modulate the stress response at the molecular level by altering gene expression, transcription, and translation of target genes (O'Connor et al., 2000).
Figure 1: The hypothalamic pituitary adrenal (HPA) axis. The onset of stress stimulates corticotrophin releasing hormone (CRH/CRF) secretion from the hypothalamus which acts on the pituitary to cause the release of adenocorticotropin releasing hormone (ACTH). Elevated levels of circulating ACTH triggers the synthesis and release of glucocorticoids (GCs) from the adrenal glands, which act on the hypothalamus and pituitary in a negative feedback loop. Reprinted from Nature Neuroscience. (Hyman, 2009 from Nature Neuroscience, Retrieved February 21 2017).
GC levels fluctuate throughout the day even without stress events, due to circadian rhythm. This fluctuation generally triggers the activation of the type -1 high affinity receptor, the mineralocorticoid receptor (MR), predominantly located in the hippocampus. During situations which elicit a stress response, GCs are elevated and bind to GR, which has a lower affinity for GCs and is more widely expressed through many tissue types, when compared to MR (Liberzon et al., 1999). GR belongs to a highly conserved superfamily of nuclear hormone receptors. The binding of GCs to the receptor leads to the dissociation of heat shock proteins, which induces a conformational change in turn normally leading to nuclear translocation of the receptor (O'Connor et al., 2000); however, this is not always the case. There is ligand-independent activation of GR that occurs, for example some β2-adrenergic receptor agonists induce nuclear translocation (Eickelberg et al., 1999), hormone-independent phosphorylation of the Ser134 site has also been shown to activate GR (Galliher-Beckley et al., 2011). In the nucleus the GR DNA binding domain binds to specific genes that contain glucocorticoid response elements (GREs) which leads to either activation or repression of the target gene.

It has been well established that GCs exert negative feedback on the HPA activity at various levels including hypothalamic and pituitary activity in addition to the hippocampus; disruption of this feedback can have major effects on the stress response (Pariante and Lightman, 2008; Pariante and Miller, 2001). Major depressive disorder is characterized by persistent feeling of sadness or a lack of interest in outside stimuli, affecting approximately 20% of women and 10% of men in the United States (Pariante and Lightman, 2008). GC-mediated feedback inhibition has been shown in many studies to be impaired in people suffering from depression. A multitude of studies have utilized a Dexamethasone (DEX) suppression test to assess HPA axis function; where DEX is a power GC agonist. In healthy individuals, a potent feedback inhibition
is observed, showing a drastic decrease in circulating cortisol levels, whereas in depressed
patients this feedback inhibition is impaired, showing a less drastic decrease in circulating
cortisol levels (Pariante and Lightman, 2008). Interestingly many successful treatments for
depression involve resolving the impairment of the negative feedback in the HPA axis (Spijker
and van Rossum, 2012). A properly functioning HPA axis is required for everyday activities in
addition to many aspects of pregnancy, including parturition and lactation and the maternal care
response (Windle et al., 1997). Pregnancy exerts a large stress on the body, including the
neuroendocrine system. Therefore, during late pregnancy and the postpartum period, the HPA
responses to stress are attenuated to prevent premature labor, which can be initiated by a rise in
GCs. This attenuation also allows the mother to properly care for her young. It has been shown
that prolonged activation of the HPA axis can contribute to poor maternal care (Catalani et al.,
2011). However, the mechanism by which this occurs is unclear.

During stressful situations the activation of GR plays a primary role in initiating downstream
signaling events. For example, GCs inhibit cytokine production through altering the stability of
cytokine mRNA, which in turn has inhibitory effects on the immune response during stress
(O'Connor et al., 2000). In the brain, GCs help regulate neurogenesis and neuron survival, which
affect the size and function of complex structures such as the hippocampus. Alterations in
hippocampal structure can affect a person’s ability to acquire new memories as well as the
manner in which they appraise emotional events (Fitzsimons et al., 2013). Alterations in HPA
axis function can affect day-to-day living but is notably detrimental during pregnancy, including
excessive exposure to GCs leading to low birth weight and shorter gestation, as well as long-term
effects of neurodevelopmental and cardiometabolic problems in the offspring (Duthie and
Reynolds, 2013). A hypo- or hyper- active HPA axis has also been implicated in a number of
psychological disorders including major depressive disorder, postpartum depression, post-traumatic stress disorder (PTSD) (Yehuda et al., 2015; Yehuda et al., 2004a; Yehuda et al., 2004b) and bipolar affective disorder (BAD) (Watson et al., 2004); however it is important to note that it is unclear if altered HPA axis function is a cause of the disorder or if it is a symptom. Moreover, despite the prevailing hypothesis that excess GCs are responsible for the adverse physiological effects of prolonged stress, there is growing evidence suggesting that low GC levels are also detrimental. In the long-term stress response, GCs exert negative feedback to constrain the processes to which they initially activate, such as activation of the sympathetic nervous system (SNS) and stimulation of CRH pathways. Therefore, conditions that are characterized by prolonged activation of the stress response might be more likely to lead to a pathological outcome when GC signaling is insufficient, which would impair the negative feedback system (Diamond et al., 1992; Salehi et al., 2010). In addition to the dysregulation of the HPA axis, recent research has focused on looking for a genetic basis for some of the major stress related disorders, particularly genes that might predispose an individual to develop a disorder.

1.2 Nuclear Receptors

Nuclear receptors (NR) are a family of ligand-regulated transcription factors activated by steroid hormones and various other lipid-soluble signals (Huang et al., 2010). NRs contain three main functional domains; the carboxy-terminal binds the ligand, the DNA binding domain and an N-terminal transactivation domain. In the absence of ligand, an inhibitory complex often associates with the ligand-binding domain. When the ligand binds it causes a conformational change so that the inhibitory complex dissociates (Labeur and Holsboer, 2010). This dissociation allows the receptor to travel to the nucleus, bind to DNA, and associate with the co-
regulator protein complex (Fig. 2). However, NRs generally exist in both the cytoplasm and nucleus, this equilibrium is shifted towards nuclear localization when a ligand binds the receptor. Genes that are regulated by NRs contain particular DNA sequences called response elements in their promoter regions, where the NR binds (Osguthorpe and Hagler, 2011). Although there are many different types of NRs serving a variety of functions, this basic mechanism is conserved among them. Ligand independent activation of some nuclear receptors has been characterized and can be induced by modulation of kinase/phosphatase activity, including the estrogen receptor (ER), progesterone receptor (PR) and androgen receptors (AR), while GR remains resistant to ligand independent activation.

**Figure 2: The action of steroid hormone receptors.** The steroid receptor remains in the cytoplasm, inactive, until a ligand (hormone) binds and activates it, translocating the receptor complex to the nuclear where is binds to specific response elements altering gene transcription. Image reprinted from Purves et al., Life: The Science of Biology, 4th Edition.
The activation of NRs is often modulated by various cofactors that can bind directly to the receptor, often recruiting other factors to modulate its activity. These factors can either increase (co-activator) or decrease (co-repressor) the activity of the NR it interacts with (Dasgupta et al., 2014). This interaction is often facilitated through a consensus peptide sequence LxxLL (L is leucine and X is any amino acid) in a region called the NR box, which binds to the groove in steroid hormone receptors (Darimont et al., 1998). The groove is a hydrophobic pocket generated by the carboxyl terminal part of H3, the loop L3-4 and H4 (Fig.3) (Bourguet et al., 2000). Cofactors will be discussed in more detail in future sections.
Figure 3: Structure of Nuclear Receptors and their interaction with co-factors. Each member of the class 3 steroid nuclear receptors form homodimers and interact with various co-factors that contain an LxxLL binding motif. SRC (Steroid Receptor Cofactor), AF-2 (Activating factor 2), CBP (Creb binding protein). (Figure adapted from Perissi & Rosenfeld, 2005 from Nature reviews).
1.3 The glucocorticoid receptor

GR is a member of the class 3 steroid nuclear receptors which also includes the androgen receptor (AR), the progesterone receptor (PR) and the estrogen receptor (ER). GCs are steroid hormones regulated in a circadian and stress-associated manner to maintain various metabolic and homeostatic functions that are necessary for life. As previously discussed GCs bind to two main types of nuclear receptors in the brain, GR and MR (Labeur and Holsboer, 2010). MR has a higher affinity for GCs and is more sensitive to circadian changes when compared to GR, which is alternatively more responsive to the increase in GC levels induced by stress (Gaeggeler et al., 2005). GR is composed of three major domains: an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand binding domain (LBD) (Kumar and Thompson, 2005). The DBD and LBD are separated by a flexible region often referred to as the hinge region; this region also contains two nuclear localization signals. The DBD is the most conserved domain in all nuclear receptors, containing two zinc finger motifs that recognize and bind target genes containing GREs (Kumar and Thompson, 2005). The NTD interacts with co-factors and basal transcription machinery and is the primary site for post-translational modifications. The LBD, forms a hydrophobic pocket used in binding GCs, and is also involved with the interaction of co-factors (Bledsoe et al., 2002). In the absence of the ligand, GR is inactive and resides predominantly in the cytoplasm as part of a complex including chaperone proteins (hsp90, hsp70, and p23) and immunophilins of the FK506 family (FKBP51 and FKBP52) (Grad and Picard, 2007). MR has a similar structure to the other members of the nuclear receptor superfamily explained above. The NTD is a region that mediates ligand-independent interactions of the receptor with other nuclear proteins that initiate transcription of target genes.
While GR exerts many of its effects through its action as a transcription factor, this is not the only mechanism through which GR works. In order to bind to GRE, GR forms homodimers, which allows it to bind to the palindromic GRE sequence (Reichardt et al., 1998). GR Knockout (KO) mice die within minutes of birth due to lack of lung maturation (Bird et al., 2015). However, GR $^{\text{dim/dim}}$ mice have a mutation inhibiting the ability of GR to homodimerize, resulting in defective DNA binding and transcriptional activation, and these mice are viable, suggesting alternative mechanisms to DNA binding are at play; otherwise the GR $^{\text{dim/dim}}$ would not survive (Reichardt et al., 1998). It has been shown that many of the repressive effects of GR are mediated through protein-protein interactions with other transcription factors, including the overall repression of the immune response (Quax et al., 2013).

One possible explanation for the previously discussed differential effects of GCs may be due to the dose-response curve having an inverted U shape, where both low and high levels appear to give similar results (Baldi and Bucherelli, 2005; Joels et al., 2006). In this system low levels of GCs or GR activation are required for many processes occurring in the body, including neurogenesis, in contrast high levels of activation can be detrimental for example reducing the growth and survival of newborn neurons. Furthermore, when GC levels are low the effects are predominantly mediated through the MR, whereas when GC levels are high the effects are mediated through GR. This paradoxical relationship is also seen in mouse hippocampal neural stem cells where both high and low levels of DEX, a GR agonist, induce pro-apoptotic caspase-3 activity (Yu et al., 2011). Hippocampal cell survival is also directly influenced by GC levels, showing the same inverted U shape relationship (Fitzsimons et al., 2013).
1.4 The Androgen Receptor (AR)

AR is part of the same group of class 3 steroid nuclear receptors as GR, resulting in a similar structure and mechanism of action, with the exception of a few minor differences. The expression of AR is widespread and therefore it has a number of diverse biological functions including important roles in reproduction, immune response and neural systems. AR mediates the actions of androgens, such as testosterone, in a ligand-dependent manner (Culig et al., 1998). Additionally, AR can exert its effects in a ligand independent manner, likely through variation in phosphorylation, chaperone proteins and cofactors (Weigel and Zhang, 1998). AR can exert its effects in both a DNA binding-dependent manner, regulating target gene transcription, and a DNA-binding independent manner, initiating signal cascades through intermediate messenger molecules (Dehm and Tindall, 2007). Lending to its widespread affects, knocking out the AR results in a number of effects including reduced bone thickness, absence of male sexual and aggressive behavior, reduced heart size, increased fat storage, as well as a lack of prostate gland development (Yeh et al., 2002).

The AR gene is highly conserved among most vertebrate species; the first exon codes for the NTD that encodes the transcriptional regulatory region, exons 2 and 3 code for the most highly conserved region the DBD, while exons 4 to 8 code for the LBD (Davey and Grossmann, 2016). Interestingly the first exon contains several regions of CAG repeats; the length and location of these repeats varies among species and is believed to have developmental and behavioural implications for more advanced species (Choong and Wilson, 1998). As a result of these CAG repeats, the length of this region is highly polymorphic within the population due to the slippage of DNA polymerase resulting in variability in the final number of CAG repeats. There are multiple genes that contain the CAG triplet repeat. When the length of the CAG repeat is increased substantially, this can result in neuromuscular degenerative diseases (Choong and
Wilson, 1998). Specifically for AR, an extension of CAG repeat length from 40-62 repeats often results in the development a neuromuscular condition called Kennedy’s disease (La Spada et al., 1991). This condition is characterized by progressive neurological impairment caused by degeneration of spinal motor neurons and muscle wasting in adults over 40 years of age (Kouroku et al., 2000; Rubinsztein et al., 1995). The neurological symptoms are caused by the AR sequences containing the excessively long CAG repeats inducing apoptosis in neurons (Rubinsztein et al., 1995). AR has many biological functions that vary based on tissue type, control by other nuclear receptors, in addition to having age dependent variations in AR transcription (Culig et al., 1998).

The AR promoter contains DNA binding sites recognized by AR, GR and the progesterone receptor and therefore AR action is, to some degree, regulated by AR negative feedback mechanisms that control the transcription of the AR gene itself (Davey and Grossmann, 2016). Similar to many NRs, when AR is inactive it normally resides in the cytoplasm sequestered by binding to various chaperone proteins. When the ligand testosterone binds to AR it causes a conformational change, releasing the chaperone proteins which allow it to be translocated to the nucleus. Regulation of AR action occurs, in large part, by post-translational modifications such as phosphorylation (Moudgil, 1990). This is a common point of regulation for many of the steroid hormone nuclear receptors; however, phosphorylation occurs under different conditions and for different reasons depending on the receptor in question. For example, the phosphorylation of the progesterone receptor is dependent on progesterone levels and is important for the transcriptional activation of the receptor (Denner et al., 1990a; Denner et al., 1990b). The phosphorylation of GR is not required for activation, despite the fact the receptor contains multiple phosphorylation sites. On the other hand, AR is phosphorylated at
multiple sites; however, not all are necessary for activation and the significance of each site is not fully understood (Culig et al., 1998). Many hormone receptors have been shown to form homodimers in solution, however the formation of homodimers has not been observed with AR in the absence of DNA (Sack et al., 2001; Wong et al., 1993). A large number of naturally occurring mutations in the Human AR gene have provided important information about its molecular structure and mechanism, which has been briefly discussed above, as well as intermolecular interactions, some of which will be discussed in an upcoming section (1.7).

1.5 The Progesterone Receptor (PR)

PR is another member of the nuclear steroid hormone receptors and the basic mechanism of action and general structure is similar to that described in the above sections. Briefly, PR is a ligand dependent transcription factor; in response to the binding of progesterone, it dissociates from its cytoplasmic complex, translocates to the nucleus and binds to target genes altering the rate of transcription (Denner et al., 1990b). PR is expressed primarily in female reproductive tissues as well as the central nervous system, where it regulates gene expression in order to control development, differentiation and proliferation (Li et al., 2003). There are two PR protein isoforms that are coded for by the same gene through the use of two different promoters. The PRA isoform contains a truncated NTD compared to PRB, interestingly these isoforms exhibit distinct transcriptional regulation through targeting different subsets of genes (Grimm et al., 2016). PRA is primarily located in the nucleus, whereas PRB is continuously moving between the nucleus and cytoplasm. Altered PR expression is often found in breast cancer cells, where altering the ratio of PRA/PRB is generally associated with poor prognosis (Grimm et al., 2016). In addition to its role in cancer, PR plays a central role in the reproductive events associated with pregnancy establishment and maintenance. Knockout studies have shown that in the absence of
PRA severe abnormalities in ovarian and uterine function are found, leading to infertility, while responses of the mammary glands and thymus remain unaltered (Gadkar-Sable et al., 2005). Knockout of PRB results in reduced mammary ductal morphogenesis, while leaving the ovarian, uterine or thymic responses to progesterone intact. Thus, it is evident that PRA is both necessary and sufficient to elicit the reproductive responses required for fertility in females; while PRB is responsible for normal proliferative responses of the mammary glands to progesterone (Conneely et al., 2002; Gadkar-Sable et al., 2005).

1.6 The Estrogen Receptor

There are two classic receptors that mediate the response elicited by 17β-estradiol, estrogen receptor α (ERα) and estrogen receptor β (ERβ). Both receptors are encoded by unique genes, but still possess the hallmark molecular structure and function characteristics of steroid hormone nuclear receptors. Estrogens bind to the estrogen receptor forming complexes that act as transcription factors and bind genes containing estrogen response elements (EREs) (Sheppard et al., 2017). These are classical effects and typically occur within a few hours-days of activation. There are also non-classical effects that begin within minutes of activation and are the result alternative estrogen receptor activation through G protein-coupled estrogen receptor 1 (ER1) (GPER1, previously GPR30), or Gq-coupled membrane estrogen receptor (Gq-mER). Activation of these receptors trigger signaling cascades affecting cell function through altering local protein synthesis, post-translational protein modifications, or downstream gene regulation (Choleris et al., 2008; Ervin et al., 2015; Galea et al., 2017; Laredo et al., 2014; Luine et al., 2003). Importantly, classical and non-classical mechanisms are not mutually exclusive and products from the activation of both can regulate each mechanism.
ERα and ERβ receptors exhibit a high degree of homology in the DBD and LBD while showing very low homology in the N-terminal portion (Hall et al., 2001). This would indicate that ERα and ERβ interact with the same DNA response elements and exhibit similar ligand binding properties. Tissue localization studies have revealed that each isoform has distinct expression patterns. ERα is predominately expressed in the breast, uterus, cervix and vagina; whereas ERβ exhibits a more widespread expression pattern being found primarily in the ovary, prostate, testis, spleen, lungs and certain parts of the brain (Hall et al., 2001). ERα knockout mice were found to be insensitive to estrogen in the reproductive tract leading to lack of mammary gland development, hypergonadotropic hypergonadism and excess fat accumulation, while in male mice testicular degeneration and epididymal dysfunction were observed, leading to infertility in both sexes. In the ERβ KO mouse model, males show no distinct phenotype and are fertile; however, a neuronal deficit in the cerebral cortex of both male and female ERβ KO mice has been found. This deficit has been shown to be, in part, as a result of disturbances during development due to abnormal neuronal migration and increased level of apoptotic neuronal death (Wang et al., 2003). Additionally, female ERβ KO exhibit inefficient ovarian function resulting in lowered fertility (Emmen and Korach, 2003).

1.7 Nuclear Receptor Co-regulators

NR co-regulators are required by NRs for efficient transcriptional regulation. Broadly, coactivators are defined as molecules that interact with nuclear receptors and enhance their transactivation, whereas co-repressors (NCoRs) lower the transcription rate of their target genes (Darimont et al., 1998). Most co-regulators are rate limiting for NR activity, but do not significantly alter basal transcription. Historically, co-regulators have been placed into either the category of co-activator or co-repressor, but there is now some disagreement over this
classification (Hermanson et al., 2002). For instance, RIP140 appeared to be an inverse co-regulator, in that it interacts with ligand-bound ER-alpha yet represses transcription. Subsequent studies have shown that RIP40 has both co-activator and co-repressor properties, which allow it to function as a central regulator in development, inflammation and metabolism (Tazawa et al., 2003).

Co-regulators occupy the driving seat for actions of all NRs, and consequently, are viable targets for selective receptor modulator drugs. Numerous transcriptional co-regulators are known to alter the maximal transcriptional activity in gene induction and repression, by steroid receptors in general and glucocorticoid receptors in particular (Leonardsson et al., 2004). Recent data has also shown these factors modify other parameters of GR transcriptional activity; such as the potency of agonists and the partial agonist activity of anti-steroids. Thus, studying these various parameters could reveal new therapeutic targets at various stages of receptor action for adjusting GR actions in pathological situations. As previously mentioned the LxxLL binding motif is also referred to as the NR box, and it is necessary and sufficient to mediate binding of various cofactors to NRs.

Steroid receptor coactivators (SRCs) were one of the first gene families to be discovered and characterized as coactivators for NRs (NCoA). There are three main members of this gene family; SRC-1 (NCOA1), SRC-2 (NCOA2, GRIP1) and SRC-3 (NCOA3, ACTR, RAC3, TRAM-1) (Tetel and Acharya, 2013). All three genes contain homologous domains and share sequence similarities of around 50%, while the expression pattern varies among the three genes. Each member of the SRC family enhances the transcriptional activity of NRs through assisting with protein-protein interactions between the NR and other co-regulators, in addition to facilitating assembly of transcriptome complexes on the promoter of target genes (Leo and Chen,
2000). There are three distinct structural domains that are normally present in NR cofactors, including the N-terminal basic helix-loop-helix, the central NR interacting domain, which contains 3 LxxLL motifs and two C-terminal activation domains. The N terminal domain is the most conserved among the three SRC family members and it can interact with various transcription factors including STAT6 and p53 among others (Leo and Chen, 2000). The three LxxLL motifs form amphipathic alpha helices and are involved in the direct interaction between the ligand and the ligand binding domain of the NR (Fig. 3). The C-terminal activation domain recruits additional co-regulators to alter transcriptional regulation, in addition to chromatin remodeling of the promoter region of specific genes (Tetel and Acharya, 2013).

Chromatin remodeling is a dynamic process that allows the condensed DNA to be accessed by transcription machinery which alters gene expression. Here, we enter the field of epigenetics and will briefly discuss its role in NR signaling. Epigenetic modifications lead to alteration in NR gene expression. DNA methylation is one of these modifications that results in decreased gene expression (Green and Han, 2011). Methylation is the most abundant epigenetic modification that consists of the addition of a methyl group on the carbon 5 of the cytosine, creating 5-methylcytosine (5mC) acting to repress transcription (Auclair and Weber, 2012). This repression occurs through either preventing the binding of certain transcription factors, or by recruiting methyl-binding proteins (MBPs) and generating a repressed chromatin environment (Auclair and Weber, 2012). DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from S-adenosyl-l-methionine to cytosine. There are three active DNMTs: DNMT1, DNMT3A and DNMT3B. DNMT1 has a preference for hemi-methylated CpG sites and therefore is primarily responsible for copying pre-existing methylation patterns to newly synthesized DNA (Legube and Trouche, 2003). DNMT3A/B do not show a preference for hemi-
methylated CpG sites and are therefore mainly responsible for establishing methylation patterns during early development. Studies have shown that a number of mental disorders, such as depression, are characterized by an increase in methylation of the GR promoter leading to reduced GR expression (Palma-Gudiel et al., 2015).

Another epigenetic mechanism that alters gene expression is histone acetylation, it occurs on specific lysine residues which create a neutral charge that causes a loosening of the DNA-histone interactions, permitting binding of transcription machinery (Georgiakaki et al., 2006; Spencer et al., 1997). Histone acetyltransferases (HATs) catalyze the transfer of an acetyl group to lysine from acetyl-coenzyme A. The previously discussed SRC family belong to this group. Conversely the acetyl group can be removed from lysine by histone deacetyltransferases (HDACs), and many NR corepressor complexes belong to this group (Spencer et al., 1997). Histone deacetylation is mostly associated with gene silencing through corepressors and HDACs have been found to bind to genes that are primed for activation, suggesting they are involved in maintaining genes that are ready for rapid activation (Georgiakaki et al., 2006). Many of the functions of the SRC family members were discovered to play key roles in fertility, cancer, metabolism and other fundamental processes (Dasgupta et al., 2014).

Alterations in the cellular concentration of a co-regulator can cause genetic dysfunction which usually leads to a pathologic outcome. For example, many cancers overexpress co-activators, such as SRC1-3, and are associated with growth, proliferation and metastasis of cancer cells (Dasgupta et al., 2014). SRC1 and 3 are often overexpressed in endocrine-related cancers including breast, prostate and ovarian cancers. At the molecular level, SRC-1 promotes metastasis from breast to lung by upregulating the expression of factors such as Twist and polyoma enhancer activator 1, which promote growth, migration and invasion. Overexpression
of SRC-1 is correlated with poor survival in breast cancer patients (Leo and Chen, 2000). More
NR co-regulators are over- rather than under-expressed in cancers; in leukemia and lymphoma
nearly all of the co-activators that have been identified have altered expression (Anzick et al.,
1997; Leo and Chen, 2000). Taking this into account, it is possible that inhibition of an
oncogenic co-activator, such as SRC-1 and SRC-3, could simultaneously silence downstream
target genes; which, together may be responsible for the accelerated growth of the oncogenic
cell. Alterations in NR co-regulators, in particular the ones that interact with GR, can alter the
stress response. Considering the vast role that the stress response system plays in day-to-day life,
changes in the concentration of these cofactors of GR could alter many important biological
functions and potentially contribute to pathological outcomes.

1.8 Stress Resilience

As classically defined, stress is a condition that seriously alters the physiological and/or
psychological balance of an individual (McEwen and Magarinos, 1997). Stress can precipitate
the onset of a number of disorders, including major depressive disorder, anxiety and
posttraumatic stress disorder; however, not everyone responds to stress in the same way
(Franklin et al., 2012). Some individuals adapt poorly to stress, expressing an inappropriate
response which can lead to a persistent stressful state; while others are resilient to stress and
develop an adaptive response. The mechanism behind the differential responses between
individuals is known to be a result of both genetic and non-genetic factors that interact in a
complex manner, however, the exact mechanisms have yet to be elucidated.

There are two types of stress-coping strategies, passive and active. Coping is considered
active when an individual deals with the challenge that is causing the stress, whereas passive
coping involves denial and avoidance of the stressor (Franklin et al., 2012). The passive coping
strategy is maladaptive and plays a central role in determining if a stress-related disorder will
develop. Variations in coping styles can influence how the neuroendocrine and immune systems
are activated in response to stress. The biological basis of stress sensitivity is not fully
understood and further investigation is essential to allow for a more comprehensive approach in
treating and preventing disorders precipitated by stress (Pfau and Russo, 2015). Animal models
are most often used in this research, as rodents can express both active and passive coping
mechanisms, and these behaviours can be reliably measured (Franklin et al., 2012).

There are mouse models used to investigate the stress response, including GR knockout
and overexpression mice. These mice are also used to examine specific disorders such as
depression and PTSD. Whole body GR knockout is embryonic lethal, and therefore studies use
alternative expression levels and mutations to study GR function regarding stress sensitivity. GR
heterozygous (GR\(^{+/−}\)) mice show a 50% GR gene dose reduction and exhibit increased
helplessness after exposure to stress, a behaviour which is correlated with depressive-like
symptoms (Ridder et al., 2005). Additionally, the GR\(^{+/−}\) mice have an altered HPA axis response
to the DEX suppression test, which is a manifestation of depression in humans, along with lower
levels of brain derived neurotrophic factor (BDNF). When looking at GR overexpression mice
reduced helplessness was observed along with enhanced feedback regulation in the HPA axis
(Ridder et al., 2005). Therefore the GR\(^{+/−}\) mice may represent a mouse model for depression,
while the GR overexpression mice may be a model for stress resilience. One common feature of
many stress-related disorders is the dysregulation of the HPA axis, often, but not always,
facilitated through altered GR expression.

The HPA axis has been strongly linked to variations in stress resilience and vulnerability.
Like the stress response, HPA axis activity is extremely variable both within and between
individuals (Pfau and Russo, 2015). A study using male rats showed that altered HPA axis activity, characterized by increased CRH secretion, are quicker to adopt a passive coping strategy (submissive posture) when compared to rats with a normal functioning HPA axis (Franklin et al., 2012). Furthermore male rats with reduced CRH secretion, leading to lower GC levels, show more proactive resisting behaviours. Additionally, depletion of CRH can attenuate social avoidance suggesting that it plays a critical role in stress sensitivity (Franklin et al., 2012). The development of the HPA axis is strongly influenced by external factors in early life, including maternal care. In mice, active maternal behaviours, such as licking and grooming, reduce the responsiveness of the HPA axis and sensitivity to stress; as well as decreasing CRH mRNA expression and increasing GR mRNA expression, while increasing GC feedback sensitivity (Franklin et al., 2012). This suggests that the level of maternal care an individual receives at an early age may, in part, underlie the inter-individual differences in HPA axis activity and stress sensitivity.

As discussed previously, alterations in GR expression can induce dramatic effects on an individual’s response to stress. However, it is not only the expression level of GR that can alter the stress response but also post translational modifications, as well as epigenetic factors (Duma et al., 2006). Studies have shown an increase in the level of site specific methylation of the GR promoter in the hippocampus of suicide victims that were abused as children, when compared to control individuals. This same study showed that the increase in methylation was accompanied by decreased levels of GR mRNA in the abuse victims (McGowan et al., 2009). GR also has multiple phosphorylation sites and some studies show that hyper-phosphorylation of GR can increase stress sensitivity (Bamberger et al., 1996; McGowan et al., 2009). Stress sensitivity is clearly a complex phenomenon, with much of the variation surrounding GR and molecules that
alter the GR pathway; however secretion of GCs and various neuropeptides can also play a large role in the variability of the stress response. Secretion of these factors is often most highly induced during stress, which requires a selective upregulation in the secretory capacity during specific cell types under stress. In order to examine this facet of the stress response, first it is important to understand how secretion works during basal conditions.

1.9 Cellular Secretion

The secretory pathway is comprised of the rough endoplasmic reticulum (ER), ER exit sites, the ER-to-Golgi intermediate compartment (ERGIC), the Golgi complex and post-Golgi carriers (Farhan and Rabouille, 2011). In certain cells, the secretion of a specific set of proteins is not continuous, but rather it relies on a stimulus to trigger exocytosis, this is referred to as regulated secretion; continuous secretion is termed constitutive secretion (Turner et al., 1992). Secretion occurs in all cells, ranging from relatively low levels in most cells to extremely high levels in specialized secretory cells, such as those of the pancreas, salivary, and mammary glands. The mechanism to selectively up-regulate secretory capacity in specialized secretory cells is unknown (Fox et al., 2010). In order to explore this phenomenon one first must examine the major components and events that occur in secretion.
Figure 4: The Cellular Secretory Pathway. Proteins are synthesized in the endoplasmic reticulum (ER) and exported at ER exit sites (ERESs). COPII vesicles form and fuse with the pre-formed ER-Golgi intermediate complex (ERGIC) making their way to the Golgi complex, where they are further modified, sorted and dispatched towards their correct final destinations or returned to the ER through COPI-coated vesicles. (Farhan and Rabouille, 2001, The Journal of Cell Science).
The first step in preparing most secretory proteins entails translocating the polypeptide into the ER. The ER is responsible for protein translocation, protein folding and protein post-translational modifications that allow proteins to move to the Golgi apparatus and ultimately to vesicles for secretion or display on the plasma surface (Barlowe and Miller, 2013). The co-translocation process occurs while the protein is still being translated by the ribosome and is initiated when a hydrophobic signal sequence or transmembrane sequence is translated and recognized by the signal-recognition particle (SRP) for targeting to a ER translocation site. In the case of post-translational translocation the protein is fully translated in the cytosol and is then transported into the ER. Chaperone proteins play a critical role in binding hydrophobic targeting signals to maintain the protein in an unfolded competent state until delivery to the ER membrane (Johnson et al., 2013). Once in the ER, protein maturation takes place; signal sequence processing, protein glycosylation and bond formation are some of the processes that occur. Additionally, chaperone proteins and foldases aid in the folding of proteins, while any misfolded proteins are disposed of through ER associated degradation (ERAD) (Barlowe and Miller, 2013).

ERES are defined as smooth projections of the ER that are coated with COPII coat components giving rise to COPII-coated vesicles. ERES are relatively stable and therefore likely to generate numerous COPII vesicles during their existence, these vesicles then move to the ERGIC (Szul and Sztul, 2011). The ERGIC is distinct in biochemical composition from the ER and the Golgi (Schweizer et al., 1990) and is not continuous with either the ER or the Golgi (Breuza et al., 2004). Individual ERGIC units appear to use rapidly forming and disappearing tubules to communicate with each other (Szul and Sztul, 2011). The ERGIC sorts proteins into ones that will be retained in the ERGIC, transported to the Golgi or transported back to the ER. These proteins are packaged into different transport vesicles depending on the target.
compartment. Transport vesicles are first created from a donor compartment. Once fully formed, the vesicles are then actively transported to, and subsequently fuse with, an acceptor compartment where they deliver the cargo. It is generally accepted that COPI vesicles are responsible for retrograde transport from the Golgi back to the ER (or a sub-compartment) and COPII vesicles are responsible for anterograde transport from the ER to the Golgi (Gomez-Navarro and Miller, 2016). The formation of both types of vesicles is mechanistically conserved among species. The COPII coat machinery consists of five cytosolic proteins: Sar1, Sec23, Sec24, Sec13 and Sec31. In cells, Sec23 and Sec24 are found in tight heterodimers, which form the inner COPII coat and are responsible for cargo selection. Sec13 and Sec31 are found in stable heterotetramers of two subunits of each, which form the outer COPII coat (Barlowe et al., 1994). Sar1, and these two stable complexes, are sequentially recruited to the ER membrane and form a complete COPII vesicle. Some ER resident proteins either require modifications that occur in the Golgi or they are transported accidentally and require retrograde transport back to the ER (Jensen and Schekman, 2011). These proteins contain a specific signal sequence called a KDEL sequence which targets the protein to the ER. The process of retrograde transport using COPI vesicles is very similar to anterograde transport however ARF is used instead of SAR1 to recruit COPI components, COPIα and COPIβ (Capitani and Sallese, 2009).
**Figure 5: COPII vesicle formation and fusion.** COPII coat assembly occurs by the stepwise deposition of Sar1-GTP, Sec23p-Sec24p, and Sec13p-Sec31p at ER exit sites (ERES). Sar1-GTP recruits the Sec23/Sec24p heterodimer, which forms the pre-budding complex. Sec13/Sec31p heterodimers polymerizes onto Sec23/Sec24p to form a mesh-like scaffold and crosslink the pre-budding complex. COPII vesicles then travel towards the Golgi where they fuse and the coat is removed. (Barrowman et al. 2003, from The Journal of Biological Chemistry).
The Golgi is morphologically distinct in different mammalian species. Generally, the Golgi is organized into a single ribbon of stacked cisternae. The Golgi complex is flanked on the cis-side by a tubular cis-Golgi network (CGN) and on the trans-side by the TGN. The CGN may consist of fused and concentrated transport intermediates approaching the Golgi from the ER. The TGN consists of tubular elements that are distinct from the trans-Golgi cisterna (Wei and Seemann, 2010). The TGN appears to be a complex compartment consisting of distinct subdomains that contain different coiled-coil proteins called golgins (Derby et al., 2004; Diao et al., 2008). From the TGN, transport intermediates can either traffic directly to the plasma membrane or can deliver cargo to the endosomal system or to secretory granules. Some cell types are specialized to sustain extremely high secretory loads such as pancreatic cells, cells of the salivary gland and some specific neuron types that secrete neuropeptides (Derby et al., 2004).

Cells that are specialized to sustain a high secretory demand must produce large amounts of peptides and balance that demand with a comparably high secretory capacity. Furthermore, the secretory products must be readily available to be released from the storage and they must recognize and respond to specific triggering stimuli causing a release of a large fraction of the stored peptide in a short period of time (Hadzic et al., 2015). These complex high volume features of regulated protein secretion are not present in all cell types. Several transcription factors play instrumental roles in these specialized cells, including XBP1 which promotes expansion of the rough ER to help regulate the UPR. ATF6 activates expression of chaperone proteins required for efficient protein folding (Lee et al., 2003) in addition to many of the lipid components of secretory organelles (Bommiasamy et al., 2009). REST in PC12 cells controls a set of genes that coordinate and support neurosecretion (Pance et al., 2006). Additionally, CREB3L1/OASIS and CREB3L2/BBF2H7, two bZip transcription factors have been shown to
be required for efficient bone deposition and cartilage matrix secretion, respectively (Fox et al., 2010). Furthermore, CREB-A, the CREB3 homologue in Drosophila, promotes a prosecretory program in constitutively secreting tissues such as the salivary glands. A question that still remains however, is whether these transcription factors function in a broad sense to up-regulate the entire secretory pathway or if the up-regulation is restricted to a subset of secretory genes in a few specialized cells (Fox et al., 2010). Importantly, an increase in secretory demand will lead to increased protein load in the ER; in order to maintain ER homeostasis an increase in the mechanisms such as the unfolded protein response is required to maintain proper secretory function.

1.10 ER stress

The ER plays an important role in the folding and maturation of newly synthesized proteins. Perturbations in ER function, termed ER-stress, trigger the unfolded protein response (UPR), a highly conserved and tightly orchestrated set of intracellular signals designed to restore protein homeostasis (Bravo et al., 2013). The UPR is characterized by the action of three signaling proteins: IRE1α (inositol-requiring protein-1α), PERK (protein kinase RNA (PKR)-like ER kinase), and ATF6 (activating transcription factor 6). Under baseline conditions PERK and ATF6 proteins are bound to the chaperone GRP78/BiP (Glucose related protein 78/Binding immunoglobulin Protein), which keeps them in an inactive state (Sano and Reed, 2013). When unfolded proteins accumulate in the ER, BiP is released and assists with the folding of accumulated misfolded or unfolded proteins. IRE1α appears to become activated when unfolded proteins bind directly to it; however, BiP desensitizes IRE1α to low levels of stress aiding in its deactivation once ER homeostasis is reestablished. When active, PERK, IRE1α and ATF6 induces various signal transduction events that work to alleviate the accumulation of misfolded
proteins in the ER by increasing expression of ER chaperones, inhibiting translation, and stimulating retrograde transport of misfolded proteins from the ER into the cytosol targeting them for ubiquitination and destruction by the ERAD process (Sano and Reed, 2013).

IRE1α is a transmembrane kinase that consists of an N-terminal luminal domain, used for sensing ER stress, a single transmembrane domain, and C-terminal cytosolic effector that is responsible for both protein kinase and endoribonuclease activities. When activated, IRE1α oligomerization occurs in ER membranes and autophosphorylation of the cytosolic domain (Pincus et al., 2010). The RNase domain removes an intron from the X box-binding protein-1 (XBP-1) mRNA to allow production of the active XBP-1 protein. IRE1α also controls its own expression by cleaving its own mRNA (Hassler et al., 2012). The activated XBP-1 protein binds to promoters of several genes involved in UPR and ERAD to restore protein homeostasis and promote cellular protection. High levels of chronic ER stress can lead to the recruitment of TNF-receptor-associated factor 2 (TRAF2) by IRE1 and the activation of apoptosis-signaling-kinase 1 (ASK1). Activated ASK1 phosphorylates c-Jun N-terminal protein kinase (JNK) and p38 MAPK, promote apoptosis by regulating the BCL2 family of proteins (Han et al., 2009; Hollien et al., 2009).

The second UPR protein ATF6 has two isoforms, ATF6α and ATF6β. ATF6α is involved in the UPR, and has been extensively studied in the context of ER Stress. Upon activation ATF6α moves to the Golgi where it is cleaved by site 1 (S1) and site 2 (S2) proteases, generating an activated b-ZIP factor (Oslowski and Urano, 2011). The active form of ATF6α translocates into the nucleus where it binds to ATF/cAMP response elements (CRE) and ER stress-response elements (ERSE-1) to activate target genes such as BiP, Grp94 and CHOP and other genes involved in protein folding, processing, and degradation (Haze et al., 1999).
PERK is the third UPR protein and its main responsibility is to attenuate mRNA translation under ER stress, preventing influx of newly synthesized proteins into the already stressed ER (Oslowski and Urano, 2011). This translational attenuation is mediated by phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) which inhibits the recycling of eIF2α to its active form. The active form of eIF2α is required for the initiation phase of polypeptide chain synthesis, thus attenuation of eIF2α leads to preferential translation of UPR-dependent genes such as the activating transcriptional factor 4 (ATF4) while decreasing translation of other proteins (Sano and Reed, 2013). Some of the important targets activated by ATF4 are CHOP, GADD34 and ATF3. PERK is also capable of phosphorylating nuclear erythroid 2 p45-related factor 2 (NRF2), which promotes expression of genes containing antioxidant response elements (ARE), helping prevent oxidative stress (Oslowski and Urano, 2011).

If the UPR-induced mechanisms fail to restore ER homeostasis various mechanisms help to induce apoptosis. The induction of CHOP, a transcription factor activates GADD34 promoting the dephosphorylation of eIF2α reversing translational inhibition (Sano and Reed, 2013). This contributes to the accumulation of unfolded proteins in the ER in addition to permitting translation of mRNAs encoding pro-apoptotic proteins. A second pathway that activates TRAF2 (tumor necrosis factor receptor associated factor 2) stimulating the pro-apoptotic signaling kinase JNK and downstream pro-apoptotic transcription factors c-Jun and p38 MAPK (Wajant et al., 2003). Ero1α (endoplasmic reticulum oxidoreductase-1) hyperoxidizes the ER, and it also activates the inositol triphosphate receptor, which stimulates excessive calcium transport from the ER to the mitochondria, cell death is induced by each of these (Li et al., 2009).
Protein folding in the ER is essential to the survival of each cell, which explains the need for development of the UPR in unicellular organisms such as yeast. Dysregulation of ER homeostasis can cause chronic diseases in humans (Olsowski and Urano, 2011). Therefore, it is important to study ER stress and the UPR using mammalian cells to understand the UPR and ER stress-related diseases.

Figure 6: ER stress activates the 3 arms of the unfolded protein response. (A) Endoplasmic reticulum (ER) stress activates protein kinase RNA-like endoplasmic reticulum kinase (PERK) which then phosphorylates eukaryotic initiation factor 2 α (eIF2α), leading to inhibition of protein synthesis and a reduction in the ER protein folding load. Reduced protein synthesis increases levels of activating transcription factor 4 (ATF4), inducing the transcription factor C/EBP homologous protein (CHOP), which induces growth arrest and DNA damage-inducible protein (GADD34) and several pro-apoptotic genes. (B) ER stress activates activating transcription factor 6 (ATF6) increasing protein-folding capacity by inducing chaperones, including binding protein (BiP) and GRP94 (glucose regulating protein 94). (C) ER stress activates Inositol-requiring Enzyme 1α (IRE1α) which causes X-Box binding protein 1 (XBP1) mRNA to be spliced, which increases the protein-folding capacity and the turnover of misfolded proteins by inducing chaperone protein and ER-associated degradation (ERAD) genes as well as altering mRNA decay and translation. (Andruska et al, 2015, from PNAS).
1.11 CREB3 Proteins

Following the discovery of ATF6 another group of ER associated proteins was discovered, the CREB 3 family which consists of 5 different proteins with similar structure. This family includes LUMAN/CREB3, in addition to 4 other CREB3-like proteins; CREB3L1/OASIS, CREB3L2/BBF2H7, CREB3L3/CREBH, and CREB3L4/AlbZIP/CREB4/Tisp40 (Fox et al., 2010). The five proteins are collectively identified as the CREB3-like family due to their structural and functional similarities. These proteins are all ER transmembrane proteins with an N terminus facing the cytoplasm containing a highly basic DNA-binding domain and an amino-terminal transcriptional-activation domain next to a carboxyl terminus (Fig. 7).

![Figure 7: The CREB3 family of transcription factors.](image)

The bZIP domains of the members are aligned. TA: transcriptional activation domain. bZIP: basic leucine zipper domain. TM: transmembrane domain (Chan et al, 2011, from Cell & Bioscience).
They also contain well-conserved cleavage sites for protease S1P and S2P indicating that they are all subject to regulated intramembrane proteolysis (RIP) (Lu and Misra, 2000; Shen et al., 2004). It is thought that the CREB3 family of proteins exhibit functional redundancy in order to compensate if one of them is dysfunctional or missing.

The initial studies looking at the CREB3 proteins were completed using cell culture and ER stress treatments to trigger activation. UPR (Fox and Andrew, 2015). All CREB3 proteins undergo RIP which is a common feature of proteins involved in the UPR. A number of mouse knockout models have been characterized over the last 5-10 years leading to the conclusion that CREB3 proteins play a key role in normal organ development (Asada et al., 2011). While there are a number of mouse knockout models for the CREB3 proteins, the work in this thesis is the first to generate and describe the LUMAN knockout mouse model. There are clearly some functional redundancies within the CREB3 family, however, in the absence of LUMAN the mice are not able to fully compensate for its loss. Genetic model systems including knockout mouse models for each CREB3-like protein and the simpler drosophila CREB A knockout have been key to investigate how each of these factors function during normal physiology and under stress in the regulation of biological processes.

CREB3L1 was initially discovered in cultured astrocytes and was discovered to be an ER stress response protein. CREB3L1 is a glycoprotein that undergoes glycosylation which can serve as a sensor for ER stress, a similar mechanism to ATF6. It has been shown that CREB3L1 contributes to both maximal induction of the UPR (chaperone capacity), in addition to maintaining extracellular matrix protein expression in some cell lines (Iseki et al., 2012). CREB3L2 is preferentially expressed in chondrocytes (Chan et al., 2011), when the N-terminal is cleaved and translocated into the nucleus where it promote the expression of target genes such as
Sec23a. In contrast, after RIP the C-terminus is extracellularly secreted and has been shown to activate Hedgehog signaling. Both the N-terminal and C-terminal pathways are essential for the normal development of cartilage in mice (Iwamoto et al., 2015). CREB3L3 is highly expressed in the liver and small intestine. In the liver, it contributes to glucose and triglyceride metabolism through regulation of fibroblast growth factor 21. Intestinal CREB3L3 has been shown to regulate the flow of dietary cholesterol from the small intestine through regulating the expression of various transporters (Kikuchi et al., 2016). CREB3L4 has been shown to play a key role in adipocyte differentiation; with overexpression leading to inhibition of adipocyte differentiation. Knockdown of CREB3L4 caused differentiation from pre-adipocytes into mature adipocytes, increasing adipogenesis as well as improved glucose tolerance and insulin sensitivity (Kim et al., 2014).

*Drosophila* has only a single CREB3 protein, called CREBA, however its structure varies slightly when compared to its mammalian orthologs. The DNA binding domain shows 48% homology with the corresponding region in mammalian *Luman* sequence, however, the leucine zipper is unusual in that it has a tyrosine substitution at the third leucine position (Dwarki et al., 1990). CREBA also has an extended leucine zipper region containing 6 hydrophobic residue iterations where normal CREB zippers contain 4. It has been shown that CREBA is both necessary and sufficient for expression of high level secretory pathway component genes (SPCGs) in the secretory tissues of the *Drosophila* embryo (Fox et al., 2010). Interestingly, it is not only general secretory machinery components that are effected by CREBA but also cell type–specific secreted cargo. The role of LUMAN/CREB3 in regard to general or cell-type specific secretion has yet to be investigated (Fox and Andrew, 2015).
LUMAN is an ER bound transcription factor that was originally identified as a neuronal stress response protein involved in the reactivation of the herpes simplex virus (HSV) from latency. LUMAN is capable of interacting with other bZIP proteins forming homodimers and heterodimers and is often associated with a cell cycle regulator, Host Cell Factor 1 (HCF-1) (Lu and Misra, 2000). It was observed that the translocation of HCF-1 from cytoplasm to nucleus, which is required for reactivation of latent HSV, coincided with the disassociation of LUMAN from the ER membrane (Kristie et al., 2010).

Full-length LUMAN resides on the ER membrane with the carboxyl terminus in the ER lumen and the amino terminus extended in the cytoplasm. The N-terminus of LUMAN contains the acidic activation domain, the basic leucine zipper domain and the transmembrane domain. RIP involves 2 cleavage events and causes the release of the N-terminus of LUMAN from the ER allowing it to translocate into the nucleus, acting as a transcription factor. N-terminal LUMAN directly alters the transcription of downstream targets or is sequestered and repressed by LUMAN recruiting factor (LRF) (Fig. 8) (Audas et al., 2008).
Figure 8: The activation mechanism of LUMAN. LUMAN is located on the endoplasmic reticulum (ER) membrane. When activated, it is translocated to the Golgi where it undergoes regulated intramembrane proteolysis (RIP). After 2 distinct cleavage events, the N-terminal is translocated to the nucleus where it either alters gene transcription through interaction with host cell factor 1 (HCF-1) or is functionally repressed by Luman recruitment factor (LRF). (modified from Audas et al, 1998, MCB).
LUMAN has been proposed to play a role in ER stress due to its structural similarities with ATF6. However, activation of LUMAN is not effectively induced by ER stressors such as thapsigargin (Tg) and tunicamycin (Tm) (Roy and Lee, 1999; Rutkowski and Kaufman, 2004). Activation of LUMAN is strongly induced by treatment of brefeldin A (BFA), a regent that inhibits protein transport from the ER to the Golgi apparatus indirectly by preventing formation of the COPII-mediated transport vesicles (Lu and Misra, 2000; Roy and Lee, 1999; Rutkowski and Kaufman, 2004). This suggests that LUMAN may not be a classic ER stress response protein but rather that it may be involved in protein transport and secretion-related ER stress. However, LUMAN has been shown to play a role in the UPR during ER stress and has been shown to directly interact with Herp (homocysteine-induced ER protein), a protein involved the ERAD pathway. LUMAN binds to and activates the ER stress response element II (ERSE-II) enhancer element in the herp promoter, which provides a role for LUMAN in the ERAD pathway (Sai et al., 2003) in preventing ER stress-induced apoptotic cell death through herp activation.

LUMAN appears to play multiple roles in the cell, many of which are not fully understood. Analysis of various domains of the protein may help shed some light on its possible functions, and the mechanisms through which it works. The N-terminus of LUMAN contains two LxxLL motifs that are known for mediating protein-protein interactions, in particular with the NRs (Luciano and Wilson, 2000). LxxLL motifs are present in most cofactors of NRs, such as steroid receptor coactivator 1 (Lu et al., 1997) and the thyroid hormone receptor-binding protein (Shen et al., 2002). The LxxLL motifs are also found in other transcription factors (Shen et al., 2001) and are well conserved among both Human and Murine LUMAN homologues (Luciano and Wilson, 2000). It is therefore possible that LUMAN acts as a cofactor for one or more of the NRs,
altering their activity. From this, we have proposed that LUMAN may be involved in the stress response at both the cellular and organism level.

1.12 Rationale and Objectives

LUMAN is highly conserved between mice and humans, showing 67% homology at the amino acid level. Therefore, using mice to investigate the biological function of LUMAN is an important step in understanding how it can affect important aspects of human physiology. Research in this thesis utilized the LUMAN gene trap reporter knockout (KO) mouse model in order to characterize the physiological function of LUMAN, in addition to elucidating the molecular mechanism through which it works. Behavioural and hormonal profiles were compared to wildtype mice to assess differences that could be attributed to LUMAN. Additionally, LUMAN KO and WT Mouse Embryonic Fibroblast (MEF) cells were used to explore its molecular function before moving to an in vivo model.

Chapter 2: LUMAN/CREB3 is a key regulator of glucocorticoid-mediated stress responses

ABSTRACT

Altered glucocorticoid sensitivity is believed to contribute to a number of human diseases, including inflammatory and autoimmune conditions as well as disorders characterized by abnormal hypothalamic-pituitary-adrenal axis (HPA) function. LUMAN (or CREB3), originally identified through its interaction with a cell cycle regulator HCFC1, is an endoplasmic reticulum membrane-bound transcription factor that is involved in the unfolded protein response. Here we demonstrate that LUMAN changes the glucocorticoid response by modulating the expression of the glucocorticoid receptor leading to an overall increase in GR activity. Luman-
deficient mice exhibited a blunted stress response characterized by low levels of both anxiety and depressive-like behaviour and low circulating corticosterone levels. These mice also have reduced dendritic branching in the CA3 region of the hippocampus, consistent with increased GR responses. These findings are consistent with the notion that elevated GR activities are the primary cause of the observed phenotype in these LUMAN-deficient mice. We thus postulate that LUMAN is a key regulator of GR-mediated signaling and modulates HPA axis reactivity.

2.1 INTRODUCTION

Variations in glucocorticoid (GC) sensitivity are believed to contribute to a wide range of human diseases (Chrousos, 2009; McEwen and Gianaros, 2010). The underlying mechanisms are not well understood, but may involve heterogeneity in glucocorticoid receptor (GR) structure as well as epigenetic regulation of GC sensitivity through various cellular mechanisms (Charmandari et al., 2013; Keenan and Hipwell, 2015; McEwen and Gianaros, 2010; Quax et al., 2013). Importantly, some of these regulatory mechanisms appear to have effects that may continue for some time after the initial triggering event, even across generations via the transplacental transmission of stress-related signals (Turecki and Meaney, 2014; Yehuda et al., 2014). An increase in GR activity has a number of downstream effects, including alterations in neurogenesis, immune response in addition to behavioural abnormalities as well as subsequent increase in negative feedback in the hypothalamic-pituitary-adrenal (HPA) axis (Reichardt et al.).

The HPA axis, is a major branch of the neuroendocrine system that regulates the stress response and many other processes, including the immune system and inflammation as well as mood, memory, sexuality and reproduction (Pariante and Lightman, 2008). The central effector
of the stress response is corticotrophin releasing hormone (CRH), synthesized in the hypothalamus. Both physical and psychological stress can rapidly stimulate the release of CRH, which induces adrenocorticotrophic hormone (ACTH) secretion in the anterior pituitary, subsequently leading to glucocorticoid (GC; cortisol or corticosterone in rodents) secretion in the adrenal cortex. GCs exert negative feedback at various levels of the HPA axis including the hypothalamus, anterior pituitary in addition to the hippocampus. Mice with GR overexpression showed enhanced negative feedback of the HPA axis characterized by low levels of GCs as well as a reduced response to stress (Reichardt et al.). Altered function of the HPA axis, in particular the negative feedback, has been implicated in many prevalent human conditions including depression, anxiety, bipolar disorder along with a number of autoimmune disorders (Pariante, 2009).

One of the potential factors involved in GR regulation is LUMAN/CREB3, a stress related endoplasmic reticulum (ER)-bound cellular transcription factor, originally identified as a neuronal stress response protein involved in herpes simplex virus (HSV) reactivation from latency (Freiman and Herr, 1997; Lu and Misra, 2000; Lu et al., 1997; Lu et al., 1998). LUMAN is most highly expressed in the hypothalamus, hippocampus, anterior pituitary and adrenal gland (Lein et al., 2007). LUMAN is known to play an important role in cellular stress responses, particularly signaling from the ER to the cell nucleus. LUMAN contains two LxxLL nuclear receptor binding motifs (Luciano and Wilson, 2000) and also interacts with a regulatory protein, LUMAN/CREB3 Recruitment Factor (LRF or CREBRF) (Audas et al., 2008). Deletion of LRF alters the attenuation of stress responses normally observed at parturition in mice; evidence supporting this includes below-normal corticosterone levels along with an increase in GR activity. In addition, a maternal defect is observed in LRF-deficient females characterized by
maternal neglect and a significant reduction in pup survival to LRF KO females when compared to the wild type (WT) females (Martyn et al., 2012). Given that LRF regulates the activity of LUMAN we hypothesized that these effects might actually result from dysregulation of LUMAN. To test this hypothesis and elucidate the biological function of LUMAN, we created a Luman-knockout (KO) mouse line.

In the present study, we report that down-regulation of Luman reduces basal corticosterone levels and blunts stress responses, via dysregulation of the HPA axis. Luman-deficient animals exhibit behavioural abnormalities, altered patterns of hormone secretion and structural changes in the hippocampus accompanied by elevated GR activities, suggesting a dramatically enhanced responsiveness to circulating corticosterone levels. Variations in LUMAN-mediated signaling may thus contribute to the regulation of GR-activated stress responses.

2.2 Materials and Methods

Animals. This study followed the Canadian Council of Animal Care guidelines and was approved by the Animal Care Committee at the University of Guelph. The LUMAN gene knockout mouse line was generated in collaboration with the International Gene Trap Consortium (Nord et al., 2006). Chimeric mice were backcrossed to C57BL/6 mice (Charles River, Montreal, Canada) to produce a 99.9% congenic mouse strain. C57BL/6NTac mice were group housed with same-sex siblings and maintained on a 12-h light/dark cycle (10:00-22:00). Temperature was maintained at 21-24 °C, and food (2014 Teklad Global 14% protein rodent maintenance diet) and tap water were provided ad libitum. To obtain sufficient mice in certain circumstances pups from LUMAN KO/HET mice were cross fostered onto CD1 dams. Due to low LUMAN KO pup survival, heterozygote mice were used in all experiments except the
behaviour assays where LUMAN KO mice were used. Mice were euthanized by cervical dislocation and tissues were collected either in liquid nitrogen for protein and mRNA extraction or in 4% Paraformaldehyde for histological analysis.

**Behaviour tests.** All tests were performed with virgin female mice 2-5 months of age under red light, counterbalanced for placement where applicable, with the exception of the dark-light test, which was performed in a brightly lit room, and tests were videotaped for subsequent analysis with Observer Video Analysis (Noldus, Wageningen, The Netherlands). Data were scored blindly by a single, impartial, third party. All testing apparatus was cleaned between each mouse with Alconox detergent (Prolab Scientific, Laval, Quebec, Canada) in water, followed by an aqueous baking soda solution.

To examine if altering the level of LUMAN had an impact on mood-related behaviours four tests were performed, the elevated plus maze test (EPM), the light/dark test, the forced swim test (FST) and the tail suspension test (TST). The EPM and light/dark test are often use to assess differences in anxiety levels and were performed at described in Karl et al. (2003). The FST and TST are used to assess the level of depressive-like behavior in mice and was performed as described in Porsolt et al. (1997).

**In brief:**

**EPM:** to assess anxiety related behaviour, where the dependent variable is the time spent in the open vs closed arms, the elevated plus-maze consisted of 2 open arms (50 cm × 10 cm) and 2 enclosed arms of the same size with 30 cm walls. The arms were elevated 50 cm off the floor. Mice behaviour was recorded for 10 minutes. Less time in the light is interpreted as higher anxiety. Mice were allowed to move freely for 10 minutes in the plus-shaped apparatus with 2
open and 2 enclosed arms. Each mouse receives one trial in the test apparatus. Illumination at the center was maintained at 100 lux. Times spent in the light arms, the numbers of entries into the light arms, and total numbers of entries into all arms were recorded. The time and entries were counted when mice had all four paws in the relevant area.

**Light/dark test**: to assess anxiety related behaviour, where the dependent variable is time spent in the dark vs light compartment, the apparatus used for light/dark transition test was a clear polycarbonate square box divided by a black insert into dark and light rectangular compartments of equal sizes (16” L x 8” Hx5” W) with an entry opening of 4” x 1.25” (AccuScan Instruments, Colombus, OH, USA). Mice were released in a corner of the dark area, and their horizontal activities (in centimeters) was tracked by a set of three photo beam arrays for 10 minutes. After each trial, all chambers are cleaned with 75% ethanol to prevent a bias based on olfactory cues. All data was collected using the VersaMax Analyser (AccuScan Instruments).

**FST**: to assess despair/depressive-like behaviour, where the dependent variable is time spent immobile, the forced swim tests were carried out using a procedure modified from the method reported by Ademet al. in 2012 (194). The mice were individually placed into a glass cylinder (30 cm height, 20 cm diameter) containing 15 cm of water at 23 to 25°C. Behaviour was recorded for six minutes using a video camera placed to give a side view of the cylinder. Only data from the last four minutes of a total of six minutes were used for mobility evaluation, defined as any movements other than those necessary to balance the body and keep the head above water. Small foot movements to keep head above water are not scored as mobility. Drifting due to earlier momentum is not scored as mobility. The time of immobility was determined by: time of immobility (seconds) = 240 seconds – time of mobility (seconds).
TST: to assess despair/depressive-like behaviour, where the dependent variable is time spent immobile, mice were suspended 30 cm above the service of the table by the use of adhesive tape applied to the tail for a period of 6 minutes and observed for struggling (mobility) vs. time of immobility. Because it is easier to mark and detect movements (mobility) rather than the lack of movement (immobility), only the time of movement was recorded. Time for immobility was calculated as: 360 seconds – time (seconds) mobility = time (seconds) immobility. Longer periods of immobility are characteristic of a depressive-like state (195). This experiment was therefore performed to assess level of depression in Luman-/- female mice using Luman+/+ counterparts as controls.

Buried Food Test: to test for olfactory deficits, where the dependent variable is latency to find a food pellet, mice were deprived of food with water supplied ad libitum 24 hours before testing. All mice were tested in the dark cycle (10:00a.m. - 22:00p.m.). In the test, individual mouse were placed into a clean cage (46x 24 x 14.5 cm) in which a food pellet was hidden under 1.5 cm bedding at one corner of the cage. The mice was positioned at the opposite end of the cage, and the time it takes to find the food, i.e., the time from the moment the mice was placed into the cage to the time it located the pellet and initiated burrowing, was recorded. A fresh cage was used for each trial, and all mice underwent identical testing procedures. The Luman+/+ and Luman+/- females used in the buried food-finding test were age-matched naïve littermates of 2.5 month old. The Luman-/- females used included both naïve and parous females ranging from 2.5 month to 6 month old.

Hotplate test: a measure of stress-induced analgesia, dependent variable: latency to paw lift, each mouse was placed on a hotplate set at 50 °C until a pain response was recorded; the mouse was then placed in a 50 mL falcon tube for 30 minutes. After which blood was drawn from the
saphenous vein and the mouse was placed back on the same hotplate and pain responses were recorded a second time. The hotplate was cleaned with Alconox detergent (Prolab Scientific, Laval, Quebec, Canada) in water. RU486 Rescue: The previous protocol was repeated 3 hours after an injection of RU486 (mifepristone; Sigma) at 7.5 mg/kg.

Cell culture. All cell types were grown in monolayer culture in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 g/ml streptomycin. All cultures were maintained in a 5% CO2 humidified atmosphere at 37 °C and passaged every 2 to 3 days. Cells were plated 24 h prior to transfection and allowed to grow to 60% confluence prior to transfection. Cells were transfected by polyjet transfection reagent (SignaGen Laboratories) as per the manufacturer’s instruction.

Immunohistochemistry (IHC) staining of LUMAN: Brain tissues were harvested immediately following euthanasia, cut sagittally in half and fixed for 24 hours in 4% paraformaldehyde, processed, and embedded in paraffin. IHC staining for LUMAN (CREB3) was performed on 4μm tissue sections (sagittal section) mounted on charged slides using an automated immunostainer (Dako Autostainer, Dako, Mississauga, ON, Canada). Following manual deparaffinization and rehydration, sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity and with Proteinase K (Dako) for antigen retrieval. Sections were incubated with rabbit anti-LUMAN (7281.3) antiserum (1:100 dilution; pacific immunology, USA) for 30 minutes. A goat anti-rabbit polymer detection system (EnVision + Dako) was used with Nova Red (Vector Laboratories, Burlington, ON, Canada) as chromogen. For negative reagent controls, duplicate tissue sections were subjected to the same IHC staining procedure, with the substitution of nonimmune rabbit serum at similar protein concentration for the anti-LUMAN rabbit polyclonal antiserum.
**Nissl staining (cresyl violet):** Naïve male littermates of two-month old (3 Luman+/+ and 3 Luman +/-) were sacrifice by cervical dislocation. Brain tissues were harvested immediately following euthanasia, fixed for 24 hours in 4% paraformaldehyde, processed, frozen in cryoprotectant and stored at -80 °C. Sections of 15 μm mounted on charged slides were subjected to Nissl staining. Sections were first dehydrated via soaking in descending concentrations of ethanol to reduce background staining and then they were rehydrated, and stained in 0.02% cresyl violet solution for 10 minutes. Slides were rinsed quickly with distilled water and then dehydrated, cleared and mounted.

**ELISAs.** Blood samples (100 to 150 μl) were collected in the active cycle between 1100 to 1300 h from the saphenous vein of the hind limb; serum was separated and stored at -80 °C. Hormone levels were detected using a PRL enzyme-linked immunosorbent assay (ELISA) kit (Calbiotech, Spring Valley, CA) and corticosterone ELISA kits (Enzo life sciences, Farmingdale NY), as per the manufacturers’ instructions, and detected using a POLARstar Omega plate reader (BMG Labtech GmbH, Offenburg, Germany). Statistical analysis was performed using two-way repeated measures analysis of variance (ANOVA) and a pairwise multiple comparison procedure where applicable. Data were deemed significant at a $P$ value <0.05.

**Evaluation of the Hypothalamic-Pituitary-Adrenal Axis Function.** DEXamethasone Suppression test: The mice received an intraperitoneal injection of DEXamethasone (3g/100g body weight; Sigma, Schnelldorf, Germany) 6 h before blood was drawn from the saphenous vein. DEX/CRH test: Six hours after application of DEXamethasone, the mice were injected intraperitoneally with 0.5 mg of CRH (Sigma) 30 minutes later blood was drawn from the saphenous vein. RU486/CRH/ACTH test: Mice were injected with CRH at 0.5 mg/mouse (Sigma), ACTH at 0.5 mg per mouse (Phoenix pharmaceuticals) and RU486 (mifepristone;
Sigma) at 7.5 mg/kg 3 hours prior to blood being drawn from the saphenous vein. All blood samples were immediately spun to separate serum and stored at -80 °C until determination of corticosterone levels was complete using a CORT ELISA kit (Enzo life sciences, Farmingdale NY).

**RNA analysis and qRT-PCR.** Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from adult mouse tissues. cDNA was synthesized from total RNA using SuperScriptIII reverse transcriptase (Invitrogen) and oligo(dT) (Roche Diagnostics, Laval, QC, Canada). Transcript levels were measured by quantitative RT-PCR (qRT-PCR) using PerfeCTa SYBR green Supermix with 6-carboxy-X-rhodamine (ROX) (Quanta Biosciences, Inc., Gaithersburg, MD) and primers against the mouse genes. Samples were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and subjected to standard curve analysis, and arbitrary values were represented, adjusting for primer efficiencies. For primer sequences see Appendix, Table 1.

**Protein extraction and western blot analysis.** Tissues were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) RNA was extracted and the phenol phase was frozen at -80 °C until protein extraction was done. Isopropanol precipitation was performed to isolate the protein. Total protein was quantified using Pierce® BCA protein assay reagent (Thermo Scientific, USA) according to the manufacturer’s instructions. Primary antibodies were used at the following dilutions: GR polyclonal antibody (sc-1004; Santa Cruz) at 1:400, a custom polyclonal anti-LUMAN antibody (Pacific Immunology, California USA) at 1:400. Secondary horseradish peroxidase (HRP)-conjugated antibodies were used at 1:10 000 (Promega). The blots were visualized using ECL (GE Healthcare, Piscataway, NJ) on Amersham Hyperfilm ECL (GE Healthcare).
**Histological assessment of mouse mammary gland tissue.** The right inguinal mammary gland was taken within 24 hours of delivery. Tissues were fixed in 4% PFA in 0.1 M PBS (pH 7.2) and embedded in paraffin. Hematoxylin and eosin stains were performed on 6 μM paraffin embedded sections. All mammary tissues were blind coded and scored by a clinical pathologist. Images were captured on a Leica DMRA2 microscope with a Hamamatsu ORCA-ER digital camera and Openlab imaging software (PerkinElmer).

**Sholl Analysis.** After sacrificing the animals through cervical dislocation, brains were removed and processed using the Golgi-Cox method as described by Markham et al. (2005). Briefly, the whole brain was submerged in the Golgi-Cox solution for 28 days in light sealed vials. After 24 hours in 30% sucrose the whole brains were surrounded in 4% agarose gel and sliced in 300 μm coronal sections using a vibratome (Leica Microsystems, Richmond Hill, Ontario, Canada). Sections were developed according to Glaser and Van der Loos (1981). Briefly, sections were placed in 6% sucrose solution overnight, and then processed using the following steps, all while rocking gently at room temperature: 15 minutes in 2% paraformaldehyde, 5 minutes in double deionized water, 15 minutes in 2.7% ammonium hydroxide, two 5 minute rinses in double deionized water, 25 minutes in Kodak Fixative A, and a 5 minute rinse in double deionized water. Sections were then mounted on gelatinized slides, any remaining agar was removed and excess moisture was wicked away, and the slides were left to dry for 30-45 minutes. Sections were dehydrated using an array of increasingly concentrated ethanol solutions, cleared in xylene, and coverslipped with Permount solution. Serial images, taken 1 μm apart, were acquired along the z-axis of the sections using an Olympus BX53 microscope and Olympus UPlanSAPO 1.05NA silicone immersion 30× objective (Olympus, Richmond Hill, ON, Canada). Image stacks were compiled and 3-dimensionally traced using Neurolucida software (Version 10 MBF
Bioscience, Williston, VA, USA). After the tracing, Sholl Analysis (Sholl, 1953) was performed to assess the dendritic lengths and dendritic branching of neuron tracings using Neurolucida Explorer (NBF Biosciences). Concentric circles were fitted 20\(\mu\)m apart, from the soma out to the distal ends of the apical and basal dendritic arbours for 5 neurons in each of CA1 and CA3, per mouse, with \(n=4\) mice per group. The data is displayed with each mouse as an individual sample. In order to be included in the analysis, neurons had to be completely traceable with no sizable breaks or interruptions, and the full dendritic arbour had to be clear for tracing without considerable interference from surrounding neurons. Analysis was completed used an ANCOVA (analysis of covariance) to assess statistical significance while controlling for the distance from the stoma not being a discrete or continuous variable.
2.3 RESULTS

Figure 9: Luman-deficient mice show evidence of increased GR activity. (A) Mouse embryonic fibroblast (MEF) cells, Luman (+/+, +/- and -/-) and protein extracts from adrenal
glands (Het and WT female mice) were assessed via western blotting for levels of Glucocorticoid receptor (GR) both with and without DEXamethasone treatment (DEX; 100 nM) for 8 hours. n = 6 adrenal glands (2 pooled per sample) 3 blots were quantified and normalized to WT levels. Presented as mean ± SEM; *p < 0.05 calculated by a one-tailed t-test. (B) RNA was extracted from the hypothalamus, pituitary and adrenal gland of both WT and Het female mice that have undergone DEX treatment as well as an untreated control group, RNA was reverse transcribed and RT-PCR was performed (n = 4). Data are presented as proportion of WT values (indicated by the dotted line). Presented as mean ± SEM; * P<0.05 calculated by a one-tailed t-test, assessing difference between Het and WT levels. (C) Representative pictures of EM sections were taken of the adrenal medulla showing storage vesicles predicted to contain catecholamine from Het and WT female mice. (D) RNA from the adrenal glands of female mice was analyzed for levels of Tyrosine hydroxylase (TH) and phenylethanolamine N-methyl transferase (PNMT), data presented as proportion of WT values. Presented as mean ± SEM; *p <0.05 calculated by a one-tailed t-test (n = 4). (E) Blood samples were taken from the saphenous vein of the Het and WT male mice and the serum was run on a CORT ELISA. Presented as mean ± SEM; *p <0.05 calculated by a one-tailed t-test (n = 10). (F) A DEX Suppression test and CRH Rescue were performed on female mice. Presented as mean ± SEM; *p <0.05 calculated by a one-tailed t-test comparing Het values with WT values (n = 6). (G) Mice received a primary injection of mifepristone or saline and a subsequent injection of CRH or ACTH. All blood samples taken from the saphenous vein and were spun down and serum was stored at -80 °C until it was run on a CORT ELISA kit. Presented as mean ± SEM; *p <0.05 calculated by a one-tailed t-test comparing Het values with WT values (n = 6). (H) Both Het and WT male mice were placed on the hotplate, and measurements were taken for latency to paw lift both before and after a 30-minute restraint stress. (I) The hotplate test was repeated 2 hours after the mice were injected with mifepristone n =10. (* P-value <0.01, ** P <0.001). Values are mean ± SEM. P values calculated using a one tailed t test.

2.3.1 *Luman*-deficient mice show altered GR expression.

The effects of LUMAN on GR expression were first analyzed using mouse embryonic fibroblasts (MEFs) in Wildtype (WT, +/+ ) and *Luman*-null (-/-) cell lines. In the LUMAN null cells, GR expression was clearly increased in comparison with WT MEFs. Moreover, a graded response was observed between WT, heterozygous (Het, +/-), and null cell lines, with an inverse relationship between LUMAN expression and GR levels (Fig. 9A).

To assess downstream effects of elevated GR activation, we treated the MEF cells with the synthetic glucocorticoid DEXamethasone (DEX; 100 nM). Following this treatment, the difference in GR expression between *Luman* null and WT cells was diminished, assessed through
western blot, indicating that when the receptor is saturated the effect of LUMAN is minimal (Fig. 9A). Differences in GR protein levels translated into significant differences in the expression of GR sensitive genes. Quantitative reverse-transcription PCR (qRT-PCR) was used to measure the levels of two representative GR-inducible genes (FKBP5 and SGK1). Both of these genes were found to be increased when LUMAN was downregulated (FKBP5 $T= 3.21$, $P= 0.024$, $DF=3$; SGK1 $T= 2.5$, $P= 0.04$, $DF=3$; GR: $T= 2.19$, $P=0.048$, $DF=3$), indicating that not only do Luman-deficient mice have higher GR levels but GR biological activity is also increased (Fig. 9B).

2.3.2 Luman-deficient mice have increased GR activity

Consistent with previous qRT-PCR and in situ hybridization studies (Ying et al., 2015), we found high levels of LUMAN expression in the hippocampus, hypothalamus, and pituitary, in addition to the adrenal medulla (Sup Fig. 1). Proteins extracted from the adrenal glands of the mice showed a GR increase in the Luman-deficient animals similar to those found in MEF cells were (Fig. 9A). Further evidence of an enhanced GR-mediated response in the adrenal glands were obtained by measurement of the enzymes responsible for adrenal medullary catecholamine synthesis. Tyrosine hydroxylase (TH) and phenylethanolamine N-methyl transferase (PNMT), two enzymes involved in catecholamine synthesis that are induced by glucocorticoids (Betito et al., 1994), were significantly increased in the Luman-deficient mice (TH $T= 3.26$, $P= 0.02$, $DF=3$; PNMT $T=.2.56$ $P=0.04$, $DF=3$) (Fig. 9C). Circulating Catecholamine (CA) levels were only marginally different between WT and Het mice (Het$=181.08\pm 2.99$ pg/mL, $n =10$; WT$=171.99\pm 2.91$ pg/mL, $n =7$; $p=0.08$ by two tailed $t$-test, Sup Fig. 2). Electron microscopic (EM) analysis of the adrenal medulla suggested a possible explanation of this apparent paradox (Fig. 9D). Substantially more storage granules were found in the WT adrenal medulla, compared
to the *Luman*-deficient mice (qualitative analysis only), suggesting that the observed elevations in the activity of TH and PNMT in the *Luman*-deficient animals did not result in an elevation in adrenal medullary CA stores. LUMAN, being an ER associated protein, may be involved in the packaging of secretory vesicles and therefore this process is defective when LUMAN is downregulated.

Despite the evidence of an enhanced GR-mediated response in the *Luman*-deficient mice in both males (Fig 9E) and females (Fig. 10D), circulating CORT levels were only approximately half of those observed in WT controls (Females T= -5.2, P= 0.003, DF=5; Males T= -3.78, P=0.01, DF=5). We postulated that the low circulating CORT levels in *Luman*-deficient mice might result from enhanced GR mediated negative feedback control of pituitary ACTH release. To test this, we performed a DEX suppression test. In both *Luman*-deficient and WT mice, a significant decrease in CORT levels was observed following DEX treatment controls (Het T=11.59, P= 0.0001, DF=5; WT T= 5.24, P= 0.003, DF=5) (Fig. 9F), but the reduction was more pronounced in *Luman*-deficient than that in WT controls (F= 5.62, P= 0.04, DF=5). Six hours following treatment with DEX, corticotrophin release hormone (CRH) was injected to assess the pituitary-adrenal axis response to exogenous CRH stimulation (Fig. 9F). CRH administration increased CORT levels in both *Luman*-deficient and WT mice, but the response was greater in *Luman*-deficient mice (F= 11.52, P= 0.009, DF=5). Pre-treatment with the GR antagonist mifepristone eliminated this difference, so that responses of *Luman*-deficient and WT mice became indistinguishable (F= 2.92, P=0.13, DF=5), suggesting that the hypersensitivity to CRH in the *Luman*-deficient animals may have been secondary to enhanced GR-mediated negative feedback on the hypothalamus and pituitary. Finally, to determine the ACTH sensitivity of the adrenal gland, mice were injected with ACTH, and their CORT responses determined.
Luman-deficient mice had significantly lower CORT levels compared to the WT (F= 5.1, P= 0.049, DF=5), indicating a blunted response to ACTH (Fig. 9F).

The preceding data suggested that Luman deficiency is associated with enhanced GR mediated suppression of CRH and hence ACTH release. A corollary of this hypothesis is that Luman-deficient mice should also have an altered response to a nociceptive stimulus; stress-induced release of ACTH reduces pain sensitivity because it is co-released with endorphin (Herbert and Uhler, 1982). To test this, we examined the animals using the hotplate test to measure nociception. Luman-deficient mice did not exhibit the stress-induced analgesia observed in WT animals (first measurement: T= 0.16, P= 0.43, DF= 9; Second measurement: T= 5.6, P= 0.001, DF= 9) (Fig. 9G). Injection of mifepristone prior to testing eliminated the difference between the genotypes in stress-induced analgesia (first measurement: T= 0.64, P= 0.27, DF= 9; Second measurement: T= 0.53, P=0.30, DF= 9) (Fig. 9H). These results are consistent with the view that stress-induced CRH and ACTH release was impaired in Luman-deficient animals because of the aberrant GR-mediated negative feedback.

2.3.3 Luman-deficient mice exhibit low corticosterone, low body weight and decreased pup survival.

We have previously shown that LRF deficiency results in deficits in maternal behavior as a result of increased stress sensitivity post-partum (Martyn et al., 2012). In Luman-deficient mice there was also a maternal deficit evident by low pup survival and apparent maternal neglect, which was much more severe than in the LRF KO mice. Survival of pups in litters from Luman-deficient dams was significantly lower than in litters from WT dams (p>0.01, df= 11), while the survival rate of pups born to a KO dam was zero (Fig. 10A). We tracked the overall health of the pups from week one until week ten after birth. The average body weight of Luman-deficient pups was lower than that of WT pups (see appendix table 2 for descriptive statistics) (Fig. 10B), but
no other obvious defects were present. Histological analysis of mammary glands collected from the dams within 24 hours after parturition revealed secretory alveolar structures in both genotypes indicating that milk production was not a factor in the pup death (Fig. 10E and 10F). *Luman*-deficient female mice were also able to locate a buried food pellet using an olfactory cue as fast as WT female mice (p>0.05 in a Kruskal-Wallis test), indicating that no major olfactory abnormalities were present that might interfere with their ability to recognize pups (Sup Fig. 3).
Figure 10: Luman-deficient mice exhibit altered hormone profiles during pregnancy and low pup survival

(A) The survival rate of pups born to our WT C57BL/6N was determined to be approximately 80%, survival rate of pups born to our Luman-deficient mice is 20%; while if we cross foster these pups onto CD-1 mice we have a survival rate of 100%. Presented as mean ± SEM; *p <0.05 calculated by a one-tailed t-test (n = 6). (B) Luman-deficient mice are smaller than WT mice. WT, n = 5; Het, n = 7; Null, n = 6; for full descriptive stats see Sup. Table 2. (C, D) Prolactin (C) and Corticosterone (D) levels in Luman-deficient mice are chronically decreased over the duration of pregnancy and immediately after birth when compared to WT mice. Presented as mean ± SEM, * P ≤0.05; ** P ≤ 0.01 calculated by a two way repeated measures ANOVA (n = 6). (E, F) Mammary gland development and milk production are intact in Luman-deficient mice (postpartum day 1). n = 5. (G, H) A typical observation of the cage at postpartum day 2 of WT (G) and the Luman-deficient mice (H).
When pups from *Luman*-deficient females were cross-fostered to CD1 dams, 100% of the cross fostered pups survived (Fig 10A), indicating that a maternal defect was likely responsible for the differences in post-natal pup survival and development. Levels of both prolactin (F= 26.1192, P=0.000026, DF= 1) and corticosterone (CORT) (F= 31.802, P= 1.771e-08, DF=1) were chronically decreased during pregnancy and at parturition in the *Luman*-deficient mice (Fig. 10C and 10D), consistent with previous observations in *LRF*-deficient females (Martyn et al., 2012).
2.3.4 *Luman*-deficient mice have altered behavioural responses to stress.

Figure 11: *Luman*-deficient mice exhibit a blunted response to stress. (A, C) Summed time spent in the open arms. *Luman*-deficient mice spent significantly more time in the open arms as compared to the WT mice. Ratio of open arm entries indicated by open arm entries/total entries. The ratios of open arm entries of WT mice were significantly lower than those of *Luman* deficient mice. (D-F) Mice were placed in a specialized box containing lasers to measure position and movement of the mice. Time spent in the light vs dark compartment of the box was measured over a 10 minute duration. (G) Summed immobility score for female mice in the forced swim test. *Luman*-deficient mice showed significantly less immobility as compared to the WT controls. (H) Summed immobility score for female mice in the tail suspension test. (* P ≤ 0.05; ** P ≤ 0.01). n = 4 for each group. All behaviour tests were done using both WT and Null female mice. Values are mean ± SEM. P values calculated using a two tailed t test.
The preceding data suggested that *Luman*-deficient mice exhibit an abnormal response to stress associated with increased GR activity and consequent reductions in hypothalamic CRH release. To further test this hypothesis, four tests of stress and anxiety were performed.

The elevated plus maze (EPM) test assesses the level of anxiety and is particularly sensitive to intracerebral CRH levels, as CRH is anxiogenic (Martins et al., 2000). *Luman*-deficient mice spent significantly more time in the open arms when compared to WT animals. After controlling for overall increased movement by normalizing the data to the number of total entries, the *Luman*-deficient mice still showed results that were indicative of reduced anxiety (Fig. 11A-C).

Similarly, in the light/dark test of anxiety, *Luman*-deficient mice spent significantly more time in the light when compared to WT animals, indicating a lower level of anxiety (Fig. 11D-F). This was particularly prevalent in the first minute of the test, with a diminution in the difference between the groups in the latter half of the test.

Chronic mild stress represents one of the most reliable and widely used animal models of depression (Willner, 2005). We postulated that if *Luman*-deficient mice were expressing chronically elevated GR-mediated responses, differences might be observed between them and WT controls in terms of depressive behavior. Depression-like behaviour were assessed using the forced swim test (Fig. 11G) and the tail suspension test (Fig. 11H). While both results suggest a reduced level of depression-like behaviour in *Luman*-deficient mice, only the forced swim test showed statistically significant differences between the genotypes, with the *Luman*-deficient mice showing less immobility when compared to WT mice.
2.3.5 *Luman*-deficient mice have reduced branching of hippocampal CA3 pyramidal neurons.

**Figure 12: Hippocampal CA3 dendrites are truncated in *Luman*-deficient mice.** (A, B) Representative Nissl stained hippocampi from a *Luman*-deficient mouse and a WT mouse (n = 4) per group. (C, D) Sholl analysis of both apical and basal dendrites of the CA3 region of the hippocampus (E, F) Sholl analysis of both apical and basal dendrites of the CA1 region of the hippocampus. (G, H) Representative dendritic trees from a *Luman*-deficient mouse and a WT mouse from CA3 and CA1 regions of the hippocampus. These experiments were completed using both WT and Het male Mice. Data presented are averages of 5 neurons per mouse (n = 4). P<0.01, calculated using a one-way ANCOVA with a Tukey post hoc test.
The combination of enhanced GR-mediated feedback and altered behavioral stress responses suggested that the *Luman*-deficient mice might exhibit a syndrome that can be compared to that observed in WT animals exposed to chronically elevated corticosterone levels. In WT mice sustained high glucocorticoid levels can lead to excessive activity in the mossy fiber system innervating the CA3 pyramidal neurons, resulting in retraction of the apical dendritic tree of these neurons (Magarinos et al., 2011; McEwen and Magarinos, 2001). We reasoned that if a deficiency in LUMAN resulted in increased GR-mediated signaling, while the underlying cause is different, similar effects might be observed in the hippocampus of *Luman* deficient mice. Nissl staining revealed no overt changes in overall cell number in the principal cell fields of the hippocampus (Fig. 12A-B); however, Sholl analysis (Sholl, 1953) demonstrated marked reduction of branching in the dendritic trees of pyramidal layer neurons in area CA3, in *Luman*-deficient mice as compared to WT controls (Fig. 12C, D) (Apical: T=20.920, P=0.0001842, DF= 3; Basal T= 4.12, P= 0.000072, DF= 3). In contrast, no significant differences were found in the CA1 region between the two genotypes (Apical T= 0.974, P=0.332, DF= 3; Basal T=1.007, P=0.319, DF= 3), indicating that the impact of LUMAN did not extend throughout the entire *cornu ammonis* pyramidal cell layer (Fig. 12E, F).

2.4 DISCUSSION

Hypo- and hyper-reactivity of the GR system are believed to contribute to a wide range of disease states, functioning either as a predisposing factor or contributing to the onset of the disorder (Chrousos, 2009). Many of these disorders involve changes in central nervous system (CNS) glucocorticoid sensitivity and hence altered feedback control of hypothalamic and
pituitary hormone release, which can lead to the onset of psychopathologies including depression and post-traumatic stress disorder (PTSD) (Yehuda et al., 2004a; Yehuda et al., 2004b). Although some inherited disorders have been associated with changes in either the sequence or regulation of the GR gene, there is a substantial body of evidence indicating that other regulatory factors are also involved (Keenan et al., 2015). Here we demonstrate that deficiency of the stress-related protein LUMAN in both null and heterozygous mice alters GC and GR levels, leading to changes of the responsiveness of the HPA axis, as well as animals’ response to stress stimuli. Luman-deficient mice exhibit low levels of both anxiety and depressive-like behavior in all test paradigms that we have performed. As previously reported for LRF-deficient animals, the Luman-deficient dams have an increased rate of pup death compared to WT dams regardless of the genotype of the pups, pointing to possible altered maternal behavior and the associated excessive GC responsiveness at the time of parturition as the potential cause. By analogy with the LRF deficient model, we hypothesize that under normal physiological conditions the increased GR activity observed in Luman-deficient animals enhances the corticosterone-mediated negative feedback control of CRH and ACTH release, thereby impairing normal stress responses. During parturition, however, when GC release is independent of the pituitary (Barlow et al., 1974; Chida et al., 2011), the peripartum release of corticosterone may have an exaggerated effect in Luman-deficient mice, precipitating deficits in maternal behavior.

Altered HPA axis responsiveness has been repeatedly observed in patients suffering from disorders varying from hypertension and cardiovascular disease to some of the most prevalent psychopathologies. The mechanisms through which GR mediates the onset and symptoms of these disorders are not entirely clear (Pariante, 2009; Rosmond, 2005). Luman-deficient mice exhibit an altered response to stress that appears to be caused by exaggerated negative feedback
that is associated with high GR expression. Although $Luman$-deficient mice display a condition resembling chronic stress, this similarity may be superficial, given that the underlying mechanisms are not the same. Chronic stress results in hyper-activation of the CRH-ACTH-adrenal axis, leading to elevated GC levels, which cascade into the myriad of other symptoms associated with GC excess. In $Luman$-deficient animals, circulating corticosterone levels are below normal, because of enhanced GR-mediated negative feedback, presumably leading to reduced CRH release. $Luman$-deficient mice thus exhibit the unusual combination of low CORT levels, reduced anxiety (reflecting reduced anxiogenic effects of CRH), reduced depressive-like behavior, but enhanced GR-mediated responses. The reduction in dendritic branching in the apical and basal dendrites of CA3 pyramidal neurons in the hippocampus mirrors what has been seen in mouse models of chronic stress (McEwen, 2001); but again, the underlying defect may be different. Elevated GC levels (Magarinos et al., 1997) and CRH-mediated impairment of synaptic plasticity (Chen et al., 2013) have both been implicated in the truncation of hippocampal dendrites observed after stress, but neither are present in $Luman$-deficient animals. The changes in $Luman$-deficient mice presumably reflect increased GR mediated signaling, mimicking the effects of elevated corticosterone in WT animals, which is supported by observed increase of GR target genes in the mutant mice (Fig. 9B). Since it is more common to observe apical, not basal, CA3 dendrite reduction after chronic stress (Magarinos et al., 2011; Magarinos et al., 2006; Magarinos et al., 1997; Watanabe et al., 1992; Watanabe et al., 1993), it is possible that other mechanisms may also be involved. These possible explanations include, decreased secretion of neuroprotective elements like brain derived neurotrophic factor (BDNF) (Bath et al., 2012) or increased glutamatergic activity (Popoli et al., 2012), suppression of the expression of nucleoparin 62 (NUP-62) (Kinoshita et al., 2014) and increased polysialylated neuronal cell
adhesion molecule (PSA-NCAM) (McCall et al., 2013; Sandi and Bisaz, 2007) or alterations in other cell-surface molecules (van der Kooij et al., 2014). However, based on our current data, we believe the increase in GR activity is primarily responsible for the observed phenotype. While we acknowledge the alternative explanations, we have shown that an increase in GR activity exists in the Luman-deficient mice and previous research has shown that this increase can result in the behavioural, physiological and molecular changes we observed. However, these alternative explanations should be investigated to confirm or refute our hypothesis.

A question that remains is whether the initiating event is if the increase in GR expression, leading to the increase in the HPA negative feedback and decreased levels of GCs, or is a defect in GC secretion which subsequently led to the compensatory increase in GR expression. Our current data cannot rule out either possibility. As CORT is a membrane permeable steroid hormone and does not involve the vesicle-mediated secretory pathway, defects in secretion should not be responsible for the observed phenotype. It is, however, a possibility that a defect upstream of CORT secretion in the signalling pathway may exist. We have noted that the Luman-deficient mice can respond normally when given exogenous CRH (Fig. 9G); therefore, it is possible that the Luman-deficient mice do not exhibit normal stress-induced CRH release. In support of this, CREB3-Like protein 3 (CREB3L3, or CREB-H) and its Drosophila counterpart CREBA, have been shown to play a key role in regulating secretory capacity, especially during high secretory demand (Barbosa et al., 2013; Abrams and Andrew, 2005; Fox et al., 2010; Bailey et al., 2007). In addition, all mammalian CREB3-like proteins can activate the expression of secretory pathway component genes (Barbosa et al., 2013). LUMAN has also been implicated in Golgi stress responses (Reiling et al., 2013). We are currently investigating the possible role of LUMAN in secretory function of both neurons and non-neuronal cells that have a high demand
for protein production. Whichever event initiates the alterations in the pathway, the common outcome is the enhanced GR sensitivity resulting in an altered GR stress response and HPA axis dysregulation which is the focus of this chapter.

Accumulating evidence suggests that a number of human diseases result from alterations in GC sensitivity. For example, the onset of PTSD is associated with an altered stress response. In affected individuals, studies have repeatedly found alterations in GR levels and enhanced GC negative feedback, resulting in low GCs, demonstrated through an exaggerated response to the DEX suppression test (Liberzon et al., 1990). In addition, two studies have shown that enhanced induction of FKBP5 with cortisol, and altered negative feedback in controls, are associated with increased risk of developing PTSD (Koenen, 2005; Ozer et al., 2003). Stress during development programs GC sensitivity later in life, affecting susceptibility to neurological and psychiatric disorders (Babenko et al., 2015) as well as metabolic and cardiovascular disease (Moisiadis and Matthews, 2014a, b). While, in some cases, alterations in GR sensitivity have been associated with changes in the structure of the GR gene in either the coding or promoter regions (Huizenga et al., 1998), others appear to be linked to factors that regulate GR itself, or are involved in the HPA axis (Chen et al., 2013). The demonstration that LUMAN, and its associated regulatory protein LRF, affect GR regulation, thereby altering in vivo stress responses, raises the possibility that these factors could also contribute to the physiological regulation of GC sensitivity. Further work will be required to determine whether alterations in LUMAN and/or LRF play a role in disease states associated with altered GR function.
In conclusion, Luman-deficient mice exhibit a blunted stress response, with low levels of anxiety and less depressive-like behaviour, associated with a hypoactive HPA axis, which is caused by enhanced GR sensitivity. We thus postulate that LUMAN plays a key role in regulating GR activity and may therefore be involved in the pathology of disorders involving alterations in GR sensitivity.

Chapter 3: LUMAN plays a dual role in altering the stress response by acting as a coactivator of GR and a regulator of secretion

Abstract

LUMAN/CREB3, originally identified through its interaction with a cell cycle regulator HCFC1, is a transcription factor involved in the unfolded protein response during endoplasmic reticulum (ER) stress. Previously we have shown that LUMAN modulates the glucocorticoid (GC) response by regulating the expression of the glucocorticoid receptor (GR), leading to enhanced GR activity and lower circulating GC levels. Consequently, the central stress response system, the hypothalamic pituitary adrenal (HPA) axis, is dysregulated leading to a blunted stress response in the Luman-deficient mice. These observations have led to two hypotheses: (1) a decrease in LUMAN expression causes an increase in GR levels, resulting in increased negative feedback in the HPA axis, leading to low GC secretion, and (2) LUMAN deficiency results in defective secretion of GCs, leading to a compensatory increase in GR during development which persists through life. These two possibilities are not mutually exclusive, and a variety of experiments were conducted to delineate the mechanism by which LUMAN affects HPA axis function. We found that LUMAN interacts with GR through the NR box and activates GR in the absence of a ligand. Further investigation showed that, when activated, LUMAN binds to the GRE, increasing the activity of GR exponentially compared to GR-ligand binding alone. On the
other hand, we also found that in the absence of LUMAN, cells were more sensitive to cellular stress, with decreased secretory capacity. Hence our current data suggest that LUMAN may function both as the transcription cofactor of GR and a hormone secretion regulator. We thus postulate that LUMAN plays dual roles as a key regulator of animal sensitivity and reactivity to stress.

### 3.1 Introduction

The secretion of glucocorticoids (GCs) is induced by neuroendocrine responses to stress in mammals and has numerous downstream effects. Aberrations in this response have been linked to several highly prevalent mental disorders such as depression, anxiety, as well as various metabolic diseases and cancers (Pariante, 2009). Understanding the underlying mechanisms in GC dysregulation is key in preventing and treating these diseases. Dissecting and analyzing factors involved in the primary stress response, the hypothalamic pituitary adrenal (HPA) axis, will help gain critical knowledge of these mechanisms. GCs are released following a cascade of events that occur in the HPA axis. Corticotropin releasing hormone (CRH) is released from the hypothalamus triggering the release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary, which acts on the adrenal cortex to initiate the synthesis and secretion of GCs. GCs then negatively regulate their release by inhibiting the secretion of CRH and ACTH from the hypothalamus and pituitary respectively (O'Connor et al., 2000). One of the primary differences between GCs and CRH/ACTH is that, as steroid hormones, GCs can move freely into cells while CRH/ACTH are peptide hormones and therefore are synthesized, stored in vesicles and rapidly released when triggered by stress (Stevens and White, 2010).

Secretion takes place at low levels in most cells types and at extremely high levels in specialized cells, such as cells found in the pancreas, the salivary gland and various
neuroendocrine tissues including neurosecretory cells in the hypothalamus and pituitary (Barlowe and Miller, 2013). When the stress response is elicited, a number of important changes occur, including increased secretion of CRH, ACTH and GCs, all of which has to occur quickly in order to initiate an appropriate response (Pariante and Lightman, 2008). Importantly, while these hormones are the main effectors of the stress response, many other factors must be selectively upregulated in order to coordinate this response, including enzymes responsible for synthesizing GCs such as 3β-hydroxysteroid dehydrogenase and 21-hydroxylase (Whirledge and Cidlowski, 2010). The mechanism by which secretory capacity is selectively upregulated in specific cell types is not well understood. CREBA is a transcription factor found in Drosophila that has been implicated in secretion; the mammalian homologues are proteins belonging to the CREB3 family (Fox et al., 2010). It has been shown that CREBA and CREB3 family members of bZip transcription factors can function to up-regulate expression of protein machinery required in all cell types for basal secretion and in specialized cells with an increased secretory capacity (Dalle et al., 2011). Furthermore, over half of the genes that require CREBA encode identifiable secretory components and phenotypes associated with the loss of CREBA are consistent with the role it plays in secretion (Abrams and Andrew, 2005).

CREB3, also known as LUMAN, is an endoplasmic reticulum (ER) membrane-bound transcription factor that is involved in ER stress and the related unfolded protein response (UPR) (Audas et al., 2008) as well as being involved in the Golgi stress response (Taniguchi and Yoshida, 2017). Cellular stressors can decrease the secretory capacity of cells as well as decrease the efficacy of the protein folding and modification machinery in the ER, leading to an accumulation of misfolded proteins in the ER (Moore and Hollien, 2012). The UPR is a highly conserved mechanism that is activated in response to accumulation of misfolded proteins. The
main purpose of the UPR is to restore normal function of the cell by halting protein translation, degrading misfolded proteins and increasing the production of molecular chaperones involved in protein folding (Schroder and Kaufman, 2005). LUMAN is known to play an important role in cellular stress responses including parts of the UPR. It is also involved in the physiological stress response altering GC and GR activity in mice. LUMAN contains two LxxLL nuclear receptor (NR) binding motifs common to co-factors in some NRs (Luciano and Wilson, 2000; Penney et al., 2017). LUMAN is most highly expressed in the hypothalamus, hippocampus, anterior pituitary and adrenal gland. We have previously shown that Luman-deficient mice have low levels of corticosterone and high levels of its receptor, the glucocorticoid receptor (GR), resulting in enhanced GR activity (Penney et al., 2017). However, the mechanism by which LUMAN deficiency causes this phenotype is not completely understood. The question that remains is whether the initiating event is the increase in GR expression which would result in the increase in negative feedback in the HPA axis decreasing GC levels, and/or if a secretion defect causes low GCs which could subsequently lead to the compensatory increase in GR expression. It is important to note that these mechanisms are not mutually exclusive.

In this chapter, I present data that indicates LUMAN binds to GR through the LxxLL motif and that it alters GR activity through this interaction as well as binding to the glucocorticoid response element (GRE). Also, in the absence of LUMAN, the cells are more sensitive to cellular stress, decreasing the secretory capacity of the cell. I also present data that indicates LUMAN binds to the unfolded protein response element (UPRE) found in two of the proteins found in COPII secretory vesicles. To help discern the mechanism through which LUMAN alters GR activity, I performed an assay to look at the secretion of CRH when LUMAN
is deficient. This assay shows no difference in circulating CRH levels between Luman-deficient and wildtype mice.

3.2 Materials and Methods

Animals. This study followed the Canadian Council of Animal Care guidelines and was approved by the Animal Care Committee at the University of Guelph. The LUMAN gene knockout mouse line was generated in collaboration with the International Gene Trap Consortium (Nord et al., 2006). Chimeric mice were backcrossed to C57BL/6 mice (Charles River, Montreal, Canada) to produce a 99.9% congenic mouse strain. C57BL/6NTac mice were group housed with same-sex siblings and maintained on a 12-h light/dark cycle (10:00-22:00). Temperature was maintained at 21-24 °C, and food (2014 Teklad Global 14% protein rodent maintenance diet) and tap water were provided ad libitum. To obtain sufficient mice in certain circumstances pups from LUMAN KO/HET mice were cross fostered onto CD1 dams. Due to low LUMAN KO pup survival, heterozygote mice were used in all experiments except the behaviour assays where LUMAN KO mice were used. Mice were euthanized by cervical dislocation and tissues were collected either in liquid nitrogen for protein and mRNA extraction or in 4% Paraformaldehyde for histological analysis.

Cell culture. All cell types were grown in monolayer culture in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 g/ml streptomycin. All cultures were maintained in a 5% CO2 humidified atmosphere at 37 °C and passaged every 2 to 3 days. Cells were plated 24 h prior to transfection and allowed to grow to 60% confluence prior to transfection. Cells were transfected by polyjet transfection reagent (SignaGen Laboratories) as per the manufacturer’s instruction.
**ELISAs.** Blood samples (100 to 150 μl) were collected in the active cycle (1100 to 1300 h) from the saphenous vein of the hind limb; serum was separated and stored at -80 °C. Hormone levels were detected using a CRH enzyme-linked immunosorbent assay (ELISA) kit (Aviva Biosystems, San Diego, CA) as per the manufacturers’ instructions, and detected using a POLARstar Omega plate reader (BMG Labtech GmbH, Offenburg, Germany). Statistical analysis was performed using a two way ANOVA with repeated measures. Data were deemed significant at a P value <0.05.

**RNA analysis and qRT-PCR.** Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from adult mouse tissues. cDNA was synthesized from total RNA using SuperScriptIII reverse transcriptase (Invitrogen) and oligo(dT) (Roche Diagnostics, Laval, QC, Canada). Transcript levels were measured by quantitative RT-PCR (qRT-PCR) using PerfeCTa SYBR green Supermix with 6-carboxy-X-rhodamine (ROX) (Quanta Biosciences, Inc., Gaithersburg, MD) and primers against the mouse genes. Samples were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and subjected to standard curve analysis, and arbitrary values were represented, adjusting for primer efficiencies. For primer sequences see Appendix, Table 1.

**Protein extraction and western blot analysis.** Tissues were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) RNA was extracted and the phenol phase was frozen at -80 °C until protein extraction was done. Isopropanol precipitation was performed to isolate the protein. Total protein was quantified using Pierce® BCA protein assay reagent (Thermo Scientific, USA) according to the manufacturer’s instructions. The blots were visualized using ECL (GE Healthcare, Piscataway, NJ) on Amersham Hyperfilm ECL (GE Healthcare) or using the ChemiDoc XRS+ imaging system (Biorad).
**Antibodies:** Primary antibodies were used at the following dilutions: GR polyclonal antibody (sc-1004; Santa Cruz) at 1:400, Creb3 polyclonal antibody (Proteintech), Lamin polyclonal antibody (abcam, ab26300) at 1:1000 and Tubulin monoclonal antibody (abcam, ab7291). Secondary horseradish peroxidase (HRP)-conjugated antibodies were used at 1:10,000 (Promega).

**Dual Luciferase Assay.** HEK293 cell cultures were grown to approximately 70% confluence prior to transfection using polyjet (SignaGen Laboratories) using manufacturer’s instructions. The cells were co-transfected in a 12 well plate with 0.3 μg of MMTV-luc, 0.3ug of GR, 0.05ug of pRL-SV40 (promega) and 0.2ug of either pcDNA3.1, pcLUMAN, pc N terminal LUMAN, LxxLL KO N-terminal LUMAN, Δ AD N terminal LUMAN or ΔDBD N Terminal LUMAN . At 16-18 h post-transfection, the medium was replaced to allow the cells to recover for 8 h. Dexamethasone was then added and incubated for 12h. The cells were harvested, and dual luciferase assays were carried out according to the manufacturer's instruction (Promega). Reporter activity was calculated as relative luciferase activity (firefly luciferase/Renilla luciferase) to correct for transfection efficiency. Assays were independently repeated at least five times, and results are shown with standard error. Statistical analysis was done using a one-way ANOVA post hoc Tukey test, the data had to be log transformed to meet the assumptions of normality.

**Cellular Fractionation** HEK293 cells were grown to 70% confluency and transfected with Polyethyleneimine as per manufacturer’s instructions (Santa Cruz, CA), 36 hours after transfection the cells with treated with Dexamethasone (100nm) (or ETOH for control), 12 hours after treatment the cells were collected using the Genetex: Fractionation of Membrane/Cytoplasmic and Nuclear Proteins protocol. In brief, cells were collected in cold PBS, spun
down and resuspended in a hypotonic buffer, after a 15 minute incubation detergent (NP40) was added, mixed and the samples were centrifuged, the supernatant was kept as the cytoplasmic fraction. The nuclear pellet was re-suspended in cell extraction buffer, incubated for 30 minutes after which the sample was centrifuged and the supernatant was transferred to a new tube as the nuclear fraction. These samples were then either stored at -80 freezer or run immediately on an SDS page gel.

**Coimmunoprecipitation** For lysis and co-immunoprecipitation of various LUMAN constructs and the GR, HEK293 cells were transfected with indicated vectors using Polyethyleneimine as per manufacturer’s instructions (Santa Cruz, CA). Media was changed after 6h, 40 hours after transfection cells were crosslinked using 1% paraformaldehyde (Sigma) for 10 minutes and were then lysed in RIPA buffer (150mM NaCl, 1% (V/V) triton x-100, 0.5% (V/V) sodium deoxycholate, 0.1% (V/V) SDS, 50mM Tris) supplemented with 1mM PMSF as well as 10ug/ml aprotinin and leupeptin at 4°C for 10min. After centrifugation (4°C, 13,000 RPM, 10min), 10% of the lysate was taken as input and the indicated antibody was immediately added to the rest of the supernatant and incubated on a rotator at 4°C for 4h. Immunoprecipitation was performed using Sera-Mag SpeedBead Protein A/G (GE Healthcare) following the manufacturer’s protocol. The lysates and immunoprecipitates were detected by western blot using the antibodies indicated by measurement with Pierce ECL western blotting substrate (Thermo Fischer, Rockford, IL).

**Chromatin immunoprecipitation** m-Hippo-E14 cells were cultured in 10-cm plates and either left untreated or were treated with 100nM of DEXamethasone. The chromatin immunoprecipitation (ChIP) assay was performed using the Chromatrap ChIP-seq Protein G kit following the manufacturer’s instructions. Briefly, after cross-linking in 1% formaldehyde, the cells were lysed and sonicated yielding fragments 200-600bp. A 10% aliquot of the precleared
chromatin was taken as input, and the rest was incubated with either 2μg of CREB3 (Proteintech) or 2 μg of Rabbit IGG followed by immunoprecipitation. After reversing the formaldehyde-induced cross-linking, the chromatin DNA was used in Q-RTPCR, using primers that bind in the promoter region of each gene within 200bp of a GRE site, for primer sequences see Appendix, supplemental Table 1.

**Viral infection and trafficking** Mouse embryonic fibroblasts were infected with an adenoviral vector expressing GFP–VSV-G<sup>ts0–45</sup> (VSVG; X. Zha and R. Parks, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada) and incubated at 40.5°C for 18–20 h to allow VSVG accumulation in the ER. Under treatment cells were incubated with 200nM Brefeldin A for 3 hours. Cells were then transferred to 32°C and incubated for specific time periods (10 min, 20 min and 1hr) to allow the VSVG to move to the Golgi/PM. The cells were fixed the specific time period using 4% PFA. Cells were subsequently visualized and analyzed with a confocal microscope (Quorum Diskovery Spinning Disk Confocal System). VSVG was considered ER associated if VSVG could be visualized within ER compartments, whereas VSVG was considered Golgi associated once VSVG entirely co-localized with the Golgi marker GM130. Data were expressed as a percentage of total cell number in each condition. Lentiviral infection of Gaussia Luciferase (GLuc) (B. Tannous, Harvard Medical School, Boston, MA) was done in mouse embryonic fibroblasts and the conditioned media was collected and assayed to assess the level of secretion. The efficiency of infection was calculated and used to standardize the data. 48 h after infection, media were changed, and 50-µl media samples were harvested at various time points for analyses for GLuc activity to determine the rate of secretion. For the Gluc assay Statistical analysis was done using a two-way ANOVA post hoc Tukey; for the VSVG a two way ANOVA post hoc Dunnett’s T test was used.
Immunostaining: Cells were fixed for 5 min in ice-cold methanol and blocked for 60 min in 10% goat serum at room temperature. Antibody incubations (Creb3 1:200, proteintech; Alexa594, anti-mouse IgG 1:400, Molecular Probes and Alexa488, anti-rabbit IGG 1:400) were for 30 min at 37 °C with 5% CO2. Glass cover slips were mounted in 50% glycerol/500 pmol DAPI solution and sealed with nail polish. Images were visualized with a confocal microscope (Quorum Diskovery Spinning Disk Confocal System).
3.3 Results

3.3.1 LUMAN alters GR activity.

**Figure 13: LUMAN alters GR activity.** (A) Activation of the MMTV promotor by LUMAN. HEK293 cells were transiently transfected with pGL3-MMTV-Luciferase reporter together with the reference *Renilla* luciferase plasmid pRL-SV40, GR and indicated effector plasmid. Luciferase values from five independent experiments were normalized to *Renilla* luciferase activity before being referenced to control values. Cell lysates from the luciferase assay were subjected to western blot analysis using the following antibodies CREB3 (1:1000), GR (G-5) (1:400). Presented as mean ± SEM; *p* <0.05 calculated by a one way and a Tukey test (Untreated compared to pcDNA control, treated compared to pcDNA + DEX). (B) A cellular fractionation shows the localization of both GR and LUMAN using Lamin (1:1000) and Tubulin (1:1000) as controls. (C) Using immunofluorescence we confirm the localization of GR (red-594) and CREB3 (green-488) as well as looking at the mutant LUMAN constructs (Zoomed out pictures can be seen in Sup. Fig. 9). Scale bars: 10µM.
To assess how LUMAN alters GR activity a dual luciferase assay was performed in HEK293 cells to allow for the overexpression of various LUMAN constructs. The data shown indicates that N-terminal LUMAN can activate the MMTV (mouse mammary tumor virus) reporter, which contains GREs (Belikov et al., 2001), in the absence of a ligand (DEX) (T= 19.313, P= 2e\(^{-16}\), DF=4) (Fig. 13A). When both N-terminal LUMAN and DEX is administered the reporter activity increases dramatically compared to either factor alone (T= 5.773, P= 1.80e\(^{-05}\), DF=4). When full-length LUMAN is overexpressed there is no activation seen when compared to control (T= 1.390, P= 0.175, DF=4) (pcDNA), however, once DEX is added activation is observed (T= 1.94, P= 0.004, DF=4) but the effect appears to be dampened compared to DEX treatment alone (DEX alone vs DEX+ FL: T=4.741, P= 0.000163, DF=4) (Fig. 13A).

To determine the regions required for activation, various deletion mutants of LUMAN, LxxLL KOLxxLL KO (NR box), ΔAD (activation domain) and ΔDBD (DNA binding domain mutated) were tested (Fig. 13A). Using the LxxLL KOLxxLL KO mutant without treatment there is activation compared to pcDNA control (T= 12.161, P= 3.99e\(^{-13}\), DF=4). When treated with DEX a dramatic increase in activation was seen compared to DEX alone similar to cells overexpressing N terminal and treated with DEX (T= 5.773, P= 1.80e\(^{-05}\), DF=4). With overexpression of ΔAD, no difference in activation was seen in the absence or presence of DEX when compared to the control values (pcDNA and pcDNA +DEX respectively) (pcDNA: T= 0.425, P= 0.674, DF=4; pcDNA + DEX: T= 0.703, P= 0.491118, DF=4) (Fig. 13A). However, the ΔAD mutant appears to produce a very unstable protein that is not detectable through western blot or immunocytochemical techniques but expression of the construct has been confirmed through q-RT-PCR (Sup. Fig.12). The ΔDBD mutant does not affect GR activity in the absence
of DEX (T= 1.117, P= 0.273, DF=4), however, under DEX treatment the ΔDBD mutant induces GR activity significantly when compared to DEX alone (T= 2.502, P= 0.022222, DF=4). Interestingly, this enhanced activation seen with the ΔDBD mutant and DEX is significantly lower when compared to N terminal+ DEX or the LxxLL KO mutant + DEX (N-term: T= 5.48, P= 0.002, DF=4; LxxLL KO T= 7.37, P=0.008, DF=4) (Fig. 13A), indicating different mechanisms may be at play.

Through immunostaining (Fig. 13C) and sub-cellular fractionation (Fig. 13B) we show that full length LUMAN appeared to be predominantly located in the cytoplasm, N terminal LUMAN was localized in the nucleus, LxxLL KO and ΔDBD were also in the nucleus. While GR was predominantly localized in the cytoplasm until treated with DEX which caused it to translocate to the nucleus (Fig. 13B, C).

3.3.2 LUMAN binds to GR through the LxxLL motifs

Mouse embryonic day 18 hippocampal cells (m-hippo-E18) were used to look for LUMAN targets due to the relatively high LUMAN expression found in these cells. As well, these cells are physiologically relevant when investigating HPA axis regulation. Using chromatin immunoprecipitation, we found that when the cells were not treated (NT) LUMAN did not bind to any of the GR responsive genes examined. However, when the cells were treated with DEX (100nM) LUMAN bound to the promoter region of each of the genes that contain glucocorticoid respond elements (GREs): period homolog 1 (Per1) and dopamine decarboxylase (DDC) and FK506 Binding Protein 5 (FKBP5) (Fig. 14A). To investigate if LUMAN interacts with GR at the protein level, coimmunoprecipitation experiments were performed, both to look for endogenous interaction (m-hippo-E18 cells) (Fig. 14B), and in transfected HEK293 cells (Fig. 14C) to allow mapping of the interaction sites (Fig. 14C).
Figure 14: LUMAN binds to GR through the LxxLL motifs. (A) Chromatin immunoprecipitations were performed using mHippo-E18 cells showing that with DEX treatment, LUMAN binds to the promoter of FKBSP5, Per1 and DDC all containing GREs. Primers for GAPDH and IGG pull downs were both used as negative controls. All data is representative of 3 independent replicates, presented as mean ± SEM. (B-C) Coimmunoprecipitation experiments were performed in both mHippo-E14 cells (B), looking for endogenous interaction, as well as HEK293 cells transfected with various constructs to help map the interaction (C). Here we show that GR interacts with both full-length and N-terminal LUMAN but not the LxxLL KO N-terminal LUMAN mutant. CREB3 antibody (proteintech) and GR (G-5 Santacruz) was used to pulldown their respective proteins, IGG was used as a negative control. Full blots can be seen in Appendix: Sup. Fig. 6.
3.3.3 In the absence of LUMAN, cells have reduced secretory capacity under stress

To investigate potential differences in the cellular secretion pathway two lines of mouse embryonic fibroblasts (MEFs) were used in these experiments, a wildtype (WT) strain and a Luman knockout (KO) strain. We found that in the absence of LUMAN the cells were more sensitive to Brefeldin A (BFA), while no other stressors showed a significant difference between WT and KO cells (tunacamycin, thapsigargin, monensin, MG132, BFA and DEX were all tested – data not shown). In the Gaussia Luciferase (Gluc) assay (Fig. 15 A-C) assay, several ER and Golgi stressors were used (listed above) (Fig. 15C). The results from the Gluc assay (Fig. 15A-C) indicate that in control experiments (no treatment or ethanol) there was no difference in flux through the secretory pathway (F=0.0307, P=0.8622, DF=1) (Fig. 15B). When these cells were treated with a low level of BFA (200nm), secretion was delayed in the KO cells when compared to WT (F=13.2471, P=0.001188, DF=1) (Fig. 15C). (Fig. 15C, G). These results were consistent with the VSVG assay (Control F= 0.5832, P= 0.4540, DF=1) (BFA treated F=20.920, P=0.0001842, DF=1) (Fig. 15D-G). Confirmation that treatment with 200nM BFA induced LUMAN cleavage is shown in Sup. Fig. 4 to indicate that LUMAN is active under these conditions.
Figure 15: LUMAN -/- cells have reduced secretory capacity under stress. (A) Infection efficiency of Gaussia Luciferase (GLuc) virus shown through co-staining with DAPI (A-C) GLuc was delivered to cells using an adenovirus, and its secretion was monitored by measuring luciferase activity in the media at specific time points under control conditions (B) as well as treatment with brefeldin A (BFA) (C). GLuc data shown is presented as the average of 5 independent experiments, showing mean ± SEM; *p < 0.05 calculated by a two way ANOVA, post hoc Tukey (n=4) (D-E) Trafficking of VSVG was unaffected by removing LUMAN under control or vehicle conditions, (E) No difference was seen in Golgi localization after 20 minutes or plasma membrane localization after 60 minutes. (F-G) An exaggerated delay in trafficking of VSVG from the ER to the Golgi in the absence of LUMAN was shown under 3h treatment with BFA (200nM) (F) The percent of VSVG stuck in the ER was higher in the KO cells when compared to WT. Representative images shown from 3 independent experiments completed in duplicate, counting a total of 300 cells per group (50 per coverslip), presented as mean ± SEM; *p <0.05 calculated by a two way ANOVA post hoc Dunnett’s T test. Scale bar: 10µM. GM130 co-staining can be seen in Sup. Fig. 15.
3.3.4 In the absence of LUMAN, expression of COPII components are altered during stress

To investigate if LUMAN affects COPII vesicle formation I assessed the expression of the major proteins that make up these vesicles and how LUMAN may affect them. Chromatin immunoprecipitation was performed using M-Hippo-E18 cells under control conditions (no treatment or ethanol) and cells treated with BFA (1uM) (Fig. 16A). Under control conditions, LUMAN was not bound to the promoter region of the genes that were examined however under treatment with BFA there was binding to genes containing UPREs. All of these genes examined encode proteins that are integral components of COPII vesicles. The expression of the five main components of COPII vesicles were then examined using Q-RTPCR from both the hippocampus of WT and Luman-deficient mice (Fig. 16B) as well as WT and KO MEF cell lines (Fig. 16C). Without treatment, the only genes that showed a significant difference between Luman-deficient and WT samples were Sec23 and SAR1 in the mouse hippocampus (Fig. 16B) (SAR1: T= 4.64, P= 0.02, DF=3; Sec23: T= 3.8, P= 0.016, DF=3) (Fig. 16C). However, in WT cells (Fig. 16C), when treated with BFA, we see a significant increase in all COPII components compared to control or untreated levels (F= 18.2929, P= 3.117e^-08, DF=3), this overall increase in expression of these genes is not observed in the Luman KO cells; (F= 1.801, P=0.1797, DF=3) (Fig. 16C).
Figure 16: In the absence of LUMAN, expression of COPII components are altered during stress. (A) Chromatin immunoprecipitation was performed using mHippo-E18 cells showing that, when treated with Brefeldin A, LUMAN binds to the promoter region of SEC13 and SEC23 genes. Primers for GAPDH and IGG pull downs were both used as negative controls. (B-C) Transcript levels of the COPII components were measured from RNA extracted from the hippocampus (B) of wildtype and Luman-deficient mice, data represented as a mean proportion of WT ± SEM. The expression level of the same transcripts were measured in RNA collected from MEF cells (C) Transcript level is presented as a ratio of BFA treated / control; where the controls for WT and KO samples were untreated/vehicle samples from WT and KO cells respectively to show the effect of BFA on these components. This data was collected from extracted RNA reverse transcribed into cDNA and analyzed using Q RTPCR. The dotted line indicates all control data were set to one. *p>0.05 calculated using a Two way ANOVA post hoc Tukey (n=4).
3.4 Discussion

Variation in HPA axis reactivity has been shown to play an important role in precipitating the onset of many prevalent mental disorders (Chrousos, 2009). Although these variations in HPA axis reactivity may not always be associated with a pathological outcome, they can provide a possible mechanism for differences in stress sensitivity (Quax et al., 2013). Thus, identifying and examining factors that affect HPA axis function is important to understand the underlying mechanism of stress sensitivity. We have previously identified the stress-induced transcription factor LUMAN as one such factor. Here, we examine the molecular mechanism of LUMANs effect on the HPA axis, showing that it alters GR activity through acting as a cofactor of GR and possibly altering the secretory capacity of cells during stress-induced increase in secretory load.

Previously, we have shown that in the absence of LUMAN the expression of GR increases, whereas circulating CORT levels decrease (Penney et al., 2017). In comparison to these observations that result in the blunted stress response observed in the Luman-deficient mice, patients suffering from depression show the opposite trend of low GR expression and increasing circulating CORT levels (Pariante and Lightman, 2008). Here, we present evidence that LUMAN binds to GR through the LxxLL binding motif and that this interaction results in increased activity of GR both in the presence and absence of a ligand (DEX). When the LxxLL motif was mutated (LxxLL KO), the activation without DEX was still present, but not to the same extent as seen with the N-terminal domain. Under DEX treatment a dramatic increase in activation was comparable to that seen with N-terminal LUMAN. This suggests that LUMAN acts as a coactivator of GR through binding at the protein level as well as alternative mechanisms. However, when LUMAN is unable to bind to GR it cannot activate it to the same extent in the absence of a ligand. When activated via DEX, LUMAN translocate to the nucleus,
and act as both a transcription factor binding to GRE, and a cofactor, binding to GR. When
LUMAN is unable to bind directly to DNA (ΔDBD), there is no activation seen without DEX
treatment, suggesting that the ligand-independent activation of GR seen with N-terminal
LUMAN is facilitated through LUMAN’s transcriptional activity. However, the ΔDBD mutant
can still enhance GR activity under DEX treatment. Taken together these data suggests that
LUMAN plays a dual role as a transcription factor, binding to promoter regions in DNA and as a
co-factor of GR, altering the GR-mediated stress response.

Altered GR function has been a consistent finding when LUMAN is deficient, but a
paradoxical relationship exists; when LUMAN is deficient, GR activity is enhanced, while here
evidence suggests that LUMAN acts as a coactivator of GR. It is important to note that GR is
essential during embryonic development, and when it is absent there are many defects including
lack of lung maturation resulting in death shortly after birth (Reichardt et al., 1998). Therefore, it
is possible that when an important coactivator of GR is absent, such as LUMAN, GR levels
increase to compensate for the absent factor to allow for survival. When specific NCoAs are
absent, such as SRC-1, it has been shown that the activity of the NR is altered (Tetel, 2009). This
includes the progesterone (PR) and estrogen receptor resulting in decreased fertility consistent
with the Luman-deficient mice (Tetel and Acharya, 2013). While the estrogen receptor and PR
are integral to many biological functions, they are not necessary for survival; however, GR
function is required during development and therefore its activity must be preserved for survival
(Tetel, 2009; Tetel and Acharya, 2013). This increase in GR expression would lead to enhanced
negative feedback in the HPA axis, resulting in decreased circulating CORT levels. Each of these
characteristics are observed in the Luman-deficient mice. However, this is not the only
mechanism through with LUMAN can exert its effects and alternative mechanisms need to be explored, including the potential role for LUMAN in altering the secretory capacity under stress.

The HPA axis is the major neuroendocrine system that is responsible for the stress response in mammals (Pariante and Lightman, 2008). This system contains many cell types that have a high secretory demand, particularly during stress. Therefore, in order to mount a normal response to stress there are factors that function to selectively increase secretory capacity (Turner et al., 1992). The mechanism through which secretory capacity is selectively increased is not completely understood, and therefore identifying new potential factors that could be involved is of great interest. Previous research has shown that proteins belonging to the CREB3 family are involved in cellular secretion, including CREBL1, CREB3L2 and the Drosophila counterpart CREB A. CREB3L1 is essential for bone formation, through activating the secretion of bone matrix proteins, while CREB3L2 is responsible for the secretion of collagens and other extracellular matrix proteins during normal chondrogenesis (Chan et al., 2011; Fox and Andrew, 2015; Fox et al., 2010). CREB3 proteins are essential in the secretion pathway and their functions differ primarily due to different expression patterns.

We have identified LUMAN, which is highly expressed in neuroendocrine tissues, as a potential factor that can selectively increase the secretory capacity in cells that play an important role in HPA axis function. There was no significant difference in the stress-induced secretion of CRH between Luman-deficient and WT mice (Sup. Fig. 5); however, this does not eliminate secretion as a possible mechanism through which LUMAN works. Regulation of CRH secretion is complex and occurs through numerous mechanisms, including immune regulation through interleukin-1β, in addition to peptide hormones such as norepinephrine (NE), serotonin (Wei et al., 2002) and brain derived neurotrophic factor (BDNF) (Jeanneteau et al., 2012). In the Luman-
deficient mice I found 2-3X lower expression of BDNF in the hippocampus when compared to WT mice (Sup. Fig. 10), which could contribute to CRH secretion regulation. Previously we have shown that in the Luman-deficient mice there appear to be less storage vesicles found in the adrenal medulla, which are believed to contain catecholamines (CA). However, no difference was found in the level of circulating CA, indicating other mechanisms may be at play (Penney et al., 2017). Taking this into account, it is possible that many points of control for CRH secretion are responsible for maintaining normal levels of circulating CRH in the absence of LUMAN. Data collected using LUMAN KO and WT MEF cells suggest that LUMAN increases the secretory capacity through enhancing elements of the UPR during times of high secretory demand. Data show that secretion is stunted in the absence of LUMAN when cellular stress is elicited. Furthermore, the key proteins that make up COPII vesicles do not show the normal stress-induced increased expression in the absence of LUMAN. This suggests that the COPII components may be downstream targets of LUMAN which are activated in response to cellular stress, to maintain ER homeostasis during high secretion demand during animal stress responses.

In conclusion, LUMAN alters GR activity through binding GR via the LxxLL motif, and its actions as a transcription factor, binding GREs independently of GR. Additionally, LUMAN alters the secretory capacity of cells when the secretion demand is high through altering expression of the components of COPII vesicles. We therefore postulate that LUMAN plays dual roles in the stress response, regulating secretion at the cellular level, and acting as a cofactor of GR. It is clear that LUMAN plays key roles in the stress response, altering stress sensitivity; this suggests LUMAN as a potential factor that may be involved in the development of stress-related pathologies.
Chapter 4: LUMAN’s role in Metabolism and Obesity

In addition to the previously presented data studying LUMAN’s role in the stress response, potential connections with metabolism were also investigated. Here I present preliminary data that suggests the role of LUMAN in this research direction, providing a basis for future research.

4.1 Introduction

The prevalence of obesity has increased dramatically in North America over the past two decades (Bose et al., 2009). Obesity is one feature of metabolic syndrome, additional clinical findings including glucose intolerance and hypertension; all posing a significant threat to one’s health and amplifying the risk of diabetes and cardiovascular disease (Cornier et al., 2008; DeFronzo and Ferrannini, 1991; Lindsay and Howard, 2004). The ever growing epidemic of obesity and metabolic syndrome highlights the need for a better understanding of the specific mechanisms that link these conditions to diabetes, the development of cardiovascular disease and other metabolic disorders. It has been shown that pro-inflammatory cytokines are high in obese individuals and can stimulate the HPA axis; whereas GCs are known to decrease the production of cytokines and other inflammatory mediators (Schleimer, 1993). The pathology of obesity is multifactorial with a number of contributing causes and resulting conditions, but it is clear that there is cross talk with the HPA axis and this has been of great interest for research.

Over the past 15 years many studies have investigated the cross talk between the HPA axis, the inflammatory response and obesity; while this connection has been explored for many years, the results are not conclusive (Lucassen and Cizza, 2012). It has been shown that impairments in HPA axis activity correlates with enhanced susceptibility to inflammatory and autoimmune diseases, often characterized by hypercortisolism and GC resistance (Kopf et al.,
Increased levels of GCs have been shown to promote gluconeogenesis, fat breakdown and to maintain circulating levels of glucose necessary to mount a response to the stressor (Masserini et al., 2015). Research often focuses on how the GC/GR relationship is altered to elucidate the molecular mechanism that contribute to disorders that are associated with this relationship. Despite extensive research, the mechanism remains unclear; therefore, investigating and identifying novel factors that affect the GC/GR relationship has become imperative. Here I present LUMAN as one possible factor that can alter this GC/GR relationship.

With the initial observation of the lack of visible fat in the Luman KO mice and data showing that LUMAN alters the stress response through changing GC and GR levels, I decided to examine if there were any metabolic effects when LUMAN is deficient. In this chapter I present evidence that LUMAN alters a variety of factors involved in metabolism; however, it has yet to be investigated if it is a direct effect or if it is a result of the alterations in the stress response. The most evident phenotype was the total lack of visceral fat in the LUMAN KO mice; however, this was only observed qualitatively without being quantified. This led me to investigate if there were any differences in insulin sensitivity, glucose metabolism, blood pressure and alterations in the expression of genes related to metabolism.

4.2 Materials and Methods

Animals. This study followed the Canadian Council of Animal Care guidelines and was approved by the Animal Care Committee at the University of Guelph. The LUMAN gene knockout mouse line was generated in collaboration with the International Gene Trap Consortium (Nord et al., 2006). Chimeric mice were backcrossed to C57BL/6 mice (Charles River, Montreal, Canada) to produce a 99.9% congenic mouse strain. C57BL/6NTac mice were group housed with same-sex siblings and maintained on a 12-h light/dark cycle (10:00-22:00).
Temperature was maintained at 21-24 °C, and food (2014 Teklad Global 14% protein rodent maintenance diet) and tap water were provided ad libitum. To obtain sufficient mice in certain circumstances pups from LUMAN KO/HET mice were cross fostered onto CD1 dams. Due to low LUMAN KO pup survival, heterozygote mice were used in all experiments except the behaviour assays where LUMAN KO mice were used. Mice were euthanized by cervical dislocation and tissues were collected either in liquid nitrogen for protein and mRNA extraction or in 4% Paraformaldehyde for histological analysis.

**Insulin and Glucose Tolerance Test**: Intraperitoneal (I.P.) glucose (GTT) and insulin tolerance tests (ITT) were performed as an assessment for whole body glucose homeostasis. For the GTT, mice were fasted for 6 h prior to an I.P. injection of glucose (2 g/kg body weight). For the ITT, mice had free access to food prior to an I.P. injection of insulin (0.75 U/kg body weight). Blood glucose levels were determined by tail vein sampling at the indicated intervals using a glucometer. Changes in glucose over time were plotted, and the area under the curve (AUC) was calculated for each. To assess differences at each time point a one-tailed t-test was performed.

**High Fat Diet (HFD)**: Male C57BL/6N mice 2-3 months of age were fed a 60 kcal% fat diet (ENVIGO) for 10 weeks. Control mice we fed the normal diet (2014 Teklad Global 14% protein rodent maintenance diet), both food and tap water were provided ad libitum. After being on the HFD for 10 weeks the mice were sacrificed via cervical dislocation and tissues were collected for RNA isolation.

**RNA isolation and q-RT-PCR**: Cells were treated with glucose (33mM) for 24 and 36 hours respectively before RNA was collected. RNA isolation was done using E.Z.N.A. Total RNA Kit II (Omega Biotek) following manufacturer’s instructions. The RNA was treated with DNasel and
reverse transcribed using Qscript (Quanta Biosciences, Inc., Gaithersburg, MD). Transcript levels were measured by quantitative RT-PCR (qRT-PCR) using PerfeCTa SYBR green Supermix with 6-carboxy-X-rhodamine (ROX) (Quanta Biosciences, Inc., Gaithersburg, MD) and primers. Samples were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and subjected to standard curve analysis, and arbitrary values were represented, adjusting for primer efficiencies. For primer sequences see Appendix, Table 1.

4.3 Results

In figure 10B we weighed the mice over a period of 10 weeks and found that Luman-deficient are smaller in size compared to WT controls. Here I present data indicating that an altered glucose concentration as measured by the glucose and insulin tolerance tests (Fig. 17A, B). The overall response to the injection of insulin or glucose was not significantly different ($F= 0.4092, \ P= 0.5278, \ DF=1$). Although when analyzing the data baseline (Time 0) glucose levels in the Luman-deficient mice are higher in both tests when compared to WT ($GTT: T= 3.2, \ P= 0.009, \ DF=5; ITT: T= 3.73, \ P= 0.01, \ DF= 5$). Furthermore, when dissecting LUMAN KO mice there was no visible fat (Fig. 17E). Figure 17C-D shows initial data obtained regarding the expression of genes related to glucose metabolism, many of which are altered in the absence of LUMAN. Normally under metabolic stress sirtuin 1 (SIRT1) expression increases, however, in the absence of LUMAN, this increase in expression was not significant (WT response to treatment $T= 3.0, \ P= 0.047, \ DF=3; \ Het$ response to treatment: $T= 0.78, \ P= 0.26, \ DF=3$). Solute Carrier Family 2 Member 1 (SLC2A1) is a major glucose transporter in the mammalian blood-brain barrier (Ooi and Gomperts, 2015) and its expression increased in response to high glucose treatment in WT cells, whereas in the KO cells this increase was not observed to the same extent ($F= 13.74, \ P= 0.001, \ DF=3$).
P=0.002, DF=1). This is a preliminary subset of genes that are involved in glucose transport and metabolism chosen to provide initial data that can serve as a basis for in vivo studies.
Figure 17: LUMAN alters metabolism through gene regulation. (A-B) Luman-deficient mice have altered blood glucose levels during the insulin tolerance test (A) and the glucose tolerance test (B). Presented as mean ± SEM, *p<0.05 calculated by a one-tailed t-test; by a one-way ANOVA p>0.05 with a Tukey post hoc test for both GTT and ITT (n=6). (C-D) In absence of LUMAN the expression of genes associated with glucose metabolism are altered in response to treatment with high glucose (33mM). Untreated controls are denoted by NT and Glucose treatment denoted by G (n=4). A two way ANOVA was used to assess if the change induced by the HFD was significant between WT and het mice, (*p < 0.05). (E) There was no visible abdominal fat in each Luman-KO mouse we dissected; Qualitative observation (n=6), female mice, age 4-6 months.
To further investigate if this alteration in gene expression occurred in vivo I set up a high fat diet study using WT and *Luman*-deficient mice. I looked at a set of genes that are often altered in response to a high fat diet in the liver and found differences in the response of the *Luman*-deficient mice when compared to WT. Acyl-CoA Synthetase Short-Chain Family Member 2 (ACSS2), Squalene Epoxidase (SQLE) and Cytochrome P450 Family 3 Subfamily A Member 4 (CYP3A4) all followed a similar pattern where in WT mice the expression of each gene was increased in response to the HFD, whereas in the *Luman*-deficient mice this pattern was altered. In WT mice expression of both ACSS2 and SQLE was significantly increased in response to the HFD, and this response was absent in the *Luman*-deficient mice (ACSS2: $F=37.4403$, $P=1.249e^{-07}$, $DF=2$; SQLE: $F=4.12$, $P=7.21e^{-05}$, $DF=2$). It is important to note that SQLE expression is increased in the *Luman*-deficient mice fed the control diet compared to WT mice fed the control diet ($T=3.01$, $P=0.047$, $DF=2$), so while the HFD did not induce an increase in expression it may be due to the fact it was already high. In WT mice expression of CYP3A4 increases in response to the HFD, however, in the *Luman*-deficient this response is opposite, in that expression decreases in response to the HFD ($F=26.328$, $P=2.082e^{-05}$, $DF=2$). Interestingly, on the control diet the *Luman*-deficient mice have increased expression when compared to WT mice on the control diet ($T=4.4$, $P=0.023$, $DF=2$). In WT mice FOXO1 increases in response to the HFD, this response is absent in the *Luman*-deficient mice; however, when fed the control diet *Luman*-deficient mice have high expression of FOXO1 when compared to WT mice on the control diet ($T=3.9$, $P=0.03$, $DF=2$).
Figure 18: Altered gene expression in response to HFD in Luman-deficient mice. (A) In response to the metabolic stress of the HFD ACSS2 transcript levels increase in WT mice but not in Luman-deficient mice. (B) SQLE expression is higher in the Luman-deficient mice fed control and high fat diets when compared to WT mice. (C) CYP3A4 expression increases in response the HFD in WT, while the Luman-deficient mice show the opposite pattern. (D) FOXO1 expression is increased in the Luman-deficient mice compared WT control. N=3 for each group; *p> 0.05 calculated by a two way ANOVA assessing differences in the response to the HFD between Het and WT mice.
4.4 Discussion

Stress can be caused by external factors such as social strains or by intrinsic stressors such as sleep deprivation (Bose et al., 2009). Although an acute short-term stress response is adaptive, chronic or prolonged stress responses can be harmful and has been associated with several disease states (Peeke and Chrousos, 1995). Studies have shown a correlation between stress and risk for obesity in nonhuman primates (Bose et al., 2009; Shively et al., 2009). In female monkeys fed an atherogenic diet and housed in social groups, subordinate animals (more likely to receive aggression) showed increased visceral adipose tissue, incidence of atherosclerosis, and ovarian dysfunction compared with non-subordinate animals (Shively et al., 2009). Here I demonstrate that the transcription alterations normally induced by metabolic stress are altered when Luman is deficient.

In mammals, SIRT1 controls metabolic processes in specific tissues and has been shown to increase lipolytic rates in white adipose tissue as well as insulin secretion in β-pancreatic cells (Purushotham et al., 2012). SIRT1 is a NAD-dependent protein deacetylase that links transcriptional regulation directly to intracellular energetics. It plays important roles in the coordination of several cellular functions including cell cycle, response to DNA damage, metabolism, apoptosis and autophagy (Ooi and Gomperts, 2015). SIRT1 deacetylates a broad range of transcription factors and co-regulators, resulting in both positive and negative regulation of target genes. In particular it is involved in the deacetylation of FOXO1, resulting in nuclear retention and enhancing its transcriptional activity leading to an increase in gluconeogenesis in the liver (Boily et al., 2008; Lu et al., 2017; Oliva et al., 2008). Here I present data that indicates SIRT1 expression is increased in response to the metabolic stress of treatment with a high concentration of glucose in WT cells but this increase is not observed in the Luman KO cells. A
similar expression pattern is observed in the SLC2A1 gene, which is a major glucose transporter expressed in the mammalian blood brain barrier (Lee and Hur, 2016). From these data it appear that these genes may be downstream target genes of LUMAN; however, this may be a simplistic explanation.

Both SIRT1 and SLC2A1 are NAD+ dependent enzymes, it is possible that NAD+ is depleted in the Luman-deficient mice which would result in the lack of response that normally occurs in HFD fed mice (Liu et al., 2008). Generally, high glycolytic rates lead to higher NAD+ reduction rates. Given that Luman-deficient mice have increased blood glucose concentrations, this may be, in part, the mechanism that accounts for the lack of response of SIRT1 and SLC2A1 expression in the glucose treated group. This is a known mechanism by which metabolism would be directly coupled to the activity of SIRT1 and down-stream pathways (Canto and Auwerx, 2012; Liu et al., 2008). Interestingly, SIRT1 overexpression mice fed a high-fat diet show lower lipid-induced inflammation and better glucose tolerance. The SIRT1 overexpression mice are almost entirely protected from hepatic steatosis, exhibiting less and smaller lipid droplets compared to WT mice (Pfluger et al., 2008). The paired WT mice fed a HFD had severe hepatosteatosis, including massive accumulation of large lipid droplets (Pfluger et al., 2008). This seems to suggest that the Luman-deficient mice may have enhanced hepatic steatosis with increase lipid droplet accumulation. While this has not been investigated, we did find increased lipid droplet in the adrenal cortex of the Luman-deficient mice, although this was not quantified, representative pictures shown in supplemental Figure 7.

ACSS2 is an enzyme that belongs to the acyl-CoA synthetase family, responsible for the formation of fatty acid-Coenzyme A or acyl-CoA. ACSS2 and SQLE are both important enzymes in sterol biosynthesis and activation of both is controlled by SREBP proteins. The latter
is believed to be the rate limiting step (Nagai et al., 1997). Free fatty acids must be converted to acyl-CoAs in order to be shuttled into nearly all fatty acid metabolic processes, including protein acetylation, membrane phospholipid biosynthesis, energy storage, oxidation for energy production, and the synthesis of signaling lipids (Ellis et al., 2015). Dysregulation of lipid metabolism is the basis of multiple diseases including obesity and diabetes. A decrease in ACSS2 expression lowers nuclear acetyl-CoA levels and histone acetylation (Carrer et al., 2017). Diet induced changes on the metabolism-epigenome axis are not well understood but could alter gene expression and influence metabolic health (Carrer et al., 2017). When fed a HFD WT mice have increased expression of ACSS2 when compared to the control diet, this response is not observed in the Luman-deficient mice. ACSS2 is activated via phosphorylation and acts through forming complexes with transcription activators at the promoter regions of different sets of genes (Zlotorynski, 2017). LUMAN may be one of these transcription activators, and when it is deficient ACSS2 expression cannot increase in response to metabolic stress. Alternatively LUMAN may be required for SREBP activation of these enzymes and when absent this pathway is altered. Furthermore, SQLE expression is controlled, in part, by cholesterol levels, and given that Luman-deficient mice have high GR activity they may also have high cholesterol levels which can directly affect SQLE expression.

CYP3A4 is an enzyme that catalyzes many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. It localizes to the ER and its expression is induced by GCs (Augustin et al., 2013). This may explain why the expression of CYP3A4 is high in the Luman-deficient mice when compared to WT, as I have shown that they have high GR activity. The transcriptional factor FOXO1 plays an important role in metabolic homeostasis and is the main target of insulin signaling. FOXO1 signaling leads to an increase in glucose
levels and triggering glucose intolerance and insulin insensitivity. While the FOXO1 gene does not follow the same pattern as the other genes examined, its expression is increased in the Luman-deficient mice when compared to WT on the control diet. This could be caused by the increase in blood glucose levels or/and an increase in cytokine levels that is normally attributed with an increase in GR activity.

Here I present data that suggests when LUMAN is deficient some important metabolic processes are altered under baseline levels and/or under metabolic stress. In particular, genes involved in lipid synthesis and glucose use were affected, which may be a by-product of the altered GR responses or it may be an independent effect of LUMAN. It is important to understand these pathways and how they can become dysregulated resulting in conditions such as obesity and diabetes. While these data are preliminary it provides a basis for future research to explore these important pathways and the role LUMAN may play in them.

**Chapter 5: Discussion and Future Directions**

**5.1 Significance of Research**

The stress response plays an important role in everyday life and varies dramatically between individuals. Identifying factors that underlie differences in how individuals react to stress is paramount in being able to alter this response to ensure it is adaptive rather than detrimental. Here I present LUMAN as a factor that can alter the stress response through affecting the main stress system, the HPA axis. The discovery of LUMAN’s involvement in the stress response opens a new area of research and represents a novel factor that can alter stress sensitivity.
5.2 Potential role of LUMAN in Neurogenesis

Neurogenesis is defined as the process of generating functional neurons from precursors, it was originally thought to occur solely during embryonic development in mammals (Ming and Song, 2005). However, it has been shown that neurogenesis does occur in adults but is generally restricted to two specific areas; the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles where new neurons are produced before they migrate to the olfactory bulb (Kempermann and Gage, 2000). Neurogenesis is believed to be very limited in other parts of the adult central nervous system (Gould, 2007). It is well known that stress inhibits cell proliferation, and adult neurogenesis is no exception. Many studies have used chronic psychosocial stressors to induce stress and they have shown a decrease in hippocampal cell proliferation and survival, in addition to depression-like symptoms in many types of rodents (Gould, 2007; Jacobs et al., 2000; Kempermann and Gage, 2000). However, artificially reducing adult neurogenesis in the absence of stress does not generally induce a depression-like phenotype, suggesting that the depression-like symptoms observed are related to the impairment of stress modulation (Jacobs et al., 2000).

Studies have shown that depression is associated with a reduction in neuron survival and hippocampal volume, this observation may be a result of alterations in the stress response affecting adult neurogenesis (Wood et al., 2004). Prolonged stress results in a decrease in the rate of cell proliferation in the adult hippocampus, negatively affecting neurogenesis (McEwen and Magarinos, 1997). Interestingly, treatment for chronic depression up-regulates hippocampal neurogenesis, primarily through altering GR activity (Seckl and Fink, 1992). There are 2 possible ways in which LUMAN may alter neurogenesis: high GR activity has been observed in the Luman-deficient mice; we have also shown that LUMAN plays a protective role in the cell
during cellular stress. Given the role LUMAN plays in the UPR this is not a surprising function; cytoprotection may also be a mechanism through which LUMAN could alter neurogenesis and neuron survival in the hippocampus, and potentially elsewhere in the brain as well.

Morphological neuronal maturation is differentially affected by experience, in particular stress, investigating variations in maturation morphology may be of interest when looking at the effects of stress (von Bohlen Und Halbach, 2007). In the future, it would be beneficial to use neuronal markers to look for differences in neuron distribution at different levels of maturation. Investigating the differential distribution of neurons could show us if LUMAN alters neurogenesis in the hippocampus, and potentially if altering LUMAN levels could be used as a tool to manipulate neurogenesis. Looking for the presence of stem/progenitor markers (Nestin and Musashi1), immature neuron markers (calretinin) as well as mature neuron markers (MAP-2 and NeuN) can be used to assess differences in the amount of neurons present at each level of maturation and to label astrocytes Glial fibrillary acidic protein (GFAP) can be used (von Bohlen Und Halbach, 2007; Ziabreva et al., 2006). If LUMAN promotes the survival of neurons at a specific level of maturation manipulating LUMAN levels may be used as a tool to increase or decrease neurogenesis in specific situations. It has been shown that patients suffering from depression tend to have decreased neurogenesis in the hippocampus (Jacobs et al., 2000), additionally, patients afflicted with autism have been shown to have altered neurogenesis patterns, although they are not well characterized (Kaushik and Zarbalis, 2016), suggesting that being able to manipulate this process would be extremely valuable.

5.3 LUMAN and Stress-Related Pathologies

Generally, when the stress response is acute, and limited in duration, it induces adaptive mechanisms to promote survival. Some of these mechanisms include a decrease in the secretion
of both reproductive and growth hormones, reducing the nociceptive sensitivity, along with various immunosuppressive effects; obviously, if the stress response is prolonged, these mechanisms can have deleterious effects (Schneiderman et al., 2005). The HPA axis acts as an interface between stress and neurological function, it is therefore not surprising that abnormal functioning of this axis has been found in many psychiatric disorders (Baumeister et al., 2014). In many of these disorders, it appears that altered HPA axis function leaves an individual more susceptible to developing stress-related pathologies, rather than being a direct cause of the disorder (Pariante and Lightman, 2008). Some of the disorders most commonly associated with stress include depression, anxiety, PTSD and bipolar disorder, which combined affect millions of people worldwide (Yehuda et al., 2004b). Without a known cure, research is ongoing, investigating new avenues for prevention and treatment of these disorders.

In this thesis, I have presented evidence that when Luman is deficient, the HPA axis activity is dysregulated, characterized by low GC levels and high GR expression. Luman-Deficient mice also have an altered response to the DEX suppression test, which is often used as a diagnostic tool of depression in humans. A normal response to the injection of DEX is a drastic decrease in GC secretion, caused by an increase in negative feedback in the HPA axis. In depressed patients the drastic decrease in GC level is not observed, which is thought to be caused by impaired negative feedback (Pariante, 2009). In the Luman- deficient mice we see the opposite effect, in that, the decrease in circulating GCs is even more drastic than seen in WT mice, leading us to believe that they have enhanced negative feedback in the HPA axis. The enhanced negative feedback in the HPA axis could result in the decrease in anxiety and depressive-like behavior observed in the Luman-deficient mice. This enhanced feedback is likely mediated through the increased GR expression seen in the Luman-deficient mice, as similar
findings have been observed using GR over-expression mice (Ridder et al., 2005). In the future, it would be important to investigate further into this mechanism to gain a better understanding of how LUMAN alters HPA axis negative feedback, to allow for a novel way to manipulate the stress response. A starting point would be to make a Luman overexpression mouse and perform the DEX suppression on these mice to assess their HPA axis function. Repeating some of the experiments from this thesis on the Luman overexpression mice, such as assessing GR expression and activity, any behavioural defects and if they also exhibit a significant maternal defect, would help better understand the mechanism through which LUMAN works.

To further examine the role LUMAN plays in the HPA axis it will require fine mapping of LUMAN’s structure. This may allow us to pinpoint mutations that can alter HPA axis activity to a degree that will decrease stress sensitivity, lowering one’s susceptibility to develop a stress-related pathology, while limiting the negative consequences such as the apparent maternal deficit. I have used a number of Luman mutant plasmids in this thesis, however, in each of them a large portion of a specific domain was altered, giving us a crude map of LUMANs interaction with GR. It would also be important to knockdown LUMAN expression in GR +/- mice, often used as models for depression, to investigate if it has similar effects of treating with antidepressants. In the big picture, LUMAN represents a novel factor to examine when looking at the stress response and may have potential as a treatment for depression in the future. Furthermore, it would be imperative to assess the level of LUMAN and if any consistent polymorphisms are observed in depressed patients, if any alterations were identified (compared to controls) manipulating Luman expression may have anti-depressive effects.

5.3 LUMAN and Secretion
While previous research has shown that CREBA, a homologue of LUMAN, plays a key role in secretion in drosophila, this thesis presents the first evidence of LUMANs’ ability to alter the secretory capacity in mammals. From the data presented, it appears that LUMAN is not required for basal levels of secretion but rather is important when the secretory load increases due to stress, or in certain cell types with high secretory capacity. There is evidence that each of the CREB3- like proteins are involved in secretion, while their functions vary due to differences in expression patterns (Fox et al., 2010). It was originally thought that the CREB3 family of proteins were redundant in order to provide compensation if one is non-functional, however, this may not be entirely true (Chan et al., 2011). Although their functions may overlap, their expression patterns are very different lending to the fact that they cannot all compensate for each other.

Interestingly, not all of the CREB3 family proteins are activated in response to ER stress, despite the consensus that they are ER stress response proteins (Chan et al., 2011). LUMAN is not activated by the classic ER stressors tunacamycin (Tm) or thapsigargin (Tg) but is highly activated by Brefeldin A, which works through inhibiting COPII vesicle formation, essentially causing the contents of the golgi to relocate to the ER, causing both ER and Golgi stress (Miller et al., 1992). This suggests that LUMAN may alter COPII vesicle formation altering stress-induced secretory capacity. LUMAN is also activated by lipopolysaccharides (LPS), and mildly activated by both DEX and monensin (disrupts Trans Golgi function), suggesting the function of LUMAN is more widely spread than just activation of the UPR (Asada et al., 2011).

Here, I have shown that LUMAN is required during high secretory demand in cell-based assays. It appears to be functionally important during cellular stress under BFA treatment, and in the whole-body response to stress through altering the GR response. To determine if this is the
primary mechanism through which LUMAN works, further investigation is required. This thesis presents evidence for LUMAN’s role as a cofactor of GR as well as its’ role in secretion. While I found that CRH secretion was not affected in *Luman*-deficient mice, there are many factors that can affect this system; to delineate the mechanism of action, we must first separate out these factors. Primary neurons can be collected from the hypothalamus, cultured, elicit CRH secretion through interleukin-1 treatment and assess CRH secretion levels. This would take out the element of HPA axis negative feedback control and allow us to assess if LUMAN directly affects the secretion of CRH. Importantly, we can collect cells from the floxed LUMAN mice and use a CRE virus to reduce LUMAN expression (or a control virus). This would allow us to bypass the obstacle of using the heterozygous mice that exhibit a severe maternal defect, limiting the number of pups available. Furthermore, using the virus will allow us to obtain cells that have a 90% or greater knockdown of LUMAN, which would yield cleaner results.

**5.4 LUMAN and Fertility**

While deletion of LUMAN does not appear to be embryonically lethal, as some *Luman* KO pups do survive to adulthood, there is clearly a deleterious effect given the low proportion of KO pups born. The KO mice have significantly decreased fertility and if they do give birth to live pups the litter size is extremely small (1-4 pups) and a *Luman* null dam has never successfully raised any pups. Thus, it is clear that LUMAN plays a role in reproduction, however it may be an indirect interaction.

In addition to the dramatic changes in the physiology and function of multiple tissues, increases in GCs can also inhibit reproduction (Whirledge and Cidlowski, 2010). GCs are required for normal reproductive function, however, when the levels of GCs are disrupted, so is fertility. Elevated GCs lead to decreased synthesis and release of gonadotropin releasing
hormone (GnRH), luteinizing hormone (LH) and follicle stimulating hormone (FSH), in addition to modulating steroidogenesis and gametogenesis in the testis/ovary (Whirledge and Cidlowski, 2010). Therefore, LUMAN may alter fertility indirectly through its effects on GC levels and GR activity. Another important observation to note is that there has not been a Luman null male pup born during my studies (4 years) and therefore, a null × null mating has not been set up to assess viability. Additionally, Luman heterozygous male mice have 5X lower testosterone levels when compared to WT mice (Sup. Fig. 11). Previous research in our lab has shown that LRF-/- × LRF -/- mating pairs are 100% infertile. Taking into account that the Luman-deficient mice show a similar, but more severe, phenotype to the LRF-deficient mice, I believe that a Luman -/- × Luman -/- mating would also not result in pregnancy.

To explore LUMAN’s role in fertility we could use conditional KO mice, selectively knocking out LUMAN from the testis, ovaries, uterus, pituitary, hypothalamus or adrenal glands. This would allow us to see if it is a direct effect on fertility through altering secretion of important hormones and neuropeptides affecting the reproductive system or if it is a secondary effect from the alterations of the stress response system. Additionally, knocking out LUMAN at different time points in development would allow us to assess if there are differential effects of manipulating LUMAN expression at various points in development.

5.5 LUMAN, Obesity, and Metabolism

It has been shown that increased GCs result in decreased sex hormones, leading to regional fat metabolism and distribution (Peckett et al., 2011). Additionally, GCs have both direct and insulin-mediated effects on adipose tissues, with extended exposure leading to increased visceral adiposity, insulin resistance and hypertension (Smart et al., 2006). In the Luman-deficient mice we see changes in each of these aspects except for blood pressure (hypertension) (data not shown),
which was measured and no significant differences were found when compared to WT mice. The interface between LUMAN and metabolism requires further investigation and to explore this there are a few experiments that can be performed. Completing full blood chemistry will allow us to assess differences, in particular, measuring the level of circulating triglycerides in the blood. It would also be important to measure the fat content in the liver of Luman-deficient mice through oil red O staining, this will give a more complete picture of LUMAN’s role in fat deposition.

A recent study looked at a modulator of LUMAN, LRF, in a population in Samoa that has an obesity rate of higher than 80% (Minster et al., 2016). They found a mutation in the LRF gene and it was shown to be associated with a highly increased risk of obesity through promoting fat storage (Minster et al., 2016). It will be important to understand the functional significance of this mutation on LRF activity to assess if it alters the function of LUMAN. It is possible that the observed phenotype is due to a reduction in LRFs ability to repress LUMAN; higher LUMAN may lead to increased deposition of fat may consistent with observations made in the Luman-deficient mice.

5.6 General Future Directions

One short-coming that must be addressed is that, while each experiment used a single sex (male or female), both sexes were used in this thesis for different experiment. This is an important thing to note as the stress response differs between males and females. In order to rectify this these experiments could be completed using the sex that has not yet been tested. However, in some cases both sexes were used, in particular measuring baseline Cort levels was completed using both males and females in separate experiments.
In order to further delineate the molecular mechanism of LUMAN we must first manipulate its expression in specific tissues and at specific points in development. Recently we have obtained *Luman* floxed mice allowing for spatial and temporal deletion of *Luman*, which will help by bypassing the maternal defect and low availability of null mice. To allow for temporal control of *Luman* deletion, I have validated a CRE associated adenovirus (AAV), this virus can be injected into a specific part of the brain (or body) at a predetermined point in development to help elucidate if LUMAN plays a role in development. This may allow us to pinpoint a time in which it is most effective to alter LUMAN levels to reduce stress sensitivity to a level that avoids the accompanying maladaptive consequences. However, to achieve this, we would first need to gain a better understanding of where LUMAN exerts its’ effects that alters stress sensitivity. This can be done using the CRE AAV, injecting into various parts of the mouse where LUMAN is known to be highly expressed, or by using mice that express CRE in specific body regions. While the CRE mice eliminate the need for and variability of injections, setting up and maintaining a breeding colony of CRE mice has its own hurdles.
5.7 Working Models

![Diagram showing the hypothalamic-pituitary-adrenal (HPA) axis and the effects of stress on glucocorticoid receptor (GR) expression.

**Figure 19: Working physiological model.** In the absence of LUMAN glucocorticoid receptor (GR) expression increases to compensate for the loss of an integral co-activator. This increase in expression leads to enhanced negative feedback in the hypothalamic pituitary adrenal (HPA) axis, resulting in low corticosterone levels and a hyper-reactive HPA axis.
**Figure 20: Working cellular model.** When LUMAN is activated it is transported to the Golgi where it undergoes regulated intramembrane proteolysis (RIP) and translocates to the nucleus where it enhances transcription of glucocorticoid inducible genes through acting as a co-activator and as a transcription factor binding to glucocorticoid response elements (GRE). LUMAN also plays a role in the unfolded protein response through binding to unfolded protein response elements (UPRE) enhancing the transcription of various genes including the components of COPII vesicles.
5.8 Conclusion

It is clear that the biological role of LUMAN is widespread and varied, likely due to its effects on the stress response, which can have whole body implications. This thesis provides the first evidence that LUMAN is important for many important biological processes, affecting the stress response, reproduction and metabolic systems. Additionally, this work provides the first evidence that LUMAN is involved in the HPA axis through altering GC/GR levels and may be implicated in many prevalent stress-related pathologies. Using the *Luman*-deficient mice I was able to delineate some of the important biological functions of LUMAN in addition to characterizing two possible mechanisms of action.
References


glucocorticoid receptor activity and is essential for prolactin-mediated maternal instinct. Mol Cell Biol 32, 5140-5150.


### Tables

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### ChIP Primers

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Table 1: Primer sequences used in this thesis
Table 2: Descriptive statistics for Figure 10B

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1. IQR: interquartile range. * p < 0.05
Supplemental Figures

Sup. Fig. 1. LUMAN expression. A) Western blot analysis of LUMAN expression in brains of wildtype females. The blot represents three independent experiments. B) Luman mRNA detection by RT-PCR in tissues from adult Luman +/+ and Luman +/- mice. C) Immunohistochemistry staining of LUMAN was performed using paraffin-embedded whole brain slides. D) In situ hybridization data from the Allen Brain Atlas showing Luman mRNA expression.
Sup. Fig. 2. Circulating Catecholamine levels. Blood was taken from the saphenous vein of each mouse, spun down, serum removed and frozen until a catecholamine ELISA was performed. N=10; P-value >0.1 by a two tailed t-test.
Sup. Fig. 3. Olfaction Test. The time from the moment the mouse was placed into the cage to the time it located the pellet and initiated burrowing was recorded as latency to find. There was no difference in latency to find a buried food pellet among mice of three genotypes. p>0.1 in a Kruskal-Wallis test. n=5 for Luman null, n=8 for Luman heterozygous, n=10 for Luman wildtype. Values for each subject are presented with median and interquartile range.
Sup. Fig. 4. Low brefeldin A (BFA) concentration induces LUMAN cleavage. Luman knockout and wildtype MEF cells were treated with 200nM BFA for 3 hours, after which they were collected and run on an SDS gel and analyzed via western blot for LUMAN.
Sup. Fig. 5. Circulating CRH levels. Blood was taken through cardiac puncture, spun down, serum removed and frozen until a CRH ELISA was performed. N=8; P-value >0.1 by a two tailed t-test.
Sup. Fig. 6: Full blots used in Figure 14.
Sup. Fig. 7: Electron microscope images taken from 2 WT mice in the adrenal cortex and adrenal medulla.
Sup. Fig. 8: Electron microscope images taken from 2 heterozygous mice in the adrenal cortex and adrenal medulla.
**Sup. Fig. 9: Immunofluorescence of GR and LUMAN.** The localization of GR (red-594) and LUMAN (green-488) in the presence and absence of DEX, as well as looking at the mutant LUMAN constructs. Scale bars: 30µM.
Sup. Fig. 10: BDNF expression in the hippocampus. *Luman*-deficient mice have significantly lower BDNF expression when compared to WT mice. Measured via Q-RTPCR from RNA extraction from the hippocampus. *p= 0.003, T= 7.08, DF=3; calculated by a one-tailed t-test and values are mean ± SEM.
Sup. Fig. 11: Testosterone levels in male WT and Luman-deficient mice. Luman-deficient mice had significantly lower testosterone levels when compared to WT mice.
Sup. Fig. 12: ΔAD expression for DLA. HEK293 cells were transfected with either pcDNA, as a control, or ΔAD, RNA was collected, treated with DNase and reverse transcribed into cDNA and qPCR was performed using SYBR green reagents. *Luman* primers were designed to detect only the transfected ΔAD construct, pcDNA control CT values for *Luman* were ‘undetermined’ and were set to 40 for calculation purposes (PCR program runs 40 cycles). The construct was detected in the ΔAD samples but not in the pcDNA control.
Sup. Fig. 13: Western blot showing LUMAN expression in mouse embryonic fibroblasts using a wildtype (+/+ ) and a LUMAN KO strain (-/-).
Sup. Fig. 14: Construct Map of Mutants
Sup. Fig. 15: GM130 Co-staining with VSVG. GM130 (594-red) is used as a Golgi marker and VSVG (488-green) is the protein being tracked through the secretory pathway. Staining performed in Mouse embryonic fibroblasts (MEFs), both Wildtype and LUMAN null cells were assessed under control conditions and under treatment with brefeldin A (BFA).