Stress Hormone Regulation of Splicing in the Hippocampus

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ABSTRACT

OBJECTIVES: The regulation of splicing by glucocorticoids in the hippocampus has not been well studied. Hence, this study aims to determine the transcripts/exons that are regulated by stress hormones in the hippocampus. **RESULTS:** Hypophysectomy was confirmed by weight reduction in experimental animals. Serum corticosterone was also reduced upon hypophysectomy which was rescued by administration of CORT or ACTH. Using RNA-Seq, we identified transcripts/exons that are changed upon hypophysectomy and successfully rescued by CORT or ACTH. Specifically, edgeR and DEXSeq identified 32 genes with >2 fold changes and 889 exons with >1.5 fold changes respectively that were changed upon hypophysectomy and rescued by CORT or ACTH administration. RT-PCR validated the regulation of several target exons by stress hormones. We further identified a decline and an increase in the transcripts containing the target exon of the *Sept8* gene in a time-course dependent manner upon hypophysectomy and in normal groups respectively. **CONCLUSION:** These findings demonstrate a specific exon that is regulated by stress hormones in the hippocampus.

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DEDICATION

This work is dedicated to God, my father, my siblings and my late mum.

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AD	Alzheimer's disease
APP	amyloid precursor protein
APPL	adaptor protein phosphotyrosine and leucine zipper 2
ARGLU1	Arginine and glutamate-rich protein 1
ASC	activating signal coregulator
AT	Ataxia Telangiectasia
ATM	Ataxia telangiectasia-mutated
ATP	Adenosine triphosphate
Aβ	amyloid β-protein
BDNF	brain-derived neurotrophic factor
BK	Big Potassium
CA	Cornu ammonis
cAMP	Cyclic adenosine 3',5'-monophosphate
CAPER	coactivator of activating protein-1 and estrogen receptors
CaRRE	CaMKIV-responsive RNA element
ChIP	Chromatin immunoprecipitation

CIS	Chronic immobilization stress
CORT	Corticosterone
CRF	Corticotropin-releasing factor
CRH	Corticotropin releasing hormone
DAVID	Database for Annotation, Visualization and Integrated discovery
DBD	DNA binding domain
ddH_2O	double-distilled water
DEX-Seq	Differential exon sequencing
DG	Dentate gyrus
DNMT	DNA methyltransferase
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ETC	Electron transport chain
GC	Glucocorticoid
GH	growth hormone
GILZ	Glucocorticoid-induced leucine zipper
GnRH	gonadotropin-releasing hormone

GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
hGR	human glucocorticoid receptor
hnRNP	heterogenous ribonucleoprotein
HPA	hypothalamic-pituitary-adrenal
IGV	integrative genomics viewer
MDD	Major depressive disorder
MR	Mineralocorticoid receptor
mtDNA	Mitochondria DNA
NADH	Nicotinamide adenine dinucleotide hydrogen
nGRE	negative glucocorticoid response element
NOVA1	Neuro-Oncological Ventral Antigen 1
ORF	Open reading frame
PCR	polymerase chain reaction
PD	Parkinson’s disease
PER 1	Period 1
PKC	protein kinase C
POMC	Proopiomelanocortin

PP1	protein phosphatase 1
pre-mRNA	precursor messenger RNA
PS1	Presenilin 1
PTB	polypyrimidine tract-binding protein
PTSD	Post-traumatic stress disorder
RBM	RNA binding motif
RNA-Seq	RNA Sequencing
ROS	Reactive oxygen species
ROS	Reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SF	splicing factor
SGK 1	serum- and glucocorticoid-regulated kinase 1
SMN2	survival motor neuron
snRNP	small nuclear ribonucleoprotein
STREX	stress axis-regulated exon
TF	Transcription factor
TIF2	Transcriptional intermediary factor 2
TPA	Tissue plasminogen activator

Ttr	Transthyretin
U2AF	U2 auxiliary factor
UTR	Untranslated region
UV	Ultraviolet

CHAPTER I

INTRODUCTION

1. STRESS

Stress refers to a non-specific reaction by the body in response to physiological challenges [1]. There are many stressful events faced by different individuals in their day-to-day life activities ranging from stress at the workplace, health trauma and psychosocial stress [2]. These stressors could be acute or chronic, for instance, finding out about the loss of a loved one or getting fired from a job, living with dangerous neighbors or having constant financial or marital problems [3, 4]. The impact of these stressors on the overall health during an individual's lifespan could result in the development of diseases [5-7]. One of the central organs affected by stress and adaptation is the brain. This is because it determines the stressor or threatening situation, stores memories, and regulates the physiological and biological response that may be harmful or protective [8]. When stress is in its positive form, it could increase performance and biopsychosocial health [5], particularly for motivation and adaptation. All organisms are exposed to stressors at various points in time which can serve as internal or external challenges to homeostasis that would require adaptation [9]. The idea of allostasis which argues that certain levels of stress can be handled by organisms before returning to equilibrium, suggests that individuals who recover from the stress successfully are resilient without experiencing any negative effects physiologically or behaviorally. However, if the stress goes beyond a certain level, the homeostasis compensation could be compromised and allostasis will result in negative impact on the human health and this will make the organism vulnerable to subsequent stressors [8, 10].

2. STRESS HORMONE AND HPA AXIS

The HPA (hypothalamus-pituitary-adrenal) axis is responsible for stress hormone production, in response to stressful conditions. The HPA axis serves as the central stress response system. It requires the coordinated effort of the three organs: hypothalamus, anterior pituitary gland, and adrenal gland. In the paraventricular nucleus of the hypothalamus, there are CRH neurons that serves as the initiator of the stress response. The neurons travel to the median eminence to stimulate the release of CRH and other peptides such as vasopressin into the hypophyseal portal vein. When the CRH is released, it binds to G-protein coupled receptors on the corticotropes to activate adenylate cyclase in the anterior pituitary gland to initiate ACTH production [11]. Binding of CRH enhances the transcription of POMC (Proopiomelanocortin) gene which encodes for ACTH. When the POMC is synthesized, the enzyme prohormone convertase-1 cleaves it into ACTH and Beta-lipotropin [12]. The ACTH is packaged into vesicles and then released into the systemic circulation.

The zona fasciculata of the adrenal cortex contains melanocortin 2 receptors which ACTH binds, to produce adenylate cyclase and increase intracellular levels of cAMP and stimulate cholesterol biosynthesis, which will finally lead to the production of glucocorticoids and mineralocorticoids. Once the glucocorticoids are produced, they are released into the circulation to bind to different target cells in the body to exert their actions [13], including adrenal medulla chromaffin cells to produce adrenaline (epinephrine).

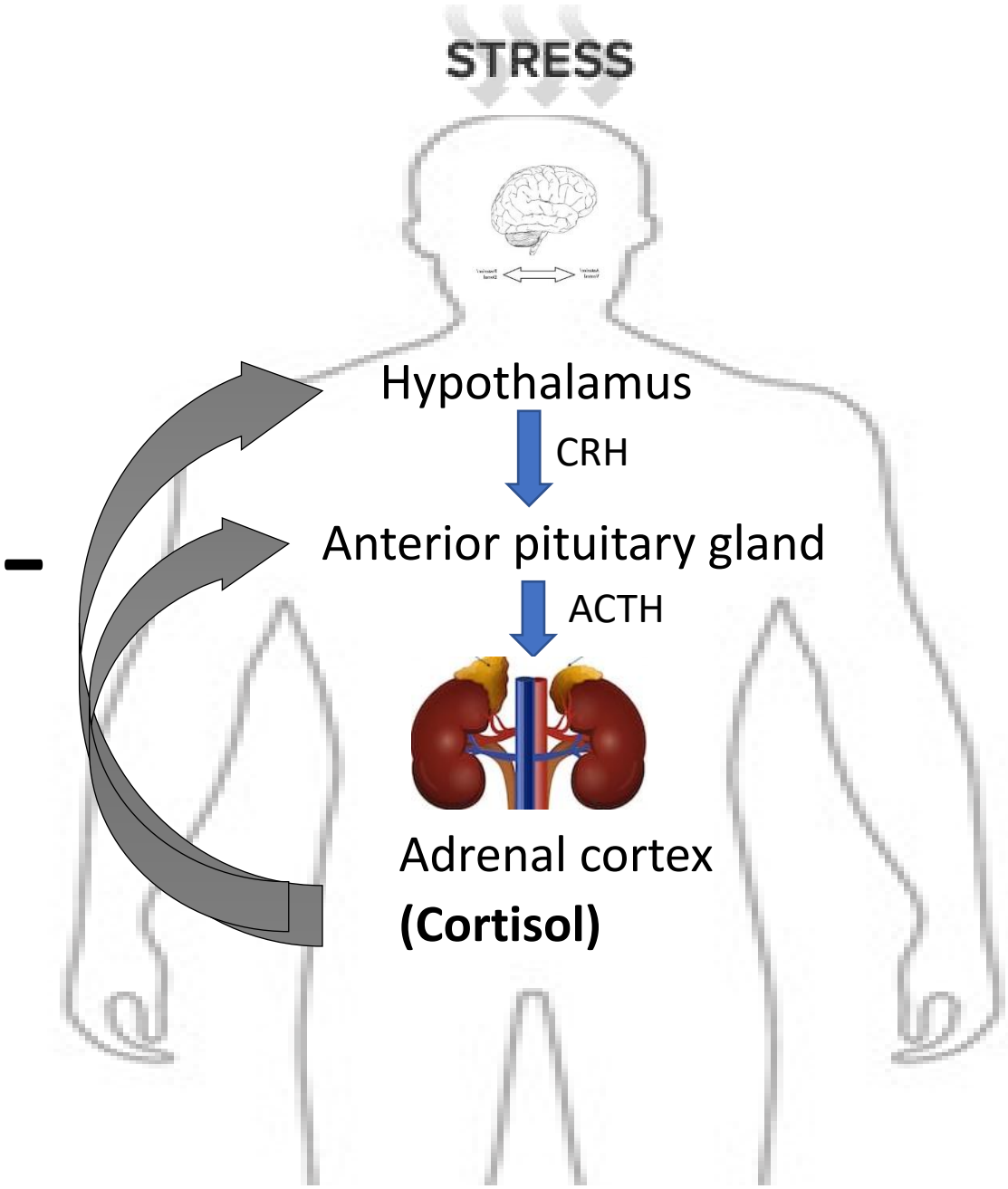


Fig 1: Diagram of the hypothalamic-pituitary-adrenal axis (HPA axis)

3. ANATOMY OF THE HIPPOCAMPUS

The hippocampus is a structure embedded in the medial temporal lobe (limbic system) of the brain [14]. The hippocampus functions in new memory formation and helps to switch short term memories to long term memories [14, 15]. Other functions of the limbic system are cognition, episodic memory, spatial navigation, and learning [16-21]. The hippocampal structure resembles the sea horse hence its name was coined with the interpretation (*hippos*-horse, *kampos*-sea monster). The hippocampal system comprises two parts namely: dentate gyrus (DG) and Cornu ammonis (CA). The two parts are separated by a sulcus of which the subiculum lies below it. The entorhinal cortex serves as the initial input to the DG via the performant pathway to function in recognizing patterns and encoding memories. The CA regions are divided into three regions; CA1, CA2, and CA3. The DG projects neurons to the CA3 through the mossy fiber pathway. The CA3 extends to the CA1 through the Schaffer collateral pathway, which functions in the formation of memories and emotional network [22]. To make it a complete loop, the CA1 projects to the entorhinal cortex via the subiculum [23]. The predominant cell layer is the pyramidal cell layer, which is densely packed in the CA1 region and less in the other two regions: CA2 and CA3 [24].

4. GLUCOCORTICOID RECEPTOR ACTIVATION

Glucocorticoids (GCs) act on two main receptors in many organs of the body including the brain, namely: GR and MR. These two receptors have been found in several regions of the brain, for example, amygdala, hippocampus, and prefrontal cortex, which are related to cognition [25]. Under baseline conditions, glucocorticoids bind to the MR but during chronic stress, glucocorticoids bind to GR to exert its action. The MR and GR when occupied with the ligand

binds to the same GRE sequences on the target genes such as *Per1*, *Sgk1*, *Gilz*, and *Fkbp5* [26-28].

GR is present as a protein complex in the cytoplasm, consisting of heat shock proteins (hsp90 and hsp70), immunophilin [29]. Binding of GC stimulates a conformational change causing hsp proteins to be dissociated. The GR complex then enters the nucleus to exert its effects to either induce or suppress transcription of target genes (Fig. 2) [30, 31]. The GR has a domain structure consisting of a hinge region, carboxy-terminal ligand-binding domain (LBD), a zinc-finger DNA binding domain (DBD), and an amino terminal domain (NTD). These domains contain regions of regulatory activity such as activation function domain 1 and tau2 (transactivation domain 2) [32]. Steroid receptor coactivator 1 (SRC1) and glucocorticoid receptor interacting protein (GRIP1) or TIF2 are examples of coactivators that ligand-bound GR interacts with, to affect the chromatin structure by recruiting histone acetyltransferase enzymes [33-35]. Recruitment of RNA polymerase II and gene transcription initiation occurs as a result of the interactions between coactivators, transcription factors and other regulatory proteins [36]. GR could enhance gene transcription through chromatin remodeling and recruiting RNA polymerase II to the site of local DNA unwinding [37]. In 2011, a study identified a negative glucocorticoid response element (nGRE) that is responsible for the repression of target genes by recruiting corepressors and histone deacetylase [38]. The sequence in nGRE differs from that of the activating GRE and upon GR binding, they recruit corepressors NcoR and SMRT to the promoters of the genes that contain nGRE such as Bcl-2, insulin, and the insulin receptor [38]. To identify the mechanism in which GR causes transrepression of target genes through the nGRE, thymic stromal lymphopoetin promoter (TSLP) was assessed. In response to GR agonists, TSLP mRNA level reduction (50%) was mediated by the nGRE which contains 850 base pairs

upstream of the transcription site [38, 39]. This demonstrates a mechanism of how transrepression happens via the GR in target genes.

The GR is produced from the glucocorticoid receptor (hGR) gene in humans, which generates two isoforms hGRα and hGRβ due to alternative splicing of exon 9 [40]. The most studied isoform is GRα which contains 777 amino acid polypeptide while GRβ contains 742 amino acids polypeptide [41]. The other isoform GRβ changes its ligand binding properties by the LBD alterations it carries to probably compete with GRα in certain experiment settings [42]. GR deletion in the forebrain of mice (Camk2a-Cre) resulted in hyperactivity and disrupted feedback regulation of the HPA axis. Depression-like behaviors were observed in these mice, which was normalized by antidepressant [43, 44]. In contrast, GR deletion in the glutaminergic neuron (Nex-Cre) in mice, stimulated hyperactivity of the HPA axis and reduced fear but GR deletion in the GABAergic neurons (Dlx5/6-Cre) in mice did not affect both responses to fear and the HPA axis [45]. These demonstrate that GR plays a role in regulating depression or fear via the HPA axis.

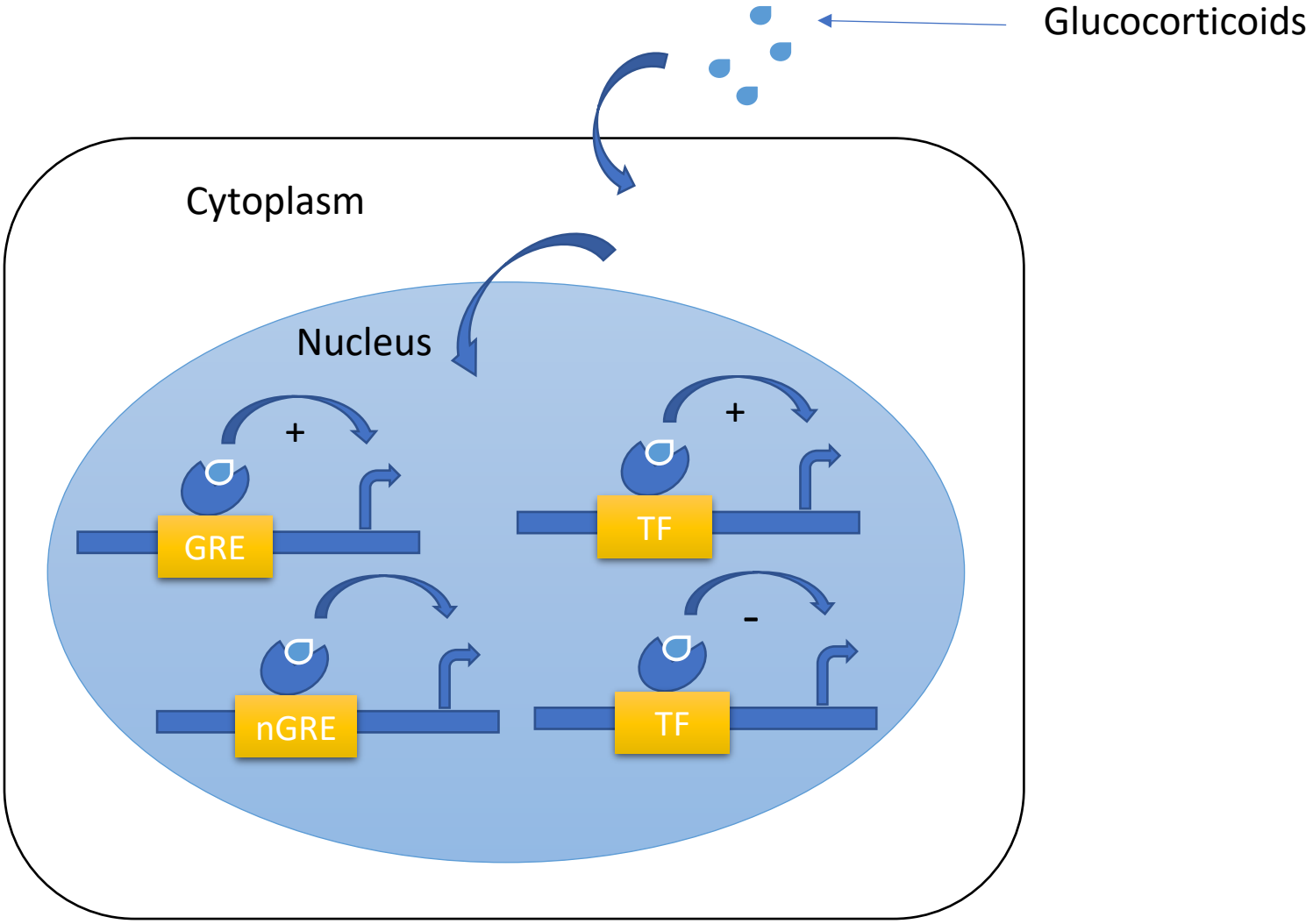


Fig 2: Diagram of the mechanism of glucocorticoids. When glucocorticoids bind to its receptor, it regulates transcription through the physical interaction with other transcription factors to either activate or repress gene expression. *TF- transcription factors, nGRE- negative glucocorticoid response element, GRE-glucocorticoid response element*

5. STRESS HORMONE EFFECTS ON THE HIPPOCAMPUS

The effect stress has on the hippocampus includes the following:

5.1. Structural plasticity:

In the hippocampus, stress hormones could play a role in structural plasticity [46] which could be neuron replacement [47-49]. For instance, a mouse strain C57BL/6 was subjected to chronic immobilization stress (CIS) for ten (10) days, and this induced dendrite retraction in CA3 short-shaft pyramidal neurons and dorsal CA1 pyramidal neurons [50]. Animal models of psychosocial stress have revealed some insights into the relationship between stress and structural plasticity. For example, subjecting the animals to chronic restraint stress for 21 days, apical dendritic atrophy was observed in the CA3 region of the hippocampus [46, 51]. In assessing the stress paradigm in tree shrews, repeated psychosocial stress resulted in apical dentritic atrophy in the CA3 pyramidal cells, which was reversed upon administration with an antiepileptic drug, phenytoin [52]. This phenytoin suppresses the release of excitatory amino acid and prevent T-type calcium channel activation, inhibit corticosterone and stress-induced dendritic atrophy [53, 54].

The synapse within the brain that underlies learning and memory is affected by stress particularly in the advent of long term potentiation (LTP) and long term depression (LTD) within the hippocampus [55, 56]. For instance, *in vivo* and *in vitro* studies reveals that stress impaired the LTP and primed burst potentiation [57-60]. The effects of stress on the synaptic plasticity could be changed by blocking the activated glucocorticoid receptor before or immediately after stress [61-63]. Adrenal steroids have long been implicated in hippocampal plasticity and other neurochemical systems such as glutamate, calcium current. Serotonin, endogenous opioids, and GABA-benzodiazepine receptors can also be involved [46, 64]. For instance, rats were subjected

to chronic restrain stress (CRS) to observe its regulation on glial glutamate transporter in the hippocampus. CRS increased GLT-1 expression in the CA3 region and was inhibited by an antidepressant, tianeptine. This reveals the impact of stress on glutamate levels [65]. In addition to the above mentioned, transmembrane glycoprotein, corticotropin-releasing factor, BDNF and tissue plasminogen activator (tPA) are molecular players in the stress-induced structural remodeling [66]. These suggest that stress could induce structural changes in the neurons of the hippocampus.

5.2. Hippocampal neurogenesis:

The development of new neurons by neural stem cells is referred to as neurogenesis [67]. In this process, stem cells undergo several stages of development. As a result of aging, exposure to acute or chronic stress, phases of the neurogenic process could be suppressed. [68, 69]. Several studies have shown the effect of GR activation on neuronal differentiation and hippocampal cell proliferation. For example, high glucocorticoid levels resulted in GR activation to reduce hippocampal cell proliferation and neuronal differentiation *in vivo* and *in vitro* and under depression and stressful conditions [70-74]. Early environmental stress has also been shown to impact adult neurogenesis [75-78]. In the hippocampus, stress and glucocorticoid hormones have been shown to have a high impact on the regulation of neurogenesis. Similarly, an adaptor protein, phosphotyrosine and leucine zipper 2 (APPL) activates GR signaling and hence alters neurogenesis. APPL transgenic mice decreased neurogenesis and the effect was reversed upon administration of GR antagonist [79].

Under stressful conditions, high levels of corticosteroids could reduce the number of pyramidal neurons in the hippocampus. Decreased neurogenesis in the adult hippocampus plays

a role in depressive behaviors [80-82]. Transgenic mice (GFAP-TK) or irradiation method was used to inhibit neurogenesis in the hippocampus. The neurogenesis deficient mice were subjected to stress and the glucocorticoid level was less suppressed by dexamethasone compared to the wildtype mice [80], suggesting that the HPA response is controlled by the hippocampal neurons. Research has shown that stress activation of GR leads to decreased neurogenesis in the hippocampus [83-85]. For example, antidepressant, sertraline was tested in human progenitor cells to assess its effect on neurogenesis. It was observed that sertraline increased neuronal differentiation via GR, in which its effect was eradicated when GR antagonist was administered [84]. These suggest that glucocorticoid decreases neurogenesis in the hippocampus. The molecular pathway in which increased glucocorticoids cause impairment of neurogenesis in cellular neural stem/progenitor cells is elucidated below. The two receptors, MR and GR can differentially affect the differentiation and proliferation of NSPC. For example, a low concentration of cortisol levels caused an increase in cell proliferation of the hippocampus and differentiation into astroglia but suppressed differentiation into neurons through the MR. Upon high concentrations of cortisol, there was a decrease in hippocampal cell proliferation and differentiation through the GR. These suggest that neurogenesis is modulated via MR at basal levels of glucocorticoids but inhibited via GR at high levels of glucocorticoids [86].

Other types of NSPC have been reported to have inhibited neurogenesis via the GR [87-89]. Bose et al 2010 revealed the possibility of glucocorticoids causing heritable alterations in neural stem cells through an epigenetic mechanism. Exposure of rat embryonic neural stem cells to the synthetic glucocorticoid (dexamethasone) revealed changes in expression of genes related to cellular senescence (upregulated) and mitochondria functions (downregulated). However, there was a decrease in global DNA methylation and its enzyme (*Dnmt*) to suggest an epigenetic

change [87]. Also, the effects of stress on granule cell proliferation can be achieved through the NMDA receptor. NMDA receptor functions as a site of action (glucocorticoids) for learning, memory, and inhibiting cell proliferation in the hippocampus. Hence, stress could inhibit granule cell proliferation through NMDA receptor activation [73, 90]. Another mechanism by which GR activation can reduce neurogenesis is the role of serum- and glucocorticoid-regulated kinase 1 (SGK1). For instance, in a human hippocampal progenitor cell line, an inhibitor of SGK1 was able to prevent the inhibition of neurogenesis in the presence of cortisol by stimulating the translocation of the nucleus and GR phosphorylation. Also, cortisol-induced inhibition of the neurogenic hedgehog pathway was SGK1 dependent [91]. These suggest that SGK1 is involved in the effect of cortisol-induced neurogenesis.

5.3. Behavioral and Cognitive effects of stress

Several studies have shown the consequence of continuous exposure to stress on behavior in rats which includes; reduced exploratory activity, anxiety, and aggressive like behaviors [92-94]. Rats that have undergone chronic stress displayed impaired learning and memory in spatial challenges, for example, morris water maze, y-maze, and radial arm water maze [95-99]. To identify the mechanism between stress and cognition, CRH is secreted upon stress and activates CRF_1 in pyramidal cell dendrites of which high expression of CRH was observed in rats subjected to chronic early-life stress (CES). The high expression of CRH ultimately results in high occupancy of CRF_1 to produce morphological effects and poor memory in these rats subjected to CES. Upon the administration of CRF_1 blocker, there was a restoration of memory performance and dendritic atrophy was prevented, to suggest that hippocampal CRH-CRF_1 interactions are involved in cognitive impairment associated with chronic early-life stress [100].

5.4. Stress hormones and its effect on aging in the hippocampus

Aging is one area of science that has been shown by researchers to be affected by stress hormones and the hippocampus is one major target tissue. As already stated earlier, the hippocampus contains high levels of glucocorticoids making it a target tissue for stress-related disorders. However, does the hippocampal structure or function deteriorate with age? Do stress hormones such as glucocorticoids have a role to play? In 25-month-old female mice, neuronal loss (significant reduction of NeuN positive neurons) occurs in the hippocampus during the aging process compared to 7-month-old counterparts which could suggest hippocampal neuronal loss in the elderly [101].

Aging mice (C57BL/6) revealed a reduction in learning, memory, and locomotion but an increase in depressive-like behaviors and anxiety in several studies [102-106]. For instance, a study examined the effect of aging under a controlled environmental condition in C57BL/6 mice for locomotion activity, anxiety, depressive-like behaviors, cognition, gene expression, and immune function in the hippocampus. Healthy mice of ages 4, 9, and 14 months were subjected to an established behavior test for four weeks. In comparison to the younger counterparts, 14 month-old mice revealed a reduction in locomotion, impaired spatial memory, and increased anxiety. Aging in these mice revealed certain genes with significant expression in the hippocampus. These suggest that aging is associated with changes in anxiety-like behaviors, locomotor activity, cognition, and gene expression [106].

Exposure to high levels of glucocorticoids that occurs with age may be a partial cause of the deterioration of the hippocampus. Evidence from previous studies reveals that age-related structural changes can be associated with the HPA axis [107]. For example, high levels of

cortisol were observed in older individuals compared to younger adults [108] suggesting involvement of glucocorticoids in the process of aging.

6. GENE EXPRESSION REGULATION BY GLUCOCORTICOIDS

Gene expression is a process in which genes utilize information (genetic code) to produce RNA and/or proteins. This process can be regulated at different stages: transcription, RNA processing, alternative splicing; mRNA stability and translation. Regulation of gene expression by glucocorticoids has been well known to occur at the transcription level. Many genes have been identified to be targets for glucocorticoid regulation, especially with the advent of massive experimental approaches. For example, chromatin immunoprecipitation (ChIP) and RNA-Seq analysis were performed on cultured A549 lung epithelial carcinoma to identify 234 genes regulated by dexamethasone, including *Period 1* that is associated with circadian rhythm [109]. In adrenalectomized rats, corticosteroid pellets were implanted to produce high or low blood glucocorticoid levels to identify 393 genes that were changed in the hippocampal CA1 region by microarray/pathway analyses [109]. Some of these genes are involved in memory/learning, plasticity, differentiation, glucose metabolism, and inflammatory/immune response [110]. Experiments conducted in male tree shrews to assess the effects of chronic psychosocial stress revealed the reduction of NGF, CLK-1, M6a, and GNAQ transcript levels. Upon treatment with antidepressants, the expression of the above genes except NGF was restored [111].

Gene expression microarrays were used to analyze the hippocampal transcriptome of stressed mice. The longer the exposure to stress, the more genes were altered in expression. The functions of the genes affected include vascular system, injury response, and inflammation [112]. Furthermore, pathway analysis, gene expression, and microarrays revealed the mechanism in

which stress hormone regulates hippocampal neurogenesis, through molecular signaling pathways such as Notch/Hes-signaling, TGFβ-SMAD2/3-signaling, and the Hedgehog signaling. Administration of cortisol (low concentration) caused Noth/Hes signaling to be enhanced in an immortalized human hippocampal cell line, whereas high concentrations of cortisol inhibited TGFβ-SMAD2/3-signaling. When the concentrations of cortisol (low and high concentration) were administered, Hedgehog signaling was inhibited [86]. In the same study, TGFβ-SMAD2/3-signaling and Hedgehog signaling were inhibited in prenatally stressed rats [86]. Together these suggest the possible molecular mechanisms (signaling pathways) through which glucocorticoids regulate gene expression and neurogenesis.

7. REGULATION OF ALTERNATIVE SPLICING BY GLUCOCORTICOIDS

Alternative splicing allows the production of numerous protein products from a gene [113], which can be controlled by glucocorticoids. For example, an examination of the *Slo1* BK potassium channel gene shows that hypophysectomy reduced *Slo* transcripts that contain the stress-axis-regulated exon (STREX). When ACTH was administered, the reduction was inhibited. Hence, ACTH regulates the splicing of STREX [82]. Also, there was a sharp drop in plasma corticosterone during the stress-hyporesponsive period of rat development. A reduction in transcripts containing STREX was observed in the adrenal and pituitary gland. Administration of CORT increased the transcripts containing STREX [114].

Furthermore, the application of glucocorticoids decreased STREX inclusion in bovine cells (*in vitro*) which was reversed on the administration of GR antagonist [115]. In bovine cells, STREX inclusion was increased upon glucocorticoid administration which was reversed when glucocorticoid receptor antagonist RU38486 was administered [115]. In assessing the effect of

dexamethasone on the alternative splicing of the insulin receptor, dexamethasone caused a switch in expression from A to B isoform increasing the exon 11 inclusion transcripts in a dose-dependent manner in HepG2 cells [116, 117]. In a rare disorder, ataxia-telangiectasia (AT), a genetic disorder characterized by mutations in its gene (ATMutated), dexamethasone is shown to induce alternative splicing. Lymphoblastoid cell lines from AT patients treated with dexamethasone caused the skipping of mutations upstream of the ATM coding sequence to produce the ATMdexa1 transcript variant. This resulting transcript provides an ORF that can be translated into a protein that possesses the complete kinase domain for a new ATM variant [118]. Together, these suggest glucocorticoids regulate splicing and could further provide a therapeutic solution to splicing mutations.

Table 1: Showing the summary of exons regulated by stress hormones

Genes	**Exon**	**RNA source**	**Ref**
Slo1	STREX	Adrenal and pituitary gland of rats, Bovine chromafin cells,	[82, 114, 115]
INSR	Exon 11	HepG cells	[116, 117]

8. ALTERNATIVE SPLICING AND MECHANISM OF ALTERNATIVE SPLICING REGULATION

The mechanism that contributes to the diversity and complexity of the proteome is largely dependent on alternative splicing. The process involves genes producing multiple proteins. Splicing begins with the involvement of a protein complex called Spliceosome. The spliceosome comprises of five small nuclear ribonucleoprotein particles (snRNP) and auxiliary proteins that work together to identify the splice sites and initiate the splicing reaction [119, 120].

For the spliceosome assembly to begin, U1 snRNP is recruited to recognize the 5'ss then SF1 binds to the branch point. [121]. E complex formation (ATP independent) occurs when U2AF heterodimer (U2AF35 and U2AF65) binds to the polypyrimidine tract and 3'AG terminal [122]. This E' complex is converted to pre-spliceosomal A complex (ATP-dependent) after the SF1 is replaced by U2 snRNP on the branch point. Furthermore, U4, U5, and U6 tri-snRNPs will be recruited for B complex formation. The B complex contains the spliceosomal units required for pre-mRNA splicing. Conformational and remodeling changes will occur such as, the loss of U1 and U4 snRNPs for the catalytically active spliceosome (C complex formation) [123]. Two trans-esterification reactions will be performed afterward. In the first trans-esterification reaction, the intron branch point contains a 2'OH group of adenosine in which a nucleophilic attack is carried out on 5'ss. The site will be cleaved and the 5' end will ligate with the branch adenosine to result in a lariat structure. The 5' exon has a 3'OH group that attacks the 3'ss in the second reaction, which leads to the ligation of the 5' and 3' exon to form the mRNA and the intron is released [124]. Alternative splicing occurs in over 90% of human genes [113]; therefore it will be important to understand the regulation of this process by external factors to appreciate how genes are regulated.

8.1. Types of alternative splicing events

- **Exon skipping:** This is the process whereby an exon could be spliced out of the transcripts in like manner as the introns [125].
- **Mutually exclusive**: In this type of alternative splicing event, the mRNA transcripts could contain only one of two exons [125].
- **Alternative 3' splice site (acceptor site):** In this event, the 3' acceptor site is used within an exon to change the 5' boundary [125].
- **Alternative 5' splice site (donor site):** In this event, the 5' donor site is used within an exon to change the 3' boundary [125].
- **Intron retention:** In this event, the mRNA transcripts could contain introns [125].

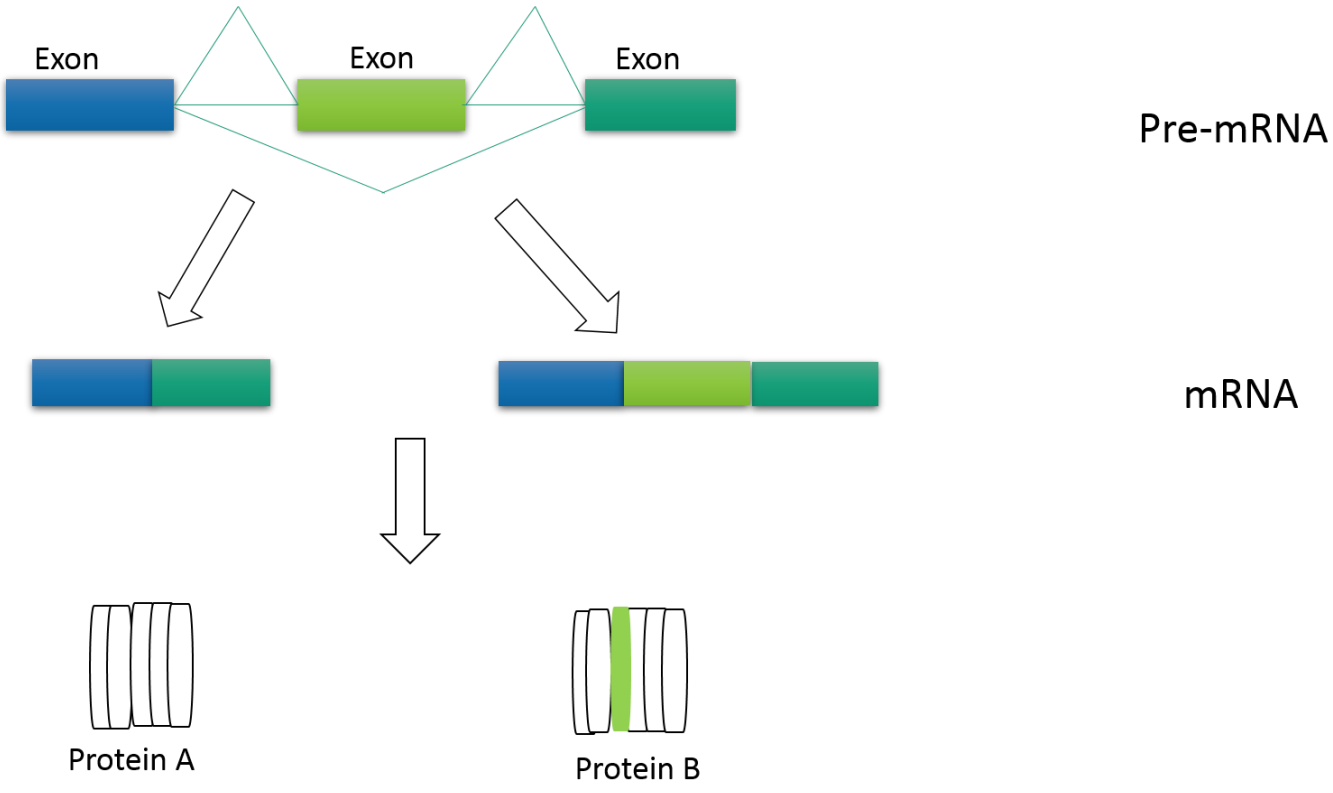


Fig 3: Diagram of the process of alternative splicing

9. MECHANISM OF ALTERNATIVE SPLICING REGULATION

Alternative splicing regulation occurs by trans-acting factors which is mainly RNA-binding proteins binding to cis-acting elements [126]. For example, exonic splicing enhancers (ESE) are pre-mRNA sequences located within exons that enhance accurate splicing. Members of the Serine-Arginine family (SR) binds to the ESE through their N-terminal RNA recognition motif. They could mediate protein-protein, protein-RNA interactions through their RS (Arg-Ser repeats) domains. These domains are phosphorylated and function as activators to promote exon splicing [127]. Another example is exonic splicing silencers (ESS), which are short regions of an exon that inhibits or silences splicing of pre-mRNA. Splicing repressors of hnRNP class often bind to ESS. The hnRNPs contain splicing inhibitory domains such as glycine-rich motifs [128] and RNA-binding motif. Intronic splicing enhancer (ISE) and splicing silencer (ISS) are sequences on the intron often within the polypyrimidine tract or close to the branch point or 5' splice site that are bound by regulatory elements for either a positive or negative regulation [119]. The UGCAUG, is a common ISE element, which when duplicated, increases splicing of exons in the c-src, fibronectin and calcitonin transcripts [129-132]. SR proteins bind to intronic sequences near the branch site of the adenovirus L1 mRNA [133]. During early infection, when SR proteins bind to the sequence, the use of 3' splice site is blocked, and the splicing is shifted to a nearby site. But during late infection, dephosphorylation of the SR proteins occurs and not active during repression of splicing thereby allowing the site usage [134]. Studies have shown that mutations in cis-elements and trans-acting factors can disrupt the splicing code and result in diseases [135, 136].

9.1. RNA SEQUENCES REGULATING ALTERNATIVE SPLICING

How do the sequences regulate splicing? Xie and Black 2001 examined the BK potassium channel transcripts for the alternative splicing of STREX. GH3 pituitary cells were depolarized to observe its effect of the signal on splicing. CaMKIV overexpression resulted in a decrease in the inclusion of STREX in the mRNA. To identify sequences in the STREX exon that caused the cells to be responsive to CaMKIV, they made mutations in STREX minigene and applied transfection into cells with or without CaMKIV. They identified a CaMKIV-responsive RNA element (CaRRE) that mediates alternative splicing of ion channel pre-mRNA [137]. Similar results were obtained when assessed in cerebellar neurons [138] to suggest that RNA-responsive elements within exons could be a possible mechanism in which regulation of alternative splicing occurs.

9.2. SR-PROTEINS

One of the SR protein family members responsible for constitutive splicing is the SRSF1 or SRSF2 [139, 140]. U1 snRNP and U2 snRNP binding to the 5’ splice site and 3’ splice site respectively, is promoted by the SR protein family [141]. SR proteins required for alternative splicing undergoes modifications such as phosphorylation to change its activity and thereafter regulate splicing. For instance, the SRp40 protein was phosphorylated to regulate alternative splicing of protein kinase C (PKC) Beta II mRNA via phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathway [142]. The SR proteins have an RNA recognition motif (RRM) in their N-terminus and Arg/Ser rich peptides in the C-terminus also called RS domains. For example, SRSF1 has a short RS domain and two RRMs compared to other SR proteins. Phosphorylation of the C-terminal RS domains by two protein kinases result in distinct phosphorylation states [141]. The ratio of SR and hnRNP proteins was influenced by stress-

induced signaling via the mechanism that phosphorylates hnRNP A1 as well as its cytoplasmic accumulation to result in changes of the alternative splicing activity [143]. Similarly, 3',5'-cAMP dependent protein kinase regulates nucelo-cytoplasmic transport of polypyrimidine tract binding protein (PTB), which is vital for alternative pre-mRNA splicing [144]. In *SMN2* exon 7 and intron 7, hnRNP A1 binds to exonic and intronic silencers. It was proposed that interactions between hnRNPA1 and SRSF1 molecules, would suppress the inclusion of exon 7 in *SMN2* [145-147]. These suggest that phosphorylation of SR proteins enables the regulation of splicing.

9.3. Other RNA-binding proteins

There are also tissue specific RNA-binding proteins such as *Nova* [148] *Rbfox* family [149, 150], neuronal PTB/hnRNP1 [148] involved in alternative splicing regulation. PTB, a splicing factor repressor functions by blocking the transition of exon-intron definition [151]. For instance, N1 splicing of c-src pre-mRNA was examined using extracts from non-neuronal HeLa cells and WERI-1 retinoblastoma cells. As a result of CU rich element that flanks N1 binding to PTB, PTB mediated the repression of N1 splicing in HeLa cells [152]. However, in WERI-1 extracts, the nPTB homolog replaced PTB which allows N1 splice efficiently [153].

Another example is RBM5 also known as LUCA15, a tumour repressor protein stimulates exon 6 exclusion of CD95 pre-mRNA. RBM5 achieves this by binding to sequences within exon 6 to inhibit the U4/U6-U5 tri-snRNP complex incorporation on the intron that flanks exon 6 hence resulting in the blockage of prespliceosome maturation. Furthermore, U1 and U2 snRNP pairing at the distal splice site is stimulated by RBM5 to contribute to exon 6 exclusion [154]. These suggest that RNA-binding proteins regulate alternative splicing by inhibiting the spliceosome assembly.

10. MECHANISM OF GLUCOCORTICOID REGULATION OF SPLICING

10.1. Splicing factors

Studies have shown that glucocorticoids regulate splicing. However, the mechanism of this effect is still poorly understood. Park et al 2009, showed that *Nova-1* could be involved in the reduction of pre-mRNA splicing of GnRH transcripts induced by glucocorticoid. Dexamethasone (DEX) was injected in rats or GT1-1 and NIH3T3 cells, and it was observed via splicing assays that pre-mRNA GnRH splicing was reduced. This was reversed on the administration of a GR antagonist. The splicing regulator, *Nova-1* mediated excision of intron A in pre-mRNA GnRH when DEX was injected into the rats or GT1-1 and NIH3T3. *Nova-1* was knocked out and there was a reduction in the excision of intron A, but overexpression of the gene reversed the effect [155]. *Nova-1* mediated the excision of intron A in GnRH by binding to ACAU sequence on exon 1 of GnRH. *Nova* has also been shown to bind to primary transcripts to regulate target genes [156, 157]. For example, a genome-wide analysis of alternative splicing events was carried out using a custom affymetrix microarray in the brains. Relative to the immune tissues, differentially spliced changes were observed in the brain of which RT-PCR validation was observed for 49 exons with predicted *Nova*-dependent splicing changes [157]. In another study, cortisol upregulated the splicing factor SRSF3 that promotes GRα isoform in THP-1 cells [158]. These suggest that splicing factors could mediate glucocorticoid regulation of splicing.

10.2. Epigenetic regulation

Epigenetic modifications are modifications that affect the DNA or histones without altering the DNA sequence [159]. Epigenetic modifications have been shown to be involved in stress-associated altered gene transcription [160, 161].

Some studies have shown that stress leading to decreased hippocampal gene expression could be mediated by epigenetic mechanisms. In mammals, methyl groups are added to the cytosine by DNA methyltransferase. The enzymes for DNA methylation could be affected by stressors. For instance, rats subjected to fear conditioning revealed high expression of DNA methyltransferases particularly DNMT3A and DNMT3B. When these enzymes were inhibited, the consolidation of fear memories was impaired. An example of genes implicated in memory formation, PP1 and reelin had altered DNA methylation. The PP1 genes remove phosphorylation from histone 3 at serine 10, which could be a basis for its function in hippocampal long term memory and synaptic plasticity [162]. Exposure to prenatal stress caused alterations in BDNF via methylation changes in exon IV and exon VI. The decrease in BDNF expression was accompanied by an increase in DNA methylation of exon IV in the hippocampus and amygdala which could suggest that through DNA methylation in exon IV of BDNF, the decrease in BDNF expression by prenatal stress occurs [163].

Another study assessed the impact of glucocorticoids on epigenetic variations observed in the hippocampus, hypothalamus, and blood. Corticosterone was administered to mice through drinking water for a month to assess the physiological and behavioral changes. Some of the HPA axis genes examined includes *Hsp90, Nr3c1*, *Crh, Fkbp5,* and *Crhr1*. Specifically, DNA methylation was decreased in *Fkbp5* with increased expression and anxiety-like behaviours to suggest that glucocorticoid's effect on *Fkbp5* function can be mediated by DNA methylation

[164]. Histone H3 acetylation and histone H3-lysine 4 trimethylation was increased which shows activation of chromatin [165]. Neuron-specific exon 17 of *Nr3c1* promoter in adult rodents, contains an epigenetic modification [166, 167]. These suggest that glucocorticoids alter methylation patterns or histone modification of genes.

Some stressors could trigger a decrease in DNA methylation of genes in the brain. For example, neuronal plasticity genes such as genes that codes for BDNF. For instance, a rat model of PTSD was exposed to predator stress and BDNF DNA methylation patterns at exon IV was assessed. It was found that in BDNF exon IV, there was an increase in DNA methylation but a decrease in BDNF expression in the hippocampus due to psychosocial stress [168]. Therefore, these suggest that DNA methylation and other epigenetic mechanisms could be a possible mechanism in which glucocorticoids regulate splicing.

10.3. RNA-binding property

Proteins such as GR could regulate genes by binding to RNA. Some of the RNAs include binding to tRNA, mRNA, and long non-coding RNA [169, 170]. For instance, GR interacts with CCL2 mRNA in rat smooth muscle cells [171, 172]. The (DBD) DNA binding domain of GR is shown to interact with RNA. An example is a Gas5-GR interaction, which GR-DBD is a structure-specific binding domain to RNA through electrostatic force [170, 173]. There is thus a possibility that GR-DBD can implicate other RNA structures other than Gas5 to regulate GR-mediated gene expression.

10.4. Co-regulator recruitment

Steroid receptors such as GR, recruit coregulators to modulate gene expression. For instance, CAPER-alpha and CAPER-beta which are cofactors for steroid receptors [174] coactivates

progesterone receptor in transcription assays and alters alternative splicing of calcium/calcitonin gene. Other studies suggest that steroid receptors recruit coregulators that bind to promoters. These promoters could be involved in the splicing of some target genes and hence lead to its regulation [175]. For instance, steroid hormone target genes had increased production of its transcripts and alternative splicing regulation of these transcripts by transcriptional coregulators such as activating signal coregulator (ASC-1 and ASC-2) [176]. Together these suggest that splicing regulation can occur via the recruitment of coregulators by steroid receptors.

11. STRESS-RELATED DISORDERS AND ITS ASSOCIATION TO SPLICING

Exposure to traumatic experiences can result in memory impairment leading to a pathological disorder known as post-traumatic stress disorder (PTSD) [31]. Stress hormones such as glucocorticoids have been implicated in the pathophysiology of PTSD. For example, glucocorticoids were infused into the hippocampus of mice after subjecting them to a fearful situation. PTSD memory impairment was observed when the mice were unable to identify the context or situation as the correct predictor of the threat [177]. With the advent of RNA sequencing, studies have analyzed the gene expression profiles of PTSD in human blood and postmortem brains [178, 179]. Studies identified FKBP5 and STAT5B, which have a role in inflammation and GC response, to be downregulated in PTSD patients [180-185]. FKBP5 protein is a chaperone to GR which can prevent the translocation of GR to the nucleus when regulating many genes [29].

A progressive neurodegenerative disease called Parkinson's disease (PD), has been shown to be affected by stress. Previous studies have revealed some genes that are affected by aberrant

splicing in patients with PD [186]. For example, PARK2 gene produces several isoforms as a result of alternative splicing. Transcript variants that arise from the aberrant splicing could be as a result of in-frame deletion of exons 3-5 and exons 2-7. These changes were shown to increase in PD. [187-189]. Similarly, Johnson et al 2007 sequenced exons of LRRK2 gene in North American patients of PD. *In vitro* analysis revealed an intronic deletion at the splice site of exon 19 which disrupts normal splicing [190].

Alzheimer's disease (AD) has also been implicated to be associated with increased levels of glucocorticoids. *In vivo* and *in vitro* experiments revealed that glucocorticoids contribute to the development of AD, causing an increase in amyloid beta-peptide and tau protein accumulation. The amyloid precursor protein (APP) and its cleaving enzyme are increased for this to occur [191], suggesting that high glucocorticoid levels contribute to the development and progression of AD. Another study used a knock-in mouse model of AD that expressed mutants of APP, human beta-amyloid (hAβ) and presenilin 1 (PS1) to show an increase in corticotropin-releasing factor (CRF) levels which were normalized when a copy of CRF receptor was lost, which reduced the level of corticosterone [192]. Similarly, post-traumatic stress disorder-like induction which increased β-amyloid (Aβ) levels, exacerbating the progression of AD was observed in the above mouse model. An increase in Aβ levels hyperexcited the CRF neurons which led to PTSD-like phenotypes and stress-related symptoms. However, γ-secretase inhibitor which inhibits the production of Aβ and reduces its levels were injected into the mice and the PTSD-like phenotype was reduced [193]. Tau gene *MAPT* undergoes alternative splicing to produce six isoforms that contain microtubule-binding repeats (3R or 4R) [194]. In the normal brain, these repeats exist in equal ratio but altered in several tauopathies [195]. Studies have shown that mutations in the intron of the tau gene enhance the splicing of exon 10, which has been

implicated in neurodegeneration and dementia [196]. Together these suggest that glucocorticoids contribute to the pathophysiology of stress-related disorders.

12. Questions leading to the current hypothesis of the research

Previous studies focused on the regulation of transcription by glucocorticoids and their underlying molecular mechanisms. For instance, dexamethasone reduced the mRNA levels of GnRH most likely by reducing the activity of the GnRH promoter [197, 198]. It was further identified that GCs could also down-regulate GnRH gene expression at the splicing level. Dexamethasone, a synthetic glucocorticoid appears to prevent the expression of a splicing regulator *Nova-1* which stimulates excision of intron A by binding to exon 1 of GnRH pre-mRNA [155]. GR is highly expressed in the hippocampus with many studies showing that under chronic stress conditions, there are elevated glucocorticoid levels which leads to the onset of depression [199]. Furthermore, aberrant splice variant has been linked to stress-related conditions, neurodegenerative disorders [200, 201]. Therefore, in the field of alternative splicing and its regulation by stress hormones such as glucocorticoids, studies have shown a correlation that stress hormones control alternative pre-mRNA splicing [82, 115]. For instance, the regulation of STREX of *Slo1* gene by stress hormones of which splicing factors, RNA elements, phosphorylation site, and the mechanism for its regulation has been identified [202-206]. In the study on STREX regulation by stress hormones, a unique observation for the decline of STREX upon hypophysectomy in a time-dependent manner was made [82].

However, whether glucocorticoids regulate alternative pre-mRNA splicing in the hippocampus has not been clearly shown. Recently, Lilia et al 2019 revealed alternative splicing changes in genes by stress hormones in neural cells and found ARGLU1 as a potential mechanism for this regulation [207]. Furthermore, the underlying molecular mechanism, the

elements/factors regulated by glucocorticoids and GR of splicing in the hippocampus is not still clear yet. Hence, our study provides a reproducible approach (hypophysectomy/rescue system) to identify specific exons that are regulated by stress hormones in the hippocampus. Therefore, investigating the exons that are regulated by stress hormones in the hippocampus will shed more light on the above questions. This study aimed to identify the exons that are regulated by stress hormones in the hippocampus.

13. Hypothesis

Based on the previous finding that stress hormones control splicing, we hypothesized that glucocorticoids regulate alternative splicing of specific exons of genes in the hippocampus with an effect in a time-dependent manner.

CHAPTER II

Materials and Methods

Materials

Rats:

5 week-old Sprague-Dawley rats obtained from Charles River Laboratories were used as in a previous experiment [82].

Chemicals and Reagents

Name	Sources
Agarose RA	VWR
Ethidium Bromide	Fisher Biotech
Ethylenediamine Tetraacetic Acid (EDTA)	Fisher scientific
Potassium Chloride (KCl)	Fisher scientific
Glycerol	Fisher scientific
Bromophenol blue	Fisher scientific
Magnesium Chloride (MgCl)	Fisher scientific
Tween 20	Fisher Biotech
Sucrose	Acros organics
Xylene Cyanol	Fisher Biotech
Tris Base	Fisher scientific

Enzymes and Buffers

Product	**Source**
Dithioreitol (DTT), 1M	Invitrogen
5X First Strand Buffer	Invitrogen
M-MLV Reverse Transcriptase	Invitrogen
SUPERase• In™ RNase Inhibitor	Invitrogen
Taq DNA polymerase	Xie Lab
dNTP Set	Invitrogen
Oligo d(T)	Integrated DNA Technologies

Kits

Kit	**Source**
RNeasy extraction kit	QIAGEN

Buffers and Solutions

1M Tris. HCl

Tris	60.97g
ddH_2O	400ml

pH is adjusted to 7.5 with 6 N HCl

Make up to 500ml with ddH_2O, autoclave and stored at 4˚C

10X PCR Buffer (40ml)

200 mM	Tris. HCl (pH 8.3)	8.0 ml
15 mM	$MgCl_2$	6.0 ml
250 mM	KCl	2.5 ml
0.5% mM	Tween-20	667 ul
1 mg/ml	BSA (Inactivated)	800 ul
	ddH_2O	22.033 ml

Stored in aliquots at -20˚C

PCR MIX 1 (21.64 ml)

10X PCR Buffer	2.5 ml
dNTP (100 mM) 2.5 ul each	50 x 4 ul
$MgCl_2$	125 ul
ddH_2O	18.815 ml

Stored in aliquots at -20˚C

RT MIX 1

5 x First Strand buffer	400 ul
dNTP (100 mM) 2.5 ul each	2.5 ul x 4 ul
DTT (0.1 M)	200 ul

ddH_2O	90 ul

Store at -20˚C

Methods

Animal Experiment

The experiment was as conducted in [82]. Four groups of 5 week-old Sprague-Dawley rats were used, namely: Normal, Hypophysectomized (H), H+CORT and H+ACTH. Each group had at least three rats. The serum corticosterone was monitored using radioimmunoassay of ether-extracted fractions as carried out in [82]. Right after the hypophysectomy, ACTH (4u/100g body weight subcutaneous injection every other day post surgery) was injected, and CORT (100 mg/100 g body weight) was implanted subcutaneously.

RNA Sequencing

After extraction of total RNA from the hippocampus with the RNA extraction kit, the RNA samples (at 21 days) from the four groups (Normal, Hypophysectomized (H), H+CORT, H+ACTH) in triplicates were used as templates for cDNA library construction and RNA-Sequencing. The quality of RNA samples was analyzed with the UV-spectrophotometer.

RNA quality, library preparation, Illumina Hi-Seq 2000/2500 paired-end ~100bp sequencing were conducted by McGill University and Genome Quebec Innovation Centre (Montreal, Quebec, Canada). Reference mapping to the *Rattus norvegicus* genome (rn6) and following the expression analysis were done in our laboratory.

Bioinformatics analysis for RNA-Seq data

The gene expression and the intronic splicing were calculated using Bioconductor package edgeR [208, 209]. DEXSeq was used to calculate the differential alternative exon usage [210]. Visualization of the sequencing data was done with the Integrative genomics viewer (IGV) (created at the Broad Institute of MIT and Harvard, (http://www.broadinstitute.org/igv) [211]

Classification of significant genes and functional annotation was performed with a Database for Annotation, Visualization and Integrated Discovery (DAVID) developed at the U.S. National Institute of Allergy and Infectious diseases, (http://david.abcc.ncifcrf.gov) [212].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For 10ul of reverse transcription reaction, about 600 ng of RNA was used. *Sept8* was amplified for 32 cycles in the PCR reaction. 3.0% agarsoe gels containing ethidium bromide was employed to view the PCR products under ultraviolet (UV) light with a camera.

Table 2. Primers for semi-quantitative RT-PCR

Sequence amplified	**Primers**	**PCR conditions (Annealing temp)**
TDRD7	F- CCTCAGACAACCAGGATTTGC R- CAGGGATGACTTTGGACTGAAC	60°C
Sept8	F-TCTGAGGAAAGACAAGGACAAG R1-TCACCAGAGCGGCACATAGC R2-CCGTGTGGAGGAACTGTTGC	65°C

Kcnd3	F- CCACCTGCTACACTGCTTAG R- TCTGCTCATCAATAAACTCGTGG	60°C

Image data analysis

Image J software (created by the US National institutes of Health and its available at http://rsb.info.nih.gov/ij/) [213] was used to quantify the data images.

Statistical analysis

Data was presented as means ± S.D. for experiments carried out independently and repeated in triplicates. *P* values < 0.05 is regarded as statistically significant.

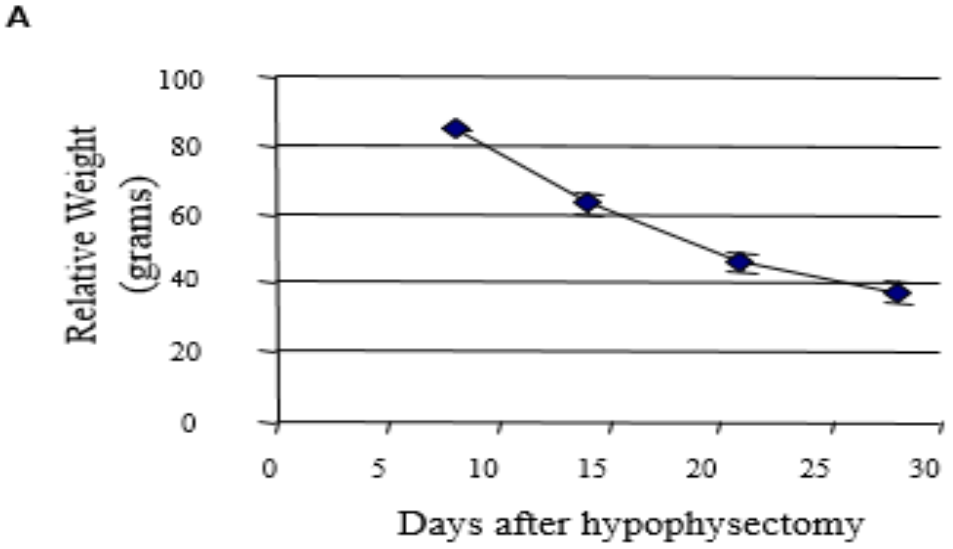
CHAPTER III

Results and Discussion

1. Reduced stress hormone in hypophysectomized rats and successful rescue

Previous studies have applied hypophysectomy to ascertain a reduced hormonal production [82, 214, 215]. The pituitary gland produces hormones: posterior pituitary gland- antidiuretic hormone, oxytocin; intermediate zone- melanocyte-stimulating hormone; anterior pituitary gland- prolactin, thyroid-stimulating hormone, growth hormone, follicle-stimulating hormone, adrenocorticotropic hormone, and luteinizing hormone. The removal of the pituitary gland was performed to inhibit the production of the hormones (particularly stress hormones) to assess its effect on the hippocampus. Hence, we examined the weight loss of the rats after hypophysectomy to confirm the successful surgery (Fig 4A).

We confirmed the hypophysectomy effect on stress hormones by assessing the serum corticosterone concentration across the groups; Normal, Hypophysectomised, H+ACTH, H+CORT.



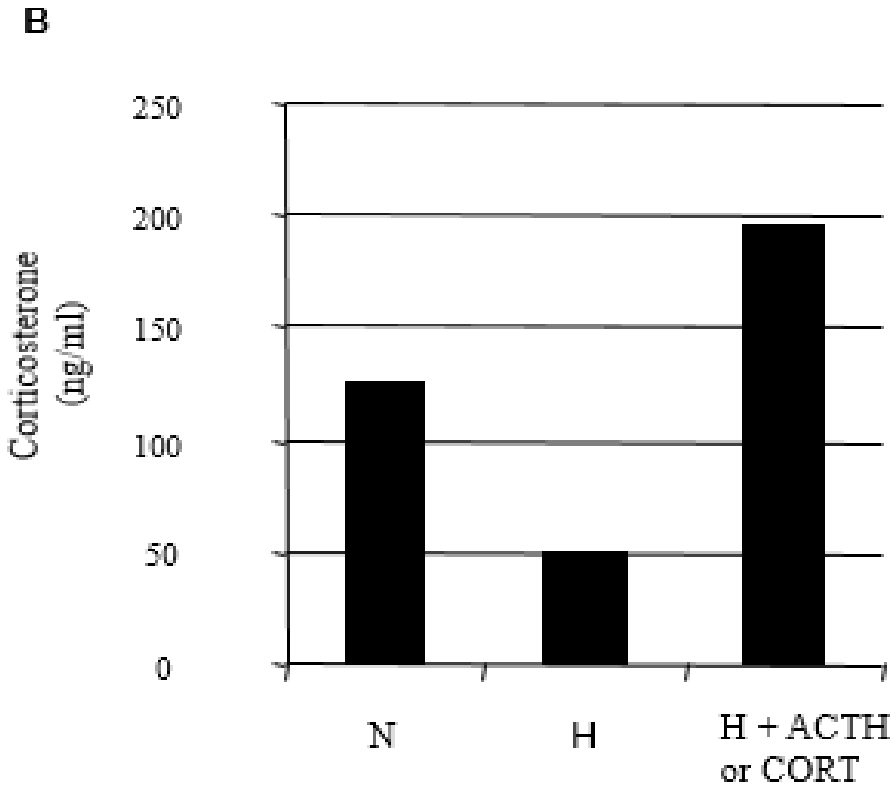


Fig. 4: Effect of hypophysectomy on the production of stress hormones in rats. (A) Rats showed reduced weight (grams) as the day goes by **(B)** Graph summarizing the serum corticosterone level in normal (N), hypophysectomized (H) rats and rescue with either ACTH or CORT.

The corticosterone level in hypophysectomized rats (Fig 4B) mimics cortisol insufficiency such as Addison's disease [216] because the corticosterone level is below the normal. In our rescue system, the corticosterone level is above the normal which could mimic cortisol overproduction such as Cushing's syndrome [217]. There has been a relationship between high cortisol levels and hyperactive HPA axis in major depressive disorder [108, 218]. Cushing's disease has been related to reduced hippocampal volume and function [219]. As the

hippocampus contains a high level of the glucocorticoid receptor, high levels of cortisol could result in hippocampal abnormality [220]. Furthermore, It has been observed as well that depressed patients have a high plasma concentration of cortisol and many studies have related high glucocorticoid concentration to stress-induced atrophy of the hippocampus [221, 222]. Neuroimaging studies have also shown hippocampal reductions in depressive patients, nonmedicated depressed patients and in PTSD patients [223-227]. Together, these suggest that cortisol overproduction negatively impacts the hippocampus.

2. Gene expression changes in the hippocampal RNA samples

To determine the genes whose expression changes in the hippocampus due to hypophysectomy and rescued by CORT or ACTH, we carried out an RNA-Seq analysis on the four groups (Normal, hypophysectomized (H), H+CORT, H+ACTH). The edgeR analysis revealed 1,530 genes with total transcripts changes in expression due to hypophysectomy and rescued. (Fig 5A). Moreover, 32 genes had fold changes >2 (Fig 5B).

The 32 genes changed with fold changes > 2.0: *Ttr, RNaseP_nuc, Enpp2, Opalin, Hmgb1, AABR07070043.1, Plekhb1, LOC100361854, Rps14, Atp1b2, Fam65a, SCARNA13, Nrgn, Scaf1, Kcnc3, Fam107a, Shank1, LOC100911911, Slc25a23, SCARNA2, Mt-nd5, Mt-cox3, Mt-nd4l, Mt-nd4, AABR07000398.1, Rn7sl1, Metazoa_SRP, Mt-atp6, Mt-nd1, Mt-nd2, Mt-cytb, Mt-atp8.*

In the hypophysectomized rats, one of the most up-regulated genes is *Ttr*. *Ttr* is expressed higher in the hypophysectomized sample over the normal. Previous studies have shown the relationship between *Ttr* and stress hormones. A study was carried out on rat choroid plexus and choroid plexus epithelial cell lines. These cell lines were incubated with varying concentrations

of hydrocortisone and estradiol. *Ttr* mRNA levels and protein increase in a concentration-dependent manner was observed [228]. Administration of glucocorticoid receptor and mineralocorticoid receptor antagonist suppressed the effect. It has also been shown that the *Ttr* gene in rats possesses GRE at the 3' region of the first intron via *in silico* analysis [229]. Hence it was concluded that the upregulation of *Ttr* gene expression by stress hormones is via the glucocorticoid responsive element. Oxidative stress could cause neurodegenerative diseases such as Alzheimer's disease [230]. Oxidative stress has been known to be the cytotoxicity of beta-amyloid deposits [231].

The three most downregulated genes in the hypophysectomy samples are *Mt-nd2, Mt-cytb, and Mt-atp8*. They are genes of the mitochondria, which is the main energy generation site of the cell. Mitochondria contains ~16.6kb circular chromosomes with about 37 genes of which about 13 of them code for proteins related to electron transport chain [232-234]. The energy required for the adaptation to stressful conditions at different levels such as intracellular processes (e.g. gene transcription), tissue structural changes (synaptic change in the brain), and endocrine system (hormonal changes) is the mitochondria [235]. The ability of the mitochondria to produce energy (ATP) from food substrate and oxygen via the electron transport chain (ETC) makes it essential to many cell types, tissues, and transcriptional regulation [236]. Mitochondria is not only valuable in the production of energy during stressful conditions, but it is also integrated into the HPA axis by synthesizing steroid hormones including glucocorticoids [237].

When ACTH arrives at the adrenal cortex to stimulate glucocorticoid production, a steroidogenic regulatory protein facilitates the movement of cholesterol across the mitochondria membrane [237]. Pregnenolone conversion occurs in the matrix. In the endoplasmic reticulum, pregnenolone is converted to deoxycorticosterone in rodents. Eventually, in the mitochondrial

matrix, 11β-hydroxylase produces corticosterone which will be exported to the circulation [235]. Studies have revealed that GR is present in the mitochondria which could suggest the action of glucocorticoid in the mitochondria [238-243]. The activation of GR in the mitochondria to enhance or repress gene expression seems to act by binding to mtDNA D-loop [244].

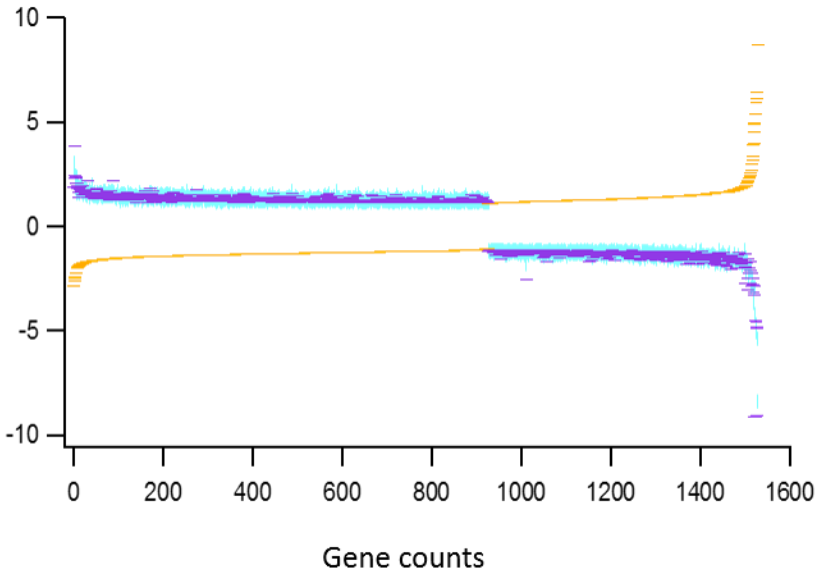
In the hippocampus, GR was observed to bind to mitochondria D-loop upon the administration of corticosterone acutely. This was observed via ChIP-sequencing. PCR and RNA sequencing confirmed the altered expression of mtRNA by acute restraint stress in the same study. There was down-regulation of mtDNA-encoded genes with significant effects on the expression of *mtND-3, mtND-1, mtATP-6* and *mtND-6* [234, 245]. Several studies have associated stress-related disorders with mitochondria dysfunction [246-251].

Table 3: Functions of some changed genes

Gene Name	**Functions**
Ttr	Encodes the protein, transthyretin, which transports thyroid hormones in the serum and cerebrospinal fluid (CSF) [252]. It can also be a regulator of retinol transportation of which retinol plays a role in hippocampal neurogenesis [253].
Enpp2	Encodes the enzyme ectonucleotide pyrophosphatase/phosphodiesterase family member 2, which hydrolyzes lysophosphatidylcholine to lysophosphatidic acid [254].
Hmgb1	Encodes the protein, high mobility group protein 1, a non-histone protein involved in DNA organization and transcription regulation [255].
Rps14	Provides instructions to make ribosomal proteins [256].
Atp1b2	Encodes a protein that belongs to the Na+/K+ ATPase beta chain family, which maintains the Na+ and K+ ions electrochemical gradient in the plasma membrane [257].

Nrgn	Encodes the protein neurogranin, which serves as a calmodulin-binding protein in the absence of calcium [258].
Kcnc3	Functions in repolarization of fast firing brain neurons [259].
Shank1	Encode scaffolding proteins that function in the development of neuronal synapses [260].
Mt-nd4	Encodes the protein, NADH dehydrogenase 4, which catalyzes NADH hydrogenation and electron transfer to ubiquinone [261].
Mt-atp6	Encodes a protein that provides information for normal mitochondrial function [262, 263].
Mt-nd1	Encodes the protein, NADH-ubiquinone oxidoreductase chain 1, important in electron transport chain in the mitochondria [264].
Mt-nd2	Encodes the protein, NADH reductase 2, which transfers electrons to the electron transport chain in the mitochondria [264].
Mt-cytb	Encodes the protein, mitochondrial cytochrome b, functions in the complex III of the mitochondrial respiratory chain. Electron transfer catalysis from coenzyme Q to cytochrome c is conducted by complex III [265].
Mt-atp8	Encodes a subunit of the mitochondria ATP synthase enzyme, which plays a role in ATP production during oxidative phosphorylation [262].

A


10
5
0
-5
-10
0
200
400
600
800
1000
1200
1400
1600
Gene counts

B


N vs H
H vs CORT
H vs ACTH

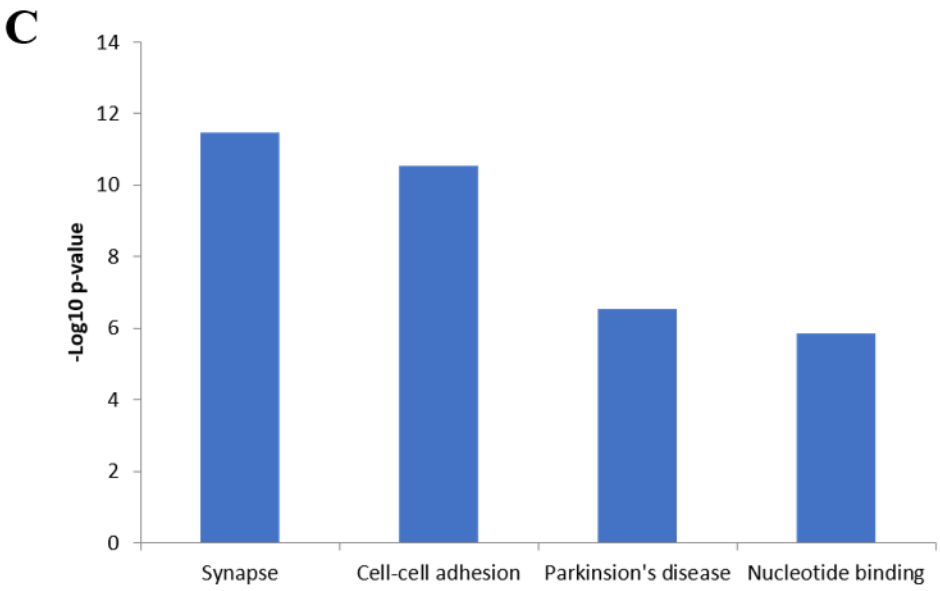


Fig 5: Total transcript changes of genes in hippocampal RNA samples (A) 1530 genes whose changes by Hypox were reversed by both ACTH and CORT, ranked according to the changes by Hypox vs Normal, analyzed by edgeR (FDR<0.1, average reads >50 **(B)** Heatmap showing 32 genes with >2 fold changes by hypophysectomy and rescued by CORT and ACTH. **(C)** Top 4 most significant function clusters of 1,530 genes with total transcripts.

Based on the edgeR results, we examined the functional cluster of these 1,530 genes using DAVID, which indicates that the changed genes cluster for specific functions. Specifically, the genes changed cluster for synapse, adhesion, Parkinson's disease and nucleotide-binding at the transcript level (Fig 5C).

Looking at the top functional annotation clusters in our study, we found that previous research had shown the relationship between stress hormones and synapse, adhesion, Parkinson's disease and nucleotide-binding. For example, AMPA glutamate receptors function in synaptic transmission and can be regulated by stress hormones to modulate synapses [266-270]. The mechanism in which AMPA receptors are regulated by stress hormones is through the activation

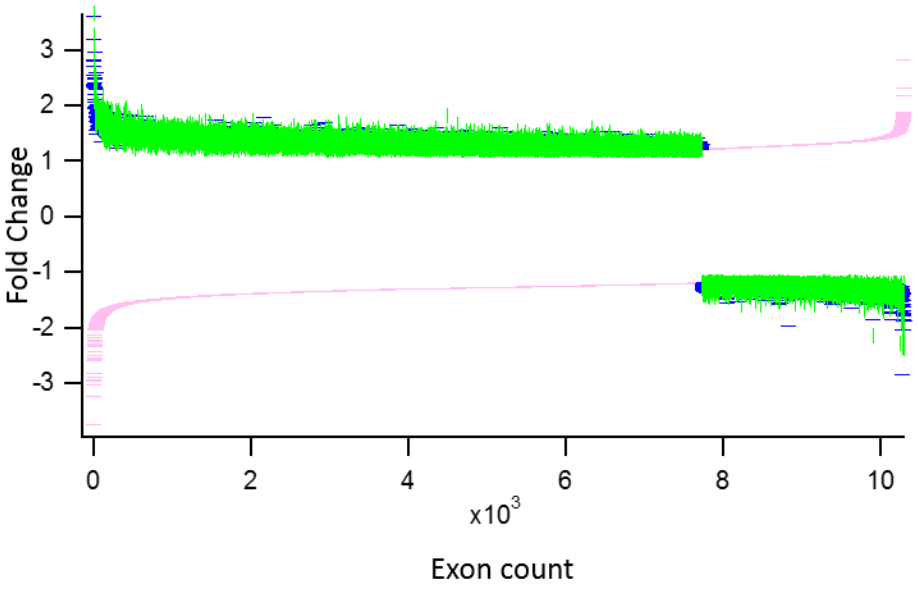
of β-adrenergic receptors to activate PKA and CaMKII to cause the GluA1 to phosphorylate at *Ser845* and *Ser831* [267, 271]. Also, corticosteroid hormones can increase hippocampal synaptic transmission within minutes of its application through the activation of MR. This could be as a result of the increase in presynaptic glutamate release where the *Erk* pathway is involved [266, 272]. Hence, activation of β-adrenergic receptors alongside corticosterone enhances AMPA receptor function [273].

Cell-cell adhesion molecules have also been shown to be involved in cognitive functions. For instance, neuronal adhesion molecules such as the immunoglobulin family and L1 mediates the impact of stress in the brain. The immunoglobulin superfamily has cell adhesion molecule that functions in synapse and circuit remodelling. Chronic stress impairs the cell-adhesion expression to lead to neuronal damage [98]. In the hippocampus, 21 days chronic restraint stress, increased expression of L1 mRNA and protein but diminished expression of neural cell adhesion molecule (NCAM) to result in cognitive and neural alterations [97, 98, 274]. There are functional implications in the hippocampus affected by the alterations of NCAM such as dendritic atrophy and structural alterations, increased aggressive and anxiety behaviors [275, 276].

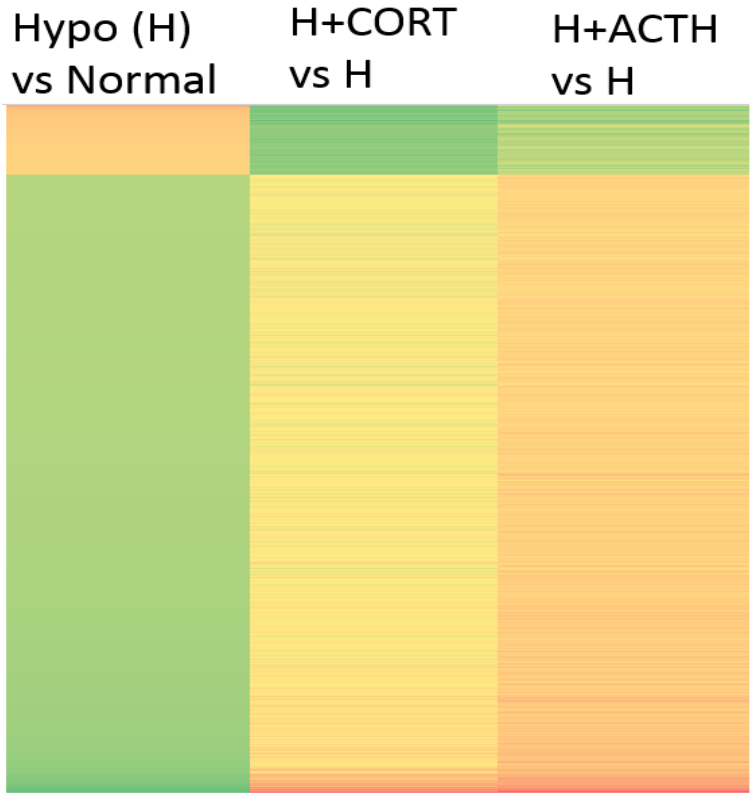
3. Exon changes of genes in the hippocampal RNA sample

To identify the exons that are changed in the hippocampus due to hypophysectomy and rescued by CORT or ACTH, we carried out an RNA-Seq analysis on the four groups (Normal, hypophysectomized (H), H+CORT, H+ACTH). In the normal vs hypophysectomized group, 889 exons had a fold change of >1.5 (Fig 6A) that were successfully rescued by either CORT or ACTH (Fig 6B).

A


Fold Change
3
2
1
0
-1
-2
-3
0
2
4
6
8
10
x10^3
Exon count

B


Hypo (H)
vs Normal
H+CORT
vs H
H+ACTH
vs H

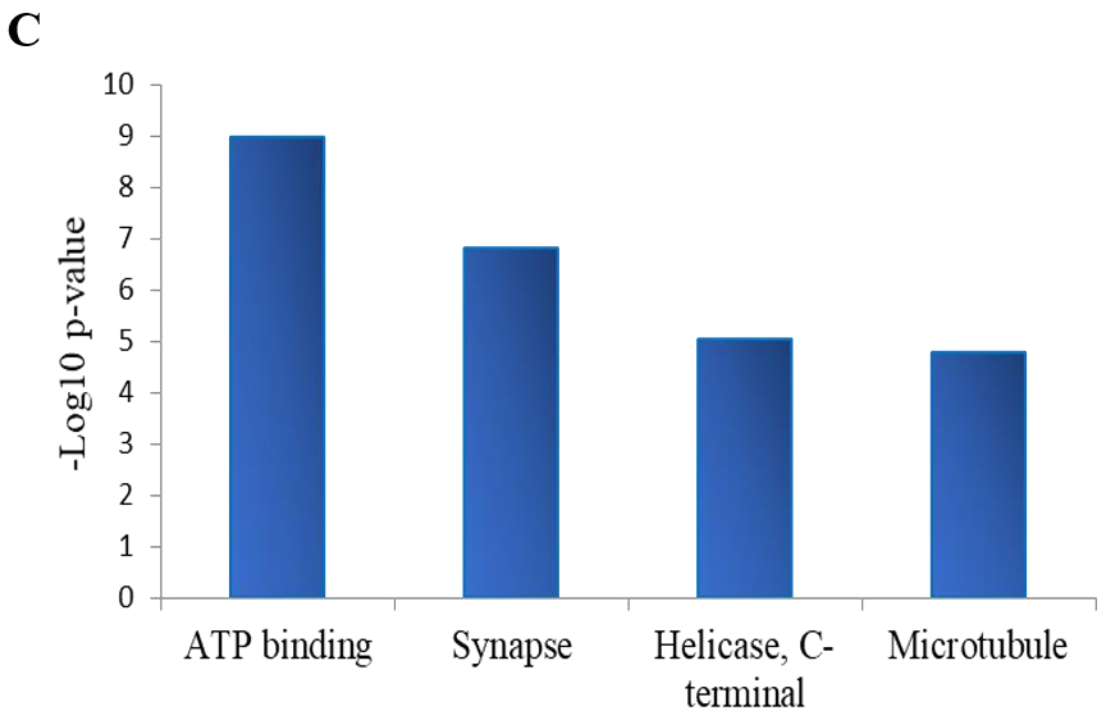


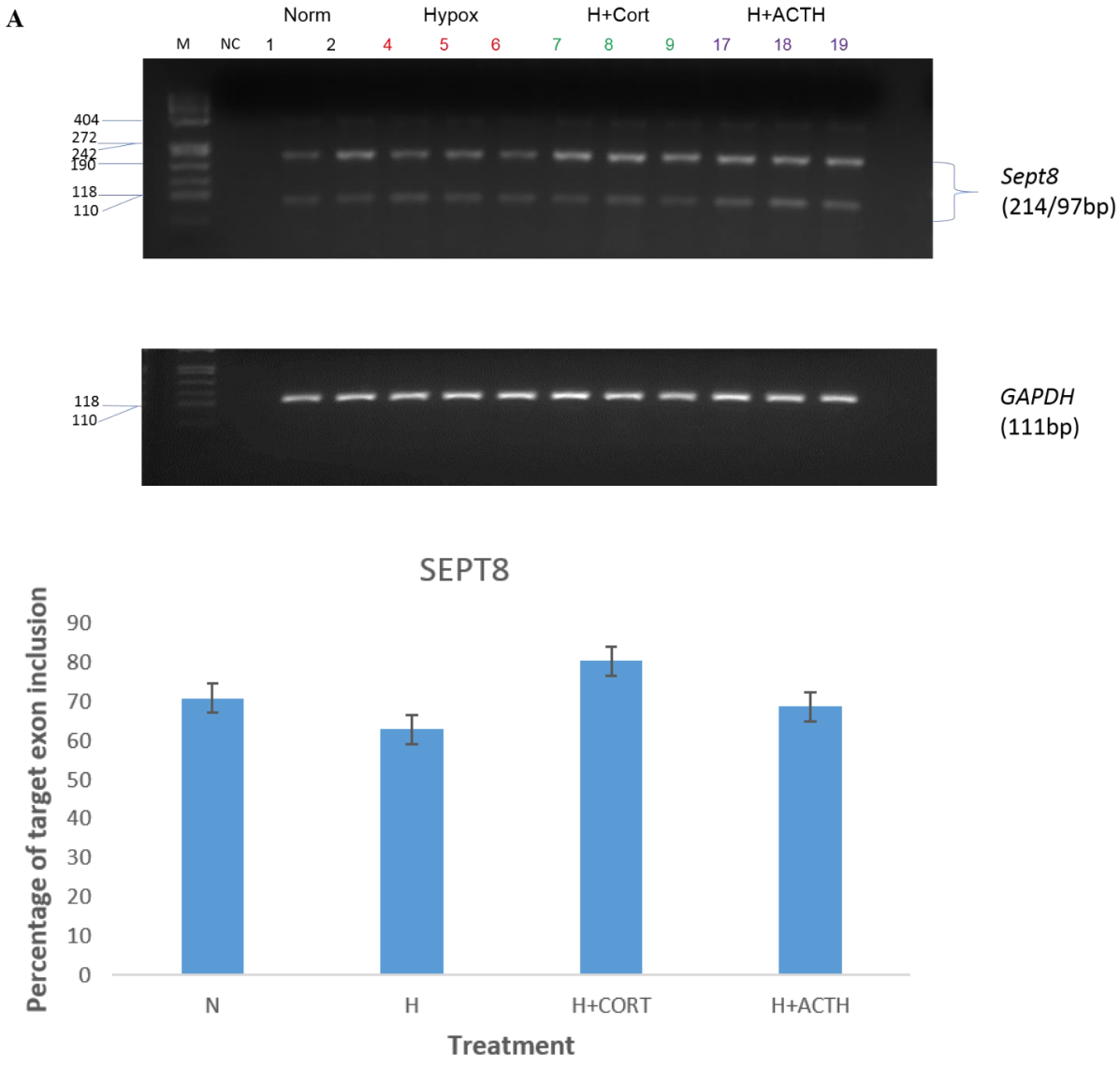
Fig 6: Exon changes in hippocampal RNA samples (A) 10,300 bins/exons (X-axis) that changed with >1.2 fold changes. Normal (horizontal), CORT (blue) vs Hypox or ACTH (green) vs Hypox groups (each of the three compared to H), ranked according to the changes by Hypox vs Normal. **(B)** Heatmap showing 889 exons changed with >1.5 fold changes. **(C)** Top 4 most significant function clusters of 970 ENSEMBL genes whose exons were altered by hypophysectomy and rescued by both ACTH and CORT.

Further analysis of the biological functions of the 970 ENSEMBL genes whose exons are altered by hypophysectomy and rescued by both CORT and ACTH revealed the most significant four clusters, as analyzed by DAVID. The functional clusters are: ATP binding, synapse, helicase, and microtubule (Fig 6C).

4. Validation of exons regulated by stress hormones

To identify the exons that are changed in the DEXSeq analysis, we examined the exons in IGV and validated using semi-quantitative RT-PCR. We designed primers for alternative exons in *Sept8*, *Kcnd3* and *Tdrd7* genes. RT-PCR demonstrates that upon hypophysectomy, the

transcripts containing the target exon in the *Sept8* and the other two genes were reduced or increased (Fig 7). In the H+CORT and H+ACTH groups, the exon seems to be rescued successfully, which indicates that the splicing events are regulated by corticosterone and ACTH.



B

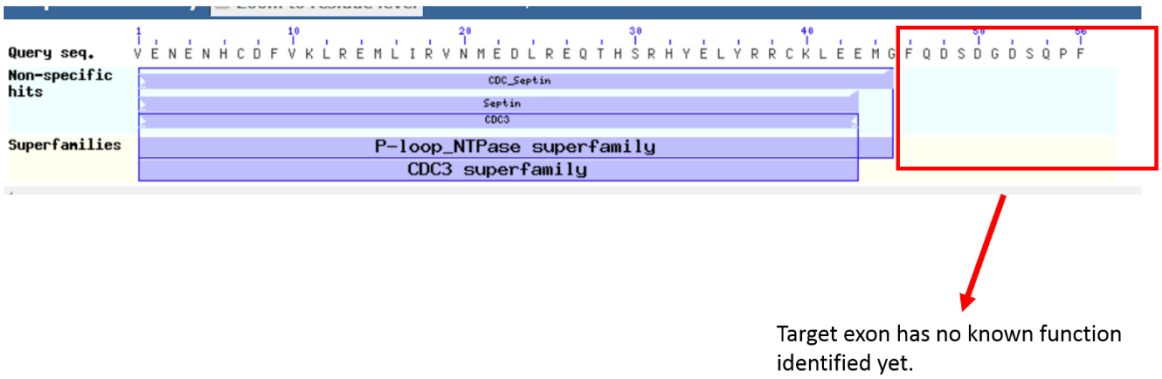
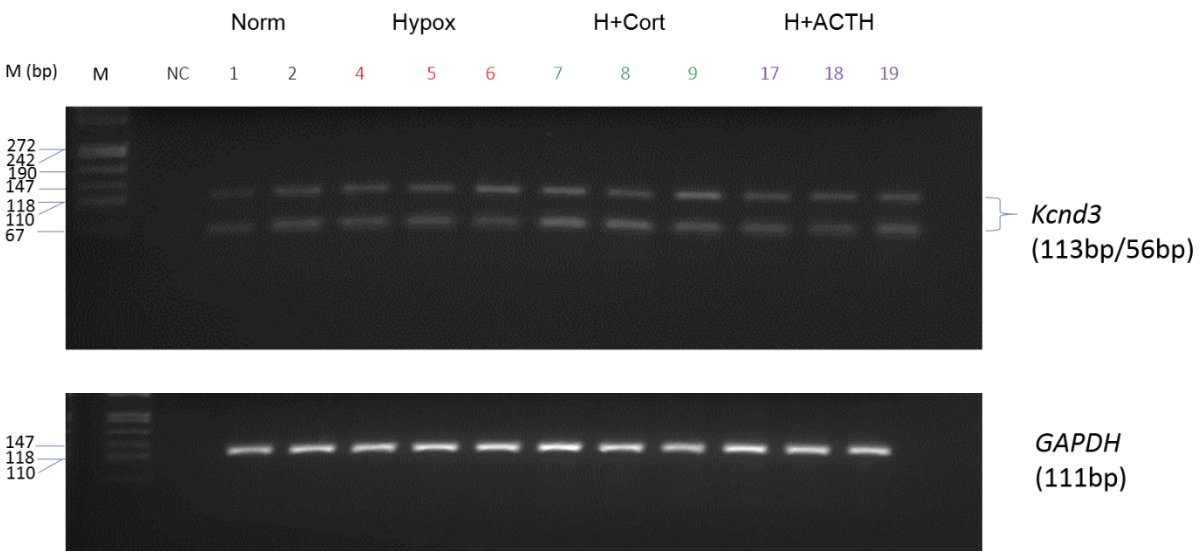
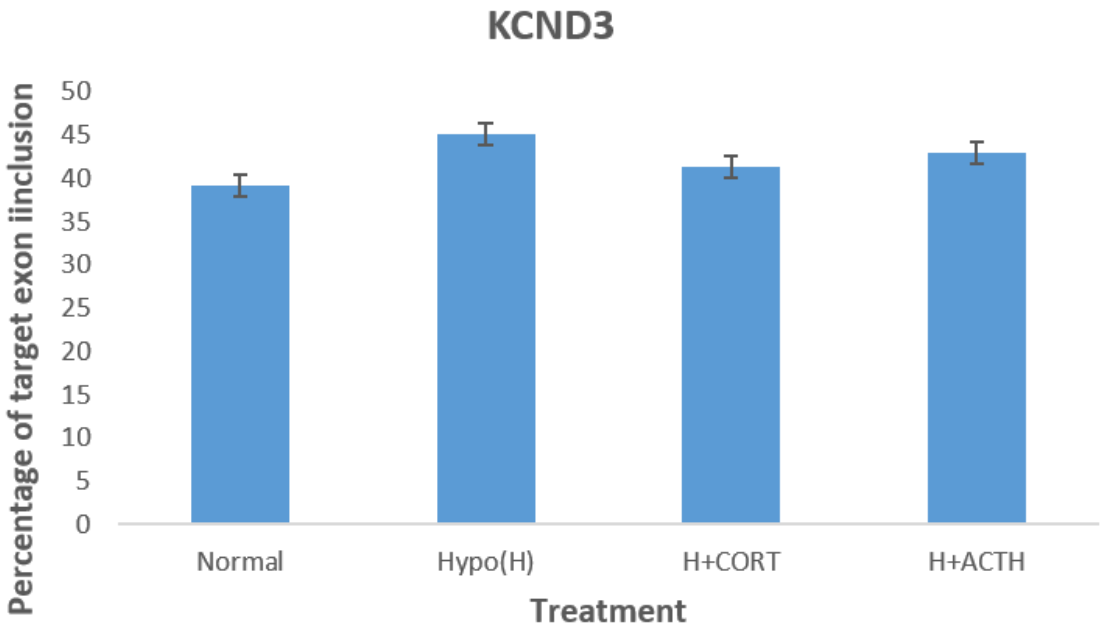


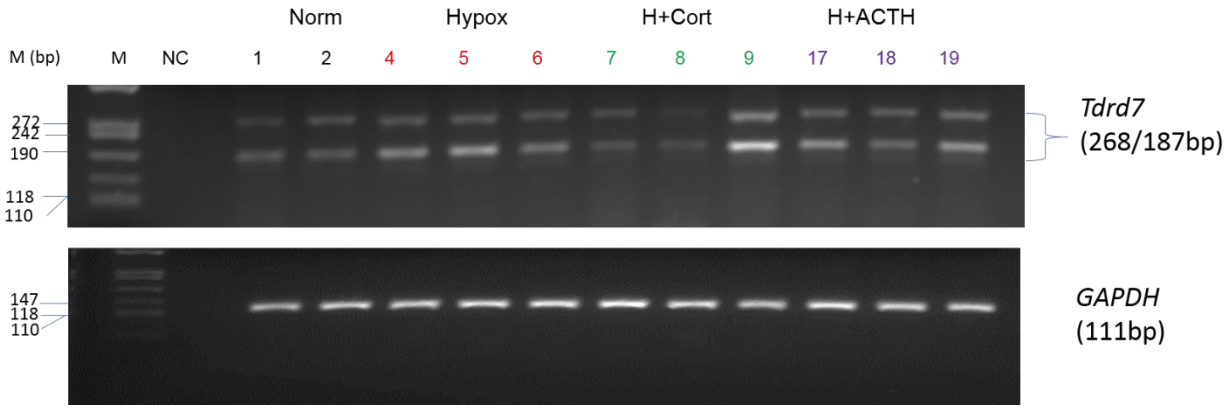
Image obtained from NCBI BLASTP

C




KCND3
Percentage of target exon iinclusion
50
45
40
35
30
25
20
15
10
5
0
Normal
Hypo(H)
H+CORT
H+ACTH
Treatment

D


Norm
Hypox
H+Cort
H+ACTH
M (bp)
M
NC
1
2
4
5
6
7
8
9
17
18
19
272
242
190
118
110
Tdrd7
(268/187bp)
147
118
110
GAPDH
(111bp)

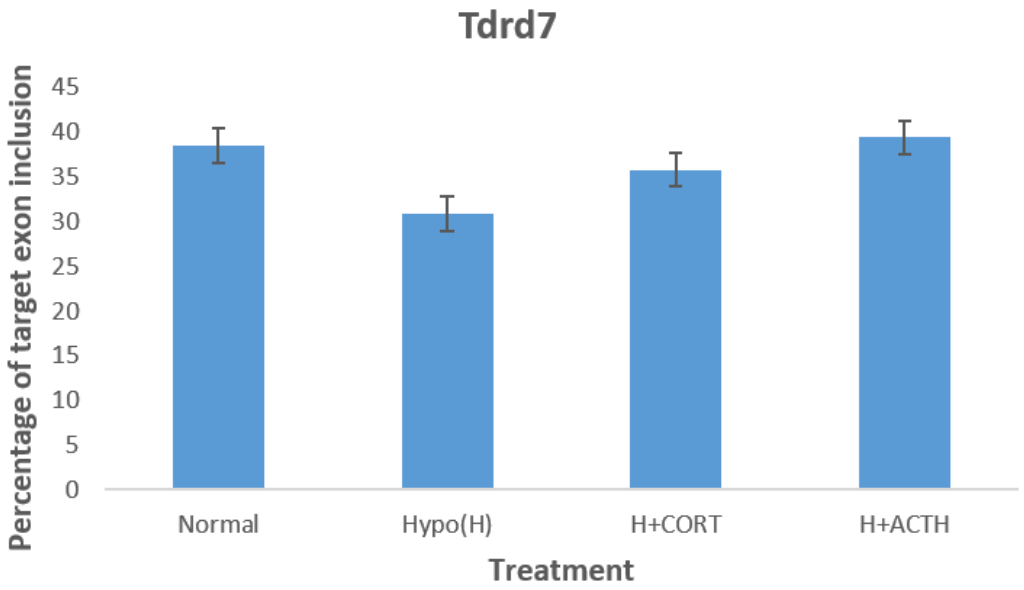


Fig 7: RT-PCR validation of regulated exons (A) RT-PCR products (agarose gel) of target exon in *Sept8* gene upon hypophysectomy and rescue by CORT or ACTH. Left, DNA size markers (bp), NC: PCR negative control. **(B)** Image from NCBI BLASTP showing the domain functions of the exon in the *Sept8* gene. **(C-D)** RT-PCR products (agarose gel) of target exon in KCND3 and TDRD7 respectively without regulation by stress hormones. Left, DNA size markers (bp), NC: PCR negative control

Sept8 gene which is expressed in the brain belongs to the septin family, and functions in vesicle trafficking, synaptic plasticity, and the stability of axons and dendrites [277-280]. Studies have shown the implication of septins in a number of neurodegenerative diseases such as Alzheimers disease and schizophrenia [281-283]. For instance, *Sept8* influences amyloid precursor protein (APP) by modulating intracellular sorting and stability of the β-site APP cleaving enzyme (BACE) in neuronal cells. This suggests *Sept8* could be linked to the cellular processes in the development of Alzheimer's disease.

Kcnd3 (Potassium voltage-gated channel subfamily D member 3) gene encodes the potassium channel Kv4.3 protein with its functions in neurotransmitter release, neuronal

excitability and heart rate. They are vital in membrane repolarization in excitable cells [284]. The target exon in this gene seems to be down-regulated by stress hormones.

Tdrd7 (tudor domain-containing protein 7) gene encodes proteins in the Tudor family, which plays a role in lens development and spermatogenesis. Mutations in this gene causes cataracts and glaucoma [285, 286]. The target exon seems to be up-regulated by stress hormones

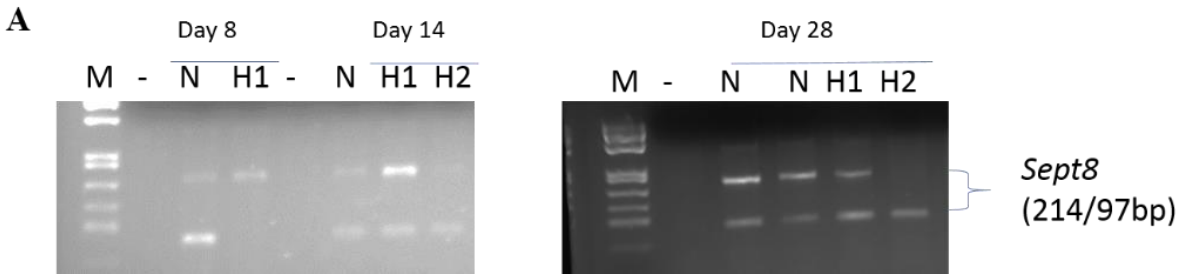
Taken together, these suggest that stress hormones regulate splicing of specific exons in specific genes.

5. Time course analysis for the regulated exon of the *Sept8* gene

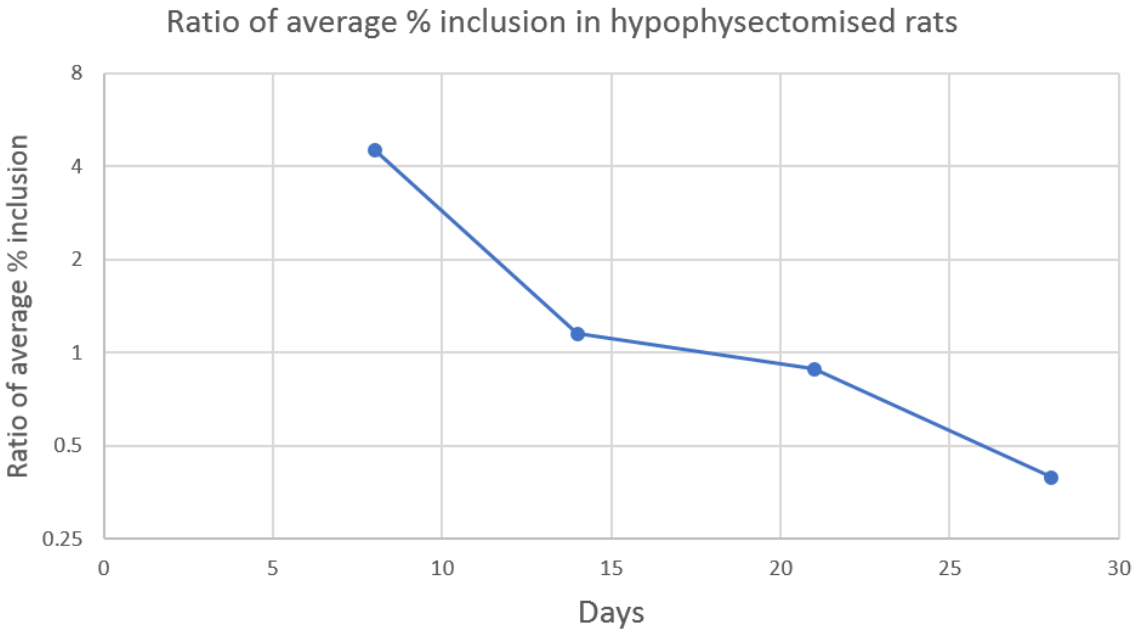
To determine if the regulated exon changes in a time-dependent way. In the normal group, we observed an increase in the transcripts containing the target exon from days 8, 14, 21, to 28. Whereas in the hypophysectomized rats, the transcripts containing the target exon were decreased from day 8 to day 28 (Fig 8). This result is similar to a previous study carried out on rats to assess *Slo* transcripts containing STREX which was reduced upon hypophysectomy and rescued successfully by the administration of ACTH. There was a decline in the transcripts containing STREX days after hypophysectomy [82].

Aging serves as one of the risk factors for some neurodegenerative diseases like dementia and Alzheimer's disease. Some studies have shown gene expression changes as rat ages [287-289]. For instance, rats of ages 6-27 month-old were assessed for age-related gene expression signatures (AGES) in different tissues (liver, muscle, kidney, gastrocnemius, and hippocampus). Genes that changed in multiple tissues and provide mechanisms of aging [289]. However, in our current study, we observed a splicing change in the animals as they age. Although we did not

look at the gene expression changes in terms of months for the rats like the above study, which will be an interesting area to look at in the future. Therefore, this study provides the possibility of a probable age signature for determining the onset of neurodegenerative disease in aging individuals. Further research will be required to confirm this suggestion.



B


Ratio of average % inclusion in hypophysectomised rats
Ratio of average % inclusion
8
4
2
1
0.5
0.25
0
5
10
15
20
25
30
Days

C

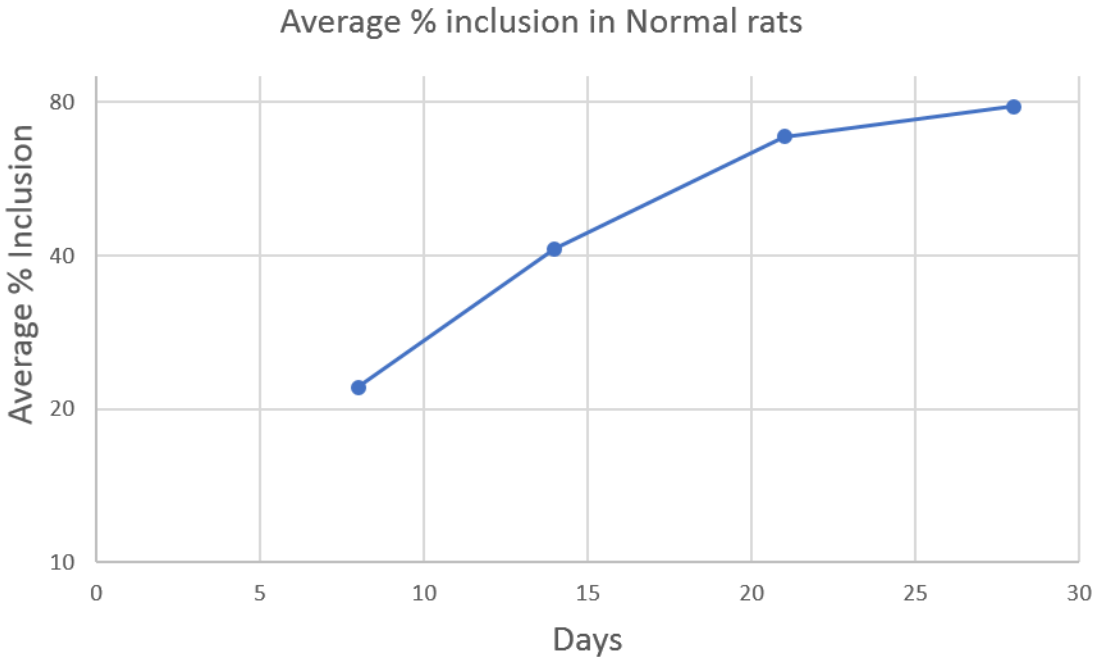
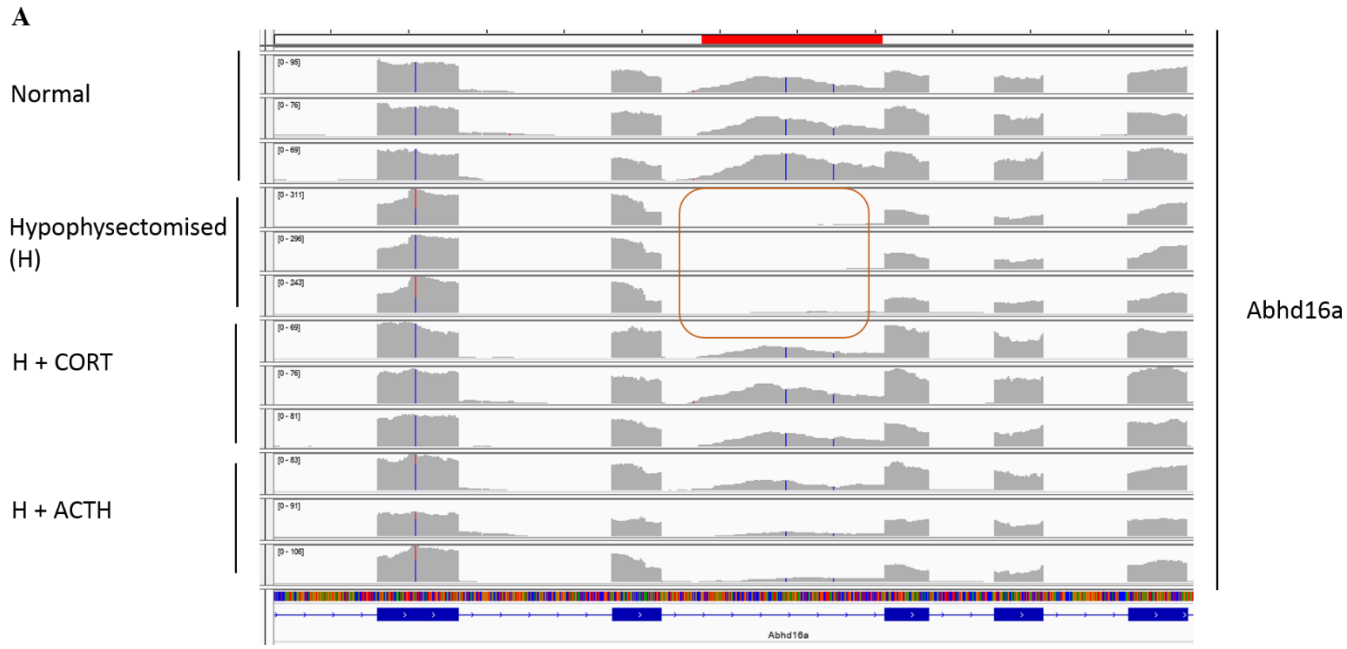

Average % inclusion in Normal rats
Average % Inclusion
80
40
20
10
0
5
10
15
20
25
30
Days

Fig 8: Time course for the target exon in *Sept8* gene (A) Agarose gels showing RT-PCR products of target exon in *Sept8* gene in a time course dependent manner in normal rats and upon hypophysectomy. N: Normal, H: Hypophysectomized **(B)** Graph showing the average percentage inclusion of target exon in *Sept8* gene upon hypophysectomy **(C)** Graph showing the increase in percentage inclusion of target exon in *Sept8* gene in normal rats

6. Intronic targets/cryptic exons identified by RNA-Seq analysis

To determine the introns/cryptic exons that emerged or eradicated in the hippocampus due to hypophysectomy and rescued by CORT or ACTH, we carried out an edgeR analysis of the intronic reads on the four groups (Normal, hypophysectomized (H), H+CORT, H+ACTH). The target intronic region of Abhd16a was eradicated upon hypophysectomy but rescued with either CORT or ACTH (Fig 9A) In *Tmem143*, cryptic exon emerged upon hypophysectomy which was rescued by either CORT or ACTH (Fig 9B).



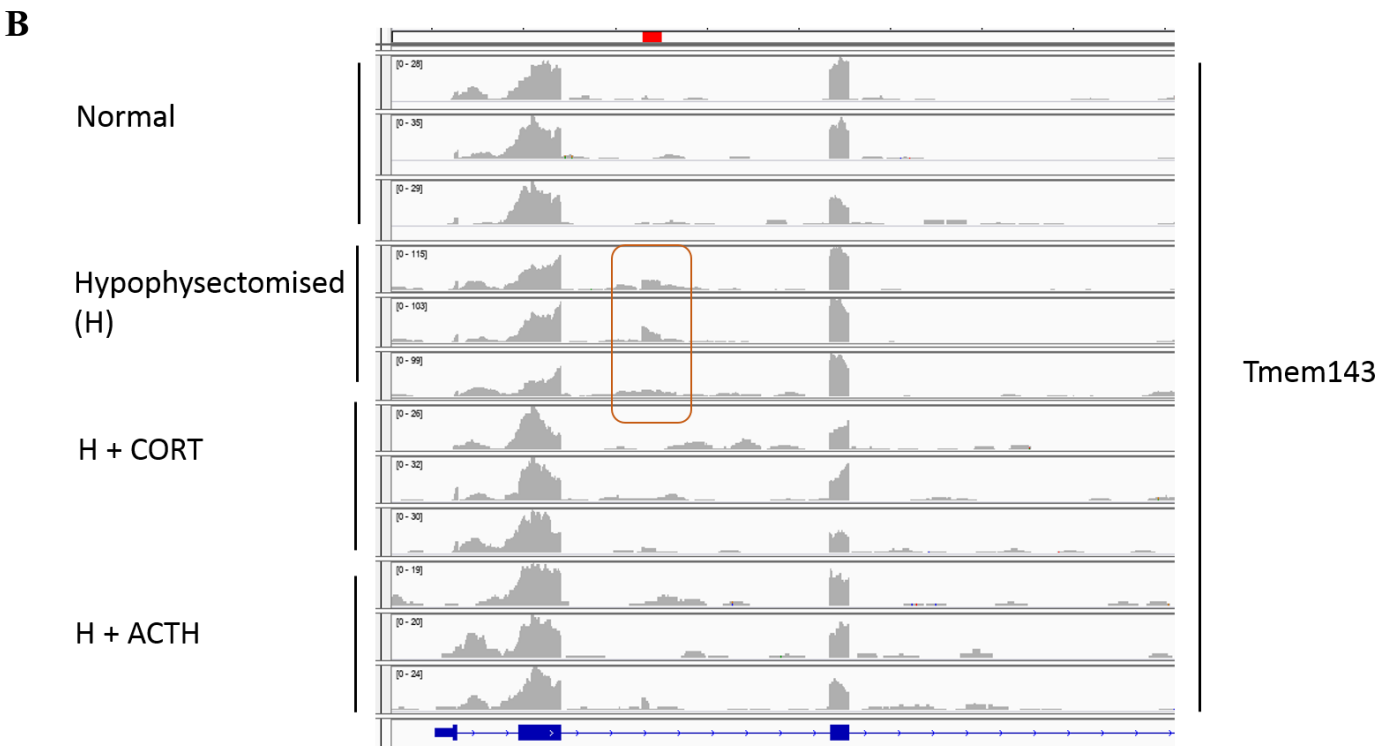


Fig 9: Intronic targets identified by RNA-Seq analysis (A-B) Integrative genomics viewer (IGV) screenshot of intronic target in Abhd16A gene and Tmem143 gene respectively (Big & small red box indicates the target of interest). Each row indicates one sample. Each of the treatment groups are separated by a thick black line. The red circle indicates the target region, the reads are the (big blue box indicates exons, small blue box indicates UTR and blue line indicates introns, the arrows indicate the direction of the transcription)

Abhd16a (Abhydrolase domain-containing 16a): This gene is involved in a cluster of genes for tumor necrosis factor alpha and beta. The human major histocompatibility complex class III (MHC III) is one the areas in which the protein encoded by this gene is clustered for, to suggest its role in immunity. In the brain, ABHD16a is the primary phosphatidylserine lipase [290].

Tmem143 (Transmembrane 143): This gene encodes the protein transmembrane protein 143, of which transmembrane proteins functions in vesicle trafficking, ion transport, and compartmentalization [291]. However, the function and detailed description of this gene remains to be fully elucidated.

CHAPTER IV

GENERAL DISCUSSION ON THE OBSERVATIONS

1. Gene expression regulation by stress hormones

Several studies have revealed genes that are regulated by stress hormones in the hippocampus [109, 110]. The genes changed are clustered for synapse, adhesion, Parkinson's disease, and nucleotide-binding at the transcript level. At the exon level, the genes clustered in ATP binding, synapse, helicase, and microtubule. Therefore, these suggest that the regulation of the gene expression by stress hormones in the hippocampus could majorly impact memory-related functions in the hippocampus.

Of the 32 genes changed upon hypophysectomy and rescued by administration of either CORT or ACTH, *Ttr* gene is the most upregulated. Previous studies have reported that *Ttr* expression is directly regulated by glucocorticoids in hippocampal neurons [228]. The evidence thus suggests that stress hormone inhibits the expression of *Ttr* gene, which can make one prone to neurodegenerative disorders under stressful conditions [292]. This might be because *Ttr* has a protective function, that its increased expression prevents a-beta caused neurodegeneration. Several studies have reported the neuroprotection function of *Ttr* gene in Alzheimer's disease. For instance, a comparative study revealed a decrease in the concentration of *Ttr* in the CSF and plasma of Alzheimer's patients compared to healthy individuals [292-295]. Hence *Ttr* gene can serve as a neuronal biomarker of neurodegenerative diseases such as Alzheimer's disease [296].

Since several studies have shown the direct impact stress and corticosteroids have on mitochondria DNA transcription [297-299]. Here, we revealed three most downregulated genes

at the transcript level to be mitochondria genes namely: *Mt-nd2, Mt-cytb,* and *Mt-atp8*. A brief description of these genes are stated below;

mt-Nd2, also known as mitochondrially encoded NADH dehydrogenase 2, located in the mitochondria inner membrane (electron transport chain) in which transfer of electrons occurs from NADH to the mitochondria's respiratory chain [264]. However, upon hypophysectomy, we observed a downregulation of *mt-ND2* which suggests that electrons won't be transferred from NADH to the respiratory chain of the mitochondria.

mt-cytb, also known as mitochondrial cytochrome b functions in mitochondrially complex III of the mitochondria respiratory chain. Electron transfer catalysis from coenzyme Q to cytochrome c is conducted by complex III [265]. Upon hypophysectomy, we observed *Mt-cyb* gene expression was downregulated, suggesting that glucocorticoids prevent the production of cytochrome b to transfer energy within cells.

mt-ATP8, also known as mitochondrially encoded ATP synthase, is involved in ATP production for the cells [262]. If there is a defect in the biogenesis of ATP synthase, there could be a dysregulation of reactive oxygen species (ROS) in the electron transport chain [300, 301]. Several studies have linked oxidative stress to the onset of neurodegenerative and neurological disorders [248-250]. Upon hypophysectomy, we observed *mt-ATP8* gene expression was downregulated which could suggest that glucocorticoid affects the production of ATP required by the cells.

2. Splicing regulation by stress hormones

Previous studies have revealed the regulation of splicing by stress hormones in adrenal glands, bovine chromaffin cells [82, 114]. Not so much has been done on the regulation of

splicing by stress hormones in the hippocampus. Hence, we identified 889 exons that are changed upon hypophysectomy and rescued by CORT or ACTH. A typical example validated was the reduction in transcripts that contains the target exon in the *Sept8* gene upon hypophysectomy which was rescued by the administration of CORT or ACTH. This is similar to the observation of the reduction of transcripts containing STREX upon hypophysectomy and rescued by ACTH [82].

Another study also showed a decrease in transcripts containing STREX when glucocorticoids were applied directly to bovine chromaffin cells, confirming that stress hormones regulate splicing [115]. According to our knowledge, we show for the first-time regulation of the target exon in the *Sept8* gene particularly in the hippocampus by stress hormones. Other exons remain to be validated. Furthermore, the target exon in *KCND3* and *TDRD7* seems to be downregulated and up-regulated by stress hormones respectively. More repeat experiments will be required to confirm this interesting observation as the RNA samples are old which can likely affect the RT-PCR consistency. The functional implication of this regulation in *Sept8* gene is that the loss of this exon in the final transcripts upon hypophysectomy could lead to a loss of function. When we inputted the ORF with the target exon into NCBI BLASTP, to identify the matched domain functions, we observed there is no known domain function attached to the target exon in the *Sept8* gene. However, other domain functions identified such as cell division control (CDC3 superfamily), could suggest its involvement in cell division control. Therefore, the loss of the exon upon hypophysectomy could suggest regulation of cell division by stress hormones. Further research will be required to validate this point.

We further examined the introns to identify if there are cryptic exons that emerged or novel exons. We identified targets of *Abhd16a* and *Tmem143* genes to assess its regulation by

stress hormones. Here we observed in *Abhd16a* that upon hypophysectomy, the exon that emerged in normal was eradicated and the reverse was the case when rescued with CORT or ACTH. But in *Tmem143*, a cryptic exon emerged upon hypophysectomy which was rescued by CORT or ACTH. This remains to be validated using RT-PCR. When we inputted the ORF with the cryptic exon into NCBI BLASTP, to identify the matched domain functions, we observed there is no known domain function attached to the target exon in both *Abhd16a* and *Tmem143* genes. Further exploration will be required for this observation.

3. Time course analysis in alternative splicing

Previous studies have revealed splicing changes that are age-related in both the normal human brain (prefrontal cortex and cerebellum) and Alzheimer's disease samples [302, 303]. We observed in our study that in the normal group, there was an increase in the transcripts containing the target exon in the *Sept8* gene in a time course dependent manner which could suggest that aging plays a role in alternative splicing. But upon hypophysectomy, we observed a decrease in transcripts containing the target exon in *Sept8* gene in a time course dependent manner. Mechanisms that could play a role in this age-related splicing change are splicing factors. A study showed that certain splicing factors such as hnRNPK, PTB, and *NOVA*1, have their expression changed which decreases with age. This is as a result of age-associated changes that occurs in alternative splicing [302, 304]. Further studies will be required to identify which splicing factor is affected in our current study. Together these could suggest that aging and stress hormone play a role in alternative splicing.

Summary

This study identified transcripts/exons that are changed upon hypophysectomy and rescued by the administration of CORT or ACTH.

CHAPTER V

Conclusions

1. Hypophysectomy was confirmed by weight loss and reduced serum corticosterone levels.
2. RNA-Seq analyses identified genes whose total transcripts/exons were changed upon hypophysectomy and rescued by the administration of either ACTH or CORT.
3. The time-course analysis revealed a decline in transcripts containing the target exons days after hypophysectomy but a reverse in normal rats.

CHAPTER VI

Further considerations

1. The regions of the hippocampus

Studies have shown that different regions of the hippocampus such as ventral, dorsal reveal distinct gene expression profiles. For instance, next-generation sequencing was employed to identify the gene expression profile of the dentate gyrus cells, granule cells, and pyramidal cells of CA1, CA2, and CA3, differences in gene expression were observed [305]. In our current study, the different regions of the hippocampus were not investigated. In the future, it will be worthwhile to investigate the different regions of the hippocampus and their relationship with stress hormones in terms of gene expression and splicing.

2. Time course analysis

In a study conducted by Tea 2019 revealed the gene expression changes observed were categorized into distinct classes of the time-course analysis. The classes include early logistics, in which the gene expression changes early in life, mid-logistics, in which the gene expression changes happen in midlife, late-logistics, in which gene expression changes happen at a later point in time [289]. In our study, we did not investigate these various classes as the time points, we only focused on days. It will be interesting to investigate the splicing change observed in these rats over the time points in months similar to the above study.

Further Directions

Identification of transcripts/exons that are changed upon hypophysectomy and rescued by the administration of CORT or ACTH will lead to the question, what is the mechanism underlying this regulation? To determine the underlying mechanism in the regulation of splicing by the stress hormone, some points highlighted in the introduction of this thesis could be assessed. In our lab, we have been studying the mechanism for the regulation of STREX to identify RNA elements, splicing factors that could be involved [137, 138, 202, 204-206, 306]. For instance, there is a CaRRE that exists within the 3' splice site of STREX, which is the mechanism in which CaMK IV repressed the splicing of STREX in the BK channel [137, 138]. Hence, it will be interesting to assess if there are conserved elements that mediate the splicing of the target exon for example in *Sept8* (Fig 8A).

Another mechanism, coregulators of the glucocorticoid receptor can be further researched. For example, in neuronal cells, a GR coactivator ARGLU1 was identified to activate GR and regulate transcription and alternative splicing. Upon administration of dexamethasone in the absence of ARGLU1, alternative splicing changes were observed [207]. Other coregulators have also been identified to be involved in RNA splicing [175, 307-311]. Therefore, it will be interesting to investigate the genes whose splicing is regulated in our study and identify if there are coregulators that could be involved. Other exons that are regulated by stress hormones are currently been assessed in our lab.

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