INVESTIGATION OF THE EFFECTS OF CENTRALLY INJECTED NESFATIN-1 ON THE HYPOTHALAMIC CYCLOOXYGENASE AND LIPOXYGENASE ENZYMES USING WESTERN BLOTTING

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ÖZET

Yüksek Lisans Tezi

MERKEZİ YOLLA ENJEKTE EDİLEN NESFATİN-1'İN HİPOTALAMUSTA SİKLOOKSİJENAZ VE LİPOKSİJENAZ ENZİMLERİ ÜZERİNE ETKİSİNİN WESTERN BLOT YÖNTEMİ İLE ARAŞTIRILMASI

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Nesfatin-1 vücut sıcaklığının düzenlenmesi, enerji dengesi, kan şekerinin düzenlenmesi, üreme sistemi, gastrointestinal sistem ve stres üzerinde fizyolojik ve metabolik etkileri olan tokluk uyarıcı bir nöropeptiddir. Bununla beraber hem merkezi hem periferik yolla uygulanan Nesfatin-1'in kan basıncı, kalp atış hızı, plazma katekolamin ve vasopresin seviyelerini etkilediğine dair bulgular vardır. Oksitosin, melanokortin ve pro-opiomelanokortin reseptörlerin Nesfatin-1'in kardiyovasküler etkilerinde rol oynadığı bilinmektedir. Bu reseptörler dışında Nesfatin-1'in kardiyovasküler etkilerine aracılık eden diğer mekanizmalar konusunda çok sınırlı literatür bulunmaktadır. Nesfatin-1 ve araşidonik asidin kardiyovasküler etkileri arasında benzerik bulunmaktadır. Araşidonik asit siklooksijenaz ve lipoksijenaz enzimleri aracılığı ile prostaglandinlerin ve leukotrienlerin sentezinde prekursor rolu olan bir molekülken, bu enzimlerin inhibe edilmesi Nesfatin-1'in de anti-inflamatuar ve stress durumunda koruyucu etkilerini bastırdığını gösteren çalışmalar bulunmaktadır. Bu çalışmanın amacı merkezi yolla enjekte edilen Nesfatin-1'in kardiyovasküler etkilerinin araşidonik asit (AA) yolağı üzerinde bulunan siklooksijenaz (COX1 ve COX2) ve lipoksijenaz (LOX) enzimlerinin ifadesini ölçerek araştırmaktır. Çalışmada kontrol, salin ve nesfatin olmak üzere üç farklı gruba ayrılmış 21 erkek Sprague-Dawley sıçan kullanılmıştır. Kontrol grubundaki sıçanlara hiçbir farmakolojik işlem uygulanmadan hipotalamusları çıkarılırken, salin ve nesfatin gruplarına merkezi yolla salin veya Nesfatin-1 verildikten 20 dakika sonra hipotalamusları izole edilmiştir. Örneklerdeki siklooksijenaz ve lipoksijenaz enzimlerin miktarı Western Blot yöntemi ile araştırılmıştır. İkinci olarak her bir deney grubundaki (kontrol, salin ve nesfatin) örneklerin birleştirilmesi ile havuz örnekler oluşturulmuştur ve aynı şekilde Western Blot ile ifadelerindeki değişimine bakılmıştır. İlk çalışmada istatistiksel olarak anlamlı bir COX1, COX2 ve LOX artışı gözlenmezken havuz çalışmada bu enzimlerde hafif bir artış gözlenmiştir.

Anahtar kelimeler: Araşidonik asit, lipoksijenaz, Nesfatin-1, siklooksijenaz
2019, ix + 69

ABSTRACT

MSc Thesis

INVESTIGATION OF THE EFFECTS OF CENTRALLY INJECTED NESFATIN-1 ON THE HYPOTHALAMIC CYCLOOXYGENASE AND LIPOXYGENASE ENZYMES USING WESTERN BLOTTING

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Nesfatin-1 is an anorexigenic neuropeptide influencing various metabolic actions in the regulation of body temperature, energy homeostasis, gastrointestinal system, glucose homeostasis, stress and reproduction. Both central and peripheral Nesfatin-1 administration have been found to alter blood pressure, heart rate and plasma catecholamine and vasopressin concentrations. The involvement of oxytocin, melanocortin and pro-opiomelanocortin receptors in Nesfatin-1's cardiovascular effects has previously been demonstrated. Apart from these mechanisms, limited knowledge is available on the central mode of cardiovascular effects of Nesfatin-1. The mediation of Nesfatin-1-evoked cardiovascular effects is similar to arachidonic acid's mode of action in exerting these effects. Arachidonic acid is a precursor molecule in the synthesis of prostaglandins and leukotrienes mediated by cyclooxygenase and lipoxygenase enzymes. Inhibiting these enzymes has been shown to suppress Nesfatin-1's anti-inflammatory effects. This study was designed to investigate the expression of cyclooxygenase (COX1 and COX2) and lipoxygenase (LOX) enzymes upon central Nesfatin-1 administration. 21 male Sprague-Dawley rats distributed among control, saline and nesfatin groups were used as test subjects. While control subjects were sacrificed without any pharmacological procedure, saline and nesfatin groups were sacrificed after 20 minutes of saline or Nesfatin-1 administration respectively. The hypothalamus was isolated and samples were tested with Western Blotting to determine the immunoreaction of cyclooxygenase and lipoxygenase enzymes. A pooled study was also performed. While Western Blotting results of individual-samples study did not show significant rise in the concentration of these enzymes, the pooled study showed a slight rise in their concentrations.

Keywords: Arachidonic acid, cyclooxygenase, lipoxygenase, Nesfatin-1
2019, ix + 69

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TABLE OF CONTENTS

Page

Page

SYMBOLS AND ABBREVIATIONS

Symbold	**Explanation**
%	Percent

Abbreviation	**Explanation**
AgRP	Agouti-related Peptide
AP	Alkaline Phosphatase
ARC	Arcuate Nucleus
BCA	Bicinchronic Acid
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CART	Cocaine- and Amphetamine- Regulated Transcript
CEA	Central Nucleus of the Amyglada
CNS	Central Nervous System
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
CVDs	Cardiovascular Diseases
CVMS	Chronic Cariable Mild Stress
DMNV	Dorsal Motor Nucleus of the Vagus
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
EWcp	Centrally Projecting Edinger-Westphal nucleus
Fc	Fragment Crystallizable
GPRC	G-protein Coupled Receptors
HpETE	Hydroperoxyeicosatetranoic Acid
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1β	Interleukine1-beta
INS	Insular Cortex
i.c.v.	Intracerebroventricular
LHA	Lateral Hypothalamic Area
LOX	Lipoxygenase
LTs	Leukotrienes
LXs	Lipoxins
MCR3/4	Melanocortin Receptor
MSH- α	Melatonin Stimulating Hormone
m-TOR	The Mammalian Target of Rapamycin
NPY	Neuropeptide Y

NSAIDs	Non-steroidal Anti-Inflammatory Drugs
NTS	Nucleus Tractus Solitarus
NUCB2	Nucleobindin2
PAD	Peripheral Arterial Disease
PAGE	Polyacrylamide Gel Electrophoresis
PC	Prohormone Convertase
PGHS	Prostagandin Endoperoxide H Synthase
PGs	Prostaglandins
PMSF	Phenylmethylsulphonyl Fluoride
POMC	Pro-Opiomelanocortin Receptors
PVDF	Polyvinylidene Difluoride
PVN	Paraventricular Nucleus
p-AMPK	Phosphorylated 5’ AMP-activated Protein Kinase
p-ACC	Phospho-acetyl-CoA Carboxylase
RIPA	Radioimmunoprecipitation Assay
SDS	Sodium Dodesyl Sulphate
SON	Supraoptic Nucleus
sRPa	Rostral Raphe Pallidus
TBST	Tris Buffer Saline Tween-20
TNF-α	Tumor Necrosis Factor-alpha
TXA2	Thromboxane A2
TXs	Thromboanes
UCP1	Uncoupling Protein 1
VLM	Ventrolateral Medulla
WAS	Water Avoidance Stress
WAT	White Adipose Tissue
β-MCE	Beta Mercapthoethanol

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

Cells use a multitude of chemically distinct substances for communicating with neighbouring cells as well as cells far away from them. Complex, precise and efficient intercellular signalling is vital in most physiological processes and their coordination. These intercellular signalling pathways are observed across all multicellular organisms. Nervous system and the endocrine system are two different but complementary communication and coordination systems across the animal kingdom. In the nervous system, direct and swift communication is observed between neurons as well as peripheral tissues through electrical signalling. These electrical signals that are generated and transmitted along the axons stimulate the release of the enzymatically altered amino acids called neurotransmitters into the synapsis, a cleft that is formed between the inter-communicating neurons. Neurotransmitters then bind to their receptors on the post-synaptic target cell that kickstarts intracellular signalling cascades, propagating the signal within the cell. A swift response to the stimuli is ensured, but the response is not long-lasting. Endocrine system is another cellular communication system in which neurons and other specialized secretory cells found in endocrine glands use a plethora of fatty acids-, cholesterol- and amino acids-derived chemical messengers, also known as hormones. Peptide hormones are the most diverse group of chemical messengers made of amino-acid chains and have more than 100 species (Hook et al. 2008). These hormones have a greater specificity for receptor binding due to their diverse linear and tertiary structural formulations. This structural uniqueness is facilitated by the disulphide bonds, common across peptide hormones. A vast majority of peptide hormones initiate intracellular signalling cascades by binding to G-protein coupled receptors (GPCRs) Others bind to ion channels to transmit the signal (O'Brien and Taghert, 1994).

Neuropeptides are the peptidergic hormones produced, packaged and released by the neuroendocrine cells. Neuroendocrine cells are the neurons that produce hormones. Because all endocrine cells (neuroendocrine, glandular endocrine and enteroendocrine) are derived from neurectoderm and regulated by the same proneural gene factors, neuropeptides can be localized in various peripheral organs, outside the central nervous system (CNS) (Hartenstein et al. 2010). Neuropeptides can regulate metabolic processes

to influence energy balance, glucose homeostasis and provide communication between enteroendocrine cells in gastrointestinal tract, reproductive organs and the CNS. Various neuropeptides that are localized in the CNS and the periphery have been linked to appetite regulation. Some of the most common members of these satiety based neuroendocrine factors include ghrelin, glucagon-like peptide-1, cholecystokinin, peptide YY, leptin, glucose-dependent insulinotropic polypeptide and adiponectin. The circulating levels of these neuropeptides help propagate the information about gut nutrient content and influence short-term hunger and satiety.

Nesfatin-1 is one of the most recent additions to the list of neuropeptides with anorexigenic functions. Oh-I et al. (2006) discovered Nesfatin-1 in the paraventricular nucleus (PVN) of the hypothalamus and cerebrospinal fluid in rats. Nesfatin-1 was reported to reduce food intake, dose-dependently, upon intracerebroventricular (i.c.v.) injection in rodents (Oh-I et al. 2006). Since that landmark study where Nesfatin-1 was first discovered, it has been researched extensively with fascinating results. Even though the initial studies (Oh-I et al. 2006; Yosten and Samson, 2009) showed leptin-independent and melanocortin-mediated mode of action for Nesfatin-1, it was later on demonstrated that leptin directly stimulates Nesfatin-1 in the paraventricular nucleus and it was suggested that leptin might be mediating anorexigenic effects of Nesfatin-1 (Darambazar et al. 2015). Nesfatin-1's receptors and its interaction with them also shows differing results in different parts of the CNS and the topic needs further illumination. Nesfatin-1 activity is reported to be GPCR-mediated in the hypothalamus (Brailoui et al. 2007) and melanocortin system-mediated in the paraventricular nucleus (Maejima et al. 2009). In the arcuate nucleus, nesfatin-1 exerts its effects by hyperpolarising neuropeptide Y (NPY) receptors (Prince et al. 2008b).

Nesfatin-1 is composed of 82 amino acid residues at the N-terminal of its precursor protein, NUCB2. NUCB2 is composed of a 396 amino acid peptide and a 24 amino acid signal chain. NUCB2 has cleavage sites for protein convertases. Prohormone convertases (PC) fragmentate NUCB2 into three peptides namely Nesfatin-1, Nesfatin-2 and Nesfatin-3 (Oh-I et al. 2006). Unlike Nesfatin-1, the other two fragments of the precursor protein NUCB2 i.e. Nesfatin-2 and Nesfatin-3 have not yet been shown to demonstrate any effect on food intake in rats (Oh-I et al. 2006). In fact, their in-vivo existence and

secretion is still unknown, which makes Nesfatin-1 particularly relevant as a satiety related peptide and other related physiological processes.

Nesfatin-1 demonstrates low lipophilicity which might be facilitating its non-saturable transmembrane diffusion across the blood-brain barrier. Upon using highly sensitive multiple-time regression analysis to determine the flow of radioactively labelled Nesfatin-1 across the blood-brain barrier in the blood-to-brain direction, Prince et al. 2007 reported a moderate, linear blood-to-brain influx of Nesfatin-1 (Price et al. 2007). This also means that peripheral Nesfatin-1 may access the CNS by crossing the blood-brain barrier thus influencing certain physiological processes. Nesfatin-1 expression in gastric mucosa, intestine and adipocytes with minor expressions in pancreas and testis have since been identified (Shimizu et al. 2009b, Stengel et al. 2009b, Gonzalez et al. 2009, Ramanjaneya et al. 2010, Kim et al. 2014). Tsuchiya et al. (2010) established a novel enzyme-linked immunosorbent assay (ELISA) to measure Nesfatin-1 expression in peripheral blood in non-obese human males for the first time. Furthermore, the study suggested a negative correlation of body mass index (BMI), body fat mass and blood glucose with peripheral concentration of Nesfatin-1 (Tsuchiya et al. 2010).

The widespread expressional profile of Nesfatin-1 suggests its extraordinary involvement in physiological actions such as gastric emptying (Stengel et al. 2009b), regulation of body temperature (Könczöl et al. 2012), inhibition of intestinal motility (Atsuchi et al. 2010), energy homeostasis (Gonzalez et al. 2011) and reproductive processes (Palasz et al. 2012). Alongside its implications in normal physiological processes, Nesfatin-1 levels have also been attributed to pathological conditions, diseases and syndromes. To mention a few, Nesfatin-1 has been found to be regulated in diabetes mellitus II (Zhang et al. 2012), epileptic seizures (Aydin et al. 2011), hyperthyroidism (Tohma et al. 2015), endometriosis (Şengül et al. 2014), prevention of obesity (Tan et al. 2011), polycystic ovary syndrome (Ademoğlu et al. 2014) and anxiety related behaviours (Merali et al. 2008). Besides, Nesfatin-1 also demonstrates antiapoptotic effects by decreasing caspase-3 activity, anti-inflammatory effects by inhibiting neutrophil infiltration and by inhibiting proinflammatory cytokines, neuroprotective effects by inhibiting lipid peroxidation and protein carbonylation alongside reducing haemorrhage-induced elevated permeation of the blood-brain barrier (Özsavcı et al. 2011, Goebel et al. 2009b, Tang et al. 2012).

Nesfatin-1 has been reported to modulate various cardiovascular processes upon both central and peripheral administration. Centrally administered Nesfatin-1 was recently reported to increase sympathetic nervous system activity and blood pressure by activating oxytocinergic system in the hypothalamus. This increase in bood pressure was reported to be mediated via the 3/4 melanocortin receptors (MCR3/4) in the hypothalamus (Yosten and Samson 2009, Yosten and Samson 2010). Another study investigated Nesfatin-1's effect on blood pressure and heart rate under normotensive and hypotensive conditions. The results suggested elevated blood pressure under both normotensive and hypotensive conditions while its effect on heart rate was bradycardiac in normotensive rats and tachycardiac in hypotensive rats (Yılmaz et al. 2015). The study also suggests the possible role of peripheric hormonal systems in the cardiovascular effects of Nesfatin-1 because central nesfatin-1 administration also increases plasma catecholamine and vasopressin concentrations alongside a heightened renin-angiotensin activity (Yılmaz et al. 2015). Similarly, peripheral administration of Nesfatin-1 also results in higher blood pressure (Osaki and Shimizu, 2014).

Arachidonic acid is a polyunsaturated fatty acid that is released upon the hydrolysis of the cell membrane phospholipids by the phospholipase A enzymes. It serves as a precursor endogenous molecule in the synthesis of prostaglandines and leukotriens, a process that is mediated by the cyclooxygenase (COX) and lipooxygenase (LOX) enzymes (Molly et al. 1998). Central administration of melittin- an enzyme that activates phospholipase A which releases arachidonic acid from the cell membrane- and arachidonic acid results in increase in blood pressure through the activation of renin-angiotensin system along with increased levels of plasma catecholamine and vasopressin in both hypotensive and normotensive rats (Yalcin et al. 2006a, Yalcin and Erturk 2007, Yalcin and Savci 2007, Aydin and Yalcin 2008, Yalcin and Aydin 2009). These cardiovascular effects of mellitin were found to be entirely annulled in both hypotensive and normotensive rat models by the administration of phospholipase A inhibitor meparin and COX inhibitor indometazin, findings that suggest the release of arachidonic acid by the activity of phospholipase A and the mediation of COX enzymes in arachidonic acid's cardiovascular effects (Yalcin et al. 2009b). Another study suggests the involvement of histaminergic receptors in the arachidonic acid-induced cardiovascular effects (Altınbaş et al. 2012, Altınbaş et al. 2014).

The mediation of peripheral catecholamine, vasopressin and renin-angiotensin systems in the cardiovascular effects of both the arachidonic acid and Nesfatin-1 suggest strickingly similar neuromodulatory profiles for both these molecules. Nesfatin-1 also colocalizes with histamin-another neurotransmitter that is shown to mediate arachidonic acid-induced cardiovascular effetcs- in the central nervous system. Furthermore, administration of non-selective COX inhibitor indomethazin, selective cyclooxygenase-1 (COX1) inhibitor SC-560 and selective cyclooxygenase-2 (COX2) inhibitor rofe-coxib have been reported to inhibit Nesfatin-1's protective and healing effects on stress-induced gastric mucosal damage (Sclachric et al. 2013). Similarly, another study where acetic acid-induced gastric ulcer models were used suggests the involvement of the cyclooxygenase enzymes in the anti-inflammatory effects of Nesfatin-1. Inhibition of COX1 and COX2 resulted in the reversal of Nesfatin-1's anti-inflammatory effects with COX2 inhibition effecting on a more significant level (Kolgazi et al. 2017).

Literature available on the mediation of the central mode of action of Nesfatin-1 in inducing its cardiovascular effects suggest the involvement of oxytocin and melanocyte receptors in the hypothalamus and pro-opiomelanocortin receptors (POMC) in the nucleus tractus solitarius (NTS) in rat experimental models (Yosten and Samson 2009, Yosten and Samson 2010). Apart from these mechanisms, there is no reported involvement of another central mechanism that mediates central Nesfatin-1-induced cardiovascular effects. In the light of the above reviewed literature knowledge, the aim of this study is to investigate the hereby hypothesized mediation of COX-1, COX-2 and LOX in the cardiovascular effects of centrally administered Nesfatin-1 in Sprague-Dawley rat experimental models.

2. THEORETICAL BASIS AND LITERATURE REVIEW

2.1. NUCB2/Nesfatin-1- An Introduction

2.1.1. NUCB2/Nesfatin-1 structure

NUCB2 was first identified as a Ca2+ binding protein that contains 396 amino acid residues and a 24 amino acid signal peptide. Besides the signal peptide, NUCB2 has a Leu/Ile rich region, basic amino acid region that contains a nuclear localization signal, two EF-hand domains for Ca2+ binding and a leucine zipper region (Barnikol-Watanabe et al. 1994, Miura et al. 1992). Prohormone convertases proteolytically cleave the basic amino acid region of NUCB2 at the 83-84 (Lys-Arg) residues to form the 82 amino acid anorexigenic Nesfatin-1 at its N-terminus. Nesfatin-2 (85-163 residues) and Nesfatin-3 (166-396) are also formed through the proteolytical cleavage of NUCB2 at the Carboxy terminus (Oh-I et al. 2006). Although no physiological roles have yet been attributed to any of Nesfatin-2 or Nesfatin-3, it is interesting to note that the Ca2+ binding domains, the nuclear localisation signal and the leucine zipper sequence all fall into Nesfatin-3 region of NUCB2. This suggests a not-yet-discovered potential metabolic role for Nesfatin-3. NUCB2 shows extraordinary conservation across species that also suggests its physiological relevance and significance. The amino acid sequence of rat NUCB2 is 87,4% homologous to that of human NUCB2 and 95% homologous to that of mice (Shimizu et al. 2009b).

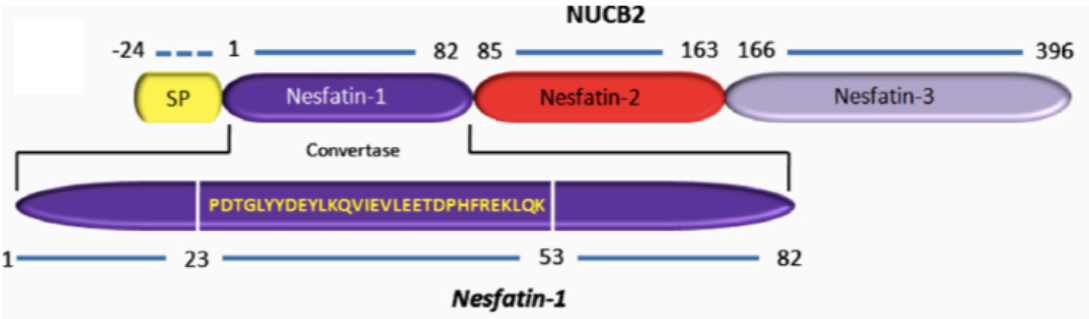

Figure 2.1. Nesfatin-1 structure: Summarized with its mid-segment from 23-53 amino acids also prominent. The signal peptide (SP), Nesfatin-1, Nesfatin-2 and Nesfatin-3 and their respective locations on the prohormone NUCB2 are also demonstrated (Palasz et al. 2012)

Nesfatin-1 itself is tripartite and contains potential proteolytic sites. The N23 segment is on the N-terminus of Nesfatin-1 and constitutes from the amino acids 1-23. The mid-segment is called M30 and contains amino acids 24-53. The final region at the C-terminus contains 53-82 amino acids and is called C29 (Shimizu et al. 2009). The mid-segment M30 is a potential candidate that inhibits food intake dose-dependently. Furthermore, the amino acid sequence of the mid-segment has astonishing similarity to Agouti-related peptides (AgRP) and Melatonin Stimulating Hormone (MSH- α) (Oh-I et al. 2006).

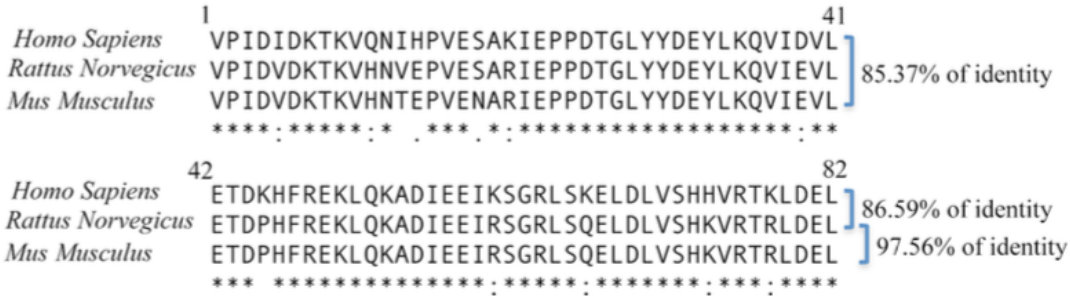

Figure 2.2. Homology between the human, rat and mouse Nesfatin-1 amino acid sequences (Feijo'o-Band' in et al. 2016)

2.1.2. Expression and distribution of Nesfatin-1

To better understand the physiological importance of a peptide in physiology and disease, it is of utmost importance to better understand its expression throughout the body of an organism. Species belonging to the phyla Mammalia and Pisces have regularly been used as model organisms for this purpose and Nesfatin-1 expression has been studied in the CNS, the peripheral organs and body fluids. Nesfatin-1 has been studied both at the mRNA and protein level, with mostly similar but occasionally differing expression patterns for both protein and mRNA. Nesfatin-1 expression may also show fluctuations among model organisms, pointing towards a probability for species-specific implications for Nesfatin-1. In this chapter a brief summary of the studies illuminating these expression patterns has been discussed. Where relevant, the physiological significance of a particular study is discussed, even though Nesfatin-1's importance in physiological processes and its possible regulation and role in diseases has been thoroughly discussed in the next chapter.

Since its discovery by Oh-I et al. (2006) in the satiety related regions of the hypothalamus i.e. paraventricular nucleus (PVN), supraoptic and tractus solitarius (SON) and the arcuate nucleus (ARC), Nesfatin-1 has been identified in many regions inside the central nervous system. Its expression has been detected in several parts of the hypothalamus, hypophysis gland, medulla oblongata and cerebellum. Many studies have presented detailed evidence for its expression in lateral hypothalamic region, tuberal hypothalamic region, dorsomedial hypothalamic nucleus, the zona inserta, in the soma of neurons in the spinal cord, in the dorsal vagus motor nuclei (Goebel et al. 2009b) and the hypophysis gland (Cowley and Grove 2006), central amygloid nuclei (Goebel et al. 2009b), ventrolateral medulla, the cerebellum (Foo et al. 2010, Gonzalez et al. 2009, Stengel and Tache 2010), thoracic and lumbar sympathetic and sacral parasympathetic spinal cord preganglionic neurons (Stengel and Tache 2010).

Like many other peptides that were considered restricted to the central nervous system (CNS), Nesfatin-1 expression was also thought to be limited to the CNS initially. Peripheral expression for Nesfatin-1 has regularly been hypothesized after a moderate, linear blood-to-brain influx of Nesfatin-1 (Prince et al. 2007) and a bidirectional flow of Nesfatin-1 reported (Pan et al. 2007). The passage of Nesfatin-1 through the blood-brain barrier was demonstrated to be non-saturable (Prince et al. 2007). This study suggested that Nesfatin-1 could actually be expressed and secreted in peripheral organs and could still influence many physiological processes by stimulating related areas inside the brain and the central nervous system. In the periphery, Stengel et al. (2009) for the first time detected Nesfatin-1 in stomach. Interestingly, Nesfatin-1 expression in the gastric mucosa of rats was an astonishing 10-fold of that in the brain (Stengel et al. 2009). These results were later replicated in mice (Kim et al. 2014), dogs (Jiang et al. 2016), fish (Gonzalez et al. 2010) and humans (Stengel et al. 2013).

Studies conducted in different model organisms suggest colocalization of Nesfatin-1 with other satiety-related hormones. Double-labelling immunofluorescence revealed colocalization of Nesfatin-1 with the hunger promoting hormone ghrelin in gastric X/A-like cells (Stengel et al. 2009). Despite being in the same type of gastric cells, both Nesfatin-1 and ghrelin were found to be localised in separate vesicles (Stengel et al. 2009). Nesfatin-1 and ghrelin was also found to colocalize in the taste buds in rats (Cao

et al. 2016). Separate vesicle localisation gives an opportunity of differential release for these two peptides, which is of utmost importance as both the peptides have opposite effects with Nesfatin-1 inducing satiety while ghrelin stimulating food intake. Later on, Stengel et al. 2013 replicated the findings on colocalization of Nesfatin-1 with ghrelin in human gastric P/D1 cells (Stengel et al. 2013). Nesfatin-1 was found to colocalize with peptide YY in large intestine and cholecystokinin in small intestine in mice (Ramesh et al. 2016). An interesting study conducted in rats indicated Nesfatin-1's colocalization with insulin in the beta cells of the islets of Langerhans pointing towards possible implications of Nesfatin-1 in glucose homeostasis (Gonzalez et al. 2009, Foo et al. 2010). Another study performed in rats and humans revealed nesfatin-1 expression in beta cells, but no expression was reported in alpha and delta cells (Riva et al. 2011)

After the discovery of its presence in the gastric mucosa, Nesfatin-1 mRNA and protein expression patterns have thoroughly been investigated throughout the gastrointestinal tract in different model organisms. Nesfatin-1 immunoreactivity has been reported in Brunner's glands of the duodenum in rats and mice (Zhang et al. 2010), in the small and large intestine of mice (Ramesh et al. 2016), endocrine cells of the gastric fundus, duodenum, ileum, ileocecal valve and colon in pigs (Varricchio et al. 2014), stomach and pancreas in dogs (Jiang et al. 2016) and at towering levels in liver in goldfish (Gonzalez et al. 2010) and zebrafish (Hatef et al. 2015).

Interestingly in dogs Nesfatin-1 immunoreactivity was not detected except in pancreas and stomach but a widespread Nesfatin-1 mRNA expression throughout the gastrointestinal tract is reported (Jiang et al. 2015). Whether the absence on immunological detection of Nesfatin-1 in the gastrointestinal tract (excluding stomach and the pancreas) even though Nesfatin-1 mRNA is widely detected, can be attributed to its post-translational modification is currently unclear. On the other hand, in goldfish minimal Nesfatin-1 expression was found in the midgut and the rectum (Gonzalez et al. 2010). These differences in the localisation of Nesfatin-1 in different species, and a clear difference between the members of the Pisces (goldfish and zebrafish) and Mammalia could mean certain differences in the physiological functionality of nesfatin-1. Nesfatin-1 could be facilitating specific functions in certain species. In humans Nesfatin-1 protein expression was detected in the pancreas (Riva et al. 2011) and gastric mucosa (Stengel et

al. 2013), but further investigation is required to know whether it has human-specific expression patterns and functions in the gastrointestinal tract.

Apart from its extensive localization in the gastrointestinal tract, immunoreactivity in reproductive, cardiovascular and adipose tissues suggest much broader functions for Nesfatin-1 in reproduction, energy homeostasis and cardiac functionality. Nesfatin-1 expressions have been detected sex-independently in the pituitary glands of mice (Kim et al. 2014), goldfish (Gonzalez et al. 2010) and rats (Stengel et al. 209). The discovery of Nesfatin-1 expression in the murine (Ramananjaya et al. 2010), dog (Nozawa et al. 2016) and human (Ramananjaya et al. 2010) adipose tissue is of particular importance, advocating its potential role in energy balance. This argument of Nesfatin-1's involvement in energy homeostasis was strengthened by the upstream regulation of Nesfatin-1 in the yin yang 1 knockout mice (Verdeguer et al. 2016). Nesfatin-1 has also been detected in the cardiomyocytes of rats (Feijoo-Bandin et al. 2013), humans (Feijoo-Bandin et al. 2013), mice (Kim et al. 2014), dogs (Nozawa et al. 2016), goldfish (Gonzalez et al. 2010) and zebrafish (Nair et al. 2016), implicating its potential physiological involvement in the regulation of cardiac functions.

2.2. Physiological significance of nesfatin-1 and implications in disease

Since its discovery as a satiety related protein, many other functions have been implicated to Nesfatin-1. Nesfatin-1's localisation in both central and peripheral organs suggest a widespread functional profile for Nesfatin-1. Nesfatin-1 has been linked to glucose and lipid metabolism, thermogenesis, modulation of gastrointestinal functions, depression and anxiety, cardiovascular and reproductive processes.

2.2.1. Nesfatin-1 and the regulation of food intake

Nesfatin-1 was discovered as an anorexigenic peptide for the first time in a ground-breaking research conducted by Oh-I et al. (2006). Upon intracerebroventricular injection, Nesfatin-1 was found to be dose-dependently reducing food intake in rodents. These findings about the satiety-related functions of Nesfatin-1 were confirmed independently by research groups in model animals such as rats, mice, pigs and goldfish (Yosten et al. 2010, Könczöl et al. 2012, Stengel et al. 2009, Goebel et al. 2011, Atsuchi et al. 2010, Lents et al. 2013, Gonzalez et al. 2010). Nesfatin-1 also reduced dark phase food consumption upon third ventricular injection. Blocking endogenous Nesfatin-1 with an antisense oligonucleotide resulted in elevated food intake and increased body weight (Oh-I et al. 2006). A study conducted on rats where hypothalamic paraventricular-specific knockdown of NUCB2 showed a rise in food consumption and body weight gain strengthened the consensus of Nesfatin-1's anorexigenic actions and its physiological importance through its central mode of action (Oh-I et al. 2006, Nakata et al. 2016). On the other hand, Nesfatin-1's peripheral mode of action presents contradictory results. While some research groups have confirmed the existence of peripheral anorexigenic mode of action for Nesfatin-1, other studies have shown minimal evidence (Shimizu et al. 2009, Stengel et al. 2009). Intraperitoneal injection of Nesfatin-1 inhibited food consumption in db/db mice and mice fed a high fat diet, suggesting leptin-independent mechanism of action for Nesfatin-1. Furthermore, the study also presented the first ever evidence of a possible POMC and cocaine- and amphetamine-regulated transcript (CART) activation in the NTS after peripheral administration of Nesfatin-1 (Shimizu et al. 2009). Chronic/long-term effects of subcutaneous Nesfatin-1 administration on energy homeostasis maintenance were researched in another study in rats. Nesfatin-1 infusion

was conducted using osmotic minipumps. Elevated spontaneous physical activity and a hike in whole-body fat oxidation was observed along with a significant decrease in cumulative food consumption in test subjects (Mortazavi et al. 2015). While low doses of central Nesfatin-1 seem to exert dominant physiologic effects, a much higher dose of Nesfatin-1 is needed in case of peripheral administration to exert such effects. These findings suggest a more dominant central mode of action for Nesfatin-1.

In a study conducted in rats, intracerebroventricularly-injected Nesfatin-1 decreased water consumption. Because Nucb2 expression in hypothalamus decreases even under normal feeding conditions but water-deprived environment, it can be hypothesized that Nesfatin-1 alters water consumption independent of its anorexigenic function. Even when the rats were exposed to high enough doses of Angiotensin II for causing thirst, Nesfatin-1 administered subjects consumed comparatively lower amount of water against control groups. Upon inhibition of Nesfatin-1 expression through an antisense oligonucleotide, a hike in water consumption is observed which clearly suggest physiologically significant water-consumption altering effects of Nefatin-1 (Yosten et al. 2012)

2.2.2. Nesfatin-1 and its modulation of the gastrointestinal system

Several satiety-related peptides have been shown to regulate physiological processes within the gastrointestinal system and their implications in gastrointestinal system related diseases have frequently been hypothesized. In a study in normally fed and conscious mice, centrally administered Nesfatin-1 was found to inhibit antral and duodenal motility (Atsuchi et al. 2010) Various subsequent studies confirmed these results. Microinjection of Nesfatin-1 into paraventricular nucleus of the hypothalamus (Guo et al. 2015), lateral hypothalamus (Xu et al. 2017) and ventromedial hypothalamus (Gao et al. 2017) also resulted in reduced gastric emptying in rodents. Nesfatin-1 also seems to effect secretory functions in the gastrointestinal tract. For instance, intracerebroventricular injection of Nesfatin-1 is shown to reduce H+/K+-ATPase related gastric acid production in obese rats. Nesfatin-1 was also shown to activate efferent vagal neurons in vivo and elevated Ca2+ signalling in dorsal motor nucleus of the vagus (DMNV) neurons in vitro (Xia et al. 2012) Moreover, intraperitoneal (Kolgazi et al. 2015) and intravenous (Kalaycı et al. 2017) injections of Nesfatin-1 have been implicated in healing of gastric ulcer in rat

models. This healing process is thought to be associated with tumor necrosis factor-alpha (TNF- α) and interleukine1-beta (IL-1β) secretion and seems to be mediated by reduced COX2 signalling. (Kolgazi et al. 2017, Szlachcic et al. 2013)

2.2.3. Nesfatin-1's role in glucose homeostasis and implications in diabetes

Glucose Nesfatin-1's expression in stomach and its colocalization with insulin in the endocrine islets of the pancreas present a strong case for its possible implications in glucose homeostasis (Zhang et al. 2010, Mohan et al. 2012, Foo et al. 2010, Morton et al. 2018). Colocalization of Nesfatin-1 with insulin in the islet of Langerhans's has been demonstrated in various studies in a wide range of model organisms like rodents, pigs and humans (Foo et al. 2010, Morton et al. 2018, Riva et al. 2011). Furthermore, in line with the idea of Nesfatin-1's involvement in glucose homeostasis and its regulation in diabetes, downregulation of NUCB2 gene expression was reported in the islets of type II diabetes mellitus human subjects (Riva et al. 2011).

Intravenously injected Nesfatin-1 is reported to increase the expression of phosphorylated 5' AMP-activated protein kinase (p-AMPK) and phospho-acetyl-CoA carboxylase (p-ACC) in skeletal muscles thus triggering a decrease in blood glucose concentrations and overall insulin resistance in type II diabetic (induced by sptreptozocin) mice models (Li et al. 2013). Nesfatin-1 was also reported to stimulate muscle glucose absorption upon i.c.v. injection (Yang et al. 2012). Wu et al. (2014) investigated the relation between Nesfatin-1 and glucose uptake or release by peripheral organs in obese and normal rat models. Hypothalamus-specific knockdown of Nesfatin-1 caused an increase in the release of glucose from liver and a lowered uptake of glucose in peripheral tissues. The results were identical in both normal and obese rats (Wu et al. 2014).

2.2.4. Nesfatin-1 and its role in lipid metabolism and energy homeostasis

Early studies have shown that intracerebroventricular injection of nesfatin-1 elevates core body temperature in rodents. (Könczöl et al. 2012) Further studies have presented evidence regarding Nesfatin-1's modulation of the energy expenditure and brown adipose tissue growth (Dore et al. 2017). NUCB2/nesfatin-1's physiological involvement in regulation of energy expenditure is suggested in one of the studies where the transcription

factor Yin Yang 1 was knocked out. This caused an increase in energy expenditure and oxygen consumption in white fat deposits along with an elevated NUCB2/nesfatin-1 expression in brown adipose tissue (Verdeguer et al. 2015). Nesfatin-1 might be functional in long-term energy expenditure changes because of its stimulation of brown adipocyte differentiation. Nesfatin-1 has been shown to stimulate brown adipose cell differentiation via the mammalian target of Rapamycin (m-TOR) signalling and uncoupling protein 1 (UCP1) expression (Wang et al. 2016). Because Nesfatin-1 creates a negative energy balance by reducing food intake and increasing energy expenditure, it is hypothesized, with limited evidence as of now, that this negative energy balance might actually be relevant in stress conditions and overnutrition.

2.2.5. Nesfatin-1 regulation in stress

Stress plays an important role in determining psychological and physiological wellness of an organism. Numerous studies have been carried out to understand mutual interplay between stressors and Nesfatin-1 levels. Studies show that Nesfatin-1 colocalizes with stress factors Corticotropin Related Factor (CRF), Cocaine and Amphetamine Regulated Transcript (CART), urocortin-1 and Neuropeptide Y (NPY) (Foo et al. 2008, Okere et al. 2010, Inholf et al. 2010). Furthermore, a study conducted in Sprague-Dawley rats showed an elevated activity in Nesfatin-1 immunoreactive neurons in Supraoptic nucleus (SON), Paraventricular nucleus (PVN), rostral raphe pallidus (sRPa) and ventrolateral medulla (VLM) when exposed to restraint stress (Goebel et al. 2009a). Another study conducted in male Sprague-Dawley rats showed similar increase in c-Fos expression (biomarker for assessing neuronal activity) in parts of brain stem and the hypothalamus but no increase in plasma Nesfatin-1 levels was reported (Yoshida et al. 2010).

Water avoidance stress (WAS) also led to increased c-Fos expression patterns in immunoreactive neurons in Nesfatin-1 expressive regions across the hypothalamus and the brain stem (Goebel-Stengel et al. 2011). Unlike restraint stress, which did not result in plasma Nesfatin-1 levels, water avoidance stress was reported to increase Nesfatin-1 concentration in the plasma (Xu et al. 2015). The results are contradicting in case of chronic stress exposure. While some studies suggest the activation of Nesfatin-1 neurons under chronic variable mild stress (CVMS) (Xu et al. 2010), others report no significant

alteration of Nesfatin-1 concentration in plasma or nesfatin-1 mRNA expression patterns in the hypothalamus under chronic stress (Xu et al. 2015).

Physical stressors like laparotomy and cecal palpation induces c-Fos expression in SON, PVN, rRPa, NTS, VLM and centrally projecting Edinger- Westphal nucleus (EWcp) in rats (Stengel et al. 2010b). Lipopolysaccharide administration increases Nesfatin-1 immunoreactive neuronal activity in PVN, ARC, SON and NST and causes an elevated Nesfatin-1 concentration in the plasma and gastric mucosa in rats (Bonnet et al. 2009).

2.2.6. Implications of Nesfatin-1 in the cardiovascular system

Cardiovascular system's functionality and the cardiovascular diseases (CVDs) are widely accepted as closely related with glucose homeostasis and obesity. Numerous studies suggest that apart from endocrine factors, paracrine and autocrine chemical mediators secreted from the adipose tissue modulate a wide range of physiological processes related to the cardiovascular system, among others. Given this relationship between adipose tissue function/total mass, and the fact that Nesfatin-1 is secreted by adipose tissue, it can be hypothesized that Nesfatin-1 levels have significant implications in the cardiovascular function.

Various studies show Nesfatin-1 expression in the cardiovascular functions regulating areas in the CNS. Oh-I et al., (2006) reported nesfatin-1 distribution in the paraventricular nucleus (PVN), arcuate nucleus (ARC), supraoptic nucleus (SON) and the lateral hypothalamic area (LHA). Later on, its presence in more brain areas regulating the cardiovascular function were discovered i.e. the dorsal motor nucleus of the vagus (DMV) (Brailoiu et al. 2007, Machhada et al. 2015), nucleus ambiguous (Goebel et al. 2009, Sharp et al. 2014), central nucleus of amyglada (CEA) (Goebel et al. 2009, Granjeiro et al. 2014), insular cortex (INS) (Goebel et al. 2009, Nagai et al. 2010) and the NTS (Foo et al. 2008, Bundzikova-Osacka et al. 2015).

Nesfatin-1 is significantly expressed in cardiomyocytes and this expression seems to be consistent across species. As of now, its expression has been demonstrated in the cardiomyocytes of humans (Feijoo-Bandin et al. 2013), rats (Feijoo-Bandin et al. 2013), mice (Kim et al. 2014), dogs (Nozawa et al. 2016), goldfish (Gonzalez et al. 2010) and

zebrafish (Nair et al. 2016), further consolidating the notion of its physiological involvement in the regulation of cardiac functions. These illuminating research developments led to the exploration of Nesfatin-1 in the regulation of blood pressure, heart rate and its effects on vasoconstriction/vasodilation related hormonal concentrations. Emphasis has also been on its regulation under pathological conditions like hypertension, hypotension, obesity and diabetes and its potential to become a biomarker in cardiovascular diseases (CVDs).

Nesfatin-1 has been reported to increase blood pressure upon lateral ventricular administration in rats. This pressor effect is reversed when a MCR3/4 (a G-protein coupled receptor) antagonist i.e. SHU9119 is introduced (Yosten et al. 2009). This reversal of hypertension-inducing effect of Nesfatin-1 through the inhibition of MCR3/4 suggest that just like its anorexigenic actions mediation (Oh-I et al. 2006), the blood pressure regulation by Nesfatin-1 could also be mediated by the MCR3/4 signalling.

Intracerebroventricular administration of Nesfatin-1 elevates blood pressure and heightens MCR3/4 dependent renal sympathetic neuronal activity in rats (Tanida et al. 2011). Another study that strengthens the idea of Nesfatin-1-induced increase in blood pressure through the renal sympathetic activity reported elevated concentrations of plasma vasopressin, renin and catecholamine upon intracerebroventricular injection of Nesfatin-1 (Yilmaz et al. 2015). The findings also suggest bradycardic effect of Nesfatin-1 in normotensive rats, tachycardic in hypotensive rats and a stimulating effect on blood pressure under both normotensive and hypotensive conditions (Yılmaz et al. 2015). Nesfatin-1 has been reported to stimulate an elevated extracellular signal-regulated kinase (ERK). Nesfatin-1 was also shown to elevate sympathetic nerve activity in kidney, liver and white adipose tissue (WAT) and increase blood pressure upon i.c.v. administration. These stimulatory effects of Nesfatin-1 on the sympathetic nerve activity and blood pressure were reported to vanish once an Extracellular Signal-Regulated Kinase (ERK) inhibitor was administered. This suggests that these effects are mediated through ERK signalling pathways (Tanida et al. 2015).

Some studies with appealing results have been conducted to investigate Nesfatin-1 and its cardiovascular implications in human as a model organism. One such study was

conducted in 80 Chinese subjects, 40 of whom were diagnosed with hypertension while the remaining half (used as the control group) had normal age-wise health parameters. Plasma Nesfatin-1 levels showed significantly elevated results compared to the control group. The results also revealed that Nesfatin-1 plasma levels also have the significance of being able to predict the risk of hypertension (Zhao et al. 2015). Another interesting study was conducted in women with polycystic ovary syndrome (PCOS). Nesfatin-1 was associated with elevated systolic/diastolic blood pressures and tachycardia in these subjects. Besides, high plasma Nesfatin-1 concentration was suggested to induce high blood glucose levels, towering luteinizing hormone levels and insulin resistance (Sahin et al. 2015).

Serum Nesfatin-1 levels can be used as a biomarker in some cardiovascular diseases (CVDs). Apart from being a potential prognostic predictor in hypertension (Zhao et al. 2015), Nesfatin-1 has recently been demonstrated as an effective biomarker for peripheral arterial disease (PAD) prognosis (Wang et al. 2015). The study revealed an inverse relation of circulating Nesfatin-1 levels with the development and severity of PAD in non-obese type-II diabetes mellitus patients (Wang et al. 2015).

2.3. Arachidonic Acid Metabolism and The Role Of Cyclooxygenase and Lipooxygenase Enzymes

Arachidonic acid is a polyunsaturated fatty acid present in the lipid bilayer of the cell. Stimuli like stress and inflammation triggers its release from the cell membrane, a process that is catalysed by phospholipase A (Van Dorp 1975). Arachidonic acid then participates in inflammation-promoting responses facilitated by COX and LOX enzymes. While arachidonic acid metabolism through the COX enzymes produce thromboxanes (TXs) and prostaglandins (PGs), LOX pathway leads to the metabolization of arachidonic acid into leukotriens (LTs) and lipoxins (LXs) (Calder 2015, Rae et al. 1982).

Cyclooxygenase enzymes, also known as prostagandin endoperoxide H synthase (PGHS) has two isosymes namely cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2). These enzymes have active sites for their two complementary enzymatic activities, the cyclooxygenase activity converts arachidonic acid into prostaglandin G2 (PGG2) which is highly unstable and is then converted to prostaglandin H2 (PGH2) by peroxidase activity of COX enzymes. PGH2 can generate many distinct prostaglandins and thromboxane A2 (TXA2) (Hawkey 1999, Smith and Song 2002). COX enzymes are important biological catalyzers with important roles ranging from cardiovascular, renal, gastrointestinal and reproduction as well as in pathological conditions such as inflammation and cancer (Lettino et al. 2001, Williams et al. 1999, Fitzgerald 2002, Kniss 1999). This makes it very important to better understand the regulation of these enzymes during certain physiological and pathological conditions, as well as its pharmacological activation regulation. Both COX enzymes, COX1 and COX2 have a molecular weight of 71kDa and 600 amino acids with 63% simlarity among them. COX1 is constantly expressed in a wide range of cell types while COX2 expressions usually alter after it is induced by a stimulus (Otto et al. 1995, Herschman 1996). COX are the targets of Nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin, drugs that inhibit the activity of these enzymes at the cyclooxygenase site, not effecting peroxidase activity (Vane 1971). COX enzymes produce the cytoprotective PGs that maintains the integrity of the gastric mucosa, thus NSAIDs's inibition of COX enzymes leads to side effects such as gastric damage and ulcer (Kargman et al. 1996).

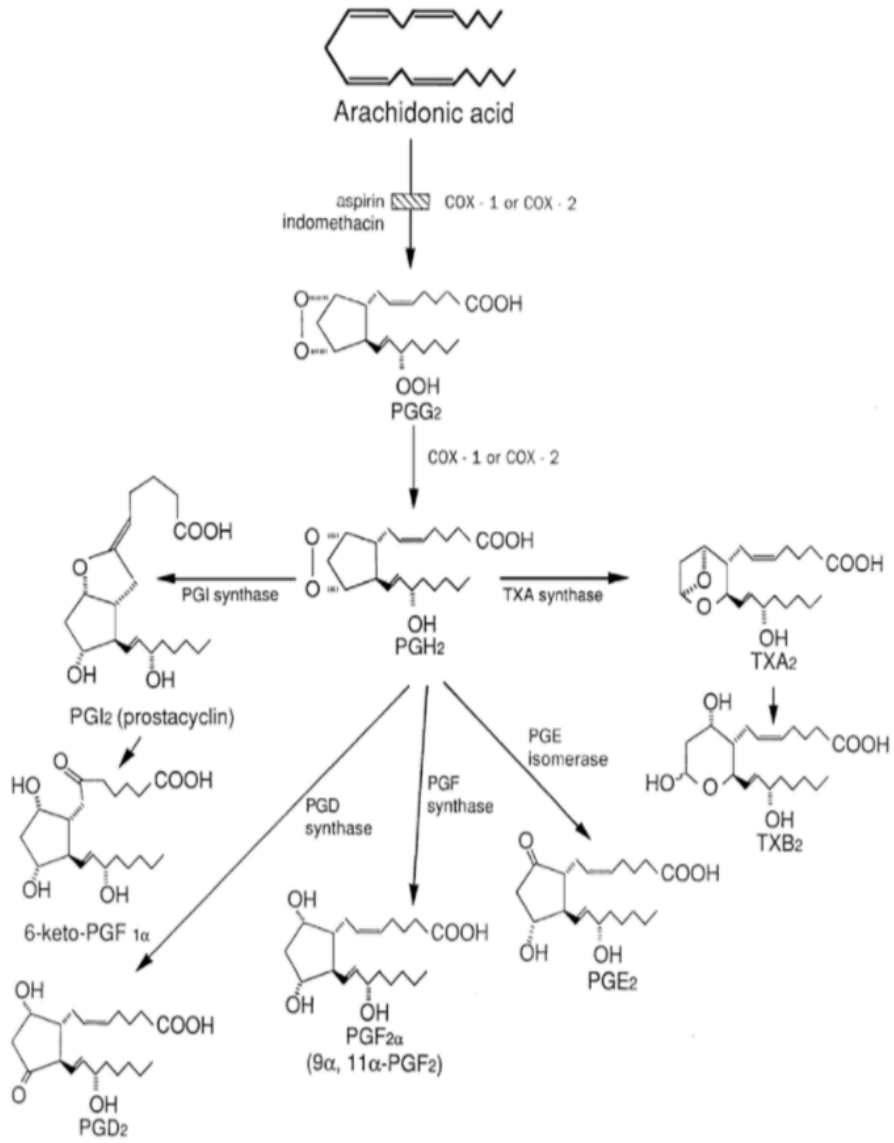

Figure 2.3. Prostaglandin synthesis by arachidonic acid mediated by cyclooxygenase enzymes: Aspirin, indomethacin and other NSAIDs inhibit the reduction of arachidonic acid to PGG2. COX enzymes metabolize arachidonic acid in a two- step process, the cyclooxygenase activity where arachidonic acid is converted into PGG2, the activity that is also a target of NSAIDs. Secondly, COX enzymes convert PGG2 into PGH2. PGH2 is then converted into Thromboxane A2 by Thromboxane A synthase while PGI synthase, PGD synthase, PGF synthase, PGE synthase catalyse the production of PGI2 and subsequently PGF-1a, PGD2, PGF-2a and PGE2 respectively (Vane et al. 1998)

At least four different types of lipoxygenase enzymes, named according to their positions of their cite of oxidation of arachidonic acid i.e. 5-LOX, 8-LOX, 12-LOX and 15-LOX, catalyse the conversion of arachidonic acid into hydroperoxyeicosatetranoic acid (HpETE). HpETE is then converted into the inflammation-promoting leucotriens upon a chain of reactions (Mittal et al. 2016).

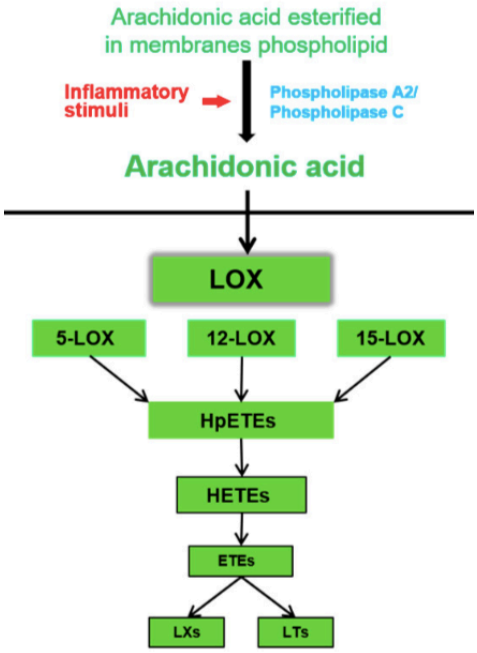

Figure 2.4. Arachidonic acid metabolism: A schematic diagram on the release of arachidonic acid from the plasma membrane in case of inflammation-related stimuli and the subsequent multi-step metabolism of arachidonic acid into the final proinflammatory leucotriens LXs and LTs mediated by LOX enzyme (Mittal et al. 2016)

2.4. The Hypothalamus

Hypothalamus, an essential member of the neuroendocrine system, is localised under the thalamus in the diencephalon and regulates the pituitary gland and its secretion of hormones (Toto KH. 1994). Composed of highly conserved cell types from annelids to vertebrates, it is one of the most crucial part of the brain regulating vital physiological processes ranging from regulating anorexia, water intake, regulation of body temperature, development and reproduction to its influence in stress, behavioural and cognitive processes. Serving as a control centre for the autonomous nervous system and the endocrine system, the hypothalamus evaluates the functionally diverse signals from both the CNS and the peripheral systems to maintain optimum metabolite levels and obtain homeostasis (Saper and Lowell 2014).

The hypothalamus is comprised of characteristically distinct nuclei along with many regions that can be less definite but well-organized to ensure coordination with each other that is vital for its complex functionality. Anatomically, it can be characterized into preoptic, anterior, tuberal and mammillary hypothalamus (Markakis 2002, Burbridge et al. 2016). The preoptic region deals with the regulation of body temperature, electrolytes and reproduction. The anterior hypothalamus is concerned with stress response, food-intake and circadian rhythm and contains various nuclei related to these physiological processes such as the supraoptic nucleus and the paraventricular nucleus. The arcuate nucleus, the lateral hypothalamic area, the median eminence, the dorsomedial hypothalamus and the ventromedial hypothalamus are parts of the tuberal hypothalamus, the region that serves as a regulatory hub for various endocrine and autonomic nervous processes e.g. feeding and sexual behaviour. The fourth region of the hypothalamus, the mammillary hypothalamus has important implications in sexual arousal and stress response. Because of its connection with the hippocampus, the mammillary hypothalamus also has a vital role in memory (Vann and Nelson 2015).

The hypothalamus has two type of cells i.e. the magnocellular and the parvocellular neurons, capable of secreting neuroendocrine hormones. The magnocellular neurons are based in the paraventricular and the supraoptic nuclei of the hypothalamus and secrete oxytocin (OXT) and vasopressin (VP). They are capable of secreting OXT and VP

directly into the bloodstream of the neurohypophysis by extending their axons to this posterior lobe of the pituitary gland (Landgraf and Neumann 2004). On the contrary, the parvocellular neurons project their axons to the median eminence, releasing the neurohormones to the blood circulation there, from where they are carried to the adenohypophysis. These neuroendocrine cells are located in the paraventricular nucleus and the arcuate nucleus of the hypothalamus and secrete corticotropin-releasing hormone that is vital for managing stress responses (Vale et al. 1983).

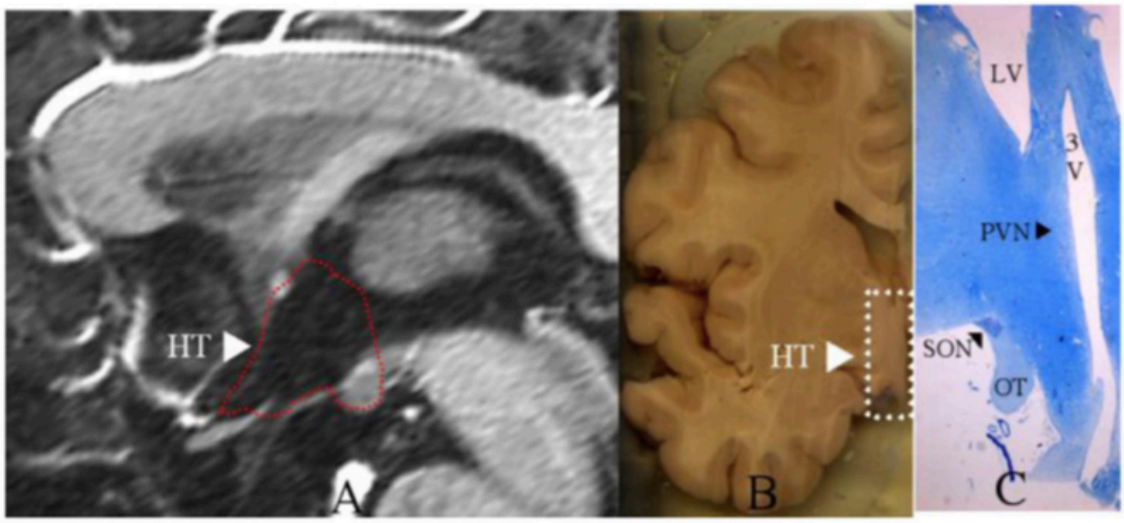

Figure 2.5. The Hypothalamus: Location of human hypothalamus *in vivo* by MRI (A), localisation of the hypothalamus in a dissected human brain hemisphere (B) and histological demonstration of the PVN and SON (Makris et al. 2013).

Other hypothalamic hormones include the gonadotropin releasing hormone (GnRH) secreted from the preoptic area (POA), gonadotropin inhibitory hormone (GnIH) released from the dorsomedial nucleus of the hypothalamus (DMH), somatostatin (SS) and thyrotropin releasing hormone (TRH) secreted from the paraventricular nucleus (PVN), growth hormone releasing hormone (GHRH) and dopamine (DP) secreted from the arcuate nucleus of the hypothalamus (Clarke 2015). Peripheral hormones and metabolites can also exert their effects on the hypothalamus, thus altering its secretions. Gastrointestinal tract hormones such as the cholecystokinin and leptin are relayed via the afferent vagus nerve to the hypothalamus through the brain stem (Smith et al. 2011).

2.5. Western Blotting

E. M. Southern revolutionised the field of molecular genetics by developing a technique for immobilizing fractionated DNA fragments, (cleaved with restriction endonucleases (Smith & Wilcox 1970, Kelly & Smith 1970), from agarose gel onto a nitrocellulose membrane. The technique was presented as a tool to efficiently and economically detect a specific DNA sequence inside the DNA fragment separated through agarose gel electrophoresis (Southern 1975). Soon after, the idea was adapted for RNA studies. Using similar technique, electrophoretically separated RNA fragments were transferred from agarose gels to diazobenzyloxymethyl-paper (Alwine et al. 1977). The main reason behind the quest for immobilizing macromolecules after electrophoresis was because hybridization of these macromolecules in a gel was time consuming and had poor resolution upon detection (Southern 1975). On the other hand, solid membranes are more versatile to work with, economical and time saving. Both nitrocellulose and diazobenzyloxymethyl-paper provided a more stable platform to conduct DNA and RNA analysis respectively.

Many techniques have been developed over time for protein analysis, purification and characterisation. Polyacrylamide gel electrophoresis (PAGE) has been one of the most frequently benefited tools in protein purification and analysis. The amount and location of a specific protein can easily be determined by the band intensity and its position respectively. When enzymatic and binding activity of a protein is under focus, a method named immunoelectrophoresis is used where substrates or ligands are electrophoretically mobilized against an antibody (Laurel 1965) forming a precipitate to indicate antigen-antibody interaction. While immunoprecipitation and other electrophoretic techniques are still under use, they also have some limitations such as their heavy dependence on antigen and antibody concentrations as well as their types as a physically immobile aggregate is difficult to achieve. Besides, these techniques are more often than not time consuming and require high expertise to perform (Towbin et al. 1979). To solve these limitations of protein characterisation and interaction studies at the time, a method for transferring ribosomal proteins from polyacrylamide gels to nitrocellulose membranes was developed in 1979. In this procedure, an exact replica of the pattern formed by proteins on the gel could be obtained on the membrane (Towbin et al. 1979). Two years later, W. Neal

Burnette modified and improved the procedure to transfer proteins from SDS-gels to nitrocellulose quantitatively (W. Neal Burnette 1981). W. Neal Burnette named the procedure as “Western Blotting”, respecting the “geographic” naming tradition for gel-membrane transfer techniques preceded by Southern Blotting and Northern Blotting, developed for DNA and RNA detection/analysis respectively.

Since its development, Western Blotting has been extensively used in biochemical studies. Western Blotting has been utilized for detecting the presence, abundance and cellular localisation of proteins. Scientists have also used Western Blotting technique for investigating post-translational modifications like methylation (Voelkel et al. 2013), phosphorylation (Nairn et al. 1982), glycosylation (Pere-Brissaud et al. 2015) and ubiquitination (Paul et al. 2012).

Western Blotting comprises of seven basic steps to accomplish the task of identifying specific proteins and quantifying them from a complex protein mixture extracted from the samples. These steps (Bass et al. 2017) are:

i. Protein extraction from cells and tissues and sample preparation
ii. Separating proteins by size through electrophoresis
iii. Transfer (blotting) to a membrane
iv. Inhibition of non-specific binding through treatment with excessive protein mixtures (blocking)
v. Incubation of the transferred proteins with primary antibodies and subsequently with labelled secondary antibodies
vi. Visualizing the signal and
vii. Quantitative analysis of the band intensities.

2.5.1. Sample preparation for Western Blotting: Basic principles and procedures

Sample preparation is one of the most important stages of a Western Blotting experiment. Careless handling of the samples can cause differential protein denaturation from sample to sample, thus effecting the ultimately quantified results. Sample preparation starts with protein extraction. The method used to extract proteins varies depending on the sample type, but the overall emphasis is on minimizing protein degradation, preserving post-

translational modifications and avoiding sample contaminations with blood and unwanted tissues apart from the ones in focus. Haemoglobin interference with colorimetric protein assays is a good example of repercussions of such contaminations. (Doumas et al. 1981). To limit degradation, proteins must not be exposed to frequent freeze thaws and should be manipulated as minimal as possible (Mahmood & Yang 2012).

Proteins are extracted from adherent cells or tissue samples through different homogenisation techniques. These techniques can be mechanical, chemical or sonication-based by principle and can be mutually used when required. For instance, sonication and mechanical homogenisation techniques are sometimes combined to achieve effective disruption of cell membranes (Autuori et al. 1982). On the other hand, some samples like muscle cell cultures do not need the same decree of mechanical homogenisation but need more vigorous pipetting techniques (Crossland et al. 2013). Cellular sub-fractionation is needed in cases where the protein of interest belongs to a certain cellular fraction or is uniquely localized inside an organelle or the plasma membrane (Wilkinson et al. 2008). In skeletal muscles, for instance, soluble proteins are first separated as the sarcoplasmic fraction from the insoluble fractions using standard Western Blotting buffers. Differential centrifugation and fraction-specific buffers are then used to further fractionate the sarcoplasmic fraction into mitochondrial and cytosolic fractions (Dimaru et al. 2012, Huff-Lonergan et al. 1995).

Due to the retained enzymatic activity of certain proteases, phosphatases and kinases caused by the disruption of cell membranes during cell lysis procedures, there is always the possibility of protein degradation (Scopes 1994). Protease inhibitors are used to suppress the catalytic activity of proteases in such cases. Different protease inhibitors have different action mechanisms. As an example, metalloproteinases require binding to Ca2+ and Mg2+ for their catalytic activity (Auld 1995). Chelation is a process where an enzyme binds with the metal present in the active site of the target enzyme, such that the catalytic activity of the target enzyme is inactivated. Metal chelators inhibit proteinase activity through this mechanism (Auld 1995). Ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) are excellent chelators (Auld 1995) and also inhibit serine/ threonine (Ser/Thr) phosphatase interactions (Huyer et al. 1997) and therefore are used frequently inside sample buffers. Aprotinin (against serine protease

(Grabski 2009)) and E-64 (against cysteine proteases (Grabski 2009)) are other examples of protease inhibitors.

Homogenisation of tissues should be done in the presence of a buffer that is calibrated to solubilise and ensure preservation of the target proteins. Isoelectric point is the pH at which proteins have neutral electric charge. Optimum solubility of proteins is obtained by buffering the solution near their respective isoelectric points. Positive and negatively charged functional groups (R) of proteins should be kept in equilibrium to avoid protein precipitation during homogenisation (Grabski 2009). Proteins that retain their tertiary and quaternary structures have centrally-oriented hydrophobic regions which keeps them soluble in water (Tanford 1962). On the other hand, non-polar proteins are insoluble in water and require the addition of non-ionic detergents such as Triton X-100 to increase polarity, which results in better solubility of these proteins. (Helenius & Simns 1975). Sodium dodecyl sulphate (SDS) is used to give a uniform negative charge to all proteins in the sample. This is very important in separating proteins only as a function of their molecular mass, without any influence of their charge density.

2.5.2. Protein quantification

The total protein content of each well should be identical. Therefore, the respective protein concentration of each sample is determined through quantification techniques. There are two major techniques for this purpose, colorimetric (Bradford 1976) and UV absorbance (Desjardins et al. 2009). M.M. Bradford developed the colorimetric which benefited from protein-dye binding in principle. (Bradford 1976). Coomassie Brilliant Blue G-250 is a dye that changes its colour from brown (465nm) to blue (595nm) once bound to proteins. Thus, the concentration of the total protein present inside the sample would be directly proportional to the absorption at 595nm. A standard curve of known concentrations is used to provide reference. Bicinchoninic acid (BCA) assay and the Lowry method are other colorimetric methods used in Western Blotting experiment for protein quantification (Noble et al. 2007). The UV absorbance technique is based on aromatic rings containing amino acids' absorbance of the UV light at 280nm (Layne 1957). Though both colorimetric and UV absorbance methods are widely used by scientists with good results, the fact that the UV absorbance techniques require much

lesser amount of sample to work with and have a much wider quantification range from 0,1 to 3000 ug at 280nm makes them more suitable to a wide range of studies.

After determining the total protein concentration through the above-mentioned quantification methods, samples are then exposed to certain denaturing procedures. These procedures linearize the protein structure by unfolding the tertiary and quaternary structures of proteins. Reducing agents such as beta-mercapthoethanol (β-MCE) or Dithiothreitol (DTT) are used to destabilize tertiary and quaternary structure of proteins by cleaving di-sulphide bonds (Anfisen 1973) formed by cysteine residues (Creighton 1988). After unravelling the non-primary structural formations of proteins, and linearizing the proteins into its primary polypeptide structure, the R groups of all proteins in the sample can be masked with a negative charge through exposure to SDS. At this stage all proteins present in the sample are in their primary structural formation and negatively charged. The charge carried by each protein is directly proportional to its molecular weight at this phase which in principle makes possible the separation of proteins solely on the basis of their relative molecular weight inside a polyacrylamide gel (Smith 1984).

2.5.3. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels have equally sized pores formed by chains of acrylamide monomers and cross-linked N,N'- Methylenebisacrylamide (Raymond and Weintraub 1959). The size of the pores is regulated by the altering the concentration of acrylamide monomers and the cross-linkers relative ratio to the monomers. Proteins with larger molecular weight show more resistance to the frictional force while passing through pores. This differential speed of migration (aided by specific frictional resistance of each protein) separates proteins from each other and proteins with similar molecular weights forms bands on the gel (Ornstein 1964).

The concentration of acrylamide can be modified according to the molecular weight of the protein of interest. If the protein of interest is heavy, e.g. mTOR, the concentration of acrylamide must be kept lower to obtain better resolution. On the other hand, lighter proteins such as 4EBP1 would require much concentrated acrylamide gel to have satisfactory results (Chrambach & Rodbard 1971). Gradient gels can be used to obtain a

more uniform resolution for a wide range of proteins having extremely different molecular weights compared to each other. These gels usually have a gradient of 4-12% acrylamide concentration (Rath et al. 2013). Polyacrylamide gels usually have two regions with different pore size. The stacking gel has large pores and allows a faster movement for proteins. When protein reach the separating gel, the second region that has relatively smaller pores, they slow down and form tight bands (Ornstein 1964).

After the gel and the electrophoresis system is ready to work, previously denatured and uniformly negatively charged proteins are then loaded on to the wells, found at the top of the gels. A marker with proteins of known molecular weights is loaded usually to the first well of the gel to validate the band containing the protein of interest and its molecular weight (Weber & Osborn 1969). A typical electrophoresis procedure is conducted by applying a voltage of 200V to the gel for 1 hour.

2.5.4. Blotting

After the proteins are resolved according to their respective molecular weights in a polyacrylamide gel electrophoresis, the next step is to transfer them to a more solid surface for further experimentation, in this case a membrane. Two common types of membranes can be used for this purpose, either a polyvinylidene difluoride membrane (PVDF) or a nitrocellulose membrane. The membrane used by Towbin et al. (1976) and Burnette (1981) for the electro-transfer of proteins was made from nitrocellulose. The transfer of proteins from the gel to the membrane is achieved by a applying a potential difference across the surface of the sandwiched gel and the membrane (Towbin et al. 1976). An exact replica of protein bands found originally on the gel is obtained on the membrane (Towbin et al. 1976, Burnette 1981).

There are numerous methods of transferring proteins from the fragile polyacrylamide gel to the relatively less fragile and easier-to-use membrane. Towbin et al. (1976) and W. Neal Burnette (1981) used a transfer method called “wet transfer”. In this method, the membrane, blotting surface and polyacrylamide gel are all sandwiched against the cassette front and back sides. This sandwiched cassette is then put in a transfer tank containing the electrodes that are immersed in a buffered solution (Towbin et al. 1976). The first systems used for the first blotting were very slow and would take more than 20

hours (Burnette 1981). Semi-dry transfer methods are also utilized nowadays for their faster application durations (Kurien et al. 2015).

2.5.5. Blocking

Membranes used in Western Blotting have a very high affinity for all types of proteins including the primary and secondary antibodies that are used in subsequent steps of Western Blotting (Jansen 2012). To prevent non-specific binding of these antibodies to the membrane, fat-free milk powder is used after being diluted in Tris Buffer Saline Tween-20 (TBST). Alternatively, Bovine Serum Albumin (BSA) can be preferred for antibodies that are not compatible with milk powder. Milk powder contains a phospho-protein namely casein which can react with phospho-protein-specific antibodies (used when the protein in focus is a phospho-protein). This causes incompatibility of milk powder with such antibodies (Mahmood & Yang 2012). Two aspects of a blocking agent make it ideal for most Western Blotting experiments. The first is the ability of the blocking solution to bind to all non-specific binding sites on the membrane and prevent antibodies from binding to the membrane, causing background staining. The second and equally important aspect is its inertness towards altercations of antibodies access to the target proteins. Optimum volume and solute to solvent ratio along with the ideal incubation period is necessary to obtain desired results. Incubation periods may vary from experiment to experiment, in some case an hour while in other experiments overnight incubation might give better results (Gershoni & Palade 1983).

2.5.6. Primary and Secondary antibody incubation and detection

After the blocking process, the membrane is incubated in primary antibodies. The most suitable antibodies should be chosen with precision as the specificity of the antibody towards the target proteins is of utmost importance. The binding site of the antibodies on the protein is called an epitope while the recognition site highly specific for the epitope found on the antibody is called a paratope (Kurien et al. 2011). Monoclonal or polyclonal primary antibodies could be used. Monoclonal antibodies bind to a solitary epitope region on an antigen (protein) while polyclonal antibodies can recognize multiple epitopes on an antigen. While both types of antibodies have their own advantages and disadvantages, overall polyclonal antibodies are cost effective and more sensitive compared to their

monoclonal counterparts (MacPhee 2010). On the contrary, monoclonal antibodies are more efficient from the aspect of specificity and are less prone to non-specific binding thanks to their single cell lineage (Lipman et al. 2005, MacPhee 2010).

To detect the antigen (target protein) after it has bound to the primary antibody, a secondary antibody is needed. These antibodies bind to the fragment crystallizable (Fc) region on a primary antibody. More than one secondary antibody can bind to a single primary antibody that results in an amplified signal. This is of high important especially if the target protein is not abundantly found in the sample. Secondary antibodies are labelled to report the presence of the antigen. The species in which the first antibody was developed in directly influences the choice of the secondary antibody. There are five major classes of antibodies raised in mammals; ImmunoglobulinM (IgM), ImmunoglobulinD (IgD), immunoglobulin E (IgE), immunoglbulin A (IgA) and immunoglobulinG (IgG). IgG are further classified within humans, mice and rats (Lipman et al. 2005).

Secondary antibodies are usually conjugated with a radio-isotopic or a fluorophore tag or a reporter enzyme. Horseradish peroxidase (HRP) (Kricka 1991) and alkaline phosphatase (AP) (Bronstein et al. 1989) are the most widely used enzymes in Western Blotting. Though HRP-linked secondary antibodies can be used for both colorimetric and chemiluminescence-based detection, chemiluminescence is the preferred method because the membrane can be used multiple times for detection. HRP is oxidized with hydrogen peroxide in case of chemiluminescence detection (Kricka 1991). The membrane is usually incubated with the substrate for 3-5 minutes to produce a signal that can last for hours (Alegria-Schaffer et al. 2009). The membrane can be rinsed over and over again and exposed to the substrate to get multiple results. Multiple results are very useful to avoid any possible detection errors or differential exposure of the membrane to the substrate.

3. MATERIALS AND METHODS

3.1. Test Subjects

Male Sprague-Dawley normotensive rats ranging from 300-350 grams by weight were used as model organisms for this study. All test subjects were bought from the "Laboratory Animals Research and Application Centre, Uludağ University". Normotensive rats were raised as groups of four in each chamber and were regularly fed. Room conditions were managed as 12-hour daylight the temperature was kept between 20-24C and humidity between 60-70%.

Test subjects were classified in the following categories:

a. Control group (n=7): These rats were not exposed to any sort of surgical procedure and no pharmacological administration was performed on them.
b. Saline group (t=20 min) (n=7): These subjects were sacrificed after 20 minutes of 5ul i.c.v. saline administration, and hypothalamus was obtained from each subject, kept at -80C until further experimentation
c. Nesfatin-1 (t=20 min) (n=7): These subjects were sacrificed after 20 minutes of 200pmol i.c.v. Nesfatin-1 administration, and hypothalamus was obtained from each subject, kept at -80C until further experimentation

3.2. Procedures

3.2.1. Surgical procedures

Subjects were anesthetized with 2-4% sevoflurane/100% oxygen and a small opening was made in the pre-anesthetized rats' skulls at 1,0mm posterior and 1,5mm lateral of the Bregman according to the Paxinos and Watson's atlas, demonstrating the stereotaxic coordinates (Paxinos & Watson 2005). In order to perform the administration of nesfatin-1 or saline, a guide cannula was placed in the opening such that it was buried 4,2mm inside the skull. Finally, the opening was sealed with the dental porcelain.

3.2.2. Nesfatin-1 and saline administration

An injection cannula made of 28G stainless steel injection was prepared such as it would reach 4,2mm inside the skull and was placed inside the guide cannula. The injection cannula was connected to the polyethylene catheter. 5ul Nesfatin-1 or saline solution was then administered to the lateral cerebral ventricle through this catheter by using 10ul Hamilton microinjector. After the i.c.v. injection and surgical procedures on groups b and c of the test subjects, the subjects were sacrificed and the hypothalamus was obtained from each subject, kept at -80C until further experimentation.

*All experimental procedures were approved by The Animal Care and Use Committee of Uludag University, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, in the Report No. B.30.2.ULU.0.8Z.00.00/118 dated 15.10.2019.

3.2.3. Tissue homogenisation and protein extraction

The following procedures were followed for homogenising the tissue samples before protein quantification:

1. Hypothalamus tissue samples that were kept at -80C were weighed in sensitive balance. The tissues weighed 65mg with minimal fluctuations across samples.
2. 650ml 1x Radioimmunoprecipitation assay (RIPA) buffer (Millipore Catalogue # 20-188, Lot # 2840486) was added to each tissue sample (1ml RIPA/100mg hypothalamus tissue sample). 1mM Phenylmethylsulphonyl fluoride (PMSF) (Cell Signalling #8553) was added for serine protease inhibition.
3. Each sample was homogenized for 5 minutes using mechanical homogenisation on ice.
4. Homogenized samples were then cooled down for 30 minutes on ice.
5. Samples were then taken to be centrifuged at 13000rpm at 4C for 30 minutes.
6. Supernatant containing the proteins was carefully separated without touching the pallet.
7. A small portion of the homogenised samples was taken in Eppendorf tubes for protein quantification while the remaining was kept at -80C until further usage.

8. Ice was used throughout the homogenisation process and carrying the samples to keep samples cool and prevent protein degradation.

3.2.4. BCA Assay for protein quantification

i. Basic principle

Bicinchronic Acid Assay (BCA) was used to determine total protein concentration of samples. BCA is a colorimetric assay in which the reduction of copper ions takes place. Copper ions coming from copper sulphate solution are reduced to cupric or cuprous forms by cysteine, tyrosine and tryptophan amino acid residues present in the protein samples. This reduction reaction results in a purple colour formation and then detected through a spectrophotometer.

ii. BCA working reagent preparation

CuSO4 solution was first prepared at 4% mass/volume in distilled water. BCA working reagent solution was prepared by mixing BCA reagent A (containing bicinchronic acid) with CuSO4 solution at 50:1 ratio. 200ul working reagent was used for each well on the microplate.

iii. Well composition on the microplate

a. Protein sample was added to the BCA working solution at the ratio of 1:20. The final composition of the wells was as follows:
   - 200ul working solution (containing 4ul CuSO4 solution and 196ul BCA reagent A)
   - 10ul protein sample in each well on the microplate
   - 40ul dH2O
b. Bovine serum albumin (BSA) standards were prepared in the series 0, 1, 2, 5, 10, 20, 50ug/ul and diluted with dH2O so that the total volume is 50ul on each well. 200ul BCA reagent working solution was then added to each well.

The samples were left at room temperature for 30 minutes before taking absorbance readings at 550nm on spectrophotometer. Taking standard BSA concentrations as reference total protein concentration of each sample was determined. Subsequently, samples were aliquoted to be stored at -80C until further usage.

3.2.5. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

After determining the total protein content of each sample, the samples were fractionated with electrophoresis in polyacrylamide gel.

i. Sample preparation for SDS-PAGE

a. To ensure equal amount of total protein content in each sample, the loading volume of each sample was determined with the help of protein quantification results by BCA assay.
b. Volumes were determined for each sample such that the total protein content in the loading solution is 15ug.
c. Sample buffer (4x) (Life Technologies # 1772823) and reducing agent (10x) (Life Technologies 1772696) were used in volumes such that they were diluted at 1x.
d. The solution containing reducing agent, sample buffer and samples (each containing 15ug total protein) was diluted with distilled water to make the final loading volume equal to 12ul.
e. SDS-PAGE loading solution containing protein sample, sample buffer and reducing agent was exposed to spin so that the content settles.
f. Samples were then incubated for 10 minutes at 70C to denature proteins while retaining sulphide bridges. This is important as all proteins must be negatively charged to move inside an electric field.

ii. Loading the samples to SDS-PAGE

Invitrogen Bolt 4-12% Bis-Tris Plus gel cassette was installed inside Life Technologies Mini Gel Tank filled with MES-SDS running buffer. Before loading the samples to the gel, the wells on the gels were cleaned with syringe with MES-SDS buffer and the gels were run for 10 minutes to maximise polarization and open pores. SeeBlue Plus2 Prestained Protein ladder (10ul) was loaded to the first well and samples in the next wells. Electrophoresis was performed for 22 minutes applying 200V potential difference. After the electrophoresis was completed, the cassette was dislodged, and the gel was taken to a bowl with distilled water to prevent drying before starting the blotting procedure.

iii. Blotting

Life Technologies iBlot2 dry blotting device was used for transferring proteins from polyacrylamide gel to a nitrocellulose membrane. ThermoFisher Scientific iBlot2 regular size transfer stacks were used that contained nitrocellulose membrane.

a. The pre-run gel was first placed on the nitrocellulose membrane present in the copper anode containing bottom stack and covered with pre-soaked filter paper before placing the cathode containing top stack.
b. Potential difference of 20V was applied for 7 minutes to perform the blotting of proteins from the gel to the nitrocellulose membrane.
c. After finishing the blotting procedure, the filter paper and the gel were discarded while the membrane was place in deionized distilled water for 2 minutes.

iv. Blocking

5% (5g/100ml) non-fat milk in pre-prepared 1X Tris-buffered saline-Tween (TBST) solution was prepared for blocking procedure. Blocking procedure was done at room temperature for one hour while the membrane was being incubated at 75rpm in shaker.

v. Primary and secondary antibody incubation

All antibodies; Abcam COX1 (catalogue no. ab109025), Abcam COX2 (catalogue no. ab15191) and LOX (catalogue no. ab74316), b-actin (Cell Signalling 8457S) primary antibodies and HRP-conjugated anti-rabbit IgG secondary antibody (Cell Signalling 7074S) were diluted 1/1000 in 5% (5g/100ml) milk powder solution in 1X TBS-T as per the recommendation of the manufacturer.

The procedure followed was as follows:

a. Membranes were left for overnight incubation at 4C inside COX1, COX2 or LOX primary antibody solution
b. Membranes were washed thrice in 1X TBS-T buffer at room temperature and 70rpm for 10 minutes each.

c. The membranes were then left for incubation for one hour and shake at 70rpm inside secondary antibody solution
d. Membranes were subsequently washed thrice in 1X TBS-T buffer at room temperature and 70rpm for 10 minutes each before adding the HRP substrate.

vi. HRP incubation and scanning

Membrane was transferred to a sterile tray after the washing process and HRP substrate was added to the tray such that the membrane was entirely covered with it. HRP substrate incubation was performed for 3 minutes. Results after the enzymatic reaction of HRP and IgG secondary antibody was visualized with Li-Cor C-Digit Blot Scanner.

vii. β-actin internal control incubation

a. After visualising the results of the target enzymes i.e. COX1, COX2 and LOX, the membrane was washed in 1X TBS-T and subsequently left in 5% milk powder solution in 1X TBS-T for blocking.
b. Membranes were left for overnight incubation at 4C inside β-actin primary antibody solution
c. Membranes were washed thrice in 1X TBS-T buffer at room temperature and 70rpm for 10 minutes each.
d. The membranes were then left for incubation for one hour and shake at 70rpm inside HRP-conjugated IgG secondary antibody solution
e. Membranes were subsequently washed thrice in 1X TBS-T buffer at room temperature and 70rpm for 10 minutes each before adding the HRP substrate.
f. Similar to the target gene, visualisation was performed in LiCor C-Digit Blot Scanner after the membrane incubation with HRP substrate for three minutes at room temperature.

viii. Determining relative band intensities and statistical analysis

Band intensities for target enzymes COX1, COX2 and LOX as well as the internal control β-actin were measured with Image J program. Relative band intensities of each enzyme was then determined with reference to b-actin.

Statistical analysis was performed with ANOVA and student t-test was performed to compare the difference between each study group. All statistical analysis and graphs were generated with GraphPad Prism 8.2.1.

4. RESULTS

Subjects were classified into control (where no pharmacological administration was erformed), saline administered and nesfatin administered groups. Samples were assigned a two character code comprising of a letter (C for control, S for saline and N for nesfatin-1) and a number (from 1-7 corresponding to the number of subjects in each group).

4.1. Protein Quantification Results

Two separate BCA protein quantification assays were performed, solely because of technical reasons. In the first assay samples C1, S1 and N1 total protein amount was quantified with reference to the standard BSA protein concentrations. In the second assay all others excep C1, S1 and N1 were quantified. OD values of both these quantifications are summarized separately in the below tables.

Table 4.1. OD raw values and total protein concentration values for the samples C1, S1 and N1

ug BSA	**0**	**1**	**2**	**5**	**10**	**20**	**50**
OD 01	115	128	170	296	468	774	1473
OD 02	111	130	169	287	496	833	1523
AVG	113	129	169,5	291,5	482	803,5	1498
AVG-BLANK	0	16	56,5	178,5	369	690,5	1385
Samples		**C1**		**S1**		**N1**	
OD 01		645		698		803	
OD 02		639		730		845	
AVG		642		714		824	
AVG-BLANK		529		601		711	
ug protein		17,6697		20,2113		24,0943	
Ul/20ug protein		11,31881		9,895455		8,300718	

Tablo 4.2. OD raw values and total protein concentration values for all the samples except C1, S1 and N1

µg BSA	**0**	**1**	**2**	**5**	**10**	**20**	**50**
OD 01	102	121	183	270	439	732	1451
OD 02	97	130	178	282	447	720	1493
AVG	99,5	125,5	180,5	276	443	726	1472
AVG-BLANK	0	26	81	176,5	343,5	626,5	1372,5

Samples	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**
OD 01	959	1025	1256	711	811	763
OD 02	959	1052	1332	752	862	739
AVG	959	1038,5	1294	731,5	836,5	751
AVG-BLANK	859,5	939	1194,5	632	737	651,5
µg protein	30,2128	33,1066	42,4068	21,9318	25,7538	22,6416
µl/20µg protein	6,6197	6,0411	4,7162	9,1192	7,7658	8,8333

Samples	**S2**	**S3**	**S4**	**S5**	**S6**
OD 01	737	759	1111	989	731
OD 02	796	784	1081	1001	820
AVG	766,5	771,5	1096	995	775,5
AVG-BLANK	667	672	996,5	895,5	676
µg protein	8,6185	8,5515	5,6819	6,3445	8,4986
µl/20 µg protein	17,2371	17,1029	11,3638	12,6891	16,997

Samples	**N2**	**N3**	**N4**	**N5**	**N6**	**N7**
OD 01	657	648	719	783	748	739
OD 02	690	695	753	865	872	960
AVG	673,5	671,5	736	824	810	849,5
AVG-BLANK	574	572	636,5	724,5	710,5	750
µg protein	10,0905	10,1277	9,0516	7,9055	8,0680	7,6257
µl/20µg protein	20,1810	20,2554	18,1032	15,8110	16,1361	15,2515

4.2. Western Blotting Results

Western Blotting was performed with COX1, COX2 and LOX-specific antibodies for all the control, saline and nesfatin-1 groups to determine whether there was a significant difference between the immunoreaction among the three groups. Western blotting was performed in two distinct ways. Firstly, hypothalamic total protein samples were run separately for each sample and a statistical analysis was performed at the end to determine whether the results were significant. In the second experiment, bulk samples were made for each group and the samples were then immunoblotted for determining the difference in intensity.

4.2.1. Western Blotting results for COX1

Figure 4.1. illustrates results for samples (15ug) Control 1, Saline 1 and Nesfatin 1 belonging to male Sprague-Dawley rats that were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated overnight with COX1 antibody (a) and b-actin as internal control (b).

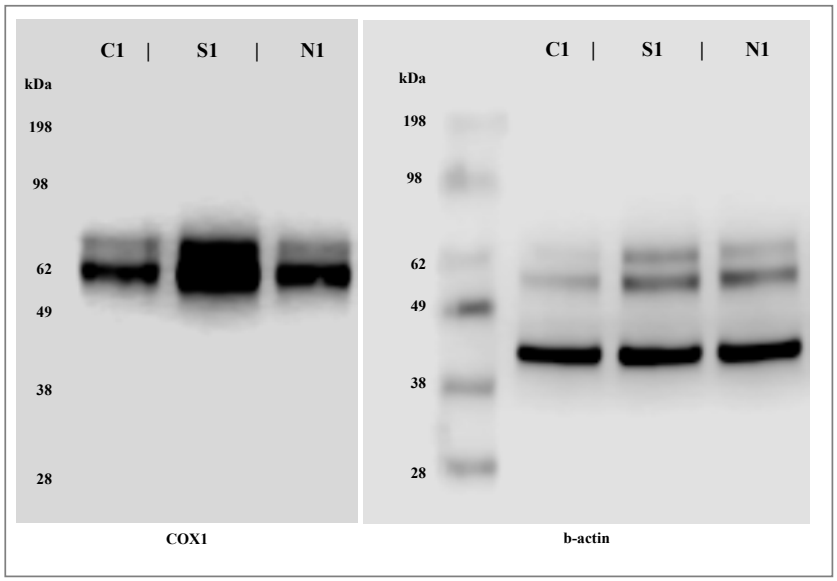

Figure 4.1. COX1 C1, S1, N1 Results

Figure 4.2. shown Western Blotting results for 15ug protein samples extracted from nine different male Sprague Dawley Hypothalamus, fractionated with SDS-PAGE in the series control 2, saline 2, nesfatin 2, control 3, saline 3, nesfatin 3, control 4, saline 4 and nesfatin 4 , transferred to a nitrocellulose membrane and incubated with COX1 (protein of interest) antibody (a) and b-actin (b).

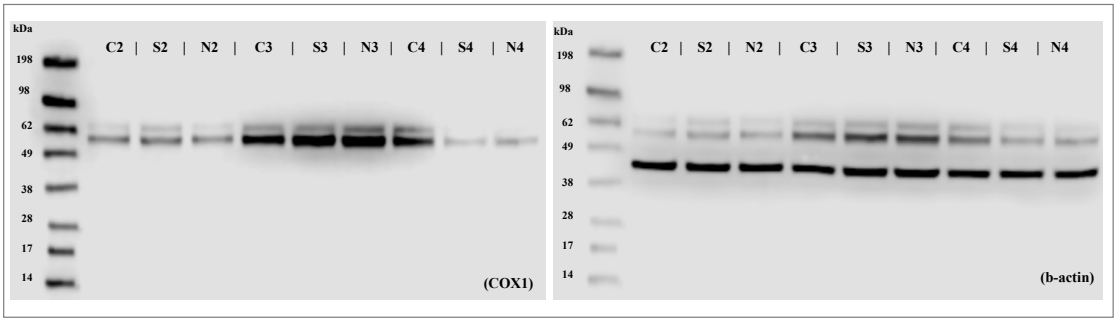

Figure 4.2. COX1 C2-4, S2-4, N2-4 results

Figure 4.3. shows gel images for the Western Blotting of 15ug protein samples that were extracted from nine different male Sprague Dawley Hypothalamus, fractionated with SDS-PAGE in the series control 5, saline 5, nesfatin 5, control 6, saline 6, nesfatin 6, control 7, saline 7 and nesfatin 7, transferred to a nitrocellulose membrane and incubated with COX1 (protein of interest) antibody **(a)** and b-actin **(b)**

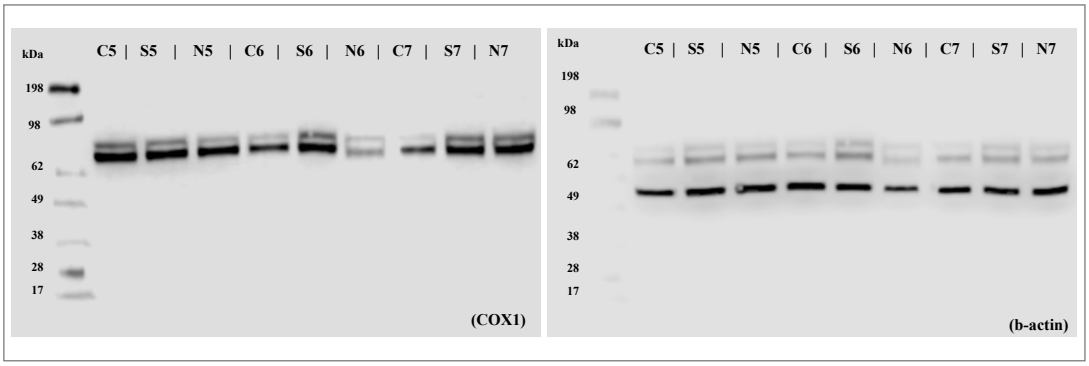

Figure 4.3. COX1 C5-7, S5-7, N5-7 results

Figure 4.4. demonstrates Western Blotting results for COX1 in Control (pooled), Saline (pooled) and Nesfatin (pooled) samples. Bulk samples for each study group (control, saline, nesfatin) were made by pooling 10ug protein from each of the seven samples of the group, making a pool of 70ug protein in each of control (pooled), saline (pooled) and nesfatin (pooled). 10ug protein was loaded to each well for SDS-PAGE and subsequently electrotransfer to a nitrocellulose membrane. After the electrotransfer, membrane was incubated with COX1 **(a)** and b-actin **(b)** antibodies.

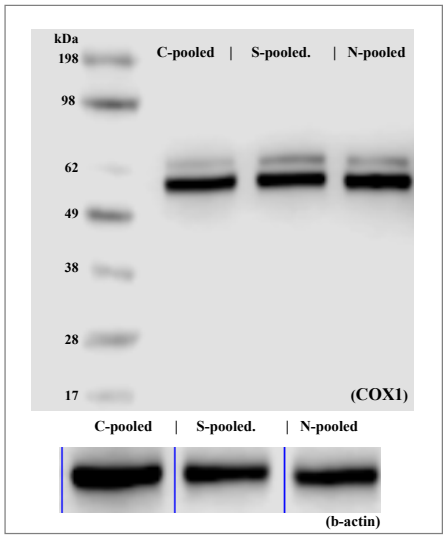

Figure 4.4. COX1 pooled results

4.2.2. Western Blotting results for COX2

Figure 4.5. shows the gel images for 15ug protein samples extracted from nine different male Sprague Dawley Hypothalamus were fractionated with SDS-PAGE in the series control 2, saline 2, nesfatin 2, control 3, saline 3, nesfatin 3, control 4, saline 4 and nesfatin 4 , transferred to a nitrocellulose membrane and incubated with COX2 (protein of interest) antibody (a) and b-actin (b).

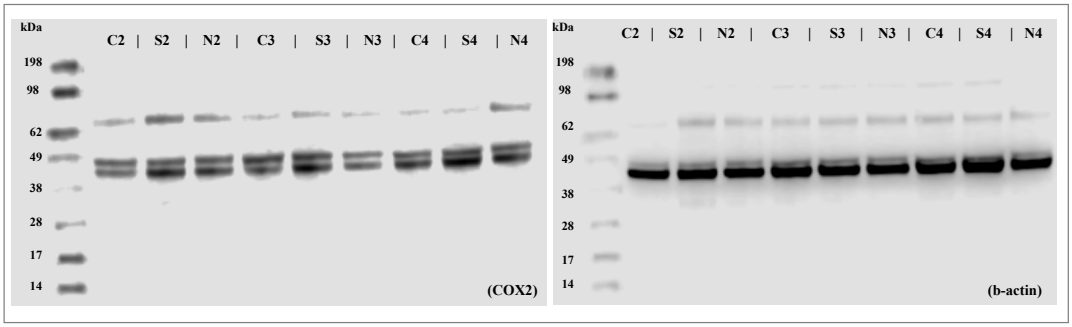

Figure 4.5.. COX2 C2-4, S2-4, N2-4 results

Figure 4.6. demonstrates Western Blotting results for 15ug protein samples extracted from nine different male Sprague Dawley Hypothalamus were fractionated with SDS-PAGE in the series control 5, saline 5, nesfatin 5, control 6, saline 6, nesfatin 6, control 7, saline 7 and nesfatin 7, transferred to a nitrocellulose membrane and incubated with COX2 (protein of interest) antibody **(a)** and b-actin **(b)**

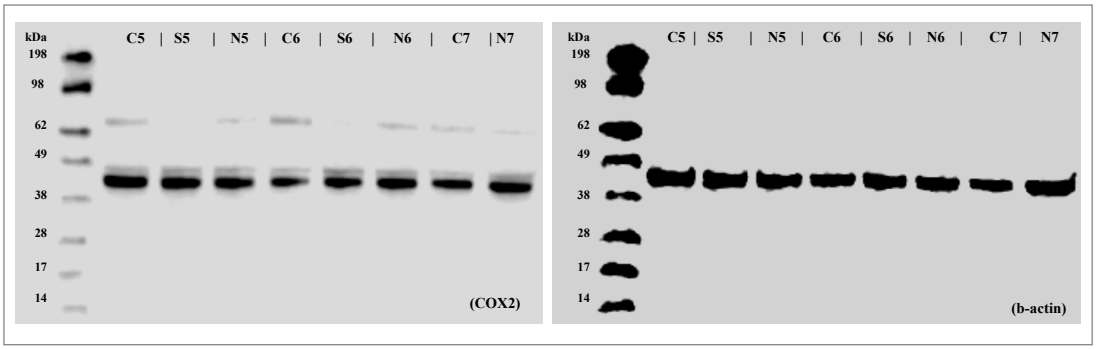

Figure 4.6. COX2 C5-7, S5-7, N5-7 results

Western Blotting results for COX2 in Control (pooled), Saline (pooled) and Nesfatin (pooled) samples are shown in figure 4.7. Pooled samples for each study group (control, saline, nesfatin) were made by pooling 10ug protein from each of the seven samples of the group, making a pool of 70ug protein in each of control (pooled), saline (pooled) and nesfatin (pooled). 10ug protein was loaded to each well for SDS-PAGE and subsequently electrotransfer to a nitrocellulose membrane. After the electrotransfer, membrane was incubated with COX2 **(a)** and b-actin **(b)** antibodies

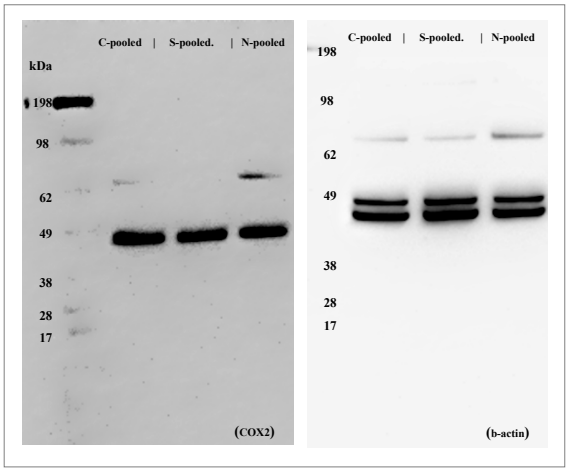

Figure 4.7. COX2 C-pooled, S-pooled, N-pooled results

4.2.3. Western Blotting results for LOX

Figure 4.8. shows Western blotting results for samples (15ug) Control 1, Saline 1 and Nesfatin 1 belonging to male Sprague-Dawley rats were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated overnight with LOX antibody **(a)** and b-actin as internal control **(b).**

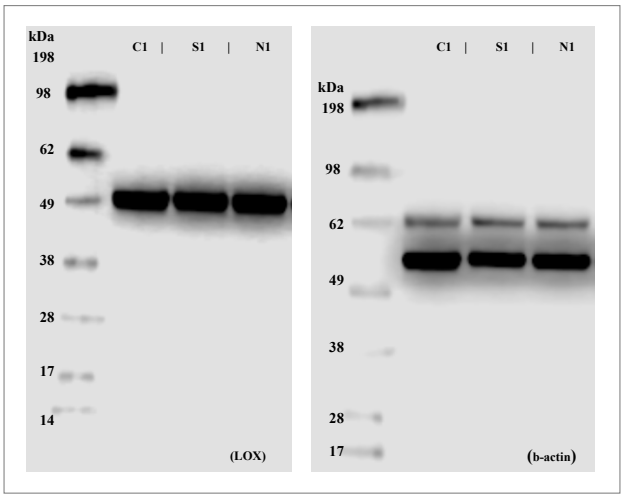

Figure 4.8. LOX C1, S1, N1 results

Shown in the figure 4.9. are the gel images for 15ug protein samples extracted from nine different male Sprague Dawley Hypothalamus were fractionated with SDS-PAGE in the series control 2, saline 2, nesfatin 2, control 3, saline 3, nesfatin 3, control 4, saline 4 and nesfatin 4 , transferred to a nitrocellulose membrane and incubated with LOX (protein of interest) antibody **(a)** and b-actin **(b).**

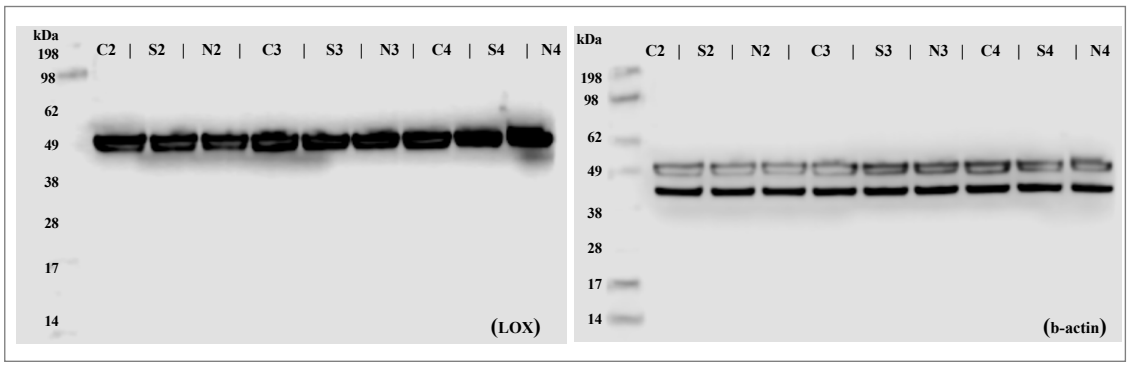

Figure 4.9. LOX C2-4, S2-4, N2-4 results

Figure 4. 10. demonstrates the gel images for 15ug protein samples extracted from nine different male Sprague Dawley Hypothalamus were fractionated with SDS-PAGE in the series control 5, saline 5, nesfatin 5, control 6, saline 6, nesfatin 6, control 7, saline 7 and nesfatin 7, transferred to a nitrocellulose membrane and incubated with LOX (protein of interest) antibody **(a)** and b-actin **(b)**

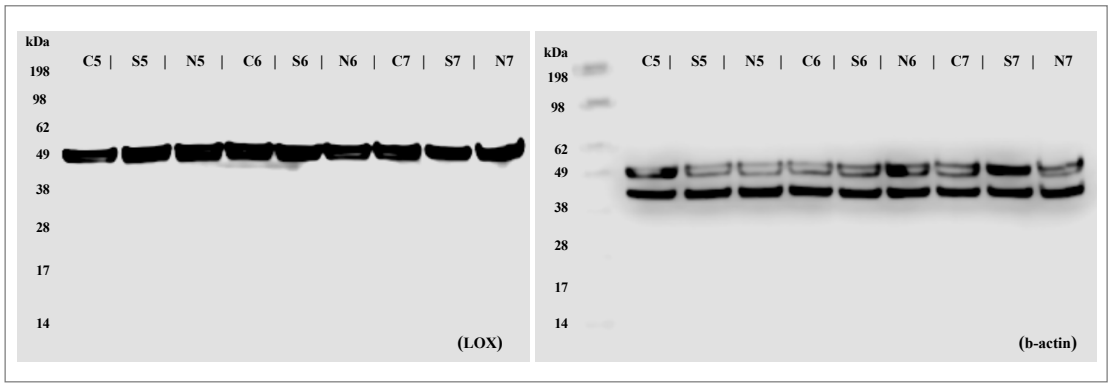

Figure 4.10. LOX C5-7, S5-7, N5-7 results

Bulk samples for each study group (control, saline, nesfatin) were made by pooling 10ug protein from each of the seven samples of the group, making a pool of 70ug protein in each of control (bulk), saline (bulk) and nesfatin (bulk). 10ug protein was loaded to each well for SDS-PAGE and subsequently electrotransfer to a nitrocellulose membrane. After the electrotransfer, membrane was incubated with COX2 **(a)** and b-actin **(b)** antibodies. The results are shown in figure 4.11.

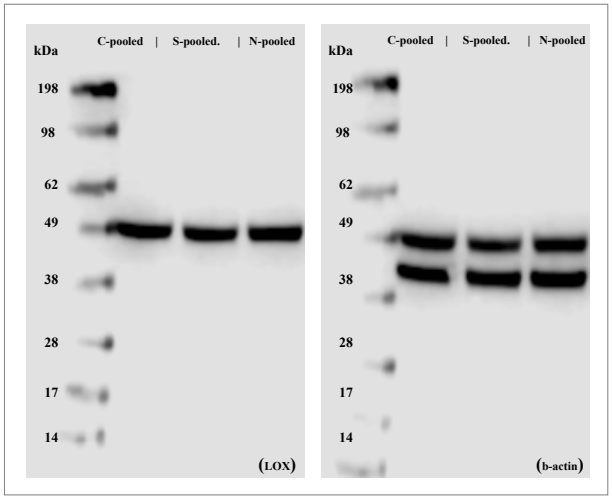

Figure 4.11. LOX C-pooled, S-pooled, N-pooled results

4.3. Stastical Analysis of Results

After calculating relative band intensities of all samples of each group to their respective internal control beta-actin, mean and standard deviation were determined to form a bar graph. After performing ANOVA, student t-tests were performed to compare relative band intensities of each study group. Expression of target proteins in Nesfatin-1 administered group was expected to be higher compared to saline administered group. A significant difference between the target protein expressions (COX1, COX2 and LOX) was not found among study groups, as shown in Figure 4.12.

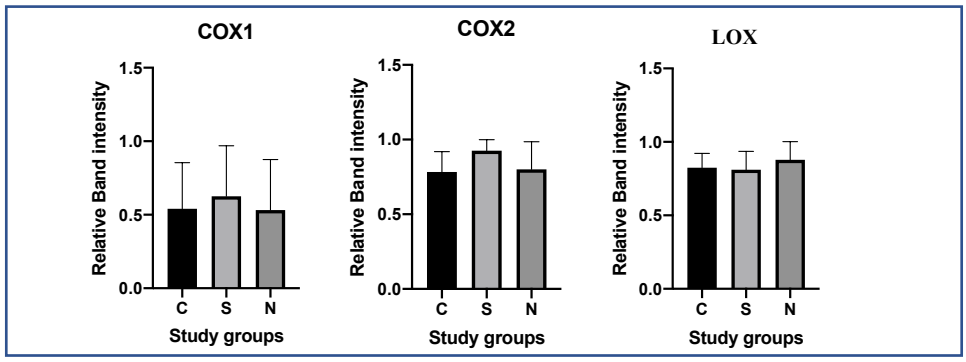

Figure 4.12. Comparative relative band intensities analysis for COX1, COX2 and LOX among groups

As illustrated in Figure 4.13., pooled relative band intensities of COX1, COX2 and LOX show a slight increase in the nesfatin-1 administered group compared to saline administered and control groups. While this increase is more prominent in COX1 and COX2 expressions, it is negligible in LOX expression.

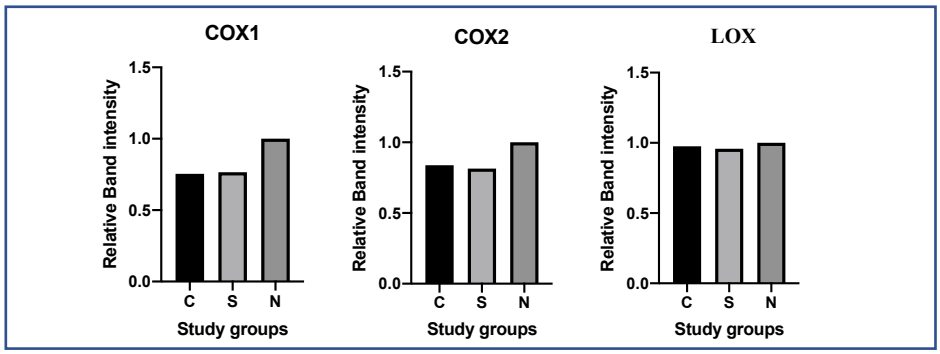

Figure 4.13. Comparative relative band intensities analysis for pooled COX1, COX2 and LOX study groups

5. DISCUSSION

Studies suggest pressor effects of centrally-injected arachidonic acid through the mediation of cyclooxygenase and lipooxygenase enzymes (Erkan et al. 2017). The pressor effect is caused by prostaglandins, a product of a cyclooxygenase-catalysed and arachidonic acid-induced reaction. Centrally-administered Nesfatin-1 induces similar pressor effects to arachidonic acid. Furthermore, both Nesfatin-1 and arachidonic acid-induced cardiovascular effects are mediated by catecholaminergic, renin-angiotensin system and vasopressinergic system in the periphery and both stimulates histaminergic system in the central nervous system.

There is very little knowledge in literature considering the contribution of COX enzymes in Nesfatin-1 induced physiological and pharmacological effects and no research paper is available on its effects on LOX enzyme expression. Anti-inflammatory effects of Nesfatin-1 have been shown to function by altering the oxidant-antioxidant balance. The effects of Nesfatin-1 on COX enzymes concentration and their respective mRNA expression have been shown both *in-vitro* and *in-vivo*. The mediation of Nesfatin-1's protective effects against water immersion and restraint stress (WRS) induced gastric mucosal injury through COX pathway was first demonstrated by Sclachcic et al., 2013. The study demonstrated that both COX-1 and COX-2 suppression led to the attenuation of Nesfatn-1 induced anti-inflammatory effects (Szlachcic et al. 2013).

Similarly, nesfatin-1's anti-inflammatory response against acetic-acid induced ulcer was abolished when COX-1 and COX-2 were inhibited (Kolgazi et al. 2017). Having said that, Nesfatin-1 only induced COX2 expression when injected together with IL-1 in chondrocytes *in-vitro.* Nesfatin-1 did not show any effect on COX2 when pharmacologically administered alone (Scotece et al. 2014). Except COX contribution towards Nesfatin-1's anti-inflammatory response, literature on COX and LOX pathways' contribution towards Nesfatin-1 induced effects is rather scarse and no paper has yet been published regarding the mediation of Nesfatin-1 induced cardiovascular effects through COX and LOX enzymes. Further research is required in order to illuminate the role of these enzymes.

Considering the similarity of Nesfatin-1's and arachidonic acid's mediation of cardiovascular functions, and very little understanding on how these effects are mediated, we aimed to investigate whether centrally administered Nesfatin-1 induced cardiovascular effects were mediated by cyclooxygenase and lypoxygenase pathways. The study comprised of three groups of male Sprague-Dawley rats i.e. control, saline and Nesfatin-1 administered groups. An increase in the level of COX-1, COX-2 and LOX enzymes was hypothesized in Nesfatin-administered groups. Two studies, one based on the Western Blotting results of individual test subjects and the other based on a pooled sample, were conducted. Though the individual study does not show a clear rise in the concentration of these enzymes upon Nesfatin-1 administration, an increase was reported in the pooled experiment.

This experiment was designed such that the test subjects were sacrificed after 20 minutes of pharmacological administration of saline or Nesfatin-1, as the high blood pressure and change in heart beat have been recorded between 15-20 minutes (Aydin et al. 2018) of its central administration. A much more extensive study can be performed where the subjects are sacrificed after 5-30 minutes to see whether the enzymes do mediate these physiological effects of Nesfatin-1 but can the peak concentration can be seen at a different time after central Nesfatin-1 administration. The possibility that the hypothalamic concentration of these enzymes start to decrease towards baseline after mediating the cardiovascular effects should also be considered. Posttrasciptional and posttranslational modifications of COX1, COX2 and LOX enzymes might also be involved in mediating Nesfatin-1's cardiovascular effects. Through these modifications, these enzymes might be influencing other pathways and physological cascades, causing the effects at the end. Considering this, mRNA expression levels for COX1, COX2 and LOX could also be investigated in further studies.

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APPENDICES

APPENDIX 1 COX1 band intensities of gels upon Western Blotting

COX1

	Control	Saline	Nesfatin-1
COX1	3.401.761	6.869.045	7.792.974
	3.717.832	3.905.075	3.331.175
	9.166.095	10.539.217	9.759.167
	7.224.874	1.184.426	1.875.033
	9.475.530	9.618.288	9.553.459
	7.180.853	8.414.409	2.970.640
	5.737.296	7.704.409	7.627.309

	Control	Saline	Nesfatin-1
b-actin	13.373.095	14.285.045	13.684.217
	10.515.296	10.020.933	10.243.468
	9.308.761	10.619.296	10.587.711
	8.970.225	9.126.933	8.795.347
	7.754.589	9.675.711	9.873.660
	9.757.761	8.646.054	6.594.711
	7.471.054	7.805.589	8.540.589

COX1 (pooled study)

COX1		b-actin	
C (pooled)	46.098.782	**C (pooled)**	16.701.095
S (pooled)	47.385.468	**S (pooled)**	16.891.602
N (pooled)	52.073.702	**N (pooled)**	14.223.167

APPENDIX 2 COX2 band intensities of gels upon Western Blotting

COX2

	Control	Saline	Nesfatin-1
COX2	13.909.116	19.584.380	17.256.087
	18.782.622	23.060.593	11.049.338
	15.683.359	23.211.986	18.819.966
	19.548.016	17.645.309	15.537.652
	10.465.510	14.780.359	16.095.945
	13.030.459		18.045.794

	Control	Saline	Nesfatin-1
b-actin	14.554.794	16.103.673	16.776.158
	18.283.108	17.818.158	16.959.187
	17.750.037	18.474.652	14.369.945
	21.484.832	18.397.347	17.024.296
	14.882.619	16.038.054	15.296.104
	12.485.255		20.394.004

COX2 (Pooled Study)

COX2		b-actin	
C (pooled)	19.540.681	**C (pooled)**	59.090.660
S (pooled)	18.189.146	**S (pooled)**	56.633.388
N (pooled)	21.094.179	**N (pooled)**	53.447.338

APPENDIX 3 LOX band intensities of Western Blotting

LOX

	Control	Saline	Nesfatin-1
LOX	58.197.317	62.653.468	64.424.711
	17.742.154	15.811.548	14.498.205
	17.639.912	15.632.497	16.309.669
	16.853.648	18.219.891	19.341.255
	15.602.619	17.831.841	19.336.719
	19.765.426	18.498.790	15.604.012
	16.801.497	14.573.477	17.299.912

	Control	Saline	Nesfatin-1
b-actin	60.374.681	54.598.468	56.306.589
	19.079.790	17.803.012	17.222.426
	18.421.719	20.075.841	19.740.083
	19.919.305	19.530.376	16.593.083
	19.369.447	19.271.255	19.015.426
	17.495.355	17.335.134	18.127.255
	17.033.497	19.055.669	18.034.255

LOX (Pooled Study)

LOX		**b-actin**	
C (pooled)	57.495.723	**C (pooled)**	57.431.572
S (pooled)	58.922.924	**S (pooled)**	59.947.409
N (pooled)	60.197.045	**N (pooled)**	58.680.016

RESUME

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