# NEUROPROTECTIVE ROLE OF APELIN RECEPTOR (APLNR) IN GT1-7 NEURONS

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# ABSTRACT


## NEUROPROTECTIVE ROLE OF APELIN RECEPTOR (APLNR) IN GT1-7 NEURONS

Neurotoxicity is known as the detrimental effect on the structure or function of the nervous system and a consequence of oxidative stress induction in the body due to being exposed to biological or synthetic substances. The most important brain region in regulating homeostasis known as hypothalamus is implicated in the pathogenesis of various neurotoxic disorders including Parkinson's Disease, Alzheimer's Disease, Cerebral Ischemia, Multiple Sclerosis, ALS and epilepsy. For this reason, targeting potential protective signaling pathways in hypothalamic neurons are crucial for promoting neuronal cell survival and treating neurotoxic disorders.

In the current study the potential protective action of Aplnr signaling was evaluated in an oxidative stress induced neurotoxicity *in vitro* model conducted by $H_20_2$ and hypoxia induced cell stress using GT1-7 hypothalamic neuronal cell line. The effect of Aplnr activation via Apelin and ML-233, inhibition with ML-221 and downregulation via siAplnr against neurotoxicity was assessed by performing cell proliferation analysis, gene expression analysis on apoptotic and inflammatory markers, oxidative stress enzyme activity analysis and immunostaining assays.

Overall, the activation of Aplnr signaling with Apelin and ML-233 was shown to protect GnRH neurons against $H_20_2$ and hypoxia induced cell stress by increasing anti-apoptotic and cell proliferation markers, and the activity of antioxidant enzymes. Transient overexpression of Aplnr showed a protective role in viability of GT1-7 cells under $H_2O_2$ and hypoxia induced cell stress. However, inhibition with ML-221 and knocking down of Aplnr caused a significant decrease in aforementioned parameters, and reversed its protective action against neurotoxicity. Furthermore, Aplnr signaling showed a regulatory role in the release of GnRH for the first time in literature. As a result, Aplnr signaling was shown to be essential for GnRH neuronal cell survival against oxidative stress induced neurotoxicity.

# ÖZET


## GT1-7 NÖRONLARINDA APELİN RESEPTÖRÜNÜN (APLNR) NÖROPROTEKTİF ROLÜ

Nörotoksisite, biyolojik veya sentetik maddelere maruz kalınması sonucu ile vücuttaki oksidatif stres indüksiyonunun sinir sistemi üzerindeki zararlı bir etkisi olarak bilinmektedir. Homeostaziyi düzenlemede görevli olan hipotalamus olarak bilinen en önemli beyin bölgesi, Parkinson Hastalığı, Alzheimer Hastalığı, Serebral İskemi, Multiple Skleroz, ALS ve epilepsi dahil olmak üzere çeşitli nörotoksik hastalıkların patogenezinde rol oynadığı gösterilmiştir. Bu nedenle, hipotalamik nöronlardaki potansiyel koruyucu sinyal yolaklarını hedeflemek nöronal hücrelerin hayatta kalmasını sağlamak ve nörotoksik bozuklukların tedavisi için oldukça önemlidir.

Bu çalışmada, $H_20_2$ ve hipoksi ile indüklenmiş hücresel stres ile oluşturulmuş oksidatif stres kaynaklı *in vitro* nörotoksisite modelinde GT1-7 hipotalamik nöronal hücre hattı kullanarak Aplnr sinyalizasyonun potansiyel koruyucu etkisi değerlendirildi. Apelin reseptörünün Apelin ve ML-233 ile aktivasyonunun, ML-221 ile inhibisyonunun ve siAplnr ile susturulmasının nörotoksisiteye karşı etkisi hücre proliferasyon analizi, apoptotik ve enflamatuar markerler üzerinde gen ekspresyonu analizi, oksidatif stres enzim aktivitesi analizi ve immün boyama testleri yapılarak değerlendirildi.

Aplnr sinyalizasyonunun Apelin ve ML-233 ile aktivasyonu sonucu anti-apoptotik ve hücre proliferasyon markerlarını ve antioksidan enzimlerin aktivitesini arttırarak GnRH nöronlarını $H_20_2$ ve hipoksi ile indüklenmiş hücresel strese karşı koruduğu gösterilmiştir. Apelin reseptörünün kısa süreli aşırı ekspresyonu, $H_20_2$ ve hipoksi ile indüklenmiş hücresel stres koşulları altında GT1-7 hücrelerinin canlılığında koruyucu bir rol göstermiştir. Ancak, Aplnr susturulması ve ML-221 ile inhibisyonu az önce belirtilen parametrelerde önemli derecede bir azalışa neden oldu ve nörotoksisiteye karşı koruyucu etkisini tersine çevirdi. Ayrıca, literatürde ilk defa Aplnr sinyalizasyonu GnRH salınımında düzenleyici bir rol göstermiştir. Sonuç olarak, Aplnr sinyalizasyonunun oksidatif stres ile indüklenmiş nörotoksisiteye karşı GnRH nöronal hücre sağkalımında önemli olduğu gösterilmiştir.

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# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| 6-OHDA | 6-hydroxydopamine |
| 8-OHdG | 8-hydroxy-2′-deoxyguanosine |
| AD | Alzheimer's disease |
| Aβ | Amyloid-β peptide |
| Akt | Protein kinase b |
| ANOVA | One-way analysis of variance |
| ALS | Amyotrophic lateral sclerosis |
| APJ | Putative receptor protein related to the angiotensin receptor 1 |
| Aplnr | Apelin receptor |
| ARC | Arcuate nucleus |
| BAX | Bcl-2-associated x protein |
| BCL-2 | B-cell lymphoma 2 |
| BTM | Beta-thalassemia major |
| CAT | Catalase |
| CBPs | Calcium binding proteins |
| cDNA | Complementary DNA |
| CNS | Central nervous system |
| Cu | Copper |
| DAPI | 4′,6-Diamidino-2-phenylindole dihydrochloride |
| DMEM | Dulbecco's modified eagle's medium |
| DMN | Dorsomedial nucleus |
| DMSO | Dimethyl sulfoxide |
| dUTP | Deoxyuridine triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Fetal bovine serum |
| Fe | Iron |
| $Fe^{3+}$ | Ferric cation |
| FSH | Follicle stimulating hormone |
| G | Guanine nucleotide-binding |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |

| | |
|---|---|
| GFP | Green fluorescent protein |
| GnRH | Gonadotropin-releasing hormone |
| GPCR | G protein coupled receptor |
| GPx | Glutathione peroxidase |
| GSH | Glutathione |
| GSHPx | Glutathione peroxidase |
| h | Hour |
| $H_2O_2$ | Hydrogen peroxide |
| HEK | Human embryonic kidney |
| HIV-1 | Human immunodeficiency virus type 1 |
| HNE | 4-hydroxy-2-nonenal |
| $HNO_2$ | Nitrous acid |
| HOBr | Hypobromous acid |
| HOCl | Hypochlorous acid |
| HUGO | Human genome organization |
| IL-2 | Interleukin-2 |
| IUPHAR | International union of pharmacology |
| LHA | Lateral hypothalamic area |
| LH | Luteinizing hormone |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MS | Multiple sclerosis |
| mTOR | Mammalian target of rapamycin |
| MTS | 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H tetrazolium |
| NF-κB | Nuclear factor kappa B |
| $NO^{\cdot}$ | Nitrogen monoxide |
| NO | Nitric oxide |
| $NO^+$ | Nitrosyl cation |
| $NO^-$ | Nitroxyl anion |
| $NO_2^{\cdot}$ | Nitrogen dioxide |
| $N_2O_3$ | Dinitrogen trioxide |
| nNOS | Nitric oxide synthase |
| NSAIDs | Non-steroidal anti-inflammatory drugs |

| | |
|---|---|
| $O_2^{\cdot-}$ | Superoxide |
| $O_2^{\cdot\cdot}$ | Oxygen radical |
| $O_3$ | Ozone |
| $^1O_2$ | Singlet oxygen |
| OH· | Hydroxyl radical |
| $ONO_2-$ | Peroxynitrite |
| PBS | Phosphate-buffered saline |
| PD | Parkinson's disease |
| PFA | Paraformaldehyde |
| PI3K | Phosphoinositide 3-kinase |
| PSA | Penicillin/streptomycin/amphotericin b |
| PVN | Paraventricular nucleus |
| qPCR | Quantitative polymerase chain reaction |
| RIPA | Radioimmunoprecipitation assay |
| RNS | Reactive nitrogen species |
| RO· | Alkoxy radical |
| ROO· | Peroxyl radical |
| ROS | Reactive oxygen species |
| RSS | Reactive sulphur species |
| RT-PCR | Reverse transcription polymerase chain reaction |
| siRNA | Small interfering RNA |
| SOD | Superoxide dismutase |
| TdT | Terminal deoxynucleotidyl transferase |
| TUNEL | Terminal deoxynucleotidyl transferase enzyme-mediated deoxyuridine triphosphate nick-end labelling |
| VMN | Ventromedial nucleus |
| Zn | Zinc |

# 1. INTRODUCTION

## 1.1. APELIN RECEPTOR

Apelin receptor (Aplnr) is a type of a guanine nucleotide-binding (G) protein coupled receptor that is a member of the class A rhodopsin-like receptor family. Aplnr comprises of 380 amino acid residues, and has 7 α-helical transmembrane segments (Figure 1.1) [1].

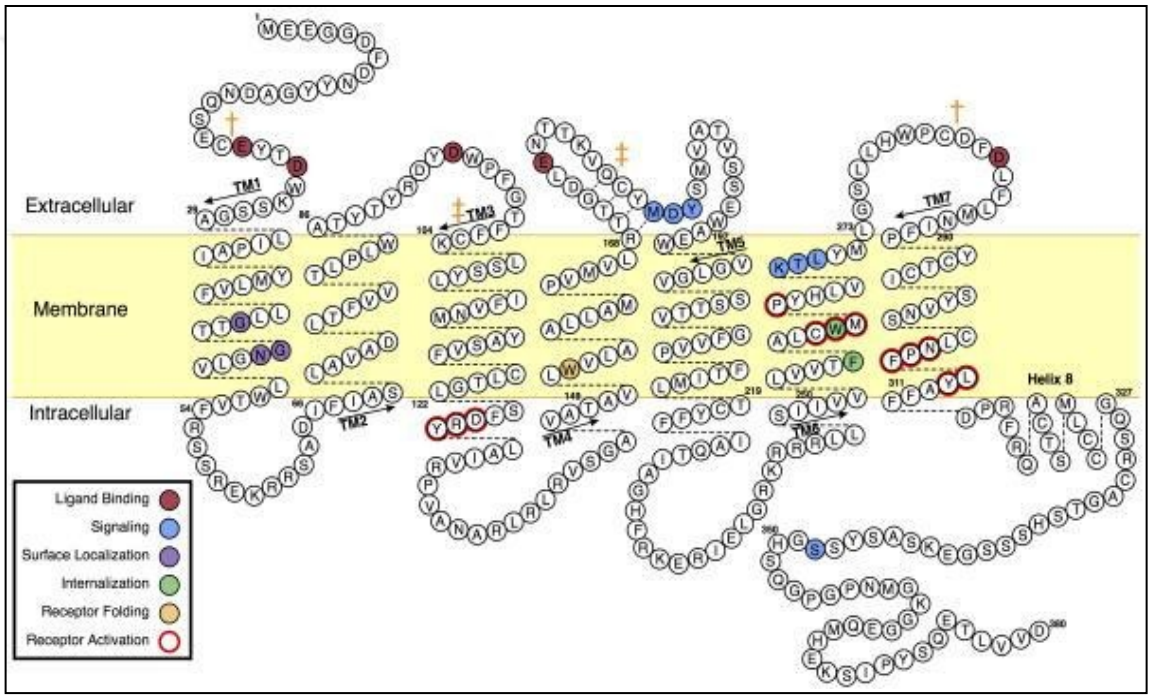


Figure 1.1. The structure of human apelin receptor [2]

It comprises consensus sequences for glycosylation, phosphorylation with protein kinase A and palmitoylation. Aplnr was first discovered in 1993 through a homology-based cloning method by O'Dowd and his co-workers. According to the findings, Aplnr was found on the long arm (q) of chromosome 11 and located at the band 12.1 and had a 40-50 per cent of sequence homology with the transmembrane regions of angiotensin AT1 receptor. Because Aplnr was found to be not activated by the binding of angiotensin II it was primarily defined as an "orphan" GPCR and named APJ (putative receptor protein related to the angiotensin receptor 1) [1]. Until the finding of Aplnr's endogenous ligand, Apelin in 1998 which was firstly identified from the tissue extracts of bovine stomach, it remained with the name APJ

[3]. The discovery of apelin guided the International Union of Pharmacology (IUPHAR) to name the receptor after its endogenous ligand, and now APJ is known as apelin receptor. Furthermore, the gene symbol for apelin receptor is approved by the Human Genome Organization (HUGO) as APLNR [4]. Apelin receptor has been detected and studied in different types of eukaryotes, including rodents[5, 6], cow[7], rhesus macaque[8], *Xenopus laevis*[9], chickens, turtles and zebrafish [10, 11]. The preliminary studies of Aplnr mRNA expression in humans by performing northern blot and quantitative PCR analysis have shown that it is widely present in the central nervous system (CNS). Aplnr expression has found in brain regions including the corpus callosum, hippocampus, caudate nucleus subthalamic nucleus, substantia nigra, medulla and also in the spinal cord [12-14]. In 2007, Hansen and his co-workers have demonstrated that Aplnr mRNA is expressed in the cortex and hippocampus of the human brain by utilizing a specific gene expression profiling assay for GPCRs [15]. Moreover, Aplnr transcripts have been observed in human bone marrow stromal cell lines [15]. According to transcriptomic studies of human brain donors, the expression of Aplnr mRNA has reported to be highly present including in the hippocampus, paraventricular nuclei of the thalamus, habenular nuclei, supraoptic nucleus of the hypothalamus as well as in the hindbrain structures [16]. The expression of Aplnr has also observed in the peripheral nervous system. Human mRNA expression of Aplnr was reported highly in the spleen by Edinger and his co-workers in 1998. They also reported that Aplnr has low expression in the colonic mucosa, small intestine and ovary [12]. In a detailed qPCR study, it has been revealed that Aplnr is strongly expressed in the spleen and placenta but found low levels in organs including the stomach, intestine and lung [14]. Furthermore, immunohistochemical analyses for the localization of Aplnr have demonstrated that it is also distributed in cardiovascular tissues including in the intramyocardial endothelial cells, ventricular cardiomyocytes and vascular smooth muscle cells [17].

According to molecular mechanism studies, when Aplnr signaling is activated it starts numerous intracellular signaling cascades including, PI3K/Akt, JNK, ERK1/2, P70S6 kinase which are all well-known for their involvement in the proliferation and survival of the cells (Figure 1.2) [16, 18, 19]. However, Aplnr signaling pathway is not fully elucidated in the literature yet.

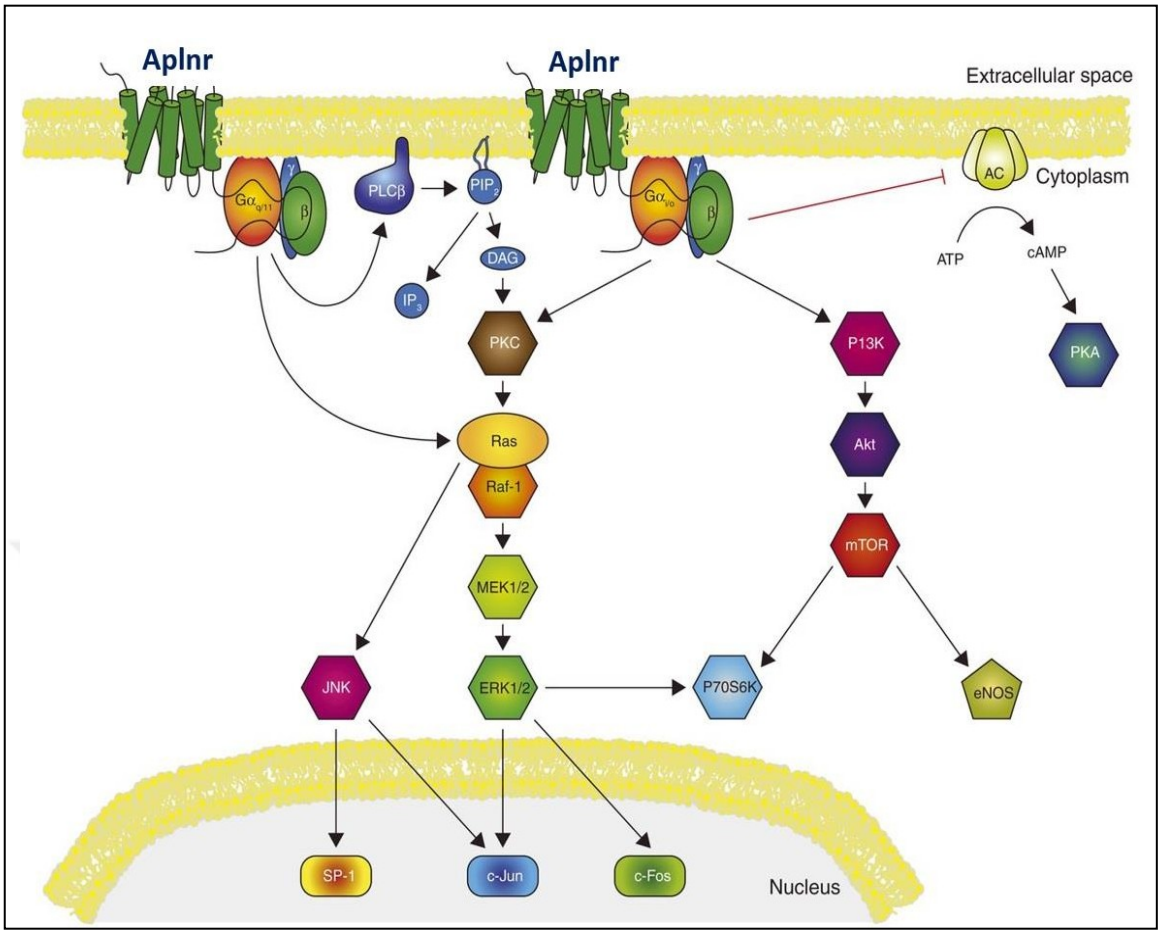


Figure 1.2. Predicted map of Aplnr signaling pathway [16].

In the late 2000s, Aplnr's synthetic small molecule activator (agonist), ML-233 [20] and inhibitor (antagonist), ML-221 [21] were developed. ML-233 activates Aplnr signaling by diminishing forskolin-mediated activation of intracellular cAMP, and as a consequence, stimulates downstream phosphorylation of the pathway [20]. Moreover, ML-221, is known to specifically cause the inhibition of apelin-13 induced activation of Aplnr [21].

## 1.2. APELIN

In 1998 Tatemoto and his colleagues isolated Apelin, the native endogenous ligand of Aplnr, from bovine stomach [3]. The human gene that is responsible for encoding Apelin is found on the long arm (q) of chromosome X and located at the band 26.1 [16]. The isolated Apelin gene was reported to encode 77-amino acid long prepropeptide which is a precursor molecule of active apelin. Upon this discovery, preproapelin was cleaved into biologically

active carboxyl-terminal peptide isoforms; apelin-36, apelin-17, and apelin-13 (Figure 1.3) by gel filtration chromatography and polypeptide sequencing [3].

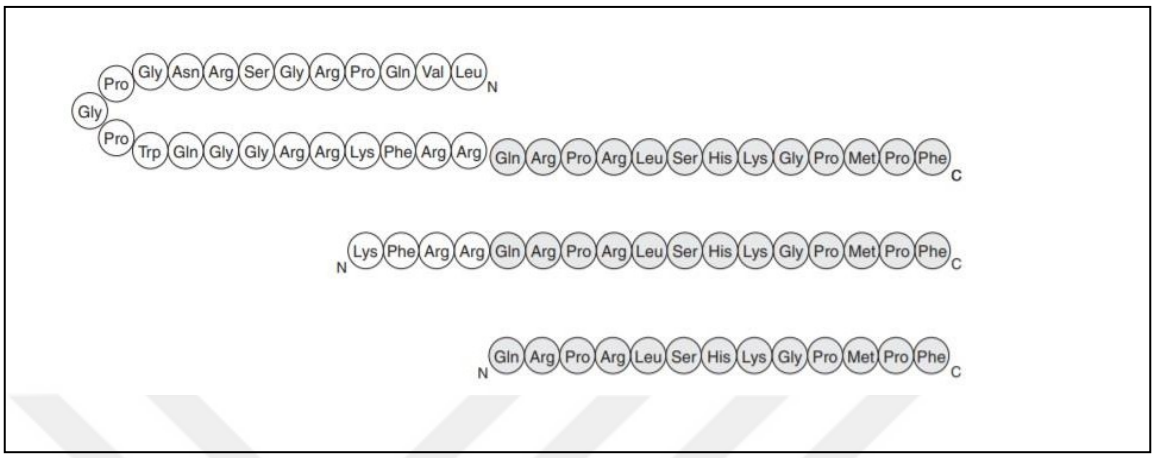


Figure 1.3. Aplnr agonists; apelin-36, apelin-17 and apelin-13, respectively [4].

Furthermore, in several *in vivo* studies, apelin peptides have been isolated and shown to bind Aplnr and activate intracellular signaling cascades (2, 5, 13, 17-19). Although all of the isoforms were shown to activate Aplnr, apelin-13 was identified as the most active one among others [3]. Also, apelin-13 can be further modified after translation by the spontaneous cyclization of its N-terminal glutamine and turned into N-terminally pyroglutamate modified apelin-13 (Pyr-apelin-13) [22, 23]. Pyr-apelin-13 has shown to have increased stability [24]. Apelin is found to be strongly conserved across various species. The 23 C-terminal residues of preproapelin is entirely identical in mammals including human, cattle, rats and mice [2]. In studies related to non-mammalian species, the 12 C-terminal amino acid sequence of the preproapelin are shown to share the same homology in a broad range of fish and *Xenopus laevis* [25]. Just like its receptor, apelin is also abundantly expressed in neurons and oligodendrocytes of the CNS [26]. According to both rodent and human studies, apelin is highly expressed and distributed in brain regions including hypothalamus, subthalamic nucleus, hippocampus, striatum, pituitary gland, medulla, amygdala, cerebellum, corpus callosum, spinal cord, substantia nigra, central gray matter, piriform cortex, olfactory tract, and dorsal raphe nucleus [1, 6, 26-32]. This indicates that apelin and its receptor have a significant role in neuronal signaling pathways. Furthermore, apelin is present in a wide variety of human peripheral tissues such as heart, lung, adipose,

kidney, and retinal endothelium. Peripheral apelin mRNA is expressed in high levels in the placenta, whereas, found at its lowest in the heart, lung and kidney [14, 33-36].

## 1.3. BIOLOGICAL ROLES OF APLNR/APELIN

Aplnr and its natural ligand apelin have been implicated to play an important role in primary cell signalling events including the regulation of cardiovascular system, fluid balance, energy metabolism, angiogenesis and other physiological roles. Since they act as key regulators in many of these systems it is indicated that Aplnr and apelin may also be involved in pathophysiological events [16].

### 1.3.1. Aplnr/Apelin in Cardiovascular System

Szokodi and his colleagues have demonstrated that apelin has a significant role in cardiac function. According to the research, infusion of apelin to the isolated rat heart resulted in a dose-dependent positive inotropic effect meaning that apelin strengthens the contraction of heart muscle [37]. In addition, apelin has been reported to have regulatory effects on blood pressure and shown to diminish arterial blood pressure through a nitric oxide (NO) synthase-dependent mechanism in rats [38]. Another study has revealed that apelin acts as an endothelium-dependent vasodilator in humans and as a result has demonstrated positive effects on the cardiac tissue [39]. In rat ischemic and hypoxia-induced heart failure models, apelin treatment has shown to enhance myocardial function and cardiac contractility [40, 41]. In glucose-deprived cultured rat cardiomyocytes, treatment with apelin has shown to have significant inhibitory effect on apoptosis indicating that Aplnr has cardioprotective effects [42]. According to a study in recent years, patients with ischemic heart failure have lower myocardial apelin levels and these reduced levels have shown to be linked with higher mortality rates, infarct size and inflammation suggesting that pharmacologically targeting Aplnr signalling pathway might be beneficial for the treatment of cardiovascular diseases [43].

### 1.3.2. Aplnr/Apelin in Energy Metabolism

Numerous studies have implicated the close relationship between energy metabolism and Aplnr signalling. Both Aplnr and its ligand have shown to be expressed in adipose tissue [35, 44]. Boucher and his colleagues have demonstrated that apelin is actually an adipokine that is released by adipose tissue. According to their study, obesity in mice and humans is correlated with the elevated blood plasma levels of apelin. Furthermore, they have observed a decrease in adipocyte apelin mRNA levels and plasma insulin levels in fasting mice. However, after feeding behaviour both of these levels returned to normal. This research has revealed a significant link between apelin and insulin [35]. According to a clinical study done on obese patients apelin plasma levels were found significantly higher than in the control group showing correlation between apelin expression levels and obesity [45]. In the study of Sorhede Winzell and his co-workers, treating mice and isolated pancreatic islets with apelin-36 have resulted in the inhibition of insulin secretion. Thus, it has been concluded that Aplnr is expressed in pancreatic islets and its ligand apelin might play a regulatory role in glucose homeostasis [46]. In an in vitro model stimulated by high glucose concentrations, apelin-13 has also been demonstrated to inhibit insulin secretion [47]. Moreover, administration of apelin intravenously in standard mice at low concentrations significantly cause lowered glucose levels in blood. In additionally, apelin has been reported to ameliorate glucose tolerance [48].

### 1.3.3. Aplnr/Apelin in Fluid Homeostasis

The discovery of the expression of Aplnr and apelin in regions where the control of bodies' fluid homeostasis occurs including the supraoptic nucleus and paraventricular nucleus of the hypothalamus has led scientists into investigating their role in fluid homeostasis. According to an *in vivo* study done on rats, apelin-36 has been shown to be significantly present in the paraventricular nucleus and supraoptic nucleus of the hypothalamus which is an important region of the human brain that is responsible for maintaining homeostasis within the body [27]. Antidiuretic hormone known as vasopressin is secreted by these small nuclei, and apelin's co-localization with this hormone has definitely suggested its role in regulating the fluid balance of the body. De Mota and his colleagues have given apelin to mice

intracerebroventricularly and shown that it actually has an inhibitory act on vasopressin release. Thus, apelin has lowered the plasma vasopressin levels and caused increase in diuresis [49]. Furthermore, it has been revealed that dehydration causes increase in the expression of apelin and Aplnr in rat brain. On the other hand, the expression of vasopressin has been shown to decrease in dehydration [50, 51]. Intracerebroventricular treatment of apelin-13 in rats have been reported to cause differences in their drinking behaviour. In response to apelin, rats' water intake significantly elevated albeit it has been reported that they were water-replete [52]. In addition, Aplnr mRNA has been found to be highly expressed in the glomeruli of the kidney and apelin has also shown to have regulatory effects on the network of small blood vessels of the kidney [53]. In another study, Aplnr knockout mice have exhibited abnormalities in regulating the fluid balance of their bodies [54].

### 1.3.4. Aplnr/Apelin in Angiogenesis

According to the study of Kasai and his co-workers in 2004, for the first time apelin has been reported to be involved in the angiogenesis of retinal endothelial cells [36]. Furthermore, apelin has been found to be a mitogenic peptide for endothelial cells [55]. In an animal study done on frogs, apelin and Aplnr have been both shown to be essential for the development of heart vessels. Also, in the lack of the apelinergic system, the vessels in frog embryos have been found to be disrupted [56]. Additionally, apelin has shown to be accountable for the formation of blood vessels in mice [57]. The angiogenic property of Aplnr and apelin has also been implicated in cancer pathology. The expression of apelin and Aplnr has found to be up-regulated in microvascular proliferations of brain tumours [55]. Moreover, the overexpression of apelin in tumour cell lines have shown to cause increase in tumour growth. Hence, it is indicated that apelin promotes new vessel formation in tumour growth [58]. In another cancer related research, apelin's expression has shown to be up-regulated in colon adenocarcinomas suggesting that it plays a role in tumour angiogenesis [59]. Apelin has also demonstrated vessel regeneration and endothelial cell proliferation in hypoxia-induced in vitro conditions. Thus, it is suggested that apelin might be therapeutic in the recovery of ischemia [60].

### 1.3.5. Other Physiological Roles of Aplnr/Apelin

Other than its physiological actions discussed above, Aplnr and apelin have also been studied and found to have regulatory effects in the gastrointestinal system. According to several studies, Aplnr and apelin are involved in inducing the proliferation of cells found in the stomach. Also, they are found to be responsible in regulating the secretion of gastric acid in the gastrointestinal tract [61-65]. Interestingly, in 1998 Aplnr was found to play a co-receptor role with CD4 which is a receptor that human immunodeficiency virus type 1 (HIV-1) binds in order to enter and infect cells [66]. Furthermore, Zou and his co-workers proved that apelin isoforms prevent the entry of HIV-1 and thus inhibit it from infecting the cell [67]. In recent years, Aplnr's neuroregulatory role in response to stress through the activation of hypothalamus–pituitary–adrenal axis has been demonstrated using Aplnr knockout mice [68]. Some studies have also reported apelin's protective roles in the immune system [69-71].

## 1.4. NEUROTOXICITY

In daily life, people are exposed to multitude of substances such as chemicals found in air, cosmetic ingredients, pesticides found in food, drugs and household chemical products that can potentially endanger human health. Being exposed to various types of both synthetic and natural chemical substances have been linked with neurotoxicity [72, 73]. Neurotoxicity is known as the detrimental effect on the structure or function of the central and peripheral nervous systems due to exposure to biological or manmade toxic substances [74]. Depending on the level of exposure, neurotoxic agents can cause significant adverse effects on human health. Unfortunately, it is not exactly known how many chemicals may cause neurotoxicity in humans [75]. Approximately 3 per cent to 28 per cent of all chemicals have been estimated to have neurotoxic potential according to the neurotoxicological studies in the United States [73]. Substances considered as neurotoxic can be classified as naturally occurring elements such as lead and aluminum; biological agents such as botulinum toxin (the neurotoxic compound produced by *Clostridium botulinum*) and aflatoxins (toxins produced by the fungi, *Aspergillus* spp.), synthetic substances such as pesticides; rotenone and paraquat and industrial chemicals [76]. World Health Organization has reported that annually 375,000

neurotoxicity cases in humans occur due to pesticide poisoning [77]. Furthermore, drugs that are used for therapeutic purposes including anti-cancer and anti-viral agents have also been shown to have neurotoxicological side effects [73]. Neurotoxic agents can affect the nervous system at various levels including anatomical, neurochemical, physiological and behavioural. At the anatomical level, changes can occur in the soma (the cell body of neurons') or the axon whereas at the neurochemical level neurotoxic agents can cause alterations in ion transmission across cellular membranes or play an inhibitory role in neurotransmitter release. Neurotoxic substances might also affect the speed of neurotransmission and thus cause changes at the physiological level. Behavioral level changes can be including the disturbances in the motor system or in cognitive functions including learning new information or remembering memories [78]. Most importantly, scientists are in the opinion that neurotoxic substances play a destructive role in neurodegenerative disorders such as Parkinson's disease, and Alzheimer's disease [73].

### 1.4.1. Oxidative Stress Induced Neurotoxicity

Oxidative stress is a term which was firstly described by Dr. Sies in 1991. According to his definition oxidative stress is the inequality between reactive oxygen species (also known as pro-oxidants) and antioxidants resulting in the excess production of reactive oxygen species and thus cause cellular damage [79]. Normally, atoms are surrounded by electrons that orbit around them and fill their outer layers' in pairs. However, in the case of an atom having an unpaired electron its reactivity rises leading it to have the tendency to bond with neighbouring molecules. These types of atoms are referred as free radicals. In the process of reacting with other molecules to fill their outer layers with electrons, free radicals cause oxidation. If the oxidation reactions occur several times, they can result in extensive cellular disruption and negatively affect biological molecules including lipids, proteins and nucleic acids. This harmful activity of free radicals' is known as oxidative stress. The level of damage is actually dependent on the availability of antioxidant molecules [80]. Antioxidants react with free radicals directly and donate their electrons for neutralization. Antioxidants act indirectly and inhibit enzymes that produce free radicals or increase the antioxidant enzymes' activity [81]. In metabolic reactions free radicals are usually produced and derived from oxygen molecules which are also refered as reactive oxygen species (ROS). These are Superoxide ($O_2^{\cdot-}$), Oxygen radical ($O_2^{\cdot\cdot}$), Alkoxy radical ($RO^{\cdot}$), Peroxyl radical ($ROO^{\cdot}$),

Hydroxyl ($OH^{\cdot}$), Nitrogen monoxide ($NO^{\cdot}$) and Nitrogen dioxide ($NO_2^{\cdot}$) [82]. Non-radical species such as hydrogen peroxide ($H_2O_2$), hypobromous acid (HOBr), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), ozone ($O_3$), nitrosyl cation ($NO^+$), nitroxyl anion ($NO^-$), nitrous acid ($HNO_2$), dinitrogen trioxide ($N_2O_3$), are also involved in reactions that can generate free radicals [82]. Free radicals can also be derived from nitrogen and sulphur molecules which are referred as reactive nitrogen species (RNS) and reactive sulphur species (RSS), respectively [81]. Production of free radicals alter cellular calcium homeostasis and thus cause mitochondrial dysfunction. This situation then in turn leads to the majority of ROS to be generated in the mitochondria [83]. Other than mitochondria, organelles including the endoplasmic reticulum [84] and the peroxisome[85] are also considered as a source for ROS generation. Hypoxia which is known as the state of oxygen deprivation, also causes cells to undergo oxidative stress from the excessive ROS produced in the mitochondrion [86-88]. Oxidative stress can emerge from numerous reasons such as alcohol consumption, exposure to neurotoxic chemicals, medications, air pollutants, trauma, and radiation [80].

Every major organ system can be affected by oxidative stress induced damage. However, since central nervous system (CNS) comprises of elevated levels of oxidizable lipid, demands high amounts of oxygen, and has low levels of antioxidant enzymes, it is considered to be particularly vulnerable to oxidants among others [89]. Also, the existence of redox-active metals in the brain including iron (Fe), copper (Cu), and zinc (Zn) are linked with its susceptibility to oxidative stress (Figure 1.4) [90, 91].

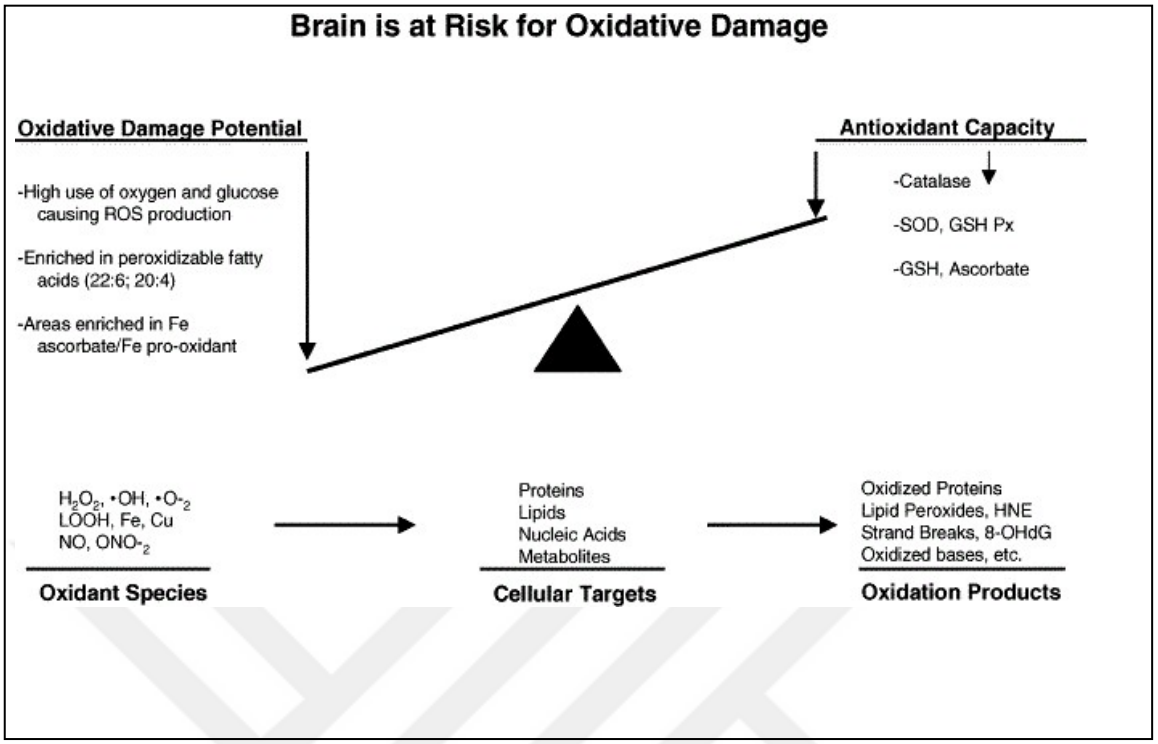


Figure 1.4. Oxidative damage risk of brain. LOOH, lipid hydroperoxide; Cu, copper ions; Fe, iron ions; NO, nitric oxide; $ONO^{2-}$, peroxynitrite; SOD, superoxide dismutase; GSH, glutathione; GSHPx, glutathione peroxidase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine and HNE, 4-hydroxy-2-nonenal [92].

The most susceptible regions to encounter attack by free radicals in the CNS are hippocampus, substantia nigra and the striatum [93, 94]. In an $H_2O_2$ treated oxidative stress rat model it has been shown that hydrogen peroxide diminishes the brain mass of rats, lowers the activity of antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, and reduces the activity of glutathione in the brain tissue suggesting that oxidative stress plays a significant role in neurotoxicity [95]. Numerous *in vitro* neurodegeneration models have shown that exposing neuronal cells to the prooxidant $H_2O_2$ cause oxidative stress-induced neurotoxicity leading to neuronal cell death [96-99]. Moreover, in a global forebrain ischemia rat model $H_2O_2$ have been observed to be in neurotoxic levels leading to significant loss of neurons [100]. In additionally, hydrogen peroxide accumulation has been reported to be neurotoxic in acute hypoxia-induced cerebral ischemia mouse model [101].

## 1.5. NEUROTOXIC DISORDERS

Oxidative stress has been associated with various neuronal disorders and shown as one of the major factors in the pathogenesis of neurotoxic disorders including Parkinson's Disease, Alzheimer's Disease, Multiple Sclerosis, ALS, epilepsy as well as cerebral ischemia [102].

### 1.5.1. Parkinson's Disease

Parkinson's Disease (PD) is defined as the progressive degeneration and death of dopamine producing neurons in the substantia nigra pars compacta region of the brain leading to dysfunctionalities in voluntary movement [103]. According to studies, the antioxidant glutathione has been observed dramatically in low levels in PD brain indicating that oxidative stress is significantly involved during disease progression [104]. The iron levels were found significantly high in PD brain and as a redox active metal it was shown to trigger the formation of ROS in dopaminergic neurons [105]. The deposition of iron can cause oxidation of dopamine and the formation of a neurotoxic compound called 6-hydroxydopamine (6-OHDA). This molecule then can go through an auto-oxidation process in the presence of superoxide and turn into the cytotoxic quinone form [106]. Ultimately, these events increase oxidative stress induced dopaminergic neuronal cell damage in PD patients [107]. Furthermore, it has been shown that several neurotoxic agents, including paraquat, rotenone, and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) lead to the formation of ROS and cause neuronal cell death in a similar pattern observed in PD [108-111].

### 1.5.2. Alzheimer's Disease

Alzheimer's disease (AD) is a common neurodegenerative disease characterized with the deposition of the peptide called β-amyloid and neurofibrillary tangles comprising of hyperphosphorylated tau protein in the brain. AD results in impairments in cognitive functions including memory and thinking [112]. The Amyloid-β peptide (Aβ) is known to chelate with metals associated with free radical generation including $Cu^{2+}$, $Zn^{2+}$ and $Fe^{3+}$ and lead to the production of $H_2O_2$ and form highly toxic reactive hydroxyl radical [113]. Iron

has been found to accumulate in AD brain and its ferric cation form ($Fe^{3+}$) has been reported to trigger hyperphosphorylated tau protein aggregation and eventually form neurofibrillary tangles leading to neuronal loss [114, 115]. Furthermore, in AD patients the presence of oxidative stress biomarkers such as 3-nitrotyrosine and protein carbonyls related to protein damage have been observed [112].

### 1.5.3. Cerebral Ischemia

Ischemic stroke is the most commonly known disease which is characterized by a substantial decrease in the cerebral blood flow resulting in oxygen and glucose deficiency related brain damage [116]. Ischemic brain damage is not only considered as an outcome of inadequate oxygen supply but also as harmful processes where the production of free radicals and other oxidizing toxic chemicals cause oxidative stress and disrupt the brain tissue [117]. Several oxidants and their by-products including superoxide anions ($O_2 \cdot -$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($\cdot OH$) are formed after stroke [117]. After superoxide anions are generated by pro-oxidant enzymes such as NADPH oxidase (NOX) and xanthine oxidase they can interact with nitric oxide (NO) and thus cause the production of a toxic oxidative radical known as peroxynitrite. Furthermore, peroxynitrite causes the nitration and dysfunction of proteins [118]. On the other hand, nitric oxide generated by neuronal nitric oxide synthase (nNOS) during brain ischemia/reperfusion, induces protein nitrosylation leading to cellular dysfunction [119]. Moreover, antioxidant enzymes against free radicals including superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT) detoxify oxidants [117]. However, from these reactions highly reactive hydroxyl radical is produced causing further toxic effects within the cell including lipid peroxidation, protein, DNA and RNA oxidation (Figure 1.5) [116].

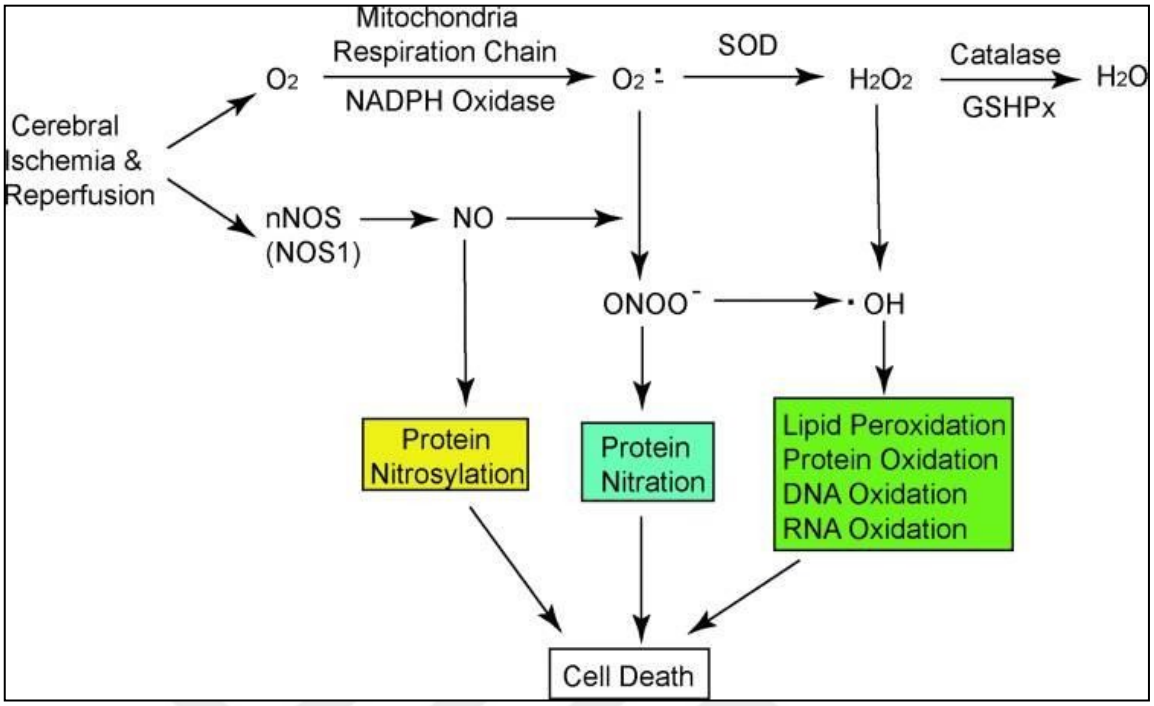


Figure 1.5. Oxidative stress in Cerebral Ischemia/Reperfusion [116]

### 1.5.4. Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune neuronal disorder defined by demyelination of neurons leading to nerve transmission impairment in the central nervous system. Activated microglia and macrophages is linked with the initiation of MS and considered to be responsible for myelin destruction in the CNS [120]. Furthermore, this activation is regarded as the major source for the production of ROS that can induce lipid peroxidation, and as a result demyelination occurs and neurons get damaged [120]. Also, besides ROS generation, impairments in iron metabolism have been shown to be responsible in the pathogenesis of MS [121].

### 1.5.5. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a serious neurodegenerative disease characterized by cellular damage and death of motor neurons that eventually leads to dysfunctionalities in voluntary muscle movement and even paralysis within several years from the beginning of the disease. The underlying causes of ALS are complex and have not been fully elucidated

yet [122]. However, several studies have observed increased oxidative stress related damage in the cerebrospinal fluid and the spinal cord of ALS patients. These studies have indicated oxidative stress to play an underlying role in motor neuron degeneration [123-125]. Moreover, studies have revealed that mutations in the antioxidant enzyme encoding gene, SOD1, cause nearly 20 per cent of familial ALS [126]. Although oxidative stress mechanism has been observed in the pathogenesis of ALS, there is still not enough evidence showing the precise cause of motor neuron degeneration.

### 1.5.6. Epilepsy

Epilepsy is a complicated CNS disease caused by primarily due to imbalance between excitation and inhibition in the brain. Epilepsy is characterized by repetitive and spontaneous seizures [127]. According to studies, both oxidative and nitrative stress are mechanisms that contribute to the pathogenesis of epilepsy [128]. Liang and Patel have shown prolonged seizures in rat models to change redox status, and thus cause oxidative damage to biomolecules [129]. Furthermore, number of studies have also observed an elevation in oxidative stress related cellular damage after recurrent seizures [130-133].

### 1.5.7. Other Disorders

Apart from the commonly known neurotoxic disorders discussed above several neurological disorders including bipolar disorder, depression, and schizophrenia have also been linked with high levels of ROS and RNS in the brain [134]. In additionally, patients with bipolar disorder and major depression have been reported to have neuronal cell death specifically in the hypothalamus region of the brain [135]. However, it is not known exactly whether this selective hypothalamic neuron loss is due to oxidative stress in the brain.

## 1.6. TREATMENT OF NEUROTOXIC DISORDERS

There is no available treatment that can cure neurotoxic disorders including PD, AD, MS cerebral ischemia, ALS and epilepsy. However, there are only symptomatic treatments of these diseases [136]. There are no drugs that can halt the destruction of nerve cells. In the

symptomatic treatment of PD, patients are usually given drugs that can increase dopamine levels in the brain including: levodopa and dopamine receptor agonists such as ropinirole pramipexole and bromocriptine [137]. Current symptomatic therapy for AD involves medications including donepezil, galantamine, and rivastigmine which are all known as acetylcholinesterase inhibitors. These are mainly used for improving cognitive functions including memory and thinking [138]. Furthermore, to slow the progression of AD, non-steroidal anti-inflammatory drugs (NSAIDs) are also used for treatment [139]. MS patients are usually given drugs called baclofen and tizanidine to diminish the spasticity which is the most common symptom seen in this disorder [140]. Also, other than pharmacological treatment MS patients are given physiotherapy for the management of spasticity [140]. For the treatment of cerebral ischemia, antithrombotic drugs including aspirin and alteplase are given to patients [141]. Epilepsy patients are usually given anti-epileptic drugs such as carbamazepine and levetiracetam to control seizures [142]. In the recent years, a drug called riluzole which might potentially slow the progression of ALS has been approved by FDA [143]. Today, research is still ongoing for developing new drugs for these disorders. In additionally, antioxidant compounds are considered to be a therapeutic approach in slowing the disease progression in neurodegenerative disorders as well as cerebral ischemia [144].

## 1.7. OXIDATIVE STRESS IN HYPOTHALAMUS

The hypothalamus is a significant region located in the ventral brain and made up of several small nuclei including the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), arcuate nucleus (ARC), the ventromedial nucleus (VMN) and the dorsomedial nucleus (DMN) with each of them having different functions [145-147]. Basically, hypothalamus controls and regulates various autonomic nervous system functions including nutrient intake, body weight, reproduction, glucose homeostasis, and sleep-wake patterns [145, 148]. Its anatomical localization allows hypothalamus to receive inputs from the thalamus and send them to a small pea sized gland called the pituitary. After receiving the signals, the pituitary gland then in turn releases hormones in response to changes in the autonomic nervous system [149]. Both in hypothalamic neurons and in pituitary cells voltage-gated $Ca^{2+}$ channels are expressed and responsible in controlling the entrance of calcium ions [150-152]. Calcium (Ca2+) is an important ion in neurons and its signalling has been known to generate various cascade of events including neurotransmitter release,

synaptic plasticity, and gene expression [153-155]. Intracellular $Ca^{2+}$ homeostasis is maintained by the control of $Ca^{2+}$-ATPase activity, calcium binding proteins (CBPs), and $Ca^{2+}$ uptake mechanisms [153, 156, 157]. In the review of Erika Gyengesi and his colleagues, excessive amounts of $Ca^{2+}$ ions are indicated to lead to the production of ROS and as a result cause oxidative stress in hypothalamic neurons specifically involved in energy homeostasis [148]. The relationship between oxidative stress and hypothalamus has also been studied in primary hypothalamic neuron cultures obtained from fetal rat brains. In the study, oxidative stress induction by ethanol has been reported to cause apoptosis in developing hypothalamic neurons [158]. According to an in vitro study done on GT1-7 mouse hypothalamic neuron cells, overexpressing alpha-synuclein which is a protein involved in the neuropathology of PD, causes mitochondrial dysfunction along with increased formation of free radicals and thus lead to oxidative stress. As a result of this hypothalamic neuronal stress, it has been shown that the secretion levels of gonadotropin-releasing hormones are significantly diminished [159]. Interestingly, studies have revealed the importance of iron-induced oxidative stress in the hypothalamus of women who suffer from anemia known as beta-thalassemia major (BTM) [160]. Since BTM patients are constantly dependent on blood transfusions, it has been shown that over-transfusions result in high amounts of iron deposition in endocrine system related organs including hypothalamus and pituitary gland and cause infertility in these patients [161]. According to a clinical study done on BTM patients, the levels of gonadotropin-releasing hormones were found significantly lower when compared to those in the control group [162]. Although the role of oxidative stress is implicated in hypothalamic neurons in various studies, there is still not enough evidence on oxidative stress induced hypothalamic degeneration and its relationship with neurodegenerative disorders.

## 1.8. GNRH NEURONS

Gonadotropin-releasing hormone (GnRH) is one of the significant hormones released from the hypothalamus, and known to regulate the reproductive function of the body [163]. In the beginning of 1970s, GnRH was isolated from pig hypothalamus and shown to have a structure as a decapeptide (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH(2) [164-166]. From these discoveries, GnRH is now known to be secreted from hypothalamus and bind to the GnRH receptors found on the anterior of the pituitary gland [166-168]. This

binding then causes the synthesis and the release of gonadotropins including the luteinizing hormone (LH) and follicle stimulating hormone (FSH) [166-168]. LH and FSH bind to their specific receptors LHR and FSHR, respectively, and act on the gonads (i.e. ovaries or testes) to stimulate the production of steroidal sex hormones[163, 168]. The gonadal sex hormones produced from the ovaries are estrogen and progesterone and the one that is produced from the testes is called the testosterone [163, 168]. The gonadal sex hormones, including estrogen, progesterone, and testosterone give negative feedback to hypothalamus and also to pituitary gland to inhibit the further release of GnRH, LH, and FSH [168]. Furthermore, the gonadotropins, LH, and FSH also provide negative feedback on the pituitary gland to block their further production (Figure 1.8) [168].

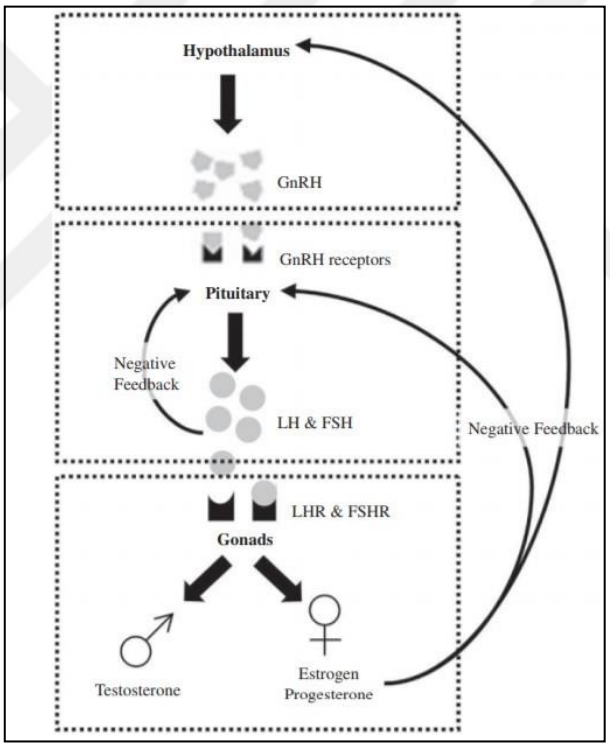


Figure 1.6. Reproductive hormone homeostasis

### 1.8.1. Oxidative Stress and GnRH Neurons

Due to different responses of women and men to oxidative stressors, reproductive hormones have been suggested to play a role in modulating the pro-oxidant/antioxidant balance [168]. Thus, it is implicated that gender differences affect the susceptibility to oxidative stress. In one clinical study, it has been found that women have higher levels of antioxidant glutathione

peroxidase (GSH-Px) erythrocyte activity in comparison to the same aged men suggesting that sex hormones related differences make individuals more susceptible to oxidative stress [169]. According to animal studies, the male brain has been shown to be more vulnerable to oxidative stress mediated ischemic injury when compared to the female brain [170]. These studies indicate that reproductive hormones play a major role in gender difference related susceptibility in oxidative stress. Furthermore, other studies have shown that not only gender differences but also pre- and post-menopausal hormonal changes in women can affect their proneness to oxidative stress [169, 171, 172]. Post-menopausal women have been shown to have higher levels in lipid peroxidation markers, as well as lower amounts of antioxidants including glutathione peroxidase, ascorbic acid (Vitamin C), and α-tocopherol (Vitamin E) when compared to pre-menopausal women [169, 171, 172].

## 1.9. AIM OF THE STUDY

The aim of this study is to elucidate the protective role of Apelin receptor (Aplnr) signaling in GnRH neurons against neurotoxicity by constructing an oxidative stress induced *in vitro* model. GT1-7 mouse hypothalamic (GnRH) neurons were used as a neuronal cell line and neurotoxicity was conducted by $H_2O_2$ and hypoxia induced cell stress. The goal of the current study is to explore the potential protective role of Aplnr signaling in GnRH neurons *in vitro* for the first time in the literature. This study will not only identify the Aplnr signaling as a therapeutic target in hypothalamic hormone releasing neurons but also comprises a new oxidative stress induced cell model in the literature which is using GT1-7 cells.

# 2. MATERIALS AND METHODS

## 2.1. CELL CULTURE

GT1-7 mouse hypothalamic (GnRH) neurons [173] were kindly provided by Prof. Dr. Fikrettin Şahin at Yeditepe University, Istanbul, Turkey. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Gibco, UK) containing high glucose (4.5 g/L) supplemented with 10 per cent fetal bovine serum (FBS) and 1 per cent of antibiotics including penicillin, streptomycin and amphotericin B (PSA, Invitrogen, Gibco, UK). GT1-7 cells were incubated in an 80 per cent humidified incubator with a temperature at 37°C and 5 per cent $CO_2$ in atmosphere. The medium was replenished two or three times per week, as needed. For sub-culturing of the cells, 0.25 per cent trypsin-EDTA (Invitrogen, Gibco, UK) was used after they reached approximately to 80 per cent confluency.

## 2.2. PREPARATION OF PEPTIDE AND SMALL MOLECULES

Apelin-13 (natural Aplnr ligand), ML-233 (synthetic Aplnr small molecule agonist) and ML-221 (synthetic Aplnr small molecule antagonist) were dissolved in DMSO with a final stock concentration of 10mM. Selected concentrations were freshly prepared in cell culture medium before application.

## 2.3. PREPARATION OF FUSION CONSTRUCTS

To determine whether Aplnr signaling can be activated with Apelin and ML-233 in HEK-293 cells (ATCC, CRL-1573) a GFP-Aplnr fusion construct was designed. Aplnr was cloned to the C-terminal part of sfGFP-C1 (Addgene plasmid # 54579) with restriction enzymes EcoRI and KpnI (Figure 3.1). The expression and internalization of Aplnr was detected after Apelin and ML-233 treatments for the confirmation of the activity of the peptide and small molecule, ML-233, in HEK-293 cells. Briefly; HEK-293 cells were seeded in 12-well cell culture plates with a cell density of $10^4$ cells per well. Afterwards, cells were incubated for 24 h at 37°C and 5 per cent $CO_2$ in humidified chambers. After 24 h, cells were exposed to

1 μM Apelin and 1 μM ML-233. Aplnr internalization was determined at 30 min and 120 min using fluorescence microscope (Axio Vert.A1; Zeiss, Heidelberg, Germany). After 30 min of exposure, flow cytometry analyses were performed for the detection of GFP positive cell ratio utilizing a Becton Dickinson FACSCalibur flow cytometry system (Becton Dickinson, San Jose, CA, USA).

## 2.4. HYPOXIA *IN VITRO* MODEL

For hypoxia induced in vitro neurotoxicity model experiments, GT1-7 cells were maintained in a hypoxia incubator under 2 per cent $O_2$ and 5 per cent $CO_2$ levels at $37^0$C in a humidified chamber.

## 2.5. $H_2O_2$ *IN VITRO* MODEL

In order to construct a $H_2O_2$ induced neurotoxicity model GT1-7 were incubated with different concentrations of $H_2O_2$. Briefly, 30 per cent (w/w) hydrogen peroxide solution (#H1009, Sigma-Aldrich, USA) were diluted in cell culture medium to obtain different concentrations including 10 μM, 50 μM, 100μM, 200μM, 500μM for further cell viability analysis. The moderate toxic concentration was selected for further experimental system.

## 2.6. SMALL INTERFERING RNA

For siRNA applications, Aplnr specific siRNA was purchased from ThermoFisher Scientific, USA (#AM16704). Briefly, GT1-7 cells were plated in 12-well plates (#CLS3512, Corning Plasticware, Corning, NY) with a density of $5 \times 10^4$ cells per well, and maintained in an incubator overnight. Next day, cells were transfected with siRNAs (Control siRNA #AM4611, ThermoFisher Scientific, USA or siAplnr) using Lipofectamine®RNAiMAX (#13778150, ThermoFisher, Invitrogen, USA) according to the manufacturer's instructions. Opti-MEM (#31985070, ThermoFisher, Gibco, USA) was used as a serum free environment during transfection applications. Following the transfection, cells were incubated for 48 hours at $37^0$C in a humified chamber. Then, cells were induced

with 200μM concentration of $H_2O_2$ and maintained in an incubator. Hypoxia treatment was applied by incubation of cells in a humidified hypoxia incubator.

## 2.7. TRANSIENT OVEREXPRESSION

GT1-7 cells were plated in 12-well plates with a cell density of 70 x $10^3$ cells per well for transient overexpression of Aplnr. Opti-MEM (#31985070, ThermoFisher, Gibco, USA) was used as a serum free environment during transfection applications. Cells were transfected with GFP-Aplnr fusion construct using lipofectamine. Next day, cells were maintained either in hypoxia incubator or exposed to $H_20_2$ (200μM) for oxidative stress induced conditions. After 24h of incubation, cells were collected for further analysis.

## 2.8. CELL VIABILITY ASSAY

Cell viability assays were performed on GT1-7 cells to analyse the cytotoxic effects of oxidative stress models and protective role of Aplnr signalling.

Cell viability analysis were performed by the 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H tetrazolium (MTS) assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to the manufacturer's instructions. For each time interval, cell viability was analysed at the absorbance value of 490 nm with an ELISA plate reader (Biotek, Winooski, VT).

### 2.8.1. Determination of $H_20_2$, Apelin, ML-233 and ML-221 Concentrations

Cells were seeded in a 96-well plate (#13485, SPL, Korea) at a density of 5x$10^3$ cells per well and incubated in three different experimental conditions including normoxia (standard cell culture conditions), $H_20_2$ (200 μM) and hypoxia for 24 h (2 per cent $O_2$) for generation of experimental conditions of oxidative stress in comparison with normal culture conditions. Apelin, ML-233, and ML-221 was dissolved in dimethyl sulfoxide (DMSO, #D4540, Sigma-Aldrich, USA) at a stock concentration of 10mM and diluted to 100 μM of working solution in DMEM containing 10 per cent FBS and 1 per cent of PSA. 30 per cent (w/w) $H_20_2$ solution (9.8 M) (#H1009, Sigma-Aldrich, USA) was diluted to 9800 μM of working

solution in complete growth medium. Cells were administered with various concentrations (0.1µM, 0.5 µM, 1µM, 2µM and 3µM) of Apelin, ML-233, ML-221 and $H_20_2$ (50µM, 100µM, 200µM and 500µM) for determining the cell viability effects of these compounds in GT1-7 cells. All of the concentrations were prepared in complete cell culture medium. At the end of 24 hours, cells were administered with 10 per cent MTS reagent and incubated in a humidified incubator for 2 h at 37 $^0$C and 5 per cent $CO_2$. Afterwards, Apelin (1 µM), ML-233 (1 µM), ML-221 (0.5 µM), and $H_2O_2$ (200µM) were determined and applied to the three different experimental conditions (hypoxia, normoxia and $H_2O_2$ application). For positive control, cells were only treated with 20 per cent DMSO.

## 2.9. CELL PROLIFERATION ASSAY

GT1-7 cells were seeded in 12-well plates at a density of $5x10^4$ cells per well and transfected with siRNAs (Control siRNA or Aplnr siRNA). After 48 hours of incubation cells were treated with 200µM $H_2O_2$ for 24 h and incubated in standard culture conditions. Control siRNA or Aplnr siRNA transfected cells with no $H_2O_2$ treatment were maintained in hypoxic conditions (2 per cent $O_2$ and 5 per cent $CO_2$ levels with a temperature at $37^0$C) for 24 h. At the end of 24 h incubation, cell morphology was determined by light microscope (Axio Vert.A1; Zeiss, Heidelberg, Germany). The cell proliferation was determined by staining cells with trypan blue solution. Afterwards, cells were counted using a hemocytometer.

## 2.10. QUANTITATIVE REAL TIME PCR ANALYSIS

Apelin, Aplnr, Caspase-3, BAX, BCL-2, AKT, p53, IL-2, NF-kB, COX-2 and GAPDH primers (Table 2.1) were designed with Primer-BLAST software from the National Center for Biotechnology (Bethesda, MD, USA). All of the primers were synthesized by Macrogen (Seoul, Korea). GAPDH was used as a housekeeping gene. The reagents and conditions of the RT-PCR analysis were illustrated in Table 2.2 and 2.3, respectively. Total RNAs were isolated using RNAeasy plus mini kit (#74136, Qiagen, Hilden, Germany) following the manufacturer's instructions. High Fidelity cDNA synthesis kit (#05081955001, Roche, USA) was utilized for cDNA synthesis. For the detection of mRNA levels of the desired genes, reverse transcription polymerase chain reaction (RT-PCR) was performed using

SYBR Green method. Synthesized cDNAs were mixed in PCR tubes with a final volume of 20µl with primers, SYBR-mix (#K0221, Fermentas, USA) and PCR grade distilled water (#SH30538.02, Hyclone, Utah, USA). CFX96 RT-PCR system (Bio-Rad, Hercules, CA) was utilized in all RT-PCR experiments.

Table 2.1. Primer designs used in RT-PCR analysis

| **Gene** | **Species** | **Primer Sequence** | **Product Length** |
|---|---|---|---|
| Caspase-3 | Mouse | F 5' GGGAGCAAGTCAGTGGACTC 3'<br>R 5' CCGTACCAGAGCGAGATGAC 3' | 136 bp |
| BAX | Mouse | F 5' CTCAAGGCCCTGTGCACTAA 3'<br>R 5' CACGCAGGAAGTCCAGTGTC 3' | 73 bp |
| BCL-2 | Mouse | F 5' CCACCTGTGGTCCATCTGAC 3'<br>R 5' CAATCCTCCCCCAGTTCACC 3' | 175 bp |
| AKT | Mouse | F 5' GGGACCTGAAGCTGGAGAA 3'<br>R 5' CCTGGTTGTAGAAGGGCAGG 3' | 240 bp |
| GAPDH | Mouse | F 5' TGGTGAAGCAGGCATCTGAG 3'<br>R 5' TGAAGTCGCAGGAGACAACC 3' | 78 bp |
| P53 | Mouse | F 5' CAGTGGGAACCTTCTGGGAC 3'<br>R 5' CTTCTGTACGGCGGTCTCTC 3' | 77bp |
| IL-2 | Mouse | F 5' ATGAACTTGGACCTCTGCGG 3'<br>R 5' GTCCACCACAGTTGCTGACT 3' | 170 bp |
| NF-KB | Mouse | F 5' ACACGAGGCTACAACTCTGC 3'<br>R 5' GGTACCCCCAGAGACCTCAT 3' | 164 bp |
| Aplnr | Mouse | F 5'GCTCATTCCTGCCATCTACA 3'<br>R 5'GTGGCAAAGTCACCACAAAG3' | 164 bp |

| | | | |
|---|---|---|---|
| Apelin | Mouse | F 5'TCCAGATGGAACAGGACTAGAA3'<br>R 5'CTGTCTGCGAAATTTCCTCCT3' | 112 bp |
| COX-2 | Mouse | F 5' ACCCTGCCCTGCTGGTGGAA 3'<br>R 5' CGCCTCCAAAGGTGCTCGGC 3' | 147 bp |

Akt: Protein Kinase B, Bax: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2, IL-2: Interleukin-2, NF-KB: Nuclear factor kappa B, COX-2: Cyclooxygenase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2.2. Reagents of RT-PCR analysis

| **Reagents** | **Volume** |
|---|---|
| Maxima™ SYBR Green qPCR Master Mix | 10 µl |
| Primer Forward (10pmol) | 1 µl |
| Primer Reverse (10pmol) | 1 µl |
| Distilled water | 5 µl |
| Template (100ng/ml) | 5 µl |

Table 2.3. Conditions of RT-PCR analysis

| **Cycle** | **Repeats** | **Step** | **Standby Time** | **Set Point** |
|---|---|---|---|---|
| Initial Denaturation | 1 | 1 | 3 min | 93 °C |
| Denaturation | 36 | 1 | 30 sec | 93 °C |
| Annealing | | 2 | 40 sec | 61 °C |
| Extension | | 3 | 45 sec | 72 °C |
| Final Extension | 1 | 1 | 10 min | 72°C |
| Melt Curve | 110 | 1 | 12 sec | -0.5 °C/cycle |
| Hold | 1 | 1 | ∞ | 4 °C |

## 2.11. OXIDATIVE STRESS ENZYME ACTIVITY

For the detection of oxidative stress markers, super oxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activity were measured in GT1-7 cells treated with Apelin (1µM), ML-233 (1µM), ML-221 (0.5µM), Control siRNA and Aplnr siRNA at three different experimental conditions including normoxia (standard cell culture conditions), $H_20_2$ (200 µM) and hypoxia for 24 h (2 per cent $O_2$). SOD determination kit (#19160, Sigma Aldrich, USA) was used according to the manufacturer's instructions. 96-well plates were utilized during the oxidative stress marker test. 10 µg of protein samples were used. Briefly, 20 µl from sample solution was added to each sample as well as the blank 2 well. Next, double distilled water (20 µl) was added to each blank 1 and blank 3 wells. Then, 200 µl of WST working solution was added to every well and mixed. Afterwards, each blank 2 and blank 3 wells were diluted with 20 µl of dilution buffer. Following dilution, enzyme working solution (20 µl) was added to all samples and the blank 1 well, and fully mixed. Next, the 96-well plate was incubated for 20 minutes at 37 °C and 5 per cent $CO_2$ levels in humidified chambers. Absorbance at 450 nm was detected utilizing a microplate reader. SOD activity was calculated according to the formula (SOD activity (inhibition rate %) = {(Ablank 1 - Ablank 3) – (Asample - Ablank 2) / (Ablank 1 - Ablank 3)} x 100) provided in the instructions.

As an oxidative stress marker, glutathione peroxidase (GPx) activity was also detected in GT1-7 cells treated with Apelin (1µM), ML-233 (1µM), ML-221 (0.5µM), Control siRNA and Aplnr siRNA at three different experimental conditions including normoxia (standard cell culture conditions), $H_20_2$ (200 µM) and hypoxia for 24 h (2 per cent $O_2$) using Total Glutathione Peroxidase Assay kit (# 08050002, ZeptoMetrix Corporation, NY, USA). Briefly, GPx assay reagents (working solution A and start solution) and collected samples were prepared according to the manufacturer's instructions. 870 µl of working Solution A and 60 µl of start solution were added to the cuvette of the spectrophotometer, and mixed. Then, 30 µl of sample was pipetted and mixed thoroughly. Absorbance at 340 nm was recorded for 1 min utilizng a spectrophotometer.

## 2.12. IMMUNOCYTOCHEMICAL ANALYSIS

Cells were seeded in 8-well multi-chamber plates with a density of 5000 cells per well, and maintained in a humidified incubator at 37 °C and 5 per cent CO2 in atmosphere. Next day, cells were treated with Apelin (1μM), ML-233 (1μM), ML-221 (0.5μM) and $H_2O_2$ (200) μM with desired combinations for 24 hours and incubated in normoxic and hypoxic conditions. Similar methodology was used for siRNA and transient overexpression experiments. After 1 day of incubation, cells were fixed with 4 per cent paraformaldehyde (PFA) and permeabilized in PBS solution containing 0.1 per cent Triton-X 100. Afterwards, cells were rinsed with PBS and treated with 1 per cent bovine serum albumin (BSA, Santa Cruz, CA, USA) to allow the blocking of the cells. Cells were treated with the following primary antibodies against Aplnr (Thermo,USA),and GnRH (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated overnight at 4 °C. Then, cells were rinsed and incubated with secondary antibodies including AlexaFluor 488 (anti-mouse) and AlexaFluor 647 (anti-rabbit) for 30 minutes in room temperature. DAPI (0.5 lg/mL; Applichem, Darmstadt, Germany) was used for nuclei staining. The images of samples were taken using a confocal microscope (LSM 700; Zeiss, Heidelberg, Germany). Image J software was utilized for calculating the intensities.

## 2.13. STATISTICAL ANALYSIS

For statistical analysis of the data, one-way analysis of variance (ANOVA) and Tukey post hoc test were performed. The P values less than 0.05 were considered as statistically significant.

# 3. RESULTS

## 3.1. PEPTIDE ACTIVITY RESULTS

### 3.1.1. Aplnr Signaling is Activated with Apelin and ML-233 in HEK-293 Cells

For determining the time frame of Aplnr internalization, HEK-293 cells were utilized as an easily transfected cell line. 67 per cent of the HEK-293 cells were GFP positive showing the successfully transfected cell ratio in the beginning (0 min). 3 per cent increase was observed only after 30 min in Apelin and ML-233 treated groups and approximately 70 per cent of the HEK-293 cells were GFP positive. At 0 min, GFP-Aplnr fusion protein was mainly observed on surface of the cells. After 30 min in Apelin and ML-233 treated groups the internalization of Aplnr was started to appear. After 120 min later, the intracellular localization was observed more clearly, and about 35 per cent of the cells had Aplnr inside the cell following Apelin and ML-233 exposure (Figure 3.1).

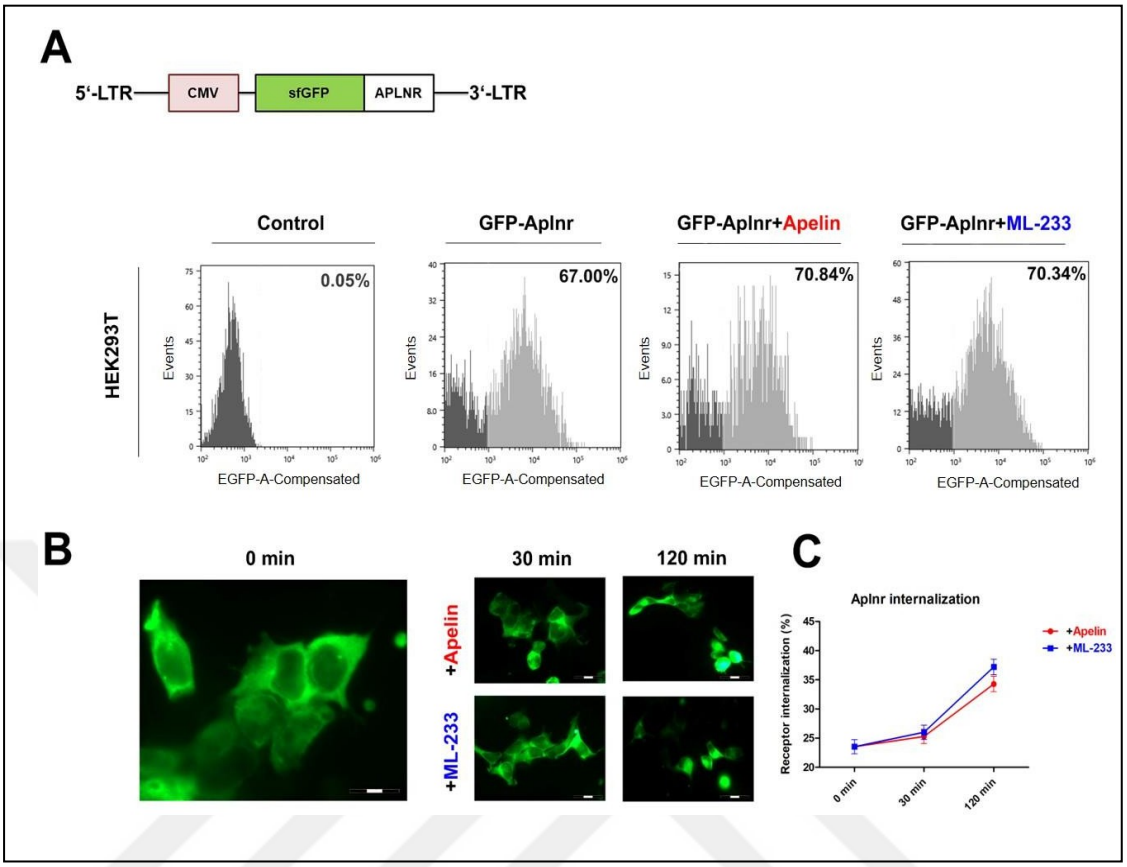


Figure 3.1. Aplnr signaling activation with Apelin and ML-233 in HEK-293. (A) Plasmid vector design for GFP-Aplnr fusion protein. Flow cytometry analysis of Aplnr signaling activation in HEK293 cells in response to Apelin (1µM) and ML-233(1µM). Activation of Aplnr was confirmed after 30 min. (B) GFP-Aplnr transfected cells were shown in fluorescence microscope after internalization at 30 min and 120 min. (C) 35 per cent of the cells showing Aplnr internalization at 30 min and 120 min following Apelin and ML-233 treatment.

## 3.2. ACTIVATION OF APLNR SIGNALING

### 3.2.1. Cell Viability and Proliferation Analysis

For determining the cytotoxic effects of Apelin, ML-233, ML-221 and $H_2O_2$ on GT1-7 cells, cell viability analyses were performed for 24 hours. DMSO (20 per cent) was used as positive control during MTS assay. After 24 hours of administration at various concentrations of Apelin, ML-233, ML-221 (0.1, 0.5, 1, 2 and 3µM) and $H_2O_2$ (10, 50, 200,

and 500 μM) cell images were taken for determined doses using a light microscope. Results have shown that treatment with Apelin and ML-233 increased the cell viability of GT1-7 cells approximately to 1.5 fold after 1 μM treatment compared to negative control indicating that Apelin and ML-233 have proliferative effects in these cells (Figure 3.2). However, doses greater than 1 μM decreased cell viability and resulted in cytotoxic effects. 1 μM was the selected concentration for further experiments. The Aplnr antagonist, ML-221, application decreased the viability of the cells at a certain dose (0.5 μM) approximately to 70 per cent, and caused fluctuations in cell viability with higher doses when compared to negative control (Figure 3.2). Treating GT1-7 cells with $H_2O_2$ significantly decreased the viability after 24 h treatment to 78 per cent at 10 μM, 61 per cent at 50 μM, 44 per cent at 100 μM, 21 per cent at 200 μM and 1.5 per cent at 500 μM (Figure 3.3). Cell proliferation analysis were performed after treatment for 24 h with the determined dosages of Apelin (1 μM) ML-233 (1 μM), ML-221 (0.5 μM), and $H_2O_2$ (200 μM) in normoxic, hypoxic and $H_2O_2$ induced conditions. Apelin (1 μM) and ML-233 (1 μM) treated cells in normoxic conditions significantly increased cell proliferation from 45 x $10^3$ to 47 x $10^3$ when compared to negative control. On the other hand, inhibiting Aplnr via ML-221 (0.5 μM) significantly decreased cell proliferation to 40 x $10^3$ (Figure 3.4). In $H_2O_2$ (200 μM) induced conditions Apelin treated cells increased cell proliferation from 43 x $10^3$ to 45 x $10^3$ compared to $H_2O_2$ only treated cells. Interestingly, ML-233 treatment in $H_2O_2$ induced cell conditions didn't cause any significant changes in cell proliferation. However, ML-221 treated cells exposed to $H_2O_2$ significantly decreased the proliferation of GT1-7 cells from approximately 43 x $10^3$ to 42 x $10^3$. When compared to hypoxia control group both Apelin and ML-233 treated cells under hypoxic conditions caused a significant increase in cell proliferation from nearly 32 x $10^3$ to 37 x $10^3$ and to 42 x $10^3$, respectively. ML-221 treatment in hypoxic conditions resulted in 35 x $10^3$ number of viable cells (Figure 3.4).

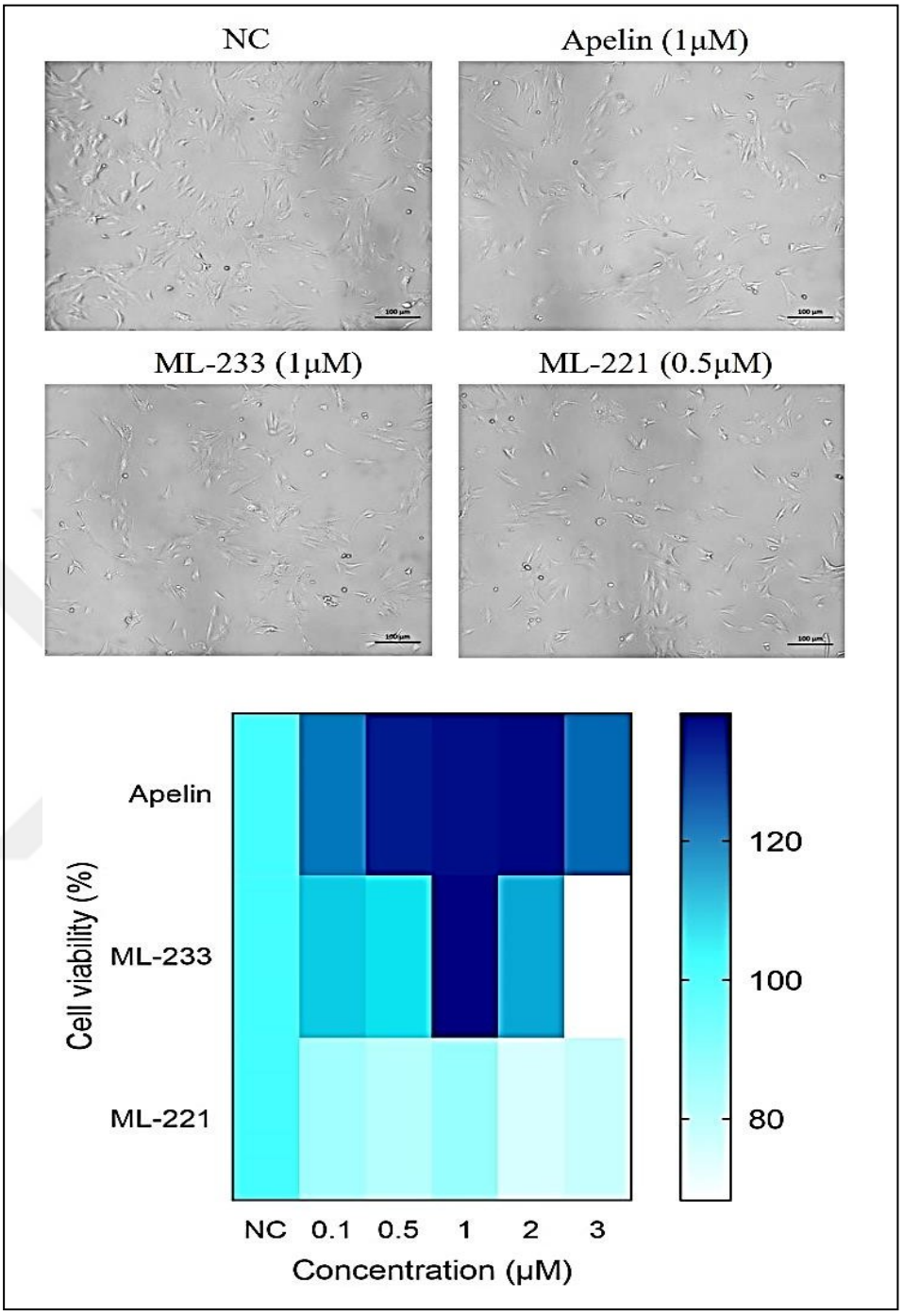


Figure 3.2. Cell viability of Apelin, ML-233 and ML-221 treated GT1-7 cells. Light microscopy images represented at the upper lane are shown as one hundred times magnified after 24 h treatment. The heat map demonstrates cell viability effects of Apelin, ML-233 and ML-221 in GT1-7 cell line at different concentrations. NC: negative control. *$p<0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1. Scale bar: 100 µm.

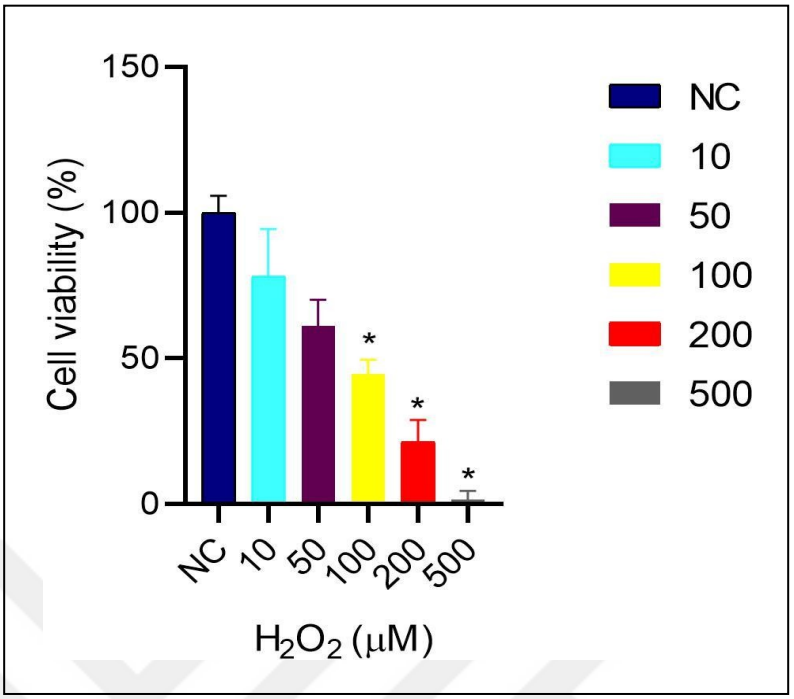


Figure 3.3. Cell viability of $H_2O_2$ treated GT1-7 cell line. The bar graph demonstrate cell viability effect of $H_2O_2$ in GT1-7 cell line at different concentrations. * $p < 0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test utilizing GraphPad Prism Version 8.0.1.

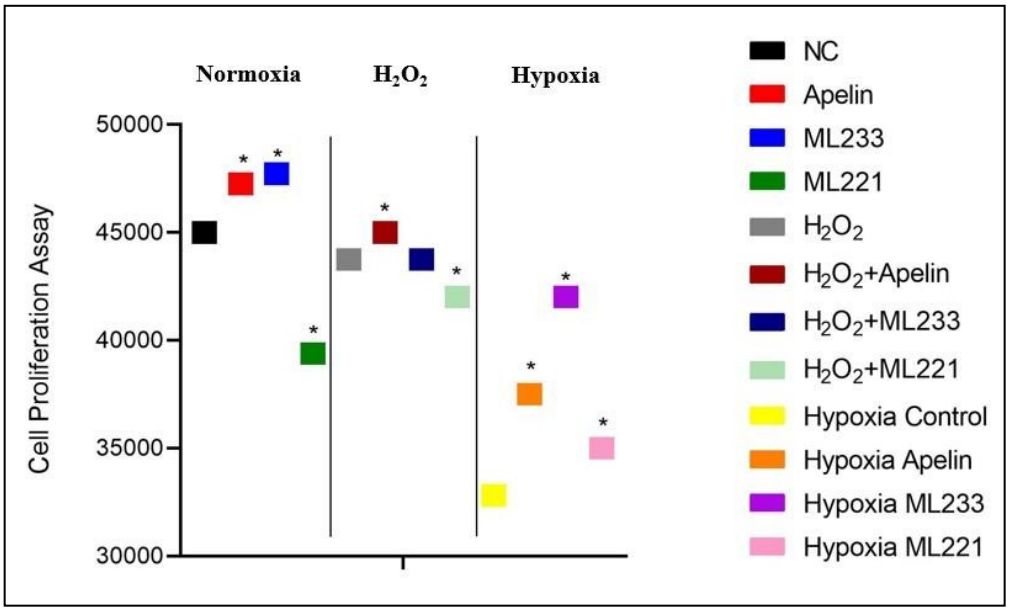


Figure 3.4. Cell proliferation analysis of Apelin, ML233, and ML221 treated GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced conditions. * $p < 0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test utilizing GraphPad Prism Version 8.0.1.

### 3.2.2. Cellular Morphology Analysis of Apelin and Small Molecule Induced Aplnr Signaling

Light microscope images were taken after 24h treatment with Apelin (1 μM), ML-233(1 μM), ML-221 (0.5 μM), and same treatment regimen in the presence of $H_2O_2$ (200 μM) to visualize and detect differences in cellular morphology. According to the microscope images, $H_2O_2$ (200 μM) treated group had fewer cells compared to negative control (only growth medium treated group). The combination of $H_2O_2$ (200μM) and Apelin (1μM) had increased numbers of cells as opposed to the only $H_2O_2$ treated cells. The similar effect was detected for $H_2O_2$ (200μM) and Aplnr agonist, ML-233 (1μM), treated group. However, $H_2O_2$ (200 μM) and Aplnr antagonist, ML-221 (1μM), treated group had decreased number of cells when compared to $H_2O_2$ (200μM) and Apelin (1μM) treated group (Figure 3.5).

Effect of hypoxia on cellular morphology was also observed in Apelin (1 μM), ML-233(1 μM), and ML-221 (0.5 μM) treated GT1-7 cells by using light microscope. Results have shown that apelin treated cells under hypoxic conditions had increased number of cells when compared to hypoxia negative control. The similar effect was detected for ML-233 (1μM) treated hypoxia group. However, ML-221 administered cells had lower number of cells as opposed to negative control under hypoxic conditions (Figure 3.5).

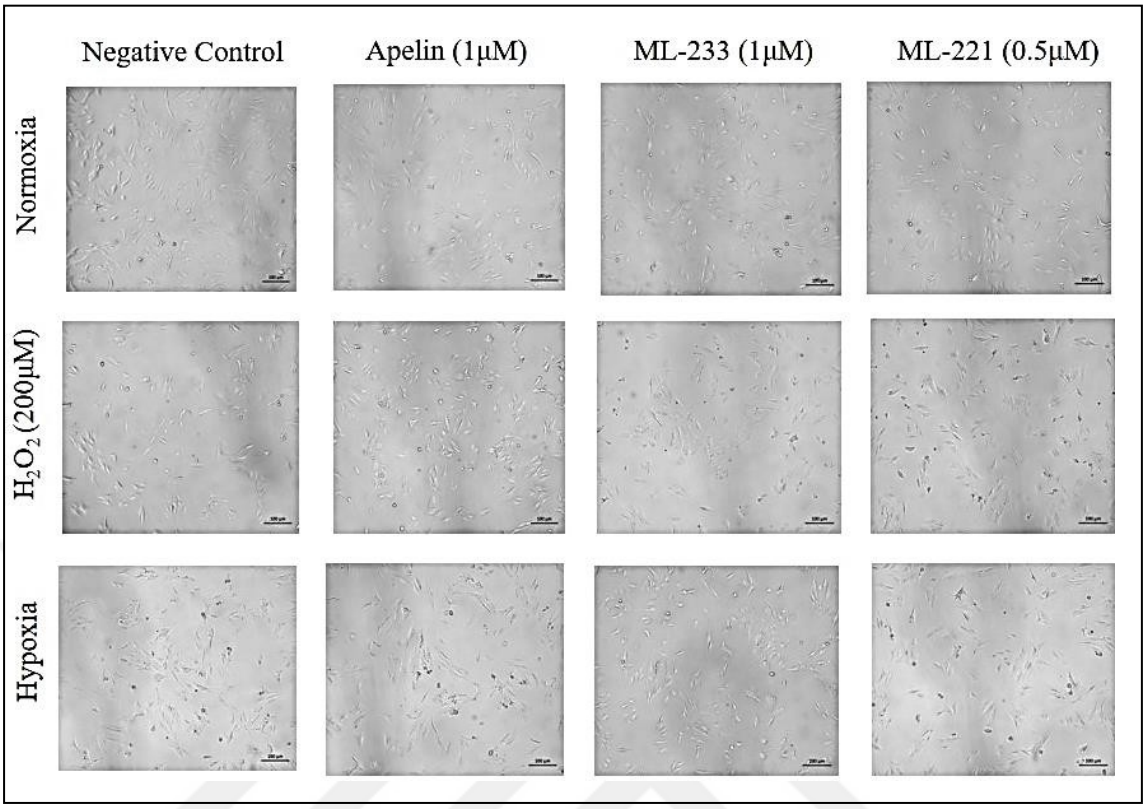


Figure 3.5. Morphological images of GT1-7 cells treated with Apelin (1 µM), ML-223 (1 µM) and ML-221 (0.5 µM) in normoxia, $H_2O_2$ (200µM), and hypoxia induced conditions. Light microscopy images are shown as one hundred times magnified. Scale bar: 100 µm.

### 3.2.3. Gene Expression Analysis

Gene expression analysis were performed in Apelin (1 µM), ML-233(1 µM) and ML-221 (0.5 µM) treated GT1-7 cells in normoxia, $H_2O_2$ (200 µM) and hypoxia induced cell stress conditions by performing RT-qPCR. As a proliferation marker, Akt, in normoxic conditions slightly increased 1.12 fold in Apelin treated cells. Aplnr agonist, ML-233, treatment caused 1.9 fold increase. However, inhibiting Aplnr with ML-221 had no significant changes in Akt mRNA levels with 0.8-fold. $H_2O_2$ and Apelin treatment resulted in significant increase of Akt mRNA levels with 46-fold change. $H_2O_2$ and ML-233 treated cells had increased Akt gene expression levels as well with 20-fold change. However, $H_2O_2$ and ML-221 treated cells had lower Akt mRNA levels with 8-fold change. Cells treated with Apelin in hypoxic conditions caused significant increase in Akt mRNA levels with 80-fold change. ML-233

treated hypoxia group had 30-fold increase whereas ML-221 treated hypoxia group had lower Akt gene expression levels with 1.5-fold change (Figure 3.6).

As an apoptotic marker, BAX, gene expression levels had no significant changes in Apelin treated cells (1.03 fold), but had showed a slight increase in ML-233 treated cells with 1.63-fold change. Treating cells with Aplnr inhibitor ML-221 had significantly increased apoptosis marker, BAX, with 2-fold change. $H_2O_2$ treatment caused increase by 42-fold in BAX gene expression levels as expected. However, $H_2O_2$ and Apelin treatment together significantly decreased BAX mRNA levels as 0.00013-fold. The similar fold change was detected for $H_2O_2$ and ML-233 treated cells. On the other hand, pharmacological inhibition of Aplnr with ML221 in $H_2O_2$ induced cell stress conditions dramatically increased BAX gene expression levels by 3.9-fold. Apelin treatment in hypoxic conditions increased BAX mRNA levels by 1.2-fold. However, ML-233 treated hypoxia group resulted in 0.63 fold decrease in BAX gene expression levels. ML-221 treated hypoxic group had higher BAX mRNA levels with 1.9-fold change (Figure 3.7).

The gene expression levels of p53 decreased by 0.7 fold in Apelin treated cells. ML-233 treatment decreased p53 mRNA levels even more by 0.3 fold. However, Aplnr antagonist, ML-221, resulted in only 0.9 fold change of decrease in p53 gene expression levels. $H_2O_2$ and Apelin treatment together caused 0.5-fold change of decrease in p53 mRNA levels. Interestingly, $H_2O_2$ and ML-233 administration significantly increased p53 gene expression levels by 8.7-fold. On the other hand, exposing cells to $H_2O_2$ and ML-221 dramatically increased p53 mRNA levels by 26-fold. Apelin treatment in hypoxic conditions had 1.07-fold change in p53 gene expression levels. On the other hand, ML-233 treatment in hypoxia caused 0.2-fold decrease in p53 mRNA levels. Inhibiting Aplnr with ML-221 in hypoxic conditions caused p53 gene expression levels to significantly increase by 2.2-fold (Figure 3.7).

Caspase-3 gene expression levels were significantly decreased by 0.0004-fold change in Apelin treated cells. ML-233 treatment decreased caspase-3 mRNA levels by 0.02-fold. However, ML-221 treatment caused caspase-3 gene expression levels to increase by 3.2-fold. $H_2O_2$ and Apelin treatment significantly decreased caspase-3 mRNA levels to 0.09-fold. However, $H_2O_2$ and ML-233 treatment together significantly decreased apoptotic marker, caspase-3, levels to 0.00002-fold. Exposing cells to ML-221 in $H_2O_2$ induced conditions resulted in 1.3-fold change of increase in caspase-3 gene expression levels.

Apelin treatment in hypoxic conditions resulted in remarkable decrease in caspase-3 mRNA levels. In hypoxic conditions, ML-233 treated cells had 0.1-fold change, whereas ML-221 treated cells had 4.3-fold change in caspase-3 gene expression levels (Figure 3.7).

Anti-apoptotic marker, BCL-2, mRNA levels were significantly elevated in cells treated with Apelin by 9.4-fold. Aplnr activator, ML-233, treatment also increased BCL-2 gene expression levels by nearly 25-fold. However, inhibition of Aplnr with ML-221 caused BCL-2 gene expression levels to change by 2.9-fold. Apelin treatment in $H_2O_2$ induced conditions significantly increased BCL-2 gene expression levels by 11-fold. $H_2O_2$ and ML-233 treatment resulted in 4.6-fold change of increase in BCL-2 mRNA levels. On the other hand, 0.2-fold of change was detected in ML-221 treated cells in $H_2O_2$ induced conditions. In hypoxic conditions BCL-2 gene expression levels were significantly increased in Apelin treated cells by 10-fold. However, ML-233 treatment decreased BCL-2 mRNA levels by 0.009-fold in hypoxia. On the other hand, ML-221 treated cells had no significant changes in hypoxic conditions (Figure 3.7).

Aplnr gene expression levels were found to significantly increase in Apelin treated cells by 2.2-fold. ML-233 treatment in normoxic conditions caused Aplnr mRNA levels to change 35-fold. Interestingly, ML-221 treated cells had the highest Aplnr gene expression level with 1000-fold. Apelin treatment with $H_2O_2$ resulted in 10-fold change of increase in Aplnr mRNA levels. $H_2O_2$ and ML-233 treatment together significantly increased Aplnr gene expression levels by 68-fold. On the other hand, inhibiting Aplnr with ML-221 in $H_2O_2$ treated cells caused 0.03-fold change of decrease in Aplnr gene expression levels. Treating cells with Apelin in hypoxic conditions caused significant increase in Aplnr mRNA levels by 663-fold, and even higher in ML-233 treated cells with 1269-fold. Inhibiting Aplnr in hypoxic conditions caused Aplnr mRNA levels to change 3.2-fold (Figure 3.8).

Inflammatory marker, IL-2, mRNA levels were found to be increased in Apelin and ML-233 treated cells with 4.3-fold and 1.43-fold, respectively. However, ML-221 treatment in normoxic conditions decreased IL-2 mRNA levels by 0.2-fold. On the other hand, Apelin treated cells in $H_2O_2$ induced conditions caused IL-2 gene expression levels to decrease by 0.01-fold. $H_2O_2$ and ML-233 treatment resulted in 0.2-fold change of decrease in IL-2 gene expression levels. IL-2 mRNA levels were significantly increased by 16-fold in cells exposed to ML-221 in $H_2O_2$ induced conditions. Apelin treated cells in hypoxic conditions decreased IL-2 gene expression levels significantly by 0.4-fold. Interestingly, ML-233

treatment in hypoxia group caused a slight increase in IL-2 mRNA levels by 1.2-fold. On the other hand, inhibiting Aplnr with ML-221 in hypoxia induced conditions resulted in 11-fold change of increase in IL-2 gene expression levels (Figure 3.9).

Pro-inflammatory marker, NF-KB, gene expression levels were found to be significantly diminished in Apelin treated cells in normoxia by 0.2-fold. However, ML-233 treated cells had 6 x $10^6$ -fold change of increase in NF-KB mRNA levels in normoxic conditions. ML-221 treatment caused NF-KB gene expression levels to significantly increase by 56 x $10^3$-fold in normoxia. Apelin treatment in $H_2O_2$ induced conditions significantly decreased NF-KB gene expression levels by 0.0007-fold. $H_2O_2$ and ML-233 treatment resulted in 0.002-fold change of decrease in NF-KB mRNA levels. On the other hand, the inhibition of Aplnr with ML-221 in $H_2O_2$ induced conditions slightly increased pro-inflammatory marker, NF-KB, levels by 1.16-fold. Exposing cells to Apelin in hypoxic conditions significantly decreased NF-KB gene expression levels by 0.0000039-fold. ML-233 and ML-221 treatment in hypoxic conditions caused NF-KB mRNA levels to change 0.000032-fold and 0.0000052-fold, respectively (Figure 3.9).

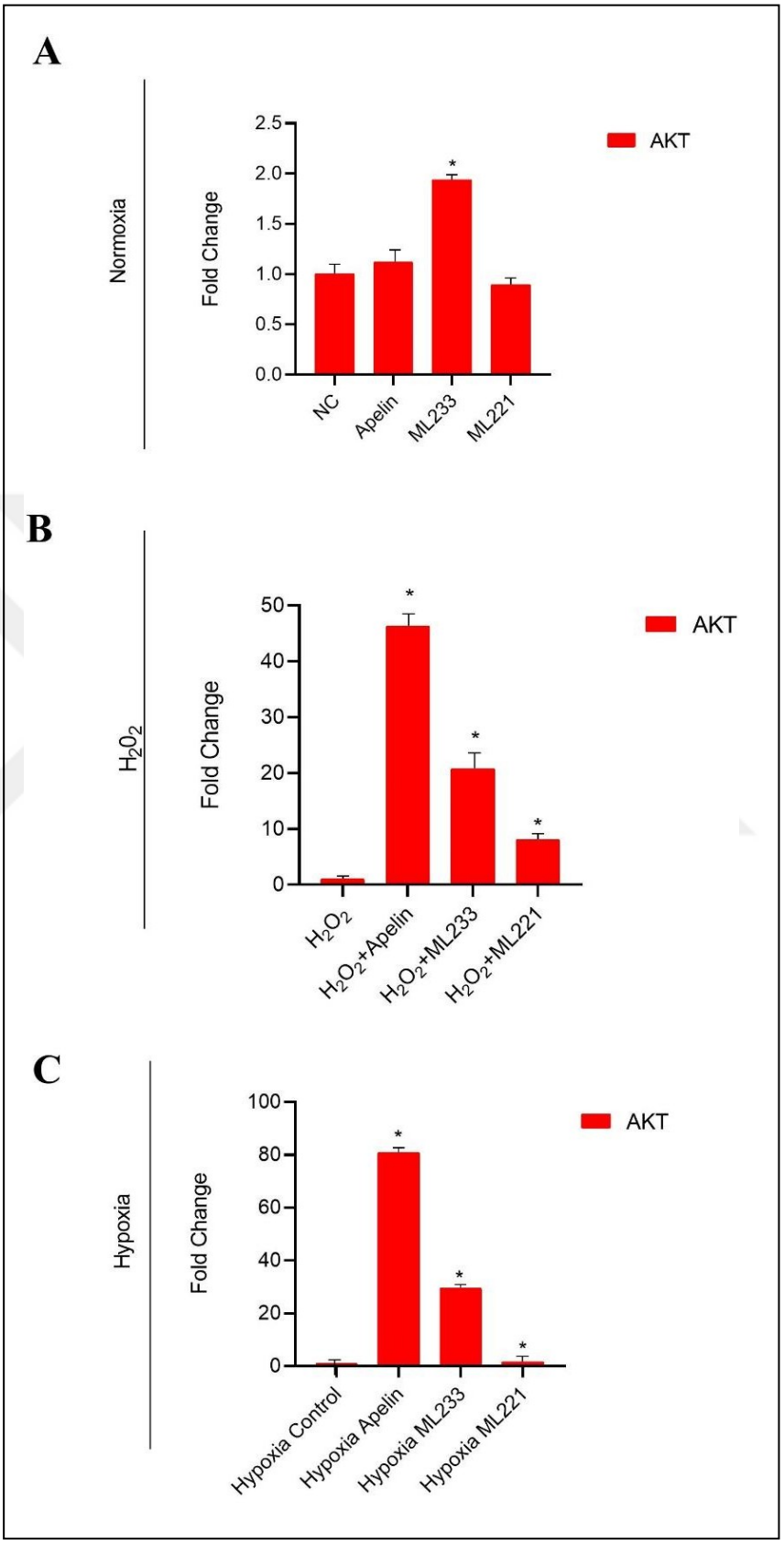


Figure 3.6. AKT gene expression profile of GT1-7 cells treated with Apelin, ML-233 and ML-221 in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p < 0.05$.

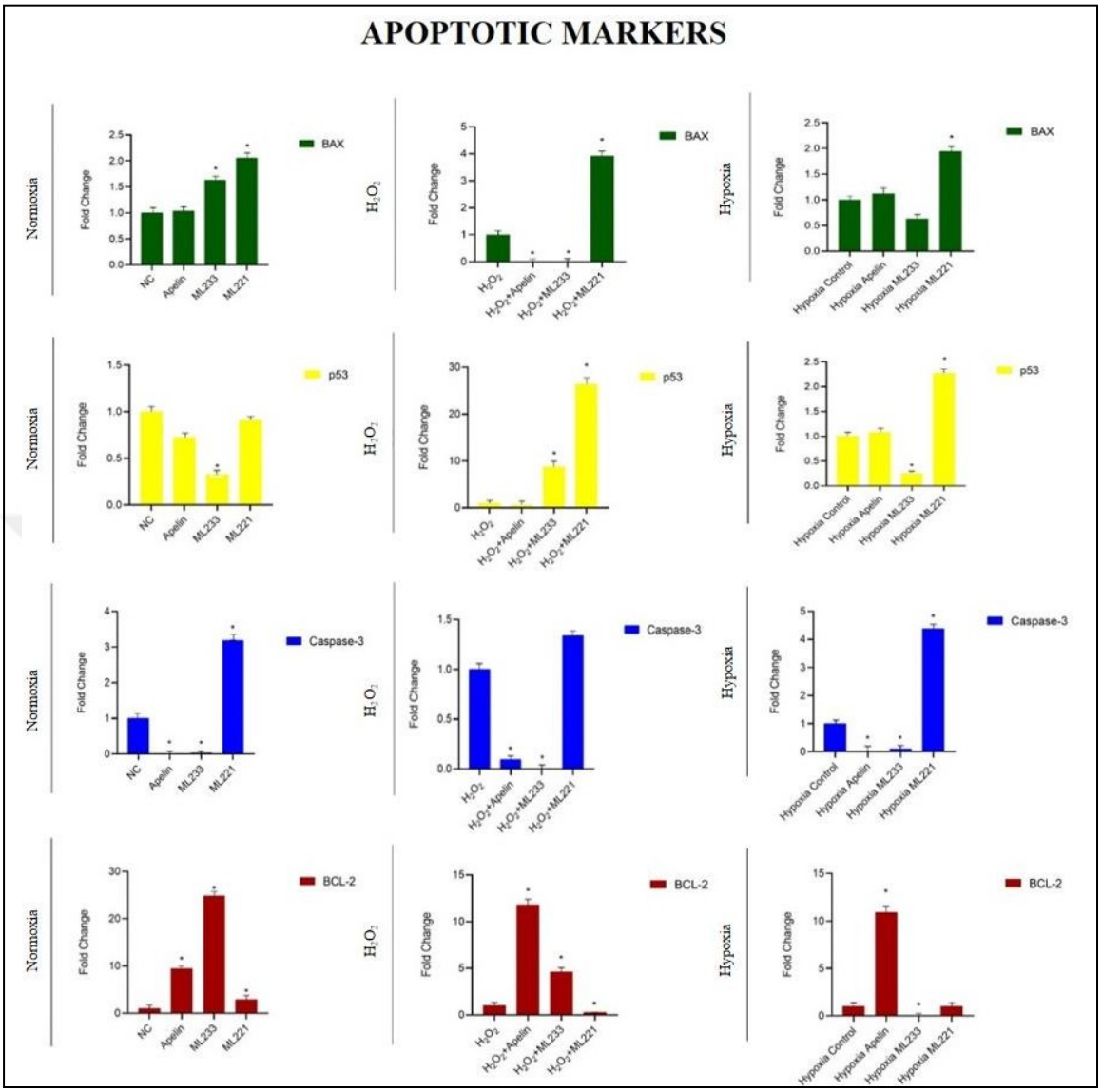


Figure 3.7. Apoptotic markers; BAX, p53, caspase-3 and BCL-2 gene expression analysis of GT1-7 cells treated with Apelin, ML-233 and ML-221 in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p < 0.05$.

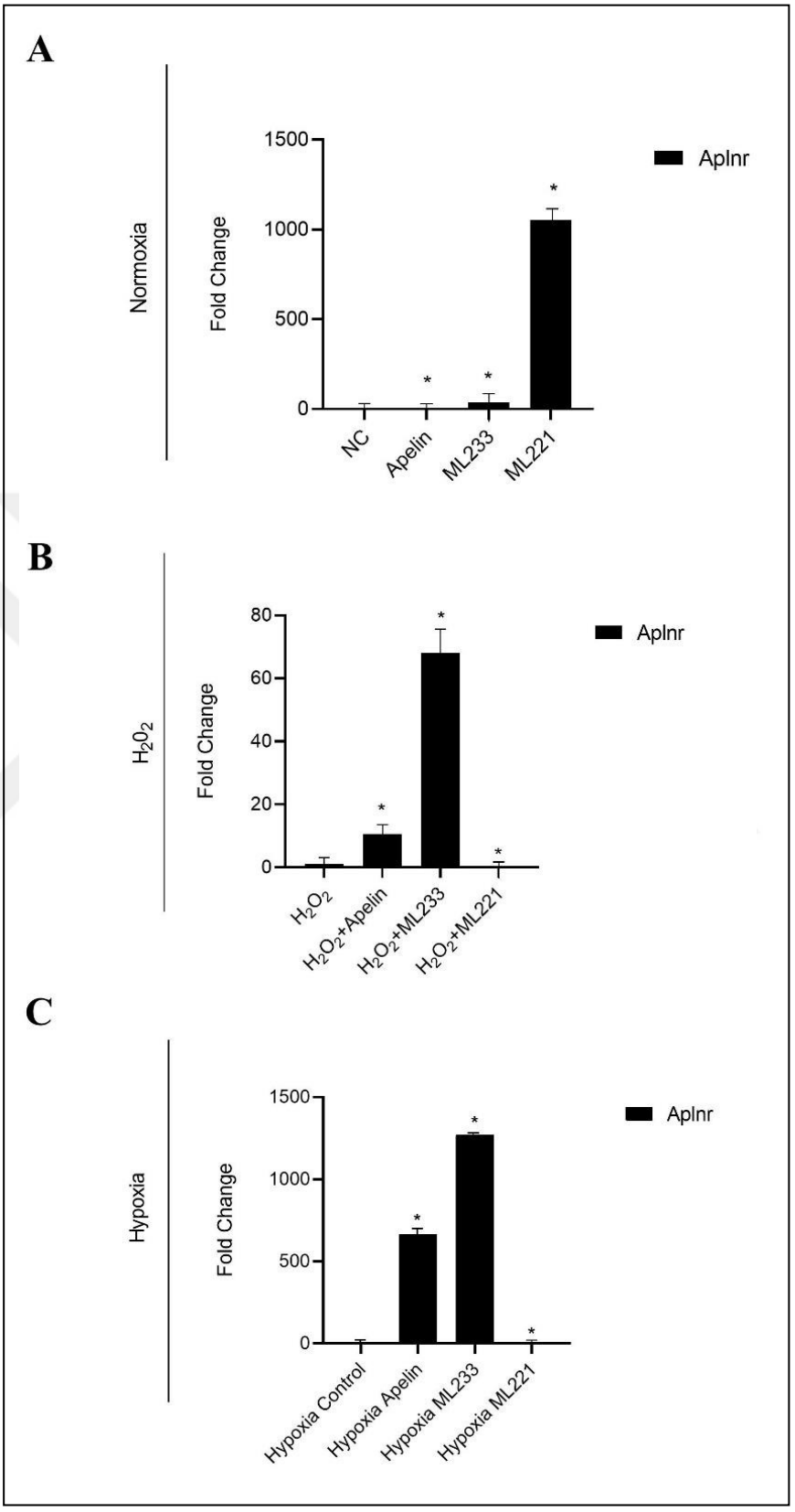


Figure 3.8. Aplnr gene expression profile of GT1-7 cells treated with Apelin, ML-233 and ML-221 in normoxia, $H_20_2$ and hypoxia induced cell conditions. * $p < 0.05$.

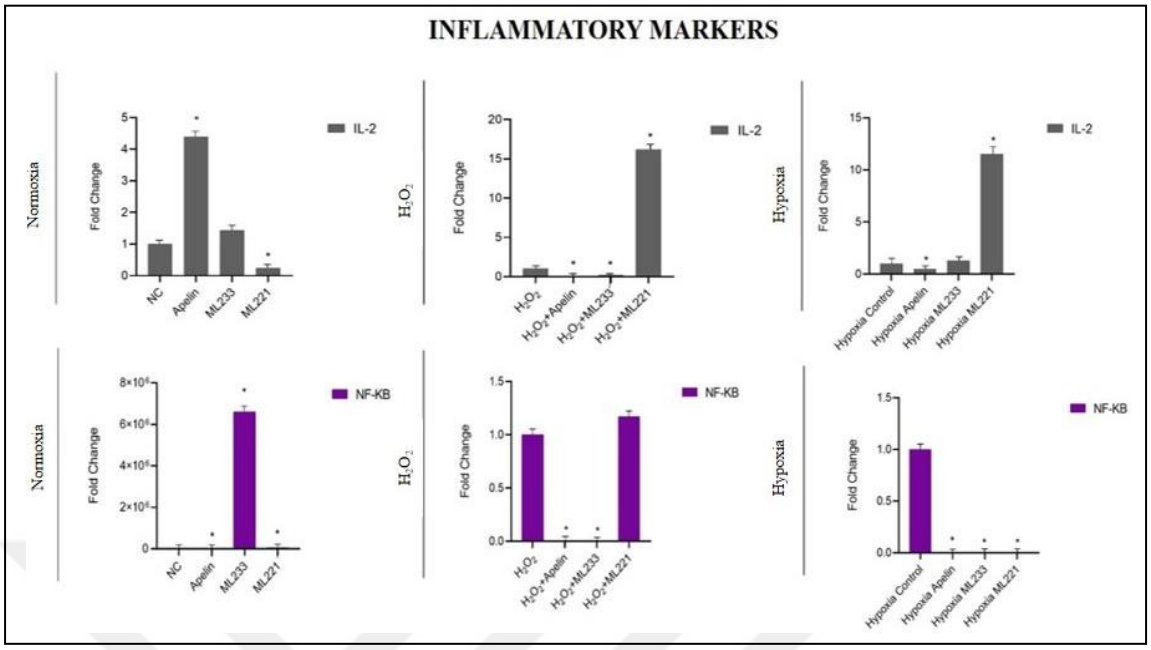


Figure 3.9. Inflammatory markers; IL-2, and NF-KB gene expression analysis of GT1-7 cells treated with Apelin, ML-233 and ML-221 in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p< 0.05$.

### 3.2.4. Oxidative Stress Enzyme Activity

As oxidative stress markers the activity of antioxidant enzymes including superoxide dismutase and glutathione peroxidase activity was detected in GT1-7 hypothalamic neurons treated with Apelin (1 μM), ML-233(1 μM) and ML-221 (0.5 μM) for 24 hours in normoxia (normal cell culture conditions), hypoxia (2 per cent $O_2$ and 5 per cent $CO_2$ levels with a temperature at $37^0$C) and $H_2O_2$ (200 μM) induced cell conditions. Results have shown that in normoxic conditions treating cells with Apelin (1 μM) for 24 hours increased SOD activity approximately to 55 per cent when compared to negative control. Nearly the same percentage (50 percent) was detected in ML-233 (1 μM) treated cells. However, pharmacological inhibition of Aplnr with ML-221 didn't cause any significant changes in SOD activity of the cells when compared to control (Figure 3.10).

In $H_2O_2$ (200 μM) administered cells, SOD activity was increased significantly to 55 percent when compared to negative control. However, treating cells together with $H_2O_2$ (200 μM) and Apelin (1 μM) caused decrease in SOD activity approximately to 36 per cent when compared to $H_2O_2$ (200 μM) treated group. $H_2O_2$ (200 μM) and ML-233 (1 μM) treatment

reduced SOD activity nearly to 40 per cent as opposed to cells treated only with $H_2O_2$ (200 μM). On the other hand, $H_2O_2$ (200 μM) and Aplnr antagonist, ML-221 (0.5 μM), administration caused no significant changes in SOD activity when compared to $H_2O_2$ (200 μM) treated group (Figure 3.10).

In hypoxic conditions treating cells with Apelin (1 μM) significantly increased SOD activity of the cells to nearly 50 per cent when compared to hypoxia control group. There were no significant changes in SOD activity found between Apelin (1 μM) and ML-233 (1 μM) treated cells in hypoxic conditions when compared to hypoxia control. ML-221 (0.5 μM) treatment caused no significant changes in SOD activity of the cells as opposed to the hypoxia control (Figure 3.10).

Glutathione peroxidase activity in normoxic conditions was significantly increased in cells treated with Apelin (1 μM) and ML-233 (1 μM) but caused decrease in ML-221 (0.5 μM) treated cells when compared to negative control (Figure 3.11).

In $H_2O_2$ (200 μM) induced cell conditions GPx activity was increased when compared to cells in negative control (normoxia). Treatment with $H_2O_2$ (200 μM) and Apelin (1 μM) caused significant increase in the catalytic activity of GPx when compared to cells treated only with $H_2O_2$ (200 μM). The same GPx activity levels were detected for $H_2O_2$ (200 μM) and ML-233 (1 μM) treated cells. However, $H_2O_2$ (200 μM) and ML-221 (0.5 μM) administration significantly decreased GPx activity levels when compared to $H_2O_2$ (200 μM) treated cells (Figure 3.11).

In hypoxic conditions GPx activity was found to be significantly increased in cells treated with Apelin (1 μM). The same GPx activity levels were detected for cells treated with ML-233 (1 μM). ML-221 (0.5 μM) administration in hypoxic conditions caused significant decrease in GPx activity when compared to hypoxia control group (Figure 3.11).

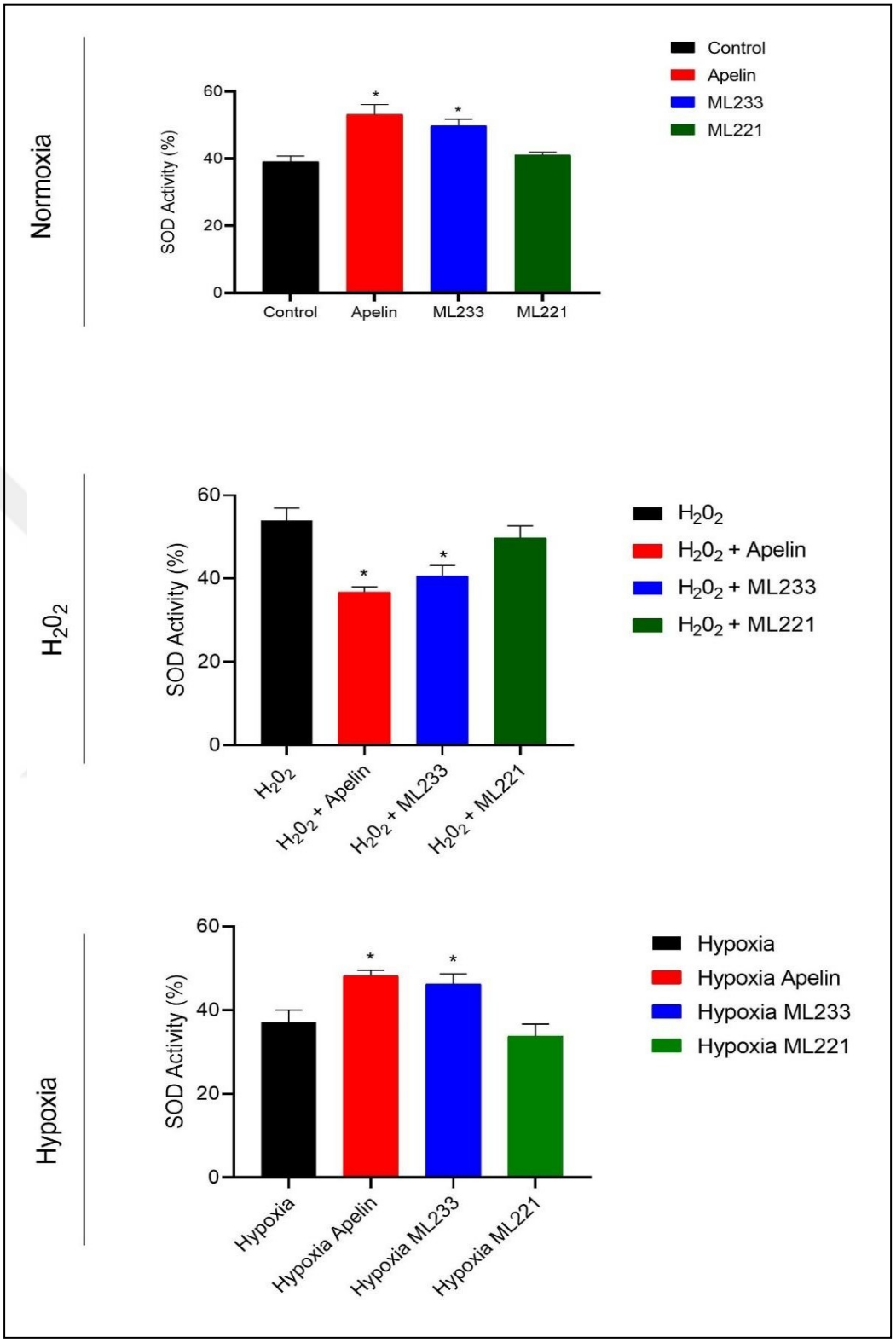


Figure 3.10. SOD activity of Apelin, ML233, and ML221 treated GT1-7 cells in normoxia, hypoxia and $H_2O_2$ applied cell culture conditions. * $p<0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1.

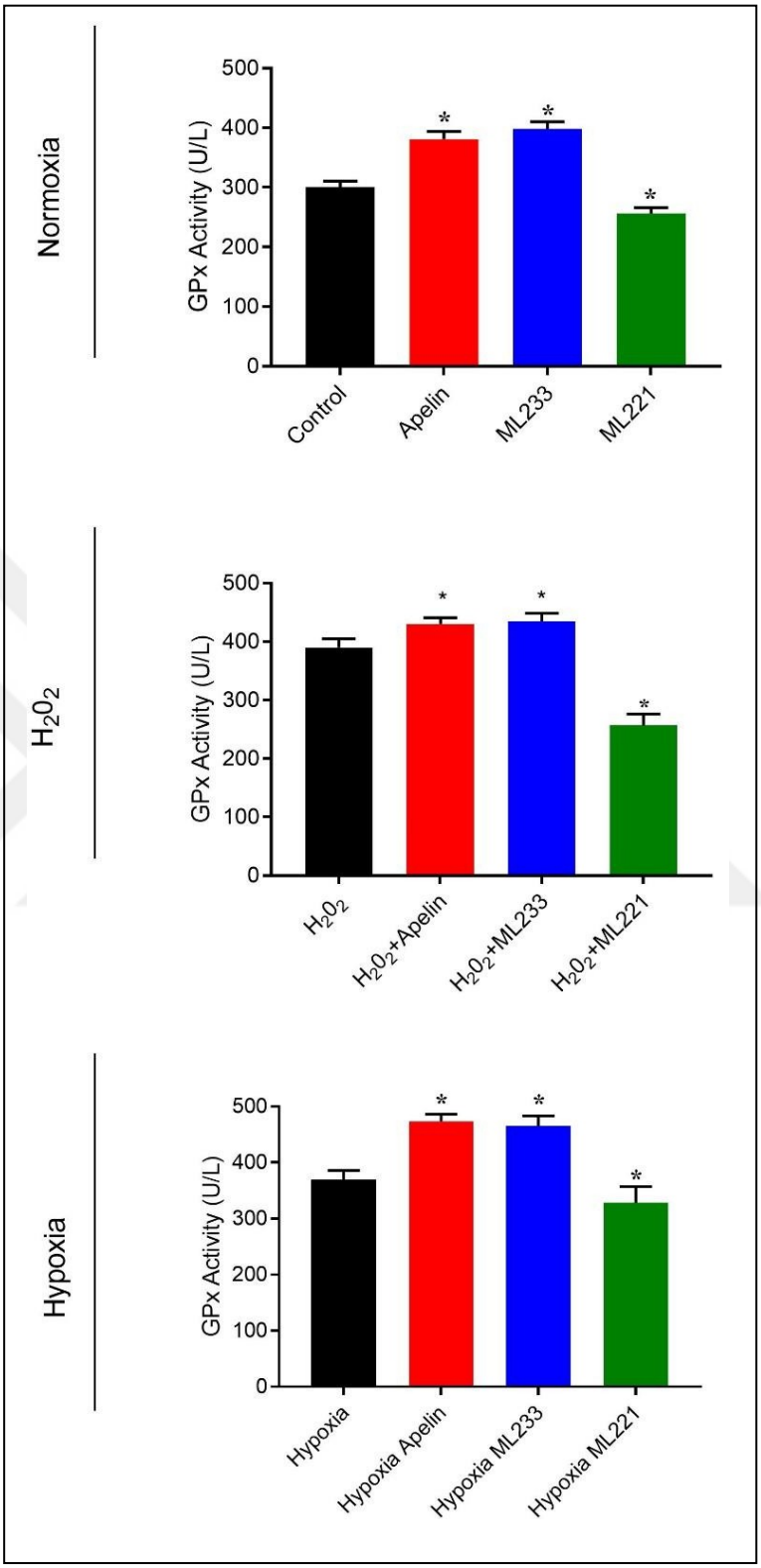


Figure 3.11. GPx activity of Apelin, ML233, and ML221 treated GT1-7 cells in normoxia, hypoxia and $H_2O_2$ applied cell culture conditions. U/L: units per liter. * $p<0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1.

### 3.2.5. Immunostaining Analysis

The protein expression level of GnRH and Aplnr was detected in GT1-7 cells treated with Apelin (1 μM), ML-233 (1 μM), and ML-221 (0.5 μM) in normoxia, $H_2O_2$ (200 μM) and hypoxia induced conditions by performing immunocytochemical analysis using antibodies against GnRH and Aplnr. The protein expression level of GnRH was significantly increased (1.5-fold) in cells treated with Apelin in normoxic conditions. Similar GnRH protein expression level (1.5-fold increase) was observed for ML-233 treated cells. However, inhibiting the activity of Aplnr with ML-221 significantly decreased GnRH protein levels (nearly 0.6-fold) in normoxic conditions. Aplnr protein expression levels increased with Apelin treatment in normoxic conditions approximately by 1.5-fold. The same protein expression level (nearly 1.5-fold) for Aplnr was detected in ML-233 treated cells. ML-221 treatment did not cause any significant changes in Aplnr protein levels (Figure 3.12).

In $H_2O_2$ induced oxidative stress conditions, Apelin treatment caused increase in GnRH protein levels with approximately 1.5-fold. ML-233 treated cells under $H_2O_2$ induced oxidative stress situation also significantly increased (nearly 1.5-fold) the protein expression levels of GnRH. However, inhibition of Aplnr with ML-221 caused no significant changes in GnRH protein expression levels. The protein level of Aplnr was increased approximately 1.5-fold in both Apelin and ML-233 treated cells. On the other hand, ML-221 treatment didn't cause any significant changes in Aplnr protein levels in $H_2O_2$ induced conditions (Figure 3.13).

In hypoxic conditions Apelin treated cells slightly increased the protein expression levels of GnRH. ML-233 treatment significantly increased GnRH protein levels. Inhibiting Aplnr with ML-221 caused no significant changes in GnRH protein expression. Aplnr protein expression levels increased with Apelin treatment in hypoxic conditions nearly by 1.5-fold. The similar protein expression level for Aplnr was detected in ML-233 treated cells. ML-221 treated cells had no significant changes in Aplnr protein levels (Figure 3.14).

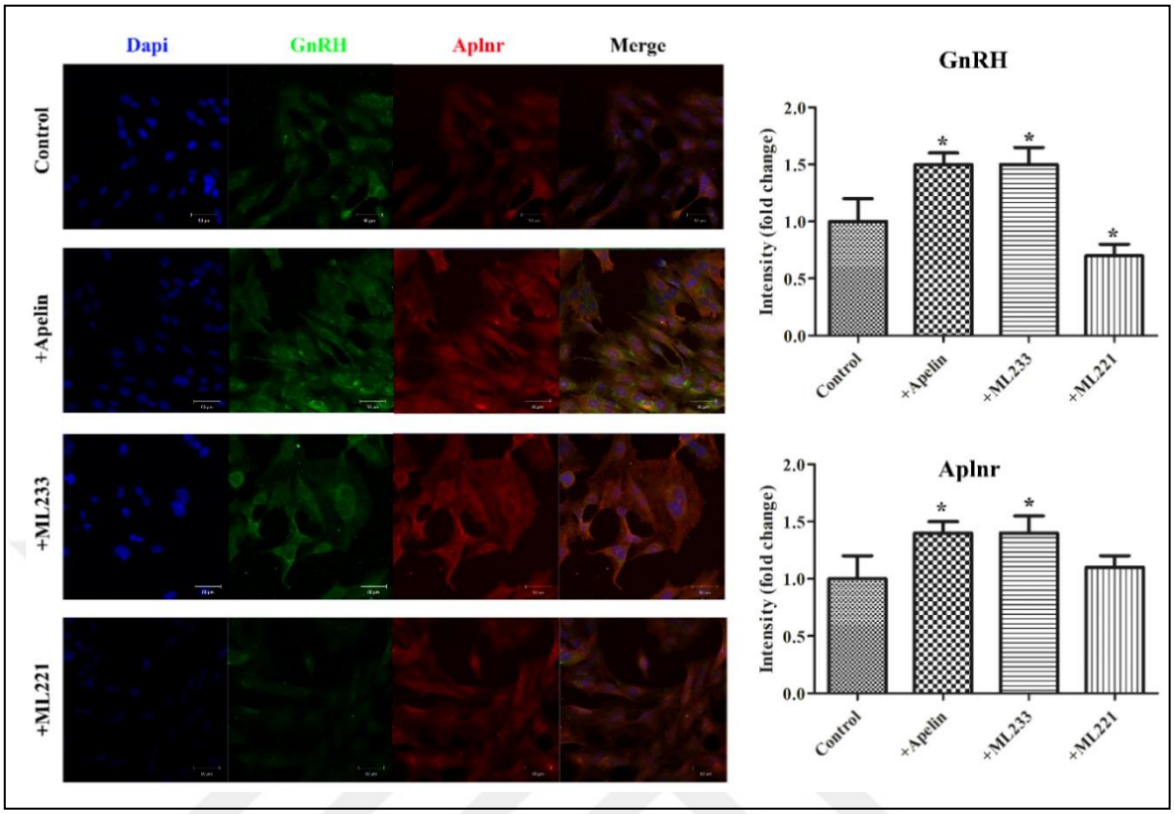


Figure 3.12. Immunocytochemical analysis of GT1-7 cells treated with Apelin, ML-233 and ML-221 in normoxic conditions. Aplnr and GnRH antibodies were used. The nuclei were stained with DAPI. The bar graphs show fold change of GnRH and Aplnr in Apelin, ML-233 and ML-221 treated cells. * $p < 0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1. Scale Bar: 50µm.

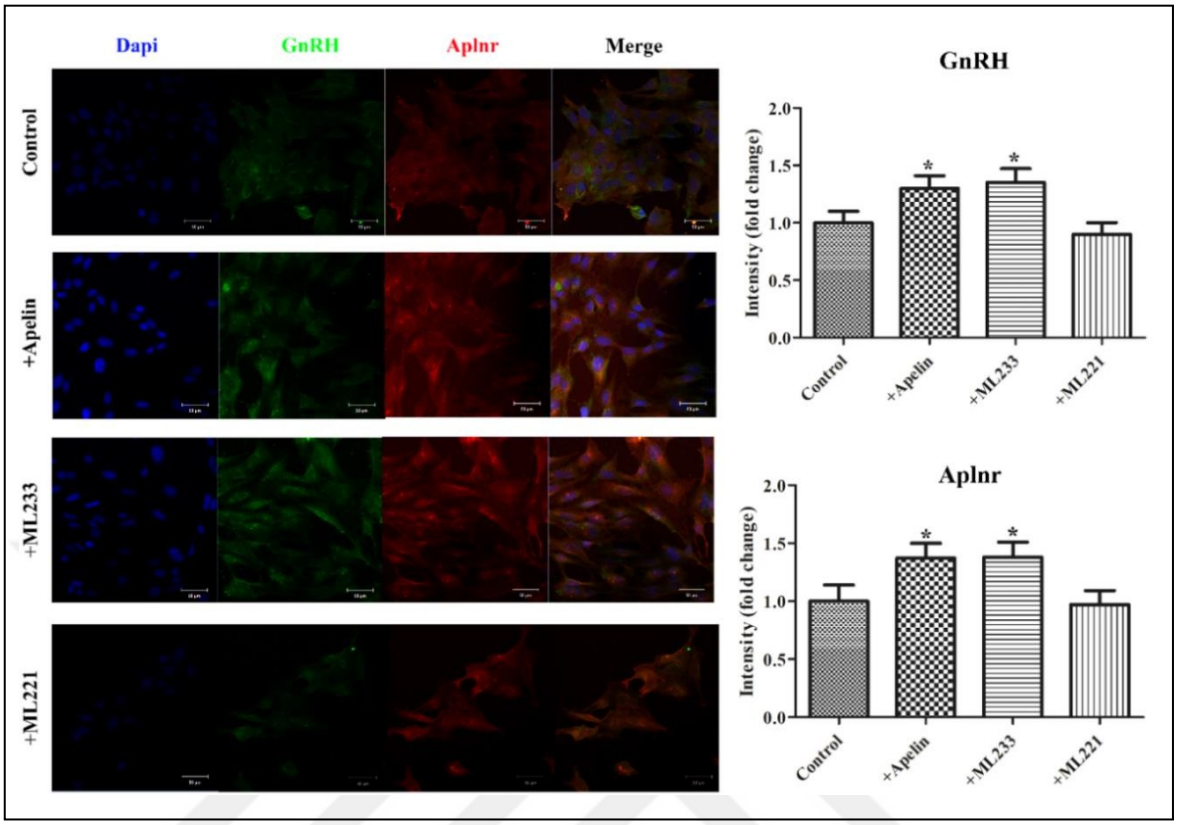


Figure 3.13. Immunocytochemical analysis of GT1-7 cells treated with Apelin, ML-233 and ML-221 in $H_2O_2$ induced conditions. Aplnr and GnRH antibodies were used. The nuclei were stained with DAPI. The bar graphs show fold change of GnRH and Aplnr in Apelin, ML-233 and ML-221 treated cells. * $p < 0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1. Scale Bar: 50µm.

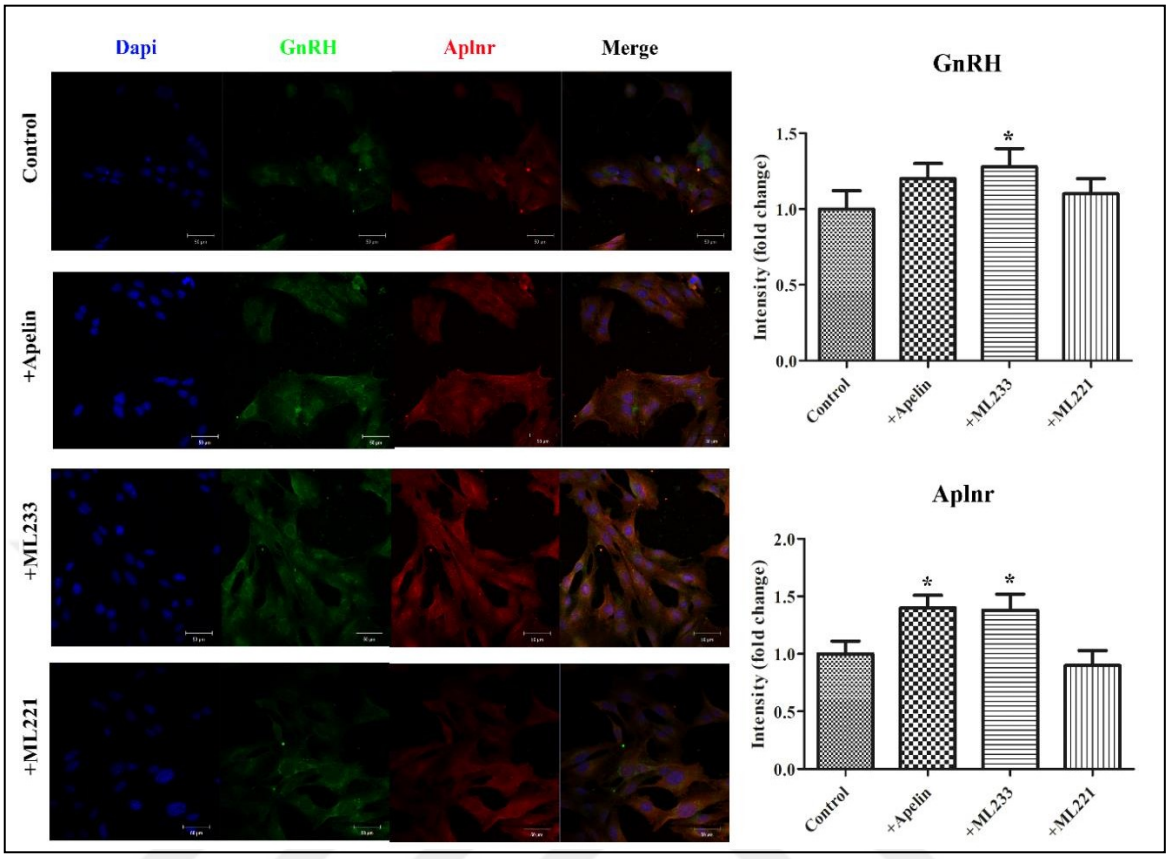


Figure 3.14. Immunostaining results of GT1-7 cells treated with Apelin, ML-233 and ML-221 under hypoxic conditions. Aplnr and GnRH antibodies were used. The nuclei were stained with DAPI. The bar graphs show fold change of GnRH and Aplnr in Apelin, ML-233 and ML-221 treated cells. * $p < 0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1. Scale Bar: 50µm.

## 3.3. TRANSIENT OVEREXPRESSION OF APLNR

### 3.3.1. Cell Viability and Morphological Analysis

The effect of Aplnr transient overexpression in viability of GT1-7 cells in normoxia, $H_2O_2$ (200 µM) and hypoxia induced conditions for 24 hours were determined with cell viability analysis. Aplnr overexpressed cells in normoxia had higher cell viability with 0.3 absorbance unit compared to control (pmax-GFP) group (0.2 absorbance unit). In $H_2O_2$ and hypoxia induced conditions Aplnr overexpressed GT1-7 cells had nearly 0.35 absorbance unit of increase in cell viability compared to their control (pmax-GFP) groups (Figure 3.15).

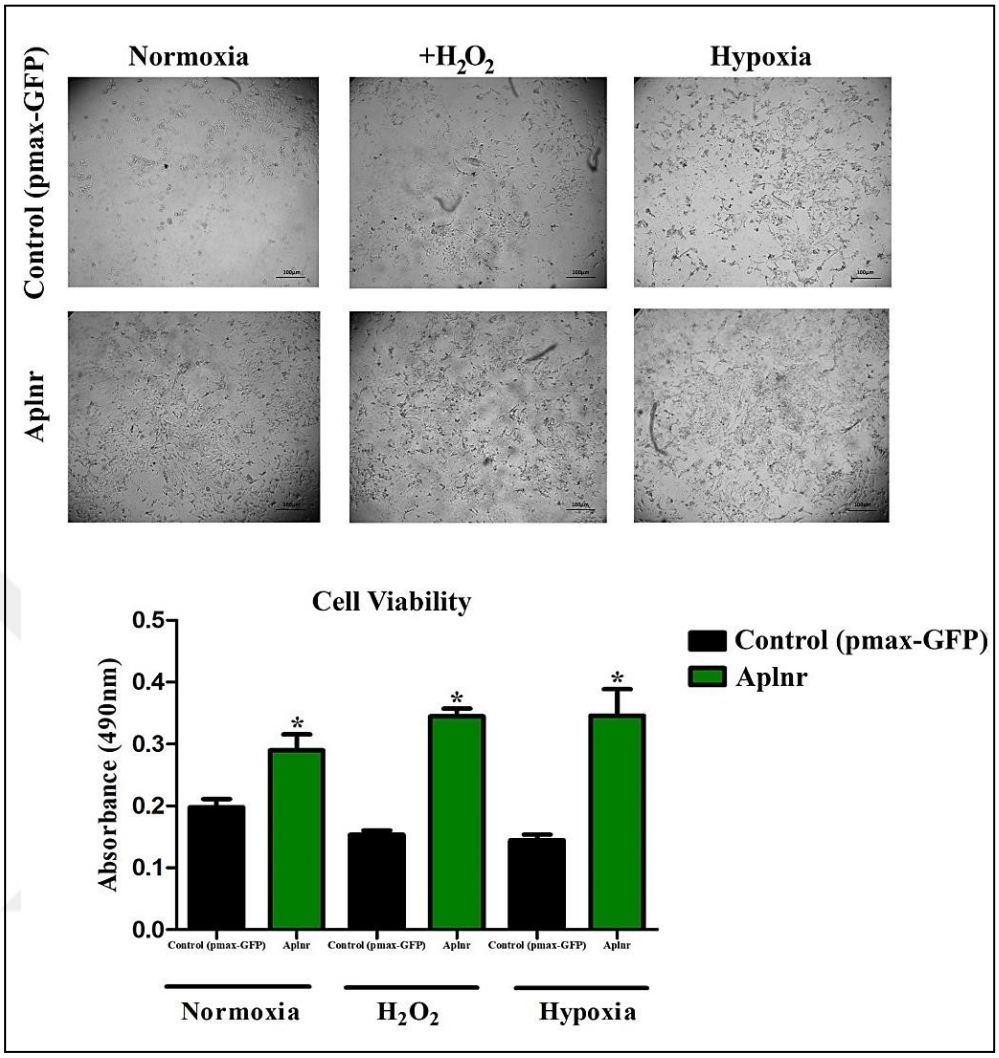


Figure 3.15. Cell viability of Aplnr transient overexpression in GT1-7 cells under normoxic, hypoxic and $H_20_2$ induced conditions. Light microscope images demonstrated the cellular morphology and cell numbers after Aplnr overexpression. Cell viability results were represented as absorbance measurements for comparison of all conditions at the same graph. * $p<0.05$. Scale bar: 100 µm.

### 3.3.2. Gene Expression Analysis

Gene expression analysis were performed with RT-qPCR in Aplnr overexpressed GT1-7 cells in normoxia and oxidative stress ($H_20_2$ and hypoxia) induced conditions. Overexpression of Aplnr caused significant increase in proliferation marker, Akt, gene expresion levels in normoxic conditions with nearly 3.5-fold. In $H_2O_2$ induced cell stress overexpression of Aplnr caused significant increase in Akt mRNA levels by 5-fold. Akt gene

expression levels were also significantly increased in Aplnr overexpressed cells under hypoxic conditions by 1.7-fold (Figure 3.16).

Aplnr overexpressed cells had no significant changes in BAX gene expression levels in normoxia. In $H_2O_2$ induced conditions overexpressing Aplnr resulted in 0.7-fold change in BAX gene expression levels. However, in hypoxic conditions BAX mRNA levels were significantly decreased by 0.4-fold in Aplnr overexpressed cells (Figure 3.17).

Overexpression of Aplnr caused a significant increase in p53 mRNA levels by approximately 6-fold in normoxia. However, p53 mRNA levels in Aplnr overexpressed cells in $H_2O_2$ induced conditions significantly decreased with nearly 0.5-fold change. Overexpression of Aplnr in hypoxic conditions decreased p53 gene expression levels by 0.3-fold (Figure 3.17).

Overexpression of Aplnr significantly increased anti-apoptotic marker, BCL-2, mRNA levels by 5.6-fold. In $H_2O_2$ induced conditions Aplnr overexpression caused significant increase by 20000-fold. Aplnr overexpression also resulted in 110-fold change of significant increase in BCL-2 mRNA levels under hypoxic conditions (Figure 3.17).

Aplnr mRNA levels were increased by 17-fold in Aplnr overexpressed cells in normoxic conditions. In $H_2O_2$ induced conditions, overexpressing Aplnr significantly increased Aplnr mRNA levels by nearly 8.4-fold. Overexpression of Aplnr in hypoxic conditions caused significant increase in Aplnr mRNA levels by 19-fold (Figure 3.18).

Apelin mRNA levels were significantly decreased in Aplnr overexpressed cells by 0.4-fold in normoxia. However, Aplnr overexpression caused Apelin gene expression levels to increase by 8-fold in $H_20_2$ induced conditions. Also, overexpression of Aplnr caused 2.2-fold change of increase in Apelin mRNA levels under hypoxic conditions (Figure 3.18).

IL-2 gene expression levels resulted in 1.2-fold change in Aplnr overexpressed cells under normoxic conditions. However, in $H_2O_2$ induced oxidative stress conditions Aplnr overexpressed cells significantly decreased IL-2 gene expression levels by 0.2-fold. On the other hand, overexpression of Aplnr in hypoxic conditions caused no significant changes (0.9-fold) in IL-2 mRNA levels (Figure 3.19).

As an inflammatory marker, NF-KB, mRNA levels were significantly increased in Aplnr overexpressed cells by nearly 3.5-fold in normoxic conditions. The gene expression levels

of NF-KB also increased significantly in $H_2O_2$ (4.5-fold) and hypoxia (1.8-fold) induced conditions (Figure 3.19).

Inflammation marker, COX-2, gene expression levels were significantly decreased (0.04-fold) in Aplnr overexpressed cells in normoxia. However, overexpressing Aplnr in $H_20_2$ induced conditions caused COX-2 levels to increase by 48-fold. On the other hand, COX-2 mRNA levels were significantly decreased (0.09-fold) in Aplnr overexpressed cells under hypoxic conditions (Figure 3.19).

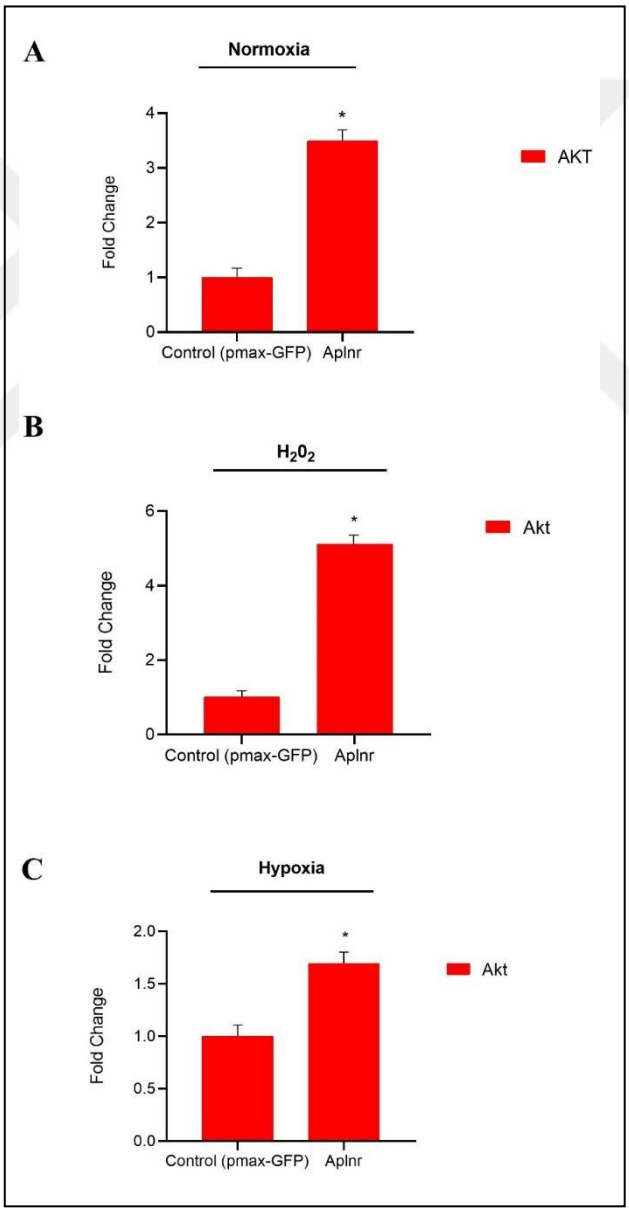


Figure 3.16. Akt gene expression profile of Aplnr overexpressed GT1-7 cells in normoxia, $H_20_2$ and hypoxia induced conditions. * $p<0.05$.

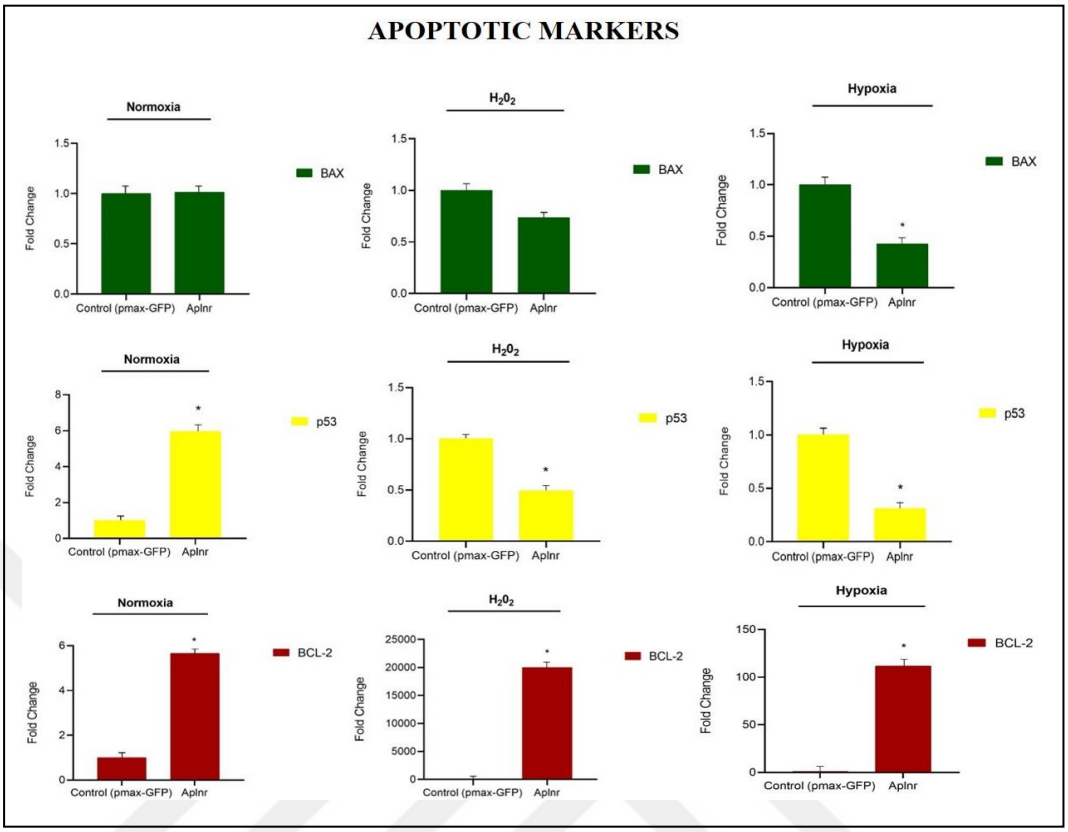


Figure 3.17. Apoptotic markers; BAX, p53 and BCL-2 gene expression profile of Aplnr overexpressed GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced conditions. * $p<0.05$.

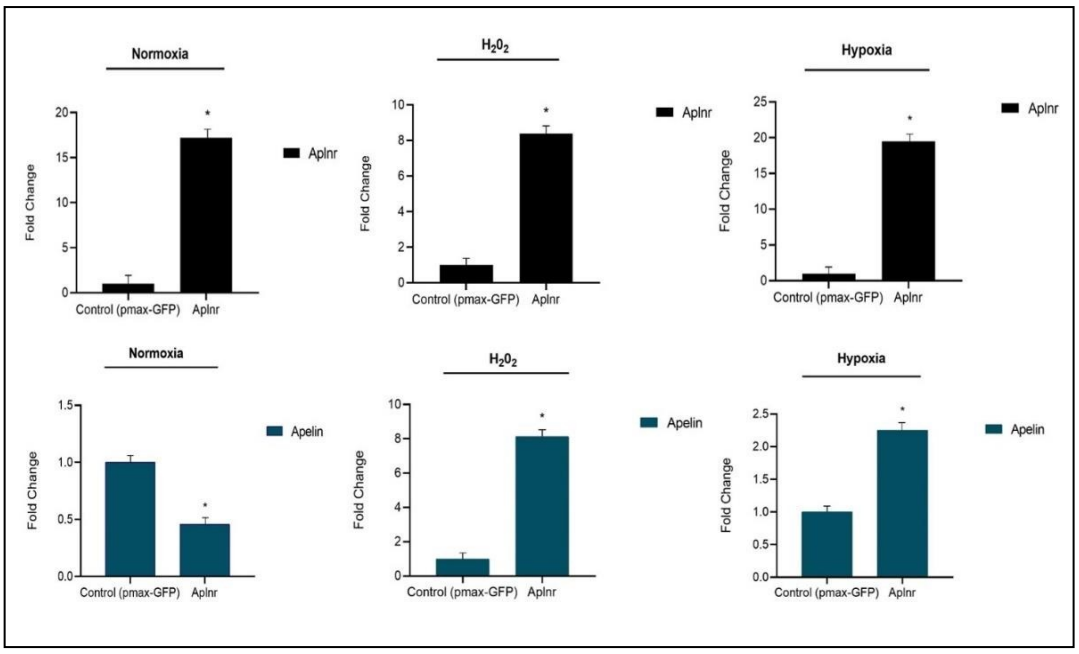


Figure 3.18. Aplnr and apelin gene expression profile of Aplnr overexpressed GT1-7 cells in normoxic, $H_2O_2$ and hypoxia induced conditions. * $p<0.05$.

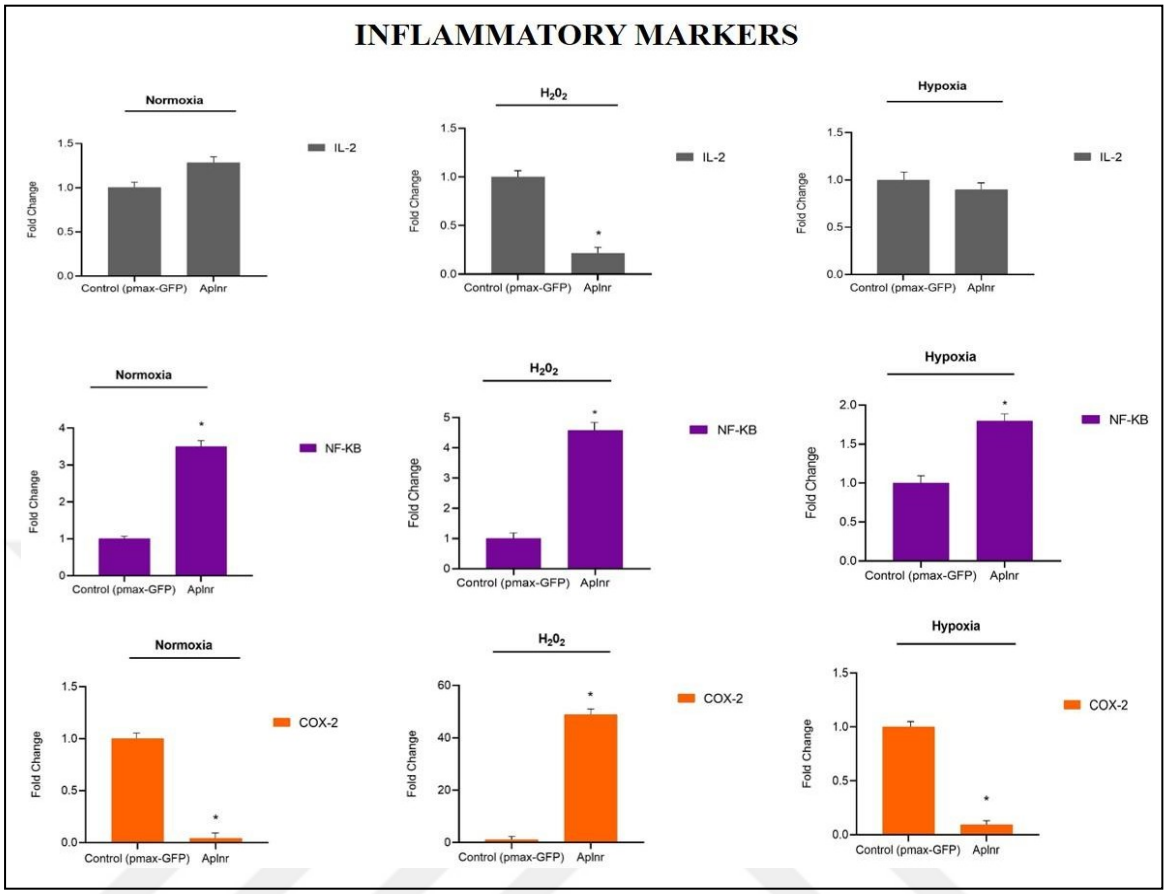


Figure 3.19. Inflammatory markers; IL-2, NF-KB, and COX-2 gene expression profile of Aplnr overexpressed GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced conditions. * $p<0.05$.

### 3.3.3. Immunostaining Analysis

The protein expression level of GnRH and Aplnr was detected in Aplnr transient overexpressed GT1-7 cells in normoxia, $H_2O_2$ (200 µM) and hypoxia induced cell culture conditions by performing immunocytochemical analysis using antibodies against GnRH and Aplnr. Transient overexpression of Aplnr in GT1-7 cells increased the protein expression levels of GnRH and Aplnr in normoxic conditions. In oxidative stress ($H_2O_2$ and hypoxia) induced conditions, overexpression of Aplnr in GT1-7 cells increased GnRH and Aplnr protein levels compared to their control (empty vector) groups (Figure 3.20).

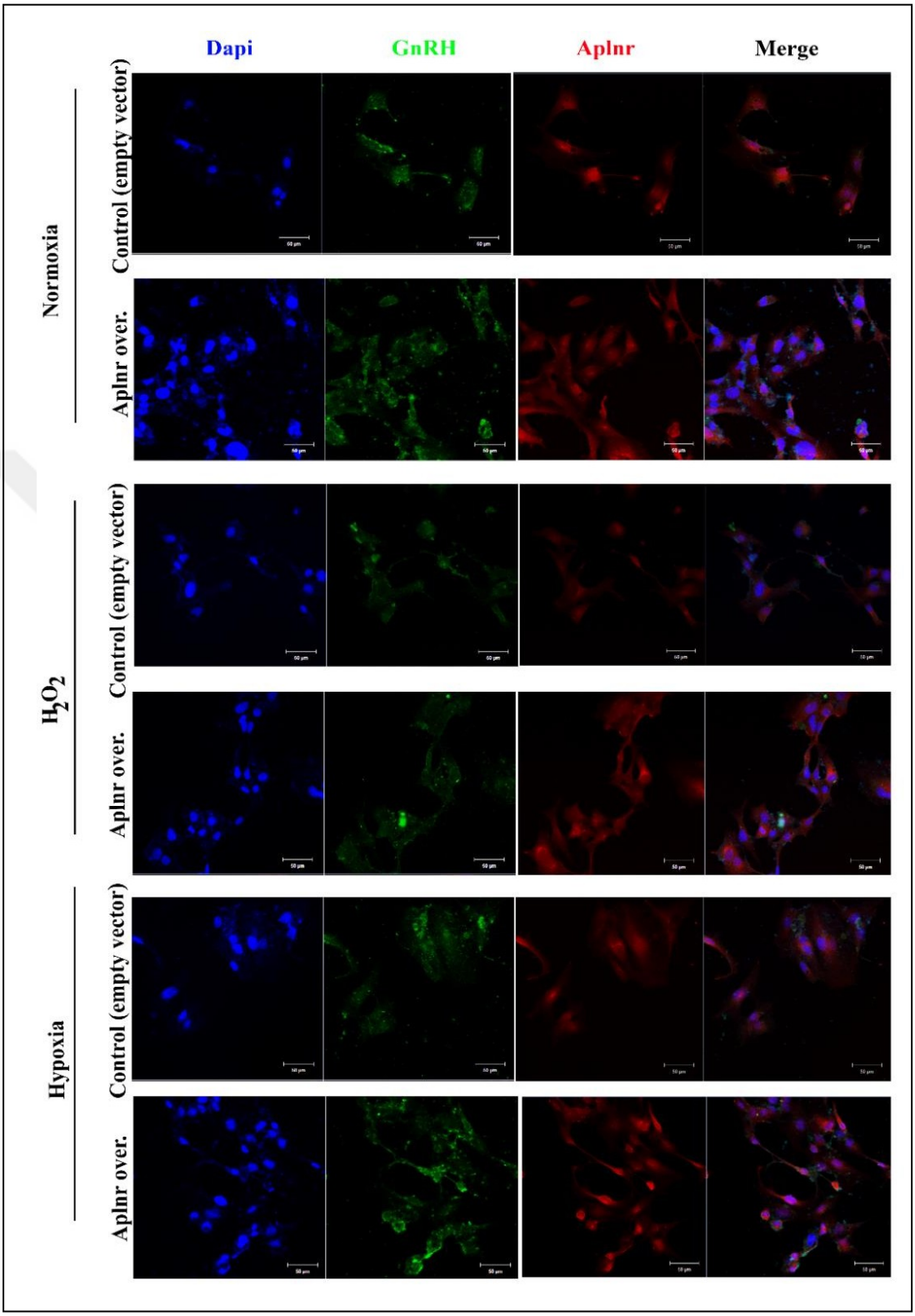


Figure 3.20. Immunostaining results of Aplnr transient overexpression in GT1-7 cells in normoxia, $H_20_2$ and hypoxia induced conditions. Aplnr and GnRH antibodies were used. The nuclei were stained with DAPI. Empty vector was used as control for transfection. Scale Bar: 50µm.

## 3.4. DOWNREGULATION OF APLNR SIGNALING

### 3.4.1. Cell Proliferation Analysis

Cell viability analysis of siRNA application (Control siRNA or siAplnr) on GT1-7 cells after 24 h treatment with $H_2O_2$ (200 µM) was determined by performing cell proliferation analysis. Results have shown that siAplnr treatment significantly decreased viable cell number from 25 x $10^3$ to 5 x $10^3$ when compared to control siRNA in normoxic condition. In $H_2O_2$ treated Aplnr siRNA group, viable cell numbers were decreased from 7,5 x $10^3$ to 5 x $10^3$ (Figure 3.21).

Effect of hypoxic condition on cell viability in siRNA (Control siRNA or siAplnr) applied GT1-7 cells was also determined by a cell proliferation assay. Down regulation of Aplnr in hypoxic conditions significantly decreased viable number of cells from approximately 22 x $10^3$ to 8 x $10^3$ cells (Figure 3.21).

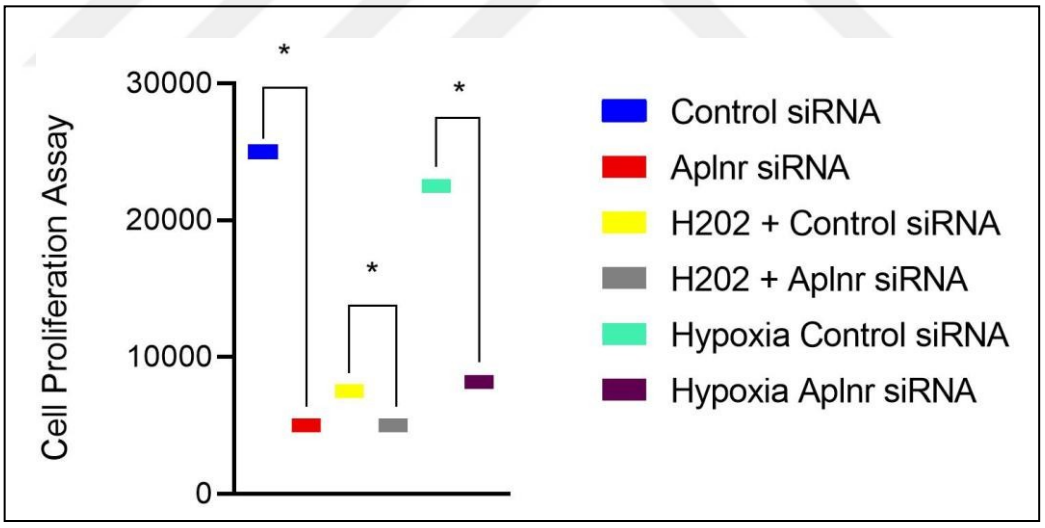


Figure 3.21. Effect of Aplnr downregulation on proliferation of GT1-7 cells in normoxia, hypoxia and $H_2O_2$ induced cell conditions. * $p<0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1.

### 3.4.2. Cellular Morphology Analysis

Light microscopy images were taken for siRNA transfected cells (Control siRNA and siAplnr) after 24 h treatment with $H_2O_2$ (200 μM) to visualize and detect differences in cellular morphology. Results have indicated that transfecting GT1-7 cells with siAplnr decreased cell viability when compared to control siRNA as confirmed by cell proliferation analysis. Treating Aplnr siRNA transfected cells with $H_2O_2$, decreased viable number of cells compared to baseline which is also confirmed by counting viable number of cells in proliferation assay (Figure 3.22).

Effect of hypoxia on siRNA (Control siRNA or siAplnr) transfected cells' morphology was also observed in GT1-7 cells by using light microscope. Results have shown that siAplnr treatment in hypoxic conditions significantly decreased cell viability as opposed to control group (hypoxia plus control siRNA treatment) (Figure 3.22).

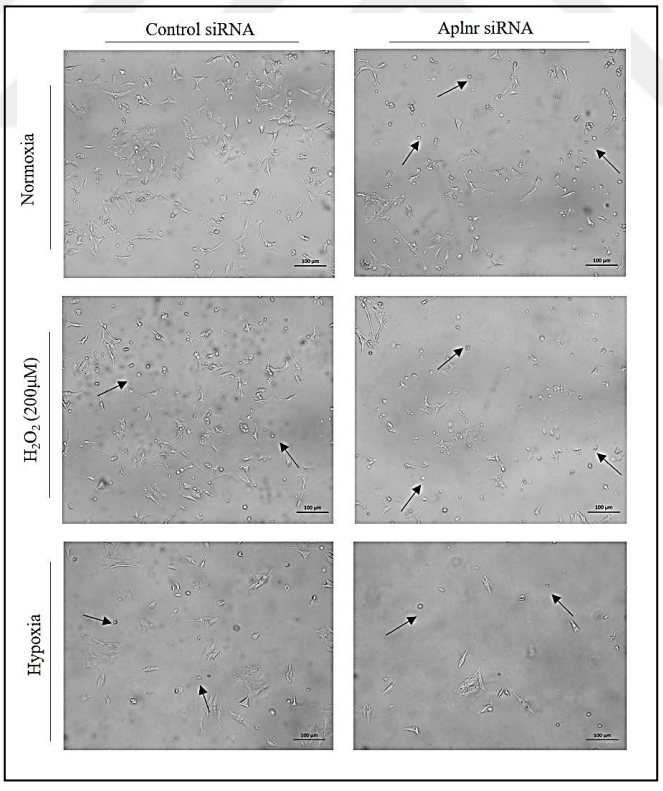


Figure 3.22. Cellular morphology of siRNA (control siRNA or siAplnr) transfected cells with 24 hours of normoxia, $H_2O_2$ and hypoxia induced conditions. Dead cells are indicated with the black arrow. Light microscopy images are represented as one hundred times magnified. Scale bar: 100 μm.

### 3.4.3. Gene Expression Analysis

The effect control siRNA or siAplnr application in GT1-7 cells in normoxia, hypoxia and $H_20_2$ induced cell stress conditions on gene expression profile was analysed with RT-qPCR analysis. Statistical results of gene expression analysis were obtained by performing one way ANOVA and Tukey's test. P values smaller than 0.05 were considered as significant. All fold changes are compared with group's negative control. Downregulating Aplnr signaling with siAplnr in normoxic conditions had significantly decreased Akt gene expression level to 0.31 fold. Aplnr siRNA transfected cells exposed to $H_20_2$ had 1.53-fold change in Akt gene expression levels. Knocking down of Aplnr with siAplnr in hypoxic conditions caused a slight decrease in Akt gene expression levels with 0.82-fold (Figure 3.23).

The gene expression levels of apoptotic marker,BAX, showed 1.13 fold change of increase in GT1-7 cells transfected with siAplnr. In $H_20_2$ induced cell conditions knocking down Aplnr caused BAX gene expression levels to increase by 1.5 fold. Hypoxia induced Aplnr siRNA treated cells didn't cause any significant changes in BAX mRNA levels (Figure 3.24).

Caspase-3 gene expression levels were dramatically increased by 1.7 fold in Aplnr siRNA treated cells. Aplnr siRNA transfected cells exposed to $H_20_2$ caused a slight fold change with 1.22 in caspase-3 mRNA levels. Knocking down Aplnr via siAplnr in hypoxia induced conditions caused a significant increase in caspase-3 gene expression levels by 2.5 fold (Figure 3.24).

The mRNA levels of p53 increased by 3.7-fold in Aplnr siRNA transfected cells in normoxic conditions. $H_20_2$ treated Aplnr siRNA group had fold change of 0.7-fold in p53 gene expression levels. Aplnr siRNA treated cells in hypoxic conditions had no significant changes (0.8-fold) in p53 mRNA levels (Figure 3.24).

BCL-2, an anti-apoptotic marker, mRNA levels were slightly increased in siAplnr treated cells in normoxic conditions by 1.2-fold. However, BCL-2 gene expression levels were decreased in cells transfected with Aplnr siRNA in $H_20_2$ induced conditions by 0.5-fold. The gene expression level of BCL-2 was found to slightly decrease in Aplnr siRNA treated cells in hypoxic conditions by 0.7 fold (Figure 3.24).

As a pro-inflammatory marker, NF-KB, gene expression levels were increased dramatically by 3.2 fold in cells transfected with siAplnr. In Aplnr siRNA transfected cells exposed to $H_20_2$, NF-KB mRNA levels were decreased by 0.8-fold. However, siAplnr applied cells in hypoxic conditions increased NF-KB gene expression levels by 1.2-fold (Figure 3.25).

IL-2 gene expression levels were significantly increased by 1.8 fold in Aplnr knocked down cells. In $H_20_2$ induced cell stress conditions transfecting cells with Aplnr siRNA resulted in 1.5 fold of increase in IL-2 mRNA levels. siAplnr treated cells in hypoxic conditions had higher fold change in IL-2 gene expression levels with 36-fold (Figure 3.25).

Aplnr gene expression levels were decreased in cells transfected with siAplnr by 0.6-fold. Cells treated with Aplnr siRNA in $H_20_2$ induced conditions caused 0.1-fold change of decrease in Aplnr mRNA levels. The gene expression level of Aplnr was decreased in Aplnr siRNA transfected cells under hypoxic conditions by 0.85-fold change, respectively (Figure 3.26).

Insterestingly, Apelin mRNA levels in Aplnr knocked down cells were increased by 2.9-fold. In in $H_20_2$ induced conditions cells transfected with siAplnr had non-significant 1.1-fold change in Apelin mRNA levels. On the other hand, under hypoxic conditions Aplnr siRNA treated cells had 1.38-fold change in Apelin gene expression levels. (Figure 3.26).

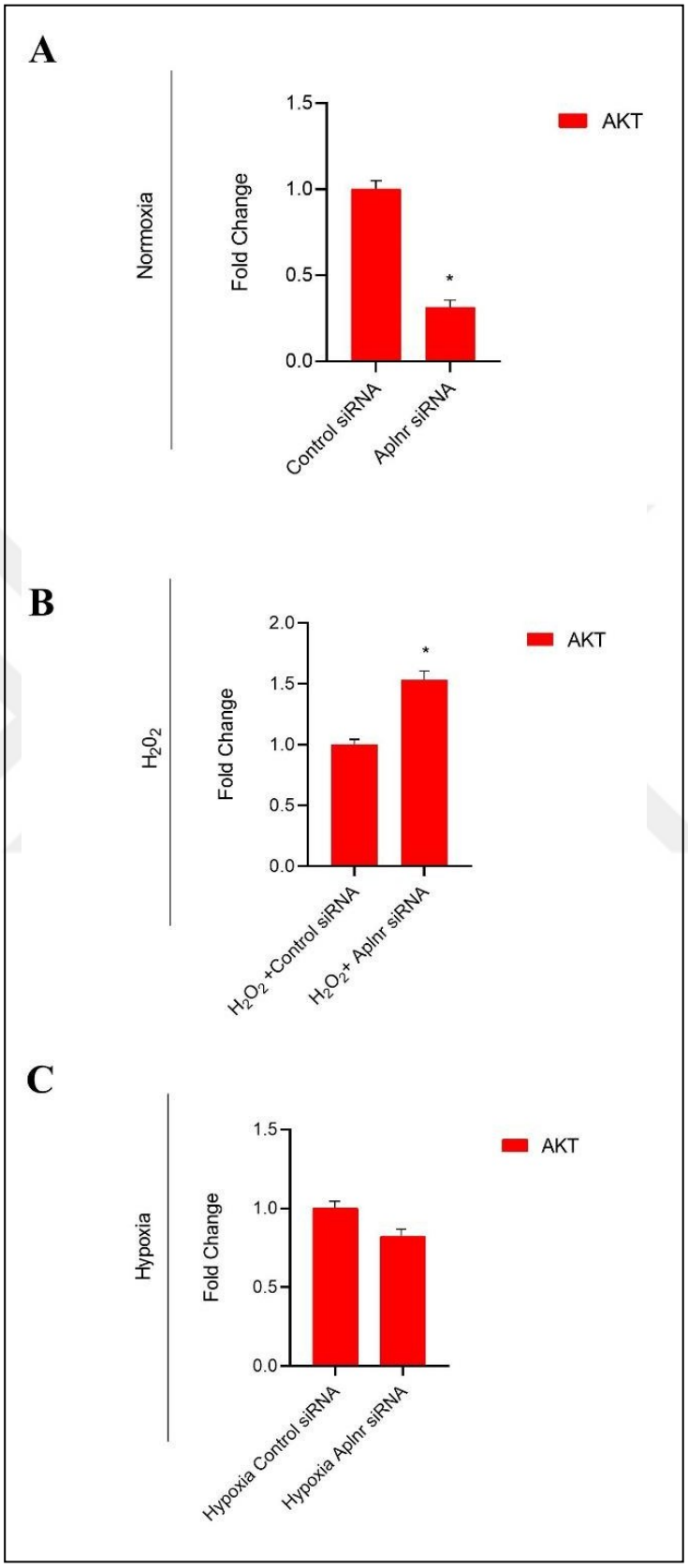


Figure 3.23. AKT gene expression profile of control siRNA or Aplnr siRNA transfected GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p < 0.05$.

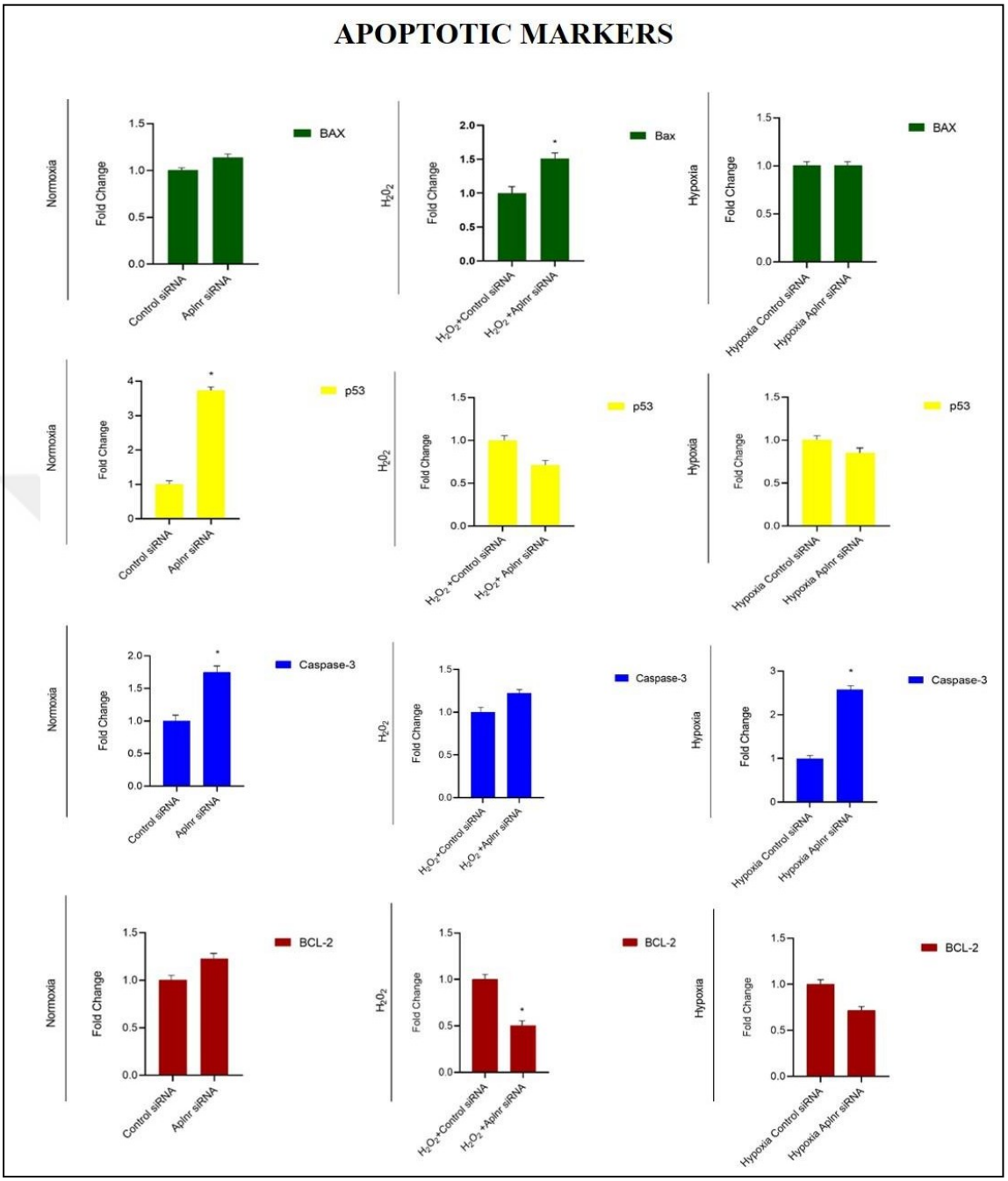


Figure 3.24. Apoptotic markers; BAX, p53, caspase-3, BCL-2 gene expression analysis of control siRNA or Aplnr siRNA transfected GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p < 0.05$.

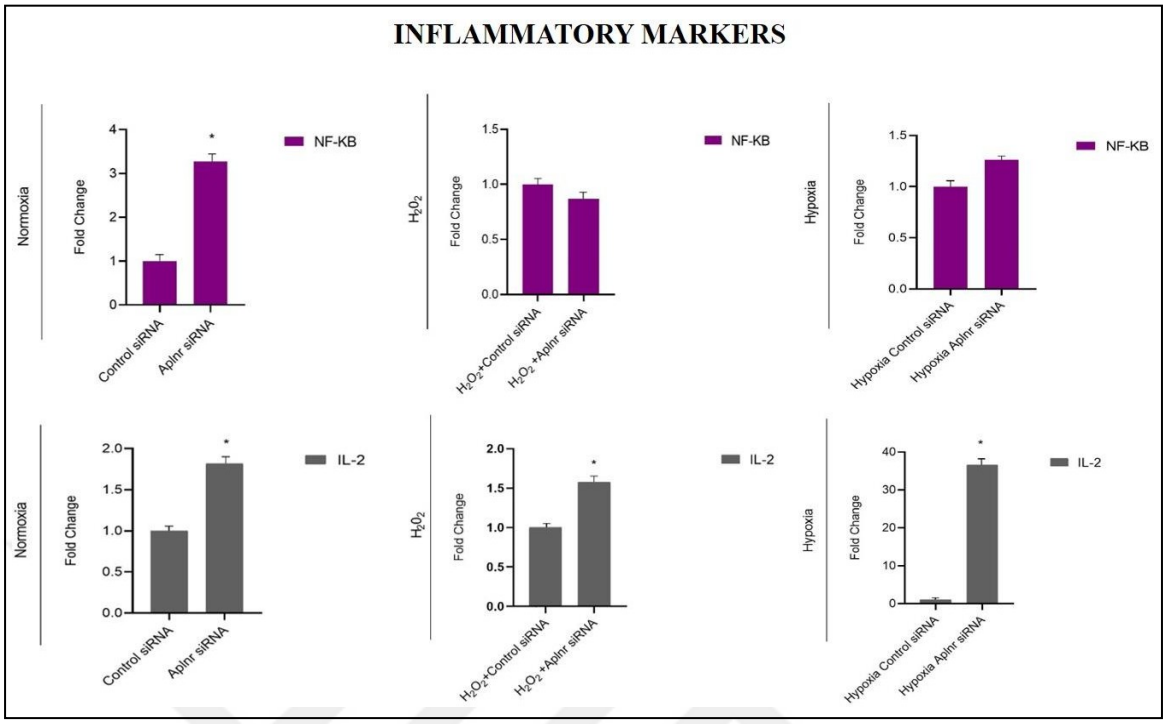


Figure 3.25. Inflammatory markers; NF-KB and IL-2 gene expression levels of control siRNA or Aplnr siRNA transfected GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p < 0.05$.

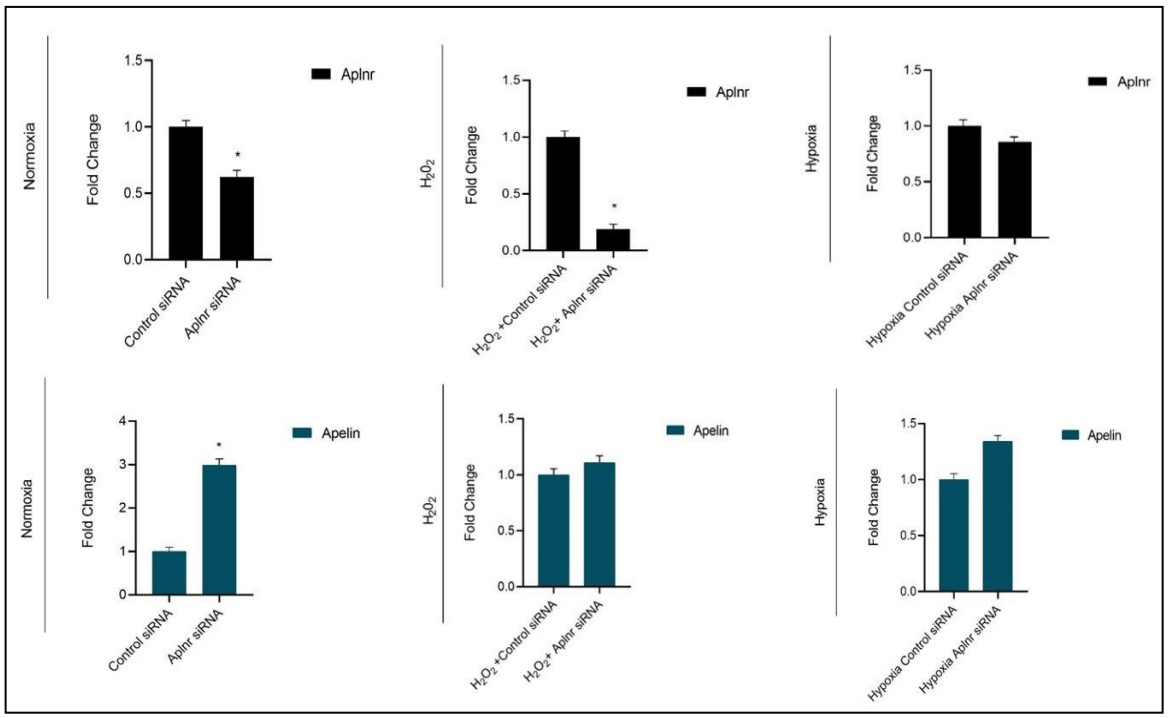


Figure 3.26. Aplnr and Apelin gene expression profile of control siRNA or Aplnr siRNA transfected GT1-7 cells in normoxia, $H_2O_2$ and hypoxia induced cell conditions. * $p < 0.05$.

### 3.4.4. Oxidative Stress Enzyme Activity

The enzyme activity of antioxidants; superoxide dismutase and glutathione peroxidase was determined in GT1-7 hypothalamic neurons transfected with control siRNA or Aplnr siRNA in normoxia (normal cell culture conditions), hypoxia (2 per cent $O_2$ and 5 per cent $CO_2$ levels with a temperature at $37^0$C) and $H_2O_2$ (200 µM) induced cell conditions. Results have shown that knocking down Aplnr with siRNA application caused significant decrease (approximately from 40 per cent to 30 per cent) in SOD activity when compared to control siRNA group. $H_2O_2$ (200 µM) treatment in cells transfected with Aplnr siRNA resulted in approximately 40 per cent SOD activity. In hypoxic conditions Aplnr knock down caused significant decrease (from nearly 45 per cent to 30 per cent) in SOD activity levels when compared to hypoxia control siRNA group (Figure 3.27).

The catalytic activity of GPx was found to be significantly decreased in Aplnr siRNA transfected cells when compared to control siRNA group. There were no significant GPx activity changes found between $H_2O_2$ (200 µM) administered cells transfected with control siRNA or Aplnr siRNA. In hypoxic conditions transfecting cells with siAplnr significantly decreased the activity levels of GPx when compared to hypoxia control siRNA group (Figure 3.28).

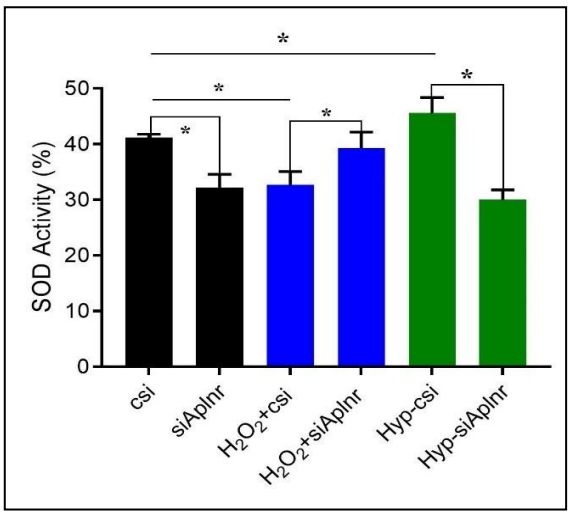


Figure 3.27. SOD activity of siRNA (control siRNA or siAplnr) transfected GT1-7 cells in normoxia, hypoxia and $H_2O_2$ oxidative stress cell culture conditions. Csi: control small interfering RNA. Hyp: hypoxia. * $p<0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1.

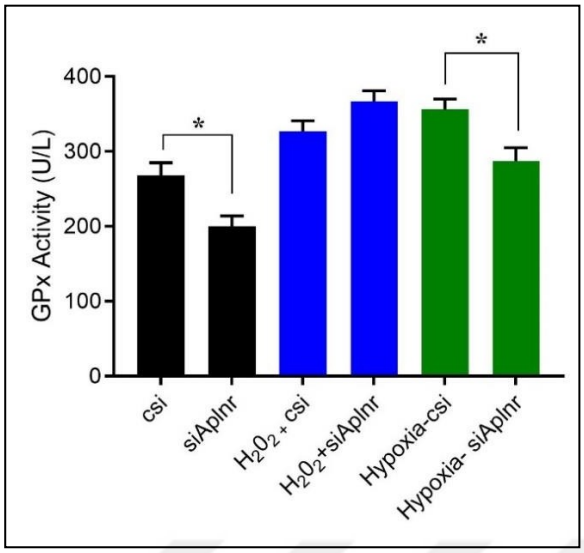


Figure 3.28. GPx activity of siRNA (control siRNA or siAplnr) transfected GT1-7 cells in normoxia, hypoxia and $H_2O_2$ induced oxidative stress cell culture conditions. U/L: units per liter. Csi: control small interfering RNA. * $p<0.05$. Data were analyzed by one-way ANOVA and Tukey's post hoc test using GraphPad Prism Version 8.0.1.

### 3.4.5. Immunostaining Analysis

The protein expression levels of GnRH and Aplnr was observed in GT1-7 cells transfected with Control siRNA and Aplnr siRNA in normoxia, $H_2O_2$ (200 μM) and hypoxia induced conditions by performing immunocytochemical analysis using antibodies against GnRH and Aplnr. The results have shown that knocking down Aplnr caused dramatically decrease in both GnRH and Aplnr protein expression levels under normoxia. Downregulation of Aplnr with siAplnr in $H_2O_2$ and hypoxia induced conditions remarkably diminished GnRH and Aplnr protein levels compared to control siRNA transfected GT1-7 cells (Figure 3.29).

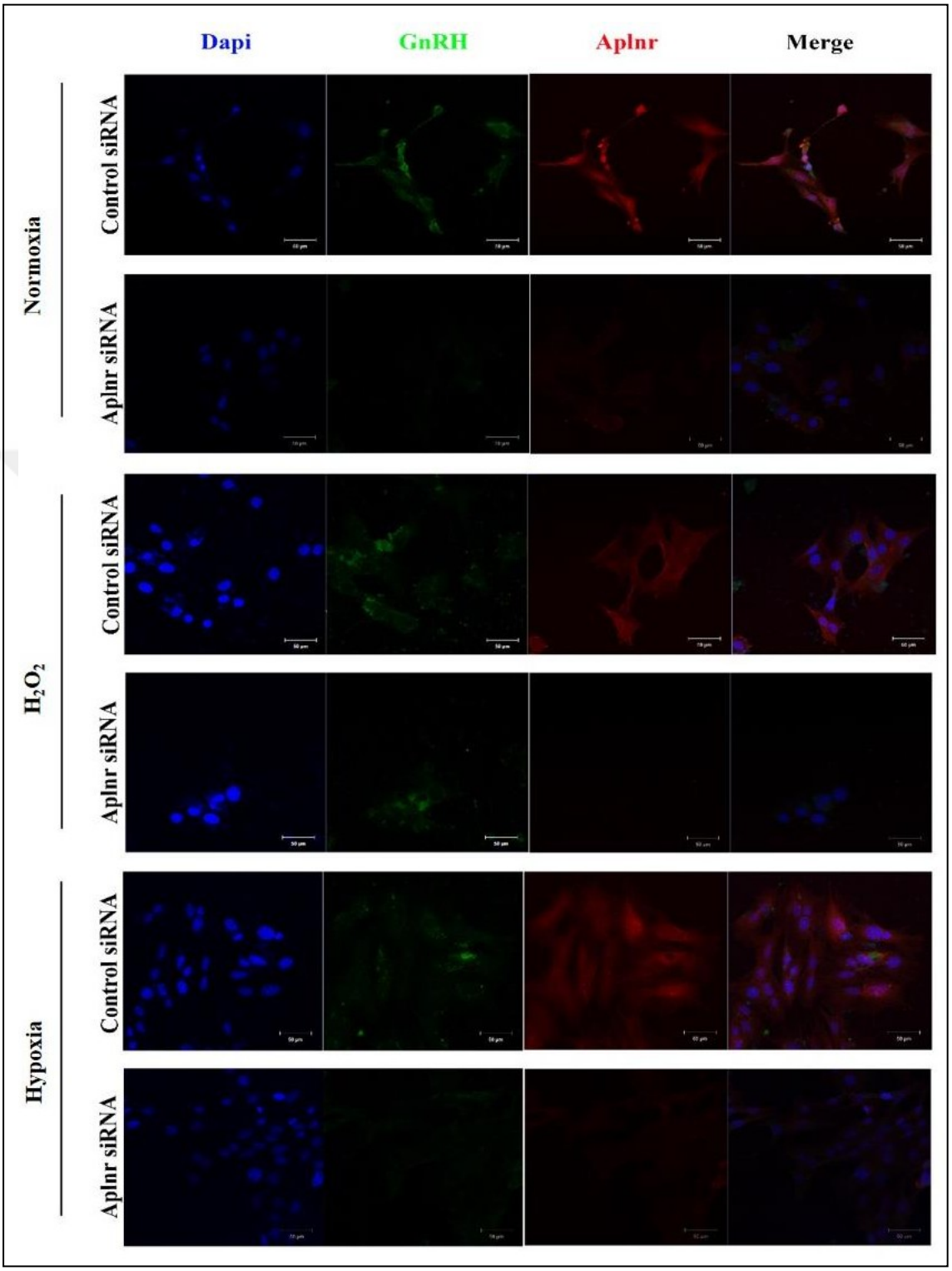


Figure 3.29. Immunostaining analysis of Control siRNA and Aplnr siRNA transfected GT1-7 cells in normoxia, hypoxia and $H_20_2$ induced conditions using primary antibodies GnRH and Aplnr. The nuclei were stained with DAPI. Scale Bar: 50 μm.

# 4. DISCUSSION

Neurotoxicity is defined as the destructive damage of the nervous system due to the exposure to biological or synthetic substances such as pesticides, organic solvents, metals, gases and drugs [74, 174]. Neurotoxicity has been shown to be the main result of oxidative stress which is correlated with common neuronal disorders including Parkinson's Disease [175], Alzheimer's Disease [176], Cerebral Ischemia [177], Multiple Sclerosis [178], ALS [122] and epilepsy [179]. Brain is known as the most susceptible organ to oxidants because of high amounts of oxidizable lipids, requirement of high levels of oxygen, and low activity levels of antioxidant enzymes [89]. Thus, brain is a vulnerable target for free radical induced damage and as a result, neurotoxicity. It is worldwide estimated that at least 50 million people are suffering from Alzheimer's disease [180], over 6.3 million from Parkinson's Disease[181], 2.3 million from Multiple Sclerosis [182] and at least 50 million people from epilepsy [183]. The global incidence of ALS cases was estimated to range from 0.4 to 2.6 within a population of 100,000 people every year [184]. The mortality rates of cerebral ischemia are nearly 3.5 million people worldwide [185]. Identification of new pathways to block oxidative stress induced neurotoxicity is the aim of interest in recent years for treatment of various neurotoxic disorders.

Hypothalamus, the most important brain region, is responsible for producing and releasing neurohormones (including stimulatory or inhibitory hormones) such as gonadotropin-releasing hormone, corticotropin-releasing hormone, thyrotropin-releasing hormone, growth hormone releasing hormone, and somatostatin. These hormones are released to pituitary gland and control numerous autonomic nervous system functions including thermoregulation, appetite, water balance, circadian rhythm, and reproduction to maintain homeostasis [186, 187]. Hypothalamus is implicated in the pathogenesis of neurotoxic disorders in number of studies [188-193]. Therefore, it is essential to study the alterations in hypothalamic neurons against neurotoxicity and find therapeutic approaches for treatment.

Apelin, an endogenous ligand of apelin receptor which is a G-protein coupled receptor that is highly expressed in various parts of the human brain [12-14], has been shown to have neuroprotective action in many studies [194-198]. Evidently, targeting apelin receptor signaling pathway has a potential therapeutic role in treating neurotoxic disorders. The role of Aplnr signalling in hypothalamus and hormone regulation is not known up to now.

The aim of the current study was to elucidate the protective role of Aplnr signaling against oxidative stress related neurotoxicity in hypothalamus GnRH neurons by using GT1-7 cells as a model system. $H_2O_2$ and hypoxia induced oxidative stress models were used in *in vitro* cell culture system and Aplnr signalling is activated and downregulated to explore the potential protective role. In this thesis, the potential therapeutic role of Aplnr signaling was shown *in vitro* for the first time in GT1-7 mouse hypothalamic neuronal cell line.

The Aplnr signaling is activated after 30 min exposure in HEK-293 in our study which is consistent with our previous research. In parallel with Dr. Doğan's previous study [199] showing Aplnr signaling activation in mouse embryonic fibroblasts (MEFs) and NIH-3T3 mouse fibroblast cells with Apelin and Aplnr agonist, ML-233, Aplnr signaling was activated after 30 min of exposure. This assay has confirmed the reliable activity of peptide and small molecule ML-233 before cell culture experiments.

According to the literature Aplnr signaling activates intracellular signaling cascades including PI3K/Akt, ERK1/2, and mammalian target of rapamycin (mTOR) which all of them are well-known for their role in cell proliferation [18, 19]. In correlation with these studies, Apelin showed cell proliferative effects in GT1-7 cells at 1 μM concentration. Small non-peptide Aplnr synthetic agonist, ML-233 [20], showed the same proliferative effects (1 μM) on GT1-7 cells as well. Aplnr inhibitor, ML-221 suppressed proliferation only with 0.5 μM of low dose indicating that blocking Aplnr signaling with this non-peptide small molecule stops the action of intracellular proliferative pathways and thus cell proliferation, as its inhibitory effects on Aplnr were shown in previous different studies [200, 201]. $H_2O_2$ is recognized in the literature as one of the most hazardous ROS to cells as it was shown for its oxidative stress related damage to cells in many studies [202-205]. In correlation with the literature, $H_2O_2$ exposure at 200 μM significantly decreased viable number of GT1-7 cells to 30 per cent.

The effect of transient overexpression of Aplnr was also determined in GT1-7 cells by cell viability analysis in normoxia, $H_2O_2$ (200 μM) and hypoxia induced conditions for 24 h. Aplnr overexpressed cells in normoxic conditions significantly increased viability of GT1-7 cells indicating that Aplnr overexpression even for short amount of time has a proliferative effect on GT1-7 cells, as expected. Since Aplnr signaling is known as the downstream activator of cell proliferation pathway, PI3K/Akt the overexpression of Aplnr is expected to stimulate cell proliferation. More importantly, under oxidative stress ($H_2O_2$ and hypoxia)

induced conditions Aplnr overexpression increased viability of GT1-7 cells suggesting that activation of Aplnr signaling with transient overexpression has a protective role in the viability of GnRH neurons under cellular stress.

Suppressing Aplnr signaling in normoxic conditions via siAplnr caused significant decrease in the proliferation of GT1-7 cells suggesting that the inhibition of Aplnr signaling causes a domino effect to PI3K/Akt pathway which was also reported in the literature [206]. On the other hand, exposing siAplnr transfected cells to $H_2O_2$ and hypoxia induced conditions decreased cell proliferation even more when compared to their control siRNA transfected groups as expected. Although it is expected to cause decrease in cell proliferation when control siRNA transfected cells were treated with $H_2O_2$ or maintained in hypoxic conditions, transfected cells with siAplnr caused even more diminishing effects on the proliferation of GT1-7 cells. This indicates that down regulation of Aplnr causes significant decrease in cell proliferation in oxidative stress induced ($H_2O_2$ and hypoxia) conditions as well.

Number of studies have revealed that activation of Aplnr signaling via its endogenous ligand Apelin causes significant changes in the gene and protein expression levels of apoptotic and proliferation markers found within the cell [206-210]. According to the study of Hui Xie and his colleagues, Apelin treatment activates proliferation markers PI3K and AKT as well as anti-apoptotic marker, BCL-2 protein. Furthermore, they showed that Apelin treatment diminishes the activity of apoptotic markers including caspase-3 and BAX indicating that it has suppressing effects in apoptotic activity [206]. Apelin treatment in GT1-7 hypothalamic neurons in normoxic conditions didn't cause any significant changes in Akt gene expression levels. However, $H_20_2$ and hypoxic cell conditions significantly increased Akt gene expression levels indicating that in cell stress induced conditions Apelin showed its proliferative effects and thus increased and protected the GnRH neuronal cell population. Non-peptide Aplnr activator, ML-233, also showed significant increase in Akt gene expression levels in normoxia, hypoxia and $H_20_2$ induced cell conditions. However, pharmacologically inhibiting Aplnr signaling with ML-221 showed no significant changes in proliferation marker Akt mRNA levels in normoxia but it showed diminishing effects in Akt gene expression levels in $H_20_2$ and hypoxia induced cell stress conditions compared to Apelin and ML-233 treated cells. More importantly, transfection with siAplnr in normoxic conditions significantly decreased Akt mRNA levels in GnRH neurons suggesting that Aplnr activation is definitely associated with cell proliferation. However, siAplnr treatment caused

an increase in Akt gene expression levels in $H_20_2$ induced conditions. This increase might be associated with 10 per cent FBS containing cell culture medium. On the other hand, knocking down of Aplnr under hypoxic conditions didn't cause any significant changes in Akt mRNA levels, indicating that Akt gene expression levels are not only associated with the presence of Aplnr. Further detailed protein expression analysis is required for identification of the molecular pathway. Furthermore, overexpression of Aplnr caused a significant increase in Akt gene expression levels in normoxia, $H_20_2$ and hypoxia induced conditions meaning that the activation of Aplnr signaling plays a protective role in GnRH cell population even under oxidative stress induced conditions.

Apelin treatment didn't cause any significant changes in apoptotic marker, BAX, in normoxic and hypoxic conditions, however, it significantly decreased BAX gene expression levels in $H_20_2$ induced cell stress. On the other hand, Aplnr agonist, ML-233, treated cells showed decrease in BAX mRNA levels in $H_20_2$ and hypoxia induced conditions. Since ML-233 is a synthetic agonist of Aplnr it is expected to be more potent than Apelin because of its molecular structure. Therefore, ML-233 showed significant decrease in BAX gene expression levels under oxidative stress induced conditions. However, interestingly, ML-233 showed increase in BAX mRNA levels under normoxic conditions. Inhibiting Aplnr with ML-221 significantly increased BAX mRNA levels in normoxic, hypoxic and $H_20_2$ induced cell conditions suggesting that Aplnr activation plays a protective role against apoptosis. Moreover, silencing Aplnr didn't cause any significant changes in BAX mRNA levels under normoxic and hypoxic conditions. However, BAX gene expression levels were significantly increased when Aplnr was knocked down under $H_20_2$ induced cell stress suggesting that the presence of Aplnr shows a protective role against $H_20_2$ induced apoptosis. Overexpression of Aplnr in GT1-7 cells caused no significant changes in BAX mRNA levels in both normoxia and $H_20_2$ induced conditions but showed a significant decrease under hypoxic conditions. For further verification of BAX levels in Aplnr overexpressed cells, immunoblotting analysis must be performed. On the other hand, Apelin treatment didn't cause any significant decrease in apoptotic marker p53 gene expression levels in normoxic conditions. However, Apelin treatment under $H_20_2$ induced conditions significantly decreased p53 mRNA levels. Moreover, p53 mRNA levels didn't cause any significant changes in Apelin treated cells under hypoxic conditions. ML-233 treated cells showed significant decrease in p53 mRNA levels in normoxic and hypoxic conditions. All together these data suggest that both Aplnr activators (Apelin and ML-233) have different effects in

p53 mRNA levels under different ($H_2O_2$ and hypoxia) cell stress conditions because of their structural (natural and synthetic) and activity differences. Inhibition of Aplnr with ML-221 under normoxic conditions didn't cause any significant changes in p53 mRNA levels. However, ML-221 treatment in $H_2O_2$ and hypoxia induced conditions significantly increased p53 gene expression levels indicating that the activation of Aplnr under oxidative stress induced conditions is important for suppressing p53 mRNA levels. Silencing Aplnr in normoxic conditions caused a significant increase in p53 mRNA levels whereas in $H_2O_2$ and hypoxia induced conditions it didn't cause any significant changes. The gene expression profile for p53 must be confirmed with western blotting analysis for further understanding of the differences. Interestingly, overexpression of Aplnr caused a significant increase in p53 gene expression levels. However, under oxidative stress ($H_2O_2$ and hypoxia) induced conditions p53 mRNA levels were significantly decreased suggesting that activation of Aplnr protects GT1-7 hypothalamic neurons from going to apoptosis in the case of oxidative stress.

Caspase- 3 mRNA levels in Apelin and ML-233 treated cells in normoxia and oxidative stress induced ($H_2O_2$ and hypoxia) conditions were significantly decreased. Furthermore, caspase-3 gene expression levels were increased in cells treated with ML-221 under normoxic, hypoxic and $H_2O_2$ induced conditions meaning that inhibition of Aplnr promotes apoptosis. Therefore, it can be concluded that activation of Aplnr signaling protects cells against apoptosis under normoxic, hypoxic and $H_2O_2$ induced conditions. In parallel with the observed data, activation of Aplnr signaling was shown to be involved in suppressing apoptotic activity in the literature [210, 211]. Moreover, knocking down Aplnr with siAplnr significantly increased caspase-3 mRNA levels under normoxic and hypoxic whereas caused no significant changes in $H_2O_2$ induced conditions. The result of caspase-3 mRNA levels shows that silencing Aplnr via siAplnr abolished its anti-apoptotic activity under normoxic and hypoxic conditions.

In literature, Apelin has been shown to have regulatory effects in pro-inflammatory pathway, NF-KB, and inhibit its induced activation in the case of an inflammation [212, 213]. Concordantly to these findings, Apelin treatment in oxidative stress induced conditions ($H_2O_2$ and hypoxia) significantly decreased the gene expression level of the inflammation marker, IL-2. Contradicting to the aforementioned observations, Apelin treatment in normoxic conditions increased IL-2 mRNA levels. The similar effect on IL-2 mRNA levels

were detected for ML-233 treated cells in normoxia. Inhibiting the activity of Aplnr with ML-221 decreased IL-2 gene expression levels. However, pharmacological activation of Aplnr signaling in $H_20_2$ induced conditions with ML-233 reduced IL-2 mRNA levels. On the other hand, ML-233 treatment under hypoxic conditions didn't cause any significant changes in IL-2 gene expression levels. Oxidative stress ($H_20_2$ and hypoxia) induced conditions in ML-221 treated cells caused IL-2 gene expression levels to increase significantly. More importantly, silencing Aplnr signaling caused significant increase in IL-2 mRNA levels in normoxic, hypoxic and $H_20_2$ induced conditions. Therefore, it can be suggested that the activation of Aplnr signaling is involved in attenuating inflammation under $H_20_2$ and hypoxia induced conditions but not in normoxia. This might be explained with different molecular mechanisms under oxidative stress induced conditions. Furthermore, overexpressing Aplnr showed no significant changes in IL-2 mRNA levels in both normoxic and hypoxic conditions. However, IL-2 mRNA levels were significantly decreased under $H_20_2$ induced cellular stress in Aplnr overexpressed GT1-7 cells indicating that the activation of Aplnr signaling is important for diminishing inflammation in the case of oxidative stress. NF-KB mRNA levels were also detected to understand Aplnr's role in pro-inflammatory pathways. Inflammatory marker, NF-KB, mRNA levels were significantly decreased in Apelin treated cells in normoxic conditions. Interestingly, activating Aplnr via ML-233 showed a significant increase in NF-KB mRNA levels in normoxia. More importantly, inhibiting Aplnr via ML-221 resulted in significantly high gene expression levels of NF-KB suggesting that Aplnr activation is essential for alleviating inflammation. NF-KB mRNA levels were also significantly decreased in Apelin and ML-233 treated cells under both hypoxic and $H_20_2$ induced conditions. However, inhibiting the activity of Aplnr signaling via ML-221 in $H_20_2$ induced condition didn't cause any significant changes in NF-KB gene expression levels. On the other hand, ML-221 treated cells in hypoxia caused a decrease in NF-KB mRNA levels which shows contradiction and needs to be searched in detail. Although NF-KB gene expression levels were significantly elevated in Aplnr silenced cells in normoxia, it didn't have any significant changes in $H_20_2$ and hypoxia induced conditions. Increased mRNA levels of NF-KB under normoxic conditions in siAplnr transfected cells suggest that the presence of Aplnr and its activation might be involved in reducing inflammation within the cell. Interestingly, overexpressing Aplnr caused a significant increase in NF-KB mRNA levels in both normoxia and oxidative stress ($H_20_2$ and hypoxia) induced conditions. Another inflammatory marker, COX-2, gene

expression levels were significantly decreased in Aplnr overexpressed GT1-7 cells under normoxic and hypoxic conditions. However, overexpression of Aplnr increased COX-2 mRNA levels in $H_20_2$ induced conditions. For further explanation of the effect of Aplnr signaling in inflammatory pathways immunoblotting analysis are needed.

Several studies have reported that Apelin treatment increases the activity of anti-apoptotic marker, BCL-2, and knocking down of Aplnr abrogates its anti-apoptotic activity [206, 210, 214]. In parallel with these findings, Apelin treatment in normoxic and $H_20_2$ induced conditions caused significant increase in BCL-2 gene expression levels. Activating Aplnr signaling with ML-233 also showed significant increasing effects in BCL-2 mRNA levels in normoxia and $H_20_2$ induced conditions. Interestingly, inhibition of Aplnr with ML-221 resulted in significant increase in BCL-2 gene expression levels in normoxic conditions. However, ML-221 treatment in oxidative stress ($H_20_2$ and hypoxia) induced conditions caused no significant changes in BCL-2 mRNA levels indicating that Aplnr activation is needed for anti-apoptotic activity in oxidative stress induced conditions. In hypoxic conditions BCL-2 mRNA levels were significantly increased in Apelin treated cells. However, ML-233 treatment caused a significant decrease in BCL-2 gene expression levels under hypoxic conditions. Furthermore, knocking down Aplnr in GT1-7 cells via siAplnr significantly decreased BCL-2 gene expression levels in $H_20_2$ induced condition whilst caused no significant change under hypoxic conditions. Similar to other gene expression analysis, oxidative stress induced conditions caused a different response compared to normoxic conditions. Knocking down Aplnr caused no significant changes in BCL-2 mRNA levels in normoxic conditions. Overexpressing Aplnr significantly increased BCL-2 gene expression levels in normoxia, hypoxia and $H_20_2$ induced conditions. Overall, it can be concluded that Aplnr activation plays an important regulatory role in anti-apoptotic activity and prevents cells from going to apoptosis under normoxic and oxidative stress ($H_20_2$ and hypoxia) induced conditions.

Aplnr gene expression levels were also determined in GnRH neurons under normoxic, $H_20_2$ induced and hypoxic conditions. Apelin treatment in normoxia caused a significant increase in Aplnr mRNA levels. The similar effects were observed for ML-233 treated cells. Apelin and $H_20_2$ treated cells caused a significant increase in Aplnr mRNA levels as expected. Activating Aplnr with ML-233 in $H_20_2$ treated cells also showed significant increase in Aplnr mRNA levels. However, inhibiting the activity of Aplnr by treating cells with small molecule

ML-221 had significantly decreased Aplnr gene expression levels in $H_20_2$ induced conditions. Aplnr mRNA levels were also increased in Apelin and ML-233 treated cells. However, ML-221 treatment under hypoxic conditions increased Aplnr mRNA levels as well which was also observed in normoxia. This could be explained by a feedback loop in Aplnr signalling which might induce Aplnr expression after blockage of signalling by ML-221. Overall findings indicate that Aplnr was successfully activated and inhibited in $H_20_2$ induced conditions using its agonist peptide, Apelin and non-peptide molecules including ML-233 and ML-221. Furthermore, knocking down Aplnr slightly diminished Aplnr gene expression levels in normoxic conditions. On the other hand, in $H_20_2$ induced cell stress, Aplnr mRNA levels were significantly decreased in siAplnr transfected cells, as expected. In hypoxia induced conditions siAplnr transfected cells had no significant changes in Aplnr mRNA levels but reduced protein expression levels. Furthermore, overexpressing Aplnr showed a significant increase in Aplnr mRNA levels, as expected. Aplnr mRNA levels were also significantly increased in Aplnr overexpressed GT1-7 cells under $H_20_2$ and hypoxia induced conditions indicating that Aplnr signaling is activated in the case of cellular stress to possibly protect cells from oxidative stress induced damage.

Apelin mRNA levels in Aplnr knocked down cells under normoxic conditions were significantly increased. The reason for this could be a negative feedback mechanism of Aplnr signaling in downregulation. On the other hand, siAplnr treatment caused no significant changes in Apelin mRNA levels in $H_20_2$ induced conditions. However, Apelin mRNA levels were slightly increased in siAplnr treated cells in hypoxia. Furthermore, Apelin gene expression levels were significantly decreased in Aplnr overexpressed GT1-7 cells in normoxia. The reason for this might be that the overexpression of Aplnr in normoxic conditions shows reduced requirement for its endogenous ligand, Apelin, or reaching of Aplnr to a saturation state and thus inhibition of the Apelin expression. On the other hand, Apelin gene expression levels were significantly increased in Aplnr overexpressed GT1-7 cells under oxidative stress induced conditions.

Many in vitro and in vivo studies have revealed that $H_20_2$ and hypoxia induced conditions cause cells to undergo oxidative stress, and as a result damaging effects to biological molecules [87, 203, 204, 215]. As a cellular defense mechanism the antioxidant enzyme, superoxide dismutase, is responsible for the conversion of reactive oxygen species known as superoxide anion into $H_20_2$ and molecular oxygen [216]. Then, the converted hydrogen

peroxide is catalysed into water and molecular oxygen by another antioxidant enzyme, glutathione peroxidase [217]. These free radical scavenging activities are important for cells to maintain their redox balance [218]. In this study, GT1-7 cells in normoxic and hypoxic conditions treated with Apelin and Aplnr agonist, ML-233, showed increased activity in the antioxidant enzymes SOD and GPx. However, inhibiting Aplnr via ML-221 didn't cause any significant changes in SOD and GPx activity levels in normoxic and hypoxic conditions. In parallel with these findings Apelin was previously reported to elevate antioxidant enzyme activities and block ROS production induced by $H_20_2$ in adipocytes [219]. Furthermore, Apelin treatment was shown to increase antioxidant enzyme catalase activity and alleviate $H_20_2$ induced oxidative stress in rat cardiomyocytes [220]. Cells treated only with $H_20_2$ had increased SOD and GPx activity due to the fact that cells want to protect themselves against free radicals, and thus in the case of ROS production they elevate their antioxidant enzyme activities as a defense mechanism which is a known fact from literature [221]. However, cells treated with $H_20_2$ and Apelin decreased SOD activity levels to approximately to negative control cells' SOD activity levels. This indicates that Apelin might have diminished the damaging effect of $H_20_2$ and protected cells from going to oxidative stress, and thus cells didn't need to increase cellular antioxidant enzyme levels. The same was detected for $H_20_2$ and ML-233 treated group. In correlation with this result, inhibition of Aplnr with ML-221 treatment in $H_20_2$ induced conditions increased SOD activity levels nearly to $H_20_2$ only treated group. Although cells treated with $H_20_2$ and Apelin showed nearly the same SOD activity as negative control, they however showed increase in GPx activity levels. Since GPx is known to convert hydrogen peroxide into $H_2O$ and $O_2$ and prevent it from causing damage to cells [217], its activity was expected to increase in this case but more interestingly inhibiting the activity of Aplnr with ML-221 caused GPx activity levels to significantly decrease. This situation indicates that Aplnr activation by Apelin and ML-233 in $H_20_2$ induced cell stress conditions increase the activity of antioxidant enzyme, GPx. Knocking down Aplnr by transfecting cells with siAplnr significantly decreased the catalytic activity of SOD and GPx in normoxic conditions which was also shown in literature [219]. In hypoxic conditions silencing Aplnr also caused a significant decrease in antioxidant enzymes; SOD and GPx indicating that both in normoxic and hypoxic conditions the presence of Aplnr plays a modulatory role in antioxidant enzymes' activity. Interestingly in $H_20_2$ and siAplnr treated cells SOD activity remained the same as the control siRNA group.

On the other hand, the activity of GPx in $H_20_2$ and siAplnr treated cells wasn't significantly changed compared to $H_20_2$ and control siRNA treated group.

In the immunostaining analysis of GT1-7 cells both Apelin and ML-233 treated cells showed significant increase in the protein expression levels of Aplnr and GnRH in normoxic conditions. From these observations, it can be concluded that Apelin and ML-233 successfully activates Aplnr at the protein level, and as a result of Aplnr activation both of them stimulates the release of GnRH in GT1-7 cells. However, pharmacological inhibition of Aplnr via ML-221 in normoxic conditions caused no significant changes in Aplnr protein levels and this in turn resulted GnRH protein expression levels to significantly decrease meaning that the activation of Aplnr signaling plays a regulatory role in the release of GnRH. This is first study in literature to show the role of Aplnr signaling in hormone regulation. In $H_20_2$ induced conditions GnRH and Aplnr protein expression levels were significantly increased in both Apelin and ML-233 treated cells. However, ML-221 treated cells in $H_20_2$ induced conditions caused no significant changes in protein levels of both Aplnr and GnRH indicating that under oxidative stress ($H_20_2$ induction) the inhibition of Aplnr signaling doesn't stimulate the production of GnRH. In hypoxic conditions Apelin treatment caused only slight increase (not significant) in the protein expression levels of GnRH. However, ML-233 treatment significantly increased GnRH protein levels under hypoxic conditions. Aplnr protein levels in hypoxia induced conditions were significantly increased in both Apelin and ML-233 treated cells. Inhibiting the activity of Aplnr with ML-221 caused no significant changes in Aplnr protein expression levels in hypoxic conditions. Furthermore, ML-221 treatment caused no significant changes in GnRH protein expression levels under hypoxic conditions meaning that Aplnr activation is associated with GnRH production. From these observations, it can be inferred that the activation and inhibition of Aplnr signaling has a regulatory effect in the release of GnRH. Also, immunostaining analysis of Aplnr overexpression in GT1-7 cells revealed correlation with the aforementioned results. Transiently Aplnr overexpressed GT1-7 cells showed increase in the protein levels of GnRH and Aplnr in normoxic conditions. More importantly, even in under oxidative stress ($H_20_2$ and hypoxia) induced conditions overexpression of Aplnr in GT1-7 cells caused GnRH and Aplnr protein levels to increase compared to their control groups indicating that Aplnr overexpression remarkably protected GnRH protein levels. On the other hand, knocking down of Aplnr with siAplnr caused a dramatic decrease in GnRH and Aplnr protein levels in normoxia, $H_2O_2$ and hypoxia induced conditions compared to their control groups. Overall

data suggest that Aplnr signaling is indeed in charge of regulating GnRH protein levels. Although the protein expression analysis should be confirmed with the western blot analysis, it is clearly observed that Aplnr signaling increases the GnRH protein levels in consistent with Aplnr protein expression itself.

Aplnr signaling was shown to be a promising target because of its neuroprotective, anti-inflammatory and anti-apoptotic properties for the treatment of neurotoxic disorders including Parkinson's disease [194], Alzheimer's disease [222], Multiple Sclerosis[223], ALS [224], epilepsy [225], and cerebral ischemia [226]. In ALS animal models, apelin deficiency has been shown to accelerate disease progression suggesting that Aplnr signaling is an important target for protecting motor neurons against degeneration [224]. According to a recent study published in 2019, women with MS have significantly lower plasma levels of apelin compared to healthy individuals. It has been further suggested that apelin not only might become a therapeutic target but also a potential biomarker for MS patients [223]. In parallel with these studies, the current study revealed the protective role of Aplnr signaling *in vitro* using GnRH neurons in two different oxidative stress conditions including $H_20_2$ and hypoxia induction. Hypothesis has been confirmed by using genetic activation-downregulation and peptide/small molecule induced activation-downregulation systems. Further investigations in Aplnr signaling are needed for in-depth understanding its potential protective effects against neurotoxic disorders by conducting *in vitro* or *in vivo* knock-out models using a CRISPR-Cas9 genome editing technology. Currently, we are trying to establish genetically engineered lines of GT1-7 cells for future studies of this thesis.

# 5. CONCLUSION

Oxidative stress induced neurotoxicity is a complex pathological situation observed in various neurotoxic disorders including Parkinson's Disease, Alzheimer's Disease, Cerebral Ischemia, Multiple Sclerosis, ALS and epilepsy due to the damage of reactive oxygen species (ROS) in multiple parts of the CNS. Hypothalamus is an important control point upon other brain regions with a hormone regulatory role. Targeting potential protective signalling pathways in order to promote neuronal cell survival in hypothalamus is crucial for treatment since it is a significant hormone-producing brain region which has various functions for maintaining homeostasis within the body.

Protective role of Aplnr signaling in an *in vitro* GnRH neuron model against $H_20_2$ and hypoxia induced neurotoxicity was used in the current study. Regulatory and protective activity of Aplnr signaling in GnRH neurons was observed for the first time in literature with this thesis research. Activation of Aplnr signaling via its neuropeptide, Apelin, and non-peptide small molecule agonist, ML-233, increased anti-apoptotic activity and proliferation at gene expression level while reducing apoptotic and inflammatory markers in GnRH neurons. Furthermore, Aplnr signaling activation protected GnRH neurons against $H_20_2$ and hypoxia induced cell stress conditions by increasing the activity of antioxidant enzymes; SOD and GPx. Transient overexpression of Aplnr showed a protective role in viability of GT1-7 cells under oxidative stress ($H_2O_2$ and hypoxia) induced conditions. On the other hand, downregulation of Aplnr signaling using siAplnr in oxidative stress induced conditions significantly reversed its protective effects indicating that activation of Aplnr is essential for GnRH neuronal cell survival against neurotoxicity.

Overall, the current study is the first one in the literature which represents the activity of Aplnr signaling in GnRH neurons. Aplnr signaling is protective against oxidative stress in GnRH neurons *in vitro* and might be a promising therapeutic candidate for the clinical applications in the future. Identification of the role of Aplnr signaling in GnRH cells might propose a new gene therapy approach for the future. Detailed molecular mechanism analyses are being conducted by our research group to elucidate the Aplnr signaling in GnRH neurons. Further in vivo studies are required to explore potential clinical applications in the future.

# REFERENCES


1. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, et al. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene*. 1993;136(1-2):355-60.

2. Shin K, Kenward C, Rainey JK. Apelinergic system structure and function. *Comprehensive Physiology*. 2017;8(1):407-50.

3. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human apj receptor. *Biochemical and Biophysical Research Communications*. 1998;251(2):471-6.

4. Pitkin SL, Maguire JJ, Bonner TI, Davenport AP. International union of basic and clinical pharmacology. lxxiv. apelin receptor nomenclature, distribution, pharmacology, and function. *Pharmacological Reviews*. 2010;62(3):331-42.

5. Devic E, Rizzoti K, Bodin S, Knibiehler B, Audigier Y. Amino acid sequence and embryonic expression of msr/apj, the mouse homolog of xenopusx-msr and human apj. *Mechanisms of Development*. 1999;84(1):199-203.

6. De Mota N, Lenkei Z, Llorens-Cortes C. Cloning, pharmacological characterization and brain distribution of the rat apelin receptor. *Neuroendocrinology*. 2000;72(6):400-7.

7. Schilffarth S, Antoni B, Schams D, Meyer H, Berisha B, Schilffarth S, et al. The expression of apelin and its receptor apj during different physiological stages in the bovine ovary. *International Journal of Biological Sciences*. 2009;5:344-50.

8. Margulies B, Hauer D, Clements J, Margulies B, Hauer D, Clements J. Identification and comparison of eleven rhesus macaque chemokine receptors. *Aids Research and Human Retroviruses*. 2001;17(10):981-6.

9. Devic E, Paquereau L, Vernier P, Knibiehler B, Audigier Y. Expression of a new g protein-coupled receptor x-msr is associated with an endothelial lineage in xenopus laevis. *Mechanisms of Development*. 1996;59(2):129-40.

10. Zhang J, Zhou Y, Wu C, Wan Y, Fang C, Li J, et al. Characterization of the apelin/elabela receptors in chickens, turtles, and zebrafish: identification of a novel apelin-specific receptor in teleosts. *Frontiers in Endocrinology*. 2018;9:756.

11. Tucker B, Hepperle C, Kortschak D, Rainbird B, Wells S, Oates A, et al. Zebrafish angiotensin ii receptor-like 1a (agtrl1a) is expressed in migrating hypoblast, vasculature, and in multiple embryonic epithelia. *Gene Expression Patterns*. 2007;7(3):258-65.

12. Edinger A, Hoffman T, Sharron M, Lee B, Yi Y, Choe W, et al. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. *Journal of Virology*. 1998;72(10):7934-40.

13. Matsumoto M, Hidaka K, Akiho H, Tada S, Okada M, Yamaguchi T, et al. Low stringency hybridization study of the dopamine d4 receptor revealed d4-like mrna distribution of the orphan seven-transmembrane receptor, apj, in human brain. *Neuroscience Letters*. 1996;219(2):119-22.

14. Medhurst A, Jennings C, Robbins M, Davis R, Ellis C, Winborn K, et al. Pharmacological and immunohistochemical characterization of the apj receptor and its endogenous ligand apelin. *Journal of Neurochemistry*. 2003;84(5):1162-72.

15. Hansen A, Chen Y, Inman J, Phan Q, Qi Z, Xiang C, et al. Sensitive and specific method for detecting g protein-coupled receptor mrnas. *Nature Methods*. 2007;4(1):35-7.

16. O'Carroll A-M, Lolait SJ, Harris LE, Pope GR. The apelin receptor apj: journey from an orphan to a multifaceted regulator of homeostasis. *Journal of Endocrinology*. 2013;219(1):13-35.

17. Kleinz M, Skepper J, Davenport A, Kleinz M, Skepper J, Davenport A. Immunocytochemical localisation of the apelin receptor, apj, to human cardiomyocytes, vascular smooth muscle and endothelial cells. *Regulatory Peptides*. 2005;126(3):233-40.

18. Masri B, Morin N, Cornu M, Knibiehler B, Audigier Y, Masri B, et al. Apelin (65–77) activates p70 s6 kinase and is mitogenic for umbilical endothelial cells. *The Faseb Journal*. 2004;18(15):1909-11.

19. Liu C, Su T, Li F, Li L, Qin X, Pan W, et al. Pi3k/akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13. *Acta Biochimica et Biophysica Sinica*. 2010;42(6):396-402.

20. Khan P MP, Hedrick M, et al. Functional agonists of the apelin (apj) receptor: nih; 2011 [cited 2019 21 May] Available from: https://www.ncbi.nlm.nih.gov/books/NBK98921/.

21. Maloney PR, Khan P, Hedrick M, Gosalia P, Milewski M, Li L, et al. Discovery of 4-oxo-6-((pyrimidin-2-ylthio)methyl)-4H-pyran-3-yl 4-nitrobenzoate (ml221) as a functional antagonist of the apelin receptor. *Bioorganic and Medicinal Chemistry Letters*. 2012;22(21):6656-60.

22. Shin K, Pandey A, Liu X-Q, Anini Y, Rainey JK. Preferential apelin-13 production by the proprotein convertase pcsk3 is implicated in obesity. *Febs Open Bio*. 2013;3(1):328-33.

23. Valle A, Hoggard N, Adams AC, Roca P, Speakman JR. Chronic central administration of apelin-13 over 10 days increases food intake, body weight, locomotor activity and body temperature in c57bl/6 mice. *Journal of Neuroendocrinology*. 2008;20(1):79-84.

24. Zhen EY, Higgs RE, Gutierrez JA. Pyroglutamyl apelin-13 identified as the major apelin isoform in human plasma. *Analytical Biochemistry*. 2013;442(1):1-9.

25. Volkoff H. Cloning and tissue distribution of appetite-regulating peptides in pirapitinga (piaractus brachypomus). *Journal of Animal Physiology and Animal Nutrition*. 2015;99(5):987-1001.

26. Choe W, Albright A, Sulcove J, Jaffer S, Hesselgesser J, Lavi E, et al. Functional expression of the seven-transmembrane hiv-1 co-receptor apj in neural cells. *Journal of Neurovirology*. 2000;6(1):61-9.

27. Brailoiu GC, Dun SL, Yang J, Ohsawa M, Chang JK, Dun NJ. Apelin-immunoreactivity in the rat hypothalamus and pituitary. *Neuroscience Letters*. 2002;327(3):193-7.

28. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, et al. Characterization of apelin, the ligand for the apj receptor. *Journal of Neurochemistry*. 2000;74(1):34-41.

29. O'Carroll AM, Selby TL, Palkovits M, Lolait SJ. Distribution of mrna encoding b78/apj, the rat homologue of the human apj receptor, and its endogenous ligand apelin in brain and peripheral tissues. *Biochimica et Biophysica Acta*. 2000;1492(1):72-80.

30. O'Donnell LA, Agrawal A, Sabnekar P, Dichter MA, Lynch DR, Kolson DL. Apelin, an endogenous neuronal peptide, protects hippocampal neurons against excitotoxic injury. *Journal of Neurochemistry*. 2007;102(6):1905-17.

31. Puffer BA, Sharron M, Coughlan CM, Baribaud F, McManus CM, Lee B, et al. Expression and coreceptor function of apj for primate immunodeficiency viruses. *Virology*. 2000;276(2):435-44.

32. Reaux A, Gallatz K, Palkovits M, Llorens-Cortes C. Distribution of apelin-synthesizing neurons in the adult rat brain. *Neuroscience*. 2002;113(3):653-62.

33. Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, et al. Molecular and functional characteristics of apj. tissue distribution of mrna and interaction with the endogenous ligand apelin. *Journal of Biological Chemistry*. 2000;275(28):21061-7.

34. Kleinz MJ, Davenport AP. Immunocytochemical localization of the endogenous vasoactive peptide apelin to human vascular and endocardial endothelial cells. *Regulatory Peptides*. 2004;118(3):119-25.

35. Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, et al. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology*. 2005;146(4):1764-71.

36. Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, et al. Apelin is a novel angiogenic factor in retinal endothelial cells. *Biochemical and Biophysical Research Communications*. 2004;325(2):395-400.

37. Szokodi I, Tavi P, Foldes G, Voutilainen-Myllyla S, Ilves M, Tokola H, et al. Apelin, the novel endogenous ligand of the orphan receptor apj, regulates cardiac contractility. *Circulation Research.* 2002;91(5):434-40.

38. Tatemoto K, Takayama K, Zou M-X, Kumaki I, Zhang W, Kumano K, et al. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regulatory Peptides*. 2001;99(2):87-92.

39. Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. *Hypertension*. 2009;54(3):598-604.

40. Atluri P, Morine KJ, Liao GP, Panlilio CM, Berry MF, Hsu VM, et al. Ischemic heart failure enhances endogenous myocardial apelin and apj receptor expression. *Cellular and Molecular Biology Letters*. 2006;12(1):127-38.

41. Dai T, Ramirez-Correa G, Gao WD. Apelin increases contractility in failing cardiac muscle. *European Journal of Pharmacology*. 2006;553(1-3):222-8.

42. Zhang Z, Yu B, Tao GZ. Apelin protects against cardiomyocyte apoptosis induced by glucose deprivation. *Chinese Medical Journal*. 2009;122(19):2360-5.

43. Wang W, McKinnie SM, Patel VB, Haddad G, Wang Z, Zhabyeyev P, et al. Loss of apelin exacerbates myocardial infarction adverse remodeling and ischemia-

reperfusion injury: therapeutic potential of synthetic apelin analogues. *Journal of The American Heart Association*. 2013;2(4):e000249.

44. Wei L, Hou X, Tatemoto K. Regulation of apelin mrna expression by insulin and glucocorticoids in mouse 3t3-l1 adipocytes. *Regulatory Peptides*. 2005;132(1-3):27-32.

45. Heinonen MV, Purhonen AK, Miettinen P, Paakkonen M, Pirinen E, Alhava E, et al. Apelin, orexin-a and leptin plasma levels in morbid obesity and effect of gastric banding. *Regulatory Peptides*. 2005;130(1-2):7-13.

46. Sorhede Winzell M, Magnusson C, Ahren B. The apj receptor is expressed in pancreatic islets and its ligand, apelin, inhibits insulin secretion in mice. *Regulatory Peptides*. 2005;131(1-3):12-7.

47. Guo L, Li Q, Wang W, Yu P, Pan H, Li P, et al. Apelin inhibits insulin secretion in pancreatic beta-cells by activation of pi3-kinase-phosphodiesterase 3b. *Endocrine Research*. 2009;34(4):142-54.

48. Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buleon M, et al. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metabolism*. 2008;8(5):437-45.

49. De Mota N, Reaux-Le Goazigo A, El Messari S, Chartrel N, Roesch D, Dujardin C, et al. Apelin, a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. *Proceedings of The National Academy of Sciences of The United States of America*. 2004;101(28):10464-9.

50. O'Carroll A-M, Lolait SJ. Regulation of rat apj receptor messenger ribonucleic acid expression in magnocellular neurones of the paraventricular and supraopric nuclei by osmotic stimuli. *Journal of Neuroendocrinology*. 2003;15(7):661-6.

51. Reaux-Le Goazigo A, Morinville A, Burlet A, Llorens-Cortes C, Beaudet A. Dehydration-induced cross-regulation of apelin and vasopressin immunoreactivity

levels in magnocellular hypothalamic neurons. *Endocrinology*. 2004;145(9):4392-400.

52. Taheri S, Murphy K, Cohen M, Sujkovic E, Kennedy A, Dhillo W, et al. The effects of centrally administered apelin-13 on food intake, water intake and pituitary hormone release in rats. *Biochemical and Biophysical Research Communications*. 2002;291(5):1208-12.

53. Hus-Citharel A, Bouby N, Frugiere A, Bodineau L, Gasc JM, Llorens-Cortes C. Effect of apelin on glomerular hemodynamic function in the rat kidney. *Kidney International*. 2008;74(4):486-94.

54. Roberts EM, Newson MJF, Pope GR, Landgraf R, Lolait SJ, O'Carroll A-M. Abnormal fluid homeostasis in apelin receptor knockout mice. *The Journal of Endocrinology*. 2009;202(3):453-62.

55. Kalin R, Kretz M, Meyer A, Kispert A, Heppner F, Brandli A, et al. Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis. *Developmental Biology*. 2007;305(2):599-614.

56. Cox C, D'Agostino S, Miller M, Heimark R, Krieg P, Cox C, et al. Apelin, the ligand for the endothelial g-protein-coupled receptor, apj, is a potent angiogenic factor required for normal vascular development of the frog embryo. *Developmental Biology*. 2006;296(1):177-89.

57. Kidoya H, Takakura N, Kidoya H, Takakura N. Biology of the apelin-apj axis in vascular formation. *Journal of Biochemistry*. 2012;152(2):125-31.

58. Sorli S, Le Gonidec S, Knibiehler B, Audigier Y, Sorli S, Le Gonidec S, et al. Apelin is a potent activator of tumour neoangiogenesis. *Oncogene*. 2007;26(55):7692-9.

59. Picault FX, Chaves-Almagro C, Projetti F, Prats H, Masri B, Audigier Y. Tumour co-expression of apelin and its receptor is the basis of an autocrine loop involved in the growth of colon adenocarcinomas. *European Journal of Cancer*. 2014;50(3):663-74.

60. Eyries M, Siegfried G, Ciumas M, Montagne K, Agrapart M, Lebrin F, et al. Hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. *Circulation Research*. 2008;103(4):432-40.

61. Han S, Wang G, Qiu S, de la Motte C, Wang HQ, Gomez G, et al. Increased colonic apelin production in rodents with experimental colitis and in humans with ibd. *Regulatory Peptides*. 2007;142(3):131-7.

62. Susaki E, Wang G, Cao G, Wang HQ, Englander EW, Greeley GH, Jr. Apelin cells in the rat stomach. *Regulatory Peptides*. 2005;129(1-3):37-41.

63. Wang G, Kundu R, Han S, Qi X, Englander EW, Quertermous T, et al. Ontogeny of apelin and its receptor in the rodent gastrointestinal tract. *Regulatory Peptides*. 2009;158(1-3):32-9.

64. Wang G, Anini Y, Wei W, Qi X, AM OC, Mochizuki T, et al. Apelin, a new enteric peptide: localization in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. *Endocrinology*. 2004;145(3):1342-8.

65. Lambrecht NW, Yakubov I, Zer C, Sachs G. Transcriptomes of purified gastric ecl and parietal cells: identification of a novel pathway regulating acid secretion. *Physiological Genomics*. 2006;25(1):153-65.

66. Choe H, Farzan M, Konkel M, Martin K, Sun Y, Marcon L, et al. The orphan seven-transmembrane receptor apj supports the entry of primary t-cell-line-tropic and dualtropic human immunodeficiency virus type 1. *Journal of Virology*. 1998;72(7):6113-8.

67. Zou MX, Liu HY, Haraguchi Y, Soda Y, Tatemoto K, Hoshino H. Apelin peptides block the entry of human immunodeficiency virus (hiv). *Febs Letters*. 2000;473(1):15-8.

68. Newson MJF, Pope GR, Roberts EM, Lolait SJ, O'Carroll AM. Stress-dependent and gender-specific neuroregulatory roles of the apelin receptor in the hypothalamic-

pituitary-adrenal axis response to acute stress. *The Journal of Endocrinology*. 2013;216(1):99-109.

69. Habata Y, Fujii R, Hosoya M, Fukusumi S, Kawamata Y, Hinuma S, et al. Apelin, the natural ligand of the orphan receptor apj, is abundantly secreted in the colostrum. *Biochimica et Biophysica Acta*. 1999;1452(1):25-35.

70. Horiuchi Y, Fujii T, Kamimura Y, Kawashima K. The endogenous, immunologically active peptide apelin inhibits lymphocytic cholinergic activity during immunological responses. *Journal of Neuroimmunology*. 2003;144(1-2):46-52.

71. Leeper NJ, Tedesco MM, Kojima Y, Schultz GM, Kundu RK, Ashley EA, et al. Apelin prevents aortic aneurysm formation by inhibiting macrophage inflammation. *American Journal of Physiology- Heart and Circulatory Physiology.* 2009;296(5):1329-35.

72. Anger WK. Workplace exposures. *Neurobehavioral toxicology*. 1986;331-47.

73. United States. Congress. Office of Technology Assessment. Neurotoxicity : identifying and controlling poisons of the nervous system : summary : new developments in neuroscience; 1990 [cited 2019 12 February] Available from: https://www.princeton.edu/~ota/disk2/1990/9031/9031.PDF.

74. Mohammad Ahmadi Soleimani S, Ekhtiari H, Cadet JL. Drug-induced neurotoxicity in addiction medicine: from prevention to harm reduction. *Progress in brain research*. 2016;223:19-41.

75. Reiter LW. Neurotoxicology in regulation and risk assessment. *Developmental Pharmacology and Therapeutics*. 1987;10(5):354-68.

76. Arlien-Søborg PS, Leif. Chemical neurotoxic agents: ilo encyclopaedia of occupational health and safety; 2011 [cited 2019 14 February] Available from: http://iloencyclopaedia.org/component/k2/item/289-chemical-neurotoxic-agents.

77. Almeida WF. The dangers and the precautions. *World health*. 1984;10-2.

78. Risk Assessment Forum USEPA. Guidelines for neurotoxicity risk assessment. 1998 [cited 2019 16 February ] Available from: https://www.epa.gov/sites/production/files/2014-11/documents/neuro_tox.pdf.

79. Sies H. Oxidative stress: from basic research to clinical application. *The American Journal of Medicine*. 1991;91(3):31-8.

80. Kelly FJ. Oxidative stress: its role in air pollution and adverse health effects. *Occupational and Environmental Medicine*. 2003;60(8):612-6.

81. Lu JM, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *Journal of Cellular and Molecular Medicine*. 2010;14(4):840-60.

82. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry*. 2015;30(1):11-26.

83. Ermak G, Davies KJ. Calcium and oxidative stress: from cell signaling to cell death. *Molecular Immunology*. 2002;38(10):713-21.

84. Cross AR, Jones OTG. Enzymic mechanisms of superoxide production. *Biochimica et Biophysica Acta - Bioenergetics.* 1991;1057(3):281-98.

85. Schrader M, Fahimi HD. Peroxisomes and oxidative stress. *Biochimica et Biophysica Acta*. 2006;1763(12):1755-66.

86. Dosek A, Ohno H, Acs Z, Taylor AW, Radak Z. High altitude and oxidative stress. *Respiratory Physiology and Neurobiology*. 2007;158(2):128-31.

87. Maiti P, Singh SB, Sharma AK, Muthuraju S, Banerjee PK, Ilavazhagan G. Hypobaric hypoxia induces oxidative stress in rat brain. *Neurochemistry International*. 2006;49(8):709-16.

88. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. Hif-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metabolism*. 2006;3(3):187-97.

89. Pollack M LC. Molecular mechanisms of oxidative stress in aging: free radicals, aging, antioxidants and disease. *Elsevier science bv handbook of oxidants and antioxidants in exercise*. 1999:881-923.

90. Huang X, Moir RD, Tanzi RE, Bush AI, Rogers JT. Redox-active metals, oxidative stress, and alzheimer's disease pathology. *Annals of the New York Academy of Sciences*. 2004;1012(1):153-63.

91. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology*. 2007;39(1):44-84.

92. Floyd RA, Hensley K. Oxidative stress in brain aging: implications for therapeutics of neurodegenerative diseases. *Neurobiology of Aging*. 2002;23(5):795-807.

93. Rivas-Arancibia S, Guevara-Guzman R, Lopez-Vidal Y, Rodriguez-Martinez E, Zanardo-Gomes M, Angoa-Perez M, et al. Oxidative stress caused by ozone exposure induces loss of brain repair in the hippocampus of adult rats. *Toxicological Sciences*. 2010;113(1):187-97.

94. Santiago-Lopez D, Bautista-Martinez JA, Reyes-Hernandez CI, Aguilar-Martinez M, Rivas-Arancibia S. Oxidative stress, progressive damage in the substantia nigra and plasma dopamine oxidation, in rats chronically exposed to ozone. *Toxicology Letters*. 2010;197(3):193-200.

95. Guesmi F, Bellamine H, Landoulsi A. Hydrogen peroxide-induced oxidative stress, acetylcholinesterase inhibition, and mediated brain injury attenuated by thymus algeriensis. *Applied Physiology, Nutrition, and Metabolism*. 2018;43(12):1275-81.

96. Desagher S, Glowinski J, Premont J. Astrocytes protect neurons from hydrogen peroxide toxicity. *Journal of Neuroscience*. 1996;16(8):2553-62.

97. Ratan RR, Murphy TH, Baraban JM. Oxidative stress induces apoptosis in embryonic cortical neurons. *Journal of Neurochemistry*. 1994;62(1):376-9.

98. Whittemore ER, Loo DT, Cotman CW. Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons. *Neuroreport*. 1994;5(12):1485-8.

99. Whittemore ER, Loo DT, Watt JA, Cotman CW. A detailed analysis of hydrogen peroxide-induced cell death in primary neuronal culture. *Neuroscience*. 1995;67(4):921-32.

100. Hyslop PA, Zhang Z, Pearson DV, Phebus LA. Measurement of striatal h2o2 by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of h2o2 in vitro. *Brain Research.* 1995;671(2):181-6.

101. Lafemina MJ, Sheldon RA, Ferriero DM. Acute hypoxia-ischemia results in hydrogen peroxide accumulation in neonatal but not adult mouse brain. *Pediatric Research*. 2006;59(5):680-3.

102. Niedzielska E, Smaga I, Gawlik M, Moniczewski A, Stankowicz P, Pera J, et al. Oxidative stress in neurodegenerative diseases. *Molecular Neurobiology*. 2016;53(6):4094-125.

103. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron*. 2003;39(6):889-909.

104. Pearce RK, Owen A, Daniel S, Jenner P, Marsden CD. Alterations in the distribution of glutathione in the substantia nigra in parkinson's disease. *Journal of Neural Transmission.* 1997;104(6-7):661-77.

105. Ayton S, Lei P. Nigral iron elevation is an invariable feature of parkinson's disease and is a sufficient cause of neurodegeneration. *BioMed Research International*. 2014;2014:581256.

106. Heikkila RE, Cohen G. 6-Hydroxydopamine: Evidence for superoxide radical as an oxidative Intermediate. *Science*. 1973;181(4098):456-7.

107. Wang X, Michaelis EK. Selective neuronal vulnerability to oxidative stress in the brain. *Frontiers in Aging Neuroscience*. 2010;2:12.

108. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. *Brain Research*. 1999;823(1-2):1-10.

109. McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, et al. Environmental risk factors and parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiology of Disease*. 2002;10(2):119-27.

110. Moratalla R, Quinn B, DeLanney LE, Irwin I, Langston JW, Graybiel AM. Differential vulnerability of primate caudate-putamen and striosome-matrix dopamine systems to the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proceedings of The National Academy of Sciences of The United States of America*. 1992;89(9):3859-63.

111. Johnson ME, Bobrovskaya L. An update on the rotenone models of parkinson's disease: their ability to reproduce the features of clinical disease and model gene-environment interactions. *Neurotoxicology*. 2015;46:101-16.

112. Butterfield DA, Perluigi M, Sultana R. Oxidative stress in alzheimer's disease brain: new insights from redox proteomics. *European Journal of Pharmacology*. 2006;545(1):39-50.

113. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology*. 2009;7(1):65-74.

114. Budimir A. Metal ions, alzheimer's disease and chelation therapy. *Acta Pharmaceutica*. 2011;61(1):1-14.

115. Good PF, Perl DP, Bierer LM, Schmeidler J. Selective accumulation of aluminum and iron in the neurofibrillary tangles of Alzheimer's disease: a laser microprobe (lamma) study. *Annals of Neurology*. 1992;31(3):286-92.

116. Chen H, Yoshioka H, Kim GS, Jung JE, Okami N, Sakata H, et al. Oxidative stress in ischemic brain damage: mechanisms of cell death and potential molecular targets for neuroprotection. *Antioxidants and Redox Signaling*. 2011;14(8):1505-17.

117. Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. *Journal of Cerebral Blood Flow and Metabolism*. 2001;21(1):2-14.

118. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of The National Academy of Sciences of The United States of America*. 1990;87(4):1620-4.

119. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, et al. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science*. 2002;297(5584):1186-90.

120. Gonsette RE. Neurodegeneration in multiple sclerosis: the role of oxidative stress and excitotoxicity. *Journal of the Neurological Sciences*. 2008;274(1-2):48-53.

121. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology*. 2009;7(1):65-74.

122. Barber SC, Shaw PJ. Oxidative stress in als: key role in motor neuron injury and therapeutic target. *Free Radical Biology and Medicine*. 2010;48(5):629-41.

123. Ihara Y, Nobukuni K, Takata H, Hayabara T. Oxidative stress and metal content in blood and cerebrospinal fluid of amyotrophic lateral sclerosis patients with and without a cu, zn-superoxide dismutase mutation. *Neurological Research*. 2005;27(1):105-8.

124. Smith RG, Henry YK, Mattson MP, Appel SH. Presence of 4-hydroxynonenal in cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis. *Annals of Neurology*. 1998;44(4):696-9.

125. Shaw PJ, Ince PG, Falkous G, Mantle D. Oxidative damage to protein in sporadic motor neuron disease spinal cord. *Annals of Neurology*. 1995;38(4):691-5.

126. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in cu/zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*. 1993;362(6415):59-62.

127. Scharfman HE. The neurobiology of epilepsy. *Current Neurology and Neuroscience reports*. 2007;7(4):348-54.

128. Chang SJ, Yu BC. Mitochondrial matters of the brain: mitochondrial dysfunction and oxidative status in epilepsy. *Journal of Bioenergetics and Biomembranes*. 2010;42(6):457-9.

129. Liang LP, Patel M. Seizure-induced changes in mitochondrial redox status. *Free Radical Biology and Medicine*. 2006;40(2):316-22.

130. Bruce AJ, Baudry M. Oxygen free radicals in rat limbic structures after kainate-induced seizures. *Free Radical Biology and Medicine*. 1995;18(6):993-1002.

131. Gluck MR, Jayatilleke E, Shaw S, Rowan AJ, Haroutunian V. CNS oxidative stress associated with the kainic acid rodent model of experimental epilepsy. *Epilepsy Research*. 2000;39(1):63-71.

132. Chuang YC. Mitochondrial dysfunction and oxidative stress in seizure-induced neuronal cell death. *Acta Neurologica Taiwanica*. 2010;19(1):3-15.

133. Cock HR. The role of mitochondria and oxidative stress in neuronal damage after brief and prolonged seizures. *Progress in Brain Research*. 2002;135(16):187-96.

134. Salim S. Oxidative stress and psychological disorders. *Current Neuropharmacology*. 2014;12(2):140-7.

135. Lei D-L, Manaye KF, Dávila-García MI, Tizabi Y, Mouton PR, Kelly PH. Selective neuron loss in the paraventricular nucleus of hypothalamus in patients suffering from major depression and bipolar disorder. *Journal of Neuropathology and Experimental Neurology*. 2005;64(3):224-9.

136. Chen X, Pan W. The treatment strategies for neurodegenerative diseases by integrative medicine. *Integrative Medicine International.* 2014;1(4):223-5.

137. Goldenberg MM. Medical management of parkinson's disease. *P & T : a Peer-r eviewed Journal for Formulary Management*. 2008;33(10):590-606.

138. scarpini E, Schelterns P, Feldman H. Treatment of alzheimer's disease; current status and new perspectives. *The Lancet Neurology*. 2003;2(9):539-47.

139. Stewart WF, Kawas C, Corrada M, Metter EJ. Risk of alzheimer's disease and duration of nsaid use. *Neurology*. 1997;48(3):626-32.

140. de Sa JCC, Airas L, Bartholome E, Grigoriadis N, Mattle H, Oreja-Guevara C, et al. Symptomatic therapy in multiple sclerosis: a review for a multimodal approach in clinical practice. *Therapeutic Advances in Neurological Disorders*. 2011;4(3):139-68.

141. Bansal S, Sangha KS, Khatri P. Drug treatment of acute ischemic stroke. *American Journal of Cardiovascular Drugs : Drugs, Devices, and Other Interventions*. 2013;13(1):57-69.

142. Liu G, Slater N, Perkins A. Epilepsy: treatment options. *American Family Physician*. 2017;96(2):87-96.

143. Miller RG, Mitchell JD, Moore DH. Riluzole for amyotrophic lateral sclerosis (als)/motor neuron disease (mnd). *Cochrane Database of Systematic Reviews*. 2012(3):Cd001447.

144. Behl C. Antioxidants as treatment for neurodegenerative disorders. *Expert Opinion on Investigational Drugs*. 2002;11(10):1407-35.

145. Vercruysse P, Vieau D, Blum D, Petersén Å, Dupuis L. Hypothalamic alterations in neurodegenerative diseases and their relation to abnormal energy Metabolism. *Frontiers in Molecular Neuroscience*. 2018;11:2.

146. Marc S, Ramon G, Marc C. Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance. *Journal of Endocrinology.* 2014;220(2):25-46.

147. Shahid Z SG. Physiology, hypothalamus. : statpearls; 2018. [cited 2019 18 March] Available from: https://www.ncbi.nlm.nih.gov/books/NBK535380/.

148. Gyengesi E, Paxinos G, Andrews ZB. Oxidative Stress in the hypothalamus: the importance of calcium signaling and mitochondrial ros in body weight regulation. *Current Neuropharmacology*. 2012;10(4):344-53.

149. Berne RM LM. *Principles of physiology*. 3rd ed. St. Louis., Mo: Elsevier Mosby-Year Book, Inc., ; 1998.

150. Perez-Reyes E. Molecular physiology of low-voltage-activated t-type calcium channels. *Physiological Reviews*. 2003;83(1):117-61.

151. Stojilkovic SS, Zemkova H, Van Goor F. Biophysical basis of pituitary cell type-specific ca2+ signaling-secretion coupling. *Trends in Endocrinology and Metabolism*. 2005;16(4):152-9.

152. Van Goor F, Zivadinovic D, Stojilkovic SS. Differential expression of ionic channels in rat anterior pituitary cells. *Molecular Endocrinology*. 2001;15(7):1222-36.

153. Berridge MJ. Neuronal calcium signaling. *Neuron*. 1998;21(1):13-26.

154. Carrasco MA, Hidalgo C. Calcium microdomains and gene expression in neurons and skeletal muscle cells. *Cell Calcium*. 2006;40(5-6):575-83.

155. Dolmetsch R. Excitation-transcription coupling: signaling by ion channels to the nucleus. *Science Signaling*. 2003;2003(166):4.

156. Andressen C, Blumcke I, Celio MR. Calcium-binding proteins: selective markers of nerve cells. *Cell and Tissue Research*. 1993;271(2):181-208.

157. Nicholls DG. Mitochondrial calcium function and dysfunction in the central nervous system. *Biochimica et Biophysica Acta*. 2009;1787(11):1416-24.

158. Boyadjieva NI, Sarkar DK. Microglia play a role in ethanol-induced oxidative stress and apoptosis in developing hypothalamic neurons. *Alcohol, Clinical and Experimental Research*. 2013;37(2):252-62.

159. Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, et al. Alpha-synuclein promotes mitochondrial deficit and oxidative stress. *The American Journal of Pathology*. 2000;157(2):401-10.

160. Roussou P, Tsagarakis NJ, Kountouras D, Livadas S, Diamanti-Kandarakis E. Beta-thalassemia major and female fertility: the role of iron and iron-induced oxidative stress. *Anemia*. 2013;2013:617204.

161. Ansari S, Azarkeivan A, Tabaroki A. Pregnancy in patients treated for beta thalassemia major in two centers (ali asghar children's hospital and thalassemia clinic): outcome for mothers and newborn infants. *Pediatric Hematology and Oncology*. 2006;23(1):33-7.

162. Safarinejad MR. Reproductive hormones and hypothalamic-pituitary-ovarian axis in female patients with homozygous beta-thalassemia major. *Journal of Pediatric Hematology/Oncology.* 2010;32(4):259-66.

163. Tsutsumi R, Webster NJG. Gnrh pulsatility, the pituitary response and reproductive dysfunction. *Endocrine Journal*. 2009;56(6):729-37.

164. Baba Y, Matsuo H, Schally AV. Structure of the porcine lh- and fsh-releasing hormone. II. confirmation of the proposed structure by conventional sequential analyses. *Biochemical and Biophysical Research Communications.* 1971;44(2):459-63.

165. Matsuo H, Arimura A, Nair RM, Schally AV. Synthesis of the porcine lh- and fsh-releasing hormone by the solid-phase method. *Biochemical and Biophysical Research Communications*. 1971;45(3):822-7.

166. Schally AV, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, et al. Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. *Science*. 1971;173(4001):1036-8.

167. Schally AV, Arimura A, Baba Y, Nair RM, Matsuo H, Redding TW, et al. Isolation and properties of the fsh and lh-releasing hormone. *Biochemical and Biophysical Research Communications*. 1971;43(2):393-9.

168. Barron AM, Fuller SJ, Verdile G, Martins RN. Reproductive hormones modulate oxidative stress in alzheimer's disease. *Antioxidants and Redox Signaling*. 2006;8(11-12):2047-59.

169. Massafra C, Gioia D, De Felice C, Muscettola M, Longini M, Buonocore G. Gender-related differences in erythrocyte glutathione peroxidase activity in healthy subjects. *The Journal of Clinical Endocrinology and Metabolism*. 2002;57(5):663-7.

170. Hall ED, Pazara KE, Linseman KL. Sex differences in postischemic neuronal necrosis in gerbils. *Journal of Cerebral Blood Flow and Metabolism*. 1991;11(2):292-8.

171. Signorelli SS, Neri S, Sciacchitano S, Pino LD, Costa MP, Marchese G, et al. Behaviour of some indicators of oxidative stress in postmenopausal and fertile women. *Maturitas*. 2006;53(1):77-82.

172. Vural P, Akgul C, Canbaz M. Effects of menopause and tibolone on antioxidants in postmenopausal women. *Annals of clinical biochemistry*. 2005;42(3):220-3.

173. Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic gnrh neurons by genetically targeted tumorigenesis. *Neuron*. 1990;5(1):1-10.

174. Caroline MaF, D. Nervous system disorders induced by occupational and environmental toxic exposure. *Open Journal of Preventive Medicine*. 2012;2(3):272-8.

175. Blesa J, Przedborski S. Parkinson's disease: animal models and dopaminergic cell vulnerability. *Frontiers in Neuroanatomy*. 2014;8(155):9.

176. Su B, Wang X, Nunomura A, Moreira PI, Lee Hg, Perry G, et al. Oxidative stress signaling in alzheimer's disease. *Current Alzheimer research*. 2008;5(6):525-32.

177. Selim MH, Ratan RR. The role of iron neurotoxicity in ischemic stroke. *Ageing Research Reviews*. 2004;3(3):345-53.

178. Adamczyk-Sowa BAaM. New insights into the role of oxidative stress mechanisms in the pathophysiology and treatment of multiple sclerosis. *Oxidative Medicine and Cellular Longevity*. 2016;2016:18.

179. Aguiar CCT, Almeida AB, Araújo PVP, de Abreu RNDC, Chaves EMC, do Vale OC, et al. Oxidative stress and epilepsy: literature review. *Oxidative Medicine and Cellular Longevity*. 2012;2012:795259.

180. World Alzheimer Report 2018. The state of the art of dementia research: new frontiers. london: alzheimer's disease international. alzheimer's disease international (adi), london. 2018 [cited 2019 30 March]. Available from: https://www.alz.co.uk/research/WorldAlzheimerReport2018.pdf.

181. Dorsey ER, Sherer T, Okun MS, Bloem BR. The emerging evidence of the parkinson pandemic. *Journal of Parkinson's Disease*. 2018;8(1):3-8.

182. Browne P, Chandraratna D, Angood C, Tremlett H, Baker C, Taylor BV, et al. Atlas of multiple sclerosis 2013: a growing global problem with widespread inequity. *Neurology*. 2014;83(11):1022.

183. Beghi E, Giussani G, Nichols E, Abd-Allah F, Abdela J, Abdelalim A, et al. Global, regional, and national burden of epilepsy, 1990&2013;2016: a systematic analysis

for the global burden of disease study 2016. *The Lancet Neurology*. 2019;18(4):357-75.

184. Beghi E, Logroscino G, Chiò A, Hardiman O, Mitchell D, Swingler R, et al. The epidemiology of als and the role of population-based registries. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 2006;1762(11):1150-7.

185. Feigin VL, Norrving B, Mensah GA. Global burden of stroke. *Circulation Research*. 2017;120(3):439-48.

186. Miana Gabriela Pop CCaIO. Anatomy and function of the hypothalamus. Hypothalamus in health and diseases. 2018. [ cited 2019 30 March ] Available from: https://www.intechopen.com/books/hypothalamus-in-health-and-diseases/anatomy-and-function-of-the-hypothalamus.

187. Utiger RD. Hypothalamus: encyclopædia britannica, inc. [ cited 2019 30 March ] Available from: https://www.britannica.com/science/hypothalamus.

188. de Lacalle S, Iraizoz I, Gonzalo LM. Cell loss in supraoptic and paraventricular nucleus in alzheimer's disease. *Brain Research*. 1993;609(1-2):154-8.

189. Sandyk R, Iacono RP, Bamford CR. The hypothalamus in parkinson disease. *The Italian Journal of Neurological Sciences*. 1987;8(3):227-34.

190. Politis M, Piccini P, Pavese N, Koh S-B, Brooks DJ. Evidence of dopamine dysfunction in the hypothalamus of patients with parkinson's disease: an in vivo11c-raclopride pet study. *Experimental Neurology*. 2008;214(1):112-6.

191. Schultz C, Ghebremedhin E, Braak H, Braak E. Neurofibrillary pathology in the human paraventricular and supraoptic nuclei. *Acta Neuropathologica*. 1997;94(1):99-102.

192. Burfeind KG, Yadav V, Marks DL. Hypothalamic dysfunction and multiple sclerosis: implications for fatigue and weight dysregulation. *Current Neurology and Neuroscience Reports*. 2016;16(11):98.

193. Radak D, Resanovic I, Isenovic ER. Changes in hypothalamus-pituitary-adrenal axis following transient ischemic attack. *Angiology*. 2014;65(8):723-32.

194. Pouresmaeili-Babaki E, Esmaeili-Mahani S, Abbasnejad M, Ravan H. Protective effect of neuropeptide apelin-13 on 6-hydroxydopamine-induced neurotoxicity in sh-sy5y dopaminergic cells: involvement of its antioxidant and antiapoptotic properties. *Rejuvenation Research*. 2018;21(2):162-7.

195. Xin Q, Cheng B, Pan Y, Liu H, yang C, Chen J, et al. Neuroprotective effects of apelin-13 on experimental ischemic stroke through suppression of inflammation. *Peptides*. 2015;63:55-62.

196. Cook DR, Gleichman AJ, Cross SA, Doshi S, Ho W, Jordan-Sciutto KL, et al. Nmda receptor modulation by the neuropeptide apelin: implications for excitotoxic injury. *Journal of Neurochemistry*. 2011;118(6):1113-23.

197. Cheng B, Chen J, Bai B, Xin Q. Neuroprotection of apelin and its signaling pathway. *Peptides*. 2012;37(1):171-3.

198. Zeng XJ, Yu SP, Zhang L, Wei L. Neuroprotective effect of the endogenous neural peptide apelin in cultured mouse cortical neurons. *Experimental Cell Research.* 2010;316(11):1773-83.

199. Dogan A. Apelin receptor (aplnr) signaling promotes fibroblast migration. *Tissue and Cell*. 2019;56:98-106.

200. Ishimaru Y, Shibagaki F, Yamamuro A, Yoshioka Y, Maeda S. An apelin receptor antagonist prevents pathological retinal angiogenesis with ischemic retinopathy in mice. *Scientific Reports*. 2017;7(1):1-9.

201. Hall C, Ehrlich L, Venter J, O'Brien A, White T, Zhou T, et al. Inhibition of the apelin/apelin receptor axis decreases cholangiocarcinoma growth. *Cancer Letters.* 2017;100(386:)179-88.

202. Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biology*. 2017;11:613-9.

203. Wijeratne SSK, Cuppett SL, Schlegel V. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in caco-2 human colon cells. *Journal of Agricultural and Food Chemistry.* 2005;53(22):8768-74.

204. Wei T, Ni Y, Hou J, Chen C, Zhao B, Xin W. Hydrogen peroxide-induced oxidative damage and apoptosis in cerebellar granule cells: protection by ginkgo biloba extract. *Pharmacological Research*. 2000;41(4):427-33.

205. Bandyopadhyay U, Das D, Banerjee RK. Reactive oxygen species: oxidative damage and pathogenesis. *Current Science*. 1999;77(5):658-66.

206. Xie H, Yuan L-Q, Luo X-H, Huang J, Cui R-R, Guo L-J, et al. Apelin suppresses apoptosis of human osteoblasts. *Apoptosis*. 2007;12(1):247-54.

207. Jiang Y, Liu H, Ji B, Wang Z, Wang C, Yang C, et al. Apelin13 attenuates er stress associated apoptosis induced by MPP+ in SHSY5Y cells. *International Journal of Molecular Medicine*. 2018;42(3):1732-40.

208. Qin D, Zheng XX, Jiang YR. Apelin-13 induces proliferation, migration, and collagen I mRNA expression in human rpe cells via pi3k/akt and mek/erk signaling pathways. *Molecular Vision*. 2013;19:2227-36.

209. Shuang L, Jidong W, Hongjuan P, Zhenwei Y. Effects of apelin on proliferation and apoptosis in rat ovarian granulosa cells. *Clinical and Experimental Obstetrics and Gynecology*. 2016;43(3):409-13.

210. Cui RR, Mao DA, Yi L, Wang C, Zhang XX, Xie H, et al. Apelin suppresses apoptosis of human vascular smooth muscle cells via apj/pi3k/akt signaling pathways. *Amino Acids*. 2010;39(5):1193-200.

211. Boal F, Timotin A, Roumegoux J, Alfarano C, Calise D, Anesia R, et al. Apelin-13 administration protects against ischaemia/reperfusion-mediated apoptosis through the foxo1 pathway in high-fat diet-induced obesity. *British Journal of Pharmacology*. 2016;173(11):1850-63.

212. Han S, Englander EW, Gomez GA, Greeley GH, Jr. Apelin regulates nuclear factor-kappab's involvement in the inflammatory response of pancreatitis. *Pancreas*. 2017;46(1):64-70.

213. Han S, Englander EW, Gomez GA, Aronson JF, Rastellini C, Garofalo RP, et al. Pancreatitis activates pancreatic apelin-apj axis in mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2013;305(2):G139-50.

214. Yang Y, Zhang X, Cui H, Zhang C, Zhu C, Li L. Apelin-13 protects the brain against ischemia/reperfusion injury through activating pi3k/akt and erk1/2 signaling pathways. *Neuroscience Letters*. 2014;568:44-9.

215. Jayalakshmi K, Sairam M, Singh SB, Sharma SK, Ilavazhagan G, Banerjee PK. Neuroprotective effect of n-acetyl cysteine on hypoxia-induced oxidative stress in primary hippocampal culture. *Brain Research.* 2005;1046(1):97-104.

216. Fridovich I. Superoxide anion radical (o2-.), superoxide dismutases, and related matters. *Journal of Biological Chemistry*. 1997;272(30):18515-7.

217. Lubos E, Loscalzo J, Handy DE. Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. *Antioxidants and Redox Signaling*. 2011;15(7):1957-97.

218. Schieber M, Chandel NS. Ros function in redox signaling and oxidative stress. *Current Biology.* 2014;24(10):R453-R62.

219. Than A, Zhang X, Leow MK-S, Poh CL, Chong SK, Chen P. Apelin attenuates oxidative stress in human adipocytes. *The Journal of Biological Chemistry.* 2014;289(6):3763-74.

220. Foussal C, Lairez O, Calise D, Pathak A, Guilbeau-Frugier C, Valet P, et al. Activation of catalase by apelin prevents oxidative stress-linked cardiac hypertrophy. *Febs Letters*. 2010;584(11):2363-70.

221. El-Bahr SM. Biochemistry of free radicals and oxidative Stress. *Science International*. 2013;1(5):111-7.

222. Aminyavari S, Zahmatkesh M, Farahmandfar M, Khodagholi F, Dargahi L, Zarrindast MR. Protective role of apelin-13 on amyloid beta25-35-induced memory deficit; involvement of autophagy and apoptosis process. *Progress in Neuropsychopharmacology and Biological Psychiatry*. 2019;89:322-34.

223. Tehrani AR, Gholipour S, Sharifi R, Yadegari S, Abbasi-kolli M, Masoudian N. Plasma levels of ctrp-3, ctrp-9 and apelin in women with multiple sclerosis. *Journal of Neuroimmunology*. 2019:576968.

224. Kasai A, Kinjo T, Ishihara R, Sakai I, Ishimaru Y, Yoshioka Y, et al. Apelin deficiency accelerates the progression of amyotrophic lateral sclerosis. *Plos One*. 2011;6(8):e23968.

225. Kalantaripour TP, Esmaeili-Mahani S, Sheibani V, Asadi-Shekaari M, Pasban-Aliabadi H. Anticonvulsant and neuroprotective effects of apelin-13 on pentylenetetrazole-induced seizures in male rats. *Biomedicine and Pharmacotherapy*. 2016;100(84):258-63.

226. Khaksari M, Aboutaleb N, Nasirinezhad F, Vakili A, Madjd Z. Apelin-13 protects the brain against ischemic reperfusion injury and cerebral edema in a transient model of focal cerebral ischemia. *Journal of Molecular Neuroscience*. 2012;48(1):201-8.