THE POTENTIAL ROLE OF GROWTH HORMONE SIGNALING IN ROTENONE-INDUCED NEUROTOXICITY IN SK-N-AS CELLS

by

Nayat Narot Ciroğlu

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APPROVED BY:

Prof. Dr. Mehveş Ece Genç ……
(Thesis Supervisor)

Prof. Dr. Ferda Kaleağasıoğlu ……

Assoc. Prof. Dr. Elif Damla Arısan ……

DATE OF APPROVAL: …./…./2016

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ABSTRACT

THE POTENTIAL ROLE OF GROWTH HORMONE SIGNALING IN ROTENONE-INDUCED NEUROTOXICITY IN SK-N-AS CELLS.

Rotenone is a widely used pesticide, leading to neurotoxic effects and may also affect humans. Growth hormone (GH) has been reported to have neuro-protective and/or neuro-repairing effects on neuronal cells. The aim of this thesis is to demonstrate the potential neuro-protective effect of induced GH expression in rotenone treated neuroblastoma SK-N-AS cells.

In order to accomplish this purpose, we generated GH expressed SK-N-AS neuroblastoma (NB) cells by using GH gene inserted PC3.1 plasmid by lipofectamine transfection and neomycin selection. The protective role of transfected GH expression on rotenone-induced neurotoxicity in SK-N-AS cells was investigated by cell viability assay, growth assay, soft agar and colony forming assay, the latter for determination of dose- and time-dependent rotenone effects. On protein level, rotenone effects on caspase 3, 7, 9 and Bcl-2 family members were investigated by immunoblotting in SK-N-AS wild type (wt) and SK-N-AS GH transfected cells for the evaluation of biological protective and repairing effect of GH signaling.

In cell viability assay, the amounts of vital cells were significantly higher in GH transfected SK-N-AS cells than wt SK-N-AS cells, but GH was not effective to completely protect NB cells from apoptosis. In addition, the antioxidative agent N-acetyl-L-cysteine (NAC) was effective in prevention of rotenone-induced apoptosis only in SK-N-AS wt cells. Immunoblotting of JAK/STAT signaling pathway proteins demonstrated a STAT1 increase in rotenone treated SK-N-AS GH+ transfected cells. The preference of SK-N-AS cells for the caspase-dependent (caspase 9 activation) intrinsic apoptosis pathway and the mitochondrial involvement including ROS generation could be confirmed.

ÖZET

BÜYÜME HORMON SİNYAL YOLAĞININ ROTENON TARAFINDAN SK-N-AS NEUROBLASTOMA HÜCRE HATTINDA İNDÜKLENEN NÖROTOKSİSİTE ÜZERINDEKİ POTANSİYEL ROLÜ

Rotenon, nörotoksik etkilerinden ötürü tarımda sıkça kullanılan bir pestizitdir. Bu pestizitle çalışan kişilerde uzun vaadede nörolojik problemler gelişebilir. Büyüme hormonunun (Growth hormone, GH) nöronal hücreleri koruyucu ve onarıcı rolü olduğu tespit edilmiştir. Bu tezin amacı GH'nun rotenon uygulanan SK-N-AS neuroblastoma hücreleri üzerinde göstereceği koruyucu ve onarıcı etkisini incelemektir.

Bu amaç doğrultusunda, birinci basamak GH geni PC3.1 plazmidi lipozom türevli bir ajan ile aktarılmış ve neomisin uygulanarak GH salgılayan SK-N-AS hücreleri üretilmiştir. GH'nin Rotenon tarafından tetiklenen nörotoksik etkisini koruyucu aktivitesini GH aktarilmiş SK-N-AS hücrelerinde görebilmek ve araştirmak için MTT hücre canlılık testi, büyüme testi, yumuşak agar ve koloni oluşturması deneyleri kullanılmıştır. Bu testlerle, rotenonun SK-N-AS hücreleri üzerindeki doza ve zamana bağlı olan ilişkisi de incelenmiştir. GH'nin ve rotenonun biyolojik etkileri ise protein seviyesinde immunoblotting tekniğiyle kaspaz 3, 7, 9 ve Bcl-2 ailesi üzerinde gözlendi.

Sonuç olarak, GH salgılanması SK-N-AS hücrelerinin büyümesini ve çoğalmasını sağlamasına ve bazı testlerde rotenona karşı belli bir koruyucu etki oluşturmasına rağmen, rotenonun negatif etkilerine tamamıyla nötralize etmeye ve apoptozdan korumaya yeterli olmadığı gözlenmistir. Ayrıca rotenona ilaveten antioksidatif ajan N-asetilsistein (NAC) kullanıldığında NAC'in sadece GH aktarılmamış SK-N-AS hücrelerinde apoptozu engelleyebildigi tespit edilmiştir. JAK/STAT sinyal yolağı proteinlerin immunoblotla incelenmeside sadece STAT1 proteininde dikkate değer bir artış gözlenmiştir. Buna ilaveten yine immunoblot analizinde SK-N-AS hücrelerinin apoptozda kaspaz 9 üzerinden mitokondriyal içsel apoptoz yolağını tercih ettikleri onaylanmıştır.

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ALK	Anaplastic lymphoma kinase
APS	Ammonium persulfate
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology
bHlH	Basic helix-loop-helix
BSA	Bovine serum albumin
Ca^{++}	Calcium
CLB	Cell lysis buffer
CO_2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
CARD	Caspase recruitment domain
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DED	Death effector domain
DISC	Death Inducing Signaling Complex
DMs	Double minutes
DIOC6	3,3'-Dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
ETC	electron transport chain
FADD	Fas associated death domain
GH	Growth hormone
GHR	Growth hormone Receptor
GHRH	Growth hormone releasing hormone
GHRHR	Growth hormone releasing hormone receptor
HSR	Homogeneously staining region
INRG	International neuroblastoma risk group
INSS	International neuroblastoma staging system
JAK	Janus kinase
kDa	Kilo Dalton

LOH	Loss of heterozygosity
MAPKs	Mitogen-activated protein kinases
µL	Microliter
mL	Milliliter
µM	Micromolar
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test
MYCN	N-myc proto-oncogene protein
NAC	N-acetyl-L-cysteine
NB	Neuroblastoma
NEAA	Non-Essential amino acid
nM	Nanomolar
PARP	Poly ADP ribose polymerase
PBS	Phosphate-buffered saline
PD	Parkinson disease
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Sodium dodecyl sulfate
STAT	Signal transducer and activator of transcription
PUMA	p53 upregulated modulator of apoptosis
p-STAT	Phosphor Signal transducer and activator of transcription
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TNF	Tumor Necrosis Factors
Wt	Wild Type

1. INTRODUCTION

Neuroblastoma (NB) is a small round blue cell malignant tumor originating from the embryonal sympathetic nervous cells of the neural crest. NB is the most common extracranial solid tumor with a fatal course and the second most common abdominal neoplasm in young children, mostly diagnosed at an age of 17 months, and occurs slightly more common in boys than among girls. Stage IV NB is the most aggressive and fatal form. Therefore, new treatments are urgently needed and being currently investigated.

Rotenone is a well-known and widely used broad spectrum pesticide and was originally isolated from plant roots or leaves. It is a high-affinity inhibitor of complex I and binds to the mitochondrial electron transport chain (ETC) setting free lots of electrons that react with oxygen molecules and produce O_2^-. Increasing O_2^- leads to increasing formation of reactive oxygen species (ROS) which damage mitochondria irreversibly. In dopaminergic neuronal cells, rotenone-induced cumulative oxidative damage leads to an increase of intracellular calcium (Ca^{+2}) levels and directs the cells to apoptosis *via* activating of caspases.

Growth hormone (GH) is not solely responsible for the individual organ and/or whole body growth but also for the regulation of carbohydrate, protein and lipid metabolism. On the other hand, GH has been reported to have a neuro-protective and/or neuro-reparative effect on neuronal cells as well. The aim of this thesis is to demonstrate the potential role of neuro-protective effect of forced GH expression in rotenone treated neuronal cells.

In this study, the toxic effect of rotenone was investigated in wild type (wt) and GH transfected (GH+) SK-N-AS NB cancer cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test) cell viability assay, growth assay, soft agar, colony forming assay. Furthermore, the changes in the expression levels of the Bcl-2 (B-cell lymphoma 2), caspase and Janus kinase/ Signal transducer and activator of transcription, JAK/STAT, family members were detected by immunoblotting method. Additionally, apoptosis was investigated by PI (Propidium iodide) staining and DNA fragmentation by DAPI (4',6-diamidino-2-phenylindole) staining. 3,3'-Dihexyloxacarbocyanine iodide (DIOC6) dye was used for the specificstaining of the mitochondrial membrane and to visualize the mitochondria membrane potential (also transmembrane potential or

membrane voltage defined as the difference in electric potential between the interior and the exterior site of the cell). In addition rotenone induced ROS were shown by cell viability determination and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) fluorescent dye.

As a result, rotenone decreased cell viability in GH+ SK-N-AS cells more than in wt SK-N-AS cells after 24 h of application. Rotenone down-regulated p-STAT1 (phosphorylated STAT1) and JAK2 expression and increased caspase activity on protein level in GH+ SK-N-AS cells more than in wt SK-N-AS cells resulting in a higher apoptosis rate in GH+ SK-N-AS cells. In MTT assay, addition of N-acetyl cysteine (NAC) increased cell viability in wt SK-N-AS cells more than in GH+ SK-N-AS cells, probably by more effectively reduction of rotenone induced ROS generation. However, there was no difference between wt and GH+ SK-N-AS in the fluorescent microscopic staining and pictures in regard with cell death, DNA fragmentation and destruction of mitochondrial membranes.

This study presents the novel results of the potential role of GH expression in SK-N-AS neuroblastoma cells in response to rotenone toxicity.

1.1. AIM

Rotenone leads to an increased level of cellular ROS generation and kills the dopaminergic neurons. GH has been shown to have a significant role in regulation of neuro-protective and/or neuro-restorative mechanisms in the brain, especially in natural aging or neuro-degenerative diseases. GH is one of the neurotrophic factors with neuro-developmental role after injury. The aim of this thesis is to demonstrate the potential role of neuro-protective effect of forced GH expression in rotenone treatment in neuronal cells.

2. GENERAL INSTRUCTIONS

2.1. NEUROBLASTOMA

Neuroblastoma (NB) is a malignant embryonal tumor and the most common fatal extracranial solid tumor in children (Table 2.1). NB is originating from the neural crest of the sympathetic nervous system. It generates 8-12% of all childhood cancers and approximately 15% of childhood deaths caused by pediatric tumors [1]. NB is a small round blue cell tumor and resembles to rhabdomyosarcoma, non-hodghin's lymphoma and Ewing's sarcoma [2, 3]. There are four types of neuroblastoma tumors described under 2.3 in detail that can be classified according to the prognosis in three risk groups as low, intermediate and high risk group [4]depending on the age of affected children at the time point of diagnosis, the tumor stage and biology, amplication of N-myc proto-oncogene and DNA ploidy [5].

In Turkey as in most other European countries, neuroblastoma incidence is 1 in 7000 live births per year.

Table 2.1. The most common types of cancer in childhood [6].

Name of disease	Incidence (%)
Acute leukemia	27.5
Central Nervous System Tumors (CNS)	20.7
Lymphoma	11.3
Neuroblastoma	7.3
Kidney tumors	6.1
Osteoma	4.7
Rhabdomyosarcoma	3.4
Retinoblastoma	2.9
Other tumors	16.1

2.1.1. Epidemiology

Neuroblastoma manifests in infants generally at an age of 17 months. Neuroblastic tumors metastasize in 40% of cases into adrenal gland, in 25% of cases into liver, in 15% into thoracic organs, in 5% into cervical and pelvic sympathetic ganglia. More than 36% of the patients are diagnosed before the age of 1, 90% before the age of 5 and 97% of by the age of 10. 1-2% of the cases are family inherited (Serap Aksoylar, Department of Pediatric Oncology, Medical Faculty of Ege University).

- Frequency: Neuroblastoma is the fourth most common pediatric cancer among all pediatric neoplasms [7, 8]. NB is also the most frequent solid tumor in children under 4 years [9]. Incidences of the different NB stages at the time point of diagnosis are shown below.

Table 2.2. Tumor stages and their percentages [10, 11]

Tumor Stage	**frequency %**
I	6 - 10%
II	7 - 10%
III	22 - 26%
IV	45 - 59%
IVS	9 - 10%

With increasing tumor stages the prognosis of NB turns poorer except in case of stage IVS which has a less poor prognosis than stage IV.

- Age: The median age of infants with neuroblastoma for stage I is 7 months, stage II 8 months, stage III 21 months, stage IV 32 months and stage IVS is 3 months. However, familial neuroblastoma tumors are found in 9 month old babies. Although NB is a pediatric tumor it occasionally occurs also in adults [12, 13].
- Sex: Neuroblastoma is slightly more common in boys than girls with a rate of 1.2:1 [14, 15].

- Ethnic difference: Incidence rate is higher in Caucasian than in Hispanic and black population [15, 16].
- Geographical differences: Neuroblastoma incidence varies according to geographical regions. The frequency of NB in equatorial Central America, India and China is lower than e.g. in Pakistan and the United Arab Emirates' where the incidence rate is similar to the European population. In contrast, North America, Israel and Australia show the highest rates of NB [8].
- Survival: Children under 1 year of age have significant higher survival rate.
- Prophylaxis: Since the pathogenesis and the etiology are not fully recognized yet, there are no realistic prophylactic measurements.
- Early detection: An active mass screening program of hormonal biomarkers of neuroblastoma (either elevated vanillylmandelic acid, VMA and/or homovanillic acid, HVA, in urine investigated by high-pressure liquid chromatography with electrochemical detection method, HPLC-ECD) in infants between 4-6 months of age in France have shown a beneficial effect in early detection of neuroblastoma in totally 69,551 screened cases. In the group of due to these tests positively screened infants, the following clinical and radiological examination revealed 8 truly positive patients in different neuroblastoma stages, 2 false negative and 25 false positive cases. Therefore, the authors suggested such a screening at 1 year of age in developed countries for early neuroblastoma diagnosis to avoid high false positivity rate [17].

2.1.2. Etiology

The actual etiology of the neuroblastoma tumor is still a mystery. Any exposure to environmental factors linked to neuroblastoma's etiology has not yet been identified.

- Familial occurrence: Neuroblastoma is primarily not a hereditary disease. However, some cases show hereditary characteristics. Probably, transmission of a dominant gene with a low penetrance causes neuroblastoma formation [18, 19] in inherited cases.

- Co-existing diseases: Neuroblastoma has been linked to von Recklinghausen's disease and lumbar hernia [20, 21].
- Hypothetical carcinogens: Some chemicals such as hydration, phenobarbital or alcohol may be linked to increased risk of neuroblastoma. Maternal exposure to these drugs during pregnancy may contribute to the ethiopathogenesis of neuroblastoma. Other environmental factors may be biological carcinogenesis and some viral infections [22, 23].
- Cytogenetic findings: Deletion of short arm in the chromosome 1 (1p36) [24] and translocation between chromosome 1 and 17 have been shown to cause NB. Additionally, deletions in chromosome 11q, 14q, and/or 17q loci may also play a predictive role in neuroblastoma development [25, 26]. DNA Ploidy Index refers to the DNA content of a cell. This amount differs in regard with the phase of cell cycle, e.g. diploid and tetraploid, in healthy cells. In tumor cells, DNA aneuploidy, triploidy or pentaploidy may be observed [27, 28]. An aneuploid cell population may show a DNA index of < 1.0 (hypodiploid) or > 1.0 (hyperdiploid). Aneuploid cells with a DNA index near 2.0 must be differentiated from diploid cells. Patients with aneuploid tumors without N-myc gene amplification have a good survival prognosis than patients with diploid or tetraploid tumors with N-myc amplification [29].
- Genetic factors: Amplification rate of the N-myc gene is 20-27% in neuroblastoma. Poor prognosis and the aggressive stage correlate with higher N-myc amplification [30, 31].

2.2. CYTOGENETICS

In neuroblastoma tumors, the short arm of chromosome 1 (1p31-tern) may display alterations and deletions or a N-myc proto-oncogene protein (MYCN) amplification [24].

2.2.1. MYCN

MYCN, N-myc proto-oncogene protein, is a highly conserved proto-oncogene rich of GC sequences and contains 3 exons and stands frequently in collaboration with oncogenic Ras protein. Exon 1 is not encoded and 5 'UTR contains two potential TATA boxes [32]. It is located on p24 region of short arm in chromosome 2. In 1983, MYCN oncogene became a new member of the myc family [33]. It is expressed in the nervous system and other selected organs and exceedingly expression of N-Myc, a nuclear phospho-protein product with a short life-time, can e.g. recover embryonic fibroblasts from aging. N-myc contains as all Myc protein family members a transactivation domain at the N-terminal (Myc box) and the C terminal end of a basic helix-loop-helix/leucine zipper motif (bHLH-LZ) [34].

MYCN oncogene plays a pivotal role in the development of NB and can be used as a genetic marker in the diagnosis [35, 36]. Some NB patients are cytogenetically characterized by extra small chromosome fragments dms (double minutes) or a repetition of a region of the chromosome with hsr (homogeneously stained regions). NB Tumors with high MYCN amplification have more aggressive disease course [37, 38] and poor survival prognosis [39].

Addition of antisense-RNA coupled N-myc to NB cell lines may reduce cell proliferation and/or promote differentiation [40].

2.2.2. Anaplastic Lymphoma Kinase (ALK)

Anaplastic lymphoma kinase (ALK) mutations already exist in all cases of neuroblastomas [41]. ALK and MYCN work together and increase the tumorigenesis in NB [42]. ALK is a crucial regulator of stem cell functions, STAT3 regeneration and target of transcriptional MYCN gene [43].

2.2.3. 17q Translocation

Amplification of the chromosome 17 long arm is another NB-specific genomic alteration. This abnormal chromosomal function was found in 50% of patients suffering from NB

[44]. Neuroblastoma may develop if chromosome 17 shows more than one replicate in neuronal cell sub-regions. This replication can occur *de novo* or during cell divisionby an atypical translocation between chromosome 1 and 17^{th} [45].

2.2.4. 1p Deletion

Generally, human NB cells have a deletion of chromosome 1p as found by Brodeur and his colleagues for the first time in 1977 during analysis of the primary tumor [46]. Later on, 1p deletion and rearrangements in the primary tumor tissue were reported in 25-35% of the cases [24, 47]. In patients suffering from advanced NB stages, the 1p allelic loss was more prevalent and commonly associated with MYCN amplification. The end fragment of Chr 1p contains gens that display an important role for the rapid progress in NB [48]. 1p36 deletion in NB tumor suppressor gene leads to a loss and asset, that are associated with poor prognosis [49]. 1p36 was analyzed as a possible candidate for the NB suppressor gene. The p73 gene is a candidate tumor suppressor gene in NB and it is localized on the 1p36 region. The p73 gene has a structural and functional homology to p53 and inhibits apoptosis and promotes cell growth after stimulation of p53 [50].

2.2.5. 11q Deletion

Chromosome 11q deletions at 11q14-q23 lead again to a poor prognosis in NB tumors and occur at advanced stages and in patients older than 1 year. This deletion is not related to MYCN amplification but is associated with 1p deletion [51, 52].

2.3. CLASSIFICATION

Neuronal cell tumors can be classified in three main categories [53]. Firstly, ganglioneuroma, which is a benign tumor consisting of gangliocytes, mature stroma and sparse neuroblasts. However, ganglionic cells are aneuploid. They are easily diagnosed in comparison to other NB-types. The second NB type is called ganglioneuroblastoma and includes mature gangliocytes combined with immature neuroblasts, is surrounded by ganglion and schwann cells and has intermediate malignant potential. Schwann cells in

ganglioneuroblastoma do not express any DNA abnormalities. NB, as described in detail under 2.1 above refers to the third group. NB is the malignant tumor of neuronal cells and consists of immature cells. The diagnosis of NB is more difficult than that of the other two neuronal tumor types [54].

2.3.1. Stages of the Neuroblastoma

Five stages were emphasized by the International Neuroblastoma Staging System (INSS) in 1990 [5]. Stage 1 tumors are small and localized that have not infiltrated any lymph nodes. These localized tumors can fully be removed without any residue. Stage 2 consists of two different sub-types two different sides. 2A tumors are small but difficult to operate and remove completely. Lymph nodes do not include any cancer cells. Stage 2B tumors are also localized and may or may not be completely removable but have already infiltrated ipsilateral non-adherent lymph nodes. Stage 3 consists of large tumors. These are located across the midline and can therefore be incompletely resected. Lymph nodes in stage 3 may or may not be infiltrated by cancer cells. [55]. Stage 4 tumors are very aggressive and disseminate and metastasize into lymph nodes, bones, bone marrow, liver, skin or other organs. Stage 4S refers to a 'special'='S' type of NB [56]. This tumor is on its own a primary NB that can regress and disappear without any treatment but may also metastasize into skin, lymph nodes or liver It is easily operable when patients are not older than 12 months. However, these tumors may rarely metastasize into the bone marrow but the bone cortex is always spared [55, 57, 58].

As the International Neuroblastoma Risk Group (INRG) indicates, there are three risk groups which are low risk, intermediate risk and high risk group. Patient's risks groups are determined by their INRG stage, age, histology, tumor differentiation, MYCN amplification, 1p deletion, 11q loss of heterozygosity (LOH) and aberrant ploidy [5].

- The low risk group: This group contains stage 1 and 2 NB for all age of children without MYCN amplification, favorable histology and hyperdiploidy. More than 50 % of the tumors can be removed from the 4S

stage patients that are up to 18 months old and without MYCN amplification [59].

- The intermediate-risk group: This group contains stage 2A and 2B NB patients without MYCN amplification. However, less than 50% of the tumors can be removed by surgery [60]. Stage 3 diseases without MYCN amplification but favorable histopathology have good prognosis for all ages of children; stage 4 children who are younger than age 1 without MYCN amplification belong to the intermediate risk group [61]. Stage 4 children who are 12 months to 18 months old without MYCN amplification, hyperdiploidy and favorable histology belong to the intermediate risk group. Stage 4S children who are younger than age 1 out any gene amplification and either unfavorable histology or hiperdiploidy also belong to this group [62].
- The high-risk group: This group contains stage 2A and 2B with MYCN amplification. Stage 3 disease for all ages with MYCN amplification, stage 3 disease children who are older than 18 months with unfavorable histology belongs to the high risk group. Stage 4 diseases with MYCN amplification regardless of age, stage 4 disease children who are older than 18 months, stage 4 disease and children between 12 to 18 months old with MYCN amplification or diploidy or unfavorable histology belongs to the high risk group. Stage 4S disease with MYCN amplification also belongs to the high risk group [5, 63].

2.4. SK-N-AS CELL LINE

SK-N-AS cell line (available from ATCC, American Type Culture Collection=a non-profit organisation: CRL-2137) was first obtained from human bone marrow metastases but originates from neuronal cells in the brain and belongs to NB cancer type. This type of NB arises from neuronal cells either due to a p73 mutation on 1p36.33 chromosome or due to lack of dimerization of N-myc proto-oncogene protein (N-Myc=bHLHe37, basic helix-loop-helix protein 37) on MYCN gene with another bHLH in the nucleus in order to bind DNA. There are no mutations in chromosome 17 [24].

2.5. ROTENONE

Pesticides were being used in farming for a very long time and are very toxic chemicals. Pyrethrins, rotenone (Figure 2.1.), nicotine, sabadilla, hellebre and ryania are some of these insecticides. These chemicals are plant-derived structures and also they are very complex. These natural pesticides consist of two groups. First group includes only C, H and O elements such as pyrethrum and rotenone. Second group includes C, H, O and N such as nicotine, hellebore, sabadilla and ryania. These chemicals are not only being used for insects but also for fishing [64]. However, they may also affect the human health directly or indirectly. Safety and hazardability analysis should therefore be prepared in order to use these chemicals as safe as possible.

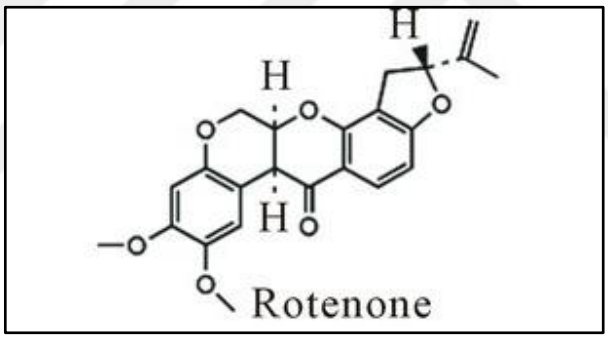

Figure 2.1. The rotenone molecule [64]

Rotenone is a well-known and a widely used broad spectrum pesticide. It is isolated from plant roots or leaves [65]. Rotenone is a member of the retonoid group and has isoforms. Rotenolone I and hydroyrotenone have some toxicity as rotenone. However, rotenolone II and dihydroxyrotenone's toxicity are less then rotenone (Figure 2.2.). Rotenone is highly lipophilic and can diffuse easily into the blood-brain barrier and cell membranes regardless from any active transporters [66, 67].

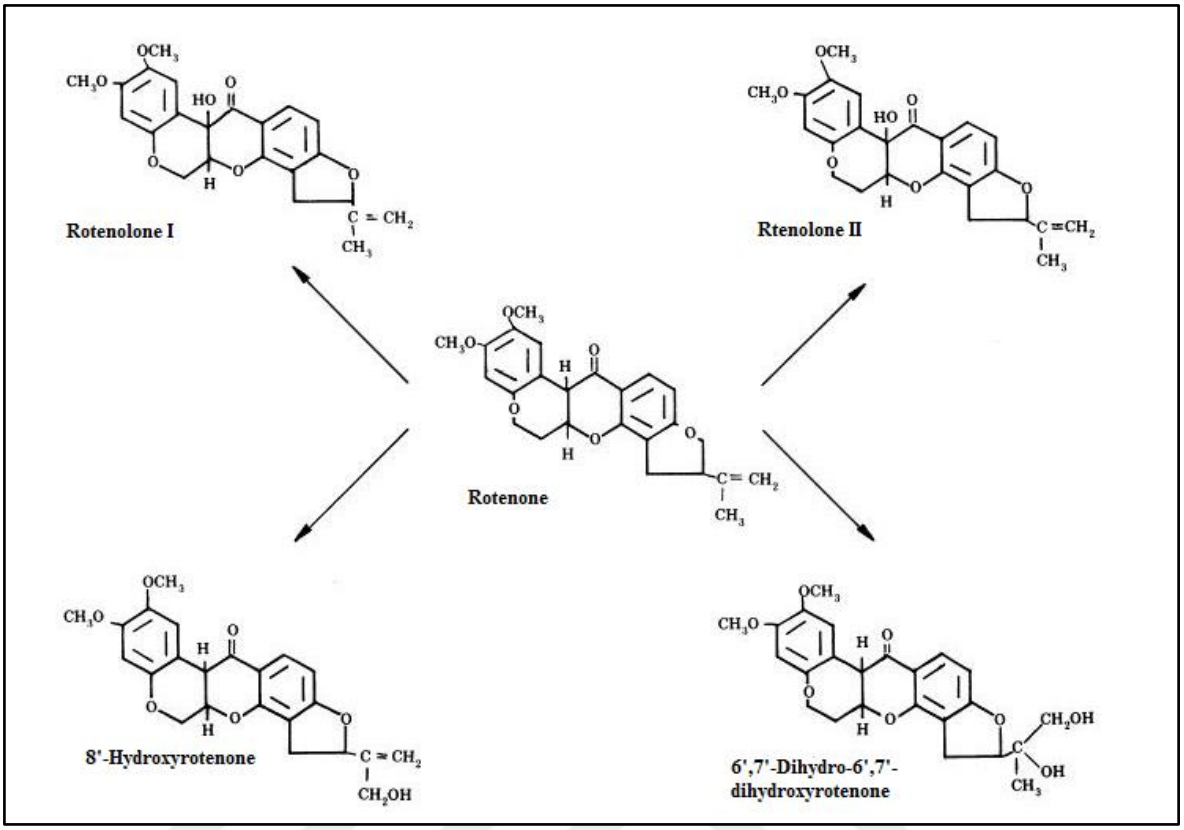

Figure 2.2. The rotenone molecule and rotenone isoforms [64]

Some studies showed that high rotenone level kills the dopaminergic neurons [68]. Several other studies with rotenone treatment on rats demonstrated that rats develope symptoms of Parkinson's disease (PD) [69]. Lower dose of rotenone is shown to inhibit mitochondrial complex I and affect mitochondrial electron transport chain [67, 70, 71]. This inhibition increases the oxidative stress in the neuronal cells, intracellular calcium levels and induces apoptosis *via* activating caspases [72].

2.5.1. Effects of Rotenone in Neuroblastoma

For oxidative phosphorylation, a potential difference is needed on both sides of the inner membrane of the mitochondria. Electron transfer chain (ETC) provides this potential difference for the transport of electrons to the transmembrane region of the mitochondrial matrix and consists of four members: Complex I, Complex II, Complex III and Complex IV. Rotenone binds to complex I and inhibits the transfer of electrons into mitochondria

leading to cumulation of electrons reacting with oxygen molecules and inducing an increase of ROS that damages mitochondria [73, 74]. This rotenone induced cumulative oxidative damage in neuronal mitochondria kills the dopaminergic neurons [75].

On the other hand, rotenone may arrest also the cell cycle [76]. Additional toxic effects of Rotenone are increase of lipid peroxidation level in the dopaminergic NB cells [77]. Increase of protein oxidation in some experiments also increases of protein carbonyls and thiol oxidation in NB cell lines [78].

2.6. GROWTH HORMONE

GH was first discovered from pituitary gland in 1921. In the following years, researchers all over the world investigated GH effects.. End of the 1950s, they managed to obtain pure GH [79]. In 1960, GH treatment was accepted for children who were diagnosed with hypopituitarism [80]. End of the 1970s, human growth hormone (hGH) was cloned and expressed [79]. Treatment with recombinant GH became available in 1980s [80].

GH is not solely responsible for body growth but also responsible of regulation of carbohydrate, protein and lipid development and metabolism [81]. GH has another vital function which is the regulation of the specific gene transcription and activation of tyrosine kinase. GH is a 22 kDa protein and contains 191 amino acids as a single-chain polypeptide. [82].

GH receptor (GHR) is a transmembrane glycoprotein and consists of 620 amino acids. It consists of a single pass transmembrane domain of 24 amino acids, an extracellular domain of 246 amino acids and an intracellular domain of 350 amino acids [82].

GH binds to the extracellular domain of GH-receptor. This binding leads to the binding of JAK2 molecules to the intracellular part of GHR and activation of JAK2. Active JAK2 phosphorylates tyrosines within itself and GH receptor. Furthermore, STATs are phosphorylated too. (Figure 2.4.) [83].

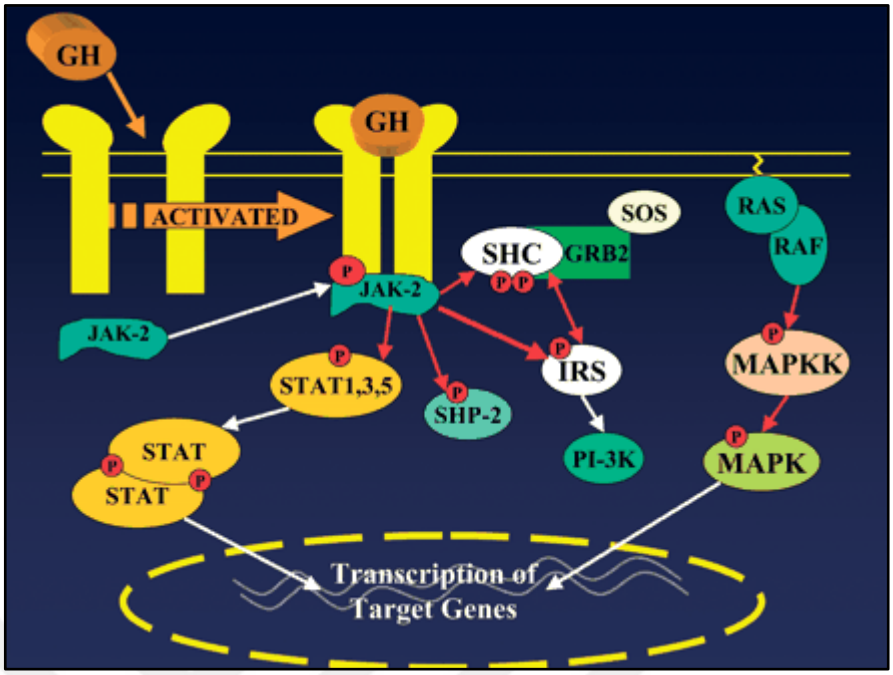

Figure 2.3. GH binding to GH receptor and its effects [84]. GH binding to GHR activates not only the receptor itself but also JAK2 by binding to the intracellular part of GH receptor. JAK2 in turn phosphorylates receptors *via* activation of many other tyrosine kinases on the receptor. This process enables STAT proteins to bind to these phosphorylated tyrosine kinases on the receptor. In turn, JAK2 phosphorylates and activates STAT 1, 3, 5A and 5B proteins. In this way activated STAT proteins detached from their receptor, get dimerized *via* their SH2 domains and are transported to the nucleus where they bind to the specific DNA region and initiate transcription of target genes.

STAT proteins are essential for GH induced regulation of the gene transcription. GH activates some signaling pathways *via* JAK2/STAT receptor complexes. JAK2 can also activate MAPKs (Mitogen-Activated Protein Kinases), IRS1 (Insulin Receptor Substrate-1), PI3K (Phosphatidylinositol- 3-Phosphate-Kinase), DAG (Diacylglycerol), PKC (Protein Kinase-C) and Ca^{2+} (intracellular calcium) [85].

2.7. JAK/STAT PATHWAY

Janus kinase (JAK) is a family of intracellular, non-receptor tyrosine kinases. Signal transducers and activator of transcription (STAT) are intracellular transcription factors and associated with JAK kinases. JAK/STAT pathway is the most important signaling pathway

of cytokines and growth factors [86]. The JAK/STAT pathway sends a signal into the nucleus to start DNA transcription. The activation of JAK/STAT pathway induces immunity, proliferation, differentiation, apoptosis and cell migration [87].

JAK/STAT pathway consists of three main components. First component is the receptor and its specific ligands such as prolactin, erythropoietin, interferons and interleukins that bind to these receptors. Second component is JAK. This enzyme uses ATP to phosphorylate other proteins. The phosphorylation of proteins can lead to their activation or deactivation. In mammals, JAK has four members, these are JAK1, JAK2, JAK3 and Tyrosine kinase 2 [88]. The third component is STATs. STAT family has seven members, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 [89]. STAT1 and STAT2 transduce signals for type I and type II IFNs. STAT 1 can also activate apoptotic genes [90]. STAT4 and STAT6 have important roles in the polarization of naive T cells. Cytokines can activate STAT3 and STAT5. STAT3 and STAT5 inhibit apoptosis in cancer cells [91-93].

After ligand binding to its receptor, ligands activate JAK2 on the intracellular part of its receptor which in turn phosphorylates receptors *via* activation of many other tyrosine kinases on the receptor. This process enables cytoplasmic STAT proteins to bind to these phosphorylated tyrosine kinases on the receptor. In return, JAK2 phosphorylates and activates STAT 1, 3, 5A and 5B proteins. After this process, activated STAT proteins get detached from the receptor and following dimerization they migrate into the cell nucleus. After their binding to the specific DNA region, transcription starts with target genes (Figure 2.3.) [88, 89].

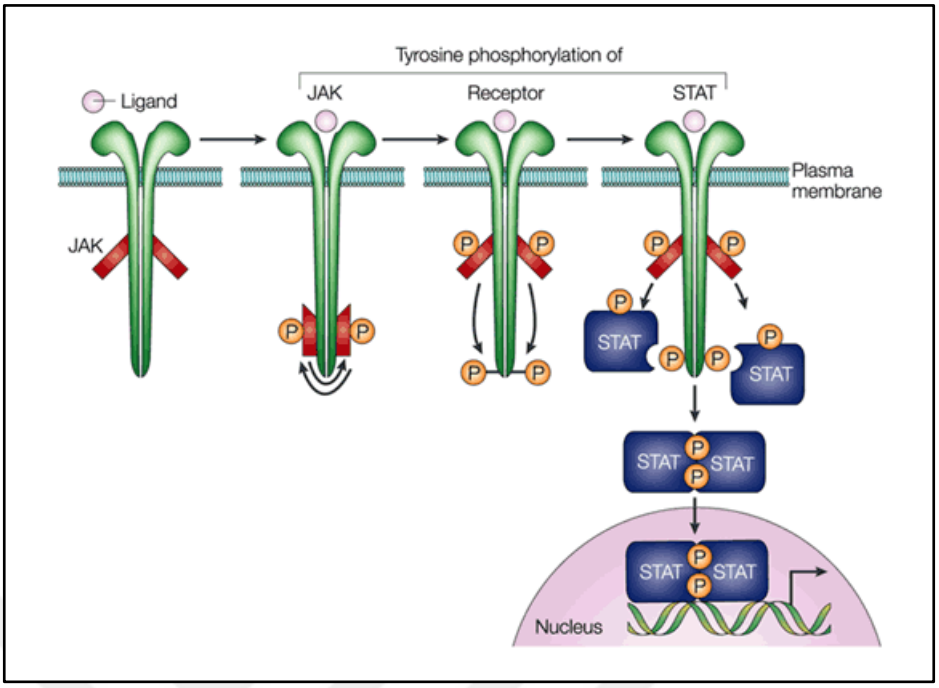

Figure 2.4. JAK/STAT pathway [94]. Ligand binding to its receptor expressed on every cell, ligand activates JAK2 on the intracellular part of receptor which in turn phosphorylates receptors *via* activation of many other tyrosine kinases on the receptor. This process enables cytoplasmic STAT proteins to bind to these phosphorylated tyrosine kinases on the receptor. In turn, JAK2 phosphorylates and activates STAT proteins. In this way activated STAT proteins detach from receptor, get dimerized and migrate into the cell nucleus and after binding to the specific DNA region transcription of target genes start.

JAK/STAT signal pathway also has additional effects on survival, growth, differentiation and regeneration of the neuronal cell [95].

2.8. CELL CYCLE

Cell cycle regulates cell division and proliferation within any healthy organism for tissue turnover of the organism and regulates its own differentiation, proliferation, regeneration, shortly its physiological homeostasis and maintenance. During cell cycle, there are different check points to avoid and monitor cell division for the prevention of incorrect and inaccurate cell proliferation [96].

In contrast, cancer development can be characterized as an abnormal or faulty cell division that ends in tumor formation. In cancer cells, cytoplasmic protein levels also differ from that of healthy cells. For example, in tumor cells cyclin D is overexpressed.

Cell division consists of 5 phases (Figure 2.6.):

1) Phase G0 is known as the resting phase with diploid DNA,

2) G1 is the growing phase of the cell. G1 is the first gap where cytoplasmic growing occurs before cell cycle enters the S phase.

3) S phase is the DNA replication phase, called also synthesis phase, where DNA content increases continuously until they reach tetraploid phase.

4) G2 is the second growing phase of the cell for the production of microtubules which are needed for mitosis and includes also an interphase after DNA synthesis completion. This phase ends with the beginning of mitosis [97].

5) M phase is cell cycle phase in which all events in regard with cell mitosis occur and also includes 4 phases, i) prophase is the first stage of mitosis or meiosis where chromosome contraction followed by migration towards 2 centrioles at the edge of nucleus occur, ii) in metaphase chromosomes get arranged and form the equatorial plate and centromeres are attached to the spindle fibers, iii) in anaphase a set of 46 daughter chromosomes move to the poles of the cell, and iv) in telophase the new daughter chromosomes installed at the 2 poles of the nucleus get longer and slim followed by the reappearance nuclear membranes around each daughter chromosome. Thereafter cytoplasm is divided surrounding each new 2 nucleus and cell division is completed [98]. In the end of this phase 2 diploid G0 cells are generated.

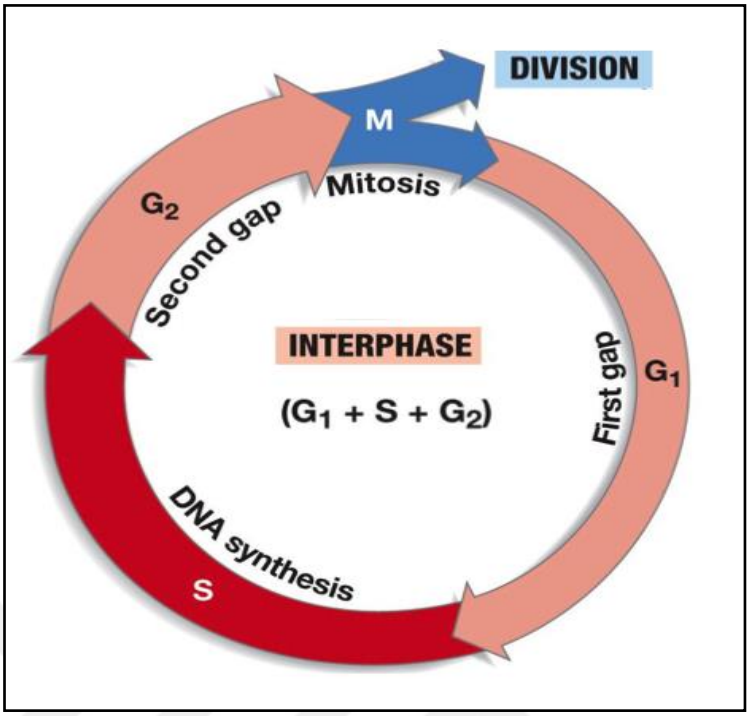

Figure 2.5. Cell cycle

2.9. APOPTOSIS

The term apoptosis, a Greek word that originally represents the “leaves falling from trees” in the nature, was first described by de Kerr in 1972. In contrast to cell division, apoptosis characterizes the physiological form of cell death which is also needed for prevention of uncontrolled growth and maintenance of healthy organism.

Apoptosis is the only possibility of the cell to eliminate itself on a natural way. It may occur as a solitary or multi cellular phenomenon in organisms and eliminate all negative effects in a cell. DNA synthesis stops just before apoptosis and indicates that apoptosis will begin shortly. The time interval depends on the size of cell, cell type and maturity with regard of cell division capacity. Apoptosis is a tightly regulated physiological process of "cell suicide", which plays an important role for the development (e.g. organogenesis and synaptogenesis), maintenance (tissue maturation) and aging of organisms. In this process, individual cells on schedule are eliminated in order to balance the number of cells. That means a lower rate of apoptosis leads to a growth of organism and an extremely high rate

of apoptosis causes cell destruction up to an undesired extent. These physiological regulatory effects make apoptosis to an important gate keeper in normal organic turnover processes.

Apoptosis also plays a physiological role in the elimination of inner tissue between fingers and toes during embryonal development, formation of spine, muller canal being destroyed in male fetuses. In addition, it has been shown that pathologic apoptosis plays a key role in some major neurodegenerative diseases such as Alzheimer's and Parkinson [99]. *In vitro* studies reveals that apoptosis develops fastly and apoptotic cells firstly lose their connection to other living cells in the organism followed by cell shrinking without loss of cell membrane integrity. In this phase, although not mandatory for apoptosis, also DNA fragmentation may rapidly occur by calcium activated endonuclease enzyme and becomes visible approximately 1 hour after the activation of apoptosis. This process can be studied by DNA laddering or made recognizable by DAPI staining. The shrinken apoptotic cells are then internalized by phagocytes e.g. macrophages and eliminated without any tissue inflammation in contrast to cell necrosis where due to cell membrane destruction cytoplasmic enzymes and organelles are scattered into healthy tissue and induce inflammation [100, 101]. The elimination process of apoptotic cells requires 12 to 18 hours which is significantly longer than the apoptosis event itself [102].

2.10. CASPASE FAMILY

Fourteen caspases have been identified in mammalian. Some members of the caspase family have a very important role in apoptosis pathway. Pro-caspase has small and large subunits. These small and large subunits connect together and form an active heterodimer caspase. Caspases are divided into three subfamilies based on their amino acid sequences. CED-3 is the cell death gene in the *Caenorhabditis elegans*, a threadworm, and it is highly homologous to the mammalian interleukin-1β-converting enzyme family proteases (ICE/ known as caspase-1) [103, 104].

Subfamily I: Inflammatory mediator (Caspase 1/4/5/13) – ICE family

Subfamily II: Activator of apoptosis (Caspase6/8/9/10) – CED-3 family

Subfamily III: Executioner of apoptosis (Caspase3/2/7) – CED-3 family

Some caspases have CARD‘s (caspase recruitment domain) or DED’s (death effector domain). All caspases have different protein-protein interaction domains, e.g. caspase 1, 2, 4, 5 include CARD and procaspase 8 and procaspase 10 include DED. The cytokine activator caspases have similar sequences. In humans, caspase 4 and 5 are cytokine activators. Some caspases act as initiators or effectors in the apoptotic pathway. Caspases 6/8/9/10 activate caspases 2/3/7. However, caspase 2 can also activate itself automatically. Caspase 6 can act like a protease. Granzyme B is a serine protease and can cleave and activate initiator caspases 8 and 10 and executioner caspases 3 and 7 that initiates apoptosis. Thereby, Caspase 7 is completely cleaved while caspases 3, 8, and 10 need further cleavage for full activation.

Additionally, Granzyme B generates high levels of mitochondrial ROS and potentiate cell death.

2.10.1. Extrinsic Pathway

Extrinsic pathway of apoptosis involves the activation of ligands and death effector domain (DED) receptors. Fas ligand or tumor necrosis factors (TNF) binds to the transmembrane death receptor. The intracellular part of the Fas receptor is called Fas associated death domain (FADD) and the extracellular part of this receptor has a specific binding site for Fas ligand. Fas ligands bind to Fas receptor and activate FADD. FADD in turn activates the FADD adaptor protein which consists of two domains. The first one is structurally identical to the FADD protein and is therefore called FADD domain and enables the binding of the FADD adaptor protein to the intracellular part of Fas receptor. The second domain of FADD adaptor protein is the so-called death effector domain (DED). On the other hand pro-caspase 8 and 10 both possess a DED pro-domain. If DED pro-domains of pro-caspases attach to the DED domain of the FADD adaptor proteins they get dimerized and DED pro-domain cleaved from pro-caspases leading to activation of caspases. Activated Caspase 8 or 10 in turn activate the pro-caspase 3 or 7 to caspase 3 or 7 again by cleaving of their pro-domains. The complex consisting of Fas receptor, FADD adaptor protein and pro-caspase 8 and 10 is named the death inducing signaling complex (DISC). These caspases activate the death substrates and apoptosis starts. (Figure 2.6.), [105, 106].

2.10.2. Intrinsic Pathway

Intrinsic pathway is not sensitive to FAS and TNF signals. DNA damage or oxidative stress causes intrinsic pathway based apoptosis. Bcl-2 family proteins bind to the mitochondria membrane and induce apoptosis.

Anti-apoptotic Bcl-2 proteins binds to pro-apoptotic BH1,2,3 proteins such as Bax and Bak proteins to prevent apoptosis as the attachment of Bax and Bak together initiates the intrinsic pathway of apoptosispreventing them to get together. The Pro-apoptotic BH3 only proteins consist of two groups, depending on the ability to binds to the anti-apoptotic Bcl-2 proteins in the cytoplasm and to inhibit apoptosis or to interact with the effector proteins Bax and Bak to initiate apoptosis. Bax and Bak proteins oligomerize on mitochondrial outer membrane. Cytochrome c is released from mitochondria. Cytocrome c and pro-caspase 9 form apoptosome in the cytoplasm. This complex activates caspase 8 and 9. Activated caspase 8 and 9 can directly cleave pro-caspase 3 and pro-caspase 7 which in turn activate . Then active caspase 3 and caspase 7 are activating death substrates (Figure 2.5.).

Death substrates can be classified as nuclear fragmentation, cytoskeleton disruption and cell membrane alterations [105, 106].

2.11. Bcl-2 FAMILY

The Bcl-2 family members play a pivotal role in caspase activation and apoptosis. Bcl-2 family members regulate the apoptosis by intrinsic pathway. Bcl-2 family has nearly 20 members and all of them include three or four Bcl-2 homology (BH) domains. BH domains mediate the interaction between proteins. Some of these Bcl-2 family members inhibit apoptosis, anti-apoptotic and others stimulate apoptosis, pro-apoptotic proteins (Table 2.3.). The pro-apoptotic and anti-apoptotic Bcl-2 family member proteins can bind each other and inhibit each other’s functions.

The first identified anti-apoptotic family member of 6 known members in mammals was Bcl-2 protein which also gave its name to the whole family (first column of the table). All members of anti-apoptotic family have hydrophobic tail and four regions of homology

consisting of BH1, BH2, BH3 and BH4 domains. These domains have also receptor function for the interaction between BH3-only and anti-apoptotic proteins.

Pro-apoptotic members consist of 2 subgroups: a) “BH3 only proteins” such as mono-domain Bad, Bim or Bid proteins, b) BH1, BH2 and BH3 containing multi-domain proteins such as Bax, Bak or Bok [107, 108]. In each group there are different proteins with the same function but they are found and are effective / responsible for the same function in different cell types. In contrast, the multi-domain subgroup of pro-apoptotic proteins possesses a hydrophobic tail (second column of the table) [109, 110].

The BH-3 only pro-apoptotic members of Bcl-2 family proteins also have a hydrophobic tail and show no significant homologies with the members of other subgroups. They are regulated at transcriptional and post-transcriptional levels and under normal conditions; their level is very low in the cells. Some members of these proteins are able to activate other pro-apoptotic members directly or indirectly regardless of binding to the anti-apoptotic proteins (third column of the table).

Table 2.3. Bcl-2 Family

Anti-apoptotic proteins	**Pro-apoptotic proteins**	
	Multi-domain	**BH3-only**
Bcl-2	Bax	Bad
Bcl-$_{XL}$	Bak	Bim
Bcl-w	Bok	Bid
Mcl-1		Bik
Bcl-B		Hrk
Bcl-2A1		Puma
		Noxa
		Bmf

BH3 only pro-apoptotic proteins generally control cell death and apoptosis dynamics or survival and can therefore develop 2 opposite functions depending on the protein that binds them: a) if members of anti-apoptotic Bcl-2 proteins such as Bcl-2 bind to BH3-only proteins they prevent and inhibit apoptosis. b) If multi-domain pro-apoptotic proteins such

as Bax and Bak bind to BH3-only protein the latter experience a conformational change that enables to the mitochondrial membrane to release cytochrome c into the cytoplasm. The subsequent binding of cytochrome c with APAF-1 and caspase 9 initiates apoptosis.

The p53 protein controls the genes which are responsible for the expression of pro-apoptotic and anti-apoptotic members in Bcl-2 family. Bcl-2 can repress the expression of the Bcl-$_{XL}$ anti-apoptotic protein [111]. Also, p53 effects PUMA (p53 upregulated modulator of apoptosis) protein which is for apoptosis induction.

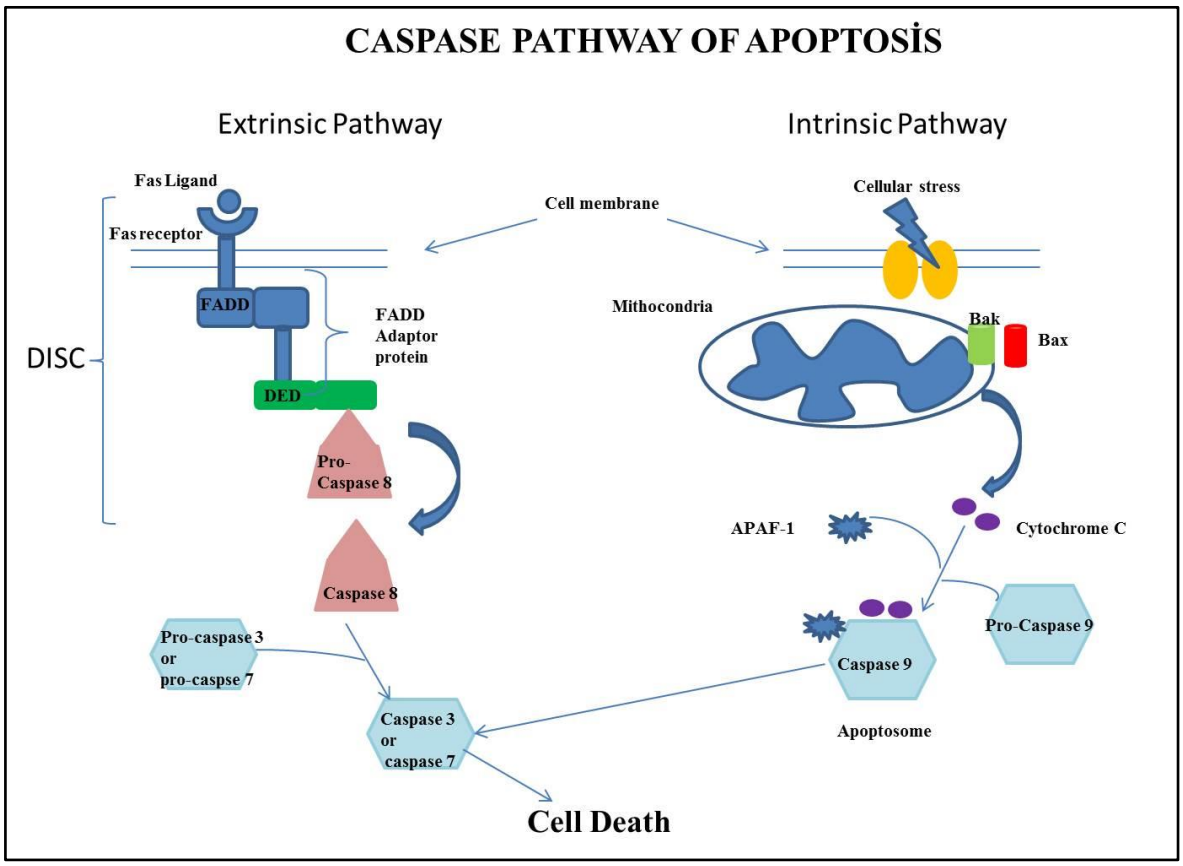

Figure 2.6. The intrinsic and extrinsic pathway of caspase driven apoptosis**.** The extrinsic pathway starts with ligand binding to the Fas receptor and formation of DISC complex and caspase 8 activated. However, intrinsic pathway starts with the cellular stress and release of the cytochrome c from mitochondria. Cytochrome c, APAF-1 and pro-caspase 9 form to apoptosome in the cytoplasm. In the following, caspase 3 or 7 are activated resulting in the cell death.

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

All the chemicals used in this thesis were given as a table in the appendix A.

3.1.2. Machinery Equipment's

All the machinery equipment's used in this thesis were given as a table in the appendix B.

3.1.3. Cell Culture Equipment's

All the cell culture equipment's used in this thesis were given as a table in the appendix C.

3.2. METHODS

3.2.1. Cell Culture

Human NB cell line (SK-N-AS) was used for all experiments in this thesis. Rotenone induced cell toxicity and the role of GH expression on rotenone toxicity in NB cells were evaluated. SK-N-AS cells were cultured at 37°C under 5% CO_2 in air in DMEM medium which included 0.1 mM Non-essential amino acids (NEAA), 1% L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin and fetal bovine serum to a final concentration of 10% before using them for different tests.

3.2.2. Growth Hormone Expressing SK-N-AS Stable Cell Line Generation

The transfection process to eukaryotic cells based on the introduction of foreign DNA into eukaryotic cells. Foreign DNA is integrated within the cell via $CaPO_4$, Lyposomal agents or electroporation. According to lyposomal transfection procedure SK-N-AS cells were seeded in 6 well plate by $2x10^5$ cells per well. Following incubation at 37°C over 48 h, GH-PC3.1 plasmid was transfected by using lipofectamine 3000 reagent diluted with transfection agent: plasmid concentration (1:3 and 1:6) ratio. Following transfection for 24 h, cells were applied with neomycin in increasing dose. After 1 month with neomycin selection, survived SK-N-AS cells were transferred to a new flask as a GH+ SK-N-AS stable cell line. Stable cell line GH+ SK-N-AS cells were cryopreserved in liquid nitrogen.

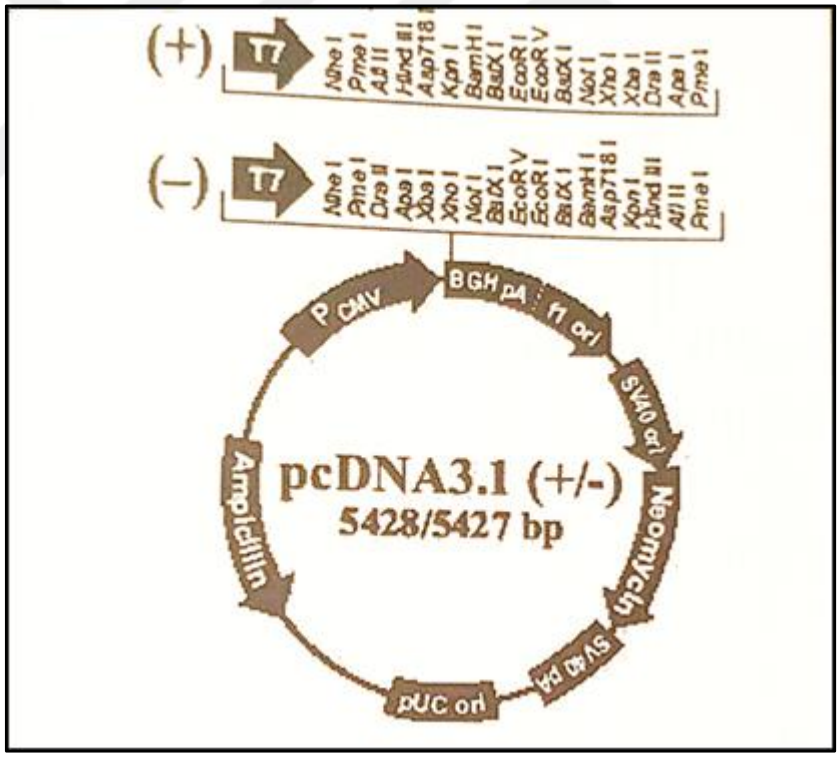

Figure 3.1. GH was inserted into this plasmid (GH-PC3.1). This plasmid was kindly provided by Assoc. Prof. Ajda Çoker Gürkan.

3.2.3. MTT Cell Viability Assay

In order to determine the dose-dependent effect of rotenone (0-100 nM) in 24 h in SK-N-AS wt and GH+ NB cells, we performed MTT cell viability assay. $1x10^4$ cells were seeded in 96 well plates and incubated over night at 37 °C. Cells were treated with (0.1 -10 μM) rotenone in dose dependent manner. Following 24 h drug treatment, cells were applied as 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) for 4h at 37°C. After 4 h exposure to MTT agent, cell's media was discarded and cells were treated with 200 μL DMSO, incubated for 5 minute in the dark. 96 well plate was measured by spectrophotometer at 570 nm and 655 nm. Absorbance measures were calculated by percentage with assuming untreated healthy cells as a 100%.

3.2.4. Cell Survival Assay

To determine the time-dependent cytotoxic/cytostatic effect of rotenone on wt and GH+ SK-N-AS NB cells, cell survival assay was performed. $1x10^5$ human NB cells were seeded 12 well petries. After incubation overnight, cells were treated with 100 nM rotenone. Every 24 h period, cells were washed with 1x PBS and trypsinezed and cell pellet was diluted by 1x PBS. Resuspended cells were diluted with trypan blue and counted at hemocytometer. The effect of rotenone on cell growth was determined (x-axis was rotenone treatment duration (0-72 h), y-axis was cell number).

3.2.5. Colony Formation Assay

Colony formation assay or clonogenic assays aimed to understand the single cell survives and growth into a colony within 14 days. This assay shows that all the cells have the ability of unlimited division in the population. $2.5x10^3$ cells were seeded in to 6 well petri dishes. After incubation overnight cells were treated with 100 nM rotenone. Following 24 h drug treatment, cell medium was applied and placed for colony formation for 14 days. Cell medium was changed every 3 days. At the end of 9 day period, cell medium was discarded and colonies were fixed with cold 100% methanol and waited for 5 min at -20°C. After fixation, cells were applied by 1 mL of 0.05% crystal violet and incubated at room

temperature for 20 minutes. Discarding the crystal violet, cells were washed with 1x PBS twice and the colony photographs were taken by light microscope.

3.2.6. Soft-Agar Colony Formation Assay

$2.5x10^3$ cells were embedded between 500 mL of 0.05% agarose and 500 mL of media containing 20% fetal bovine serum (FBS) with 0.5% agar diluted with sterile PBS into the 0.3 % agar. Following 5 days, the diameter and colony sphere formation was determined by light microscopy.

3.2.7. Methods for Determination of Apoptotic Cell Death

3.2.7.1. DiOC6 Staining

$5x10^4$ cells were seeded into 12 well plates and incubated overnight. Following cells adherence, cells were treated with 100 nM rotenone for 24 h. After drug treatment, cells were stained with 4 nM DiOC6 for 15 mins. Cells were examined under a fluorescent microscope with 10x, 20x and 40x magnification.

3.2.7.2. Propidium Iodide Staining

$5x10^4$ cells were seeded into 12 well plates and incubated overnight. Following cells adherence, cells were treated with 100 nM rotenone for 24 h. After drug treatment, cells were stained with 5 mg/mL PI for 30 min. And cells were examined under a fluorescent microscope with 10x, 20x and 40x magnification.

3.2.7.3. DAPI Staining

$5x10^4$ cells were seeded into 12 well plates and incubated overnight. Following cells adherence, cells were treated with 100 nM rotenone for 24 h. After drug treatment, cells were stained with 5 mg/mL DAPI for 10 min. And cells were examined under a fluorescent microscope with 10x, 20x and 40x magnification.

3.2.7.4. Cell Cycle Analysis

$2x10^5$ cells were seeded into 6 well plates and incubated overnight and treated with 100 nM rotenone for 24 h. Medium was discarded after 24 h rotenone treatment, cells were trypsinezed and resuspended in 1x PBS. Cell pellet was fixed with 500 µL ice cold 70% EtOH and mixed gently in order not to clamp. After fixation, cells were treated washed with 1mL 1x PBS. After centrifugation cells were resuspended in 245 µL cold 1x PBS, 2.5 µL PI, 2.5 µL RNase and incubated for 30 mins at room temperature in dark. PI stained cells were determined by flow cytometry. Cell cycle analysis was performed by FL1 channel at x-axis and cell count at y-axis by BD Accuri C6 program.

3.2.8. Immunoblotting

3.2.8.1. Protein Isolation

$1.2x10^6$ cells were seeded into the 100 mm petri dish. After waiting for adherence, cells were treated with 100 nM rotenone for 24 h. Cells were washed with cold 1x PBS. Then, cells were centrifuged at 13200 rpm for 2 min at +4°C. Pellet was lysed by using 25-50 µl Cell Lysis Buffer (CLB) and incubated at room temperature for 15 mins. in the agitator. Cells were centrifuged at 13200 rpm for 20 mins. at +4°C. The supernatant was transfered into a new eppendorf tube and stored at -80°C.

3.2.8.2. Bradford Assay

1.5 µg – 7 µg range protein standard (BSA) was applied at 96 well plate two repeats and 1 µl protein lysates were applied twice to each well and 200 µl Bradford reagent was added on the protein lysates and incubated at room temperature in dark for 5 mins. The absorbance of each well was measured in the microplate reader at 595 nm wavelength. Protein concentration was determined according to protein standard graphic.

3.2.8.3. Running and Blotting

Protein was pipetted and mixed with 1:5 loading buffer. Prepared samples were put into the heater at 95°C for 5 min. Protein samples were loaded at 12% SDS-PAGE and gel runned at 85 Volt for 3-5 h. than gel was transferred to the 0.22 µm PVDF membrane at 25

mA for 5 min. and membrane was blocked by 5% skimmed milk at room temperature or overnight at 4°C. Membranes were transferred to primary antibodies against GH, GHR, GHRH, GHRHR, JAK2, STAT1, p-STAT1, STAT3, p-STAT3, PARP, Bax, Bcl-2, Caspase 3, Caspase 7 and Caspase 9 proteins and incubated overnight at +4 °C. Membranes were washed three times with TBS-T and transferred to appropriate secondary antibodies according to primary antibody and incubated overnight at +4 °C. Then membranes were washed with TBS-T twice and treated with ECL reagent for 2 min and chemiluminescence imaged was determined by Chemidoc Gel Imager.

3.2.9. Statistical Analysis

Data sets were examined using Graph Pad and multiple data analysis was performed using ANOVA. All results were summarized below within their statistical analysis.

4. RESULTS

4.1. Expression Profiles of Growth Hormone (GH) and Growth Hormone Receptor (GHR) in SK-N-AS Cell Line

GH and GHR expression profile of SK-NA-S NB cells were determined in comparison to MDA-MB-231 GH+ breast cancer cells by immunoblotting and using GH and GHR specific primary antibodies. MDA-MB-231 GH+ breast cancer cells have already been GH transfected and known to be GHR positive. Therefore they were chosen as a positive control. GH was not expressed in SK-N-AS NB cells although they are GHR expression (Figure 4.1).

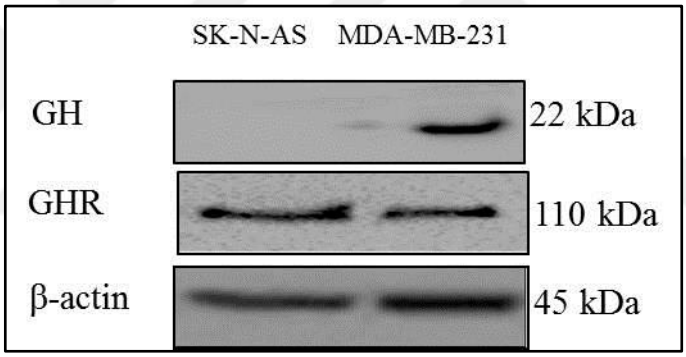

Figure 4.1. Determination of growth hormone and growth hormone receptor in SK-N-AS cell lines. 200 000 cell/well SK-N-AS cells were seeded in 6 well plates. Total protein was isolated, 30 µg of protein loaded in 12% SDS-PAGE and transferred to 0.22µm PVDF membrane and blocked with 5% skimmed milk. GH and GHR specific primary antibodies were then applied within 5% skimmed milk and anti-goat secondary antibody were applied and visualized by ECL in ChemiDoc.

Autocrine GH expression was generated by transfection of GH inserted PC3.1 plasmid (PC3.1-GH) *via* Lipofectamine 3000 transfection reagent and neomycin (G418) selection. The expression of GH was determined by immunoblotting assay. According to immunoblotting results 1:6 transfected SK-N-AS cells were shown to express GH

compared to wt SK-N-AS cells. Again MDA-MB-231 is used as a positive control for autocrine GH expression (Figure 4.2).

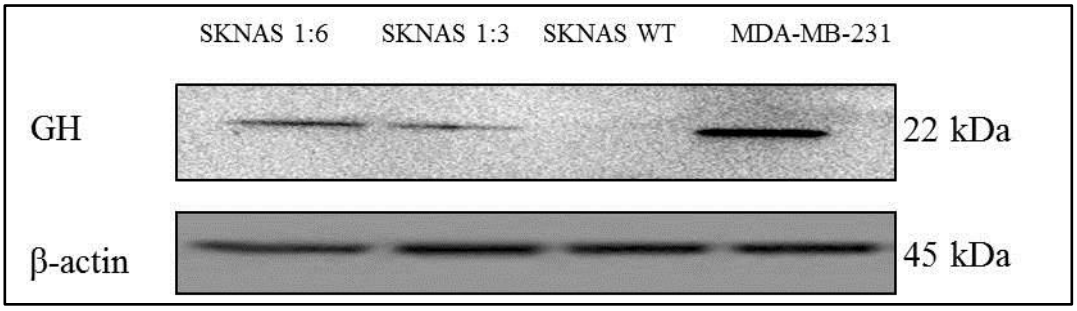

Figure 4.2. Forced GH expression in SK-N-AS cell line by immunoblotting. 200 000 cell/well SK-N-AS cells were seeded in 6 well plates. After overnight incubation at 37°C, cells were transfected with GH inserted PC3.1 plasmid with lipofectamine 3000 by 1:3, 1:6 ratio. Following dose dependent G418 selection, GH inserted cells were picked and stable cell line was generated. Detection of GH expression in selected cell lines were determined by immunoblotting.

4.2. Expression of GH / GHR / GHRH / GHRHR in wt and GH+ SK-N-AS Cells

The GH / GHR / GHRH / GHRHR expression profile of wt and GH + SK-N-AS cells were evaluated by immunoblotting. Immunoblotting revealed that wt cells express GHR and GHRHR but no GH and GHRH expression. In contrast, GH + SK-N-AS cells expressed all 4 proteins. Again MDA-MB-231 GH+ breast cancer cells were used as positive control (Figure 4.3).

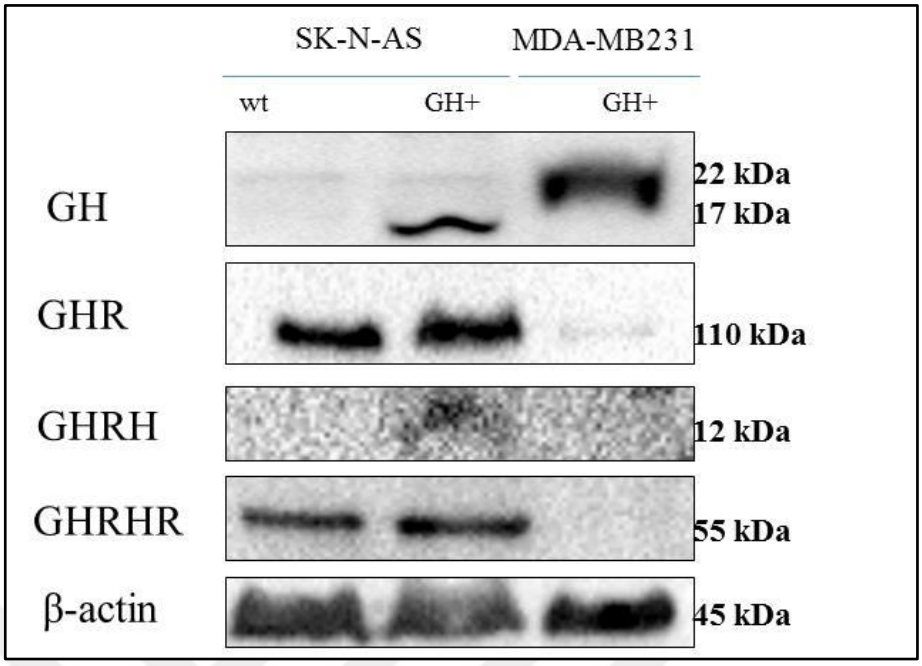

Figure 4.3. Expression of GH/GHR/GHRH/GHRHR in wt and GH+ SK-N-AS cells by Immunoblotting. 800 000 cell/well wt and GH+ SK-N-AS were seeded in 60 mm petries. Overnight incubation at 37°C, cells were treated with 100 nM rotenone. Total protein was isolated from each cell line and 30 µg protein loaded in 12% SDS-PAGE and transferred to 0.22 µm PVDF membrane and blocked with 5% skimmed milk. GH, GHR, GHRH and GHRHR specific primary antibodies were applied within 5% Skimmed milk and anti-goat secondary antibody were applied and visualized by ECL in ChemiDoc.

In Figure 4.3, two points need detailed explanation: i) GH is a 22 kDa (aa1–191) protein as can be seen in Figure 4.1, 4.2 and 4.3 for MDA-MB231 GH+ cells and in Figure 4.2 in GH+ SK-N-AS cells. In SK-N-AS GH+ cells in Figure. 4.3, there is a 17 kDa protein positive that also corresponds to GH. Through limited proteolysis, the 22 kDa GH proteins can sometimes be fragmented into a 5 and 17 kDa parts as the sequences aa1–43 and aa44–191 respectively that has happened in this experiment. ii) Additionally, MDA-MB231 GH+ cells presented only weak GHR expression in comparison to Figure. 4.1. This results only from an inadequate low protein loading as it can also be confirmed by ß-actin control.

4.3. Cell Growth in wt and GH+ SK-N-AS Cell Lines

To evaluate and compare the doubling time of wt and GH+ SK-N-AS cells, cell growth assay was performed over 4 days and cells were counted every day under light microscope. Both cell types doubled after 24 h and 48 h similarly as can be seen in figure 4.4 indicating a doubling period of 24h. Therefore, for the following experiments a time period of 24 h was chosen.

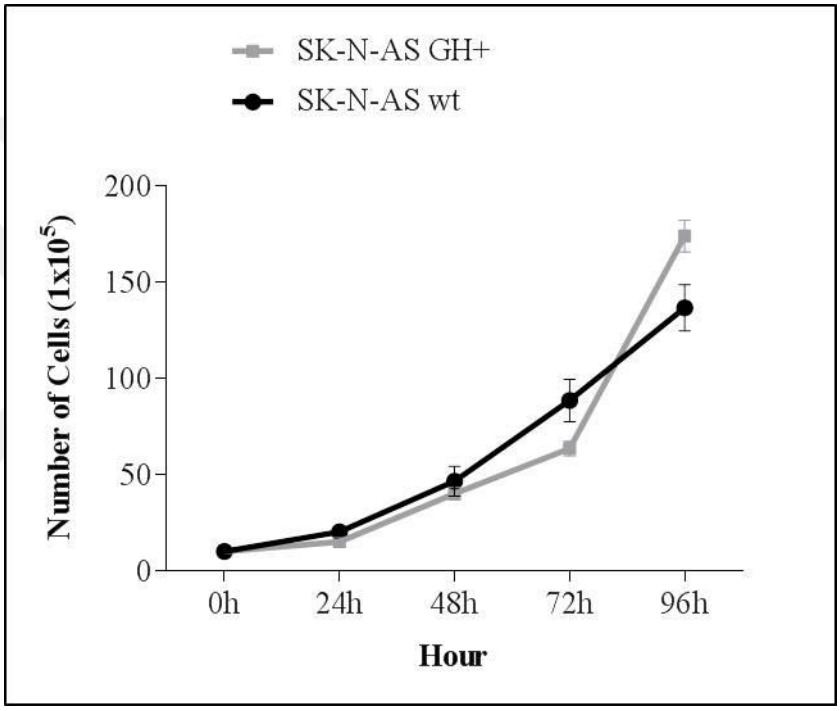

Figure 4.4. wt and GH+ SK-N-AS cells doubling time were measured.100 000 cells/well wt and GH+ SK-N-AS cells were seeded in 6 well plates. Cells were incubated overnight at 37°C. After 24 h medium was discarded, cells were trypsinized and re-suspended in 1x PBS. 10µL cell suspension was mixed with 10µL trypan blue and cells were counted under light microscope *via* hemocytometer. The same process was repeated every 24 h for 4 days.

4.4. Dose Dependent Effect of Rotenone on wt and GH+ SK-N-AS Cell Viability

wt and GH+ SK-N-AS cells were treated by dose dependent rotenone concentration (100, 500 nM, 1, 5, 10 μM) for 24 hours and cell viability was determined by MTT cell viability assay. Dose dependent rotenone treatment decrease cell viability in both wt and GH+ SK-N-AS cell lines. In all rotenone concentrations, except for 10 000 nM, there was a significant difference in viability decrease between wt and GH+ SK-N-AS NB cells in favor to the GH+ cells confirming a neur-protective effect of GH.

For example in 100 nM rotenone treatment cell viability in wt SK-N-AS cells decreased by 40%, in contrast in GH+ SK-N-AS cells, to 20% (p value: 0.037, Figure 4.4.).

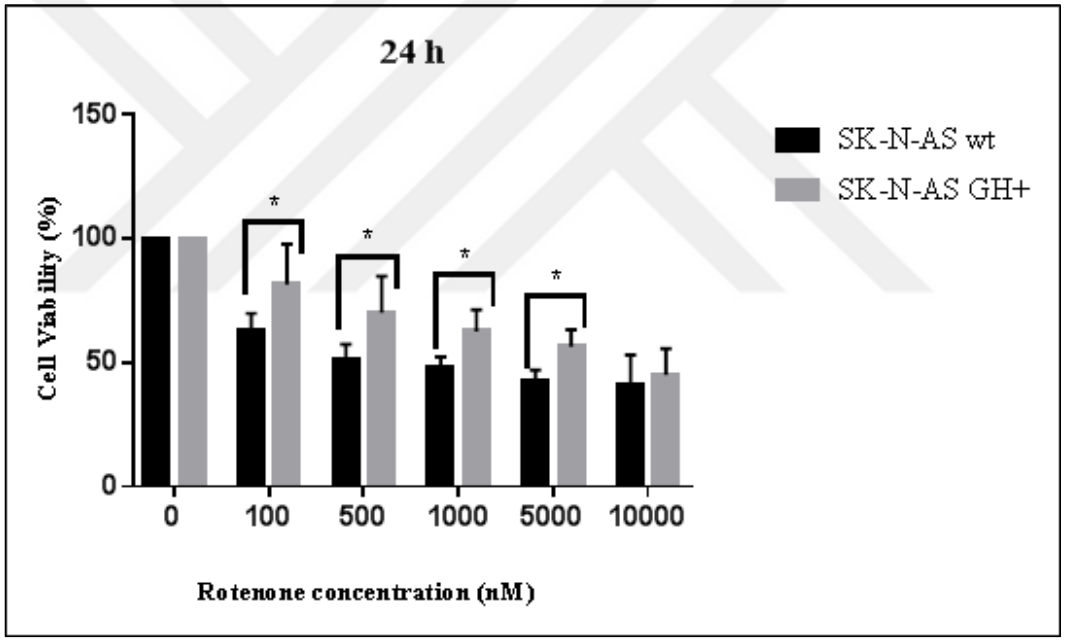

Figure 4.5. Dose dependent effect of rotenone on wt and GH+ SK-N-AS cell viability. 10 000 cells/well wt and GH+ SK-N-AS cells were seeded in 96 well plates. Following cell adherence, cells were treated with increasing doses of rotenone (100 nM-10000 nM) for 24 h at 37°C. Thereafter, cells were applied with MTT reagent for 4 h at 37°C in dark. After removal of media, 200 μL DMSO was applied to the cells for 5 minutes at room temperature in dark followed by the measurement of the colorimetric difference in ELISA Reader at 405 nm.

4.5. The Effect of Rotenone on Cell Growth in wt and GH+ SK-N-AS Cell Lines

In order to determine the effect of rotenone (100 nM) on wt and GH+ SK-N-AS cell proliferation, we performed growth assay. 100 nM rotenone treatment inhibited cell proliferation in a time-dependent manner in both wt and GH+ SK-N-AS cells. Despite GH transfection there was no significant difference in cell proliferation between wt and GH+ SK-N-AS cells after 72 h. Rotenone treatment significantly inhibited cell growth in both cell types in a similar rate in contrary to MTT assay shown above (Figure 4.5).

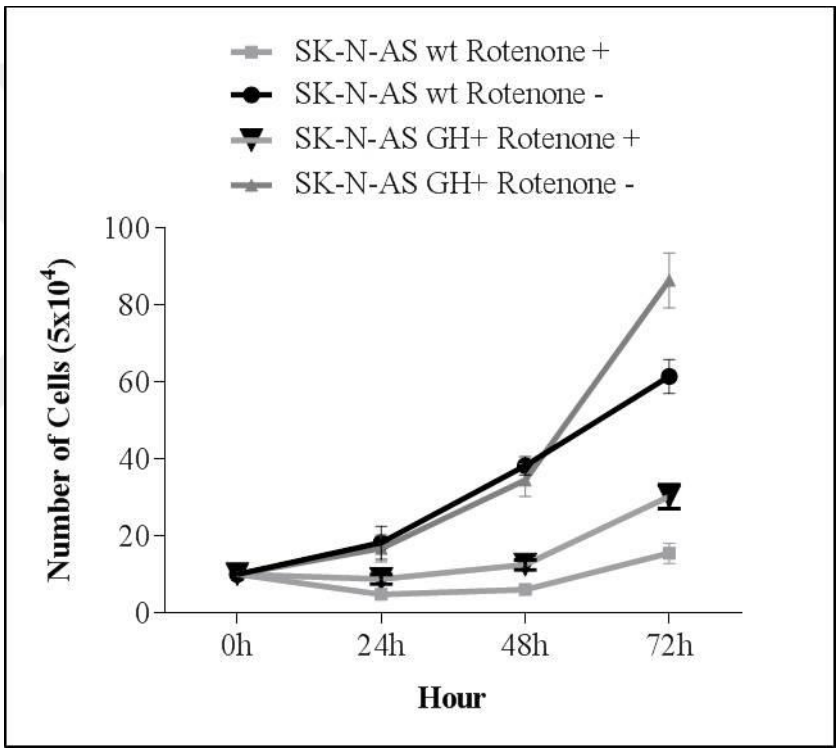

Figure 4.6. Time dependent effect of rotenone (100 nM) on cell growth in wt and GH+ SK-N-AS cell lines. 50 000 cells/well wt and GH+ SK-N-AS were seeded into 12 wells plates. After overnight incubation at 37°C, cells were treated with 100 nM rotenone. After 24 h medium was discarded, cells were trypsinized and resuspended in 1x PBS. 10µL cell suspension was mixed with 10µL trypan blue and cells were counted under light microscope *via* hemocytometer for the first time. The same process was repeated every 24 h for 3 days.

4.6. The Effect of Rotenone on Colony Formation ln wt and GH+ SK-N-AS Cells

The role of rotenone on colony formation in wt and GH+ SK-N-AS cells were evaluated by colony formation assay. Although GH expression induced slightly more colony formation in GH+ SK-N-AS cells, rotenone (100 nM) prevented colony formation in both wt and GH+ SK-N-AS cells. This experiment repeated three times. The average of colonies in the untreated and rotenone treated groups in wt SK-N-AS cells were 474 and 409, respectively. In comparison, in GH+ SK-N-AS cells averages of the colonies in rotenone – and rotenone + group were 657 and 478 respectively. There was no statistically significant difference in colony numbers of wt and GH+ SK-N-AS cells (Figure 4.6).

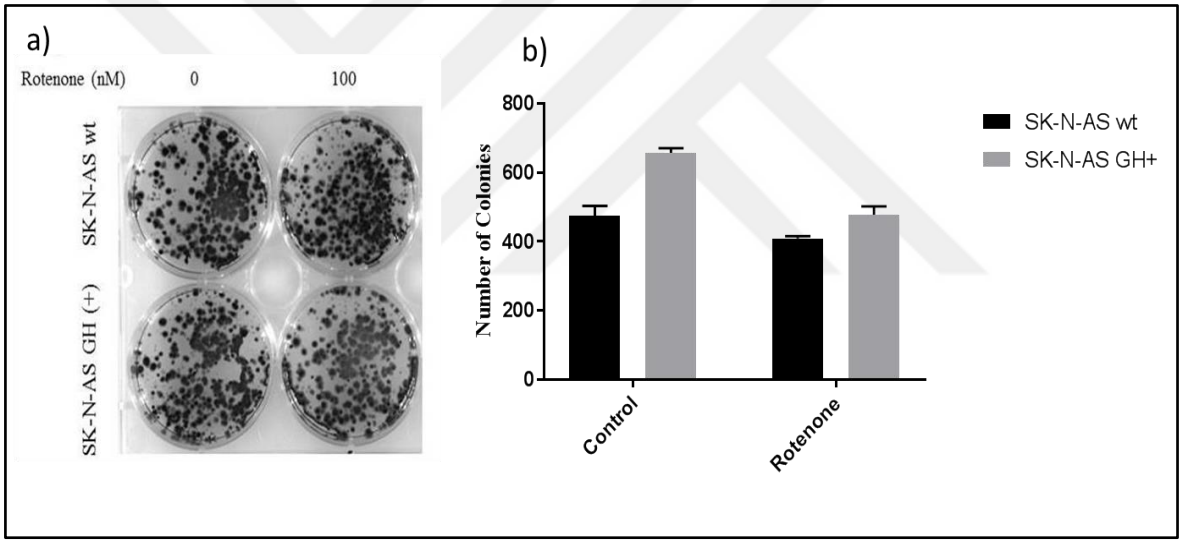

Figure 4.7. The effect of rotenone on colony formation in wt and GH+ SK-N-AS cells. a) 2 500 cell/well wt and GH+ SK-N-AS cells were seeded into 6-well plates. Following overnight incubation at 37°C cells were treated with 100 nM rotenone for 3 days. Thereafter rotenone and medium were discharged. In the following, every 3 day medium was renewed without any rotenone addition. After 9 days colonies were stained with crystal violet for visualization and counted manually under ChemiDoc. b) Number of colonies in wt and GH+ SK-N-AS cells were graphically illustrated.

4.7. Soft Agar Assay for The Evaluation of Rotenone Effect on Colony Formation of wt and GH+ SK-N-AS Cells

This experiment evaluates colony formation of wt and GH+ SK-N-AS cells starting from a single cell. wt and GH+ SK-N-AS cells were treated with 100 nM rotenone for 5 days. After this time, diameters of the colonies, 6 representative colony formations in each group were measured manually using light microscope with a ruler. Diameters of rotenone untreated GH+ SK-N-AS cells were generally larger than the wt SK-N-AS cells. The average diameter value of untreated wt SK-N-AS cells was 20.43 μm in contrast to the average value of rotenone treated colony diameter that decreased to 10.95 μm resulting in a ratio of 1.87. The comparison of the average value of colony diameters in untreated GH+ and rotenone treated GH+ cells were 23.53 μm and 12.66 μm, respectively resulting again in a similar ratio of 1.86. Thus, GH expression induced a slightly higher value of colony diameter but rotenone treatment decreased colony formation in both wt and GH+ cells in a comparable value (Figure 4.7.a).

DiOC6 dye colors endoplasmic reticulum and mitochondrial membrane only in vital cells. All colonies of rotenone treated and untreated wt and GH+ SK-N-AS cells were colored with DiOC6 and evaluated in light and fluorescence microscope for their vitality, photographed and compared with each other. The results of both microscopic pictures were always similar in all 4 experiments with both cell types (Figure 4.7.a)

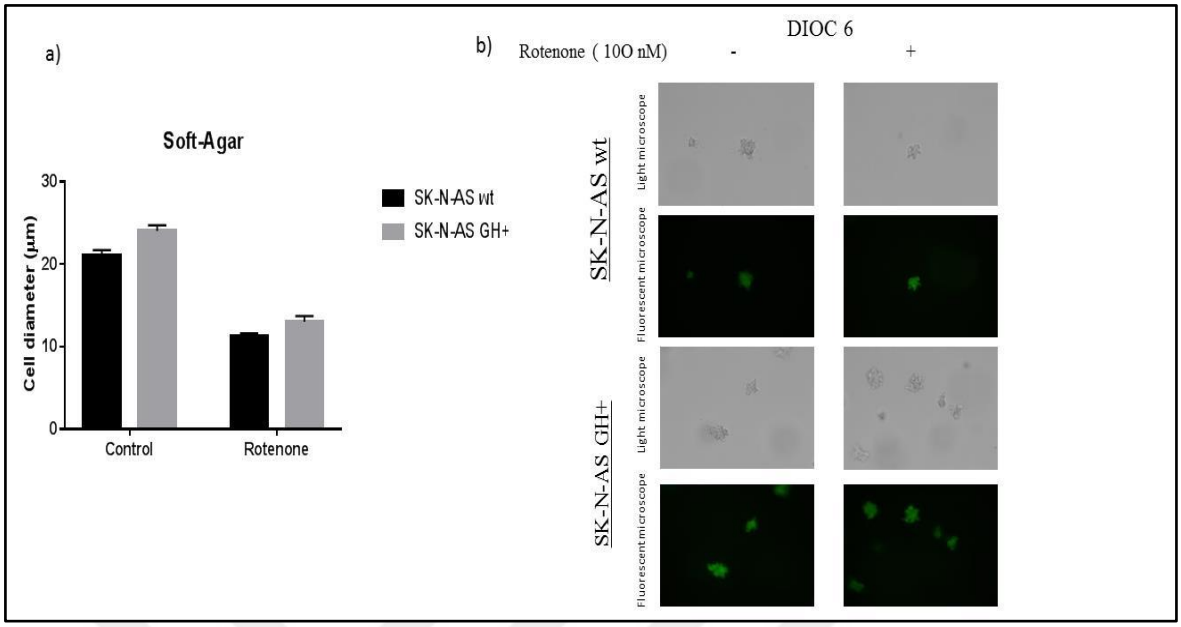

Figure 4.8.The effect of rotenone on soft agar colony formation in wt and GH+ SK-N-AS cells. 6-well plates were firstly coated with 0.5% agar. 2 500 cell/well wt and GH+ SK-N-AS cells each were mixed up with 100 nM rotenone in 0.3% agar and seeded into the previously coated 6-well plate and incubated at 37°C for 5 days. After 5 days colony diameters of each 6 representative colonies for wt and GH+ SK-N-AS cells were measured manually in light microscope and documented. a) Results are shown graphically. b) The colonies were then dyed with DiOC6 and incubated for 5min at 37°C in dark. wt and GH+ SK-N-AS cells were then visualized under light and fluorescence microscope.

4.8. The Effect of Rotenone on The Expression of JAK/STAT Pathway Key Players in wt and GH+ SK-N-AS Cells

To evaluate the role of rotenone (100 nM) on the expression of JAK2, STAT1, 3 and p-STAT1, 3 proteins and the protective effect of transfected GH expression in GH+ SK-N-AS cells on JAK/STAT signaling pathway all the above named proteins were firstly determined by immunoblotting.

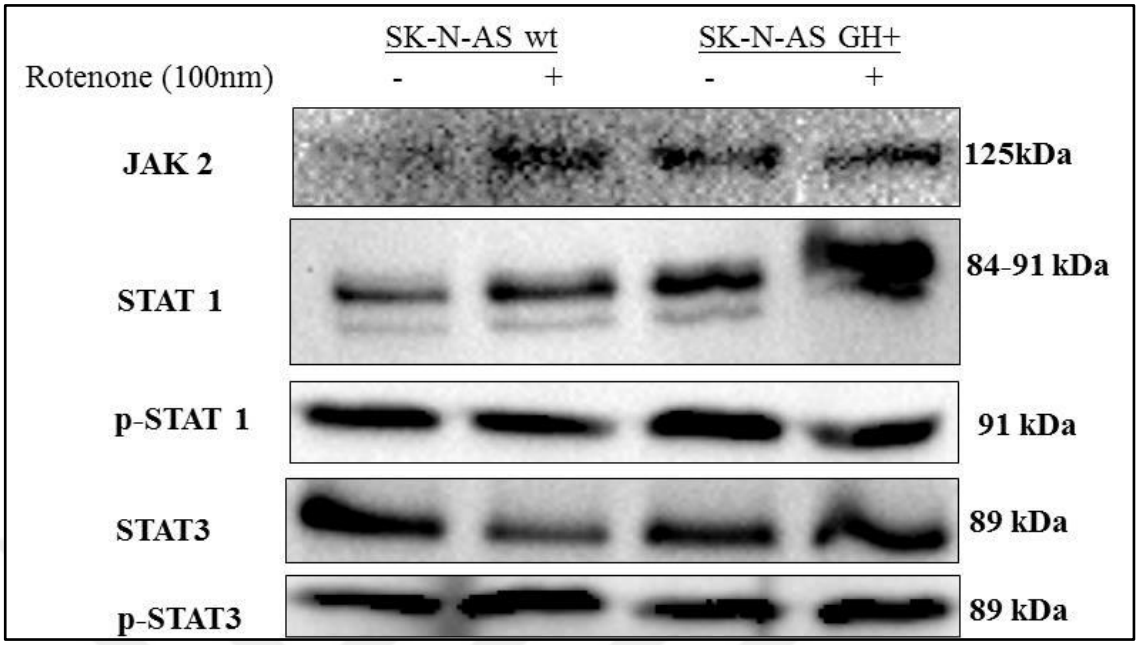

Figure 4.9. Demonstrates the expresssion of JAK/STAT pathway in wt and GH+ SK-N-AS cells by Immunoblotting. 800 000 cell/well wt and GH+ SK-N-AS were seeded in 60 mm petries. After overnight incubation (37°C), cells were treated with 100 nm rotenone. Total protein was isolated from each cell line and 30 µg protein loaded in 12% SDS-PAGE, transferred to 0.22µm PVDF membrane and blocked with 5% skimmed milk. JAK 2, STAT 1, p-STAT 1, STAT 3 and p-STAT 3 specific primary antibodies were blocked in 5% skimmed milk followed by the application of anti-rabbit secondary antibodies. Results were visualized by ECL in ChemiDoc.

In immunoblotting, JAK 2 expression on the cell membranes are demonstrated in all cell types in a similar amount except in rotenone negative wt SK-N-AS cells which may result from a technical problem during the experiment as p-STATs and STAT3 is regularly activated. In the above experiment these two bands are found with a higher amount in the rotenone treated wt and GH+ SK-N-AS cells. STAT1 protein was highest in SK-N-AS GH+ than in wt cells.

STAT1 phosphorylation into p-STAT1 is needed for the proceeding of cells into apoptosis. p-STAT1 amount was higher in GH+ SK-N-AS cells when rotenone was not applied. In wt SK-N-AS cells with or without rotenone treatment and in rotenone treated GH+ SK-N-AS cells, there was a similar amount of p-STAT1 expression. STAT3 is pre-existent in the cell

cytoplasm of all cells and in case of its activation (p-STAT3) cells can escape apoptosis and survive. As can be seen in figure 4.8, line 4, STAT3 amount was reduced in only rotenone treated wt SK-N-AS cells. However, p-STAT3 amount was increased in all; rotenone treated or untreated wt and GH+ SK-N-AS cells.

4.9. The Effect of Rotenone on The Expression of Bcl-2 Family Members in wt and GH+ SK-N-AS Cells

Apoptosis inducing proteins such as PARP, caspase3, caspase 7 and caspase 9 were analyzed by applying immunoblotting technique. PARP has a very significant role in many levels of apoptosis including and starting at cleavage of caspases from pro-caspases and caspase activation. PARP level was higher in rotenone treated and untreated wt SK-N-AS cells when compared to the GH+ SK-N-AS cells. This result confirms that rotenone has/had no effect on the cellular PARP levels in SK-N-AS NB cells.

BCL-2, a cytoplasmic anti-apoptotic protein, and Bax, a mitochondrial membrane bound pro-apoptotic protein, levels in wt and GH+ SK-N-AS cells were observed in the presence and absence of rotenone. While rotenone decreased Bcl-2 expression only in wt SK-N-AS cells it had no effect on Bax expression resulting in an increased rate of apoptosis in wt SK-N-AS cells. In contrary, rotenone didn't alter Bcl-2 and Bax expression levels GH+ in SK-N-AS cells.

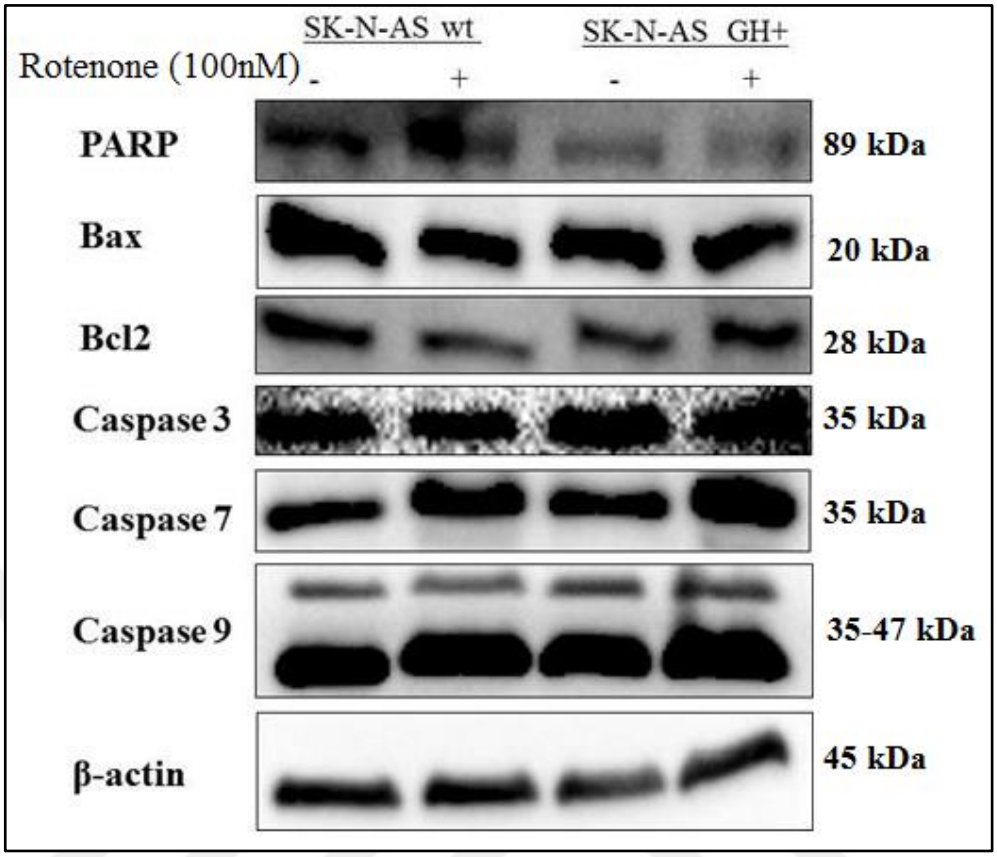

Figure 4.10. Demonstrates thre expresssion of Bcl-2 and caspase family pathway in wt and GH+ SK-N-AS cells by Immunoblotting. 800 000 cell/well wt and GH+ SK-N-AS were seeded in 60 mm petries. After overnight incubation at 37°C, cells were treated with 100 nm rotenone. Total protein was isolated from each cell line and 30 µg protein loaded in 12% SDS-PAGE, transferred to 0.22 µm PVDF membrane and blocked with 5% skimmed milk. PARP, Bax, Bcl-2, caspase 3, caspase 7 and caspase 9 specific primary antibodies were blocked in 5% skimmed milk followed by the application of anti-rabbit secondary antibodies. Results were visualized by ECL in ChemiDoc.

In case of caspase family members, Figure 4.9 lines 4-6, addition of rotenone increased the expression of caspase 7 and caspase 9 in both wt and GH+ SK-N-AS cells whereas caspase 3 expressions was only and slightly increased in GH+ cells. Additionally, the experiment shows that both wt and GH+ SK-N-AS cells have usually similar levels of caspase 3, 9 and 7 expressions without any treatment. Caspase family members may consist of 2- 3 different protein components. Caspase 3 protein may show 17, 19 and/or 35 kDa protein bands, caspase 7 protein 20 and or 35 kDa protein bands whereas caspase 9 proteins may have 35,

45 and/or 47 kDa protein bands. In Figure 4.9, in case of caspase 3 and 7 only the 35 kDa proteins were seen in contrast to 35 and 47 kDa protein bands in caspase 9.

Increased caspase 9 expression on its own activates also caspase 3 and 7 expression. In the following, the latter two again activate death substrates within the cytoplasm indicating initiation of apoptosis.

4.10. The Effect of Rotenone on Mitochondrial Membranes and Nuclear DNA wt and GH+ SK-N-AS Cell Lines Evaluated by Fluorescent Colors

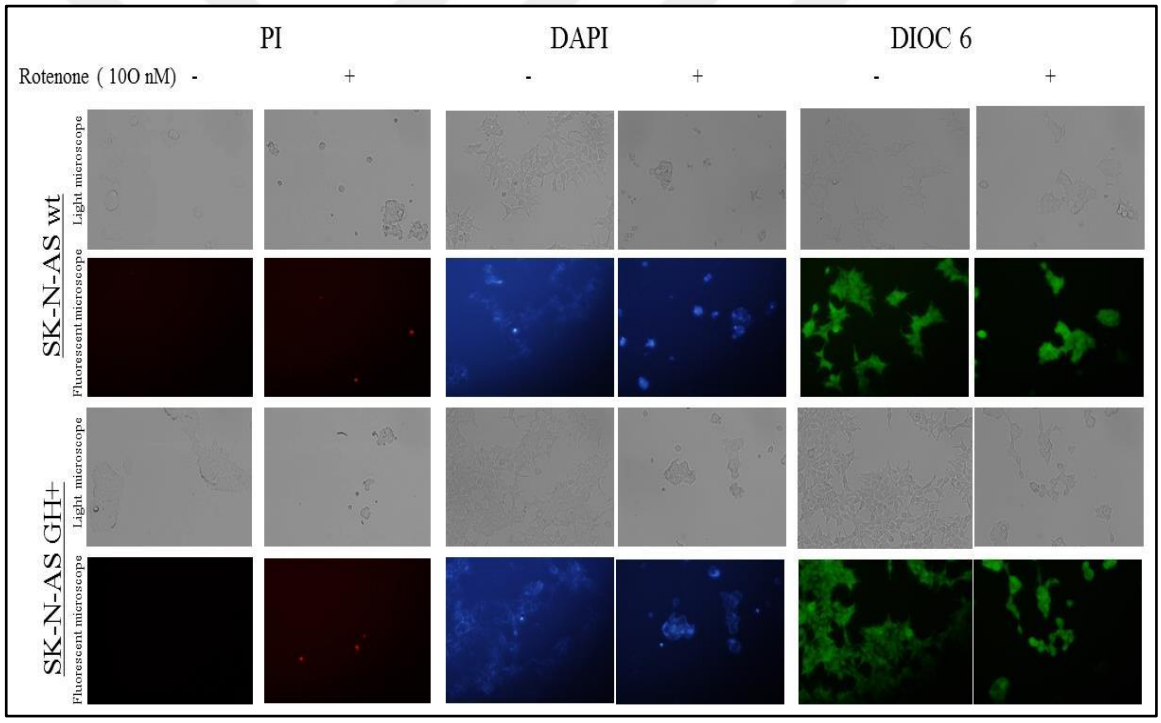

Figure 4.11. Demonstrates the death cells by PI, DNA fragmentation by DAPI and mitochondrial membranes by DiOC6 staining. 50 000 cell/well wt and GH+ SK-N-AS were seeded in 12 well plate. After overnight incubation (37°C), cells were treated with 100 nM rotenone for 24 h and stained with PI, DAPI and DiOC6. Respectively, cells were incubated for staining at 37°C with PI over 30min., with DAPI over 5min. and with DiOC6 15min. in dark. Thereafter, cells were visualized under light and fluorescence microscope**.**

Figure 4.10 shows in the first two lines the pictures of wt SK-N-AS cells either taken at light microscope (first line) or at fluorescence microscope (second line) stained with PI,

DAPI and DiOC6 dyes with or without rotenone treatment. Lines 3 and 4 show the same procedure for SK-N-AS GH+ cells.

The first two columns on the left side illustrate the apoptotic cells stained by PI for both wt and GH+ SK-N-AS cells. In both cell lines without rotenone treatment, there was no PI stained cells as expected. In case of rotenone treatment, there was a bit more apoptotic cells in GH+ SK-N-AS cells than in wt SK-N-AS cells.

The middle two columns show the DAPI colorization of DNA fragments for both cell types with or without rotenone application. In both cell lines without rotenone treatment, there were DNA fragments only in one cell which comply for a usual observation. In both treated cell lines there was a similar increase in DNA fragmentation due to rotenone toxicity.

The last two columns illustrate the same procedure for the mitochondrial membrane coloration (DiOC6) in living cells. In untreated cell lines, there were much more living cells than in the rotenone treated cell lines. After rotenone application, there were much less living wt SK-N-AS cells than GH+ cells. Even if compared to the untreated controls where GH+ SK-N-AS cell count seems to be more than wt SK-N-AS cells, the apoptosis rate in GH+ SK-N-AS cell after rotenone treatment was a bit more than in wt cells.

4.11. The Effect of Rotenone on Reactive Oxygen Species (ROS) Generation in wt and GH+ SK-N-AS Neuroblastoma Cells

In order to evaluate the effect of rotenone on ROS generation in wt and GH+ SK-N-AS NB cells and the reversibility of this effect by the antioxidant agent N-acetylcysteine (NAC), MTT cell viability assay were performed with rotenone, NAC, and rotenone and NAC combination (Figure 4.11).

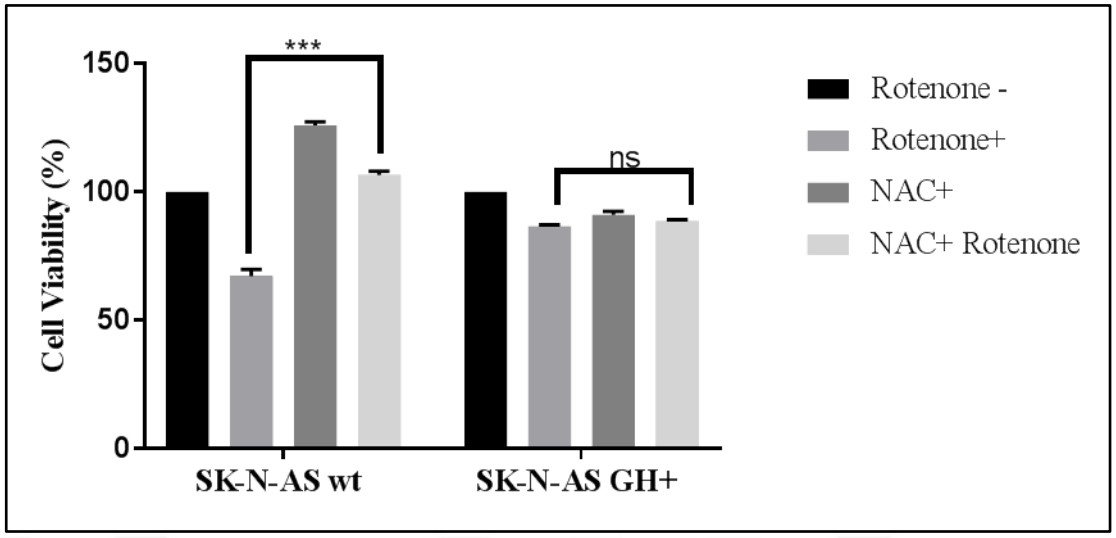

Figure 4.12. Effect of rotenone on ROS generation in wt and GH+ SK-N-AS cells. 10 000 cells/well of both cell types were seeded in 96 well plate and incubated for 24 h at 37°C to enable adherence. Then, the experiment was performed with a rotenone negative control group, a rotenone (100 nM), a NAC (10 mM) and a rotenone + NAC combination-treatment group. After 24 h, MTT reagent was applied for 4 h at 37°C in dark. After removal of media, 200 µL DMSO was applied to the cells for 5 minutes at room temperature in dark followed by the measurement of the colorimetric difference in ELISA Reader at 405 nm.

Rotenone treatment again resulted as seen in Figure. 4.4. in higher mortality of wt SK-N-AS cells. 10 mM of NAC treatment led to an increase of 11% cell viability in SK-N-AS wt cells. This effect was a bit less in GH+ SK-N-AS cells. Although rotenone alone decreased cell viability in a rate of 35% and 11% in wt and GH+ SK-N-AS cells respectively, this effect was prevented and cell viability was even though a bit increased by NAC co-treatment in only wt SK-N-AS cells after 24 hours. This difference in cell survival between rotenone mono- and rotenone + NAC combination treatment wt SK-N-AS cells was statistically highly significant (p value < 0.001, Figure 4.11). However, the addition of NAC to rotenone in GH+ SK-N-AS cells had only very low protective effect on cell viability and did not reach any significance (p value > 0.05, Figure 4.10).

4.12. The Effect of Rotenone on Cell Cycle wt and GH+ SK-N-AS Neuroblastoma Cells

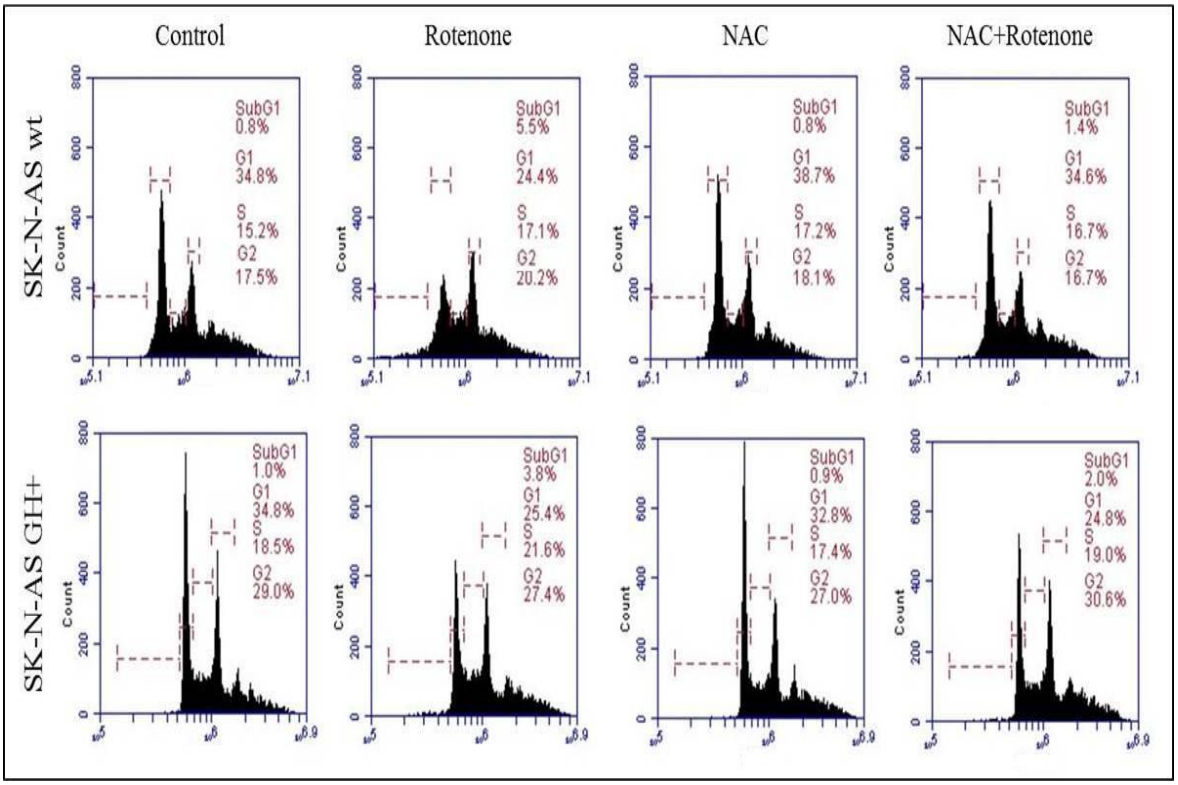

Figure 4.13. Effect of rotenone on cell cycle in wt and GH+ SK-N-AS cells evaluated by flow cytometry. 200 000 cells/well of both wt and GH+ SK-N-AS cells, were seeded in 6 well plate. After overnight incubation (37°C), cells were treated with 100 nM rotenone alone, 10 mM NAC alone and rotenone + NAC combination of the same doses. After 24 h, all cells were removed from wells with trypsin, incubated with ethanol at -20°C for 1 week, dyed with PI and incubated for 20min at room temperature in dark. Thereafter, in both cell types 10 000 cells were measured and visualized by FACS analysis.

Cell cycle analyses were performed in rotenone, NAC and rotenone + NAC co-treated wt and GH+ SK-N-AS cells by flow cytometry after previous staining with PI. Control groups for both cell types are shown as graphs in the left boxes (Figure 4.12) illustrating the cell count (y axis) in different phases of the cell cycle and PI coloration intensity (y axis). The portion between y axis and the first peak corresponds to Sub G1 phase is a hallmark of apoptosis and can be seen in FACS only if apoptotic cells are present, the first peak to

G0/G1 phase and the second peak to G2 phase. The lowered area between the two peaks complies with the S phase of cell cycle and the region after the second peak with the M phase. The percentages given for all phases in each graph corresponds to the number of cells that are found in the named cell cycle phase.

In the following, the findings of subG1 phase are described. In both controls there are a similar amount of cells found to be in apoptosis, e.g. 0.8% in wt cells and 1.0% in GH+ SK-N-AS cells. After rotenone treatment the apoptotic cells were increased to 5.5% in wt SK-N-AS cells and to 3.8% in GH+ cells. NAC mono-treatment reduced the apoptosis rate to 0.8% in wt cells whereas in GH+ cells there was almost no change (0.9%) to the rate of untreated controls. In case of NAC and rotenone combination treatment, apoptosis rate was a bit increased in comparison to untreated control (from 0.8% to 1.4%), if compared to rotenone mono-effect then NAC combination reduced the decreased apoptosis rate from 5.5.% to 1.4% indicating a high protective effect on wt SK-N-AS cells. In case of GH+ SK-N-AS cells, the combination treatment had a lower protective effect, the apoptosis rate decreased from 3.8% (rotenone alone) to 2% which is 1% more than in the untreated control.

FACS analysis of the G1 phase, also an important part of this experiment, revealed the following: In wt SK-N-AS cells, rotenone treatment reduced the cells in G1 phase from 34.8% to 24.4%, NAC treatment increased these cells from 34.8% to 38.7% whereas in NAC + rotenone co-treatment, NAC neutralized the effect of rotenone completely (34.6%). In GH+ SK-N-AS cells, rotenone treatment similarly reduced cells in G1 phase from 34.8% to 25.4%. In contrast to wt cells, NAC mono-treatment did not promote cell proliferation in GH+ neuroblastoma cells (32.8%) and NAC + rotenone co-treatment did not neutralize rotenone toxicity (24.8%).

4.13. The Effect of Rotenone on Cellular Reactive Oxygen Species (ROS) Generation in wt and GH+ SK-N-AS Cells

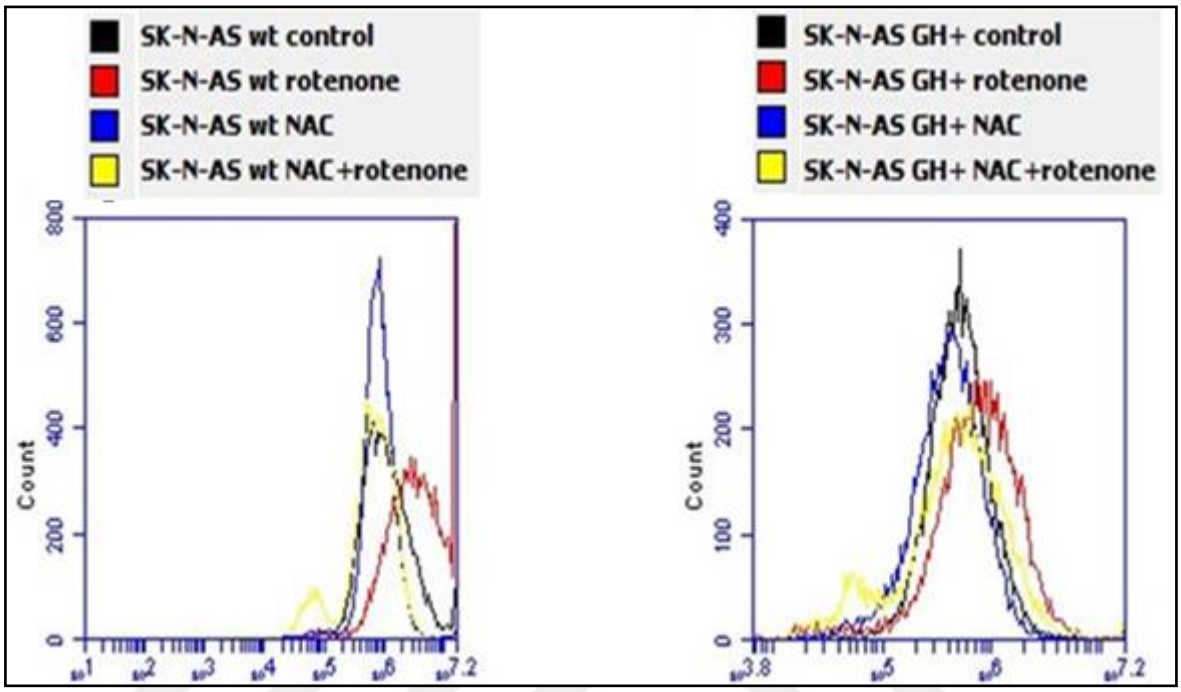

Figure 4.14. Detection of rotenone induced cellular Reactive Oxygen Species generation in wt and GH+ SK-N-AS cells by flow cytometry. 50 000 cells/well wt and GH+ SK-N-AS cells were seeded in 12 well plate and incubated overnight at 37°C. In the following, cells were treated with rotenone (100 nM) alone, NAC (10 mM) alone and rotenone + NAC combination for 24 h at 37°C. Thereafter, cells were dyed with 2′,7′-dichlorofluorescein diacetate (DCFD-DA) and incubated at 37°C for 20 min in dark. After incubation all cells were removed with trypsin and in both cell types 10 000 cells were measured and visualized by FACS analysis.

For detection of cellular ROS generation an oxidation-sensitive fluorescent dye, DCFH-DA, was used (Figure 4.13). The images show the living cell count on the y-axis and the amount of cellular ROS on the x-axis. Black colored peaks show ROS amount in untreated wt and GH+ SK-N-AS cells, left and right panel, respectively.

In wt SK-N-AS cells rotenone treatment (red line) reduced the living cells count (lower peak) but increased the individual cell ROS generation (wider and rightward shifted line).

In the same cells, NAC treatment (blue line) lead to strong cell proliferation with reduced ROS amount in individual cell (higher and narrow peak). Rotenone and NAC combination treatment, yellow line, was almost similar to the black control line indicating the neutralization of rotenone effect by NAC.

In GH+ SK-N-AS cells rotenone treatment reduced the living cell count more than in wt SK-N-AS cells but again individual cell ROS generation was increased (wider and rightward shifted red line). NAC treatment (blue line) was comparable to the own control peak.

In case of NAC + rotenone application, cells count was nearly halved and ROS generation in individual cells was slightly increased, indicating missing protective effect of NAC in GH+ SK-N-AS cells.

5. DISCUSSION

Rotenone is a widely used pesticide by farmers. Neurodegeneration such as Parkinson's disease has been frequently observed among farmers that are exposed to this chemical [75]. This pesticide has also been used by researchers to induce neurodegeneration in experimental animals.

This project focuses on the toxic effects of rotenone in SK-N-AS NB cells on cellular and protein levels as well as on the effects of possible neuro-protective and -regenerative factors on rotenone induced toxicity.

Growth hormone, GH, has been shown to have neuro-protective and neuro-reparative effects in neurodegenerative disorders [75]. Another glycoprotein, Erythropoietin, plays a massive role in survival, diversification and death in neuronal cells [75]. JNX1001 that is a small molecule protects the NB cells from oxidative stress caused by rotenone. Similar to JNX1001, GH also plays a role on protection of SK-N-AS cells from rotenone related problems [67].

SK-N-AS cell lines were therefore firstly evaluated for the presence of GH and were by immunoblotting found not to express this hormone, although they express GH receptors (Figure 4.1).

To evaluate a possibly protective and regenerative effect of GH in NB SK-N-AS cell lines as demonstrated in neurodegenerative diseases, we firstly transfected these cells with GH as to date only very few researchers did so. The successful transfection of SK-N-AS cells with GH was again demonstrated by immunoblotting after the transfection procedure (Figure 4.2). Thereafter, not processed SK-N-AS cells are called wt SK-N-AS and the transfected cells GH+ SK-N-AS in the following evaluations.

Since GH production and release is dependent of GHRH, which is secreted in mammals from the anterior lobe of the pituitary gland, wt and GH+ SK-N-AS cells were additionally examined for GHRH and growth hormone releasing hormone receptor, GHRHR.

GHRH protein was not expressed in wt SK-N-AS cells in contrast to GH transfected GH+ SK-N-AS cells as demonstrated by western blotting (Figure 4.3). This result possibly is an evidence for an autocrine GHRH production in GH producing (natural / transfected)

cells for GH production maintenance. In contrast, wt and GH+ SK-N-AS cells, both were positive for GHRHR in the same experiment (Figure 4.3).

In a next step, the dose dependent neurotoxicity of rotenone on both cell types was evaluated by MTT assay. There was a dose dependent decrease of cell viability in both cell types after 24 h of rotenone application but neurotoxicity was significantly (p value 0.037) higher in wt SK-N-AS than in GH+ SK-N-AS cells (Figure 4.4). At 100 nM rotenone treatment, cell viability decreased by 40 % in wt SK-N-AS cells, in contrast to 20% in GH+ SK-N-AS cells. Therefore, it is concluded that GH+ SK-N-AS cells are more resistant and better protected against rotenone effect as was expected according to previously published data.

The next question of interest was the time dependency of 100 nM rotenone on both cell lines. For this purpose, both cell lines were cultured, treated with rotenone for 24 h and observed over 72 h. Cells were then counted every 24 h and compared to untreated controls. The most impressive cell count reduction was seen after 24 h in both wt and GH+ SK-N-AS but unexpectedly in a similar manner without any significance (Figure 4.5). The discrepancy in lacking of protective effect in GH+ SK-N-AS cells might result from the different number of passages compared to the MTT assay result.

Another important issue was the evaluation of colony formation capability of both cell lines primarily and/or after rotenone treatment by colony formation assay over 9 days. In case of wt SK-N-AS cells, in the untreated assay there were 474 colonies in contrast to 409 colonies in rotenone treated cells. In GH+ SK-N-AS rotenone untreated assay there were 657 colonies in contrast to 478 in the rotenone group. Probably due to the protective effect of GH, there have been more colonies after 9 days in untreated GH+ SK-N-AS cells than in wt SK-N-AS cells. Although rotenone treatment caused twice as much colony loss in wt than GH transfected cells this difference was not significant (Figure 4.6).

In order to evaluate the size of the colonies, soft agar colony formation assay over 5 days was performed. Rotenone treated wt and GH+ SK-N-AS cells have developed less numbers of colonies than untreated control groups. When compared to wt cells GH+ SK-N-AS cells developed a bit more colonies. Furthermore GH+ SK-N-AS cell colony

diameters were larger than that of wt SK-N-AS cells in rotenone treated assay. Consequently, GH+ SK-N-AS cells have more colonies and larger colony diameters than wt SK-N-AS cells indicating a protective effect of GH (Figure 4.7).

Furthermore, rotenone effects on wt and GH+ SK-N-AS cells were evaluated on protein levels in regard with cell signaling pathways e.g. differences in protein levels and/or their activation state, oxidative stress induction, mitochondrial membrane integrity and cell viability by immunoblotting, florescent staining and flow cytometry.

In order to evaluate the role of rotenone on the expression of transcription related proteins (JAK2, STAT1, 3 and p-STAT1, 3) and the protective effect of transfected GH expression in GH+ SK-N-AS cells on JAK/STAT signaling pathway the existence and levels of all named proteins were firstly determined by immunoblotting in wt and GH+ SK-N-AS cells with or without rotenone application. JAK2 on cell membranes and STAT 1 were present as expected in both cell types under both conditions (Figure 4.8). The only very weak band in rotenone untreated wt SK-N-AS cells resulted probably from a technical problem during the experiment. STAT1 protein composed of two components (84 and 91 kDa) was strongly expressed in rotenone treated cells, being more pronounced GH + SK-N-AS cells indicating STAT1 activation. STAT1 phosphorylation into p-STAT1 is needed for the proceeding of cells into apoptosis. In wt SK-N-AS cells with or without rotenone treatment and in rotenone treated GH+ SK-N-AS cells, there was a similar amount of p-STAT1 expression while in untreated GH+ SK-N-AS cells; p-STAT1 amount was the highest. This result is not surprising as SK-N-AS cells are tumor cells, which should proceed into apoptosis for a natural elimination from the body. Activation of SK-N-AS cells by GH transfection may also result in p-STAT1 activation as GH and GHR are well known to activate STAT proteins inducing their phosphorylation and convention into p-STAT proteins. p-STAT molecules migrate to nucleus after being dimerized in cytoplasm and get connected to the target gene. p-STAT1 activates the apoptosis process. The amount of expressed STAT1 protein was higher in wt SK-N-AS cells than in GH+ SK-N-AS cells. This result indicates that GH+ cells may more escape apoptosis. In other words, cells were protected against the rotenone toxicity, when growth hormone was present. STAT3, a counter player of STAT1, is also pre-existent in the cytoplasm of all cells and in case of its activation (p-STAT3 generation) it prevents cells from apoptosis. STAT3 amount (Fig 4.8,

line 4) was reduced in only rotenone treated wt SK-N-AS cells. However, p-STAT3 amount was increased in all; rotenone treated or untreated wt and GH+ SK-N-AS cells. Increase of p-STAT 3 levels is an indicator of cells attempt to escape apoptosis [85].

Caspase induced apoptosis pathway was investigated by immunoblotting of Poly(ADP-ribose) polymerase (PARP), Bax, Bcl-2, caspase 3, 7, 9 proteins in wt and GH+ SK-N-AS cells with or without rotenone treatment. PARP plays a central role in the cleavage of caspases from pro-caspases and initiation of caspase pathways of apoptosis and therefore was also investigated in this study. PARP levels were higher in rotenone treated and untreated wt SK-N-AS cells than in GH+ SK-N-AS cells indicating a protective effect of GH transfection. Additionally this result also confirms that rotenone has no effect on the cellular PARP levels (Figure 4.9). On the other hand, PARP may also induce necrosis. In case of Bax, a pro-apoptotic, mitochondrial membrane bound protein, similar degree of high expression was found in both cell types with or without rotenone application, neither indicating that neither GH transfection had a protective nor rotenone had an increasing effect on this protein (Figure 4.9, line 2). If Bak, a second pro-apoptotic protein, binds to Bax, the following changing of mitochondrial membrane potential leads to an increased release of cytochrome c (Figure 2.6) from mitochondria into cytoplasm [112]. Cytochrome c binds to caspase 9 and forms a protein called apoptosome. This protein in turn activates caspase 3 and initiates the intrinsic pathway of apoptosis on cytochrome c expression. In contrary, in case of BCL-2, a cytoplasmic anti-apoptotic protein, rotenone decreased Bcl-2 expression only in wt SK-N-AS cells resulting in an increased rate of apoptosis in these cells. In case of GH+ SK-N-AS, rotenone did not alter Bcl-2 levels indicating a protective effect of GH transfection (Figure 4.9, line 3). In case of caspase family members, Figure 4.9 lines 4-6, immunoblotting showed that both wt and GH+ SK-N-AS cells have usually similar levels of caspase 3, 9 and 7 expressions without any treatment. This result is not surprising, since SK-N-AS cells are tumor cells where a certain rate of apoptosis is expected. Addition of rotenone increased firstly the expression of caspase 9 in both cell lines but in GH+ cells more than in wt SK-N-AS cells. Caspase 9 on its part activates caspase 3 and 7 in apoptosis. As immunoblotting demonstrated caspase 3 activation (increase in protein levels) was found only in GH+ cells whereas caspase 7 were activated in both rotenone treated cell types, being slightly more increased in GH transfected SK-N-AS cells (Figure 4.9, line 4 and 5) indicating that GH transfection-induced

protective effect (Bcl-2 increase in rotenone treated GH+ cells) was not sufficient to prevent apoptosis in these cells. Additionally, this result show that at least the intrinsic pathway of caspase induced apoptosis is involved rotenone treated SK-N-AS cells.

Arsenic trioxide for example causes oxidative stress and induces apoptosis *via* activation of caspase 3 [113] in SH-SY5Y and SK-N-AS cell lines and is therefore used as a chemotherapeutic agent.

In the following, the effects of rotenone on mitochondrial membrane and nuclear DNA were evaluated in both cell lines with and without rotenone treatment by fluorescent dyes, DiOC6, DAPI and PI. PI staining illustrates apoptotic cells. In both cell lines without rotenone treatment, there was no PI stained cells seen as was to be expected. In contrast, in case of rotenone treatment, there were some apoptotic cells visible in GH+ SK-N-AS cells and in wt SK-N-AS cells (Figure 4.10, second left column, line 2 and 4). DAPI staining only dye DNA fragments. In both cell lines without rotenone treatment, there were only few DNA fragments visible (Figure 4.10, third left column, line 2 and 4) which comply for a usual observation. In contrary, in treated cell lines there was a similar increase in DNA fragmentation due to rotenone toxicity as is seen in Figure 4.10, fourth column, line 2 and 4. These findings indicate that dying cells are found in a very late phase of apoptosis. The mitochondrial membrane dye, DiOC6, stains the membranes only in living cells. In untreated wt and GH+ SK-N-AS cells, there were much more living cells than in the same cell lines after rotenone application (Figure 4.10, fifth and sixth column, line 2 and 4). This effect was more pronounced in wt SK-N-AS cells than GH+ cells. Even if compared to the untreated controls where GH+ SK-N-AS cell count seems to be more than wt SK-N-AS cells, the apoptosis rate in GH+ SK-N-AS cell after rotenone treatment was a bit more than in wt SK-N-AS cells as indicated by the staining of destroyed mitochondrial membranes. This result confirmed the results of caspase immunoblotting (Figure 4.9), that apoptosis in SK-N-AS cells were triggered on the mitochondrial pathway, which means the intrinsic pathway of caspase related apoptosis.

Oxidative stress is known to affect cell cycle, increase or decrease protein levels, phosphorylation and activation of proteins, transcription factors and inducing apoptosis [114].

Rotenone interferes with the electron transport chain, ECT, in mitochondria by strong inhibition of mitochondrial membrane bound complex 1 and the subsequent electron transfer within the mitochondrial matrix. As cellular oxygen is reduced, reactive oxygen species, ROS are generated which can damage mitochondrial membrane and/or DNA. Therefore, it was of great interest to evaluate the effect of GH transfection and the effect of the antioxidant and also detoxifying agent NAC on rotenone induced ROS generation in SK-N-AS NB cells by MTT cell viability assay using rotenone alone, NAC alone and rotenone + NAC combination (Figure 4.11). NAC is also used as a detoxifying agent in paracetamol overdose. NAC have been demonstrated to reduce rotenone-induced toxicity in the SH-SY5Y NB cell line [115]. Rotenone treatment again resulted as seen in Fig. 4.4. in decreased cell viability in a rate of 35 and 11% in wt and GH+ SK-N-AS cells respectively. NAC treatment led to an increase of 11% cell viability in wt SK-N-AS cells after 24 hours. This effect was lesser in GH+ SK-N-AS cells (Figure 4.11). In case of rotenone and NAC combination, the rotenone effect was neutralized in wt SK-N-AS by NAC in a statistically highly significant (p value < 0.001, Figure 4.11) manner. This result is in concordance with previous observations regarding NAC´s protective effect from oxidative stress on SH-SY5Y NB cells [116]. However, the same combination had GH+ SK-N-AS cells only very low protective effect on cell viability and did not reach any significance (p value > 0.05, Figure 4.11).

The same protocol, rotenone alone, NAC alone and rotenone + NAC combination was used for flow cytometric analysis to evaluate the effects of rotenone and its protection in both wt and GH+ SK-N-AS cells during the cell cycles by PI staining (Figure 4.12).

Rotenone was found to increase the apoptotic cell rate in wt SK-N-AS cells from 0.8% (untreated cells) to 5.5% in subG1 phase and to 3.8% in GH+ cells. NAC mono-treatment reduced the apoptosis rate to 0.8% in apoptotic in wt cells whereas in GH+ cells there was almost no change (0.9%) in comparison to the rate of untreated controls. In case of NAC and rotenone combination treatment in wt cells, apoptosis rate was a bit increased in comparison to untreated control (from 0.8 to 1.4%), if compared to rotenone effect then NAC combination reduced the increased apoptosis rate from 5.5 to 1.4% indicating a high protective effect on wt SK-N-AS cells. In case of GH+ SK-N-AS cells, the combination

treatment had a lower protective effect, the apoptosis rate decreased from 3.8% (rotenone alone) to 2% which was 1% more than in the untreated control.

FACS analysis of the G1 phase, also an important part of this experiment, revealed that in wt SK-N-AS cells, rotenone treatment reduced cells in G1 phase from 34.8 % to 24.4%, NAC treatment alone increased these cells from 34.8 % to 38.7% whereas in NAC + rotenone co-treatment, NAC neutralized the effect of rotenone completely (34.6%). In GH+ SK-N-AS cells, rotenone treatment similarly reduced the cells in G1 phase from 34.8% to 25.4%. In contest to wt cells, NAC treatment did not promote cell proliferation in GH+ SK-N-AS cells (32.8%) and NAC+ rotenone co-treatment did not neutralize rotenone toxicity (24.8%).

Rapamycin e.g. is an anti-cancer therapeutic agent that exerts its effect through apoptosis induction in tumor cells. Detailed evaluation of its mechanism of action revealed that rapamycin arrests the cell cycle at G1 phase via specific inhibitor of the mTOR protein kinase and induces apoptosis. Rapamycin is also effective in the treatment of NB and arrests SK-N-AS cells at SubG1 phase [115] in contrast to rotenone that has been shown to arrest tumor cells at G2/M phase of cell cycle [115].

As the effects of possible rotenone induced ROS generation on mitochondrial membranes were indirectly shown by DIOC 6 staining (Figure 4.10) it was of great interest to also demonstrate the ROS generation directly which is performed by an oxidation-sensitive fluorescent dye, DCFH-DA. The experiment was performed by flow cytometry in the same experimental pattern/series as described in MTT cell viability assay and visualized by FACS (Figure 4.13).
In wt SK-N-AS cells rotenone treatment (red line) reduced the living cells count (lower peak) and increased the individual cell ROS generation (peak was shifted to the right). In the same cells, NAC mono-treatment (blue line) lead to strong cell proliferation with reduced ROS amount (peak was shifted to the left). Rotenone and NAC combination treatment, yellow line, was almost similar to the black control line indicating the neutralization of rotenone effect by NAC in wt cells.

In GH+ SK-N-AS cells rotenone treatment reduced the living cell count more than in wt cells but again ROS generation was increased (rightward shifted red line). NAC mono-application (blue line) was comparable to the control peak. In case of NAC + rotenone

application, cells count was nearly halved and ROS generation was slightly increased, indicating missing protective effect of NAC in GH+ SK-N-AS cells.

Taken all the described experiments and results together, this study demonstrates:

- Those SK-N-AS cells bears GHR and GHRHR but are not able to produce GH naturally. However, after GH transfection there was besides GH production also GHRH production possibly to some degree due to autocrine stimulation.
- In MTT assay, a dose dependent neurotoxic effect of rotenone in both wt and GH+ SK-N-AS cells was seen but with a statistically significant rate of apoptosis only in wt SK-N-AS cells indicating some neuro-protective effect of GH.
- However, in the time dependent evaluation of the rotenone effect in both cell lines the expected neuro-protective effect of GH was very limited and did not reach significance.
- In respect with the colony stimulation assay, GH again did not develop a significant protective effect against rotenone toxicity.
- In the subsequently performed soft agar assay there was an increased number of colony formation with larger diameters in GH transfected SK-N-AS cells compared to wt SK-N-AS cells.
- SK-N-AS cells upon rotenone treatment, JAK2, STAT1, p-STAT1, STAT3 and p-STAT3 protein levels were demonstrated by immunoblotting. There was an increase in p-STAT3 protein in both cells with and without rotenone application indicating that SK-N-AS cells.
- The following evaluation of the caspase pathway of apoptosis by immunoblotting revealed an increase of PARP only in rotenone treated wt SK-N-AS cells, no change in high expression rate of pro-apoptotic protein, Bax, a slight increase in anti-apoptotic protein, Bcl-2, only in rotenone treated GH transfected SK-N-AS cells in contrast to a slight decrease in rotenone treated wt SK-N-AS cells and a similar increase of caspase 3 in both rotenone treated cell lines whereas in caspase 9 and 7 there was an apparent increase in rotenone treated GH+ SK-N-AS cells more than in rotenone applicate wt SK-N-AS cells. This result indicates the involvement of the intrinsic caspase pathway in apoptosis of rotenone treated SK-N-AS cells. Although, GH transfection induced some apoptosis protective effect by increase of Bcl-2 it was not sufficient to completely prevent apoptosis.

- Mitochondrial membrane destruction illustrated by DiOC 6 staining, an early apoptotic marker, demonstrates the mitochondrial involvement in the intrinsic apoptosis pathway. In addition, PI coloration confirms the apoptosis in these cells, whereas DAPI staining for DNA fragmentation corresponds to a very late phase of cell death.
- The anti-oxidative agent NAC was used to demonstrate the protective effect against rotenone induced ROS generation. The highly toxic ROS effects of rotenone in wt SK-N-AS cells were completely neutralized by addition of NAC (p value < 0.001). Unexpectedly, this effect could not be demonstrated in GH transfected SK-N-AS cells. A possible explanation for this observation may be the increased proliferation rate of GH transfected cells according to the nature of the hormone and the increased cell vulnerability in proliferation phase.
- Rotenone induced ROS generation was additionally investigated by the oxidation-sensitive DCFH-DA staining evaluated by flow cytometry. In wt SK-N-AS cells rotenone reduced the living cells count and increased cellular ROS generation which was neutralized upon NAC addition. In GH+ SK-N-AS cells rotenone reduced the living cell count in a higher value and increased ROS generation. NAC addition did not protect GH+ SK-N-AS cells from rotenone toxicity.
- The rotenone induced apoptotic effect was also demonstrated by flow cytometric analyses of cells in different phases of cell cycles after PI staining. Rotenone was found to increase the apoptotic cell rate in both but wt SK-N-AS cells more pronounced than GH+ SK-N-AS cells. NAC addition in wt SK-N-AS cells reduced the rotenone induced apoptosis rate highly whereas in GH transfected SK-N-AS cells, this protective effect was lower.

To the results, GH transfection reduced rotenone effects in some experiments under various conditions but was not effective to completely protect NB cells from apoptosis. One of the reasons for less efficacy of its neuro-protective effect might be the inability of SK-N-AS NB cells to produce GH naturally. In addition, NAC was effective in apoptosis prevention only in wt SK-N-AS cells. The preference of SK-N-AS cells for the intrinsic pathway (caspase9 activation) of caspase induced apoptosis and the mitochondrial involvement including ROS generation could be confirmed.

As the intrinsic pathway is not exclusive and other apoptosis pathways may occur simultaneously, additional investigation of Caspase 8 activation (extrinsing pathway), should be performed to exclude the additional involvement of the extrinsing pathway of caspase 8 driven apoptosis and caspase 12 and 4 to exclude any involvement of endoplasmic reticulum involved apoptosis pathway.

Rotenone toxicity may not only cause apoptosis but eventually also cell necrosis, eg, in excessive DNA damage and overactivation of PARP that can result in cell necrosis instead of caspase 9 activation. There was a weaker induction of PARP in GH+ SK-N-AS cells in our experiments indicating the protective effects of GH on necrosis. However, to concisely exclude this possibility, Annexin V staining FACS measurement of cell cycles phases would be needed.

These investigations can provide additional important informations in the research field of SK-N-AS NB cells in regard with translational aspects expand and complete the present work by diligently studying further molecular mechanisms involved in certain physiological and/or pathological conditions.

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APPENDIX A: CHEMICALS

Table A.1. Chemicals

Chemicals	Tradename	Catalog No
Ethanol	ALKO MED	CAS 64-17-5
Absolute Ethanol 100%	Sigma	32221
Methanol	Sigma-Aldrich	24229
Isopropanol	Merck	K44518295 321
2-meraptoethanol	Merck	54805940517
Acetic acid	Sigma	27225
Acrylamide / Bis-Acrylamide of 30% solution	Sigma-Aldrich	A9099
Ammonium persulfate	Applichem	A2941
Coomassie Blue Reagent	Applichem	A3480
DMSO(Dimethyl sulfoxide)	Merck	K46505343 517
EDTA(Ethyenediaminetetraacetic acid)	Applichem	A2937
Laemmli Reagent	Sigma	S3401-1V
p-Coumaric Acid	Sigma	C9008-1G
Luminol	Applichem	A2185
MTT Solution(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Sigma	M2128-5G
PBS(Phosphate buffered saline)	Lonza	BE17-51-5F
BSA(Bovine Serum Albumin)	Sigma	A2153-106
M-PER Mammalian Extrection Reagent	Thermo	78501
Page Ruler Plus Prestained Protein Ladder	Fermentas	36619
Agarose	Sigma	A9539
Tris Base	Calbiochem	648310
Ethidium Bromide	Sigma	E1510
SDS(Sodium Dodecyl Sulfate)10% Solution	Applichem	UN1325
TEMED (Tetramethylethylenediamine)	Applichem	A1148,0100
Tween 20	Merck	S6740684 348
Dry Milk	Santa Cruz	SC-2325
Tris-HCL	Calbiochem	648317
Glycine	Calbiochem	3570
Hydrogen Peroxide	Merck	K39218400838
Sodium Chloride	Applichem	A2942
Phenol Red	Applichem	A2279,0250

Orange DNA Loading Dye 6X	Fermentas	R0631
O'GeneRuler 1kb DNA Ladder	Fermentas	SM1163
Propidium Iodide	Sigma	P4170-100MG
Lipofectamine 3000	Thermo Fisher	L3000001

APPENDIX B: EQUIPMENTS

Table B.2. Equipment's

Equipments	**Tradename**	**Product Code**
Refrigerator (No frost)	Arçelik	4263TMB
Deep Freezer	Arçelik	2041D
Vertical-flow Safety Cabinet	HeraSafe	
Distilled Water Device	TKA-Pacific	D56412
Freezer (-80^{o}C)	New Brunswick	Ultra Low
Electrophoresis System	Bio-Rad	165-8000
Electrophoresis Transfer System	Bio-Rad	170-4155
Inverter Fluorescent Attachement	Olympus	MODEL BH2-RFL
Fluorometer	Thermo	Fluoroskan
Power Source	Bio-Rad	PowerPac/B
Precision Scales	Sartorius	LE6202S
Hemocytometer	Sigma Aldrich	Z359629
Inverted Microscope	SOIF	XDS-1B
Incubator	Nüve	EN 025
Ice Maker	Scotsman	AF80
Magnetic Stirrer	Stuart/ProLab	SB162
Cooled Desktop	Eppendrof	5417R
Micropipette (0,5µl-10µl)	Thermo	EH52836
Micropipette (2µl-20µl)	Thermo	CJ17240
Micropipette (20µl-200µl)	Thermo	EH46925
Micropipette (200µl-1000µl)	Thermo	T27274
Microplate Reader	Bio-Rad	680
Autoclave	Nüve	OTO032
pH Meter	SEM/Mettler	N315
ChemiDoc	Bio-Rad	34090
PVDF Membran	Thermo	88518
Liquid Nitrogen Tank	Air Liquid	Arpege 40
Spectrophotometer	Biosciences	Ultraspec
Weighing	Sartorius	LE6202S
Tube Rotation Tool	Stuart	SRT9D
Vortex	Stuart/ProLab	SA8
X-Ray Film	34090	Thermo
Flow cytometry	BD Accuri	C6

APPENDIX C: CELL CULTURE EQUIPMENTS

Table C.3. Cell culture equipment's

Cell Culture Equipments	**Tradename**	**Catalog NO**
6 Well Petri Dish	TPP	92006
12 Well Petri Dish	TPP	92012
96 Well Petri Dish	TPP	92096
60 mm Petri	TPP	93060
100 mm Petri	TPP	93100
25 cm^2 Petri Dish	TPP	90025
Injector Kit	Astraject	216
Syringe Filter (0,22µm)	TPP	99722
Trypsin-EDTA	Gibco	25200-056
Penicillin-Streptomycin	Gibco	15140-122
FBS (Fetal Bovine Serum)	Gibco	10500-064
NEAA (Non-Essential Amino Acids)		11140050
DMEM Medium	PAN	P04-01160
SK N AS Cell line	ATCC	CRL-2137
G 418 Disulfate Salt Solution	Sigma	G8168
Rotenone	Sigma	83-79-4
Cryovial Tube	NUNC	V7634