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DEPARTMENT OF HISTOLOGY AND EMBRYOLOGY

IN-VITRO EFFECT OF MELATONIN ON HT-22 CELLS IN NEURODEGENERATIVE DISEASE MODEL

Ph.D. Thesis

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ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled **"*IN-VITRO* EFFECT OF MELATONIN ON HT-22 CELLS IN NEURODEGENERATIVE DISEASE MODEL"** was prepared by **Abair Awadalla Ahmed MAHDI** and supervised by **Doç. Dr. Mehmet Emin ÖNGER** was successful and unanimously accepted by committee members as a Ph.D. thesis, following the examination on the date 11.8.2023.

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ÖZET

NÖRODEJENERATİF HASTALIK MODELİNDE MELATONİNİN HT-22 HÜCRELERİ *İN VİTRO* ETKİSİ

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Bir epifiz bezi hormonu olan melatonin, nöroprotektif etkilere sahip olup potansiyel olarak Alzheimer ve Parkinson hastalığı gibi nörodejeneratif bozuklukların tedavisinde kullanılabilmektedir. Nörodejeneratif bozuklukların patojenik koşulları genellikle glutamat gibi toksik maddelerin kullanıldığı in vitro çalışmalarda simüle edilmektedir. Glutamat, protein sentezi için gerekli olan bir amino asittir ve beyindeki en sık görülen nörotransmiterdir. Nöral hücrelerde aşırı glutamat sentezi eksitotoksisiteye, oksidatif strese ve nöron ölümüne neden olmaktadır.

Bu çalışma, HT-22 hipokampus hücre hattında glutamat kaynaklı nörodejenerasyona karşı melatoninin nöroprotektif özelliklerini incelemektedir. HT-22 hipokampus sinir hücreleri, nöroprotektif olarak melatonine ve sitotoksik olarak glutamata maruz bırakıldı. Etkili dozlar için, IC_{50} değerleri ve hücre canlılığı MTT analizleri kullanılarak belirlendi. HT-22 hipokampal sinir hücreleri üç tekrarlı şekilde, 96 kuyucuklu plakaya ekildi ve optimizasyon için çeşitli zamanlarda (24-48-72 saat) farklı melatonin konsantrasyonları kullanılarak inkübasyona bırakıldı. Ayrıca, glutamatın toksisite dozu 24, 48 ve 72 saat gibi farklı sürelerde değerlendirdi. Her madde için IC_{50} değerleri hesaplandı.

Sonuçlar 570 nm absorbansta bir spektrofotometre kullanılarak analiz edildi. Glutamatın 2 mM ve daha yüksek konsantrasyonlarda kontrol grubuna kıyasla hücre canlılığını önemli ölçüde etkilediği bulundu ve toksisite etkisini gerçekleştirmek için 5 mM kullanıldı. Glutamat IC_{50} dozu 24 saat boyunca 2,465 mM'dir. Melatonin IC_{50} dozları ise 24, 48 ve 72. saatlerde sırasıyla 0,8399 mM, 0,48 mM ve 0,39 mM olarak bulundu. Sonuçlar melatoninin HT-22 sinir hücrelerinde glutamat toksisitesine karşı koruyucu etki gösterebileceğini gösterdi. Bu çalışmada sunulan kanıtlar, melatoninin HT-22 hücrelerinde hücre canlılığını etkileyen glutamat toksisitesine karşı nöroprotektif bir etki gösterdiğini güçlü bir şekilde ortaya koymaktadır.

Anahtar Sözcükler: Melatonin, HT-22 hipokampal hücre hattı, Glutamat, MTT, Hücre canlılığı

ABSTRACT

IN-VITRO EFFECT OF MELATONIN ON HT-22 CELLS IN NEURODEGENERATIVE DISEASE MODEL

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Melatonin, a pineal gland hormone, may have neuroprotective effects and potentially be used as a treatment for neurodegenerative disorders such as Alzheimer's and Parkinson's disease. The pathogenic circumstances of neurodegenerative disorders are usually simulated in *in-vitro* studies employing toxic substances such as glutamate. Glutamate is an amino acid required for protein synthesis and is the most frequent neurotransmitter in the brain. Excessive glutamate synthesis in the neural cells causes excitotoxicity, oxidative stress, and neuronal death.

This study examined melatonin neuroprotective properties against glutamate-induced neurodegeneration in the HT-22 hippocampus cell line. The HT-22 hippocampus neural cells were exposed to melatonin as neuroprotective and glutamate as cytotoxic. IC_{50} values and cell viability using an MTT assay were used to determine the effective doses. Three replicates of HT-22 hippocampal neural cells were seeded in a 96-well plate and incubated with different concentrations of melatonin at various times (24- 48 –72 h) for optimization. In addition, the study assessed the toxicity dose of glutamate at different durations, 24, 48, and 72 hours. IC_{50} values were calculated for each substance. Results were analyzed using a spectrophotometer at 570 nm absorbance. It is found that glutamate significantly affects cell viability compared to the control group at 2 mM concentrations and higher; 5 mM was used to perform its toxicity effect. The glutamate IC50 is 2.465 mM during 24 hours. The melatonin IC_{50} was calculated at 24, 48, and 72 hours as 0.8399 mM, 0.48 mM, and 0.39 mM, respectively.

The results demonstrated that melatonin might exhibit a protective effect against glutamate toxicity in HT-22 neural cells. The evidence presented in this study strongly suggests that melatonin displays a neuroprotective effect against glutamate toxicity in HT-22 cells, which impacts cell viability.

Keywords: Melatonin, HT-22 hippocampal cell line, Glutamate, MTT, Cell viability

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Abair Awadalla Ahmed MAHDI

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SYMBOLS AND ABBREVIATIONS

Abbreviation

AD	:	Alzheimer's disease
ALS	:	Amyotrophic lateral sclerosis
BDNF	:	brain-derived neurotrophic factor
Cont	:	Control group
CM		complete media
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide
EMEM	:	Eagle’s Minimal Essential Medium
ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal Bovine Serum
FACS	:	Flow cytometry analysis
Glu	:	Glutamate
HD	:	Huntington's disease
IC_{50}	:	The half-maximal inhibiter concentration
L-GLUT	:	L-Glutamine
Mel	:	Melatonin
MTT	:	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MS	:	multiple sclerosis
NMDA	:	N-methyl-d-aspartate receptor
OS	:	Oxidative stress
PBS	:	phosphate-buffered saline
PCR	:	Polymerase Chain Reaction
PD	:	Parkinson's disease
ROS	:	Reactive oxygen species
SD	:	Standard Deviation

Symbols

mM	:	Millimolar
µl	:	Micromolar
ml	:	Milliliter
µg	:	Microgram
g/mL	:	Grams Per Milliliter
g/L	:	Grams Per Liter
mg/mL	:	Milligrams Per Milliliter
µg/mL	:	Micrograms Per Milliliter
µg/µL	:	Micrograms Per Microliter
ng/L	:	Nanograms Per Liter
ng/µL	:	Nanograms Per Microliter
nm	:	Nanometers
°C	:	Centigrade
kb	:	Kilobyte

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1. INTRODUCTION

The primary cause of motor and cognitive impairments is a variety of irreversible and progressive disorders that may result in neuronal death. Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and (HD) are the most common neurodegenerative diseases (Yoon et al., 2022). Several factors have been identified as potential contributors to the development of these diseases, such as age, genetics, environmental toxins, and lifestyle factors. The most prominent signs of these disorders include impairment of mobility and loss of memory, behavioral and cognitive functions due to progressive neuronal death, and a range of motor symptoms, including tremors, rigidity, and bradykinesia. Research is ongoing to understand these diseases better and develop effective treatments (Myszczynska et al., 2020). Oxidative stress is a condition where the body's cellular protection is overwhelmed by harmful by-products of aerobic metabolism, resulting in the production of free radicals and ROS that can cause tissue injuries and disorders such as inflammation, arthritis, diabetes, cancer, neural tube defects, and neurodegenerative diseases. A new therapeutic approach based on scavenging excess free radicals is being developed to combat this. The specific mechanism for how these species cause chronic diseases is still unknown, but it may be related to damage to DNA, proteins, and lipids within cells (Singh et al., 2019). Neurodegenerative disorders differ in etiology, pathophysiological routes, and specific clinical signs, symptoms, and risk factors. Accurate diagnosis and appropriate management are essential for improving the quality of life of individuals affected by these conditions.

Cell lines are used for research on cellular processes and disease models. The HT-22 cell line is a popular and well-established mouse hippocampal neural cell line widely used in basic neuroscience research to study cellular mechanisms of neurodegeneration, oxidative stress, and cytotoxicity (Koszła et al., 2020). It was originally derived from embryonic mouse hippocampal tissue and has been extensively characterized for its morphology, electrophysiological properties, and gene expression profile. It has been shown to exhibit several key features of hippocampal neurons, including the expression of Glu receptors, voltage-gated ion channels, and synaptic proteins. Its susceptibility to glutamate-induced excitotoxicity allows researchers to investigate the complex mechanisms of neuronal cell death and potentially identify new therapeutic targets. Researchers have used the HT-22 cell line to study the

neuroprotective effects of various compounds, such as curcumin and resveratrol, in reducing glutamate-induced excitotoxicity (Vongthip et al., 2021). HT-22 cells are useful for research in neuroscience and drug development due to their ability to retain neuronal properties and rapid growth in culture. They are particularly useful in studying oxidative stress and excitotoxicity. These two processes are critical in developing neurodegenerative diseases such as AD and PD. They have also been utilized in studies investigating gene expression, calcium signaling pathways, mitochondrial metabolism, and other essential cellular physiological processes. Recent findings have highlighted the significance of HT-22 cells in understanding the underlying mechanisms of neurological diseases (Tuo et al., 2022). Alpha-synuclein, a protein linked to Parkinson's disease, has been linked to HT-22 cells to mitochondrial dysfunction and oxidative stress. In contrast, exposure to the protein amyloid beta can cause calcium dysregulation and neuronal death in HT-22 cells.

Toxic agents are often used to imitate the diseased circumstances seen in neurodegenerative illnesses *in vitro* research. Examples include amyloid-beta peptides, rotenone, Glu, and Lipopolysaccharide (LPS) (Vrabec et al., 2023). These chemicals are known to harm neurons and can be used to test potential cures and better understand the underlying causes of certain illnesses. Glu simulates excitotoxicity, while LPS can lead to oxidative stress, neuroinflammation, neuronal malfunction, and death. It is important to remember that the concentrations and exposure intervals employed *in vitro* research don't exactly correspond to those seen in the human body, so researchers must explore alternative models and approaches to understand better and treat neurodegenerative diseases (Silva-Reis et al., 2023).

Glu is one of the 20 amino acids involved in protein synthesis as a building block, but it is not considered an essential amino acid. Neuronal Glu biosynthesis plays a crucial role in the central nervous system. Glu is the most abundant neurotransmitter in the brain and serves as a precursor for GABA synthesis (Newsholme et al., 2023). Glu is essential for disposing of nitrogen, a metabolic waste protein metabolism produces. Neurons release Glu into the synaptic cleft to connect with ionotropic and metabotropic receptors. Astroglia cells play an important role in the uptake of Glu, which is essential to prevent accumulations of extracellular Glu toxicity. Overproduction of Glu outside the cell leads to excitotoxicity for the cell and increases the production of oxidative stress. There are two primary ways for Glu neuroexcitatory

mechanisms in the brain synaptic area: oxidative Glu toxicity and excitotoxicity (J. Wang et al., 2020). Independent process intermediated by N-methyl-D-aspartate known as ionotropic Glu receptors and other Glu receptors (Fu et al., 2018).

Studies have demonstrated that excessive Glu extracellular levels can be toxic and accelerate neuronal death by increasing oxidative stress. Another research revealed that Glu significantly enhances the generation of oxidative stress and neuronal death in the HT-22 hippocampal neural cell line by inhibiting cystine uptake via the cystine/glutamate transporter system (Hirata et al., 2018). Glu is the salt form of glutamic acid, an acidic amino acid constituent of many proteins. Glu bears a net negative charge, while glutamic acid is a neutral molecule. In the body, glutamic acid is always converted into Glu through deprotonation. L-glutamic acid is the chemical form of Glu that the body produces. Glu is an excitatory neurotransmitter in the brain and the precursor molecule for creating GABA. Monosodium glutamate (MSG) is the name given to Glu that has been linked to sodium. The Glu cycle occurs between the presynaptic neuron terminal and the postsynaptic dendritic terminal with glia. Glu is packaged into synaptic vesicles, reuptake by EAAT, and converted to glutamine again.

Dysfunction of these receptors can lead to various neurological disorders such as AD and epilepsy. Ionotropic Glu receptors are important for normal physiological processes and are implicated in various pathological conditions. Excessive activation of NMDA receptors has been linked to neuronal damage and cell death in conditions such as stroke and traumatic brain injury (Kilb and Kirischuk, 2022). At the same time, decreased activity has been associated with cognitive impairment and mood disorders. There is great interest in developing drugs that can selectively modulate the activity of these receptors, intending to treat these and other neurological disorders. AMPA receptors are independent of voltage found in the post-synapse of glutamatergic neurons and are activated by binding to Glu and triggering a cation influx into the neuron. NMDA receptors are Ion ligand-gated ion channels with a unique mode of operation, function by having a block of magnesium in its pore. These receptors play a crucial role in learning, memory, and synaptic plasticity. NMDA receptors are involved in various neurological disorders, such as AD, PD, and epilepsy. Activation of NMDA receptors leads to the influx of calcium ions, which can trigger a cascade of signaling events. KAINATE receptors are ligand-gated ion channels that conduct sodium and potassium, with a slightly higher single-channel conductance than AMPA

receptors (Gielen and Corringer, 2018). KAINATE receptor activation can result in excitotoxicity, a condition in which excessive stimulation of neurons leads to their death. Regulation of KAINATE receptor activity is essential for maintaining normal brain function.

Mel is a hormone secreted by pinealocytes of the pineal gland, which regulates the sleep-wake cycle. Mel is a free-radical scavenger and antioxidant that has been found in high concentrations in the brain after exogenous administration. It is also a neuroprotector in various conditions affecting the central nervous system (CNS). It is believed to have antioxidant abilities and may impact aging and immune system performance. The retina, bone marrow cells, platelets, lymphocytes, skin, cerebellum, and gastrointestinal tract of vertebrates have all been identified as extra-pineal sources of Mel (Mund et al., 2020). It is frequently referred to as the Dracula hormone and significantly impacts the melanin pigmentation of frog skin. Mel production varies with age and is highest in childhood and decreases as we age. The suprachiasmatic nucleus (SCN) regulates the cycle of pineal Mel synthesis through its connection to the paraventricular hypothalamic nucleus. Mel is a well-known wide-spectrum antioxidant with the capacity to scavenge free radicals. According to some research, Mel activates CREB signaling pathways involved in culture's long-term memory processing. The high-affinity G protein-coupled ML1 receptor and the low-affinity ML2 receptor have been identified as the two main Mel receptors. According to research, activating Mel receptors MT1 and MT2 has favorable effects on sleep and circadian abnormalities, mood disorders, learning and memory problems, neuroprotection, drug abuse, and cancer (J. Liu et al., 2016). Mel receptors play a crucial role in regulating circadian rhythms and neuroendocrine processes. Selective ligands that target these receptors could encourage the creation of novel drugs. The effects of high doses of Mel on the reproductive and endocrine systems should not be dismissed. It is important to consider individual differences and lifestyle factors when evaluating the risks of Mel supplementation. Individuals with certain medical conditions or taking medications that affect Mel levels may have different responses to supplemental intake than those without these factors.

Additionally, lifestyle factors and more effective medical treatments. Artificial light may also impact the effectiveness of supplemental Mel. However, some studies have shown that high doses of Mel may have adverse effects on the reproductive

system and disrupt the normal functioning of the endocrine system, such as decreased testosterone levels and alter due to Mel's direct and indirect interactions with them as well as the activation of these receptors, various tissues express MT1 and MT2 Mel membrane receptors (Xia et al., 2019). Mel, a hormone, plays a key role in regulating mitochondrial formation. Recent research has demonstrated its efficacy in preventing and treating a range of central nervous system diseases, managing physical disorders, affecting circadian rhythm, and protecting the skin. Additionally, it has been found that forming bonds with metal ions and preventing electron discharge from the electron transport chain lessens the effects of COVID-19 (Camp et al., 2021). It has been discovered that Mel promotes collagen synthesis and cell bone proliferation and inhibits bone resorption, all of which help to maintain body mass. But there is a chance of harm, including disturbed sleep patterns and exposed testicular function. It is necessary to research the potential negative effects of high doses of Mel on the endocrine and reproductive systems in both animal studies and human trials (Gurunathan et al., 2021).

The present study aims to enrich our understanding of cell viability in the context of Mel's neuroprotective effects. While past research has focused on mitochondria-depleted HT-22 cells, this study employs the MTT method to explore how varying Mel concentrations impact the survivability of the HT-22 hippocampal neuronal cell line. By determining the IC_{50} value for Mel's neuroprotective properties, we may gain valuable insights into its potential use as a therapeutic agent for various neurological disorders (Jana et al., 2022). Expanding our knowledge on these topics can have important implications for improving patient outcomes in clinical contexts where such interventions are needed most.

2. BACKGROUND AND LITERATURE REVIEW

2.1.The Neuron Cell

The neuron is the basic unit in the nervous system. Humans have a hundred billion neurons, most centered in the brain and spinal cord's central nervous system (Z. G. Zhang et al., 2019). The typical neuron cell metabolizes and uses energy. It consists of the cell body at the center; it has a nucleus, Golgi apparatus, and mitochondria, but there are extensions of those cell bodies called the dendrites; those dendrites connect to other neurons, and they are taking information inside, in the form of neurotransmitters. This whole side of the neuron receives information; also, the neuron has an axon to transmit information (Ali et al., 2020). The axon begins at the hillock and will move to the terminals, where it does a synapse that connects a neuron to other neurons. So, the neuron takes signal information from the dendrites to the cell body and then sends information to the next neuron through the axon. Specific axons in our body are very long. To travel information quickly, a Schwann cell wraps the axon with Myelin sheaths around it. There are voltage-gated in between that, which insulates that nerve. It allows the information to travel more readily down that axon, which contacts another neuron's cell body or dendrites (Tang et al., 2019). The gap between them, at the contact area, is called the synapse, so it makes a connection between neurons. All this needs an action potential to occur (Van Schaik, 2001).

2.1.1. Neuronal Classification

The neurons are classified in two ways; the first is due to their structural classification and how many poles are coming off the cell body, which means dendrites and one axon from every neuron. The types are 1- Multipolar neuron with several dendrites and one axon, the most common in the human body. 2- Bipolar neuron when the axon comes out, and dendrite comes in. Bipolar neurons are very rare; they transmit information from the receptors to the central nervous system as the receptor in the eye and the nose. 3- Unipolar neurons have only one pole coming from the cell body; it is a fused axon and dendrite; in vertebrates, it referred to as the pseudo unipolar neuron these are going to be most of the sensory neurons in the human body, it taking information in and transmitting that back into the central nervous system. 4- Anaxonic neuron: There is no axon on it, only dendrites, and it is found in the brain. There are a lot of other structural classifications, but these are the general ones (Brehmer, 2021).

The neurons can also be classified according to their functional type. 1- Sensory neurons take information from the receptor and the stimulus from the environment, such as skin; for example, they have receptors for touch, thermal, pain, etc. Those neurons that transmit information to the central nervous system are also called afferent neurons. 2- Motor neurons that send information to effectors in the body, such as muscles and glands. It also refers to efferent neurons. 3-Inter neurons that makes a connection between different neurons (Gowda et al., 2021).

2.1.2. Neurotransmitters in the Human Body

Neurotransmitters are chemical agents that transmit messages through the synapse, the tiny gap between two neurons. These chemical messengers are synthesized within the neuron and stored in vesicles, ready for release upon neural activity. Neurotransmitters play a crucial role in the functioning of responsibility for regulating various physiological processes within the neurological system, such as cognition, mood, behavior, and movement. Numerous neurotransmitters are present in the human body, including serotonin, dopamine, adrenaline, noradrenaline, acetylcholine, and Glu, each with distinct functions and effects on the body. When a nerve impulse reaches the axon terminal of a neuron, it triggers calcium ion channels to open up and release neurotransmitters into the synaptic cleft, which then binds to receptors located on the adjacent neuron or other cells in the body.

Furthermore, the functioning of neurotransmitters can be influenced by various factors such as genetics, diet, and environmental conditions. Mental health conditions, such as depression, anxiety, schizophrenia, and bipolar disorder, have been associated with neurotransmitter imbalances and dysfunctions. Some examples of neurotransmitter imbalances and dysfunctions associated with mental health disorders include the reduced activity of serotonin in people who experience depression, the overactivity of dopamine in people with schizophrenia, and the decreased production of acetylcholine in AD. Various drugs and medications have been developed that target specific neurotransmitters to regulate their levels in the brain, thereby improving mental health conditions. Neurotransmitters are essential for communication between neurons in the nervous system, and they play a critical role in regulating many physiological processes. Understanding the role and functioning of neurotransmitters is vital in identifying potential neurochemical imbalances that could contribute to a myriad of mental health disorders.

Moreover, neurotransmitters facilitate communication across neural networks

and form an integral part of the brain-body connection. Hence, any disruption in the delicate balance of neurotransmitter levels and their functioning can significantly impact brain function and overall health. In summary, neurotransmitters are chemical messengers facilitating communication between neurons and regulating cognitive, behavioral, and physiological processes. The complex interplay of neurotransmitters and their functions in the brain is an area of extensive research, as it provides insights into the underlying mechanisms of mental health disorders and the development of potential treatments. In conclusion, understanding the role and functioning of neurotransmitters is crucial in identifying potential neurochemical imbalances that could contribute to a vast array of mental health. Disorders, as well as the development of effective treatment strategies. Furthermore, it is essential to note that neurotransmitter imbalances and dysfunctions are not the sole cause of mental health disorders. Rather, they are one aspect of a complex interplay between multiple factors, including genetic predispositions, environmental influences, and lifestyle habits. Therefore, future research on neurotransmitters and their impact on mental health should adopt a holistic approach considering these multiple factors to identify personalized treatment options for individuals with mental health disorders. Additionally, it is important to note that the use of drugs targeting specific neurotransmitters for mental health treatment should be approached with caution because the complex interactions between neurotransmitters and other bodily systems mean that such drugs can have unintended side effects, especially when taken over a prolonged period. Therefore, a comprehensive assessment of an individual's mental health is necessary before selecting a drug therapy treatment strategy. Moreover, non-pharm ecological interventions such as psychotherapy, cognitive-behavioral therapy, and mindfulness-based practices have shown promising results in treating mental health disorders and should be considered alongside drug therapy. A thorough understanding of neurotransmitters is fundamental to identifying potential imbalances contributing to various mental health disorders.

However, it is important to approach treatment strategies for mental health disorders holistically and cautiously, considering individual factors such as genetic predispositions, environmental influences, and lifestyle habits. Furthermore, the use of drugs targeting specific neurotransmitters should be approached with care due to potential unintended side effects.

Additional research clarifying the complexity of neurotransmitter function could

provide possibilities for new therapeutic targets for treating nervous system disorders.

Neuronal Action Potentials

The neuron, or the nerve cell, is a vital nervous system component. Its primary function is transmitting electrical and chemical signals throughout the body, enabling us to sense, process, and respond to external stimuli.

The neuron comprises four essential components that work harmoniously to achieve its functions. The dendrites, elongated extensions of the neuron, receive signals from sensory cells or other neurons. The cell body, called the soma, processes the information received and decides whether the neuron should transmit a signal to other cells. The axon, a long fiber that stretches from the cell body, carries the signal over long distances within the neuron. Finally, the axon terminal, located at the end of the axon, relays the signal to adjoining neurons or effector cells, such as muscles or glands. Nerves are formed when multiple axons bundle together and vary in size and complexity. They range from simple sensory nerves that transmit signals from the skin to the spinal cord to complex nerves that control intricate movements, such as speech or dance. However, nerves can be lengthy and intricate, causing them to take considerable time to transmit information over long distances. Reflexes, which are rapid, reflexive reactions to stimuli, are processed in the spinal cord rather than the brain due to the reduced time a signal must travel before a response is generated. (Liu et al., 2021) The dendrite is responsible for receiving incoming signals, which can vary in strength depending on the stimulation level. When the stimulation reaches a certain threshold, it triggers an electrical impulse known as an action potential. This phenomenon allows for the rapid transmission of signals across the neurons and is crucial for properly functioning the nervous system (Alcamí and Pereda, 2019). The positively charged gradient variation, mainly the sodium and the potassium- across the plasma membranes generates and propagates these action potentials. These action potentials eventually reach the axonal terminal and depolarize adjacent cells via synapses (Raghavan et al., 2019).

The movement of ions is entirely responsible for transmitting the neural signal. The concentrations of different types of ions, such as sodium, potassium, and chloride, are distributed unevenly between the interior and exterior of the cell. The presence and movement of these ions are crucial in determining whether a neuron will activate or remain at rest (Dmitriev et al., 2019). The resting state of a neuron is crucial for its proper functioning and the transmission of signals. When a neuron is not sending a

signal, its positively charged sodium and potassium ions are at rest. In a typical resting neuron, the concentration of sodium ions is higher outside the cell than inside, while the concentration of potassium ions is higher inside the cell than outside. This creates an uneven distribution of ions across the cell membrane, resulting in the resting membrane potential essential for maintaining the cell's electrical charge. The resting membrane potential determines whether the neuron will fire an action potential, which is the electrical impulse that transmits signals along the neuron; when a neuron receives a stimulus, such as a signal from another neuron or a sensory receptor, the resting membrane potential changes, causing depolarization, which can trigger the neuron to fire an action potential and transmit a signal to other neurons or effector cells (Jiawei Zhang, 2019a).

Ionic separation occurs at the cell membrane, creating a chemical gradient across the membrane. The distribution of ions across the membrane must consider their charge since ions are charged particles. When the neuron is at rest, there are more positively charged ions outside the cell than inside, leading to a charge difference across the membrane called an electrical gradient. The electrochemical gradient results from this ionic imbalance, which combines the electrical and chemical gradients (Manoj and Tamagawa, 2022).

The resting membrane potential is the difference in charge between the inside and outside of the cell. When no signals are transmitted, the neuronal membrane has a resting potential of approximately minus 70 millivolts. That means the inside of the cell is around 70 millivolts less positive than the outside. The resting potential is established due to the contribution of both the chemical and electrical gradients. Despite the inside of the cell having a net negative charge and the outside having a net positive charge, the charges align at the cell membrane. As a result, the bulk solution on either side of the membrane is electrically neutral (McCuskey et al., 2021). The resting membrane potential is the point at which the neuron achieves electrochemical equilibrium, where each ion's concentration gradient and electrogradient are equal and opposite. Ions cannot cross the membrane at will; they require a specific protein embedded in the membrane to facilitate their movement. Most ions cross the membrane through ion channels, which allow for passive diffusion along the concentration gradient, moving from high to low concentration (Kulbacka et al., 2017).

2.1.3. Ion Channels

While some ion channels remain open, others require a signal to open or close. Voltage-gated channels, for instance, only open when the membrane potential reaches a specific value. On the other hand, Ligand-gated ion channels open when a specific molecule binds to them, while mechanically-gated ion channels open in response to physical forces like changes in length or pressure. Most ion channels are selectively permeable and only allow certain ions to pass through. For instance, voltage-gated ion channels typically permit only one type of ion to cross the membrane when they open. Therefore, separate channels are required for each ion, such as voltage-gated sodium channels and voltage-gated potassium channels (Bähring and Covarrubias, 2011).

The movement of ions through a channel across the cell membrane can cause the membrane potential to shift from its resting potential. If this change is small, it is referred to as a graded potential. Graded potentials can vary in size, be either positive or negative, are transient, and typically do not result from the opening of voltage-gated ion channels (Sengupta and Stemmler, 2014).

When ion channels open and a graded potential occurs, neurons swiftly restore their membrane potential to resting values. This process is primarily accomplished through the sodium-potassium pump, which utilizes ATP hydrolysis to transport ions across the membrane against their concentration gradient (Gupta et al., 2021). In other words, sodium is transported to the outside of the cell, where its concentration is higher, and potassium is transported back into the cell, where its concentration is higher. With one cycle, the sodium-potassium pump transports three sodium ions out of the cell and simultaneously brings two potassium ions into the cell; this unbalanced charge transfer contributes to the separation of charge across the membrane and also to the ionic concentration we see at rest, thus, restoring the chemical and electrical gradients to their resting levels (Galassi and Wilke, 2021).

Maintaining these ionic balances in neurons is so essential that this process can account for 20% to 40% of the brain's total energy use. To enable a neuron to generate an action potential when stimulated, it must maintain precise levels of resting membrane potential and ion distribution. When the outside stimulation is strong enough to increase the membrane potential in the neuron body from minus 70 millivolts to the threshold of minus 55 millivolts, it triggers an action potential at the axon hillock. The action potential travels down the axon through voltage-gated sodium channels in open, closed, and inactivated states. The sodium channel is closed at rest

(Cummins et al., 2020).

When the cell membrane reaches the threshold voltage, sodium ions rush into the cell due to the electrochemical gradient as the channel changes to an open position; this causes the membrane potential to become less negative and more positive, known as depolarization. The voltage gradient eventually exceeds zero, resulting in an overshoot of up to 30 millivolts. As the membrane potential becomes positive, the sodium channel's inactivation gate shuts, stopping sodium ions from entering the cell. The change in membrane potential also opens voltage-gated potassium channels, which close slowly due to the potassium electrochemical gradient; this causes potassium ions to flow out of the cell, making the membrane potential less positive and eventually negative. This process is called repolarization, resulting in a brief period of hyperpolarization where the membrane potential is more negative than the resting potential. The sodium-potassium pump works throughout this process to restore the chemical gradients and re-establish the potential gradient. During the absolute refractory period, the inactivated sodium channels won't respond to any stimulus, and the neuron cannot fire another action potential. This period prevents action potentials from happening too quickly and traveling backward along the axon. During hyperpolarization, the sodium channels are closed, and the inactivation gate opens, but there is no change in sodium flow. The sodium channels could open again during the relative refractory period. Still, it would require a larger than usual stimulus to reach the threshold because the cell is hyperpolarized due to the potassium leaving it (Feria Pliego and Pedroarena, 2020).

The amplitude of the action potential for a particular neuron is the maximum voltage in one neuron during an action potential. An action potential doesn't increase with more stimuli; it is all or nothing; it either happens or doesn't happen. What can change is the frequency of the action potential. Neurons might fire many more times per second in response to intense pain and less frequently in response to a gentle breeze. Some axons transmit action potentials faster than others (Abdelfattah et al., 2019).

One variable that increases conduction velocity is the presence of myelin sheets around the axon. Myelin speeds up the transmission through a process called saltatory conduction, in which the action potential signal appears to jump along the part of the axon covered by the sheets in the peripheral nervous system; the sheets are formed from glial cells known as Schwann cells. There are small gaps between Schwann cells

called the nodes of Ranvier. The action potential jumps from node to node, facilitating faster transmission in the central nervous system. The sheets are made by cells known as oligodendrocytes (Smaga, 2022).

With no stimulus, the membrane is at its resting potential. A slight stimulation causes a graded potential if the stimulation above the threshold creates an action potential, and the neuron fires.

2.1.4. Neuronal Synapses

The nervous system comprises billions of communicating neurons (Jiawei Zhang, 2019b). Neurons conduct electrical signals or action potential along their cell membrane along the axon to the terminus, where synapses are formed. The synapse consists of the membrane of the presynaptic cell, a synaptic cleft, and the postsynaptic cell membrane. A neurotransmitter must bridge the gap between adjacent cells to transmit an action potential along a neuron's membrane. The presynaptic cell releases the neurotransmitter into the synaptic cleft, binding with receptor proteins in the postsynaptic cell membrane (Postila and Róg, 2020). Two general types of synapses exist electrical synapses and chemical synapses. Electrical synapses are less prevalent in the nervous system and involve pores between two cells that allow ions to pass through, facilitating the transfer of electrical signals to neighboring cells. This structure is similar to combining two cells into a larger one, allowing for fast, high synchronicity coupling. However, most neurons are connected via chemical synapses, which are more complex (Leighton and Lohmann, 2016).

In a chemical synapse, rather than simply passing along an electrical signal from one cell to another, the action potential travels to the end of the axon, causing the release of a chemical into the synaptic cleft, which is the small space between two neurons. The downstream neuron takes up the released chemical substance on the other side of the cleft. This chemical signal can cause the downstream neuron to depolarize its membrane, converting the signal back into an electrical one, or it can have other effects on the cell. However, this chemical step is slower than the transmission across an electrical synapse. Still, it opens up an enormously diverse repertoire of different and more complex kinds of signaling, and synaptic function plays a critical role in computations performed by neurons (Martin et al., 2020). The neuron can connect with other neurons due to the action potential and neurotransmitter release. If the cell body membrane undergoes enough depolarization to initiate an action potential, it is sent down the axon to another neuron. This junction between two neurons, specifically

between one neuron's axon and another's dendrite, is known as a synapse (Guang et al., 2018).

When the action potential reaches the synapse, voltage-gated calcium channels open, causing an inward flow of calcium. This calcium binds to the vesicular membrane, activating the vesicles to fuse with the presynaptic membrane and release neurotransmitter molecules into the synaptic cleft. The neurotransmitter molecules diffuse through the interstitial fluid in the gap and may cross the synaptic cleft to bind to receptors in the postsynaptic membrane. This binding opens protein channels and allows ion flow into and out of the cell, producing an effect. If this effect excites the postsynaptic membrane, it generates an excitatory postsynaptic membrane potential. (Ramachandran et al., 2022). An excitatory postsynaptic potential (EPSP) is a localized depolarizing signal that can generate long-distance signals called action potentials in the dendrite of the postsynaptic cell. On the other hand, an inhibitory postsynaptic potential (IPSP) is a localized hyperpolarizing signal that generally prevents the formation of long-distance signals in the postsynaptic cell by inhibiting the postsynaptic membrane potential (Capogna et al., 2021). Neurotransmitters influence the postsynaptic membrane's electrical state for only a short time. They are removed from the synaptic cleft in one of three ways: diffusion away from the receptor, enzymatically degraded, or recycled by the presynaptic cell; some neurotransmitter molecules diffuse out of reach of the neurotransmitter receptors, some neurotransmitters molecules are removed by enzymatic degradation. Some neurotransmitter molecules are transported back into the presynaptic cell by neurotransmitter transporters, where they are recycled (Tukker and Westerink, 2021).

2.2.Neurodegenerative Diseases

The primary cause of motor and cognitive impairments is recognized to be a variety of irreversible and more progressive disorders that may result in neuronal death of the central or peripheral nervous systems. AD, which primarily impacts the central nervous system and is characterized by the accumulation of abnormal proteins in the brain, is one of the most well-known examples of these disorders (Kumar et al., 2021). As a result of this build-up, neurons begin to deteriorate and eventually lose their ability to perform cognitive functions like memory, language, and decision-making. Contrarily, PD primarily impacts dopaminergic neurons in the substantia nigra of the brain, resulting in bradykinesia, rigidity, and other motor symptoms. (ALS), (HD), and other progressive diseases (Amor et al., 2014).

More than thirty million people are influenced by neurodegenerative diseases, which result in death and disability. It significantly impacts a person's quality of life and can devastate their relatives. While there is currently no cure for these diseases, research is ongoing to understand them better and develop effective treatments (Miquel et al., 2014; Radi et al., 2014). In the meantime, it is important to support those affected by these diseases and raise awareness about their impact on individuals and society.

In most common neurodegenerative diseases such as PD, AD, Huntington's, and ALS, the causes are not clear (Dauer and Przedborski, 2003). However, several factors have been identified as potential contributors to the development of these diseases. One of the most significant is age, as the risk of developing a neurodegenerative disease increases as we get older. Other potential factors include genetics, environmental toxins, and lifestyle factors such as diet and exercise (Krishnendu et al., 2021). While researchers have made significant progress in understanding the underlying mechanisms of these diseases, there is still much to learn about how they develop and how they can be treated or prevented (Madetko et al., 2022).

The most prominent signs of these disorders include impairment of mobility, loss of memory, and behavioral and cognitive functions due to progressive neuronal death, and this leads to a decline in cognitive function, a loss of the ability to perform everyday tasks, and a range of motor symptoms, including tremors, rigidity, and bradykinesia (Dahodwala et al., 2020).

The main causes are excessive oxidative stress levels, an imbalance between the free radicals release, and an antioxidant defense system associated with cell death and neurological diseases (Hsieh et al., 2021). Oxidative stress is a condition where the body's cellular protection is overwhelmed by harmful by-products of aerobic metabolism, resulting in the production of free radicals and ROS that can cause tissue injuries and several disorders such as inflammation, arthritis, diabetes, cancer, neural tube defects, and neurodegenerative diseases. A new therapeutic approach based on scavenging excess free radicals is being developed to combat this. The specific mechanism for how these species cause chronic diseases is still unknown, but it may be related to damage to DNA, proteins, and lipids within cells (Walters, 2021). The main significance of these diseases is characterized by severe oxidative disturbance for DNA, lipids, and proteins, and this can cause cell death through several mechanisms, either by inhibiting essential cellular activities or by stimulating harmful

substances (Dumitras et al., 2020). Neurodegenerative disorders differ in etiology, pathophysiological routes, and specific clinical signs, symptoms, and risk factors (Choe et al., 2022). Therefore, accurate diagnosis and appropriate management are crucial for improving the quality of life of individuals affected by these conditions.

2.3.HT-22- Hippocampal Cell Line

Cell lines are used for research on cellular processes and disease models. They provide valuable tools for understanding diseases and developing treatments such as drug targets and therapy testing. Additionally, cell lines are used for large-scale protein production in research and medical applications. The HT-22 cell line is a popular and well-established mouse hippocampal neural cell line widely used in basic neuroscience research to study cellular mechanisms of neurodegeneration, oxidative stress, and cytotoxicity.

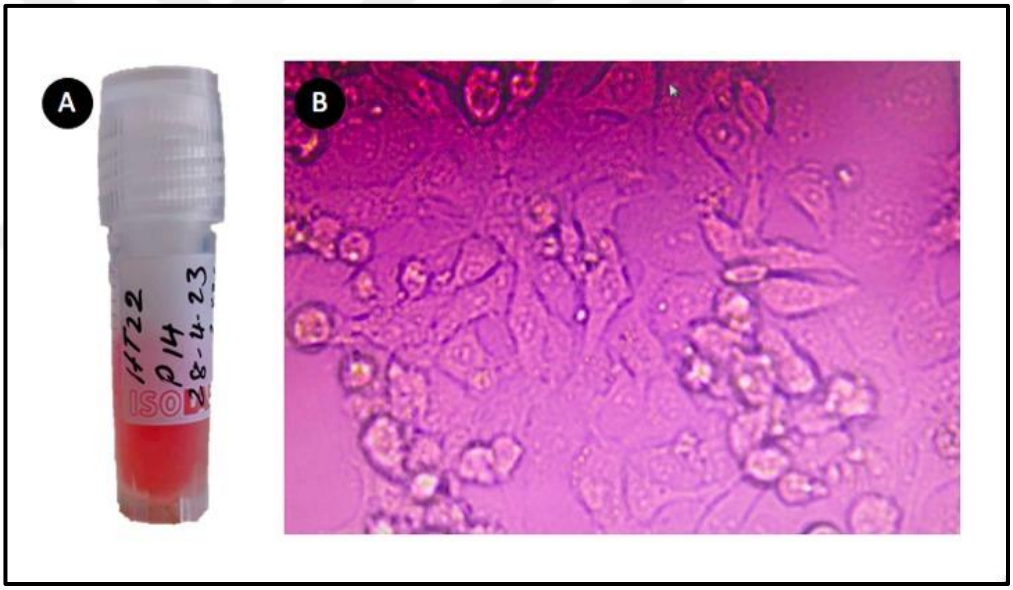


Figure 2.1. HT-22 hippocampal neural cell line

HT-22 hippocampal neural cell line in a cryovial and freezing at −86 °C (A). In culture cell flask at confluency stage (B).

The image was captured by a Leica inverted microscope Evos XI at 10x magnification.

This cell line was originally derived from embryonic mouse hippocampal tissue and has been extensively characterized for its morphology, electrophysiological

properties, and gene expression profile. The HT-22 cells have been shown to exhibit several key features of hippocampal neurons, including the expression of Glu receptors, voltage-gated ion channels, and synaptic proteins (Abe et al., 1995; Rzajew et al., 2020). The HT-22 cell line is a valuable tool for studying the mechanisms of neuronal cell death in neurodegenerative diseases such as AD and PD. Its susceptibility to glutamate-induced excitotoxicity allows researchers to investigate the complex mechanisms of neuronal cell death and potentially identify new therapeutic targets. Researchers have used the HT-22 cell line to study the neuroprotective effects of various compounds, such as curcumin and resveratrol (Silvestro et al., 2021), in reducing glutamate-induced excitotoxicity. The HT-22 cell line has also been used to investigate the role of oxidative stress and mitochondrial dysfunction in neurodegeneration (Tung et al., 2021). HT-22 cells are suitable for research in neuroscience and drug development due to their ability to retain neuronal properties and rapid growth in culture. They provide a platform for investigating cellular signaling pathways and testing the toxicity of various compounds. Additionally, they are particularly useful in studying oxidative stress and excitotoxicity. These two processes are critical in developing neurodegenerative diseases such as AD and PD (Marí et al., 2020). HT-22 cells have also been utilized in several studies investigating gene expression, calcium signaling pathways, mitochondrial metabolism, and other essential cellular physiological processes (Calvo-Rodriguez and Bacskai, 2021). These cells have been demonstrated to be a helpful resource in studying neuronal development and function and neurological disorders like Parkinson's and AD's. Recent findings highlighted the significance of HT-22 cells in understanding the underlying mechanisms of neurological diseases. Alpha-synuclein, a protein linked to PD, has been linked in one study to HT-22 cells to mitochondrial dysfunction and oxidative stress (Ikram et al., 2020). According to a different study, exposure to the protein amyloid beta, linked to AD, can cause calcium dysregulation and neuronal death in HT-22 cells. Overall, HT-22 cells have proven to be a valuable tool in understanding various cellular processes and have been widely used in research studies to state the underlying causes of neurological disorders.

2.4.Toxic Substances Used in Neurodegenerative *in Vitro* Studies

Toxic agents are often used to imitate the diseased circumstances seen in several neurodegenerative illnesses *in vitro* research. These compounds are essential for examining the processes behind nervous tissue injury and cell death, and they shed

light on the pathophysiology of various diseases (Singh et al., 2019).

To accurately mimic the cellular and molecular changes that occur in neurodegenerative disorders, the toxic agents used *in vitro* experiments are carefully chosen. These substances may be poisons found in the environment, neurotoxic agents, or pathogenic elements linked to the disorders under investigation (Y. Wang et al., 2017). Using toxic chemicals in *in vitro* research on neurodegenerative diseases is a common practice. Examples include amyloid-beta peptides, rotenone, Glu, and Lipopolysaccharide (LPS). These chemicals are known to harm neurons and can be used to test potential cures and better understand the underlying causes of certain illnesses. Glu simulates excitotoxicity, while LPS can lead to oxidative stress, neuroinflammation, neuronal malfunction, and death. It is important to remember that the concentrations and exposure intervals employed in *vitro* research don't exactly correspond to those seen in the human body, so researchers must explore alternative models and approaches to improve the understanding and treatment of neurodegenerative diseases (Zhang et al., 2019).

2.5.Glutamate

Glu is one of the most abundant non-essential amino acids in the human body. It represents the most common free amino acid in the CNS and is crucial for the metabolization of proteins (Zhang et al., 2019). It is a primary excitatory growth and develops neurotransmitters in the brain (Park et al., 2019). Therefore, it is crucial during the brain, neuronal transmission, and plasticity (Cuellar-Santoyo et al., 2023). Neurons release Glu into the synaptic cleft, binding to ionotropic and metabotropic receptors to deliver an excitatory signal across brain cells. Astroglia cells are crucial in the uptake of Glu released from brain synapses. This procedure is necessary to stop extracellular Glu toxicity from building up (Maher et al., 2018). The extracellular Glu levels are controlled by a specific family of sodium-dependent Glu transporters, mostly found on the surface of neuronal and glial cells (Suárez-Pozos et al., 2020).

Excessive production of extracellular Glu leads to cell excitotoxicity and increases oxidative stress development, consequently progressing to neurological disorders (Kamal et al., 2020). Glu neuroexcitatory is classified into two primary pathways in the synaptic area; the first is known as oxidative Glu toxicity, which results from excessive oxidative stress, while the second is known as excitotoxicity independent process intermediated by N-methyl-D-aspartate known as ionotropic Glu receptors and other Glu receptors (Pagano et al., 2021).

Many studies have revealed that elevated concentrations of extracellular Glu can act as a toxic agent and accelerate neuronal death via facilitation of oxidative stress in many types of neural line cells, such as PC12 cells, HT-22 hippocampal neural cell line, C6 glial cells (Talebi et al., 2021). Another study has shown that Glu significantly generated oxidative stress and neuronal death in the HT-22 hippocampal neural cell line by inhibiting cystine uptake via the cystine/glutamate transporter system, resulting in glutathione depletion in the cell (Guo et al., 2021).

2.5.1. The Chemical Structure of Glutamate

The amino acid Glu, which has the chemical formula C5H9NO4 and a molecular weight of 147, is an essential substance for the health of humans. Glu has a unique structure consisting of an amino group (-NH2), a carboxyl group (-COOH), and a side chain composed of a four-carbon chain ending in a carboxyl group (-CH2-CH2-COOH). Glu is the most abundant excitatory neurotransmitter in the nervous system. It facilitates the control of neuronal activity and signal transmission between neurons. Also, it helps to provide energy for cellular processes and modulates many metabolic pathways. Glu is an essential component of proteins and is involved in synthesizing other vital molecules, such as glucose (Lieu et al., 2020). Glu is an essential amino acid for many biological processes, as it can form hydrogen bonds with other molecules and participate in various chemical reactions. Its unique structure allows it to interact with other molecules in various ways, making it an essential component in many biological processes (Gasmi et al., 2021).

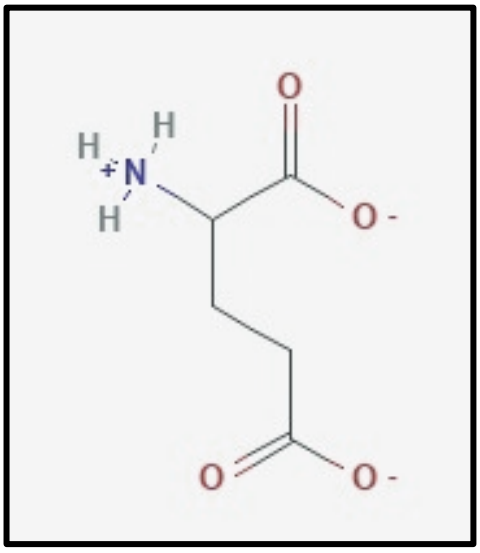


Figure 2.2. The chemical structure of Glu was modified from (Sayers et al., 2023).

2.5.2. Glutamate and Glutamic Acid

Although Glu is a common name for glutamic acid, Glu is the salt form of glutamic acid. In the human body, Glu, as well as glutamic acid, are both considered forms of biomolecules. Each molecule has two carboxyl groups and one amino group, totalling five atoms of carbon (Miller et al., 2020). The body can synthesize the amino acid form. Accordingly, the fundamental difference Between Glu and Glutamic acid is that the anion of glutamic acid is formed by losing one hydrogen atom from the molecule. While glutamic acid refers to an acidic amino acid constituent of many proteins, Glu has a net negative charge, while glutamic acid is a neutral molecule. Under physiological conditions, Glu is the form that exists in the body, and glutamic acid is always converted into the neutral form, Glu. In the body, glutamic acid is always converted into Glu through deprotonation (Khalil et al., 2017). L-glutamic acid is the chemical form of Glu naturally produced in the body. It acts as an excitatory neurotransmitter in the brain and serves as the precursor molecule for creating GABA, another significant distinction between Glu and glutamic acid. Contrarily, glutamic acid functions in proteins as a precursor to amino acids (Johnson, 1972).

Glu is vital for eliminating nitrogen, a metabolic waste produced during protein metabolism, through transamination. Due to its negative charge, Glu can bind with cations like sodium or potassium. When linked to sodium, Glu is known as MSG, a synthetic form of Glu produced outside the body. MSG contains impurities like D-glutamic acid and pyroglutamic acid, which can produce carcinogenic mono- and dichloro-propanols and convert them into L-glutamic acid. Despite this, MSG is commonly used as a food additive to enhance flavor. Glutamic acid, one of the 20 amino acid building blocks in protein synthesis, has a CH2CH2COOH side chain (Nagai and Taniguchi, 2018). While glutamic acid is not an essential amino acid because the body can produce it through metabolism, it can still be obtained from plant and animal sources as a food. Glu biosynthesis in the neuron plays a crucial role in the central nervous system. Glu is the most abundant neurotransmitter in the brain and serves as a precursor for GABA synthesis (Klotz et al., 2019).

A complex procedure happens in the glutamatergic synapses known as the Glu cycle (Lawton et al., 2000) between the presynaptic neuron terminal and the postsynaptic dendritic terminal with glia. Now, the presynaptic action potential reaches the synaptic turning terminal, which leads to the release of Glu into the synaptic cleft; this Glu binds to the Glu receptors in the post-synapse (Duarte-Silva et al., 2022).

The Glu comes from glutamine with the help of the mitochondrial enzyme glutaminase. The glia supplies the glutamine. When the Glu is synthesized, it gets packaged into synaptic vesicles with the help of a V- GLUT transporter or vesicular Glu transporters. Ultimately, these vesicles are released into the post-synapse. Then, Glu gets reuptake by the glia and neurons with the help of excitatory amino acid transporters (EAAT) (Malik and Willnow, 2019). Inside the glia, the Glu is converted to glutamine again with the help of the enzyme glutamine synthetase. That glutamine is again supplied back to the presynaptic terminal for further Glu biosynthesis. Alternatively, a small proportion of Glu is directly up taken into the synaptic terminal (Tan et al., 2022). Subsequently, that is how the Glu cycle keeps the excitatory synapses going. The Glu synapse is one of the excitatory synapses; there are many others (Falkovich et al., 2023).

2.5.3. Glutamate Receptors

There are two primary types of Glu receptors: ionotropic and metabotropic receptors. Ionotropic receptors are activated by Glu binding and act as ligand-gated ion channels, while metabotropic receptors are G protein-coupled receptors that activate intracellular signaling pathways. Both types of receptors are essential for synaptic transmission and plasticity, and Glu signaling dysfunction has been linked to Glu signaling dysfunction (Kruyer et al., 2023).

Ionotropic Glu receptors can be divided into three sub-types: AMPA, NMDA, and kainate. These receptors are crucial for excitatory neurotransmission and play a role in various physiological processes, such as learning and memory. AMPA receptors mediate fast synaptic transmission, while NMDA receptors mediate long-term potentiation and synaptic plasticity. Kainate receptors have diverse functions and are present in different brain regions. Malfunctioning of these receptors can lead to neurological disorders like AD, epilepsy, and schizophrenia (Hussaini and Jang, 2018). Therefore, understanding the function and regulation of these receptors is important for developing effective treatments for these conditions. In addition to their role in normal physiological processes, ionotropic Glu receptors have been implicated in various pathological conditions. Excessive activation of NMDA receptors is linked to neuronal damage and cell death in conditions such as stroke and traumatic brain injury. (Palop and Mucke, 2016). Conversely, decreased activity of these receptors has been associated with cognitive impairment and mood disorders. Therefore, there is great interest in developing drugs that can selectively modulate the activity of these

receptors to treat these and other neurological disorders.

AMPA receptors are independent of voltage found in the post-synapse of glutamatergic neurons. The AMPA receptor is activated by binding to Glu and triggering a cation influx into the neuron, causing a postsynaptic potential or current results from the membrane developing a positive potential. NMDA receptors are Ion ligand-gated ion channels with a unique mode of operation, function by having a block of magnesium in its pore. This block repels the magnesium ion from the channel, allowing cations to enter the post-synapse through the NMDA receptor. The NMDA receptor plays a crucial role in synaptic plasticity, learning, and memory. It is also associated with various neurological disorders, including ADs disease, Parkinson's disease, and epilepsy. Calcium ion influx is triggered by NMDA receptor activation, which can initiate a cascade of signaling events.

Other neurotransmitters, neuromodulators, and the neuron's overall state tightly regulate NMDA receptors' activity. KAINATE receptor is a ligand-gated ion channel that conducts sodium and potassium with a slightly higher single-channel conductance than AMPA receptors. These single channels have slightly higher currents than AMPA receptors due to their single channel conductance of greater than 20 picoseconds, compared to 10 to 15 for AMPA receptors. The postsynaptic potential produced by KAINATE receptors has a slightly slower rise and decay time than AMPA but less slowly than NMDA receptors. KAINATE receptor activation has been found to cause excitotoxicity, which is the overstimulation of neurons leading to their death. Therefore, it is essential to regulate KAINATE receptor activity to maintain normal brain function. KAINATE receptors are widely expressed in the brain and play a role in various physiological processes like learning and memory, synaptic plasticity, and neuronal development. They are also associated with neurological disorders such as epilepsy, depression, and schizophrenia. KAINATE receptors modulate other Glu receptors, such as NMDA and AMPA, indicating their significance in regulating synaptic transmission. KAINATE receptors show promise as a target for developing new therapies for neurological disorders due to their diverse functions and involvement in disease (Luessen and Conn, 2022).

Metabotropic Glu receptors (mGluRs) are G protein-coupled receptors that activate intracellular pathways. When Glu activates these receptors, it triggers various second messengers and intermediate players, such as IP3, DAG, and Ca^{2+}. There are eight different sub-types of mGluRs, classified into three groups based on their

sequence homology, pharmacology, and intracellular signaling processes. mGluR1 and mGluR5 belong to Group I, mGluR2 and mGluR3 are in Group II, and mGluR4, 6, 7, and 8 are in Group III. The mGluRs are present in the synapses of the hippocampus, cerebellum, cerebral cortex, and other brain regions. They are essential for physiological and pathological processes, including synaptic plasticity, neurodegenerative disorders, and addiction. Furthermore, they have potential targets for developing therapeutic drugs for neurological disorders. These receptors play a crucial role in physiological and pathological processes, and developing drugs targeting them is a promising area of research for treating neurological disorders (Jun Zhang et al., 2013).

2.5.4. Transporter

Excitatory amino acid transporters (EAATs) are membrane proteins that regulate the recycling and use of neurotransmitters in the central nervous system (CNS). Dysfunction of EAATs has been associated with neurological disorders. Understanding the molecular mechanisms underlying their function and regulation may provide new therapeutic targets. Since Glu is the primary excitatory neurotransmitter in the CNS, EAATs play a significant role in its reuptake and recycling. Research has shown that mutations or dysfunction in the EAATs can cause various neurological conditions, including epilepsy, ALS, and depression. EAATs and KAINATE receptors are crucial components of the CNS's regulation of Glu, and their dysfunction can result in several neurological disorders. More research is necessary to fully understand how these receptors contribute to the emergence and development of these conditions. According to studies, mutations in EAAT2, the brain's most prevalent subtype of EAAT, increase the risk of developing ALS (Fontana et al., 2023). EAATs have been shown to increase Glu availability and mitigate symptoms for neurological conditions, making them a potential therapeutic target. Studying EAATs and their function is important for understanding neurotransmitter regulation and identifying potential therapeutic targets. EAAT activity is considered to preserve the CNS's normal glutamatergic neurotransmission. EAATs must control extracellular Glu levels to avoid excessive excitatory receptor activation, which can injure neurons and consequently result in neuronal cell death. EAATs, expressed in different brain parts, are found substantially in the CNS; this shows that EAATs play a crucial part in preserving healthy Brain function, given their participation in controlling diverse neuronal processes at multiple levels. Research on EAATs generally yields valuable

information on controlling neurotransmission and creating viable remedies for neurological illnesses (Hindeya Gebreyesus and Gebrehiwot Gebremichael, 2020).

The signaling function of Glu depends on how cells respond to it. Glu receptor proteins act as neurotransmitters in the extracellular fluid and are located on the surface of cells. Receptor activation is controlled by releasing and removing Glu from the extracellular fluid. For proper Glu signaling, cells must be sensitive to Glu and have sufficient energy to respond to typical stimuli. Additionally, Glu must be present in appropriate amounts in the right places at the right time. Excessive activity at Glu receptors can lead to excitotoxicity, a condition where nerve cells die. The ability of a cell to tolerate glutamatergic stimulation is determined by various factors (Kostic et al., 2014). Because Glu plays a vital metabolic role in the brain, brain tissue often has higher quantities of Glu than any other amino acid, ranging from 5 to 15 mmol more per kg, depending on the specific brain area. It shows that the activity of Glu used in brain cells is extremely high. Because of the remarkable flexibility of the Glu molecule, medicinal chemists have been able to synthesize substances that can distinguish between receptors and transporters. Glutamate-binding proteins can bind Glu despite having different binding sites because of their adaptability. Furthermore, the EAAT-type transporters serve as chloride channels (Danbolt et al., 2022). EAAT4 and EAAT5 are believed to function more as inhibitory Glu receptors than transporters and have the highest chloride conductance, according to Danbolt et al. (2022) and Y. Huang et al. (2011) (Danbolt et al., 2022; Y. Huang et al., 2011). Although the complex structure of mammalian transporters has yet to be crystallized, researchers have gained substantial knowledge about their structure, as noted by Danbolt, Furness, and Zhou (2016) (Danbolt et al., 2016). EAAT2 and EAAT3 proteins are thought to be homotrimers, with subunits non-covalently connected. Glu transporters are integral membrane proteins dependent on the lipid environment and can be influenced by fatty acids and oxidation. (Yun Zhou and Danbolt, 2014). The crystalline structure of a Glu transporter homolog (GltPh) from Pyrococcus horikoshii has been recently discovered. It is a bowl-shaped trimer with a solvent-filled extracellular basin that extends halfway across the membrane bilayer, as reported by recent studies. This structure is optimized for rapid transport and was cloned in the early 1990s (Danbolt et al., 2022).

Glu may be redistributed to synaptic vesicles or mitochondria when it enters the cytoplasm (Li and Sheng, 2022). In mitochondria, Glu serves as a substrate for several enzymes. Both mitochondria and Glu translocation pathways exist. Vesicular Glu

transporters (VGLUTs) transport Glu into synaptic vesicles in glutamatergic nerve terminals (Pietrancosta et al., 2020). Additionally, they differ significantly from those in the plasma membrane (Trudeau and El Mestikawy, 2018). Several non-exocytotic mechanisms are essential for providing the extracellular fluid with Glu, including anion channels, the reversed operation of Glu transporting proteins at the plasma membrane, xCT, and the potential ability of mature brain astrocytes to release Glu into the extracellular fluid. Glu transporters are distributed cell-wide and subcellularly in healthy and mature brain tissue. EAAT1 (GLAST; slc1a3) is primarily expressed in astrocytes throughout the CNS and is the predominant transporter in the retina, inner ear, and circumventricular organs. Episodic ataxia is associated with EAAT1 mutations in humans. EAAT2 (GLT-1; slc1a2) is mostly found in astrocytes in the adult normal brain. It was the first Glu transporter to be immunocytochemically recognized. EAAT2 is the only Glu transporter essential for non-challenging situations, accounting for 1% of total forebrain protein, and is four times more common in the hippocampus than EAAT1. It accounts for 95% of Glu uptake activity in young adult forebrain tissue.

Regarding EAAT2 activity in neurons in the normal and fully developed brain, most studies agree that EAAT2 is present in hippocampus neurons and that the protein is expressed at their axon terminals. Unlike EAAT1, mice and rats have very little EAAT2 at birth and during the first postnatal week. When wild-type mice's EAAT2 levels reach 50% of adult levels at three weeks, the EAAT2-deficient mice are easily distinguished because they are hyperactive, epileptic, and smaller than their wild-type littermates. EAAT3 (EAAC1; slc1a1) has been particularly difficult to identify. EAAT3 is a neuronal transporter that is not found in glia. It is expressed in most neurons throughout the CNS if not all. Still, it has a distinctive sorting pattern that directs it to soma and dendrites while avoiding axon terminals. The hippocampus and neocortex have the highest levels of EAAT3; however, the total tissue content in young adult rat brains is around 100 times lower than that of EAAT2. It is also found in the kidney and the ileum. EAAT4 (slc1a6) is found in cerebellar Purkinje cells, forebrain neurons, vestibular hair cells, and calyx endings.

On the other hand, localizes specifically to the cerebellum. EAAT4 knockout mice are viable and appear normal with some alteration of receptor activation. EAAT5 (slc1a7) is expressed primarily in the retina, with low levels in the brain. It is an inefficient transporter with excellent chloride channels, suggesting it may be more

significant as an inhibitory Glu receptor than transporters. Research has attempted to pinpoint its precise localization, but no EAAT5 knockout animal has been created.

Glu taken up by astroglial cells can be metabolized via the tricarboxylic acid cycle and be used in protein synthesis or converted to glutamine. Glutamine can be released to the extracellular fluid by a sodium-neutral amino transporter in the astrocytic membrane. The nervous system isolates itself from the blood via barriers, which are important for physiological and pharmacological reasons. Endothelial cells do not express EAAT1-3, but Glu transporters are detected in astrocytic endfeet surrounding blood arteries. Glu has an efflux mechanism due to blood-mediated scavenging.

2.5.5. Glutamate Transporters at the Blood-Brain Barrier

The blood-brain barrier isolates blood from the brain's interstitial fluid. It is created by endothelial cells under the influence of brain cells. The choroid plexus epithelium, which secretes cerebrospinal fluid (CSF), has another barrier. These barriers are significant from a physiological and pharmacological perspective because they restrict substances from infiltrating brain tissue, which is vital for maintaining brain homeostasis (Alajangi et al., 2022). Yet, the astrocytic feet surrounding the blood arteries have many Glu transporters. The preparations to isolate brain microvessels will likely be contaminated with endfeet, which might account for part of the results. So, a healthy and undamaged blood-brain barrier does not allow for any major Glu transit. In concurrence, radiolabeled aspartate and Glu injections do not cause radioactivity to build up in the brain (Fu et al., 2018).

2.5.6. Excitotoxicity of Glutamate

Excitotoxins can be defined as substances that lead to overexcitation of neurons, causing exhaustion and neuronal death. Excitotoxicity is the phrase employed to characterize the neuronal damage caused by excess Glu within the nervous system. Almost all excitotoxins mainly work through increased cation influx, particularly calcium; therefore, calcium serves as the main mediator of excitotoxicity. According to the source, excitotoxins can be classified as endogenous and exogenous (Rajda et al., 2017).

Endogenous excitotoxins are excitatory neurotransmitters that are naturally present in the body and conduct many vital biological functions, including learning,

memory, pain perception, immunological function, and the activation of many special senses. But so far, if their concentrations are too excessive, it can cause excitotoxic effects. Glu is a good example of this type. Exogenous excitotoxins are present in the environment, and after entering the body, they either directly cause excitotoxic damage or increase the sensitivity of receptors to endogenous excitatory neurotransmitters, which then become excitotoxic even at low levels. An example of this type is MSG. MSG is found almost inevitably in all processed foodstuffs, especially instant products and low-calorie supplements.

In the presence of associated risk factors, long-term exposure to excitotoxins has been proved in the etiology and pathogenesis of neurodegenerative disorders like AD, PD, ALS, and MS (Maddison and Giorgini, 2015). Substantial evidence shows that excitotoxic damage has a large share in neuronal death associated with status epilepticus, cerebral ischemia, stroke, and head trauma (Mishra et al., 2015). Glu toxicity is assumed to be responsible for memory loss, confusion, and mild intellectual disorientation that frequently occurs in late middle age or old age. They are involved in neurodegeneration because of the following mechanisms:

1. After metabolizing, most excitotoxins bind to various glutaminergic receptors, mainly ionotropic receptors like NMDA, AMPA, and kainate, making those receptors more sensitive to local Glu.
2. They impair the mitochondria's ability to handle oxidative metabolism, leading to increased free radicals or reactive oxygen species (ROS) generation.
3. ROS affects the membrane potential, lifts the magnesium tors, and induces persistent receptor activation, which significantly increases calcium influx and activates a variety of enzymes, including phospholipase A2, nitric oxide synthase II, calpain, phosphatases, proteases, endonucleases, etc.

These substances created and similarly stimulated platelet-activating factor (PAF) to set up a vicious auto-stimulatory positive cycle. Free radicals, together with endonucleases, cause DNA fragmentation and induce apoptosis. If the damage is severe enough, a lysosome rupture and extracellular release of intact and enzymatically changed cellular organelles results in inflammation and necrosis.

Exogenous excitotoxins, such as MSG, are consumed in large quantities under the guise of flavourful, processed food products. If combined with genetic disposition, aging, or any other factor causing oxidative metabolic stress, they eventually result in an auto-stimulatory glutaminergic vicious cycle that causes the apoptosis and necrosis

of specific neurons and the creation of neurodegenerative disorders (Belov Kirdajova et al., 2020).

2.6.Melatonin

Mel is the primary hormone secreted by pinealocytes of the pineal gland; it is produced in response to darkness and controls the sleep-wake cycle. It is also thought to possess antioxidant properties and may influence aging and immune function. Light exposure, aging, and some medications are just a few variables affecting Mel's production. Extra-pineal sources of Mel were also reported (Mayo et al., 2018) in the retina, bone marrow cells, platelets, lymphocytes, skin, cerebellum, and the gastrointestinal tract of vertebrates. It profoundly affects the melanin pigmentation of frog skin, so it was given its name (Mannino et al., 2021). It is often called the Dracula hormone, the hormone of darkness and sleep. It is important to transmit the daily cycle of light and dark to the body, which leads to regulating the circadian rhythm (Cardinali et al., 2022).

2.6.1. The Chemical Structure of Melatonin

Mel (N-Acetyl-5-methoxytryptamine) is an important neurohormone with a chemical structure resembling serotonin (Raghav et al., 2021). From a physicochemical perspective, Mel is a yellowish-white powder with a weight of 232.28 g/mol and a density of about 1.175 g/cm3. It melts at a temperature of 116.5 and 118 degrees. Mel has the molecular formula C13H16N2O2, which can be used to identify it from a chemical standpoint. A 3-amide group and a 5-alkoxy group are used to functionalize the chemical framework of the indole. Additionally, because it started as a tryptophan molecule, it is categorized as an indolamine compound (Jones et al., 2022). This molecule's amphiphilicity is also primarily due to the 3-amide group and 5-alkoxy group. Mel can penetrate all cellular and subcellular compartments due to its characteristics (Mannino et al., 2021).

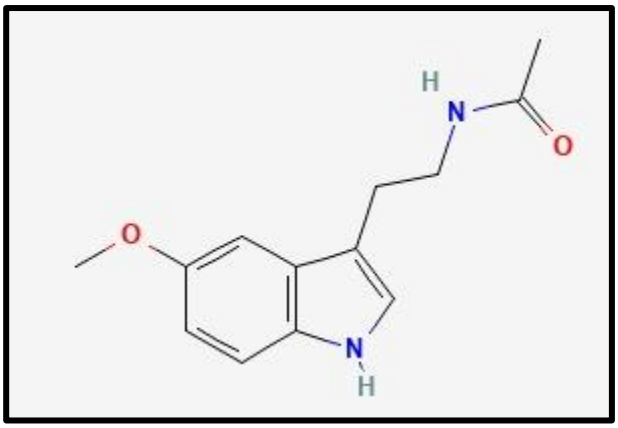


Figure 2.3. The chemical structure of Mel is modified by (Sayers et al., 2023).

2.6.2. Biosynthesis of Melatonin

Synthesis and secretion of Mel are enhanced by darkness and inhibited by light. Mel is created from L-tryptophan, mainly in the pineal gland and outside tissues such as the retina, skin, digestive, and immune system cells (Zagajewski et al., 2021). According to recent research, the pinealocytes, which constitute a large portion of the pineal gland, serve as the principal Mel producers, with astrocytes and microglia acting as its support cells by stimulating nuclear factor κB (NF-κB) and guiding tumor necrosis factor (TNF) (Pires-Lapa et al., 2018). Based on previous studies, the mechanism of Mel production by pineal cells depends on the conversion of tryptophan into serotonin and the inhibition of Mel by the enzyme N-acetyl transferase (NAT) and hydroxy indole-O methyltransferase (HIOMT). (Skarlis and Anagnostouli, 2020). Age-related variations in Mel production show it is highest in childhood and declines as older people (Öztürk et al., 2020). Several studies on Mel show that the suprachiasmatic nucleus (SCN) controls the cycle of pineal Mel synthesis through its branches to the paraventricular hypothalamic nucleus, which communicates with the higher thoracic segments of the intermediolateral spinal column. According to the superior cervical ganglion, information is sent to the pineal gland, where sympathetic postsynaptic fibers release norepinephrine only during the dark portion of the night, causing the enzymatic conversion of tryptophan to Mel (Wurtman, 2020). The biosynthesis and metabolism of Mel are described as Tryptophan, an amino acid. Tryptophan is then converted to 5-hydroxyptophan (Jeong et al., 2022). Hepatic cytochrome P450 enzymes (CYPs), especially the 6-hydroxylate CYP1A2, participate in plasma Mel metabolism and are responsible for the demethylate of Mel to its precursor, NAS (Zhou, 2018).

2.6.3. Receptors

The literature confirmed that the Mel receptors are found in the hippocampus of various animals. It has been suggested that Mel induces response element-binding protein (CREB) signaling pathways associated with long-term memory processing *in vitro* (Yu et al., 2022). Two major Mel receptors have been indicated: the ML1 high-affinity G protein-coupled receptor and the ML2 receptor, which has a low affinity (Nikolaev et al., 2021). The ML1 receptor is divided into Mel1a and Mel1b. Reports show that Mel1a, or MT1, contains 351 amino acids and is on human chromosome number 4 (4q35.1). It is characterized by its wide distribution as the tuberous part of the anterior pituitary gland and the SCN of the hypothalamus, which is known as the site of the circadian clock, as well as in the cortex, thalamus, substantia nigra, nucleus accumbens, amygdala, hippocampus, cerebellum, cornea, and retina. Mel1b, or MT2, contains 363 amino acids and is on human chromosome 11 (11q21-q22). It is found in the retina, hippocampus, cortex, paraventricular nucleus, and cerebellum (Samanta, 2022). In recent years, research has shown that Mel receptors, MT1 and MT2, play a crucial role in regulating circadian rhythms and neuroendocrine processes, and their activation has been linked to beneficial effects in sleep and circadian abnormalities, mood disorders, learning and memory, neuroprotection, drug abuse, and cancer. Selective ligands targeting these receptors may promote the development of novel and more efficacious therapeutic agents (Wang et al., 2022). In the same context, they have an active role in developing novel antidepressants. Early indications of Mel receptor involvement in depression have been found through studies on animal models and humans. These findings suggest that targeting these receptors may provide a new avenue for developing effective antidepressant drugs (Liang and Olsen, 2014). The Mel membrane receptors MT1 and MT2 are expressed in different tissues through the interaction of Mel with them directly and indirectly, in addition to activating the Gi/o-cAMP pathways, and the receptors activate the Gi-ERK or cAMP/PKA pathway in addition to the Gq/11-PLC-Ca2+ pathway and recruit β-arrestins with quite different downstream pathways. In diseases of glucose metabolism, MT1/MT2 has received extensive research (Mao et al., 2021).

2.6.4. Biological Activities of Melatonin

Mel is a powerful hormone with therapeutic properties that have been studied for decades, and it is known as a wide-spectrum antioxidant with the ability to scavenge free radicals. Mel reduces the activity of pro-oxidant enzymes while

increasing the activity of antioxidant enzymes and neutralizing ROS. Furthermore, it bonds with metal ions and impedes electron discharge from the electron transport chain. Additionally, Sirtuin 1 (SIRT1) and 3 (SIRT3), as well as peroxisome proliferator-activated receptor gamma 1-alpha (PGC1α), are activated by Mel, which is essential for regulating the formation of mitochondria (Huang et al., 2022).

Recent studies have confirmed the effectiveness of Mel in improving physical disorders such as temporomandibular joint, fibromyalgia, functional dyspeptic, and irritable bowel disorder (Zisapel, 2018). In addition, Mel protects against various diseases of the central nervous system, including ischemic brain injury, AD, and PD. It has been proven to correlate with memory and the ability to control bodily balance and posture. These findings provided Strong evidence indicating that Mel supplementation plays a critical role in cardioprotection and helps the cardiovascular system (Bonomini et al., 2018) in addition to its beneficial effects on blood coagulation and enhancing vascular endothelial dysfunction caused by atherosclerosis, hypertension, diabetes, and nicotine-induced morbidity. Mel has been shown to influence the circadian rhythm, protect the skin, and treat diabetes in humans. Furthermore, it protects lymphocytes from DNA damage brought on by ionizing radiation. Based on recent research, it was found that it helps reduce the effects of COVID-19 (Zimmerman and Reiter, 2022). In another context, Mel has been found to contribute to body mass by encouraging bone cell proliferation and collagen synthesis and limiting bone resorption by suppressing the creation and activation of osteoclasts by Rankl (Yi Zhou et al., 2020).

The potential adverse effects on the reproductive and endocrine systems. The potential adverse effects of high doses of Mel on the reproductive and endocrine systems should not be dismissed, as evidence supports these concerns. However, it is important to consider individual differences and lifestyle factors when evaluating the risks of Mel supplementation. In conclusion, while Mel supplements have been linked to menstrual irregularities and decreased fertility in women, the potential adverse effects of high Mel on the reproductive and endocrine systems should be evaluated on a case-by-case basis. Individual differences, such as medical conditions and lifestyle factors, may impact Mel supplementation's effectiveness and potential risks. It is important to consider these factors when deciding whether to use Mel supplements. Mel is a neuroprotector for individuals differently based on their health status and lifestyle factors. For example, individuals with certain medical conditions or taking

medications that affect Mel levels may respond differently to supplemental intake than those without these factors. Additionally, lifestyle factors such as sleep habits and exposure to artificial light may also impact the effectiveness of supplemental Mel.

2.6.5. Melatonin as Neuroprotective

Mel is a free-radical scavenger and possesses antioxidant properties. Previous studies revealed that it easily crosses the blood-brain barrier, and it has been found in high concentrations in the brain after exogenous administration (Meng et al., 2021). It is also a neuroprotector in various conditions affecting the central nervous system CNS due to its free-radical scavenging activities and lipophilic-hydrophilic properties. Therefore, it can protect the cell membrane, organelles, and core against free-radical damage. Neuroprotective actions have been discovered *in vitro* and *in vivo* (Verteramo et al., 2022). Recent evidence indicates that autophagy-mediated mitochondrial homeostasis is crucial for oxidative stress-related brain damage and repair. The highest concentration of Mel is in the mitochondria of cells, and Mel exhibits well-known antioxidant properties (Gurunathan et al., 2021). Some studies have shown that high doses of Mel may have adverse effects on the reproductive system and disrupt the normal functioning of the endocrine system. For example, a study on rats showed that high doses of Mel led to decreased testosterone levels and altered testicular function. Additionally, in humans, excessive intake of Mel supplements has been linked to menstrual irregularities and decreased fertility in women.

3. OBJECTIVES OF THE STUDY

3.1.General Objective:

This study aimed to determine the effects of Mel as a neuroprotection against glutamate-induced neurodegeneration in the HT-22 hippocampal cell line.

3.2.Specific Objectives:

To investigate the effect of different concentrations and the impact of durations of Mel on HT-22 hippocampal neuronal cell line using the MTT assay and determine its IC_{50} value.

To evaluate the cytotoxic effect of various Glu concentrations on the HT-22 hippocampal neuronal cell line and to measure its IC_{50} value.

To investigate the neuroprotective effect of Mel on HT-22 cells subjected to Glu using the recommended concentrations.

4. MATERIAL AND METHODS

4.1. Chemical Reagents

Melatonin, glutamate, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 100 U/ml penicillin 10mg/ml streptomycin, L-glutamine 200 mM (29.2mg/ml), Trypsin-Ethylene Diamine Tetra Acetic Acid (EDTA) and Dulbecco's phosphate-buffered saline (PBS), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). Chemical reagents were purchased from (biological industries Israel Beit Haemek Ltd and Gibco BRL (Grand Island, NY, USA).

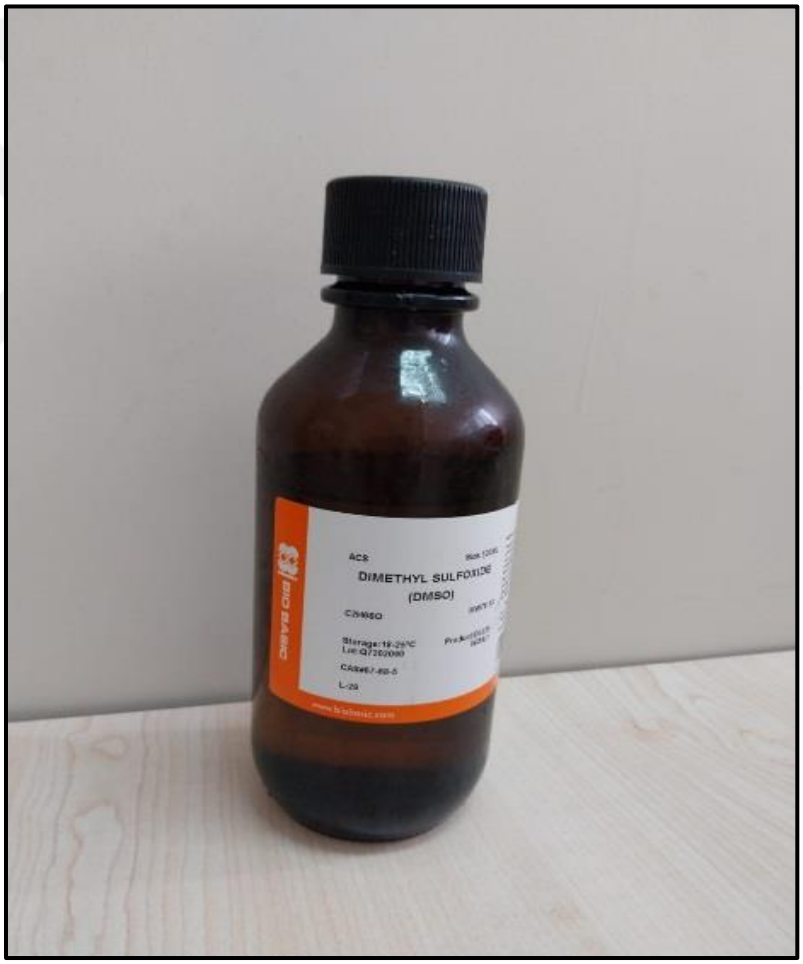


Figure 4.1. Dimethyl Sulfoxide (DMSO)

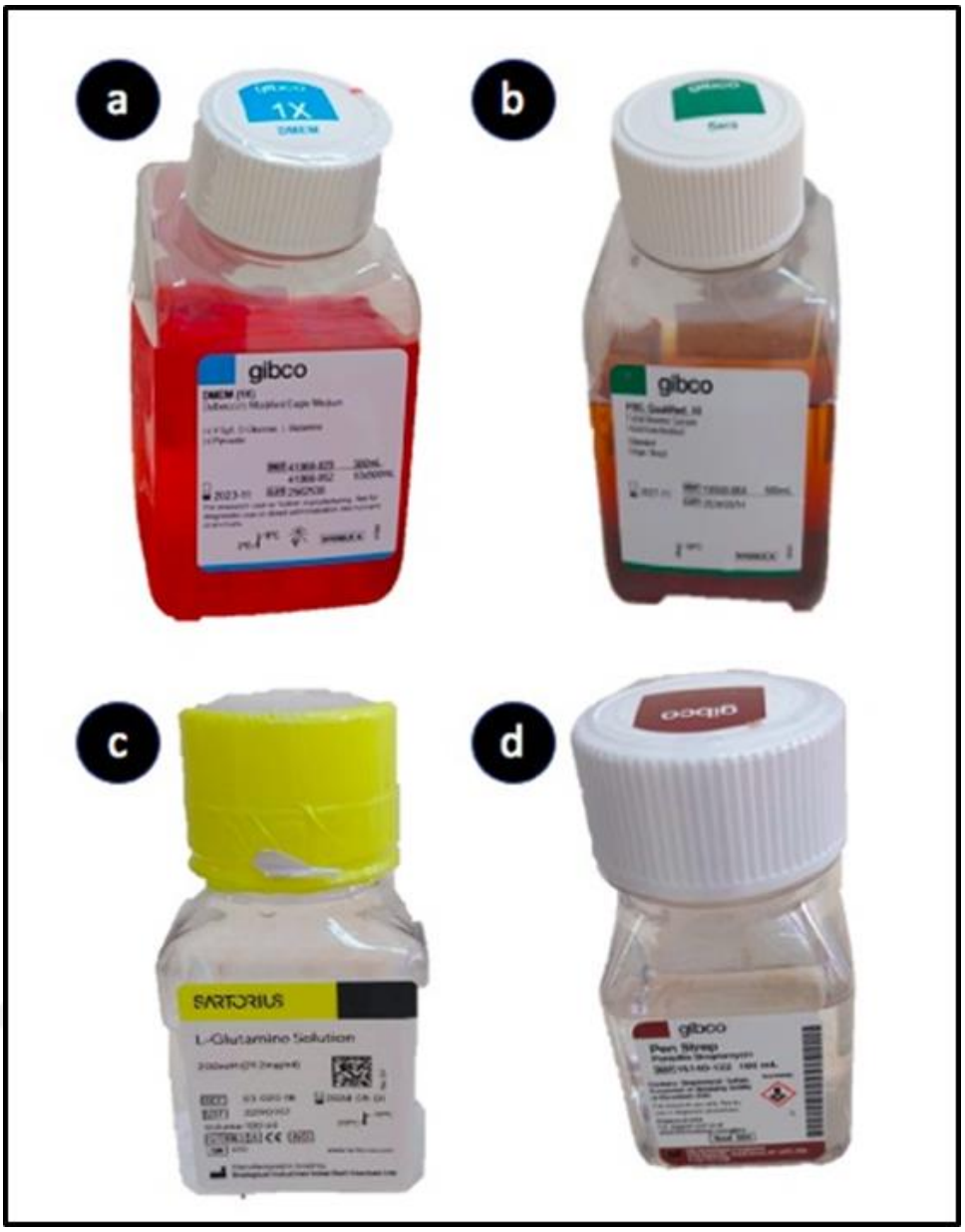


Figure 4.2. (a) Dulbecco's Modified Eagle Medium (DMEM). Supplementary (b) FBS, (c) L glutamine, (d) penicillin-streptomycin solution.

4.2. The Cell Lines

HT-22 hippocampal neural cell line was kindly gifted from Doç. Dr. Mehmet Emin ÖNGER (Histology and Embryology Department - Ondokuz Mayis University-Samsun -Türkiye).

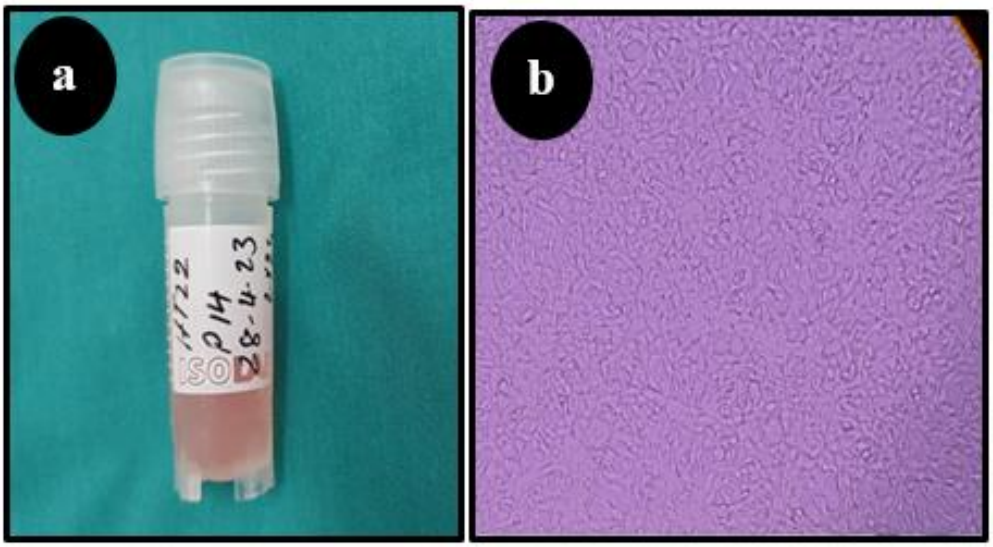


Figure 4.3. HT-22 hippocampal neural cell line (a) in a cryovial and freezing at −86 °C. (b) in culture cell flask at the confluence stage.

The incubator (SANYO Electric Co. Ltd., Japan) environment under conventional culture conditions is 5% carbon dioxide in 95% humidified at 37◦C to allow the cell to attach to the surface of the flask.

Figure 4.4. Incubator: (5% of atmospheric carbon dioxide CO_2, in 95% humidified at 37◦C) with UV system installed.

4.3.Seeding Protocol

Vials of frozen HT-22 cells were obtained and warmed in a water bath. Complete media was pre-prepared and pre-warmed. The cells were put in a falcon tube, and complete media were added. The cells were centrifuged, and the supernatant was removed. Then, complete media was added to the pellet, and the cells were seeded into 25cm^3 flasks.

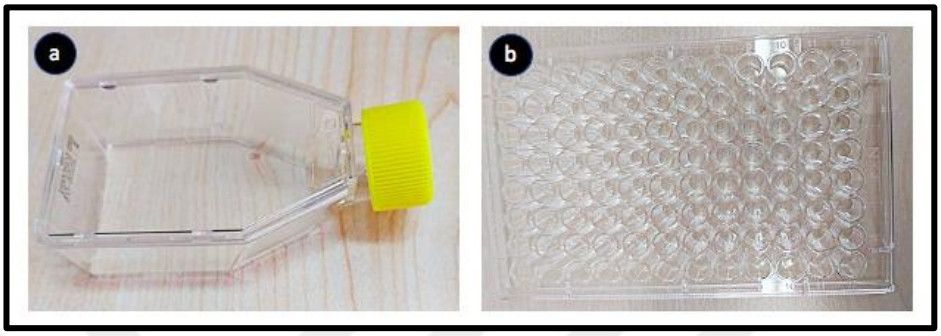


Figure 4.5. (a) Flasks (25, 75, 175cm^2) (b) Sterile cell culture well-plates, 96-well

4.4.Feeding Protocol

This protocol determines the need for media replacement; several factors should be considered. These include a drop in pH, color change of the media, rate of pH decline, cell concentration, and cell type. Regular examination and familiarity with the cell line are essential to identify morphological deterioration. If not addressed in time, irreversible damage may occur as cells enter apoptosis (Phelan and May 2015).

4.5. Passaging Protocol

Upon reaching 80% confluence, cells were detached using Trypsin/EDTA. After eliminating the culture media in the flask, the cells were rinsed with PBS. Then, 1-3 ml Trypsin/EDTA was added and kept for 3 minutes until the cells were completely detached. The trypsin was inhibited by adding 3 ml of complete media, followed by centrifuging at 2500 rpm for 5 min, and the supernatant was removed.

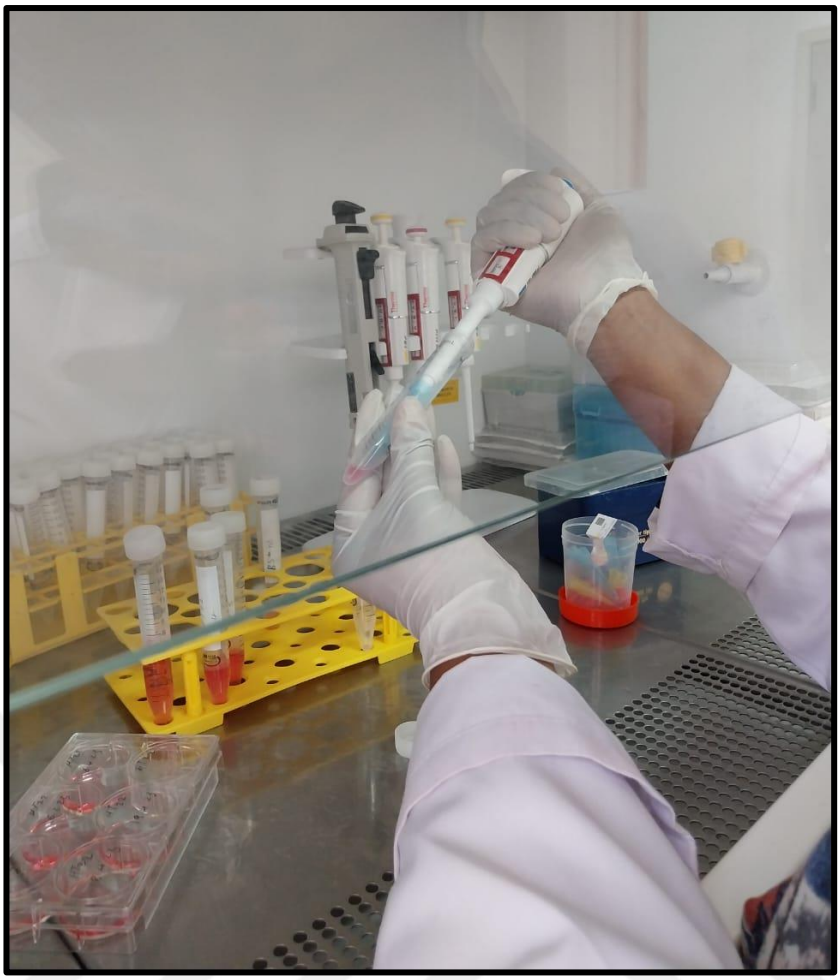


Figure 4.6. Passaging process

4.6.Seeding in the Well Plate

The cells were seeded in 96-well plates. Each well is contained in a 100 µl complete media and cells and incubated for 24 hours before administration of the experimental treatment.

4.7.MTT Assay

To evaluate the cell viability and determine the IC_{50}, we used the MTT assay known as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium). It is used for multiple applications such as cell viability, cytotoxicity, cell proliferation, cell activity, and determining the IC_{50}. The reaction of this assay depends on the ability of the mitochondria metabolic activity of viable cells to transform the water-soluble yellow tetrazolium salt into a water-insoluble formazan (purple); this action did not occur in dead cells.

Figure 4.7. MTT Protocol Reagents. (a) DMSO, (b) MTT

Before going into the process of the MTT assay, the HT-22 hippocampal neural cells must be seeded in 96-well plates, then expanded in 25cm^2 culture flasks until 80-90% confluency. 5000-6000 cells should be distributed equally in each well plate, suspended in 100 µl of complete media; then, they should be incubated overnight or 24 hours at 37°C under 5% CO_2 and 95% humidity.

4.7.1. MTT Assay Protocol

Three replicates of HT-22 were seeded in a 96-well plate at various concentrations. The complete media was aspirated after 24 hours, and cells were washed with PBS. After applying various doses of the toxic and protective substances, the 96-well plate was incubated for 24 hours.

MTT solution was added, and the well plates were incubated for 2 hours, followed by careful aspiration. DMSO was added, and aggressive pipetting was performed. The plate was covered with folio and incubated or left at room temperature for 10 minutes. The results were analyzed at 570 nm absorbance using a spectrophotometer.

Table 4.1. MTT assay protocol.

1.	Removed all the complete media.
2.	Rinsed the cell with 3ml of PBS
3.	Added trypsin EDETA 1 to 3ml (according to flask volume) for 3 minutes
4.	Added 4 to 5 ml fresh media to deactivate the trypsin reaction
5.	Transferred the cell using a pipette, put it in a falcon tube, and centrifuged in 2500 for 5 minutes.
6.	Removed all the supernatant, added 3.5 ml of new complete media, pipetted the cell in the falcon tube, and then distributed it to the 96-well plates.
7.	Then, incubate for 24 hours to attach.
8.	Serial dilutions of different doses of Glu concentrations were prepared within complete culture media. After incubation, the culture media in a 96-well plate was removed gently, washed with PBS, and added 200 µl fresh medium with various drug concentrations, and this step was triplicated.
9.	Returned the well plate to the incubator for 24 hours or more according to the experimental duration needs. All experiments were conducted within 5-13 passages of original thawed cell culture stocks.
10.	An added MTT kit must be prepared for it. The DMSO will be added.

https://www.abcam.com/kits/mtt-assay-protocol.

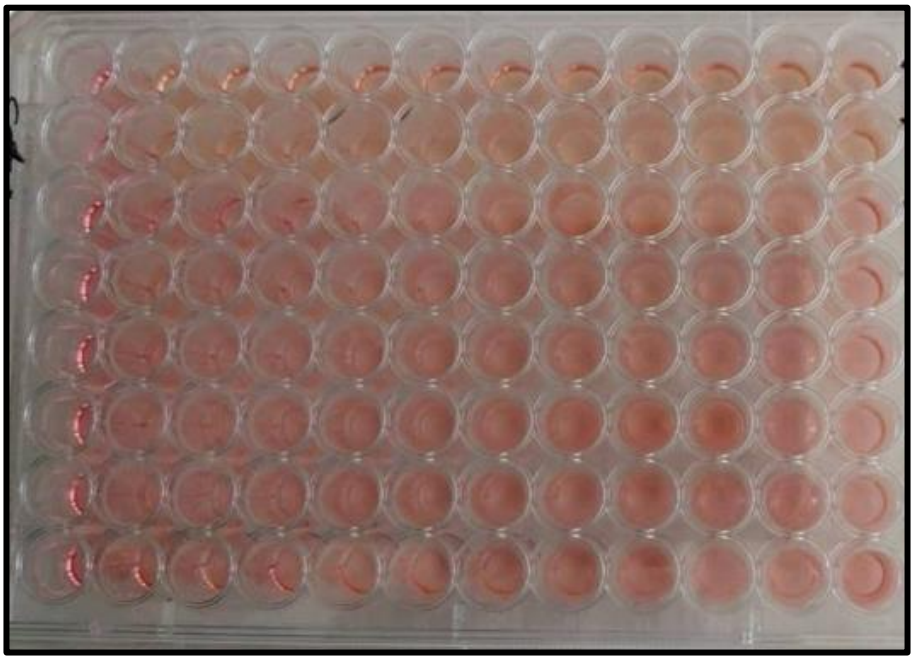


Figure 4.8. 96-well-plates with media

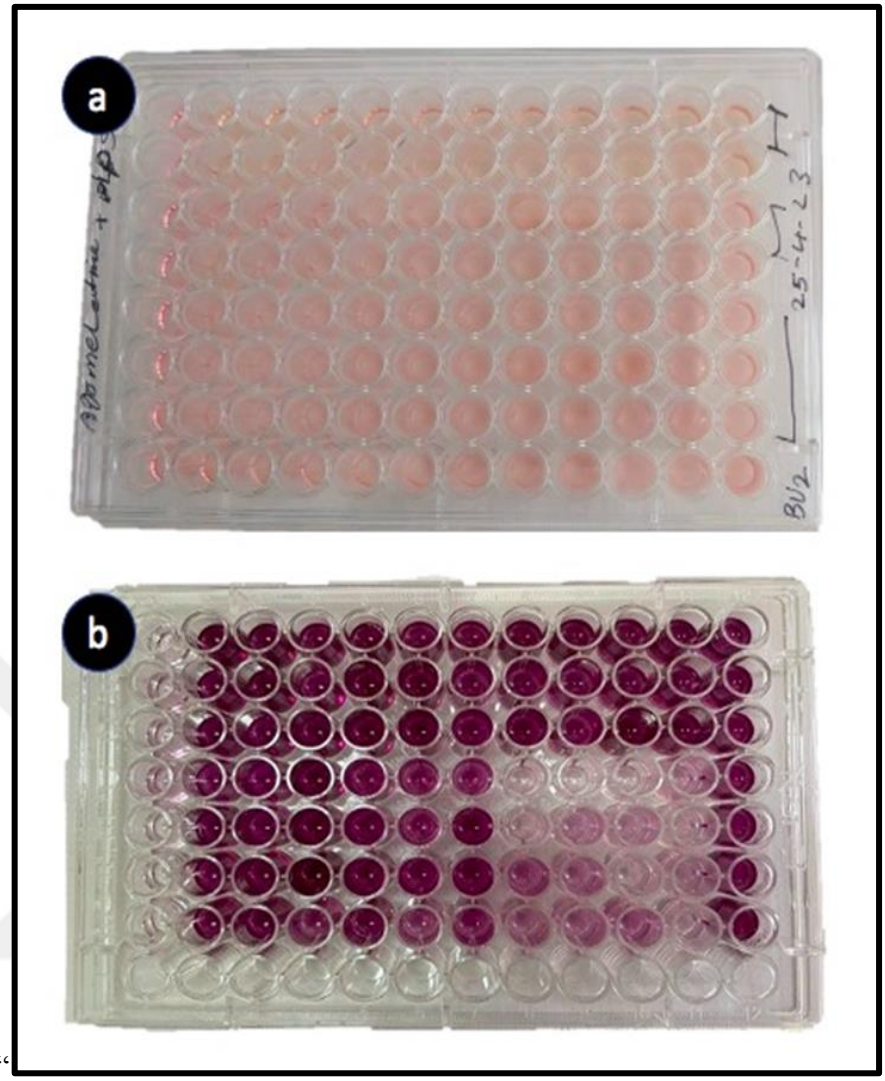


Figure 4.9. 96-well-plates with (a) media, (b) DOMSO

4.7.2. Elisa Absorbance Reader

Absorbance (calorimetric measurement of MTT reduction) was recorded at Elisa microplate reader 570 nm to measure cell samples. All treatments were seeded in triplicates, and the mean viability was calculated from three independent experiments.

4.7.3. Dosage Determination

In this experiment, HT-22 neural cells were exposed to Mel as a protective or treatment substance. The HT-22 neural cells were subjected to different Mel concentrations to assess their neuroprotective or toxic effects and determine these substances' effects; we performed MTT assays with different doses and durations. The MTT assay is commonly used to evaluate cell viability. One of the main objectives

was to find the IC_{50} value for the neuroprotective substance, which represents the concentration at which 50% of the cells' viability is affected. Based on our pilot studies, the IC_{50} value of the protective substances on Mel's HT-22 neural cell line is 0.8 mM. These values indicate the concentrations of protective substances significantly affecting cell viability.

Additionally, MTT assays were conducted to determine the ideal toxic concentrations of Glu. The results indicated that the perfect doses for the project are 2.465 mM for Glu in HT-22 cells. By determining the IC_{50} values and toxic concentrations, we established the appropriate dosage range for the subsequent experiments in our study.

4.7.3.1.Melatonin

We use the MTT process to get the most effective protection concentrations, determine the IC_{50} value of different doses of Mel as neuroprotective, and investigate their effect on HT-22 hippocampal neuronal cell line viability. To find that, we cultured the HT-22 cell line in 96-well plates, and after 24 hours, cells were treated with different concentrations of the protective Mel substance and incubated later for 24 hours. Three triplicates of the experiment were executed. To determine the effect of various concentrations on cell viability and calculate the IC_{50} of Mel dose. Cell viability was detected using the MTT assay and Elisa absorbance reader. Different dosages of Mel were applied to the cell treatment (0.1, 0.25, 0.5, 0.75, 0.9, 1, 2, 3, 4, 5 mM). However, it revealed that the IC_{50} of the Mel after 24 hours presented at IC_{50}= 0.8399 mM. IC_{50} means the half maximal inhibitory concentration is the concentration of a substance at which cell viability or growth is reduced by 50%.

Table 4.2. Different doses of Mel

Mel concentration (mM)	Mel from the complete solution (µL)	Diluted by Complete Media (µL)
0.1	350.0	350.0
0.25	280.0	420.0
0.5	210.0	490.0
0.75	140.0	560.0
0.9	70.0	630.0
1	63.0	347.5
2	52.0	647.5
3	35.0	665.0
4	17.5	682.5
5	7.0	693.0

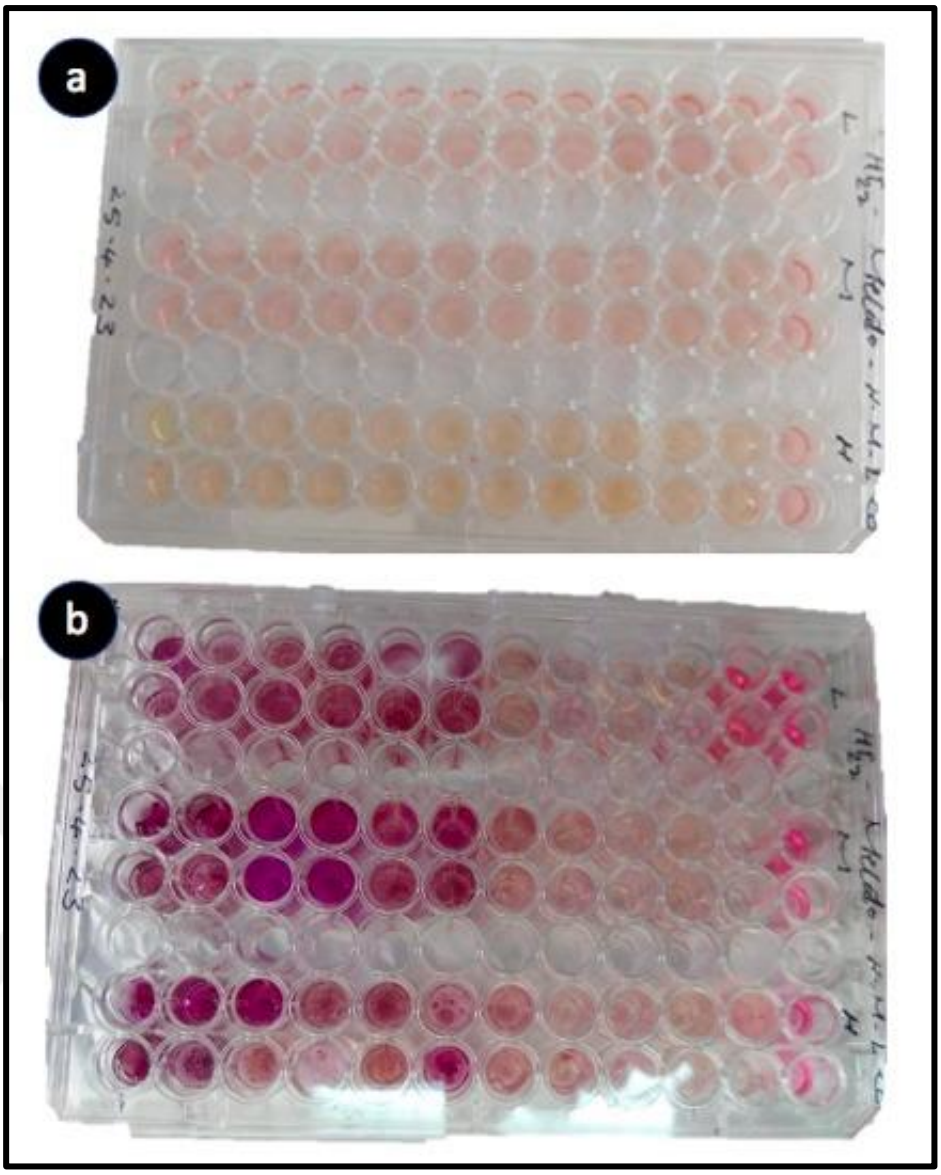


Figure 4.10. HT-22 hippocampal neuronal cell line in 96-well plates in different doses with Mel for 24 hours.

(a) After adding MTT, (b) After adding DMSO.

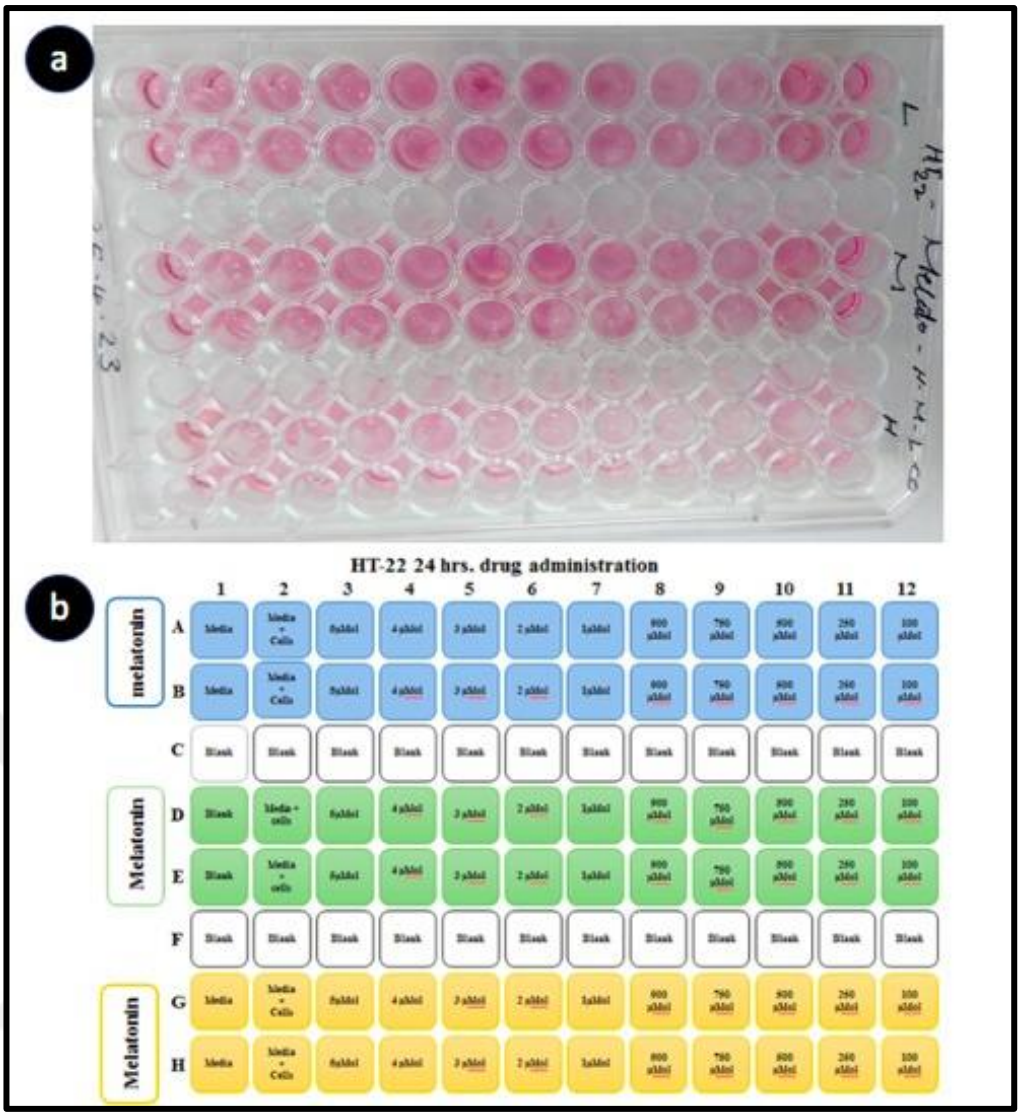


Figure 4.11. HT-22 hippocampal neuronal cell line in 96-well plate for Mel dose adjustment in 24 hours.

(a) The 96-well plate contains Mel with HT-22 hippocampal neuronal cell line and media.
(b) Study design of 96-well plate with Mel at different doses.

4.7.3.2. Glutamate

The MTT method was used to explore the cytotoxicity of different Glu doses on HT-22 neural cells, the IC_{50} value of Glu as a neurotoxin agent, and its impact on the viability of the HT-22 hippocampus neuronal cell line. The HT-22 cell lines were cultured in 96-well plates. After 24 hours, the cells were exposed to various concentrations of Glu (1,2,3,4,5 mM) and then incubated for another 24 hours. Then, the IC_{50} of the Glu dose was calculated to determine the impact of the various concentrations on cell viability. Elisa absorbance reader and the MTT test were used to find cell viability, and it was conducted to determine the ideal toxic concentrations

of Glu. The results indicated that the perfect doses for the project are 2.465 mM for Glu in HT-22 cells; by determining the IC_{50} values and toxic concentrations, we established the appropriate dosage range for the subsequent experiments in the study.

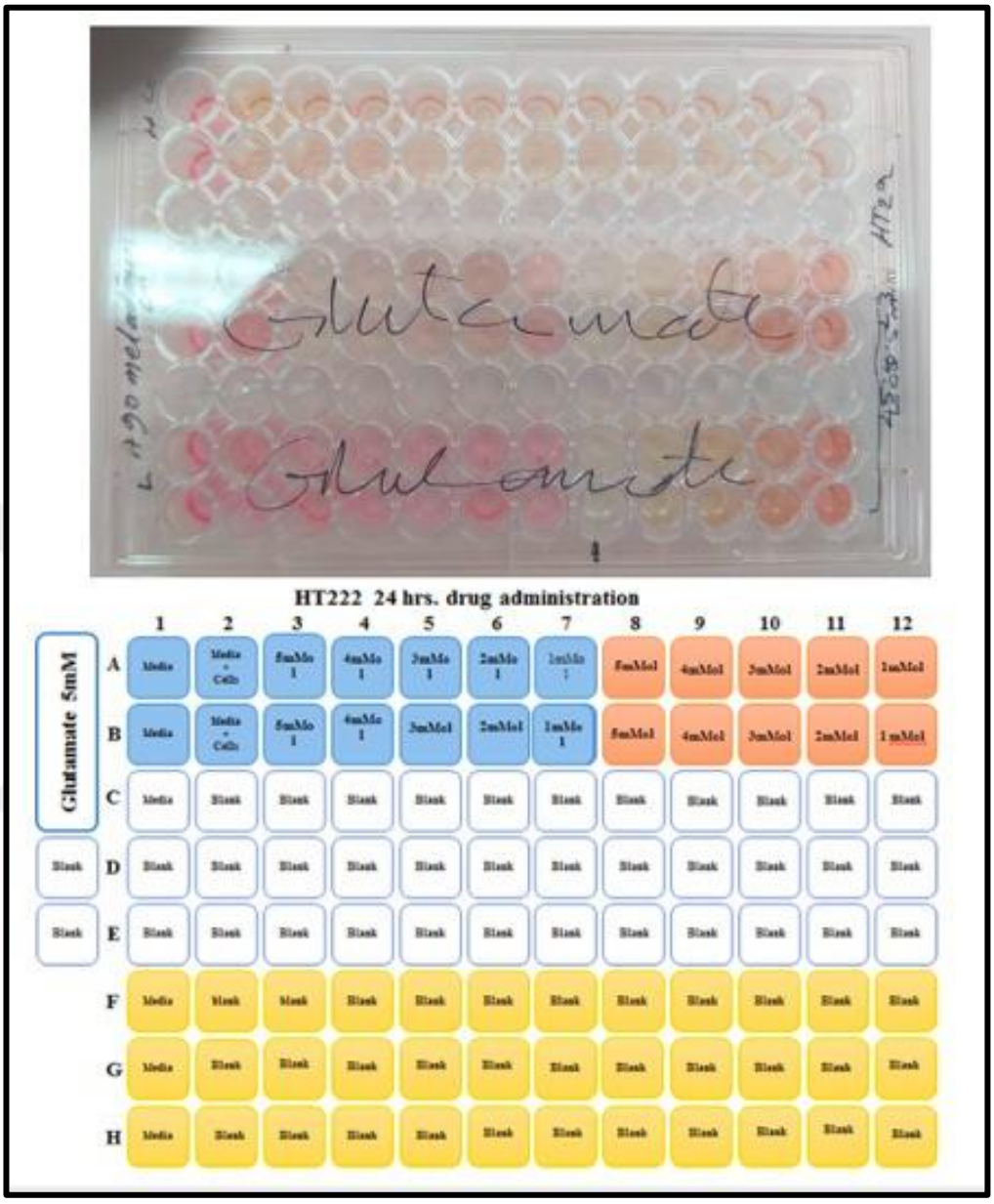


Figure 4.12. HT-22 hippocampal neuronal cell line in 96-well plate for Glu dose adjustment in 24 hours.

(a) The 96-well plate contains Glu with HT-22 hippocampal neuronal cell line and media.
(b) Study design of 96-well plate with Glu at different doses.

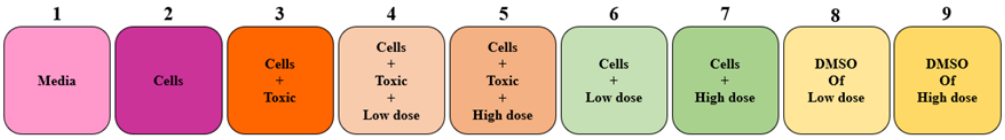
Table 4.3. Different doses of Glu

Glu concentration (mM)	Complete media (µl)	Glu from the complete solution (µL)
5	0.00	250
4	50	200
3	100	150
2	150	100
1	200	50

4.7.3.3.Melatonin Protective Effect Against Glutamate Toxicity

Cells were seeded in 96-well plate groups in all experiments, remained for 24 hours, and were put in an incubator to grow at 37 °C with 5 % CO_2 and 95% humidity before cell treatment. Then, the HT-22 hippocampal neural cell line group's experimental well plates were formed.

The HT-22 hippocampal cell line samples were subdivided randomly into nine groups for treatment as follows:



1. In the first column, 100 µl complete media was added.
2. In the second through the ninth columns, HT-22 was seeded in 100 µl complete media and then incubated for 24 hours.

3. After 24 hours of incubation, all the media was aspired, and 80 µl complete media was added.
4. In the third, fourth, and fifth columns, 10 µl Glu 5 mM was added as a toxic substance.
5. In the first, second, sixth, seventh, eighth, and ninth columns, 10 µl complete media was added.
6. Then, the well plate was incubated for an hour.
7. In the fourth column, 10 µl of Mel 0.5 mM was added as a low-dose protective substance.
8. In the fifth column, 10 µl of Mel 1 mM was added as a high-dose protective substance.
9. In the sixth column, 10 µl Mel 0.5 mM was added as a low-dose protective substance.
10. In the seventh column, 10 µl Mel 1 mM was added as a high-dose protective substance.
11. In the eighth column, 9.5 µl CM+ 0.5µl DMSO were added based on the volume used to prepare 10 µl of Mel 0.5 mM.
12. In the ninth column, 9 µl CM + 1µl DMSO was added based on the volume of preparing 10 µl of Mel 1 mM.
13. An additional 10 µl complete media was added to all other columns except the fourth and fifth columns.

Table 4.4. The experimental groups

NO	GROUP	DETAILS OF THE GROUP
1	Blank	Complete medium only
2	Control	Control group (Cells + media)
3	Glu	Glu (5mM) as a toxic substance for 1 hour
4	Glu+ Low Mel	Glu (5mM) + Low Mel (0.5 mM) for 24 hours
5	Glu+ High Mel	Glu (5mM) + High Mel (1mM) for 24 hours
6	Low Mel	Low Mel (0.5 mM) for 24 hours
7	High Mel	High Mel (1mM) for 24 hours
8	Low DMSO	DMSO
9	High DMSO	DMSO

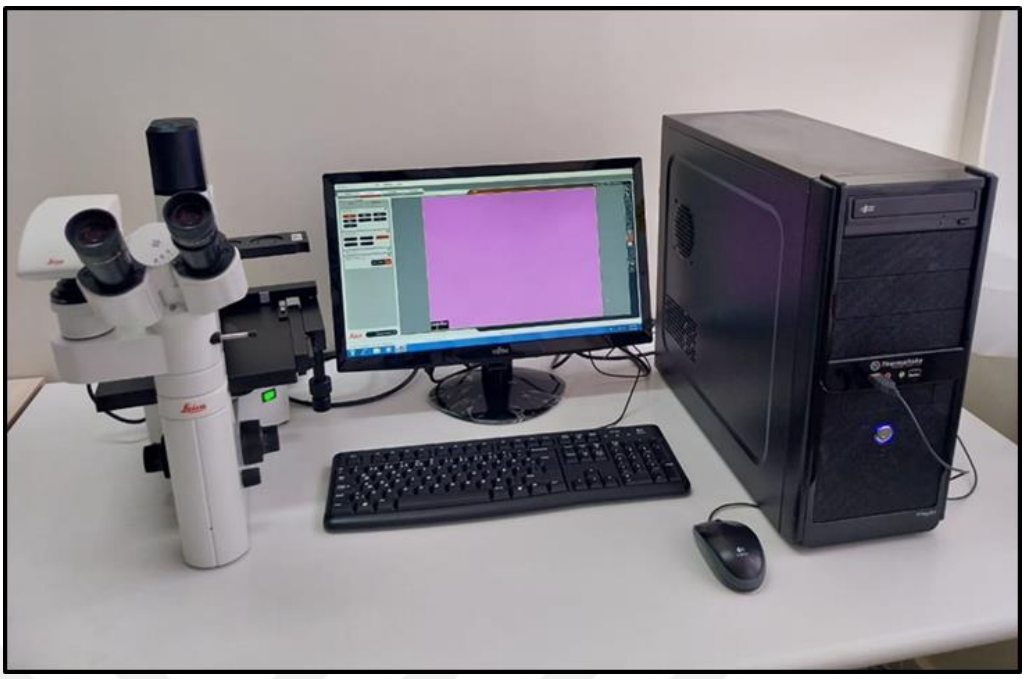


Figure 4.13. A Leica inverted microscope Evos XL core (AMG, WA, U.S.A).

Statistical Analysis:

- Statistical analysis was performed using SPSS 21.0 (IBM Corporation, Armonk, NY, USA) software.
- Microsoft Excel 365 and Graph Pad Prism version 9.5.1
- The data were presented as Mean ±SD
- This study used the one-way ANOVA and Tukey's multiple comparison tests to compare the groups' results.

5. RESULT

The study was designed to investigate Mel`s therapeutic and neuroprotective effects on HT-22 hippocampal neural cell line against Glu-induced excitotoxicity in *in-vitro* models. In this study, the HT-22 cells were subjected to different concentrations of the experimental substances Mel and Glu, and to determine the effects of these substances, MTT assays were performed with different durations and doses of these substances and to calculate the IC_{50} value for each substance, which represents the concentration at which 50% of the cells' viability is affected.

5.1. MTT Assay for HT-22 Cell Viability

MTT assays were conducted with various doses and exposure times to ascertain the effects of the Mel and Glu on the HT-22 cells. The IC_{50} value for each substance was calculated, which denotes the concentration at which 50% of the viability of the cells is affected.

5.1.1. Melatonin

5.1.1.1. The Dose-Dependent Effect of Melatonin on Cell Viability

The mean number of the cell viability of Mel dose groups and the degree of significance are shown in (Table 1) and (Figure 1) below, respectively, after 2 hours of MTT examinations.

The findings showed that the cells started to lose viability in the low doses, and the death rate increased with the increase in the Mel doses. At the concentration of 0.5 mM, the percentage of mean cell viability was around 59.75; at the concentration of 0.8 mM, nearly half of the cells died.

Table 5.1. Illustrates the mean number of cells with viability and the significant degrees of the various Mel concentrations after 24 hours of cell treatment.

Different Doses Group (mM)	Mean cell viability %	SD	p-value
0.1	84.64	4.21	0.6528
0.25	76.17	25.64	0.1297
0.5	59.75	6.051	0.0012
0.75	55.45	13.97	0.0003
0.9	61.8	7.122	0.0022
1	57.75	3.296	0.0007
2	6.913	1.891	<0.0001
3	5.559	1.515	<0.0001
4	4.952	1.305	<0.0001
5	5.823	0.7851	<0.0001

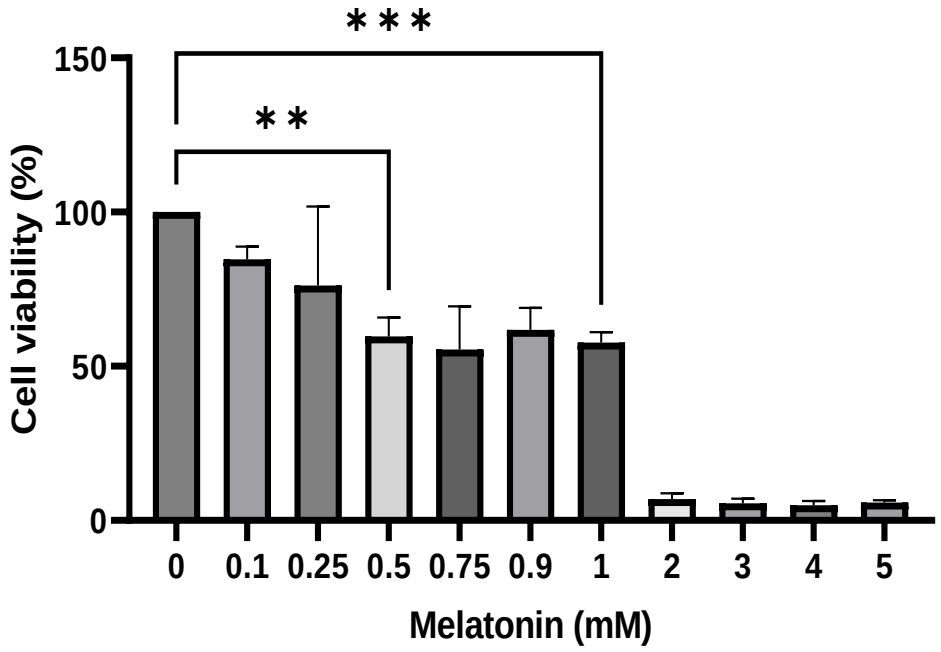


Figure 5.1. Cell viability after 24 hours of Mel's effects on the HT-22 neural cell line.

No significant difference was seen at Mel doses under 0.5 mM compared to the control. However, the significance was present starting from 0.5 mM ($p = 0.0012$).

The Mel dose of 1 mM showed very high significance compared to the control group ($p = 0.0007$).

The values were presented as the mean and standard deviation. Compared to the untreated control, no substantial cytotoxicity was seen at Mel doses of 0.1 and 0.2 mM; however, the cytotoxicity was seen from 2 mM and above. The concentration of a drug at which cell viability is reduced by 50% is known as the IC_{50}. The IC_{50} value for Mel after one day is 0.8399 mM.

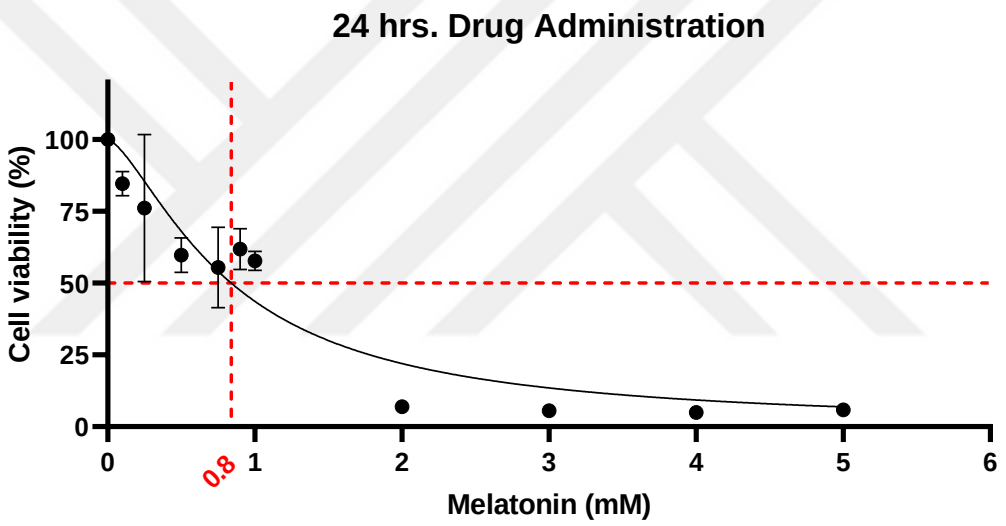


Figure 5.2. The IC_{50} value for Mel after one day is 0.8399 mM.

All the images were acquired to meet the requirements of the study. The Leica inverted microscope Evos XL core captured the images at 10x magnification. Figure 4.3 shows the control group containing media with HT-22 hippocampal neural cell line in culture cell well-plate with no treatment. Figure 4.4 shows significant effects on the cell viability during 24 hours when treated with Mel at a dose of 0.5 mM. The cells start to detach, lifting empty areas. At the same time, there are no significant effects on cell viability after being treated with Mel at the dose of 0.25 mM for one day.

There are significant effects on the cell viability at dose 1 Mm (Figure 4.5). The cell death increased further during 24 hours after Mel at the dose of 2 mM and above (Figure 4.6); more cells detached, and the empty areas increased.

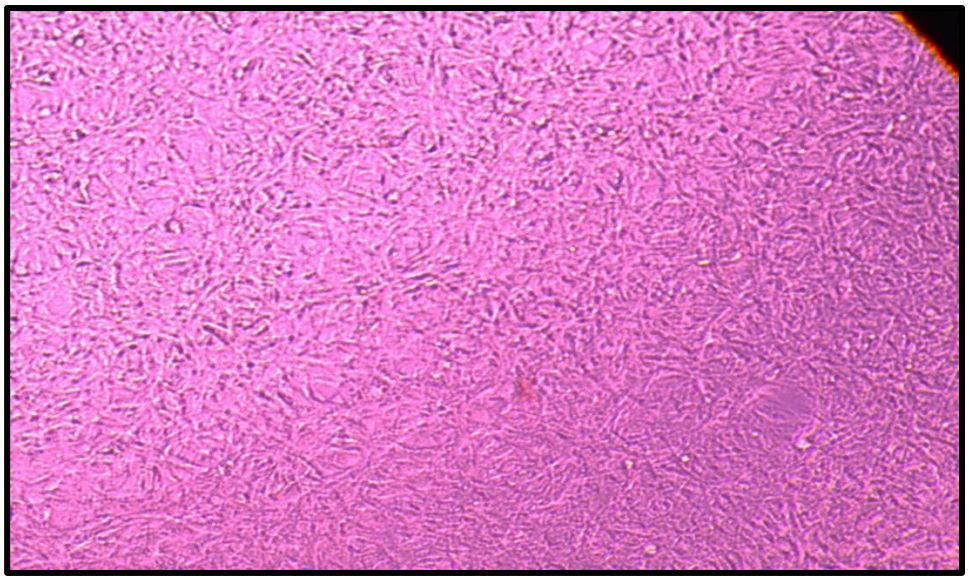


Figure 5.3. HT-22 hippocampal neural cell line in culture cell well-plate taken from the control group.

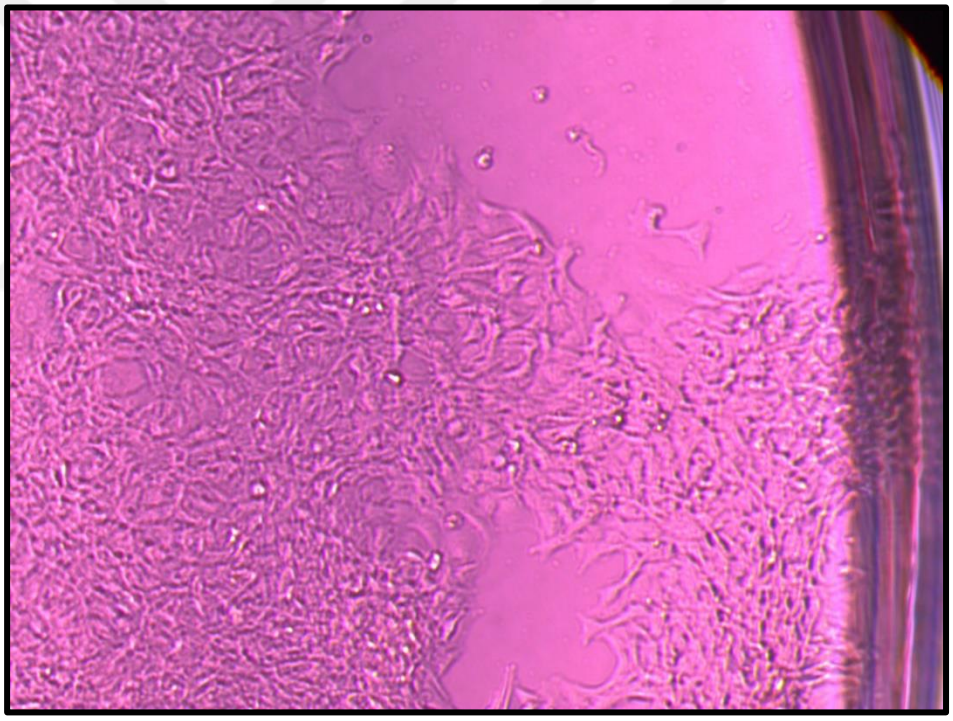


Figure 5.4. HT-22 hippocampal neural cell line in culture cell taken from the well-plate. Mel at dose 0.5 mM.

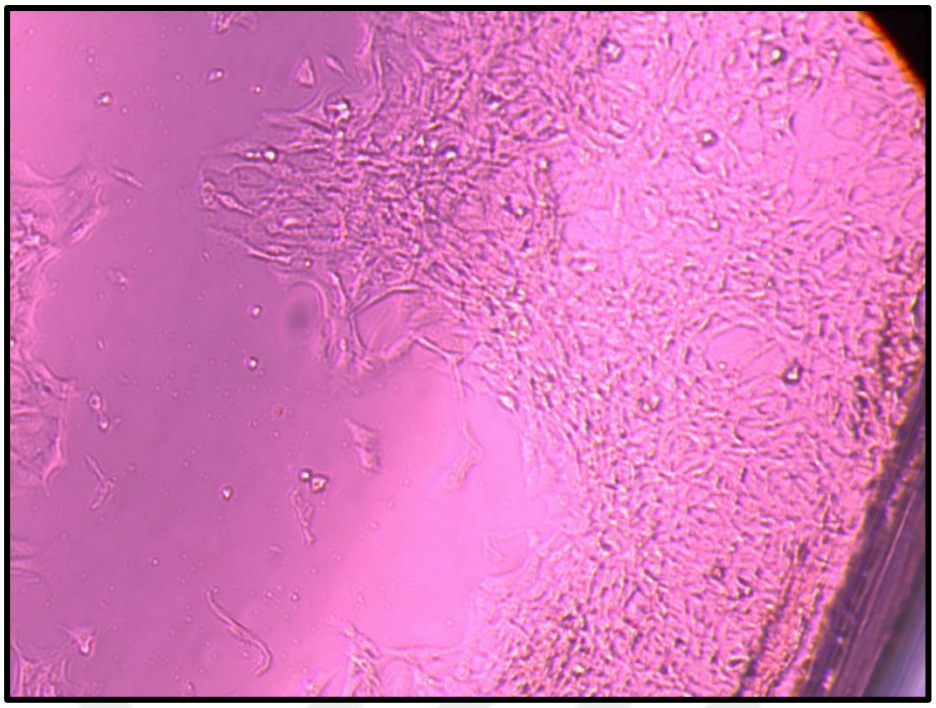


Figure 5.5. HT-22 hippocampal neural cell line in culture cell taken from the well-plate, Mel at dose 1 mM.

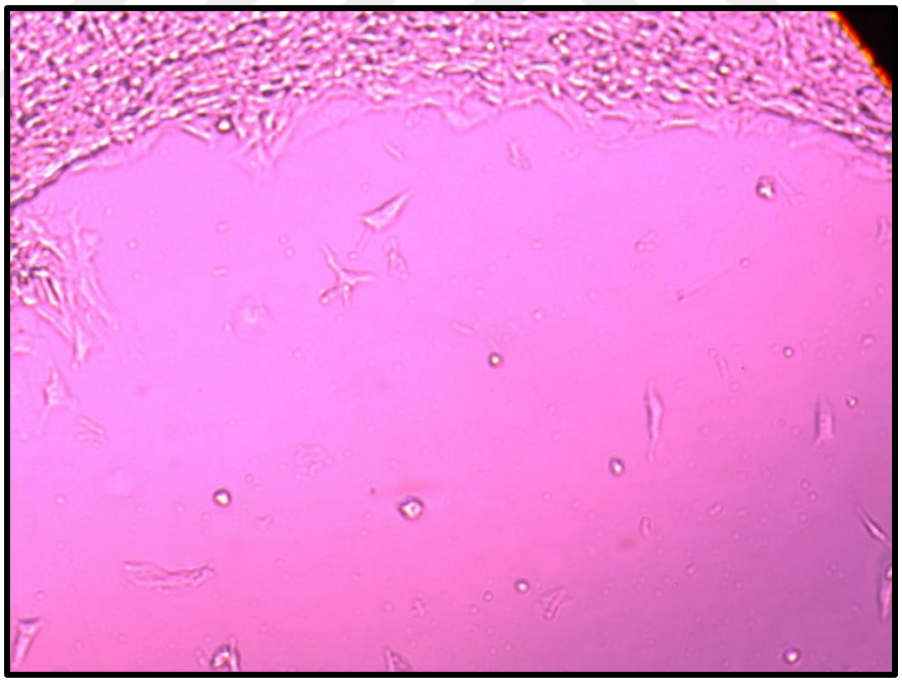


Figure 5.6. HT-22 hippocampal neural cell line in culture cell taken from the well-plate, Mel at dose 4 mM.

5.1.1.2. The Impact of Melatonin Duration of Exposure on Cell Viability

This experiment estimated the effect of different concentrations on cell viability at various durations and subsequently calculated the IC_{50} of the Mel doses (Figure 4.7). Cell viability was determined using the MTT test and the Elisa absorbance reader. At 24 hours, gradient doses started from 0.1 mM to lower than 0.5 mM do not significantly affect cell viability. Increasing the dose concentration by more than 0.5 mM would significantly decrease or lower the cell viability. The IC_{50} for 24 hours is 0.8399 mM in Figure 4.7. The values were the mean ± standard deviation. Cell viability at 24 hours was compared to 48 and 72 hours. At 48 hours, the IC_{50} for Mel was calculated as 0.48 mM. At 72 hours, the IC_{50} for Mel was calculated as 0.39 mM.

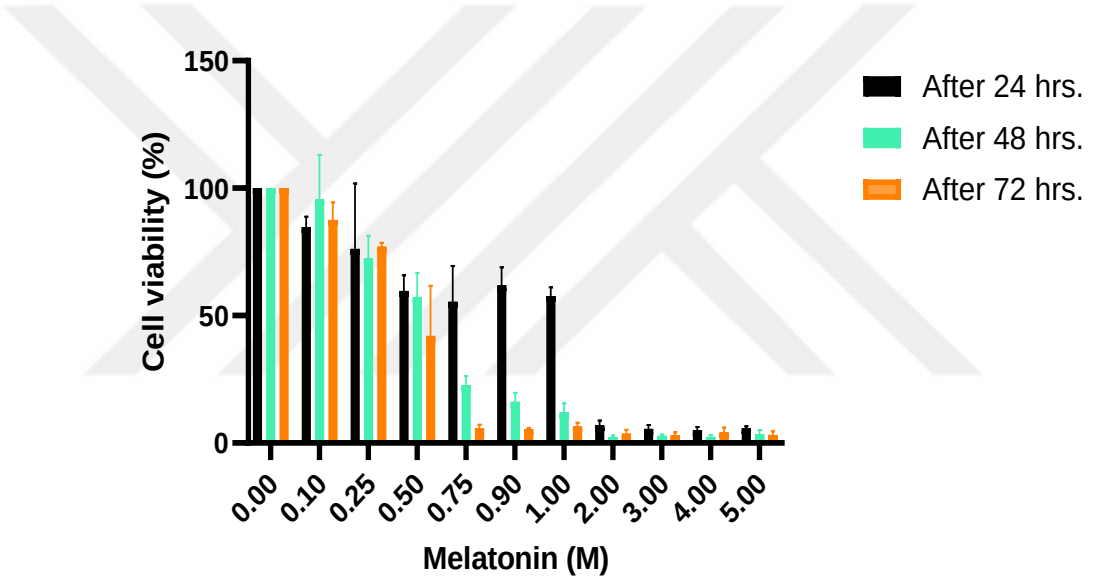


Figure 5.7. The bar chart represents the means of different concentrations of Mel on HT-22 cell viability after 24, 48, and 72 hours.

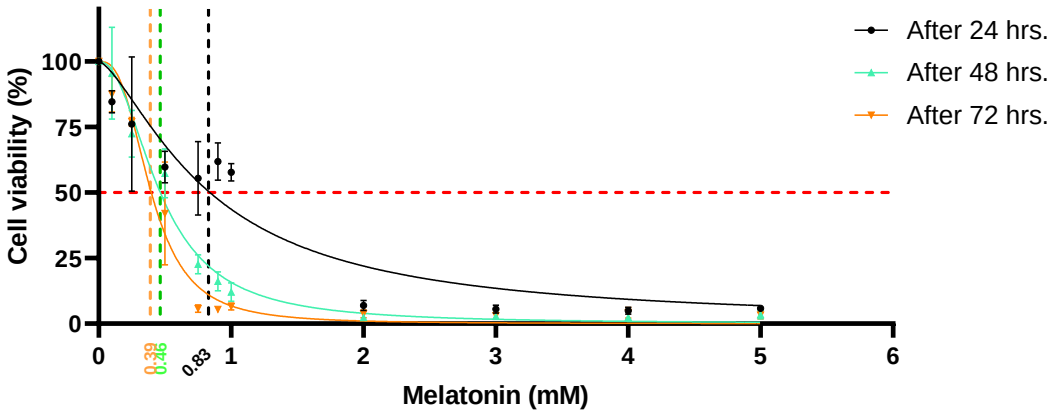


Figure 5.8. Comparison of the IC_{50} values of MEL administration on HT-22 neural cell line after different times 24, 48, and 72 hours.

5.1.1.3.The Effective Dose of Glutamate as a Toxic Substance

Table 4.2 and Figure 4.9 demonstrate no significant effects at low concentrations of 1 mM and below. In comparison, there are significant effects on the cell viability at 2 mM ($p = 0.030$). The cell viability was highly significant at 3 mM ($p = 0.0001$) or greater concentrations. The Glu IC_{50} = 2.465 mM during 24 hours, as shown in Figure 4.10. The values were given as the mean ± standard deviation, compared with positive control untreated for 24 hours treatment of Glu.

Table 5.2. Shows the mean numbers of the cell viability and SD of Glu toxicity in different concentrations for 24 hours.

Different Doses Group (mM)	Mean (%)	SD	p-value
1	88.63	18.04	0.8456
2	66.72	20.14	0.0307
3	40.16	19.64	0.0001
4	9.681	1.546	0.0001
5	10.71	4.043	0.0001

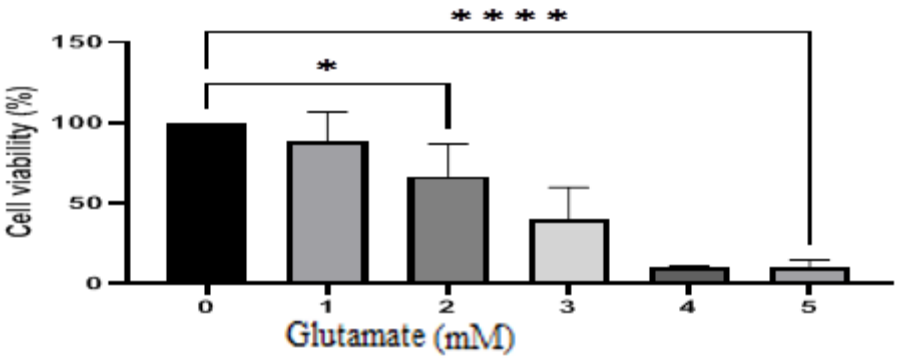


Figure 5.9. Glu induces neuronal cell death in HT-22 cells in a 24-hour and dose-dependent manner.

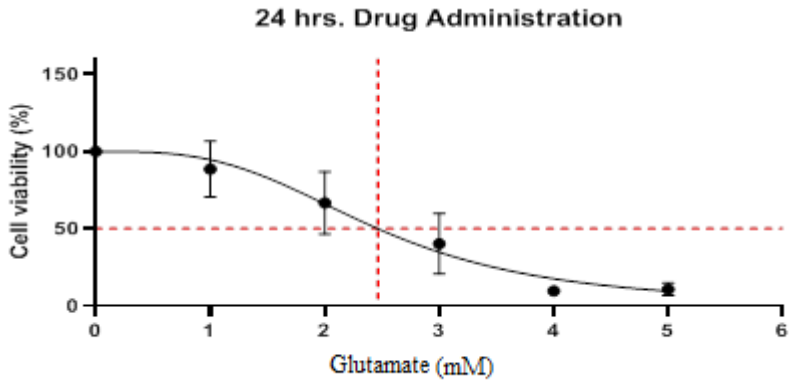


Figure 5.10. The IC_{50} value of Glu is 2.465 mM after 24 hours.

The cell death and loss of viability were examined using a Leica microscope at 10x magnification. Glu exhibited no substantial impact on the viability of the cells at 1 mM as compared to the untreated control group (Figure 4.11); at a dose of 2 mM, the cells started to lose viability during 24 hours (Figure 4.12). Glu at a dose of 3 mM increased cell death and decreased viability during 24 hours (Figure 4.13). Large amounts of cell death occur at 4 and 5 mM (Figure 4.14).

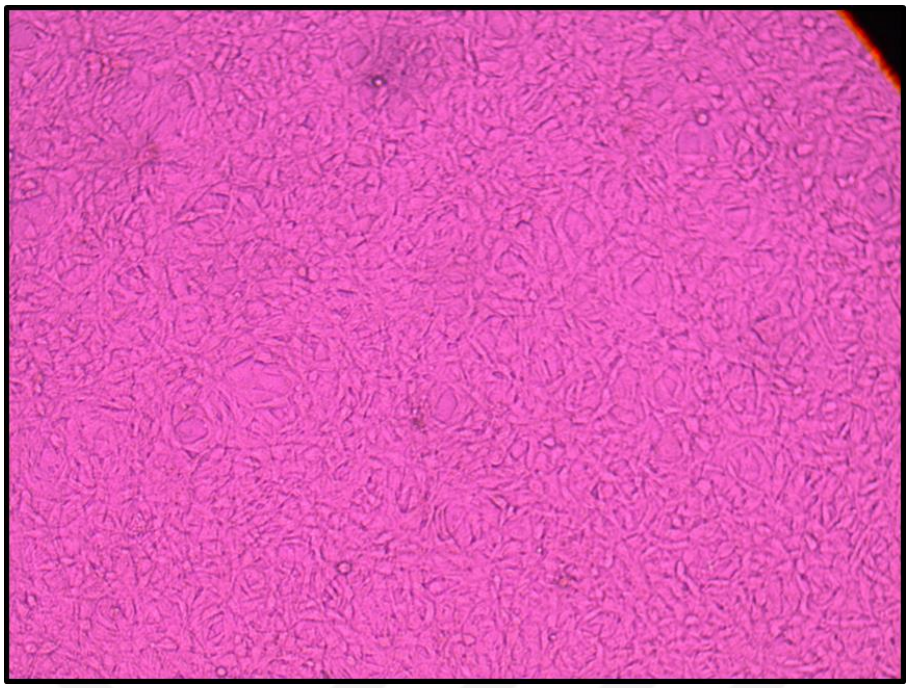


Figure 5.11. HT-22 hippocampal neural cell line in culture cell taken from the well-plate with Glu at a dose of 1 mM, and there are no significant effects during 24 hours.

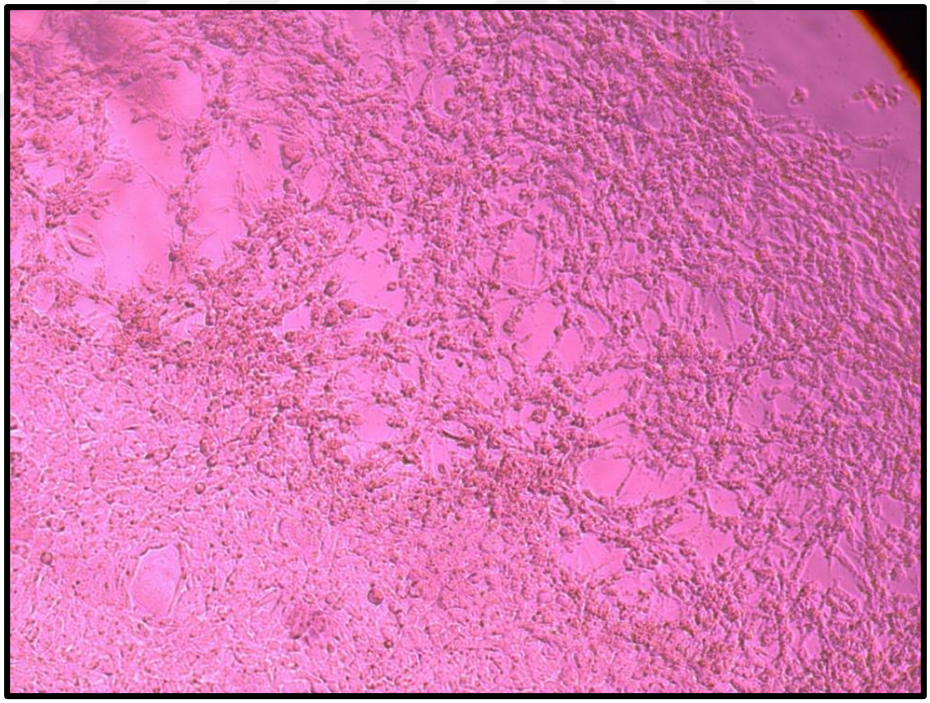


Figure 5.12. HT-22 hippocampal neural cell line in culture cells taken from the well-plate with Glu at dose 2 mM showed significant effects within 24 hours.

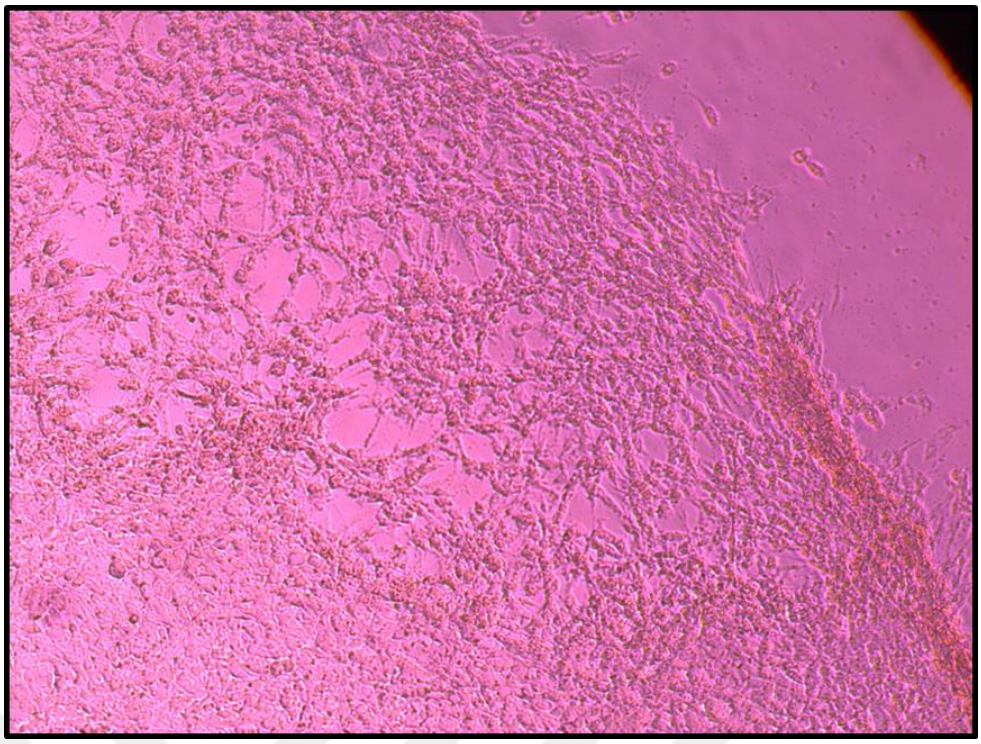


Figure 5.13. HT-22 hippocampal neural cell line in culture cell taken from the well-plate with Glu at dose 3 mM during 24 hours.

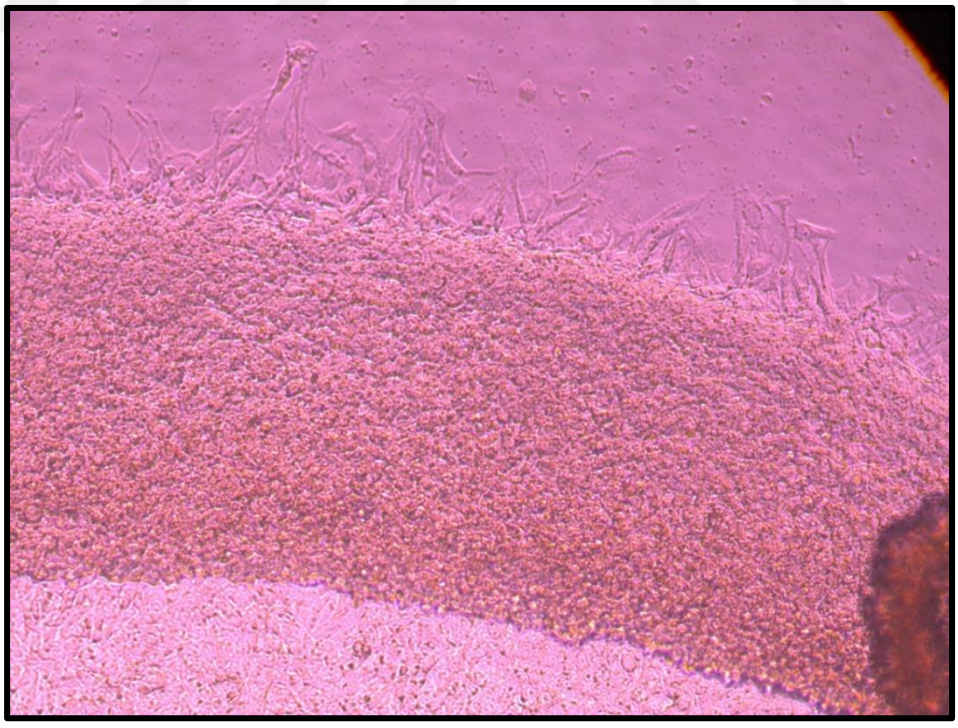


Figure 5.14. HT-22 hippocampal neural cell line in culture cell taken from the well-plate with Glu at dose 4 mM during 24 hours.

5.1.2. Melatonin + Glutamate

HT-22 was seeded in different concentrations in a 96-well plate with three replicates. After 24 hours, the complete media was aspirated, and cells were washed with PBS. The suitable toxicity dose and the effective protective dosage were applied, and the 96-well plate was incubated for another 24 hours.

The mean and SD are illustrated in Table 4.3 and (Figure 4.15). Induce of the HT-22 cells with 5 mM Glu for one hour completely reduced the cell number and changed the normal morphology of cells compared to control cells. Cell viability was significantly affected in the Mel-treated groups when administered at a concentration of 0.5 mM against Glu toxicity, with a p-value of 0.0112 compared to the control group. In comparison to the control group, Mel-treated groups receiving a dose of 1 mM against Glu toxicity revealed a significant difference in cell survival p-value = 0.0052. Compared to the control, the Mel at extremely low concentrations, as a dose of 0.25 mM, decreased cell number and cell death but did not significantly prevent the 5 mM Glu toxicity.

The findings also indicated that very high Mel concentrations at 2 Mm and higher doses could increase toxicity and cause cell death.

Table 5.3. Shows the mean number of cell viability and SD among the experimental groups in different concentrations for 24 hours.

	Cell viability (%)	SD	p-value
Cont	100	0	<0.0001
Glu 5 mM	12.19	6.143	<0.0001
Glu 5 mM + Mel 0.5 mM	35.84	2.360	<0.0015
Glu 5 mM + Mel 1 mM	38.07	10.44	<0.0009
Mel 0.5 mM	59.75	6.051	<0.0001
Mel 1 mM	57.75	3.296	<0.0007
DMSO 2.5 µL	36.65	8.719	<0.0001
DMSO 5 µL	14.44	9.875	<0.0001

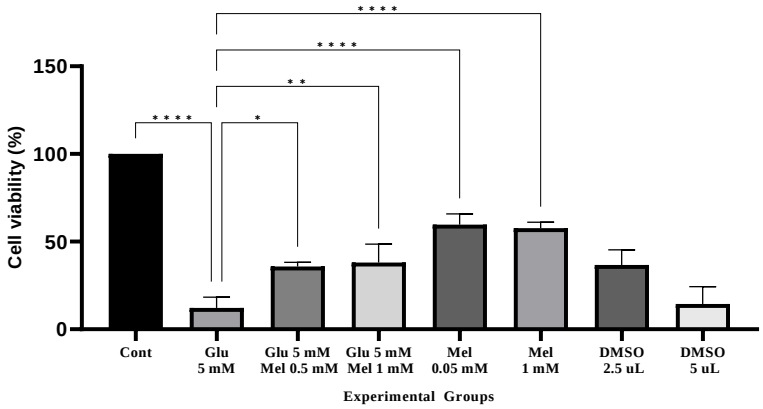


Figure 5.15. Administration of Mel at doses 0.5 and 1 mM treated groups against Glu toxicity showed a significant difference in cell viability compared to the Glu group, with the p-value = 0.0001.

For this study's experimental requirements, all images were captured using a 10x Leica Evos XL core inverted microscope. (Figure 4.16) illustrates the untreated control group, which contains media with the HT-22 cells in culture cell well-plate. In (Figure 4.17) and (Figure 4.18), the HT-22 cells were administered with 5 mM Glu; this resulted in an extensive decrease in the cell amount and modified shape. In (Figure 4.19), the HT-22 cells were treated with 5 mM Glu and Mel dose 0.25 mM. The results showed that a low dose of Mel could not inhibit Glu-induced cell death, morphological changes, and increased cell death. This was shown by the change in cell morphology in the group induced with 5 mM Glu in the presence of a concentration dose of Mel.0.25.

Cell viability was significantly different in the Mel 0.5 mM treated groups against Glu toxicity compared to the Control and toxic groups, as shown in (Figure 4.20) A dose of 2 Mm of Mel was given to the HT-22 cells, which substantially decreased the number of cells and changed the morphology of the cells and (Figure 4.21) illustrates that. Also, (Figure 4.22) shows the considerable difference in cell survival between the Mel 1 mM treated groups against Glu toxicity compared to the Control and toxic groups.

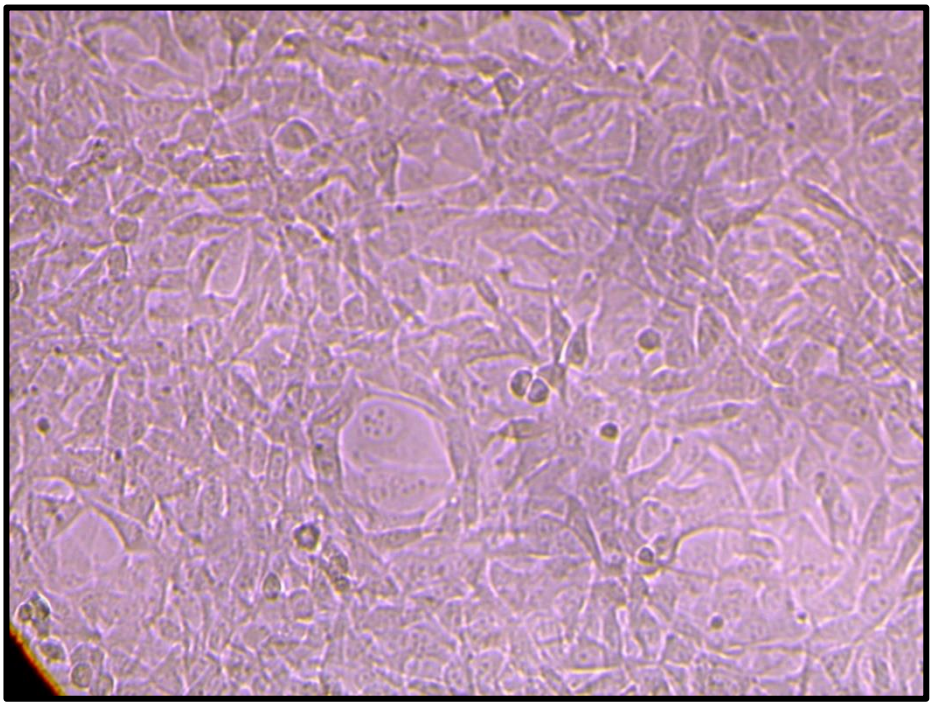


Figure 5.16. HT-22 hippocampal neuronal cell line in 96 well plates taken from the cont group.

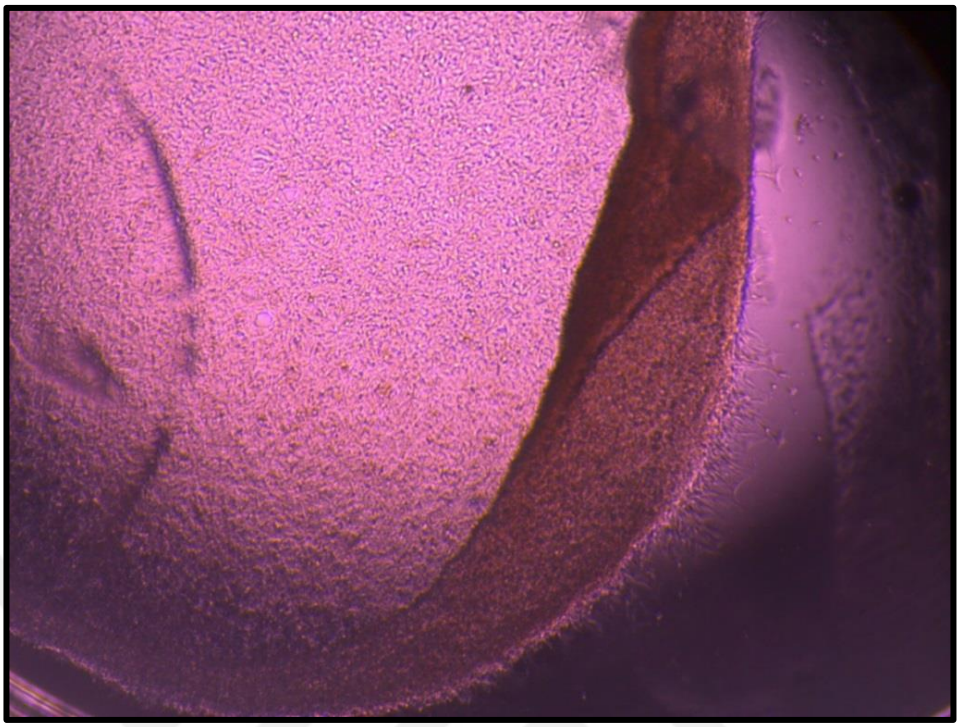


Figure 5.17. The HT-22 cell line was administered with 5 mM Glu.

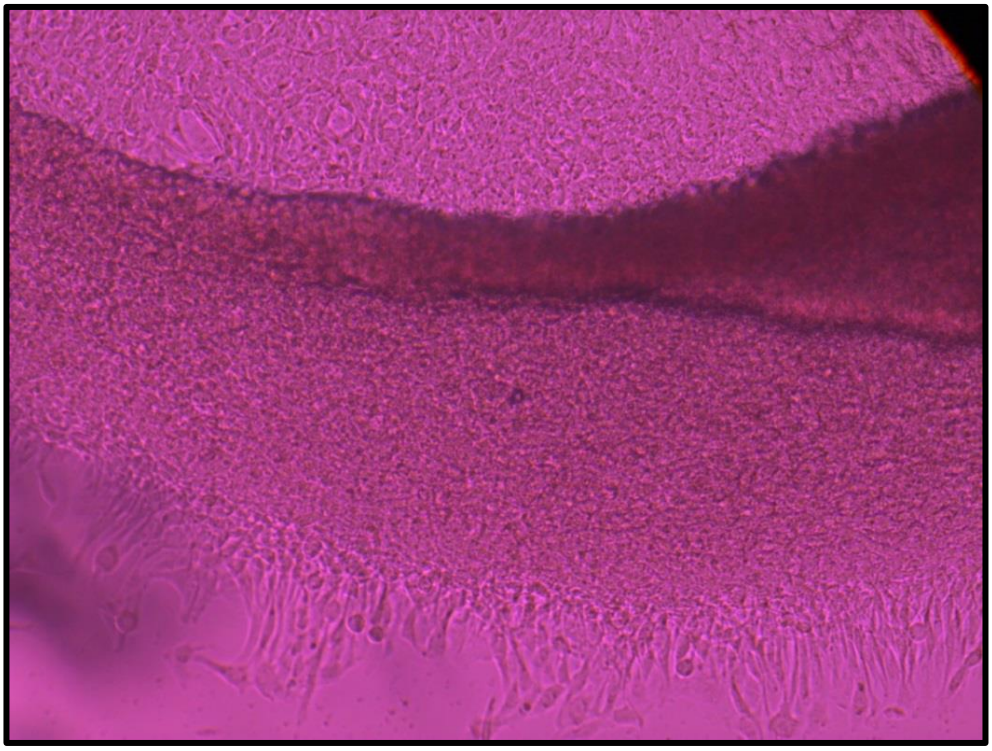


Figure 5.18. The HT-22 cell line was treated with 5 mM Glu.

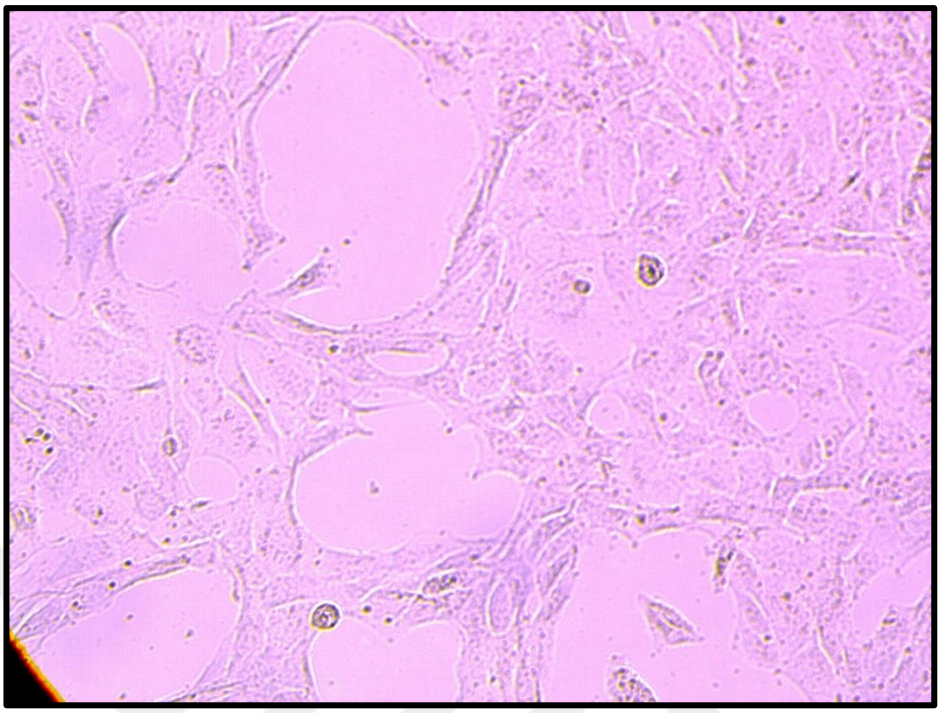


Figure 5.19. Glu in the presence of a low concentration dose of Mel.0.25.

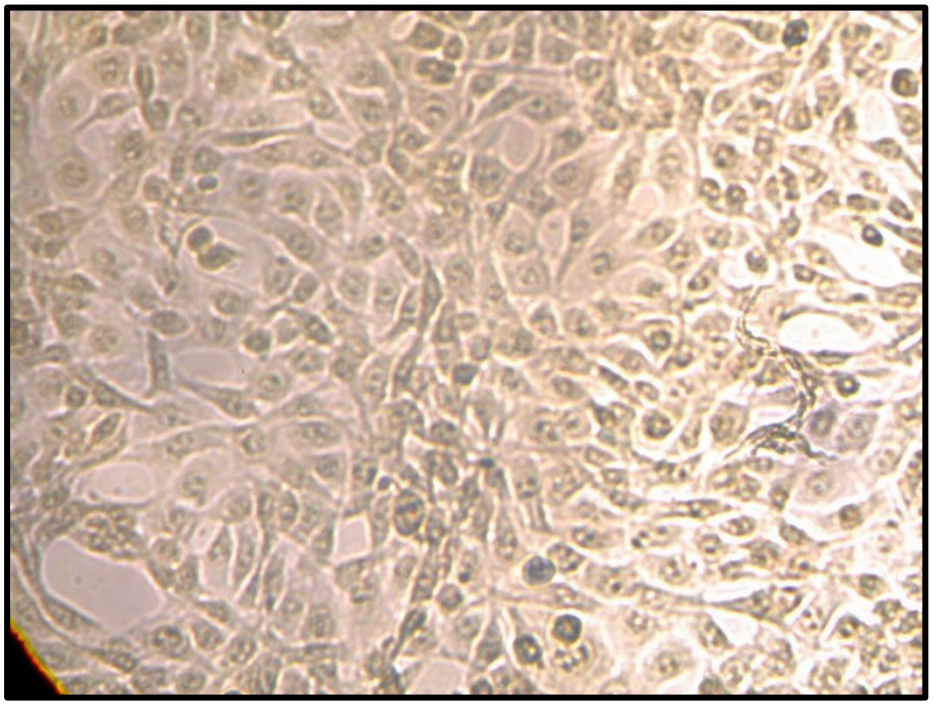


Figure 5.20. HT-22 hippocampal neural cell line in culture cell taken from the well-plate with Glu at dose 5 mM treated with Mel 0.5 mM.

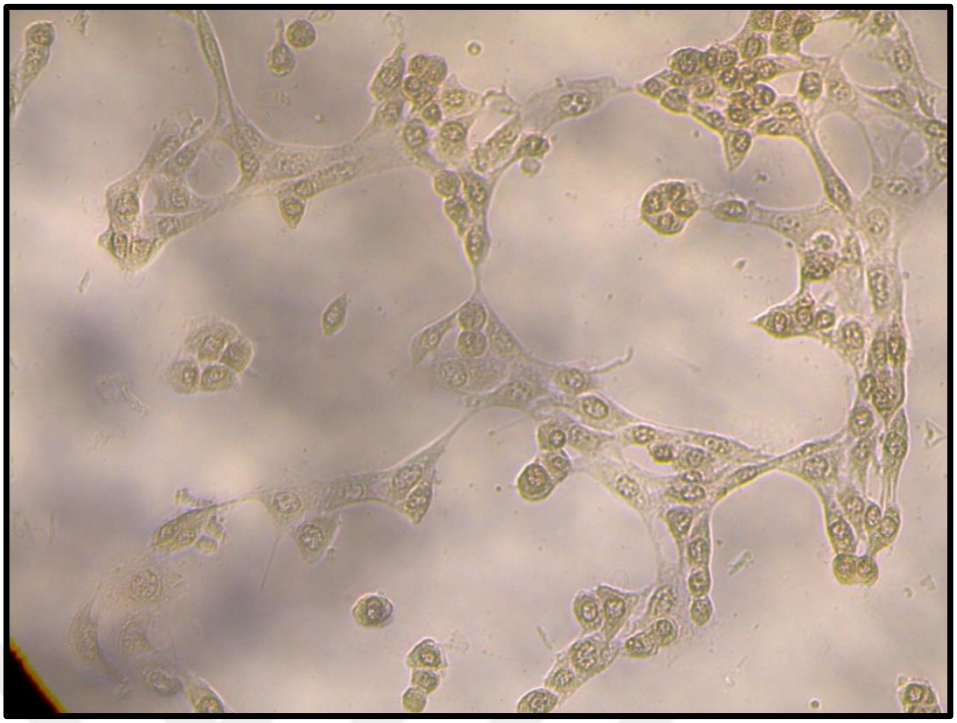


Figure 5.21. A higher dose of 2 mM of Mel was given to the HT-22 cells.

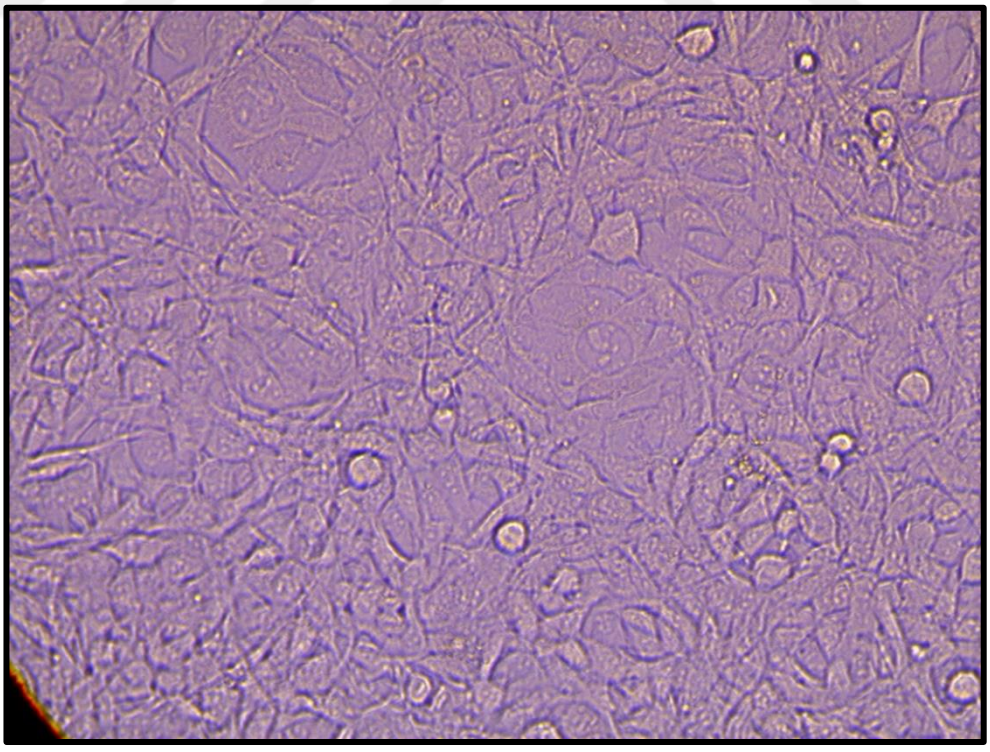


Figure 5.22. The HT-22 cells were administered with a 1 mM dose of Mel.

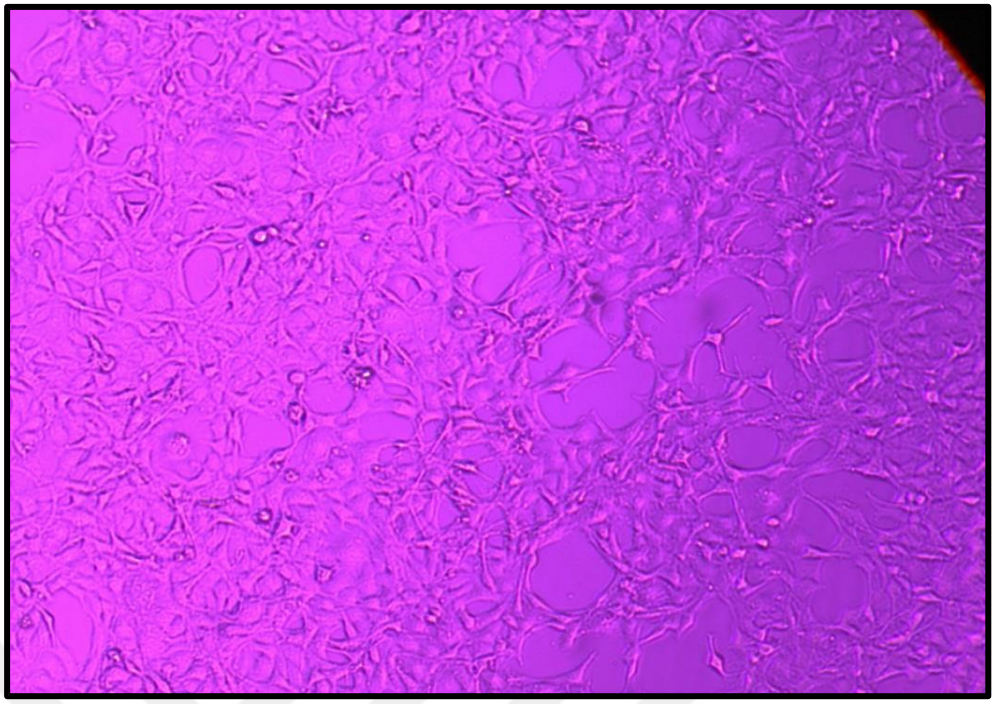


Figure 5.23. A 96-well plate containing the HT-22 hippocampus neuronal cell line with DMSO

6. DISCUSSION

Neurodegenerative disease is a neurological disorder in which the cells of the central nervous system cease working or die. Incurable and frequently getting worse over time, most neurodegenerative disorders. The origins of neurodegenerative conditions are not fully established. Still, high oxidative stress and an imbalance between the generation of free radicals and the antioxidant defense system have been linked to etiology (Shohag et al., 2022). A tumor or stroke may cause them, or they may be hereditary. People can get neurodegenerative disorders if they drink much alcohol or are exposed to certain viruses or poisons. PD, AD, Huntington's, and ALS are merely some of the illnesses affecting millions of individuals globally (X. Liu et al., 2022). Challenges with mobility and memory loss are among the major characteristics of these illnesses, which are brought on by increasing neuronal death and consequent behavioural and cognitive abnormalities and neurodegenerative disorders(Cariccio et al., 2019). Our research used HT-22 cell lines to recreate diseased circumstances, investigate neurodegenerative disease mechanisms, and discover possible therapeutic approaches. We used Glu, a toxic chemical for HT-22, to generate cellular alterations similar to neurodegenerative diseases. In addition, we determined the efficacy of the protective chemical Mel in moderating the negative effects of these toxins.

The HT-22 cell line is a valuable tool for studying the mechanisms of neuronal cell death in neurodegenerative diseases such as AD and PD. Its susceptibility to glutamate-induced excitotoxicity allows researchers to investigate the complex mechanisms of neuronal cell death and potentially identify new therapeutic targets. Researchers have used the HT-22 cell line to study the neuroprotective effects of various compounds, such as curcumin and resveratrol (Vishwas et al., 2022), in reducing glutamate-induced excitotoxicity. The HT-22 cell line has also been used to investigate the role of oxidative stress and mitochondrial dysfunction in neurodegeneration (Sabogal-Guáqueta et al., 2019). These cells have been utilized to analyse several aspects of neuronal function and serve as an *in vitro* model for studying the brain's neuronal cells (Liu et al., 2018). Due to the lack of ionotropic Glu receptors and a cell death program, HT-22 cells are particularly vulnerable to glutamate-induced oxidative damage (Kim et al., 2017).

Our assessment methods will involve measuring cell viability using the MTT

assay. Many studies have demonstrated that the MTT assay, which evaluates cytotoxicity and cell proliferation based on the efficacy of living cells to decrease MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), is a technique that is commonly employed to establish cell viability. For this method to operate properly, MTT salt is reduced to formazan dye, which is inversely correlated to mitochondrial activity and cell viability (Nur Nadia, 2022). Studies in toxicology and pharmacology depend on *in vitro* cytotoxicity and cell viability procedures because they assess how substances affect cellular health (N. Martin et al., 2022). To measure changes in metabolic activity and cell viability in response to a range of chemical stimuli or medications, the MTT examination has been utilized widely (Aslantürk, 2018). Our current study examined how Mel protects HT-22 cells against glutamate-induced cytotoxicity.

6.1.Glutamate as a Toxic Substance

Nowadays, several toxic compounds are used to induce neurodegeneration *in vitro,* to imitate the clinical circumstances, and to examine the mechanisms driving neuronal damage and cell death seen in neurodegenerative disorders. (Moujalled et al., 2021). Glu is often used to cause excitotoxicity, amplifying Glu receptors and resulting in neuronal injury. It is a basic excitatory neurotransmitter found in the brain (Fouad et al., 2018). Glu is the main excitatory neurotransmitter in the central nervous system, reaching 1–10 mM concentrations in the synaptic cleft and intraneuronal compartments (Khan et al., 2022). Disturbance of Glu levels is the primary cause of neuronal death in stroke, mechanical trauma, and seizure, and it is considered to play a role in some chronic neurodegenerative disorders (Khan et al., 2022). Many studies have demonstrated that excessive levels of Glu extracellular can serve as a toxic agent and accelerate neuronal death by generating oxidative stress in some types of primary and neural lines cell cultures, such as HT-22 hippocampal neural cell line (Tonsomboon et al., 2021). Another research revealed that Glu significantly enhances the production of ROS and promotes cell death in HT-22 hippocampal neural cell line by inhibiting cystine uptake via the cystine/glutamate transporter system, thus leading to glutathione depletion in the cell (Yan et al., 2021).

These potent substances provide valuable resources for investigators to accurately analyze the complicated factors involved in the cause and outcome of neurodegenerative disorders within a controlled laboratory setting. This allows researchers to conceptualize potential therapeutic strategies and deepen our

comprehension of these complex afflictions. In this investigation, we employed Glu as an experimental toxin known for its ability to provoke excitotoxicity and initiate oxidative stress within neurons. Subjecting the HT-22 cell line to this substance, we induced and examined the progressive degeneration of cell conditions in neurodegenerative diseases like PD and AD.

6.2.Melatonin as a Neuroprotection Substance

Mel is a neuroprotective agent and plays a vital role in therapeutic neurological disorders. Mel is a powerful hormone with medicinal properties that have been studied for decades, and it is known as a wide-spectrum antioxidant with the ability to scavenge free radicals (Juybari et al., 2020). Recent studies have confirmed the effectiveness of Mel in improving physical disorders such as temporomandibular joint, fibromyalgia, functional dyspeptic, and irritable bowel disorder (Shiha and Aziz, 2021). In addition, Mel protects against various diseases of the central nervous system, including ischemic brain injury, AD, and PD. It has been proven to correlate with memory and the ability to control bodily balance and posture (Gunata et al., 2020). The current study tested Mel for its neuroprotective effect against glutamate-induced neurotoxicity in mouse hippocampal HT-22 cells. The results showed that this compound has a neuroprotective effect against glutamate-induced neurotoxicity in HT-22 cells. Mel-containing substances also resist glutamate-induced neuronal cell death in the hippocampus neuronal cell line HT-22 in a concentration-dependent manner.

Through the process and the results of this research, we were able to determine how the protective substance Mel prevented the damaging effects of Glu on HT-22 cells. We provide compelling evidence that the properties of Mel contribute to its ability to protect HT-22 cells against Glu toxicity.

Mel has an inhibitory effect on cell proliferation, and its concentration levels have a significant role to play. That is supported by earlier research, which confirmed that Mel inhibited NOS activity with an IC_{50} value of roughly 0.1 mM. (Gonzalez-Candia et al., 2019). Additionally, Jinjing Wu demonstrated that Mel at the same doses of 1 mM inhibits many biological activities (Wu et al., 2021). According to studies, cells tended to lose viability at a dosage of 2 mM, and cell death accelerated further at higher doses of 5 mM; too many viable cells emerged when the Mel inhibitory concentration was IC_{50} = 0.8399 mM. To get the most affected protection doses of Mel, the HT-22 cell line was seeded and treated with different concentrations of the

protective Mel, and the cells were incubated with varying concentrations of Mel (0.1, 0.25, 0.5, 0.75, 0.9, 1,2,4,5 mM) for various times (24- 48 –7 2 h). Following cell exposure to these doses, the results of the MTT and ELISA 570 reader assays were immediately evaluated. Prism analysis was used to get the IC_{50} values for the drug. Subsequently, this study demonstrated that Mel's therapeutic potential could promote cell viability when the dose is less than 1 mM. Accordingly, Mel can be used as a protective substance for therapeutic neurodegeneration. The MTT assays were used to determine the affected concentration doses of Mel on HT-22 hippocampal neuron viability cells; the study's results clearly show that Mel's effect on cell viability correlates with concentration. It also showed decreased cell viability while using large doses of Mel. Since excessively high concentrations of Mel have been shown to have detrimental effects on cellular viability and survival. Our findings highlight the need to carefully establish the ideal dosage of Mel that may be utilized in research. Previous studies have demonstrated that increased extracellular Glu exposure might result in prolonged neuronal death, especially in sensitive regions like the hippocampus during epilepsy (Komoltsev et al., 2023). Glu is the most commonly found excitatory neurotransmitter in the central nervous system. Intraneuronal compartments and the synaptic cleft have Glu concentrations ranging from 1 to 10 mM (Iovino et al., 2020). Glu-level alterations are thought to contribute to various chronic neurodegenerative disorders. They are the main cause of neuronal death in stroke, mechanical trauma, and seizures (Ladak et al., 2019). A rapid removal of Glu from the synaptic cleft is essential to maintain low, non-toxic extracellular Glu concentrations and ensure the fidelity of synaptic transmission. When extracellular Glu concentrations reach toxic levels, the glutamate-induced excitotoxicity process occurs; this may prevent cells from taking cystine, which would diminish intracellular cysteine and cause loss of glutathione (Pavlů et al., 2022). In another context, using different concentrations of Glu as a toxic substance on HT-22 hippocampal cells leads to the degeneration of HT-22 hippocampal cells (Olasehinde et al., 2023). The concentration of a substance at which cell viability or growth is reduced by 50% is called the IC_{50} (half maximal inhibitory concentration). The study aimed to evaluate the IC_{50} value of different Glu concentrations as a toxic substance. To determine the affected protective dosages, HT-22 hippocampus neural line cells were seeded and cultured with various concentrations of Glu substance multiple times (24–48–72 h). Additionally, to establish an adequate toxicity dosage, this study implemented different doses of Glu as a neurotoxic agent for 24 hours. The MTT and ELISA reader test results were assessed immediately after

cell exposure to these concentrations. Furthermore, each drug's IC_{50} values were calculated.

7. CONCLUSION AND RECOMMENDATION

7.1.Conclusion

Upon thorough examination, the evidence presented in our study strongly suggests that Mel's antioxidant properties play a crucial role in its neuroprotective effect against Glu toxicity in HT-22 cells. Interestingly, this antioxidant effect specifically targets certain components within the cell.

It is worth noting that previous studies have explored Mel's effectiveness against glutamate-induced toxicity in the HT-22 hippocampal cell line; however, these studies primarily focused on mitochondria-depleted cells. In contrast, our present study aims to shed light on various aspects of cellular function, such as cell viability. This expanded approach allows for deeper insights into how Mel may beneficially affect different mechanisms within brain cells under oxidative stress conditions.

7.2.Recommendation

The preliminary results provided in this study strongly suggest that Mel's antioxidant capabilities play a critical role in its neuroprotective impact against Glu toxicity in HT-22 cells.

Investigations of the potential involvement of specific pathways in mediating Mel's neuroprotective effects may provide a novel target for future therapeutic interventions against neurodegenerative diseases.

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