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Theory, Methodology and Practice

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Assoc. Prof. Bülent Işık, MD.

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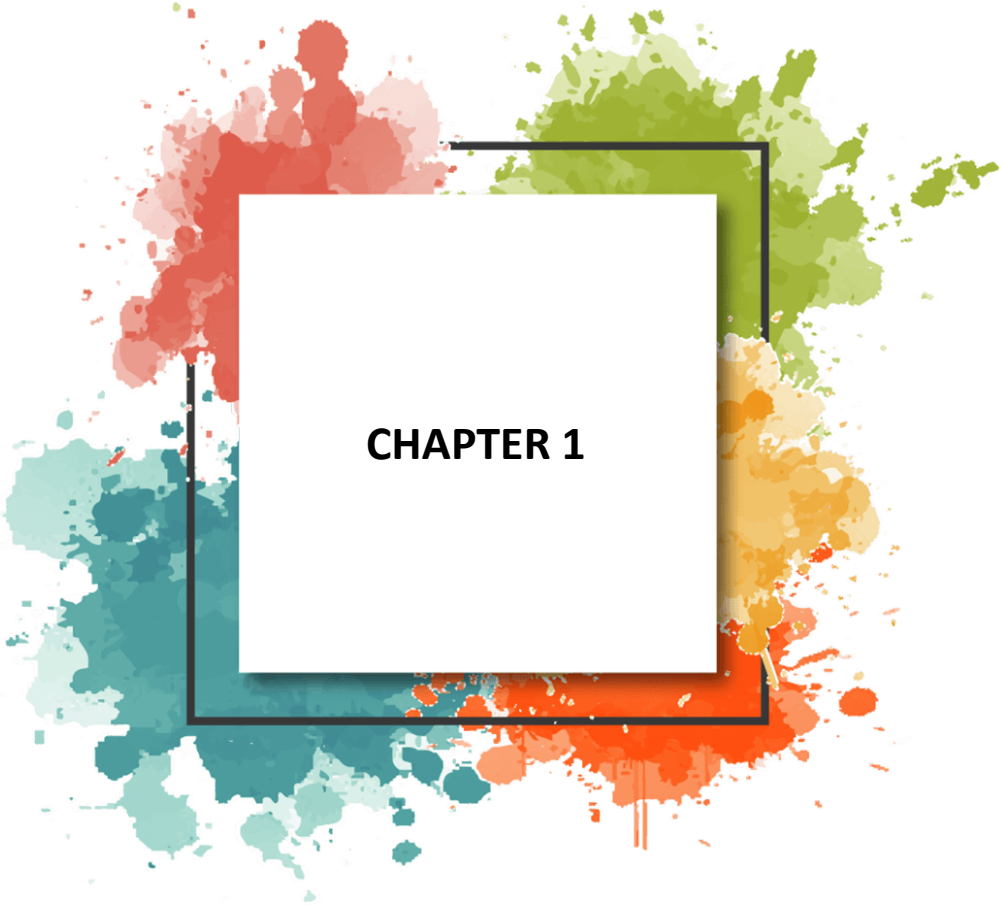
CHAPTER 1

Physiological Roles of Trace Elements in Cognitive Functions

Raviye Ozen Koca[1]

Introduction

Although trace elements are present in the body only in minute amounts, they are indispensable for a wide range of biological processes. Elements such as iron (Fe), zinc (Zn), copper (Cu), and selenium (Se) play particularly critical roles in the development and functioning of the nervous system. Numerous neural processes including enzymatic activity, neurotransmitter synthesis, antioxidant defense, and myelin formation are dependent on these micronutrients. Consequently, disturbances in trace element balance can impair cognitive performance, both during childhood and in adulthood. For example, iron or iodine deficiency in early life has been associated with persistent deficits in learning and intellectual capacity (Jáuregui-Lobera, 2014). Similarly, in adults, inadequate intake or metabolic dysregulation can lead to impairments in attention, memory, and executive functions.

Emerging evidence further suggests that disruption of trace element homeostasis contributes to the pathogenesis of neurodegenerative conditions such as Alzheimer's and Parkinson's disease. Importantly, not only deficiency but also excess poses risks: excessive accumulation may enhance the production of reactive oxygen species, which can damage neurons. In particular, abnormal deposition of Zn^{2+} and Cu^{2+} in the brain has been shown to promote pathological aggregation of amyloid and tau proteins (Kawahara et al., 2023). For these reasons, maintaining proper trace element balance is considered essential for healthy cognitive aging.

Iron (Fe)

Iron is essential for functions in the nervous system, ranging from oxygen transport to neurotransmitter synthesis. As a component of hemoglobin, iron ensures the delivery of oxygen to the brain, and adequate iron intake during early life is crucial for preventing anemia and supporting normal cognitive development (Ferreira et al., 2019). Iron deficiency anemia is strongly associated with diminished attention, memory, and learning performance in children and adolescents.

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At the cellular level, Fe^{2+} ions serve as cofactors in numerous neurochemical reactions and form essential components of mitochondrial cytochrome enzymes, thereby sustaining ATP production. This ensures that neurons meet their high energy demands, which are fundamental for cognitive processes. Conversely, iron deficiency may manifest as shortened attention span, reduced memory capacity, and impaired perception (Jáuregui-Lobera, 2014). In adults, iron-deficiency anemia leads to cerebral hypoxia and delayed nerve conduction, both of which compromise cognitive performance.

Due to its redox-active nature, excess iron readily participates in electron transfer, generating oxidative stress. Free Fe^{2+} catalyzes the formation of hydroxyl radicals from hydrogen peroxide, causing damage to neuronal membranes and DNA. Under normal conditions, ferritin binds and stores iron in a safe form, but elevated ferritin levels in the cerebrospinal fluid (CSF), observed in aging and certain pathological states, indicate excessive iron accumulation. Longitudinal studies have shown that individuals with higher CSF ferritin levels experience faster cognitive decline (Reichert et al., 2020; Ayton et al., 2015).

One of the principal pathways through which iron excess exerts toxicity is ferroptosis, a regulated form of cell death dependent on iron. It is initiated by lipid peroxidation in neuronal membranes, leading to mitochondrial dysfunction, membrane rupture, and ultimately neuronal death (Dixon & Stockwell, 2014). In memory-related structures such as the hippocampus, ferroptosis has been implicated in neuronal loss that contributes to deficits in learning and memory (Masaldan et al., 2019; Bao et al., 2021).

In summary, iron is vital for maintaining normal cognitive function, yet both deficiency and overload disrupt neuronal physiology and impair cognition. Preserving balanced iron metabolism is therefore a cornerstone of healthy cognitive performance.

Zinc (Zn^{2+})

Zinc serves as a cofactor for hundreds of enzymes and is involved in a wide variety of structural and functional processes in the nervous system. It plays a central role in brain development and in maintaining synaptic plasticity throughout adulthood (Nuttall & Oteiza, 2014). In particular, zinc is highly concentrated in presynaptic vesicles of glutamatergic neurons within the hippocampus. During synaptic activity, it is released into the synaptic cleft, where it modulates the activity of NMDA and AMPA receptors. Disruptions in zinc signaling have therefore been linked to impairments in learning and memory (Szewczyk, 2013).

When zinc is deficient, neurogenesis declines, neuronal apoptosis increases, and cognitive performance deteriorates. Animal studies have shown that

inadequate zinc intake impairs memory and learning, while mice lacking the ZnT3 transporter exhibit age-related memory loss (Adlard et al., 2010). Additionally, zinc forms part of the Cu/Zn-superoxide dismutase enzyme, working in conjunction with copper to neutralize free radicals. In the absence of sufficient zinc, oxidative stress increases, rendering neurons more vulnerable to damage (Nuttall & Oteiza, 2014).

Excess zinc can also be detrimental. Conditions such as stroke, traumatic brain injury, or seizures may trigger uncontrolled zinc release, causing postsynaptic neurotoxicity. To protect against both deficiency and overload, the brain relies on finely tuned homeostatic mechanisms (Szewczyk, 2013).

In conclusion, zinc supports synaptic transmission, antioxidant defense, and neuronal plasticity, making it indispensable for the maintaining of cognitive functions.

Copper (Cu)

Copper is an essential trace element involved in numerous biochemical pathways, including those that sustain nervous system function. Its roles extend from neurotransmitter synthesis to energy metabolism and antioxidant defense. A key example is dopamine-β-hydroxylase, the enzyme responsible for converting dopamine to norepinephrine, which requires copper as a cofactor; in its absence, this conversion cannot occur (Gale & Aizenman, 2024; Fan et al., 2024). Copper also supports the maturation of neuropeptides, further highlighting its contribution to neuronal signaling (Gaetke et al., 2014).

Within mitochondria, copper is an integral part of cytochrome c oxidase, a critical enzyme in the electron transport chain. By sustaining ATP production, copper enables neurons to meet the high energy demands of processes such as memory and attention. Moreover, as a constituent of Cu/Zn-superoxide dismutase, it plays a role in neutralizing superoxide radicals, thereby shielding neurons from oxidative stress (Scheiber et al., 2014).

Deficiency in copper is most clearly illustrated by genetic disorders. Menkes disease, characterized by impaired copper transport, results in severe developmental delay, seizures, and neurological dysfunction from infancy (Kaler, 2011). Conversely, Wilson's disease involves copper accumulation, particularly in the basal ganglia, leading to motor abnormalities, tremors, personality changes, and, in some patients, cognitive impairment (Ala et al., 2007; Członkowska et al., 2018). These conditions underscore the necessity of copper homeostasis for brain health.

Disturbances in copper balance have also been implicated in Alzheimer's disease. A meta-analysis reported that while serum and free copper levels are

elevated in patients, total copper levels in CSF and brain tissue may be reduced. This paradox suggests systemic accumulation alongside functional deficits within the brain (Squitti et al., 2021). Furthermore, copper binds to amyloid-β (Aβ) peptides, promoting their aggregation into toxic plaques and thereby accelerating disease progression (Kawahara et al., 2023).

In summary, copper is critical to enzymatic reactions, energy production, and antioxidant defense in the brain. Both deficiency and excess disrupt neuronal function and contribute to cognitive decline.

Selenium (Se)

Although required only in trace amounts, selenium is essential for nervous system function. Its biological effects are largely mediated through selenoproteins, which play a pivotal role in protecting neurons from oxidative stress. Among these, glutathione peroxidase (GPx) is particularly well studied; it reduces hydrogen peroxide and organic peroxides, thereby preventing the accumulation of reactive molecules that would otherwise damage neuronal structures. When selenium levels are insufficient, the activity of GPx and other selenoproteins declines, leaving neurons vulnerable to oxidative injury. In elderly populations, epidemiological studies have linked low serum selenium concentrations to poorer performance on cognitive assessments (Cardoso et al., 2014).

Selenium also contributes to thyroid hormone regulation as a cofactor of deiodinase enzymes, which convert thyroxine (T4) into the biologically active triiodothyronine (T3). Because thyroid hormones are crucial for brain development and for the regulation of neurological processes, selenium deficiency can impair cognitive functions indirectly through altered thyroid activity (Drutel, Archambeaud, & Caron, 2013; Bano et al., 2025). Furthermore, combined supplementation with antioxidant vitamins such as C and E, along with selenium, has been suggested as a potential strategy to slow age-related cognitive decline. Evidence from Alzheimer's disease patients indicates that selenium levels, as well as overall antioxidant capacity, are often reduced compared to healthy individuals (Socha et al., 2021).

Excess selenium is rare but may lead to a toxic condition known as selenosis. Reported symptoms in chronic exposure or high-dose supplementation include irritability, fatigue, hallucinations, and peripheral neuropathy; in some cases, cognitive impairments have also been described (Vinceti et al., 2013). However, the direct impact of selenium toxicity on cognition remains unclear. In addition, epidemiological findings suggest that very high selenium levels could increase the risk of type 2 diabetes, further underscoring the importance of maintaining balance (Casanova et al., 2023).

In summary, selenium makes a crucial contribution to brain health primarily by safeguarding neurons against oxidative stress. While deficiency weakens antioxidant defenses and accelerates age-related cognitive decline, excessive intake may pose its own toxic and metabolic risks.

Trace Elements in Neurodegenerative Disorders

Neurodegenerative diseases are progressive clinical syndromes that typically emerge in later life and are characterized by gradual neuronal loss. Conditions such as Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies, Huntington's disease, and prion disorders present with distinct clinical features but share several cellular mechanisms in common. One of these shared mechanisms is the disturbance of metal homeostasis in the brain. Imbalances in intracellular levels of essential metals such as iron, zinc, and copper are frequently observed and are thought to play a direct role in disease pathogenesis (Kawahara et al., 2023).

Excess iron and copper contribute to oxidative damage through the Fenton reaction, generating hydroxyl radicals. In Parkinson's disease, iron accumulation in the substantia nigra has been linked to accelerated degeneration of dopaminergic neurons. Similarly, in Alzheimer's disease, elevated iron levels in the hippocampus and cortex have been shown to enhance oxidative stress and neuronal death (Lee et al., 2010; Ashraf et al., 2020). These processes are closely tied to ferroptosis, a form of iron-dependent cell death, which may exacerbate neuronal loss in memory-related brain regions (Masaldan et al., 2019).

Another hallmark of neurodegenerative disorders is the abnormal accumulation of misfolded proteins. High levels of copper and zinc have been shown to facilitate the transition of Aβ from soluble oligomers into insoluble plaques. Likewise, iron and copper can induce oxidative cross-linking of Aβ, making these aggregates more resistant to clearance mechanisms (Kawahara et al., 2023; Bush & Tanzi, 2008). In Parkinson's disease, iron buildup further promotes α-synuclein aggregation, contributing to Lewy body formation (Masaldan et al., 2019).

It is not solely the excess of trace elements, but also their deficiency, that plays a role in driving pathological mechanisms. Lack of selenium diminishes the activity of antioxidant enzymes such as GPx, thereby exacerbating oxidative injury observed in Alzheimer's disease and other dementias (Cardoso et al., 2014). Similarly, insufficient zinc lowers metallothionein levels, weakening the brain's defense against heavy metal toxicity (Szewczyk, 2013).

Metal imbalance affects not only neurons but also glial cells such as microglia and astrocytes. Iron overload, for instance, can over-activate microglia and stimulate the release of pro-inflammatory cytokines including TNF-α and IL-6,

amplifying neuroinflammatory responses (Urrutia et al., 2013). Disturbances in zinc homeostasis destabilize synaptic microenvironments, which in turn can provoke secondary inflammatory cascades (Kawahara et al., 2023).

Recent discoveries highlight ferroptosis (iron-dependent cell death) and cuproptosis (copper-dependent cell death) as novel mechanisms through which trace elements directly regulate neuronal survival. Ferroptosis, in particular, has been identified in Alzheimer's and Parkinson's disease models, where its inhibition was shown to reduce neuronal loss and preserve cognitive function (Masaldan et al., 2019; Weiland et al., 2019).

Current research underscores the therapeutic potential of targeting metal homeostasis. Chelation strategies designed to bind and remove accumulated metals are under active clinical investigation. Supplementation with antioxidant elements such as selenium and zinc is also being explored as supportive therapy, especially in early cognitive decline. Moreover, dietary patterns rich in trace elements are associated with better cognitive outcomes and may offer a protective effect (Barnes et al., 2023).

Conclusion

Trace elements play an essential role in maintaining the healthy functioning of the nervous system. Among them, iron, zinc, copper, and selenium contribute to fundamental biological processes such as energy production, neurotransmitter synthesis, preservation of myelin integrity, and antioxidant defense. When the balance of these elements is disturbed, oxidative stress rises, inflammatory responses in the brain intensify, and the structural integrity of neurons can be compromised. These changes create a background that favors the development of neurodegenerative conditions.

In many disorders, particularly Alzheimer's disease, disruptions in metal balance constitute central pathological mechanisms. For example, iron accumulation is linked to ferroptotic cell death, copper and zinc promote the stabilization of amyloid-β aggregates, and selenium deficiency weakens antioxidant defenses. Imbalances of this kind, especially within the hippocampus and cortical areas, provide the biological foundation for cognitive decline observed with aging and disease.

From a practical standpoint, strategies that focus on the regulation of trace elements are increasingly seen as promising in both prevention and treatment. Chelation therapies that reduce excessive metal load, supplementation with protective elements such as selenium and zinc, and dietary models rich in essential micronutrients are all gaining attention. Together, these approaches suggest a future in which maintaining trace element balance may help slow the progression of neurodegenerative disorders and support cognitive health during the aging process.

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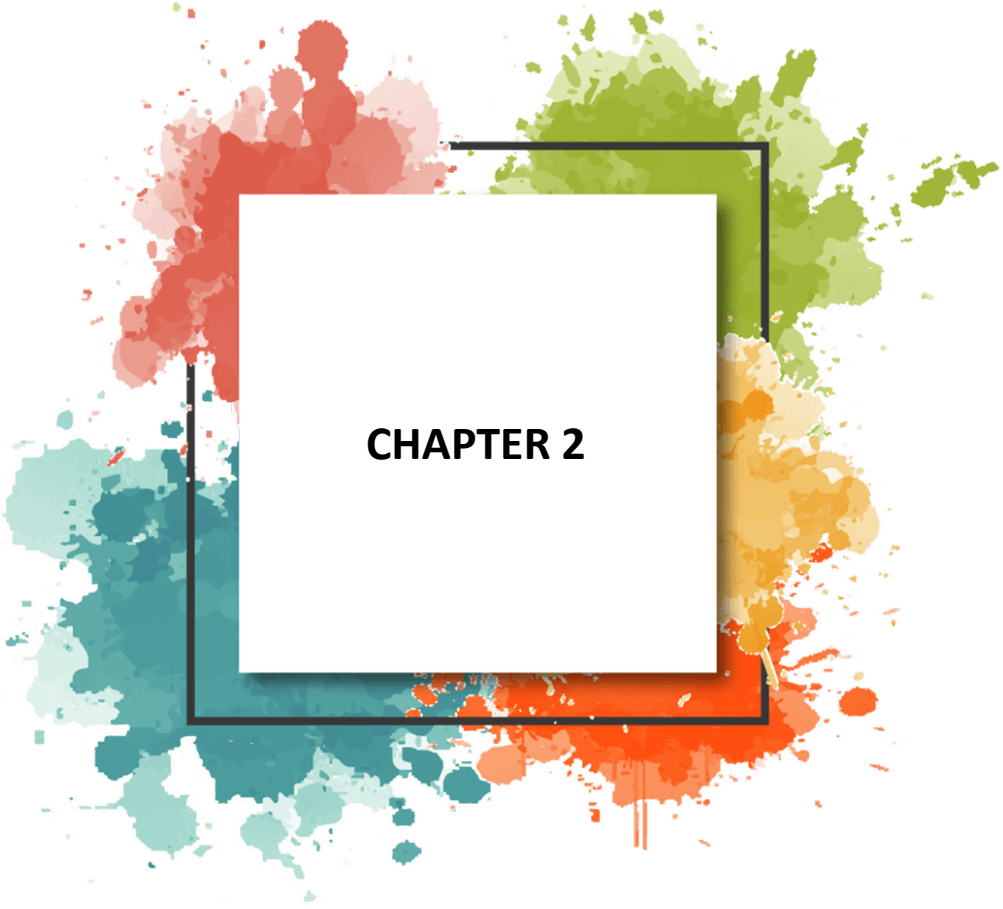
CHAPTER 2

Neuronal Plasticity

Ayşe Çiğel[1]

1. INTRODUCTION

The term *"plasticity"* derives from the Greek word *"plaistikos,"* meaning to shape or form, and refers to the central nervous system's ability to adapt to environmental changes. It encompasses the structural and functional changes exhibited by neurons in the brain and the synapses they form in response to stimuli from within and outside the body. Neuroplasticity, on the other hand, refers to the brain's ability to learn, remember, and forget. Adult hippocampal neurogenesis has been demonstrated in many species, including humans. The discovery in 1998 that the adult human brain can generate new brain cells has revolutionized our understanding of the human brain and led us to wonder about the extent of plasticity it exhibits throughout life. The number of progenitor cells varies across species. However, experiments have focused on rats, mice, and primates, whose cell behavior is similar to humans. Studies on human subjects have focused on stem cell behavior in the degenerative effects encountered in depression, epilepsy, Alzheimer's disease, Huntington's disease, and Parkinson's disease (Katz & Dwyer, 2021).

The hippocampus is a part of the limbic system and is the central hub for many functions, such as emotion, movement, and memory. The identification of these newly formed cells in the hippocampus has been greatly accelerated by the discovery of markers such as BrdU *([3H] thymidine and 5-bromo-20-deoxyuridine)*. The hippocampus continuously produces new neurons for cognitive functions such as learning and memory. A study in rats reported that 9.000 new cells form in the hippocampus daily, and approximately 3.3% of these cells develop into neurons in one month. The subgranular zone *(SGZ)* and subventricular zone *(SVZ)* of the hippocampus are the most active areas for neural stem cell formation. Furthermore, it is known that new neurons are actively generated in the amygdala, frontal cortex, and olfactory bulb, and that their plasticity is higher than in other regions (Ji, Guo, & Zhang, 2023).

Dendrites are the structures of neurons most susceptible to change. Therefore, changes in their structure are thought to reflect neuroplasticity. Changes in dendrite structure are associated with increased synaptic communication. The

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increase in synaptic areas is manifested by an increase in environmental stimuli. Environmental stimuli trigger the release of neurotrophic factors such as NGF, BDNF, NGF, NT-3, and NT-4. Neural growth factor *(NGF)*, the first neurotrophic factor, was discovered in studies investigating the maintenance and growth of neurons (Cheng et al., 2024). These factors allow the cell to adapt to stimuli and also prevent atrophy by preventing apoptosis. Neural stem cells are another important player in neuroplasticity. After stem cells form, they undergo a series of changes, provided the appropriate environment and stimuli are present, to reach a mature neuronal structure (Yuan et al., 2023).

Cellular processes form first, followed by polarization. They establish afferent and efferent connections with other neurons. Spines form on dendrites. These steps occur at different times, depending on the species. During this period, surface receptors are also formed in maturing neurons. Glutamate is the neurotransmitter that participates in the formation of surface receptors and is known to function most effectively in plastic synapses. Glutamate is an amino acid that also participates in the structure of other proteins in our bodies (Demartini et al., 2023).

There are four types of glutamate receptors. Three of these, AMPA, NMDA, and kainate, are ionotropic receptors. The fourth type is a metabotropic receptor, called mGluR. Although all types of glutamate receptors respond to the same neurotransmitter, they perform very different functions. Ionotropic glutamate receptors use their own ion channels to generate excitatory postsynaptic potentials, while metabotropic glutamate receptors modify the nature and extent of this response in ways similar to neuromodulatory effects (Danielson et al., 2025). While all types are important for synaptic plasticity, AMPA and NMDA receptors are the most well-known and are often thought of as memory molecules. While all types are important for synaptic plasticity, AMPA and NMDA receptors are the most commonly thought of as memory molecules. Much of this knowledge has been gained through pioneering work developing new drugs that alter the activity of these receptors (Zimmerman et al., 2024).

2. FACTORS THAT DECREASE AND INCREASE NEUROPLASTICITY

In 1965, Altman and colleagues first demonstrated the presence of newly formed neurons in the adult rat hippocampus after birth (Altman & Das, 1965). Neurogenesis in adults is dynamically triggered by physiological stimuli. The most important factor that positively influences neuroplasticity is breaking away from familiar life or behaviors. Activities that have not been performed before or

breaking away from routine are among the most important factors that trigger neuronal restructuring. Neuroplasticity can be classified as developmental plasticity *(learning and memory)*, reactive plasticity *(after short-term exposure)*, adaptive and degenerative plasticity *(after long-term exposure)*, and reparative plasticity *(recovery of damaged neurons)*. Developmental plasticity has been the most emphasized type of plasticity in recent years. Van Praag, Kempermann, & Gage, 1999) emphasized that physical exercise increases cell proliferation in the SGZ, while a study published in 1997 demonstrated that a stimuli-rich environment is a key factor in maintaining the viability of new neurons (Parent et al., 1997).

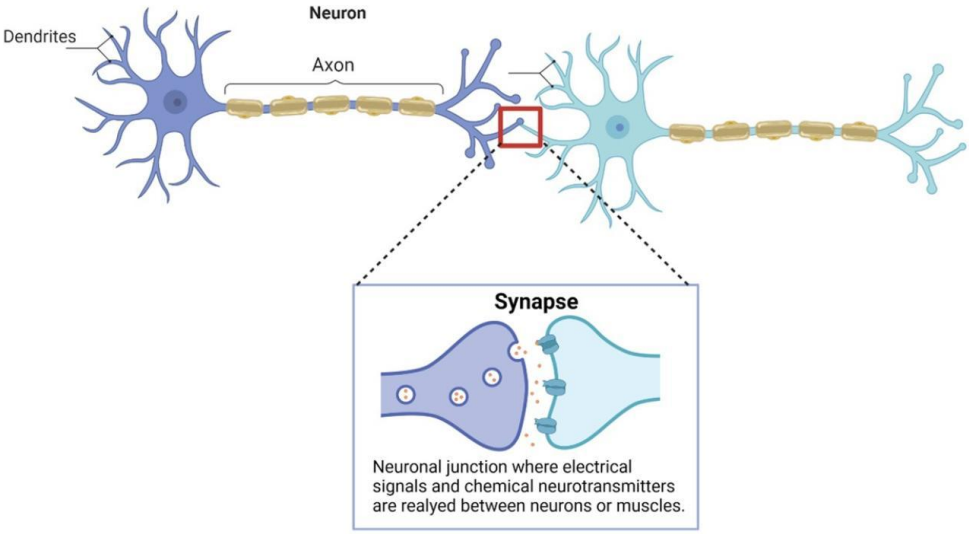

Figure 1: Structure of a neuron and synapses. Image created with Biorender.com.

Understanding the interactions between genes, development, and specific experiences is another important area of research in plasticity studies. The most significant influence of this interaction is the sensitive period, defined early in brain development. The sensitive period is the time during which experience exerts differential effects on behavior and the brain during development. Research on musicians has shown that those who began musical training at an earlier age perform better at work and experience positive changes in the motor and processing regions of the brain. However, these studies do not control for the musical experience of musicians who received early and later training. This view suggests that those who received early training can use auditory and visual rhythms simultaneously (Blanchette et al., 2025). This suggests that the sensitive period has a very strong influence on sensorimotor integration during brain

development. The effect of the sensitive period has also been examined in studies on movement learning in children. These studies define what is known about the sensitive period in animals and humans. For example, anatomical and psychological findings, studies on deafness, and structural and functional studies have been examined in musicians. The underlying mechanism of the sensitive period for music education is being debated. In this regard, the low-level influence of sensory experience and the high-level influence of the cognitive process have been identified as an important theory (Figueroa-Vargas et al., 2024).

While the variety of physical exercise experiences and the richness of stimuli in the hippocampus are effective in differentiating and maintaining neuronal viability, the most important factor negatively affecting neural stem cell formation and synaptic plasticity is stress. This is followed by aging and neurodegenerative diseases. However, stress is the most important factor causing decreased cell proliferation in the subgranular zone *(SGZ)* and subventricular zone *(SVZ)*, regardless of age (Jiang, Jang, & Zeng, 2023).

The hippocampus contains a high number of adrenal steroid receptors and is highly sensitive to the effects of stress hormones. Glucocorticoids, known to increase with stress, have been reported to cause extensive damage in the CA3 region of the hippocampus. Chronic stress has been shown to disrupt signaling pathways in the brain, affecting neuroplasticity and leading to neuronal atrophy and cell death in the hippocampus. Stress has also been observed to reduce hippocampal neurogenesis and cause deterioration in dendrite structure. Furthermore, stress can suppress the survival and proliferation of neurons in the hippocampus. These effects caused by stress are also evident in the aging process and neurodegenerative diseases. Studies in the literature using experimental learning models have highlighted the effect of increasing cell proliferation, which decreases during aging and in neurodegenerative diseases (Pushchina, Kapustyanov, & Kluka, 2024).

One striking example in this regard is the evaluation of patients with early-stage Alzheimer's disease. Alzheimer's disease is a degenerative disease characterized by declines in cognitive functions, visual and spatial memory. Histologically, neurofibrillary degradation and atrophy in the hippocampus and temporal lobes are observed. A study evaluating aging and neural plasticity used young and old macaque monkeys. Two behavioral tests were administered to groups of young *(16 years in humans)* and old *(70 years in humans)* monkeys to compare learning abilities and platysity abilities. In the first test, only one of 13 picture cards was assigned as a reward card. An apple was given as a reward for selecting the correct card. In the second test, administered immediately after the first, only one of the 13 picture cards was given as a reward, and participants

received an apple for selecting the other card. Forty trials were considered a block, and 15 blocks were repeated daily. The score for selecting the reward card was used, and Ki-67 and cell proliferation indices were assessed in sections taken from the hippocampus. Visual tests revealed that the older group learned more slowly than the younger group, and accordingly, fewer Ki-67-positive cells were present in the hippocampus. The striking finding here is that new neurons continue to form during aging (Aizawa et al., 2009).

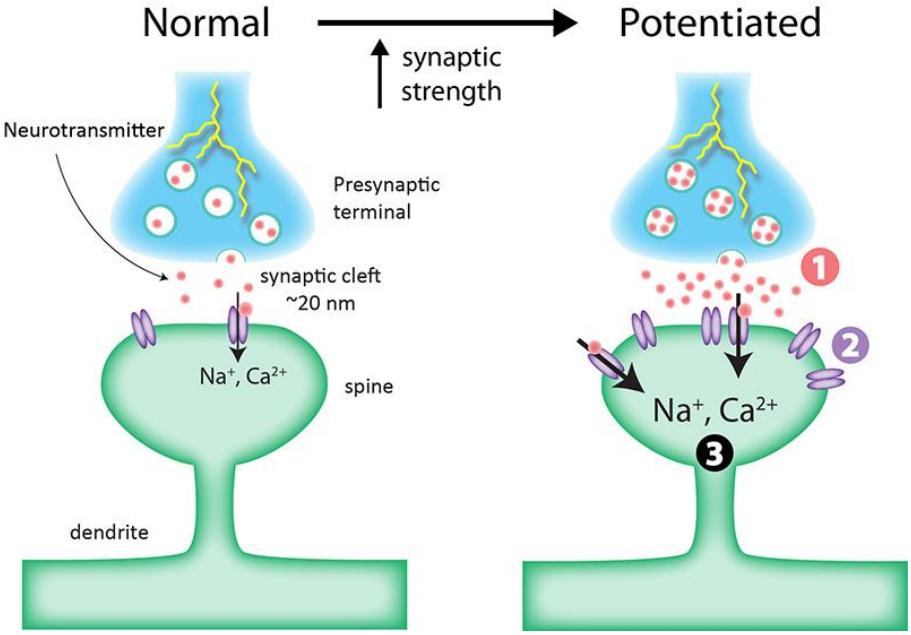

Figure 2: Synaptic plasticity can change either the amount of neurotransmitter released (1) or the number of postsynaptic receptors available (2), resulting in changes in synaptic strength. Image created with Biorender.com.

Neural plasticity is the structural and functional changes in neurons and synaptic connections caused by environmental stimuli. Through neural plasticity, the brain develops new neurons and increases the number of synaptic connections, enabling neurophysical and neurochemical adaptation to environmental changes and potential damage. It has been proposed that neural plasticity, which is effective in the acquisition of important functions such as learning and memory, occurs in two ways (Mouret et al., 2008). These are:

- An increase in neuronal structure and number.
- An increase in synapses between neurons.

Increased visual-spatial, visual-sensory, and motor skills at 1-2 years of age occur primarily through the growth of fibers in the corpus callosum and other connections between the cerebral hemispheres. In later ages *(between 2 and 12 years of age)*, plasticity in the speech centers is particularly notable (Caicedo-Acosta et al., 2021).

The formation of new synaptic connections is crucial for learning, where neural plasticity is highly effective. For this purpose, studies indicate that new information can be rehearsed 400 times a day to ensure its retention. Studies have also shown that repetition, along with diversification and modification, creates more synaptic connections (Bhattacharya et al., 2025).

The brain regions most affected by neural plasticity, which governs learning, recall, and forgetting, are the hippocampus, amygdala, olfactory bulb, and frontal cortex. Furthermore, the subgranular zone (SGZ) and subventricular zone (SVZ) within the hippocampus are regions where neural stem cells form and where neural plasticity is intense. Increases in hippocampal volume and neural plasticity are observed with any type of mental exercise. Furthermore, studies have found that the number of connections in the frontal cortex in early childhood is much greater than in adults (Nogueira et al., 2022).

3. EVOLUTION OF NEURONAL PLASTICITY

The vast majority of human neurons form by the end of the second trimester of prenatal life. Neuronal migration begins in the first weeks of pregnancy and is largely complete by birth. Synapse formation is quite rapid following birth until around age 6. Beginning at age 14, a gradual decrease in synapse number is observed. This decrease slows and continues throughout life. While the number of synapses decreases, the ability of neurons to regenerate or repair themselves and the formation of new neurons continue. While it was once believed that in addition to the decrease in synapse number, neurons also lose their ability to regenerate themselves, and that new neurons do not form, this idea has now been proven to be completely different (Kveim et al., 2024).

The central nervous system is capable of adapting to internal and external stimuli. This adaptation allows many important central functions to be carried out, or inadequate adaptation can lead to the emergence of certain diseases. Neuroplasticity can be briefly defined as changes in the structural properties and functions of neurons and the synapses they form in the brain, in response to various internal and external stimuli. If these changes are not limited to a single neuron but reach the synapse level, the resulting adaptive response can also be called *"synaptic plasticity."* The ability to change synaptic efficacy plays a role in the adaptation of the nervous system. Adaptation to environmental changes can only be achieved through *"learning."* Learning, in turn, occurs through synaptic

plasticity. Learning is the most powerful and important adaptive response of the central nervous system to internal and external stimuli. For learning to occur, long-term potentiation *(LTP)*, a long-term increase in synaptic transmission in neurons, must occur. While chronic and severe stressors, such as depression, cause negative neuroadaptive changes, short-term, moderate levels of stress are necessary for LTP, which forms the basis for learning. Neuroplasticity can lead to physical changes in a specific part of a neuron, such as its dendrites, or in the entire neuron.

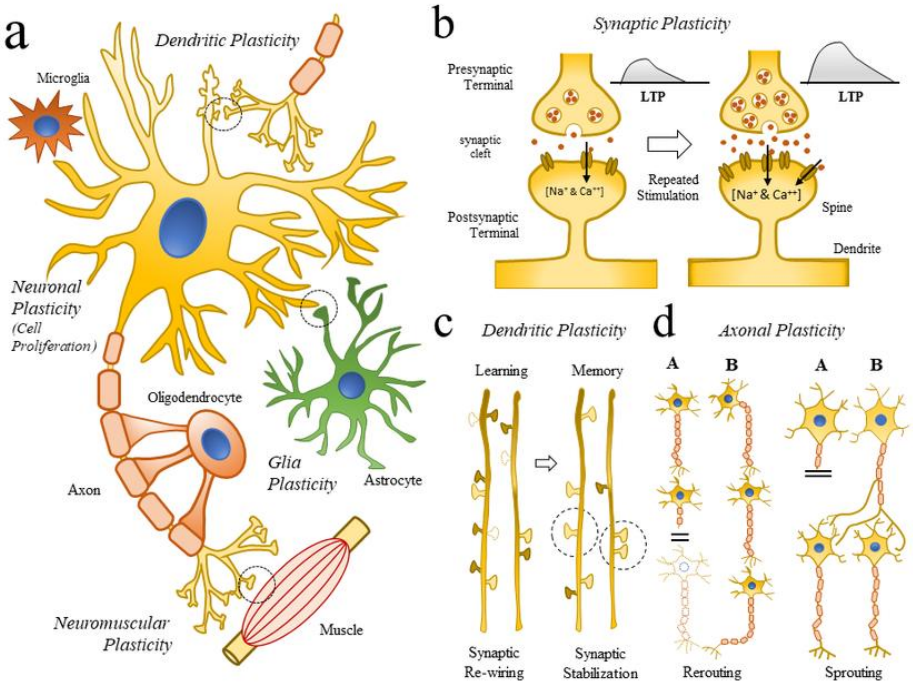

Figure 3: Changes in cellular structures related to neuroplasticity. (2a) Schematic representation of key cellular elements (neuronal and glial cells) involved in the neuroplasticity (NP) process (Neuronal and Glial Plasticity) as well as subcellular compartments (Synaptic, Dendritic, Axonal, Neuromuscular Plasticity). (2b) Schematic diagram representing how repetitive synaptic stimulations repetitive LTPs are linked to molecular changes (Doted circle in (Fig. 2a), and the generation of dendrite and spine remodeling. (2c) Schematic illustration showing some of the changes in dendrite formations (dotted dendrite spines) following learning (synaptic re-wiring) and memory formation (synaptic stabilization). (2d) Schematic illustration showing axonal mechanism of neuroplasticity and repair (rerouting and sprouting) following brain injury. Abbreviations: LTP, long-term potential. Image created with Biorender.com.

Additionally, new neuron formation, changes in the resistance of neurons to adverse factors such as chronic severe stress, and increases or decreases in synaptic efficacy can occur. Changes associated with neuroplastic responses in the central nervous system can be listed as follows (Pan et al., 2023):

- Decreased or increased branching in dendrites
- Dendrite breakage
- Lengthening of dendrites
- Formation of new synapses or disappearance of existing synapses
- Changes in the effectiveness of existing synapses (increase or decrease)
- Formation of new neurons (Neurogenesis) g. Neuronal death (Apoptosis)
- Changes in essential brain metabolites
- Changes in the survival time of existing neurons
- Increased resistance of existing neurons to deterioration under stress
- Changes in the postsynaptic potentials of existing neurons in response to stimulation
- Changes in the effectiveness of neurotrophic factors.

One, several, or all of these changes may occur depending on the intensity and duration of the incoming stimulus, as well as the characteristics of the region of the central nervous system that will primarily respond. The nature of the resulting neuroplasticity and the resulting remodeling also depend on these factors. The hippocampus is one of the brain regions with the highest neuroplasticity. While any form of mental exercise increases hippocampal volume and neurogenesis, persistent stress causes a decrease in these processes (Dellaferrera et al., 2022).

NEUROTROPHIC FACTORS

Neurotrophic factors are molecules that are crucial for the development and protection of neurons. They not only enhance cell survival by providing the trophic support required for growth, but also exhibit inhibitory effects on cell death cycles. They perform these functions by binding to cell membrane receptors and regulating intracellular signaling cycles.[12] The first neurotrophic factor, neuron growth factor (NGF), was discovered by Levi-Montalcini, Hambuger, and Cohen in their studies investigating the maintenance of neuronal morphology and growth. NGF is secreted by the target organ (neuron) and, after binding to receptors on the axon terminals, is transported backwards via retrograde

transport. While this system differs from other endocrinological systems in that the target organ is also the secretory organ, it is more similar to cells involved in the body's defense system. In other words, neurons require the neurotrophic factors they secrete for survival, differentiation, and neuroplasticity (Costa et al., 2022).

The best-defined group of neurotrophic factors is the neurotrophins, consisting of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4). Neurotrophic factors act through two different receptors: the tyrosine kinase receptor (Trk), to which they show high binding, and the pan-neurotrophic receptor p75, to which they show lower binding. P75 forms a complex with Trk receptors to modulate signal transduction. NGF binds to the Trk A receptor, BDNF and NT-4 bind to the Trk B receptor, and NT-3 binds to the Trk C receptor. After neurotrophic factors bind to their receptors, they are taken into the cell together with their receptors (Gao et al., 2022).

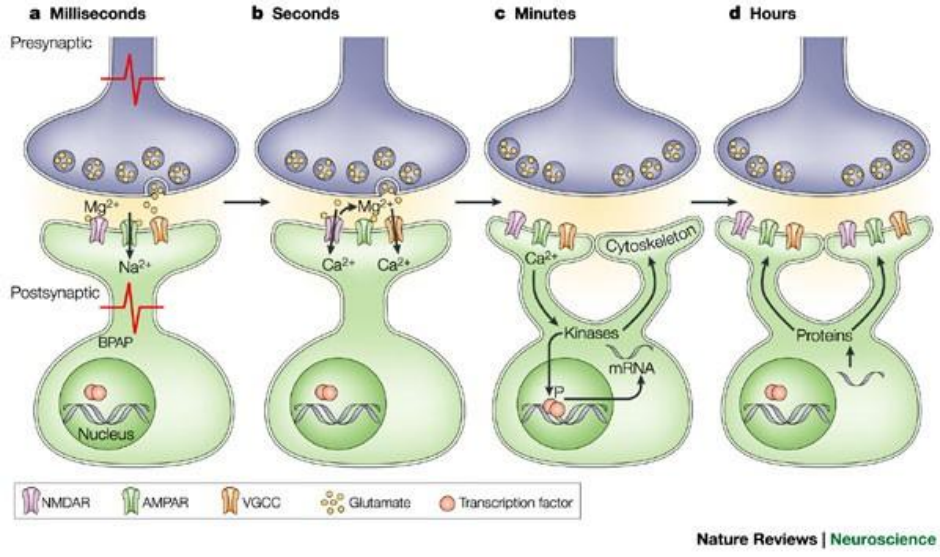

Figure 4: A model in which glutamate transmission and adhesion molecules regulate neuronal morphogenesis initiated by stimulation that leads to LTP and long-term memory. These structural changes are mediated and stabilized by the Rho GTPases and the actin cytoskeleton. Alterations in synaptic morphology and stabilization of these changes are hypothesized to be involved in memory consolidation and persistence. Image created with Biorender.com.

Currently, most neurotrophic factors are believed to exert their effects by affecting the following pathways: the mitogen-activated protein kinase (MAPK) cycle, the phosphatidylinositol-3 kinase (PI3K)-Akt cycle, and the phospholipase C cycle. Activation of the MAPK cycle inhibits apoptosis by inducing phosphorylation of Bad, a key proapoptotic protein, and increasing expression of Bcl-2, a major antiapoptotic protein. CAMP response element-binding protein (CREB) plays a key role in this effect. CREB is a protein that enhances the positive effect of cAMP on gene transcription. CREB acts as a transcription factor and accelerates the genetic transcription of certain proteins. Increased gene transcription increases the production of neurotrophins or related proteins necessary for neuroplasticity. It has been observed that CREB levels increase in the hippocampus with long-term antidepressant treatment, and the time required for CREB induction is equivalent to the 10–21 days required for antidepressant effects to emerge. The effects of CREB and BDNF in the hippocampus are observed in the same group of neurons. It has been reported that decreased CREB function may contribute to BDNF deficiency. Antidepressants have been found to activate CREB and BDNF in the hippocampus, which protects it from stressful stimuli such as high glucocorticoid levels (Zhang et al., 2021).

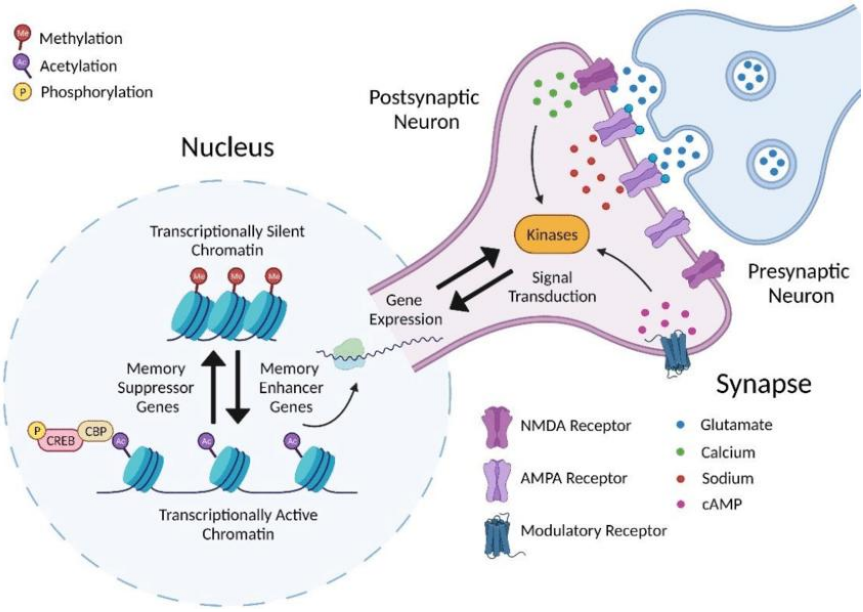

Figure 5: Molecular mechanisms of synaptic plasticity bridge the synapse and nucleus. A series of molecular events spanning the nucleus and synapse result in plasticity at glutamatergic synapses. Neuronal stimulation leads to a postsynaptic influx of Ca 2+ and cAMP, which stimulate kinases that signal to the nucleus. Chromatin remodeling in the nucleus alters patterns in gene expression, which in turn affects synaptic function.

4. CLASSIFICATION OF NEURONAL PLASTICITY

4.1. Developmental Plasticity

Refers to the changes that occur in neural connections as a result of environmental interactions during development, as well as the neural changes induced during the learning process. While initially genetically controlled, environmental stimuli also become influential in later stages. It includes cell proliferation, migration, synapse formation, pruning, and myelination (Yousef & Li, 2025).

4.2. Reactive Plasticity

It occurs through increased neurotransmitter release with repetitive neuronal activity. While increased practice and repetition increase this process, depression and anxiety have been reported to decrease this process (Yousef & Li, 2025).

4.3. Degenerative Plasticity

It occurs through changes at the genetic level in postsynaptic applications with long-term practice. It results in the activation of transcription factors and the genetic differentiation of new growth factors, ion channels, structural molecules, and other proteins necessary to alter neuronal organization (Yousef & Li, 2025).

4.4. Repair Plasticity

This type of neural plasticity allows for the sequential degeneration, regeneration, reorganization, and ultimately, recovery of function in damaged brain tissue (Yousef & Li, 2025).

5. CONCLUSION

Until the 20th century, nerve cells were thought to lack the ability to regenerate. However, Santiago y Cajal, who also defined nerve cells, identified the neuron as the fundamental unit of the nervous system and coined the term neuronal plasticity (Santiago Ramón y Cajal, 2012). Neuronal plasticity is the nervous system's ability to adapt to damage. In other words, it refers to the nervous system's ability to adapt. Thus, the brain reorganizes itself and establishes new connections. Humans are born with almost all of their neurons. However, a sufficient number of synapses have not yet formed between these neurons. Perceptual functions, speech skills, and other learned behaviors increase at birth. Synaptic connections increase in the brain from the earliest years of life. The more frequently synaptic connections in the brain are used, the stronger they become. These synaptic connections disappear when not used. When a neuron is damaged, regeneration and neuroplasticity begin at and around the lesion site. In

neurons and glial cells, axon and dendrite regeneration initiates synaptic restructuring. Adaptive plasticity is defined as the plasticity exhibited by synaptic activity at the border of an ischemic lesion, as evidenced by increased synaptic protein expression and related growth factors, enabling adaptation to epilepsy and repair. Adaptive plasticity has been demonstrated in MRI spectroscopy with the N-Acetyl Aspartate *(NAA)* peak and in motor mapping studies using transcranial magnetic stimulation. Positron Emission Tomography also provides information about plasticity through increased activity. Repetitive rehabilitation movements and activation repetitions have been shown to be effective in managing neuroanatomical recovery. Rehabilitation is a retraining technique that facilitates the plasticity process by performing inhibitory tasks alongside positive reinforcement.

Despite its positive properties, plasticity may not always yield the desired results. A disrupted neuronal environment can result from excessive plasticity. For example, abnormal muscle activity, neuropathic pain, phantom pain mechanisms, and tinnitus are conditions that manifest as maladaptive plasticity. Persistent cortical overstimulation creates maladaptive plasticity. Damage to the motor cortex creates excessive dendrite growth and growth in the contralateral hemisphere, increasing excitability. Increased excitability creates maladaptive plasticity, which in turn leads to undesirable outcomes. Minimizing the use of the healthy side and strengthening the affected side are among the rehabilitation methods.

Today, it has been proven that neurons can repair and regenerate themselves and possess the capacity for plasticity. Neuroplasticity results from various complex processes that occur in our brains throughout life. Neuroplasticity can be used in the rehabilitation of injuries or diseases that cause loss of mental function. While neuroscience research currently recognizes that the brain retains its plasticity in advanced age and across various diseases, one of the most pressing questions remains how to identify and maintain the long-term viability of neural stem cells in vivo in experimental studies. Finding answers to these questions will enable understanding the functions of neural stem cells in pathological and psychological settings, enabling the development of therapies that remain inadequate for human neurodegenerative diseases, and will help minimize the effects of Alzheimer's disease, Huntington's disease, Parkinson's disease, multiple sclerosis, stroke, brain tumors, and many other degenerative diseases.

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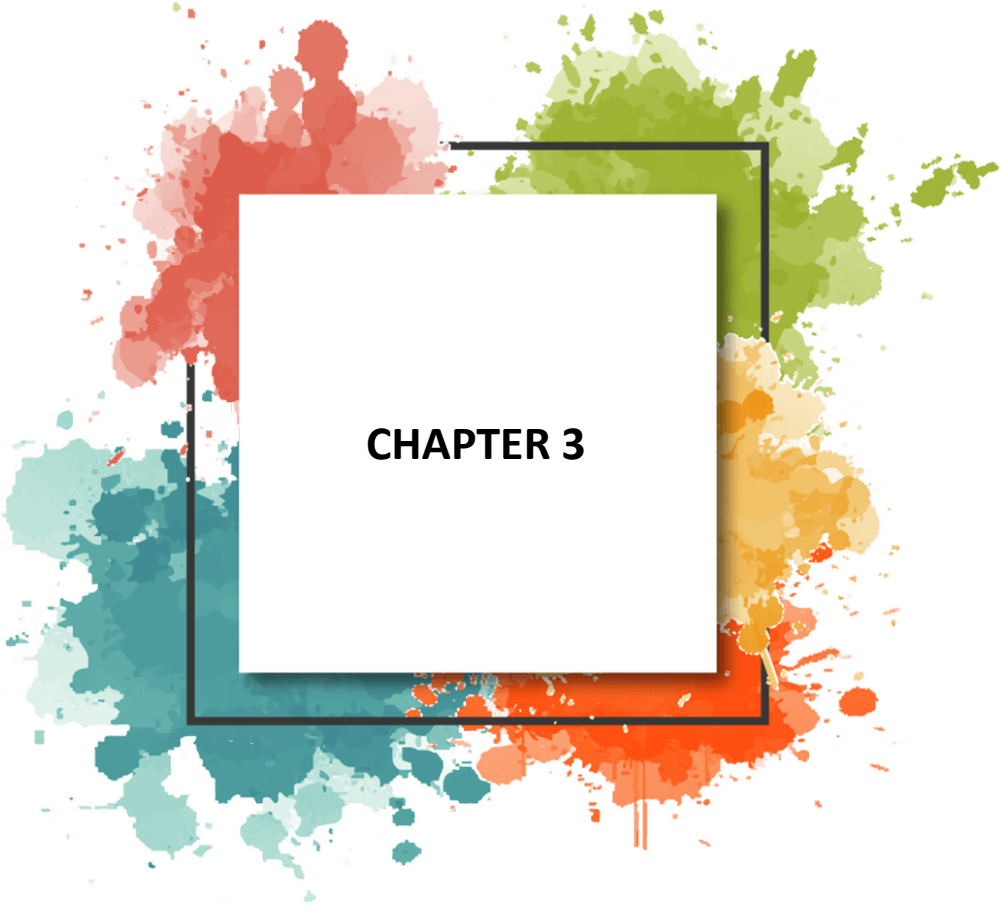
CHAPTER 3

ELISA Techniques in Experimental Physiology: Principles, and Applications

Seyhan Taşkın[1]

Introduction

The exceptionally high specificity of antibodies and antigens, along with their strong binding affinities and large association constants, has made them highly valuable for the detection and quantification of analytes within complex biological matrices such as clinical samples. Immunoassays are analytical methods based on the use of an antibody or an antigen as a reactive component for the identification and measurement of target molecules (Sakamoto vd, 2018).

The foundation of immunoassay techniques was established in the 1960s with the development of the Radioimmunoassay (RIA), a method based on the formation of an antigen–antibody complex labeled with a radioactive isotope. In clinical practice, plasma hormone levels were accurately and rapidly quantified using the RIA technique, and this diagnostic potential earned its inventors the Nobel Prize. However, the use of radioactive isotopes in RIA generated radioactive waste and posed health risks, while the technical difficulties and optimization challenges associated with handling radioactive materials led to the development of alternative methods (Ergen & Ataş, 2020; Kim, Lee & Lee, 2021).

The discovery that enzyme–substrate complexes can produce measurable colorimetric changes paved the way for the creation of analytical antibody–enzyme conjugates and enzyme–substrate systems. In other words, enzymes began to replace radioactive isotopes as labels bound to antibodies or antigens (Shah & Maghsoudlou, 2016). The first quantitative applications of enzyme-based immunoassay techniques were introduced in 1971 with the Enzyme-Linked Immunosorbent Assay (ELISA), which was used to determine immunoglobulin G and human chorionic gonadotropin (hCG) levels (Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971).

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The main factors that facilitate the design and execution of ELISA analyses are the high binding affinity of the antigen–antibody complex and the immobilization of one of these components onto a solid phase. For antibody immobilization, solid supports such as tubes or microplates made of rigid polystyrene, polyvinyl, or polypropylene are commonly used. Among these, 96-well or 384-well microplates are most widely employed, as they allow efficient adsorption of antibodies while minimizing nonspecific adsorption of other components in subsequent steps. Immobilization of the antibody onto the microplate surface simplifies the separation of bound analytes (target molecules) from unbound materials during the assay (Engvall & Perlmann, 1971; Aydin, 2015).

In ELISA reactions, monoclonal or polyclonal antibodies derived from various mammalian species (e.g., mouse, goat, sheep, or rabbit) are utilized (Sequeira, 2019). The enzymes conjugated to these antibodies typically include β-galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. The alkaline phosphatase conjugate uses *p*-nitrophenyl phosphate as its substrate, producing a yellow chromogenic product, whereas the peroxidase conjugate commonly employs 5-aminosalicylic acid or *o*-phenylenediamine to yield a brown-colored chromogen (Engvall, 2010).

The enzyme–substrate reaction generally reaches completion within 30–60 minutes and can be terminated by the addition of sodium hydroxide, hydrochloric acid, or sulfuric acid (Hornbeck, 2015). The chromogenic reaction products formed as a result of enzyme-catalyzed substrate conversion are measured spectrophotometrically at wavelengths between 400 and 600 nm. The use of high-affinity antibodies, effective washing to remove nonspecifically bound materials, and the catalytic activity of the enzyme toward its substrate collectively make

ELISA a powerful in vitro diagnostic tool with high specificity, sensitivity, and analytical efficiency.

As a fundamental principle of the ELISA technique, when the objective is to measure antibodies in a biological sample, the microplate surface should be coated with an antigen specific to the target antibody. Conversely, when the goal is to determine antigen concentrations, an antibody specific to that antigen must be immobilized onto the microplate surface.

Based on their procedural design and detection strategies, ELISA methods are classified into four main categories: Direct ELISA**,** Indirect ELISA**,** Sandwich ELISA**,** and Competitive (Inhibition) ELISA**.**

Direct ELISA Method

The direct ELISA represents the earliest and most straightforward form of enzyme-linked immunosorbent assay, serving as the foundation for subsequent ELISA techniques (Shah & Maghsoudlou, 2016; Engvall & Perlmann, 1971). This method is particularly suitable for the quantitative determination of high–molecular-weight antigens. Compared with other ELISA variants, the direct format involves the fewest procedural steps, making it both simple and rapid to perform.

The basic principle is as follows: the wells of a microplate are coated with a solution containing the target analyte (antigen) to be measured. After an appropriate incubation period, an enzyme-labeled antibody specific to the antigen is added to the wells, allowing for antigen–antibody binding. Following a second incubation, unbound antigens and antibodies are removed by thorough washing to prevent nonspecific or cross-reactive interactions. Subsequently, a substrate specific to the enzyme is introduced, generating a measurable signal or chromogenic product. The optical density of this signal, read at an appropriate wavelength, is directly proportional to the antigen concentration (Engvall, 2010; Hornbeck, 2015) (Figure 1).

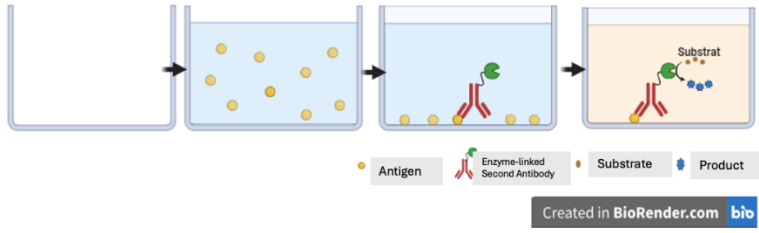

Figure 1. Direct ELISA Method

In the direct ELISA method, a bicarbonate buffer is commonly used to facilitate the adsorption of the antigen of interest onto the microplate surface. The alkaline pH of the buffer (pH > 9) ensures that antigens remain soluble while carrying a net negative charge, allowing efficient binding to the positively charged microplate. Standards containing known concentrations of the analyte, as well as positive and negative controls, undergo the same procedures as the biological samples (e.g., serum, tissue extracts, cell lysates) to verify assay optimization and the absence of contamination.

When performing direct ELISA on serum samples, it is often recommended to include a second, diluted standard curve to evaluate whether other serum proteins interfere with the measurement or affect extreme values (outliers). After antigen adsorption onto the microplate, uncoated regions are blocked to minimize nonspecific binding and prevent interference. Common blocking agents include bovine serum albumin (BSA) or casein, although detergents such as Tween 20 or Triton X-100 can also be employed. The choice of blocking buffer is primarily influenced by the composition of the biological sample and the chemistry of the plate surface. Therefore, preliminary testing is necessary to determine the optimal blocking buffer and to optimize the protocol (Shah & Maghsoudlou, 2016).

Between the antigen coating and blocking steps in the direct ELISA protocol, the microplate is washed with phosphate-buffered saline (PBS) at neutral pH. Washing is performed by completely filling and emptying the wells, as specified in the experimental protocol, and is typically repeated multiple times. This process serves to remove unbound analytes, nonspecific interactions, and residual reagents from the wells. Additionally, washing minimizes background noise during signal detection, thereby enhancing assay specificity.

Following washing, an enzyme-conjugated antibody is added to the antigen-coated wells. A second incubation allows antigen–antibody binding, after which unbound conjugates are removed through repeated washing. Subsequently, a substrate specific to the enzyme is introduced, and the reaction is terminated using an acidic solution. The resulting chromogenic signal is measured using a

microplate reader, and the analyte concentration is calculated based on the established standard curve (Shah & Maghsoudlou, 2016).

In direct ELISA, the absence of a secondary antibody eliminates the potential for cross-reactive interactions between secondary antibodies and immobilized antigens. While this represents a major advantage, the method exhibits lower sensitivity compared with other ELISA formats. The requirement for a specific antibody for each assay, coupled with the labor-intensive and costly nature of the procedure and its limited sensitivity, restricts its routine application in clinical diagnostics (Ergen & Ataş, 2020; Shah & Maghsoudlou, 2016).

Indirect ELISA Method

The terms “direct” and “indirect” ELISA refer to the manner in which the immobilized analyte (antigen adsorbed to the microplate surface) is detected. In the direct ELISA technique, an enzyme-conjugated detection antibody is applied directly to the antigen-coated microplate. In contrast, indirect ELISA employs a two-step detection strategy: a primary antibody binds the target antigen, followed by an enzyme-conjugated secondary antibody that recognizes the primary antibody. In other words, it is the secondary antibody bound to the antigen–antibody complex—not the primary antibody alone—that determines and discriminates the presence or quantity of the antigen. While the direct method offers the advantages of a shorter and simpler protocol, it requires the development of analyte-specific antibodies for each individual target. The indirect approach, on the other hand, benefits from the ability of the secondary antibody to recognize and bind multiple epitopes on the primary antibody, thereby amplifying the signal and increasing assay sensitivity (Drijvers et al., 2017).

The indirect ELISA method was developed in 1978 by Lindström and Wager, inspired by the direct ELISA approach (Lindström & Wager, 1978). In this technique, after antigen immobilization, incubation, blocking, and washing steps, the primary antibody is added to the microplate. The plate is incubated to allow antigen–antibody binding within the biological sample, followed by washing. Unbound regions may be blocked again to minimize nonspecific interactions. Subsequently, an enzyme-conjugated secondary antibody is introduced, the plate is incubated once more, and washing is repeated. An enzyme-specific substrate is then added, and the enzymatic reaction is terminated using an acidic solution. The resulting chromogenic signal is measured with a microplate reader, and analyte concentrations are calculated based on a standard curve (Shah & Maghsoudlou, 2016) (Figure 2).

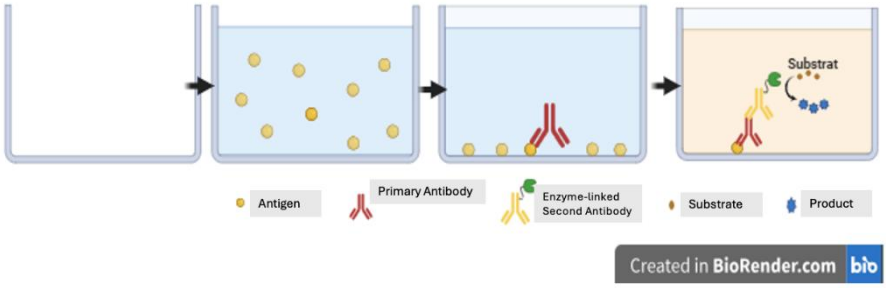

Figure 2. Indirect ELISA Method

While direct ELISA represents a suitable approach for the detection of high-abundance analytes in biological samples, it exhibits limited performance when measuring low-concentration analytes. This limitation arises from weak or absent signal amplification, resulting in reduced sensitivity (Shah & Maghsoudlou, 2016). In contrast, the indirect ELISA method facilitates the detection of low-abundance analytes through the use of signal-amplifying systems, such as the biotin–streptavidin/avidin complex. In this configuration, biotin is conjugated to the primary antibody, while streptavidin or avidin molecules are conjugated to the secondary antibody. Streptavidin binds biotin with high affinity, enabling stronger signal detection and consequently increasing assay sensitivity. Each avidin or streptavidin molecule can bind up to four biotin moieties, allowing multiple secondary antibodies to attach to a single primary antibody, thereby further amplifying the signal (Shah & Maghsoudlou, 2016; Lin, 2015).

Another major advantage of indirect ELISA, in addition to its high sensitivity, is its flexibility in employing multiple primary antibodies of the same species. All primary antibodies can be detected using the same secondary antibody, forming consistent complexes, which contributes to cost reduction. However, the use of secondary antibodies may also introduce cross-reactivity, leading to nonspecific binding and undesired signal generation, which can result in inaccurate quantification of the target analyte (Shah & Maghsoudlou, 2016).

Sandwich ELISA Method

The sandwich ELISA technique was developed in 1977 by Kato and colleagues (Kato, Hamaguchi, Okawa, Ishikawa, & Kobayashi, 1977). This method is primarily employed for the detection of specific antigens within complex biological samples. It can be implemented using either direct or indirect approaches (Kabala & Çelik, 2022). Depending on the procedural design, three different antibodies are utilized: a capture antibody immobilized on the microplate surface, a primary antibody, and a secondary antibody (Ergen & Ataş, 2020; Kohl & Ascoli, 2017).

The sandwich ELISA format is particularly suitable for the analysis of complex biological materials. The term “sandwich” derives from the positioning of the target antigen between two antibodies—the capture antibody and the primary antibody—effectively “sandwiching” the antigen within the assay (Shah & Maghsoudlou, 2016).

In the sandwich ELISA technique, an antigen-specific capture antibody is applied to the microplate surface using a coating buffer solution. The plate is incubated to facilitate the adsorption of the capture antibody onto the positively charged microplate surface. Following incubation, the microplate is washed, and uncoated regions are blocked to prevent nonspecific binding. Commercial sandwich ELISA kits typically provide microplates pre-coated with the capture antibody.

The biological sample containing the target antigen is added to the antibody-coated wells and incubated for a defined period to allow antigen binding to the capture antibody. In addition to the biological sample (e.g., serum, tissue extract, cell lysate), standards with known antigen concentrations as well as positive and negative controls are subjected to the same procedure. After incubation, unbound antigens are removed through washing to minimize nonspecific interactions. Depending on the chosen detection strategy (direct or indirect), either an enzyme-conjugated antigen-specific primary antibody (direct method) or an unconjugated primary antibody (indirect method) is added. The formation of the capture antibody–antigen–primary antibody complex (the “sandwich”) is allowed through incubation, followed by repeated washing.

Wash buffers may vary depending on the properties of the antibodies used, the solvents, the type of biological material, and the chemical characteristics of the microplate surface; phosphate-buffered saline (PBS) is generally employed. After these steps, an enzyme-conjugated secondary antibody is added, followed by incubation and washing. An enzyme-specific substrate is then introduced, and the resulting color change is monitored. The enzymatic reaction is terminated using an acidic solution, and the color intensity is measured spectrophotometrically with a microplate reader (Shah & Maghsoudlou, 2016; Aydin, 2015; Kohl & Ascoli, 2017) (Figure 3).

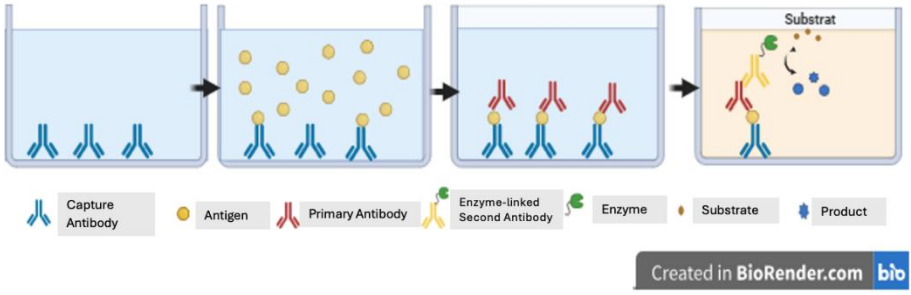

Figure 3. Sandwich ELISA Method

The sandwich ELISA method is 2–5 times more sensitive than other ELISA formats (Aydin, 2015). The capture antibody immobilized on the microplate surface binds and sequesters the target antigen from the biological sample. The binding of the primary antibody to the antigen–antibody complex, forming the "sandwich," is a principal factor contributing to the high sensitivity of the assay. Additional factors enhancing sensitivity include the use of signal-amplifying systems, such as the biotin–streptavidin/avidin complex. However, a notable limitation of the method is the requirement for two antibodies capable of simultaneously binding distinct epitopes on the same antigen. Furthermore, the antigen must possess a minimum size to accommodate binding by both antibodies.

Sandwich ELISA is predominantly employed for the detection of macromolecular antigens, including proteins, nucleotides, vitamins, and polypeptides (Shah & Maghsoudlou, 2016; Qu et al., 2016). Consequently, the size of the antigen imposes inherent limitations on the assay. Additionally, the production of antibodies targeting multiple epitopes increases cost and complexity. Despite these limitations, sandwich ELISA remains the most widely used ELISA format in both clinical diagnostics and basic research applications.

Competitive (Inhibition) ELISA Method

The competitive ELISA method is more complex than other ELISA formats. Compared with sandwich ELISA, it is capable of detecting smaller-molecule analytes, resulting in higher sensitivity. The technique was first developed in 1976 by Yorde and colleagues (Yorde, Sasse, Wang, Hussa, & Garancis, 1976). In this assay, the immobilized antigen on the microplate surface competes with the target antigen in the biological sample for binding to the primary antibody, which gives the method its "competitive" designation.

In competitive ELISA, the biological sample containing the target antigen is incubated with the primary antibody to allow the formation of antigen–antibody complexes. The greater the antigen concentration in the sample, the more antigen–antibody complexes are formed. This mixture of antigen–antibody

complexes and unbound primary antibodies is then added to the microplate previously coated with the antigen. Unbound primary antibodies bind to the immobilized antigen on the plate, forming additional antigen–antibody complexes. The more primary antibodies that are already bound to the antigens in the biological sample, the fewer are available to bind to the plate-bound antigen. Thus, antigens in the sample and the immobilized antigens "compete" for the primary antibody. After a defined incubation period, washing is performed to remove unbound antibodies. Antibodies that have formed complexes with antigens in the biological sample are also removed, as they cannot bind to the microplate.

Subsequently, an enzyme-linked secondary antibody binds to the primary antibody attached to the plate-bound antigen. Following incubation and washing, a substrate specific to the enzyme is added, and the resulting color change is measured spectrophotometrically. Because antigen–primary antibody complexes from the biological sample cannot bind to the plate, the intensity of the color change is inversely proportional to the concentration of the target antigen in the sample (Shah & Maghsoudlou, 2016; Vashist & Luong, 2018) (Figure 4).

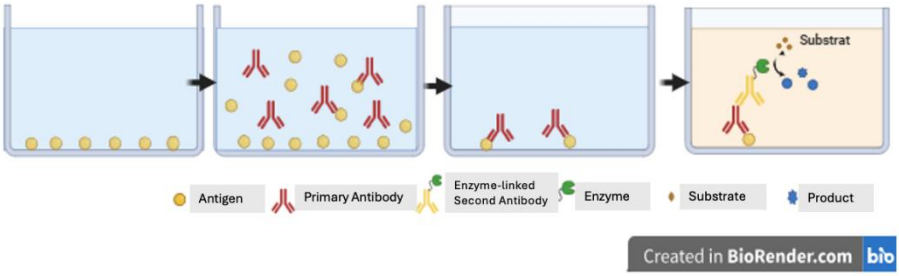

Figure 4. Competitive (Inhibition) ELISA Method

An alternative approach to competitive ELISA, often referred to as the direct method, involves immobilizing the microplate surface with either an antigen-specific antibody or an antibody-specific antigen. The biological sample and an enzyme-labeled antigen or antibody are added to the wells simultaneously. Labeled and unlabeled molecules (i.e., the analyte in the biological sample) compete for binding to the immobilized antibody or antigen on the microplate. Following a defined incubation period, the wells are washed to remove unbound components. Upon addition of the enzyme substrate, the resulting color change is measured using a microplate reader.

In this configuration, the intensity of the color signal is inversely proportional to the concentration of the analyte in the biological sample. Specifically, low concentrations of the target antigen or antibody in the sample yield high absorbance, whereas high concentrations produce low absorbance (Aydin, 2015).

Calculating ELISA Results

The successful execution of an ELISA assay depends critically on the precise performance of each procedural step. Factors such as incubation time, washing procedures, and volumetric accuracy during the addition of test reagents directly influence assay performance. As with all ELISA steps, the calculation and interpretation of results are equally important. ELISA outcomes can generate three distinct types of data: qualitative (presence or absence), quantitative (absolute measurement), and semi-quantitative (comparative relative levels).

In experiments employing the ELISA technique, samples, standards, and positive and negative controls should always be assayed in duplicate or triplicate. For each set, the mean absorbance is calculated. The absorbance of the blank or "zero" standard, typically the diluent or a solution containing no analyte, is subtracted from the standard readings. The standard curve is then constructed according to the characteristics of the chosen ELISA method. Typically, data are plotted using optical density versus the logarithm of analyte concentration.

The four-parameter logistic (4PL) curve is a widely used regression model for ELISA data analysis. The resulting sigmoidal curve provides an optimal representation of bioanalytical measurements, as these assays generally exhibit linearity only over a defined concentration range. Outside this range, responses rapidly plateau, approaching minimum and maximum values. Standard curves can be generated using specially designed millimeter or logarithmic paper or with software integrated into ELISA microplate readers. Additionally, calculations can be performed using software programs such as Microsoft Excel, CurveExpert, OriginLab, and GraphPad.

Conclusions and Recommendations

- The detection range of commercial ELISA kits may not correspond to the concentration range of the analyte in the biological sample. Therefore, the expected analyte concentration should be estimated based on previous studies and the literature, or preliminary experiments should be conducted to determine whether sample dilution is required.
- Hemolyzed samples can lead to unwanted color formation and assay interference; therefore, careful collection of serum or plasma samples is essential.
- It is important to ensure that the analyte is present in the relevant fraction of the serum or plasma. The specific centrifugation speed and duration required for the analyte to partition into the serum fraction should be known. Sample processing should follow the centrifugation

protocols recommended by the ELISA kit or reported in the literature. The same approach should be applied to other biological matrices, and attention must be paid to pre-analytical errors.

- Repeated freeze–thaw cycles of biological samples can degrade proteins; therefore, samples intended for multiple tests should be aliquoted appropriately.
- Bacterial contamination of biological samples can result in false-positive outcomes. Samples should be free from contamination, not stored for extended periods at +4 °C, and, if possible, fresh samples should be used.
- For high sensitivity, commercial kits containing signal-enhancing reagents are recommended.
- Unless otherwise specified, all reagents should be maintained at room temperature. If not all wells of a microplate will be used for measurement, unused strips should be removed from the plate and stored at the recommended temperature. During incubation, moisture accumulation in empty wells or unintended contamination during washing may compromise well integrity.
- Adding standards and samples to the microplate using a multichannel pipette minimizes time discrepancies between the first and last additions. Applying the same incubation period for all samples ensures more consistent results.
- During pipetting, tips should be held at an angle and should not touch the bottom of the wells.
- Although pipette tips do not need to be changed for replicate wells of the same sample, tips should be replaced between different samples to prevent cross-contamination. In particular, tips should be changed between replicate standards, as residues inside the tip may cause variability between replicates. Pipettes should be calibrated regularly.
- Manual washing may result in higher background signals; therefore, the use of an automated microplate washer is recommended.
- Adherence to the incubation times specified in the ELISA protocol is critical. Incubation durations should not deviate by more than ±5 minutes per hour from the recommended time.

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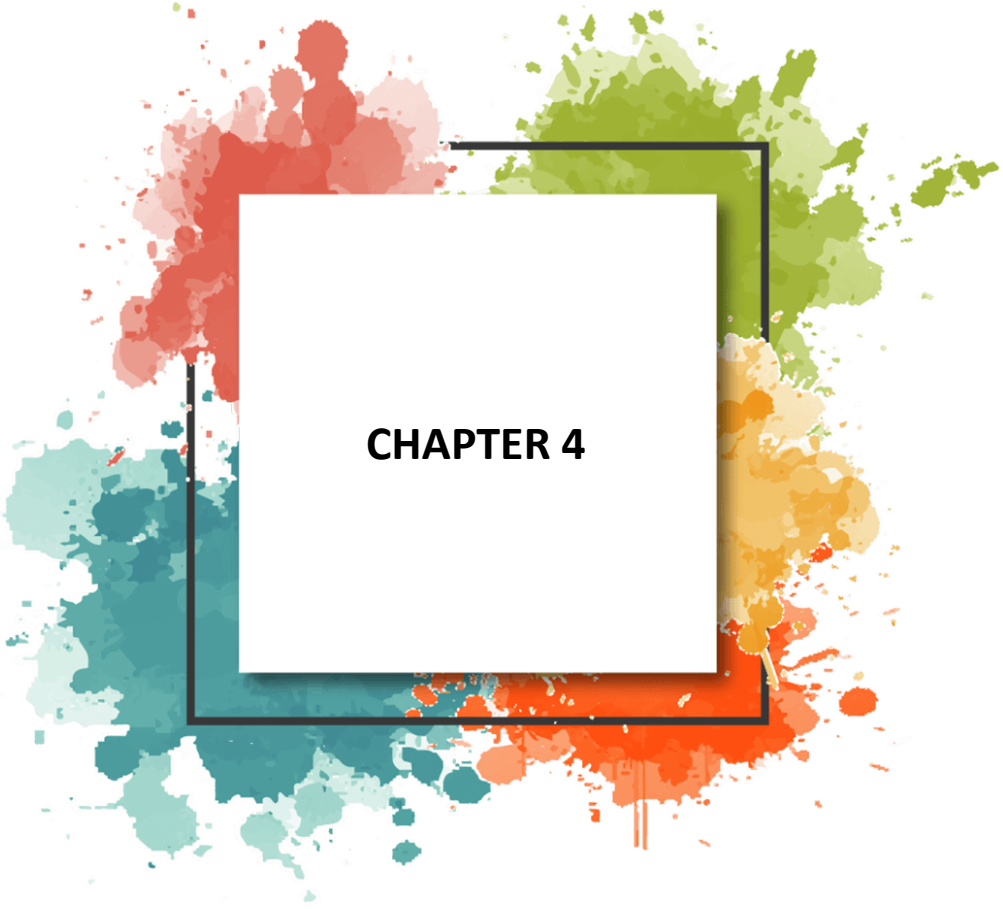
CHAPTER 4

Aposinin ve Diaposinin'in Pankreas Kanser Hücrelerine Etkileri

Mehmet Kirazlar[1]

Giriş

Aposinin, aynı zamanda acetovanillone olarak isimlendirilen; 4. pozisyonda bir hidroksi grubu ve 3. pozisyonda bir metoksi grubu ile ikame edilmiş 1-feniletanon olan aromatik bir ketondur. Narkotik olmayan analjezik, steroidal olmayan antiinflamatuar, antiromatizmal, periferik sinir sistemi ilacı olarak rol oynar. NAD(P)H oksidaz ($H_2O2_{,2}$ oluşturan) inhibitörü ve bir bitki metabolitidir. Asetofenonlar, metil ketonlar ve aromatik ketonların bir üyesidir.

Aposinin (4-hidroksi-3metoksi-asetofenon) ilk defa 1883'de Oswald Schmiedeberg isimli bir farmakolog tarafından tariflenmiş ve doğal yolla metoksi katekol yer değişimi meydana getiren bir bitki olan Apocynum Cannabinum (Kanada keneviri) bitkisinin köklerinden elde edilmiştir. Sonraki dönemde, özellikle Asya'da yetişen bir bitki olan Picrorhiza kurroa kök ekstratından 1971 yılında aposinin elde edilerek kullanılmıştır. Elde edilen ekstratlar ödem atmayı kolaylaştırıcı ve kalp sorunları için iyileştirici etki beklenerek kullanılmıştır. Aposinin, 166,17 molekül ağırlığına sahiptir ve asetofenon olarak gruplandırılır. Hafif bir vanilya kokusuna sahiptir ve 115 °C'lik bir erime noktasına sahiptir. (Luchtefeld et al., 2007).

Diaposinin, Aposinin molekülünün homo-dimeridir. 330.33 molekül ağırlığına sahip bileşiğin kapalı formülü $C_{18}H_{18}O_6$ şeklindedir. Erime noktası 310 °C olup kaynama noktasının 540 seviyelerinde olduğu hesaplanmıştır. Hücre içinde Myeloperoxidaz etkinliği ile Aposinin dimerleşerek Diaposinin'e dönüşür (Ismail, Scapozza, Ruegg, & Dorchies, 2014).

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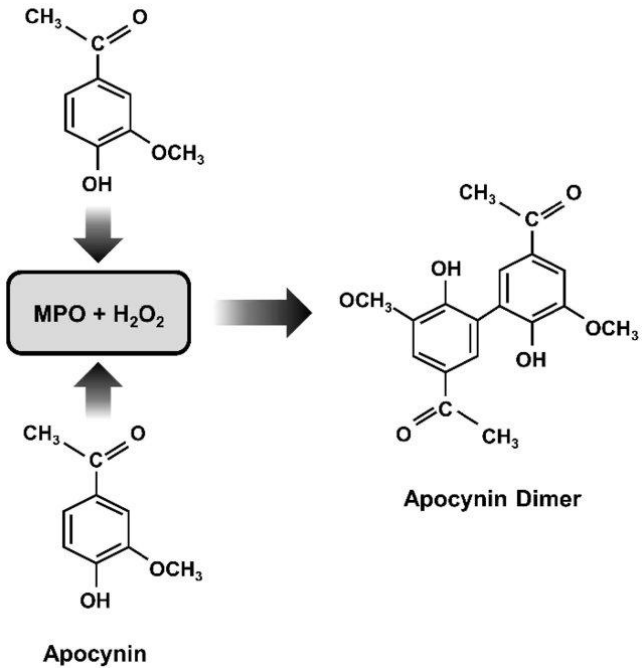

Şekil: Aposinin moleküler yapısı ve dimerizasyonu sonucu Diaposinin moleküler yapısı (Kim et al., 2017)

Dünya çapında kansere bağlı ölümlerde dördüncü sırada yer alan pankreas kanseri, en ölümcül hastalıklardan biridir. Hastaların %80'inden fazlası tanı sırasında lokal veya uzak metastaz gösterdiği görülmüştür ve bu hastalarda küratif cerrahi tedavi için uygun görülmemektedir. Bununla birlikte, son yıllarda yapılan istatistiklere göre 5 yıllık sağkalım oranı %6'dan düşüktür. Cerrahi operasyona uygun hastaların takibinde ise %70 oranda bir yıl içinde metastatik hastalık geliştiği kaydedilmiştir. Pankreas kanseri tanı ve tedavisindeki bu zorluklardan ötürü 21. yüzyılda hala baş etmesi oldukça güç bir kanser türü olarak karşımıza çıkmaktadır. Sonuç olarak, pankreas kanseri tedavisinde yeni yöntemlerin veyahut yardımcı moleküllerin keşfi çok önemlidir (Du, Liu, You, Wu, & Zhao, 2016).

Günümüz kanser çalışmalarında bitki derivelerinin antioksidan özelliğini kullanarak kanser yükünü azalttığı yoğunlaşılan bir çalışma alanıdır. Aposinin kullanılan çalışmalar var olsa da Diaposinin kullanılmış çalışmalar çok kısıtlıdır. Yapılan çalışmalarda Aposinin yerine Diaposinin kullanıldığında NOX inhibisyonu etkinliği, antiinflamatuar ve antioksidan etkinlik çok daha yüksek görülmüştür (Ismail, Scapozza, Ruegg, & Dorchies, 2014).

Biz de hem etkinlik avantajını hem de literatürdeki eksikliği gördüğümüz için bu çalışmayı yapmakta karar kıldık. Bu bölümde literatürde bizi çalışmaya iten yayınları inceleyeceğiz.

Literatür

Liu ve arkadaşları tarafından yapılmış bir çalışmada $CoCl_2$ ile hasarlanan PC12 hücrelerine aposinin etkileri incelenmiştir. Aposinin, hasarlı PC12 hücrelerinde MDA oluşumunu azaltmış ve Süper Oksit Dismutaz aktivitesini artırmıştır. Elde edilen veriler, hücre hasarına bağlı indüklenen oksidatif stresin regülasyonunda endojen p38-MAPK-kaspaz-3 ekspresyonunu azalttığını, önceden aposinin uygulanmış hücrelerde oksidatif stresi azalttığını göstermiştir. (Liu et al., 2018).

El-Ghafar et al. (2021) tarafından yayınlanmış bir makale, aposinin'in metotrexate kaynaklı hepatotoksisite modelinde hepatositleri koruyucu etkisinin varlığına dair bulgular sağlamıştır. Karaciğer hücrelerini korumasının arkasında altı çizilen mekanizmalar arasında, NADPH oksidazın down regülasyonu ve Nrf2/ARE, SIRT1, PPARy ve sitoglobin sinyallerinin up regülasyonu yoluyla metotrexate'ın neden olduğu oksidatif hasarın azaltılması öne sürülmüştür. Ek olarak AV, IL-6/STAT-3 ve NF-κB/AP-1 sinyallerinde down regülasyonu yoluyla hepatik inflamasyonu azalttığı gözlenmiştir. Ayrıca aposinin, metotrexate'ın Prostat kanseri (PC-3), Serviks adenokarsinomu (HeLa) ve kronik miyeloid lösemi (K562) hücre hatlarına karşı in vitro sitotoksik aktivitesini arttırdı (El-Ghafar et al., 2021).

Aposinin kullanılan in vitro bir başka çalışmada prostat kanseri (PLS10) hücrelerinin hücre proliferasyonunu önemli ölçüde inhibe ettiği hücre döngüsünün G1de tutulduğu gözlenmiştir. Bu çalışmada NADPH oksidaz kompleksinin bir bileşeni olan Rac1'in fosforilasyonunu inhibe ettiği ancak şaşırtıcı bir şekilde reaktif oksijen türlerini (ROS) etkilemediği izlenmiştir. Aposinin Rac1 ve NF-kB'nin yanı sıra siklin D1'in fosforilasyonunu azaltmıştır. In vivo doza bağlı olarak, PLS10'lu prostat kanserinin ksenograft modelinde aposinin, tümörlerdeki hücre çoğalmasını ve damar sayısını azaltmıştır. Böylelikle tümör büyümesini ve metastazı önleme özelliği vurgulanmıştır. In vivo ve in vitro deneylerde aposinin tedavisi,vasküler endotelyal büyüme faktörünün (VEGF) ekspresyonu ve salgılanmasını azaltmıştır. Sonuç olarak, androjenden bağımsız prostat kanseri patofizyolojisinde oksidatif stres ile doğrudan bir ilişki olmamasına rağmen, aposinin in vitro ve in vivo olarak kanserin büyümesini durdurduğu gösterilmiştir (Suzuki et al., 2013).

Diaposinin ROS üretimini azalttığını gösteren bir çalışmada EDL-MDX-2 miyoblast hücreleri kullanılarak deney kurgulanmıştır. Hücre içi Ca+2 artışı, aşırı ROS üretimi ve artan fosfolipaz A2 aktivitesi, kastaki distrofi eksikliği üzerindeki patolojiye katkıda bulunan üç faktörün, kastaki distrofi modelinde etkileri incelemek için yapılan çalışmada bulunmuştur. Ek olarak, Ca+2, ROS ve fosfolipaz A2, özellikle iPLA2'nin pozitif geri besleme döngülerinde birbirini güçlendirir. Kastaki ROS'un ana kaynağı olarak kabul edilen NADPH oksidazlar

(NOX) ve MDX farelerinin kaslarında aşırı eksprese edildiği söylenir. Distrofik miyotübüllerde, diaposinin ROS üretimini durdurduğu, iPLA2 aktivitesini ortadan kaldırdığı ve distrofik kasta aşırı Ca+2 girişinden sorumlu iki ana yol olan gerilerek aktive edilen ve depo ile çalıştırılan kanalların aktivitesini azalttığı gösterilmiştir. Diaposinin ayrıca mdx kasının vahşi tip kas değerine yakın eksantrik kasılmalarının kuvvet kaybını önlediği ve membran hasarını azalttığı kaydedildi (Luchtefeld et al., 2007).

Diaposinin ile ilgili çalışan bir grup araştırmacı, LRRK2 mutant farelerde yürüttüğü bir çalışmada, reaktif oksijen substratlarının Parkinson hastalığının gelişiminde önemli bir rol oynadığını ve Diaposinin, ROS'un oluşumunu engelleyerek Parkinson hastalığına karşı kullanılabileceğini öne sürmüşlerdir. Bu fareler genellikle on aylık olduklarında parkinson semptomları gösteriyor olmalarına rağmen, bu çalışmada koloniler ancak 16 aylık bir süre sonunda motor koordinasyon sorunları sergilediler. 12 haftadan itibaren fareler farklı gruplara ayrıldı ve her bir gruba diaposinin (200 mg kg^{-1}) oral gavaj ile haftada üç kez verildi. Diaposinin verilen grup direğe tırmanma testi ve rotor-rod testi, kontrol grubuna kıyasla motor koordinasyonunu koruduğunu göstermiştir. Nöroprotektif bir ajan olarak parkinson hastalığına karşı kullanılabileceğini öne sürmüşlerdir. (Dranka et al., 2014).

A549 akciğer karsinomu ve BEAS2B normal hava yolu epitel hücre hatları üzerinde yapılan çalışmada Metoksifenol grubu moleküllerin uygulandığı çalışmada diaposinin ve aposinin uygulanmış. Antiinflamatuar etkiler değerlendirilirken hücreler TNF-a ile uyarılarak ROS üretimleri CCL2 ekspresyonu gibi seviyeler değerlendirilmiştir. Aposinin veya Diaposinin uygulanarak salınan sitokinlerin kontrol grubu hücrelere oranla düşük olduğu, CCL2 üretiminin Diaposininde çok daha düşük olmakla birlikte aposininde de anlamlı şekilde düşük olduğu kayda alınmıştır (Houser et al., 2012).

Marin ve arkadaşları tarafından yürütülmüş bir deneyde aposinin ile diaposinin; in vitro lipopolisakkarid ile stimüle edilmiş RAW264.7 makrofajlarında çeşitli inflamatuar belirteçler üzerindeki etkinliği ve farelerde dekstran sodyum sülfat tarafından indüklenen ülseratif kolit üzerindeki antiinflamatuar etkisi gözlenmiştir. Diaposininin aposininden daha yüksek anti inflamatuar etki ortaya koyduğu gözlenmiştir. Diaposinin, ROS üretimini, TNF-a, IL-6 ve IL-1β seviyelerini azaltmıştır. Bunun yanında iNOS ve COX-2 ekspresyonunu inhibe ettiği görülmüştür. Lipopolisakkarit ile uyarılmış RAW264.7 hücrelerinde PGE2 üretimini down regüle ettiği görülmüştür. Diaposininin antiinflamatuar mekanizması, NF-κB aktifleşmesinin engellenmesiyle açıklanmıştır. Haftada üç kez oral gavaj yoluyla uygulanan aposinin ve diaposinin (100 mg kg^{-1}) Dekstran sodyum sülfatın sebep olduğu deneysel inflamatuar barsak hastalığına karşı koruyucu etkiler izlendiği

belirtilmiştir. Ancak diaposinin dozunun (0,3 mmol kg^{-1}) aposinin dozunun yarısı miktardayken yakın etkinlik ortaya koyduğu gözlenmiştir. (Marín et al., 2017).

Amyotrofik lateral skleroz hastalığı transgenik modeli olan SOD1G93A farelerde yapılmış deneylerde aposinin ve diaposinin (a: 200 μM ve d:10 μM) izole nöron kültüründe inflamasyona bağlı hücre kaybının önemli şekilde azalttığı gözlenmiştir. Ek olarak diaposinin uygulamasının nöroinflamasyonu azaltmakla birlikte ele alınan farelerin yaşam sürelerini arttırmadığı not düşülmüştür (Trumbull et al., 2011).

Hepatik karsinom HepG2 hücre hattı üzerinde vanillin, divanillin, aposinin ve diaposinin molekülleri uygulanarak hücreler üzerinde hücre göçünü azaltıcı etkisi değerlendirilmiştir. Nontoksik (tedavi edilen hücrelerin canlılığı %90'dan fazla kaldığı durumlarda konsantrasyonlarda) (vanilin ve aposinin için 0.5-2.0 mM ve divanilin ve diaposinin için 10-100 μM;) tüm uygulamaların invazyonu önemli şekilde azalttığı görülmüş ve bu engelleme doz bağımlı şekilde %20 ila %80 arasında olduğu gözlenmiştir. Vanilin (1.1 mM) ve aposinin (0.8 mM) IC50'leri arasında istatistiksel olarak belirgin bir fark olmadığı ancak, diaposinin'in (23.3 μM) IC50'si, divanilin'in (41.3 μM) IC50'sinden daha düşük olduğu kaydedilmiştir. Vanilin ve aposinin'in monomerik ve dimerik formlarının hücrelerde invazyonu engelleme modeli üzerinde etkileri incelendiğinde dimer form olan divanilin ve diaposinin'in kanser hücresi invazyonunu engelleme açısından vanilin ve aposinin'den daha etkili olduğunu görülmüştür. İnvazyon ve migrasyon gerçekleşmesi için, hücre yüzeyi ekstraselüller matrikse integrinler aracılığıyla bağlanır. Integrin-ECM etkileşimleri FAK, PI3K/Akt, ERK ve NF-κB sinyal yollarını içeren sinyalleşme aktifleşir. Bu aşağı yönlü sinyallemenin çalışamaması kanser invazyonunu ve migrasyonunu azalttığı gösterilmiştir. Araştırmacılar divanilin ve diaposinin'in hücre-ekstraselüller matriks bağlantısının aktifleştirdiği intrasellüler sinyalleri engelleme etkinliğini incelemek için, hücrelere 30 dakika boyunca Matrigel'e bağlanma süresi tanınmış. Sonrasında, hücre lizatındaki proteinler, sinyal proteinlerinin aktifleşme miktarını gözlemleme amacıyla immünoblüt analizi uygulanmıştır. İmmünoblüt analiz sonuçları, Matrigel bağlanmasının HepG2 hücrelerinde FAK, Akt ve ERK proteinlerinin fosforilasyonunu stimüle ettiğini, ancak p65 NF-κB fosforilasyonu üzerine etkisi olmadığı kaydedilmiştir. Çalışmada kullanılan tüm vaniloid moleküllerin FAK ve Akt fosforilasyonunu spesifik olaran azalttığı gözlenmiştir. Ek olarak, dimer form bileşiklerin monomer formlara kıyasla Matrigel tarafından stimüle edilen Akt fosforilasyonunu azaltmada daha etkin olduğu görülmüştür. Böylelikle, divanilin, diaposinin, vanillin ve aposinin'in hücre-ekstraselüller matriks bağlanması ile stimüle edilen FAK ve PI3K/Akt yolağını inhibe ederek aynı yol üzerinde etkili olduğu görülmüştür (Jantaree et al., 2017).

Pek çok kanser türünde rekatif oksijen substratları üretimi yüksektir ve onkojenik fenotipin korunmasına ve tümörlerin ilerlemesine katkıda bulunur. Ek olarak, rektif oksijen substratlarının tümör gelişiminde ve karsinogenezde önemli bir rol oynadığı bilinmektedir (Klaunig et al., 2009). Diaposinin, doğal olarak bulunan bir NOX inhibitörü olduğu için, antioksidan işlevi sayesinde tümör büyümesini baskılayabileceği düşünülmüştür. Sonuç olarak Diaposinin üzerinde çalışmaların kısıtlılığı ve ümit vaad edici oluşu araştırma grubu olarak bizim bu konuya yönelmemizi sağlamıştır.

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