東京工科大学

博士学位論文

Inhibitory effect of thymoquinone against amyloid beta and synuclein-induced neurotoxicities in rat primary and human induced pluripotent stem cells-derived neurons

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ALHIBSHI AMANI HASAN A

Contents

Chapter 1

Introduction

1.1 Alzheimer's disease (AD)

Progressive mental deterioration in old age has been recognized and described throughout history. However, it was not until 1906 when Dr. Alois Alzheimer, a German physician, specifically identified a collection of brain cell abnormalities as a disease. One of Dr. Alzheimer's patients died after years of severe memory problems, confusion and difficulty understanding questions. Upon her death, while performing a brain autopsy, the doctor noted dense deposits surrounding the nerve cells (neuritic plaques). Inside the nerve cells he observed twisted bands of fibers (neurofibrillary tangles). Today, this degenerative brain disorder bears his name, and when found during an autopsy, these plaques and tangles mean a definite diagnosis of Alzheimer's disease (AD). Since its discovery more than 100 years ago, there have been many scientific breakthroughs in AD research.

In the 1960s, scientists discovered a link between cognitive decline and the number of plaques and tangles in the brain. The medical community then formally recognized Alzheimer's as a disease and not a normal part of aging. In the 1970s, scientists made great strides in understanding the human body as a whole, and AD emerged as a significant area of research interest. This increased attention led in the 1990s to important discoveries and a better understanding of complex nerve cells in the brains of AD patients. More research was done on AD susceptibility genes, and several drugs were approved to treat the cognitive symptoms of the disease. Specific genes related to both the early-onset and late-onset forms of AD have been identified, but genetic risk factors alone do not fully explain its causes, so researchers are actively exploring environment and lifestyle to learn what role they might play in the development of this disease.

1.2 Amyloid beta peptide (Aβ)

One of the hallmarks of AD is the accumulation of amyloid plaques between neurons in the brain. Amyloid is a general term for protein fragments that the body produces normally. Amyloid beta (Aβ) is a peptide of 36–43 amino acids that is

processed from the amyloid precursor protein (APP). Aβ is the main component of amyloid plaques found in the brains of patients with AD. In a healthy brain, these protein fragments are broken down and eliminated. In AD, the fragments accumulate to form hard, insoluble plaques**.**

1.3 Amyloid beta formation and neurotoxicity

APP is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. Its primary function is not known, though it has been implicated as a regulator of synapse formation [1], neural plasticity [2] and iron export [3]. APP can be processed in different ways by different sets of enzymes one pathway leads to amyloid plaque formation (amyloidogenic), while another does not (non-amyloidogenic). Usually about 90% of APP enters the non-amyloidogenic pathway, and 10% the amyloidogenic one, but these ratios can change due to mutations, environmental factors, as well as the age of the individual. Cleavage products from both these pathways may play important roles in neural development and function (Fig.1-1).

In the non-plaque-forming pathway, APP is cleaved first by α-secretase a proteases in the secretase family to yield a soluble N-terminal fragment (sAPPα) and a C-terminal fragment (CTFα). sAPPα may be involved in the enhancement of synaptogenesis, neurite outgrowth and neuronal survival, and are considered to be neuroprotective. CTFα is retained in the membrane, where it is acted upon by presenilin-containing γ-secretase to yield a soluble N-terminal fragment (p3) and a membrane-bound C-terminal fragment (AICD, or APP intracellular domain). AICD may be involved in nuclear signalling via transcriptional regulation as well as axonal transport through its ability to associate with a host of different proteins.

In the plaque-forming pathway, APP is cleaved first by a different enzyme, β-secretase (a transmembrane aspartic protease), yielding a soluble N-terminal fragment (sAPPβ) and a membrane-bound C-terminal fragment (CTFβ). This cut is made closer to the N-terminal end of APP than with α-secretase, making CTFβ longer than CTFα. CTFβ is then acted upon by γ-secretase (as occurred in the previous pathway), yielding a membrane-bound C-terminal fragment (AICD) the same as before, and a soluble N-terminal fragment Aβ that is longer than p3. The γ-secretase can generate a number of isoforms of 36-43 amino acid residues in length. The most common isoforms are $A\beta_{40}$ and $A\beta_{42}$; The $A\beta_{40}$ form is the more common of the two, but $A\beta_{42}$ is the more fibrillogenic and is thus associated with disease states. Mutations in APP associated with early-onset Alzheimer's have been noted to increase the relative production of $A\beta_{42}$, and

thus one suggested avenue of Alzheimer's therapy involves modulating the activity of β and γ secretases to produce mainly $A\beta_{40}$ [4]. γ-Secretase is a large multi-subunit complex whose components have not yet been fully characterized, but includes presenilin, whose gene has been identified as a major genetic risk factor for Alzheimer's [5]. The progressive accumulation of $A\beta_{1\text{-}42}$ (Fig.1-2) aggregates is widely believed to be fundamental to the initial development of neurodegenerative pathology and to trigger a cascade of events such as, lipid peroxidation in brain cell membranes [6], mitochondrial dysfunction through free radical generation, and resultant oxidative stress [7], synaptic plasticity impairment [8], and chronic inflammatory reactions [9]. Importantly, Aβ oligomers have been demonstrated to potentiate synaptic loss and inhibit hippocampal Long-term potentiation (LTP) [10]. It has been also suggested that neurotoxic amyloid aggregates may lead to synaptic dysfunction and eventually synaptic loss [11], leading finally to neuronal death [12].

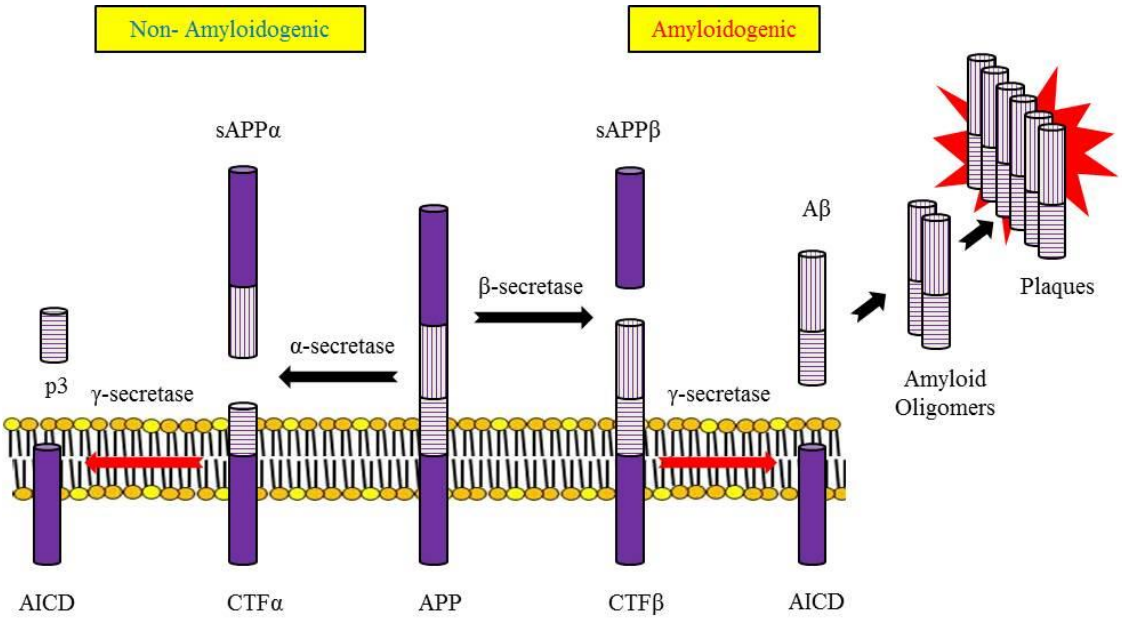

Figure 1-1. APP Processing: α-secretase and γ-secretase produce non-plaque forming p3, while β-secretase and γ-secretase produce amyloid plaque-forming Aβ

H_2N-Asp[1]-Ala[2]-Glu[3]-Phe[4]-Arg[5]-His[6]-Asp[7]-Ser[8]-Gly[9]-Tyr[10]-Glu[11]-Val[12]-His[13]-His[14]-Gln[15]-Lys[16]-Leu[17]-Val[18]-Phe[19]-Phe[20]-Ala[21]-Glu[22]-Asp[23]-Val[24]-Gly[25]-Ser[26]-Asn[27]-Lys[28]-Gly[29]-Ala[30]-Ile[32]-Gly[33]-Leu[34]-Met[35]-Val[36]-Gly[37]-Gly[38]-Val[39]-Val[40]-Ile[41]-Ala[42]-COOH

Figure 1-2. Primary sequence of $A\beta_{1-42}$

1.4 Alpha Synuclein (αSN)

Synucleins (SNs) are small (113-143 amino acids) proteins, highly conserved among vertebrates [13], with up to 87% homology between the human and the zebra finch sequence [14-17]. Three different genes, α, β and γ, encode αSN, βSN, and γSN, respectively. SNs were first described in 1988 when a novel protein was isolated in the electrical organ of *Torpedo Californica* and in rat brain [14]. The protein was named synuclein because of its synaptic and nuclear localization. A second intrinsic peptide component of human AD amyloid, named 'non-amyloid-beta-component precursor protein' (NACP), belonging to the SN family, was reported in 1993 [15]. Later, in 1995, data were presented documenting the presence of αSN in the telencephalic song control circuit of the zebra finch [17]. αSN was subsequently found in Parkinson's Disease (PD) neuropathologic hallmarks, named Lewy Bodies (LBs) and Lewy neurites, and in cytoplasmic inclusions of patients affected by Dementia with Lewy Bodies (DLBs) and multiple system atrophy [18-25]. αSN-positive-LBs have also been found in LBs variant of AD, familial AD and Down's syndrome [26, 27]. Because of the presence of αSN deposits, all these neurodegenerative disorders are now grouped under the name of synucleinopathies [28].

αSN has received much attention because of the discovery that a mutation of the protein is involved in familial PD [29]. αSN is expressed throughout the brain: the molecular layer of cerebellum, hippocampus, amygdala, retina, neocortex, nucleus accumbens, striatum and olfactory mucosa. The primary sequence of αSN consists of 140 residues. The mechanism(s) by which αSN plays a role in the pathogenesis of synucleinopathies is (are) not yet clear. This is due, at least in part, to the fact that the physiologic function of αSN still remains unknown. Numerous studies strongly support the idea that αSN plays a role in neurotransmission. Indeed, the protein is located in proximity to synaptic vesicles in presynaptic terminals [30] where it co-localizes with βSN [31]. Interestingly, a small fraction of αSN is associated with vesicular membranes [32]. Moreover, recent studies, examining structural synaptic changes after treatment of cultured hippocampal neurons with an antisense oligonucleotide, a manipulation that reduces αSN expression, have demonstrated a marked selective reduction of the presynaptic vesicle distal pool size in treated neurons [33, 34]. These findings are interpreted as support for a role of αSN in interaction and regulation of vesicles storage and turnover that might be compromised in synucleinopathies [33, 34].

1.5 Human induced pluripotent stem cells (hiPSC)

Human Induced Pluripotent Stem Cells (hiPSC) are a type of pluripotent stem cell that can be generated directly from adult cells. The iPSC technology was pioneered by Shinya Yamanaka's lab in Kyoto, Japan, who showed in 2006 that the introduction of four specific transcription factors could convert adult cells into pluripotent stem cells [35]. He was awarded the 2012 Nobel Prize along with Sir John Gurdon "for the discovery that mature cells can be reprogrammed to become pluripotent.

hiPSC hold great promise in the field of regenerative medicine. Because they can propagate indefinitely, as well as give rise to every other cell type in the body (such as neurons, heart, pancreatic, and liver cells), they represent a single source of cells that could be used to replace those lost to damage or disease. An important subject of future research in this area involves the use of human iPSC-based strategies to study the morphology and electrophysiological behavior of neurons from patients with synucleinopathies and compare them with those of healthy cells. There are many potential causes for the failed translation of drug discovery from levels of molecular and animal models to human therapeutics. In particular, the success of preclinical phases of drug development is based on animal models [36]. Furthermore, less than 10% of the compounds that enter the clinical phase of testing reach the stage of market approval; the estimated cost of the entire drug development process is 1.2–1.7 billion US$ per drug [36-38].

Drug discovery/development platforms using hiPSC- based disease models could be useful in filling the gap between animal models and clinical trials. Cell lines and animal models contribute to the exploration of disease mechanisms and drug development for various diseases. However, the animal models do not always demonstrate the same phenotypes as those seen in humans [39]. For instance, in mice the type and/or distribution of cardiac ion channels are different from those in humans, demonstrating a relatively shorter duration of action potential and higher heart rate (600 bpm) [40]. An in vitro analysis of human cardiomyocytes is therefore critical to an understanding of the mechanism of genetics-related arrhythmias (irregular heartbeat) in humans [40]. Also, compounds that demonstrate significant benefit in animal models may fail to show effectiveness in clinical trials in humans [39, 41, 42]. The use of transgenic mice of mutant superoxide dismutase (SOD1), a gene found to be associated with amyotrophic lateral sclerosis (a specific disorder that involves the death of neurons) [43], enabled the identification of several compounds that relieve the disease phenotype, including vitamin E and creatine [44-46]. However, when these compounds

were tested in humans, no clinical improvements were observed [44-46]. The toxicity of compounds is sometimes missed in cell lines and animal models because specific interactions with human biological processes cannot be recapitulated in these systems [36] Also, the use of animal models for toxicity assays may be ethically problematic, the animals may be expensive to purchase and maintain, and the process may be difficult to automate [36]. Clearly, different drug screening models that complement these systems and represent the human condition with high fidelity are required [47]. hiPSC are expected to fulfill these requirements and are amenable to the demands of drug development.

1.6 Nigella Sativa

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Historical and current studies and surveys indicate that the Eastern region of the Mediterranean has been distinguished throughout the generations with a rich inventory of natural medicinal herbs [48]. Among the promising medicinal plants, *Nigella Sativa* (also known as the black seed or black cumin), a dicotyledon of the Ranunculaceae family, has been used for medicinal purposes for centuries, both as a herb and pressed into oil, in Asia, Middle East and Africa. It has been traditionally used for a variety of conditions and treatments related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support, and for general well-being [49-51]. In Islam, it is regarded as one of the greatest forms of healing medicine available. The Islamic prophet Muhammad once stated that the black seed can heal every disease except death. Black cumin seeds were found in the tomb of Egyptian Pharaoh Tutankhamun, who ruled Egypt from 1332 BC to 1323 BC. He was the son of Akhenaten and Nefertiti and his tomb is the most complete Egyptian tomb ever discovered. The Egyptians supposedly put the seeds in his tomb so that he may have excellent health in the afterlife [52].

The black cumin herb goes by many different names. For example, in old Latin it is called as 'Panacea' meaning 'cure all' while in Arabic it is termed as 'Habbat Al Baraka' translated as 'Seeds of blessing'. The plant belongs to the *Ranunculaceae* family of flowering plants and genus of about 14 species including *Nigella arvensis, Nigella ciliaris, Nigella damascene, Nigella hispanica, Nigella integrifolia, Nigella nigellastrum, Nigella orientalis and Nigella sativa, respectively*. Among these, *Nigella sativa* is the species most exhaustively investigated for therapeutic purposes although other species have also been implicated for therapeutic uses [53]. The species grow to 20-30 cm tall, with finely divided leaves wherein the leaf segments are narrowly linear to threadlike. The flowers are white, yellow, pink, pale blue or pale purple, with 5-10 petals. The fruit is a capsule composed of several united follicles, each containing numerous seeds. The parts of the plant most commonly used for the therapeutic purposes in the "Alternative Medicinal" systems are the seeds (Fig.1-3), which are contained in an inflated capsule formed from the united follicles containing considerable amount of oil having pungent and bitter taste. Commonly the seeds are used primarily as a spice and food preservative. In folk medicinal practices they are ingested with food or mixed with honey and are primarily used as galactogogues and anthelmintic agents. The seeds have also been used as diuretics, anti-hypertensive, muscle relaxants and as immunity enhancers in immune compromised people. There are

reports that the oil from the seeds can be used to treat dermatitis topically [54]. Several beneficial pharmacological effects have been attributed to various crude or purified components of these seeds including antihistaminic [55], antihypertensive [56], hypoglycemic [57], antifungal [58], anti-inflammatory [59], along with significant anti-neoplastic activities [60]. These studies collectively provide early indication that further development of agents derived from *Nigella sativa* seeds could be useful in modern medicine.

Figure 1-3. Nigella Sativa Seeds

1.7 Thymoquinone (TQ)

The chemical composition of the black *Nigella sativa* seed is diverse and contains amino acids, proteins, carbohydrates, fixed and volatile oils, alkaloids, saponins and many other compounds. Thin Layer Chromatography (TLC) screening of the oil samples showed the presence of four main components, viz. thymoquinone, carvacrol, tanethole and 4-terpineol, which demonstrated respectable radical scavenging property. These four constituents and the essential oil possessed variable antioxidant activity when tested in the 2,2′-diphenyl-p-picrylhydrazyl (DPPH) assay for non-specific hydrogen atom or electron donation. The oil samples showed variable antioxidant activity which was ascribed mainly to the variable composition of these constituents [61]. According to a previous study [62], the bioactive constituents of the volatile oil of black seed (54%) were identified showing that Thymoquinone or, in short, TQ (Fig.1-4) was the main active constituent of volatile oil of the black seed although it is accompanied by other analogous compounds such as Thymol and Thymoquinone dimer named as Dithymoquinone.

TQ has a variety of beneficial properties including analgesic and anti-inflammatory actions [63, 64], neuroprotection [65], and suppression of oxidative stress-induced neuropathy [66]. Moreover, it has been reported that TQ prevents oxidative injury in hepatocytes induced by carbon tetrachloride [67]. TQ also caused morphological improvements and prevented neurodegeneration against chronic toluene exposure [68], and showed significant anti-anxiety like activity through possible modulation of NO and GABA [69], and suppress nuclear factor kappa B (NF-KB) activation in brain and spinal cord [70]. Additionally, TQ exerts inhibitory effects on the cell proliferation in many types of cancer cells, including breast and ovarian adenocarcinoma and on angiogenesis and tumor growth at low dosages by blocking tumor angiogenesis [71, 72].

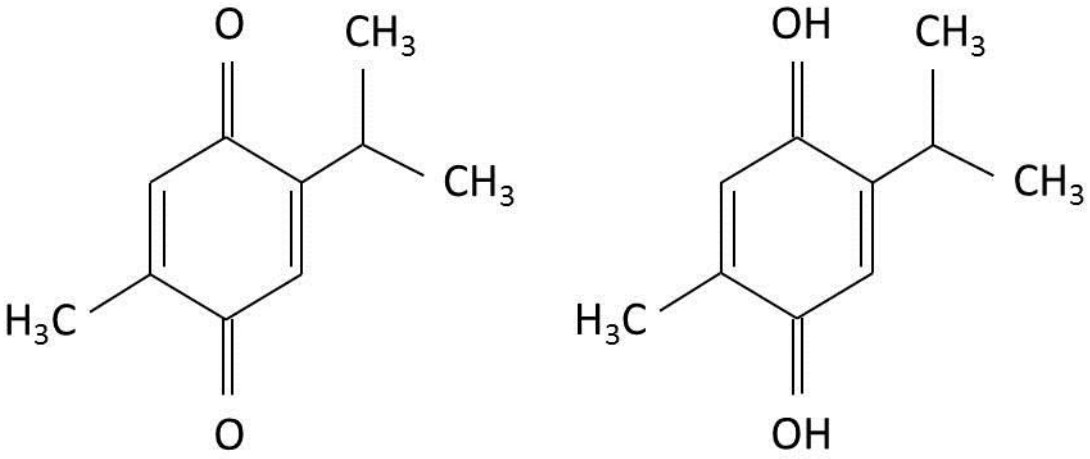


Figure 1-4. Chemical structure of Thymoquinone and Thymohydroquinone

1.8 Outline of this thesis

The present study was designed to investigate first as will be indicated in the second chapter of this thesis the possible protective effect of TQ against $A\beta_{1-42}$-induced neurotoxicity using rat primary and hiPSC-derived cholinergic neurons. We investigated cell viability, caspase 3/7 activities, mitochondria membrane potential (MMP), Glutathione (GSH) levels, reactive oxygen species (ROS) generation, synaptic activity, spontaneous firing activity, and the effect of TQ on the aggregation state of $A\beta_{1-42}$. In the third chapter of this thesis we investigated the possible protective effect of TQ against alpha synuclein (αSN)-induced synaptic damage using both rat hippocampal and hiPSC-derived neurons. We investigated synaptic protein (synaptophysin) levels, synaptic activity, and spontaneous firing activity. In the fourth chapter of this thesis we summarized our studies and the suggested mechanism for TQ protective effects.

Chapter 2

Inhibitory effect of thymoquinone against amyloid beta-induced neurotoxicity in rat primary and hiPSC-derived cholinergic neurons

2.1 Introduction

AD is the most common form of dementia in the elderly. It is a slowly progressive neurodegenerative disorder of the central nervous system, characterized by profound impairment of cognitive function and memory [73]. The Aβ peptide was initially identified and biochemically characterized in 1984 [74] as a peptide that aggregated and was deposited outside neurons in the brain tissue of Alzheimer's patients, leading to the formation of neuritic plaques in the AD brain. The presence of these neuritic plaques is the major pathological hallmark of AD. The Aβ peptide, a principal component of these plaques, is thought to play a central role in AD and is regarded as the causative agent in development of the disease. Soon after the identification of Aβ as the major component of amyloid plaques in AD, reports of both toxicity and trophic activity appeared [75]. The toxicity was reported to be related to the aggregation state of Aβ, with aggregation being required for toxicity [76, 77]. Aβ aggregate-mediated toxicity has been documented in vitro and in vivo.

Initial reports in 1994 showed that elevated oxidative stress, one of the early pathological events of AD, was mediated by hydrogen peroxide (H_2O_2) produced through the reduction of metal ions by Aβ peptides [78]. Furthermore, Aβ aggregate-mediated toxicity impairs synaptic function, which leads to the progressive memory loss and cognitive failure associated with AD which has been also well documented. Synaptic dysfunction is triggered by changes in synaptic structure and neurochemicals induced by oligomerized Aβ rather than amyloid plaques [79].

In addition to oxidative damage and synaptic failure, Aβ aggregates can induce mitochondrial dysfunction, which is another pathological hallmark of AD. The alteration of synaptic mitochondria, as a result of the buildup of Aβ, may underlie the synaptic pathology in AD [80]. Reported mitochondrialmal functions as a consequence of membrane localized Aβ include the inhibition of protein transport into mitochondria,

the disruption of the electron transport chain leading to impaired glucose utilization in neurons, and mitochondrial damage due to an increase in ROS production [81].

In recent years, the cholinergic system of neurons has been a main focus of research in aging and neural degradation, specifically as it relates to AD [82]. In addition, it is known that the dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections are among the earliest pathological events in AD [83]. In presenile (early onset), and in the advanced stages of late-onset AD, a severe loss of cortical cholinergic innervation has extensively been documented [84]. The brain of an individual with AD exhibits extracellular plaques of aggregated Aβ, intracellular neurofibrillary tangles that contain hyperphosphorylated tau protein and a profound loss of basal forebrain cholinergic neurons that innervate the hippocampus and the neocortex. Aβ accumulation may trigger or contribute to the process of this neurodegeneration [85]. It has been hypothesized that Aβ peptides induce neurodegenerative changes at cholinergic terminals [86], while other studies revealed that oligomeric Aβ causes cell death [87], encourage apoptosis by physically piercing the cell membrane, causes neurotoxic cascade and neurodegeneration that leads to AD [88, 89]. As previously reported by many studies, both AD and mild cognitive impairment brains have significantly decreased levels of antioxidant enzymes, making the brain more vulnerable to Aβ induced toxic effects [90]. Oxidative stress is also evident in AD brain by marked levels of protein, lipid, DNA, and RNA oxidation, neuronal dysfunction and death [91, 92]. Consequently, antioxidants have long been considered as an approach to slow down AD progression.

As an established historical and religion-based remedy for a wide range of health problems, *Nigella sativa* is one of the herbal medicines that is being actively investigated and is thus gaining worldwide recognition [93]. The seeds of *Nigella sativa L.*, commonly known as black seed or black cumin, has been used for medicinal purposes for centuries, both as herb and pressed into oil, in Asia, Middle East, and Africa. It has been traditionally used for various conditions and treatments related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support, and for general well-being [51]. Reviews have reported *Nigella sativa* as having antioxidant and neuroprotective effects in addition to many other therapeutic effects, such as antitumor, immune potentiation, anti-inflammatory, and antimicrobial [50, 68, 94]. Consuming antioxidant nutrients, such as *Nigella sativa*, could be one of the promising health strategies to help prevent the oxidative damage to cells, particularly in the brain regions, which are related to memory functions [95]. The seeds contain fixed and essential oils, proteins, alkaloids, and saponin. Much of the

biological activity of the seeds has been shown to be due to TQ, the major component of essential oil, which is also present in the fixed oil [49].

TQ is known to be the active principle responsible for many of the seed antioxidant and anti-inflammatory effects [96], which is frequently used in herbal medicine. TQ seems promising due to its many biological effects, which include antioxidant, anti-inflammatory, anticancer, and neuroprotection characteristics [97, 64, 65, 98] that may be beneficial in the management of AD. TQ was reported to counteract the induced oxidative stress in rats' brain tissue by reducing the levels of peroxidation, and enhancing the activities of enzymatic and non-enzymatic antioxidants, and protects PC12 cells against cytotoxic agents via attenuation of oxidative stress [99, 100]. A study demonstrated that TQ has a protective role against ethanol-induced neuronal apoptosis in primary rat cortical neurons [101], and suppression of oxidative stress-induced neuropathy [66]. TQ also causes morphological improvements and prevents neurodegeneration by chronic toluene exposure [67], shows significant anti-anxiety-like activity through possible modulation of nitric oxide (NO) and brain GABA content [68], and suppresses nuclear factor kappa B (NF-KB) activation in brain and spinal cord [69].

Searching for new compounds that slow down or stop the progression of Aβ is an important target of research in central nervous system drug development. And identifying a way to reduce the Aβ induced neurotoxicity would be beneficial for AD treatment. Although the precise mechanisms underlying Aβ induced neurotoxicity remains obscure, several lines of evidence suggest that they are associated with oxidative stress-dependent apoptosis [102]. Therefore, antioxidants are considered as potential drug candidates for amyloid hypothesis based therapy.

Accordingly, this study is designed to evaluate the possible protective effects of TQ against $A\beta_{1-42}$ induced neuronal toxicity using embryonic rat primary hippocampal and cortical neurons and hiPSC-derived cholinergic neurons. First, cell viability was assessed using the CellTiter-Glo assay. Then, we evaluated apoptosis measuring caspase-3, -7 activations. Next, we measured mitochondrial membrane potential (MMP) using Rhodamine 123, glutathione (GSH) level using GSH-Glo Glutathione assay, the production of ROS using DCFH-DA and ROS-Glo H_2O_2 assays . Because synaptic degeneration is one of the mechanisms underlying the neurotoxicity of Aβ, we investigated the role of TQ and $A\beta_{1-42}$ on synaptic vesicle recycling using FM1–43 dye, and on spontaneous firing activity using a multielectrode array system. To further clarify our results, we investigated the role of TQ in the cellular defense against $A\beta_{1-42}$ using a thioflavin T binding assay.

2.2 Materials and methods

2.2.1 $A\beta_{1-42}$ and TQ Preparation

1mM stock solutions of Amyloid β-Protein 1-42 (Peptide Institute, INC), molecular weight (4514.0), was made by dissolving the content of Aβ1-42 in the vial in 120µl Dimethyl Sulphoxide (DMSO, Wako) and store it at -20°C. 10mM stock solution of Thymoquinone (Sigma-Aldrich), molecular weight (164.20), was prepared by dissolving 1.642 mg of TQ in a solution of 0.3 ml DMSO and 0.7 ml culture medium. Final concentrations of TQ were prepared in culture medium. Cultures were treated in every experiment with a freshly prepared TQ.

2.2.2 Neurons viability

Brain atrophy caused by neuronal loss is a prominent pathological feature of AD. Aβ, the major component of senile plaques, is considered to play a central role in neuronal cell death. In addition the removal of the toxic Aβ, direct suppression of neuronal loss is an essential part of AD treatment; however, no such neuroprotective therapies have been developed. Excess amount of Aβ evokes multiple cytotoxic mechanisms, involving increase of the intracellular Ca^{2+} level, oxidative stress, and receptor-mediated activation of cell-death cascades. Such diversity in cytotoxic mechanisms induced by Aβ clearly indicates a complex nature of the AD-related neuronal cell death [103].

In order to investigate the protective effect of TQ against $A\beta_{1-42}$ induced neurotoxicity, we first investigated its effect against $A\beta_{1-42}$ induced cell death. We used CellTiter-Glo Luminescent Cell Viability Assay, a homogenous method for determining the number of viable cells in a culture based on quantitation of ATP, which signals the presence of metabolically active cells. The CellTiter-Glo Luminescent Cell Viability assay uses luciferase as the detection enzyme because of the absence of endogenous luciferase activity in mammalian cells. The Luciferase used in the CellTiter-Glo Luminescent Cell Viability Assay kit generates a stable, glow-type signal that has a half-life of greater than four hours. Luciferase enzyme requires ATP in order to generate light. Metabolically active cells produce ATP as energy for respiration and other vital processes. After an equal volume of CellTiter-Glo Reagent is added to the cell culture, luminescence is measured. Light signal is proportional to the amount of ATP present which correlates with the number of viable cells present. The CellTiter-Glo assay was used to determine the effect of various concentrations of $A\beta_{1-42}$ on the cell viability and the protective effect of TQ on the cell death.

CellTiter-Glo (Promega); reagent was prepared by transferring the contents of one bottle of CellTiter-Glo Buffer to one bottle of CellTiter-Glo Substrate, mixed by inversion until the substrate is thoroughly dissolved. Reconstituted reagent was used on the same day it is prepared or stored at -20°C. Primary embryonic Wistar rat hippocampal neurons were prepared and harvested on a Poly-$_D$-lysine (PDL, 0.1 mg/ml in deionized water, MW150,000 - 300,000, Sigma–Aldrich) coated 96-microwell plate (Nunc) in Neurobasal Medium, density at 2×10^4 cell/well. Plates were incubated at 37°C, 5% CO_2. $A\beta_{1-42}$ was applied to cultures on 13 DIV in a series of concentrations (2, 5, 10 μM) and cell survival was assessed 72 hours later. To investigate the effect of TQ on the survival rate of hippocampal neurons, cultures were treated with TQ (0.1, 1,

10,100 nM) on culture day 13 for 72 hours. Finally, to investigate the neuroprotective potential of TQ against $A\beta_{1-42}$ toxicity, cultures were treated with TQ and $A\beta_{1-42}$ simultaneously for 72 hours.

hiPSC-derived cholinergic neurons (RCESDA001, ReproCell) were cultured at a density of 3.0×10^4cell/well in 96-microwell plates, treated with $A\beta_{1-42}$ (5 μM) with or without TQ (100 nM) for 48 hours on culture day 13. On experiment day, the plate and contents were equilibrated to room temperature for approximately 30 minutes. An equal volume to the culture medium of CellTiter-Glo Reagent was added, and mixed gently for 2 minutes, then incubated at room temperature for 10 minutes to allow luminescent signal to stabilize. Microplate reader (TECAN) was used for measuring Relative Light Units (RLU) values.

2.2.3 Caspase-3 and -7 activities

Caspases (cysteinyl aspartate-specific proteases) are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation. Caspases are essential in cells for apoptosis in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. Some caspases are also required in the immune system for the maturation of lymphocytes. Failure of apoptosis is one of the main contributions to tumor development and autoimmune diseases; this, coupled with the unwanted apoptosis that occurs with ischemia or AD, has stimulated interest in caspases as potential therapeutic targets since they were discovered in the mid-1990s. Caspases have been broadly classified by their known roles in apoptosis (caspase-3, -6, -7, -8, and -9 in mammals), and in inflammation (caspase-1, -4, -5, -12 in humans and caspase- 1, -11, and -12 in mice). Caspases involved in apoptosis have been sub classified by their mechanism of action and are either initiator caspases (caspase- 8 and -9) or executioner caspases (caspase-3, -6, and -7) [104].

Caspase-3 is a caspase protein that plays a central role in the execution-phase of cell apoptosis. A previous study revealed that comparing to other proteinases; caspase-3 is the primary activator of apoptotic DNA fragmentation [105] which is the best recognized biochemical event of apoptosis [106, 107]. This result was consistent with another study that demonstrated that caspases-3 and -7, but not caspases-6 and -8, inactivate recombinant DFF45, releasing active DFF40 which lead to chromatin condensation and DNA fragmentation and therefore apoptosis [108]. Neuronal death in a variety of neurodegenerative diseases, including AD, has been associated with deregulated caspase activation [109]. However, several lines of evidence suggest that the role of caspases in AD may involve more than just action as cellular executioners driven by upstream disease processes. Caspase-mediated cleavage of APP has been reported [110], as has Aβ peptide induced apoptosis by the activation of caspase-3 [111, 112].

Under normal circumstances, caspases recognize tetra-peptide sequences on their substrates and hydrolyze peptide bonds after aspartic acid residues. Caspase 3 and caspase 7 share similar substrate specificity by recognizing tetra-peptide sequence DEVDG (Asp-Glu-Val-Asp-Gly) with cleavage occurring on the carboxy side of the second aspartic acid residue (between D and G) [113, 114]. The C-terminal Asp is absolutely required while variations at other three positions can be tolerated [115]. Caspase substrate specificity has been widely used in caspase based inhibitor and drug

design [116].

In this experiment we investigated the effect of $A\beta_{1-42}$ and TQ on caspase-3 and -7 activates using Caspase-Glo 3/7 Assay (Promega), a homogeneous, luminescent assay that measures caspase-3 and -7 activities. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetra-peptide sequence "DEVD", which is preferentially recognized by caspase-3 and -7 in a reagent optimized for caspase activity, luciferase activity and cell lysis. Adding a single Caspase-Glo 3/7 Reagent results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

hiPSC-derived cholinergic neurons were treated with $A\beta_{1-42}$ (5 μM) with or without TQ (100 nM) for 48 hours on culture day 13. On the day of the experiment, Caspase-Glo3/7 Buffer and lyophilized Caspase-Glo 3/7 Substrate were equilibrated to room temperature. The contents of the Caspase-Glo 3/7 Buffer bottle were transferred into the amber bottle containing Caspase-Glo 3/7 Substrate, mixed by swirling or inverting the contents until the substrate was thoroughly dissolved to form the Caspase-Glo 3/7 Reagent. Cell culture plate was removed from the incubator and plate was allowed to equilibrate to room temperature for 10 minutes. 100 μl from Caspase-Glo 3/7 Reagent was added to each well. The contents of wells were gently mixed using a plate shaker at 300–500rpm for 30 seconds, and then incubated at room temperature for 1 hour. Luminescence was measured using a microplate reader (TECAN).

2.2.4 Mitochondrial membrane potential

Biochemical and morphological alterations of mitochondria may play an important role in the pathogenesis of AD. Particularly, mitochondrial dysfunction is a hallmark of Aβ-induced neuronal toxicity in AD. Studies of postmortem brains from AD patients and transgenic mouse models of AD suggest that oxidative damage, induced by Aβ, is associated with mitochondria early in AD progression [81]. Evidence from AD postmortem brain as well as cellular and animal AD models showed that Aβ triggers mitochondrial dysfunction through a number of pathways such as impairment of oxidative phosphorylation, elevation of ROS production, alteration of mitochondrial dynamics, and interaction with mitochondrial proteins [117]. A decline in protein import seems to precede increased ROS and decreased MMP suggesting a gradual failure of mitochondria [117]. Moreover, Aβ interacts with mitochondrial matrix components inducing an improper mitochondrial complex function leads to a decreased MMP of the organelle [118] and impairing ATP formation [119]. Previous data demonstrated mitochondrial dysfunction through reduction of MMP in a novel triple transgenic AD mouse model [120]. Cationic probes appear to exhibit a potential-dependent interaction with the native mitochondria of living cells and may reflect intercellular variations in MMP based on variations in the intensity of mitochondria-associated fluorescence [121].

Rhodamine123 **(**Rh123) is a cationic cell-membrane permeable fluorescent dye that selectively localizes in mitochondria of viable cells. Rh123 also is positively charged at physiological pH and does not appear to have cytotoxic effects. In screening a number of Rhodamine compounds it has become apparent that only those that are positively charged at physiological pH-namely, 123, 6G, and 3B-are able to stain mitochondria specifically whereas uncharged Rhodamines (such as B, 19, 110, and 116) and the negatively charged compound fluorescein do not. These results suggest that an attraction of cationic Rhodamine molecules by the relatively high negative electric potential across the mitochondrial membrane may be the basis for the selective staining of mitochondria by Rh123 in living cells [56].

Therefore, in this study we used Rh123 to investigate the effect of $A\beta_{1-42}$ and TQ on MMP in hippocampal neurons. In this experiment primary hippocampal neurons were cultured on a PDL coated 96-microwell plate (density at 2×10^4 cell/well) for 13 days in MACS Neuro Medium, and incubated at 37°C, 5% CO2. Rhodamine123 (Sigma-Aldrich), molecular weight (380), stock solution of (1 μg/ml) was made by dissolving the whole content into 400 μl DMSO, covered with aluminum foil and kept

in -20°C. On the experiment day final (1 nM/ml) solution was made in Phosphate buffer saline (PBS). Cultures were treated with $A\beta_{1-42}$ (10 μM) and TQ (100 nM) on culture13 DIV for 72 hours. On experiment day, after the medium was removed, the cells were incubated with Rh123 (1 nM) by adding 100 μl in each well for 15 minutes at 37°C, and then the cells were washed with PBS to remove the extracellular Rh123. The cells were then suspended in PBS. The fluorescence intensity (relative fluorescence unit) was monitored using TECAN microplate reader with excitation at 485 nm and emission at 535 nm.

2.2.5 Glutathione levels

GSH (L-gamma-glutamyl-L-cysteinylglycine) is a tripeptide present in large quantities in all mammal cells and in small amounts extracellularly [122], being mainly located in the cytosol, mitochondria, and endoplasmic reticulum [123]. It plays a very important role in many biological processes involved in organism homeostasis, most notably, in neutralizing the free radicals that produce ROS (due to its great antioxidant activity) [124-127], since oxidation is a basic process in the genesis of neurodegenerative disorders [128].Glutathione exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms [129]. GSH is the most important component of the antioxidant mechanism of the brain [130]. An increase in cellular GSH concentration makes the neurons more resistant to cytotoxic injuries [131-133]. The progressive decrease of GSH levels resulting from aging and related illnesses is of great interest for investigators [134]. The decrease found with aging is linked to an increase of ROS [135]. There is scientific evidence of a relationship between aging and a number of neurodegenerative processes due to the excessive production of free radicals and the imbalance between the oxidant species and antioxidant defenses [136, 137]. GSH levels are decreased in diseases with oxidative stress -including AD- and with age [138]. Oxidative stress, the imbalance between antioxidants and ROS, has been increasingly implicated as an important causative factor in various neurodegenerative diseases including AD [139].

Aβ has been shown to be a source of free radical oxidative stress that may lead to neurodegeneration. Aβ, perhaps in concert with bound redox metal ions, initiates free radical processes resulting in protein oxidation, lipid peroxidation, ROS formation, cellular dysfunction leading to calcium ion accumulation and subsequent neuronal death [140]. A previous study showed the potential role of free radical toxicity and the damage of cholinergic neurons in the Aβ-treated mice as there was an increased lipid peroxidation by elevated malondialdehyde – a reactive species and a marker of oxidative stress- and decrease of GSH levels [141]. Neuronal cell dysfunction and oxidative cell death caused by Aβ contribute to the pathogenesis of AD [142] According to the oxidative stress hypothesis of AD, the Aβ inserts into the neuronal membrane bilayer and generates oxygen-dependent free radicals and then causes the lipid peroxidation and protein oxidation. The loss of membrane integrity leads to cellular dysfunction, such as loss of Ca^{2+} homeostasis, disruption of signal pathways and activation of nuclear transcription factors and apoptotic pathways. The neuronal death is the ultimate consequence of these cellular dysfunctions [143].

In this study we investigated the effect of $A\beta_{1-42}$ and TQ on oxidative stress by assessing GSH levels in hiPSC-derived cholinergic neurons. Oxidative stress was assessed through measurement of the GSH using the GSH-Glo™ Glutathione assay (Promega). The GSH-Glo™ Glutathione assay is a luminescence-based assay for the detection and quantification of GSH. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH, catalyzed by glutathione S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH present in the sample.

In this experiment hiPSC-derived cholinergic neurons were treated with $A\beta_{1-42}$ (5 μM) with or without TQ (100 nM) for 48 hours on culture day 13. On experiment day the culture medium was removed from the wells and 100 μM form the prepared GSH-Glo reagent was added to each well and then, the contents were mixed briefly using a plate shaker and incubated for 30 minutes at room temperature. Then, 100 μM of reconstituted luciferin detection reagent was added to each well. The contents were mixed briefly using a plate shaker. Plate was incubated for 15 minutes and luminescence signals were measured using a microplate reader (TECAN).

2.2.6 ROS generation

AD brain is under extensive oxidative stress as measured by protein oxidation and lipid peroxidation. $A\beta_{1-42}$ has been shown to induce protein oxidation and lipid peroxidation in vitro and in vivo [144]. A study demonstrated that both oligomers and fibrils induced ROS production inside the cells [145]. Moreover, the incubation of cortical neurons with low concentrations of soluble $A\beta_{1-40}$ results in an early time-dependent increase in the ROS production, preceding both microtubule perturbation and caspase activation [146]. Additionally, increasing data suggest that age-related oxidative stress contributes to degenerative changes in basal forebrain cholinergic systems as levels of Aβ increased [147]. It has been reported that Aβ impairs mitochondrial redox activity and increases the generation of ROS [148]. Several studies also suggest that Aβ-induced oxidative stress leads to apoptotic neuronal cell death that can be inhibited by antioxidants [149]. Taken together, these findings support the central role of $A\beta_{1-42}$ in the pathogenesis of AD as a mediator of oxidative stress.

Numerous studies demonstrated that TQ protect against oxidative stress through its antioxidant properties. Treatment with TQ increased resistance to oxidative stress and proved to be beneficial in restoring declined superoxide dismutases (SOD) and catalase due to ischemia insult. Moreover, TQ significantly improved antioxidant status and reduced lipid peroxidation in rat hippocampus [150]. Furthermore, TQ have inhibitory effects against lipid peroxidation process during cerebral ischemia-reperfusion injury in rat hippocampus [151]. In addition, TQ protected liver and kidney tissues of diabetic rats from peroxidative changes through increase in the levels of antioxidant enzymes and low molecular weight antioxidants in TQ treated diabetic rats [152]. Additionally, TQ supplementation prevents the development of diethylnitrosamine -induced initiation of liver cancer by decreasing oxidative stress and preserving both the activity and mRNA expression of antioxidant enzymes [153].

To examine whether the protective effect of TQ on the toxicity of $A\beta_{1-42}$ is mediated by antioxidant ability, first the level of intracellular ROS was determined in rat cortical neurons by the change of the fluorescent probe Dichlorofluorescin diacetate (DCFH-DA). When apply to intact cells, DCFH-DA readily diffuses through the cell membrane and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescin (DCF). DCF Fluorescent intensity is proportional to the amount of ROS formed intracellulary.

In this experiment primary cortical neurons were cultured in a PDL coated 96-microwell plate (density at 2×10^4 cell/well) for 13 days in MACS Neuro Medium, incubated at 37°C, 5% CO_2. Dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, molecular weight: 487.29) was prepared by dissolving 0.0097 g in 2ml DMSO to make a 100 μM stock solution. Cultures were treated with $A\beta_{1\text{-}42}$ (10 μM) and TQ (100 nM) on culture day 13. After the medium was removed, the cells were incubated with 100 μM DCFH-DA for 30 minutes at 37°C, and the cells were washed with PBS to remove the extracellular DCFH–DA. The cells were then suspended in PBS and the fluorescence signals were determined using a TECAN microplate reader at the excitation wavelength of 485 nm and emission wavelength of 535 nm [154].

Next, the effect of $A\beta_{1\text{-}42}$ and TQ on intracellular ROS level was determined in hiPSC-derived cholinergic neurons by ROS^{TM} H_2O_2 assay (Promega). H_2O_2 is a reactive oxygen species that is measured in cells as a marker of oxidative stress. It is also measured as a marker of enzyme activities that either consume or produce H_2O_2. It is desirable to screen chemical compounds for their capacity to alter H_2O_2 levels in cultured cells or for their effects on H_2O_2 levels in enzyme reactions. The assay determines ROS level by measuring H_2O_2 concentration. An H_2O_2 Substrate is employed that reacts directly with H_2O_2 to generate a luciferin precursor. Upon addition of ROS-Glo™ Detection Reagent containing Ultra-Glo™ Recombinant Luciferase and D-Cysteine, the precursor is converted to luciferin by the D-Cysteine, and the produced luciferin reacts with Ultra-Glo™ Recombinant Luciferase to generate a luminescent signal that is proportional to H_2O_2 concentration.

hiPSC-derived cholinergic neurons were treated with $A\beta_{1\text{–}42}$ (5 μM) with or without TQ (100 nM) for 48hours on culture day 13. After 42 hours of treatment, H_2O_2 Substrate solution was prepared and added to the cells. Then, the plate was incubated at 37 °C for 6 hours. 100 μl of ROS-Glo™ Detection Solution was added to each well. The plate was incubated for 20 minutes at room temperature. Relative luminescence units were recorded using a microplate reader (TECAN).

2.2.7 Synaptic vesicles recycling

Communication between neurons involves the fusion of a subpopulation of synaptic vesicles with the cell membrane at a specialized region of the nerve terminal called the active zone or release site, thereby releasing their contents (exocytosis or synaptic vesicle fusion). The released synaptic vesicles are then retrieved from the plasma membrane (endocytosis or synaptic vesicle retrieval), refilled with fresh neurotransmitters, and trafficked back to rejoin the existing pool of 200–500 vesicles to wait to do it all again. The whole process is often called synaptic vesicle recycling.

Styryl dyes are typically used in the same manner to investigate synaptic vesicle recycling [155]. When incubated with neuronal preparations, the dyes insert into the outer leaflet of the plasma membrane and are internalized during vesicle retrieval after stimulation of exocytosis. The remaining external dye can be washed quickly away, leaving fluorescently labeled synaptic vesicles inside the nerve terminals (Fig.2-1). There is a range of styryl dyes available. The more hydrophilic probes FM1-43 (Fig.2-2) and FM2-10 are most widely used, as they possess a short half-time ($t1/2$) in membrane, allowing non-internalized dye to be washed off neurons quickly. This is an important consideration for reliable quantification of exocytosis because the dye has to dissociate fully from the vesicle membrane upon fusion. Styryl dyes allow visualization of the fusion of synaptic vesicles with the plasma membrane in nerve terminals by fluorescent imaging of regions loaded with previously accumulated dye, followed by monitoring of a stimulated dye release. Styryl dyes can also reveal a locus of action of proteins or drugs in synaptic vesicle recycling, often at steps previously assumed to be unaffected. For example, the addition of $A\beta_{1\text{-}42}$ oligomers affected αSN protein–induced inhibition of synaptic vesicle recycling. Thus, pre-mixing αSN with $A\beta_{1\text{-}42}$ oligomers enhanced αSN -induced inhibition of FM1-43 uptake into synapses [156]. Also the exocytosis of FM1-43 was significantly reduced by chronic application (24h) of 500nM Aβ [157].

Therefore, using FM1-43 dye, we investigated the protective effect of TQ against $A\beta_{1\text{-}42}$ induced synaptic damage. In this experiment, primary cortical neurons were cultured on a PDL coated 24-microwell plate (IWAKI) in Neurobasal medium, density at 3.36×10^5 cell/well, incubated at 37°C, 5% CO_2. On culture day 13 cortical neurons were treated with $A\beta_{1\text{-}42}$ (2 μM) only or with TQ (100 nM) simultaneously for 72 hours. FM1-43 (Molecular Probes) was prepared by dissolving 100μg from the dye in 100μl methanol and stored at -20°C. Artificial cerebrospinal fluid (ACSF) is commonly used when sampling from brain interstitial fluid. This solution closely matches the electrolyte

concentrations of CSF. It is prepared from high purity water and analytical grade reagents: NaCl 126 mM, KCl 90 mM, KH_2PO_4 1.3 mM, Glucose 10 mM, $NaHCO_3$ 2.6 mM, $MgSO_4$ 1.4 mM, $CaCl_2$ 4.2 mM. On experiment day, HEPES 10 mM was added to prepared ACSF solution; pH was adjusted to 7.4, and then kept at 4°C. ACSF and FM1-43 (1 μg/ml) solution was made by adding 1 μl from the dye to each 1ml of ACSF and warmed in water bath at 37°C. Culture medium was removed from each well and treated cultures were incubated at 37°C with ASCF/FM1-43 solution for 10 minutes, washed 5 times in ice cold PBS and solubilised in methanol at 1×10^6 neurons/ml. Soluble extracts were transferred into black 96-microwell plate. The fluorescence intensity was determined using a TECAN microplate reader at the excitation wavelength of 485 nm and emission wavelength of 612 nm.

hiPSC-derived cholinergic neurons were treated with $A\beta_{1-42}$ (5 μM) with or without TQ (100 nM) for 72 hours on culture day 13. On experiment day, the culture medium was removed from the treated samples and the prepared ACSF/FM1-43 solution added to each well and incubated for 5 minutes at 37°C. The dye solution was removed from the dish and the samples were washed twice with cold PBS, suspend in PBS. Fluorescent signal was measured; excitation at 480 nm, emission at 612 nm using a microplate reader (TECAN).

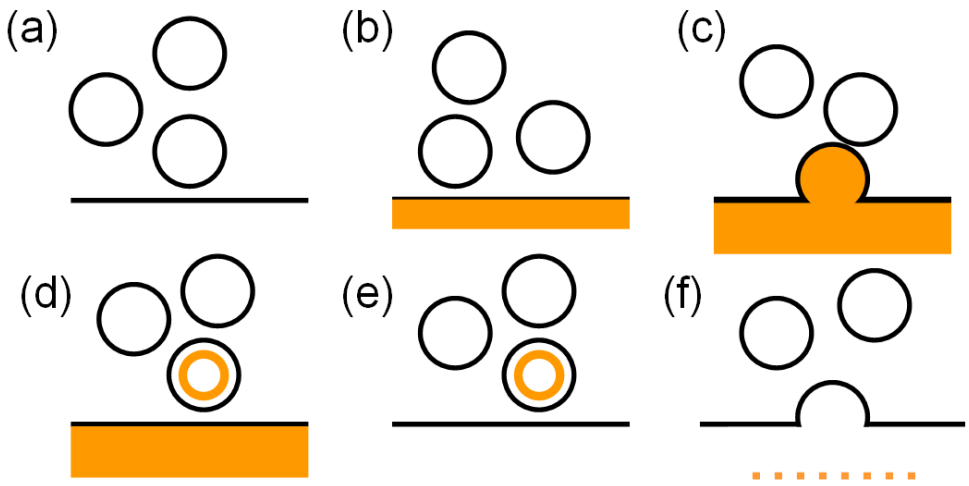


Figure 2-1. Typical FM dye experiment. (a) Synaptic vesicles near the plasma membrane. (b) FM dye is added, binds to the outer membrane, and becomes fluorescent. (c) The preparation is stimulated and a vesicle fuses with the plasma membrane exposing the luminal membrane to the FM dye. (d) The vesicle is endocytosed with FM dye inside. (e) The FM dye is washed out of the bath and labelled vesicle is imaged. (f) The preparation is stimulated again in a dye-free medium and vesicles exocytosis is measured as dye leaves the fusing vesicle.

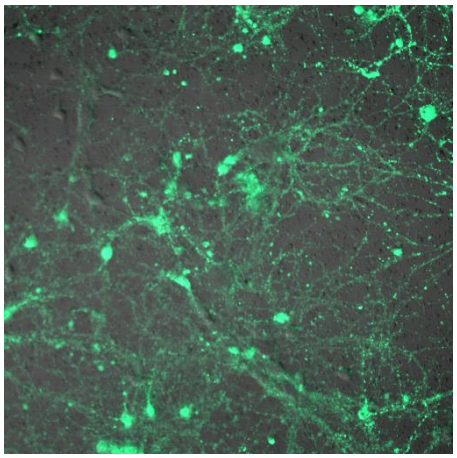


Figure 2-2. Labeling of synaptic vesicles recycling. Note that the FM1-43 fluorescence is concentrated at sites where neurites cross over and interact, indicating the sites of active synaptic vesicle recycling.

2.2.8 Spontaneous firing activity

Many fields of neuroscience research investigating long term processes share a common problem. Although numerous methods are available to assess parameters like cell vitality, neurite outgrowth or the expression of proteins, long term monitoring of electrophysiological properties is often not possible. Traditional electrophysiological *in-vitro* techniques are generally limited to recording periods of a few hours at most. However, by combining substrate integrated microelectrode arrays (MEA) with cell and tissue culture techniques, this limitation can be overcome [158]. Since its introduction in 1972 [159], MEA technology and the related culture methods for electrophysiological cell and tissue assays have been vastly improved. MEA is an electrochemical biosensor developed to detect the action potential in the extracellular microenvironment of cells. On an MEA, a thin metallic film is fabricated between a substrate of glass or silicon and a passivation layer with several electrode sites exposed for sensing the extracellular field potential changes generated by the objective cells (Fig.2-3). When spreading on the microelectrodes, cultured cells adhere to the substrate. But there is still a minute volume of electrolyte between the cells and the microelectrodes; thus, a solid-liquid interface on the electrode surfaces is formed. The electrochemical properties of the interface are the basis of the sensing mechanism of MEA [160]. Thus, MEA electrodes facilitate non-invasive stimulations and recordings from cells and networks, enabling repeated recordings from the same cells over extended periods of time.

Neurons create ion currents through their membranes when excited, causing a change in voltage both inside and outside the cell. When recording, the electrodes on MEA transduce the change in voltage from the environment carried by ions into currents carried by electrons (electronic currents). When stimulating, electrodes transduce electronic currents into ionic currents through the media. This triggers the voltage-gated ion channels on the membranes of the excitable cells, causing the cell to depolarize and trigger an action potential. MEA have been in use to study multiple aspects of electrically excitable cells. In particular, MEA have been applied to explore the pharmacological and toxicological effects of numerous compounds on spontaneous activity of neuronal and cardiac cell networks. The MEA system enables simultaneous extracellular recordings from as many as 64 sites in the network in real time, increasing spatial resolution and thereby providing a robust measure of network activity. Neuronal network grown on MEA responds to neuroactive compound sensitively with changes in their native, spontaneous activity patterns. This altered activity is often substance- and

concentration-specific. The influence includes direct metabolic effects, specific synaptic effects, transmission effects that stop action potential propagation and generic mebrance effects mediated through non-synaptic Ca^{2+} or K^{+} channels or by the generation of new channels [160].

AD patients show alterations in both neuronal network oscillations and the cognitive processes associated to them. Related to this clinical observation, it has been found that Aβ could lead to differential changes in neural network activity (even overexcitation) by forming aggregates with different sizes that produce differential effects on network activity [161]. Aβ was also found to inhibit spontaneous network activity in the olfactory bulb of mice and rats [162]. Using MEA a study demonstrated that at all measured concentrations, Aβ completely abolishes spontaneous spiking activity. The same study showed that MEA can be used to reliably detect functional effects of low doses of Aβ (100 nM) as well as screen for the rescue effect of the Aβ oligomerization inhibitor, curcumin [163]. Moreover, it has been well established that neurons cultivated on MEA form networks where extracellular action potentials or spikes can be monitored noninvasively to quantify the functional effects of neuroactive compounds [164].

Therefore, we used the MEA system (ALPHA MED SCIENTIFIC, Japan) to evaluate the effect of TQ and $A\beta_{1\text{-}42}$ on the action potentials of hippocampal neurons and hiPSC-derived cholinergic neurons. MEA probes (MED-P530A, ALPHA MED SCIENTIFIC, Japan) were PDL-coated for 24 hours at 37°C. After rinsing PDL, the MEA probe was dipped into solid state 2.25% (w/v) agarose (BM Bio, Japan) and spread using a spin-coater (MIKASA CO. LTD) at 2500 rpm for 20 seconds to form a 5μm thick agarose layer. To obtain the best recording results, the portion of agarose layer upon each electrode was etched by spot heating using a 1064-nm infrared laser to form micro chambers [165,166]. Hippocampal neurons were cultured at a density of 1×10^{6} neurons/mm^2. The MEA probe was placed in sterile-petri dish, and then incubated at 37 °C, 5% CO_2. After 24 hours. On culture day 13, cultures were exposed to $A\beta_{1\text{–}42}$ (2 μM) or to $A\beta_{1\text{–}42}$ and TQ (100 nM). Measurements were performed before applying TQ and $A\beta_{1\text{-}42}$ for 24 hours and directly after applying TQ and $A\beta_{1\text{-}42}$ for 72 hours using Mobius software. In every hour the measurements were obtained for 5 minutes and the firing frequency average for the 5 minutes measured was calculated.

hiPSC-derived cholinergic neurons were plated on the MEA probes (MED-P515A, Alpha MED Scientific) seeded at 1.0×10^{6} cells/cm^2. Recordings were obtained for 10 minutes at four times per day using MED64 software (Alpha MED Scientific). Cultures were treated with $A\beta_{1\text{-}42}$ (5 μM) with or without TQ (100 nM) on culture day 13. Firing

analyses were performed for number of firings, which obtained at 64 electrodes.

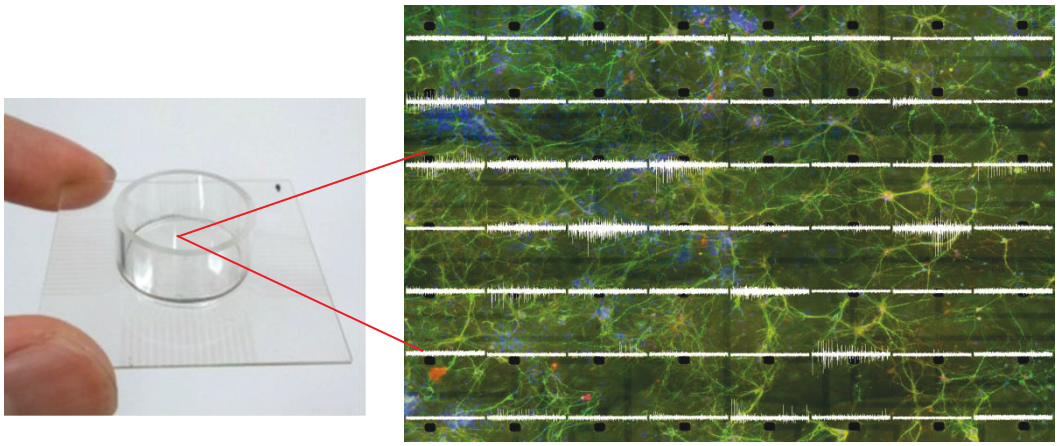


Figure 2-3. Microelectrode array probe with 64 microelectrodes embaded in the bottom of the dish (rat hippocampal neurons (20DIV)).

2.2.9 Effect of TQ on $A\beta_{1-42}$ aggregation

A number of small organic molecules have been developed that bind to amyloid fibrils, a subset of which also inhibit fibrillization. Among these, the benzothiol dye Thioflavin T (ThT) has been used for decades in the diagnosis of protein-misfolding diseases and in kinetic studies of selfassembly (fibrillization). Since its introduction in 1959, the benzothiol dye ThT has been a cornerstone in diagnosing amyloid fibrils [167]. Its dramatic increase in fluorescence upon binding to β-sheet-rich deposits has made it an essential tool for staining amyloid in tissue samples, as well as in real-time monitoring of self-assembly kinetics in vitro [168-170]. It is also used regularly to quantify the formation and inhibition of amyloid fibrils in the presence of anti-amyloidogenic compounds such as polyphenols [171].

The Aβ aggregation into insoluble amyloid fibrils occurs through a number of intermediate structural forms such as soluble oligomers or protofibrils. All these Aβ species may differentially affect neuronal function and viability in vitro and in vivo. The Aβ fibrils induced neurodegenerative changes, including neuronal cell death, apoptosis, oxidative stress, and calcium deregulation [172]. Therefore, prevention of Aβ aggregation and/or decomposition of existing oligomers and fibrils by small molecule compounds are promising strategies for the prevention and treatment of AD. Many compounds have been found to reduce Aβ aggregation or neurotoxicity in vitro, such as oligopeptides, polyphenols, antibodies, antioxidants and non-steroidal anti-inflammatory drugs. While there are no effective treatments for AD currently, substances that can efficiently inhibit the amyloid formation by interfering with the process have been sought as drug candidates for treating AD.

Therefore, we investigated the effect of TQ on $A\beta_{1-42}$ amyloid formation by using ThT induced fluorescence. 1 mM ThT (Sigma, molecular weight 318.86) stock solution was prepared by dissolving 0.00159g from the dye into glycine-NaOH buffer, (50 mM, pH 8.5), stored at room temperature covered with aluminum foil. At the day of experiment a working solution of 100nM in glycine-NaOH buffer, (50 mM, pH 8.5) was prepared. $A\beta_{1-42}$ (10 μM) was incubated in an eppendorf tube alone or with TQ (100 nM) in PBS, and then incubated at 37°C, 5% CO_2 for 72 hours. After 72 hours samples were vortex for 10 seconds and tubes content was moved to a 96-microwell plate. 100nM ThT was added to each well, mixed gently, and analyzed using TECAN microplate reader at the excitation wavelength of 450 nm and emission wavelength of 535 nm.

2.3 Results

2.3.1 Effect of $A\beta_{1-42}$ on hippocampal neurons viability

Treatment of cultures with $A\beta_{1-42}$ (2, 5, 10 μM) for 72 hours induced a decrease in hippocampal neurons viability in a dose dependant manner with a reduction in viability (15%, 25%, 42%) respectively comparing with control (Fig.2-4).

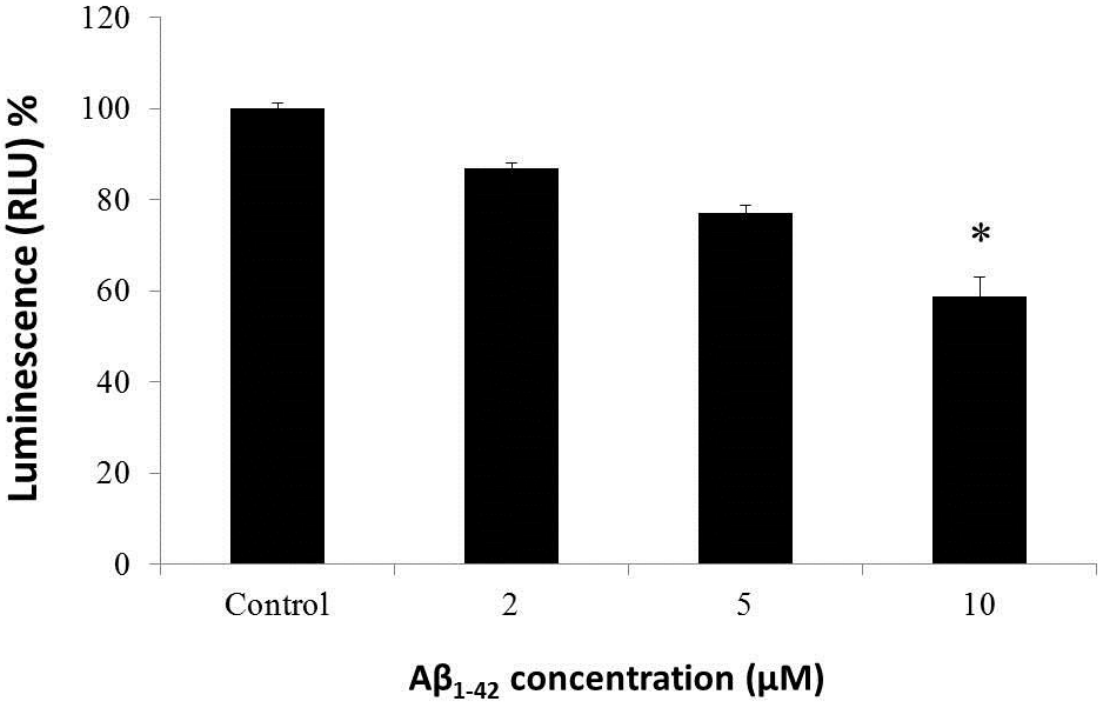


Figure 2-4. Effect of different concentrations of $A\beta_{1-42}$ on hippocampal neurons viability. Hippocampal neurons were treated with $A\beta_{1-42}$ (2, 5, 10 μM) for 72h. Cell viability was assessed by CellTiter-Glo assay. (*P< 0.01 vs. control), n = 6. Values shown are the mean percent luminescence (where 100% = luminescence in control hippocampal neurons), ± Standard Error (SE).

2.3.2 Effect of TQ on hippocampal neurons viability

In (Fig.2-5) treatment of cultures with TQ (0.1, 1, 10,100 nM) produced no significant effect on the survival rate of hippocampal neurons.

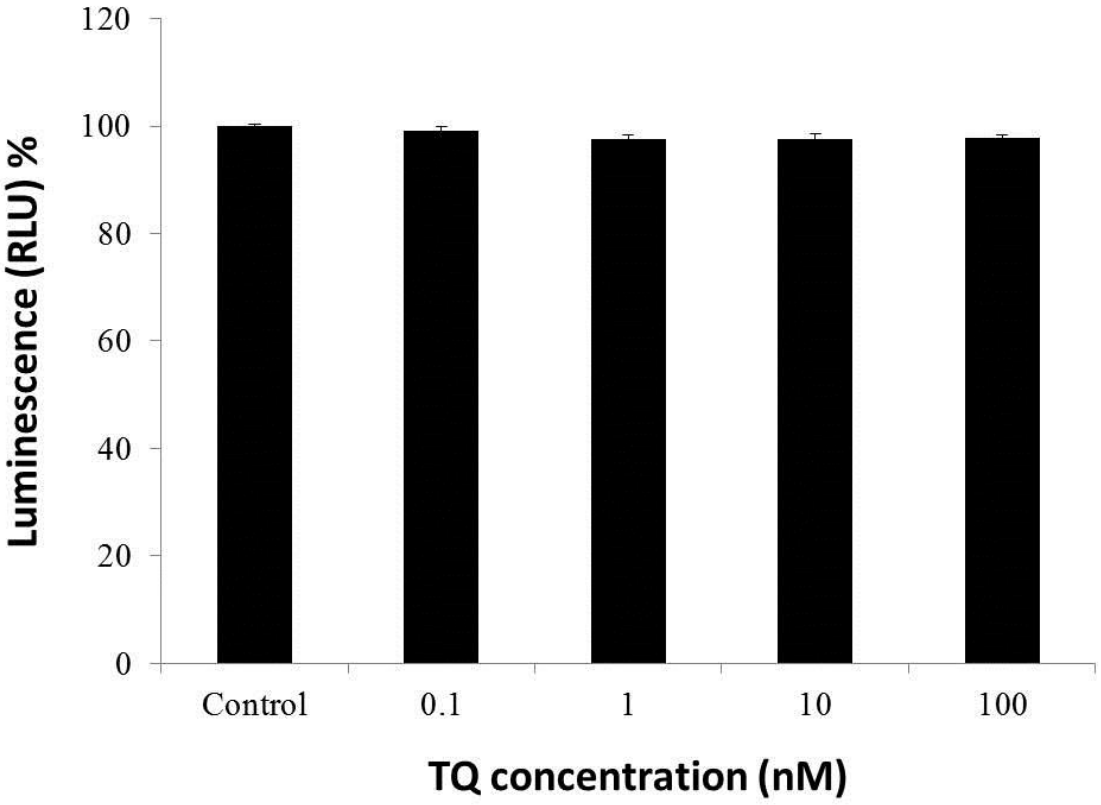


Figure 2-5. Effect of different concentrations of TQ on hippocampal neurons viability. Hippocampal neurons were treated with TQ (0.1, 1, 10, 100nM) for 72h. Cell viability was assessed by CellTiter-Glo assay. Values shown are the mean percent luminescence (where 100% = luminescence in control hippocampal neurons) ± SE, n = 6.

2.3.3 Effect of TQ against $A\beta_{1-42}$ –induced cell death

Treatment of cultures with $A\beta_{1-42}$ (10 μM) induced cytotoxicity in hippocampal neurons. Co-treatment with TQ (0.1, 1, 10,100 nM) resulted in the reduction of $A\beta_{1-42}$-induced cytotoxicity and significantly increased cell viability (Fig.2-6). TQ increased cell viability in a dose-dependent manner with only 10% loss in viability in samples treated with TQ (100 nM) comparing to 45% cell loss in $A\beta_{1-42}$ treated samples.

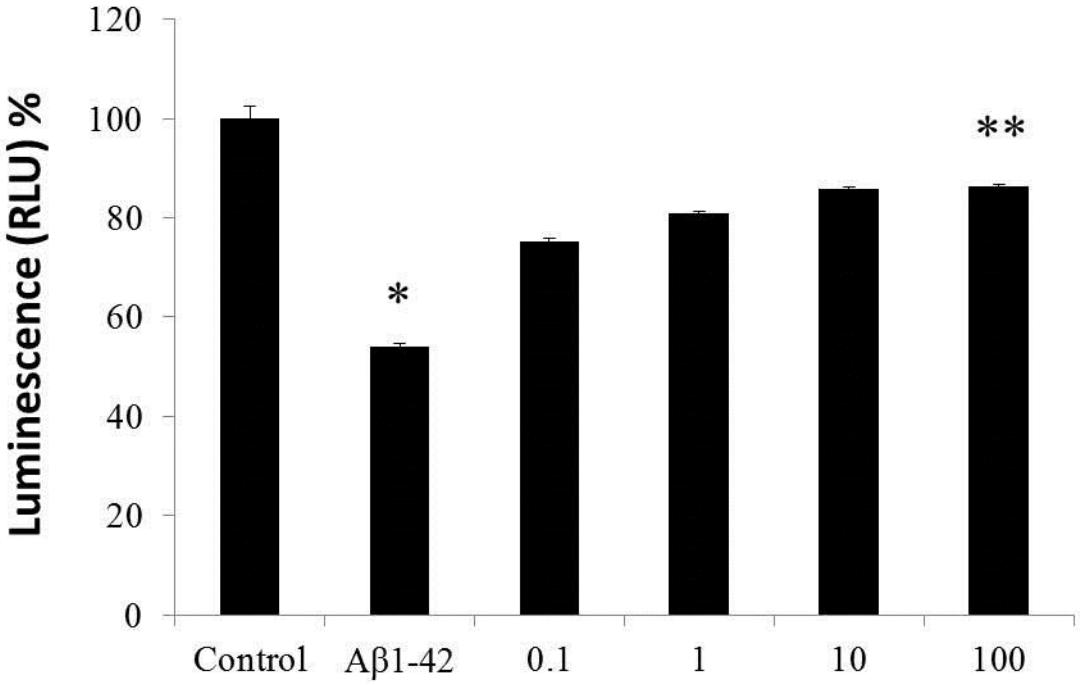


Figure 2-6. Effect of different concentrations of TQ on $A\beta_{1-42}$ induced cell death. Hippocampal neurons were exposed to $A\beta_{1-42}$ (10 μM) alone or with TQ (0.1, 1, 10,100 nM) for 72h. Cell viability was assessed by CellTiter-Glo assay. (*P< 0.01 vs. control, **P <0.01 vs. the group exposed to $A\beta_{1-42}$ alone), n = 6. Values shown are the mean percent luminescence (where 100% = luminescence in control hippocampal neurons), ± SE.

Fig. 2-7 shows the effect of $A\beta_{1-42}$ (5 μM) with or without TQ (100 nM) on hiPSC-derived cholinergic neurons viability. Treatment with $A\beta_{1-42}$ for 48 hours decreased cell viability to 65% as compared to control. However, TQ (100 nM) significantly restored Aβ induced loss of cell viability to 95% and protected the cells. The luminescent signal is proportional to the amount of ATP present which is directly proportional to the number of live cells present in culture.

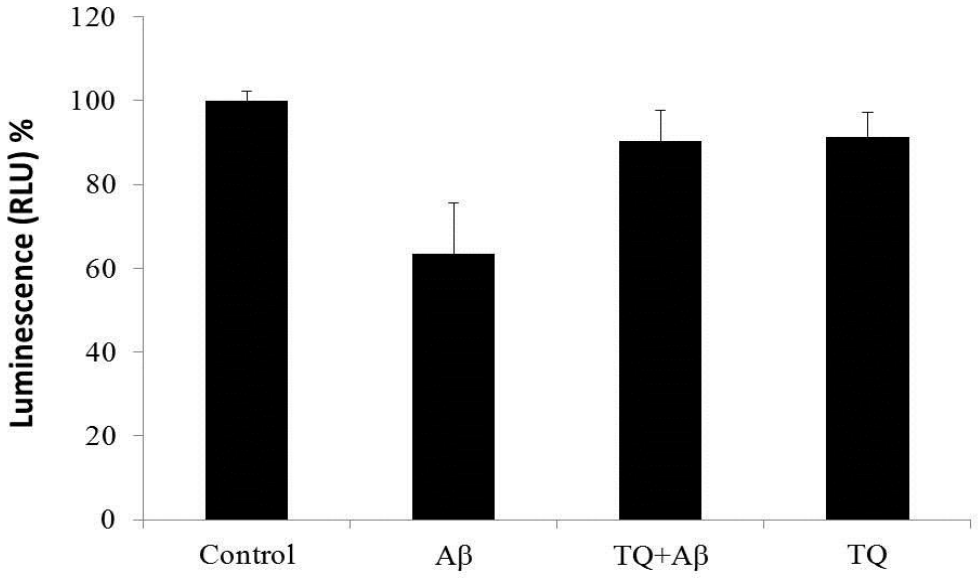


Figure 2-7. Effect of TQ against $A\beta_{1-42}$-induced cytotoxicity in hiPSC-derived cholinergic neurons. hiPSC-derived cholinergic neurons were exposed to $A\beta_{1-42}$ (5 μm) with or without TQ (100 nM) for 48h. Cell viability was assessed by the CellTiter-Glo assay. Values shown are the mean percent luminescence (where 100% = luminescence in control hiPSC-derived cholinergic neurons), ± SE, n=4.

2.3.4 Effect of TQ against $A\beta_{1-42}$-induced caspase 3/7 activities

Further studies were performed to determine if TQ protected hiPSC-derived cholinergic neurons against apoptosis induced by Aβ. Several studies have shown that in AD brains and in cultures of neurons exposed to Aβ, the dying cells display the characteristics of apoptosis [173-175]. Particularly caspase 3 has been shown to play a pivotal role in the execution phase of apoptosis induced by Aβ [176]. In (Fig.2-8) treatment of hiPSC-derived cholinergic neurons with $A\beta_{1-42}$ (5 μM) induced about 1 fold increase in the caspase 3/7 activities. However, when co-treated with TQ (100 nM), TQ was able to attenuate $A\beta_{1-42}$ toxicity and inhibit caspase activity induced by Aβ.

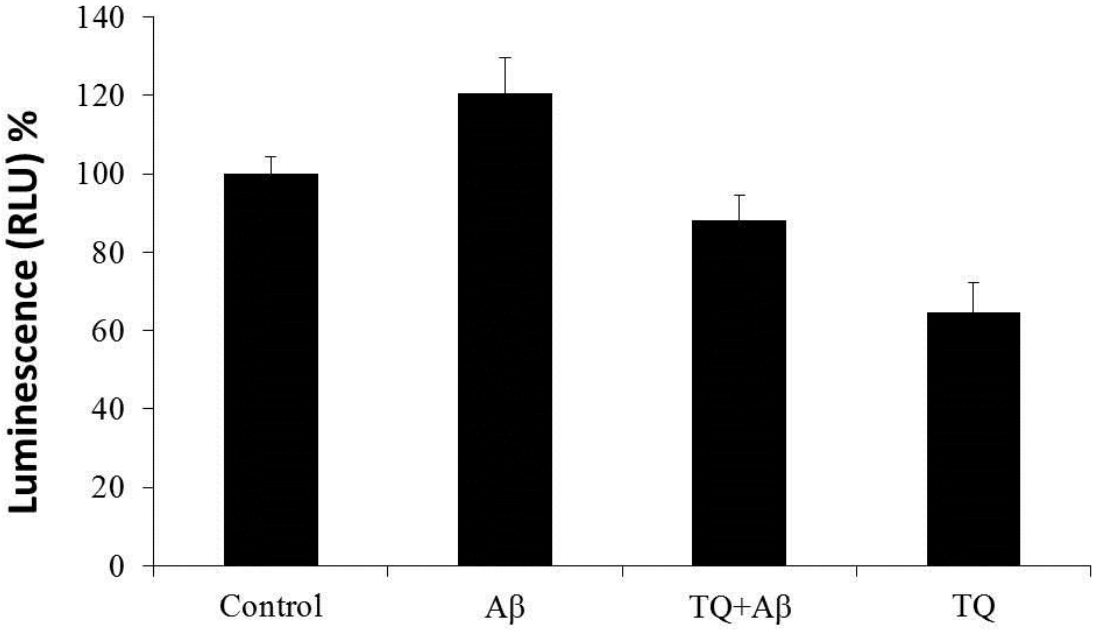


Figure 2-8. Effect of TQ against $A\beta_{1-42}$ induced caspase 3/7 activity in hiPSC-derived cholinergic neurons. hiPSC-derived cholinergic neurons were exposed to $A\beta_{1-42}$ (5 μm) with or without TQ (100 nM) for 48h. Caspase-3/7 activity was assessed by the Caspase-Glo 3/7 assay. Values shown are the mean percent luminescence (where 100% = luminescence in control hiPSC-derived cholinergic neurons), n=5, ± SE.

2.3.5 Effect of TQ against $A\beta_{1-42}$-induced MMP loss

As shown in (Fig.2-9), treatment of cultures with $A\beta_{1-42}$ (10 μM) induced about 30% decrease in Rh123 fluorescence intensity. When compared to $A\beta_{1-42}$ treated sample, TQ (100 nM) co-treatment enhanced Rh123 fluorescence intensity up to 90% comparing to controls.

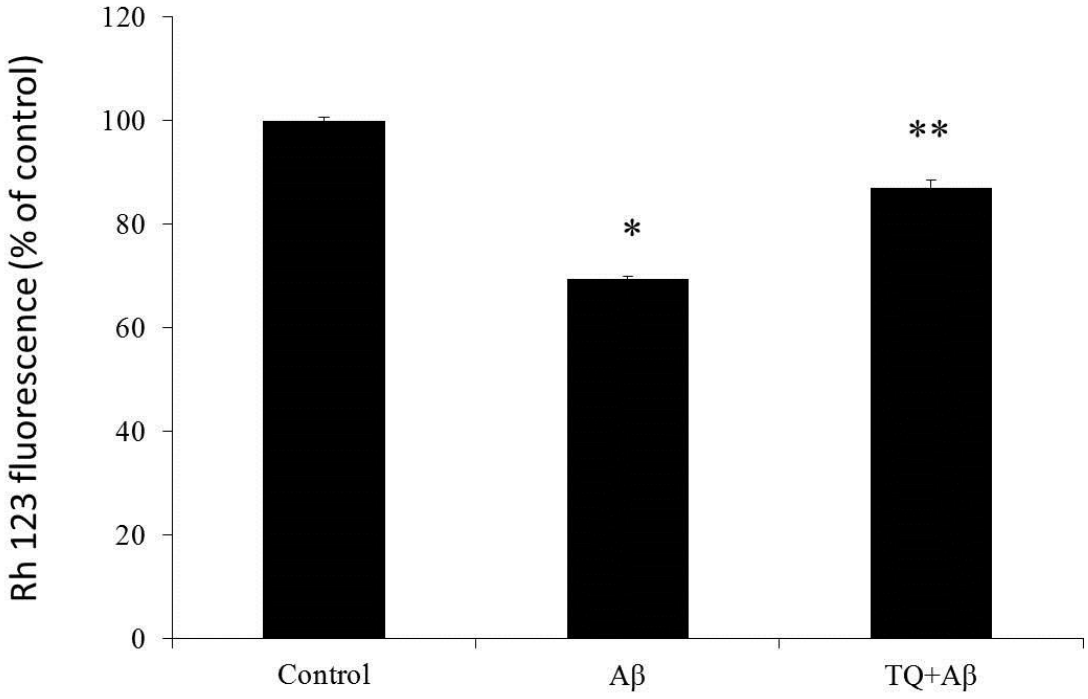


Figure 2-9. Effect of TQ on MMP changes induced by $A\beta_{1-42}$. Hippocampal neurons were treated with $A\beta_{1-42}$ (10 μM) in the presence or absence of TQ (100 nM) for 72 h. MMP was measured as Rh123. (*P < 0.05 vs. control, **P < 0.01 vs. the group exposed to $A\beta_{1-42}$ alone), n = 5. Values shown are the mean percent fluorescence (where 100% = fluorescence in control hippocampal neurons), ± SE.

2.3.6 Effect of TQ against $A\beta_{1-42}$-induced GSH level reduction

GSH is an important antioxidant prevents damage to important cellular components caused by reactive oxygen species [177]. In (Fig. 2-10) GSH level was significantly decreased in $A\beta_{1-42}$ treated cells (about 53%) as compared to control cells. Co-treatment with TQ (100 nM) has protected GSH level significantly with a 37% increase in GSH level comparing to control.

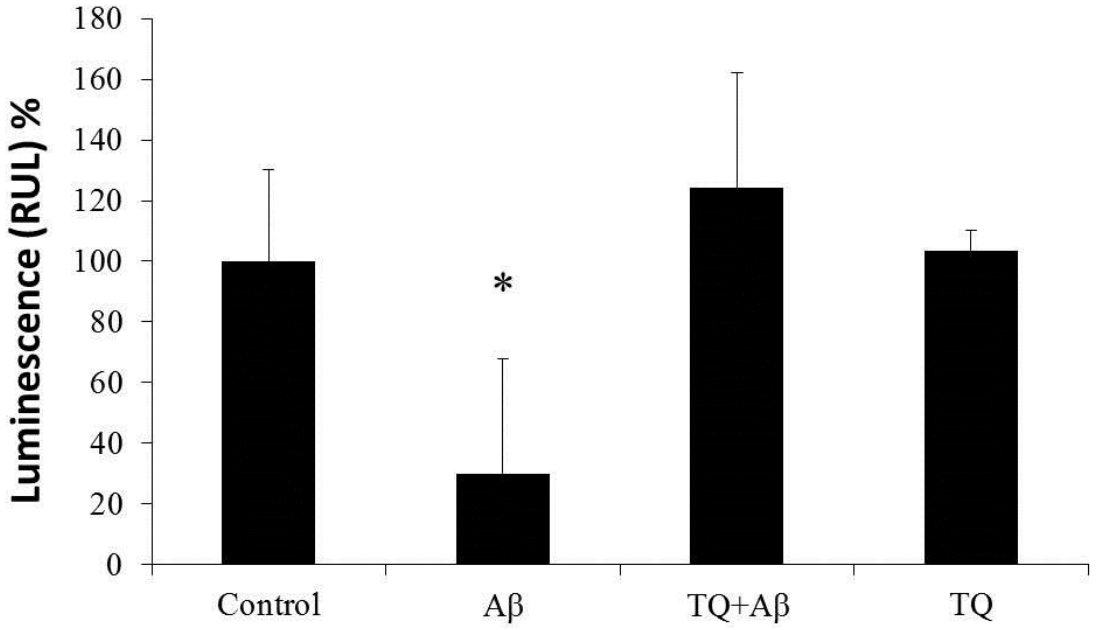


Figure 2-10. Effect of TQ against $A\beta_{1-42}$ induced oxidative damage in hiPSC-derived cholinergic neurons. hiPSC-derived cholinergic neurons were exposed to $A\beta_{1-42}$ (5 µm) with or without TQ (100 nM) for 48h. Glutathione level was assessed by the GSH-Glo™ Glutathione assay. (*P<0.05 vs. control), n=5. Values shown are the mean percent luminescence (where 100% = luminescence in control hiPSC-derived cholinergic neurons), ± SE.

2.3.7 Effect of TQ against $A\beta_{1-42}$-induced ROS generation

When rat cortical neurons were exposed to $A\beta_{1-42}$ (10 μM) the intracellular ROS level was significantly increased with 2.5 fold comparing to control, revealing that $A\beta_{1-42}$ enhanced ROS generation in cultured cortical neurons. However, treatment with TQ (100 nM) effectively reduced intracellular ROS level to 1.65 times that of control levels in neurons treated with $A\beta_{1-42}$ (Fig.2-11).

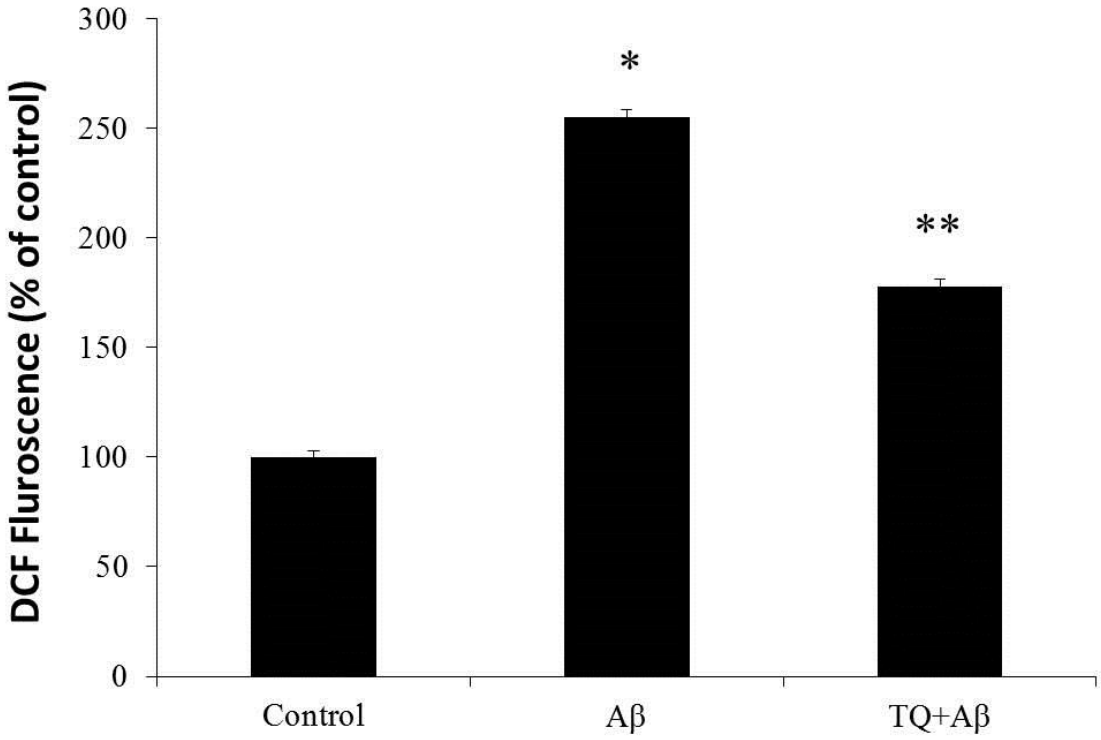


Figure 2-11. Effect of TQ on alterations in ROS generation induced by $A\beta_{1-42}$ in cortical neurons. Cortical neurons were exposed to $A\beta_{1-42}$ (10 μM) with or without TQ (100 nM) for 72h. ROS generation was assessed using the DCFH-DA assay. (*P < 0.01 vs. control, **P < 0.01 vs. the group exposed to $A\beta_{1-42}$ alone), n = 5. Values shown are the mean percent fluorescence (where 100% = fluorescence in control cortical neurons), ± SE.

To clarify the possible antioxidant effect of TQ, the accumulation of ROS (H_2O_2) was evaluated in hiPSC-derived cholinergic neurons. As illustrated in (Fig.2-12), the treatment with $A\beta_{1-42}$ caused a significant increase of more than 1.15 fold in H_2O_2 concentration comparing to controls. TQ co-treatment has attenuated $A\beta_{1-42}$ effect and decreased H_2O_2 concentration significantly to 137% comparing to control samples.

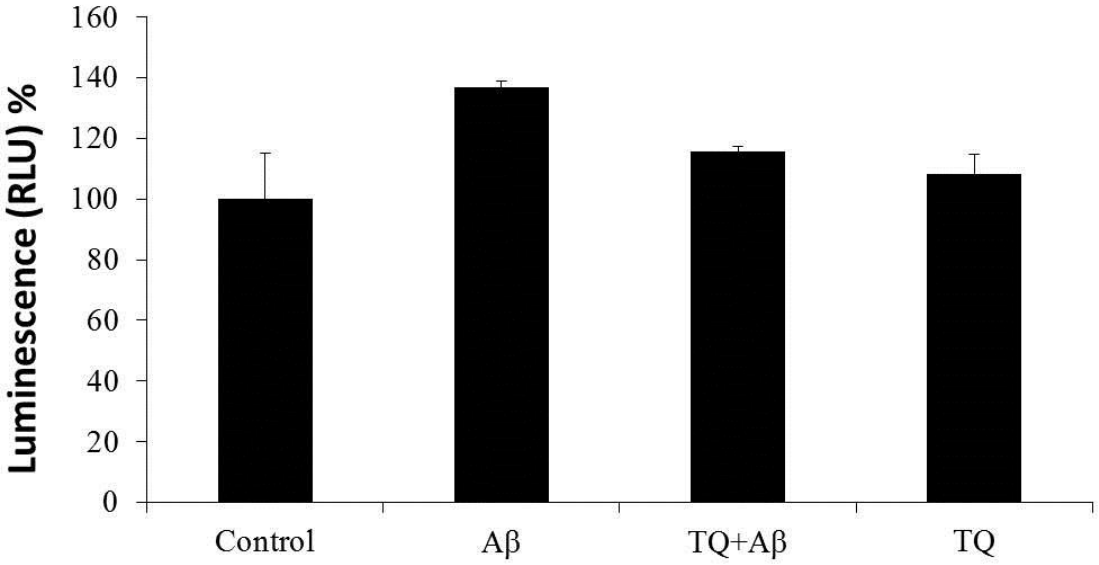


Figure 2-12. Effect of $A\beta_{1-42}$ and TQ on intracellular ROS level. hiPSC-derived cholinergic neurons were exposed to $A\beta_{1-42}$ (5 µm) with or without TQ (100 nM) for 48h. H_2O_2 was measured using the ROS-Glo™ H_2O_2 assay. Values shown are the mean percent luminescence (where 100% = luminescence in control hiPSC-derived cholinergic neurons), n=3, ± SE.

2.3.8 Effect of TQ against $A\beta_{1-42}$-induced synapse damage

The addition of $A\beta_{1-42}$ (2 μM) affected synaptic vesicle recycling, as indicated by a 50%-reduction in FM1–43 uptake, compared with control samples. However, co-administration of TQ (100 nM) enhanced the uptake of FM1–43 by 15% compared with control levels in cortical neurons and thus enhanced neurotransmission (Fig.2-13).

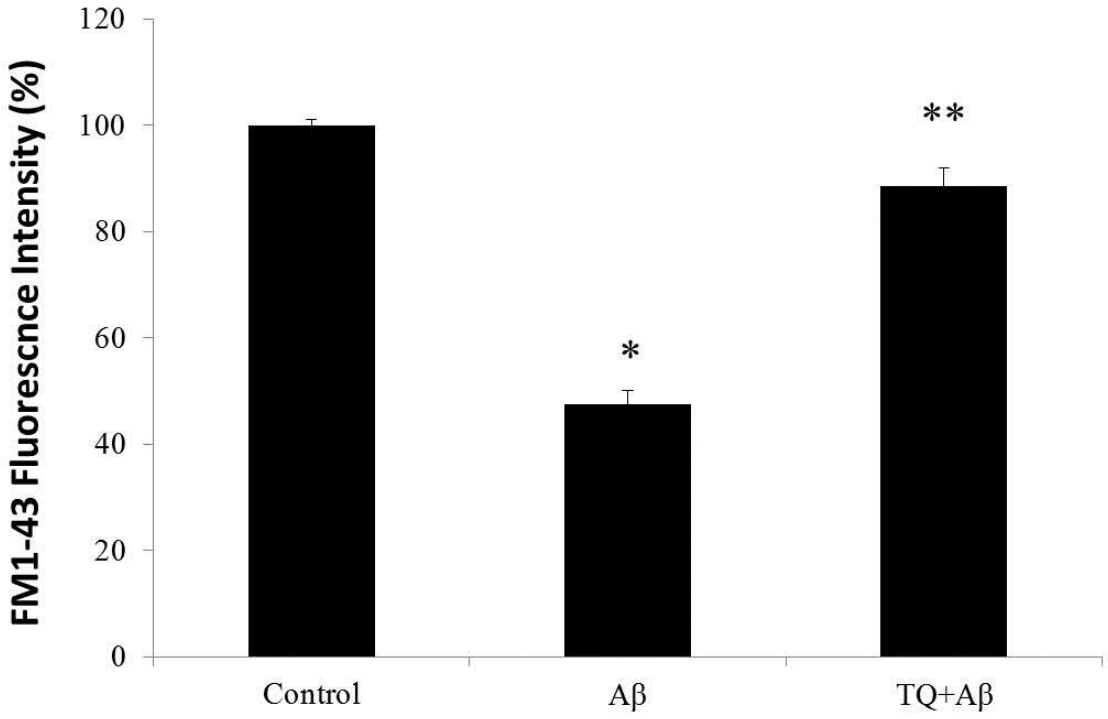


Figure 2-13. Effect of $A\beta_{1-42}$ and TQ on synaptic vesicle recycling in cortical neurons. Cortical neurons were treated $A\beta_{1-42}$ (2 μM) with or without TQ (100 nM) for 72h. Synaptic vesicle recycling activity was assessed using the fluorescence dye FM1-43. (*P < 0.01 vs. control, **P < 0.05 vs. the group exposed to $A\beta_{1-42}$ alone), n=4. Values shown are the mean percent fluorescence (where 100% = fluorescence in control cortical neurons), ± SE.

In Fig.2-14 (A), we demonstrated that the addition of $A\beta_{1-42}$ (5 μM) induced a 4.5 fold increase in the synaptic activity comparing to control. The co-treatment with TQ (100 nM) reduced the increase in the activity to 4 fold comparing to control sample.
(B) We additionally investigated the cell viability of the same sample and we found that $A\beta_{1-42}$- induced approximately 55% reduction in cell viability. However, the co-treatment with TQ (100 nM) attenuated the toxic effect of $A\beta_{1-42}$ with 20% reduction in viability comparing to controls.

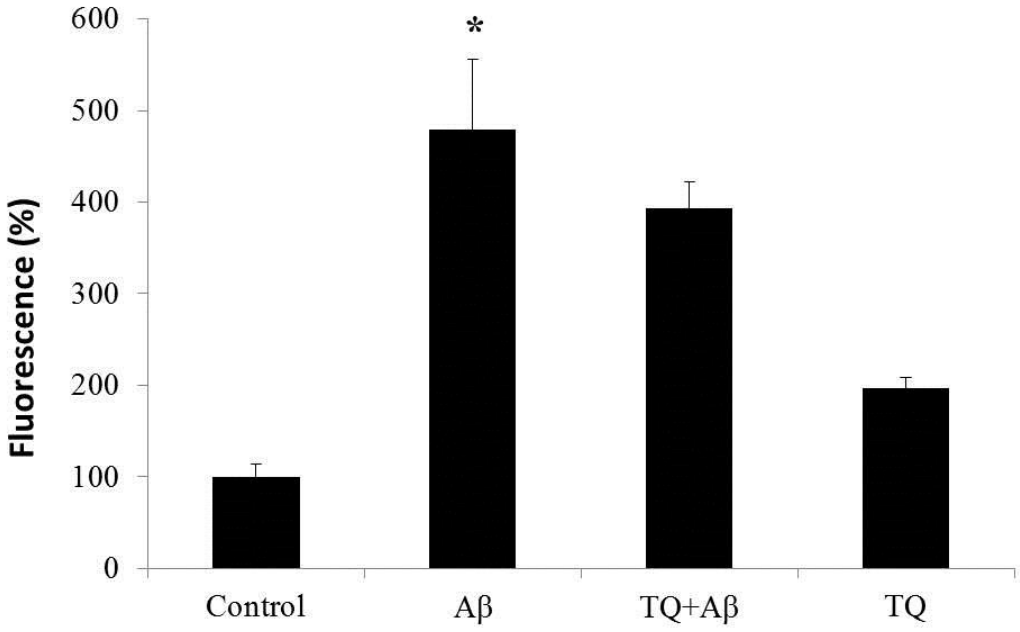
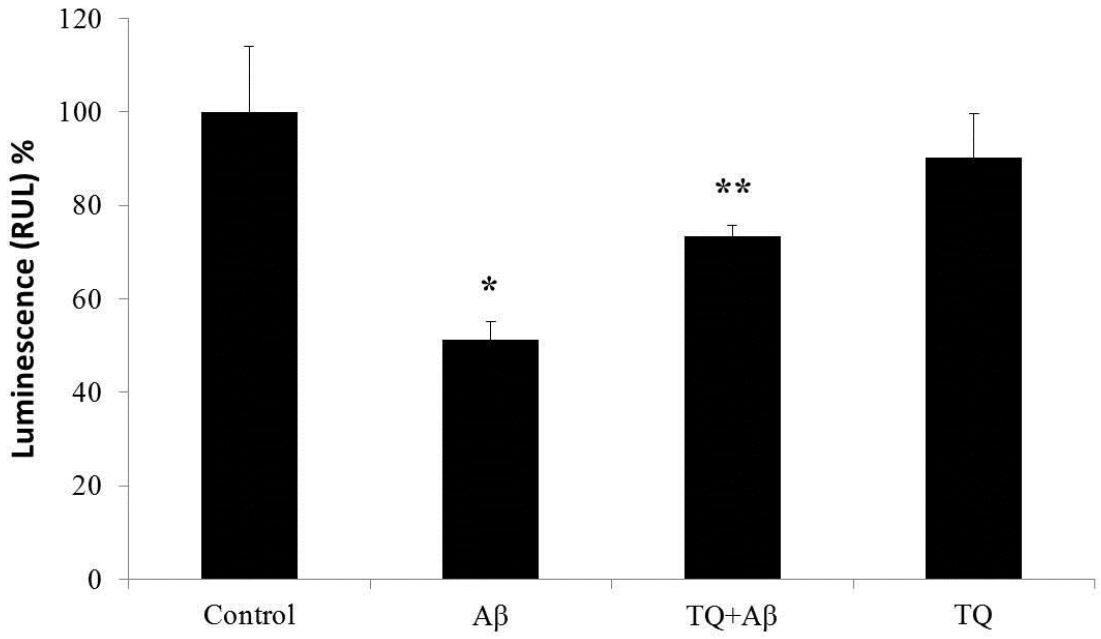


Figure 2-14. (A) Effect of $A\beta_{1-42}$ and TQ on synaptic activity of hiPSC-derived cholinergic neurons. Neurons were treated for 72h with $A\beta_{1-42}$ (5 μM) in the presence or absence of TQ (100 nM). $A\beta_{1-42}$ induced a significant 4.5 fold increase in FM1-43 fluorescence intensity. Administration of TQ together with $A\beta_{1-42}$ reduced the increase in FM1-43 fluorescence intensity to 4 fold. (*P<0.01 vs. Control), n=5. Values shown are the mean percent fluorescence (where 100% = fluorescence in control hiPSC-derived cholinergic neurons), ± SE.



(B) Protective effect of TQ against $A\beta_{1-42}$ induced toxicity in hiPSC-derived cholinergic neurons. hiPSC-derived cholinergic neurons were exposed to $A\beta_{1-42}$ (5 µm) with or without TQ (100 nM) for 72h. Cell viability was assessed by the CellTiter-Glo assay. (*P<0.05 vs. control, **P<0.01 vs the group exposed to $A\beta_{1-42}$ alone), n=4. Values shown are the mean percent luminescence (where 100% = luminescence in control hiPSC-derived cholinergic neurons), ± SE.

2.3.9 Effect of TQ on $A\beta_{1-42}$-induced spontaneous firing activity inhibition

Fig.2-15. (A) shows the pattern of spontaneous firing activity of hippocampal cells before and after the simultaneous administration of $A\beta_{1-42}$ (2 μM) and TQ (100 nM). TQ enabled the cells to maintain their spontaneous firing frequency even 72 hours after administration. (B) The addition of $A\beta_{1-42}$ (2 μM) abolished spontaneous firing activity of hippocampal neurons; no spontaneous action potential was recorded in the exposed cells at 72 hours after exposure. However, when we co-administrated TQ (100 nM), cells were able to maintain approximately 60% of their baseline firing activity, as opposed to the complete loss of functionality that resulted from treatment with $A\beta_{1-42}$ only.

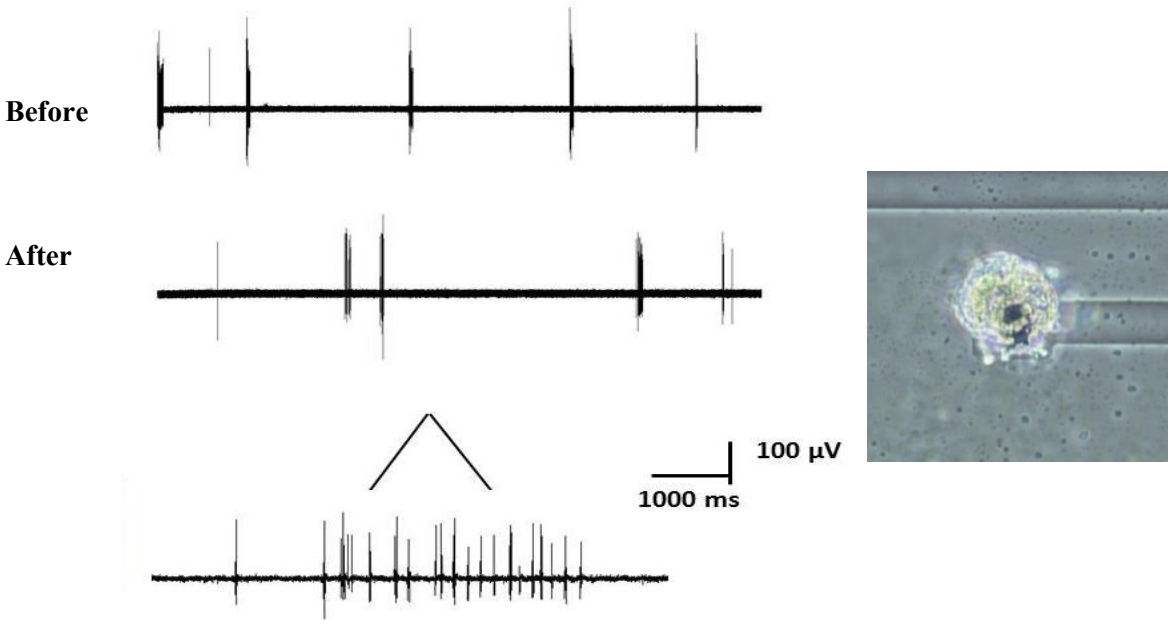
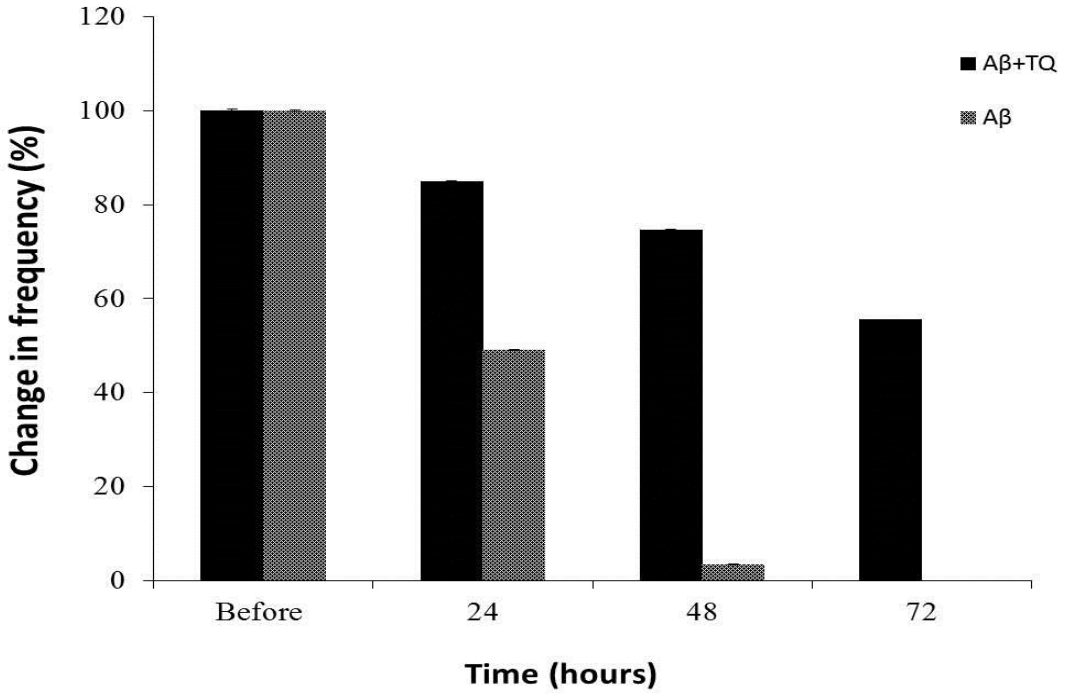


Figure 2-15. Effect of TQ against $A\beta_{1-42}$-induced inhibition of spontaneous firing activity (A) Spontaneous firing activity pattern before and after the simultaneous treatment of cells with $A\beta_{1-42}$ and TQ. The firing activity of hippocampal neurons had not disappeared by 72 h after the exposure. The phase contrast image shows hippocampal neurons (13 DIV) cultured inside the agarose micro chamber and attached to the microelectrode probe of MEA 72h after treatment with $A\beta_{1-42}$ and TQ.



(B) Time course of the effect of TQ on spontaneous firing frequency when co-administrated with $A\beta_{1-42}$. When the cells were exposed to $A\beta_{1-42}$ spiking activity started to decline 24h after the exposure and the firing ceased after 72h, n = 29. However, co-administration of TQ partially reversed the loss of spontaneous firing activity even 72h after the exposure, n = 51. (100% implies baseline values before exposure to $A\beta_{1-42}$), ±SE.

Fig.2-16-A shows the pattern of the spontaneous firing activity of hiPSC-derived cholinergic neurons before and 6 days after the treatment with $A\beta_{1-42}$ (5 μm) only or with $A\beta_{1-42}$ and TQ (100 nM). The treatment with $A\beta_{1-42}$ abolished the firing activity after 6 days. In contrast, TQ helped the cells to maintain their firing activity even 6 days after the treatment with $A\beta_{1-42}$. The change in firing frequency was observed as shown in Fig.2-16-B; the samples treated with TQ were able to maintain 91% of their firing frequency even 6 days after the treatment with $A\beta_{1-42}$ (5 μM). The samples treated with $A\beta_{1-42}$ (5 μM) had 24% firing activity after 6 days.

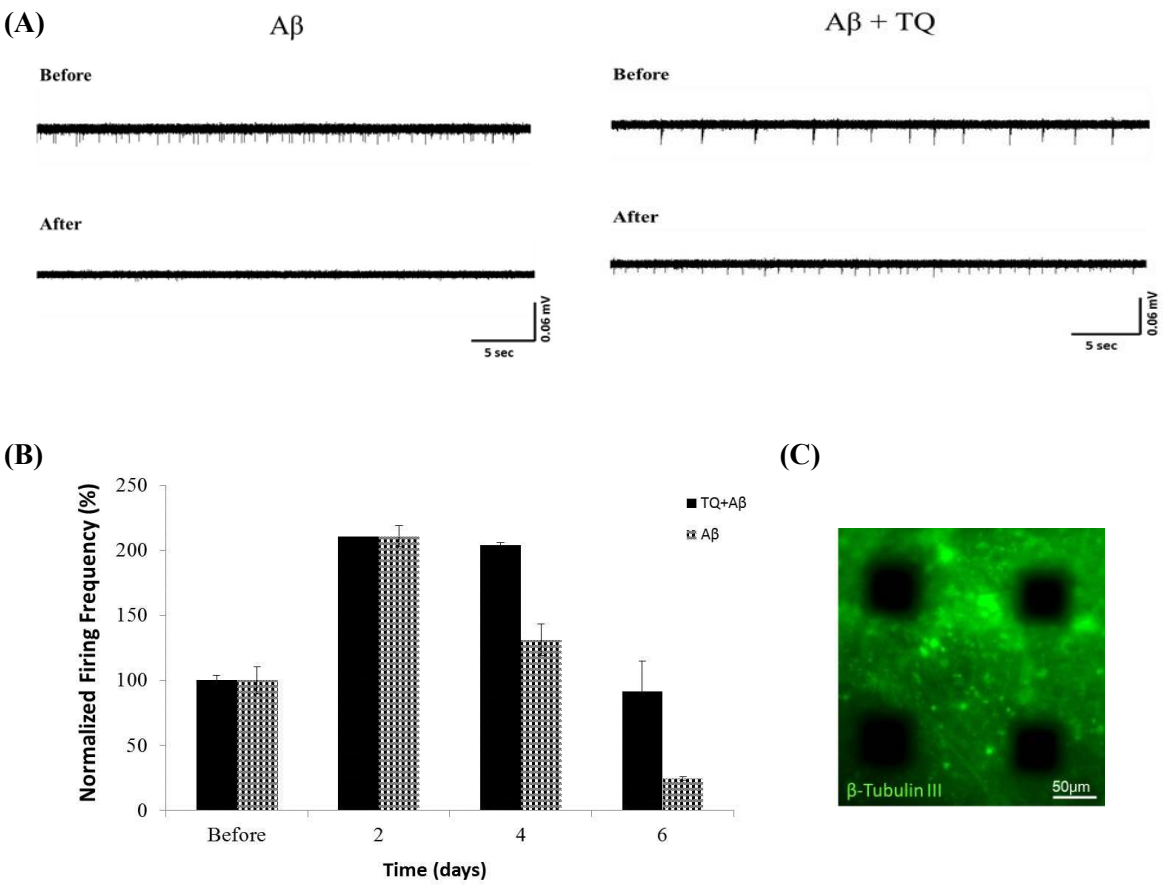


Figure 2-16. Effect of TQ against $A\beta_{1-42}$ induced inhibition of spontaneous firing activity in hiPSC-derived cholinergic neurons. Neurons were exposed to $A\beta_{1-42}$ (5 μm) only or with TQ (100 nM) for 6 days. (A) The waveforms show typically spontaneous firings before and after 6 days of $A\beta_{1-42}$ administration or $A\beta_{1-42}$ and TQ administration. (B) Time course of the effect of TQ on spontaneous firing frequency when administered with $A\beta_{1-42}$ showing the reversal of the effect of $A\beta_{1-42}$ on the firing frequency of hiPSC-derived cholinergic neurons by TQ. When the cells were administered to $A\beta_{1-42}$, there was about 24% firing activity detected 6 days after the exposure. However, co-administration of TQ and $A\beta_{1-42}$

partially reversed the loss of spontaneous firing activity with 91% firing activity even 6 days after administration. (100% represents baseline values before exposure to $A\beta_{1-42}$) ± SE, n = 1. (C) A fluorescence image of fixed human hiPSC-derived cholinergic neurons (18 DIV) cultured on the microelectrode probe of the MEA system 6days after incubation with $A\beta_{1-42}$ and TQ. The neuronal marker β-tubulin III is shown in green.

2.3.10 TQ inhibited $A\beta_{1-42}$ aggregation in vitro

While there is currently no effective treatment for AD, substances that can efficiently inhibit amyloid formation have been sought as drug candidates for treating AD. Therefore, to further clarify our previous results and to understand the mechanism by which TQ inhibited the neurotoxicity of Aβ, we investigated its effect on $A\beta_{1-42}$ amyloid formation. Induced fluorescent change was observed for 72 hours in the presence of $A\beta_{1-42}$ (10 μM), with or without TQ (100 nM). Compared with the control ThT sample, the sample incubated with $A\beta_{1-42}$ increased ThT fluorescence to 2.6-fold. However, the sample co-incubated with TQ reduced the fluorescence increase to 1.6-fold compared with the control sample, indicating that fewer cross β-sheets formed in this sample (Fig.2-11).

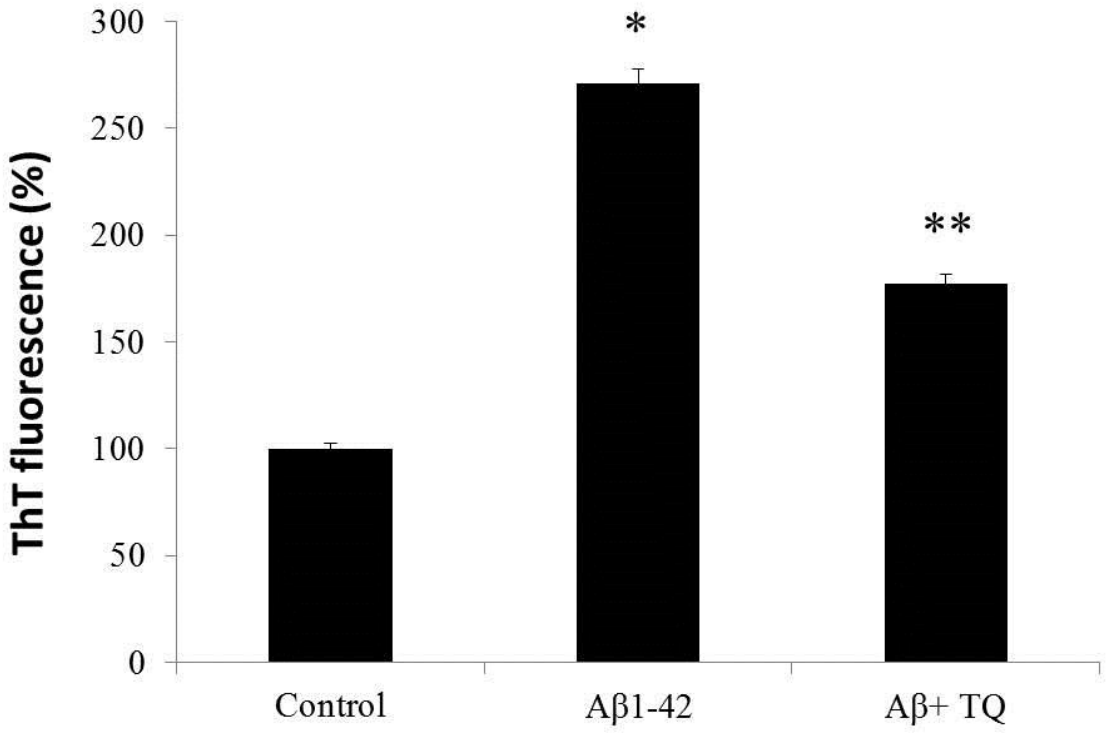


Figure 2-17. Effect of TQ on $A\beta_{1-42}$ aggregation. Aβ (at a concentration of 10 μM in PBS) was incubated at 37°C for 72h in the presence or absence of TQ (100 nM) and peptide aggregation was measured by ThT fluorescence assay. (*P < 0.01 vs. control; **P < 0.01 vs. Aβ), n = 4. Values shown are the mean percent fluorescence (where 100% = fluorescence), ± SE.

2.4 Discussion

The brain is vulnerable to oxidative stress owing to its high lipid content, its relatively high oxygen metabolism, and its low levels of antioxidant defenses [178]. One of the most interesting events in AD is that mitochondrial oxidative stress occurs early in AD progression, before the onset of Aβ pathology [179, 180]. Mitochondrial dysfunction and synaptic damage are early pathological features of AD. Aβ oligomers were found in synaptosomal mitochondrial fractions and decreased energy metabolism in AD transgenic mice [181]. Abnormalities of mitochondrial function, including decreased mitochondrial respiration, ROS generation, and hypometabolism, occur in the AD brain [182, 183] and in brains of AD mouse models [184, 185]. Aβ accumulation in the synapses directly disturbs mitochondrial function, causing oxidative stress, decreased ATP, and increased Ca^{2+} influx [186, 187]. Further, it was showed that Aβ impaired synaptic mitochondrial distribution, axonal mitochondrial mobility, and increased axonal mitochondrial fragmentation. These promising findings are in agreement with those studies indicating that Aβ causes rapid and severe impairment of mitochondrial transport [188] and alters mitochondrial dynamics [189] inducing neurodegeneration of cortical and hippocampal neurons through oxidative stress and a wide range of molecular events that disturb neuronal homeostasis [190]. Furthermore, it is well established that oxidative stress is involved in an apoptotic mechanisms by which excessive ROS production could lead to neuronal apoptosis in neurodegenerative disorders, such as Aβ-induced neuronal apoptosis [191-193].

Although the exact mechanisms of Aβ cytotoxicity is still not fully understood, lots of studies tried to find how to inhibit the Aβ toxicity on nerve cells in the brain. Antioxidants have been shown a beneficial effect in neurodegenerative disorders and Aβ-induced neurotoxicity [194-196]. Thus, considerable attention has been focused on identifying naturally occurring antioxidants that are able to protect against Aβ-mediated neurotoxicity's. TQ, the most active constituent of *Nigella sativa* seed oil, was reported to display potent antioxidant and neuroprotective properties. The antioxidant properties of TQ appeared to play an important role in rescuing THir neurons against MPP+ and rotenone-induced cell death in dopaminergic cell cultures relevant to PD [65]. It has also been reported that TQ significantly reduced neuronal cell death in the hippocampal CA1 region from ischemia-induced brain injury [150, 151], and caused morphologic improvement on neurodegeneration in the hippocampus in rats after chronic toluene exposure [68]. Moreover, TQ has been demonstrated to protect organs against oxidative

damage induced by a variety of free radical generating pathologies [197-201]. In addition, TQ improved behavioral and cellular abnormalities and markers of oxidative stress in an experimental model of early PD in rat [202]. Moreover, TQ prevented cell death and apoptosis in rat cerebellar granule neurons and restored acetylcholine esterase activity induced by Aβ [203, 204].

Cholinergic dysfunction in the basal and rostral forebrain is associated with even early cognitive impairments observed in AD, correlates with cognitive decline, and forms the basis of the 'cholinergic hypotheses of AD [205]. hiPSC-derived neurons are a great promising tool for its applications in cell transplantation, human disease modeling and drug discovery [35, 206]. Therefore, we investigated the effect of TQ on the neurotoxicity induced by $A\beta_{1\text{-}42}$ in rat primary and hiPSC-derived cholinergic neurons.

In the present study we demonstrated that TQ prevented $A\beta_{1–42}$-induced cell death in primary hippocampal and in hiPSC-derived cholinergic neurons. Cell viability was determined by measuring the amount of ATP in cells and TQ was able to protect cell viability as indicated by the higher amount of ATP found in the sample treated with TQ and $A\beta_{1–42}$ comparing to $A\beta_{1–42}$ treated samples and thus higher viability.

Caspases are implicated in the accomplishment of apoptotic cell death, while the inhibition of their activation is favorable for cell survival. Their modulation by exogenous stimuli could prevent or promote apoptosis. The activation of caspase-3 induces DNA fragmentation, nuclear chromatin condensation, and cell apoptosis [207]. Additionally, caspase-3 activation is increased in AD patients, and triggered synaptic failure [208, 209] and autophagy, which may contribute to cognitive dysfunction in AD development [210]. In our study an increase of level of caspase -3,-7 activities induced by $A\beta_{1\text{-}42}$ was observed in hiPSC-derived cholinergic neurons. However, TQ was able to suppress the activity of caspase-3, -7 with 12% reduction comparing to the control sample and the obtained results had significant difference when normalized.

Excessive ROS, which mainly derives from electron leakage from the mitochondrial respiratory chain complexes, causes free radical attack of membrane phospholipids, leading to loss of mitochondria membrane potential [73]. We demonstrated that TQ significantly inhibited $A\beta_{1–42}$-induced mitochondria membrane potential collapse in cultured hippocampal neurons. GSH is the most prevalent antioxidant in the brain, it plays a vitally important role in cellular function; in fact the maintenance of GSH homeostasis is essential for the organism to perform its many functions. GSH levels can be monitored as a non-specific indicator of cellular injury [177]. Decreased GSH activity overtime leads to an accumulation of H_2O_2 and lipid

peroxidation, possibly leading to the pathological alterations characteristic of AD [138, 211]. Consequently, one way of boosting defenses in the brain is by assisting the antioxidant defense system particularly GSH and GSH-related enzymes. In this study, treatment of hiPSC-derived cholinergic neurons with $A\beta_{1\text{-}42}$ induced a significant reduction in GSH content. Intriguingly, the co-treatment with TQ restored the content of GSH significantly, and the obtained results had significant difference when normalized.

We also tested the intra-cellular ROS levels and demonstrated that the increased ROS level produced by incubation of primary cortical neurons with $A\beta_{1–42}$ is markedly reduced in cells subjected to TQ. Next, we investigated the effect of TQ and $A\beta_{1\text{-}42}$ on H_2O_2 levels in hiPSC-derived cholinergic neurons. H_2O_2, produced during the oxidative deamination of catecholamines, has been identified as likely involved in neurodegenerative disorders, such as AD and PD, presumably via oxidative damage to the mitochondrial membrane [178/81]. The administration of $A\beta_{1\text{-}42}$ to the cells induced an increase in H_2O_2. However, TQ co-treatment inhibited this apparent increase and the obtained results had significant difference when normalized. This effect could be due to the effect of $A\beta_{1\text{-}42}$ and TQ on GSH levels. Based on these findings, we proposed that the anti-oxidative action of TQ may contribute to its protection against Aβ-induced oxidative damage.

It is now accepted that diverse forms of Aβ are responsible for producing synaptic failure. Previous findings have demonstrated that soluble Aβ can also cause functional toxicity; it inhibits spontaneous firing of hippocampal neurons without causing significant cell death at low concentrations. This toxicity will eventually also lead to loss of the synapse [156, 157, 163]. We report here that the uptake of FM1–43 dye in cortical neurons was reduced following the addition of $A\beta_{1–42}$ and thus $A\beta_{1\text{-}42}$ induced a reduction in synaptic vesicle recycling. However, in simultaneous treatment TQ was able to enhance the uptake of FM1-43 and therefore, TQ can reduce the inhibition of synaptic vesicle recycling caused by $A\beta_{1–42}$. We also investigated the effect of TQ and $A\beta_{1\text{-}42}$ on synaptic vesicle recycling in hiPSC-derived cholinergic neurons. The administration of $A\beta_{1\text{-}42}$ induced a significant increase (approximately 4.5 fold) in the uptake of the fluorescent dye FM1-43 and therefore induced an increase in synaptic activity. When TQ was administrated with $A\beta_{1\text{-}42}$ there was a 0.5 decrease in the synaptic activity comparing to $A\beta_{1\text{-}42}$ treated samples. We investigated the cell viability of the same sample to find that $A\beta_{1\text{-}42}$ -induced about 55% reduction in cell viability. However, TQ was able to attenuate $A\beta_{1\text{-}42}$-induced cytotoxicity and protected the cells with 25% cell loss and therefore; the increase in the synaptic activity could be due to excitotoxicity.

In support of our results, several reports have shown an Aβ-dependent increase in the number of vesicles available at the presynaptic active zone [212-214]. It has been shown that in neurons exposed to $A\beta_{1\text{-}42}$ the fluorescent dye FM1-43 uptake was more pronounced at a fraction of synaptic contacts, indicative of an increase in the number of vesicles primed to the presynaptic membrane. These results strongly suggest that $A\beta_{1\text{-}42}$ increases the amount of primed vesicles at the presynaptic terminal [2015]. Furthermore, it has also been shown that $A\beta_{1\text{-}42}$ induces an extensive depletion of the synaptic vesicle pool, indicative of an increased participation of synaptic vesicles in neurotransmitter release [216].

We also report here that treatment of hippocampal neurons with $A\beta_{1\text{–}42}$ abolished spontaneous activity completely, while co-administration of $A\beta_{1\text{–}42}$ and TQ partially reversed this loss of spontaneous activity. Therefore, this functional deficit can be reversed through use of TQ, as proved by MEA. Additionally, we assessed the effect of $A\beta_{1\text{-}42}$ on spontaneous activity of hiPSC-derived cholinergic neurons using MEA. When applied to hiPSC-derived cholinergic neurons cultured on the MEA probe, $A\beta_{1\text{-}42}$ had a pronounced effect on the spontaneous firing of the cells with 1 fold increase in the activity when applied alone or with TQ 2 days after the treatment. However, after 6 days of application, $A\beta_{1\text{-}42}$-induced about 76% reduction in the firing activity, however; TQ when applied with $A\beta_{1\text{-}42}$, the cells were able to retain about 91% of their firing capability compared to only 24% activity in the $A\beta_{1\text{-}42}$ treated samples. Therefore, TQ is strongly capable of protecting neural activity. The initial increase in firing frequency we observed at $A\beta_{1\text{-}42}$ treated samples could be due to an earlier reported direct depolarizing effect of $A\beta_{1\text{-}42}$ on the membrane potential or to the reputed ability of $A\beta_{1\text{-}42}$ to enhance glutamate-mediated excitotoxicity [217, 218] by its action on NMDA receptors and consequently, through an increased influx of Ca^{2+}. Additionally, mitochondrial number is very high in neurons, and mitochondria are especially enriched in synapses. Due to the limited glycolytic capacity of neurons, neurons are highly dependent on mitochondria function for energy production [219]. Notably, when ATP is generated it's subsequently used for a large repertoire of functions including neurotransmitter production, and synaptic plasticity. Therefore we thought that the protective effects of TQ could be due to the enhancement of ATP levels and therefore the enhancement of synaptic functionality.

Furthermore, using ThT fluorescence, we showed that TQ can inhibit $A\beta_{1\text{–}42}$ aggregation. Although the precise mechanism of TQ neuroprotection remains unclear, the antioxidant ability, inhibition of functional toxicity, and disaggregation of $A\beta_{1\text{–}42}$ may be involved in the neuroprotective effects of TQ. In conclusion, the results strongly

suggest the intracellular pathway of TQ to protect against the Aβ-induced toxicity on rat primary and hiPSC-derived cholinergic neurons of the central nervous system.

Chapter 3

Inhibitory effect of thymoquinone against alpha synuclein-induced synapse damage in rat primary and hiPSC-derived neurons

3.1 Introduction

Synapse degeneration is a common feature in patients with neurodegenerative diseases who exhibit dementia, including those with PD, AD, and dementia with DLBs [220]. These patients display an accumulation of proteins, including αSN aggregates, in cortical and subcortical regions of the brain [221]. αSN is predominantly expressed in the central nervous system neurons where it localizes to presynaptic terminals, regulates synaptic vesicle formation and neurotransmitter release [222], and affects synaptic plasticity during learning [13]. Evidence indicates that this pathology occurs in response to aggregates of αSN that accumulate at pre-synaptic terminals and trigger synapse degeneration in patients with PD and DLB [223]. The stereotypical progression of αSN pathology throughout the brain may be because of the transfer of αSN to neighboring neurons [224], which is similar to that of tau pathology in AD [225]. The loss of synapses in the hippocampus is characteristic of patients with PD who develop dementia [220], and in a rat model of α-synucleinopathies, synaptic degeneration precedes neuronal loss [226]. Thus, synapse degeneration is a feature shared by patients with PD and DLB.

hiPSC express αSN [227], and because αSN is particularly involved in the synaptic compartment, alterations in its expression there will be of great interest [228]. The first iPSC line-derived neurons isolated from a patient with a synucleinopathy harbors a triplication of the gene synuclein, alpha (non A4 component of amyloid precursor) or (SNCA) encoding αSN and expresses higher levels of αSN compared with healthy control cells [229]. In addition to a study on relatively young neurons, another study demonstrates nuclear as well as vesicular staining patterns of αSN in mature iPSC-derived neurons. These cells also express SNCA at a higher rate compared with iPS or neural stem cells [230].

Because dementia in patients with PD and AD is closely associated with synaptic abnormalities, the protective effect of TQ against αSN-induced synapse damage in cultured rat hippocampal and hiPSC-derived neurons (RCESDA001, ReproCell) was determined by first quantifying the level of synaptophysin using immunostaining in cultured rat hippocampal and hiPSC-derived neurons. Synaptophysin is a presynaptic membrane protein associated with recycling vesicles that are essential for neurotransmission [231], and synaptophysin levels are used to assess synaptic density in the brain [232] and in cultured neurons [233]. Next, we determined the effect of TQ and αSN on synaptic activity in cultured rat hippocampal and hiPSC-derived neurons using the fluorescent dye FM1-43. Because alterations of β-synuclein (βSN), βSN contributes to the pathogenesis of a broad range of α-synucleinopathies and enhances neurodegeneration [234], we investigated the effect of mutant P123H-βSN on synaptic vesicle recycling in hippocampal neurons using the fluorescent dye FM1-43. To further clarify our results, we investigated the effect of αSN on spontaneous spiking activity in hiPSC-derived neurons (iCell Neurons, Cellular Dynamics international) using MEA system.

3.2 Materials and methods

3.2.1 Preparation of αSN and P123H-βSN

Recombinant human αSN and P123H-βSN (Sigma–Aldrich) were reconstituted in sterilized water and stored at −20◦C.

3.2.2 Synaptic protein expression level

We first determined the effect of TQ and αSN on Synaptophysin expression level. Synaptophysin is a protein that exists in the membrane of synaptic vesicles in presynaptic terminals. Synaptophysin synaptic function includes: Exocytosis, synapse formation, biogenesis, and endocytosis of synaptic vesicles. In this experiment hippocampal and hiPSC-derived neurons were treated with αSN (1 μM) with or without TQ (100 nM). After 72 hours, the cells were fixed in 4% cold paraformaldehyde for 10 minutes and then in methanol (-20◦C) for 10 minutes. The fixed cells were washed with preblock buffer [0.05% Triton-X, 5% goat serum in PBS] for 10 minutes, and were then incubated with the primary antibodies anti-MAP2 or anti- β-tubulin III or anti-synaptophysin (Millipore) at 4◦C overnight. The cells were then washed twice with preblock buffer and incubated with secondary antibodies anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor568 (Molecular-Probes) and Hoechst 33258 (Sigma–Aldrich) at room temperature for 1 hour followed by two 5-minutes washes with PBS. Fluorescence was measured using a microplate reader (TECAN) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Immunostained specimens were viewed using an inverted fluorescence microscope (TE-2000, Nikon), and images were captured using an electron multiplying CCD camera (iXon Ultra 897, Andor).

3.2.3 Synaptic vesicle recycling

Next, we investigated the effect of TQ and αSN on synaptic activity by measuring synaptic vesicles recycling. The styryl dye FM1-43 is a powerful tool to track exocytosis, endocytosis and recycling vesicles. Therefore, it was used to determine synaptic vesicle recycling. Primary hippocampal and hiPSC-derived neurons were treated with αSN (1 μM) with or without TQ (100 nM). On the day of the experiment, the culture medium was removed; neurons were incubated with 1μg/ml ACSF/FM1-43 solution for 5 minutes, washed thrice in ice-cold PBS, and suspended in PBS. Fluorescence was measured using a microplate reader (TECAN) at excitation and emission wavelengths of 480 nm and 612 nm, respectively.

3.2.4 Synaptic vesicle recycling (P123H-βSN)

The βSN protein is a 134 amino acids protein seen mainly in presynaptic terminals and is homologous to αSN and found colocalized with αSN in the brain [235]. Both αSN and βSN have been characterized on the basis of their natively unfolded structures [236], but βSN is distinct from αSN in that βSN lacks 11 of the 35 amino acids in the central of the hydrophobic region of αSN, which was previously known as the nonamyloid-AD component of the AD amyloid (NAC) region [237]. Because of the presence of this highly amyloidogenic nonamyloid-AD component, αSN is prone to self-aggregate and form toxic protofibrils, whereas βSN is less prone to form insoluble aggregates [238]. Several lines of evidence suggest that βSN may have a protective role against α-synucleinopathies. In support of this notion, mixing with βSN reduced the loss of synaptophysin induced by αSN is consistent with the idea that molecular interactions between αSN and βSN affect the toxicity of αSN [156]. Additionally, previous reports from a transgenic mouse model of PD showed that the expression of βSN reduced the accumulation of αSN and neurodegeneration in mice expressing human αSN [239, 240].

Due to its unique structure, βSN had not been believed to play a critical role in the stimulation of neurodegeneration until two missense mutations of βSN were discovered in unrelated DLB patient pedigrees [241]. A valine to methionine substitution at position 70 (V70M) was found in a sporadic DLB case in Japan, while a proline to histidine mutation (P123H) was identified in a familial DLB pedigree in Seattle. These amino acid changes occurred at highly conserved residues in important domain regions of βSN, and did not appear to be single nucleotide polymorphisms, as they were not found in more than 330 controls. Furthermore, based on cosegregation analysis of the Seattle pedigree, the inheritance of the P123H mutation suggested that it could be a dominant trait with incomplete penetrance [241]. Histopathological analysis of the autopsy brain in the Seattle case failed to detect any βSN immunoreactivity in Lewy body inclusions. Furthermore, biochemical analysis failed to detect aggregation of βSN in the detergent- insoluble fractions of the brain extracts. Thus, it was predicted that βSN might have lost protective functions rather than having obtained a toxic gain-of-function through the P123H gene mutation, thereby accounting for the neurodegeneration observed in P123H-βSN brain [242]. Supporting this notion, a previous study showed that both P123H- and V70M-recombinant βSN proteins were prone to aggregate *in vitro* and that expression of these mutant βSN proteins in neuroblastoma cells resulted in lysosomal pathology associated with protein aggregation [243]. Furthermore, aggregates of P123H-βSN have been seen in both cortex and

hippocampal neurons in a P123H-βSN over-expressing mouse which was characterized by neuritic pathology, associated with astrogliosis and behavioral abnormalities [234]. On the other hand, cross-breeding of P123H-βSN transgenic mice with αSN transgenic mice resulted in enhanced phenotypes, including neuronal cell loss and dopaminergic dysfunction. These results strongly suggest that P123H-βSN is by itself pathogenic and cooperates with pathogenic αSN to stimulate neurodegeneration [234].

Therefore, in this study we investigated the effect of TQ and P123H-βSN on synaptic activity using FM1-43 dye in hippocampal neurons. In this experiment primary hippocampal neurons were treated with P123H-βSN (1 μM) with or without TQ (100 nM). On the day of the experiment, the culture medium was removed; neurons were incubated with 1μg/ml FM1-43 for 5 minutes, washed thrice in ice-cold PBS, and suspended in PBS. Fluorescence was measured using a microplate reader (TECAN) at excitation and emission wavelengths of 480 nm and 612 nm, respectively.

3.2.5 Spontaneous firing activity

Next, we used the Multielectrode array system to investigate the effect of αSN (2 μM) and TQ (100 nM) on the spontaneous firing activity of hiPSC-derived neurons. hiPSC-derived neurons were cultured on the MEA probe which have a 64 electrodes to record from and recordings were obtained using Mobius software and the average of the spontaneous firing activity was calculated. Measurements were taken before applying αSN to the neurons for 80 minutes as the firing activity for each second was recorded for every other 10 minutes during the 80 minutes. Then, the same measurements were taken after applying αSN for 11 days. The same measurements were taken before and after the treatment with αSN and TQ.

3.3 Results

3.3.1 Effect of TQ against αSN-induced synaptic protein inhibition

The loss of synapses is a common pathological feature in dementia associated with PD and DLB [220], in which aggregates of αSN accumulate at synapses [221, 222]. These disorders are considered to be induced by responses to αSN oligomers and the addition of recombinant human αSN-triggered synapse damage in cultured cortical neurons [156]. In the present study, treatment of hippocampal neutrons with αSN (1 μM) induced a 25% reduction in synaptophysin. However, the co-administration of αSN (1 μM) and TQ (100 nM) protected neurons against αSN-induced synapse damage and restored the synaptophysin level to 98% of the control value (Fig.3-1-A). This effect accrued without causing cell death, which was determined using the CellTiter-Glo luminescent cell viability assay (98% cell survival, n = 6, data not shown). The same experiment was performed to determine the effect of αSN and TQ on the level of Synapsin I, which serves as a marker of synaptic vesicle trafficking. The results demonstrated that adding αSN to hippocampal neurons induced a 35% reduction in Synapsin I levels. In contrast, co-administration of αSN and TQ enhanced the Synapsin I level by 15% compared with cells only treated with αSN (n = 6, data not shown). Fig.3-1-B shows the immunostaining of hippocampal neurons with synapses after incubation with both αSN and TQ.

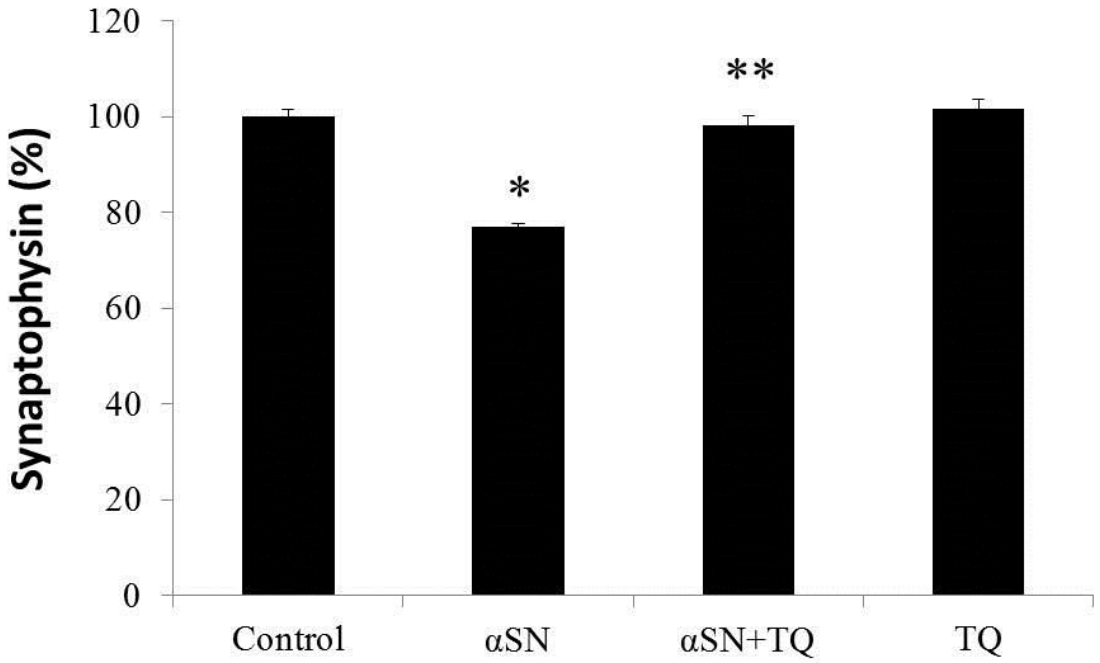
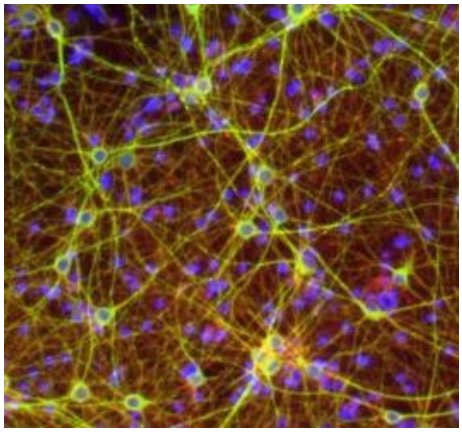


Figure 3-1. Effect of TQ and αSN on the synaptic protein (synaptophysin) level in cultured hippocampal neurons. (A) The synaptophysin content of hippocampal neurons treated with αSN (1 μM) or with αSN and TQ (100nM) incubated for 72h and assessed using immunostaining. (*P < 0.01 vs. control, **P < 0.01 vs. the group exposed to αSN alone), n=6. Values shown represent the mean percent fluorescence (where 100% = fluorescence in control hippocampal neurons), ± SE.



(B) A fluorescence image of fixed hippocampal neurons after incubation with αSN and TQ. The pre-synaptic marker synaptophysin is shown in red and the neuronal marker MAP2 in green. The Hoechst 33258 nuclear stain is blue.

As shown in Fig.3-2-A, the addition of αSN (1 μM) to hiPSC-derived neurons induced a 20% reduction in synaptophysin levels. However, co-administration of αSN (1 μM) and TQ (100 nM) protected the neurons against αSN-induced synapse damage and enhanced the synaptophysin level by 6% compared with controls. Fig.3-2-B shows the immunostaining of hiPSC-derived neurons with synapses after incubation with both αSN and TQ.

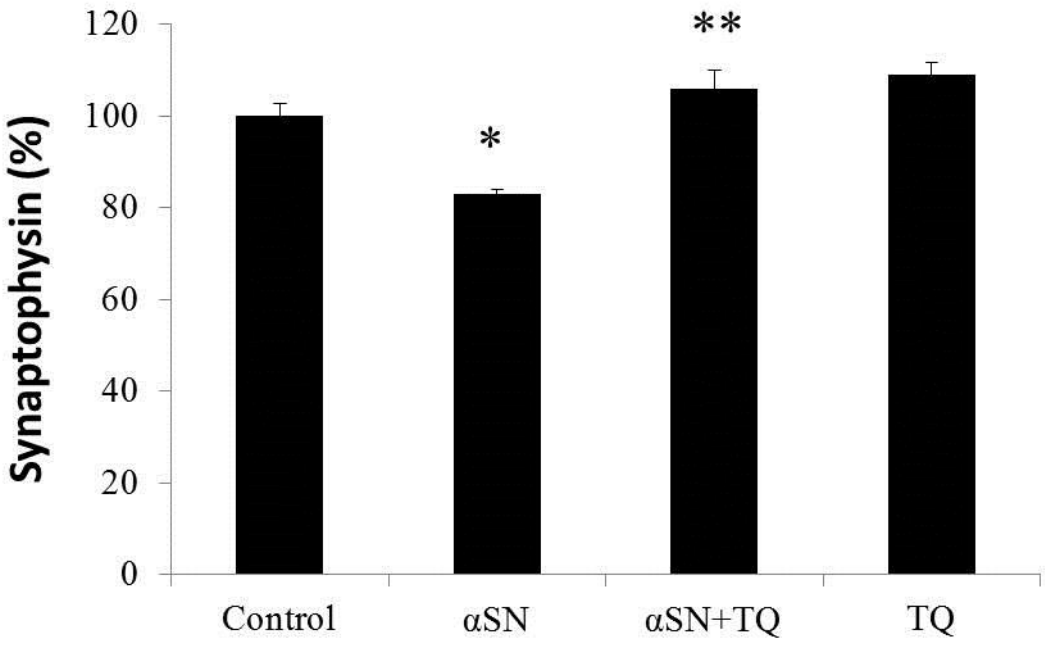
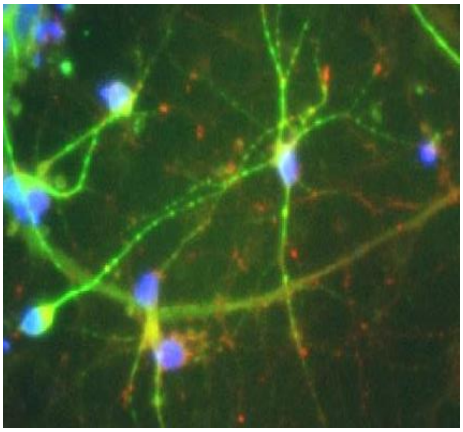


Figure 3-2. Effect of TQ and αSN on the synaptic protein (synaptophysin) level in cultured hiPSC-derived neurons. (A) The synaptophysin content of hiPSC-derived neurons treated with only αSN (1 μM) or αSN and TQ (100 nM) incubated for 72h and assessed by immunostaining. (*P < 0.01 vs. control, **P < 0.05 vs. the group exposed to αSN alone), n=5. Values represent the mean percent fluorescence (where 100% = fluorescence in control hiPSC-derived neurons), ± SE.



(B) A fluorescence image of fixed hiPSC-derived neurons after incubation with αSN and TQ. The pre-synaptic marker synaptophysin is shown in red and the neuronal marker MAP2 in green. The Hoechst 33258 nuclear stain is blue. Scale bar, 20µm.

3.3.2 Effect of TQ against αSN-induced synaptic vesicle recycling inhibition

We investigated the effect of TQ on αSN-induced inhibition of synaptic vesicle recycling. The uptake of the fluorescent dye FM1-43 into synaptic vesicles was used to determine synaptic vesicle recycling and thereby synaptic activities. The addition of αSN (1 μM) reduced FM1-43 uptake by 50% compared with that of controls. However, co-administration of αSN (1 μM) and TQ (100 nM) restored the uptake of FM1-43 by 40% compared with αSN-treated cells, and thus maintained synaptic activities in hippocampal neurons (Fig. 3-3-A).

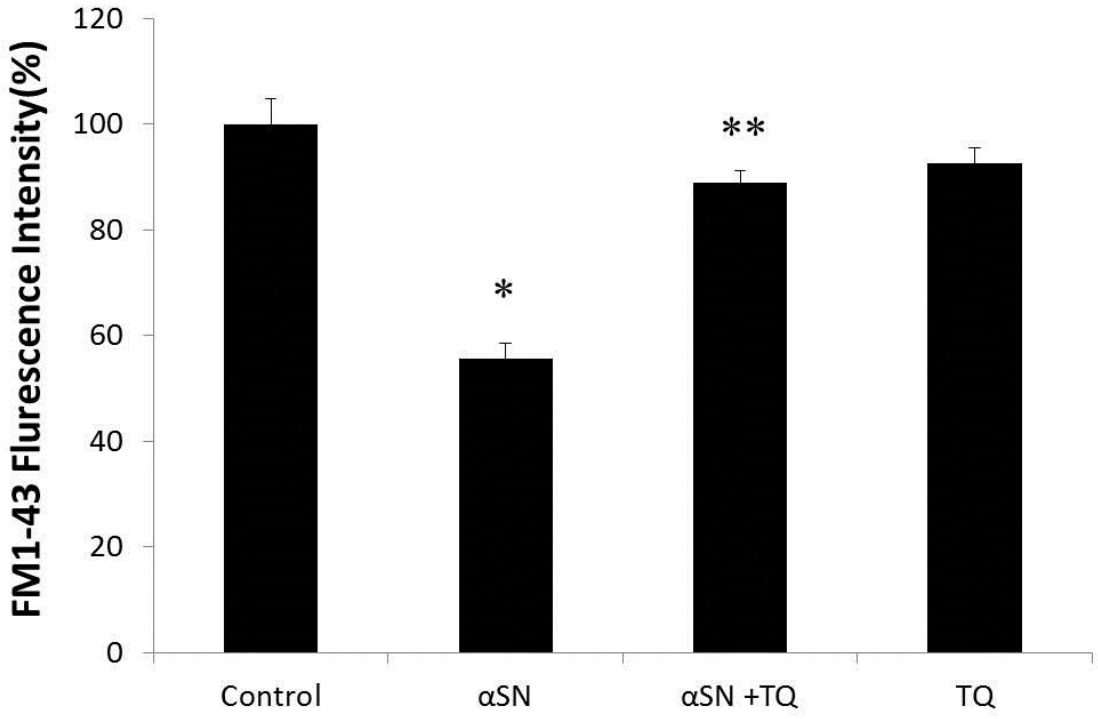
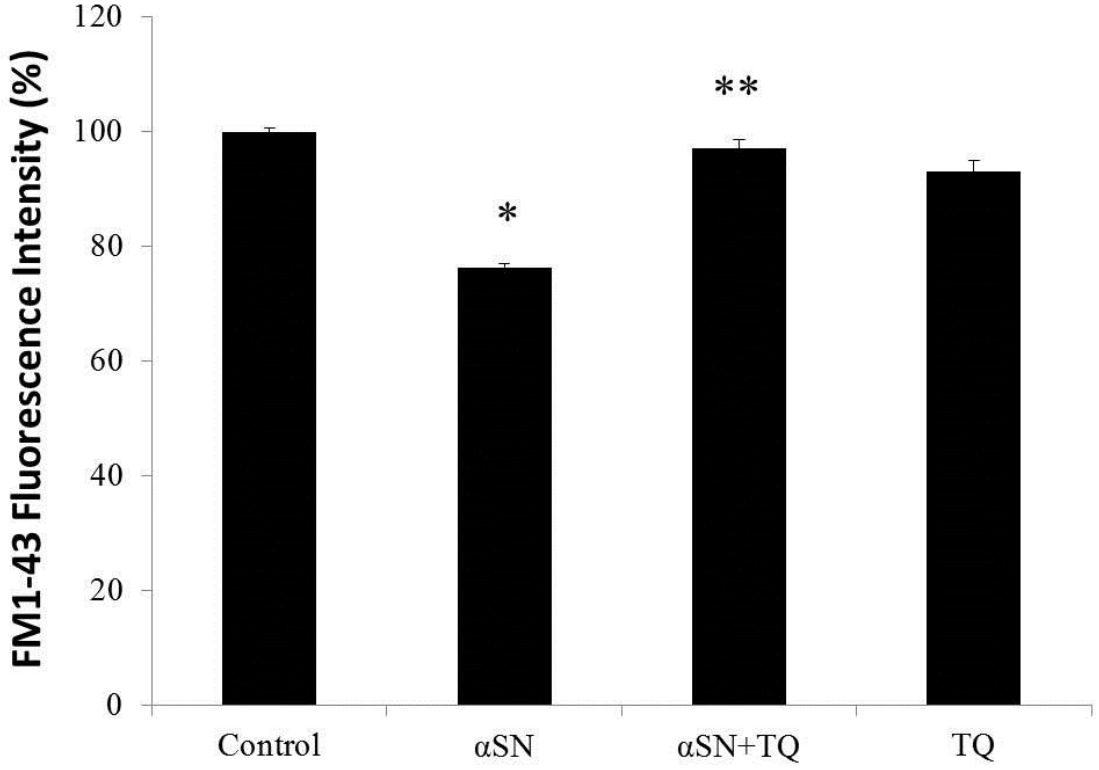


Figure 3-3. Effect of TQ against αSN-induced inhibition of synaptic vesicle recycling in cultured neurons. (A) Hippocampal neurons were treated for 72h with αSN (1 μM) in the presence or absence of TQ (100nM). Synaptic vesicle recycling was assessed using the fluorescence dye FM1-43. (*P < 0.01 vs. control, **P < 0.01 vs. the group exposed to αSN alone), n=6. Values shown are the mean percent fluorescence (where 100% = fluorescence in control hippocampal neurons), ± SE.

Addition of αSN (1 μM) to hiPSC-derived neurons induced a 25% reduction in FM1-43 uptake compared with controls. However, the co-administration of αSN (1 μM) and TQ (100 nM) restored the uptake of FM1-43 by 20% compared with αSN-treated neurons, and thus maintained synaptic activities (Fig. 3-3-B).



(B) hiPSC-derived neurons were treated for 72h with αSN (1 μM) in the presence or absence of TQ (100 nM). (*P < 0.01 vs. control, **P < 0.01 vs. the group exposed to αSN alone), n=5. Values shown are the mean percent fluorescence (where 100% = fluorescence in control hiPSC), ± SE.

3.3.3 Effect of TQ against P123H-βSN-induced synaptic vesicle recycling inhibition

Here, we investigated the effect of P123H-βSN on synaptic vesicle recycling using FM1-43. The addition of P123H -βSN (1 μM) affected synaptic vesicle recycling, as indicated by a 40% reduction in FM1-43uptake compared with controls. However, co-administration of P123H -βSN (1 μM) and TQ (100 nM) restored the uptake of FM1-43 by 30% compared with P123H -βSN-treated cells, and thus maintained synaptic activities in hippocampal neurons (Fig.3-4).

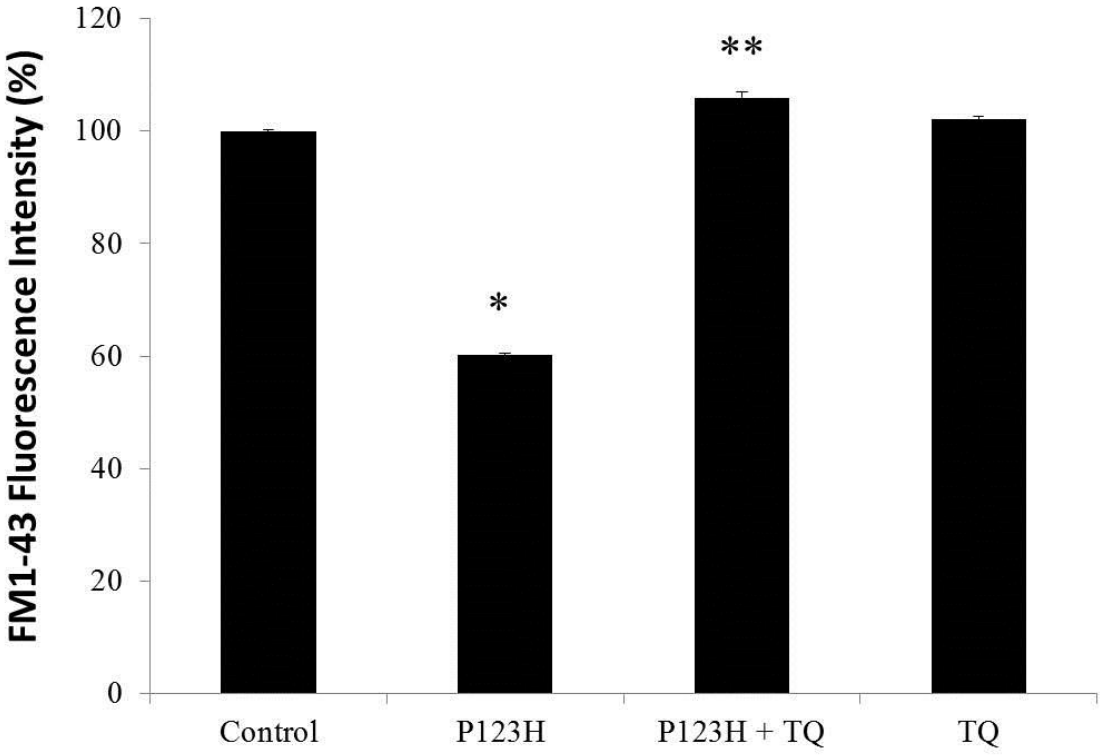


Figure 3-4. Effect of TQ against P123H- βSN-induced inhibition of synaptic vesicle recycling. Hippocampal neurons were treated for 72 h with P123H-βSN (1 μM) in the presence or absence of TQ (100 nM). (*P < 0.01 vs. control, **P < 0.01 vs. the group exposed to βSN (P123H) alone), n=6. Values shown represent the mean percent fluorescence (where 100% = fluorescence in control hippocampal neurons), ± SE.

3.3.4 Effect of TQ against αSN-induced spontaneous firing activity inhibition

In Fig. 3-5-A, on the left is the pattern of spontaneous firing activity of hiPSC-derived neurons before and after the administration of αSN (2 μM); αSN induced a reduction in spontaneous firing activity. On the right is the pattern of spontaneous firing activity of hiPSC-derived neurons before and after the simultaneous administration of αSN (2 μM) and TQ (100 nM). The cells maintained their spontaneous firing activity even 11 days after the co-administration of αSN and TQ. Moreover, in fig.3-5-C the addition of αSN (2 μM) induced more than 80% reduction in spontaneous firing activity at 11 days after exposure. However, when co-administered with TQ (100 nM), the cells maintained approximately 85% of their baseline firing activity 11 days after exposure (more than 4000 spikes for 10 min before administration of αSN or αSN and TQ treated samples).

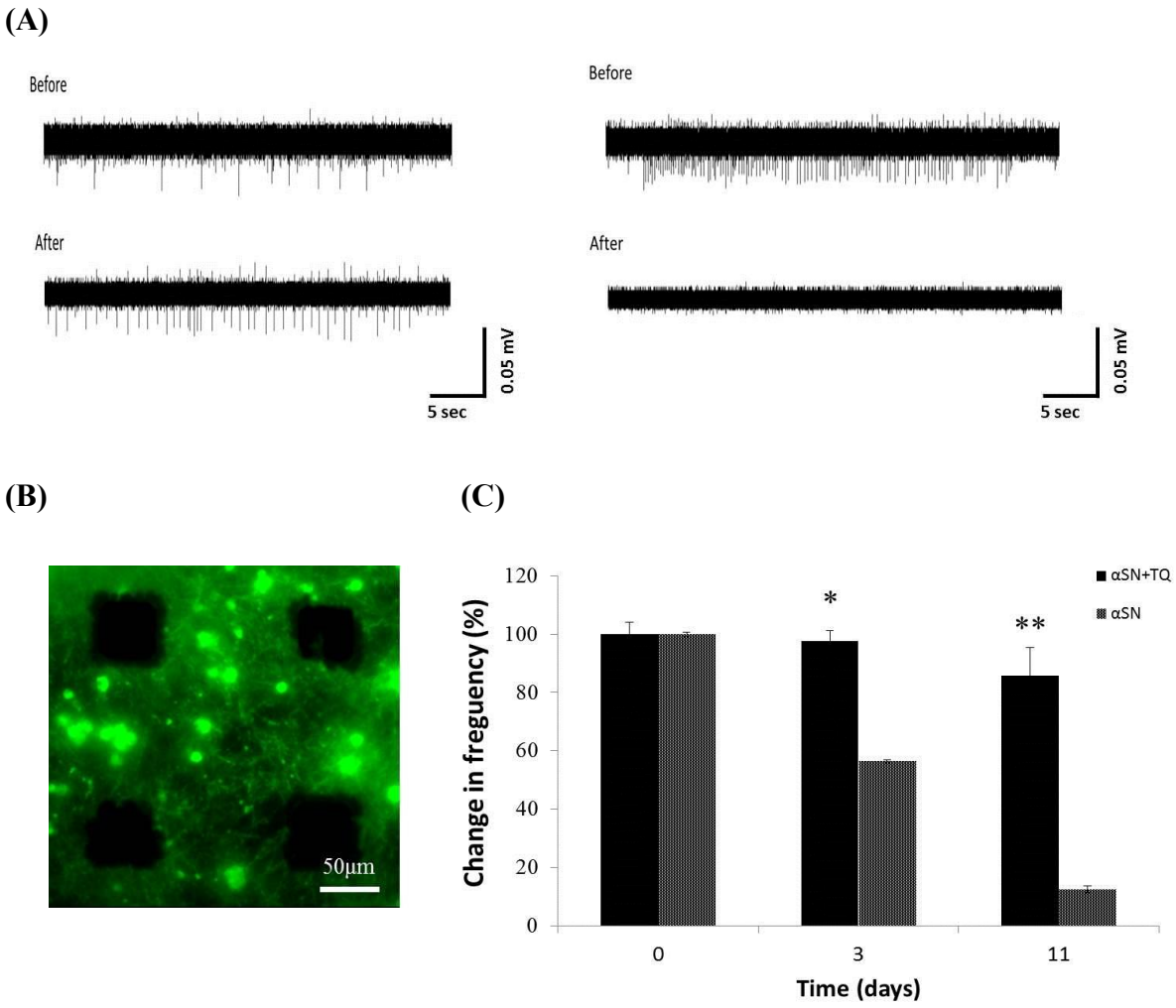


Figure 3-5. Effect of TQ against αSN-induced inhibition of spontaneous firing activity. hiPSC-derived neurons were treated with αSN (2 μM) in the presence or absence of TQ (100 nM). (A) The waveforms show typically spontaneous firings before and after 11 days of αSN administration (right). The waveforms before and after 11 days of αSN and TQ administration (left). (B) A fluorescence image of fixed human hiPSC-derived neurons (19 DIV) cultured on the microelectrode probe of the MEA system 11 days after incubation with αSN and TQ. The neuronal marker β-tubulin III is shown in green. (C) Time course of the effect of TQ on spontaneous firing frequency when administered with αSN showing the reversal of the effect of αSN on the firing frequency of hiPSC-derived neurons by TQ. (*P < 0.01 vs. control, **P < 0.01 vs. the group exposed to αSN alone), n=1. (100% represents baseline values before exposure to αSN), ± SE.

3.4 Discussion:

The discovery of new compounds that act on the central nervous system processes will stimulate not only their clinical use but will also contribute useful information for validating animal models of diseases and cell-based therapies. Among promising medicinal plants, *Nigella sativa* is an exceptional herb with a rich historical and religious background [244]. TQ is the major active component of *Nigella sativa* and constitutes approximately 30% of its volatile oil. In addition, it accounts for many of *Nigella sativa'*s pharmacodynamics properties [245].

The present study investigated whether TQ protects against αSN-induced synapse damage, and we showed that TQ effectively protected cultured hippocampal neurons against αSN-induced synapse damage and inhibition of synaptic activity. We also showed that TQ protected hiPSC-derived neurons against αSN-induced synapse damage, inhibition of synaptic activity, and inhibition of spontaneous firing activity. Moreover, mutated P123H-βSN induced the inhibition of synaptic vesicle recycling, and that inhibition was restored by the addition of TQ in hippocampal neurons. The loss of synapses is a prominent feature of many neurodegenerative diseases, including AD, PD, and DLB. The main mediators of neuropathology in PD and DLB are considered to be oligomers of αSN [17].

Here, we showed that αSN impaired synapse function and triggered a loss of synaptophysin from hippocampal neurons. These findings are consistent with reports stating that SN causes synapse damage in cultured hippocampal neurons [22]. We demonstrated that αSN reduced the synaptophysin levels of hiPSC-derived neurons, which was effectively attenuated by TQ. Moreover, treatment with αSN reduced synaptic vesicle recycling and therefore inhibited synaptic activity by hippocampal and hiPSC-derived neurons. However, the simultaneous addition of TQ with αSN effectively protected these cells from αSN and restored the recycling of synaptic vesicles. Finally, the effect of αSN on electrophysiological activities was investigated. αSN significantly reduced the spontaneous firing activity of hiPSC-derived neurons after 11 days of exposure. However, TQ partially restored the spontaneous firing activity of hiPSC-derived neurons even after11 days of exposure to αSN. We consider that these results were caused by αSN-induced synapse damage and TQ-induced protection. It is possible that the DLB-linked αSN mutants may have lost their ability to protect against αSN-induced neurotoxicity [223]. However, given the autosomal dominant trait of patients with the P123H-βSN mutation [223], toxic gain-of-function of

αSN through gene mutations may contribute to the pathogenesis of familial DLB. We demonstrated that the addition of P123H-βSN to hippocampal neurons induced the inhibition of synaptic vesicle recycling and thus inhibited synaptic activity. The use of hiPSC-derived neurons can be crucial to evaluate the exact path mechanisms involved in formation of synucleinopathies. αSN is involved in the pathogenesis of several so-called synucleinopathies, including PD. To date, a variety of models have been assessed. hiPSC provide a patient- and disease-specific model for in vitro studies, pharmacological screening, and hope for future cell-based therapies. We demonstrated for the first time that TQ effectively protects hiPSC-derived neurons from αSN-induced synapse damage and it could be effective if used with iPSC-derived neurons from patients with PD or from those with other α-synucleinopathies. In conclusion, our results suggest that TQ has potential therapeutic value for treating PD, DLB, and other neurodegenerative disorders.

Chapter 4

Summary

TQ has been chemically synthesized for years by oxidation of thymol with hydrogen peroxide. Last year marks the 50th anniversary of TQ's first isolation from a natural product when in 1963 it was identified in the essential oil of the *Nigella sativa L.* black seed, one of the most used plants in folk medicine in the Mediterranean region and West Asia [36]. Later, TQ was isolated from other plants with therapeutic properties namely *Eupatorium ayapana* [246], the leaves of several *Origanum* species [247], the heartwood essential oils of *Calocedrus decurrens* [248], the oil of different *Satureja* species [249], the aerial flowering parts of *Thymus vulgaris L.* [250] and from *Nepeta distans Raul* [251]. What makes TQ interesting is that it is readily available from a plant source and is not toxic to normal tissues. *Nigella sativa* and TQ's anti-inflammatory potential account for the observed analgesic, antidiabetic, and antihistaminic effects, and ability to alleviate respiratory diseases, rheumatoid arthritis, multiple sclerosis, and PD [252]. Three patents have been filed on behalf of TQ for the treatment of cancer, sepsis syndrome and urinary tract infections [253]. Moreover, TQ has demonstrated great potential against many microbes and parasites [252] and was shown to prevent atherosclerosis, osteoporosis, hypertension, and epilepsy [254, 255]. It reacts with the antioxidant enzyme GSH, nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) to yield the two strong antioxidants, glutathionyl-dihydrothymoquinone and dihydrothymoquinone [256]. Additionally, TQ was found to effectively ameliorate neurodegeneration [65, 68, 70, 99-101, 202-204]. Chitosan nanoparticles of TQ were described [257]. In vivo, the nanoparticles distribution was assessed in different organs after intranasal or intravenous administration and showed high targeting of the brain after intranasal administration. Those findings might be due to existence of direct nose-to-brain transport by passing the blood brain barrier. As of May 2013, the term ‘‘thymoquinone’’ brings up 345 search results in the Pubmed database. The yellow crystalline molecule TQ (2-methyl-5-isopropyl-1,4- benzoquinone) was isolated using thin layer chromatography on silica gel [108]. TQ has a basic quinone structure consisting of a para substituted dione conjugated to a benzene ring to which a methyl and an isopropyl side chain groups are added in positions 2 and 5, respectively (Fig.1-4). The crystal structure of TQ was later determined by high-resolution X-ray powder diffraction [258].

TQ was found to exert its biological functions by modulating the physiological and biochemical processes involved in ROS generation. In normal tissues, TQ acts as a strong antioxidant and inhibits the production of superoxide radicals and lipid peroxidation, or increases the activities of the antioxidant enzymes SOD, catalase, GSH, GSH transferase and quinone reductases [254, 255, 259, 260]. In tumors, however, TQ induces ROS generation [261, 262] and decreases GSH levels in a dose-dependent manner [262, 263]. Thus TQ has a dual role, and depending on the cellular microenvironment, it may act as an antioxidant or a pro-oxidant. TQ has shown efficacy in cancer cells when used in higher concentrations ranging from (20 to 60μM) or more, however; it showed efficacy in neural cells when used in concentrations (0.1 and 1μM), therefore its dual action is concentration dependent.

According to the current view on the molecular pathological mechanisms of AD, the accumulation and aggregation of Aβ initiates a cascade of cellular changes that gradually leads to memory loss [264]. It has been shown in several studies that small and soluble, non- fibrillary oligomers, rather than the large Aβ fibrils, are toxic [265-267]. It was reported that Aβ oligomers inhibit neuronal viability 10-fold more than fibrils and ~40-fold more than unaggregated peptide [268]. *In vitro*, oligomeric and protofibrillar forms of Aβ have been shown to be directly neurotoxic [269], inhibit electrophysiologic activity that may be necessary for the formation and maintenance of memory , and disrupt synaptic plasticity in AD affected neurons [10]. Furthermore, it has been reported [270] that freshly prepared $A\beta_{1-42}$ oligomers rapidly induce endoplasmic reticulum stress which results in activation of caspases and apoptosis of cortical neurons in culture, whereas aged fibrillar preparations of $A\beta_{1-42}$ were much less toxic to neurons. Additionally, it was demonstrated that small oligomers of $A\beta_{1-42}$ were toxic to both cerebellar and cortex neurons, causing cell death and LDH release when compared with $A\beta_{1-42}$ monomers and fibrils [271]. It was also found that cultures of rat cortical neurons exhibit a dose dependent increase in cell death following Aβ oligomer administration that was associated with an increase in ROS [272]. Recently, $A\beta_{1-42}$ oligomers were found to be toxic to hiPSC-derived neurons inducing apoptosis and cell death [273].

In agreement with the above mentioned previous results, we found that Aβ-induces a range of neurotoxicity's in embryonic rat primary neurons and hiPSC-derived neurons including cell death, apoptosis, MMP loss, GSH reduction, ROS generation, synaptic dysfunction, and firing activity inhibition. However, when administrated with Aβ, TQ was able to protect rat primary neurons and hiPSC-derived neurons against Aβ toxicity significantly. To understand the mechanism of which TQ

was able to perform its protective action, we investigated its effect on $A\beta_{1-42}$ aggregation using the ThT assay. We demonstrated that TQ was able to prevent the aggregation of $A\beta_{1-42}$ in vitro [274]. And therefore, the neuroprotective effects of TQ in our studies could be due to the inhibition of Aβ aggregation by TQ.

A study demonstrated that TQ could be a promising Aβ inhibitor. Its inhibitory effects were slightly similar to the anti-amyloidogenic tannic acid [275]. TQ is a non-polar molecule which was dissolved well in organic solvent (acetone). Therefore, it is postulated that the binding between TQ and Aβ could be induced by hydrophobic interactions of TQ and hydrophobic region of Aβ. Thus blocking the associations between Aβ molecules and thereby inhibiting the fibril formation [275]. These interactions could be reinforced by the H-bond acceptor group of TQ with some donor groups from Aβ. The same interactions have been suggested for resveratrol [276]. In addition, the hydrophobic regions of Aβ could interact with lipophilic chain of TQ, as occurred between Aβ and rifampicin [277]. On the other hand, resveratrol and curcumin were among the small molecules that were effective as anti-amyloidogenic agents [276, 278]. The molecular weight of TQ was much smaller than resveratrol and curcumin. TQ may have other mechanisms in inhibition of Aβ fibrils formation since its structure does not contain any hydroxyl groups and it is smaller in size than resveratrol and curcumin. Aromatic ring of TQ may also be suspected to be responsible for inhibition. Thus, TQ may have some properties in such a way to prevent Aβ aggregation. These results indicate the protective effects of TQ on neurons may be due to its contribution in disaggregating the neurotoxic Aβ accumulation, thus protecting cells from Aβ induced neurotoxicity.

Besides the presence of Aβ-peptides, the major component of senile plaques corresponds to a component now referred to as NAC (non-amyloid component of AD plaques) known as αSN. αSN is a small protein recognized in central nervous system neurons where it is localized to pre-synaptic terminals, regulates synaptic vesicle formation and neurotransmitter release, and can affect synaptic plasticity during learning. αSN was found to be the primary structural component of LBs which are the abnormal aggregates of protein that develop inside the neuron and are seen in PD, LBs, multiple system atrophy, familial AD and Down's syndrome. Because of the presence of αSN deposits, all these neurodegenerative disorders are grouped under the name of synucleinopathies. Evidence indicates that aggregates of αSN accumulate at presynaptic terminals and trigger synapse degeneration in patients with PD and DLB causing synaptic loss and reduction in the level of synaptic proteins. These effects of αSN on synapses were modified by interactions with other proteins. The addition of $A\beta_{1-42}$

exacerbated the effects of αSN on synaptic vesicle recycling and synapse damage. Similarly, the addition of αSN increased synapse damage induced by $A\beta_{1-42}$ [156]. Moreover, another report indicated that αSN enhances the release and toxicity of Aβ leading to nitric oxide mediated irreversible mitochondria dysfunction and caspase-dependent programmed cell death [279].

In our study we were able to demonstrate for the first time that TQ was able to protect rat hippocampal and hiPSC-derived neurons against the synapse damage induced by αSN effectively by restoring synaptic proteins levels, synaptic vesicle recycling activity and spontaneous activity [280]. While the role of αSN in the pathogenesis of AD remains unclear, indirect evidence suggests that this protein may interact with Aβ and increase its toxicity. TQ targets several proteins and is one of the rare natural products that can inhibit protein–protein interactions [281]. The inhibitory effect of amyloid formation in the presence of quinone molecules such as PQQ, EGCG and Baicalein is currently understood that the formation of quinone adducts of the parental amyloid forming proteins inhibits the further protein-protein oligomeric complex formation [282]. These quinone molecules were bound with peptide by forming Schiff-base to produce PQQ, EGCG or Baicalein adducts. Schiff-base formation between α-Syn and PQQ or Baicalein was occurred at the Lys residues in the α-SN molecule. Therefore, we suggest that TQ protection could be due to its quinone structure that inhibits protein-protein oligomeric complex formation through Schiff-base formation between TQ and αSN or Aβ [282]. And therefore our study have demonstrated that among several natural antioxidant compounds, we identified TQ as a potent agent that can prevent Aβ and αSN neurotoxicity and synapse damage respectively in not only rat primary neurons but most importantly in hiPSC-derived neurons that could help in the drug development and the treatment of neurodegenerative diseases. Further work should be carried out to ascertain whether TQ could be developed as a potential pharmacological agent for the prevention and treatment of AD and other related neurodegenerative disorders as well as explore the molecular mechanism of protective effect of TQ on the singling pathways of Aβ and αSN-mediated neurotoxicities.

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