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ORIGINAL PAPER

The Binding Mechanisms and Inhibitory Effect of Intravenous Anesthetics on AChE In Vitro and In Vivo: Kinetic Analysis and Molecular Docking

Mesut Işık[1]

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Abstract

Inhibitors of acetylcholinesterase (AChE), which have an important role in the prevention of excessive AChE activity and β-amyloid (Aβ) formation are widely used in the symptomatic treatment of Alzheimer's disease (AD). The inhibitory effect of anesthetic agents on AChE was determined by several approaches, including binding mechanisms, molecular docking and kinetic analysis. Inhibitory effect of intravenous anesthetics on AChE as in vitro and in vivo have been discovered. The midazolam, propofol and thiopental have shown competitive inhibition type (midazolam > propofol > thiopental) and Ki values were found to be 3.96.0 ± 0.1, 5.75 ± 0.12 and 29.65 ± 2.04 μM, respectively. The thiopental and midazolam showed inhibition effect on AChE in vitro, whereas they showed activation effect in vivo when they are combined together. The order of binding of the drugs to the active site of the 4M0E receptor was found to be midazolam > propofol > thiopental. This study on anesthetic agents that are now widely used in surgical applications, have provided a molecular basis for investigating the drug-enzyme interactions mechanism. In addition, the study is important in understanding the molecular mechanism of inhibitors that are effective in the treatment of AD.

Keywords Alzheimer's disease · AChE inhibition · Anesthetic drugs · Molecular docking

Introduction

The practice of anesthesia, which has a significant place in medicine with its widespread use in surgery, is widely accepted by the adoption of modern technologies and the discovery of new drugs. However, anesthetic applications still cause some concerns about the results, because the effects of various anesthetics on specific diseases and macromolecules are not well known [1]. Many organ systems such as cardiovascular and bronchoalveolar systems and liver are known to be affected by general anesthesia with breathing or non-volatile anesthetics. This can be attributed to the changes in the normal redox balance and the deterioration of protective mechanisms in mammals. In addition, it has been proposed that biomolecular changes may occur by anesthetic drugs in different pathophysiological cellular functions and in cases such as apoptosis, angiogenesis and proliferation [2, 3].

Ischemic periods such as transplant surgery, aortic aneurysm repair and coronary artery bypass graft surgery are known to cause the production of reactive oxygen species. Therefore, the use of anesthetic agents, which have antioxidant and free radical scavenging, has been thought to be effective in removing the harmful effects caused by the increase of reactive species [4]. In many studies, anesthetics with antioxidant properties such as propofol and thiopentone have been reported to have a mechanism of action that inhibits lipid peroxidation in tissue and alleviates reperfusion injury [5, 6]. In addition, It is known that many compounds, such as polyphenols or mushrooms, which have antioxidant and anti-inflammatory action, have neuroprotective properties. Many anesthetics drugs such as isoflurane, propofol and sevoflurane are reported to have antioxidant and anti-inflammatory effects in all cells with the exception of neuronal cell lines. Intravenous anesthetics are also known to have neuroprotective effects [7–9].

✉ Mesut Işık
misik@harran.edu.tr; mesutisik16@gmail.com

[1] Department of Pharmacy Services, Vocational School of Health Services, Harran University, 63000 Şanlıurfa, Turkey

These findings support the assumption that anesthesia can affect the molecular process and /or cellular by some unknown mechanisms. However, the effect of anesthetic drugs on the development of certain diseases and many metabolic enzymes are still unclear. It is known that many of the known diseases take place as a result of metabolic damage resulted from changes in enzyme activity with various effects. Therefore, it is important to know the effects of commonly used drugs on metabolic enzymes.

Acetylcholinesterase (AChE), which acts on the cholinergic system, is a very important enzyme for cholinergic function [10]. Alzheimer's disease (AD), one of the most common neurodegenerative diseases, is related to the change and degeneration of cholinesterase metabolism. The loss of basal forebrain neurons and decreased hippocampal and cortical acetylcholine (ACh) levels are one of the major causes of AD. The relationship between AD and cholinergic dysfunction is important for the therapeutic use of AChE inhibitors [11, 12]. Inhibition of AChE, which is responsible for the degradation of ACh, has proven to have a successful effect on the alleviation of some behavioral symptoms and cognitive of AD [13, 14]. Many studies have shown that AChE inhibitors used in the symptomatic treatment of AD are effective in both protecting the cells from oxidative damage and the production of cellular antioxidants [15]. The inhibitors (Aβ self-assembly and AChE inhibitors) have reduced the strong effect of multiple β-amyloid (Aβ) neurotoxic products on disease development [16].

In this study, the effect of anesthetic agents such as propofol, thiopental and midazolam on AChE was investigated both in vitro and in vivo. The binding mechanisms of the anesthetic drugs with inhibitory effect were determined by multiple approaches, including molecular docking and enzyme kinetic analysis. The data obtained with different approaches have a good agreement with each other.

Materials and Methods

Chemicals

All commercially available reagents required for the study were obtained from Merck and Sigma.

In Vivo Studies

This study was approved by Erzurum Regional Training and Research Hospital Clinical Research Ethics Committee (Decision number 2015/04-25). After samples are taken, they were brought to the laboratory with cold chain. Subsequently, they were divided into two parts. The samples that it will be used in this study were stored at − 80 °C. The samples were taken to − 20 °C just before this study. Patients who have been given anesthetic medication were divided into four groups containing an equal number of individuals: The first group (A) for induction was propofol 2 mg kg^{-1}, the second group (B) 4 mg kg^{-1} was thiopental sodium, the third group (C) were 1 mg kg^{-1} propofol and 0.1 mg kg^{-1} midazolam, the fourth group (D) was 2 mg kg^{-1} thiopental and 0.1 mg kg^{-1} midazolam in combination. There were 60 patients (20–40 years old, male) with 15 in each group. The use of anesthetic drugs and the characteristics of individuals in each group are shown in Table 1. There was no significant difference between the control groups and patient with regard to age or gender ($p > 0.05$).

All anesthetic procedures were performed by the same experienced anesthesiologist. All blood samples were taken under sterile conditions from 20G broth which was opened onto the hand as standard. Blood samples were taken before surgery and at 5 and 60 min after induction of anesthesia. Patients who were hospitalized for tonsillectomy, septoplasty and rhinoplasty were included in the study. A 20–40 years old male patients, who were selected for surgery due to having no additional disease, were taken to general anesthesia [17].

Measurement of AChE Activity

The inhibitory effects of anesthetic drugs on AChE enzymes both in vitro and in vivo were tested by Ellman's spectrophotometric method [18]. First, 50 µl 5,5′-dithio-bis(2-nitrobenzoic)acid compound (DTNB) and 100 µl of Tris–HCl solution (1 M, pH 8.0), and 50 µl AChE solution were incubated and mixed for 15 min at 30 °C. Next, the reaction was started by adding 50 µl of acetylthiocholine iodide (AChI), which was used as substrates. The enzymatic hydrolysis of

Table 1 The use of anesthetic drugs and the characteristics of individuals in each group

Characteristic of patients	A group	B group	C group	D group
Age (years)[a]	29.10 ± 5.66	34 ± 8.42	32.46 ± 6.74	33 ± 7.42
Male	15	15	15	15
Propofol (mg kg^{-1})	2	–	1	–
Thiopental sodium (mg kg^{-1})	–	4	–	2
Midazolam (mg kg^{-1})	–	–	0.1	0.1

[a]Data are expressed as the mean ± S.D

the substrate was recorded spectrophotometrically at a wavelength of 412 nm [19].

Partial Purification of AChE Enzyme from Human Serum

$(NH_4)_2S0_4$ precipitation was performed by slowly adding ammonium sulfate to human serum at 0–20%, 20–40%, 40–60%, 60–80% and 80–100% ranges, respectively. After each precipitation, it was centrifuged for 20 min at + 4 °C, 10,000 rpm. The pellets obtained at the end of the centrifugation were dissolved in 0.1 M sodium phosphate (pH 7.4) buffer containing 1 mM EDTA and 0.5% Triton X-100. The activity of supernatant and precipitate was measured at each interval and then the precipitation range was determined between 20 and 80%. The sample, obtained as a result of ammonium sulfate precipitation, was centrifuged at 10,000 rpm for 20 min. The pellets obtained after centrifugation were dissolved in 0.1 M sodium phosphate (pH 7.4) buffer, containing 1 mM EDTA and 0.5% Triton X-100 [20]. The obtained sample was first placed in the dialysis bag and then dialyzed for 1 h in dialysis buffer (50 mM Na-phosphate pH 7.4) at + 4° C for three hours.

In Vitro Inhibition Studies (Kinetic Studies)

The kinetic studies of anesthetic drugs on AChE were performed using partially purified AChE from serum. Enzyme activities were determined at 30 °C by using five concentrations of AChI and three concentrations of propofol, thiopental and midazolam. The inhibition percentage for each compound, the IC_{50} values, the inhibition types and the Ki values with Lineweaver–Burk curves were determined to reveal the mechanism of inhibition [21, 22].

Molecular Docking for Drug-Enzyme Interactions

The docking studies were performed using the Receptor Grid Generation [23], LigPrep [24], Protein Preparation Wizard [25], and Ligand Docking [26] panels implemented in Schrodinger Suite (Schrödinger Release 2019–1: Glide, Schrödinger, LLC, New York, NY, 2019). The crystal structure of acetylcholinesterase with the co-crystallized dihydrotanshinone-I (PDB ID: 4M0E) with the resolution of 2 Å was obtained from the protein data bank (https://www.rcsb.org/pdb). The ligands were prepared using LigPrep at pH 7.0. The energy minimization was done by using OPLS3e force-field [27]. The Glide standard-precision mode (Glide SP) was used for ligand docking. Based on the calculated Glide gscore with the best pose were ranked.

Statistical Analysis

The results were analyzed statistically via SPSS. Statistical comparison among different groups was performed by using one-way ANOVA tests. LSD post hoc pairwise comparison tests were also performed. Statistical significance was defined as $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Table 2 IC_{50} and Ki values of anesthetic drugs and inhibition types for AChE

Inhibitor	IC_{50} (μM)	R^2	K_i (μM)	Inhibition type
Thiopental	21.00 ± 1.83	0.9840	29.65 ± 2.04	Competitive
Propofol	4.70 ± 0.71	0.9783	5.75 ± 0.12	Competitive
Midazolam	3.51 ± 0.14	0.9819	3.96 ± 0.10	Competitive

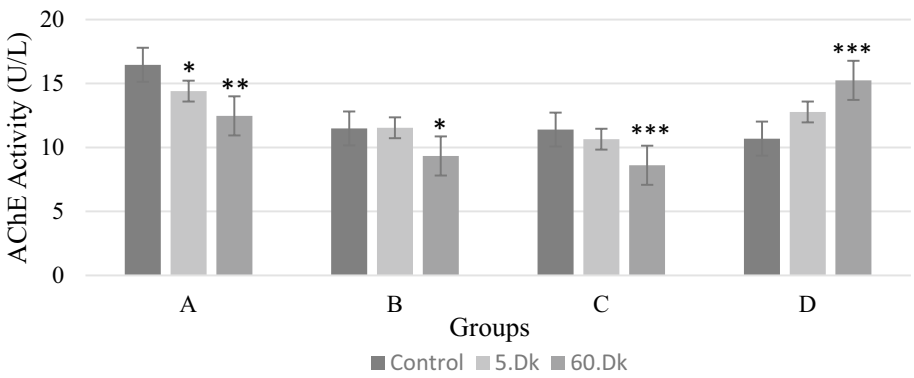

Fig. 1 The in vivo effects of *A* (propofol), *B* (thiopental), *C* (propofol-midazolam) and *D* (thiopental + midazolam) on serum AChE activity. Bars columns indicate AChE activity in serum samples taken before surgery (control) and at 5 and 60 min after induction of anesthesia as indicated. ***P < 0.001, **P < 0.01, *P < 0.05 versus control group

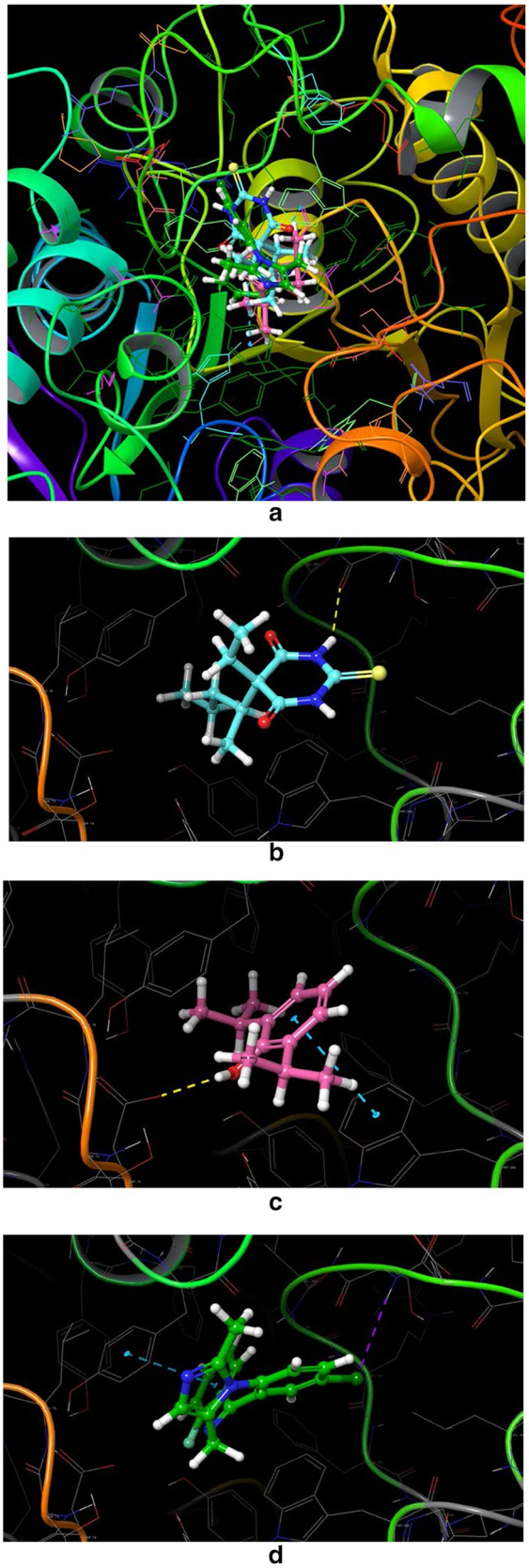

Fig. 2 Positions of thiopental, propofol, and midazolam anchored by ▶ the interaction with 4M0E (**a**). 3D schematic views of the thiopental (**b**), propofol (**c**), and midazolam (**d**) in the interactions with AChE (PDB ID: 4M0E). The interactions are depicted with different colors: pi-pi stacking (blue dotted line), H bond (yellow dotted line) and halogen bond (violet dotted line) (Color figure online)

Results and Discussion

The antioxidant property of propofol, which is commonly used in anesthetic applications, is partly due to its phenolic chemical structure, as shown in both in vivo and in vitro studies [28]. It has been reported that propofol has an important role in the inhibition of lipid peroxidation, increasing the antioxidant capacity of human plasma and the protection of cells against oxidative stress [29–31]. Furthermore, thiopental and midazolam inhibit lipid peroxidation and shows an antioxidant property. Its can also protect the brain from ischemia by acting as a free radical scavenger [32, 33]. The midazolam is a benzodiazepine derivative that is widely used. It acts on γ-aminobutyric acid (GABA) receptors by increasing neuronal permeability against chloride ions, and this leads to cell hyperpolarization. The midazolam is known to inhibit certain aspects of immune function and modulates metabolism.

It is known that hormetic dose responses are mediated by many receptors and cell signaling pathway (mediation/system, cell, organ). In a study, the effects of propofol and halothane on tissue and red blood cell (RBC) were investigated. High doses of propofol have been shown to significantly increase antioxidant capacity in tissue. As a result, a high correlation has been reported between changes in RBC sensitivity to against oxidative damage and related changes in tissues [34–36]. A group of researchers investigated the effect of ketamine, thiopental and midazolam (at different concentrations) on human neutrophil functions. The anesthetics have been found to significantly reduce reactive oxygen species, chemotaxis, and production of neutrophils in a dose-dependent manner [37]. Moreover, researchers compared the effects of thiopental, midazolam and propofol on brain ischemia with the oxygen-glucose withdrawal (OGD) model in rat brain cortical slices. High doses of midazolam and thiopental have neuroprotective effects against OGD damage in rat cerebral cortical slices, while high dose of propofol has been shown to increase OGD damage [38].

In human studies, it is known that isoflurane and sevoflurane have no effect on oxidative stress, inflammation and DNA damage in patients undergoing small incision operations. Anesthetics such as isoflurane and sevoflurane have antioxidant and anti-inflammatory effects in various cell lines, rodents and human studies. Some anesthetics and polyphenols, which have antioxidant and anti-inflammatory action, have also neuroprotective properties. For example,

Table 3 Binding free energy calculation results for thiopental, propofol, and midazolam bound with AChE (PDB ID: 4M0E)

Inhibitor	Glide XP GScore	ΔG vdW (kcal/mol)	ΔG Coulomb (kcal/mol)	Glide energy (kcal/mol)	Glide model (kcal/mol)
Thiopental	−5.36	−32.87	−6.89	−39.76	−51.57
Propofol	−6.31	−32.46	−13.08	−45.54	−61.70
Midazolam	−6.88	−38.67	−17.17	−55.84	−73.99

propofol is known to regulate the expression of Nuclear transcription factor kappa B (NFkB), which plays a key role in the formation of oxidative stress and inflammatory which is activated during ischemia/reperfusion, and induces cell damage. It has been reported that the anesthetic may have neuroprotective properties due to this effect [9, 39–41].

The molecular mechanisms of the anesthetics, that affect enzymatic metabolism, are currently not fully understood. In addition, most of the studies on the topic were performed in vitro. Many studies emphasize that anesthetic drugs cause the inhibition of important enzymes in metabolic pathways [42]. Metabolic damage, which caused by changes in enzyme activity, causes many diseases. For example, degeneration and changes in cholinesterase metabolism are associated with Alzheimer's disease (AD). The AChE inhibitors, used in the symptomatic treatment of the disease, are known to increase the production of antioxidants and protect cells from oxidative damage [15]. It was also emphasized that some AChE inhibitors used for AD have a role in increasing angiogenesis in cardiovascular patients [43].

Acetylcholinesterase (AChE), which is found in the cholinergic neuron, brain and muscles, causes neurodegenerative disorders by hydrolyzing neurotransmitter acetylcholine (ACh). The AChE also plays a role in the formation of β-amyloid (Aβ) accumulated in extracellular toxic plaques in AD brains, thus, the inhibition of the enzyme has inhibited the formation of Aβ aggregation [44, 45]. The change in concentration or activity of AChE has an important role in determining the degree of neurotoxicity [10, 46]. AChE inhibitors provide symptomatic relief and improve cognitive function in the treatment of AD by slowing or balancing ACh hydrolysis. They are also known to be widely used drugs [47]. A group of researchers studied donepezil-treated and general anesthesia for AD. They concluded that the donepezil of esterase inhibitor was effective on the muscle plaque and blocking the hydrolysis of ACh [48]. Butanol, pentanol and ketamine also inhibit AChE activity in total erythrocytes by altering both Km and Vmax [49]. Moreover, in the study on the effects of local anesthetics such as lidocaine, tetracaine and procaine on human erythrocyte AChE, the drugs were found to inhibit enzymatic activity (the concentration causing 50% inhibition). The IC_{50} was about 7.0 mM for lidocaine, 0.05 mM for tetracaine and 0.40 mM for procaine. The procaine and tetracaine show a competitive inhibition for the activity of AChE, whereas lidocaine showed mixed type inhibition kinetics [50]. The inhibitory effect of anesthetic drugs was at μM level in our study, in contrast to the previous studies where the level of the inhibitory effect of the drugs was around mM. Many researchers have observed that the ketamine inhibits both bovine brain AChE and human serum ChE in a mixed kinetic type [51]. The study showed that anesthetics such as isoflurane, enflurane, methoxyflurane, fluroxene, ether, halothane and trichloroethylene have a mixed-type inhibition effect on the AChE enzyme. It was found that ether and methoxyflurane inhibited AChE activity more efficiently in comparison to isoflurane and enflurane [52]. The effects of midazolam and thiopental on nicotinic acetylcholine receptor (nAChR) in mouse myotubes and PC12 cells were investigated. On the nAChR, midazolam acts as a competitive inhibitor at concentrations of about 10^{-6} M, while thiopental has been reported to inhibit neuronal nAChR mediated current [53, 54].

In the context of this information, we investigated the effects of intravenous anesthetics on AChE enzymes both in vivo and in vitro. According to our in vivo study, The propofol, thiopental and propofol-midazolam (A, B and C) showed inhibitory effect on AChE enzyme activity. On the other hand, AChE was activated when thiopental and midazolam were combined together. Inhibition effect on AChE of A, B and C was significantly higher at the sixtieth min ($P < 0.001$, $P < 0.05$). Moreover, thiopental and midazolam showed inhibition effect on AChE in vitro, whereas the activation effect was observed when thiopental and midazolam were combined together (Fig. 1). This is attributed to the fact that the change may be due to the synergistic effect as a result of drug-drug interaction. In our in vitro study, the IC50 and Ki parameters of the anesthetics drugs were determined with activity%-[Inhibitor] graphs and Lineweaver–Burk graphs (1/V-1/[S]). Midazolam has a high inhibitory effect in comparison to other drugs. The midazolam, propofol and thiopental have shown competitive inhibition type (midazolam > propofol > thiopental) and Ki values were found to be $3.96.0 \pm 0.1$, 5.75 ± 0.12 and 29.65 ± 2.04 μM, respectively (Table 2).

According to the results, the anesthetics drugs may be attached to the functional ends of the amino acids in the active site of the AChE enzyme. It was mentioned in the

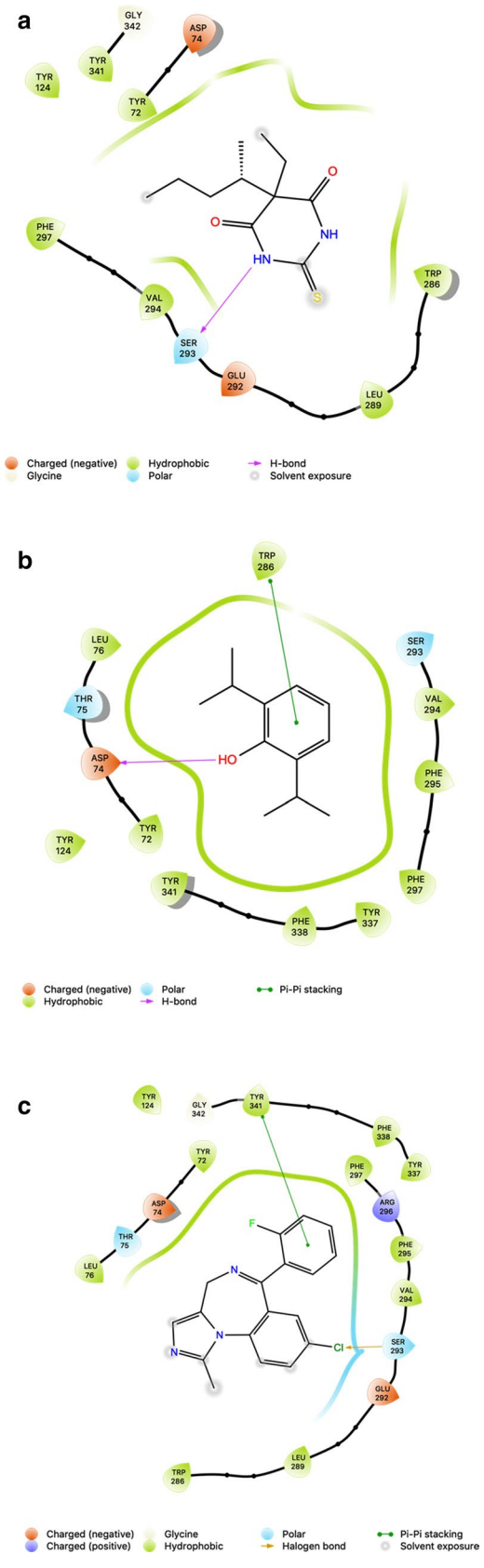

Fig. 3 2D schematic views of the thiopental (**a**), propofol (**b**), and midazolam (**c**) in the interactions with AChE (PDB ID: 4M0E) obtained from Glide XP calculations. The interactions are depicted with different colors: pi-pi stacking (green line), H bond (violet line) and halogen bond (orange line). The thiopental (**a**), propofol (**b**), and midazolam (**c**) also showed hydrophobic interactions with amino acids (Color figure online)

discussion section that anesthetics, which have antioxidant and anti-inflammatory effects, inhibit Aß aggregation formation and have neuroprotective properties. In our study, it was reported that midazolam, propofol and thiopental, which have antioxidant and anti-inflammatory effects, may have a neuroprotective effect by inhibiting the AChE. The information is consistent with the literature.

In this study, the molecular docking studies after experimental results (in vivo and in vitro) were performed in order to determine the probable binding mechanism of the drugs into the active site of the AChE. Based on the results obtained from in vitro enzymatic assays, molecular modeling studies were performed to understand the binding mode of the drugs into the active site of the 4M0E receptor (Fig. 2a). The docked molecules were ranked according to the Glide XP Gscore (Table 3). Thiopental (third most potent drug) showed one hydrogen bond interactions with amino acid Ser293. Furthermore, thiopental formed hydrophobic contact with Trp286, Leu289, Val294, Phe297 Tyr341, Tyr72 and Tyr124 (Figs. 2b and 3a). In propofol (second most potent drug), the functional group (-OH) of the phenol ring showed one hydrogen bond formation with amino acid Asp74. Additionally, it showed hydrophobic interactions with Val294, Phe295, Phe297, Tyr337, Phe338, Tyr341, Tyr124, Trp286, Tyr72 and Leu76. Pi–pi interaction appeared between the phenol ring and Trp286 residue (Figs. 2c and 3b). Midazolam is the most active drug. This drug formed one halogen bond with Ser293. Midazolam composed a hydrophobic cloud with, Tyr337, Phe338, Tyr341 Trp286, Leu289, Val294, Phe295, Phe297, Tyr72, Leu76, and Tyur124. Also, the benzene ring was found to have pi-pi stacking with Tyr341 (Figs. 2d and 3c).

In addition to our results, the molecular docking method has shown the possible binding mechanism of how the drugs bind to the active site of AChE. The analysis of interactions between anesthetics drugs and the binding site of 4M0E is shown in Table 3. The midazolam, propofol and thiopental showed the highest Glide gscores (− 6.88, − 6.31, and − 5.36, respectively). The free binding energies into the active site of AChE supports the experimental results due to the remarkable affinity of the drugs. The results indicated how the drugs can show inhibitory effect with molecular interaction mechanism.

In conclusion, the results obtained from this study provides information about interaction of AChE, protein structure and drug-enzyme interactions and molecular design role

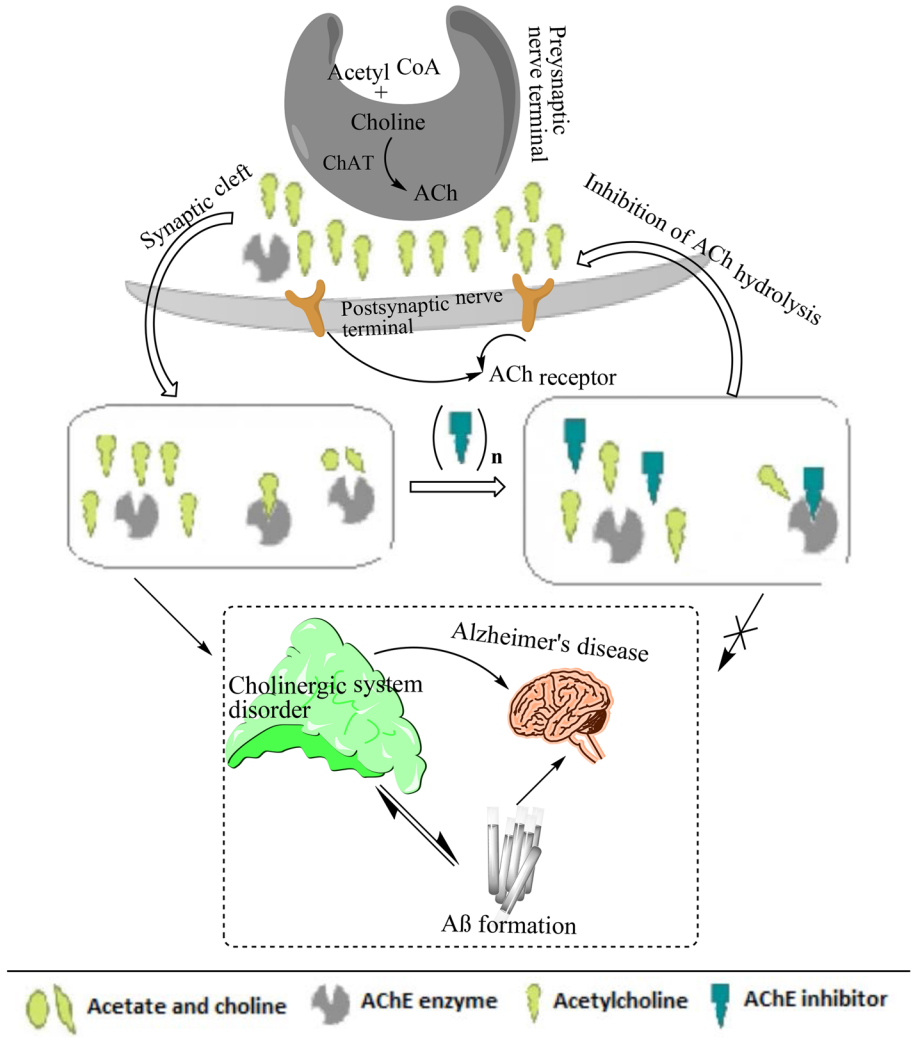

Fig. 4 Successful synaptic transmission depends on the regulation of the amount of ACh in the synapse. A portion of the neurotransmitter ACh released into the synaptic cleft, binds to postsynaptic receptors. The ACh in the synaptic space that can't bind to the receptors is hydrolyzed by excessive AChE activity. This may slow the nerve conduction and cause the development of Alzheimer's disease. The anesthetic drugs with inhibitory effect on AChE bind to the enzyme and regulate the cholinergic system by slowing the hydrolysis of ACh, and may also play a role in inhibiting the formation of Aβ

in drug development. The experimental data also provides the opportunity to explain the structural properties of inhibition by drug-enzyme interactions and to provide the main benefits of molecular docking. It has been concluded that anesthetic agents can act in a specific way to reduce AChE activity by binding to the active site of the enzyme both in vivo and in vitro (Fig. 4).

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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