

## Reversible acetylcholinesterase inhibitory effect of *Tabernaemontana divaricata* extract on synaptic transmission in rat CA1 hippocampus

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**Background & objectives:** Acetylcholinesterase inhibitors (AChE-Is) are used for the treatment of Alzheimer's disease (AD). These drugs including galanthamine have been shown to modulate synaptic activity in hippocampus and improve memory processes. Although *Tabernaemontana divaricata* extract (TDE) has been used as traditional medicine for various pharmacological effects, its effect in enhancing cholinergic activity provides additional benefit to its known effects. We investigated whether TDE can modulate the synaptic function in hippocampus and compared its effects to those of galanthamine.

**Methods:** Hippocampal slices were prepared from male Wistar rats, functional effects of TDE were characterized by using pharmacological tools and extracellular recordings of field excitatory postsynaptic potentials (fEPSPs).

**Results:** TDE significantly reduced fEPSPs. The fEPSPs reduction was prevented by atropine, but not pancuronium. These TDE effects were similar to those of galanthamine.

**Interpretation & conclusions:** Our findings indicate that TDE can effectively modulate synaptic responses in the hippocampus similar to galanthamine, suggesting that this traditional medicine could be beneficial in ageing with ACh deprivation in the brain.

**Key words** Acetylcholinesterase inhibitor - field excitatory postsynaptic potentials- hippocampus - *Tabernaemontana divaricata*

Acetylcholine (ACh), acting through neuronal muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs), is an important modulator of electrical activity in the brain. It is involved in numerous cellular processes underlying cognition including development, synaptic transmission, neuronal excitability and systems-level rhythmicity<sup>1-3</sup>. Stimulation of cholinergic inputs to the hippocampus demonstrates a decrease in the size of synaptic responses in glutamatergic projections

in different regions of the hippocampus<sup>1,4-8</sup>. An acetylcholinesterase inhibitor (AChE-I), physostigmine, has been shown to enhance cholinergic transmission and depress glutamate release in hippocampal pathways<sup>9</sup>. Modulation of the synaptic strength of excitatory glutamate synapses in the hippocampus is believed to be involved in memory processing<sup>10</sup>.

The loss of cholinergic function, particularly in the hippocampus, has been implicated in Alzheimer's

disease (AD)<sup>11</sup>. Current treatment is mainly based on the use of AChE-Is. Although some beneficial effects of these drugs on cognition have been reported, these are costly and have undesirable side effects<sup>12</sup>. Several drugs commonly used today for AD treatment were developed from local and traditional medicine, such as galanthamine, an AChE-I which was developed from alkaloids in 'Snowdrop'<sup>13</sup>.

*Tabernaemontana divaricata* (L.) R. Br. Ex Roem. & Schult (*T. divaricata*), a garden plant in tropical countries, is a rich source of alkaloids with various pharmacological properties<sup>14</sup>. *T. divaricata* has been used in the folk medicine for anti-infection, anti-inflammation, analgesic effect, anti-tumour effect, antioxidative effect and the effect in neuronal activity<sup>15</sup>. Ingkaninan *et al*<sup>16</sup> have shown *in vitro* that ethanol extracts from *T. divaricata* root (TDE) at a concentration of 0.1 mg/ml inhibit >90 per cent of AChE activity. Recently, we have demonstrated that TDE acts as a reversible neuronal AChE-I in rats<sup>17</sup>. These findings suggest that this traditional medicine could possibly help to improve memory, particularly in ageing with ACh deprivation in the brain. Despite this advancement in our understanding of the effects of TDE, its functional effect in the hippocampus, a brain region critical for learning and memory, has never been investigated. Therefore, in the present study we tested whether TDE can modulate dendritic field excitatory postsynaptic potentials (fEPSPs), which indicate the neuronal synaptic function, in *Cornu Ammonis* 1 (CA1) hippocampal slices of normal rats as found in current AChE-I drugs used for AD therapy. We also compared the effects of TDE with galanthamine, a well-known AChE-I drug used to treat AD patients.

### Material & Methods

**Plant materials and *T. divaricata* extract:** *T. divaricata* (collection no. Changwijiit 0020 at the PBM herbarium, Fac. Pharmaceutical Sciences, Mahidol University, Thailand) was collected from Phitsanulok, Thailand. Roots were extracted as described previously<sup>17</sup>. To validate the quality of TDE in each experiment, each lot was analyzed for inhibitory effects of AChE activity *in vitro* and *in vivo* before being used.

**Hippocampal slice preparation:** The study protocol was approved from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee. Hippocampal slices (400  $\mu$ m) were prepared from 4-5 wk old male Wistar rats obtained from the National Animal Center, Salaya Campus, Mohidol

University, Thailand (n=23) using standard methods. Rats were anaesthetized with halothane, decapitated, and the brain removed and placed in ice-cold "high sucrose" artificial CSF (aCSF) containing (mM): NaCl 85; KCl 2.5; MgSO<sub>4</sub> 4; CaCl<sub>2</sub> 0.5; NaH<sub>2</sub>PO<sub>4</sub> 1.25; NaHCO<sub>3</sub> 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95 per cent O<sub>2</sub>/5 per cent CO<sub>2</sub> (pH 7.4). This solution enhanced neuronal survival during the slicing procedure. Hippocampal slices were cut using a vibratome (Vibratome Company, St. Louis, MO, USA). Following a 30-min post-slice incubation in high sucrose aCSF, slices were transferred to a standard aCSF solution containing (mM): NaCl 119; KCl 2.5; CaCl<sub>2</sub> 2.5; MgSO<sub>4</sub> 1.3; NaH<sub>2</sub>PO<sub>4</sub> 1; NaHCO<sub>3</sub> 26; and glucose 10, saturated with 95 per cent O<sub>2</sub>/5 per cent CO<sub>2</sub> (pH 7.4) for an additional 30 min. For recordings, the slices were transferred to a submersion recording chamber and continuously perfused at 3-4 ml/min with standard aCSF warmed to 25-28°C.

**Stimulation and recording:** CA1 fEPSPs were recorded (Axopatch 200B, Axon Instruments, CA, USA) using standard methods described previously<sup>18</sup>. A stainless steel bipolar stimulating electrode (FHC, Bowdoinham, ME, USA) was placed in stratum radiatum to stimulate the Schaffer collaterals. A glass microelectrode (Sutter Instrument, Novato, CA, USA) filled with 2M of NaCl (Sigma, St. Louis, MO, USA) was placed in CA1 stratum radiatum to record fEPSPs. The stimulus frequency was 0.1 Hz. The stimulus intensity was adjusted to yield a fEPSP of 0.8-1.0mV in amplitude and produce ~50 per cent of maximal fEPSP responses. The delivery of two stimuli in rapid succession (50 msec interstimulus interval) elicited paired-pulse facilitation (PPF).

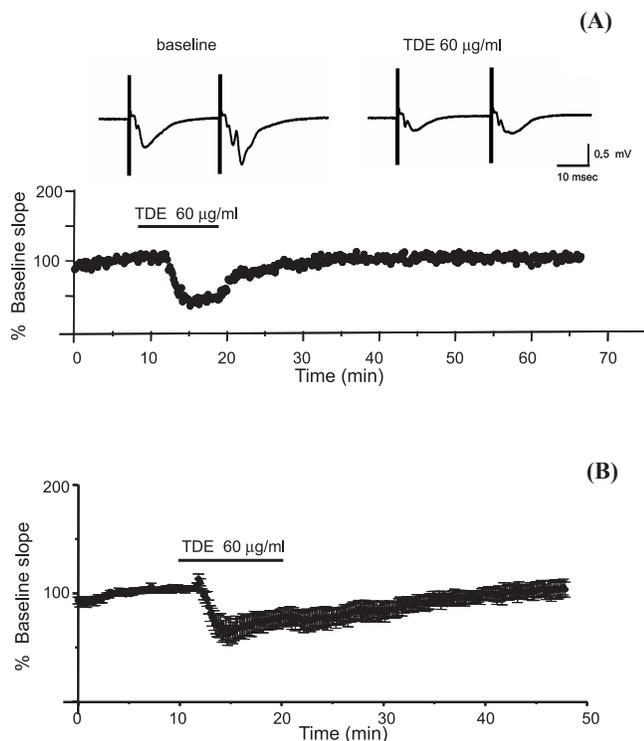
**Drug application:** Substances used in this experiment included TDE (dissolved in ethanol), atropine, ACh and galanthamine (dissolved in ddH<sub>2</sub>O). Appropriate concentrations of specific substances in solution were determined experimentally<sup>17</sup>. In baseline and wash conditions, hippocampal slices were perfused with standard aCSF. The same amount of ethanol was added to standard aCSF in baseline and wash conditions as was used for dissolving TDE. All chemicals were applied to the slices in a bath chamber via gravity perfusion. All chemicals were obtained from Sigma, St. Louis, MO, USA.

**Data analysis:** Data were filtered at 3 kHz, digitized at 10 kHz, and stored on a computer using pClamp 9.2 software (Axon Instruments, CA, USA). The initial slope of the fEPSP was measured and plotted vs. time. The initial slope is the first slope of the field

potential detected following stimulus artifact and fiber volley. The initial slope is used for measurement of synaptic strength in the synaptic experiment. Statistical significance between the groups was determined with the Student's *t*-test. Only experiments with less than a 10 per cent change in the original baseline were included in the analysis.

## Results

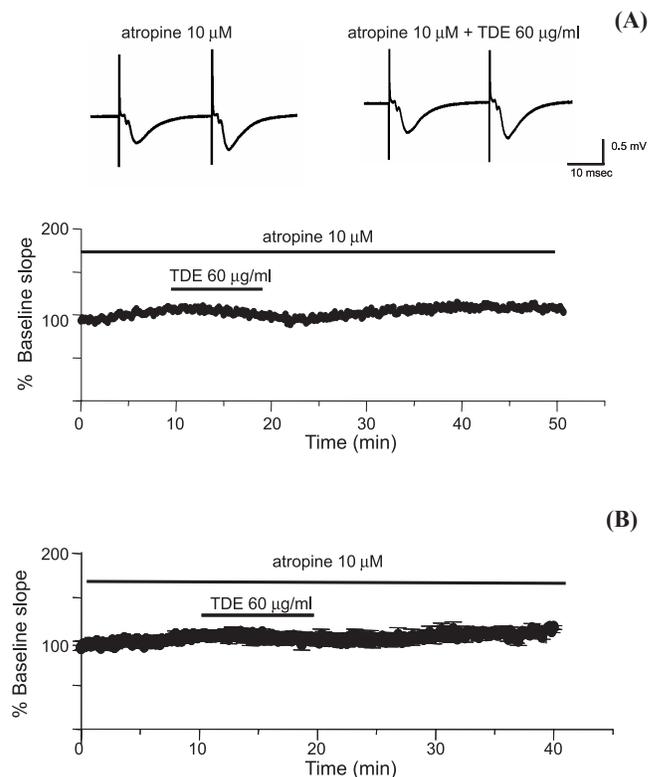
TDE's effect on fEPSPs was recorded in the stratum radiatum of the CA1 hippocampus, in response to stimulation of Schaffer collaterals. Paired-pulse facilitation (PPF), in response to paired stimulation pulses, was used to clarify Schaffer collateral pathways (Fig. 1A). TDE (60  $\mu\text{g/ml}$ ) reduced the size of the responses beginning 2-3 min after the start of infusion, with maximum effects appearing over the following 5-7 min. In our set-up, compounds added to the infusion line required approximately 1 min to reach the slices. The fEPSP depression was prominent and did not appear to be accompanied by distortion of waveform (Fig. 1A, inset). The fEPSP responses returned to



**Fig. 1.** TDE effects on CA1 hippocampal responses. (A) TDE-induced depression of synaptic responses. Traces were obtained from the experiment shown in 1A (inset). Each trace shows the average of 20 consecutive sweeps, recorded 2 min before (baseline) and during TDE application (TDE 60  $\mu\text{g/ml}$ ). (B) Average of 7 TDE applications shown as mean  $\pm$  SEM.

the same level as baseline responses after 15 min of washout (Fig. 1A). The mean depression of fEPSPs following the application of 60  $\mu\text{g/ml}$  of TDE was  $47 \pm 4$  per cent ( $n=7$ , Fig. 1B). We used 60  $\mu\text{g/ml}$  of TDE in this study since this concentration demonstrated maximum effect on the depression of fEPSPs in this study. TDE with concentrations higher than 10  $\mu\text{g/ml}$  transiently reduced synaptic responses. The range of fEPSP depression in TDE concentrations of 10-100  $\mu\text{g/ml}$  was 46-75 per cent ( $n=5$  per dose, the dose response curve of TDE was not shown).

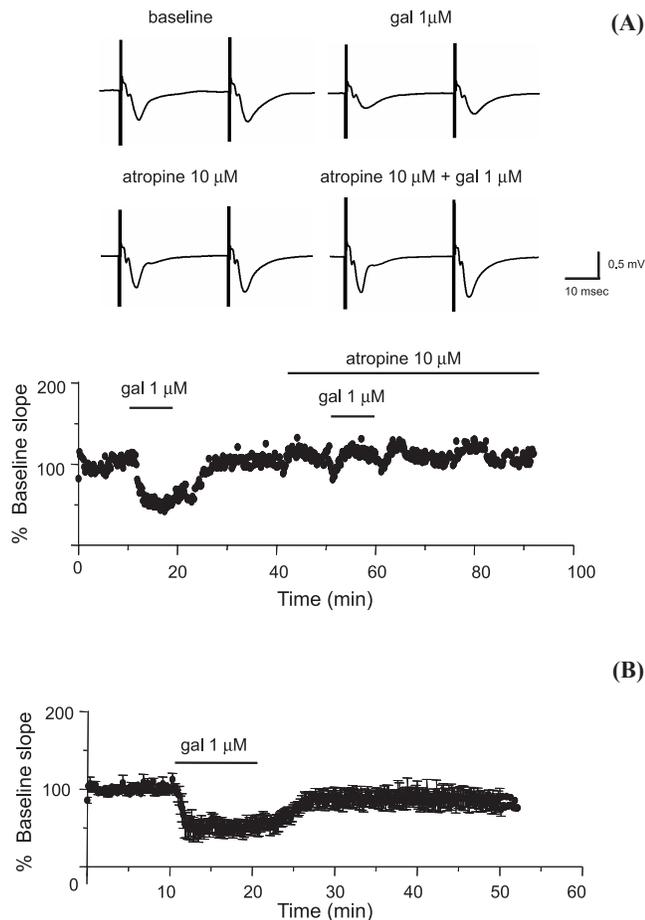
The effects of 60  $\mu\text{g/ml}$  of TDE were completely blocked by 10  $\mu\text{M}$  atropine, an mAChR antagonist (Fig. 2A and 2B), suggesting that the TDE effect was mediated by mAChRs. We used a high concentration of atropine (10  $\mu\text{M}$ ) since it can completely block the TDE effect on reducing synaptic response in all hippocampal slices. To investigate whether the nAChRs were involved in the synaptic modulation of TDE, pancuronium



**Fig. 2.** The non selective muscarinic antagonist, atropine (10  $\mu\text{M}$ ), completely blocked the acute synaptic depression caused by TDE. (A) Atropine blocked the TDE-induced synaptic depression. Traces were obtained from the experiment shown in 2A (inset). Each trace shows the average of 20 consecutive sweeps, recorded 2 min before the application of TDE (atropine 10  $\mu\text{M}$ ) and during the application of TDE (atropine 10  $\mu\text{M}$  + TDE 60  $\mu\text{g/ml}$  application). (B) Average of 7 TDE applications shown as mean  $\pm$  SEM.

bromide, a non selective nAChR antagonist, was used. In contrast to the effect of atropine, the TDE-induced reduction in Schaffer collateral fEPSPs persisted despite pretreatment of the slices with pancuronium bromide (100  $\mu$ M). The mean depression of fEPSPs following the application of 60  $\mu$ g/ml of TDE with and without 100  $\mu$ M pancuronium was not significantly different ( $n=6$ ). We used a high concentration of pancuronium (100  $\mu$ M, the dose response curve of pancuronium with 60  $\mu$ g/ml was not shown in this study) to completely block all subtypes of nAChR in hippocampal regions without having any effects on synaptic transmission<sup>19</sup>.

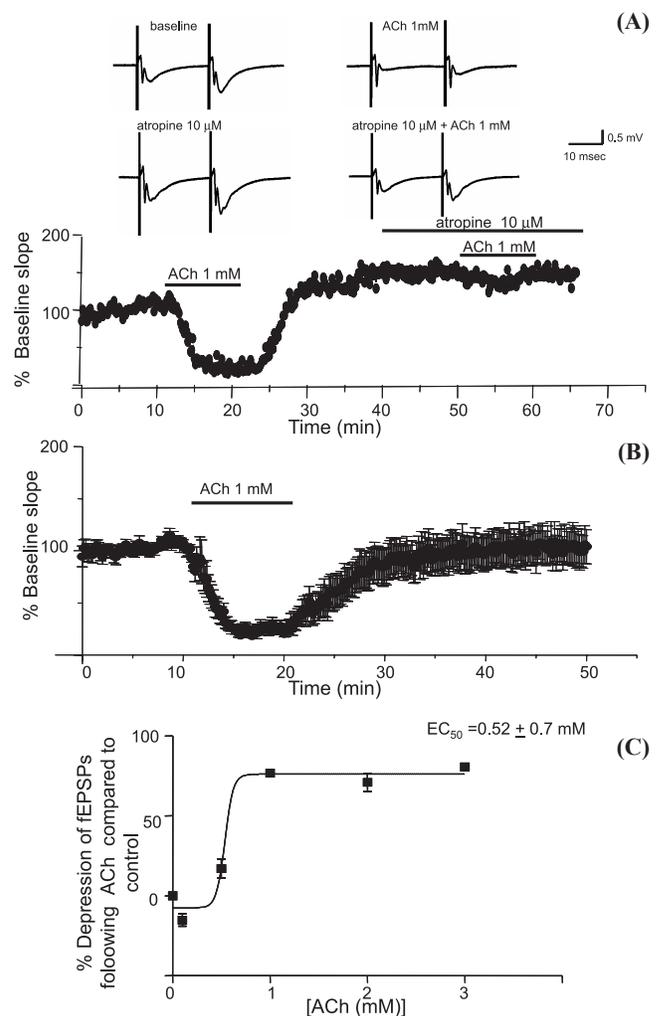
The TDE effect of fEPSPs that we observed in CA1 was similar to the effect of galanthamine, a reversible AChE-I, and of ACh. One  $\mu$ M galanthamine application transiently depressed fEPSPs (Fig. 3). The mean



**Fig. 3.** Effect of 1  $\mu$ M galanthamine (gal) on CA1 hippocampal responses. (A) Galanthamine-induced depression of synaptic responses. Depression was blocked by atropine (10  $\mu$ M). Traces were obtained from the experiment shown in 3A (inset). Each trace shows an average of 20 consecutive sweeps. (B) Average of 20 consecutive sweeps. (C) Average of 4 galanthamine applications shown as mean  $\pm$  SEM.

fEPSP depression following the application of 1  $\mu$ M galanthamine was  $42 \pm 8$  per cent (Fig. 3B,  $n=4$ ). We used 1  $\mu$ M galanthamine in this study since it has been shown to modulate glutamatergic synaptic transmission<sup>20</sup>. The effect of galanthamine in suppressing fEPSPs was also completely blocked by 10  $\mu$ M atropine (Fig. 3A). Our findings suggest that TDE acts to suppress CA1 synaptic responses as same as galanthamine does.

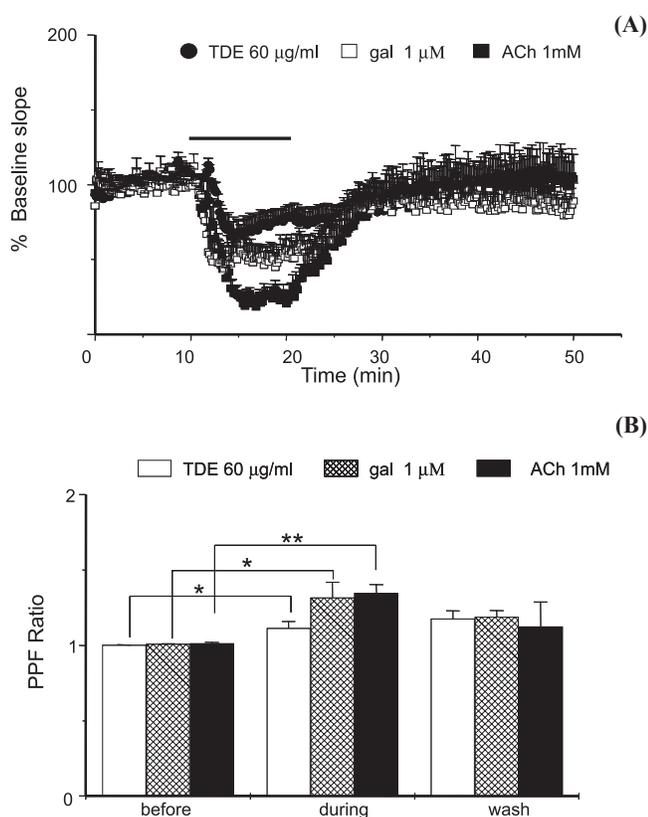
ACh (1 mM) transiently depressed fEPSPs, similar to that of TDE application (Fig. 4). The ACh effect in suppressing fEPSPs was also completely blocked by 10  $\mu$ M atropine (Fig. 4A). The mean fEPSP depression following the application of 1 mM ACh was  $77 \pm 3$  per



**Fig. 4.** Acetylcholine (ACh) caused a large depression of hippocampal synaptic response. Its effect was blocked by atropine. (A) ACh-induced depression of synaptic responses. ACh effect was blocked by atropine. Traces were obtained from the experiment shown in 4A (inset). Each trace shows an average of 20 consecutive sweeps. (B) Average of 7 ACh applications shown as mean  $\pm$  SEM. (C) The dose response of ACh in depressing synaptic responses ( $n=5$ ).

cent ( $n=7$ , Fig. 4B). We used 1 mM ACh since it had maximal effect on fEPSPs depression (Fig. 4C). The depression of fEPSPs by ACh was dose-dependent and the  $EC_{50}$  in fEPSPs depression occurred at  $0.52 \pm 0.7$  mM of ACh ( $n=5$  per dose, Fig. 4C).

The depression of synaptic transmission caused by both TDE and galanthamine application was less and slower than that by ACh (Fig. 5A). This finding suggests that TDE depresses fEPSPs in a similar way to galanthamine and TDE might not directly suppress fEPSPs in the same fashion as ACh application. Fig. 5B demonstrates the paired-pulse facilitation (PPF), a simple and sensitive measure of changes in presynaptic neurotransmitter release probability. We used PPF to test the hypothesis that TDE, galanthamine and ACh reduce synaptic responses by presynaptically depressing glutamatergic release. Mean paired-pulse facilitation of the CA1 hippocampus was increased



**Fig. 5.** Comparison of average depression of synaptic responses between 60 µg/ml of TDE, 1 µM galanthamine (gal) and 1 mM of ACh. **(A)** TDE- and galanthamine-induced suppression of CA1 hippocampal synaptic responses appeared to be less and slower than ACh-induced suppression. **(B)** TDE, galanthamine and ACh significantly increased paired-pulse facilitation (PPF) (\* $P < 0.05$ , \*\* $P < 0.01$  before comparison during TDE, galanthamine or ACh application).

during the application of TDE (60 µg/ml), galanthamine (1 µM) and ACh (1 mM) (Fig. 5B). Mean facilitation of the response slope with 60 µg/ml of TDE during infusion was  $11 \pm 4$  per cent greater than that before TDE infusion ( $P < 0.05$ ). Mean facilitation of the response slope with 1 µM galanthamine during infusion was  $13 \pm 1$  per cent greater than before galanthamine infusion ( $P < 0.05$ ). The increase of the PPF response slope with 1 mM ACh during infusion was  $35 \pm 6$  per cent greater than that before ACh infusion ( $P < 0.01$ ).

## Discussion

The major finding of this study was that TDE suppressed synaptic transmission at hippocampal CA1 synapse. We demonstrated that TDE-enhanced cholinergic transmission in hippocampal circuits affected synaptic glutamatergic transmission by depressing neurotransmitter release in a similar manner to galanthamine and ACh. In this study, the TDE effect was blocked by atropine, but not pancuronium, suggesting that TDE may modulate synaptic transmission via the muscarinic cholinergic function.

A massive glutamatergic input from the cortex depends on a collection of afferents releasing neurotransmitters other than glutamate for synchronizing rhythms in the hippocampus. ACh is one of powerful presynaptic modulators at the glutamatergic synapses<sup>6,21</sup>. The cholinergic innervation in the hippocampus is provided from the medial septal nucleus<sup>22</sup>. Stimulation of cholinergic innervation in different regions of the hippocampus reduces glutamatergic synaptic transmission<sup>1,4-8,23</sup>. Much evidence has been provided on cholinergic interaction with glutamatergic transmission<sup>11,24</sup>. A study showed that coincident glutamatergic and cholinergic inputs transiently depress glutamatergic release at the CA1 synapse<sup>3</sup>. The interaction between cholinergic and glutamatergic transmission was suggested to be important in memory processing<sup>23</sup>.

We have shown that TDE acts as a reversible AChE-I and is capable of increasing neuronal activity in the cerebral cortex, possibly by increasing cholinergic function<sup>17</sup>. In this study, we showed that TDE caused a transient depression of synaptic transmission in Schaffer collateral pathways of the CA1 response. The modulation of synaptic response by TDE was similar to the effect of galanthamine and ACh. These findings suggest that TDE possibly elevates the endogenous ACh level at the cholinergic synapses in hippocampal circuits. The synaptic

modulation of TDE may occur via cholinergic regulation of neurotransmission. This possibility is supported by our findings where the transient depression resulting from TDE application was prevented by mAChRs blockade. Atropine alone had no effect on hippocampal synaptic transmission. Also, TDE effects were similar to those of galanthamine, as demonstrated in this study, and those of physostigmine in a previous study<sup>9</sup>.

Changes in a paired-pulse ratio by drug typically indicate a presynaptic mechanism of drug action<sup>25,26</sup>. TDE acutely reduces the fEPSPs slope and the depression is accompanied by an increase in the paired-pulse ratio, suggesting that TDE transiently depressed synaptic transmission by decreasing neurotransmitter release at the presynaptic site. This effect is similar to those observed in physostigmine application<sup>9</sup>. TDE's effect on the paired-pulse ratio was similar to those of galanthamine and ACh. The paired-pulse results provide insights into the functional changes arising from cholinergic suppression of glutamatergic synapses by TDE. The different fEPSPs depression and PPF effects between ACh and TDE/galanthamine found in this study could be due to the fact that TDE and galanthamine caused an increase in the endogenous ACh level at the synaptic sites. However, the increased amount of ACh in the synaptic regions caused by TDE and galanthamine application was less than the high-concentration exogenous ACh application in our study.

In conclusion, the present findings demonstrated that TDE showed similar effects to those of galanthamine. This important evidence indicates that consumption of this natural product (TDE) could be beneficial in slowing the process of learning and memory loss particularly in old age people or Alzheimer's patients. Our findings also suggest that TDE could possibly be used to develop a new acetylcholinesterase inhibitor for the treatment of Alzheimer's disease. Further, our findings support the beneficial effects of TDE and demonstrate the additional benefits of this traditional medicine on the learning and memory process with future clinical significance.

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