Cholinergic modulation of hippocampal long-term potentiation in chronic cerebral hypoperfused rats

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Abstract: Vascular dementia (VaD) is one of the most common types of dementia in Alzheimer's disease (AD). Two-vessel occlusion (2VO), also known as permanent bilateral occlusion of the common carotid arteries, induces chronic cerebral hypoperfusion (CCH) in rats, resulting in neuronal loss and inflammation (particularly in the cortex and hippocampus). The 2VO rat model has been widely used to represent VaD conditions similar to those seen in humans. Synaptic plasticity or long-term potentiation (LTP) is one of the most important neurochemical foundations in learning and memory, deficits of which occur as a result of VaD. The aim of this study is to evaluate the role of cholinergic transmission in LTP impairment of CCH rat model. There is a significant impairment of LTP following the induction of 2VO surgery ($p < .05$). Treatment with oxotremorine and tacrine cause significant enhancement of LTP and potentiation levels ($p < .05$). There are also significant effects of paired-pulse facilitations when treated with cholinergic agonists and baseline synaptic transmission with increasing stimulation intensity ($p < .0001$). AChE activity was only found to increase significantly in the hippocampal region ($p < .05$). The role of cholinergic neurotransmission has been clearly demonstrated in LTP impairment of the CCH rat model. Augmentation of synaptic transmission was clearly observed in this model via changes of basal synaptic transmission and neurotransmitter release presynaptically.

Keywords: vascular dementia; long-term potentiation; tacrine; oxotremorine; carbachol;

1. Introduction

Vascular dementia (VaD) is one of the most common types of dementia in Alzheimer's disease (AD). In 2015, it was estimated that 46.8 million people worldwide are living with dementia, with numbers predicted to double every 20 years [1]. VaD typically develops when the blood supply to the brain is reduced or blocked by damage to the vascular system [2]. Continuous

reduction of cerebral blood flow (CBF) affects neural structures regulating memory and cognitive processes and eventually leads to the development and progression of VaD [3]. However, there are currently no approved drugs that can stop the progression of VaD. Even though the accurate mechanisms involved in VaD warrant further study, evidence is accumulating in various experimental models of cerebral ischemia, which indicate that cholinergic dysfunction may be implicated in the pathogenesis of this condition [4,5]. Furthermore, choline acetyltransferase (CAT) was found to be significantly reduced in the brain of post-mortem VaD patients in all areas except for the thalamus [6]. In addition, acetylcholinesterase (AChE) activity has been found to be significantly higher in VaD rats compared to SHAM-surgery rats [7]. Hence, regulating the cholinergic pathways may prove to be an effective therapeutic strategy in attenuating VaD.

Two-vessel occlusion (2VO), also known as permanent bilateral occlusion of the common carotid arteries, induces chronic cerebral hypoperfusion (CCH) in rats, resulting in neuronal loss and inflammation particularly in the cortex and hippocampus [8,9] with associated impairments in learning and memory [3,10]. In addition, occlusion of the vessels in the rat causes a dramatic fall in CBF, creating hypoxic-ischemic conditions. This condition closely resembles the reduced CBF in human ageing and dementia [3]. Moreover, this rat model has been considered a representational model of VaD relative to the symptomatology seen in humans [11,12].

Synaptic plasticity is defined as the ability of the synapses to strengthen or weaken over time in response to neuronal stimulation. Synaptic plasticity is one of the most important neurochemical foundations in learning and memory. Memory has been postulated as a representation of widely interconnected synapses within the brain [13]. The most studied model of synaptic plasticity is that of long-term potentiation (LTP). LTP has been widely explored to examine the synaptic strength between two neurons both *in vivo* and *in vitro* [13,14]. It has been suggested that LTP has

roles in both learning and memory functions [15,16]. Previous studies have shown that the induction of CCH in the VaD rat model cause impairments in LTP [17,18]. Therefore, maintaining the strength of synaptic plasticity via the cholinergic pathway may offer a potential therapeutic strategy for the prevention of cognitive deficits in VaD.

Activation and restoration of cholinergic function remains a major objective in the development of pharmacological approaches towards the treatment of cognitive dysfunctions associated with ageing and dementia [19,20]. Acetycholinesterase inhibitors (AChEI) such as tacrine have been shown to ameliorate the cognitive impairments seen in AD patients [21]. In addition, cholinergic agonists such as oxotremorine have been shown to increase the steady state concentration of brain ACh [22] and treat the spatial memory deficits in rats [23]. Another cholinergic drug, carbachol, has been seen to increase brain activity via the production of theta rhythms in the rat brain [24]. However, the effect of these drugs on *in vivo* LTP has not been extensively studied. Therefore, the present study seeks to investigate the role of the cholinergic system in LTP in the VaD rat model.

2. Materials and Methods
2.1 Animal and housing conditions
All experimental procedures and animals were subjected to approval by the Animal Ethics Committee, USM, Penang, Malaysia with the reference number USM/Animal Ethics Approval/2014 (91) (538). Male Sprague-Dawley rats weighing between 250-300g were obtained from the Animal Research & Service Center (ARASC), USM, Penang, Malaysia. The rats were kept in plastic cages, with ventilated stainless-steel covers, and were given one week to adjust to conditions (maintained at 24 ± 1 ºC and a 12:12 light/dark cycle) before experiments commenced. The rats were fed with standard rodent food pellets and water which were allowed *ad libitum*.

2.2 Surgery of 2-vessel occlusion

The rats were divided into two equal groups, the SHAM-operated group and 2VO group. Permanent occlusion of the rat's common carotid arteries was performed as described previously [25]. The rats were anaesthetized with a mixture of ketamine hydrochloride (80 mg/kg; Troy Laboratories Pty. Ltd., Australia) and xylazine hydrochloride (10 mg/kg; Troy Laboratories Pty. Ltd., Australia) intraperitoneally, and the body temperature was maintained at 37 °C using a homeothermic blanket (Harvard Apparatus, USA). The common carotid arteries (CCA) were exposed via a ventral midline incision and carefully isolated from the surrounding connective tissue and vagus nerves. Next, both common carotid arteries were permanently ligated with suture silk 6/0 (UNIK Surgical Sutures, Taiwan). The skin incision was closed and the rat was kept in a cage at room temperature (25 °C). The SHAM-operated control rats underwent the same procedure, but without ligation. All rats were kept individually in their cages and were given one week to recover prior to commencement of LTP experiments.

2.3 Electrophysiological recording

The rats were anaesthetized with urethane (Sigma; 2.0 g/kg, administered in four doses 0.5 g/kg 20 min apart; supplements of 0.5 g/kg were given when necessary) intraperitoneally before being secured on the stereotaxic frame. Local analgesic xylocaine (5mg/kg; AstraZeneca, Australia) was administered subcutaneously on the head. The rats were placed on the stereotaxic apparatus and the ear bar was fixed into the auditory canal of each ear respectively. The rat's body temperature was maintained between 36 °C and 37 °C using a homeothermic blanket (Harvard Apparatus). Subsequently, a small incision on the head was made, and the connective tissue adhering to the bone was carefully removed. The bone surface was cleared by using 30% hydrogen peroxide (Sigma) until bregma and lambda were clearly visible. Two small holes were drilled on the skull overlying the hippocampal CA1 (AP: -4.2. ML: +3, V: -3) and CA3 (AP: -4.2, ML: -3, V: -4) regions, and two small holes were drilled overlying the frontal region for placement of ground and reference electrodes. All stereotaxic placements were based on the anatomical rat brain atlas of Paxinos and Watson [26].

After drilling, a recording electrode (Perfluoroalkoxy [PFA] Insulated Steel Wire, A-M Systems, USA) was lowered ventrally into the CA1 region and a concentric, bipolar stimulation electrode (SNE 100, MicroProbes, USA) was used to stimulate the CA3 region. The final ventral placement of the CA3 stimulating electrode and recording electrode were adjusted to yield maximum field excitatory postsynaptic potential (fEPSP) amplitude in stratum pyramidale of CA1 in response to CA3 stimulation. The CA3 stimulation electrode was connected to a stimulus isolator providing constant current output (ML 180 Stimulus Isolator, ADInstruments, Australia). The recording, ground and reference electrodes were connected to an amplifier (Model 1800, A-M Systems Inc., Sequim, WA; half amplitude filters set at 0.1-500 Hz) which digitised at 100 Hz and stored the recorded signal for offline analysis (PowerLab 16/s System, ADInstruments, Australia).

For each rat, the intensity required to elicit the maximal fEPSP amplitude was determined via input-output curves. The stimulation was given between 0.1 mA and 1.0 mA (in 0.1 mA increments). The stimulation intensity eliciting 50-60% of maximal fEPSP amplitude was used for the remainder of the experiment. Paired-pulse facilitation was measured by delivering pairs of stimulation pulses at inter-stimulus intervals of 20, 50, 100, 200, 500 and 1000 ms.

For LTP experiments, a baseline of the fEPSP was recorded over a 60-minute period. Baseline recordings were considered stable when fEPSP amplitude stayed within a range of 90-100% of the average fEPSP amplitude over the 60-minute baseline period. Once a stable baseline was established, theta burst stimulation (TBS) was applied as a train of ten bursts (each burst consisting of 5 pulses at 100 Hz), repeated every 200 ms for each burst. Following the TBS, single pulse stimulation of the CA3 continued every 30 sec for a 3-hour recording period.

2.4 Drugs administration

In order to assess the role of cholinergic transmission in the 2VO rat model, separate groups of rats received either oxotremorine (Oxo) 0.1 mg/kg [27], tacrine (Tac) 3 mg/kg [20], or carbachol (Cch) 10µg/kg [28]. All drugs were dissolved in saline and injected intraperitoneally (i.p). Oxotremorine, tacrine and carbachol were obtained from Sigma. The rats were divided into groups as followed: **Group 1**: SHAM + saline; **Group 2**: 2VO + saline; **Group 3**: SHAM + Oxo; **Group 4**: 2VO + Oxo; **Group 5**: SHAM + Cch; **Group 6**: 2VO + Cch; **Group 7**: SHAM + Tac; **Group 8**: 2VO + Tac. Each group consists of 5 to 6 rats. The drugs were given intraperitoneally before the start of the experiments.

2.5 Determination of cholinesterase in brain samples

Different groups of rats were used to determine the level of cholinesterase enzyme in 2VO model. The cholinesterase activity was measured in rat's brain samples on day 28 of 2VO surgery. The brain tissues were extracted and homogenized. Protein concentration was determined using Bradford's assay and was subsequently followed by a cholinesterase assay.

2.6 Tissue extraction

The rats were anaesthetized using carbon dioxide (CO_2) and decapitated. CO_2 has been widely used for short-acting anaesthesia and euthanasia for small laboratory animals [29]. The brains were removed immediately and dissected according to the areas of interest. The areas selected were the hippocampus, frontal cortex and remaining cortex, sliced using a using tissue block (Kopf Instrument) on ice. Next, the brain tissue was homogenized (40% w/v) in ice cold phosphate buffer (0.1M, pH 8.0) with glass/Teflon tissue homogenizer (Thomas Scientific, USA). The tissues were centrifuged at 1000 g for 10 minutes at 4 °C to obtain the supernatant [30].

2.7 Determination of cholinesterase activity

The assessment of cholinesterase activity was measured using the method from Ellman and colleagues [31] with minor modifications [32]. Theoretically, the presence of esterase in the brain samples would hydrolyse either acetylthiocholine or butyrylthiocholine respectively to produce thiocholine. Next, 5-dithiobis-2-nitrobenzoate (DTNB; 3mM) reacted with thiocholine produced from the hydrolysis of acetylthiocholine/butyrylthiocholine, leading to the formation of a yellow coloured product that could be read at 412nm using a spectrophotometer.

For determination of acetylcholinesterase (AChE) activity, acetylthiocholine iodide (ACTi; 14mM) was used as a substrate, whereas for determination of butyrylcholinesterase (BuChE), butyrylthiocholine iodide (BCTi; 14mM) was used as a substrate. Specific butyrylcholinesterase inhibitor i.e. iso-OMPA (tetraisopropyl pyrophosphoramide; 2mM) was used in addition to acetylthiocholine. Brain samples were loaded into the wells and the reaction was initiated by adding the substrate accordingly. The summary of volume and components of this assay were described briefly (Table 1). The microplate was shaken for ~5 seconds and incubated for 15 minutes at 37 °C. After incubation, the absorbance was read at 412 nm using a microplate reader (Multiskan Go, USA). Both AChE and BuChE enzyme activity were expressed as net absorbance to protein amount optical densities/mg [30]. Protein was determined using Bradford's assay [33,34].

2.8 Data analysis

For input-output curve, the fEPSP amplitude was computed by measuring the difference between activity immediately preceding each stimulation pulse and the peak of the fEPSP elicited by the stimulation. fEPSP amplitudes recorded during input-output curve experiments were analysed with stimulus intensity as the within-subject's variable. Paired-pulse facilitation was computed by measuring the ratio of the first and second fEPSP in each pair (amplitude of second fEPSP/amplitude of first fEPSP). Ratios were analysed with inter-stimulus intervals as the within-subjects variable.

Table 1: Summary of reaction volume and component for cholinesterase assay.

Test	Type of test	Volume of tissue (µl)	Volume of inhibitor (µl)	Volume of buffer/DTNB[b] (µl)	Volume and type of substrate
1	Substrate blanks	-	-	195	5 (ACTi[c])
2	Tissue blanks	10	-	190	-
3	BuChe	10	-	180	10 (BCTi[d])
4	AChe	10	10 (iso-OMPA[a])	175	5 (ACTi)

[a] iso-OMPA - 2mM solution; [b] DTNB - 3 mM solution; [c] ACTi - 14mM solution; [d] BCTi - 14mM solution.

For the LTP study, amplitude data was averaged over 10-min epochs and normalised by dividing all data points by the average baseline fEPSP amplitude (pre-TBS) of the respective animal. The data was expressed in terms of the mean ± S.E.M. Maximal fEPSP amplitude was computed offline using LabChart software (version 7.3.7, ADInstrument, Australia). In this study, the fEPSP amplitude was used in reference to previous studies [35-37]. Statistical analyses were carried out using Prism 5 for Windows software version 5.01 (GraphPad Software Inc., USA). Comparison between two groups was analysed using an unpaired t-test. A One-way ANOVA was also used when comparing more than three groups. The comparison between 2 variables was done using a two-way ANOVA. Alpha was set at the 5% level, therefore differences between conditions required $p < 0.05$ to evidence statistical significance.

3. Results

3.1 Input-output curves

Figure 1A shows the mean fEPSP amplitudes for rats administered with oxotremorine (0.1mg/kg), with fEPSP amplitude increasing similarly in all groups with increasing stimulation intensity. There was a significant group effect ($F_{3, 340} = 35.58$, $p < .0001$) and intensity effect ($F_{9,340} = 81.98$, $p < .0001$) but there was no interaction effect ($p > .05$). Figure 2A shows the mean fEPSP amplitudes of rats treated with carbachol (10µg/kg). Similarly, there was significant group effect ($F_{3,340} = 31.30$, $p < .0001$) and intensity ($F_{9,340} = 91.12$, $p < .0001$), however not the interaction effects ($p > .05$). Treatment with tacrine (3mg/kg) resulted in significant groups effect ($F_{3,320} = 34.98$, $p < .0001$) and intensity
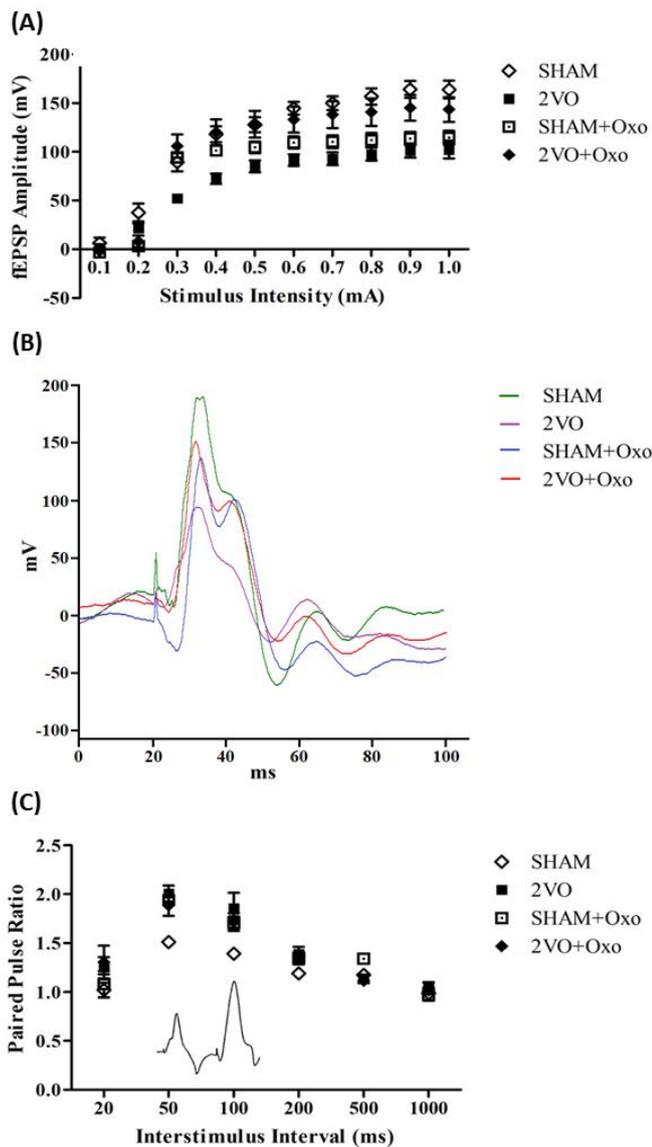
effects ($F_{9,320} = 69.03$, $p < .0001$) but not the interaction ($p > .05$; Figure 3A).

3.2 Paired-pulse facilitation

Two successive stimulation pulses were applied to CA3 at short stimulus intervals (20-1000 ms), the second of the two resulted in fEPSPs in CA1 exhibiting a larger amplitude relative to the first fEPSP in each pair (Figures 1C, 2C and 3C). This facilitation effect disappeared with longer inter-stimulus intervals (200-1000ms). There was a significant main effect between groups when treated with oxotremorine ($F_{3,72} = 10.66$, $p < .0001$), carbachol ($F_{3,72} = 12.44$, $p < .0001$) and tacrine ($F_{3,60} = 6.74$, $p < .001$). There are significant effects of the main interaction when the rats were administered with oxotremorine ($F_{15,72} = 2.01$, $p < .05$), or carbachol ($F_{15,72} = 1.88$) but not with tacrine ($p > .05$). There was significant effect of inter-stimulus interval in all groups ($p < .05$).

3.3 The role of cholinergic neurotransmission in LTP of CCH rat model

Figure 4 depicts the changes of fEPSP amplitudes following the administration of oxotremorine. The two ways ANOVA revealed a significant improvement in LTP after the administration of oxotremorine [Interaction: ($F_{69,384} = 1.58$, $p < .005$), between groups ($F_{3,384} = 84.65$, $p < .0001$) and time effect ($F_{23,384} = 34.33$, $p < .0001$)] (Figure 4A). In addition, the potentiation level of the 2VO rats treated with oxotremorine significantly higher compared to 2VO alone ($p < .05$; Figure 4C).

(A)

(B)

(C)

Another group of rats were tested with carbachol (Cch), a cholinergic agonist. Administration of carbachol showed a significant group effect ($F_{3,360}$=30.46, p <.001) and time effect ($F_{23,360}$=16.85, p < .0001) however, no significant interaction effect was found (p > .05; Figure 5A). Carbachol facilitated maintenance of LTP throughout the 3-hour LTP recording in the 2VO group, but not in the SHAM group (p > .05). However, no significant changes of the potentiation level were found between groups at the end of the LTP recording (p > .05; Figure 5C).

In order to assess the contribution of cholinesterase enzyme in the hippocampus, tacrine (Tac), a cholinesterase inhibitor was used. Administration of tacrine (3 mg/kg) caused a robust LTP induction following TBS in both SHAM and 2VO groups (Figure 6A), with a significant interaction effect ($F_{69,336}$ = 3.68, p < .0001), groups effect ($F_{3, 336}$ = 231, p < .0001) and time effect ($F_{23,336}$ = 33.98, p < .0001). Furthermore, both 2VO and SHAM treated with tacrine show significant enhancement in potentiation level of fEPSP amplitudes compared to non-treated groups (Figure 6C).

Figure 1. Field excitatory postsynaptic potentials (fEPSPs) in CA1 elicited by CA3 stimulation in four experimental groups (2-vessel occlusion [2VO], sham [SHAM], 2VO treated with oxotremorine [2VO+Oxo], sham treated with oxotremorine [SHAM+Oxo]). **(A)** Input-output curves of all groups towards increasing stimulation intensity. **(B)** The waveforms of fEPSP are illustrated with the presence of stimulus artifact, fiber volley and fEPSP taken at 1 mA stimulus intensity of each group. **(C)** Paired-pulse facilitation, elicited by application of two successive stimulation pulses to CA3 (interstimulus intervals between 20 to 1000 ms), was measured as the amplitude ratio of the second fEPSP divided by the first fEPSP in a pair. The insets depict a typical fEPSP recorded in CA1 following a paired-pulse facilitation applied to CA3 **(A)** as well as for an interstimulus interval of 50ms; note the

### 3.4 Determination of cholinesterase activity in CCH rat model

Determination of the cholinesterase activity was done on day 28 following the onset of the 2VO surgery. An unpaired Student *t*-test was conducted to measure the activity between 2VO and SHAM groups at different brain area *i.e.* hippocampus, frontal cortex and remaining cortex. The level of AChE enzyme activity in 2VO group at the hippocampus region (p < .05) was significantly higher compared to the other regions (Figure 7A). In addition to AChE, level of BuChE enzyme activity was also measured in the same regions. However, no significant changes were found in any of the respective regions (p > .05; Figure 7B).
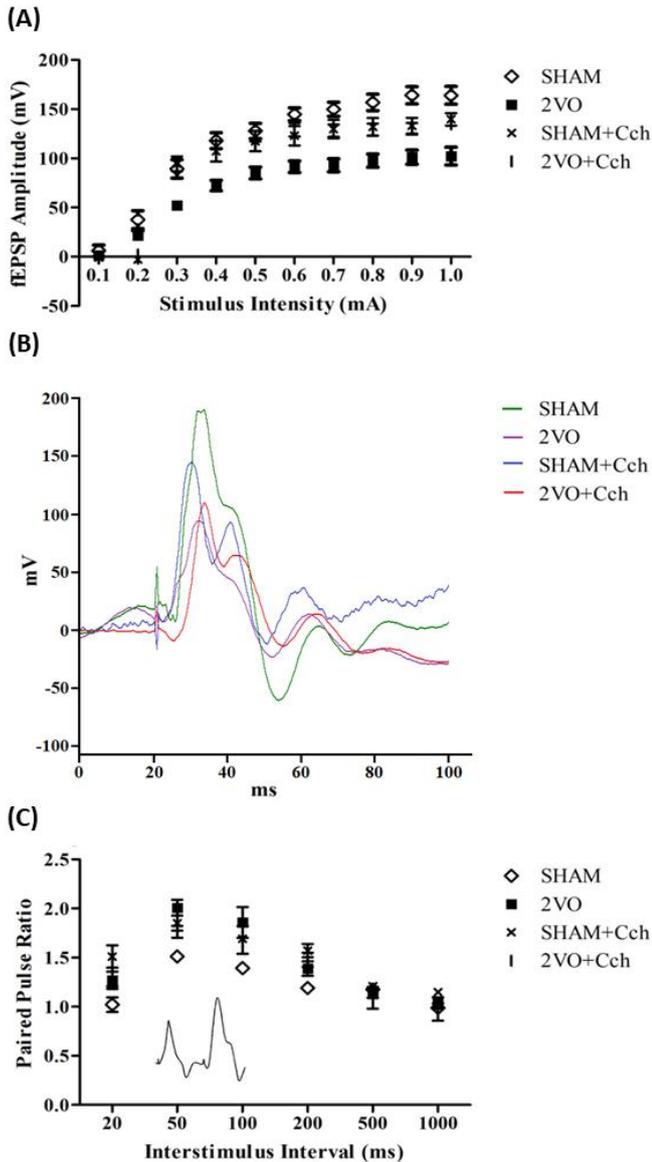
Figure 2. Field excitatory postsynaptic potentials (fEPSPs) in CA1 elicited by CA3 stimulation in four experimental groups (2-vessel occlusion [2VO], sham [SHAM], 2VO treated with carbachol [2VO+Cch], sham treated with carbachol [SHAM+Cch]). **(A)** Input-output curves of all groups towards increasing stimulation intensity. **(B)** The waveforms of fEPSP are illustrated with the presence of stimulus artifact, fiber volley and fEPSP taken at 1 mA stimulus intensity of each group. **(C)** Paired-pulse facilitation, elicited by application of two successive stimulation pulses to CA3 (interstimulus intervals between 20 to 1000 ms), was measured as the amplitude ratio of the second fEPSP divided by the first fEPSP in a pair. Data expressed as mean ± SEM of SHAM (n=5), 2VO (n=5), SHAM+Cch (n=4) and 2VO+Cch (n=4).
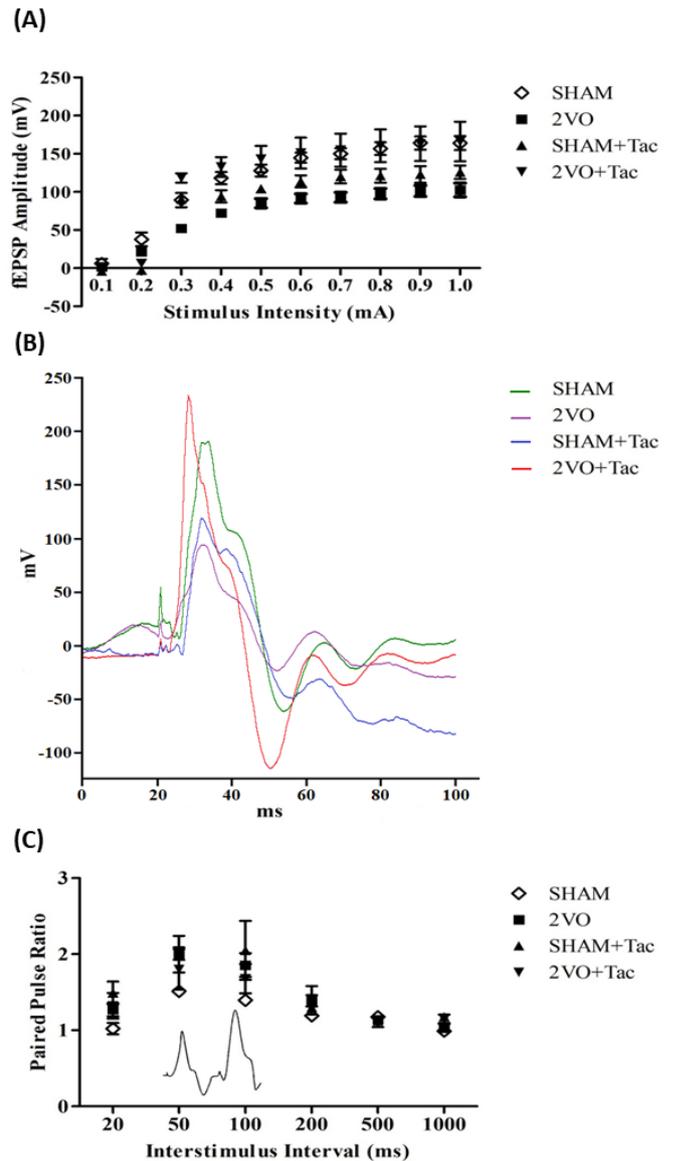
Figure 3. Field excitatory postsynaptic potentials (fEPSPs) in CA1 elicited by CA3 stimulation in four experimental groups (2-vessel occlusion [2VO], sham [SHAM], 2VO treated with tacrine [2VO+Tac], sham treated with tacrine [SHAM+Tac]). **(A)** Input-output curves of all groups towards increasing stimulation intensity. **(B)** The waveforms of fEPSP are illustrated with the presence of stimulus artifact, fiber volley and fEPSP taken at 1 mA stimulus intensity of each group. **(C)** Paired-pulse facilitation, elicited by application of two successive stimulation pulses to CA3 (interstimulus intervals between 20 to 1000 ms), was measured as the amplitude ratio of the second fEPSP divided by the first fEPSP in a pair. Data expressed as mean ± SEM of SHAM (n=5), 2VO (n=5), SHAM+Tac (n=4) and 2VO+Tac (n=4).

(A)

(B)

(C)
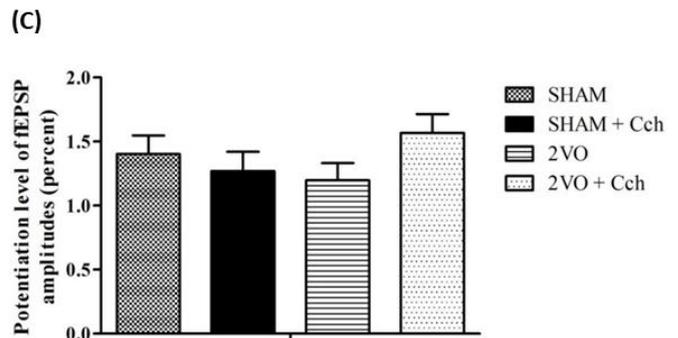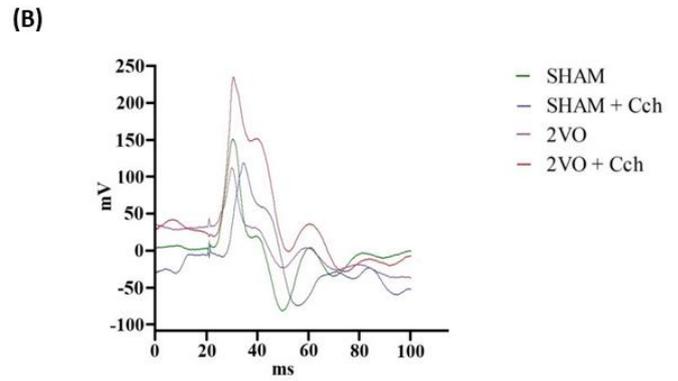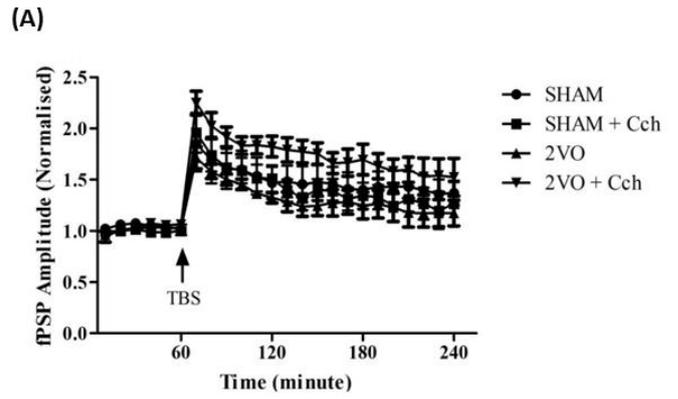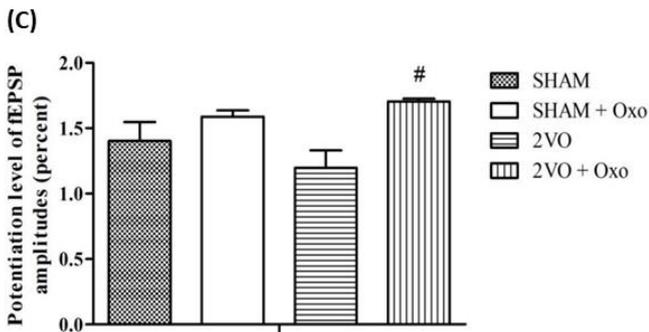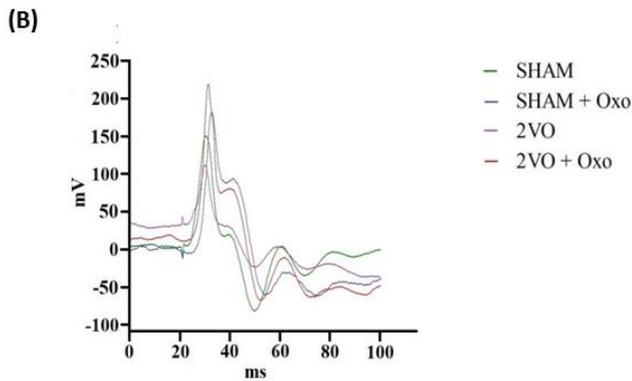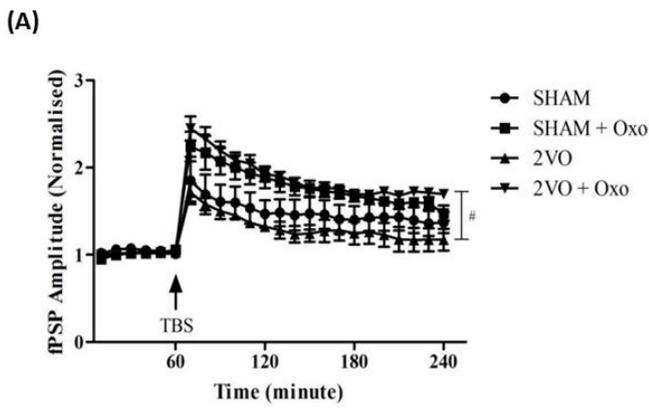
Figure 4. Changes of fEPSP amplitude following TBS in 2VO and sham-operated group treated with oxotremorine (0.1mg/kg). The fEPSP amplitude was plotted as a percent change against the baseline. **(A)** Each point represents the changes of LTP following the TBS after the administration of oxotremorine (0.1mg/kg). **(B)** The waveforms of fEPSP are illustrated with the presence of stimulus artifact, fiber volley and fEPSP taken after 2 hours following TBS of all groups. **(C)** The potentiation level of LTP at the end of the recordings which was taken during the last 60 minutes of the 3 hours LTP recording following the TBS. Data are expressed as mean ± S.E.M,* p < 0.05 compared to sham-operated group and # p < 0.05 compared to 2VO from 2VO (n=5), sham (n=5), 2VO + Oxo (n=4) and sham + Oxo (n=4) groups.

Figure 5. Changes of fEPSP amplitude following TBS in 2VO and sham-operated group treated with carbachol (10 µg/kg). The fEPSP amplitude was plotted as a percent change against the baseline. **(A)** Each point represents the changes of LTP following the TBS after the administration of carbachol (1 µg/100g). **(B)** The waveforms of fEPSP are illustrated with the presence of stimulus artifact, fiber volley and fEPSP taken after 2 hours following TBS of all groups. **(C)** The potentiation level of LTP at the end of the recordings which was taken during the last 60 minutes of the 3 hours LTP recording following the TBS. Data are expressed as mean ± S.E.M,* p < 0.05 compared to sham-operated group and # p < 0.05 compared to 2VO from 2VO (n=5), sham (n=5), 2VO + Cch (n=4) and sham + Cch (n=4) groups.

(A)

(B)

(C)
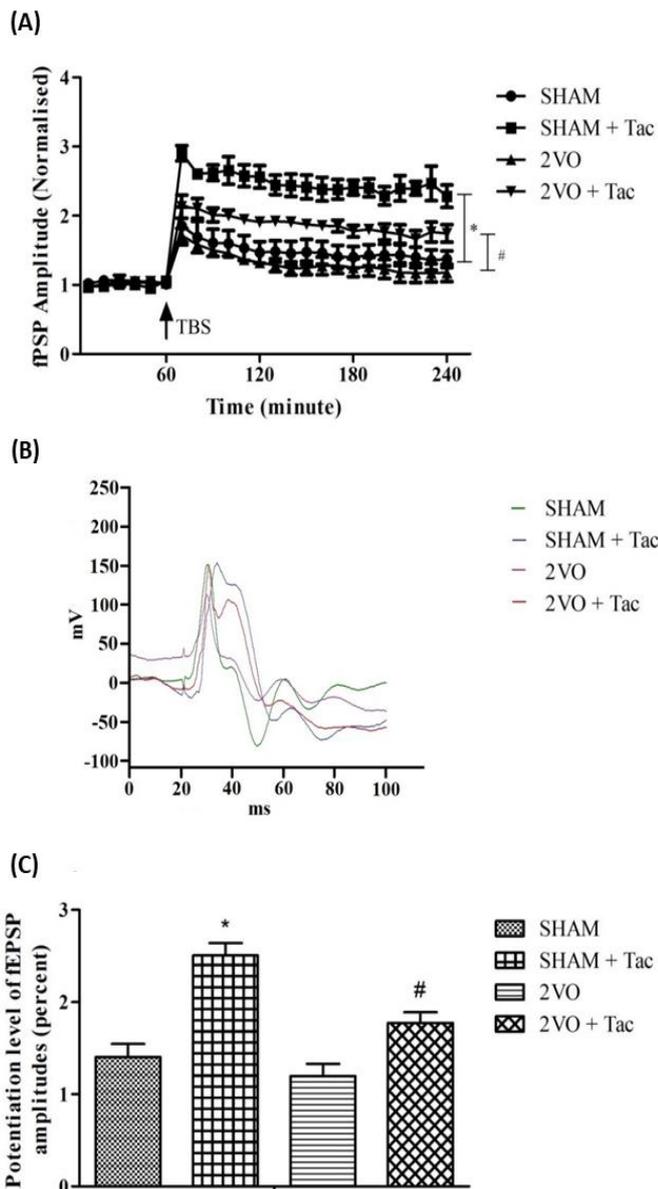
Figure 6. Changes of fEPSP amplitude following TBS in 2VO and sham-operated group treated with tacrine (3 mg/kg). The fEPSP amplitude was plotted as a percent change against the baseline. **(A)** Each point represents the changes of LTP following the TBS after the administration of tacrine (3 mg/kg). **(B)** The waveforms of fEPSP are illustrated with the presence of stimulus artifact, fiber volley and fEPSP taken after 2 hours following TBS of all groups. **(C)** The potentiation level of LTP at the end of the recordings which was taken during the last 60 minutes of the 3 hours LTP recording following the TBS. Data are expressed as mean ± S.E.M,* $p < 0.05$ compared to sham-operated group and # $p < 0.05$ compared to 2VO from 2VO (n=5), sham (n=5), 2VO + Tac (n=4) and sham + Tac (n=4) groups.

4. Discussion

In this paper, we investigate the role of cholinergic transmission in cellular and molecular mechanisms altering synaptic strength in chronic cerebral hypoperfused rats. LTP is the most commonly studied at the synapse between the Schaffer collateral axons of the CA3 neurons and the CA1 pyramidal cell [38]. In order to evaluate the basal synaptic transmission at the CA1 region of the rat after stimulating the CA3 region of the Schaffer-collateral pathway, an input-output curve was plotted. The fEPSP amplitudes in input-output curve reveal significant effects of the experimental and SHAM models treated with cholinergic drugs (Figures 1A, 2A and 3A). Many aspects of the baseline synaptic transmission at CA3-CA1 synapses indicate responses analogous to the SHAM group. Previous research studies indicate that there was no impairment of basal synaptic transmission in the 2VO model [39,40]. On the contrary, others reported that induction of 2VO caused significant impairment of basal synaptic transmission [41]. The latter was found to be in line with our findings. The reason for these discrepancies may be due to some alterations in the molecular mechanisms involved during post-surgery, or indeed variation between animals in terms of species or age [18].

Treatments with oxotremorine and carbachol showed significant effects on the level of paired-pulse facilitation (PPF) as an indirect measure of calcium levels and transmitter release from the presynaptic terminal. The degree of PPF is inversely proportional to the release of neurotransmitter from presynaptic terminals [42]. Therefore, altered levels of LTP following the 2VO surgery are not likely to result from changes in presynaptic release. Hence, we propose that a postsynaptic (rather than a presynaptic) mechanism predominates following the 2VO surgery. However, the altered LTP following cholinergic agonists (*i.e* oxotremorine and carbachol) may derive from both pre- and postsynaptic mechanisms. On the contrary, treatment with tacrine may involve the postsynaptic mechanism given that the drug depends on the level of AChE enzymes present in the synaptic cleft.
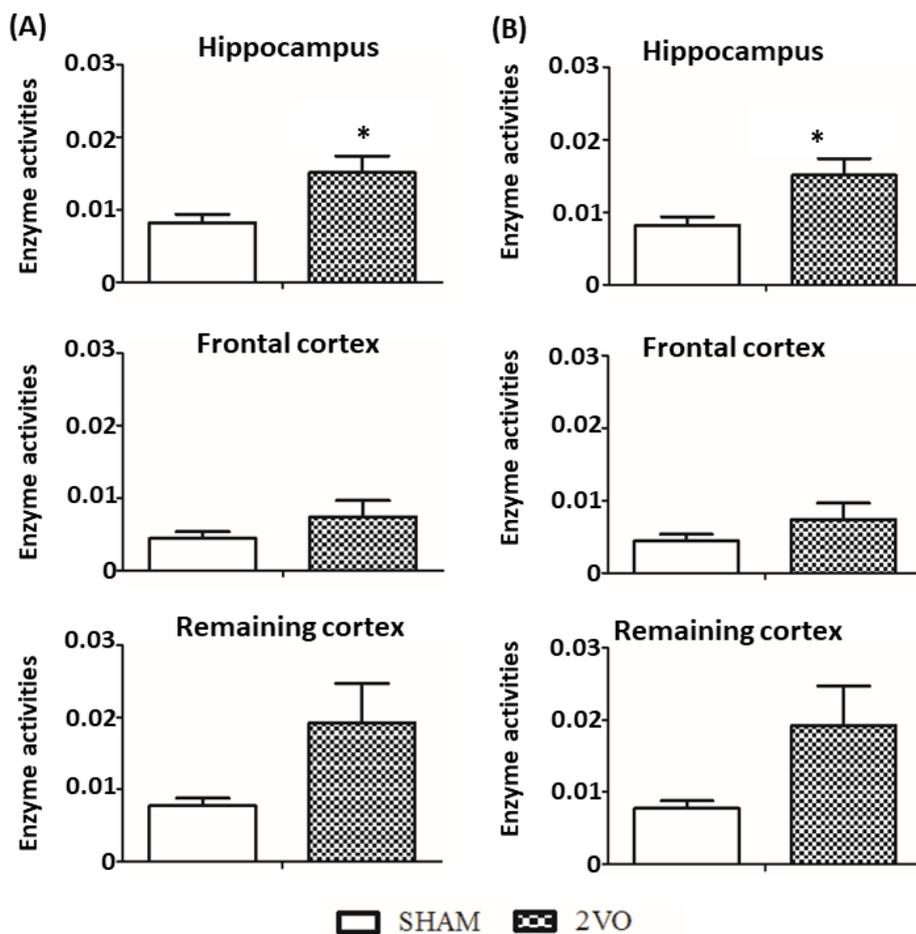
Figure 7. Determination of **(A)** AChE and **(B)** BuChE activity between 2VO and sham-operated group on day 28 after the onset of the surgery. Data are expressed as mean ± S.E.M with n=5 each group. The data was analyzed using unpaired student t-test for each region.

As evidenced in Figure 4A, the LTP of the 2VO group was significantly impaired compared to the SHAM-operated group following TBS. The result is in accordance with the findings of previous studies suggesting that 2VO caused significant reduction of LTP [9,18,40,43]. The causal mechanisms underlying LTP impairment in this model are currently unclear. However, there are few possible explanations that may contribute to the changes of the synaptic plasticity in this model. Occlusion of both common carotid arteries will lead to reduction of the cerebral blood flow and oxygen supplied to the brain. Consequently, this would alter the biochemical metabolism of the neuron and potentially cause neuronal cell death. This ischemic cell death may be prevalent in the hippocampus, with respect to hippocampal pyramidal neurons. Further,

permanent impairment of LTP may develop in the rat hippocampal Schaffer-collateral CA1 which connects pyramidal neurons [44]. Furthermore, cAMP response element-binding (CREB) protein was reported to be significantly reduced in the 2VO model [40,45,46]. CREB protein plays a role in neuronal plasticity and memory formation. In addition, synaptophysin present in synaptic vesicles [17,47] and microtubule-associated protein 2 (MAP-2) [40,48] were significantly reduced in 2VO model. Synaptophysin is a membrane protein in synaptic vesicles and localized at the axon terminals while MAP-2 is a protein that functions for dendritic outgrowth, branching and post-lesion dendritic remodelling [40]. These proteins are thought to play a crucial role in maintaining the LTP and any

malfunctioning of those proteins may lead to long term depression (LTD) [49,50].

Induction of LTP is dependent on the protein phosphorylation and dephosphorylation while maintaining LTP requires the cascade of molecular process [14,49]. In the present study, induction of LTP was given by theta-burst stimulation (TBS). TBS was given as 5-pulses burst at 100 Hz to mimic complex-spike burst and repeated for 10 times at five bursts per second to approximate the theta rhythm frequency. TBS is an effective stimulus for LTP for several reasons. Firstly, it shows the pattern of neuronal firing (complex-spikes) that occurred spontaneously during behaviour. Secondly, the optimal repetition rate corresponded to hippocampal theta rhythm (indirectly correlating with memory processes in EEG), the stimulation given will allow uncovering various events that contribute to LTP induction [51]. Furthermore, TBS is highly sensitive towards LTP. For example, TBS is vulnerable in aging [52], experimentally-induced stress [53], has become more sensitive to the effect of brain-derived neurotrophic factor (BDNF) [54], serotonin [55] or adenosine receptor-dependent signalling mechanisms [56]. Altogether, TBS of LTP may provide a better model for the memory effects than tetanus-induced LTP.

Previous studies had shown that induction of LTP at the Schaffer collateral-CA1 synapse was enhanced through activation of cholinergic receptors [57-59]. For example, a study by Shimoshige and colleagues (1997) had examined the effects of carbachol, acting as a muscarinic receptor agonist with respect to LTP. They reported that carbachol significantly increased the magnitude of LTP [58]. In addition, a study by Doralp and Leung (2008) on the cholinergic modulation of hippocampal CA1 LTP resulted in enhanced LTP *in vivo* when the rats were treated with the anticholinesterase drug, serine sulfate [59]. Another study by Ovsepian and colleagues (2004) examined the role of cholinergic on the induction of LTP using non-selective and selective muscarinic antagonist drugs, scopolamine and pirenzepine respectively [60]. These authors

concluded that the CA1 area is a muscarinic ACh receptor dependent and medial septum pre-conditioning transiently facilitates LTP induction in these synapses. Furthermore, muscarinic antagonist atropine caused significant decrease of LTP after the strong tetanization [61]. In the present study, the non-selective muscarinic agonist oxotremorine is able to enhance LTP in the CA1 of the 2VO rat. The potentiation level of LTP at the end of the recordings in 2VO treated with oxotremorine remained higher compared to 2VO group alone (Figure 4).

Carbachol, a cholinergic agonist and tacrine, an anticholinesterase was also used in the present study. Both drugs enhanced fEPSP amplitude in the CA1 region of the 2VO rat. However, the potentiation level of carbachol was unchanged when in the sham group, suggesting that the cholinergic receptors were not affected (Figure 5). The administration of tacrine produced robust LTP in both 2VO and sham groups (Figure 6). Thus, there is contribution of AChE in this model. The level of ACh in the brain (especially in hippocampus) may be low due to rapid hydrolysis by AChE [62]. This could be seen via the AChE assay in the hippocampal region (Figure 7A). It can therefore be suggested that an increased level of AChE can lead to rapid hydrolysis of ACh to choline and acetate. Thus, it may reduce the binding of ACh to the cholinoceptors. As a consequence, critical decrease in the level of ACh may contribute to the cognitive deficits evident in the 2VO rats [63]. As a summary, these results imply that central cholinergic impairment induced by 2VO may play a critical role in the development of cognitive dysfunction. Therefore, ameliorating central cholinergic dysfunction may be an auspicious strategy for managing cognitive impairment associated with cerebral hypoperfusion.

Impairment in learning and memory is one of the characteristic features of dementia in AD and VaD. The Morris water maze has become one of the most extensively accepted test models for testing learning and memory in rodents [64]. Data from our laboratory have demonstrated that 2VO rats showed significantly

longer escape latencies during the 5-days training period, as well as during probe trials [10]. This suggest that this model exhibits spatial learning and memory deficits. Impairment in memory and learning was associated with the decreased level of acetylcholine (ACh) [5] and also increase level of AChE [65] in the brain. ACh is a well-known neurotransmitter that functions in many parts of human body and especially involved in some central functions *i.e.* walking, cognition control or memory [66]. Since the discovery of rapid colorimetric to determine the AChE activity by Ellman and colleagues, there are growing number of studies have been developed using this approach as a validation method for cognitive functions [31]. In the present study, the cholinesterase enzyme tested resulted in significant increase of AChE in hippocampus region but not other region (Figure 7A). However, no significant changes were found for BuChE (Figure 7B).

In summary, our results showed the contributions of AChE inhibitor, tacrine and cholinergic agonists, oxotremorine and carbachol in ameliorating the LTP impairment in CCH rat model. The CCH rat model may be correlated with the increase of the cholinesterase enzyme activity. High level of cholinesterase enzyme activity was found to be the major problem in memory impairment of VaD model. Therefore, targeting the cholinergic pathways may be a plausible manner of affecting the cognitive deficits in VaD. These findings are useful for the development of new potential neuroprotective agents associated with dementia or cerebral hypoperfusion targeting the cholinergic neurotransmission.

5. Conclusions

The role of cholinergic neurotransmission has been clearly demonstrated in LTP impairment of the VaD rat model. Drugs such as tacrine, oxotremorine and carbachol are effective in improving the LTP deficit in CCH model. Therefore, these results can be used as a guideline in producing new therapeutic agents targeting on the cholinergic pathways.

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