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Acetylcholinesterase inhibition reveals endogenous nicotinic modulation of glutamate inputs to CA1 stratum radiatum interneurons in hippocampal slices

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Abstract

The involvement of brain nicotinic acetylcholine receptors (nAChRs) in the neurotoxicological effects of soman, a potent acetylcholinesterase (AChE) inhibitor and a chemical warfare agent, is not clear. This is partly due to a poor understanding of the role of AChE in brain nAChR-mediated functions. To test the hypothesis that AChE inhibition builds sufficient acetylcholine (ACh) in the brain and facilitates nAChR-dependent glutamate transmission, we used whole-cell patch-clamp technique to record spontaneous glutamate excitatory postsynaptic currents (EPSCs) from CA1 stratum radiatum interneurons (SRI) in hippocampal slices. First, the frequency, amplitude and kinetics of EPSCs recorded from slices of control guinea pigs were compared to those recorded from slices of guinea pigs after a single injection of the irreversible AChE inhibitor soman (25.2 µg/kg, s.c.). Second, EPSCs were recorded from rat hippocampal slices before and after their superfusion with the reversible AChE inhibitor donepezil (100 nM). The frequency of EPSCs was significantly higher in slices taken from guinea pigs 24 h but not 7 days after the soman injection than in slices from control animals. In 52% of the rat hippocampal slices tested, bath application of donepezil increased the frequency of EPSCs. Further, exposure to donepezil increased both burst-like and large-amplitude EPSCs, and increased the proportion of short (20–100 ms) inter-event intervals. Donepezil's effects were suppressed significantly in presence of 10 µM mecamylamine or 10 nM methyllycaconitine. These results support the concept that AChE inhibition is able to recruit nAChR-dependent glutamate transmission in the hippocampus and such a mechanism can contribute to the acute neurotoxicological actions of soman.

Keywords

Soman; Donepezil; Hippocampus; Interneuron; Acetylcholinesterase; EPSC

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Alkondon, Pereira, and Albuquerque participated in research design. Alkondon conducted experiments. Alkondon and Albuquerque performed data analysis. Alkondon, Pereira, and Albuquerque contributed to the writing of the manuscript. Albuquerque acquired funding for the research.

1. Introduction

Soman, one of the known chemical warfare agents, is a potent neurotoxicant affecting peripheral and central nervous system. Soman inhibits acetylcholinesterase (AChE) irreversibly leading to accumulation of ACh at synapses and causes excessive activation of both muscarinic (mAChR) and nicotinic acetylcholine receptors (nAChRs). Seizures and motor convulsions are common symptoms observed in animals that originate from the AChE inhibitory effects of soman in the brain, and are therefore used as routine parameters to assess the degree of toxicity by the agent and the level of protection offered by therapeutic agents (Shih et al., 1999; Albuquerque et al., 2006). The role of mAChRs in soman toxicity has been well established as atropine provides some protection in soman-induced convulsions (McDonough and Shih, 1997; Albuquerque et al., 2006). Although the involvement of nAChRs at the neuromuscular junction has been well known, the role played by brain nAChRs is less well understood in the actions of soman. The use of mecamylamine (MEC), a non-selective nAChR antagonist, to suppress the effects of soman has yielded controversial results. For example, MEC was found to be ineffective in blocking or stopping seizure activity in rats (Shih et al., 1999). Soman-induced epileptiform discharges in guinea pig hippocampal slices are found to be insensitive to the actions of MEC (Harrison et al., 2004). Yet studies conducted in rabbits (Heyl et al., 1980), in mice (Hassel, 2006), and in rats (Myhrer et al., 2011) suggest that MEC is effective to some extent to suppress soman-induced symptomatology and lethality. These discrepancies have been attributed to the use of different doses of MEC in various studies (Hassel, 2006).

The role of glutamatergic system in soman-induced seizures has been well documented (McDonough and Shih, 1997; Lallement et al., 1998). Studies in control animals show that glutamate inputs to CA1 and CA3 pyramidal neurons and CA1 stratum radiatum interneurons (SRI) in the hippocampus are under the influence of nAChR activity (Gray et al., 1996; Sharma et al., 2008; Alkondon et al., 2011; Banerjee et al., 2012a, b). Both methyllycaconitine (MLA)-sensitive ($\alpha 7$) and MEC-sensitive ($\alpha 3\beta 4\beta 2$) nAChR subtypes have been implicated in the above studies. Also, nicotine-induced seizures in control animals have been attributed to excessive activation of neuronal nAChRs (Damaj et al., 1999; Roshan-Milani et al., 2003). As soman-induced increase in the extracellular glutamate levels in the brain has been explained as a consequence of seizures (McDonough and Shih, 1997), it is unclear whether nAChR activation per se is able to modulate glutamate transmission in soman-injected animals.

In view of the above, we designed our experiments to address the following questions: (i) Does in vivo administration of soman modulate glutamate synaptic transmission by virtue of AChE inhibition?; (ii) Does in vitro exposure of brain slices to AChE inhibitors reproduce the effects of soman on glutamate transmission?; (iii) Do AChE inhibitors modulate glutamate transmission via recruitment of various subtypes of nAChRs?

To achieve the above objectives, we studied the spontaneous excitatory postsynaptic currents (sEPSCs) recorded from CA1 SRI in hippocampal slices. To study soman effects in vivo, we used guinea pigs, which mimic the characteristics of humans (Albuquerque et al., 2006). We studied the effect of soman at 24 h, a time at which significant AChE inhibition is present

(Lintern et al., 1998) and the free form of soman has dissipated from the system. Soman-injected animals were compared with age-matched control animals. To reproduce the AChE inhibition in in vitro experiments, we used rat hippocampal slices and exposed them to the reversible AChE inhibitor donepezil. Hippocampal slices from rat rather than guinea pig were chosen because most of our previous studies have been carried out using this preparation. Further, rat hippocampal slices have the same advantage as the guinea pig hippocampal slices because they are rich in cholinergic afferents (Aznavour et al., 2005), contain nAChR subtypes including the ones that modulate glutamate transmission (Gray et al., 1996; Alkondon and Albuquerque, 2004; Albuquerque et al., 2009; Alkondon et al., 2009; Banerjee et al., 2012b), and are used as in vitro model system to study epileptic seizures (Harrison et al., 2004; Roshan-Milani et al., 2003). The rat slice experiments allowed us to compare the status of glutamate transmission before and after achieving AChE inhibition, and reversal effects in the same preparation, all within a short period of time (~1 h). This strategy also avoided the possible interference of a long-lasting effect of soman due to its adhesion to the solution exchange system in electrophysiological recordings. Most importantly, the rat hippocampal slices allowed us to test the involvement of nAChR subtypes during the period of AChE inhibition.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs [Crl(HA)Br] that were 9 days old were purchased from Charles River Laboratories (Wilmington, MA) and housed for two days in groups of 3–5 per cage before the experiments in a temperature- and light-controlled animal-care unit. Guinea pigs were divided into two groups. The “control” group consisted of animals not injected with any agents and was used at postnatal days 11–12 (referred as P11) and 16–19 (referred as P18). The “soman” group consisted of animals (11–12 days old) that received a subcutaneous (s.c.) injection of 25.2 µg/kg soman (1xLD50) between the shoulder blades, and these animals were used at 1 and 7 days after the injections. Sprague-Dawley rats (mother with 8-day old male pups) were purchased from Charles River Laboratories (Wilmington, MA). Pups were weaned at postnatal day 21 and kept in groups of three per cage. These male rats were used between postnatal days 29 and 34. Male animals were used in this study to avoid the possible variability arising from the use of both genders. The ages of animals selected here have been used successfully to get electrophysiological data on nAChRs in our laboratory (Alkondon et al., 2009, 2011; Banerjee et al., 2012a, b). Handling of the animals was according to the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care, complied with the standards of the Animal Welfare Act, and adhered to the principles of the “1996 Guide for the Care and Use of Laboratory Animals.”

2.2. Hippocampal slices

Guinea pigs and rats were euthanized by asphyxia in a CO₂ atmosphere followed by decapitation. Their brains were removed and the hippocampus dissected out in cold artificial cerebrospinal fluid (ACSF). To reduce cell swelling, removal of the brains as well as dissection and slicing of the hippocampi were performed in an ice-cold solution consisting

of a mixture of equal parts of regular ACSF and sucrose-containing ACSF. Regular ACSF was composed of (in mM): NaCl, 125; NaHCO₃, 26; KCl, 2.5; NaH₂PO₄, 1.25; CaCl₂, 2; MgCl₂, 1; and glucose, 25. Sucrose-containing ACSF was composed of (in mM): sucrose, 230; KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 26; CaCl₂, 0.5; MgSO₄, 10; and glucose, 10. Hippocampal slices, 300 µm-thick, were cut using a vibratome (Leica VT 1000S). Slices were stored at 30 °C in regular ACSF, which was bubbled with 95% O₂/5% CO₂. After 30 min incubation in regular ACSF, some rat hippocampal slices were transferred to a 50-ml chamber containing ACSF with 100 nM donepezil and continuously bubbled with 95% O₂/5% CO₂. The chambers were maintained in a water bath at 30 °C. Time of incubation of the slices with donepezil ranged from 1 to 5 h. Biocytin labelling was used for post-recordings morphological identification of the neurons.

2.3. Patch-clamp recordings

CA1 SRI in hippocampal slices was visualized using the 40 X water-immersion objective of a Zeiss upright microscope and the images were viewed on a monitor using a digital camera. Slices were superfused with ACSF at 2 ml/min at room temperature. The ACSF contained atropine (0.5 µM) to block muscarinic receptors and bicuculline (10 µM) to block GABA_A receptors. Spontaneous EPSCs were recorded from the soma of SRI according to the standard patch-clamp technique using an EPC9 amplifier and PULSE software (ALA Scientific Instruments, Westbury, NY). Antagonists were applied via the bath perfusion. ACh was applied to the neurons via a U-tube. The internal pipette solution contained 0.5% biocytin in addition to (in mM): ethylene-glycol bis(β-amino-ethyl ether)-N-N'-tetraacetic acid, 10; HEPES, 10; Cs-methane sulfonate, 130; CsCl, 10; MgCl₂, 2; and lidocaine N-ethyl bromide (QX-314), 5 (pH adjusted to 7.3 with CsOH; 340 mOsm). Membrane potentials were corrected for liquid junction potential. All experiments were carried out at room temperature (22–24 °C). In each slice, only a single neuron was recorded, therefore, the number of cells remains the same as the number of slices in each group.

2.4. Data analysis

The frequency, peak amplitude, 10–90% rise time, and decay-time constants of glutamate EPSCs were analysed using WinEDR V2.3 (Strathclyde Electrophysiology Software, Glasgow, Scotland). Data are expressed as mean ± SEM and statistical significance was analysed using Student's *t* test or the Fisher's exact test in the StatsDirect software.

2.5. Test compounds used

Stock solution of soman (approximately 1.9 mg/ml) was obtained from the U.S. Army Edgewood Chemical Biological Center via an agreement with the U.S. Army Medical Research Institute of Chemical Defense. Soman (methylphosphonofluoridic acid 1,2,2-trimethylpropyl ester) was stored, handled, and disposed of according to the regulations set forth by the U.S. Army Medical Research Institute of Chemical Defense. Donepezil HCl was obtained from Janssen Pharmaceutical Inc (Titusville, NJ). (-)Bicuculline methochloride was purchased from Tocris (Ellisville, MO). Atropine sulfate, N-(2,6-Dimethylphenyl-carbamoylmethyl) triethylammonium bromide (QX-314), and 2-amino-5-phospho-novaleric acid (APV) were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Research Biochemicals (Natick,

MA). MLA citrate was a gift from Professor M.H. Benn (Dept. Chemistry, Univ. Calgary, Alberta, Canada).

3. Results

3.1. Effect of soman on glutamate EPSCs

To identify the possible effects of soman on glutamatergic transmission, EPSCs were recorded from CA1 SRIs in hippocampal slices obtained at various times from male guinea pigs following a single injection of 1xLD50 soman and compared to those recorded from slices obtained from control animals.

Spontaneous EPSCs recorded from SRIs at -60 mV in guinea pig hippocampal slices were glutamatergic in nature because they were blocked by the AMPA/kainate receptor antagonist CNQX (10 μ M) (Fig. 1A). The mean frequency of glutamate EPSCs was significantly higher in slices obtained at 24 h after the soman injection than in control slices (Fig. 1B). At 7 days after the injection, the frequency of glutamate EPSCs was comparable to that recorded from slices of age-matched control animals (Fig. 1B). Neither the mean rise time nor the decay time constant of the EPSCs was affected by the injection of soman (Fig. 1B and C).

The distribution of the peak amplitude of glutamate EPSCs revealed a higher percent of large amplitude events in SRIs of soman-challenged guinea pigs than in SRIs of control animals (Fig. 2). Pooled results from 13 cells in control and 18 cells in soman indicated significantly higher ratio of number of large amplitude events to total number of events in soman compared to control (Fig. 2). For example, glutamate EPSCs with peak amplitude > 40 pA increased from 3.9% of total events in control to 10.9% in soman at 24 h after treatment ($p < 0.0001$ by Fisher's exact test).

3.2. Effect of donepezil on glutamate EPSCs

In the following experiments, we investigated the acute actions of the reversible and potent AChE inhibitor donepezil on glutamate EPSCs. To achieve effective inhibition of the enzyme in the hippocampal slices, we tested donepezil at 100 nM, a concentration much higher than the IC_{50} of 6.7 nM found at inhibiting AChE in brain homogenates under prolonged equilibrium conditions (Ogura et al., 2000). To verify the effectiveness of bath-applied donepezil (100 nM), we studied its effects on $\alpha 7$ nAChR currents induced by submaximal concentration of ACh. U-tube application of ACh (50 μ M for 5 s) induced inward currents that showed a peak followed by a decaying phase during the agonist pulse. Application of ACh plus donepezil at 10 and 20 min after bath applied donepezil induced currents with higher peak amplitudes and prolonged duration (Fig. 3A). Washing of slices with donepezil-free ACSF for 10 – 20 min resulted in recovery of the response towards control levels. Donepezil exposure also resulted in enhancement in the frequency of ACh-induced EPSCs in three out of eight neurons (see Fig. 3B). The increase in the peak amplitude of ACh-evoked currents in presence of donepezil remained statistically significant at 10 and 20 min of exposure (Fig. 3C). A subsequent application of MLA (10 nM) blocked ACh-evoked currents completely (see Fig. 3A).

In the next set of experiments, glutamate EPSCs were recorded from CA1 SRI of rat hippocampal slices at -60 mV under control conditions for 10–15 min, followed by bath application of 100 nM donepezil. In 11 out of 21 cells in which a complete protocol was successfully carried out (consisting of recordings under control conditions, followed by donepezil bath application and a subsequent wash), donepezil caused a reversible increase in the frequency of glutamate EPSCs (Fig. 4A and B). Pooled data from five to eight cells indicated that donepezil increased significantly the frequency of EPSCs in a time-dependent manner (Fig. 4C). During bath application of donepezil, the mean peak amplitude of EPSCs (Fig. 4D) and the frequency of large-amplitude EPSCs (>40 pA events; Fig. 4E) increased in a time-dependent manner. The percent of large-amplitude EPSCs in relation to the total number of events recorded at 11–20 min and 21–30 min after exposure of the slices to donepezil was significantly higher than that recorded under control conditions (Fig. 4E). In four of five cells sampled, application of donepezil also resulted in the appearance of burst-like activity (EPSCs separated by intervals <100 ms), which existed at a low frequency before the drug application (see traces in Fig. 4). Burst-like activity was inferred from the short (20–100 ms) inter-event intervals, which in relation to total number of events increased significantly during donepezil bath application (Fig. 5).

In another experimental protocol, rat hippocampal slices ($n = 27$) were pre-incubated with 100 nM donepezil for 1–5 h, and EPSCs were recorded in the continuous presence of the drug. Control slices ($n = 13$) were incubated and recorded only in donepezil-free ACSF. Glutamate EPSCs were recorded from CA1 SRI of these two set of slices at -60 mV and the results analysed for the potential effects of donepezil. Similar to bath-applied condition, incubation with donepezil also caused a significant increase in the mean frequency of glutamate EPSCs compared to control (Fig. 6). Incubation with the drug had no significant effect on the mean peak amplitude, mean rise time and mean decay-time constant of glutamate EPSCs (Fig. 6). Burst-like EPSCs were also observed frequently in donepezil-incubated slices, but not in control slices (see traces in Figs. 6 and 7).

Next, we determined the role of nAChRs in the actions of donepezil as the latter by virtue of AChE inhibition is expected to increase the basal levels of ACh in the slices. As the muscarinic receptors are blocked by the presence of atropine in the ACSF during the recordings, it is likely that enhanced level of ACh acts on the nAChRs present on the glutamate neurons. To this end, two nAChR antagonists were used: MLA (10 nM) to block $\alpha 7$ nAChRs, and MEC (10 μ M) to block $\alpha 3\beta 4\beta 2$ nAChRs (Alkondon et al., 2009). In naïve slices incubated only in control ACSF, bath application of 10 nM MLA decreased the frequency of spontaneous EPSCs in five out of seven neurons tested. MLA produced a significant inhibition with a mean $\pm$ SEM value of $26.5 \pm 8.6\%$ ($p < 0.05$ by one sample t test). However, in naïve control slices, MEC was able to decrease the frequency of spontaneous EPSCs in four out of ten cells tested. MEC produced a mean $\pm$ SEM inhibition of $27.2 \pm 9.8\%$ that was found to be statistically insignificant ($p > 0.05$ by one sample t test).

In subsequent experiments, all recordings were carried out in the continuous presence of donepezil in the ACSF. In all 11 neurons studied, the frequency of EPSCs recorded in the presence of donepezil-plus-MEC was significantly lower (about 25%) than that recorded in the presence of donepezil only (Fig. 7). Thus, in presence of donepezil, 100% of neurons

became responsive to MEC compared to only 40% of neurons in the absence of donepezil, the difference between the two groups was statistically significant (Table 1; $p < 0.01$ by Fisher's exact test). In contrast, MLA reduced the frequency of EPSCs in only five of nine cells studied with a mean inhibition of about 33%. In three out of the four remaining cells, MLA caused an increase in the frequency of events. In one cell, MLA had no significant effect on the frequency of EPSCs.

Although the burst-like EPSCs occurred in a pattern of two–three events in succession (see traces in Fig. 6 and left panel of Fig. 7), in a rare case, it occurred in a burst of 9–12 events per burst (see traces in right panel of Fig. 7). Interestingly, this high frequency of EPSC bursts was completely abolished by both MEC and MLA in a reversible manner. In addition to decreasing the frequency of EPSCs, both MEC and MLA also decreased the incidence of short inter-event intervals (20–100 ms) in a reversible manner (Fig. 8). Both antagonists decreased (about 45% of control in donepezil-treated slices) the percent incidence of short intervals to a similar degree (Fig. 8).

4. Discussion

The present results provided evidence that AChE inhibitors can recruit endogenous nicotinic cholinergic pathway and thereby activate glutamate inputs in hippocampal slices. In vivo exposure of guinea pigs to the irreversible AChE inhibitor soman and in vitro exposure of rat hippocampal slices to the reversible AChE inhibitor donepezil resulted in an increase of glutamatergic synaptic activity in SRIs (Table 2). The present results predict that the acute neurotoxicological effects of soman are also mediated via excessive activation of brain nAChRs.

4.1. Mode of action of soman and donepezil on glutamate activity to CA1 SRI

Both soman and donepezil shared a common effect in this study, i.e., to increase the frequency of glutamate EPSCs in CA1 SRIs without changing the kinetics of the events. The data shown here suggest that the observed effect of soman on glutamate transmission is mediated via inhibition of AChE in the slices. First, it is known that soman induces significant inhibition of AChE at 24 h after injection of guinea pigs (Lintern et al., 1998), time at which there was a significant enhancement of frequency of glutamate EPSCs in CA1 SRIs (see Fig. 1). Our previous study (Alkondon et al., 2009) showing an increased potency of ACh in comparison to choline to activate $\alpha 7$ nAChR currents in CA1 SRIs 24 h after injection of soman in guinea pigs is consistent with this view. Second, the inhibition of AChE tends to recover substantially at 7 days after a single injection of soman in guinea pigs (Lintern et al., 1998), a time at which we did not observe changes in the frequency of glutamate EPSCs in soman animals compared to age-matched controls (see Fig. 1). The observation that EPSC frequency is back to normal (see Fig. 1) at a time when AChE activity has recovered only to 70% of control (Lintern et al., 1998) at 7 days after soman injection suggests that partial recovery of enzyme activity is enough to sustain normal synaptic function. Third, the common effect of soman and a structurally unrelated compound donepezil supports the contention that AChE inhibition is the main mode by which both compounds produced their stimulatory action on glutamate transmission. The observation

that donepezil-induced increase in EPSC frequency was found only in 50% of the slices tested suggests that only a subset of glutamate inputs to SRI is sensitive to the actions of the drug. This suggests that the close appositions of AChE and cholinergic terminals in relation to glutamate neurons are achieved only in 50% of the hippocampal slices in which donepezil was tested. While a similar scenario is applicable to the guinea pig hippocampal slices in which soman effects were tested, our protocol of using different sets of animals makes it difficult to assess the percent of slices showing enhancement of glutamate transmission. It is however possible that under in vivo conditions where all the presynaptic cholinergic neurons and contacts are intact, the impact of AChE inhibition could be much larger than that observed in the in vitro slices (Table 2).

4.2. How AChE inhibition leads to an increase in glutamate activity in the CA1 SRI?

Previous studies reported that the AChE inhibitors paraoxon and ambenonium increase the frequency and amplitude of spontaneous EPSCs recorded from hippocampal granule cells; however, their effects were blocked by the muscarinic receptor antagonist atropine (Kozhemyakin et al., 2010). Also, exposure of guinea pig hippocampal slices to 100 nM soman produces epileptiform burst of activity in extracellular recordings, a finding explained as a result of enhanced ACh level in the slices acting predominantly on muscarinic receptors (Harrison et al., 2004). However, as the muscarinic receptor activity is completely suppressed by the continuous presence of atropine in the ACSF in our recordings, we are left with the option that enhanced levels of ACh act on nAChRs present on the neurons of the hippocampal slices. The observations that both MEC and MLA decreased the frequency of glutamate EPSCs in donepezil-incubated slices in our study (see Fig. 7) indicate that two subtypes of nAChRs ($\alpha 3\beta 4\beta 2$ and $\alpha 7$, respectively) are involved in the effect of donepezil on glutamatergic transmission. Interestingly, the ability of donepezil to prevent glutamate toxicity in primary cultures of cerebral cortical neurons was attributed to its effects on MEC- and MLA-sensitive nAChR subtypes (Takada-Takatori et al., 2006). The observation that both MLA and MEC decreased the frequency of EPSCs in control slices (i.e., ACSF-incubated slices) indicates that under basal conditions, there is tonic activation of both nAChR subtypes by endogenous agonist in the slices. This mechanism is further amplified in presence of donepezil as revealed by a higher percentage of cells responding to the nAChR antagonist MEC in donepezil-treated slices compared to control slices. It is also important to note that MEC-sensitive nAChRs played a dominant role whereas MLA-sensitive nAChR played less prominent role in presence of donepezil as all neurons responded to the inhibitory effect of MEC and only subset of neurons responded to the inhibitory effect of MLA (see Table 1). It is possible that enhanced level of ACh after exposure to donepezil increased the probability of activation of MEC-sensitive nAChRs, whereas it caused either increase or decrease (due to desensitization) in the activation of MLA-sensitive nAChR. A previous study by Fayuk and Yakel (2004) also concluded that use of several AChE inhibitors was effective in potentiating ACh-evoked currents in CA1 interneurons at the non- $\alpha 7$ nAChR and slowing the rate of recovery of $\alpha 7$ nAChR from desensitization. The observation that AChE inhibition produced enhancement and prolongation of $\alpha 7$ nAChR currents in our study (see Fig. 3) unlike an absence of such effects in the study of Fayuk and Yakel (2004) can be explained by the fact that we used submaximal concentration of ACh to evoke these currents. It is unlikely that the inhibitory

effect of MEC is mediated via $\alpha 7$ nAChRs because of the following reasons. MLA increased frequency of glutamate EPSCs in three out of nine neurons, whereas MEC decreased the frequency in all cells, including one in which MLA increased the frequency. Also, exogenously applied nAChR agonists increase glutamate activity in the CA1 SRI via MEC-sensitive, MLA-insensitive nAChR (Alkondon et al., 2003). Further, we have previously reported that MEC is able to decrease the excitability of CA1 SRI via a blocking action on non- $\alpha 7$ nAChRs present on glutamate neurons (Alkondon et al., 2011).

4.3. Pattern of glutamate activity induced by donepezil and its significance

The effects of donepezil on glutamate activity can be characterized by (i) an increase in the frequency of EPSCs, (ii) an increase in the burst activity, and (iii) an increase in the proportion of large-amplitude EPSCs in relation to the total number of EPSCs. The presence of higher frequency of EPSCs including large-amplitude EPSCs in donepezil-exposed slices than in control slices suggests that the presynaptic glutamate neurons (predominantly CA3 and CA1 pyramidal neurons) are excited by ACh-induced activation of nAChRs that in turn induce action potential-dependent release of glutamate onto the CA1 SRI (see Fig. 9). Therefore, the burst-like EPSCs induced by donepezil in SRI may indicate the presence of action potentials occurring at the same frequency at the presynaptic glutamate neurons. It is interesting to note that bursts of EPSCs occurred at a frequency range of 10–50 Hz in our experiments that were conducted at about 24 °C. If corrected for higher body temperature, the frequency could reach about 20–100 Hz, as it has been demonstrated for change in oscillation frequency with temperature in hippocampal slices (Dickinson et al., 2003). Thus, it is conceivable that the frequency of glutamate EPSCs may represent brain oscillations in the gamma frequency range during exposure to donepezil. The ability of both MEC and MLA to suppress the intra-burst intervals (see Fig. 8) indicates that both nAChR subtypes are involved in the activation of presynaptic glutamate neurons to initiate oscillations in the gamma frequency range. These results are partly in line with a previous study that indicated the involvement of $\alpha 7$, but not non- $\alpha 7$ nAChR, in tetanic stimulation-induced gamma oscillations in rat hippocampal slices (Song et al., 2005).

4.4. Toxicological relevance

The apparent similarity in the effects of soman and donepezil on the glutamatergic EPSCs suggests that these effects may be related to the toxicological actions of anti-AChE agents, particularly seizures induced by soman and donepezil (Harrison et al., 2004; Kumlien and Lundberg, 2010). Soman-induced seizures have been associated with several neurochemical changes in the hippocampus and other regions of the brain (Shih and McDonough, 1997). Generally, it is accepted that there is an initial cholinergic phase involving brain muscarinic receptors, the activation of which initiates seizures. When seizures continue, there is recruitment of glutamate systems and neurotoxicity that are associated with it. As some of the initial experiments revealed that MEC was ineffective against soman-induced seizures (Shih et al., 1999; Harrison et al., 2004), the role of nAChRs in the actions of soman has been neglected and the therapeutic importance of nAChR antagonists unexplored. However, our results suggest that nAChR subtypes present on glutamate neurons are being recruited by the actions of AChE inhibitors. The effect of both soman and donepezil coincides with their ability to inhibit AChE in the slices. Therefore, it is reasonable to predict that soman

exposure while causing intense AChE inhibition would recruit nAChRs and thereby activate the glutamate pathway even within minutes and hours after exposure to soman. Although the previous neurochemical techniques failed to detect significant increase in glutamate levels in the hippocampus within minutes and hours after soman (McDonough and Shih, 1997; Lallement et al., 1998), it is possible that nAChR-dependent glutamate EPSCs maintained higher excitability of the slices without increasing the extracellular level of glutamate. Such a scenario is possible, because on most occasions extracellular measurement of glutamate has multiple origins such as neuronal activity, neuronal damage, and glia.

5. Conclusion

In conclusion, the AChE inhibitors soman and donepezil enhanced spontaneous glutamate activity to CA1 SRI in hippocampal slices. Such activity depended on the buildup in slices of endogenous ACh, which in turn activated both $\alpha 7$ and non- $\alpha 7$ nAChRs in presynaptic glutamate neurons. Increased glutamate activity may partly contribute to the toxicological actions of soman.

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Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
EC50	concentration that produces 50% effect
EPSC	excitatory postsynaptic current
LD50	concentration that produces death in 50% of animals
LTP	long term potentiation
MEC	mecamylamine
MLA	methyllycaconitine
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
SRI	stratum radiatum interneuron

TTX tetrodotoxin

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Fig. 1. Soman increases the frequency of glutamate EPSCs in SRI of guinea pig hippocampal slices

(A) Sample recordings at -60 mV under control and 5 min after bath application of CNQX.

(B) Graphs represent the mean $\pm$ SEM values of frequency, peak amplitude, 10–90% rise time and decay time constant of glutamate EPSCs recorded at -60 mV in control and soman-injected animals. The frequency of EPSCs 1 day after soman injection was found to be significantly higher than that of control. $*p < 0.05$ compared to control by unpaired t test. Number of neurons = 13 in control, 18 in soman at P11, and 24 in control, 19 in soman at P18. (C) Traces represent averaged events from a single neuron in each group.

Fig. 2. Soman increases the proportion of large-amplitude glutamate EPSCs in SRI of guinea pig hippocampal slices

Graphs represent the distribution of peak amplitude of glutamate EPSCs recorded at -60 mV in control animals (A) and in animals 1 day after soman injection (B). Events with amplitudes < 40 pA are shown in grey, and events with amplitudes > 40 pA are shown in black. In control, 3.9% of the population belonged to >40 pA, whereas, in soman animals, 10.9% of the population belonged to >40 pA. The difference between the two groups of animals was found to be statistically significant ($p < 0.0001$ by Fisher's exact test). Total number of events = 559 from 13 slices in control and 2998 from 18 slices in soman.

Fig. 3. Donepezil potentiates ACh-evoked currents in SRI of rat hippocampal slices

(A) Sample recordings of ACh (50 μM)-evoked currents before, 20 min after bath application of 100 nM donepezil, and 20 min after wash. (B) Sample recordings of ACh-induced currents and EPSCs from another neuron under similar conditions. All recordings were done at -60 mV. ACh (5 s pulse) was applied to the neurons via a U-tube. Donepezil was included in the U-tube solution for the middle traces. MLA (10 nM) blocked ACh-induced current as illustrated by superimposed grey trace in panel (A). (C) Graph shows the mean ± SEM peak amplitude of ACh-evoked currents from four neurons under various conditions. ** $p < 0.01$ compared to control by One way repeated measures of ANOVA.

Fig. 4. Brief exposure to donepezil increases the frequency of glutamate EPSCs in SRI of rat hippocampal slices

(A) Sample traces represent recordings of glutamate EPSCs at -60 mV obtained in a single neuron under control, 10 and 20 min after bath application of 100 nM donepezil, and 10 min after wash with ACSF. Note that donepezil increased the frequency of EPSCs in a time-dependent manner. Traces in grey show identified events (marked by arrows) in an expanded scale. (B) Graph illustrates the mean frequency of EPSCs under various conditions in the experiment shown in (A). (C) Graph shows the mean $\pm$ SEM EPSC frequency (expressed as percent of control) obtained from five to eight neurons before and after donepezil bath application at various time points. * $p < 0.02$ by paired t test compared to control. (D) Graph shows the mean $\pm$ SEM peak amplitude of EPSCs obtained from five neurons before and different times after exposure to donepezil. * $p < 0.05$; ** $p < 0.01$ by paired t test compared to control. (E) Graph shows the relative incidence of large amplitude (>40 pA) EPSCs in

relation to total number of events. Data were from five neurons as above. *** $p < 0.0001$ compared to control by Chi-square test.

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Fig. 5. Brief exposure to donepezil increases the proportion of short inter-event intervals

(A) Graphs represent double logarithmic plots of distribution of inter-event intervals among glutamate EPSCs under control, 10 and 20 min during bath application of donepezil, and 13 min during wash with ACSF in a typical experiment. Data are from the same neuron as in Fig. 4. Inter-event intervals < 10 ms were excluded in the analysis. Inter-event intervals > 10 s are not shown. (B) Graph represents the proportion of 20–100 ms inter-event intervals as a percent of total number of events under various conditions in the same cell as in (A). (C) Graph shows the mean values of 20–100 ms inter-event intervals as a percent of total from five neurons. *** $p < 0.0001$ by Chi-square test compared to control.

Fig. 6. Prolonged exposure to donepezil increases the frequency of glutamate EPSCs in SRI of rat hippocampal slices

(A) Top three traces represent segments of recordings from a single neuron from a slice incubated and superfused with control ACSF. Bottom three traces represent segments of recordings from another neuron from a slice incubated (2 h) and superfused with ACSF containing 100 nM donepezil. Note the more frequent appearance of closely occurring events, indicating the presence of bursts of glutamate EPSCs in donepezil-treated slice. (B) The bottom four graphs illustrate the mean $\pm$ SEM values of frequency, peak amplitude, 10–90% rise time and decay time constant of glutamate EPSCs in control and donepezil-incubated slices. In donepezil-incubated slices (1–5 h), same concentration of the drug was present in the ACSF during the entire recording session. Number of neurons = 13 in control and 27 in donepezil. * $p < 0.05$ compared to control by unpaired t test.

Fig. 7. Antagonists of nAChRs decrease the frequency of glutamate EPSCs in SRI of donepezil-treated rat hippocampal slices

(A) Panel of traces was obtained from a single neuron in the presence of donepezil (top three traces), 10 min after 10 μM mecamylamine plus donepezil (middle three traces), and 10 min after wash with donepezil-containing ACSF (bottom three traces). (B) Panel of traces was obtained from another neuron in donepezil-treated slice. In presence of donepezil alone (top trace), two types of events are visible, single EPSCs and bursts of EPSCs (shown at bottom as grey traces in expanded scale). After 10-min bath application of 10 μM mecamylamine plus donepezil, the frequency of both individual EPSCs and bursts of EPSCs was reduced

(traces in rows third and fourth). A 10-min washing with donepezil-containing ACSF resulted in the restoration of both types of events (traces in rows five and six). A subsequent application of 10 nM MLA also reduced the frequency of EPSCs including the bursts (trace in bottom row). All slices were pre-incubated in donepezil-containing ACSF for 1–5 h. (C) and (D) Graphs illustrate the mean $\pm$ SEM values of EPSC frequency, and the percent inhibition by MEC and MLA. Number of neurons = 11 in MEC and 5 in MLA. $*p < 0.05$ compared to respective control by paired t test. $**p < 0.05$; $***p < 0.01$ by single sample t test.

Fig. 8. Antagonists of nAChRs decrease the incidence of short inter-event intervals among glutamate EPSCs in SRI of donepezil-treated rat hippocampal slices

Summary of the data (mean $\pm$ SEM) on 20–100 ms inter-event intervals of EPSCs recorded in donepezil-treated slices before, 10 min after bath application of MEC (10 μ M) or MLA (10 nM), and 10 min after wash. Donepezil (100 nM) was present throughout. The number of inter-event intervals between 20 and 100 ms is expressed as percent of total number of events under each condition. The percent inhibition by MEC or MLA was calculated by comparing the value in presence of the antagonist to the average value of both control and wash phases. Data were obtained from 6 neurons in each antagonist group. * $p < 0.05$, compared to mean of control and wash values by paired t test. ** $p < 0.01$; *** $p < 0.0001$ by one-sample t test.

Fig. 9. Scheme illustrating hippocampal neurocircuitry in relation to nAChRs and AChE
Soman causes sustained inhibition of AChE that is present at 24 h after injection in guinea pigs. Exposure of rat hippocampal slices to donepezil causes inhibition of AChE within minutes. Enhanced level of endogenous ACh activates both $\alpha 7$ (MLA-sensitive) and $\alpha 3\beta 4\beta 2$ (MEC-sensitive) nAChRs at various sites in the pyramidal neurons resulting in increased glutamate release at the interneurons.

Table 1

Donepezil increases proportion of neurons responding to MEC.

Treatment of slices	MLA (10 nM)	MEC (10 μ M)
ACSF	5/7 (71%)	4/10 (40%)
Donepezil, 100 nM	5/9 (56%)	11/11 (100%)*

Numbers in numerator indicate number of neurons in which antagonists decreased frequency of AMPA EPSCs. Numbers in denominator indicate the number of neurons studied. Numbers in parentheses indicate the percent of neurons responding to the antagonists.

* $p < 0.01$ by Fisher's exact test compared to ACSF group.

Table 2

Summary of effects of AChE inhibition on EPSC frequency in CA1 SRI of hippocampal slices.

Preparation	Treatment	Time after treatment	EPSC frequency (Hz, mean $\pm$ SEM)		Number of cells
			Control	Treatment	
Guinea pig (in vivo)	Soman, 25.2 μ g/kg, s.c.	24 h	0.264 $\pm$ 0.058	0.660* $\pm$ 0.170	150 13–18
Rat (in vitro)	Donepezil, 100 nM bath exposure	20 min	0.476 $\pm$ 0.135	0.671** $\pm$ 0.124	70 6
Rat (in vitro)	Donepezil, 100 nM incubation plus bath exposure	2–5 h	0.349 $\pm$ 0.087	0.593* $\pm$ 0.072	70 13–27

* $p < 0.05$ by unpaired t test.

** $p < 0.02$ by paired t test.