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RESEARCH PAPER

Neuroprotective efficacy of caramiphen against soman and mechanisms of its action

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BACKGROUND AND PURPOSE
Caramiphen is a muscarinic antagonist with potent anticonvulsant properties. Here, we investigated the efficacy of caramiphen against behavioural seizures and neuropathology induced by the nerve agent soman, and revealed two mechanisms that may underlie the anticonvulsant efficacy of caramiphen.

EXPERIMENTAL APPROACH
Rats were given caramiphen at 30 or 60 min after treatment with soman. Neuronal loss in the basolateral amygdala (BLA) and neuronal degeneration in the amygdala, hippocampus, piriform cortex, entorhinal cortex and neocortex, were investigated 24 h after soman, using design-based stereology and FluoroJade-C staining. The effects of caramiphen on NMDA-, AMPA- and GABA-evoked currents were studied in the BLA region of *in vitro* brain slices from un-treated rats, using whole-cell recordings.

KEY RESULTS
Caramiphen given either 30 min or 60 min after soman, suppressed behavioural seizures within 10 min, but required 1~4.5 h for complete cessation of seizures. Neuronal loss and degeneration were significantly reduced in the caramiphen-treated, soman-exposed rats. Postsynaptic currents evoked by puff-application of NMDA on BLA principal cells were reduced by caramiphen in a dose-dependent manner (100 μM, 300 μM and 1 mM), while GABA-evoked currents were facilitated by 100 μM and 300 μM, but depressed by 1 mM caramiphen. AMPA-evoked currents were not affected by caramiphen.

CONCLUSIONS AND IMPLICATIONS
Caramiphen offered partial protection against soman-induced seizures and neuropathology, even when given 60 min after soman. NMDA receptor antagonism and facilitation of GABAergic inhibition in the BLA may play a key role in the anticonvulsive and neuroprotective properties of caramiphen.

Abbreviations
BLA, basolateral amygdala; DL-AP5, DL-(2*R*)-amino-5-phosphonovaleric acid; FJC, Fluoro-Jade C; HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride; IQR, interquartile range; TTX, tetrodotoxin; VX, *O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate.

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Introduction

Organophosphorus compounds are potent neurotoxic chemicals that are widely used in industry and agriculture. Furthermore, organophosphorus compounds such as sarin, soman and VX, are nerve agents used in chemical warfare (Bajgar, 2005). Nerve agents affect cholinergic transmission in the peripheral and central nervous systems by inhibiting acetylcholinesterase. This inhibition causes accumulation of acetylcholine in the synaptic cleft leading to overstimulation of muscarinic and nicotinic receptors, hyperexcitability in the brain, and, ultimately, intense generalized seizures/status epilepticus, which produce brain damage and death (Hayward *et al.*, 1990; Shih *et al.*, 2003), or long-term neurological and behavioural deficits (Brown and Brix, 1998).

The standard treatment for nerve agent intoxication is a combined administration of an acetylcholinesterase reactivator, a cholinergic receptor antagonist and an anticonvulsant (Jokanović, 2009). However, if the treatment is delayed, the effectiveness of these pharmacological interventions in preventing nerve agent-induced seizures is dramatically reduced (Lallement *et al.*, 1998; Gilat *et al.*, 2005; McDonough *et al.*, 2009, 2010). In the search for antidotes against nerve agent toxicity, it has been found that anticholinergic drugs with antiglutamatergic properties show a marked effectiveness against nerve agent intoxication when used either prophylactically or therapeutically (Weissman and Raveh, 2008). In comparisons between anticholinergic drugs versus compounds with both anticholinergic and antiglutamatergic activity, administered at 5, 10 or 20 min after the onset of nerve agent-induced status epilepticus, caramiphen, an anticholinergic that also interferes with glutamatergic transmission, was the only drug that offered significant protection against seizures and cognitive impairment when the treatment was delayed to 20 min (Raveh *et al.*, 2008). At this time (20 min), all drugs, including caramiphen, failed to produce significant reduction in brain damage, as assessed by measurements of peripheral benzodiazepine receptor densities in forebrain/midbrain homogenates (Raveh *et al.*, 2008). However, the protection conferred by caramiphen against learning deficits (Raveh *et al.*, 2008) leaves open the possibility that it also protected against brain damage, but different techniques may be needed to reveal this effect.

The exact mechanism of the anticonvulsant action of caramiphen is not well understood. The anticholinergic properties of caramiphen (Hudkins and DeHaven-Hudkins, 1991a; Hudkins *et al.*, 1991b, 1993; Gao *et al.*, 1998) probably play an important role in its anticonvulsant efficacy against nerve agents. However, NMDA receptor antagonistic properties (Apland and Braitman, 1990; Pontecorvo *et al.*, 1991; Fletcher *et al.*, 1995; Thurgur and Church, 1998; Raveh *et al.*, 1999), blockade of Ca^{2+} channels (Church and Fletcher, 1995; Thurgur and Church, 1998), or inhibition of glutamate release (Annels *et al.*, 1991) may also be involved. Facilitation of GABAergic inhibition by caramiphen has also been suggested (Thurgur and Church, 1998), but so far there has been no direct evidence for this effect.

To further elucidate the effectiveness of caramiphen against nerve agent-induced seizures and neuropathology, we evaluated its protective efficacy against soman-induced behavioural seizures and neuronal damage, when caramiphen was given relatively long after soman exposure. Such assessment of the antidotal efficacy of compounds administered long after nerve agent exposure is necessary because, in a real case scenario, medical assistance may not be immediately available. In addition, to better understand the mechanisms underlying the anticonvulsant properties of caramiphen, we tested the hypothesis that caramiphen antagonized the activation of NMDA receptors or AMPA receptors, and facilitated GABAergic inhibitory activity. We found that caramiphen administered at 30 or 60 min after soman exposure, significantly suppressed behavioural seizures, reduced neuronal degeneration in a number of brain regions, and prevented neuronal loss in the basolateral nucleus of the amygdala (BLA), an area that plays a central role in the generation and spread of seizures (Aroniadou-Anderjaska *et al.*, 2008), including seizures induced by nerve agents (McDonough *et al.*, 1987; Apland *et al.*, 2009; Aroniadou-Anderjaska *et al.*, 2009; Skovira *et al.*, 2010). We also demonstrated that caramiphen reduced NMDA-evoked currents and facilitated GABA-evoked currents in the BLA, with the facilitation of the inhibitory currents occurring only at lower than millimolar concentrations. We found no significant effect of caramiphen on AMPA-evoked currents. Thus, caramiphen has both anticonvulsant and neuroprotective efficacy against soman, which is probably due to a concomitant effect on the cholinergic, glutamatergic (NMDA receptor) and GABAergic systems.

Methods

Animals

The animal care and use programmes at the US Army Medical Research Institute of Chemical Defense and the Uniformed Services University of the Health Sciences are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, the National Research Council USA, and were in accordance with the guidelines of both of our Institutions, after obtaining approval of the Institutional Animal Care and Use Committees. Male, Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA), weighing 150–250 g at the start of the experiments were individually housed in an environmentally controlled room (20–23°C, 12 h light/12 h dark cycle, lights on 06:00 am), with food and water available *ad libitum*.

Soman exposure and caramiphen administration

Soman was diluted in cold saline and administered via a single subcutaneous injection. We used a dose of soman ($154\ \mu g \cdot kg^{-1}$, $1.4 \times LD_{50}$) that was sufficiently high to induce status epilepticus in a high proportion of the exposed rats, without killing most of the rats that did not receive anticonvulsant treatment; a good survival rate in the soman-exposed rats that did not receive caramiphen was necessary because we wanted to study neuropathology at 24 h after exposure, and compare with the soman-exposed groups that received

caramiphen. To increase survival rate, all rats were treated with HI-6 (125 mg·kg^{-1}; i.p.), 30 min prior to soman exposure. HI-6 is a bispyridinium oxime that reactivates inhibited acetylcholinesterase, primarily in the periphery (Bajgar, 2005). All rats received an intramuscular injection of atropine sulphate (2 mg·kg^{-1}) 1 min after soman exposure, in order to minimize peripheral toxic effects. A total of 41 rats were used in these experiments. The rats were divided into four groups, according to soman and caramiphen administration: (i) CONTROL group (n = 5), rats that received saline injection in place of soman; (ii) SOMAN group (n = 21), soman-exposed rats that were injected with saline in place of caramiphen; (iii) SOMAN + CRM 30 group (n = 8), soman-exposed rats injected with caramiphen (100 mg·kg^{-1}) at 30 min after injection of soman; and (iv) SOMAN + CRM 60 (n = 7), soman-exposed rats injected with caramiphen (100 mg·kg^{-1}) at 60 min after injection of soman. Caramiphen was injected i.m.. The decision to use 100 mg·kg^{-1} caramiphen was based (i) on the study by Sparenborg *et al.* (1990), where pretreatment with 100 mg·kg^{-1} caramiphen protected guinea pigs against lethality, seizures, and brain damage induced by 2 × LD_{50} soman, while lower doses of caramiphen were not as effective; and (ii) on the observations that 20 mg·kg^{-1} of caramiphen had only few beneficial effects when administered with a delay, at 20 min after the onset of seizures induced by 1.2 × LD_{50} of sarin (Raveh *et al.*, 2008), a nerve agent that requires lower doses of anticonvulsants compared with soman (Shih *et al.*, 2003). Thus, as our aim was to terminate already ongoing status epilepticus induced by 1.4 × LD_{50} soman, with delayed caramiphen administration, at 30 or 60 min after soman exposure (approximately 20 and 50 min after seizure onset), we reasoned that we had to use at least 100 mg·kg^{-1} caramiphen. In addition, we compared three doses of caramiphen (30 mg·kg^{-1}, 50 mg·kg^{-1} and 100 mg·kg^{-1}) and observed that the dose of 100 mg·kg^{-1} was more effective in suppressing behavioural seizures (see *Results*, Figure 1A).

Animals that received soman were monitored behaviourally until indications of seizure activity stopped in the rats that received caramiphen. Behavioural seizures were classified according to the Racine scale (Racine, 1972) with minor modifications: stage 0, no behavioural response; stage 1, behavioural arrest; stage 2, oral/facial movements, chewing, head nodding; stage 3, unilateral/bilateral forelimb clonus without rearing, straub tail, extended body posture; stage 4, bilateral forelimb clonus plus rearing; stage 5, rearing and falling; and stage 6, full tonic seizures.

Fixation & tissue processing

Twenty-four hours after soman administration, rats were deeply anesthetized with pentobarbital (75–100 mg·kg^{-1}, i.p.) and transcardially perfused with phosphate buffered saline (PBS: 100 mL) followed by 4% paraformaldehyde (200 mL, FuDu Neurotechnologies, Baltimore, MD, USA). The brains were removed and post-fixed overnight at 4°C, transferred to a solution of 30% sucrose in PBS for 72 h, and frozen with dry ice before storage at –80°C until sectioning. A 1-in-5 series of sections from the rostral extent of the amygdala to the caudal extent of the entorhinal cortex was cut at 40 µm on a sliding microtome. One series of sections was mounted on slides (Superfrost Plus, Daigger, Vernon Hills, IL, USA) in PBS for

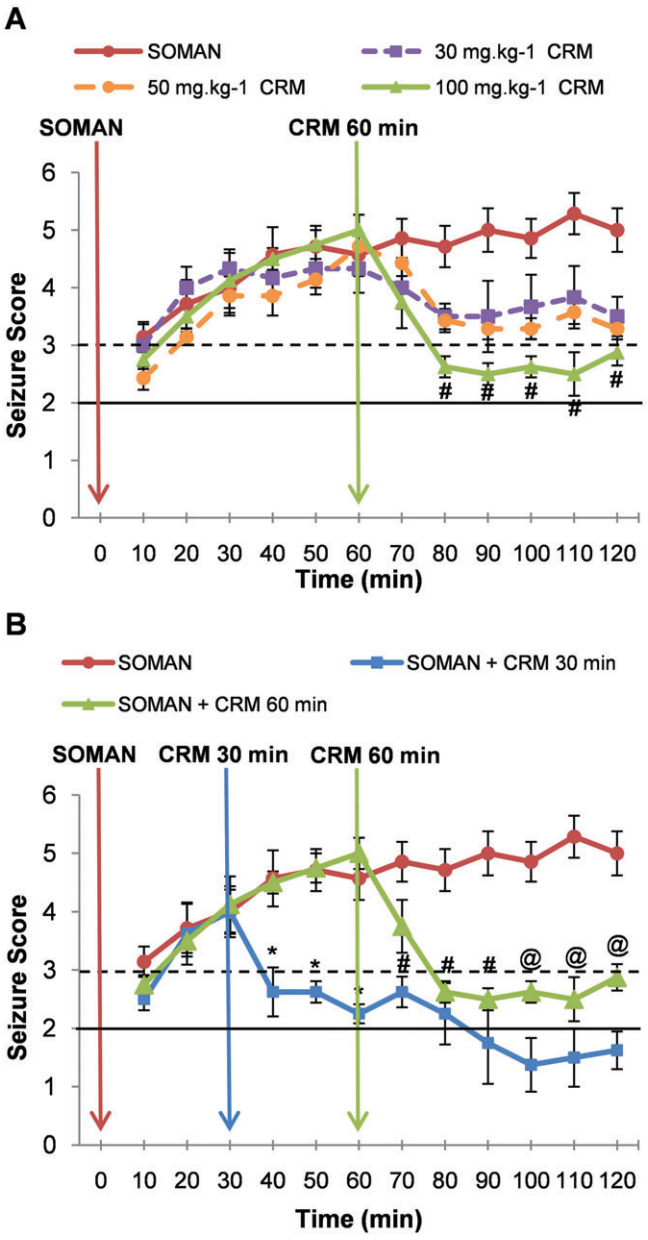

Figure 1

Efficacy of caramiphen against soman-induced behavioural seizures. The graphs show group means and standard errors of the maximum behavioural seizure score in blocks of 10 min. Arrows indicate the time of soman and caramiphen (CRM) injections, at 30 min or 60 min, after soman exposure. (A) Comparison of the anticonvulsant efficacy of three doses of caramiphen (30, 50 or 100 mg·kg^{-1}), given at 1 h after soman exposure. $^{\#}P < 0.05$, significantly different from the SOMAN-only group (n = 6–7 per group). (B) Anticonvulsant efficacy of 100 mg·kg^{-1} caramiphen, administered at 30 min or 1 h after soman exposure. $^{*}P < 0.05$, significantly different from the SOMAN group (n = 7) and the SOMAN + CRM 60 group (n = 7); $^{\#}P < 0.05$, SOMAN + CRM 30 (n = 8) and SOMAN + CRM 60 groups significantly different from the SOMAN group; $^{@}P < 0.05$, behavioural seizure scores for all three groups are significantly different from each other (ANOVA, least significant difference *post hoc* test).

Nissl staining with cresyl violet. One adjacent series of sections was also mounted on slides for Fluoro-Jade C (FJC) staining, while the remaining series of sections were stored at –20°C in a cryoprotectant solution. Analysis of neuronal loss from Nissl-stained sections was performed in the BLA. Analysis of neuronal degeneration from FJC-stained sections was performed in all the amygdala nuclei, a neocortical region, the piriform cortex, the entorhinal cortex, and the CA1, CA3 and hilar areas of the ventral hippocampus. We studied the ventral hippocampus because it displays significantly more severe neurodegeneration after soman exposure than the dorsal hippocampus (Apland *et al.*, 2010).

Evaluation of neurodegeneration

FJC-stained slices were evaluated for neuropathology as described previously (Figueiredo *et al.*, 2010; Qashu *et al.*, 2010). Briefly, from an adjacent series of Nissl-stained sections, tracings were made of the amygdala, piriform cortex, entorhinal cortex, CA1, CA3 and hilar areas of the ventral hippocampus, as well as the neocortex. The tracings from the Nissl-stained sections were superimposed on the FJC-stained sections. Five sections per animal were used for qualitative analysis of FJC-stained sections using the following rating system to score the extent of neuronal degeneration in each structure and substructures: 0 = no damage; 1 = minimal damage (1–10%); 2 = mild damage (11–25%); 3 = moderate damage (26–45%); and 4 = severe damage (>45%). We have previously shown that qualitative assessment using this scale produces results which are in agreement with quantitative assessments (Qashu *et al.*, 2010).

Stereological quantification

Design-based stereology was used to quantify the total number of neurons in Nissl-stained sections of the BLA as described by Fritsch *et al.* (2009). Briefly, sections were viewed with a Zeiss Axioplan 2ie (Oberkochen, Germany) fluorescent microscope with a motorized stage, interfaced with a computer running StereoInvestigator 9.0 (MicroBrightField, Williston, VT, USA). The BLA region was identified on slide-mounted sections, and delineated for each slide of each animal under a 2.5 × objective, based on the atlas of Paxinos and Watson (2005). All sampling was done under a 63 × oil immersion objective. Nissl-stained neurons were distinguished from glia cells by their larger size and pale nuclei surrounded by darkly stained cytoplasm containing Nissl bodies. The total number of Nissl-stained neurons was estimated using the optical fractionator probe and, along with the coefficient of error, was calculated using the Stereo Investigator 9.0.

Amygdala slice electrophysiology

Male, Sprague-Dawley rats that were not exposed to any of the *in vivo* experimental treatments were anesthetized with halothane before decapitation. Coronal brain slices (400 μm thick) containing the amygdala were cut using a Vibratome series 1000 (Leica Microsystems, Bannockburn, IL, USA) and prepared in ice-cold cutting solution. The cutting solution was a 50/50 mixture of the following two solutions: (i) 230 mM sucrose, 1 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 1 mM $CaCl_2$, 7 mM $MgCl_2$ and 25 mM D-glucose; and (ii) 144 mM N-methyl-d-glucamine (NMDG), 1.5 mM KCl, 1.25 mM NaH_2PO_4, 30 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 25 mM D-glucose, with the pH adjusted to 7.4 using 50% D-gluconic acid and $NaHCO_3$. We have observed that this mixture of cutting solution produces high viability slices with healthy neurons. All the solutions were saturated with 95% O_2, 5% CO_2 to achieve a pH near 7.4. The slices were then transferred to a holding chamber containing the bath solution: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 21 mM $NaHCO_3$, 2.1 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM D-glucose. Slices were maintained at 33°C for 20 min, and subsequently were kept at room temperature (23°C). The recording chamber (0.7 mL capacity) had continuously flowing bath solution (~5 mL·min^{-1}) at 32~33°C. The osmolarity of the external solution was adjusted to 325 mOsm with D-glucose. Neurons were visualized under infrared light, using Nomarski optics of an upright microscope (Zeiss Axioskop 2, Thornwood, NY, USA) equipped with a CCD-100 camera (Dage-MTI, Michigan City, IN, USA). Principal neurons in the BLA were identified based on their large size and pyramidal-like shape. The patch electrodes had resistances of 3.5–4.5 MΩ when filled with the internal solution: 60 mM $CsCH_3SO_3$, 60 mM KCH_3SO_3, 10 mM KCl, 10 mM EGTA, 10 mM HEPES, 5 mM Mg-ATP, 0.3 mM Na_3GTP (pH 7.2), 290 mOsm. Ionic currents were amplified and filtered (1 kHz) using the Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) with a four-pole, low-pass Bessel filter, digitally sampled (up to 2 kHz) using the pClamp10 software (Axon Instruments), and further analysed using Origin (OriginLab Corporation, Northampton, MA, USA). Pressure application of receptor agonists was conducted with the help of a 'Picospritzer' (Parker Hannifin Corporation, Fairfield, NJ, USA).

Statistical analysis

ANOVA followed by *post hoc* test was used to analyse both stereological estimations of the total number of neurons in the BLA and behavioural seizure scores. To calculate the group mean of the behavioural seizure scores at different time points (10 min intervals, see Figure 1) we used the highest behavioural seizure stage score observed within the 10 min interval for each rat. The statistical values from these tests are presented as the mean and SEM. Neurodegeneration scores were compared between groups for each structure separately using the Mann–Whitney *U*-test. The statistical values are presented as the median and interquartile range (IQR, the values at the 25th and 75th percentiles). Pearson's chi-square and Friedman's exact test were used to compare the survival rate between the groups. Student's *t*-test was used for statistical comparisons in the *in vitro* electrophysiological experiments. Differences were considered significant when $P < 0.05$. Sample sizes (n) refer to the number of animals in the *in vivo* studies, and to the number of cells in the electrophysiological experiments.

Materials

Atropine sulphate, caramiphen, bicuculline methiodide ($GABA_A$ receptor antagonist) and kynurenate (ionotropic glutamate receptor antagonist) were purchased from Sigma-Aldrich (St Louis, MO, USA). GYKI 52466 (AMPA receptor

antagonist), (2*R*)-amino-5-phosphonovaleric acid (DL-AP5; NMDA receptor antagonist), LY 341495 (metabotropic glutamate group II/III receptor antagonist), SCH50911 ($GABA_B$ receptor antagonist) and tetrodotoxin (TTX; sodium channel blocker) were purchased from Tocris (Ellisville, MO, USA). Soman (pinacoyl methylphosphonofluoridate) was obtained from Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA. HI-6 (BN44621) was obtained from Starks Associates (Buffalo, NY, USA).

Results

Seizure termination by caramiphen

In the experiments described below, the soman-exposed rats that were given anticonvulsant treatment, received caramiphen at 100 mg·kg^{-1}. In a separate set of experiments, we compared the dose of 100 mg·kg^{-1} with 30 and 50 mg·kg^{-1} caramiphen, for their efficacy in suppressing soman-induced seizures within 1 h after administration of the anticonvulsant. Caramiphen was administered at 1 h after exposure to soman. Although all three doses of caramiphen reduced the severity of status epilepticus (Figure 1A), the 100 mg·kg^{-1} dose reduced it to a level below stage 3 (which is the first stage of behavioural status epilepticus) in 71.5% of the animals (five out of seven), versus 15% of the animals that received 30 mg·kg^{-1} (two out of six) or 50 mg·kg^{-1} caramiphen (zero out of seven).

Eight out of the 36 rats that were exposed to soman did not develop behavioural status epilepticus (stage 3 and above) and were not included in the study. Twenty-eight rats developed status epilepticus within 10 to 15 min after soman injection. The survival rate for the animals that did not receive the anticonvulsant treatment (SOMAN group) was 53% (seven out of 13), while the animals that received caramiphen (SOMAN + CRM 30 and SOMAN + CRM 60 groups) had a 100% survival rate (n = 15). This difference in survival rate was statistically significant ($P < 0.001$). Caramiphen significantly attenuated behavioural seizures to a level below stage 3, within 10 min, even when administered at 60 min after soman exposure (Figure 1B). Behavioural seizures were terminated (Racine scale score less than 2) within 60 to 120 min when caramiphen was administered at 30 min after soman injection (Figure 1B), and within 4 to 4.5 h when caramiphen was administered at 60 min after soman.

Neuronal degeneration

Neuronal degeneration in the hippocampus (based on FJC staining) was severe in the SOMAN group (CA1, median = 4, IQR = 3~4; CA3, median = 4, IQR = 3~4; hilus, median = 4, IQR = 3.5~4), but absent in the SOMAN + CRM 30 group (median = 0, IQR = 0 ~ 0 in CA1, CA3 and hilus) and minimal in the SOMAN + CRM 60 group (Figure 2). The neurodegeneration score for the amygdala was moderate (median = 3, IQR = 3~4) in the SOMAN group, minimal (median = 1, IQR = 0~2.5) in the SOMAN + CRM 30 group, and mild (median = 2, IQR = 2~3) in the SOMAN + CRM 60 group. The neurodegeneration scores for the neocortical sample from the temporal cortex and for the entorhinal cortex in the SOMAN + CRM 30 min group (median = 1, IQR = 0~1 for neocortex, median = 0, IQR = 0~1 for entorhinal cortex, median = 1, IQR = 0.25~2.5 for piriform cortex) were significantly lower ($P < 0.05$) than those in the SOMAN group (median = 2, IQR = 1~2 for neocortex, and median = 3, IQR = 2~4 for entorhinal cortex, median = 3, IQR = 3~4 for piriform cortex; Figure 2). However, there was no significant difference between the SOMAN group and the SOMAN + CRM 60 group (median = 1, IQR = 1~1.5 for neocortex; median = 2, IQR = 1.75~2.5 for entorhinal cortex, median = 2, IQR = 1~3.5 for piriform cortex). The control group did not show any FJC-positive staining.

Neuronal loss in the BLA

Estimation of the total number of neurons in the BLA using an unbiased stereological method in Nissl-stained sections, showed that one day after soman exposure, the SOMAN + CRM 30 and the SOMAN + CRM 60 groups had a significantly higher ($P < 0.05$) total number of neurons compared with the SOMAN group (Table 1; Figure 3). The number of neurons in the BLA of rats receiving caramiphen did not significantly differ from the number of neurons in the BLA of the control group. In contrast, the SOMAN group had a 20% neuronal loss in the BLA compared with the control animals ($P < 0.05$).

Caramiphen modulation of NMDA and GABAergic currents in the BLA in vitro

Caramiphen displays NMDA receptor antagonistic properties (Fletcher *et al.*, 1995), and some studies suggest that this is involved in its anticonvulsant and neuroprotective effects (Pontecorvo *et al.*, 1991; Raveh *et al.*, 1999). To determine if caramiphen antagonized activation of NMDA receptors in the BLA, we tested its effect on NMDA-evoked currents in principal BLA neurons, in bath solution containing low Mg^{2+} concentration (200 μM) to relieve the Mg^{2+} block of NMDA receptors. Whole-cell patch clamp recordings at the holding potential of −70 mV demonstrated a dose-dependent antagonistic action of bath-applied caramiphen on inward cationic currents evoked by pressure-application of NMDA (100 μM, 400 ms duration), in the presence of TTX and antagonists of AMPA receptors, metabotropic glutamate receptors, $GABA_B$ receptors and $GABA_A$ receptors (Figure 4A and B). Caramiphen dose-dependently depressed NMDA-evoked currents and the IC_{50} of caramiphen on NMDA-evoked currents (derived from Figure 4B) was 550 μM.

Previous evidence has suggested that caramiphen may modulate AMPA receptors (Raveh *et al.*, 2002), but in mouse cultured hippocampal neurons, caramiphen had no effect on AMPA-evoked currents (Fletcher *et al.*, 1995). To determine if caramiphen directly affects AMPA-evoked currents in the BLA, we tested the effect of 1 mM caramiphen on currents evoked on principal cells by AMPA puff-application (10 μM, 100 ms duration, V_h = −70 mV) in the presence of TTX and antagonists of $GABA_A$, $GABA_B$, NMDA, and metabotropic glutamate receptors (Figure 4C). In three cells caramiphen had no effect, in two cells there was a slight depression of the AMPA-evoked currents, and in one cell there was a slight facilitation. The mean percentage of change for the six cells was 0 ± 3%. Thus, caramiphen had no significant effect on AMPA-evoked currents on principal cells in the BLA.

Next, we explored the possibility that caramiphen affects GABAergic mechanisms in the BLA. The experiments were

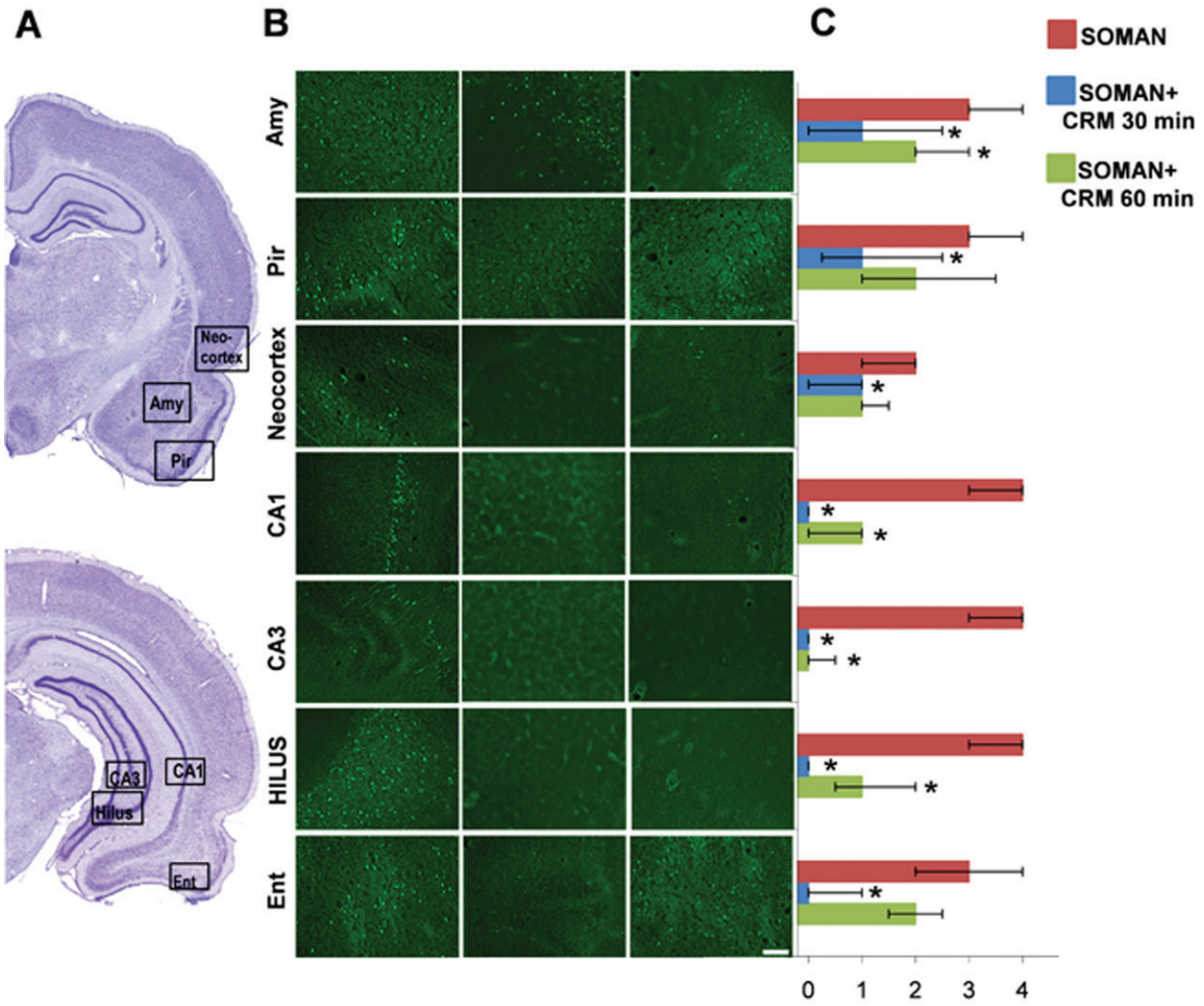

Figure 2

Caramiphen (CRM) protects against neuronal degeneration, 1 day after soman-induced status epilepticus. (A) Panoramic photomicrographs of Nissl-stained sections showing the brain regions evaluated in the Fluoro-Jade C (FJC) stained sections. (B) Representative photomicrographs of FJC stained sections from the brain regions where neuronal degeneration was evaluated, for the SOMAN (left column), the SOMAN + CRM 30 (middle column) and the SOMAN + CRM 60 (right column) groups. Total magnification is 100×. Scale bar is 50 μm. (C) Bar graphs showing the neuropathology scores (median and interquartile range) for the amygdala (Amy), piriform cortex (Pir), neocortex, CA1, CA3, hilus and entorhinal cortex (Ent), in the SOMAN (n = 7), SOMAN + CRM 30 (n = 8) and soman + CRM 60 (n = 7) groups; *P < 0.05 (Mann–Whitney U-test).

Table 1

Stereological estimation of the total number of Nissl-stained neurons in the basolateral amygdala from untreated rats (control) or after treatment with soman and caramiphen

	Control (n = 5)	SOMAN (n = 7)	SOMAN + CRM 30 min (n = 8)	SOMAN + CRM 60 min (n = 7)
Mean	81 301*	64 904	81 095*	75 647*
SEM	1 591	3 664	3 307	3 307
Mean Gundersen CE (m = 1)	0.054	0.066	0.060	0.060
Mean Schmitz–Hof CE (second estimation)	0.053	0.065	0.059	0.058

*P < 0.05 in comparison to the SOMAN group (ANOVA, Bonferroni *post hoc* test).
n, number of rats per group; CE, coefficient of error as calculated by Gundersen *et al.* (1999) and Schmitz and Hof (2000); CRM, caramiphen.

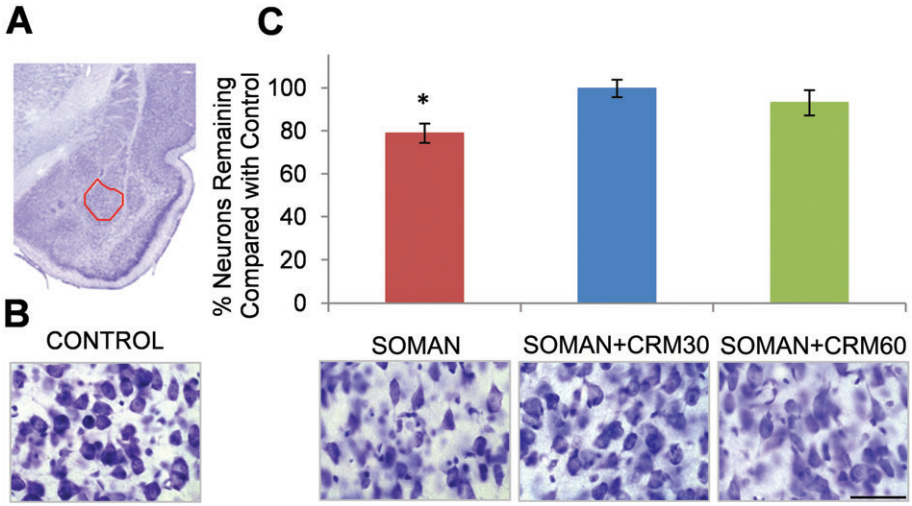

Figure 3

Caramiphen (CRM) protects against neuronal loss in the BLA, 1 day after soman-induced status epilepticus. (A) Panoramic photomicrographs of Nissl-stained sections, outlining the amygdalar nucleus where design-based stereological analysis was performed. (B) Representative photomicrographs of Nissl-stained sections showing BLA cells from the control, SOMAN, SOMAN + CRM 30, and SOMAN + CRM 60 groups. Total magnification is 630× and scale bar is 50 μm. (C) Group data (mean ± SEM) of the percentage of the neurons remaining in comparison to control group. *$P < 0.05$, significantly lower than the control group (ANOVA, Bonferroni *post hoc* test).

conducted in the whole cell-patch clamp mode, with pressure application of GABA to BLA pyramidal neurons. In our experimental conditions, anionic currents mediated via inhibitory $GABA_A$ channels had inward direction because the reversal potential for chloride ions was more positive than the holding potential of −70 mV. At 100 μM, caramiphen facilitated the GABA-evoked currents by 13 ± 2% ($n = 7$, $P < 0.05$), while at 300 μM the facilitation was 29 ± 4% increase in amplitude ($n = 5$, $P < 0.05$, Figure 5). However, 1 mM caramiphen reduced the $GABA_A$ receptor-mediated currents by 15 ± 3% ($n = 5$, $P < 0.05$, Figure 5).

Discussion

In the present study, we have demonstrated that caramiphen suppressed soman-induced behavioural seizures and protected against neuronal loss and degeneration when administered at either 30 min or 60 min after soman exposure. Caramiphen treatment at 30 min was more effective against both seizures and neuropathology, reducing neuronal degeneration in all brain regions examined, while treatment at 60 min reduced neuronal degeneration only in the hippocampus and the amygdala. We also provide direct evidence that caramiphen antagonizes NMDA receptor activation and, at low concentrations, facilitates GABAergic inhibitory currents in the BLA, suggesting that these mechanisms may be involved in the anticonvulsant and neuroprotective effects of caramiphen.

Administration of caramiphen at either 30 min or 60 min after exposure to soman, significantly suppressed behavioural seizures within 10 min. However, in the SOMAN + CRM 30 group, it required 1 to 2 h for the behavioural seizure score to fall below stage 2, whereas in the SOMAN + CRM 60 group, it required at least 4 h. Previous studies have shown that when caramiphen is administered immediately after the onset of soman-induced seizures, electrographic seizure activity is completely abolished within 5 min (Raveh *et al.*, 2003) and the longer the delay in treatment with caramiphen after sarin-induced seizures, the less pronounced is the seizure-suppressing effect (Raveh *et al.*, 2008). It appears, therefore, that the effectiveness of caramiphen against nerve agent-induced seizures is reduced with time after exposure. This is in contrast to our observations with the kainate/AMPA receptor antagonist LY293558, which blocks soman-induced seizures within 20 to 30 min, whether it is administered at 20 min, 60 min or more than 90 min after exposure (Figueiredo *et al.*, 2010, and unpublished observations). The reason for this difference may be that the anticholinergic properties of caramiphen play an important role in its anticonvulsant efficacy against nerve agents, and cholinergic hyperactivity is important in seizure generation only at the early stages after nerve agent exposure (Lallement *et al.*, 1998; Shih and McDonough, 1999; Raveh *et al.*, 2008). It is particularly notable, however, that even with caramiphen administration at 60 min after soman exposure, suppression of behavioural seizures was still significant, and survival rate was 100%; these observations point to the importance of the actions of caramiphen that are not related to cholinergic antagonism.

Different lines of evidence have led to the view that cholinergic – primarily muscarinic – hyperactivity initiates seizures after nerve agent exposure, but it is the ensuing excessive glutamatergic activity that is responsible for sustaining and reinforcing seizures, and for excitotoxic neuronal damage (McDonough and Shih, 1997). Consistent with this view, centrally acting antimuscarinic compounds with no antiglutamatergic activity can reduce seizures only when administered within a few minutes after exposure (Lallement *et al.*, 1998; Shih and McDonough, 1999; Raveh *et al.*, 2008).

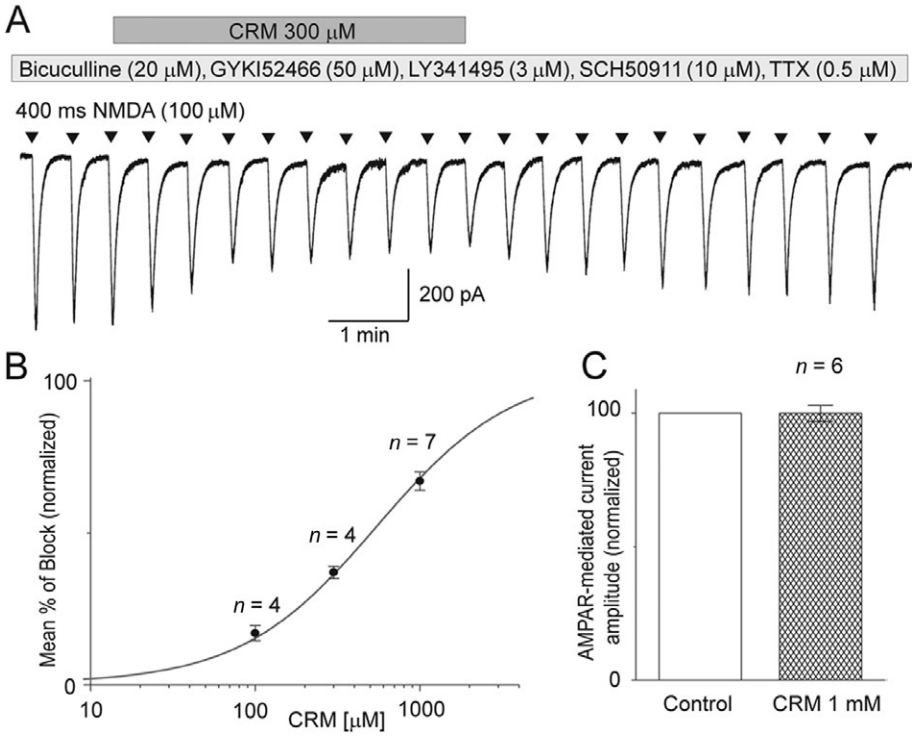

Figure 4

Caramiphen (CRM) reduces NMDA-evoked currents but not AMPA-evoked currents recorded from principal neurons in the BLA. (A) Example of cationic currents evoked by pressure application of NMDA onto BLA pyramidal neurons (arrowheads mark the time-point the agonist was applied). Bath applied caramiphen (300 μM) induced about 44% block of the NMDA receptor-mediated postsynaptic currents. The slice medium contained the antagonists shown on the long horizontal bar, and was low in Mg^{++} (200 μM). (B) Dose (concentration)–response relationship of the effect of caramiphen on NMDA-evoked currents. 100 μM, 300 μM and 1 mM caramiphen were tested. The suppression of the NMDA currents was significant at all three caramiphen concentrations ($P < 0.01$). The curve had a sigmoid fit, with a Hill coefficient of 1. (C) Caramiphen had no effect on AMPA-evoked currents. AMPA (10 μM) was puff-applied for 100 ms. The slice medium contained TTX (0.5 μM), bicuculline (20 μM), DL-AP5 (50 μM), SCH50911 (10 μM), LY341495 (3 μM) and normal concentration of Mg^{++} (1 mM). The graph shows the normalized amplitude of the AMPA-evoked currents in the absence (control) and presence of caramiphen (1 mM).

Therefore, the persistent – albeit reduced – ability of caramiphen, a muscarinic M_1 receptor antagonist (Hudkins *et al.*, 1991b), to block nerve agent-induced seizures, even when it is given relatively long after the nerve agent, has been attributed to its antagonism of NMDA receptor activation (Raveh *et al.*, 1999). Some studies are consistent with this notion (Pontecorvo *et al.*, 1991), while others do not completely agree (Apland and Braitman, 1990; Fletcher *et al.*, 1995). Because caramiphen had no effect on epileptiform activity induced by NMDA application in *in vitro* hippocampal and olfactory cortex slices, while it did block epileptiform activity induced by Mg^{++}-free medium, Apland and Braitman (1990) concluded that the antiepileptiform effect of caramiphen may not involve blockade of NMDA receptors. Fletcher *et al.* (1995), using hippocampal cultured neurons, found that caramiphen directly affects NMDA-evoked currents, but only at micromolar concentrations; it was concluded, therefore, that NMDA receptor antagonism is unlikely to account for the potent antiepileptiform effects of caramiphen, while antagonism of voltage-activated calcium channels may play a more important role (Church and Fletcher, 1995; Fletcher *et al.*, 1995). Others have stressed the importance of both NMDA receptor- and voltage-gated calcium channel antagonism in the anti-seizure effects of caramiphen (Thurgur and Church, 1998).

In the present study, caramiphen reduced postsynaptic currents evoked on pyramidal-shaped cells in the BLA by puff-application of NMDA. Whether or not the NMDA receptor antagonism revealed by these experiments plays an important role in the anticonvulsive and neuroprotective effects of caramiphen cannot be clearly answered. Such answers would require administration of caramiphen in an animal model where status epilepticus is ongoing in the presence of an NMDA receptor antagonist, and/or measurements of caramiphen concentrations in the brain to determine if they reach the concentrations required for NMDA receptor antagonism as seen in the *in vitro* studies, and correlations of such caramiphen concentrations with its anticonvulsant activity. From the results of the present study, we know that, *in vitro*, caramiphen inhibits NMDA currents even at relatively low micromolar concentrations. However, we do not know the blood and brain concentrations of caramiphen after *in vivo* injection of 100 $mg \cdot kg^{-1}$ of body weight. If we attempt to make a very rough estimate based on the blood concentrations of caramiphen after repeated or continuous administration (Levandoski and Flanagan, 1980; Levy *et al.*,

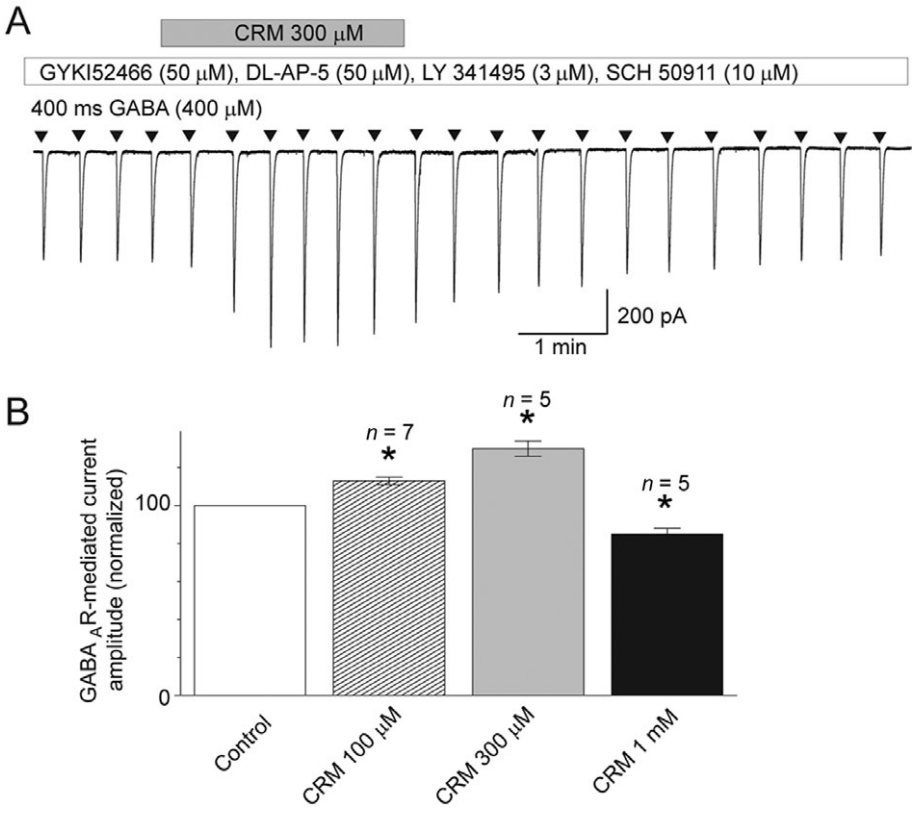

Figure 5

Effects of caramiphen (CRM) on GABA-evoked currents. Postsynaptic currents were recorded from BLA principal cells in response to puff-application of GABA (400 μM, 400 ms duration), in the presence of antagonists of AMPA/kainate, NMDA, metabotropic glutamate, and $GABA_B$ receptors. (A) Example of anionic postsynaptic currents evoked by pressure application of GABA (arrowheads). Bath applied 300 μM caramiphen caused about 36% facilitation of $GABA_A$ receptor-mediated currents. Antagonists present in the bath are indicated in the bars over current traces; V_{hold} = −70 mV. (B) Group data showing the effects of caramiphen on GABA-evoked currents. Both the facilitation of the currents with 100 μM or 300 μM caramiphen and the reduction of the currents with 1 mM caramiphen were statistically significant (*$P < 0.05$).

2007), we would suggest that a single injection of 100 mg·kg^{-1} caramiphen produced a blood concentration in the micromolar range. It is likely, therefore, that the NMDA receptor-antagonistic properties of caramiphen contributed to its anticonvulsant efficacy; previous studies have suggested that antagonism of NMDA receptors plays an important role in the termination of nerve agent-induced seizures (Braitman and Sparenborg, 1989; Dorandeu *et al.*, 2007). Regardless, however, of the extent of similarity between the *in vitro* and the *in vivo* caramiphen concentrations in the present study, the finding that caramiphen antagonizes NMDA receptor activation in a structure like the BLA, is likely to have significant implications in understanding the anticonvulsant mechanisms of this compound, as the BLA plays a central role in seizure generation and propagation (Aroniadou-Anderjaska *et al.*, 2008), including seizures induced by nerve agents (McDonough *et al.*, 1987; Apland *et al.*, 2009; Aroniadou-Anderjaska *et al.*, 2009; Skovira *et al.*, 2010). The molecular mechanism by which caramiphen antagonizes NMDA receptor activation is not clear, but it may involve direct interaction with the NMDA ion channel (Fletcher *et al.*, 1995).

In hippocampal slices, Thurgur and Church (1998) found that caramiphen, at 100 μM, reversed the paired-pulse facilitation of the field EPSP in the CA1 hippocampal area to paired-pulse depression. The authors suggested that the most plausible interpretation of this effect is that caramiphen facilitates GABAergic transmission. The present study provides direct evidence for this effect of caramiphen, in the BLA. In the presence of glutamate and $GABA_B$ receptor antagonists, caramiphen, at 100 or 300 μM, facilitated the currents evoked by puff-application of GABA, suggesting that caramiphen interacts with the $GABA_A$ receptor complex facilitating its activation. This mechanism of action can be expected to play a major role in the anticonvulsant effects of caramiphen. However, when caramiphen was increased to 1 mM, GABA-evoked currents were depressed. These findings suggest that there is more than one site on the $GABA_A$ receptor complex where caramiphen binds with different affinity; binding to the high-affinity site(s) produces a conformational change in the receptor that facilitates the binding of GABA, whereas binding to the low-affinity site(s) (when caramiphen concentration is high) produces the opposite effect, which may again involve a different conformational change of the receptor that inhibits GABA binding, or a direct blockage of the channel.

Raveh *et al.* (2002) found that caramiphen, along with its beneficial effects in preventing soman-induced behavioural

deficits, also prevented the down-regulation of AMPA receptors that follows exposure to soman. Fletcher *et al.* (1995) examined if caramiphen directly interacted with AMPA receptors, in mouse hippocampal cultured neurons, and concluded that it did not. Similarly, in the present study we found that caramiphen had no significant effect on AMPA-evoked currents on principal cells in the BLA. Thus, the protection conferred by caramiphen against soman-induced down-regulation of AMPA receptors (Raveh *et al.*, 2002) was probably an indirect effect associated with the protection against soman-induced seizures.

The suppression of behavioural seizures by caramiphen was accompanied by protection against neuronal loss, as examined in the BLA, and neuronal degeneration in the ventral hippocampus, the amygdala, and three cortical regions, piriform cortex, entorhinal cortex, and a neocortical sample from the temporal area. However, when caramiphen was administered 60 min after soman exposure, protection in the cortical regions was not significant; nonetheless, neurodegeneration in the amygdala and all three hippocampal subfields was still significantly lower than that in the SOMAN group. Thus, caramiphen protects brain regions that play a central role in emotional behaviour and cognitive functions, even when administered with a long latency after soman exposure, and, therefore, it may reduce nerve agent-induced behavioural deficits related to memory functions and emotion.

An important consideration is whether the present findings can be applicable to humans. It must be emphasized that, at present, there is no satisfactory pharmacological treatment available that can effectively stop nerve agent-induced seizures if administered with long delays after exposure and such delayed administration may be inevitable in a scenario of mass exposure. In the present study, caramiphen showed promising results even with delayed administration, but the dose required is high. Whether or not corresponding doses in humans are toxic cannot be answered at present. All rats that received caramiphen survived the status epilepticus induced by soman and we have rats that have received 100 $mg \cdot kg^{-1}$ caramiphen and appear to be healthy 3 months later. In addition, the rats that were injected with soman but did not develop status epilepticus and, therefore, were excluded from this study, were also injected with caramiphen. In these rats, there were no degenerating cells in any of the brain areas examined, 24 h later, suggesting that at least in the short term, 100 $mg \cdot kg^{-1}$ caramiphen is not toxic to brain cells. Nevertheless, behavioural and toxicological investigations will be necessary to determine possible long-term effects of receiving a high dose of caramiphen.

Together with previous studies (Raveh *et al.*, 2008), the results suggest that caramiphen is a promising antidote against nerve agent toxicity, but when administration of the anticonvulsant treatment is delayed, caramiphen should be co-administered with a faster acting anticonvulsant, due to its slow effect on seizure suppression. In regard to the anticonvulsant mechanisms of caramiphen action, the present study provides the first demonstration that caramiphen antagonizes NMDA receptor activation and has a dose-dependent bidirectional effect on $GABA_A$ receptor activation, in *in vitro* brain slices, in the BLA region. The antagonism of NMDA receptor activation by caramiphen and the facilitation of $GABA_A$ receptor activation (at micromolar caramiphen concentrations) in the BLA are likely to play an important role in the anticonvulsant properties of this muscarinic antagonist.

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Conflicts of interest

None.

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