The GluK1 (GluR5) Kainate/ α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor Antagonist LY293558 Reduces Soman-Induced Seizures and Neuropathology

Taiza H. Figueiredo, Felicia Qashu, James P. Apling, Vassiliki Aroniadou-Anderjaska, Adriana P. Souza, and Maria F. M. Braga

Departments of Anatomy, Physiology, and Genetics (T.H.F., F.Q., V.A.-A., A.P.S., M.F.M.B.) and Psychiatry (V.A.-A., M.F.M.B.), Uniformed Services University of the Health Sciences, Bethesda, Maryland; and Neurobehavioral Toxicology Branch, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Aberdeen, Maryland (J.P.A.)

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ABSTRACT

The possibility of mass exposure to nerve agents by a terrorist attack necessitates the availability of antidotes that can be effective against nerve agent toxicity even when administered at a relatively long latency after exposure, because medical assistance may not be immediately available. Nerve agents induce status epilepticus (SE), which can cause brain damage or death. Antagonists of kainate receptors that contain the GluK1 (formerly known as GluR5) subunit (GluK1Rs) are emerging as a new potential treatment for SE and epilepsy from animal research, whereas clinical trials to treat pain have shown that the GluK1/ α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist LY293558 [(3*S*,4*aR*,6*R*,8*aR*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid] is safe and well tolerated. Therefore, we tested whether LY293558 is effective against soman-induced seizures and neuropathology, when administered 1 h after soman exposure,

in rats. LY293558 stopped seizures induced by soman and reduced the total duration of SE, monitored by electroencephalographic recordings within a 24 h-period after exposure. In addition, LY293558 prevented neuronal loss in the basolateral amygdala (BLA) and the CA1 hippocampal area on both days 1 and 7 after soman exposure and reduced neuronal degeneration in the CA1, CA3, and hilar hippocampal regions, entorhinal cortex, amygdala, and neocortex on day 1 after exposure and in the CA1, CA3, amygdala, and neocortex on day 7 after exposure. It also prevented the delayed loss of glutamic acid decarboxylase-67 immuno-stained BLA interneurons on day 7 after exposure. LY293558 is a potential new emergency treatment for nerve agent exposure that can be expected to be effective against seizures and brain damage even with late administration.

Introduction

Nerve agents are organophosphorus compounds whose primary action is the irreversible inhibition of acetylcholinesterase (Bajgar, 2005). Clinical manifestations after exposure include intense generalized seizures/status epilepticus (SE) and can result in death or brain damage with long-term

neurological and behavioral consequences (Brown and Brix, 1998). Because nerve agents are relatively easy to transport and deploy, they could be used in a terrorist attack, and, therefore, there is an urgent need for the discovery of antidotes that can be effective even when administered with a substantial latency after exposure, because emergency assistance may not be immediately available.

Control of seizures after exposure is crucial for the prevention of brain damage or death. After inhibition of acetylcholinesterase and accumulation of acetylcholine, seizures are initiated by hyperstimulation of muscarinic receptors (McDonough and Shih, 1997). However, it seems that it is the ensuing glutamatergic hyperactivity that is primarily responsible for reinforcing and sustaining seizures, resulting in

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ABBREVIATIONS: SE, status epilepticus; BLA, basolateral amygdala; GluK1R, GluK1-containing kainate receptor; FJC, Fluoro-Jade C; LY293558, (3*S*,4*aR*,6*R*,8*aR*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EEG, electroencephalogram; HI-6, (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane di-chloride; PBS, phosphate-buffered saline; GAD-67, glutamic acid decarboxylase-67; CE, coefficient of error; dH₂O, distilled water; ANOVA, analysis of variance; LSD, least significant difference; IQR, interquartile range.

excitotoxic brain damage (McDonough and Shih, 1997). Accordingly, muscarinic receptor antagonists are effective against nerve agent-induced SE only if administered early after the onset of seizures (Lallement et al., 1998). Benzodiazepines are considered to be the first line of defense against nerve agent-induced SE, but the effectiveness of benzodiazepines is transient (seizures recur), and it is reduced or lost if administration is delayed beyond 30 to 40 min after nerve agent exposure (Lallement et al., 1998; Gilat et al., 2005; McDonough et al., 2010).

An emerging potential new treatment against seizures and epilepsy involves antagonism of kainate receptors containing the GluK1 subunit (previously designated as GluR5; see Jane et al., 2009). GluK1-containing kainate receptors (GluK1Rs) play an important role in the regulation of GABA_A receptor-mediated inhibitory transmission in the hippocampus (Christensen et al., 2004; Clarke and Collingridge, 2004) and the amygdala (Braga et al., 2003), two brain structures that are well known for their high propensity to generate seizure activity. The main evidence that GluK1Rs are involved in the generation of seizures is as follows: 1) The function of GluK1Rs and the expression of the GluK1 subunit are altered in patients with temporal lobe epilepsy (Li et al., 2010) and in epileptic rats (Ullal et al., 2005). 2) When the GluK1R agonist (*R,S*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (Jane et al., 2009) is infused intravenously or directly into the rat amygdala it induces clonic seizures (Rogawski et al., 2003; Kaminski et al., 2004). 3) The anticonvulsant properties of the well known antiepileptic topiramate are due in part to blockade of GluK1Rs (Gryder and Rogawski, 2003; Kaminski et al., 2004; Braga et al., 2009). 4) GluK1R antagonists block hippocampal epileptiform activity in vitro and limbic seizures in vivo induced by electrical stimulation or by the muscarinic agonist pilocarpine (Smolders et al., 2002).

Pilocarpine and nerve agents share common mechanisms of seizure induction and expression (Tetz et al., 2006). Both initiate seizures by muscarinic receptor overstimulation, followed by excessive glutamatergic activity (McDonough and Shih, 1997). Because GluK1R antagonists are effective against pilocarpine-induced seizures (Smolders et al., 2002), we hypothesized that they may also be effective against nerve agent-induced seizures. After obtaining promising results from testing GluK1R antagonists against soman-induced epileptiform activity in the amygdala and hippocampus in vitro (Apland et al., 2009), we proceeded to the in vivo experiments described here where the GluK1R antagonist LY293558 [(3*S*,4*aR*,6*R*,8*aR*)-6-[2-(1(2*H*-tetrazole-5-yl)ethyl) decahydroisoquinoline-3-carboxylic acid] was administered 1 h or longer after soman exposure. We used LY293558 because this compound, in addition to being highly selective for the subtype of kainate receptors that contains the GluK1 subunit, antagonizes AMPA receptors (Bleakman et al., 1996; Jane et al., 2009). We considered that the broader spectrum of glutamate receptor antagonism might enhance its anticonvulsant effectiveness. Furthermore, from all GluK1R antagonists, only LY293558 has been tested clinically and found to be safe and well tolerated (Gilron et al., 2000; Sang et al., 2004), which can facilitate its approval process for use in humans in an emergency case.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories Inc., Wilmington, MA), weighing 150 to 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 6:00 AM), with food and water available ad libitum. The animal care and use programs at the U.S. 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, National Research Council and were in accordance with the guidelines of our institutions, after obtaining approval of the Institutional Animal Care and Use Committees.

Experimental Procedures

Soman Administration and Drug Treatment. Soman (pinacolyl methylphosphonofluoridate) was obtained from Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), diluted in cold saline, and administered via a single subcutaneous injection (154 µg/kg, 1.4 × LD₅₀). To increase survival rate, rats were administered HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride; 125 mg/kg i.p.) 30 min before soman exposure. HI-6 is a bispyridinium oxime that reactivates inhibited acetylcholinesterase, primarily in the periphery (Bajgar, 2005). Within 1 min after soman exposure, rats also received an intramuscular injection of atropine sulfate (2 mg/kg; Sigma-Aldrich, St. Louis, MO) to minimize peripheral toxic effects. One hour after soman exposure, a group of rats was administered LY293558 (50 mg/kg i.p.; SOMAN+LY293558 group); these rats were used for the neuropathological analysis (see below) and were compared with the soman-exposed rats that received HI-6 and atropine but did not receive LY293558 (SOMAN group). LY293558 was kindly provided by Raptor Pharmaceutical Corp. (Novato CA). Seizures in these rats that were used to study neuropathology were monitored behaviorally and classified according to the Racine scale (Racine, 1972) with minor modifications: stage 0, no behavioral response; stage 1, behavioral 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. We did not implant the rats studied for neuropathological damage with electrodes for electroencephalographic (EEG) monitoring because the implantation procedure causes some damage, which could affect the neuropathology results. Control animals received HI-6 and atropine, but were injected with saline instead of soman. An additional control group received HI-6 and saline instead of soman, atropine, and LY293558 (100 mg/kg i.p.) to determine the effects of the anticonvulsant treatment alone.

A group of animals was implanted with electrodes for EEG monitoring and 1 week later were exposed to the same treatment as described above (HI-6 administration followed by soman 30 min later, followed by atropine sulfate 1 min after soman). Some of these rats were administered LY293558 (50 mg/kg i.p.) at 90 to 190 min after soman exposure to determine whether the GluK1R/AMPA antagonist was effective in stopping soman-induced seizures even at longer latencies; these rats were not used for neuropathological analysis.

Electrode Implantation for Electroencephalographic Recordings. Rats were anesthetized with ketamine (80 mg/kg i.p.) and xylazine (10 mg/kg). Five stainless-steel, cortical screw electrodes were stereotactically implanted by using the following coordinates [after Paxinos and Watson (2005)]: two frontal electrodes, 2.0 mm posterior from bregma and 2.5 mm lateral from the midline; two parietal electrodes, 5.0 mm posterior from bregma and 2.5 mm lat-

Fig. 1. LY293558 stops soman-induced generalized seizures and reduces the total duration of SE in the 24 h-period after soman exposure. *a–c*, in the example shown in *a*, administration of the GluK1R/AMPA antagonist LY293558 at 2 h after soman exposure suppressed electrographic seizure activity within 15 min (bottom set of traces in *a*), whereas seizures persisted in rats that did not receive LY293558 (an example is shown in *b*); the numbers 1, 2, 3, 4 refer to the electrodes/sites from where electrical activity was sampled, as shown diagrammatically in *c* (1, left frontal; 2, right frontal; 3, left parietal; 4, right parietal; 5, cerebellar reference electrode). *d*, left, the duration of the initial SE (the SE that started 5 to 15 min after soman exposure and was terminated by LY293558 in the SOMAN+LY293558 group, or spontaneously in the SOMAN group). Right, the duration of SE throughout the 24 h-period after soman exposure. For the SOMAN group, $n = 4$; for the SOMAN+LY293558 group, $n = 7$. **, $p < 0.01$; ***, $p < 0.001$ (unequal variance t test).

eral from the midline. A cerebellar reference electrode was implanted 1.0 mm posterior to lambda (Fig. 1C). Each screw electrode (Plastics One Inc., Roanoke, VA) was placed in a plastic pedestal (Plastics One Inc.) and fixed to the skull with dental acrylic cement.

EEG Recordings and Analysis. The rats with implanted electrodes for EEG recordings were placed in the EEG chamber and connected to the EEG system (Stellate, Montreal, Canada; 200-Hz sampling rate). Video-EEG recordings were performed in the freely moving rats. Recordings were visually analyzed offline with filter settings set to 0.3 Hz for the low-frequency filter, 60 Hz for the notch filter, and 70 Hz for the high-frequency filter, using the Harmonie Viewer 6.1e from Stellate. EEG was recorded for 24 h after soman injection. The termination of the soman-induced SE was defined as the disappearance of large amplitude, repetitive discharges (>1 Hz with at least double the amplitude of the background activity). If SE recurred at any time during the remainder of the 24-h recording time, the duration of this seizure activity was included in the total duration of SE.

Fixation and Tissue Processing. Neuropathological analysis was performed in the amygdala, piriform cortex, entorhinal cortex, hippocampus, and neocortex of the rats that were not implanted with EEG electrodes. One or 7 days after soman administration, rats were deeply anesthetized with pentobarbital (75–100 mg/kg i.p.) and transcardially perfused with PBS (100 ml) followed by 4% paraformaldehyde (200 ml). The brains were removed and postfixed overnight at 4°C, then 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) in PBS for Nissl staining with cresyl violet. Two adjacent series of sections were also mounted on slides for Fluoro-Jade C (FJC) staining, whereas the remaining series of sections were stored at -20°C in a cryoprotectant solution. Analysis of neuronal loss from Nissl-stained sections and GAD-67-immunostained sections was performed in the basolateral amygdala (BLA)

and the CA1 hippocampal area along the rostrocaudal extent of the hippocampus. Analysis of neuronal degeneration from FJC-stained sections was performed from the nuclei of the amygdala, from 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). All neuropathological analysis was done in a blind fashion.

Fluoro-Jade C Staining. FJC (Histo-Chem, Jefferson, AR) was used to identify irreversibly degenerating neurons. Mounted sections were air-dried overnight, and then immersed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min. The slides were then rinsed for 2 min in 70% ethanol, 2 min in distilled water (dH_2O), and incubated in 0.06% potassium permanganate solution for 10 min. After a 2-min rinse in dH_2O , the slides were transferred to a 0.0001% solution of FJC dissolved in 0.1% acetic acid for 10 min. After three 1-min rinses in dH_2O , the slides were dried on a slide warmer, cleared in xylene for at least 1 min, and coverslipped with dibutyl phthalate xylene (Sigma-Aldrich).

GAD-67 Immunohistochemistry. To label GAD-67 immunoreactive neurons, a 1-in-5 series of free-floating sections was collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M PBS, and then incubated in a blocking solution containing 10% normal goat serum (Millipore Bioscience Research Reagents, Temecula, CA) and 0.5% Triton X-100 in PBS for 1 h at room temperature. The sections were then incubated with mouse anti-GAD-67 serum (1:1000, MAB5406; Millipore Bioscience Research Reagents), 5% normal goat serum, 0.3% Triton X-100, and 1% bovine serum albumin overnight at 4°C. After rinsing three times for 10 min each in 0.1% Triton X-100 in PBS, the sections were incubated with Cy3-conjugated goat anti-mouse antibody (1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 0.0001% 4,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) in PBS for 1 h at room temperature. After a final rinse in PBS for 10 min, sections were mounted on slides, air-dried for at least 30 min, and coverslipped with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA).

Stereological Quantification. Design-based stereology was used to quantify the total number of neurons in Nissl-stained sections and interneurons in GAD-67-immunostained sections in the BLA and CA1 area. Sections were viewed with a Zeiss (Oberkochen, Germany) Axioplan 2ie fluorescent microscope with a motorized stage, interfaced with a computer running StereoInvestigator 8.0 (MicroBrightField, Williston, VT). The BLA and CA1 regions were identified on slide-mounted sections and delineated for each slide of each animal, under a $2.5\times$ objective, based on the atlas of Paxinos and Watson (2005). All sampling was done under a $63\times$ 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 and GAD-67-immunostained neurons was estimated by using the optional fractionator probe, and, along with the coefficient of error (CE), were calculated by using StereoInvestigator 8.0 (MicroBrightField). The CE was calculated by the software according to the equations of Gundersen et al., (1999) ($m = 1$) and Schmitz and Hof (2000) (second estimation).

For Nissl-stained neurons in the BLA, a 1-in-5 series of sections was analyzed (seven sections on average). The counting frame was $35 \times 35 \mu\text{m}$, the counting grid was $190 \times 190 \mu\text{m}$, and the dissector height was 12 μm . Nuclei were counted when the cell body came into focus within the dissector, which was placed 2 μm below the section surface. Section thickness was measured at every counting site, and the average mounted section thickness was 20 μm . An average of 345 neurons per rat was counted, and the average CE was 0.05 for both the Gundersen et al. and Schmitz-Hof equations.

For Nissl-stained neurons in the CA1 area, a 1-in-10 series of sections was analyzed (eight sections on average). The counting

frame was $20 \times 20 \mu\text{m}$, the counting grid was $250 \times 250 \mu\text{m}$, and the dissector height was $10 \mu\text{m}$. Nuclei were counted when the cell body came into focus within the dissector, which was placed $2 \mu\text{m}$ below the section surface. Section thickness was measured at every counting site, and the average mounted section thickness was $17 \mu\text{m}$. An average of 243 neurons per rat was counted, and the CE was 0.07 for the Gundersen et al. ($m = 1$) equation and 0.065 for the Schmitz-Hof (2nd Estimation) equation.

For GABAergic interneurons immuno-labeled for GAD-67 in the BLA, a 1-in-10 series of sections was analyzed (on average five sections). The counting frame was $60 \times 60 \mu\text{m}$, the counting grid was $100 \times 100 \mu\text{m}$, and the dissector height was $20 \mu\text{m}$. Nuclei were counted when the top of the nucleus came into focus within the dissector, which was placed $2 \mu\text{m}$ below the section surface. Section thickness was measured at every fifth counting site, and the average mounted section thickness was $27 \mu\text{m}$. An average of 235 neurons per rat was counted, and the average CE was 0.08 for both the Gundersen et al. and Schmitz-Hof equations.

For GABAergic interneurons immuno-labeled for GAD-67 in the CA1 area, a 1-in-10 series of sections was analyzed (on average seven sections). The counting frame was $50 \times 50 \mu\text{m}$, the counting grid was $130 \times 130 \mu\text{m}$, and the dissector height was $20 \mu\text{m}$. Nuclei were counted when the top of the nucleus came into focus within the dissector, which was placed $2 \mu\text{m}$ below the section surface. Section thickness was measured at every fifth counting site, and the average mounted section thickness was $26 \mu\text{m}$. An average of 232 neurons per rat was counted, and the average CE was 0.068 for both the Gundersen et al. and Schmitz-Hof equations.

Evaluation of Fluoro-Jade C. From an adjacent series of Nissl-stained sections, tracings were made of the BLA, piriform cortex, entorhinal cortex, hippocampal subfields CA1, CA3, and hilus, as well as neocortex. The tracings from the Nissl-stained sections were superimposed on the FJC-stained sections. For qualitative analysis of FJC-stained sections, the following rating system was used 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 that are in agreement with quantitative assessments (Qashu et al., 2010). The scores for neurodegeneration present on FJC-stained sections were assessed considering the density of cells from Nissl-stained sections, along the anterior to posterior extent, at 600- μm intervals. The stereotaxic coordinates for each brain region were the following: neocortex, between -2.04 and -6.36 mm from bregma; amygdala and piriform cortex, between -2.04 and -3.36 mm; ventral hippocampal subfields CA1, CA3, hilus, and entorhinal cortex, between -5.4 and -6.36 mm. All the coordinates and tracings of brain structures and substructures were based on Paxinos and Watson (2005).

Statistical Analysis

SE duration was compared between the SOMAN and the SOMAN+LY293558 groups, using the unequal variance *t* test. Analysis of variance (ANOVA) followed by post hoc least significant difference (LSD) test was used to analyze stereological estimations of the total number of neurons and interneurons. The statistical values from these tests are presented as mean and standard error of the mean. Neurodegeneration scores were compared between groups for each structure separately using the Mann-Whitney *U* test. The statistical values are presented as median and the interquartile range (IQR; the values at the 25th and 75th percentiles). Pearson's χ^2 and Friedman's exact test were used to compare the survival rate between the groups. Differences were considered significant when $p < 0.05$. Sample sizes (*n*) refer to the number of animals.

Results

Seizure Termination by LY293558. Behavioral SE (stage 3 seizures progressing to higher stages) developed within 5 to 15 min after soman injection. Five of 65 rats that were exposed to soman did not develop seizures and were not included in the study. The survival rate for the animals (nonimplanted with EEG electrodes) that did not receive the anticonvulsant treatment (SOMAN group) was 55% (11 of 20), whereas for the animals that received LY293558 (SOMAN+LY293558 group) 1 h after soman challenge the survival rate was 100% ($n = 12$). This difference in survival rate was statistically significant ($p \leq 0.01$; Pearson's χ^2 and Friedman's exact test). LY293558 terminated behavioral SE within 20 to 30 min after administration; the rats then fell asleep for 3 to 4 h. Four rats that were not exposed to soman but received LY293558 (at double the dose of LY293558 administered to the soman-exposed rats; see *Materials and Methods*), fell into deep sleep within 30 min after the drug administration and were completely awake 3 h later.

For the rats that were implanted with EEG electrodes ($n = 26$), the survival rate was 54.5% for the SOMAN group (6 of 11) and 66.6% for the SOMAN+LY293558 group (10 of 15). From the 16 rats that survived the initial SE (the SE that was induced by soman and terminated by LY293558, or terminated spontaneously in the SOMAN group), four more animals (two from each of the two groups) died within 3 h after the cessation of the initial SE. Therefore, EEG monitoring for 24 h was completed in 12 animals (four in the SOMAN group and eight in the SOMAN+LY293558 group). In the SOMAN+LY293558 group, the initial SE was terminated electrographically and behaviorally within 19 ± 1.9 min ($n = 7$) after LY293558 administration (data used are only from the rats that were administered LY293558 at 1.5 h after soman exposure), whereas in the SOMAN group, the initial SE continued for another 542 ± 29.2 min ($n = 4$), until it stopped spontaneously (Fig. 1). The total duration of the initial SE in the SOMAN group (10.4 ± 1.0 h) versus the SOMAN+LY293558 group (1.78 ± 0.5 h) is shown in Fig. 1d, left ($p < 0.001$). When repetitive spikes disappeared from the EEG in the SOMAN+LY293558 group (after administration of LY293558) the rats fell asleep (for approximately 4 h). SE recurred in six of the seven rats that received LY293558 and in one of four rats that did not receive the anticonvulsant. The recurring SE displayed EEG characteristics similar to the initial SE, but was not accompanied by convulsions. The total duration of SE within the 24-h period after soman exposure was significantly lower in LY293558-treated rats (6.4 ± 1.1 h) compared with the rats that did not receive LY293558 (10.9 ± 0.6 h; $p < 0.01$) (Fig. 1d, right).

Neuronal Loss and Degeneration 1 Day after Soman Administration. Estimation of the total number of neurons in the BLA and the CA1 hippocampal subfield using an unbiased stereological method in Nissl-stained sections showed that 1 day after soman exposure the SOMAN+LY293558 group ($n = 6$) had a significantly higher ($p < 0.05$) total number of neurons in the BLA ($83,773 \pm 5833$) and CA1 ($624,513 \pm 25,312$) compared with SOMAN group (BLA, $57,560 \pm 1458$; CA1, $435,796 \pm 15,198$; $n = 5$) (Fig. 2). The number of neurons in the BLA and CA1 area of the SOMAN+LY293558 group did not differ significantly from the number of neurons in the BLA ($90,019 \pm 1568$) and CA1 ($671,840 \pm 40,922$) of the control

Fig. 2. LY293558 protects against neuronal loss in the BLA and the CA1 hippocampal area 1 day after soman-induced SE. A, panoramic photomicrographs of Nissl-stained half-hemispheres outlining the amygdalar nucleus and the hippocampal subfield where stereological analysis was performed. B, representative photomicrographs of Nissl-stained sections showing BLA and CA1 cells from the control, SOMAN, and SOMAN+LY293558 groups. Total magnification is 630 $\times$. Scale bar, 50 μ m. C and D, group data (mean and standard error; $n = 6$ for the control group, $n = 5$ for the SOMAN group, and $n = 6$ for the SOMAN+LY293558 group) of stereological estimation of the total number of Nissl-stained neurons in the BLA (C) and CA1 area (D). **, $p < 0.01$; ***, $p < 0.001$ in relation to the other groups (ANOVA, LSD post hoc test).

group ($n = 6$). In contrast, the SOMAN group had a 35% neuronal loss in the BLA and 30% neuronal loss in the CA1 area compared with the control animals ($p < 0.05$). These and all neuronal loss and degeneration results are summarized in Table 1.

Neuronal degeneration (based on FJC staining) in the amygdala and ventral hippocampus (CA1, CA3, and hilus) of the SOMAN+LY293558 group was significantly less extensive ($p < 0.05$) compared with the SOMAN group (Fig. 3). The neurodegeneration score for the amygdala was mild (median = 2, IQR = 1.75–2.25) in the SOMAN+LY293558 group and moderate (median = 3, IQR = 3–3.5) in the SOMAN group. Neuronal degeneration in the CA1, CA3, and hilar regions of the ventral hippocampus was absent or minimal in the SOMAN+LY293558 group (CA1, median = 0, IQR = 0–1.5; CA3, median = 0, IQR = 0–0.75; Hilus, median = 1, IQR = 0.75–1.75), but it 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). The neurodegeneration scores for the neocortical sample from the temporal cortex and for the entorhinal cortex in the SOMAN+LY293558 group (median = 1, IQR = 0–1.25 for neocortex, and median = 2, IQR = 0.75–2 for entorhinal cortex) were significantly lower compared with the SOMAN group (median = 2, IQR = 2–2.5 for neocortex, and median = 3, IQR = 3–4 for entorhinal cortex) (Fig. 3). There was no significant difference between the two groups in the neurodegeneration score for piriform cortex (median = 3, IQR = 1.75–4 for the SOMAN+LY293558 group, and median = 3, IQR = 3–3.5 for the SOMAN group). The control group and the group that was not administered soman but received LY293558 did not show any FluoroJade-C positive staining.

Neuronal Loss and Degeneration 7 Days after Soman Administration. Seven days after soman exposure, the

TABLE 1

Summary of neuropathology results for the SOMAN and the SOMAN + LY293558 groups at 1 and 7 days after soman exposure

Shown is neuronal loss in the BLA and CA1 area for the total population of neurons and for the GABAergic neurons, expressed as percentage remaining compared with the controls. Also shown are neurodegeneration results (neuropathology scores from FJC stained-sections) in the amygdala (Amy), piriform cortex (Pir), entorhinal cortex (Ent), the CA1 and CA3 subfields of the hippocampus, hilus, and neocortex (Neo-Ctx). For neuropathology scores: R = range of scores; 0 = no FJC + cells; 1 = minimal damage (1–10%); 2 = mild damage (11–25%); 3 = moderate damage (26–45%); and 4 = severe damage (>45%).

	1 day after soman		7 days after soman	
	SOMAN $n = 6$	SOMAN + LY293558 $n = 5$	SOMAN $n = 6$	SOMAN + LY293558 $n = 6$
Percentage remaining compared with control				
Total number of neurons				
BLA	65**	93	65***	88
CA1	70**	92	61**	89
GABAergic neurons				
BLA	88	100	67*	91
CA1	88	96	89	94
Irreversible neuronal degeneration				
Amy	Moderate (R = 3–4)	Mild [†] (R = 0–3)	Moderate (R = 2–4)	Minimal ^{††} (R = 0–2)
Pir	Moderate (R = 3–4)	Moderate (R = 0–4)	Moderate (R = 3–4)	Minimal (R = 0–4)
Neo-Ctx	Mild (R = 2–3)	Minimal [†] (R = 0–2)	Mild (R = 1–3)	Minimal [†] (R = 0–2)
CA1	Severe (R = 3–4)	Minimal ^{††} (R = 0–3)	Severe (R = 3–4)	No FJC+ ^{††} (R = 0–0)
CA3	Severe (R = 3–4)	Minimal ^{††} (R = 0–3)	Moderate (R = 1–4)	No FJC+ ^{††} (R = 0–0)
Hilus	Severe (R = 3–4)	Minimal [†] (R = 0–4)	Mild (R = 0–4)	No FJC+ (R = 0–0)
Ent	Moderate (R = 3–4)	Mild ^{††} (R = 0–2)	Moderate (R = 2–3)	Mild (R = 0–3)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in relation to the control group (ANOVA, LSD post hoc test).

[†] $P < 0.05$, ^{††} $P < 0.01$ (Mann-Whitney U test) compared with SOMAN group.

Fig. 3. LY293558 protects against neuronal degeneration 1 day after soman-induced SE. A and B, panoramic photomicrographs of Nissl-stained sections showing the brain regions evaluated on the FJC-stained sections. C, representative photomicrographs of FJC-stained sections from the brain regions where neuronal degeneration was evaluated for the SOMAN and the SOMAN+LY293558 groups. Total magnification is 100 $\times$. Scale bar, 50 μ m. D, bar graph showing the median neuropathology score and interquartile range ($n = 5$ for the SOMAN group, and $n = 6$ for the SOMAN+LY293558 group) for the amygdala (Amy), piriform cortex (PIR), entorhinal cortex (Ent), the CA1 and CA3 subfields of the hippocampus, hilus, and neocortex (neo-Ctx). *, $p < 0.05$; **, $p < 0.01$ (Mann-Whitney U test).

SOMAN+LY293558 group ($n = 6$) still had a significantly higher total number of neurons in the BLA ($79,694 \pm 4766$) and CA1 area ($587,892 \pm 34,456$) compared with the SOMAN group (BLA, $58,528 \pm 3051$; CA1, $397,825 \pm 15,832$; $n = 6$), whereas it did not differ significantly from the control group

(BLA, $90,195 \pm 2343$; CA1, $654,408 \pm 30,156$; $n = 6$). In contrast, the SOMAN group displayed a 35% neuronal loss in the BLA and 39% neuronal loss in the CA1 area compared with the controls (Fig. 4).

The qualitative analysis of ongoing neurodegeneration showed

Fig. 4. LY293558 protects against neuronal loss in the BLA and the CA1 hippocampal area 7 days after soman-induced SE. A, panoramic photomicrographs of Nissl-stained half-hemispheres outlining the amygdalar nucleus and the hippocampal subfield where stereological analysis was performed. B, representative photomicrographs of Nissl-stained sections showing BLA and CA1 cells from the control, SOMAN, and SOMAN+LY293558 groups. Total magnification is 630 $\times$. Scale bar, 50 μ m. C and D, group data (mean and standard error; $n = 6$ for each of the three groups) of stereological estimation of the total number of Nissl-stained neurons in the BLA (C) and CA1 area (D). **, $p < 0.01$ in relation to the other groups (ANOVA, LSD post hoc test).

Fig. 5. LY293558 protects against neuronal degeneration 7 days after soman-induced SE. A and B, panoramic photomicrographs of Nissl-stained sections showing the brain regions evaluated on the FJC-stained sections. C, representative photomicrographs of FJC-stained sections from the brain regions where neuronal degeneration was evaluated for the SOMAN and SOMAN+LY293558 groups. Total magnification is 100 $\times$. Scale bar, 50 μ m. D, bar graph showing the median neuropathology score and interquartile range ($n = 6$ for each of the two groups) for the amygdala (Amy), piriform cortex (PIR), entorhinal cortex (ENT), the CA1 and CA3 subfields of the hippocampus, hilus, and neocortex (neo-Ctx). *, $p < 0.05$; **, $p < 0.01$ (Mann-Whitney U test).

that in the SOMAN+LY293558 group neuronal damage was minimal in the amygdala (median = 1, IQR = 0~2) and neo-cortex (median = 0.5, IQR = 0~1.25), whereas in the same brain regions of the SOMAN group it was moderate (median = 3, IQR = 2.75~4 for the amygdala, and median = 2.5, IQR = 1~3 for neocortex) (Fig. 5). Furthermore, there were no degenerating cells in any of the studied hippocampal subfields (CA1, CA3, and hilus) in the SOMAN+LY293558 group, whereas the SOMAN group displayed severe (CA1, median = 4, IQR = 3~4), moderate (CA3, median = 3, IQR = 1~3.25), or mild (hilus, median = 2, IQR = 0~4) damage in these regions (Fig. 5). Neurodegeneration in the piriform cortex and entorhinal cortex was also less in the SOMAN+LY293558 group (piriform cortex, median = 1, IQR = 0~3.25; entorhinal cortex, median = 1.5, IQR = 0~2.5) than the SOMAN group (piriform cortex, median = 3, IQR = 3~4; entorhinal cortex, median = 3, IQR = 2.25~3), but these differences were not statistically significant.

Loss of GABAergic Interneurons. The estimation of the total number of GABAergic interneurons in the BLA and CA1 hippocampal area, using an unbiased stereological method on GAD-67-immuno-stained sections, showed that 1 day after soman-induced SE the number of interneurons in the SOMAN group (BLA, $10,432 \pm 627$; CA1, $19,434 \pm 3098$; $n = 5$) and the SOMAN+LY293558 group (BLA, $11,844 \pm 766$; CA1, $21,104 \pm 2042$; $n = 6$) did not differ significantly and also did not differ from the controls (BLA, $11,764 \pm 1028$; CA1, $21,936 \pm 2829$; $n = 6$). However, 7 days after soman exposure, the number of GABAergic interneurons in the SOMAN group (BLA, 7306 ± 368 ; CA1, $18,096 \pm 1911$; $n =$

6) was significantly lower than that in the BLA, but not the CA1 area, of the control group (BLA, $10,837 \pm 988$; CA1, $19,240 \pm 1800$; $n = 6$) and the SOMAN+LY293558 group (BLA, 9963 ± 359 ; CA1, $18,096 \pm 1911$; $n = 6$) (Fig. 6). The loss of GABAergic interneurons in the BLA of the SOMAN group was 32% compared with the control animals. However, the percentage of interneurons in the BLA of the SOMAN group ($12.61 \pm 1.9\%$ of the total number of neurons) was not significantly different from the percentage of interneurons in the BLA of the control group ($12.01 \pm 1.3\%$ of the total number of neurons) or the SOMAN+LY293558 group ($12.62 \pm 1.3\%$ of the total), suggesting a proportional loss of both interneurons and principal cells in the SOMAN group.

Discussion

Brain damage after exposure to nerve agents is caused primarily by the intense seizure activity. Thus, the extent to which seizures are controlled correlates with the extent to which brain damage is prevented (Shih et al., 2003), and when a small proportion of rats does not develop SE after exposure to toxic levels of soman, these rats also do not exhibit neuropathology (de Araujo Furtado et al., 2010). Previous studies have been successful in blocking or suppressing nerve agent-induced seizures by a number of regimens, such as muscarinic antagonists (Shih and McDonough, 1999), benzodiazepines (Shih et al., 2003), a combination of AMPA and *N*-methyl-D-aspartate receptor antagonists (Lallement et al., 1994), or galantamine (Pereira et al., 2010), but cessation of

Fig. 6. Delayed loss of GABAergic interneurons in the BLA within the first week after soman-induced SE. A, panoramic photomicrograph of immunohistochemically stained section for GAD-67, indicating the BLA and the CA1 hippocampal area (outlined in white). B, representative photomicrographs of GABAergic interneurons in the BLA and CA1 area 7 days after soman injection. Total magnification is 630 $\times$. Scale bar, 50 μ m. C and D, group data showing the mean and standard error of the stereologically estimated total number of GAD-67-positive cells in the BLA (C) and CA1 (D) 1 and 7 days after soman-induced SE. Sample size was $n = 6$ for each group at each of the two time points, except for the SOMAN group where $n = 5$ on day 1 after soman exposure. *, $p < 0.05$ compared with the other groups (ANOVA, LSD post hoc test).

nerve agent-induced SE when the anticonvulsant is administered 1 h or longer after exposure has not been reported so far. The availability of an antidote that can be effective at a relatively long latency after exposure is clearly of major importance in an emergency case of mass exposure. In the present study, LY293558, a GluK1R/AMPA antagonist, was effective in terminating soman-induced SE and reducing neuronal degeneration and loss in a number of brain regions that play a key role in seizure generation, when administered 1 h after soman exposure.

LY293558 was the first subunit-specific kainate receptor antagonist developed, inhibiting with high selectivity the kainate receptors that contain the GluK1 subunit, but having only limited selectivity between GluK1Rs and AMPA receptors (Bleakman et al., 1996; Jane et al., 2009). Why did we expect a glutamate receptor antagonist to be effective against an anticholinesterase such as soman, which induces seizures caused by the increased levels of acetylcholine and strong stimulation of muscarinic receptors? The answer to this question is that the excessive muscarinic activity induced by soman exposure is followed by derangements in the balance between glutamatergic and GABAergic synaptic transmission, which reinforces and sustains seizures (McDonough and Shih, 1997). For this reason, muscarinic receptor antagonists are effective only when administered at a short latency after exposure (Lallement et al., 1998). Benzodiazepines can stop nerve agent-induced seizures, at least temporarily; however, these drugs also start losing their efficacy if administered with a latency longer than 30 to 40 min after the initiation of seizures (Lallement et al., 1998; Gilat et al., 2005; McDonough et al., 2010). This may be caused, in part, by internalization of benzodiazepine-sensitive GABA_A receptors, which is known to occur in some brain regions during prolonged seizures (Naylor et al., 2005; Goodkin et al., 2008).

It is presently unknown whether kainate receptors, and GluK1Rs in particular, undergo dynamic changes in location and function during prolonged seizure activity. However, AMPA receptors not only do not undergo internalization, but, instead, internal AMPA receptors move to synaptic sites during prolonged seizure activity, thus reinforcing seizures (Chen et al., 2007).

In the present study, the relative contribution of the GluK1R versus AMPA receptor antagonism by LY293558 to the cessation of seizures is not known. We used LY293558 because both AMPA receptor antagonists and GluK1R antagonists are effective against seizures, and, therefore, this compound could provide the benefit of antagonizing both glutamate receptor subtypes. Considering that 1) competitive AMPA receptor antagonists may require prolonged intravenous infusion to maintain their efficacy against seizures (Pitkänen et al., 2007), whereas LY293558, in the present study, required only a single injection despite the fact that it is a competitive antagonist (Bleakman et al., 1996; Jane et al., 2009), and 2) selective GluK1R antagonists alone are able to block seizures induced by the muscarinic agonist pilocarpine (Smolders et al., 2002), it is likely that antagonism of GluK1Rs contributed significantly to the effectiveness of LY293558 against soman-induced seizures. The proconvulsive role of GluK1Rs can be attributed to the reduction of GABA release that these receptors mediate presynaptically in the hippocampus (Christensen et al., 2004; Clarke and Collingridge, 2004), neocortex (Ali et al., 2001), as well as in the BLA when they are activated by high concentrations of glutamate (Braga et al., 2003). Furthermore, in the BLA GluK1Rs also excite principal neurons (Gryder and Rogawski, 2003) and facilitate glutamate release presynaptically (unpublished observations), which may significantly contribute to seizure generation.

By stopping the initial SE induced by soman, and reducing

the total duration of SE within 24 h after exposure, LY293558 also prevented neuronal loss in the BLA, the amygdala nucleus that plays a primary role in seizure generation and spread (for a review see Aroniadou-Anderjaska et al., 2008), and in the CA1 hippocampal area. This protective effect of LY293558 was evident on both days 1 and 7 after soman exposure. Administration of LY293558 also reduced the number of irreversibly degenerating cells in the amygdala, ventral hippocampus, entorhinal cortex, and a neocortical region, but not the piriform cortex, 1 day after soman-induced SE. Seven days after exposure neuronal degeneration was still ongoing in all of these regions in the group that received only soman, whereas those rats that also received LY293558 had significantly less neurodegeneration in the amygdala and neocortex and no neurodegeneration in the CA1, CA3, and hilar areas of the ventral hippocampus. For the hilus, as well as for the piriform and entorhinal cortices, the neuroprotective effect of LY293558 was not statistically significant on day 7. Why different brain regions were protected to a different extent by LY293558 cannot be answered at present. It is unlikely that it has to do with the intensity of seizure activity in these regions, at least as reflected in the neurodegeneration score (e.g., the CA1 and CA3 areas of the ventral hippocampus displayed more neurodegeneration, and therefore probably more severe seizures, than the piriform cortex, yet they were remarkably protected by LY293558). The density and function of GluK1Rs in these regions should play an important role in determining the extent to which these areas will be protected by LY293558. Although GluK1Rs are present in all of the brain areas examined (Bettler et al., 1990), their location and function is partly known only in the BLA and the CA1 and CA3 areas (usually in the dorsal portion of the hippocampus). As for the between-animals variability in the extent of neuronal degeneration within each group, the total duration of SE, including SE that returns at later times (see de Araujo Furtado et al., 2010), which probably varied significantly in different rats, must have contributed to this variability.

The loss of inhibitory neurons relative to the loss of principal cells after exposure to a nerve agent will certainly play a critical role in shaping the network excitability of the damaged brain regions. It is noteworthy that inhibitory neurons in the BLA were not lost 1 day after soman-induced SE, but their number was significantly reduced 1 week later. Such a delayed loss of GABAergic interneurons in the BLA may play an important role in the development of epileptogenesis and the appearance of spontaneous recurrent seizures, as well as in the appearance of behavioral deficits. In regard to epileptogenesis after soman exposure, spontaneous recurrent seizures appear 5 to 12 days after soman-induced SE (de Araujo Furtado et al., 2010), a timing that seems consistent with the delayed loss of GABAergic neurons in the BLA observed in the present study. LY293558 prevented this late interneuronal loss, which could have a significant impact on the development of spontaneous recurrent seizures. Because at 7 days after soman exposure the loss of GABAergic neurons was approximately proportional with the loss of principal cells, this probably implies that the interneurons in the BLA are not more resistant to soman-induced SE than principal neurons, but rather they are affected and die by different mechanisms, which induce a delayed loss. Alternatively, the BLA GABAergic interneurons may indeed be resistant to soman-induced seizures, but they may die gradu-

ally, in part as a consequence of losing their connections with the damaged principal cells. The interneurons in the CA1 hippocampal area did seem to be more resistant to soman-induced SE compared with the overall neuronal loss in CA1 and, also, compared with the BLA interneurons, at least as determined by stereological quantification of GAD-67-immuno-stained interneurons on days 1 and 7 after soman exposure.

The discovery that LY293558 can stop soman-induced seizures, which are more difficult to treat compared with seizures induced by other nerve agents (Shih et al., 2003), when administered 1 h or longer after exposure, and the neuroprotection this GluK1R/AMPA receptor antagonist offers to a number of brain regions that play a key role in seizure generation and emotional/cognitive behavior, is a significant step toward improving the emergency response capabilities in the event of a mass exposure to nerve agents. It is noteworthy that in contrast to benzodiazepines, which have adverse cardiovascular effects (Sunzel et al., 1988), or *N*-methyl-D-aspartate receptor antagonists, which can have toxic side effects (Olney et al., 1991), LY293558 has already been tested clinically, as an analgesic, and has been proven to be safe and well tolerated (Gilon et al., 2000; Sang et al., 2004; Tomillero and Moral, 2008). Certainly, however, many questions still have to be answered before it can be concluded that the doses of LY293558 used in animals to stop nerve agent-induced seizures can be safely administered to humans. It is noteworthy, in this regard, that although the rats that were administered LY293558 at 1 h after soman exposure had 100% survival rate, the survival rate was lower in the rats that were administered LY293558 at later times, even though seizures were stopped. This indicates the importance of early treatment and the need for additional efforts to prevent respiratory depression when anticonvulsant treatment has to be delayed. Another important issue that must be resolved is whether LY293558 offers significant protection even when administered without oxime pretreatment, and, also, with late atropine administration, because immediate administration of atropine would not be possible in a real-case scenario.

Authorship Contributions

Participated in research design: Figueiredo, Qashu, Apland, Aroniadou-Anderjaska, and Braga.

Conducted experiments: Figueiredo, Qashu, Apland, Souza, Aroniadou-Anderjaska, and Braga.

Contributed new reagents or analytic tools: Aroniadou-Anderjaska and Braga.

Performed data analysis: Figueiredo, Qashu, Souza, Aroniadou-Anderjaska, and Braga.

Wrote or contributed to the writing of the manuscript: Aroniadou-Anderjaska, Figueiredo, Qashu, Apland, and Braga.

Other: Braga, Aroniadou-Anderjaska, and Apland acquired funding for the research.

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Address correspondence to: Dr. Maria F. M. Braga, Department of Anatomy, Physiology, and Genetics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814. E-mail: mbraga@usuhs.mil