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Caramiphen edisylate as adjunct to standard therapy attenuates soman-induced seizures and cognitive deficits in rats

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ABSTRACT

The progression of epileptiform activity following soman (GD) exposure is characterized by a period of excessive cholinergic activity followed by excessive glutamatergic activity resulting in *status epilepticus*, which may lead to neuropathological damage and behavioral deficits. Caramiphen edisylate is an anticholinergic drug with antiglutamatergic properties, which conceptually may be a beneficial therapeutic approach to the treatment of nerve agent exposure. In the present study, rats were exposed to 1.2 LD_{50} GD or saline, treated with atropine sulfate (2 mg/kg, im) and HI-6 (93.6 mg/kg, im) 1 min after GD exposure, and monitored for seizure activity. Rats were treated with diazepam (10 mg/kg, sc) and caramiphen (0, 20 or 100 mg/kg, im) 30 min after seizure onset. Following GD exposure, performance was evaluated using a battery of behavioral tests to assess motor coordination and function, sensorimotor gating, and cognitive function. Caramiphen as adjunct to diazepam treatment attenuated GD-induced seizure activity, neuropathological damage, and cognitive deficits compared to diazepam alone, but did not attenuate the GD-induced sensorimotor gating impairment. These findings show that physiological, behavioral, and neuropathological effects of GD exposure can be attenuated by treatment with caramiphen as an adjunct to therapy, even if administration is delayed to 30 min after seizure onset.
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1. Introduction

Soman (GD), an organophosphorus (OP) nerve agent, is a chemical weapon of mass destruction (Cannard, 2006) that exerts its toxic effects by irreversibly inhibiting acetylcholinesterase (AChE) (Junge and Krisch, 1975). The resulting cholinergic imbalance manifests into a cholinergic syndrome characterized by muscle weakness, fasciculations and paralysis, miosis, salivation, respiratory distress, and mental and behavioral alterations such as apnea and seizure activity (reviewed in Cannard, 2006). Seizures that progress to a prolonged and self-sustained activity level (*status epilepticus* [SE]) result in severe and extensive neuropathological damage (de Araujo Furtado et al., 2010; de Araujo Furtado et al., 2009). Behavioral effects of nerve agent exposure include impairments in motor activity and function, spatial memory acquisition and fear memory, and sensorimotor function (Langston et al., 2012; Lumley et al., 2006; Moffett et al., 2011; Raveh et al., 2008).

The current treatment strategy to prevent the lethal effects of nerve agent exposure includes the muscarinic antagonist atropine sulfate, a benzodiazepine as an anticonvulsant, and an oxime (such as 2-PAM or HI-6) to reactivate AChE inhibited by nerve agent (Cannard, 2006). If treatment is delayed, however, protection from epileptiform activity and the subsequent cognitive and behavioral deficits is not complete (de Araujo Furtado et al., 2010; de Araujo Furtado et al., 2009; Langston et al., 2012; Moffett et al., 2011; Weissman and Raveh, 2008). During the first phase of intoxication in the 3-phase model of nerve agent exposure proposed by McDonough and Shih (1997), excessive cholinergic stimulation initiates seizure activity, which can be treated with an anticholinergic drug. If left untreated (until ~40 min post-exposure), the glutamatergic system begins to maintain seizure activity in phases 2 and 3, thereby rendering anticholinergic treatment ineffective. The increased activation of NMDA receptors by elevated concentrations of extracellular glutamate may be one of the mechanisms responsible for the accumulation of intracellular Ca^{2+} and subsequent initiation of cell death (Delorenzo et al., 2005). This elevation of intracellular Ca^{2+} in OP-exposed hippocampal neurons plateaus for more than a week following the initial exposure and is still elevated at 1 month (Deshpande et al., 2010). The impact of nerve agent-induced seizure activity on these two neurotransmitter systems suggests that drugs with anticholinergic and antiglutamatergic properties may be effective adjunct treatments for nerve agent exposure (Weissman and Raveh, 2008).

Caramiphen edisylate is an anticholinergic drug with antiglutamatergic properties and has anticonvulsant effects against nerve agent in rats

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(Figueiredo et al., 2011; Schultz et al., 2012). Caramiphen displaces [^{3}H]-pirenzepine and [^{3}H]-quinuclidinyl benzilate from M1 and M2 muscarinic receptors, respectively (Hudkins and DeHaven-Hudkins, 1991; Hudkins et al., 1993), and inhibits nicotine-induced tremors (Gao et al., 1998). In addition, caramiphen's antiglutamatergic properties may be attributed to an interaction with the Zn^{2+} binding site of the NMDA receptor (Raveh et al., 1999). Caramiphen antagonizes voltage-gated Ca^{2+} channels (Church and Fletcher, 1995; Fletcher et al., 1995) and inhibits NMDA-evoked currents in mouse hippocampal neurons (Fletcher et al., 1995) and in rat amygdalar neurons (Figueiredo et al., 2011). Based on electrophysiology findings, Figueiredo et al. (2011) suggest that the facilitation of GABA inhibition in the basolateral amygdala by caramiphen may also contribute to its anticonvulsant effects. To our knowledge, the pharmacokinetic properties of caramiphen have not been published in rats. Using published data of blood concentrations of caramiphen following repeated oral administration in humans (Levandoski and Flanagan, 1980) and following a subcutaneous injection in rabbits (Pulver, 1951) we grossly estimated the elimination half-life of caramiphen to be ~2.5 h.

Caramiphen treatment administered 5 or 10 min after GD exposure blocks EEG seizure activity in rats (Raveh et al., 2003; Schultz et al., 2012). Caramiphen treatment in the absence of diazepam 5, 10 or 20 min after the onset of sarin-induced convulsions reduces signs of toxicity, spatial memory impairment in the Morris water maze, and neuroinflammatory response (5 and 10 min treatment only; Raveh et al., 2008). At delayed time points (20 or 30 min) following GD-induced seizure onset, caramiphen is less effective as an anticonvulsant than when used in combination with diazepam (Schultz et al., 2012). The goal of the present study is to investigate the use of caramiphen as an adjunct treatment to standard therapy to prevent or ameliorate behavioral deficits that follow GD exposure in rats.

2. Methods

2.1. Subjects

Male Sprague–Dawley rats (250–300 g) from Charles River Laboratories (Kingston, NY, USA) were individually housed and maintained on a reverse light–dark cycle (lights on 2100–0900) with *ad libitum* access to food and water. The rats were surgically implanted with F40-EET telemetry transmitters (Data Sciences International [DSI], Inc., St. Paul, MN, USA) for the continuous monitoring and collection of cortical EEG activity, body temperature, and general motor activity. Rats were weighed daily, and treatment groups were counterbalanced according to pre-exposure weight. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89–544), as amended.

2.2. Chemicals

GD (pinacolyl methylphosphonofluoridate) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA). Atropine sulfate was purchased from Sigma Aldrich (St. Louis, MO, USA). HI-6 dimethanesulfonate salt was prepared by Starkes Associates (Buffalo, NY, USA) under contract with the Walter Reed Army Institute of Research (Silver Spring, MD, USA). Caramiphen edisylate was a generous gift from Dr. James Apland at the USAMRICD and was originally purchased from Sigma Aldrich. Nuclear magnetic resonance analyses conducted by Dr. Benedict Capacio's laboratory (USAMRICD) demonstrated that the sample of caramiphen was >97% pure. Diazepam (United States Pharmacopia, USP) was purchased from Hospira Inc. (Lake Forrest, IL, USA). Buprenorphine hydrochloride was purchased from Reckitt Benckiser Pharmaceuticals Inc. (Richmond, VA, USA). Chemicals used for transcardial perfusion (4% paraformaldehyde in 0.1 M phosphate buffer [PB]), as well as 20% sucrose in 0.1 M PB, were purchased from FD Neurotechnologies Inc. (Columbia, MD, USA).

2.3. Surgery

EEG transmitters were subcutaneously implanted in rats as described by Moffett et al. (2011). Briefly, rats were administered isoflurane (3% induction, 1.5–2% maintenance) and secured in a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Four cortical stainless steel screw electrodes were implanted on the skull 2 mm bilaterally to the midline and 1.6 mm anterior and 4 mm posterior to bregma. Stainless steel wires from the F40-EET transmitters were implanted subcutaneously, wrapped around the electrodes, and secured in place using dental acrylic. The incision sites were sutured, treated with topical bacitracin, and sealed with Vetbond™. Rats were administered buprenorphine (0.03 mg/kg, sc) immediately after removal from anesthesia. The rats were given 2 weeks to recover prior to GD exposure. On post-exposure day (PED) 9 following the acoustic startle response trial, transmitters were surgically removed. Anesthesia and treatments were performed as stated above.

2.4. Telemetry equipment

Each F40-EET telemetry device transmits biopotential data and was associated to an individual model RPC-1 physiotel receiver (DSI) placed under the rat's home cage in the colony room for EEG acquisition (24 h/day). Data were digitized at 250 Hz, 60 Hz notch filter, 0.1 Hz hi-pass filter, 1 KHz low-pass filter, and recorded using Dataquest ART 4.1 (Acquisition software; DSI). Body temperature was also continuously recorded from the implanted transmitter.

2.5. Exposure and treatments

After 2 weeks of recovery from surgery, 1.2 LD_{50} GD (132 μg/kg; saline, 0.5 ml/kg) or vehicle was injected subcutaneously into the flank. The LD_{50} dose used is the same as cited by Shih et al. (Shih et al. (1991a, b)). One min after GD exposure, a combination of HI-6 (93.6 mg/kg, im) and atropine (2 mg/kg, im; sterile water, 0.5 ml/kg) was injected into the right hindlimb. This standard therapy given with GD exposure maximized survival while still allowing the occurrence of SE and neuropathological damage (Moffett et al., 2011). Thirty min after the onset of seizure activity, diazepam was administered into the flank (10 mg/kg, sc; 2 ml/kg). Caramiphen (20 or 100 mg/kg, im; sterile water, 0.5 ml/kg) or vehicle was administered at the time of diazepam treatment into the left hindlimb. GD-negative controls (SAL/ST + VEH; n = 10) received only standard treatment of atropine and HI-6 1 min after exposure and diazepam 40 min after exposure. GD-positive controls (GD/ST + VEH; n = 9) received GD (1.2 LD_{50}; 132 μg/kg) and standard treatment. Caramiphen-treated animals received the same treatment as GD/ST + VEH with the addition of 20 mg/kg caramiphen (GD/ST + CED20; n = 10) or 100 mg/kg caramiphen (GD/ST + CED100; n = 12). All rats received atropine, HI-6, and diazepam. Rats were given a palatable wet mash of food, water, and sugar for 3 days following exposure. Three rats given 100 mg/kg caramiphen in the absence of diazepam 30 min after seizure onset died within 1 h of exposure; no additional rats were tested without diazepam. Two rats exposed to GD did not develop seizures and were excluded from statistical analyses.

2.6. Body weight

Rats were weighed daily between 0800 and 0830. Any rat that lost more than 10 g from the previous day's weight was treated with 3 ml saline (0.9% NaCl, sc) to prevent dehydration.

2.7. EEG scoring

EEG data were collected continuously from 3 days prior to GD exposure to 9 days following GD exposure. Initial seizure activity was viewed using Neuroscore (DSI) by an observer blind to the treatment conditions and determined as rhythmic high-amplitude spikes that lasted at least 10 s (D'Ambrosio et al., 2009; de Araujo Furtado et al., 2009). Seizures were considered terminated when the EEG no longer displayed this rhythmic high-amplitude spiking. The time points for initial seizure onset and termination were recorded, and the duration was calculated as the difference between onset and termination. For statistical analyses, seizures lasting longer than 24 h after onset were given a maximal duration score of 1440 min.

Scoring of EEG for the full duration of the recordings was performed using a high-throughput seizure detection algorithm as previously described by de Araujo Furtado et al. (2009). Briefly, seizure activity was determined in 2-s epochs through correspondence with detection thresholds set by the slope of a linear robust fit applied to a fast Fourier transform and normalized power spectra (0.1–10 Hz) during 24 h of baseline. During episodes of SE, the power spectra increase in magnitude and dominant frequencies are shifted to the left, increasing the slope of the linear robust fit. When both power spectra and slope reach threshold, the 2 s epoch is marked as a potential seizure. Minimum seizure duration was set at 10 s. Detection using this method generated a list of candidate seizures that an observer blind to the treatment groups inspected visually and either confirmed as seizures or rejected as false positives incurred by artifacts in the EEG recording.

2.8. Behavioral seizure

Rats were monitored for 5 h following exposure, and behavioral seizures were scored using a modified five-stage Racine scale (Racine, 1972): stage 1, mastication, tongue fasciculation, oral tonus; stage 2, head tremors, head bobs; stage 3, limb clonus or tonus, body tremor; stage 4, rearing with forelimb clonus, opisthotonus; and stage 5, rearing and falling with generalized convulsions. For analysis, rats received a score corresponding to the maximum stage reached per time interval. Observations were made continuously for the first 2 h and then every 30 min up to 5 h after exposure to GD.

2.9. Spontaneous locomotion

Total distance and rearing on PEDs 1 and 8 were measured with the Versamax Animal Activity Monitor (Accuscan Instruments Inc., Columbus, OH, USA), which uses photobeam interruptions to detect location and movement. The apparatus was a 40 × 40 × 30 cm arena with a 40 × 40 grid of photobeams to track the rat's rearing activity and movement within the field. Sessions within the chamber lasted 10 min. Tracking data were compiled using the Versamax software. Total distance refers to the centimeters in distance traveled by an animal in a given period of time, and rearing was measured using vertical beam break activity.

2.10. Balance beam test of vestibulomotor function

Balance beam testing for assessing motor coordination was completed as described by Moffett et al. (2011). Briefly, rats traversed a 2.5 × 90 cm beam and entered a dark goal box to escape a bright light. Habituation and two baseline sessions (two trials per session) were conducted the week prior to exposure. Following exposure, beam trials were conducted on PEDs 4, 7, and 14. Latency to cross and number of falls from the balance beam were recorded by an observer. Data were averaged per session. Trials in which rats fell from the beam were omitted from the latency to cross analysis.

2.11. Morris water maze

A 170 cm diameter pool was used for the Morris water maze (MWM). The pool was filled and drained on each test day, the water was dyed black using nontoxic tempera paint, and the water temperature was kept at 26 ± 1 °C. A 10 × 10 cm hidden platform was placed in a fixed position 1.25 cm below the surface of the water so that it was not visible. An overhead camera and HVS Watermaze 2100 tracking software (HVS Image, Cambridge, UK) were used to obtain data.

Before the first trial, rats were placed on the platform for 10 s. At the start of each trial, rats were placed in the pool adjacent to and facing the wall of the pool in one of four possible starting locations. Rats were allowed 60 s to explore the maze, find the hidden platform, and escape by climbing onto the platform. Starting locations were arranged in a pseudorandom order to decrease reliance on memory processes that would interfere with spatial memory such as habit formation or procedural memory. During training, rats received 8 trials per day organized into 2 sessions of 4 trials each with test sessions separated by a 30 min rest period in their home cage for 3 days (PEDs 21–23). Data analyzed for training were latency to escape, path length, swim speed, heading error (accuracy of rats initial heading in degrees), and time in target quadrant as a percent of trial time. On PED 24 (day 4 of the test), the escape platform was removed, and rats were given two 60-s probe trials. For the probe trials, rats were placed in the pool starting at the quadrant opposite from where the escape platform was previously located. Data recorded from probe trials were heading, platform passes, thigmotaxis, and time spent in the target quadrant.

2.12. Fear conditioning

Fear conditioning was conducted as previously described (Moffett et al., 2011). Briefly, the conditioning trial consisted of 15 presentations of a 10-s tone (conditioned stimulus, CS) that terminated with a 2-s, 1 mA foot shock (unconditioned stimulus, US) with a 60-sinter-trial interval. In the contextual trial, freezing behavior, characterized as movement less than 20 pixels per frame (0.133 s), was measured within the same context with no shock or tone. In the cued trial, plastic sheets were placed over the walls and floor of the chamber, and a different cleaning agent was used to change the context. Freezing behavior was measured in response to the tone. The conditioning trial was conducted on PED 28, the contextual trial was conducted on PED 29, and the cued trial was conducted on PED 30. Trials were conducted in Video Fear Sound Attenuating Cubicles using Video Freeze V 2.5.5.0 software (Med Associates Inc., St. Albans, VT, USA).

2.13. Acoustic startle response

Trials for acoustic startle response (ASR) were conducted as previously described (Langston et al., 2012). Within a session, rats received startle stimuli alone at 100 or 120 dB, prepulse trials in which the 100 and 120 dB stimuli are preceded by a 70 dB prepulse, trials with the 70 dB pulse alone, and 60 dB white noise. Each trial type was presented 10 times within a session in a randomized order. Trials were presented at 15 ± 5 s inter-trial intervals. Peak startle amplitude, time to peak startle (T_{max}), and prepulse inhibition (PPI) for each trial type were averaged within each session. Habituation and two baseline trials were conducted the week before GD exposure. Post-exposure trials were conducted on PEDs 2, 9, 16, 25, and 31.

2.14. Neuroanatomical assessments

On PED 32, rats were injected with sodium pentobarbital (75 mg/kg, ip) and perfused with 0.9% heparinized saline in 0.1 M PB followed by 4% paraformaldehyde in 0.1 M PB. Brains were removed, post-fixed for 6 h in 4% paraformaldehyde, and cryoprotected in 20% sucrose in 0.1 M PB. Histological sectioning and staining of brain tissue were

conducted at FD Neurotechnologies. Coronal 50 μm sections were stained with proprietary FD NeuroSilver™ stain to identify degenerating neuronal fibers. Select brain regions were qualitatively scored by an observer blind to treatment, for severity of neuropathology, on a scale of 0–4 with 4 being most severe as previously described (McDonough et al., 1995). Brain regions scored included the thalamus, amygdala, hippocampus, and piriform cortex at bregma −3.00 mm. The ventral hippocampus at bregma −5.28 mm was also investigated as this region has been found to be more susceptible to GD-induced neuropathology than the dorsal hippocampus (Apland et al., 2010). Consecutive coronal 50 μm sections were stained with cresyl violet to aid in structural identification.

Coronal 30 μm sections were stained for NeuN-immunoreactivity by FD Neurotechnologies. A monoclonal mouse anti-NeuN IgG (1:10,000; Millipore, Billerica, MA) was used in the NeuN immunostaining procedure. Subsequently, the immunoreaction product was visualized according to the avidin–biotin complex method of Hsu et al. (Hsu et al., 1981) with the Vectastain elite ABC kit (Vector Lab., Burlingame, CA). NeuN profile density was determined from profile count and area of each region evaluated using Imagepro Plus v7.0 (Media Cybernetics Inc., Rockville, MD, USA). Areas evaluated included the central mediodorsal thalamic nucleus (MDC), laterodorsal thalamic nucleus, ventrolateral part (LDVL), dorsolateral geniculate nucleus (DLG) basolateral amygdala (BLA), lateral amygdala (LA), piriform cortex, and the CA1 pyramidal layer of the ventral hippocampus.

2.15. Statistical analysis

All statistical analyses were performed using SPSS v16-20 (IBM Inc., Armonk, NY, USA). Graphs were compiled using Sigma-Plot v11-12 (Systat Software Inc., San Jose, CA, USA). Repeated Measures ANOVA was used to analyze body weights, body temperature, MWM, fear conditioning, and acoustic startle response amplitude, prepulse inhibition, and T_{max} data with repeated trials as the within-subjects variable and treatment as the between-subjects variable. Greenhouse–Geisser correction was applied to degrees of freedom to correct for violations of sphericity. For simplicity, sphericity-assumed degrees of freedom are shown. If an interaction was found between a within-subjects variable and a between-subjects variable, one-way ANOVAs were conducted on each level of the within-subjects variable. One-way ANOVA was used to analyze spontaneous locomotion. Violations of homogeneity of variance were corrected for by using a Kruskal–Wallis ANOVA. Kaplan–Meier analysis with log rank (Mantel–Cox) pairwise comparisons was used for initial seizure duration with a cutoff of 1440 min (24 h). Total seizure duration, behavioral seizure, balance beam falls, and platform passes in the MWM probe trial, were analyzed using Kruskal–Wallis one-way ANOVA. Silver stain neuronal damage score was analyzed using a Mann–Whitney *U* test. NeuN immunoreactivity was analyzed using a one-way ANOVA. Post-hoc tests for general linear model analyses were conducted using a Tukey's test to compare groups to the SAL/ST + VEH and to the GD/ST + VEH groups. Post-hoc pairwise comparisons for Kruskal–Wallis tests were performed using a Mann–Whitney *U* test. Data displayed graphically are mean ± standard error of the mean (SEM, standard deviation / square root of the sample size), box and whisker plots (box: 25th, 50th and 75th percentiles; whiskers: 10th and 90th percentiles) or median ± interquartile range (IQR).

3. Results

3.1. Body weight

The higher dose (100 mg/kg) of caramiphen treatment prevented body weight loss following GD exposure (Fig. 1). There was a main effect of day ($F(3,111) = 36.607$, $p < 0.001$), main effect of group ($F(9,111) = 4.772$, $p < 0.01$), and a significant interaction between day and group ($F(9,111) = 7.557$, $p < 0.01$). The significant effects of

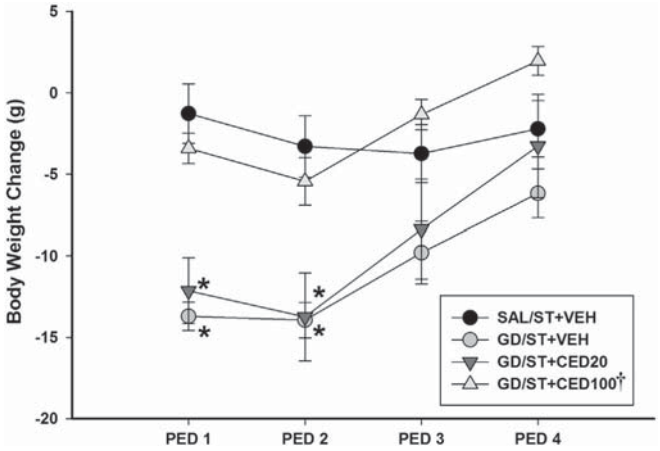

Fig. 1. Exposure to 1.2 LD_{50} GD resulted in body weight loss for rats that received standard therapy (atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset; GD/ST + VEH) and those that received standard therapy plus 20 mg/kg of caramiphen 30 min after seizure onset (GD/ST + CED20) (*$p < 0.05$). Rats exposed to GD that received standard therapy and 100 mg/kg caramiphen 30 min after seizure onset (GD/ST + CED100) had less body weight loss than GD/ST + VEH (†$p < 0.05$), and did not differ from control rats exposed to saline and treated with standard therapy (SAL/ST + VEH). SAL/ST + VEH received diazepam or 40 min after saline exposure. Data shown are mean ± SEM.

group were found on PEDs 1–4 (PED 1 $F(3,37) = 16.768$, $p < 0.001$; PED 2 $F(3,37) = 8.473$, $p < 0.001$; PED 3 $F(3,37) = 3.990$, $p < 0.05$; PED 4 $F(3,37) = 3.085$, $p < 0.05$). Further analysis showed that on PEDs 1 and 2, both the GD/ST + VEH and GD/ST + CED20 groups had significantly reduced weight compared to SAL/ST + VEH ($p < 0.05$). The GD/ST + CED100 did not differ from SAL/ST + VEH, but yet differed significantly from GD/ST + VEH on PEDs 1–4 ($p < 0.05$). The GD/ST + CED20 group did not differ from GD/ST + VEH. On PEDs 3 and 4, no group differed from SAL/ST + VEH.

3.2. Seizure

Caramiphen treatment reduced initial and total seizure activity following GD exposure, with the higher dose (100 mg/kg) more effective than the lower dose (20 mg/kg; Fig. 2A–E). Both caramiphen-treated groups had reduced initial seizure duration compared to GD/ST + VEH (Fig. 2A: Kaplan–Meier analysis: mean ± SEM: GD/ST + CED20 93 ± 16 min, $X^2 = 18.356$, $p < 0.05$; GD/ST + CED100 71.33 ± 9.34 min, $X^2 = 20.797$, $p < 0.05$). GD/ST + VEH rats can be divided into 2 subsets based on initial seizure duration: those that developed seizures lasting 3–4 h ($n = 6$, mean ± SEM = 229.17 ± 15.31 min) and those that developed seizures that continued for more than 24 h ($n = 3$).

When analyzed in 24-h bins, both caramiphen-treated groups had significantly reduced seizure activity compared to GD/ST + VEH from 0–24 h to 24–48 h, but only the GD/ST + CED100 group had significantly reduced seizure activity from 48 to 72 hr (Fig. 2B–D, Mann–Whitney *U*, $p < 0.05$). A comparison of total seizure duration over the 9 day post-exposure recording period showed that while both caramiphen-treated groups had reduced seizure activity, the GD/ST + CED20 group spent significantly more time in seizure than the GD/ST + CED100 group (Fig. 2E Kruskal–Wallis ANOVA: $X^2 = 16.066$, $df = 2$, $p < 0.001$).

3.3. Behavioral seizure

Behavioral seizures (motor convulsions) were induced by GD exposure with caramiphen treatment resulting in an earlier reduction of Racine score compared to GD/ST + VEH; the higher dose of caramiphen was more effective than the lower dose (Fig. 3). Compared to SAL/ST + VEH, GD/ST + VEH rats had higher Racine scores from 10 to

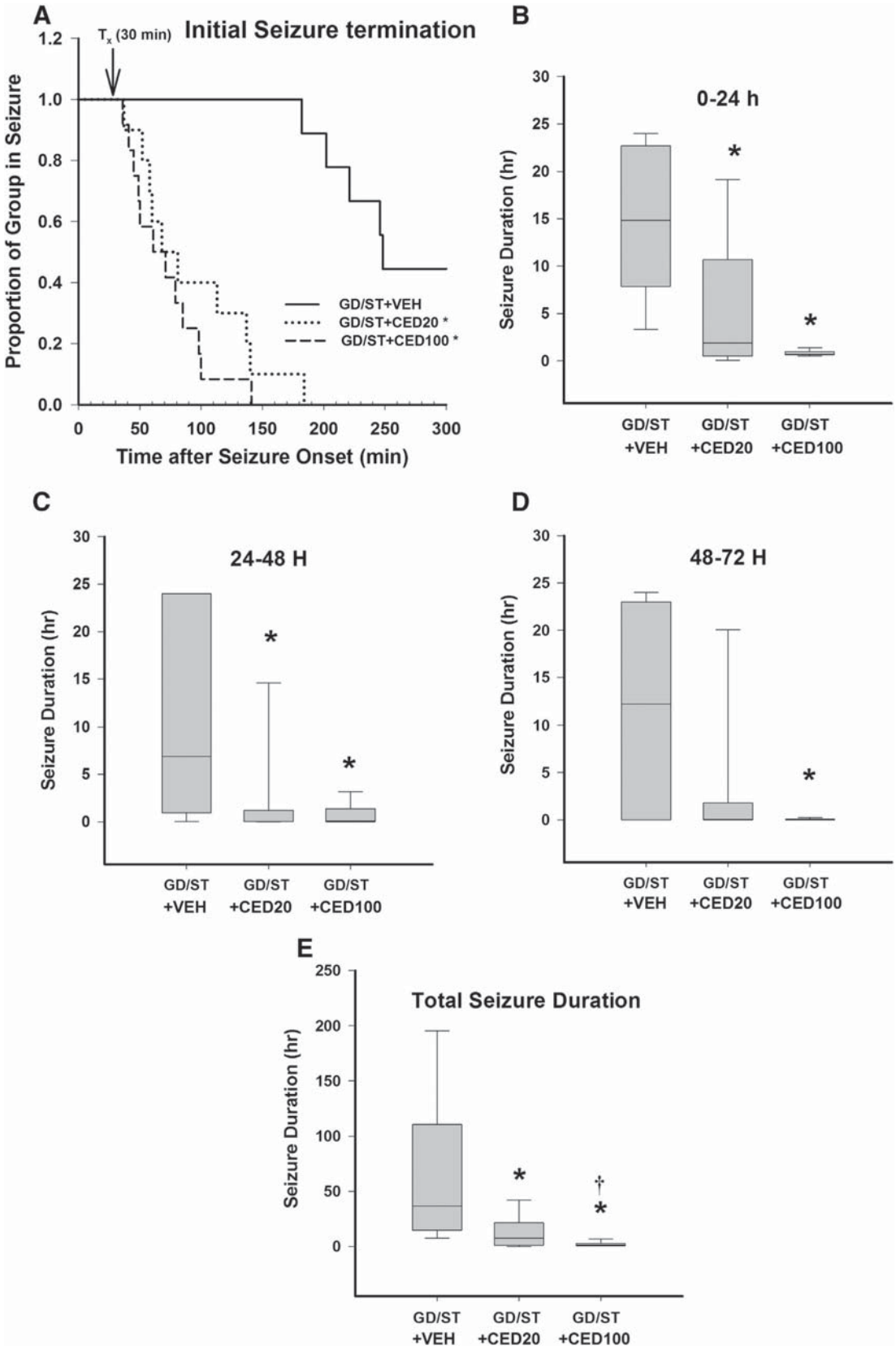

Fig. 2. Initial seizure termination times for the GD-exposed groups are plotted for the first 300 min after seizure onset (A). Any seizure lasting longer than 300 min continued for >24 h. Seizure activity was divided into 24 h bins: (B) 0–24 h, (C) 24–48 h, (D) 48–72 h. Total seizure activity for entire 9 day post exposure recording is shown in Fig. 2E. Rats exposed to 1.2 LD_{50} GD that received standard therapy (atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset; GD/ST + VEH) had sustained seizure activity. Rats that received standard therapy plus 20 mg/kg caramiphen or 100 mg/kg caramiphen 30 min after seizure onset (GD/ST + CED20 and GD/ST + CED100, respectively) had shorter seizure duration than the GD/ST + VEH group. The GD/ST + CED100 group had less seizure activity than the GD/ST + CED20 group. Data shown in Fig. 2A are individual initial seizure termination time points arranged in a Kaplan–Meier plot. Data shown in Fig. 2B–E are box and whisker plots (box: 25th, 50th and 75th percentiles; whiskers: 10th and 90th percentiles), *$p < 0.05$ compared to GD/ST + VEH, †$p < 0.05$ compared to GD/ST + CED20.

270 min after exposure. GD/ST + CED20 had higher Racine scores from 10 to 75 min and at 105 and 150 min after exposure. GD/ST + CED100 had higher Racine scores from 10 to 50 min after exposure. Both caramiphen groups had reduced Racine scores in comparison to GD/ST + VEH (GD/ST + CED20 75–240 min, GD/ST + CED100 55–270 min). Main effect was determined by Kruskal–Wallis analysis $X^2 = 9.055–32.57$; pairwise comparisons were conducted using Mann–Whitney *U*, $p < 0.05$.

3.4. Body temperature

All rats had reduced body temperature following saline or GD exposure and standard treatment, with caramiphen treatment exacerbating the reduction in body temperature (Fig. 4). Repeated Measures ANOVA showed a main effect of time ($F(24,816) = 19.804$, $p < 0.001$), no effect of group, and a significant interaction between time and group ($F(72,816) = 3.113$, $p < 0.01$). Significant effects of group were found on hours 3–9 (3 h $F(3,35) = 4.778$, $p < 0.01$; 4 h $F(3,35) = 3.23$, $p < 0.05$; 5 h $F(3,35) = 3.561$, $p < 0.05$; 6 h $F(3,35) = 5.239$, $p < 0.01$; 7 h $F(3,35) = 4.918$, $p < 0.01$; 8 h $F(3,35) = 4.918$, $p < 0.05$; 9 h $F(3,35) = 3.194$, $p < 0.05$). Body temperature in the GD/ST + VEH group did not differ from SAL/ST + VEH. The GD/ST + CED20 had lower temperature than SAL/ST + VEH at hours 6–8 ($p < 0.05$). The GD/ST + CED100 group had lower temperature than SAL/ST + VEH at hours 5–8 ($p < 0.05$). Body temperature in the GD/ST + CED20 group did not differ from GD/ST + VEH. GD/ST + CED100 had lower temperature at 3 h post-exposure compared to GD/ST + VEH ($p < 0.05$). Comparison within each group showed reduced body temperature in comparison to baseline at the following hours: SAL/ST + VEH 2–7, 17 and 24 h ($F(24,168) = 10.053$, $p < 0.001$), GD/ST + VEH 5–9 h ($F(24,192) = 2.504$, $p < 0.001$), GD/ST + CED20 3–20 h ($F(24,216) = 7.224$, $p < 0.001$), and GD/ST + CED100 3–16 h ($F(24,240) = 15.403$, $p < 0.001$).

3.5. Spontaneous locomotion

GD exposure caused a transient reduction in total distance traveled and in rearing activity, and the higher dose of CED provided partial protection against this locomotor deficit (Fig. 5). On PED 1, the GD/ST + VEH and GD/ST + CED20 groups had reduced total distance compared to SAL/ST + VEH (Fig. 5A; $F(3,37) = 6.104$, $p < 0.01$, GD/ST + VEH and GD/ST + CED20 $p < 0.01$). The GD/ST + CED100 group did not differ from SAL/ST + VEH in total distance traveled. Neither CED-treated group differed from GD/ST + VEH in total distance traveled. On PED 8, no group differed from SAL/ST + VEH in total distance traveled. On PED 1, all GD-exposed groups displayed decreased rearing (Fig. 5B; $F(3,34) = 14.699$, $p < 0.001$, 3 outliers removed); however, the high dose partially protected the rats from this deficit as the GD/ST + CED100 group differed significantly from both SAL/ST + VEH and GD/ST + VEH. The GD/ST + CED20 group did not differ from GD/ST + VEH. On PED 8, no group differed significantly from SAL/ST + VEH in rearing activity.

3.6. Balance beam

All GD-exposed rats regardless of caramiphen treatment had a higher incidence of falling from the balance beam on PED 4 (Table 1; $X^2 = 10.245$, $df = 3$, $p < 0.05$). Pairwise comparisons revealed that the GD/ST + VEH, GD/ST + CED20, and GD/ST + 100 groups all fell more than SAL/ST + VEH, but neither caramiphen-treated group differed significantly from GD/ST + VEH. Latency to cross the balance beam from rats that did not fall did not differ significantly between groups on any day of testing.

3.7. Morris water maze

3.7.1. Spatial memory acquisition

GD-exposed rats had impaired spatial memory acquisition in the Morris water maze indicated by increased escape latency (Fig. 6A), path length (Fig. 6B), decreased percent of trial time searching the quadrant (Fig. 6C) of the maze that contained the platform, and increased percent of trial time in thigmotaxis (Fig. 6D). There was no difference between groups for swim speed indicating that these differences are not likely a result of impaired swimming ability (data not shown). The higher dose of caramiphen prevented the effects of GD as the GD/ST + CED100 group did not differ significantly from the SAL/ST + VEH group, but had improved escape latency, path length, and time in target quadrant compared to GD/ST + VEH. The lower dose of caramiphen only provided partial protection from the GD-induced spatial memory deficit as the GD/ST + CED20 had higher escape latency, path length, and lower time in the target quadrant compared to SAL/ST + VEH, but reduced escape latency and path length in comparison to GD/ST + VEH. For escape latency, there was a main effect of session ($F(5,185) = 46.828$, $p < 0.001$), group ($F(3,37) = 8.759$, $p < 0.001$), and

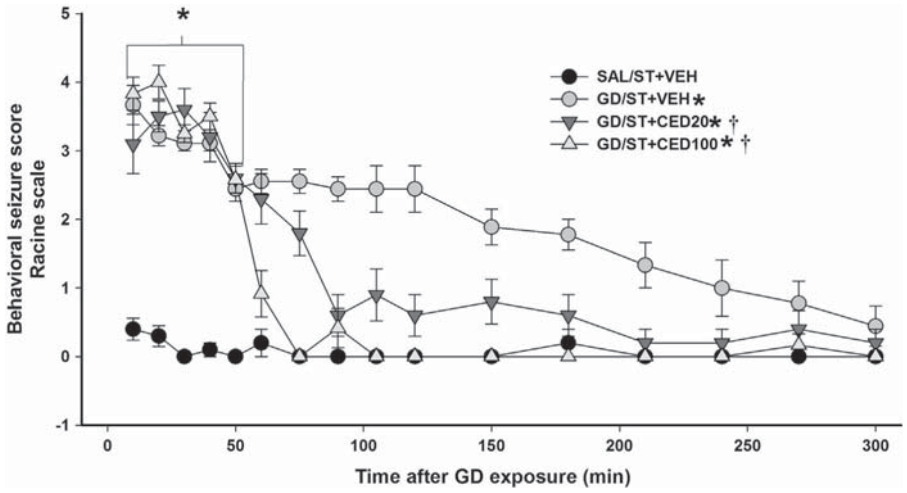

Fig. 3. Behavioral seizure was evaluated using the Racine scale for 5 h after exposure. Exposure to 1.2 LD_{50} GD followed by standard treatment (atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset; GD/ST + VEH) resulted in behavioral seizure activity which gradually decreased in intensity over the observation period. Rats exposed to 1.2 LD_{50} GD and treated with standard therapy plus 20 mg/kg or 100 mg/kg caramiphen 30 min after seizure onset (GD/ST + CED20 and GD/ST + CED100, respectively) had an earlier reduction in behavioral seizure score following GD exposure compared to GD/ST + VEH group, with the GD/ST + CED100 group more effective at terminating behavioral seizures than the GD/ST + CED20 group. Data shown are mean ± SEM; *$p < 0.05$ compared to SAL/ST + VEH; † $p < 0.05$ compared to GD/ST + VEH.

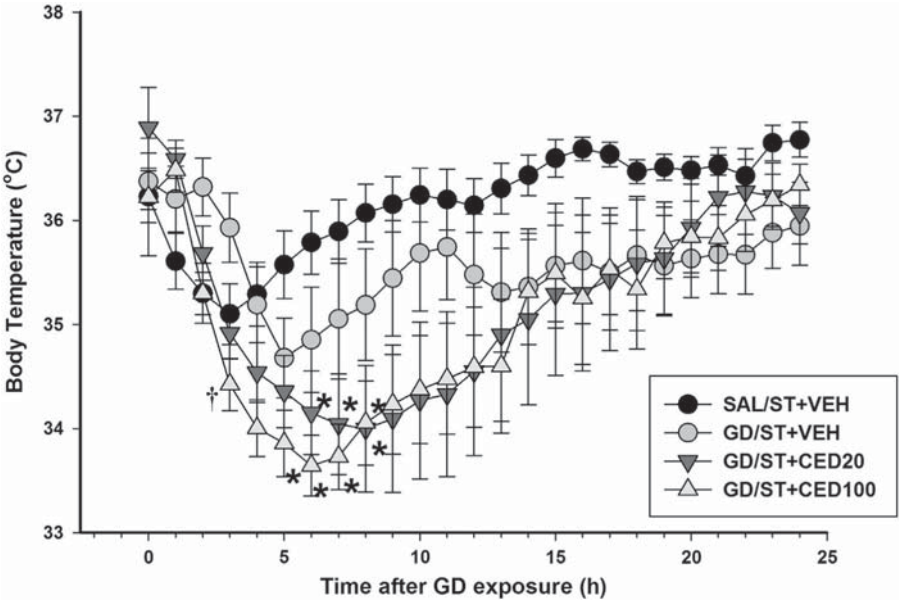

Fig. 4. All groups had transient reduction in body temperature. All rats received standard therapy of atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset; for saline control, diazepam was 40 min after exposure (SAL/ST + VEH). Rats exposed to 1.2 LD_{50} GD and treated with standard therapy plus 20 mg/kg or 100 mg/kg caramiphen (GD/ST + CED20 and GD/ST + CED100, respectively) had greater temperature reduction compared to GD-exposed rats that received only standard therapy (GD/ST + VEH). Data shown are mean ± SEM; *$p < 0.05$ compared to SAL/ST + VEH; † $p < 0.05$ compared to GD/ST + VEH.

a significant interaction ($F(15,185) = 2.464$, $p < 0.01$). The analysis of each session revealed a significant effect of group on sessions 2 through 6 (session 2 $F(3,37) = 7.725$, $p < 0.001$; session 3 $F(3,37) = 5.075$, $p < 0.01$; session 4 $F(3,37) = 10.804$, $p < 0.001$; session 5 $F(3,37) = 8.431$, $p < 0.001$; session 6 $F(3,37) = 2.861$, $p < 0.05$). GD/ST + VEH had increased escape latency on sessions 2–5 ($p < 0.05$). GD/ST + CED20 had increased escape latency compared to SAL/ST + VEH on session 2 ($p < 0.05$), and decreased escape latency compared to GD/ST + VEH on session 4 ($p < 0.05$). GD/ST + CED100 did not differ from SAL/ST + VEH in any session and had decreased latency compared to GD/ST + VEH on sessions 2, 4, and 5 ($p < 0.05$).

For path length (Fig. 6B), there was a main effect of session ($F(5,185) = 50.989$, $p < 0.001$), group ($F(3,37) = 8.391$, $p < 0.001$), and a significant interaction ($F(15,185) = 2.444$, $p < 0.01$). There were significant effects of group on sessions 2–6 (session 2 $F(3,37) = 7.354$, $p < 0.01$; session 3 $F(3,37) = 3.316$, $p < 0.05$; session 4 $F(3,37) = 11.328$, $p < 0.001$; session 5 $F(3,37) = 7.842$, $p < 0.001$; session 6 $F(3,37) = 4.470$, $p < 0.01$). GD/ST + VEH had increased path length in comparison to SAL/ST + VEH on sessions 2–6. GD/ST + CED20 had increased path length compared to SAL/ST + VEH on sessions 2 and 5 ($p < 0.05$) and decreased path length compared to GD/ST + VEH on session 4. GD/ST + CED100 path length did not differ from SAL/ST + VEH and was significantly shorter from GD/ST + VEH on sessions 2, 4, 5, and 6 ($p < 0.05$).

For percent time in target quadrant (Fig. 6C), there was a main effect of session ($F(5,185) = 6.161$, $p < 0.001$) and group ($F(3,37) = 7.568$, $p < 0.001$), but no significant interaction ($F(3,37) = 0.998$, $p = 0.459$). GD/ST + VEH and GD/ST + CED20 spent less time in the target quadrant than SAL/ST + VEH ($p < 0.05$). GD/ST + CED100 did not differ from SAL/ST + VEH and spent more time in the target quadrant than GD/ST + VEH ($p < 0.05$).

The percent of trial time in thigmotaxis (Fig. 6D) violated homogeneity of variance and was analyzed using a Kruskal–Wallis ANOVA. The significant effects of group were found on sessions 2, 4, 5, and 6 (session 2 $X^2 = 7.956$, $p < 0.05$; session 4 $X^2 = 13.050$, $p < 0.01$; session 5 $X^2 = 15.841$, $p < 0.01$; session 6 $X^2 = 9.263$, $p < 0.05$). The GD/ST + VEH group had increased thigmotaxis time compared to SAL/ST + VEH in sessions 2, 4, 5, and 6 ($U = 8–17$, $p < 0.05$). GD/ST + CED20 had increased thigmotaxis compared to SAL/ST + VEH on session 4 ($U = 21$, $p < 0.05$) and reduced thigmotaxis compared to GD/ST + VEH on session 5 ($U = 20$, $p < 0.05$). Thigmotaxis in GD/ST + CED100 did not differ from SAL/ST + VEH, but was reduced compared to GD/ST + VEH in sessions 4–6 ($U = 6–19$, $p < 0.05$). During the probe trial, when the platform was removed from the pool, no differences were seen between groups using measurements of platform passes, time in target quadrant or thigmotaxis.

3.7.2. Visual acuity

In the visual acuity trials following the probe trials, rats from the GD/ST + VEH group took significantly longer time to find the platform than the SAL/ST + VEH group ($F(3,37) = 5.495$, $p < 0.01$). Averaged over 4 trials, escape latency (mean ± SEM) is as follows: SAL/ST + VEH (11.53 ± 3.75 s), GD/ST + VEH (30.03 ± 3.95 s), GD/ST + CED20 (20.75 ± 3.75 s), and GD/ST + CED100 (11.396 ± 3.42).

3.8. Fear conditioning

There was no difference between groups during the conditioning trial. There was a significant increase in freezing from the baseline period to the repeated cue + shock presentations ($F(15,555) = 25.508$, $p < 0.001$). All groups froze in response to CS + US pairings.

3.8.1. Contextual trial

Caramiphen prevented the GD-induced reduction of freezing in response to the context. GD/ST + VEH rats displayed decreased freezing in comparison to SAL/ST + VEH ($p < 0.05$). Both caramiphen-treated groups displayed similar freezing behavior to SAL/ST + VEH and were significantly higher than GD/ST + VEH (Fig. 7A). There was a main effect of time ($F(7,273) = 10.2852$, $p < 0.001$) and group ($F(3,37) = 3.611$, $p < 0.05$), but no interaction. As GD/ST + VEH were expected to have reduced freezing behavior (Moffett et al., 2011), a one tailed Dunnett's T post hoc test was used to compare to saline control.

3.8.2. Cued trial

Caramiphen dose-dependently prevented the GD-induced reduction of freezing in response to the CS (Fig. 7A). There was a significant change in freezing behavior over repeating CS presentations ($F(7,259) = 34.097$, $p < 0.001$), a significant main effect of group ($F(3,37) = 5.609$, $p < 0.01$),

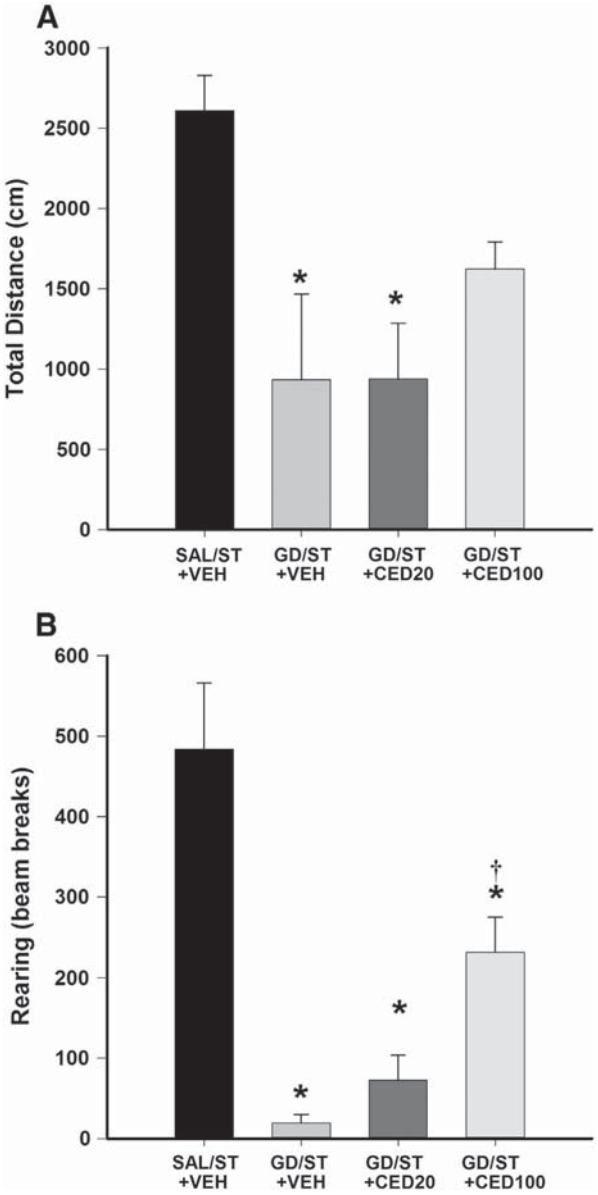

Fig. 5. Rats exposed to 1.2 LD_{50} GD exposure plus standard therapy (atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset; GD/ST + VEH) had less total distance traveled (A) and less rearing (B) in the open field test 24 h after exposure in comparison to saline control rats (SAL/ST + VEH). Rats that received 100 mg/kg caramiphen plus standard therapy (GD/ST + CED100) but not those that received 20 mg/kg caramiphen (GD/ST + CED20) were less impaired than the GD/ST + VEH group. Data shown are mean ± SEM; *p < 0.05 compared to SAL/ST + VEH. All rats received atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset (or 40 min after exposure for SAL/ST + VEH).

and a significant CS by group interaction ($F(21,259) = 1.987$, $p < 0.05$). Significant group effects were seen at all CS presentations (CS1 $F(3,37) = 4.042$, $p < 0.05$; CS2 $F(3,37) = 5.247$, $p < 0.01$; CS3 $F(3,37) = 4.327$, $p < 0.05$; CS4 $F(3,37) = 4.325$, $p < 0.05$, CS5 $F(3,37) = 4.326$, $p < 0.05$; CS6 $F(3,37) = 5.188$, $p < 0.01$; CS7 $F(3,37) = 6.456$, $p < 0.01$). During the 3 min baseline period, when rats were introduced to the novel context with no cue, there was no significant difference in freezing behavior between groups. When placed in a novel context and presented with the auditory cue from the conditioning trial, the GD/ST + VEH group had significantly reduced freezing compared to SAL/ST + VEH on CS presentations 1–7. The GD/ST + CED100 group did not differ significantly from SAL/ST + VEH and had higher freezing compared to GD/ST + VEH on CS presentations 1–3. GD/ST + CED20 had lower freezing compared to SAL/ST + VEH on CS 7 and higher freezing compared to GD/ST + VEH on CS 2.

Table 1
Falls from the balance beam.

Group	Baseline	PED 4	PED 7
SAL/ST + VEH	1/10 (10%)	1/10 (10%)	1/10 (10%)
GD/ST + VEH	1/9 (11%)	5/9 (56%)*	4/9 (44%)
GD/ST + CED20	0/10 (0%)	7/10 (70%)*	4/10 (40%)
GD/ST + CED100	3/12 (25%)	9/12 (75%)*	5/12 (42%)

# of subjects that fell on one or more trials/group size. *p<0.05 compared to SAL/ST+VEH.

3.9. Acoustic startle response

GD exposure reduced prepulse inhibition (Fig. 8A and B), increased startle amplitude (Fig. 8C and D), and decreased time to peak startle (Fig. 8E and F). Caramiphen was largely ineffective at preventing GD-induced sensorimotor gating deficits. Prepulse inhibition (PPI) was significantly reduced by GD exposure in response to both 100 and 120 dB stimuli in comparison to SAL/ST + VEH and was not affected by caramiphen treatment. For 100 dB PPI, there were main effects of day ($F(5,185 = 10.469$, $p < 0.001$) and group ($F(3,37 = 12.304$, $p < 0.001$), but no interaction ($F(3,37) = 1.180$, $p = 0.291$). For 120 dB PPI, there was a main effect of day ($F(5,185) = 21.753$, $p < 0.001$) and group ($F(3,37) = 4.854$, $p < 0.01$), but no interaction ($F(3,37) = 1.638$, $p = 0.067$).

GD exposure produced a significant increase in startle amplitude in comparison to baseline to both the 100 and 120 dB stimuli that was not affected by either dose of caramiphen. For 100 dB startle amplitude, there was a main effect of day ($F(5,185) = 17.719$, $p < 0.05$), but not group ($F(3,37) = 2.766$, $p = 0.055$), and an interaction between day and group ($F(15,185) = 2.052$ $p < 0.05$). For 120 dB startle amplitude, there was also a main effect of day ($F(5,185) = 8.310$, $p < 0.001$), but not group ($F(3,37) = 1.784$, $p = 0.167$), and an interaction between day and group ($F(15,185) = 2.301$, $p < 0.01$). For the 100 dB stimulus, SAL/ST + VEH did not differ from baseline startle amplitude on any post-exposure day. GD/ST + VEH had higher startle amplitude to the 100 dB stimuli compared to baseline on all post-exposure days (PED 2 $F(1,8) = 8.812$, $p < 0.05$; PED 9 $F(1,8) = 15.595$, $p < 0.01$; PED 16 $F(1,8) = 12.658$; PED 25 $F(1,8) = 16.726$; $p < 0.01$; PED 31 $F(1,8) = 17.627$, $p < 0.01$). GD/ST + CED20 had higher startle amplitude to the 100 dB stimuli compared to baseline on all post-exposure days (PED 2 $F(1,9) = 19.283$, $p < 0.01$; PED 9 $F(1,9) = 32.854$, $p < 0.001$; PED 16 $F(1,9) = 20.115$, $p < 0.01$; PED 25 $F(1,9) = 10.745$, $p < 0.05$; PED 31 $F(1,9) = 19.790$, $p < 0.01$). The GD/ST + CED100 group had higher startle amplitude to the 120 dB stimuli compared to baseline on all post-exposure days (PED 2 $F(1,11) = 16.655$, $p < 0.01$; PED 9 $F(1,11) = 48.002$, $p < 0.001$; PED 16 $F(1,11) = 45.178$, $p < 0.001$; PED 25 $F(1,11) = 42.637$, $p < 0.001$; PED 31 $F(1,11) = 11.269$, $p < 0.01$). For 120 dB, SAL/ST + VEH did not differ from baseline startle amplitude on any post-exposure day. GD/ST + VEH had increased startle amplitude from baseline on PED 2 ($F(1,8) = 7.200$, $p < 0.05$), PED 9 ($F(1,8) = 10.601$) and PED 31 ($F(1,8) = 8.661$, $p < 0.05$). GD/ST + CED20 had increased startle amplitude to the 120 dB stimuli compared to baseline on all post-exposure days (PED 2 $F(1,9) = 7.916$, $p < 0.05$; PED 9 $F(1,9) = 23.201$, $p < 0.01$; PED 16 $F(1,9) = 18.451$, $p < 0.01$; PED 25 $F(1,9) = 13.204$, $p < 0.01$; PED 31 $F(1,9) = 7.109$ $p < 0.05$). GD/ST + CED100 had increased startle amplitude to the 120 dB stimuli compared to baseline on all post-exposure days (PED 2 $F(1,11) = 6.378$, $p < 0.05$; PED 9 $F(1,11) = 9.845$, $p < 0.01$; PED 16 $F(1,11) = 7.492$, $p < 0.05$; PED 25 $F(1,11) = 11.752$, $p < 0.01$; PED 31 $F(1,11) = 5.456$, $p < 0.05$).

GD exposure reduced the latency to peak startle in response to the 120 dB stimulus, but this effect was not seen in caramiphen treated rats. For 120 dB T_{max}, there was a main effect of day ($F(5,185) = 5.173$,

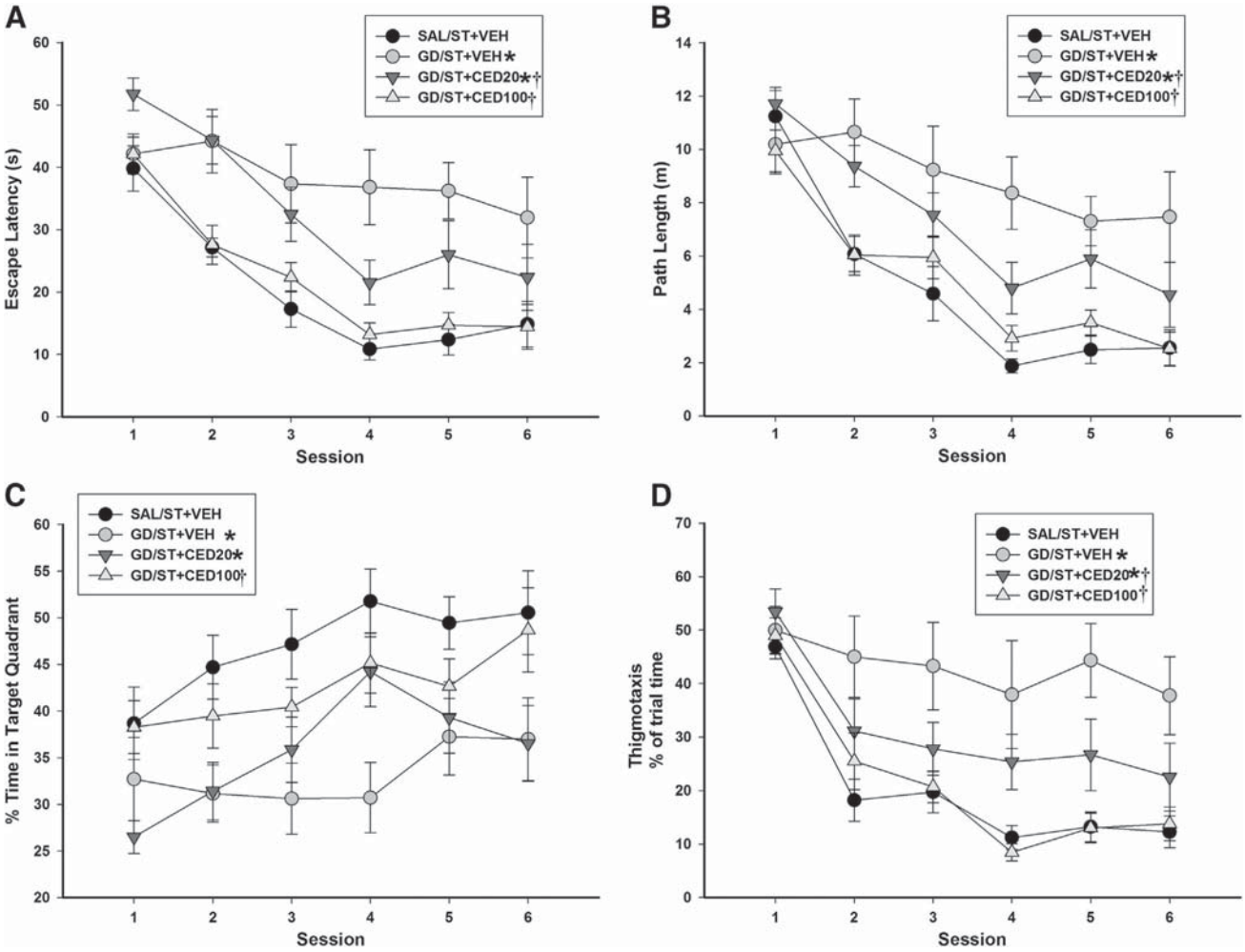

Fig. 6. Rats exposed to 1.2 LD_{50} GD exposure and standard therapy (atropine sulfate and HI-6 1 min after exposure, and diazepam 30 min after seizure onset GD/ST + VEH) had impaired spatial memory acquisition in the Morris water maze, demonstrated by increased latency to escape the water maze (A), increased path length (B), and reduced time searching the target quadrant (C) compared to unexposed controls which received standard treatment (SAL/ST + VEH). There was no effect of GD exposure on swim speed (D). Rats exposed to 1.2 LD_{50} GD and treated with standard therapy plus 20 mg/kg caramiphen 30 min after seizure onset (GD/ST + CED20) had partial protection, while those exposed to GD that received standard therapy plus 100 mg/kg caramiphen 30 min after seizure onset (GD/ST + CED100) had complete protection in this task and performed as well as SAL/ST + VEH rats. Data shown are mean ± SEM; *$p < 0.05$ compared to SAL/ST + VEH, †$p < 0.05$ compared to GD/ST + VEH).

$p < 0.001$), a main effect of group ($F(3,37) = 3.814$, $p < 0.05$), and no interaction ($F(3,37) = 1.064$, $p = 0.393$). For 100 dB T_{max}, there were no main effects of group or day and no interaction effect. GD exposure caused an increase in startle amplitude to the 70 dB prepulse stimuli on PED 9, which was prevented by both doses of caramiphen ($X^2 = 10.753$, $p < 0.05$; mean ± SEM: SAL/ST + VEH 0.076 ± 0.008, GD/ST + VEH 0.153 ± 0.027, GD/ST + CED20 0.089 ± 0.009, GD/ST + CED100 0.103 ± 0.012).

3.10. Neuroanatomical assessments

The higher dose of caramiphen administered following GD exposure prevented damage in several brain regions, as demonstrated using silver and NeuN stain. Median scores for major regions of interest were analyzed. There were significant effects of group for all regions investigated (fiber tracts $X^2 = 29.046$ $p < 0.001$; thalamus $X^2 = 28.585$ $p < 0.001$, amygdala $X^2 = 21.452$ $p < 0.001$; dorsal hippocampus $X^2 = 11.679$, $p < 0.001$, ventral hippocampus $X^2 = 14.994$ $p < 0.001$, piriform cortex $X^2 = 27.443$ $p < 0.001$). The GD/ST + VEH group had higher median neuropathology scores than SAL/ST + VEH in all major regions scored (Fig. 9; $U = 15$, $p < 0.05$ dorsal hippocampus, $U = 10$, $p < 0.01$ ventral hippocampus, $U = 0$, $p < 0.001$ fiber tracts, thalamus, amygdala, and piriform cortex). The GD/ST + CED20 group had higher median neuropathology scores than SAL/ST + VEH in fiber tracts ($U = 5$, $p < 0.001$), thalamus ($U = 5.5$, $p < 0.001$), amygdala ($U = 6$, $p < 0.001$), piriform cortex ($U = 10$, $p < 0.01$), and ventral hippocampus ($U = 15$, $p < 0.01$), but not the dorsal hippocampus. The GD/ST + CED100 did not have higher median neuropathology scores than SAL/ST + VEH in any major region. In comparison to the GD/ST + VEH group, the GD/ST + CED20 had reduced neuropathology in fiber tracts ($p < 0.05$). The GD/ST + CED100 group had lower neuropathology than GD/ST + VEH in all major regions scored ($p < 0.001$ fiber tracts, $p < 0.001$ thalamus, $p < 0.01$ amygdala, $p < 0.05$ dorsal and ventral hippocampus).

NeuN immunocytochemistry (Fig. 10) was used to evaluate neuronal loss in nuclei within the medial and lateral thalamus, including the dorsal lateral geniculate, the lateral and basolateral amygdala, the piriform cortex, and CA1 of the hippocampus. There were significant effects of group for the three thalamic regions: MDC ($F(3,37) = 13.632$, $p < 0.001$), LDVL ($F(3,37) = 20.084$, $p < 0.001$), and DLG ($X^2 = 16.146$, $p < 0.001$; Kruskal–Wallis used for DLG due to heterogeneity of variance). The GD/ST + VEH group had reduced profile density compared to SAL/ST + VEH in the MDC, LDVL, and DLG ($p < 0.001$). The GD/ST + CED20 group had reduced profile density than SAL/ST + VEH in the MDC ($p < 0.01$) and higher profile density than GD/ST + VEH in the LDVL ($p < 0.001$) and DLG ($p < 0.05$). The

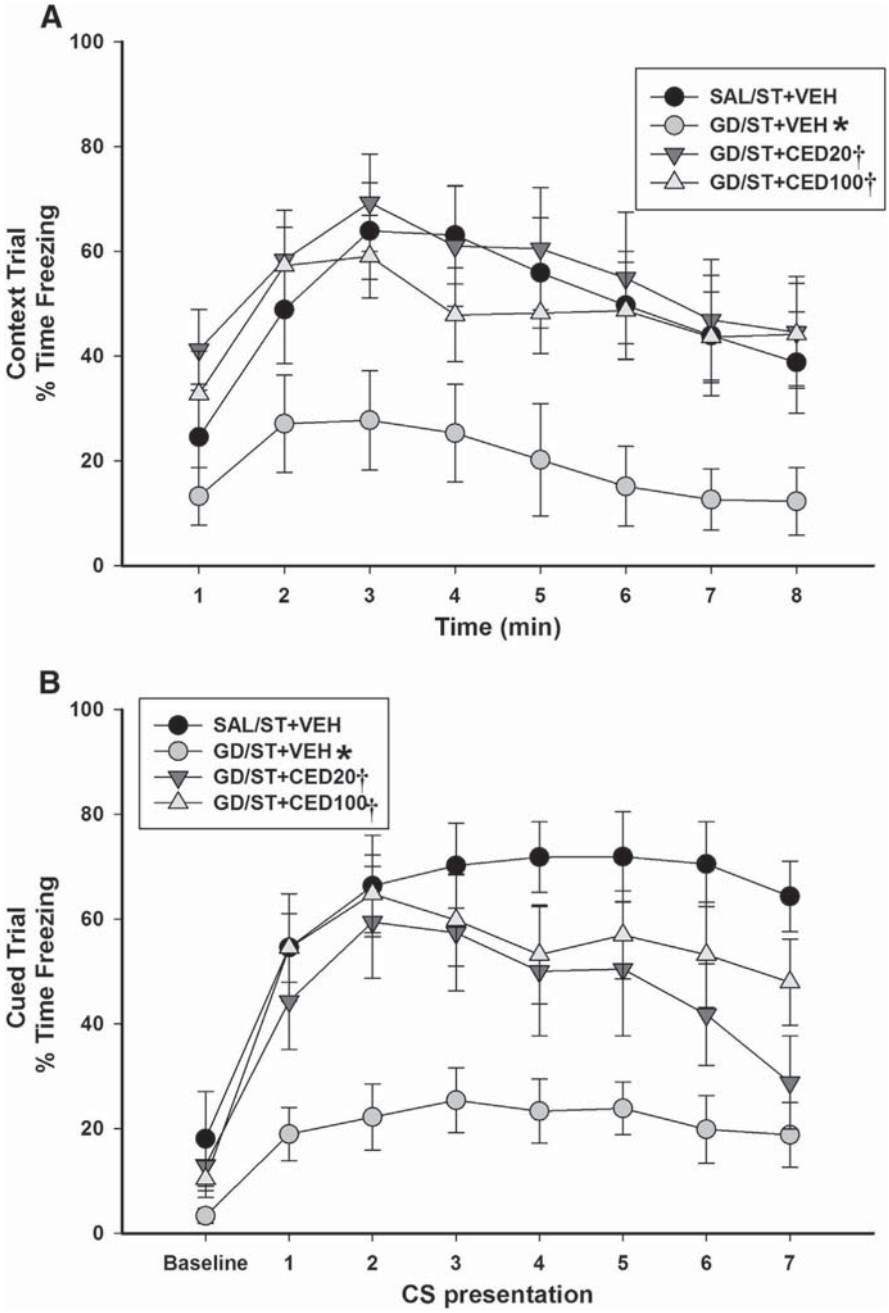

Fig. 7. Rats exposed to 1.2 LD_{50} GD that received standard therapy (atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset GD/ST + VEH) had reduced freezing behavior to both the context (A) and the auditory cue (B) in a fear conditioning test, compared to rats that received saline and were treated with standard therapy (SAL/ST + VEH). GD-induced performance deficit in fear conditioning was prevented in rats treated with standard therapy plus 20 mg/kg or 100 mg/kg caramiphen (GD/ST + CED20 and GD/ST + CED100, respectively), which had similar freezing response to SAL/ST + VEH for both trials. Data shown are mean ± SEM; *$p < 0.05$ compared to SAL/ST + VEH; †$p < 0.05$ compared to GD/ST + VEH.

GD/ST + CED100 group did not differ from SAL/ST + VEH and had higher profile density than GD/ST + VEH in the MDC ($p < 0.01$), LDVL ($p < 0.001$), and DLG ($p < 0.001$). Caramiphen protected the lateral thalamus as NeuN profile density in both LDVL and DLG regions did not differ significantly from SAL/ST + VEH in either caramiphen-treated group. Both the GD/ST + VEH and GD/ST + CED20 groups had reduced NeuN density in layer 3 of the piriform cortex (main effect $X^2 = 24.158$, df = 3, $p < 0.001$; GD/ST + VEH $p < 0.001$, GD/ST + CED20 $p < 0.001$). GD/ST + CED20 did not differ from GD/ST + VEH. GD/ST + CED100 did not differ from SAL/ST + VEH and had higher profile density than GD/ST + VEH. Two of 9 rats in the GD/ST + VEH developed visible lesions in the CA1 pyramidal layer of the ventral hippocampus, but this did not result in a significant difference CA1 NeuN density between groups. There were no noticeable hippocampal lesions in either caramiphen treated group. There was no difference between groups for profile density in the basolateral or lateral amygdala. These findings support the results seen with silver stain: that neuronal injury resulting from GD exposure and subsequent seizure activity can be ameliorated dose-dependently with caramiphen.

4. Discussion

We presently show that rats exposed to 1.2 LD_{50} GD with standard therapy (atropine sulfate, HI-6, and diazepam) displayed prolonged seizure activity, body weight loss, and behavioral deficits to include

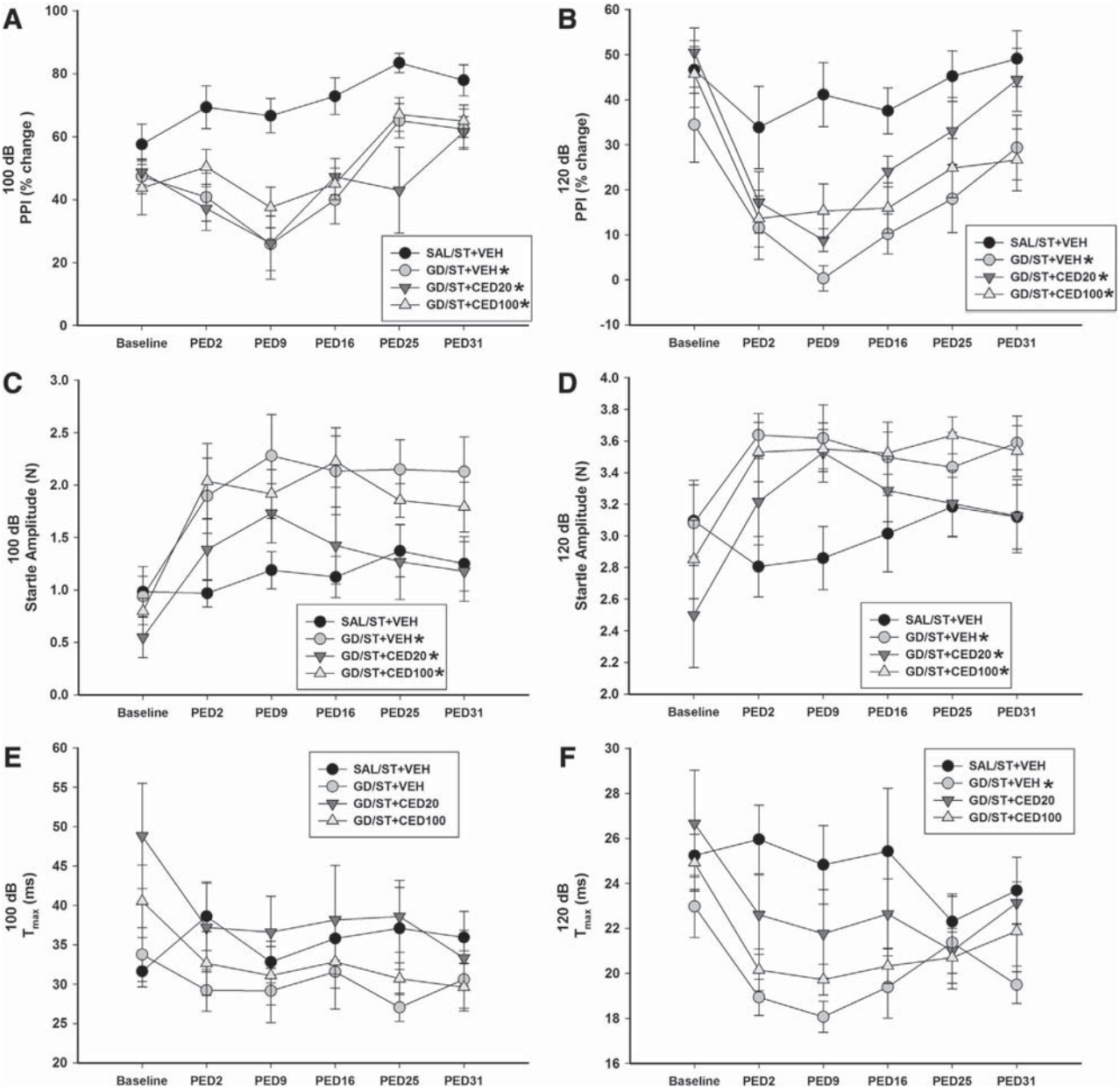

Fig. 8. Rats exposed to 1.2 LD_{50} GD followed by standard therapy of atropine sulfate and HI-6 1 min after exposure, and diazepam 30 min after seizure onset, had altered acoustic startle response, which was not attenuated in rats treated with standard therapy plus 20 mg/kg or 100 mg/kg caramiphen 30 min after seizure onset (GD/ST + CED20 and GD/ST + CED100, respectively). All GD-exposed rats displayed decreased PPI for both 100 dB (A) and 120 dB (B) stimuli in comparison with saline control rats that received standard therapy (SAL/ST + VEH; *$p < 0.05$), and increased startle amplitude in comparison to baseline for both the 100 dB stimuli (C) and 120 dB stimuli (D; *$p < 0.05$). The GD/ST + VEH group had decreased time to peak startle amplitude (T_{max}) in response to the 120 dB stimuli (F) but not the 100 dB stimuli (E). Data shown are mean ± SEM.

impairment in vestibulomotor function, locomotor activity, spatial memory acquisition in the Morris water maze, and cued fear conditioning, as well as increased acoustic startle response and decreased PPI. Caramiphen treatment as adjunct to standard therapy following onset of GD-induced SE dose-dependently reduced seizure activity, attenuated deficits in spatial memory and fear conditioning, and protected against the development of severe neuronal degeneration. Previously, we observed in a short-term (72 h) study that caramiphen (20 mg/kg, im) in combination with diazepam (10 mg/kg, sc) reduces seizure duration and neuronal degeneration following GD exposure when treatment is delayed 30 min after seizure onset (Schultz et al., 2012). Our present findings expand on those findings to demonstrate that caramiphen as adjunct to standard therapy dose-dependently attenuated the development of GD-induced cognitive impairment in the month following exposure.

A seizure-inducing dose of GD led to severe impairment in spatial memory acquisition in the Morris water maze tested three weeks after GD exposure, while caramiphen as adjunct to standard therapy dose-dependently attenuated these performance deficits. Similar impairment in Morris water maze performance occurs in mice exposed to a seizure-inducing dose of GD that leads to neuronal loss in the hippocampal CA1 field and the basolateral amygdala (Collombet et al., 2011). Caramiphen treatment (20 mg/kg, im) 5, 10 or 20 min after sarin exposure in rats partially attenuates spatial memory impairment in the Morris water maze 1 week after exposure, which is concurrent with a reduction in clinical signs of toxicity (5, 10 or 20 min after convulsions) and reduced neuroinflammatory response (5 or 10 min after convulsions) (Raveh et al., 2008). In our study, the higher dose (100 mg/kg) of caramiphen combined with diazepam was more effective than the lower dose (20 mg/kg) and completely prevented

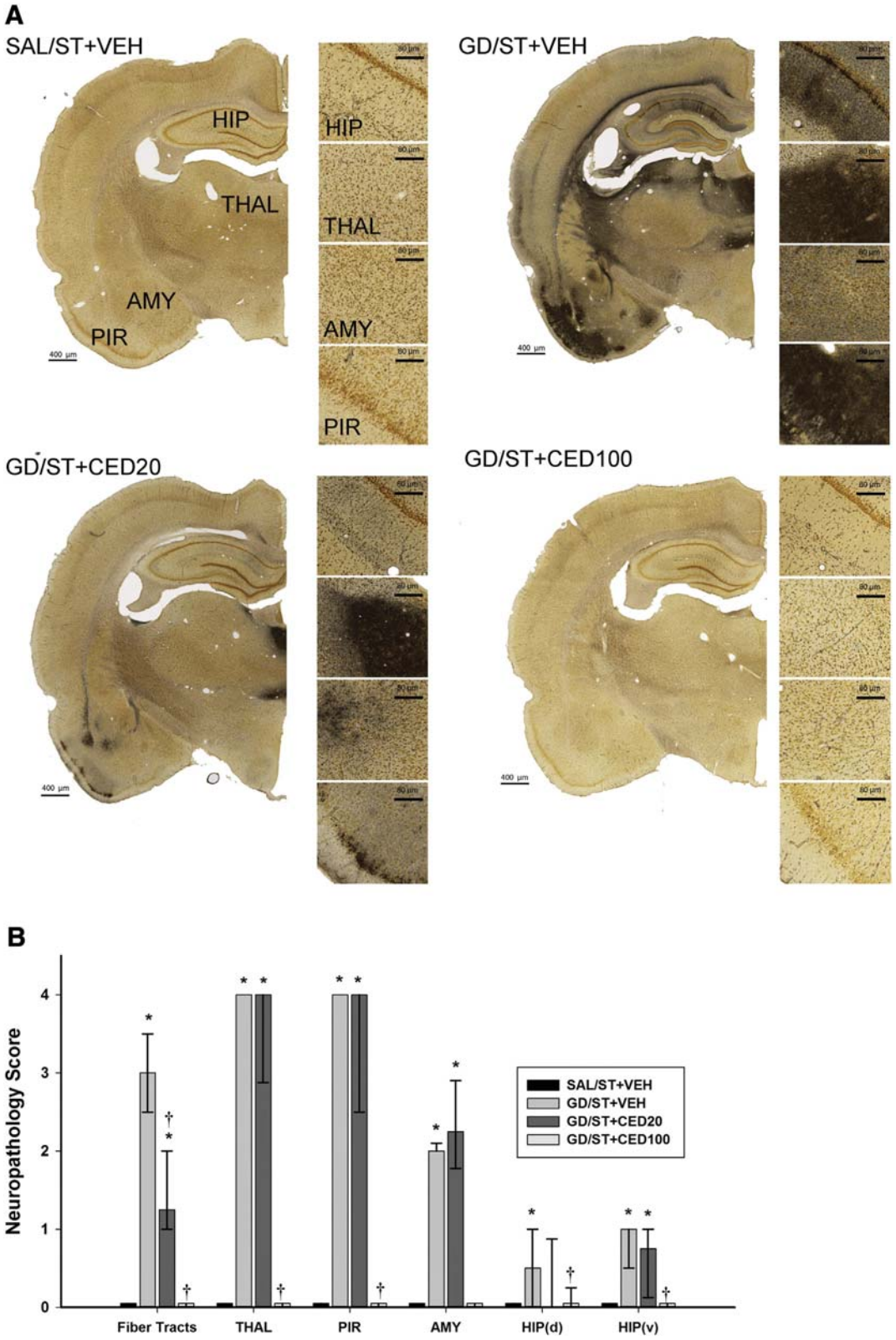

Fig. 9. Silver stain of neuronal degeneration in brains collected one month after exposure to 1.2 LD_{50} GD followed by standard therapy of atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset. In addition to standard therapy, GD-exposed rats received either vehicle (GD/ST + VEH), 20 mg/kg caramiphen (GD/ST + CED20), or 100 mg/kg caramiphen (GD/ST + CED100) 30 min after seizure onset. Negative control rats received saline and standard therapy (SAL/ST + VEH). (A) Representative images of silver-stained coronal slices showing neuronal fiber degeneration at ~3.00 mm posterior to bregma. Enlarged images are the CA1 area of the hippocampus (HIP), mediodorsal thalamic nucleus (THAL), basolateral amygdala, anterior part (AMY) and layers 1–3 of the piriform cortex (PIR). Scale bars are 400 μm left images and 80 μm for regional images. (B) The GD/ST + VEH group had extensive neuropathology in the following regions: fiber tracts, thalamus, piriform cortex, amygdala, and to a lesser extent the dorsal and ventral hippocampus. The GD/ST + CED100 group prevented the development of neuronal fiber degeneration in any region; this group did not differ from SAL/ST + VEH and were all lower than GD/ST + VEH. The GD/ST + CED20 group had reduced damage in fiber tracts compared to GD/ST + VEH, but did not differ from GD/ST + VEH in other regions investigated. Data shown are median ± IQR; *$p < 0.05$ compared to SAL/ST + VEH; †$p < 0.05$ compared to GD/ST + VEH.

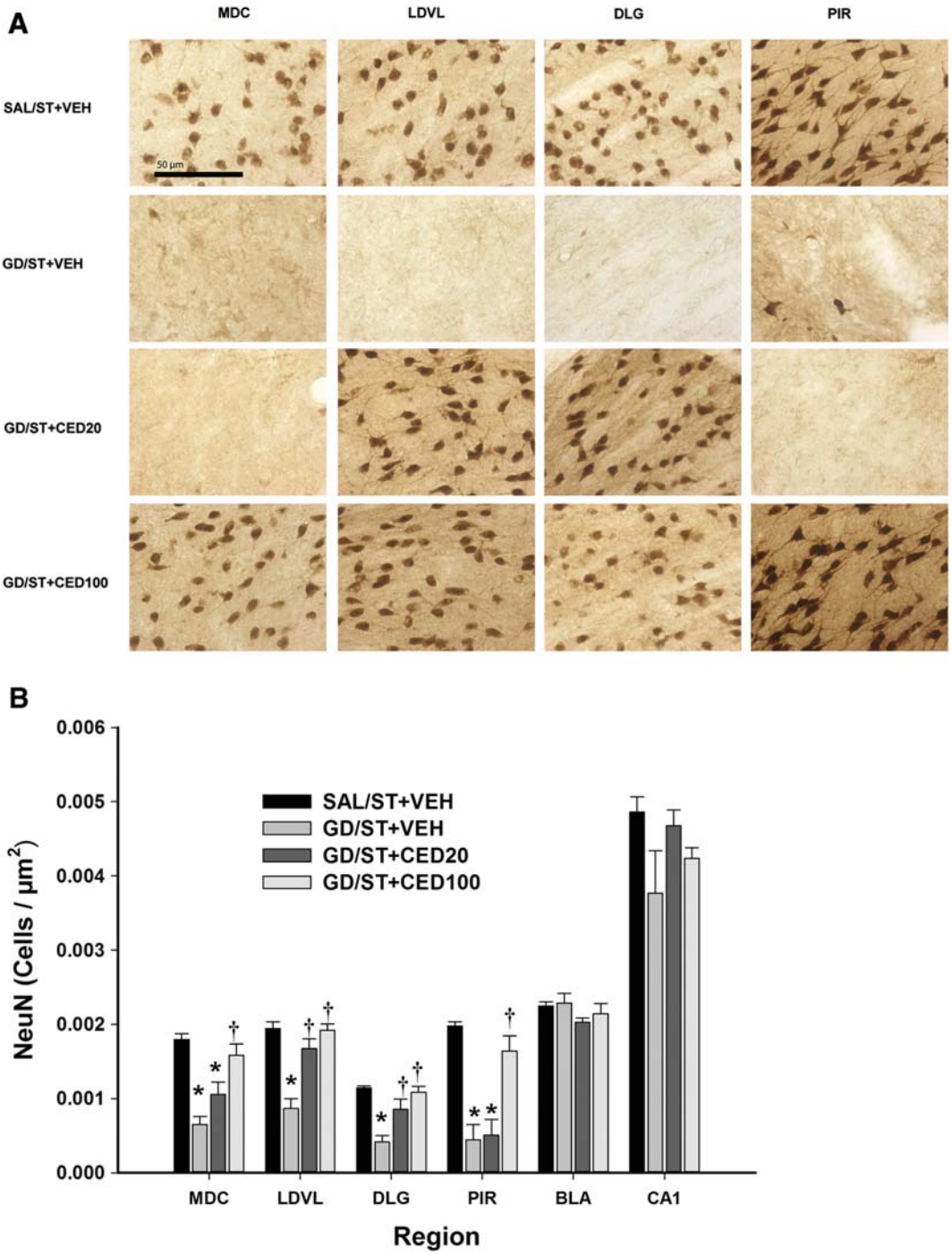

Fig. 10. NeuN stain of neurons in brains collected one month after exposure to 1.2 LD_{50} GD followed by standard therapy of atropine sulfate and HI-6 1 min after exposure and diazepam 30 min after seizure onset. In addition to standard therapy, GD-exposed rats received either vehicle (GD/ST + VEH), 20 mg/kg caramiphen (GD/ST + CED20), or 100 mg/kg caramiphen (GD/ST + CED100) 30 min after seizure onset. 1.2 LD_{50} GD with standard ATR, HI-6 and diazepam treatment and subsequent seizure activity resulted in thalamic neuronal loss measured by NeuN immunoreactivity in comparison to unexposed controls which received standard treatment. This reduction of NeuN profile density was prevented by the high dose of caramiphen (100 mg/kg im 30 min post SE plus standard treatment) (A) Representative 40× images of central mediodorsal thalamic nucleus (MDC), laterodorsal thalamic nucleus, ventrolateral part (LDVL), dorsolateral geniculate nucleus (DLG) and piriform cortex (PIR, layer 3) showing decreased profile density in GD/ST + VEH and GD/ST + CED20 groups (scale bar 50 µm) compared to SAL/ST + VEH. (B) Bar graphs of NeuN profile density showing decreased profile density in GD/ST + VEH and GD/ST + CED20 groups compared to SAL/ST + VEH (data shown are mean ± SEM; *$p < 0.05$ compared to SAL/ST + VEH; †$p < 0.05$ compared to GD/ST + VEH).

the GD-induced impairment in spatial memory acquisition. Both doses of caramiphen prevented GD-induced impairment in the visual acuity test. Both doses of caramiphen reduced initial and acute seizure activity following GD exposure, but only the higher dose was protective against recurrent seizures beyond 48 h and was more effective at preventing the development of neuropathological damage.

We observed severe neuropathological damage in regions of the thalamus, including the mediodorsal thalamic nucleus, laterodorsal thalamic nucleus, ventrolateral part, and dorsolateral geniculate nucleus following exposure to GD and *status epilepticus*. Lesions to the mediodorsal thalamus result in the perseverance of edge swim (thigmotaxis) and a 'circling' search strategy causing an indirect

platform approach and reduced rate of learning as platform crossings are reduced by this strategy (Dolleman-van der Weel et al., 2009). In the present study, GD-exposed rats that received standard treatment or caramiphen (20 mg/kg) had increased thigmotaxis and increased escape latency in the Morris water maze, as well as neuronal loss in the mediodorsal thalamic nucleus, potentially from impaired ability to adjust their search patterns during training. Mediodorsal thalamic lesions also impair performance in the visible platform test and may relate to difficulty in shifting search strategy (Dolleman-van der Weel et al., 2009). In the present study, rats that received GD exposure with standard therapy had impaired performance on the visual acuity (or visible platform) test. GD-induced damage to the dorsolateral geniculate nucleus, a thalamic relay for the visual system, receiving inputs from the retina and projecting primarily to the visual cortex (Paxinos, 2004), may also have contributed to impaired performance in GD-exposed rats. However, GD-exposed rats that received caramiphen (20 mg/kg) had impaired spatial acquisition during training trials without significant neuronal loss to the DLG and were not significantly impaired in the visual acuity test. Together, these findings suggest that GD-induced thalamic damage resulting in thigmotaxis and altered search strategy may have contributed to the impaired water maze performance in GD-exposed rats that received standard therapy or the lower caramiphen dose; however, both the neuronal damage and behavioral impairments were attenuated by the higher dose (100 mg/kg) of caramiphen.

Caramiphen treatment was also dose-dependently protective against GD-induced impairment in fear conditioning. Freezing in response to the cue was fully protected by the higher dose and partially protected by the lower dose of caramiphen. The amygdala and hippocampus are thought to be involved in cued fear conditioning and contextual fear conditioning (Goosens and Maren, 2001; Maren, 2008). In addition, the thalamus is thought to play a critical role in auditory fear conditioning beyond its role of sensory relays (Dupire et al., 2013; Parsons et al., 2006; Weinberger, 2011). Several regions of the thalamus are particularly damaged following exposure to seizure-inducing doses of GD and are postulated to be important for the impairment of auditory and contextual fear responses following GD exposure in rats (Moffett et al., 2011). In the current study, there was significant damage in the amygdala and, to a lesser extent, the hippocampus of rats exposed to GD and treated with vehicle, while GD-exposed rats treated with 100 mg/kg caramiphen (but not with 20 mg/kg) were protected in these brain regions. The higher dose of caramiphen prevented neuronal degeneration in regions of the thalamus and both doses inhibited GD-induced neuronal loss in the lateral thalamus. It is possible that caramiphen's protection of the thalamus and the amygdala prevented the development of impaired fear conditioning.

Transient motor deficits that follow GD exposure were partially protected by caramiphen in the present study. GD exposure induced a transient deficit in spontaneous locomotor activity in an open field, which was partially prevented by treatment with caramiphen (100 mg/kg). However, caramiphen treatment did not prevent performance deficits on the balance beam test. Vestibulomotor deficits occur in the first few days following GD exposure, particularly in animals that display seizures (Lumley et al., 2008; Moffett et al., 2011; Schultz, 2010). Repeated exposure to sub-lethal doses of VX that do not result in seizure activity also leads to vestibulomotor impairments, but only on the days of exposure and not 48 h after exposure (Lumley et al. (2006). Similar to previous findings, rats exposed to a seizure-inducing dose of GD had impaired vestibulomotor function demonstrated by an increased number of balance beam falls in the days following exposure with performance returning to baseline by one week after exposure, but caramiphen treatment as adjunct to standard therapy did not prevent these deficits.

We observed increased acoustic startle response and decreased PPI in GD-exposed rats treated with standard therapy, consistent with previous findings in our laboratory (Langston et al., 2012). Increased acoustic startle response following GD exposure also occurs in guinea pigs (Philippens et al., 2005; Philippens et al., 2000). Increased acoustic startle response and reduced PPI in the weeks following GD exposure depend on the incidence of seizure activity (Langston et al., 2012). In the present study, although caramiphen treatment reduced seizure duration, it did not prevent or ameliorate GD-induced behavioral deficits in acoustic startle response in the weeks after exposure. Differing models of seizure induction vary in effect on acoustic startle response and PPI (Koch and Ebert, 1998; Ma et al., 2004). Hippocampal kindling causes impairment in PPI of the acoustic startle response two weeks after hippocampal afterdischarges (Ma and Leung, 2004). In the amygdala, an area important for the propagation of nerve agent-induced seizures (Skovira et al., 2010), kindling-induced seizure disrupts PPI 2 min, but not 48 h, after convulsive episodes (Howland et al., 2007). A glutamate kainate 1 receptor (GluK1) antagonist administered into the basolateral amygdala reduces startle amplitude with no effect on PPI (Aroniadou-Anderjaska et al. 2012). In the present study, although 100 mg/kg caramiphen as adjunct to standard therapy was effective at preventing GD-induced neuronal injury, including in the basolateral amygdala and hippocampus, caramiphen had no effect on acoustic startle response and limited effect on PPI. Further study is needed on the mechanisms by which nerve agent exposure increases startle amplitude and reduces PPI.

The anticonvulsant efficacy of caramiphen against GD is dependent on the time of administration, with earlier treatment more effective than delayed treatment. Caramiphen is an effective anticonvulsant when administered within 10 min after GD-induced seizure onset (Schultz et al., 2012). However, when administered at delayed time points of 20 or 30 min after seizure onset, caramiphen (20 mg/kg, im) in combination with diazepam (10 mg/kg, sc) treatment is needed to reduce seizure duration and neuropathological damage following GD exposure (Schultz et al., 2012). Figueiredo et al. (2011) found that 100 mg/kg caramiphen (im) in the absence of diazepam reduces behavioral signs of seizure when treatment is delayed 30 or 60 min after GD exposure with the later treatment being less effective than the earlier treatment at preventing neuronal degeneration at 24 h. In the present study, rats that received 100 mg/kg caramiphen without diazepam 30 min after seizure onset had poor survival following GD exposure.

In the present study, the higher dose of caramiphen (100 mg/kg) was more effective than the lower dose of caramiphen (20 mg/kg) at reducing brain damage. Caramiphen (20 mg/kg) in combination with diazepam reduces neuronal fiber degeneration in fiber tracts, regions of the thalamus and the amygdala compared to either treatment alone when evaluated 3 days after GD exposure (Schultz et al., 2012). In the present study, neuropathology assessed one month after exposure resulted in 100 mg/kg caramiphen in combination with diazepam, reducing GD-induced brain damage in the regions of the thalamus, piriform cortex, amygdala, and hippocampus, while the 20 mg/kg caramiphen dose only reduced neuronal degeneration in fiber tracts and neuronal cell loss in thalamic regions. Some rats exposed to GD develop spontaneous recurrent seizures in the days and weeks after exposure, even when the initial seizure is effectively terminated with diazepam administration; this correlates with increased damage in the CA1 of the hippocampus (de Araujo Furtado et al., 2010). In the present study, rats exposed to GD and treated with 20 mg/kg caramiphen spent more total time in seizure than those treated with 100 mg/kg caramiphen, and increased time in seizure may have contributed to greater neuropathology in those receiving the lower dose of caramiphen.

All rats experienced a reduction of body temperature following drug treatments. GD-induced hypothermia is associated with central cholinesterase inhibition (Maickel et al., 1990). Hypothermia following GD exposure can be antagonized by atropine, but not HI-6 treatment possibly due to the inability of HI-6 to reactivate hypothalamic AChE (Clement, 1993). Diazepam (5–20 mg/kg) has been shown to

dose-dependently reduce body temperature in rats (Elliot and White, 2001), which may account for the reduction in body temperature seen in the SAL/ST + VEH group. In the current experiment, caramiphen exacerbated GD-induced hypothermia, which is consistent with our previous findings (Schultz et al., 2012). Hypothermia is potentially neuroprotective against brain injury and a consideration as treatment for drug-resistant epilepsy (reviewed in Motamedi et al., 2013). Mild hypothermia alone or in conjunction with anti-epileptic drugs has been shown to control refractory SE in clinical settings and to have anticonvulsant effects in multiple animal models.

In the present study, we demonstrated that caramiphen is capable of terminating seizure activity and preventing neuronal degeneration and subsequent cognitive deficits when administered after the development of SE; however, the dose required for this proof of concept effect is high. With respect to the potential for caramiphen toxicity, rats receiving caramiphen (100 mg/kg) were healthy 3 months after treatment, and caramiphen treatment did not cause neuronal degeneration in any brain regions examined (Figueiredo et al., 2011). The toxicity of a corresponding dose to humans is currently unknown. Previously in humans, caramiphen was taken orally at up to 1.15 mg/kg or 3.4 mg/kg over 4 days, which led to peak blood level of 60 ng/ml (Levandoski and Flanagan, 1980; Tallarida, 1982). Levy et al. (2007) extrapolated from dogs and monkeys to predict an effective dose of caramiphen against 1.6–1.8 LD_{50} sarin in humans to be 70–100 ng/ml. Our study aimed to bring about higher blood levels of caramiphen, in this range, for this later therapeutic intervention against GD exposure in our rodent model. Additional studies are needed to determine the maximum tolerated dose of caramiphen in humans *via* various routes of administration, as well as potential for drugs with similar anticholinergic and antiglutamatergic effects to provide additional protection against GD exposure.

In summary, caramiphen edisylate administered 30 min after seizure onset as an adjunct treatment to standard therapy (atropine sulfate, oxime (HI-6), and diazepam) against GD exposure reduced seizure duration, body weight loss, motor and cognitive deficits, and brain damage compared to standard therapy alone. However, caramiphen treatment did not prevent the transient impairment in vestibulomotor function or the altered acoustic startle response from developing following GD exposure. These findings suggest that drug therapies with a combination of anticholinergic, NMDA antagonistic, and GABA enhancing effects may improve the long-term outcome following exposure to a seizure-inducing dose of GD.

Disclaimers

The views expressed in this article are those of the author(s) and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government.

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