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Age-Related Susceptibility to Epileptogenesis and Neuronal Loss in Male Fischer Rats Exposed to Soman and Treated With Medical Countermeasures

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ABSTRACT

Elderly individuals compose a large percentage of the world population; however, few studies have addressed the efficacy of current medical countermeasures (MCMs) against the effects of chemical warfare nerve agent exposure in aged populations. We evaluated the efficacy of the anticonvulsant diazepam in an old adult rat model of soman (GD) poisoning and compared the toxic effects to those observed in young adult rats when anticonvulsant treatment is delayed. After determining their respective median lethal dose (LD_{50}) of GD, we exposed young adult and old adult rats to an equitoxic 1.2 LD_{50} dose of GD followed by treatment with atropine sulfate and the oxime HI-6 at 1 min after exposure, and diazepam at 30 min after seizure onset. Old adult rats that presented with *status epilepticus* were more susceptible to developing spontaneous recurrent seizures (SRSs). Neuropathological analysis revealed that in rats of both age groups that developed SRS, there was a significant reduction in the density of mature neurons in the piriform cortex, thalamus, and amygdala, with more pronounced neuronal loss in the thalamus of old adult rats compared with young adult rats. Furthermore, old adult rats displayed a reduced density of cells expressing glutamic acid decarboxylase 67, a marker of GABAergic interneurons, in the basolateral amygdala and piriform cortex, and a reduction of astrocyte activation in the piriform cortex. Our observations demonstrate the reduced effectiveness of current MCM in an old adult animal model of GD exposure and strongly suggest the need for countermeasures that are more tailored to the vulnerabilities of an aging population.

Key words: chemical warfare nerve agents; median lethal dose; soman; age; spontaneous recurrent seizures; diazepam.

Chemical warfare nerve agents (CWNAs) pose an ever increasing risk to civilian populations. However, research into medical countermeasures (MCMs) against the effects of CWNA exposure has primarily focused on drug therapies aimed to increase survival and terminate immediate seizure activity in young adult male soldiers, using young adult animal models in preclinical studies. Comparatively, few studies have addressed the impact of CWNA exposure and MCM treatment in traditionally nonmilitary populations, including particularly vulnerable segments of the population such as the elderly (Towne, 2007). The number of elderly individuals has significantly increased in the United States (U.S. Census Bureau, 2011) and the world at large (Lutz *et al.*, 2008) over the last decades, and the recent use of chemical warfare agents on unprepared civilian populations

(John *et al.*, 2018; Rosman *et al.*, 2014) demonstrates the critical importance of elucidating the unique challenges associated with CWNA MCM in the elderly.

Soman (GD) is a CWNA that leads to an excess of peripheral and central acetylcholine (ACh) at neuronal synapses and neuromuscular junctions by irreversibly inhibiting acetylcholinesterase (AChE) (Kumamoto and Shinnick-Gallagher, 1990). Overstimulation of the cholinergic system as a result of AChE inhibition results in miosis, hypersecretion, fasciculation, respiratory distress, cardiac dysfunction, and seizures progressing to *status epilepticus* (*SE*) and spontaneous recurrent seizures (SRSs) in laboratory animal models (reviewed in Cannard, 2006; de Araujo Furtado *et al.*, 2010; Zilker, 2005). Prolonged *SE*, typically defined as 30 min of sustained seizure activity, results in a severe degree of neuronal death that is primarily localized to the thalamus, amygdala, piriform cortex, and hippocampus in rodents and nonhuman primates (Carpentier *et al.*, 1990; de Araujo Furtado *et al.*, 2012; Lemercier *et al.*, 1983; Moffett *et al.*, 2011; Petras, 1994; Schultz *et al.*, 2012; Shih *et al.*, 2003). Currently, MCM consist of treatment with a muscarinic ACh receptor (mAChR) antagonist (eg, atropine sulfate), an oxime to reactivate nonfunctional synaptic AChE (eg, 2-PAM or HI-6), and a benzodiazepine (eg, diazepam or midazolam) for the treatment of seizures (Zilker, 2005). However, if MCM treatment is delayed, as is likely in a civilian mass casualty attack with CWNAs, cholinergic-related seizure activity becomes self-sustaining and develops pharmacoresistance to benzodiazepine treatment (reviewed in Niquet *et al.*, 2016).

Aging animals are more susceptible than their young adult counterparts to seizure induction and the associated neuropathological consequences, as well as seizure-induced lethality, in other non-CWNA models of *SE*. For example, kainic acid-induced seizure threshold decreases in old adult as compared with young adult rats, with an accompanying increase in the loss of pyramidal neurons within the CA1 layer of the hippocampus (Liang *et al.*, 2007). Pilocarpine, a mAChR agonist commonly used in animal models of epilepsy, results in greater mortality of 28-week-old rats compared with 5-week-old rats following *SE* (Blair *et al.*, 2009). Similar observations have been made in humans, with age-dependent increases in mortality following *SE* (Towne, 2007; Towne *et al.*, 1994). Eighteen-month-old rats also display a lower tolerated dosage to the organophosphorus pesticide parathion which, through its metabolite paraoxon, produces an inhibitory effect on AChE in a similar fashion to GD, compared with 3-month-old animals (Karanth *et al.*, 2007). In addition, senescent (30-month old) rats display more seizure-related cholinergic-mediated behaviors (eg, salivation, hypothermia, lacrimation, and tremor) when they are administered cholinesterase inhibitors, as compared with younger (7-month old) rats (Goh *et al.*, 2011). Finally, outbred rats aged to 8 months are more susceptible to the lethal effects of intramuscular exposure to GD with a lower lethal median dose (LD_{50}; 59 μg/kg) compared with that of 2-month-old rats (87 μg/kg) (Shih *et al.*, 1990). Altogether, the data demonstrate the effect of age on CWNA toxicity in animal models.

Because the aging human population may be more sensitive to the toxic effects of GD exposure, the effectiveness of novel MCMs against CWNA toxicity needs to be tested in preclinical animal models that more appropriately correlate with human life stages. Although previous studies have investigated the effects of GD exposure in 8- and 10-month-old rats, these models still only correlate to approximately 18 and 23 human years (Sengupta, 2013). We currently report on the effects of GD exposure in an aged adult 18-month old (approximately 41 years). This age was selected as a starting age to assess the effects of GD in aged rats because the use of older senescent rats (25+ months of age) may have many health complications due to advanced aging (ie, heart disease, tumors, cancer) that may impact the long-term survivability of animals after GD exposure, as well as surgical complications when undergoing anesthesia for implantation of telemetry transmitters. To our knowledge, the present study is the first to examine the effects of GD exposure in an aged adult 18-month old (approximately 41 human years) rat model.

The objective of the present study was to evaluate the effectiveness of current MCM treatment in an aged rodent model of GD exposure, when treatment with diazepam is delayed to 30 min after seizure onset. Treatment efficacy was determined by measuring epileptogenesis or SRS development and assessing neuropathology following an equitoxic dose of GD and administration of MCM (GD + MCM) in young adult and old adult F344 rats. Our investigations reveal that old adult rats are more susceptible to the acute and chronic toxic effects of GD compared with young adult rats, even after both age groups are administered the same MCM paradigm. GD-exposed old adult rats that receive MCM display a general increase in their susceptibility to develop SRS as compared with young adult rats. These findings may be related to more pronounced decreases in the density of mature neurons in the thalamus, as well as a decrease in the density of GABAergic interneurons within the hippocampus and basolateral amygdala of old adult rats. Our results emphasize the need for further research into the consequences of CWNA exposure in aging animal models for the purpose of identifying more effective therapeutics in elderly victims of CWNA exposure.

MATERIALS AND METHODS

Subjects. Male Fischer 344 (F344) rats were chosen for this study as this strain is provided by the National Institute on Aging breeding colony, and thus is a commonly used animal model in aging research (Gallagher *et al.*, 2011). F344 young adult (2 months of age; initially 300–350 g) and old adult (18 months of age; initially 400–500 g) rats were maintained on a reverse (lights on from 21:00 to 09:00) light–dark schedule, with *ad libitum* access to food and water. A total of 24 young adult and 14 old adult rats were used in the first experiment that focused on estimating the LD_{50} of GD. In the second experiment that examined the efficacy of current MCM on an aged rat model of GD, a total of 19 ($n = 11$, GD; $n = 8$ saline) young adult and 17 ($n = 9$, GD; $n = 8$, saline) old adult rats implanted with telemetry transmitters were used. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense (USAMRICD) 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.

LD_{50} of GD in young adult and old adult male rats. To determine the LD_{50} of GD for each age group, young adult and old adult rats were exposed subcutaneously to GD (70–200 μg/ml, 0.4–0.63 ml/kg) in a stage-wise manner using the methods described by Feder *et al.* (1991a, b,c). GD (pinacolyl methylphosphonofluoridate) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Initially, rats were administered GD dosages expected to span the predicted range of lethality from 0% to 100% for both age groups. Each stage included 1–3 animals randomly assigned to each of the GD dose

levels, and the results from each stage were used to guide dose selection for subsequent stages. This stage-wise approach continued until the ratio of the half width of the 95% confidence interval (CI) defined as (upper bound – lower bound)/(2 × LD_{50}), for the LD_{50} was less than 0.40. The LD_{50} for each age group was calculated by applying a probit regression analysis using the maximum likelihood procedure to the combined data from all stages. The 95% CIs for the LD_{50} were calculated using the delta method.

Surgery. Rats were anesthetized with isoflurane (4%–5% induction, 2.5%–3.5% maintenance) and surgically implanted with a telemetry transmitter (F40-EET, Data Sciences International, Inc [DSI]; New Brighton, MN) for the continuous monitoring and collection of electroencephalographic (EEG) activity and body temperature. For surgical methods, see Moffett, *et al.* (2011) and Schultz, *et al.* (2014). Rats had 2–3 weeks surgical recovery prior to exposure to GD.

Telemetry equipment. For EEG acquisition, animal home cages were placed on top of PhysioTel RPC-1 receivers (DSI) located in the animal colony room, and data were continuously recorded from 2–3 days prior to GD exposure and for 3 months after GD exposure. 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).

GD exposure and treatments. To examine the efficacy of current MCM on an aged rat model of GD exposure, young adult and old adult F344 rats implanted with telemetry transmitters were exposed to 1.2 LD_{50} GD (83.0 and 60.0 μg/kg, respectively) or saline. One minute following exposure, GD-exposed rats received treatments of atropine sulfate (2.0 mg/kg, IM; Sigma Aldrich, St. Louis, MO) and HI-6 dichloride (93.6 mg/kg; IM; Sigma Aldrich); rats in the control group were given saline injections in lieu of MCM. Thirty minutes following seizure onset, diazepam (10.0 mg/kg, SC; Hospira, Inc, Lake Forrest, IL) was administered. Following the exposure day, rats were administered daily subcutaneous injections of saline (6 ml) and given Nutra Gel Diet (BioServ, Flemington, NJ) cubes as needed for up to 2 weeks to reduce dehydration and aid recovery. Animal weights were monitored daily.

EEG scoring. Seizure activity (*SE* and SRS) was defined as rhythmic high-amplitude spikes (>2× baseline) lasting at least 10 s (Nissinen *et al.*, 2001). Seizures were considered terminated when the EEG signal no longer displayed a rhythmic high-amplitude spiking; these determinations were made by an observer that was blind to treatment conditions. Data collected were analyzed using MATLAB algorithms developed for seizure analysis (de Araujo Furtado *et al.*, 2009) and included the time points for initiation, termination, and duration of *SE*, as well as onset of SRS activity. For statistical analyses, seizures lasting longer than 24 h were given a maximal duration score of 1440 min.

Behavioral seizure. The severity of behavioral seizures was assessed using modified Racine scale scores (Racine *et al.*, 1972): 0—no behavioral seizures; 1—masticatory movements, chewing; 2—head myoclonus; 3—forelimb myoclonus; 4—forelimb myoclonus followed by rearing; and 5—falling or generalized tonic–clonic convulsions. Non-Racine scale observations including salivation, lacrimation, urination, diarrhea were also annotated. Observations were made continuously for the first 2 h following GD exposure, and every 30 min from 3 to 5 h after exposure.

Immunohistochemistry. At 3 months after GD exposure, rats were euthanized for collection of whole brains to be processed by immunohistochemistry. Following euthanasia with sodium pentobarbital (75 mg/kg, IP, Fatal Plus; Patterson Veterinary, Greely, CO), rats were perfused with 0.9% sodium chloride in 0.1 M PBS (FD Neurotechnologies, Columbia, MD), followed by 4% paraformaldehyde (PFA, FD Neurotechnologies,) in 0.1 M phosphate buffer (PB). Heparin sodium injection (0.1 ml/l of 1000 Units per ml; Henry Schein Animal Health, Dublin, OH) was added to the PBS used in perfusion. Brains were immediately removed and postfixed for 6 h in 4% PFA at 4°C, cryoprotected for 72 h in 20% sucrose (FD Neurotechnologies) in 0.1 M PB at 4°C, and stored at –80°C until sectioning and staining for neuropathological assessment. Brains were coronally sectioned at a thickness of 50 μm and immunostained for the mature neuronal marker NeuN and the inhibitory neuronal marker glutamate decarboxylase 67 (GAD67). To visualize the neuroinflammatory response following GD exposure and standard MCM treatment, brains 30 μm thick brain slices were processed for immunohistochemistry using an antibody against the astrocytic marker glial fibrillary acidic protein (GFAP). Following the inactivation of endogenous peroxidase activity with hydrogen peroxidase, sections were incubated (free-floating method) for 42 h at 4°C in 0.01 M PBS (pH 7.4) composed of 1% normal horse serum (Vector Laboratories, Burlingame, CA) for NeuN and GAD67 (Vector Laboratories) or normal donkey serum for GFAP (Jackson ImmunoResearch Labs, West Grove, PA), 0.3% Triton X-100 (Sigma-Aldrich), and either mouse anti-NeuN IgG (1:10 000; Millipore, Billerica, MA, Cat. # MAB377), mouse anti-GAD67 IgG (1:2000; Abcam, Cambridge, MA, Cat. # ab26116), or polyclonal rabbit anti-GFAP IgG (1:10 000; DAKO, Carpinteria, CA, Code # Z0334). Immunoreactive tissue was then visualized with the VECTASTAIN Elite ABC kit (Vector Laboratories), according to the manufacturer's instructions (Hsu *et al.*, 1981). Briefly, sections were incubated for 1 h in PBS containing their respective normal blocking serum, Triton X-100 and biotinylated horse antimouse IgG (for NeuN and GAD67) or biotinylated goat anti-rabbit IgG (for GFAP), and then in PBS containing avidin-biotinylated horseradish peroxidase complex for another hour. This was followed by incubation of the sections for 5 min in 0.05 M Tris (TrizmaR base [Sigma-Aldrich]; hydrochloric acid [Fisher Scientific, Pittsburgh, PA]) buffer (pH 7.2) containing 0.03% 3′,3′-diaminobenzidine (Sigma-Aldrich) and 0.0075% hydrogen peroxide (Sigma-Aldrich). All steps were followed by thorough washes in PBS. Sections were mounted on precleaned microscope slides, dehydrated in ethanol (Pharmco, Brookfield, CT), cleared in xylene (Pharmco), and coverslipped in Permount (Fisher Scientific, Fair Lawn, NJ). Sectioning and chromogen immunohistochemical staining were completed by FD Neurotechnologies (Columbia, MD).

Cell counts. Immunostained slides were scanned using an Olympus BX16IVS microscope and photographed with a Pike F-505 camera (Allied Vision, Exton, PA). Surveyor (Objective Imaging Inc; Kansasville, WI) was used to capture images, and Image-Pro Plus (Media Cybernetics, Inc; Rockville, MD) was used to obtain counts of NeuN, GFAP, and GAD67-positive cells in the dorsomedial thalamus, hippocampus, basolateral amygdala, and/or piriform cortex. GFAP cell counts were divided into lightly (nonactivated) and darkly (active) stained cells

(Arisi *et al.*, 2011) to determine the percent of activated astrocytes among the population.

Data analysis. GD LD_{50} was estimated by probit analysis and compared using SAS (SAS Institute Inc; Cary, NC) nonlinear regression and the specialized programs of Feder *et al.* (1991a,b,c). Probit slopes were statistically compared using a Student's *t*-test. Survival curves were compared between ages at each GD dose using the Gehan-Breslow-Wilcoxon method. SPSS v20 (IBM Inc; Armonk, NY), GraphPad Prism v5 (GraphPad Software, Inc; La Jolla, CA), and SigmaPlot v12.5 (Systat Software Inc; San Jose, CA) were used. A Kaplan-Meier survival analysis with Breslow model statistics was used to compare data for time to *SE* and first SRS onset between age groups. Cell counts from immunohistochemistry experiments, as well as *SE* and SRS activity data were examined by means of either Student's *t*-test or 2-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons. Assumptions for Student's *t*-test and 2-way ANOVA were tested. Statistical significance was defined as $p < .05$ for all tests. The senior biostatistician at the USAMRICD was consulted for all statistical analyses performed in the present study.

RESULTS

GD LD_{50} Estimates in Young Adult and Old Adult Male F344 Rats

Studies to determine the LD_{50} for GD in an outbred albino rat strain have previously been performed in 8-month old rats (Shih *et al.*, 1990), but to our knowledge no GD lethality data exists in the F344 strain, one of the aged rat colonies made available by the National Institutes of Aging (National Institutes of Health, Bethesda, MD) for aging-related research. Data from our dose-lethality response study show that 18-month old adult F344 rats were more sensitive to the lethal effects of GD, with an estimated LD_{50} of 50.0 μg/kg (95% CI; 37.8–66.1 μg/kg), compared with young adult rats which showed an estimated LD_{50} of 70.5 μg/kg (95% CI; 63.8–78.0 μg/kg) (Figure 1). There was no statistically significant difference between the slopes of the GD dose–response curves for young adult (13.6 ± 6.3) and old adult (7.3 ± 3.2) rats.

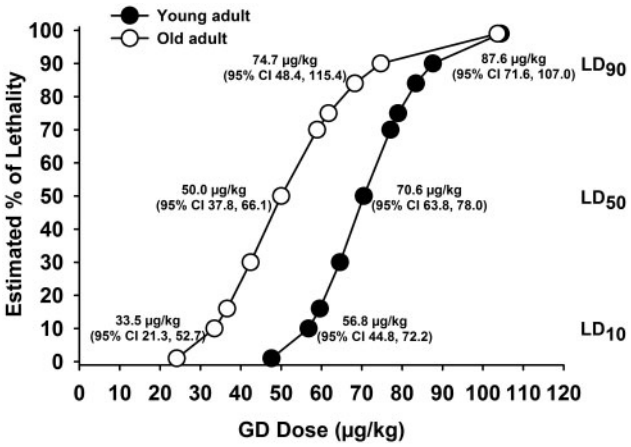

Figure 1. Dose–response curve for GD-induced lethality in young adult and aged male F344 rats. Each point represents the average percent of lethality for young adult (closed circle) and old adult (open circle) F344 rats exposed to a particular dose of GD. At 70.5 μg/mg (95% confidence interval [CI]: 63.8–78.0 μg/kg), the lethal dose (LD_{50}) of young adult rats was 1.41 times greater than that of old adult rats whose LD_{50} was estimated to be 50.0 μg/mg (95% CI: 37.8–66.1 μg/kg). The LD_{10} and LD_{90} are also shown in the graph. No statistically significant difference was found between the slopes of the curves [young adult: 13.6 ± 6.3 (CI: 0.5–27.8); old adult: 7.3 ± 3.2 (CI: 0.9–15.6)]. Young adult, $n = 24$; old adult, $n = 14$.

GD-Induced Seizure Activity in Young Adult and Old Adult F344 Rats Following MCM Treatment

Data from our LD_{50} experiment showed that old adult rats are more susceptible to GD-induced lethality; however, it remained unknown whether there are age-related differences in the effectiveness of MCM when anticonvulsant treatment is delayed to 30 min after seizure onset and seizures become refractory to benzodiazepines. Old adult and young adult rats were exposed to equitoxic 1.2 LD_{50} doses of GD (60.0 μg/kg and 83.0 μg/kg, SC, respectively) followed by atropine sulfate and HI-6 at 1 min after GD exposure, and diazepam was administered at 30 min after onset of *SE*. Using a modified Racine scale score, the degree of severity (highest score) of the GD-induced toxic signs was evaluated for each animal. The severity of toxic signs was similar between GD-exposed young adult (median highest score = 4) and old adult rats (median highest score = 5). In addition, there was no significant difference in the time-to-onset of the first-observed toxic sign (ie, Racine scale score of 1) between old adult (4.8 ± 1.2 min) and young adult (6.5 ± 1.1 min) rats.

The time spent in seizure activity in the first 72 h following GD + MCM was also evaluated. *SE* occurred in 72.7% (8/11 rats) of young adult-GD + MCM animals and 66.7% (6/9 rats) of old adult-GD + MCM animals; no significant difference in the percentage of *SE* occurrence was detected between the young adult-GD + MCM and old adult-GD + MCM rat groups. In addition, no significant difference in the median *SE* onset time was found between young adult-GD + MCM (6 min; $n = 8$) and old adult-GD + MCM (5 min; $n = 6$) F344 rats (Figure 2A). There was no significant difference in the median time spent in seizures during the first 0–24 h, 25–48 h, 49–72 h, and total of 3 days between young adult-GD + MCM and old adult-GD + MCM rats (Figure 2B).

In rats that experienced *SE*, SRS activity was also analyzed and compared between age groups after GD + MCM (Figure 3); development of SRS was not observed in rats that did not present *SE*. A higher percentage of animals in the old adult-GD + MCM with *SE* group (5/6; 83.3%) compared with the young adult-GD + MCM with *SE* group (4/8; 50.0%) developed SRS after GD exposure. It is important to note that 2 young adult-GD + MCM rats died at 3 and 35 days, and 1 old adult-GD + MCM rat died at 20 days after GD exposure and before developing SRS. A significant ($p < .05$) difference in the distribution of latency to the first-observed SRS between young adult-GD + MCM and old adult-GD + MCM rats following GD exposure with old adult-GD + MCM rats presenting SRS at earlier time points, suggesting an effect of age on the time-to-onset of SRS (Figure 3A). No statistically significant difference was found in the number of SRS between young adult-GD + MCM and old adult-GD + MCM rats (Figure 3B).

Differences Between Young Adult and Old Adult Rats in GD-Induced Neuronal and GABA Interneuronal Loss in the Presence of MCM Treatment

Given our observed age-related differences in GD-induced toxicity and propensity for SRS activity despite administration of MCM, we next examined the underlying neuronal loss in young adult and old adult F344 rats. We concentrated our efforts on the examination of the piriform cortex, dorsomedial thalamus, basolateral amygdala, and dorsal hippocampus because GD

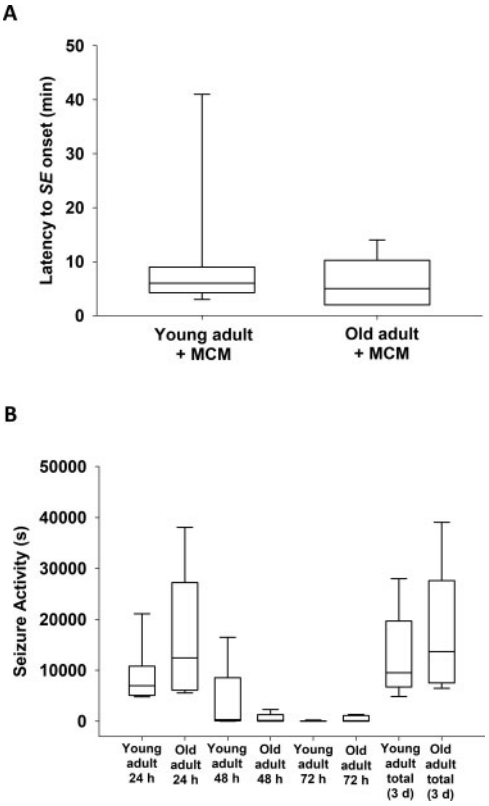

Figure 2. Latency to *status epilepticus* (SE) and early seizure activity in young adult and old adult F344 male rats exposed to 1.2 LD_{50} GD and medical countermeasures (MCMs), followed by delayed administration of diazepam. Graphics represent the median (50th percentile) with 25th and 75th percentiles of (A) latency to SE onset, and (B) time spent in seizure activity during the first 3 days after GD + MCM. Seizure activity was greatest in the first 24 h after GD + MCM, with no statistically significant difference in the median seizure activity between old adult relative to young adult rats. Young adult, $n = 8$; old adult, $n = 6$.

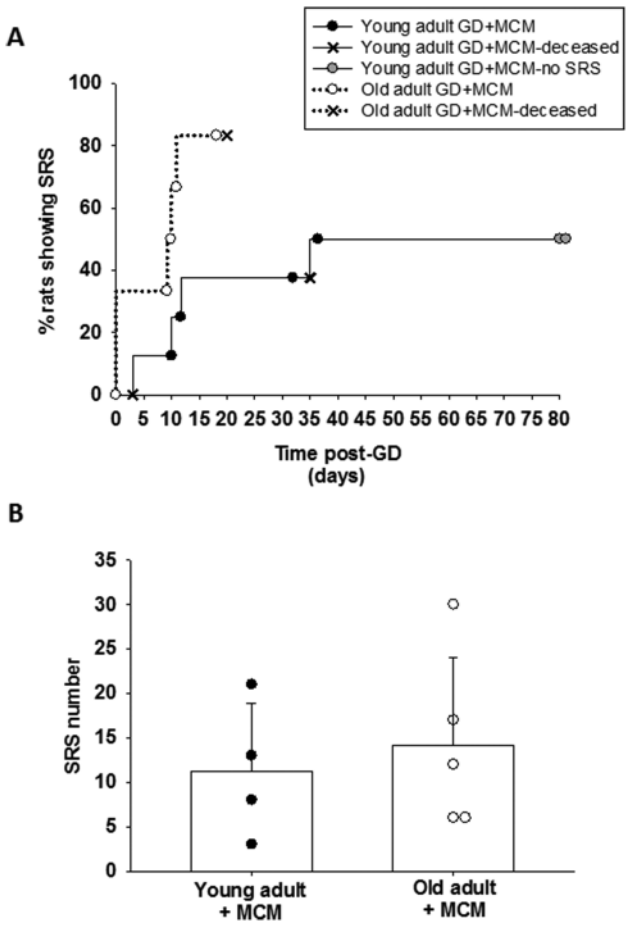

Figure 3. Spontaneous recurrent seizure (SRS) activity in young adult and aged F344 male rats exposed to 1.2 LD_{50} GD and MCMs, followed by delayed administration of diazepam. A, Out of a total of 8 young adult rats that presented SE, 4 rats developed SRS (represented by closed black circle symbol in graph), 2 rats died at 3 and 35 days post GD + MCM (represented by an X symbol in graph), and 2 rats did not develop SRS (represented by gray circle symbol in graph). Out of a total of 6 old adult rats that presented SE, 5 rats developed SRS (represented by open circle symbol in graph) and 1 rat died at 20 days post GD + MCM (represented by X symbol in graph). A significant main effect of GD + MCM on the onset of SRS was found, with the old adult group of rats having a higher risk of SRS onset; young adult $n = 8$, aged $n = 6$; $^*p < .05$, Kaplan-Meier survival analysis with Breslow test. Old adult rats showed a shorter time-to-onset of first-observed SRS. B, There was no significant difference in the mean number of SRS ± SD up to approximately 55 days following exposure to 1.2 LD_{50} GD + MCM. Young adult, $n = 4$; old adult, $n = 5$.

exposure in rats that leads to prolonged seizure activity induces neuronal damage in these areas (Carpentier *et al.*, 1990; de Araujo Furtado *et al.*, 2012; Lemercier *et al.*, 1983; Petras, 1994; Schultz *et al.*, 2014, 2012). Using antibodies against a neuron-specific marker (NeuN) and a GABAergic neuronal marker (GAD67), we visualized the overall populations of mature neurons and inhibitory interneurons to determine if the neuronal damage caused by GD exposure differed between our old adult and young adult groups following MCM treatment. GABAergic interneurons play a crucial role in excitotoxicity and seizure activity (Botterill *et al.*, 2017; During and Spencer, 1993; Shetty and Upadhya, 2016); thus, we evaluated the loss of inhibitory neurons in young adult-GD + MCM and old adult-GD + MCM F344 rats.

GD + MCM had a significant main effect on the density of NeuN-positive (NeuN+) cells in the dorsomedial thalamus (Figs. 4A and 4B; $F(1, 1) = 40.7$, $p < .001$), layer 3 of the piriform complex (Figure 4C; $F(1, 1) = 21.1$, $p < .001$), and the basolateral amygdala (Figure 4D; $F(1, 1) = 31.8$, $p < .001$), indicating that both age groups present significant GD-induced loss of mature neurons in these regions even after administration of MCM. In the medial thalamus, young adult-GD + MCM ($p = .006$) and old adult-GD + MCM ($p < .001$) rats showed a reduction in NeuN+ cell density compared with rats in the age-matched saline groups (Figure 4B). In layer 3 of the piriform cortex, young adult-GD + MCM ($p < .001$) and old adult-GD + MCM ($p = .017$) rats showed a reduction in NeuN+ cell density compared with rats in the age-matched saline groups (Figure 4C). In the basolateral amygdala, young adult-GD + MCM ($p = .001$) and old adult-GD + MCM ($p < .001$) rats showed a reduction in NeuN+ cell density compared with rats in the saline groups (Figure 4D). Although no main effect of age was detected in the piriform cortex and basolateral amygdala, a significant interaction between the age and exposure factors was detected in the dorsomedial thalamus ($F(1, 1) = 11.3$, $p = .003$). Multiple group comparisons show that in the medial thalamus old adult-GD + MCM rats had a more pronounced decrease in the density of mature neurons compared with young adult-GD + MCM rats ($p < .001$) (Figure 4B).

An antibody against GAD67, commonly used as a selective marker of neurons that synthesize the neurotransmitter GABA (Almeida-Suhett *et al.*, 2015; Di Maio *et al.*, 2015), was used to evaluate the loss of inhibitory neurons resulting from

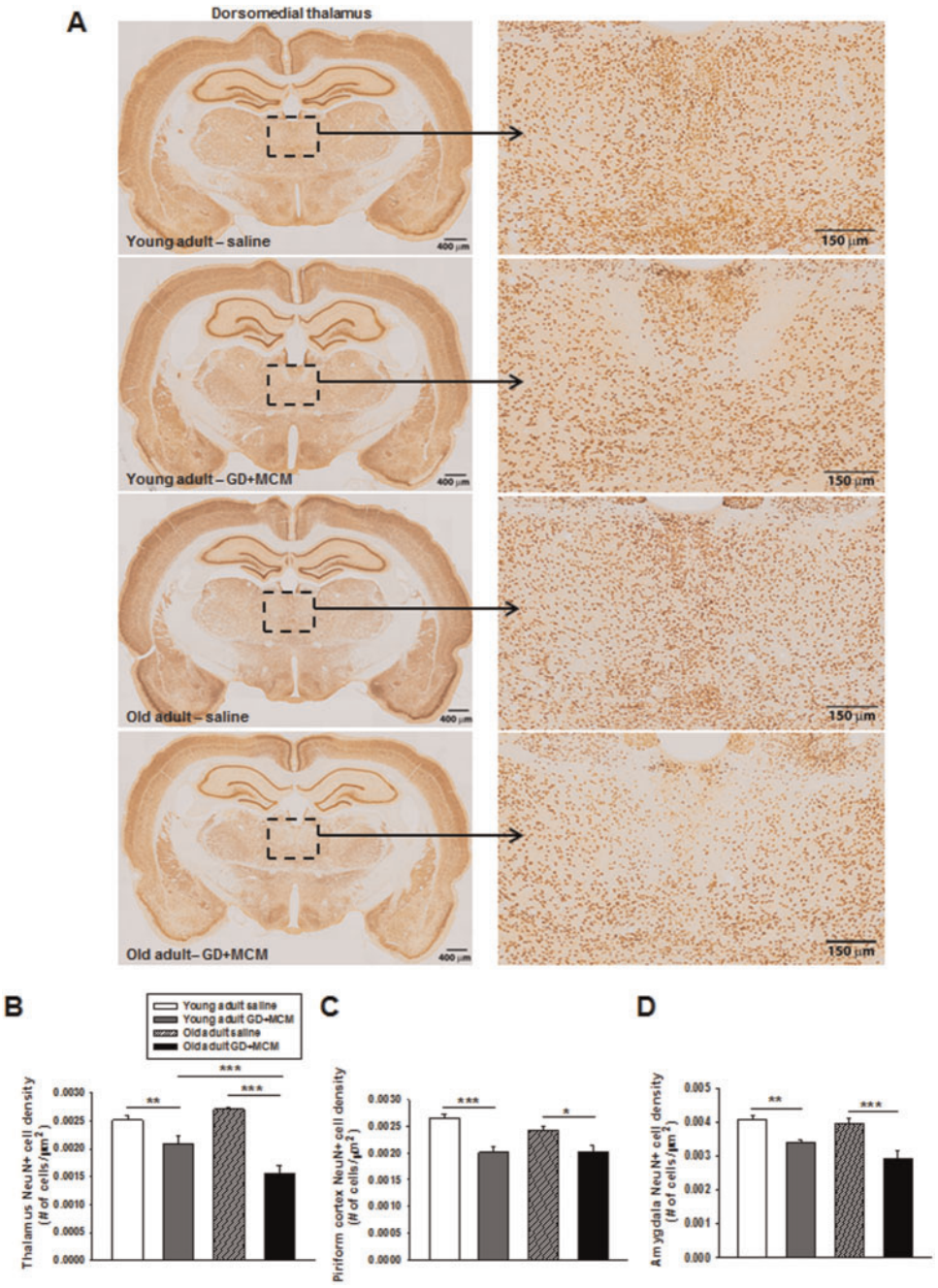

Figure 4. Increased neuronal cell loss in the medial thalamus of aged F344 male rats at 90 days after exposure to 1.2 LD_{50} GD and MCMs. A, Brain sections from young adult and aged rats exposed to GD + MCM (young adult-GD + MCM and old adult-GD + MCM, respectively) and presenting SE and SRS were immunostained for NeuN to visualize mature neurons; representative images of coronal sections (approximately bregma −3.20 mm) and medial thalamus (close-up) are shown. Graphs show mean ± SD of NeuN-positive (NeuN+) cell density in (B) dorsomedial thalamus, (C) piriform cortex, and (D) basolateral amygdala. GD-induced neuronal loss in young adult-GD + MCM and old adult-GD + MCM rats in the medial thalamus, piriform cortex, and basolateral amygdala as compared with age-matched saline counterparts. In the dorsomedial thalamus, old adult-GD + MCM rats GD + MCM presented a significantly greater loss of neurons as compared with young adult GD + MCM animals. $^{**}p < .01$, $^{***}p < .001$, 2-way ANOVA with Tukey's test. Young adult ($n = 4$, GD + MCM; $n = 8$ saline); old adult ($n = 4$, GD + MCM; $n = 6$ saline).

GD + MCM exposure in young adult and old adult rats (Figure 5). GD + MCM administration had a main effect on the density of GAD67-positive (GAD67+) neurons in the piriform cortex ($F(1, 1) = 29.2$, $p < .001$) (Figs. 5A and 5D) and basolateral amygdala ($F(1, 1) = 26.7$, $p < .001$] (Figs. 5B and 5E) of young adult and old adult rats 3 months after exposure. Multiple group comparisons show that in layer 3 of the piriform cortex, young adult-GD + MCM ($p = .011$) and old adult-GD + MCM ($p = .001$) rats showed a reduction in GAD67+ cell density compared with rats in age-matched saline groups (Figs. 5A and 5D), albeit old adult rats presented a more pronounced loss of GAD67+ cells in this brain region than young adult rats in GD + MCM groups ($p = .05$). In the basolateral amygdala, young adult-GD + MCM ($p < .001$) and old adult-GD + MCM ($p = .01$) rats showed a reduction in GAD67+ cell density compared with rats in the saline groups (Figs. 5B and 5E). A statistically significant age-related effect in GAD67+ cell density

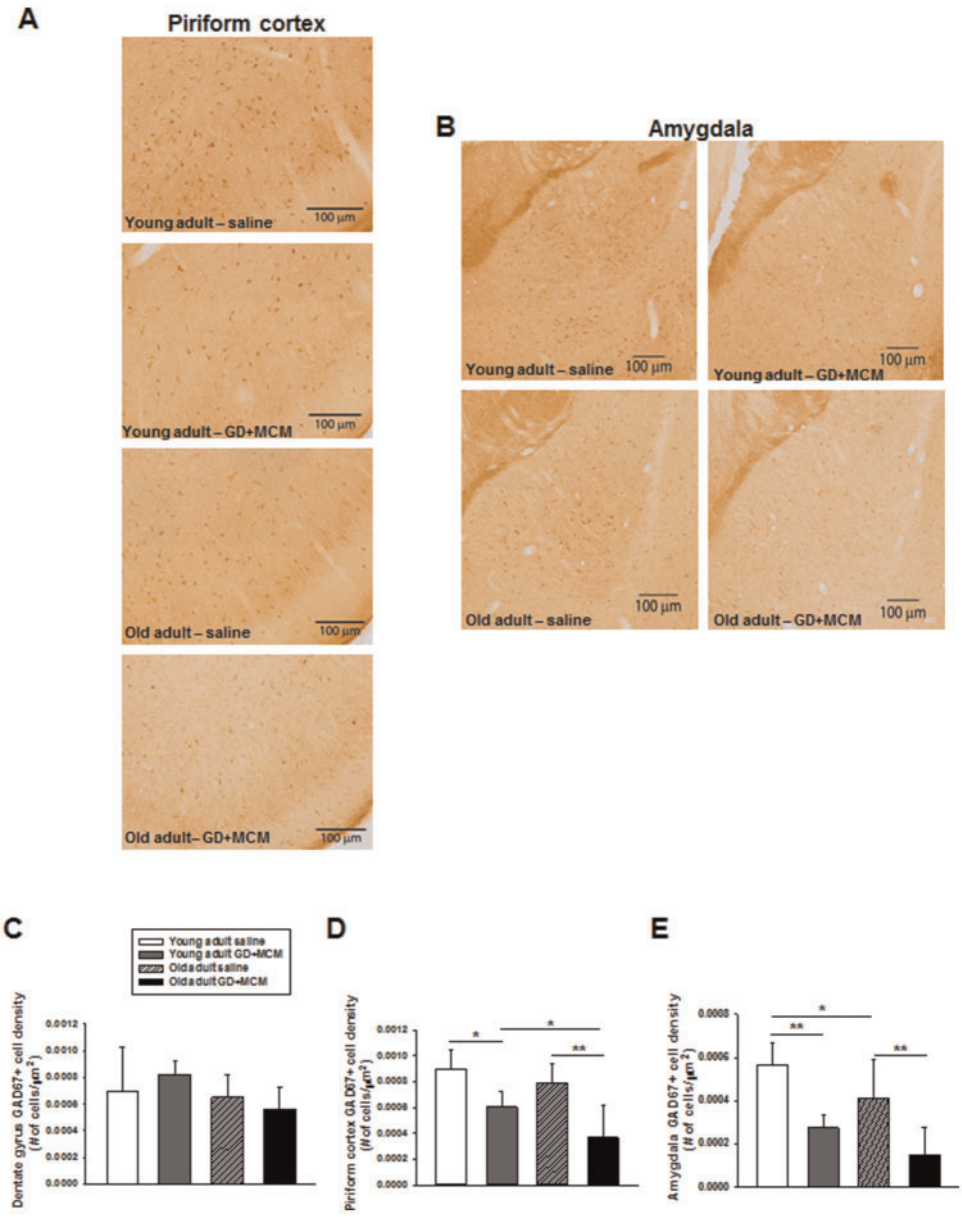

Figure 5. Decreased inhibitory input in the piriform cortex of aged F344 male rats at 90 days after exposure to 1.2 LD_{50} GD and MCMs. A and B, Brain sections from young adult and old adult rats exposed to GD + MCM and presenting SE and SRS were immunostained for glutamic acid decarboxylase 67 (GAD67) to visualize inhibitory interneurons; representative images (Bregma –3.20 mm) of the piriform cortex and basolateral amygdala are shown. Graphs show mean ± SD of GAD67-positive (GAD67+) cell density in the dentate gyrus of hippocampus (C), the piriform cortex (D), and the basolateral amygdala (E). GD + MCM induced the loss of GABA interneurons in both young adult and old adult rats in the piriform cortex and basolateral amygdala, with significantly greater loss of interneurons in the piriform cortex of old adult rats compared with young adult rats. In addition, old adult (saline) control rats had fewer GABA interneurons in the basolateral amygdala compared with young adult (saline) control rats. $^{*}p < .05$, $^{**}p < .01$, two-way ANOVA followed by a Tukey's test. Young adult ($n = 4$, GD; $n = 8$ saline); old adult ($n = 4$, GD; $n = 6$ saline).

was also found in the basolateral amygdala ($F(1, 1) = 5.26$, $p = .036$; Figure 5E), with a multiple group comparison analysis showing that old adult-saline rats had a lower density of GAD67+ cells compared with young adult-saline rats ($p = .04$). No significant main effect of GD + MCM on GAD67+ cell density was found in the dentate gyrus of the hippocampus (Figure 5C).

GD-Induced Astrocyte Activation in Young Adult and Old Adult Rats in the Presence of MCM

Astrocytes become reactive as a result of CNS neuropathology and this activation is commonly used to evaluate the degree of the neuroinflammatory response (Pekny and Nilsson, 2005). We evaluated the magnitude of the GD-induced neuroinflammatory response in the hippocampus, piriform cortex, and basolateral amygdala after administration of MCM by measuring the percent of activated (darkly GFAP-stained) astrocytes at 3 months after GD exposure to determine how GD + MCM and age, as well as the interaction of the two, affect the neuroinflammatory response (Figure 6). Although the hippocampus displayed no statistically significant changes in astrocytic activation from GD + MCM (Figure 6B), there was a main effect of GD + MCM on the percentage of activated GFAP-positive (GFAP+) cells in layer 3 of the piriform cortex ($F(1, 1) = 41.7$, $p < .001$] (Figs. 6A and 6C) and the basolateral amygdala

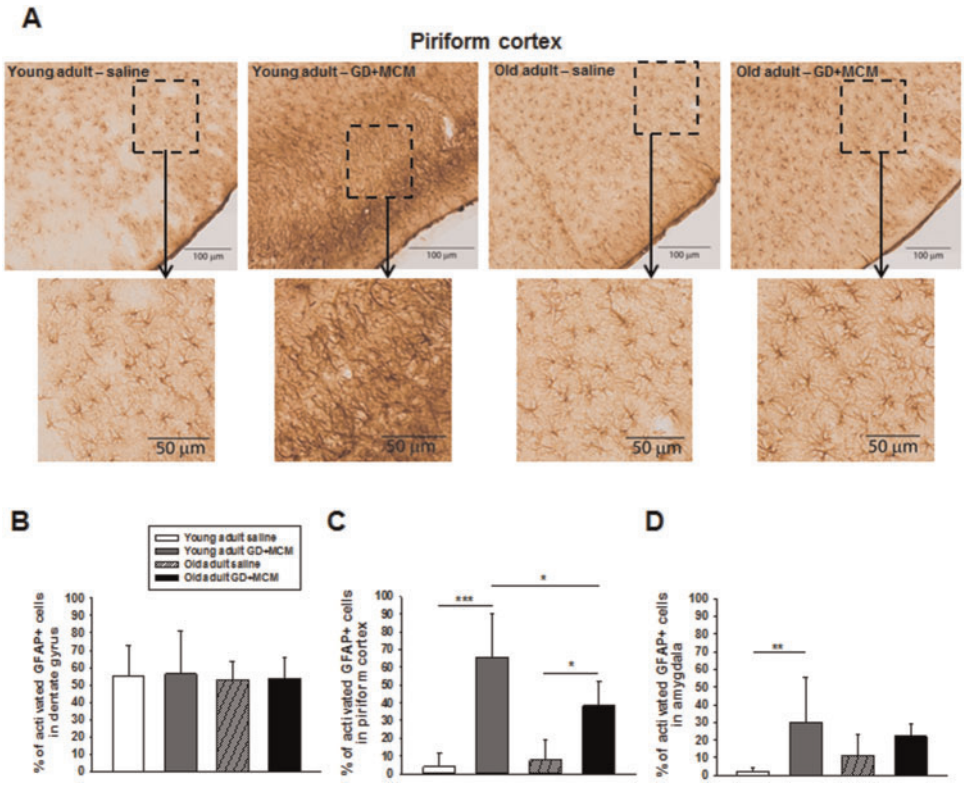

Figure 6. Representative images (approximately Bregma –3.20 mm) of GFAP+ neurons in the piriform cortex, and graphics of mean ± SD percent of activated GFAP+ cell density in (B) hippocampus, (A and C) piriform cortex, and (D) basolateral amygdala of young adult and old adult F344 male rats 90 days following exposure to 1.2 LD_{50} GD and administration of MCM. Astrocyte activation was determined by the observation of changes in their appearance from lightly stained and thinner (resting state), to darker and thicker (activated state). GD + MCM resulted in an increased percentage of activated GFAP+ cells in the piriform cortex of both young adult and old adult rats, with aged GD + MCM animals displaying a significantly less astrocyte activation compared with young adult GD + MCM. Only young adult rats displayed an increase in astrocyte activation in the basolateral amygdala (D). No significant difference in the percentage of astrocyte activation was detected in the hippocampus of either young adult or old adult rats after GD + MCM (B). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, 2-way ANOVA followed by a Tukey's test. Young adult ($n = 4$, GD; $n = 8$ saline); old adult ($n = 4$, GD; $n = 6$ saline).

($F(1, 1) = 7.63$, $p = .012$] (Figure 6D). GD + MCM resulted in a significant increase in the percent of activated astrocytes in the piriform cortex (Figs. 6A and 6C; $p < .001$) and basolateral amygdala (Figure 6D; $p = .007$) of young adult-GD + MCM rats, whereas in old adult-GD + MCM rats astrocytic activation was significantly increased only in the piriform cortex ($p = .01$).

DISCUSSION

The aims of the present study were to evaluate the effectiveness of delayed anticonvulsant treatment in aged rodent model of GD exposure, and compare the outcomes to those observed in a young adult rat model. Three overall measurements were evaluated in old adult and young adult F344 male rats as a way to assess the effectiveness of the delayed treatment paradigm: (1) the onset and duration of behavioral seizures, *SE*, and early recurrent seizures; (2) the percentage of animals developing SRS, the time of SRS onset and SRS frequency; and (3) the degree of mature neuronal cell loss, inhibitory interneuron loss, and activation of the neuroinflammatory response in key brain regions involved in seizure activity.

The determination of each age group's LD_{50} dose of GD was imperative in the present study for the subsequent comparison of toxic effects using an equitoxic dose. Our first experiment demonstrated that old adult F344 rats are more susceptible to lethality compared with young adult F344 rats following GD exposure. The LD_{50} doses for GD in F344 young adult and old adult rats were determined to be 70.5 and 50.0 µg/kg, respectively. A similar increase in susceptibility to GD toxicity with age occurs in aged outbred male albino rats with 2-month-old rats showing a higher LD_{50} (87 µg/kg) compared with that of 8-month-old rats (59 µg/kg) (Shih *et al.*, 1990). The slight differences in LD_{50} doses between outbred male albino and F344 rats could be due to differences in age (8-month vs 18-month old rats, the latter considered an aged model), variability in genetic background (inbred F344 vs outbred albino strain) or differences in routes of nerve agent administration (SC vs IM), the latter is considered to have faster physiological distribution (Taylor *et al.*, 2011).

The delay of anticonvulsant administration leads to the development of benzodiazepine-resistant seizures that exacerbate the neuropathology and cognitive deficits that result from CWNA exposure. Because delayed anticonvulsant treatment is highly likely in cases of CWNA poisoning of unprepared civilian populations, our second set of experiments focused on evaluating the efficacy of standard MCM with delayed anticonvulsant treatment in the F344 rats after exposure to an equitoxic dose of GD. The consequences of GD exposure on toxic signs, acute seizure activity, epileptogenesis, and neuropathology in the old adult group of rats were compared with those observed in the young adult animal model. We did not observe a higher severity of toxic signs as measured by the Racine scale in our old adult

rat group, as was reported by Shih *et al.* (1990). This difference is likely a result of the fact that our animals received atropine and HI-6 1 min following exposure to GD and diazepam 30 min after *SE* onset, whereas rats in the Shih *et al.* study received no treatment, although strain differences and age of "aged" rats may also have factored into the different findings. Similarly, Apland *et al.* (2017) did not observe a higher seizure severity score in 10-month-old rats (equivalent to 25 human years) compared with 2-month-old rats exposed to GD; rats in their study received atropine and HI-6 20 min after seizure onset. Similar to our study, the older adult rats were administered an equitoxic dose of 1.2 LD_{50} with the older rats receiving less GD compared with the younger adult rats based on differing LD_{50} between the ages. Greater GD-induced lethality occurred in 10-month-old rats compared with the 2-month-old rats that developed *SE* despite fewer older rats developing *SE*. In contrast to Apland *et al.* (2017), old adult rats in our study did not have a significant difference in time to onset of *SE*. We also did not observe any difference between groups in the time spent in seizures.

Novel about our study is that we evaluated the development of SRS over the months following GD exposure in 18-month-old rats compared with 2-month-old rats, demonstrating increased susceptibility to epileptogenesis in GD-exposed old adult rats. Old adult animals that experienced *SE* displayed an increase in their susceptibility to developing SRS, demonstrated by the higher percentage of old adult rats presenting with SRS and shorter time-to-onset of SRS compared with young adult rats. Our observations are similar to reports of aged animal models of traumatic brain injury that show an increased vulnerability to developing chronic epileptogenic activity after administration of ferric chloride (Jyoti *et al.*, 2009). One explanation for the higher percentage of animals in the old adult-GD + MCM group developing SRS is the comparatively greater loss of mature neurons in the dorsomedial thalamus of aged rats compared with young adult rats. The dorsomedial thalamus is known to play a critical role in seizure activity in both animals and humans, with neuronal damage in this region being a known contributor to the onset and reoccurrence of seizures (Bertram *et al.*, 2001; Cassidy and Gale, 1998; Kelemen *et al.*, 2006). Furthermore, *SE* leads to neuronal damage in the dorsomedial thalamus (Kubova *et al.*, 2001; Moffett *et al.*, 2011), which may help explain why a larger percentage of old adult rats experienced SRS following the development of *SE*. More time in initial *SE* correlates with development of SRS and greater damage in the hippocampus (de Araujo Furtado *et al.*, 2010). In the current study, old adult rats had a tendency to spend more time in initial seizure, but this did not reach statistical significance.

Another possible contributor to the higher incidence of SRS development and a shorter time-to-onset to first SRS in the old adult-GD + MCM group is the age-related reduction of inhibitory interneurons within the basolateral amygdala of old adult-saline rats compared with young adult-saline. Studies from Aroniadou-Anderjaska *et al.* (2016) have suggested that the massive GD-induced loss of inhibitory neurons in the basolateral amygdala results in hyperexcitability of this brain region which underlies the development of SRS. Even though both young adult- and old adult-GD + MCM rats suffered a GD-induced loss of inhibitory neurons in the basolateral amygdala, the reduction in inhibitory input in the basolateral amygdala of old adult rats may have had a more severe outcome in this population than in the young adult rat group, as GABAergic neurons play a vital role in neuronal excitation and seizure activity (Botterill *et al.*, 2017; During and Spencer, 1993; Shetty and Upadhya, 2016). However, it is important to note that the observed decrease in the number of GAD67+ cells within the basolateral amygdala could also be due to a reduction in the expression of GAD which would result in lighter staining of the inhibitory interneurons. We are unaware of any reports on the effect of aging on the expression of GAD protein in the amygdala; mRNA and immunohistochemical analyses show decrease in GAD protein expression in the hippocampus of aged rats (Stanley and Shetty, 2004; Vela *et al.*, 2003). As GABAergic signaling within the amygdala plays a vital role in seizure regulation, a reduction in GAD protein levels could result in an overall decrease in GABA production within these critical brain nuclei, likely contributing to the increased presence of seizures in our middle-age group. In support of this suggestion are the observations that: (1) GAD67 critically controls GABA synthesis (Lau and Murthy, 2012), with GAD67 knockout mice expressing a greater than 90% reduction in basal GABA levels in the brain (Asada *et al.*, 1997; Condie *et al.*, 1997; Heldt *et al.*, 2012) and (2) GAD67 levels sharply decline before convulsions (Li *et al.*, 2008). Additionally, middle-age animals exposed to GD and given standard MCM exhibited a significantly more pronounced loss of inhibitory neurons in layer 3 of the piriform cortex. The piriform cortex has been implicated in the propagation of seizures within the limbic system (Loscher and Ebert, 1996; Vaughan and Jackson, 2014) and, thus, the reduction of inhibitory input by the greater loss of GABAergic neurons in old adult-GD + MCM compared with young adult-GD + MCM rats may also underlie the etiology of the increase in the potential for the development of chronic spontaneous seizure activity.

A third possible explanation of increased number of old adult rats developing SRS is that astrocyte function appeared compromised within the piriform cortex of old adult-GD + MCM rats. GD exposure is known to activate astrocytes within the piriform cortex (Zimmer *et al.*, 1997). Furthermore, astrocyte gliosis is believed to be an adaptive response to seizures (Khurgel and Ivy, 1996), and dysfunctional astrocytes are an important contributor to seizure activity (Coulter and Steinhauser, 2015), which may partially account for the increase in SRS in our old adult animal group.

Our work reveals age-related increases in the susceptibility to GD-induced lethality, and development of chronic spontaneous seizure activity and neuropathology in old adult F344 rats exposed to GD and treated with MCM. Coupled with studies investigating an age-related increase in human mortality following *SE* (Towne, 2007), this cumulative information suggests that an aged human population is likely more vulnerable to the lethal and toxic effects of GD exposure and may respond more poorly to delayed treatment with diazepam than would young adults.

CONCLUSION

The elderly are expected to increase as a percent of population (Lutz *et al.*, 2008; U.S. Census Bureau, 2011), generating a critical need for research into the unique challenges associated with MCM for CWNA exposure in the elderly. Based on our findings in animal studies, aged human populations are likely more vulnerable to GD-induced toxicity and neuropathology, even when MCM are administered. Because civilians do not carry CWNA antidotes, delayed therapy with anticonvulsants is anticipated in the event of a terrorist attack against civilians. As *SE* is refractory to benzodiazepine anticonvulsants when treatment is delayed (Lallement *et al.*, 1998; Niquet *et al.*, 2016; Shih *et al.*, 1999) and aged populations are more susceptible to *SE* (Mauricio and Freeman, 2011), future studies should also include both old

adult and senescent animal models in assessing the efficacy of MCM against CWNA exposure. In addition, as there are increasing numbers of females in the military and because females make up a large percentage of the civilian population, future studies should also include female subjects (perimenopausal, postmenopausal and senescent) when evaluating chemical agent toxicity and efficacy of MCM.

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