CURRICULUM VITAE

Erica Kundrick

Degree and Date to be Conferred: Master of Science, 2019

Collegiate Institutions Attended:

University of Maryland, Baltimore
Master of Science, Toxicology
December 2019

University of Pittsburgh
Bachelors of Science, Neuroscience
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April 2015

Research Experience:

February 2016-Present
Student Research Intern.
U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD
Mentor: Dr. Lucille Lumley Lange

- Performing rodent surgeries for the implantation of EEG transmitter devices.
- Collection and analysis of signs of toxicity in rodents that have been exposed to chemical warfare nerve agent.
- Performing euthanasia and perfusions on rodents for brain extraction and other tissue collection.
- Analysis of immunostained brain tissue using stereology microscopy technique.

January 2014-April 2015
Student Researcher.
University of Pittsburgh, Pittsburgh, PA
Mentor: Dr. Sarah Ross

- Kept a laboratory notebook and attended weekly lab meetings.
- Sectioned tissue samples using a cryostat and performed subsequent immunohistochemistry.
- Imaged stained slides using a fluorescence microscope and subsequent identification of cell types imaged.
- Production of a senior thesis using the results from the independent project.

January 2012-April 2012
Student Researcher.
Mentor: Dr. Raymond Pitetti and Dr. Kim Horner

Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center, Pittsburgh, PA

- Compiled a list emergency room patient records from the years 2007-2011 that indicated the patient experienced an apparent life-threatening event.
- Examined the records further for information on lab results, age at time of visit and patient demographics.
- Presented poster on research at the First Experiences in Research student symposium.

Professional Publications

Publications

Marrero-Rosado B, de Araujo Furtado M, Schultz CR, Stone M, **Kundrick E**, Walker K, O'Brien S, Du F, Lumley LA. *Soman-induced status epilepticus, epileptogenesis, and neuropathology in carboxylesterase knockout mice treated with midazolam.* Epilepsia. 2018; 59:2206-2218.

Abstracts and Posters

Stone M, **Kundrick E**, Marrero-Rosado B, Schultz C, Walker K, Matson E, DeBus S, de Araujo Furtado M, Cadieux C.L, Lumley L. *Soman-induced cell death and neuroinflammatory response in human acetylcholinesterase knock-in serum carboxylesterase knockout mice.* Abstract and Poster. *Society for Neuroscience Annual Meeting.* Chicago, IL. October 2019

Kundrick E, Marrero-Rosado B, De Araujo Furtado M, Stone M, Schultz S, Walker K, O'Brien S, Lee R, Lumley L. *Delayed treatment with midazolam increases survival but is not fully protective against soman-induced epileptogenesis and neuropathology in male and female carboxylesterase knockout mice.* Abstract and Poster, *Society of Toxicology Annual Meeting*. Baltimore, MD. March 2019

Walker K, Pennington M, Armstrong S, Litvin S, Stone M, Schultz C, O'Brien S, **Kundrick E**, Marrero-Rosado B, Lumley L. *Comparison of plasma pharmacokinetics of intravenous and intramuscular administration of midazolam in male Sprague-Dawley rats.* Abstract and Poster, *Society of Toxicology Annual Meeting*. Baltimore, MD. March 2019

Marrero-Rosado B, Rossetti F, Schultz C, **Kundrick E**, Stone M, O'Brien S, Walker K, Lumley L. *Evaluation of cannabinoids for anticonvulsant and antiepileptic efficacy in a rat model of soman-induced status epilepticus.* Abstract and Poster, *Society for Neuroscience Annual Meeting*. San Diego, CA. November 2018

Schultz C, Marrero-Rosado B, Stone M, **Kundrick E**, Walker K, O'Brien S, De Araujo Furtado M, Lumley L, *Epileptogenesis, Neuroinflammation and Neuronal Loss Following Soman Exposure in Carboxylesterase Knockout Mice Treated with*

Midazolam. *Abstract and Poster, Bioscience Review.* Aberdeen Proving Grounds, MD May 2018

Marrero-Rosado B, Schultz C, Stone M, Rossetti F, Walker K, O'Brien S, **Kundrick E**, De Araujo Furtado M, Lumley L, *Rodent Models of Soman-Induced Epileptogenesis and Brain Pathology for Assessment of Neuroprotectants.* Abstract and Poster, *Bioscience Review*. Aberdeen Proving Grounds, MD. May 2018

Lumley L, Marrero-Rosado B, Du F, Schultz C**,** Stone M, **Kundrick E**, Rice M, De Araujo Furtado M, *Somen-Induced Epileptogenesis, Neuroinflammation and Neuronal Loss in Carboxylesterase Knockout Mice Treated with Midazolam.* Abstract and Poster, *American Epilepsy Society*. Washington D. C. December 2017

Marrero-Rosado B, Schultz C, Stone M, **Kundrick E**, Stone M, O'Brien S, Walker K, Rossetti F, Lumley L. *Evaluation of cannabinoids for anticonvulsant and neuroprotective efficacy in a rat model of soman-induced status epilepticus.* Abstract and Poster, *Society for Neuroscience Annual Meeting*. Washington, DC. November 2017

Marrero-Rosado B, Schultz C, Stone M, **Kundrick E**, Walker K, O'Brien S, Lumley L *Evaluation of Cannabinoids as Adjunct to Standard Therapy for Soman-Induced Toxicity in Rats*. *E-Poster*, CounterACT. Boston, MA June 2017

Lumley L, Franco Rossetti, Stone M, Schultz C, **Kundrick E**, Walker K, O'Brien S, Marrero-Rosado B, Rice M, Niquet J, Wasterlain C. *Efficacious treatment of soman-induced status epilepticus with synergistic drug combinations. Abstract,* CBRNE 2nd International Conference. Lyon, France. May 2017

Stone M, Schultz C, Rossetti F, Walker K, **Kundrick E**, O'Brien S, Marrero-Rosado B, Niquet J, Wasterlain C, Lumley L. *Anticonvulsant Drug Polytherapy Stops status epilepticus and Prevents Neuronal Loss in Soman-exposed Rats. Abstract and* Poster, Experimental Biology Conference. Chicago, IL. April 2017

Professional Certifications and Development

Medical Management of Chemical and Biological Casualties Course Certificate *Aug 2016*
Licensed Pharmacy Technician *Sept 2015- Jan 2017*

Honors and Awards

Dean's List Spring Semester *2015*
Sigma Alpha Lambda National Leadership and Honors Organization *2012*
Maryland Distinguished Scholar Honorable Mention *2011*

ABSTRACT

Title of Thesis: Sex differences in soman-induced toxicity and response to medical countermeasures in serum carboxylesterase knockout mice.

Erica Kundrick, Master of Science, 2019

Thesis Directed by: Dr. Lucille Lumley, Principal Investigator, U.S. Army Medical

In rodents, exposure to chemical warfare nerve agent soman leads to status epilepticus and extensive neuronal loss. Mice and rats are less sensitive to nerve agent toxicity compared to primates since high levels of plasma carboxylesterase, which acts as a bioscavenger against soman, increase resistance of these rodents to organophosphorus poisoning. One objective of this research project was to determine the LD_{50}s of soman in female plasma carboxylesterase knockout ($ES1^{-/-}$) mice at the different stages of their estrous cycle and to compare toxicity across estrous and with male mice. Female mice in estrus were less susceptible to the soman lethality compared to female mice in proestrus and to male mice. The second objective was to evaluate dose-response effects of delayed midazolam treatment in soman-exposed $ES1^{-/-}$ mice. Delayed midazolam dose-dependently increased survival and reduced seizure severity but did not prevent epileptogenesis or brain pathology in seizure-sensitive brain regions, independent of sex.

Sex Differences in Soman-Induced Toxicity and Response to Medical Countermeasures in Serum Carboxylesterase Knockout Mice.

by
Erica Kundrick

Thesis submitted to the faculty of the Graduate School
of the University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
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Disclaimer.

The views expressed in this thesis are those of the author and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. The experimental protocol is approved by the Institutional Animal Care and Use Committee at the 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.

Table of Contents

List of Tables

List of Figures

CHAPTER 1: INTRODUCTION

Chemical warfare nerve agents such as soman are organophosphorus compounds that irreversibly inhibit acetylcholinesterase, an enzyme that breaks down the neurotransmitter acetylcholine. This inhibition causes buildup of acetylcholine and overstimulation of cholinergic receptors in both the central and peripheral nervous systems (reviewed in McDonough and Shih 1997). This cholinergic receptor overstimulation can lead to peripheral symptoms including hypersecretions, tremors, and muscular fasciculations. This cholinergic overstimulation also occurs in cortical and limbic regions of the brain, leading to seizures which can develop into status epilepticus, a form of prolonged and self-sustaining seizure activity. The current treatment regimen for nerve agent poisoning includes the muscarinic receptor antagonist atropine, an oxime (e.g. 2-PAM or HI-6) to reactivate nerve agent- inhibited acetylcholinesterase, and a benzodiazepine (e.g. diazepam) as needed for treatment of seizures (reviewed in Zilker 2005).

Treatment of nerve agent-induced seizures can be difficult as status epilepticus becomes refractory to benzodiazepine treatment, and even after termination of exposure, seizures can last for hours and spontaneously recur (Niquet et al. 2019 a,b). Nerve agent-induced status epilepticus can lead to brain damage in neocortical and limbic regions via excitotoxicity, oxidative stress and neuroinflammation (de Araujo Furtado et al. 2012). Following delayed benzodiazepine treatment in rodent models of nerve agent-induced status epilepticus, long-term behavioral and cognitive deficits as well as spontaneous recurrent seizures ensue (de Araujo Furtado et al. 2010, Moffett et al. 2011, Schultz et al. 2012, Schultz et al. 2014, reviewed in Aroniadou-Anderjaska et al. 2016, Marrero-Rosado et al. 2018). There is also evidence that status epilepticus induces a

neuroinflammatory response that can contribute to sustain epileptogenesis and neuronal cell loss (Block et al. 2007, Vezzani et al. 2011, Wang and Chen 2018, Terrone et al. 2019).

Approval by the U.S. Food and Drug Administration (FDA) of novel medical countermeasures against nerve agent poisoning relies on the "Animal Rule" which allows the use of efficacy data exclusively from animal models for advancement of treatments against conditions that make human efficacy trials unethical and unfeasible. It is vital that animal models exhibit similar levels of toxicity as well as manifestation of symptoms and pathologies of nerve agent poisoning (reviewed in Pereira et al. 2014). Mice and rats are commonly used for studies of nerve agent-induced toxicity in part because of their short gestation time and life span, as well as the availability of robust behavior and neurological characterization and validation of behavioral tests in these animals. One issue with mouse and rat models in toxicity studies of organophosphorus compounds, however, is that these rodents have high levels of plasma carboxylesterase, whereas humans do not (Li et al. 2005). Carboxylesterases irreversibly bind, and, as such, reduce the bioavailability of organophosphorus compounds to bind to and inhibit acetylcholinesterase (Maxwell et al. 1987). This bioscavenger detoxification mechanism causes mice and rats to be less susceptible to the toxic effects of nerve agents, including soman as compared to other species, including humans, which have lower levels of plasma carboxylesterase. Recently, a mouse model was developed that specifically lacks plasma carboxylesterase (Duysen et al. 2011). In male plasma carboxylesterase knockout (ES1-/-) mice, the median lethal dose (LD_{50}) of soman is approximately 4-fold lower than that of male wild-type mice (Marrero-Rosado et al. 2018).

Most preclinical research is carried out in male rodents, but there is increasing evidence that sex differences occur in susceptibility and severity as well as neuronal sensitivity to toxic insults, highlighting the need for inclusion of both sexes in research studies (Clayton and Collins 2014). The biological factor of sex and its effect on toxicity of nerve agents are typically not taken into account with very few studies examining both sexes. When studied, female rodents appear to be more sensitive to the toxic effects of different chemical insults (Lipnick et al. 1995). However, the few studies that have examined the toxic effects of nerve agent exposure in male and female rodents have reported contradicting results. In addition, even fewer studies have taken into account the potential influence of the estrous cycle on nerve agent toxicity. A recent study reported that female mice from eight strains were less susceptible to the lethal effects of sarin than their male counterparts (Matson et al. 2018). In contradiction with the results of sarin toxicity in mice, studies in rats found that females were more susceptible to the lethal effects of whole-body (Mioduszewski et al. 2002, Wright et al. 2017) and subcutaneous sarin with the latter causing a smaller increase in brain inflammatory markers following exposure (Pittel et al. 2018). These differences could be species-dependent; however, none of the former studies took into account stage of estrous. A study that did account for stage of estrous in rats found that female rats in proestrus are more resistant to the acute toxicity of sarin than male rats, female rats in other stages of the estrous cycle, and ovariectomized female rats (Smith et al. 2015). These results indicate that the estrous cycle may play an important role in nerve agent toxicity and could impact the overall findings of sex differences in studies that do not account for stage of estrous. One novel

goal of the present study was to examine sex differences and the influence of the female estrous cycle on soman-induced toxicity in ES1-/- mice.

Previous studies have demonstrated that the dose of benzodiazepines needed to suppress seizure in soman-exposed rodents increases drastically as the anticonvulsant treatment is delayed from 5 to 40 min after onset of seizure (McDonough et al. 1999, McDonough et al. 2010). In addition, studies have reported that the delayed anticonvulsant treatment is unable to effectively prevent the development of spontaneous recurrent seizure and accompany neurodegeneration (de Araujo et al. 2010, Schultz et al. 2014, Marrero-Rosado et al. 2018). We have previously shown that subcutaneous exposure to a seizure-inducing dose of soman in male ES1-/- mice followed by delayed administered of midazolam at 15 min after seizure onset is unable to prevent epileptogenesis or the extensive neuronal loss and microglial activation in seizure-sensitive brain regions. A goal of the current study was to determine whether further extending the delay of the midazolam treatment to 40 min after seizure onset in soman-exposed male and female ES1-/- mice worsens their survival and neurological outcome.

In sum, the primary objective of the present studies were to further characterize soman-induced acute toxicity in ES1-/- male and female mice; and to determine the severity of acute toxicity of soman in ES1-/- male and female mice when treatment with benzodiazepine is delayed to 40 min post-onset of seizure. The results presented here suggest that ES1-/- mice are a useful animal model to screen improved medical countermeasures against soman toxicity.

CHAPTER 2: METHODS AND MATERIALS

Experiment 1

Animals

Female (17-20 g) and male (19-22 g) ES1-/- mice 8-9 weeks of age were obtained from a breeding colony established at the United States Army Medical Research Institute of Chemical Defense (USAMRICD). Mice were exposed to soman at 10-11 weeks of age. Animals were single-housed, with food and water available ad libitum, on a 12-hour:12-hour light-dark cycle with lights on at 0600. Room humidity and temperature were monitored and controlled. Mice were weighed daily (M-F). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the 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.

Estrous Cycle Stage Determination

Vaginal cytology was performed on mice approximately 1 hour prior to exposure using methods as described in McLean et al. (2012). Vaginal cells were collected using a vaginal lavage with 0.1 M phosphate buffer, and collected cells were placed on a glass microscope slide. Once dry the slides were stained with a 0.1 % crystal violet solution. Slides were examined under an Olympus BX16IVS light microscope (Center Valley, PA) to determine cell types present, and estrous cycle stage was determined based on presence and ratio of nucleated epithelial, cornified epithelial cells and leukocytes. A board-certified pathologist trained scorers in identification of cell types indicative of different stages of the estrous cycle.

Soman Exposure

Soman was obtained from the United States Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD). All primary agent stocks used to formulate diluted solutions for experimental use were Chemical Agent Standard Analytical Reference Material (CASARM) certified at a purity of >95%. Subsequent experimental diluted agent stocks were formulated gravimetrically, and concentrations were confirmed by USAMRICD chemists using GC/MS, LC/MSMS and/or NMR. Sterile saline (0.9% NaCl) was used to dilute the stock solutions.

Median Lethal Dose (LD_{50}) Determination of Soman in ES1-/- Mice

Female mice were sorted into one of three groups based on their estrous cycle stage (proestrus, estrus, diestrus). The LD_{50} was determined based on the 24-h survival of female and male mice subcutaneously (SC) exposed to soman (pinacolyl methylphosphonofluoridate; 5 mL/kg) in a stagewise manner as previously described (Feder et al. 1991). Each dose group had 1-3 mice assigned to it with up to 5 doses per stage with a dose span of predicted lethality (0-100%) covered during the first stage. Mice that survived for 24 h following exposure were considered to have reached the experimental endpoint. Results from the first stage were used for determination of additional doses used in stage 2, which helped enhance the data from the prior stage. The LD_{50} was estimated using a probit transformation in a nonlinear regression analysis with maximum likelihood procedures using the combined data from all dosing stages; additional stages were run as needed until the stopping criterion was met. The stopping criterion was reached when the ratio of the half width of the 95% confidence interval [CI; defined as (Upper bound – Lower bound)/(2 x LD_{50})] for the LD_{50} was less than 0.40. The results of the nonlinear regression analysis were used to create a lethality dose-

response curve allowing for the estimation of the LD_{50} for each group of mice examined. The dose-response curve also provided information on the dose at which there is 90% lethality (LD_{90}), 10% lethality (LD_{10}), and the slope, which indicates the change in lethality per dose unit.

Data Analysis

The LD_{50} determination of female mice in different stages of the estrous cycle (proestrus, estrus, and diestrus) was compared among stages and with that of male mice exposed to soman. The ratios of the LD_{50}s for pairs of groups were calculated along with a 95% confidence interval and used to determine if there was a significant difference between groups. A significant difference was identified when the ratio confidence interval did not include 1.

Experiment 2

Animals

Female (17-20 g) and male (19-22 g) ES1-/- mice 8-9 weeks of age were obtained from USAMRICD breeding colony and exposed SC to soman, as in experiment 1, at 10-11 weeks of age. Mice were single-housed 1-3 days prior to surgery, under the same conditions as described in experiment 1.

Surgical Implantation of Telemetry Device

Mice were implanted with sterile ETA-F10 telemetry transmitters from Data Science International (DSI, St. Paul, MN) under 1-5% vaporized isoflurane anesthesia. Transmitters were implanted (SC) with wires wrapped around two cortical stainless steel screw electrodes placed at 1.5 mm to the right of the midline, and 1.5 mm anterior and 3.0 mm posterior of the bregma. The surgical procedure was based on that described in

Lundt et al. (2016). The analgesic meloxicam (1 mg/kg, SC) was administered prior to surgery, and buprenex SR (0.5 mg/kg, SC) was administered immediately following surgery to minimize pain. Mice were allowed 10-14 days of post-surgical recovery prior to soman exposure. Dataquest Art Acquisition software (DSI) was used to record EEG data and digitized data at 250 Hz. The EEG data were continuously recorded from 3 days prior to exposure until the experimental endpoint of 14 days post-exposure.

Soman Exposure and Medical Countermeasures

Female mice were vaginally lavaged approximately one hour prior to exposure, and then male and female ES1-/- mice were exposed (SC) to saline or 82 μg/kg of soman; to increase survivability, mice exposed to soman were treated with an admix of atropine sulfate (4 mg/kg; Sigma Aldrich, St. Louis, MO) and HI-6 (50 mg/kg; Sigma Aldrich) (IP) 1 min after exposure. Seizure onset was determined based on electroencephalograph (EEG) activity. Treatment with pharmaceutical grade midazolam (1, 3, or 9 mg/kg; IP) was given 40 min after the onset of seizure. Only animals that developed seizure were included in this study. Signs of toxicity were monitored for at least 4 h following exposure. All treatment solutions were made using sterile saline (0.9% NaCl).

Behavioral Seizures

Behavioral seizure activity was monitored and recorded by an observer blinded to treatment using the Noldus Pocket Observer program (Noldus Information Technology, The Netherlands). Behavioral seizure severity was scored using a modified Racine scale (Racine 1972) of 6 stages: 0, no abnormality; 1, mastication, tongue fasciculations, oral tonus; 2, head nodding and/or tremors; 3, forelimb clonus or tonus, body tremors, body

jerks; 4, rearing with forelimb clonus, opisthotonos; and 5, rearing and falling with convulsions.

EEG Recording and Analysis

EEG was continuously recorded between 3 days prior to exposure (baseline) and the experimental endpoint of 14 days was reached. Dataquest Art Acquisition software (DSI) was used to record and digitize EEG data at 250 Hz. A customized MATLAB algorithm that determines seizure activity based on the specified parameters was used to analyzed EEG data as described de Araujo Furtado et al. (2009), followed by visual screening of candidate seizures by a blinded observer used to analyze the seizure data. Seizure activity was defined as high amplitude (greater than 2 X baseline) and high frequency rhythmic spikes with duration of at least 10 s as described by Nissinen, et al. (2001) and de Araujo Furtado et al. (2009). The EEG data were analyzed for initial seizure duration in the first 24h to 72 h as well as for the latency to the onset of spontaneous recurrent seizure which in mice exposed to this dose occurs ~5-7 days after exposure. Frequency of spontaneous recurrent seizure was also quantified. Finally, full EEG power spectrum data were further reduced by extracting the median power (20 min bins) in 60-minute intervals to obtain EEG power integral values at baseline (24 h prior to soman or saline exposure), during status epilepticus (20 min before treatment), 1 h after treatment, and 6 h after treatment.

Brain Collection and Immunohistochemistry

On post-exposure day 14, mice were deeply anesthetized with a pharmaceutical-grade solution containing sodium pentobarbital (75 mg/kg, IP, Fatal Plus; Patterson Veterinary), and then perfused with a solution of heparinized (100 units heparin per liter)

saline, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, placed in fixative for 6 hours at 4°C and, then, placed in 20% sucrose in PB for cryoprotection. Chemicals for perfusion, post-fixation, and cryoprotection were purchased from FD Neurotechnologies (Columbia, MD). Brains were processed and immunostained by FD Neurotechnologies using methods described in Hsu, Raine et al. (1981) with a marker for neuronal nuclear protein (NeuN; mouse anti-NeuN IgG 1:600; Millipore, Billerica, MA) and ionized calcium-binding adapter molecule 1 (Iba1; rabbit anti-Iba1 IgG 1:6,000; Wako Chemicals, Richmond, USA). NeuN allows for visualization of neurons, and Iba1 allows for visualization of microglia. Sections were counterstained with Nissl.

Neuropathological Analysis

Immunostained sections were scanned using an Olympus BX16IVS microscope (Center Valley, PA) with a Pike F-505 camera (Allied Vision; Exton, PA). Determination of neuronal degeneration was done through quantification of the number of cells positive for the NeuN marker using the Image-Pro Plus program (Media Cybernetics, Rockville, MD. Regions of interest were traced at bregma -1.94 mm using anatomical landmarks, then cell density was calculated, 3 replicates were scored and averaged. In regions with high neuronal densities, stereology was used to evaluate neuronal density using the Stereo Investigator program (MBF Bioscience; Williston, VT). Approximately 5 sections (30 μm each) per mouse were counted, the counting frame was 40 × 40 μm, and the counting grid was 150 × 150 μm with a dissector height of 20 μm and a 5 μm guard zone. For identification of activated microglia and microgliosis, Iba1 immunoreactivity was evaluated to determine changes in density and cell-body-to-cell-size ratio using ImageJ

software (National Institutes of Health). Methods used were modified from Hovens, Nyakas et al. (2014).

Data Analysis

A Cox regression analysis was used to determine the main effects of midazolam dose and sex on survival followed by a Kaplan-Meier analysis. A repeated measures analysis of variance was used to determine the effects of midazolam dose and sex on changes in body weight and body temperature. A univariate general linear model was used to determine the effects of midazolam dose and sex on changes in seizure duration, latency and frequency of spontaneous recurrent seizure, density of NeuN and Iba1, and changes in cell size to cell body ratio of Iba1 positive cells, followed by a Tukey's test. When $P < 0.05$, differences were considered statistically significant. All statistical analyses were done using IBM SPSS Statistic v.21.

CHAPTER 3: RESULTS

Experiment 1

Median lethal (LD_{50}) dose of soman in female and male ES1-/- mice

We have previously determined the dose-lethality response distribution of SC exposure to soman in male ES1-/- mice (Marrero-Rosado et al. 2018), which is shown for comparison purposes (Figure 1; Table 1). Data from the present dose-lethality response study show that the estimated 24-hour SC LD_{50} of soman in female ES1-/- mice at estrus (~23.6 μg/kg) is significantly higher than that in females at proestrus (19.8 μg/kg) and also higher compared to that in males (19.2 μg/kg). See Table 1 for the soman LD_{10}, LD_{50}, and LD_{90} as well as the 95% CI and the slope of the dose response curve in male and female mice at different estrous stages.

Group	LD_{10} (μg/kg) (95% CI)	LD_{50} (μg/kg) (95% CI)	LD_{90} (μg/kg) (95% CI)	Slope
Female Proestrus	16.6 (13.5, 20.3)	19.8 (17.7, 22.1)*	23.6 (19.4, 28.6)	16.7
Female Estrus	19.4 (16.0, 23.4)	23.6 (20.8, 26.7)	28.7 (22.9, 36.0)	15.0
Female Diestrus	17.8 (15.3, 20.7)	20.8 (18.9, 22.8)	24.2 (20.1, 29.2)	19.1
Male	17.2 (15.2, 19.5)	19.2 (18.0, 20.5)*	21.4 (19.1, 24.0)*	27.1

Table 1. 24-hour SC LD_{10}, LD_{50}, LD_{90} of soman in male and female ES1-/- mice. Numbers in parentheses are the 95% CI *$P < .05$ as compared to estrus.

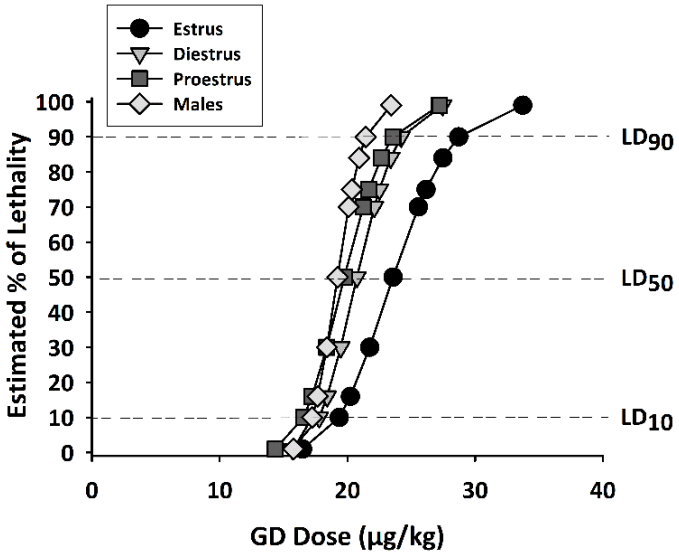

Figure 1. Dose-response curve for soman-induced lethality in adult male and female ES1-/- mice. Each data point in the graph represents the average estimated percent of lethality for adult female ES1-/- mice exposed to a particular dose of soman (GD) at different estrous cycle stages. At 23.6 µg/kg (95% CI 20.8-26.7 µg/kg), the LD_{50} of GD for female ES1-/- mice in estrus (n=17) was 1.14 and 1.19 times higher than that of female ES1-/- mice in diestrus (n=18) and proestrus (n=14), respectively. The LD_{10} and LD_{90} for mice in estrus, diestrus, and proestrus are also shown in the graph. The dose response curve for GD-induced lethality in male ES1-/- mice (gray diamond) from Marrero-Rosado, de Araujo Furtado et al. (2018) was used for comparison.

Experiment 2

Survival, body temperature and weight loss following soman exposure in mice treated with delayed midazolam

Assessment of survival data by Cox regression analysis revealed a significant effect of midazolam dose in the 14 days following soman exposure, with no effect of sex (Figure 2). Further Kaplan-Meier analysis with log rank test for group comparisons detected a dose-dependent increase in survival when midazolam was given as a delayed anticonvulsant treatment after soman exposure. The group of soman-exposed mice that received 1 mg/kg midazolam had poor survival rates with 0% females and 28.6% males surviving, which was significantly different from survival in No soman control (100%; $P < 0.001$) mice and from survival in the 9 mg/kg midazolam-treated mice (female: 85.7%;

male: 71.4%; $P = 0.001$). The 3 mg/kg midazolam-treated soman-exposed mice (female: 33.3%; male: 52.9%) also had significantly less survival compared to control ($P = 0.001$) and compared to soman-exposed mice treated with 9 mg/kg midazolam ($P < 0.05$). For body temperature, there was a significant interaction between time (hourly average) and group, independent of sex. Comparisons of group at each time point revealed that mice exposed to soman and treated with midazolam had reduced body temperature with the highest dose of midazolam (9 mg/kg) resulting in the greatest and more prolonged decrease in body temperature compared to No soman control and to the lower doses of midazolam (Figure 3). Soman-exposed mice also had a significant loss of body weight on days 1-3 after exposure compared to baseline ($P < 0.05$), with body weight returning to baseline within 1 week of exposure.

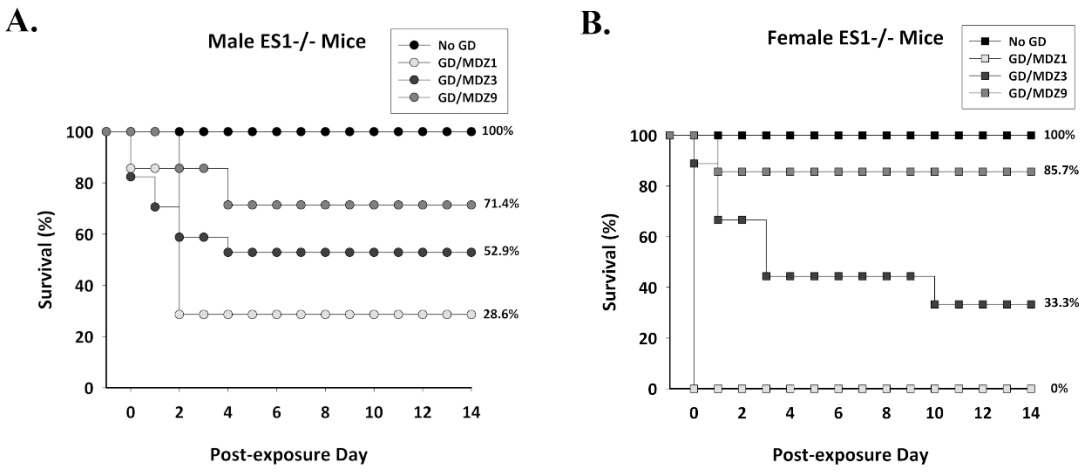

Figure 2. Dose-dependent effect of midazolam (MDZ) on survival following GD-induced status epilepticus in male (A) and female (B) ES1-/- mice. Mice exposed to 82 µg/kg of GD and treated with 1 mg/kg MDZ (GD/MDZ1; $P < 0.001$; n=11) or 3 mg/kg MDZ (GD/MDZ3; $P = 0.001$; n=26) had significantly less survival compared to control (No GD; n=14), while those treated with 9 mg/kg (GD/MDZ9; n=14) were not significantly different from control. The GD/MDZ3 group also had significantly less survival compared to the GD/MDZ9 group of mice. There was found to be no main effect of sex and thus data shown are male and female combined.

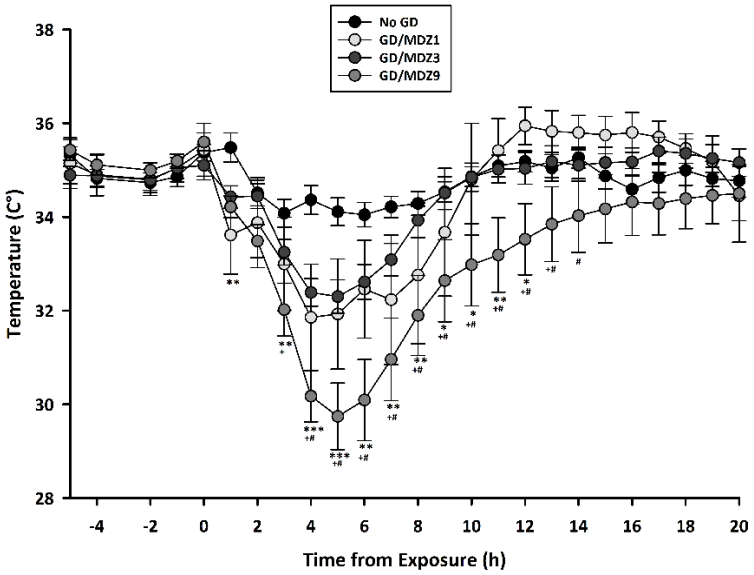

Figure 3. Dose-dependent effect of midazolam (MDZ) on body temperature following GD exposure. Mice exposed to 82 µg/kg of GD and treated with MDZ had reduced body temperature in the hours after exposure, with a greater and more prolonged decrease in those that received the highest dose (9 mg/kg) of MDZ (GD/MDZ9; n=13) compared to those that received 1 mg/kg MDZ (GD/MDZ1; #$P < 0.05$; n=6) or 3 mg/kg MDZ (GD/MDZ3; ++ $P < 0.01$; ++$P < 0.01$; n=16) or to control (No GD; ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$; n=13) that received 3 mg/kg MDZ, with effects independent of sex. Data shown are combined temperature data in male and female mice. Values shown are averages per 60 min bin.

Behavioral and EEG seizure following soman exposure in mice treated with delayed midazolam

Subcutaneous exposure to a seizure-inducing dose of soman resulted in severe cholinergic toxic signs consisting of ataxia, head bobs, and body tremors within 10 min of exposure; these toxic signs reached a maximum median severity of 3 in male and 3.5 in female ES1-/- mice during the first 40 min following exposure. Behavioral seizures progressed into status epilepticus shown on EEG as prolonged seizure of at least 30 min in both male and female mice with an average seizure onset of 4.4 ± 1.4 and 3.9 ± 2.2 min, respectively. Administration of all doses of midazolam at 40 min after onset of soman-induced seizure failed to terminate seizure activity, with animals continuing to seize for over 10 h after exposure. Male ES1-/- mice administered 1, 3, or 9 mg/kg of midazolam

spent 873.2 ± 317.9, 622.5 ± 411.9, and 581.0 ± 437.9 mins (mean ± SD) in seizure, respectively, during the first 24 h after soman exposure. Seizure activity in female ES1-/- mice during the first 24 h after soman exposure lasted 556.7 ± 320.1 min (mean ± SD) in the group that received 3 mg/kg midazolam and of 632.0 ± 545.2 min (mean ± SD) in the group that received 9 mg/kg midazolam. Since female ES1-/- mice that received 1 mg/kg midazolam after soman exposure did not survive past the end of the day of exposure, their time spent in seizure activity during the first 24 h after exposure is not included. There was no significant effect of sex or midazolam dose on duration of acute seizure activity following soman exposure.

A full-spectral power analysis of EEG activity was performed following previously described procedures (de Araujo Furtado et al. 2009). For the EEG power integral there was a significant interaction between midazolam dose and the time period following soman exposure. Therefore each time point (status epilepticus, 1 h after status epilepticus and 6 h after status epilepticus) was evaluated separately for each dose, and each treatment dose was evaluated separately over time. Comparing groups at each time revealed that soman-exposed mice had increased EEG power integral during status epilepticus (Figure 4). soman-exposed mice had increased power integral compared to no soman during the 1 h time period after midazolam (1, 3 and 9 mg/kg) treatment, but during the 6 h post-treatment time period only the two lower doses of midazolam (1, 3 mg/kg) were significantly different from no soman controls, demonstrating that the 9 mg/kg of midazolam reduced seizure severity. Evaluating each treatment dose over time revealed that while the 3 and 9 mg/kg midazolam-treated mice had reduced EEG power integral at the 1 and 6 h post-treatment times compared to the status epilepticus period, the 1 mg/kg midazolam dose

was less effective at reducing seizure severity and at the 1 h post-exposure time point was not significantly different from the status epilepticus period. In sum, the severity of seizures, as estimated by the total EEG power, was only significantly reduced 6 h post onset in soman-exposed mice that received delayed treatment with 9 mg/kg midazolam.

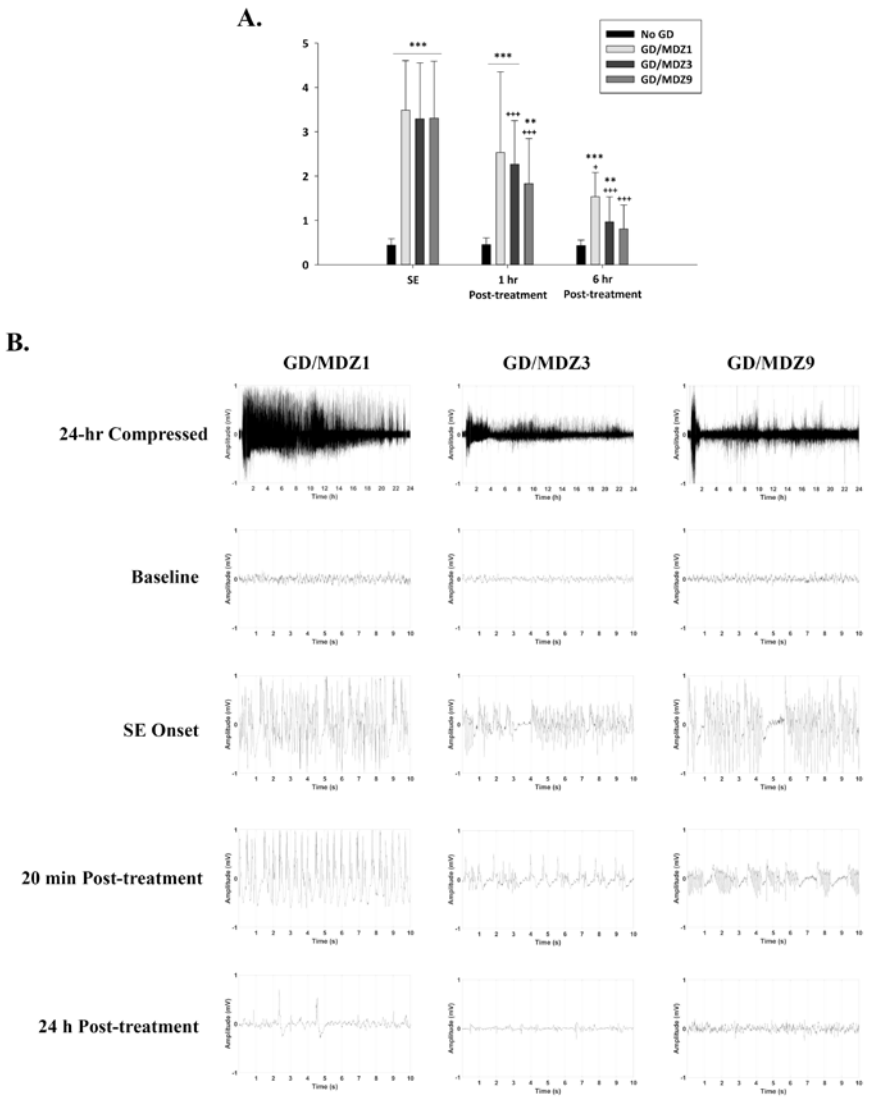

Figure 4. Effect of delayed treatment with midazolam (MDZ; 9 mg/kg) on EEG power integral, a marker of seizure severity. Acute seizure activity in ES1-/- mice following SC exposure to 82 µg/kg of GD and MDZ treatment delayed to 40 min after seizure onset. A) GD exposure increased EEG power integral during status epilepticus (SE), which continued to be increased 1 h after exposure compared to control (No GD; n=13). Midazolam [3 (GD/MDZ3; n=19) and 9 mg/kg] reduced EEG power integral at 1 h and 6 h compared to SE, while 1 mg/kg (GD/MDZ1; n=5) was only effective at 6 h. Treatment

with 9 mg/kg MDZ (GD/MDZ9; n=13) reduced EEG power integral 6 h after GD exposure and was not significantly different from control at this time. ***$P < 0.001$; **$P < 0.01$ GD/MDZ compared to No GD. +$P < 0.05$, +++$P < 0.001$ SE vs 1 h and 6 h after MDZ treatment. B) Representative EEG tracings of baseline (prior to GD exposure), onset of SE, 20 min after MDZ treatment and 24 h after treatment.

Midazolam did not prevent the development of spontaneous recurrent seizures in the days following soman-induced status epilepticus (Figure 5). In surviving soman-exposed male ES1-/- mice that showed soman-induced status epilepticus, 87.5% (n=7 out of 8) and 75% (n=3 out of 4) of those treated with 3 and 9 mg/kg of midazolam, respectively, developed spontaneous recurrent seizures (Figure 5A). Spontaneous recurrent seizures were also observed in surviving soman-exposed female ES1-/- mice; an incidence of 100% (n=4 out of 4) and 83.3% (n=5 out of 6) mice developing spontaneous recurrent seizures was observed in the group of female mice treated with 3 mg/kg and 9 mg/kg of midazolam. No effect of sex or midazolam dose on the spontaneous recurrent seizure incidence was observed. In animals that developed spontaneous recurrent seizures, there was no effect of sex or midazolam dose in the number of spontaneous recurrent seizures detected in the two weeks following soman-induced status epilepticus (Figure 5B); we observed an average (mean ± SD) of 11.7 ± 11.7 spontaneous recurrent seizures in males in the 3 mg/kg midazolam group, 11.3 ± 9.3 spontaneous recurrent seizures spontaneous recurrent seizures in males in the 9 mg/kg midazolam group, 10.5 ± 2.4 spontaneous recurrent seizures in females in the 3 mg/kg midazolam group, and 6.8 ± 5.8 events in females in the 9 mg/kg midazolam group.

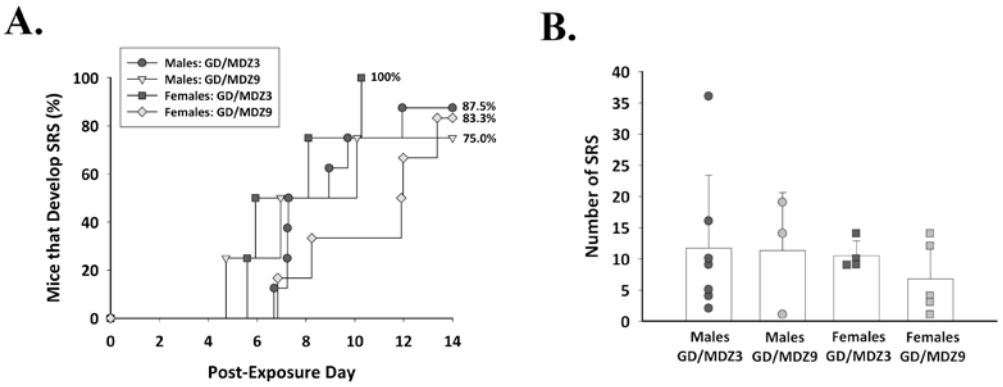

Figure 5. Delayed midazolam (MDZ) failed to prevent the development of spontaneous recurrent seizures (SRS) following GD-induced status epilepticus, independent of sex. Male and female ES1-/- mice were exposed to 82 µg/kg of GD and treated with 3 mg/kg (GD/MDZ3; n=11) or 9 mg/kg (GD/MDZ9; n=10) of MDZ at 40 min after seizure onset, and EEG activity was monitored for 14 days after exposure. (A) The onset of the first detected SRS for each surviving animal is graphed to indicate the percentage of mice in each group that developed SRS in the days following GD-induced SE and delayed MDZ treatment. (B) Graph and error bars represent the mean (± SD) of number of SRS events for each group.

Neuronal cell survival and microglia activation two weeks after soman exposure in mice treated with delayed midazolam

Soman-exposed ES1-/- mice treated with delayed midazolam (3 or 9 mg/kg) had a loss of neurons two weeks after soman exposure, compared to control mice (Figure 6). There was no effect of sex on neuronal cell density in any of the brain regions analyzed. Significant loss of neurons occurred in the dorsomedial thalamus (3 and 9 mg/kg, $P < 0.001$), dorsolateral thalamus (3 and 9 mg/kg, $P < 0.001$), basolateral amygdala (3 and 9 mg/kg, $P < 0.001$), layer 3 of the piriform cortex ($P < 0.001$, 3 mg/kg; $P = 0.01$, 9 mg/kg), and the hilus of the hippocampus (3 and 9 mg/kg, $P < 0.001$) of midazolam-treated soman-exposed mice, demonstrated by fewer NeuN-positive cells, compared to the no agent controls. Using stereology, loss of neurons was also confirmed in the CA1 of the hippocampus in soman-exposed mice treated with 3 ($P = 0.001$) and 9 mg/kg ($P < 0.01$) of midazolam.

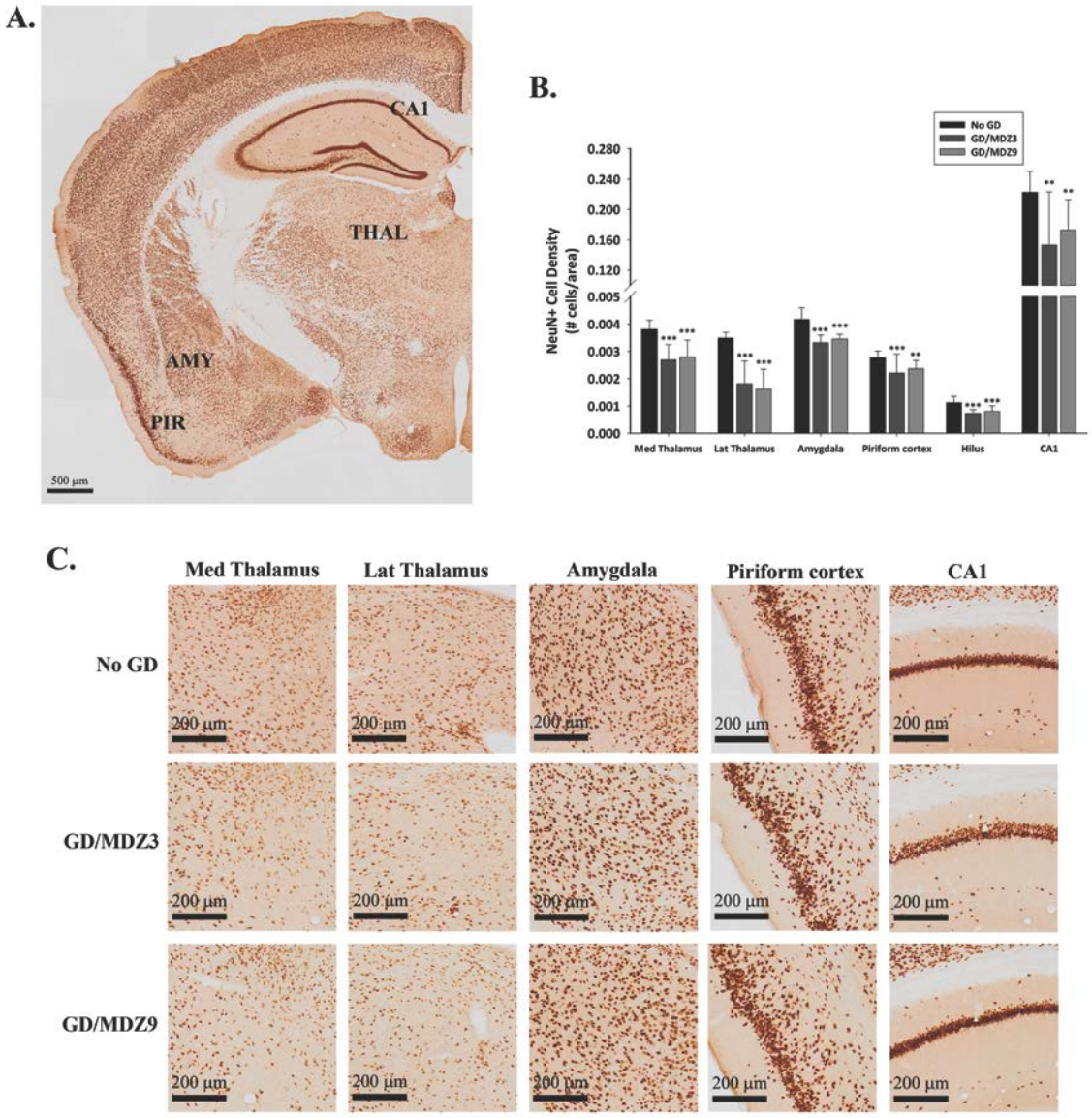

Figure 6. Midazolam (MDZ) failed to prevent neuronal loss following GD exposure, independent of sex. Dose-response effects of delayed MDZ on mature neuronal cell (NeuN) population in male and female ES1-/- mice 2 weeks after GD (82 µg/kg) exposure. Male and female GD-exposed ES1-/- mice were administered 3 mg/kg MDZ (GD/MDZ3; n=10) or 9 mg/kg MDZ (GD/MDZ9; n=11) at 40 min after GD-induced seizure onset. Control mice (No GD; n=14) received 3 mg/kg MDZ at 50 min after saline injection. A) Coronal section in control (No GD) mouse showing regions of interest. B) Graph and error bars represent the mean (± SD) of density of NeuN-positive cells is shown for the dorsomedial thalamus (Med Thalamus), dorsolateral thalamus (Lat Thalamus), basolateral amygdala (Amygdala), layer 3 of the piriform cortex, and the CA1 of the hippocampus in coronal sections in bregma range -1.28 to -1.64 mm. C) Representative images of NeuN-immunostained brain samples are shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared to control.

Soman-exposed ES1-/- mice treated with delayed midazolam (3 or 9 mg/kg) also showed signs of increased microgliosis and microglial cell activation two weeks after soman exposure, compared to no agent control mice (Figure 7A). Soman-exposed mice showed Iba1-positive cells with significantly higher cell-body-to-cell-size ratio in the dorsomedial thalamus (3 and 9 mg/kg, $P < 0.001$), dorsolateral thalamus (3 and 9 mg/kg, $P < 0.001$), basolateral amygdala (3 and 9 mg/kg, $P < 0.001$), the piriform cortex (3 and 9 mg/kg, $P < 0.001$), and the CA1 region of the hippocampus (3 and 9 mg/kg, $P < 0.001$) compared to no agent control mice (Figure 7B). In the CA1 region, we detected a lower cell-body-to-cell-size ratio in the 9 mg/kg midazolam group compared to the 3 mg/kg midazolam group ($P < 0.05$). An increase in microgliosis was also observed in soman-exposed mice, demonstrated by an increase in the density of Iba1-positive cells in the dorsomedial thalamus (3 mg/kg, $P < 0.01$; 9 mg/kg, $P < 0.001$), dorsolateral thalamus (3 and 9 mg/kg, $P < 0.001$), basolateral amygdala (3 and 9 mg/kg, $P < 0.001$), the piriform cortex (3 mg/kg, $P < 0.01$; 9 mg/kg, $P < 0.05$), and the CA1 region of the hippocampus (3 mg/kg, $P < 0.001$; 9 mg/kg, $P < 0.01$) compared to no agent control mice (Figure 7C). There were no sex differences in microglial morphology and density.

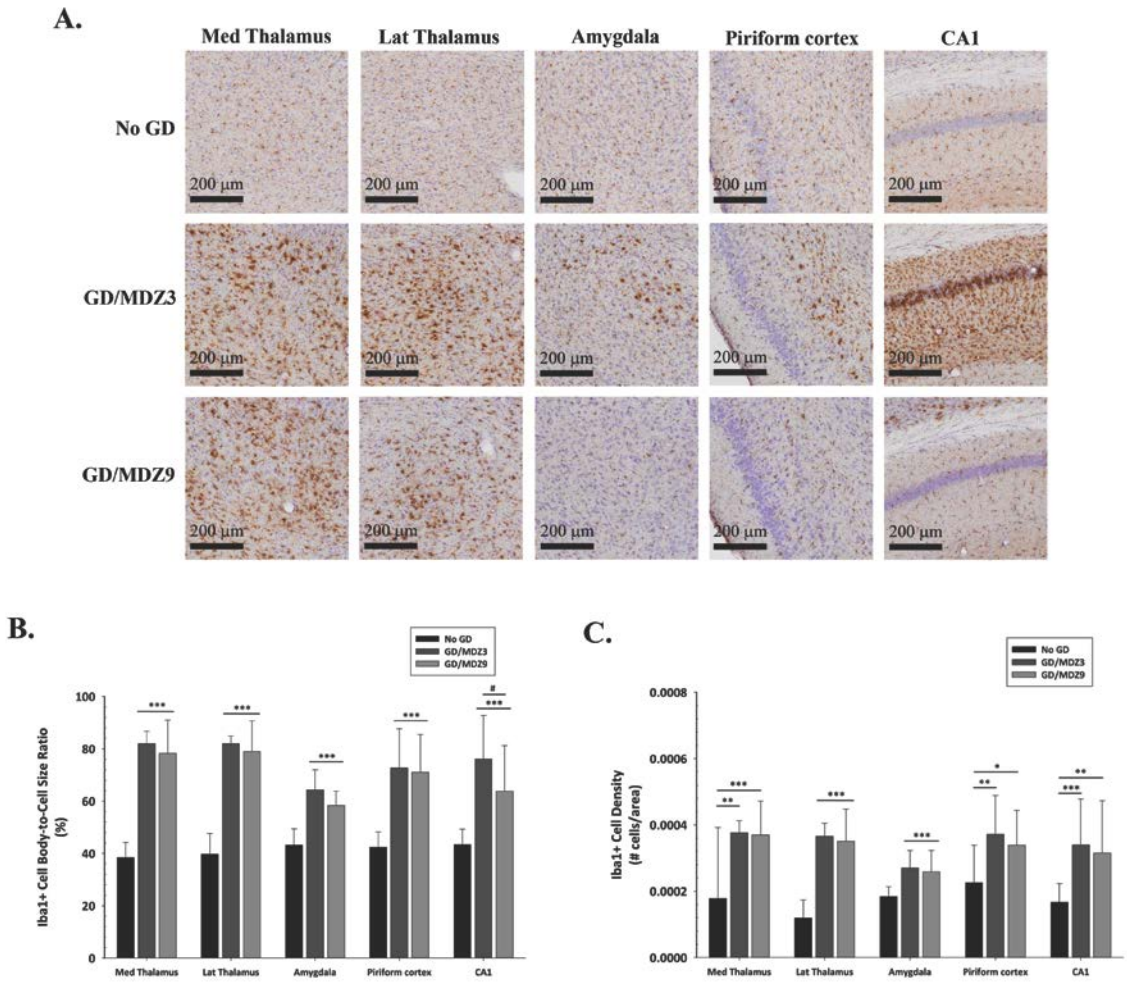

Figure 7. Midazolam (MDZ) failed to prevent microglial activation following GD exposure, independent of sex. Mice exposed to GD (82 µg/kg) received 3 (GD/MDZ3; n=10) or 9 mg/kg (GD/MDZ9; n=11) of MDZ 40 min after seizure onset. Control (No GD; n=14) mice received 3 mg/kg MDZ 50 min after saline injection. A) Representative images of Iba1-immunostained brain samples are shown. B) The) cell-body-to-cell-size ratios and C) density of ionized calcium-binding adaptor molecule 1 (Iba1), a marker for microglia, are shown for the dorsomedial thalamus (Med Thalamus), dorsolateral thalamus (Lat Thalamus), basolateral amygdala (Amygdala), layer 3 of the piriform cortex, and the CA1 of the hippocampus. Graph and error bars represent mean (± SD) of results obtained from each group. Cresyl violet (purple) counterstain was used for visualization of anatomic landmarks. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared to No GD group. #*P* < 0.05, GD/MDZ3 compared to GD/MDZ9 group.

CHAPTER 4: DISCUSSION

The goal of the present study was to characterize the toxicity of soman and to assess the effectiveness of medical countermeasures in the ES1-/- mouse model, which lacks plasma carboxylesterase and may better predict toxic effects of soman in humans compared to rats and mice that have plasma carboxylesterase. Both sexes of ES1-/- mice were evaluated to identify potential sex differences in the toxicity of soman and in response to an anticonvulsant treatment. In our first experiment, we examined the lethality of soman in female ES1-/- mice across different stages of the estrous cycle and compared the results to soman lethality in male ES1-/- mice (Marrero-Rosado et al. 2018). Results of this experiment demonstrated that female ES1-/- mice in estrus are significantly less susceptible to the lethal effects of soman than male mice and female mice in proestrus. Variation in toxic response across the estrous cycle also occurs in rats exposed to sarin, albeit with reduced toxicity during proestrus (Smith et al. 2015). In addition, pilocarpine-induced status epilepticus is reduced in rats during the estrus stage (Scharfman et al. 2005). One proposed mechanism for this protection during estrus is that brain levels of progesterone and the neurosteroid allopregnanolone are elevated (Corpechot et al. 1997). Neurosteroids such as allopregnanolone are modulators of the major inhibitory $GABA_A$ receptor (Paul and Purdy 1992) and have anticonvulsant properties (Kokate et al. 1994). Our laboratory recently reported that the neurosteroid pregnanolone in combination with diazepam quickly terminates sarin-induced status epilepticus and reduces performance deficits and brain pathology compared to diazepam monotherapy (Lumley et al. 2019). Although the effects of elevated brain progesterone and allopregnanolone levels may contribute to the increased protection against soman lethality that we observed in the female mice in estrus, there are other variations in

physiology across estrous that could also impact outcome. More research is needed to determine the mechanisms by which estrus confers some protection over the lethal effects of soman. In addition, these results further exemplify the need for studies to have inclusion of both sexes when studying toxic effects of chemical exposure.

Previously, we reported that midazolam delayed to 15 min after soman-induced status epilepticus in ES1-/- male mice induced prolonged behavioral seizure activity, development of status epilepticus, spontaneous recurrent seizures, microglia activation, and loss of neurons in seizure-sensitive brain regions (Marrero-Rosado et al. 2018). In our current study, we expand on this model to examine the effects of soman on both sexes followed by midazolam treatment delayed to 40 min after seizure onset. Male and female ES1-/- mice exposed to a seizure-inducing dose of soman and treated with standard medical countermeasures along with delayed midazolam treatment exhibited a dose-dependent increase of survival, as well as a reduction in seizure intensity by the highest dose evaluated. It did not, however, prevent prolonged behavioral and acute seizure activity, decreases of body weight and temperature, development of spontaneous recurrent seizures, and increased neurodegeneration and neuroinflammation markers.

Decreased temperature was observed in mice exposed to soman in the hours following exposure, consistent with previous studies of mice exposed to soman (Clement 1991, Clement 1993). This decrease in body temperature is theorized to be caused by disruption of normal cholinergic system signaling, specifically muscarinic receptor overstimulation in regions of the brain that help to regulate body temperature (Meeter and Wolthuis 1968, Maickel et al. 1990, Clement 1993). The largest decrease in temperature was observed in the group of mice treated with 9 mg/kg of midazolam in combination

with soman. This is in agreement with previous reports that benzodiazepines including midazolam and diazepam, also decrease body temperature (Kurz et al. 1995, Matsukawa et al. 1997, Elliot and White 2001, Mailliet et al. 2001). In models of stroke, spinal cord injury, traumatic brain injury, and hypoxic-ischemia, hypothermia has neuroprotective effects including reduction in generation of reactive oxygen species, excitotoxicity, and neuroinflammation (reviewed in Sun et al. 2019). In both clinical and pre-clinical studies hypothermia has anticonvulsive effects (reviewed in Motamedi et al. 2013). In a rat model of pilocarpine-induced status epilepticus, hypothermia in conjunction with diazepam reduces mortality and decreases neuronal damage and improves recovery following status epilepticus (Phillips et al. 2018). These results indicate that the decrease in body temperature seen in the highest dose of midazolam may offer some neuroprotective effects as well as reduce mortality following soman exposure.

Midazolam was able to increase survival in soman-exposed ES1-/- mice in a dose-dependent manner. The highest dose of midazolam (9 mg/kg) increased survival of soman-exposed female and male mice to 85.7% and 71.4 % respectively. Improved survival with midazolam following soman exposure, even when treatment is delayed to 40 min after seizure onset, is in agreement with a recent study in rats whereby delayed midazolam dose-dependently increased survival and reduced seizure severity (Lumley et al. 2019). In guinea pigs higher doses of benzodiazepines are needed to increase survival when treatment is delayed following nerve agent exposure (Shih et al. 2003, McDonough et al. 2010). However, even at the highest dose of 9 mg/kg, delayed treatment with midazolam was unable to prevent subsequent epileptogenesis. In agreement with results of studies reporting that in rats exposed to soman status epilepticus quickly becomes

refractory to benzodiazepine treatment (Shih and McDonough 1997, Schultz et al. 2012, Lumley et al. 2019), we observed that mice exposed to soman and treated with delayed midazolam continued to seize for many hours after exposure. Although delayed treatment with midazolam was unable to quickly terminate seizure activity, the EEG power integral ratio was reduced by midazolam treatment, and thus seizure severity was reduced, indicating that in addition to protecting against the lethal effects of soman exposure, delayed midazolam treatment reduced seizure severity.

Soman exposure followed by treatment with midazolam at 40 min after seizure onset led to neuronal cell loss and microglia activation in brain regions sensitive to seizure in male and female ES1-/- mice; effects were independent of sex. These results are in congruence to with those reported in our previous study in which male ES1-/- mice exposed SC to soman and treated with midazolam 15 min after seizure onset experience significant neuronal loss in brain regions sensitive to seizure as well as an increase in microglia activation in these regions (Marrero-Rosado et al. 2018). We also observed that male and female of ES1-/- mice exposed to soman had a significant increase of density and changes in cell morphology of microglia. Increased activation of microglia can lead to increases of neurotoxic or proinflammatory mediators causing neuronal cell death and neurodegeneration (reviewed in Kempuraj et al. 2016). This increase in neuroinflammatory response is also thought to contribute to hyperexcitability of neurons and contribute to epileptogenesis (Vezzani et al. 2011, Terrone et al. 2019). During a mass casualty situation there is a high likelihood that treatment with anticonvulsants would be delayed, and this study exemplifies the need for development of adjunct therapies to be administered with midazolam.

Overall, the present study found sex differences particular to stage of estrous in soman-induced lethality, with females ES1-/- mice in estrus being the most protected. Delayed midazolam treatment following soman exposure was able to increase survival and reduce seizure intensity, but was unable to terminate status epilepticus, prevent the development of spontaneous recurrent seizures, or prevent neuropathology. These results show the importance of using both sexes in studies and for developing novel medical countermeasures to give in conjunction with midazolam, particularly in a mass causality situation where treatment would most likely be delayed. Finally, the ES1-/- mouse emerges as a valuable model for screening of medical countermeasures against soman toxicity.

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