

The role of IL-1 Signaling in Neurodegeneration, Neuroinflammation, and Behavior after
Soman-induced Status Epilepticus

by

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DEDICATION

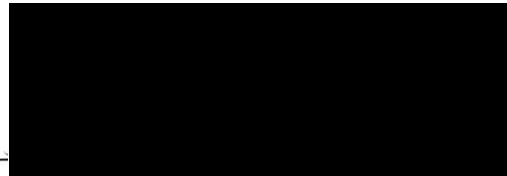
When I was a little kid, my goal was to be a medical doctor, just like my Dad. That goal changed through the years! I even remember graduating from college asking myself, “What next???” My family has been by my side through every moment, good and bad. They have watched me challenge myself, fall, and get up and try again. Mom, thank you for always lifting me up when I have felt discouraged. I will never forget you telling me to believe in myself, say a prayer, and achieve my goals. Dad, I am a “go getter” because of you. I will never be afraid to take a risk or challenge myself because of the example you have set for me. You have taught me to never settle for good, but to always go for great! My work ethic comes from you, and I am forever grateful because it has led me to this achievement. John and Paula, you two are my rock! It is such a pleasure to watch you grow and come into your own. You are both very gifted individuals with so much to offer the world. Thank you for your constant support as I have chased one dream after another. Christopher, I entered this program as your girlfriend, and you waited patiently for me to finish my qualifying exam before we said “I do!” I am forever grateful for your constant love and support. You watched me go through my Master’s program and my Ph.D. work, and there is no way I could have gotten through any of it without your encouragement. You always believe in me and that gets me through the challenges of each day. Thank you for always being by my side. This victory is ours.

My beautiful family, I dedicate this work to you.

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Teresa M. Ferrara-Bowens

November 1, 2016

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ABSTRACT

The role of IL-1 Signaling in Neurodegeneration, Neuroinflammation, and Behavior after Soman-induced Status Epilepticus

Teresa M. Ferrara-Bowens M.A., 2016

Thesis directed by: Erik Johnson, Ph.D., Research Division, Pharmacology Branch, United States Army Medical Research Institute of Chemical Defense

Soman (GD) is a nerve agent (68; 105) that blocks acetylcholinesterase, resulting in a buildup of acetylcholine in the synaptic cleft that induces a cholinergic crisis (103). GD exposure induces status epilepticus (17), that results in brain damage to the piriform cortex, amygdala, thalamus, and hippocampus (6; 24; 25; 54). This brain injury stimulates peripheral macrophages and leukocytes, and microglia and astrocytes to overexpress neurotoxic cytokines, including Interleukin-1 (IL-1), resulting in a neuroinflammatory response (36; 53; 54).

IL-1 binds to the IL-1 receptor (IL-1R1) to initiate IL-1 signaling by activating various kinase pathways, which activate the expression of other pro-inflammatory cytokines in response to injury (111). The IL-1R1 is inhibited by the IL-1 receptor antagonist (IL-1Ra), which competes with IL-1 for binding to the IL-1R1 (99). IL-1R1 and IL-1Ra knockout (KO) mice have been utilized to study various forms of CNS injury such as cerebral ischemia, β -amyloid infusion, and kainic acid induced seizures (9; 27; 120; 122). However, the role of IL-1 signaling after GD exposure has never been investigated with these KO strains.

The purpose of these studies was to determine the role of IL-1 signaling after GD-induced SE on neurodegeneration, neuroinflammation, and behavioral function. Wild type (WT), IL-1Ra KO, and IL-1R1 KO mice were exposed to GD, after which behavior was assessed with the Open Field, Zero Maze, and Barnes Maze (102). The neuroinflammatory response was examined using multiplex bead immunoassays (54). Anakinra, the human recombinant IL-1Ra (43) therapeutic, was studied to assess its neuroprotective effect after GD exposure.

Results showed that WT and IL-1Ra KO mice sustain brain injury after GD-induced SE, whereas IL-1R1 KO mice show less brain damage 24 hours after convulsion onset that does not extend up to 18 days post exposure. IL-1R1 KO mice were less anxious than the WT mice after GD exposure, but IL-1Ra KO mice showed impaired learning over time. Anakinra did not improve the neuropathological or behavioral consequences of GD exposure. Finally, WT, IL-1Ra, and IL-1R1 KO mice showed various cytokine expression after GD exposure that may reflect compensatory mechanisms of action due to the regulation of IL-1 signaling.

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CHAPTER 1: INTRODUCTION

Nerve agent exposure, whether intentional or accidental, continues to threaten the military and civilian populations (68; 105). During World War II, the Germans modified already existing organophosphate (OP) compounds to formulate soman (GD), a nerve agent that is volatile at room temperature and can be fatal if absorbed through the skin or inhaled (31; 98). GD is a unique OP because it undergoes aging within two minutes of exposure, where reactivation of the AChE cannot be reversed (113). Upon exposure, GD binds to acetylcholinesterase (AChE), resulting in a buildup of acetylcholine (ACh) in the synaptic cleft in the central (CNS) and peripheral (PNS) nervous systems (103). ACh buildup in the synaptic cleft stimulates a glutamatergic burst, where excessive amounts of glutamate bind to the AMPA and NMDA receptors (74; 134). This leads to a neurotransmitter imbalance resulting in long lasting seizures, defined as status epilepticus (SE) (30; 31; 74; 134).

Although OP intoxication results in hypersecretions, rhinorrhea, miosis, bronchial constriction, and convulsions (38; 73; 103; 134), it is the magnitude and duration of SE that is correlated with the primary brain damage that occurs after GD exposure (17). This primary brain damage is the result of excitotoxicity, characterized by overstimulation of the cholinergic receptors and the upregulation of glutamate, which initiates seizure activity that leads to neuronal swelling and damage (109). If left untreated, acute neurotoxicity occurs minutes to hours after exposure, represented by the presence of eosinophilic cells in the piriform cortex, amygdala, thalamus, and hippocampus. This primary damage is followed by neuron depletion and cell regeneration days to months after exposure to compensate for cell loss (22; 24; 25; 33; 53; 54; 113; 135).

Primary brain injury resulting from GD-induced SE stimulates microglia and astrocytes in the CNS, and activated macrophages and infiltrating leukocytes in the periphery to upregulate inflammatory cytokines, including Interleukin-1 (IL-1) (31; 36; 53; 54). IL-1 is a pleiotropic cytokine that not only mediates inflammation, but plays a role in long term potentiation and memory formation (111). In the healthy brain, IL-1 is produced at minimal levels, but after GD-induced SE, it is transiently increased and participates in a positive feedback loop resulting in secondary brain damage (8; 22; 54; 78; 110). Therefore, primary brain damage resulting from SE initiates a neuroinflammatory response that contributes to secondary brain damage (9). That secondary damage consists of eosinophilic cells, edema, and neuronal cell loss lasting days to months after injury (24). This is seen in the CA1 region of the hippocampus after GD exposure in wild type (WT) mice, where eosinophilic tissue damage found day one after injury progressively gets worse on days 15 and 30 after exposure (22).

IL-1 consists of 2 isoforms, IL-1 α and IL-1 β that are collectively referred to as IL-1 because they are functionally similar (Fig.1) (86; 107). IL-1 binds to either the IL-1 receptor (IL-1R1) or the IL-1R type II receptor (IL-1RII) (66; 110). When IL-1 binds to the IL-1R1, a conformational change recruits the IL-1R1 accessory protein (IL-1R1AcP) to form a trimer that activates the Toll and IL-1R-like (TIR) domains to activate two proteins, the myeloid differentiation primary response gene 88 (MYD88) and the Interleukin-1 receptor-activated protein kinase (IRAK) (133). These downstream events turn on the IL-1 signaling pathway, resulting in the activation of two distinct pathways leading to transcription (51). In the first pathway, the I κ B subunit of the nuclear factor kappa-B (NF- κ B) is phosphorylated leading to the release of NF- κ B (51; 91). NF- κ B

activation signals the release of other pro-inflammatory cytokines including Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF α), making it a key component in the process of inflammation (111). The second pathway activates three MAP kinase pathways: p38, JNK, and ERK (51; 80; 82). Each pathway plays a key role in the inflammatory response as they are activated by stress, can weaken cell function, and induce neuronal cell death (15; 69; 84; 91). Unlike the IL-1R1, the IL-1RII lacks a cytosolic signaling component and serves as a decoy (100; 133). IL-1 signaling can be inhibited with the IL-1 receptor antagonist (IL-1Ra), which is produced endogenously and competes with IL-1 for binding to the IL-1R1 (99; 100).

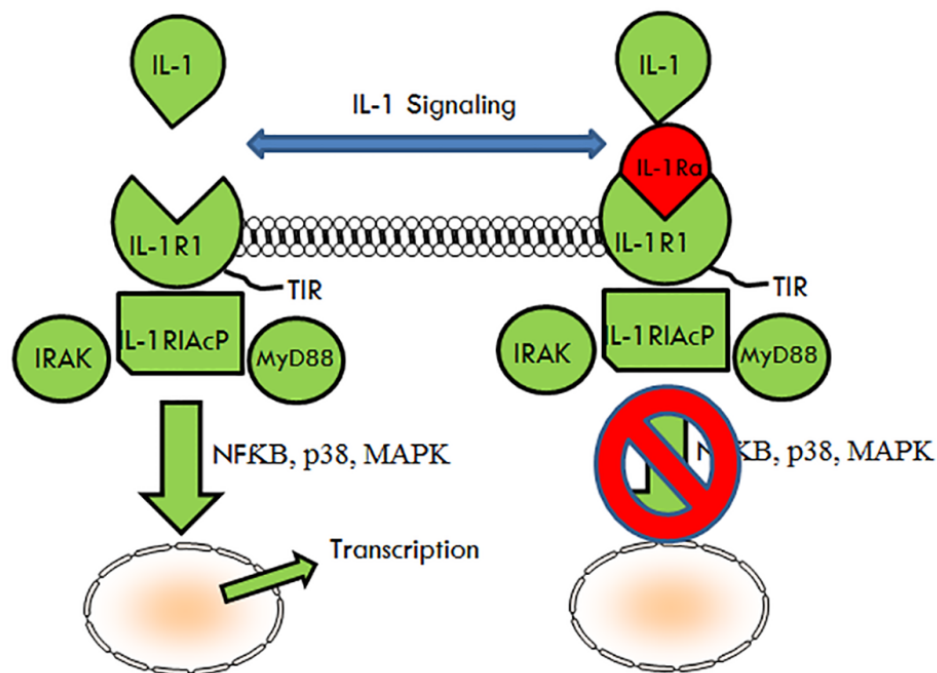


Fig. 1. The IL-1 Signaling Pathway

When IL-1 binds to the IL-1R1 receptor, various kinase pathways are activated, and transcription occurs. The endogenous IL-1Ra blocks the IL-1R1, inhibiting IL-1 signaling.

Recent studies have shown that IL-1 can also bind to the recently discovered and largely uncharacterized IL-1R3 receptor, which is derived from IL-1R1 as a shortened IL-1R1 messenger RNA (mRNA) and expressed in neural tissue (91; 125). IL-1 β binds to IL-1R3, which associates with the IL-1R accessory B protein (IL-1RAcB) that is preferentially expressed in neurons to activate the Akt kinase pathway (91). Activation of this kinase pathway is a neuroprotective mechanism against ischemic and excitotoxic injury (34). However, activation of IL-1R3 still promotes neuroinflammation (91). Whereas NF- κ B activation is MYD88-dependent in normal IL-1 signaling, Akt activation is MYD88 independent (91), so that IL-1 signaling still occurs without the presence of MYD88 when IL-1 binds to the IL-1R3. Additionally, IL-1R1 KO mice may still express IL-1R3, (91), making them prone to ischemic brain damage when IL-1R1 is not present (116).

IL-1 is known as the master cytokine of inflammation (36), and it plays a regulatory role in the expression of other inflammatory cytokines, including IL-6 and TNF α (133). IL-6 has both pro- and anti-convulsant properties (61). When IL-6 binds to its soluble or membrane-bound receptor, it associates with two molecules of gp130, which are necessary for signal transduction (2; 42). This dimerization activates the Janus kinase family (JAKs) of tyrosine kinases, which phosphorylate transcription factors of the STAT (signal transducers and activators of transcription) family, activating transcription (42). Not only is IL-6 produced by neurons, microglia, and astrocytes, as well as IL-1 β and TNF α , but it can also be blocked by the IL-1Ra (42; 112). Like IL-6, TNF α also has neurotoxic and neuroprotective properties and binds to either the TNF receptor 1 (TNF-R1) or TNF receptor 2 (TNF-R2) (59). The TNF-R1 contains cytoplasmic domains that

initiate apoptosis or activate the NF- κ B pathway upon TNF α binding (1; 11). Activation of the TNF-R2 promotes cell growth but also potentiates TNF-R1 function and NF- κ B activation (59). TNF α also works synergistically with IL-1 β to activate nitric oxide synthase expression (iNOS), which plays a role in the immune response and neuropathology after injury (32; 131). Like IL-1, TNF α activation recruits leukocytes to areas of injury and promotes further production of itself, IL-6, and IL-1 β (59).

As previously shown, the phosphorylation and ubiquitination events occurring upon IL-1 activation induce the expression of IL-6 and TNF α (133) and help progress the neuroinflammatory response. IL-1, IL-6, and TNF α upregulation have been reported at both gene and protein levels after GD exposure in small rodents between one and 48 hrs of seizure onset (33; 54; 114; 135). Neurodegeneration is also a key component of seizure activity after GD exposure (8; 22; 53; 54; 78; 110) that is mediated to some extent by the neuroinflammatory process. IL-1R1 knockout (KO) and IL-1Ra KO mouse strains have been used to study the role of IL-1 signaling in neurodegeneration using other models of CNS injury such as cerebral ischemia, β -amyloid infusion, and kainic acid and bicuculline induced seizures (9; 27; 120; 122). However, the role of IL-1 signaling in brain injury and cognitive outcomes after GD-induced SE has not been studied.

GD-induced SE results in hyperexcitation of the glutamatergic system (74; 132). Mice exposed to a convulsive dose of GD do not show spatial learning one month after exposure in the Morris Water Maze (MWM) (39). This lack of learning and memory is attributed to hippocampal neuropathology (24; 95) that occurs as a consequence of GD-induced SE and cytokine upregulation (33; 54; 114; 135). GD intoxication also results in anxiety-like behavior and hyperactivity (7; 26; 93). GD-exposed animals placed in the

Elevated Plus Maze (EPM) spend less time in the open arms of the maze, indicating anxiety (7; 26), and animals placed in the Open Field run around the outside center of the arena and spend more time in that region indicating hyperactivity (13; 93). Although the role of IL-1 signaling in cognition and anxious behavior after GD-induced SE has not been studied, the behavior of IL-1R1 KO mice has been examined. This mouse strain has been found to be less anxious, as well as active and exploratory in the Open Field and EPM (57; 78; 129). The IL-1R1 KO is also capable of spatial learning and memory in the MWM (78). Unlike the IL-1R1 KO, IL-1Ra KO mice are prone to anxiety-like behavior, which may be due to overactive IL-1 signaling (129).

Although the neuropathology, neuroinflammation, and behavioral consequences of GD-induced SE have been previously addressed, the role of IL-1 signaling as it relates to these factors has not been studied. This research is especially important because the IL-1 signaling pathway may be a therapeutic target to mitigate the consequences of long lasting seizure activity after GD exposure. The current FDA-approved treatment regimen for nerve agent poisoning includes atropine sulfate (anticholinergic that antagonizes ACh buildup), pralidoxime chloride (2-PAM: oxime that reactivates AChE), and diazepam (benzodiazepine anticonvulsant) (104), but none of these treatments are effective for the neurodegeneration and neuroinflammation occurring after GD-induced SE. One drug of interest, anakinra, is the human recombinant IL-1Ra, and is currently approved to treat rheumatoid arthritis (43). It has been reported to mitigate SE (67) and has been found to reduce peripheral inflammatory cytokines in stroke patients (43).

Therefore, the purpose of these studies is two-fold. WT, IL-1R1 KO and IL-1Ra KO mice will be used to investigate the effects of IL-1 signaling in GD-induced

neuropathology, neuroinflammation, anxiety-like behavior, and cognitive deficit.

Because the receptor is missing in the IL-1R1 KO, this mouse strain should show less neuropathology and neuroinflammatory cytokine upregulation due to the inhibition of IL-1 signaling compared to the exposed WT group. As a result, the IL-1R1 KO mice should be less anxious and capable of learning and memory after nerve agent exposure. Because of the continuous IL-1 signaling in the IL-1Ra KO mice, these animals should show severe neuropathology and neuroinflammation, as well as increased anxiety-like behavior and reduced learning and memory function compared to the exposed WT strain.

Anakinra will then be tested in the exposed WT group to determine if therapeutically targeting the IL-1 signaling pathway will improve the neuropathological and inflammatory consequences, as well as the behavioral deficit that occurs after GD-induced SE.

CHAPTER 2: Multiplex studies to evaluate cytokine expression after soman induced status epilepticus

INTRODUCTION.

Exposure to soman (GD) inhibits acetylcholinesterase (AChE), resulting in a buildup of acetylcholine (ACh) in the synaptic cleft (103). The cholinergic crisis that ensues is characterized by convulsions and continuous seizure activity (status epilepticus: SE), which damages the central nervous system (CNS) within 24 hours of exposure (6; 53; 54). SE compromises the blood brain barrier (BBB), making the brain vulnerable to leukocyte infiltration and macrophage activation (16; 53; 54). Chemokine upregulation helps guide peripheral neutrophils to damaged regions of the CNS, where microglia and astrocytes increase the expression of pro-inflammatory acute phase cytokines, including Interleukin-1 (IL-1), Interleukin-6 (IL-6), and Tumor Necrosis Factor- α (TNF α) (20; 32; 33; 53; 54; 59; 61; 135). As the master cytokine of inflammation (36), IL-1 signaling regulates the expression of IL-6 and TNF α , which are cytokines with both pro- and anti-inflammatory properties (59; 61).

IL-1 α and IL-1 β are functionally similar and are collectively referred to as IL-1 (86; 107). IL-1 binds to the IL-1R receptor (IL-1R1), activating the IL-1R1 accessory protein (IL-1R1acP). This trimer recruits the myeloid differentiation primary response gene 88 (MYD88) and the Interleukin-1 receptor activated protein kinase (IRAK) to initiate IL-1 signaling, where phosphorylation and ubiquitination events activate various kinase pathways to initiate transcription, including p38, JNK, and ERK (51; 80; 82; 121; 133). Another kinase pathway activated by IL-1 signaling is nuclear factor- κ B (NF- κ B), which induces the expression of IL-6 and TNF α , so that these cytokines work together to

produce the neuroinflammatory response (92; 133). IL-1 signaling is inhibited by the IL-1 receptor antagonist (IL-1Ra), which competitively inhibits the IL-1R1 and prevents IL-1 signaling (99; 100).

Although the presence of IL-1, IL-6, and TNF α has been reported after GD-induced SE (33; 54; 114; 135), the role of IL-1 signaling in the neuroinflammatory response has not been investigated after GD exposure. Therefore, the purpose of this study was to determine cytokine expression levels of IL-1, IL-6, and TNF α after GD-induced SE as a measure of the neuroinflammatory response seen in wild type (WT), IL-1R1 knockout (KO), and IL-1Ra KO mouse strains. Anakinra, the human recombinant IL-1Ra biologic drug, was also used in exposed WT mice to determine the effect of pharmacological inhibition of IL-1 signaling on cytokine expression. Results showed early upregulation of IL-1 followed by later downregulation in the KO strains, as well as IL-1 signaling early and later after anakinra treatment. These results show the regulatory function of IL-1 regarding neuroinflammation after GD-induced SE. Additionally, targeting the IL-1 signaling pathway with anakinra changes cytokine expression, and therefore, the neuroinflammatory response.

METHODS.

Animals

Male C57BL/6J mice (000664; wild type; n = 323), IL-1 receptor antagonist knockout (004754; IL-1Ra KO; n = 107), and IL-1 receptor (003245; IL-1R1; n = 92) KO mice (C57BL/6J background) were obtained from Jackson Laboratories (Bar Harbor, ME). A total of 522 animals were utilized for these studies. Animals were between eight and 10 weeks when testing began. Mice were group housed by strain in polycarbonate

cages in a temperature- and humidity-controlled room (68-79°F, 30-70% humidity) for at least one week prior to exposure to nerve agent. The animals were maintained on a 12-hour (hr) light-dark schedule (lights on at 0600) with ad libitum access to food and water. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The animal care program at this Institute is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

GD Seizure Model

On the day of exposure, animals received an intraperitoneal (IP) pre-treatment of the oxime HI-6 dichloride (1-(((4-(aminocarbonyl)pyridinio) methoxy) methyl)-2-((hydroxymino)methyl)pyrdinium dichloride); BN44621, Starks Associates, Buffalo, NY; 50 mg/kg) in saline (SAL) five minutes prior to sub cu (SC) GD administration (147 μ g/kg). Vehicle control animals received only HI-6 and SAL. GD (purity 98.8%) was obtained from the United States Army Medical Research Institute of Chemical Defense. The number (n) of animals per group were as follows: WT (exposed: n = 94; control: n = 62), IL-1Ra KO (exposed n = 69; control: n = 38); IL-1R1 KO (exposed: n = 40; control: n = 52). A separate group of WT animals (exposed: n = 75; control: n = 92) were exposed to GD or SAL and treated with a high dose (502 mg/kg, SC) of anakinra (Kineret^R, Swedish Orphan Biovitrum, Stockholm, Sweden) one hr after convulsion onset, and a low dose of anakinra (199 mg/kg, SC) at four, eight, and 12 hrs after

convulsion onset. SAL controls received HI-6 and anakinra at the same times. Animals were observed for convulsion onset and euthanized at one, three, six, 12, 24, and 48 hrs after exposure. At the selected time points, the mice were deeply anesthetized with sodium pentobarbital (80 mg/kg, IP) and euthanized by exsanguination with 0.9% SAL. The piriform cortex, amygdala, thalamus, and hippocampus were extracted and snap frozen in liquid nitrogen.

Multiplex bead array immunoassay

The multiplex assay was used to determine the peak cytokine response after GD-induced convulsions in all exposed and control mice. As previously reported (54) the extracted brain regions were weighed, homogenized, and centrifuged to remove the precipitate and extract the lysate. Protein counts were conducted on the samples and 100 µg of protein was added to each well for each sample. Cytokine concentrations were quantified with a mouse cytokine multiplex bead immunoassay kit (EMD Millipore, Billerica, MA) containing the IL-1 α , IL-1 β , IL-6, and TNF α . The plates were read on a MAGPix instrument (EMD Millipore), and XPonent (EMD Millipore) software was used for analysis.

Statistics

Data was analyzed by a one-way ANOVA followed by a Tukey's post hoc test (if homogeneity of variance was present) or a post hoc Dunnett's analysis (if unequal variances present) to determine differences among the exposed groups. The data is presented as a within strain comparison (compares each GD exposed mouse strain to its same strain SAL control) and a between strain comparison (compares the GD exposed KO mouse strains to the GD exposed WT group). Excluding the anakinra data, all

exposed groups were normalized to their individual SAL controls for statistical purposes to account for variability, as each SAL control had a different baseline for comparison. Therefore, each exposed group is presented as a percentage of the control group. A one-way ANOVA was appropriate to analyze the within- and between strain comparisons. For within strain, time was the main factor in comparing each exposed strain to the normalized SAL control; for between strain, time was the main factor of analysis because only the GD exposed strains were used for the analysis. For anakinra data analysis, the data was not normalized to the control, as it would have disregarded the effect of anakinra treatment in the SAL controls at the time points of interest.

RESULTS.

All data presented in this section reflects the exposed WT and KO mouse strains normalized to their individual SAL controls, so that each GD exposed group is presented as a percentage of the control group. Within strain comparison describes the expression of each cytokine of interest in each exposed strain compared to their respective controls. For between strain comparison, each exposed KO mouse strain was compared to the exposed WT group to explain cytokine expression at the selected time points. For anakinra results, only the WT exposed and SAL groups were treated with anakinra. No normalization to the SAL controls was done in order to analyze the effect of anakinra on the SAL controls and the exposed animals. For the anakinra data, within group results compare the exposed WT group treated with anakinra to the SAL group treated with anakinra, and between group results compare the untreated exposed WT to the anakinra-treated exposed WT group.

IL-1 α : within strain comparison

The following results are depicted in Table 1. In the amygdala, a significant upregulation of IL-1 α (p<0.05) was seen at 24 hrs after convulsion onset in the IL-1Ra KO exposed animals, whereas a significant downregulation of IL-1 α (p<0.05) was seen in the IL-1R1 KO exposed group. In the piriform cortex, an upregulation of IL-1 α was seen in the WT exposed at 1 (p<0.01) and 24 hrs (p<0.05) after convulsion onset. In the thalamus, the IL-1Ra KO group showed an upregulation of IL-1 α (p<0.01) at 1 hr, whereas the IL-1R1 KO showed a downregulation of IL-1 α (p<0.01) at 12 hrs. Finally, in the hippocampus, an upregulation of IL-1 α (p<0.05) was seen at 3 hrs after convulsion onset in the IL-1R1 KO group.

Table 1: IL-1 α up- or downregulation in the GD-exposed WT, IL-1Ra, and IL-1R1 KO strains as compared to their respective vehicle controls (VC).

Amy: amygdala; Piri: Piriform Cortex; Thal: Thalamus; Hipp: Hippocampus.

Amy: IL-1 α	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC					* 	
IL-1R1 Exp/VC					* 	
Piri: IL-1 α	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC	** 				* 	
IL-1Ra Exp/VC						
IL-1R1 Exp/VC						
Thal: IL-1 α	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC	** 					
IL-1R1 Exp/VC				** 		
Hipp: IL-1 α	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC						
IL-1R1 Exp/VC		* 				

IL-1 α : between strain comparison

In the amygdala, IL-1 α was significantly upregulated 1 hr after convulsion onset in the IL-1Ra KO mice ($p<0.05$; Fig. 2A) compared to WT. In the piriform cortex, IL-1 α was upregulated in the IL-1Ra KO mice 12 hrs after convulsion onset ($p<0.05$; Fig. 2B). No significant difference in IL-1 α expression was found between the KO strains and WT group in the thalamus (Fig. 2C). In the hippocampus, there was a significant upregulation of IL-1 α 12 hrs after convulsion onset in the IL-1R1 KO mice ($p<0.0001$; Fig. 2D).

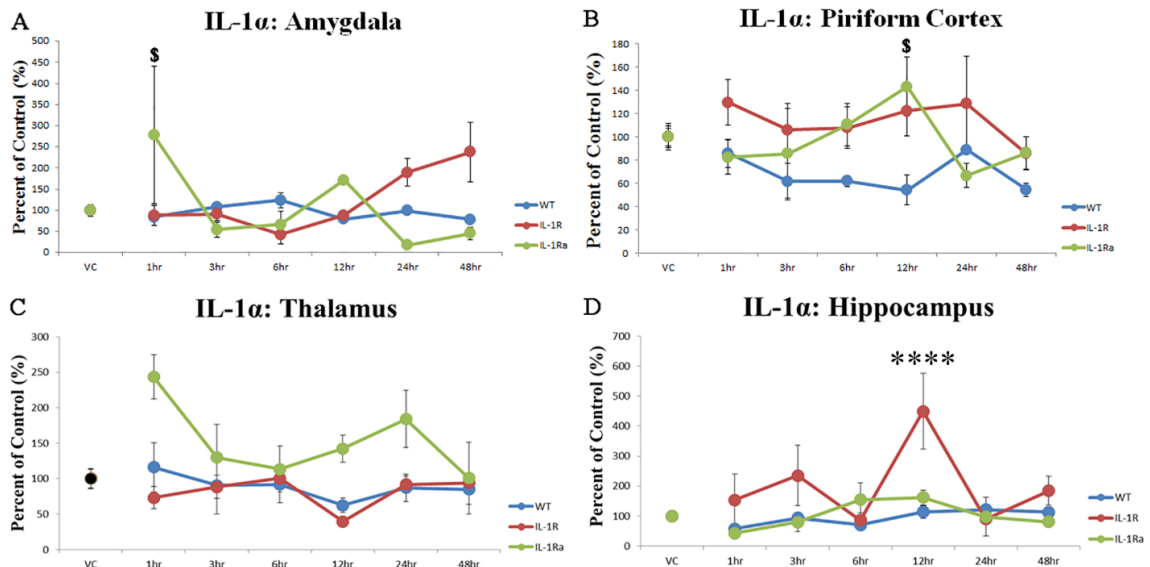


Fig. 2. Expression of IL-1 α after GD-induced SE.

Changes in IL-1 α expression in the amygdala, piriform cortex, thalamus, and hippocampus of IL-1Ra KO and IL-1R1 KO mice, as compared to WT mice. \$ represents statistical differences between the exposed IL-1Ra KO and WT groups ($p<0.05$); * represents statistical differences between the exposed IL-1R1 KO and WT groups (**** $p<0.0001$).

IL-1 β : within strain comparison

The following results are depicted in Table 2. In the amygdala, a significant downregulation of IL-1 β ($p<0.01$) was seen at 24 hrs in the exposed IL-1Ra KO and at 12 hrs in the exposed IL-1R1 KO. In the piriform cortex, a significant downregulation of IL-1 β ($p<0.01$) was seen at 24 hrs in the exposed IL-1Ra KO. In the exposed IL-1R1

KO, IL-1 β was upregulated at 3 (p<0.05) and 12 hrs (p<0.01), but downregulated at 48 hrs (p<0.01). In the thalamus, IL-1 β was upregulated at 3 hrs (p<0.001) in the exposed WT group. Finally, in the hippocampus, IL-1 β was upregulated at 1 hr (p<0.01) in the exposed WT, at 24 hrs (p<0.01) in the exposed IL-1Ra KO, and at 1 (p<0.001) and 3 hrs (p<0.01) in the exposed IL-1R1 KO group.

Table 2. IL-1 β up- or downregulation in the exposed WT, IL-1Ra, and IL-1R1 KO strains as compared to their respective vehicle controls (VC).

Amy: amygdala; Piri: Piriform Cortex; Thal: Thalamus; Hipp: Hippocampus

Amy: IL-1 β	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC					**↓	
IL-1R1 Exp/VC				**↓		
Piri: IL-1 β	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC					**↓	
IL-1R1 Exp/VC		*↑		**↑		**↓
Thal: IL-1 β	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC		***↑				
IL-1Ra Exp/VC						
IL-1R1 Exp/VC						
Hipp: IL-1 β	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC	**↑					
IL-1Ra Exp/VC					*↑	
IL-1R1 Exp/VC	***↑	**↑				

IL-1 β : between strain comparison

In the amygdala, there was a significant upregulation of IL-1 β in the IL-1Ra KO animals 1 hr after convulsion onset (Fig. 3A). No significant differences in IL-1 β expression were found in the piriform cortex between the exposed KO mouse strains and

the exposed WT group (Fig. 3B). Fig. 3C shows a significant downregulation of IL-1 β in the IL-1R1 KO group ($p<0.05$) and the IL-1Ra KO group ($p<0.01$) 3 hrs after convulsion onset. A significant upregulation of IL-1 β was found in the hippocampus of the IL-1Ra KO ($p<0.01$) 24 hrs after convulsion onset (Fig. 3D).

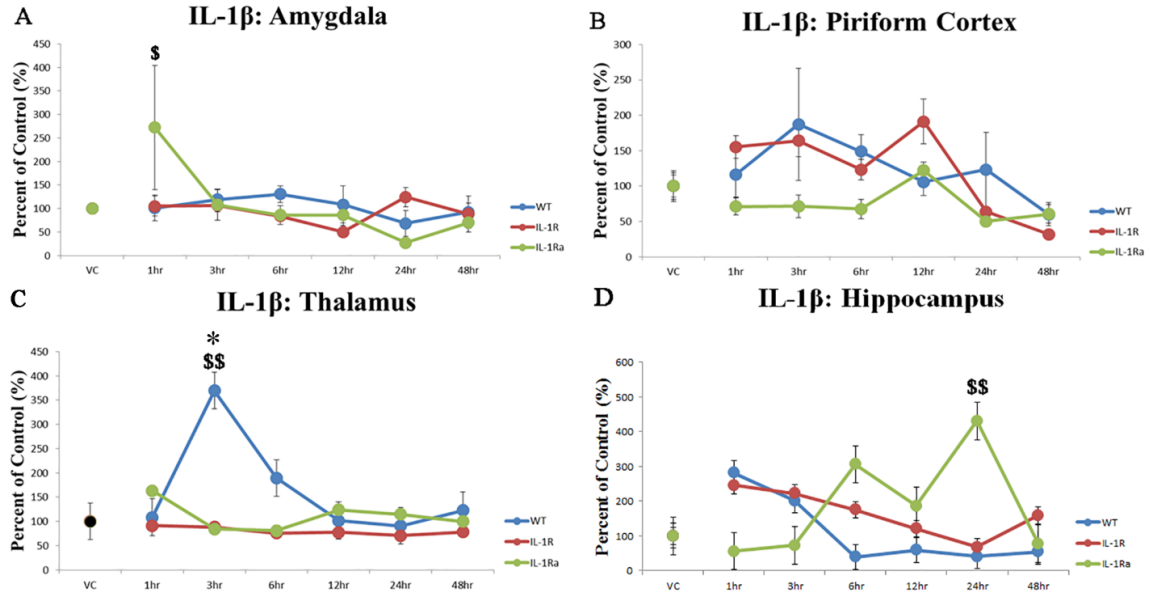


Fig. 3. Expression of IL-1 β after GD-induced SE.

Changes in IL-1 β expression in the amygdala, piriform cortex, thalamus, and hippocampus of IL-1Ra KO and IL-1R1 KO mice, as compared to WT mice. \$ represents statistical differences between the exposed IL-1Ra KO and WT (\$ $p<0.05$, ** $p<0.01$); * represents statistical differences between the exposed IL-1R1 KO and WT (* $p<0.05$).

IL-6: within strain comparison

The following results are depicted in Table 3. In the amygdala, there was a significant upregulation of IL-6 ($p<0.05$) in the exposed WT 48 hrs after convulsion onset. IL-6 was also upregulated in the exposed IL-1Ra KO ($p<0.001$) at 24 hrs. In the exposed IL-1R1 KO, an upregulation of IL-6 was seen at 3 ($p<0.001$), 6 ($p<0.01$), and 12 hrs ($p<0.01$). In the piriform cortex, IL-6 was upregulated in the exposed WT at 24

($p < 0.01$) and 48 hrs ($p < 0.001$). In the exposed IL-1Ra KO, IL-6 was upregulated at 3 ($p < 0.01$), 6 ($p < 0.001$) and 12 hrs ($p < 0.001$). In the exposed IL-1R1 KO, IL-6 was upregulated at 24 hrs ($p < 0.001$). Turning to the thalamus, IL-6 was upregulated in the exposed WT at 12 ($p < 0.05$), 24 ($p < 0.01$), and 48 hrs ($p < 0.001$). IL-6 was downregulated in the IL-1R1 KO ($p < 0.05$) 12 hrs after seizure onset. Finally, in the hippocampus, there was a significant increase in IL-6 in the exposed WT at 12 ($p < 0.05$), 24 ($p < 0.01$), and 48 hrs ($p < 0.001$). Upregulation at the same time points was seen in the exposed IL-1Ra KO (12 hrs $p < 0.05$; 24 hrs $p < 0.001$; 48 hrs $p < 0.01$). Finally, in the exposed IL-1R1 KO, IL-6 was upregulated at 6, 12, and 48 hrs ($p < 0.001$).

Table 3. IL-6 up- or downregulation in the exposed WT, IL-1Ra, and IL-1R1 KO strains as compared to their respective vehicle controls (VC).

Amy: amygdala; Piri: Piriform Cortex; Thal: Thalamus; Hipp: Hippocampus

Amy: IL-6	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						*↑
IL-1Ra Exp/VC					***↑	
IL-1R1 Exp/VC		***↑	**↑	**↑		
Piri: IL-6	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC					**↑	***↑
IL-1Ra Exp/VC		**↑	***↑	***↑		
IL-1R1 Exp/VC					***↑	
Thal: IL-6	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC				*↑	**↑	***↑
IL-1Ra Exp/VC						
IL-1R1 Exp/VC				*↓		
Hipp: IL-6	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC				**↑	*↑	***↑
IL-1Ra Exp/VC				*↑	***↑	**↑
IL-1R1 Exp/VC			***↑	***↑		***↑

IL-6: between strain comparison

In the amygdala and piriform cortex, no significant differences in IL-6 expression were found between the exposed WT and KO strains (Figs. 4A and 4B). However, in the thalamus, a significant downregulation of IL-6 was found in the IL-1Ra KO ($p<0.05$) and the IL-1R1 KO ($p<0.05$) 48 hrs after convulsion onset (Fig. 4C) relative to the increase seen in WT mice. This same downregulation of IL-6 was seen in the hippocampus at 48 hrs between the IL-1Ra KO ($p<0.0001$) and the IL-1R1 KO ($p<0.0001$) when compared to the exposed WT group (Fig. 4D).

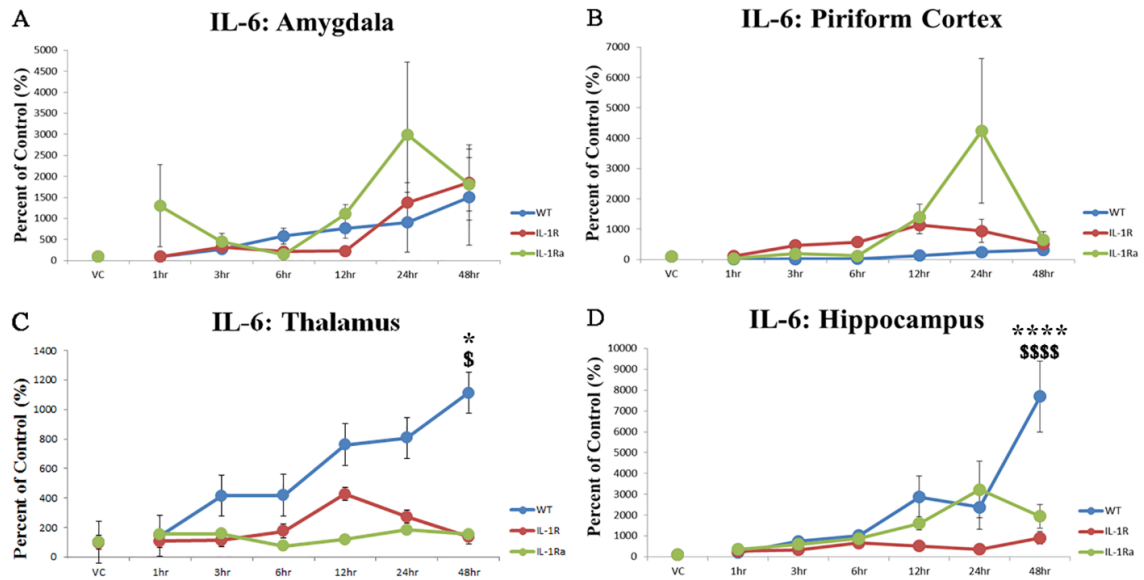


Fig. 4. Expression of IL-6 after GD-induced SE.

Changes in IL-6 expression in the amygdala, piriform cortex, thalamus, and hippocampus of IL-1Ra KO and IL-1R1 KO mice, as compared to WT mice. \$ represents statistical differences between the exposed IL-1Ra KO and WT (\$ $p<0.05$, \$\$\$\$ $p<0.0001$); * represents statistical differences between the exposed IL-1R1 KO and WT (* $p<0.05$, **** $p<0.0001$).

TNF α : within strain comparison

The following results are depicted in Table 4. In the amygdala, there was an increase in TNF α expression at 48 hrs ($p<0.001$) in the exposed WT group. There was also a significant increase in TNF α expression at 1 hr ($p<0.05$) in the exposed IL-1Ra KO. The exposed IL-1R1 KO showed a significant increase in TNF α at 6 hrs ($p<0.05$),

which then decreased at 24 hrs ($p<0.001$). In the piriform cortex, $\text{TNF}\alpha$ was upregulated in the exposed WT at 6, 12, 24, and 48 hrs ($p<0.001$). In the exposed IL-1Ra KO, an increase in $\text{TNF}\alpha$ was seen at 48 hrs ($p<0.05$). The exposed IL-1R1 KO showed an increase in $\text{TNF}\alpha$ at 12 ($p<0.001$), 24 ($p<0.05$), and 48 hrs ($p<0.001$). In the thalamus, the exposed IL-1Ra KO showed an increase in $\text{TNF}\alpha$ 1 hr after exposure ($p<0.01$). Finally, in the hippocampus, an increase in $\text{TNF}\alpha$ was seen in the exposed IL-1Ra KO at 3 and 24 hrs ($p<0.05$) after convulsion onset.

Table 4. $\text{TNF}\alpha$ up- or downregulation in the exposed WT, IL-1Ra, and IL-1R1 KO strains as compared to their respective vehicle controls (VC).

Amy: amygdala; Piri: Piriform Cortex; Thal: Thalamus; Hipp: Hippocampus

Amy: $\text{TNF}\alpha$	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						***↑
IL-1Ra Exp/VC	*↑					
IL-1R1 Exp/VC			*↑		***↓	
Piri: $\text{TNF}\alpha$	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC			***↑	***↑	***↑	***↑
IL-1Ra Exp/VC						*↑
IL-1R1 Exp/VC				***↑	*↑	***↑
Thal: $\text{TNF}\alpha$	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC	***↑					
IL-1R1 Exp/VC						
Hipp: $\text{TNF}\alpha$	1 hr.	3 hr.	6 hr.	12 hr.	24 hr.	48 hr.
WT Exp/VC						
IL-1Ra Exp/VC		*↑			*↑	
IL-1R1 Exp/VC						

$\text{TNF}\alpha$: between strain comparison

In the amygdala, a significant upregulation of $\text{TNF}\alpha$ was seen in the IL-1Ra KO group ($p<0.05$) 1 hr after convulsion onset (Fig. 5A). In the piriform cortex, a significant

downregulation of TNF α was seen in the IL-1Ra KO ($p<0.05$) 24 hrs after convulsion onset (Fig. 5B). Fig. 5C shows no significant difference in TNF α expression in the thalamus. There was a significant increase in TNF α at 24 hrs in the hippocampus of the IL-1Ra KO ($p<0.05$) when compared to WT (Fig. 5D).

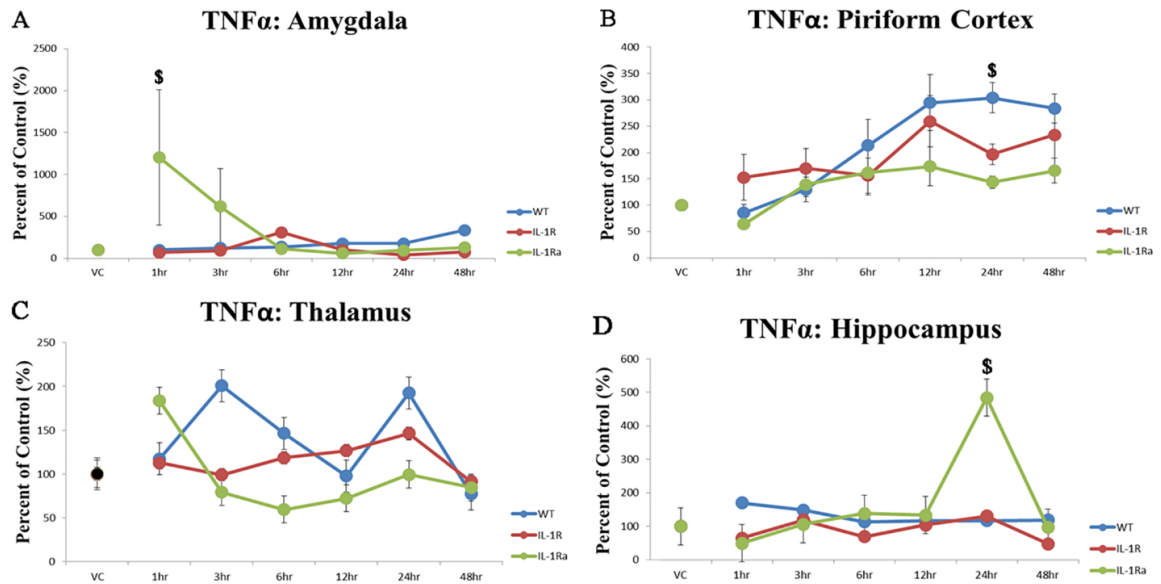


Fig. 5. Expression of TNF α after GD-induced SE.

Changes in TNF α expression in the amygdala, piriform cortex, thalamus, and hippocampus in IL-1Ra KO and IL-1R1 KO mice, as compared to WT mice. \$ represents statistical differences between the exposed IL-1Ra KO and WT (\$ $p<0.05$).

Anakinra results

For the following results, the WT animals exposed to GD and treated with anakinra are compared to the non-anakinra treated WT exposed animals from the previous study.

IL-1 α within group comparison after anakinra treatment

No significant differences were found between the exposed and SAL groups treated with anakinra.

IL-1 α between group comparison after anakinra treatment

In the amygdala (Fig. 6A), a significant downregulation of IL-1 α was seen in the anakinra-treated exposed group at 1 (p<0.001), 3, 6, 12 (p<0.0001), 24, and 48 hrs (p<0.01). There was also a significant downregulation in IL-1 α expression at 48 hrs (p<0.01) in the anakinra-treated animals compared to the untreated group (Fig. 6B). Figs. 6C and 6D show no significant differences between the untreated and anakinra-treated exposed groups for IL-1 α expression.

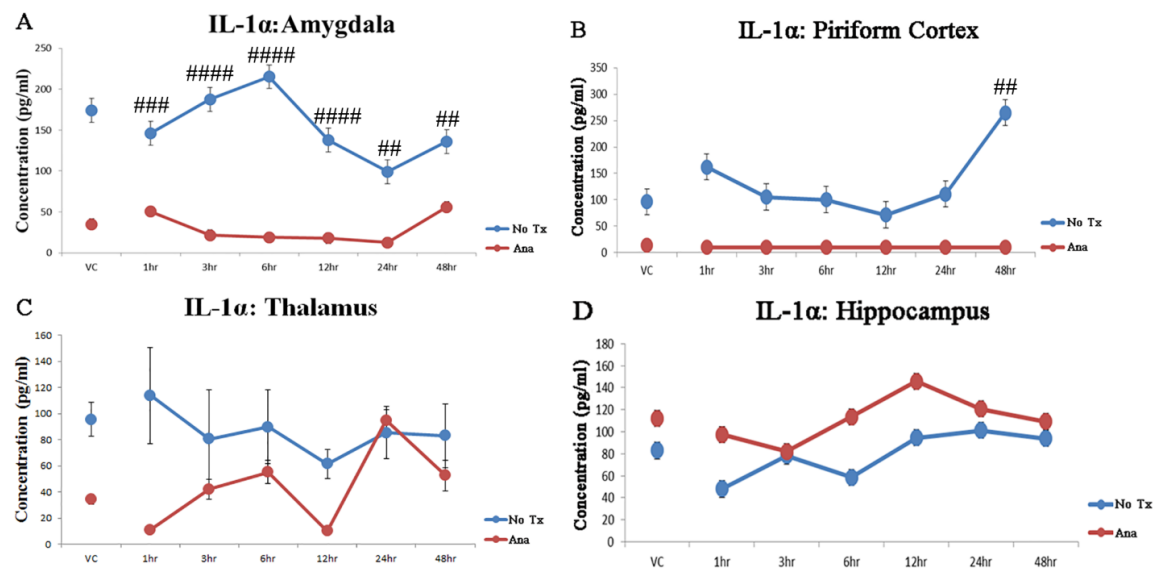


Fig. 6. IL-1 α expression after anakinra treatment.

IL-1 α expression in the amygdala, piriform cortex, thalamus, and hippocampus in exposed WT mice treated with anakinra. # represents statistical differences between the untreated exposed and the anakinra-treated exposed groups (## p<0.01, ### p<0.0001).

IL-1 β within group comparison after anakinra treatment

No significant differences were found between the exposed and SAL groups treated with anakinra.

IL-1 β between group comparison after anakinra treatment

In the amygdala, there was no significant difference between the untreated and anakinra-treated exposed groups for IL-1 β expression (Fig. 7A). In the piriform cortex, IL-1 β was significantly upregulated at 3 (p<0.01) and 6 hrs (p<0.05) in the anakinra-treated animals compared to the untreated WT group (Fig. 7B). In the thalamus, the anakinra-treated group showed a significant upregulation of IL-1 β at 1 (p<0.0001), 6 (p<0.05), 12 (p<0.001), and 48 hrs (p<0.01), unlike the untreated exposed group (Fig. 7C) relative to the untreated WT mice. In the hippocampus, there was a significant upregulation of IL-1 β in the anakinra-treated group at 6, 24, and 48 hrs (p<0.05, Fig. 7D).

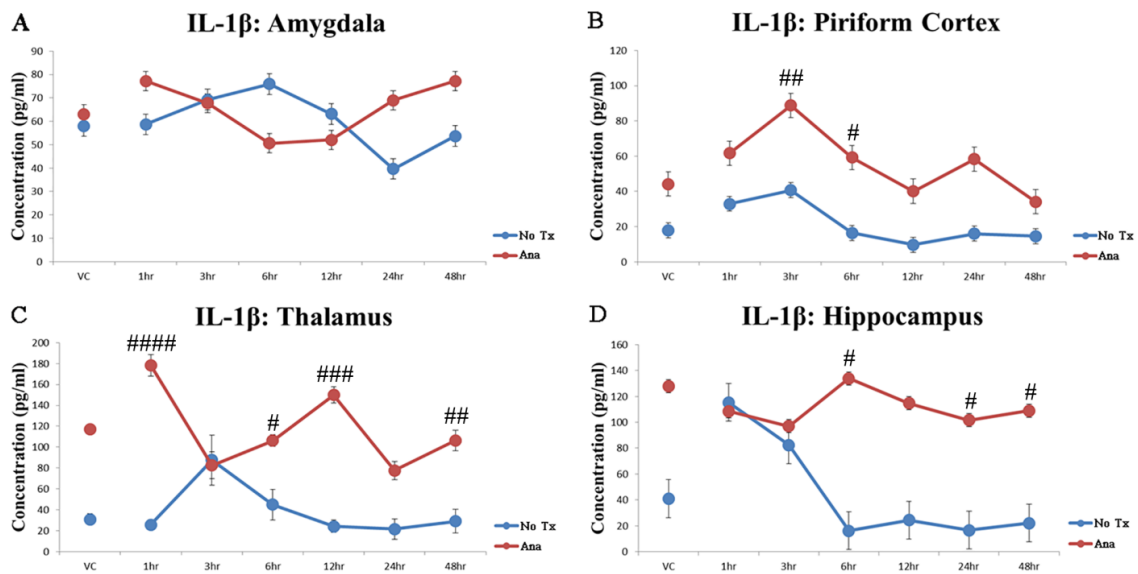


Fig. 7. IL-1 β expression after anakinra treatment.

IL-1 β expression in the amygdala, piriform cortex, thalamus, and hippocampus in exposed WT mice treated with anakinra. # represents statistical differences between the untreated exposed and the anakinra-treated exposed groups (# p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001).

IL-6 within group comparison after anakinra treatment

In the amygdala, the anakinra treated exposed group had an upregulation of IL-6 at 12 and 24 hrs ($p<0.001$) compared to the anakinra-treated SAL control (Fig. 8A).

There was also an upregulation of IL-6 at 24 hrs in the hippocampus of the anakinra-treated exposed group compared to the anakinra-treated SAL control ($p<0.001$) (Fig. 8D).

IL-6 between group comparison after anakinra treatment

There was a significant downregulation of IL-6 at 48 hrs ($p<0.05$) in the anakinra-treated exposed group compared to the untreated exposed group (Fig. 8A). There was also a significant upregulation of IL-6 at 12 and 24 hrs ($p<0.01$) in the anakinra-treated exposed group compared to the untreated exposed group (Fig. 8B). In the thalamus, no significant differences were found between the untreated exposed and anakinra-treated exposed animals (Fig. 8C). In the hippocampus, there was a significant increase at 48 hrs ($p<0.0001$) in the anakinra-treated exposed group compared to the untreated exposed group (Fig. 8D).

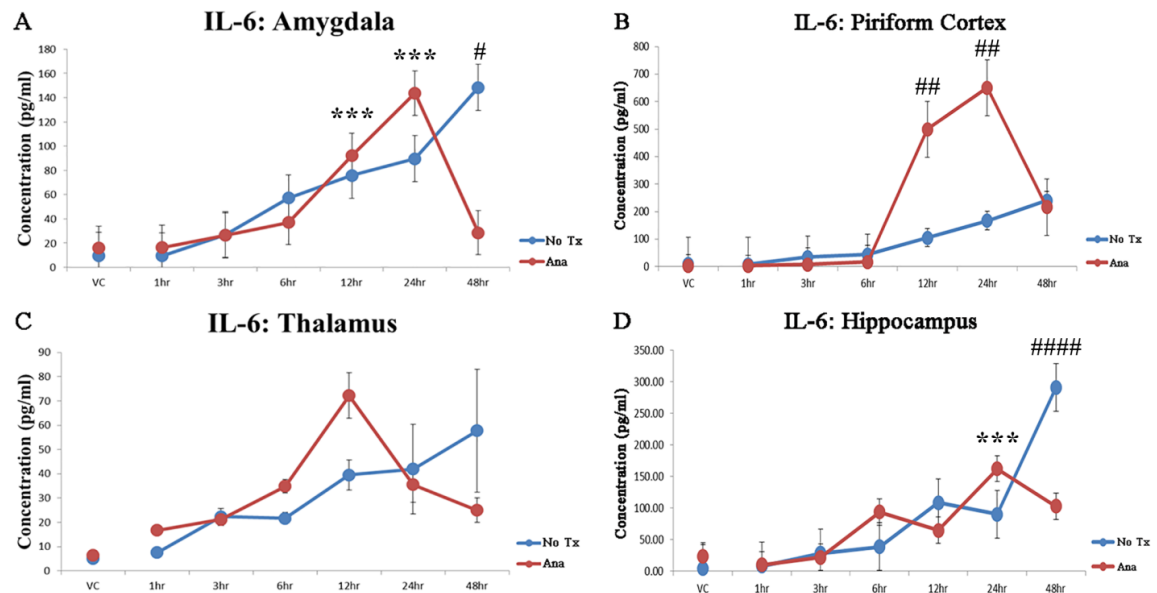


Fig. 8. IL-6 expression after anakinra treatment.

IL-6 expression in the amygdala, piriform cortex, thalamus, and hippocampus in exposed WT mice treated with anakinra. * represents statistical differences between the anakinra-treated exposed and the anakinra-treated SAL groups (** $p < 0.001$); # represents statistical differences between the untreated exposed and the anakinra-treated exposed groups (# $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$).

TNF α within group comparison after anakinra treatment

In the amygdala, there was a significant increase in TNF α in the anakinra-treated exposed group when compared to the anakinra-treated SAL control at 12 and 24 hrs ($p < 0.05$) (Fig. 9A).

TNF α between group comparison after anakinra treatment

In the amygdala, there was a significant downregulation of TNF α at 48 hrs in the anakinra-treated exposed group when compared to the untreated exposed group ($p < 0.0001$, Fig. 9A). No significant differences were seen for TNF α in the piriform cortex (Fig. 9B) or thalamus (Fig. 9C). There was a significant upregulation of TNF α at 12 hrs ($p < 0.05$) in the anakinra-treated exposed group when compared to the untreated exposed group (Fig. 9D).

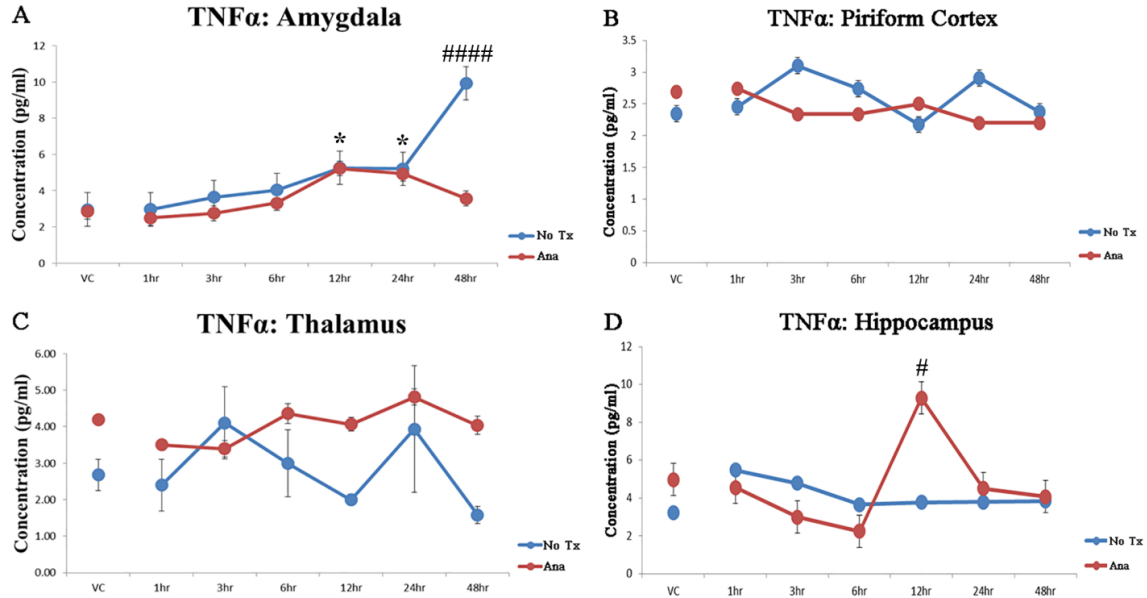


Fig. 9. TNFα expression after anakinra treatment.

TNFα expression in the amygdala, piriform cortex, thalamus, and hippocampus in exposed WT mice treated with anakinra. * represents statistical differences between the anakinra-treated exposed WT and anakinra-treated SAL groups (* $p < 0.05$); # represents statistical differences between the untreated exposed and the anakinra-treated exposed groups (# $p < 0.05$, #### $p < 0.0001$).

DISCUSSION.

The results of this study showed different patterns of cytokine expression between the WT and KO mouse strains after GD exposure. Previous work has shown that IL-1α and IL-1β are functionally similar (86; 107), which may explain the pattern of IL-1 expression seen in this work, where there was an early upregulation of IL-1α and IL-1β between one and three hrs in the WT (Tables 1, 2; Figs. 2B, 3D), IL-1Ra KO (Table 1; Fig. 2C), and the IL-1R1 KO mice (Tables 1, 2; Figs. 2D, 3B, 3D), followed by no significant IL-1 expression at six hrs, and a mix of downregulation and upregulation of IL-1 between 12 and 24 hrs. At these later time points, downregulation was seen in the IL-1Ra KO (Table 2; Figs. 3A, 3B) and the IL-1R1 KO (Tables 1 and 2; Figs. 2A, 2C,

3B), whereas upregulation was found in the WT (Table 1; Fig. 2B), IL-1Ra KO (Tables 1, 2; Figs. 2A, 3D), and the IL-1R1 KO groups (Table 2; Fig. 3B).

In terms of early IL-1 expression, cerebral ischemia research has shown that IL-1 α is released from dying cells. This causes tissue inflammation as a result of neutrophil infiltration to the injured brain (18; 66; 97). At the level of gene and protein expression in mice exposed to middle cerebral artery occlusion (MCAo), IL-1 α is upregulated earlier than IL-1 β after injury (66). Four hrs after MCAo and reperfusion injury in C57BL6/H mice, only IL-1 α expression was found in the microglia of the ipsilateral hemisphere, whereas IL-1 α and IL-1 β upregulation were found 24 hours after injury (66). Furthermore, after GD-induced SE in rats, IL-1 α is produced in activated microglia (54). These results suggest that IL-1 α mediates the early inflammatory response (66; 77). However, lipopolysaccharide (LPS), an environmental agent that induces inflammation (14) has been used in conjunction with kainic acid to study its effect on SE (4). Peripheral administration of LPS has been shown to increase IL-1 β at the mRNA and protein levels in the hippocampus, cortex, and hypothalamus between 30 minutes and six hrs of injection (14). Therefore, the early expression of IL-1 β after GD-induced convulsions may be due to the fact that IL-1 β is preferentially secreted in the CNS after brain injury (12). Also, the piriform cortex is involved in initiating soman-induced seizures, and the hippocampus is a target of propagating signal influx from the amygdala and piriform cortex (72). Therefore, the increase in IL-1 α one hr after convulsion onset in the piriform cortex (Table 1) in this study may play a role in the upregulation of IL-1 β (Table 2) in the hippocampus and thalamus early after convulsion onset.

Like the WT group, the IL-1Ra KO mice showed early IL-1 α expression in the thalamus and late expression in the amygdala (Table 1), indicating that the absence of IL-1Ra does not affect the temporal upregulation of IL-1 α after GD-induced convulsions. Since the IL-1Ra is not present to block the IL-1R1, the upregulation of IL-1 α in the exposed IL-1Ra KO is not unexpected. Furthermore, in other forms of CNS injury such as cerebral ischemia and β -amyloid infusion, IL-1Ra KO mice are more susceptible to neurodegeneration and neuroinflammation (27; 86), and the presence of pro-inflammatory cytokines like IL-1 α may contribute to that injury (66). The exposed IL-1R1 KO group showed a pattern of IL-1 β upregulation in the hippocampus (Table 2; Fig. 3D) and piriform cortex (Table 2; Fig. 3B) early after convulsion onset, followed by a downregulation of IL-1 β in the amygdala (Table 2; Fig. 3A) and piriform cortex (Table 2; Fig. 3B) later. This shows that the absence of IL-1R1 results in an increase in IL-1 β expression early after convulsion onset that decreases with time. Interestingly, previous work reported a biphasic response of IL-1 β at the mRNA level in the cortex, where WT Swiss mice exposed to GD showed an increase in IL-1 β at six hrs, a decrease at 24 and 48 hrs, and an increase at seven days (33). Early IL-1 β expression may be due to the involvement of the piriform cortex in seizure initiation (17). Not only does IL-1 β play a role in maintaining seizure activity (120; 122), but IL-1 β can induce its own transcription (36), which may account for its early expression in this study.

Later expression of IL-1 in this study is similar to previous work reporting the upregulation of IL-1 α and IL-1 β in rats exposed to GD-induced SE between 12 and 24 hrs after seizure onset in the hippocampus, piriform cortex, and thalamus (54). Unlike the IL-1Ra KO mice, IL-1 α was downregulated in the IL-1R1 KO at later time points in

the amygdala (Table 1; Fig. 2A) and thalamus (Table 1; Fig. 2C). At these time points, no increase in IL-6 or TNF α was seen in these brain regions, showing the regulatory role of this signaling pathway. With the results of this study, one can infer that without IL-1 upregulation at these time points, IL-6 and TNF α are not expressed so that the positive feedback loop resulting in neuroinflammation and secondary brain damage is inhibited. Although these results show that the missing IL-1R1 likely plays a role in attenuating IL-1 α upregulation after GD-induced convulsions, this was not the case in the hippocampus (Fig. 2D), where IL-1 α was expressed early. However, IL-1R1 KO mice have been shown to be susceptible to increases in IL-1 α , IL-1 β , TNF- α , and IL-6 after hypoxic/ischemic insult (9). Although these increases are not significantly higher than what is seen in the undamaged WT brain (9), this finding suggests that the missing IL-1R1 influences the expression of other pro-inflammatory cytokines (60). The current study supports this point as the exposed IL-1R1 KO mice also showed increases in IL-1 β (Table 2; Figs. 3B, 3D), IL-6 (Table 3; Figs. 4A, 4B, 4D), and TNF- α (Table 4; Figs. 5A, 5B), at various time points after GD exposure.

Surprisingly, the absence of IL-1Ra resulted in downregulation of IL-1 β later in the amygdala (Table 2; Fig. 3A) and piriform cortex (Table 2; Fig. 3B). This downregulation may occur for many reasons. At 24 hrs, IL-1 α is upregulated in the amygdala (Table 1; Fig. 2A) and IL-6 is upregulated in the amygdala (Table 3; Fig. 4A) and hippocampus (Table 3; Fig. 4D) of the IL-1Ra KO. One ischemia study reported that it is possible that deleting one form of IL-1 results in compensatory post-receptor signaling changes so that the effect of the initial brain injury is not reduced (12), which could explain the results of the current study. Additionally, when IL-1 binds to the IL-

1R1, several phosphorylation and ubiquitination events activate NF κ B and p38 mitogen-activated protein kinase pathways, which induce the expression of IL-1 α , IL-1 β , and IL-6 at the gene level (133). The exposed IL-1Ra KO also showed an upregulation of IL-1 β in the hippocampus at 24 hrs (Table 2; Fig. 3D), which may be due to the upregulation of IL-1 β early in the amygdala, a brain region sensitive to the seizurogenic effects of GD exposure (33). Also, in rats exposed to GD-induced SE, dystrophic microglia express IL-1 β , not IL-1 α , at later times after GD exposure (54), which could explain the later increase in IL-1 β in the hippocampus at 24 hrs.

IL-6 expression was also seen after GD-induced convulsions in the exposed WT and KO mouse strains. In the WT, a pattern of late expression was seen across all brain regions studied (Table 3; Figs. 4A-4D). Limbic seizures in rodents have been shown to upregulate IL-6 at the level of transcription (123). IL-6 mRNA was significantly upregulated in the hippocampus of GD-exposed Swiss mice between 6 and 48 hrs of exposure (33). At the level of protein expression in GD-exposed rats, IL-6 was upregulated at 12 and 24 hrs in the piriform cortex, amygdala, thalamus and hippocampus; IL-6 peaked at 24 hrs in the piriform cortex, and it peaked between 12 and 24 hrs in the hippocampus (54). Additionally, cerebral ischemia research has shown that IL-1 β and TNF α mRNA can induce IL-6 expression (42; 76). In the current study, IL-1 β was expressed at one hr in the hippocampus (Table 2; Fig. 3D) and TNF α was expressed at six hrs piriform cortex (Table 4; Fig. 5B) of the WT group, which may have induced IL-6 expression later (42; 54; 112). Additionally, the hippocampus is a main area for IL-6 and IL-6 receptor transcripts (42), so that its presence in the hippocampus is not unwarranted.

Both the IL-1R1 and IL-1Ra KO mouse strains altered their expression levels of IL-6 between three and 12 hrs in the amygdala and piriform cortex (Table 3) that was not seen in the WT mice, inferring that the absence of the IL-1R1 or IL-1Ra contributes to early IL-6 upregulation as a compensatory mechanism. More specifically, like IL-1Ra, IL-6 has neuroprotective effects (3; 112), so that in the IL-1Ra KO, IL-6 upregulation may compensate for the lack of IL-1Ra. Additionally, IL-1Ra can block IL-6 induction (42). Therefore, upregulation of IL-6 in the IL-1Ra KO is expected since the IL-1Ra is missing. In the IL-1R1 KO, IL-1Ra cannot bind to the missing receptor, so that IL-6 upregulation in the IL-1R1 KO may serve as a compensatory neuroprotective mechanism of action. Additionally, early upregulation of TNF α and IL-1 β in the IL-1Ra KO (Table 4; Figs. 5A, 5C, 5D) and IL-1R1 KO (Table 2; Figs. 3B, 3D), respectively, may have resulted in IL-6 expression. A similar pattern of expression was also seen between the WT and KO mouse strains between 12 and 48 hrs in the hippocampus (Table 3; Fig. 4D). At these later time points, IL-6 production may have been induced by damaged cells in the hippocampus (9), continuous convulsions leading to possible SE, or systemic IL-6 penetrating the compromised BBB (42). Interestingly, IL-6 was downregulated at 12 hrs in the thalamus of the IL-1R1 KO (Table 3), and this is the only time and brain region of IL-6 downregulation. No increases in IL-1 β or TNF α were found in the thalamus before this time point, so these cytokines would not have been available to induce IL-6 expression (42; 76), which may contribute to the downregulation seen.

Like IL-6 expression, TNF α was upregulated after GD-induced convulsions. A pattern of TNF α expression in the WT at later time points (Table 4; Figs. 5A, 5B) agrees with previous work in GD-exposed rats, which showed TNF α expression between six

and 12 hrs (54). Ischemic work has shown later upregulation of TNF α between 12 and 24 hrs after permanent MCAO (20; 45; 59), whereas research with kainic acid induced seizures in rodents have reported TNF α expression between two and six hrs of exposure (32; 61; 76; 123), which could explain the early upregulation of TNF α in the IL-1Ra KO (Table 4; Figs. 5A, 5C, 5D) of the current study. Like the WT group, both KO strains showed increased levels of TNF α later after exposure (Table 4; Figs. 5B, 5D). IL-1Ra KO mice have been reported to show increases in neuroinflammatory cytokines like IL-1 β and TNF α after β -amyloid infusion resulting in neuroinflammation (27). The early and later upregulation of TNF α in the current study may be due to the presence of IL-1 β in the WT and KO mouse strains, which has been reported to induce TNF α expression (120). On the other hand, whereas the upregulation of IL-1 β can maintain SE (120), increases in IL-6 and TNF α have attenuated IL-1 β release after kainic acid exposure (61). This was seen at 48 hrs in the current study with the downregulation of IL-1 β (Table 2; Fig. 3B) and the upregulation of TNF α (Table 4; Fig. 5B) in the IL-1Ra KO. This shows that the absence of IL-1Ra does not inhibit compensatory mechanisms from further damaging or protecting the CNS as TNF α and IL-6 have neuroprotective and neurotoxic properties (32; 59; 61; 94; 127).

Compared to their respective SAL control, the GD exposed IL-1R1 KO in this study showed a downregulation of TNF α (Table 4) and IL-1 α (Table 1) in the amygdala at 24 hrs as previously mentioned. Ischemic research has reported minimal increases in neuroinflammatory cytokines after injury in IL-1R1 KO mice (9), implying the missing receptor may serve as a neuroprotective mechanism. The IL-1R1 KO also showed increases in TNF α at six hrs in the amygdala (Table 4; Fig. 5A) along with IL-6

upregulation in the amygdala (Table 3; Fig. 4A) and hippocampus (Table 3; Fig. 4D). As previously mentioned, this upregulation may be a compensatory neuroprotective mechanism because IL-1Ra cannot bind to IL-1R1 to block the IL-1 signaling pathway.

Anakinra treatment also showed a pattern in IL-1 expression, where anakinra successfully blocked the upregulation of IL-1 α across all time points in the amygdala (Fig. 6A) and the piriform cortex (Fig. 6B) but did not prevent the upregulation of IL-1 β early in the piriform cortex (Fig. 7B), or at later time points in the hippocampus (Fig. 7D). This later increase of IL-1 β supports cerebral ischemia studies reporting that IL-1 α mediates the early inflammatory response (66; 77). However, early IL-1 β expression after anakinra treatment may be due to the fact that after CNS injury, IL-1 β is preferentially secreted (12). Also, the hippocampus is a target of propagating signal influx from the piriform cortex (72) so that the early increase of IL-1 β in the piriform cortex may play a role in the later upregulation of IL-1 β in the hippocampus.

The pattern of IL-6 expression after anakinra treatment was later upregulation in the amygdala (Fig. 8A) and hippocampus (Fig. 8D), compared to the anakinra-treated SAL controls. These results may serve a neurotoxic or neuroprotective role as IL-6 has pro- and anti-convulsant properties (61), and seizures trigger cytokine upregulation (33; 53; 54; 113; 118; 122; 124; 135). However, as seen in chapter 4, neuropathology after anakinra treatment improved at 24 hrs in the piriform cortex and amygdala compared to the untreated exposed, so that the significant expression of IL-6 after anakinra treatment may be neuroprotective. In this case, even though anakinra can block IL-6 expression (42; 112), its inability to do so in the amygdala may not be neurotoxic. Compared to the untreated exposed animals, the treated exposed group showed an upregulation of IL-6 in

the piriform cortex (Fig. 8B). This upregulation may be due to the presence of IL-1 β in this region (Fig. 7B), which can induce IL-6 expression (42; 112).

The fact that TNF α was upregulated in the amygdala of the anakinra-treated GD exposed mice compared to the treated SAL group (Fig. 9A) may be harmful because TNF α can work synergistically with IL-1 β to initiate iNOS expression, which may affect neurotoxicity (32; 131). Therefore, the increase in TNF α in the hippocampus of the anakinra-treated GD exposed group versus the untreated GD exposed groups (Fig. 9D), where IL-1 β was also found to be upregulated (Fig. 7B), may induce a neurotoxic response via iNOS overexpression. Interestingly, rats undergoing hippocampal stimulation to induce seizures receiving anakinra treatment did not show increases in TNF α (32), and a downregulation of TNF α was seen in the amygdala of the current study (Fig. 9A). Although IL-1 and TNF α bind to different receptors, the TNF-R1 receptor has a cytosolic domain that stimulates NF κ B, which is also activated by IL-1 signaling (59; 131). Although anakinra does not block TNF-R1 as it does the IL-1R1, the results in the amygdala show that it is possible for this drug to downregulate TNF α expression later after GD exposure, although the mechanism of action is unknown. Since IL-1 and TNF α can work synergistically and anakinra blocks IL-1 signaling, perhaps this drug also indirectly downregulates TNF α expression by an unknown mechanism of action, which could explain why less TNF α expression is found in the amygdala of GD exposed mice treated with anakinra.

CONCLUSION.

This study shows that GD-induced SE affects cytokine expression, and therefore, the neuroinflammatory response that occurs after GD exposure. It also shows that key

components of the IL-1 signaling pathway may play a role in the upregulation and downregulation of other pro- and anti-inflammatory cytokines whose expression depends on the normal functioning of the IL-1 signaling pathway. Targeting the IL-1 signaling pathway with anakinra changed cytokine expression, and therefore, the neuroinflammatory response, where the effect may not necessarily be protective due to the neurotoxic and neuroprotective effects of IL-6 and TNF α . Future studies may analyze the effect of GD-induced SE on various chemokines and cytokines affected after nerve agent exposure to further understand the mechanism of the neuroinflammatory response and the secondary neurodegeneration that occurs due to this inflammatory response.

CHAPTER 3: Neuropathological and behavioral sequelae to soman exposure in IL-1R1 or IL-1Ra gene knockout mice

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ABSTRACT.

Soman (GD) exposure results in status epilepticus (SE) that leads to neurodegeneration, neuroinflammation, and behavioral consequences including learning and memory deficits. The neuroinflammatory response is characterized by the upregulation of the pro-inflammatory cytokine, Interleukin-1 (IL-1), which mediates the expression of other neurotoxic cytokines induced after GD exposure. However, the specific role of IL-1 signaling has not been defined in terms of the consequences of GD-induced SE. Therefore, the purpose of this study was to regulate IL-1 signaling and study the behavioral deficits and neurodegeneration that occur after convulsion onset. Wild type (WT), IL-1 receptor (IL-1R1) knockout (KO) and IL-1 receptor antagonist (IL-1Ra) KO mice were exposed to a convulsive dose of GD, after which behavior was evaluated up to 18 days post exposure. Activity was studied using the Open Field, anxiety was assessed in the Zero Maze, and spatial learning and memory was evaluated with the Barnes Maze. The animals were euthanized at 24 hours and at the completion of the behavioral studies (18 days) to determine neuropathology in the piriform cortex, amygdala, thalamus, and CA1, CA2/3, and CA4 regions of the hippocampus. Unlike the IL-1Ra KO, the IL-1R1 KO showed less neuropathology at 24 hours, but moderate to severe injury was found in all strains at 18 days. Following GD exposure, behavioral testing in the Open Field showed hyperactivity in all strains studied, and the Zero Maze Test indicated all strains exhibited enhanced anxiety. Primary

latency to the goal was used in the Barnes Maze to evaluate learning performance after GD exposure. Compared to WT mice, IL-1R1 and IL-1Ra KO mice exhibited impaired performance. Therefore, the IL-1 signaling pathway affects neurodegeneration and behavior after GD-induced convulsions. Future studies will therapeutically target the IL-1 signaling pathway to mitigate the consequences of nerve agent exposure.

INTRODUCTION.

Chemical warfare nerve agents (CWNA) induce seizure activity through overstimulation of the cholinergic system, where the buildup of acetylcholine (ACh) in the synaptic cleft leads to convulsions and status epilepticus (SE) (73; 103). Soman (pinacolyl methylphosphonofluoridate, GD) is a CWNA of particular interest because it undergoes aging within two minutes of exposure, where reactivation of the blocked acetylcholinesterase (AChE) cannot be reversed (114). The resulting SE that can occur from GD exposure leads to injury in multiple brain regions including the piriform cortex, amygdala, thalamus, and hippocampus. The central nervous system (CNS) injury caused by pathogenic exposure to nerve agents results in leukocyte infiltration and macrophage activation (53; 54). Chemokines guide peripheral neutrophils to damaged regions of the CNS, where upregulation of acute phase response cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), and Tumor Necrosis Factor-alpha (TNF α) (53) can initiate and exacerbate a pro-inflammatory cascade. A similar response is seen in cerebral ischemia research, where IL-1 increases expression of proinflammatory mediators in microglia, suggesting IL-1 controls cytokine signaling in the CNS after injury (9). This pro-inflammatory process can exacerbate brain damage over time in models of nerve agent exposure, traumatic brain injury (TBI), and ischemic injury resulting in cognitive deficit (13; 24; 26; 39-41; 57; 85).

IL-1 α and IL-1 β (functionally identical and referred to as IL-1) bind to the IL-1R1, which induces downstream inflammatory upregulation (133). Since seizures induce upregulation of IL-1 signaling (120), it is of interest to understand the role of IL-1 in the behavioral response and brain damage resulting from GD-induced SE. A third cytokine, IL-1Ra, binds to the IL-1R1 to limit the activation of IL-1 signaling in a negative feedback loop (133). Upon injury, IL-1 is centrally released in activated astrocytes and microglia and binds to the IL-1R1, which is induced in neurons and astrocytes during SE (119). IL-1 signaling is a tightly regulated process and the natural inhibitor to IL-1R1, IL-1Ra, is also upregulated (99; 100). However, in severe injury states, IL-1Ra increases are not symmetrical to IL-1 release and the proinflammatory signaling continues, exacerbating brain damage (32; 35; 86; 87; 122).

Although IL-1 KO mice have been used to study the role of IL-1 in ischemic insult, beta-amyloid induced brain injury, and seizurogenic research (9; 12; 27; 60; 86; 116; 122), these mice have not been used to study behavior or brain damage after GD-induced SE. Previous work shows conflicting evidence as to whether the absence of IL-1R1 improves spatial learning and memory after ischemic injury (5; 78). Research in IL-1Ra KO mice is limited to describing brain damage only, not cognitive deficits (86). Therefore, this study investigated the role of IL-1 signaling in neuropathology and behavior after GD-induced convulsions, utilizing the Open Field to study activity, the Zero Maze for anxiety, and the Barnes Maze to analyze spatial learning and memory after GD exposure. Using wild type (WT), IL-1R1 KO, and IL-1Ra KO mice, it was found that all strains displayed hyperactive behavior after GD exposure. Additionally, the absence of IL-1R1 attenuates 24-hour neuropathology and anxious behavior over time, whereas the absence of IL-1Ra exacerbates brain damage and does not improve anxious behavior. Unlike the WT and IL-1R1 KO mice, the IL-1Ra KO mice also showed slowed learning over time.

METHODS.

Animals

Male C57BL/6J mice (000664; WT background strain), IL-1Ra KO (004754; IL-1Ra), and IL-1R1 KO (003245; IL-1R1) mice were obtained from Jackson Laboratories (Bar Harbor, ME). The background strain for the two KO mice is C57BL/6J. Animals were between eight and 10 weeks old when testing began. Mice were group housed by strain in polycarbonate cages in a temperature- and humidity-controlled room (68-79°F, 30-70% humidity) for at least one week prior to exposure to nerve agent. The animals were maintained on a 12-hour light-dark schedule (lights on at 0600) with ad libitum access to food and water. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The animal care program at this Institute is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Soman (GD) Seizure Model

On the day of exposure, animals received an intraperitoneal (IP) pre-treatment of the oxime HI-6 dichloride (1-(((4-(aminocarbonyl)pyridinio) methoxy) methyl)-2-((hydroxymino)methyl)pyrdinium dichloride; BN44621, Starks Associates, Buffalo, NY; 50 mg/kg, IP) in saline (SAL) 5 minutes prior to subcutaneous (SC) GD administration (147 μ g/kg, SC). Vehicle control animals received only HI-6 and SAL. A total of 178 animals were used in these studies. GD (purity 98.8%) was obtained from the United States Army Medical Research Institute of Chemical Defense. Animals were observed for convulsion onset, characterized by

full body tremor, and were observed intermittently throughout the day prior to return to their home cages. Beginning the day after exposure, animals were weighed each morning and given wet mash to compensate for weight loss. If the animals did not gain weight, they were given up to 0.2 ml of SAL in each flank SC twice a day (morning and evening) until they gained weight. Animals were transferred to the behavior room four days after exposure and were group housed and tested in the same room. Behavior testing began one week from the day of exposure.

Behavior Testing

Behavior testing began one week after GD exposure for the WT (exposed: n = 21; control: n = 21), IL-1Ra KO (exposed: n = 25; control: n = 18), and IL-1R1 KO (exposed: n = 13; control: n = 15) mouse strains. Behavior testing in the Open Field occurred on days seven and 11, the testing in the Zero Maze on day nine, and in the Barnes Maze on days 14 to 17 after GD exposure.

Open Field

The Open Field (Stoelting Wood Dale, IL) was a plexiglas box (40x40x35 cm) with opaque grey walls and floor used to test general activity and exploration (13). It was illuminated with two free-standing fluorescent lights on either side of the Open Field. On days seven and 11 post exposure, mice were placed in a clean polycarbonate cage with Sani-chip bedding (Harlan Teklad, Madison, WI) for 10 minutes to establish environmental acclimation to testing (48) because the animals were housed in the same room used for behavioral testing. After acclimation, mice were placed in the center of the Open Field and monitored for 10 minutes with a camera linked to ANY-maze Behavioral Tracking Software (Stoelting). This software was used to place a center region (defined as greater than 10 cm from the wall) in the Open Field to form inside center and outside center regions (13). Presence in the center or outside center fields was

determined by where the center of the animal was located (55). Total distance traveled was used to measure activity (13; 55; 90). The Open Field was cleaned with 70% ethanol between tests (55).

Zero Maze

The Zero Maze (Stoelting) was elevated 50 cm from the ground and consisted of a circular platform with a five cm-wide lane divided into two opposite open and closed quadrants. The closed quadrants were surrounded by 15 cm walls, which keep the area dark. The Zero Maze was illuminated with two free-standing fluorescent lights on either side of the maze. On day nine after exposure, the animals were acclimated for 10 minutes in a novel, clean cage, and then placed facing the entrance of a closed quadrant, and allowed to explore the Zero Maze for five minutes (52). The animals were monitored by a camera linked to ANY-maze software, which measured primary latency to exit the starting closed quadrant and time spent in the closed quadrants as measures of anxiety (52; 85). The presence in each area of the maze was determined by where the center of the animal was located. The Zero Maze was cleaned with 70% ethanol between tests (55).

Barnes Maze

The Barnes Maze was used to study spatial learning and memory (89; 102). It consists of a circular table 91 cm in diameter with 20 holes along the perimeter (5-cm diameter) elevated 91 cm from the ground (Stoelting). It was illuminated by two free-standing fluorescent lights on either side of the Barnes Maze. After a 10-minute acclimation period, the animals were transported to the maze in an opaque cup that was overturned in the center of the Barnes Maze, so that the animal stood on the maze in the dark for 30 seconds. The cup was then lifted and the animal was allowed to explore the maze for five minutes. Spatial cues (102) included posters

with black and white geometric shapes placed on the walls surrounding the maze that the animals used to find an escape tunnel. On days 14 to 17 post exposure, each animal underwent four trials with 15 to 25 minutes between trials (102). The animals were monitored by a camera linked to ANY-maze software, which recorded the latency to escape (measure of spatial learning and memory), the number of head pokes into the perimeter holes (measure of error), and the primary latency to head poke into the escape hole (41; 85; 102). If the animal found the escape tunnel within the five-minute test (defined as remaining in the tunnel for 15 seconds), the test ended and the animal was allowed to remain in the tunnel for 30 seconds to reduce anxiety (41). If the animal did not find the escape tunnel within the testing period, it was picked up and gently guided to the escape tunnel, where it was kept for one minute (19; 41). Between trials, the Barnes Maze was cleaned with 70% ethanol to eliminate odor cues (55).

Tissue Collection

At the study end points (24 hours (hrs) and 18 days post exposure), the animals were anesthetized with sodium pentobarbital (75-100 mg/kg, IP) and perfused with SAL for exsanguination. They were then perfused with 4% paraformaldehyde (FD Neurotechnologies, Columbia, MD), and post fixed in 4% paraformaldehyde for 48 to 72 hrs and paraffin processed. Sections were serially cut at five microns from Bregma -1.06mm to -1.34mm using a Leica RM225 microtome and stained with hematoxylin and eosin (H&E) for neuropathology evaluation by a board certified pathologist blinded of the conditions of exposure and strain. A scale of zero to four was used to evaluate the piriform cortex, amygdala, thalamus and hippocampal sections (0: no damage; 1: minimal damage (1-10%); 2: mild damage (11-25%); 3: moderate damage (26-45%); 4: severe damage (>45%) (79). One section per brain region was scored according to the presence or lack of acute neuronal necrosis (shrunken and angular cells

with pyknosis), dark neurons (darkly stained neurons, indistinct nucleus, and apical dendrites), or the presence of inflammation (microglial cells and reactive astrocytes).

Statistical Analyses

Assessment of mortality, the incidence or absence of convulsion, and the incidence of pathogenic convulsion across the three strains were evaluated by the Chi-Square Test. Physiological measures of body weight change overnight, and the time of onset to the presence of convulsive activity after GD treatment were analyzed with a one-way analysis of variance (1-way ANOVA), followed by Dunn's Multiple Comparison post hoc test. Neuropathology data was expressed as the median $\pm$ the first and third quartile and analyzed with the Mann Whitney or Kruskal Wallis followed by a Dunn's Multiple Comparison post hoc test. Open Field and Barnes Maze data were analyzed using a 1-way ANOVA and a Treatment Group x Time Repeated Measures analysis of variance (2-way ANOVA), respectively, followed by Bonferroni post hoc comparisons. In the Barnes Maze, a significant interaction tests whether a difference between groups is the same each day, a significant treatment difference tests whether there is a difference between groups due to treatment, and significance of time tests whether a difference was observed across testing days. Zero Maze data was analyzed with a t-test or 1-way ANOVA followed by a post hoc Dunnett test to compare the KO strains to the WT. Statistical tests were significant where $p < 0.05$ and, where applicable, significant post hoc analysis was also given a value of $p < 0.05$.

RESULTS.

Physiological Outcomes

Table 5 shows four outcomes that were measured: death within 24 hrs of exposure (% mortality 24 hrs), the absence of signs of convulsion (% no convulsion), pathology at 24 hrs (% pathology 24 hrs), and the presence of inflammation (% inflammation).

pathogenic convulsion), and convulsions without neuropathology at 24 hrs (% nonpathogenic convulsion). The IL-1Ra KO had the highest mortality rate after GD-induced SE (33.33%), whereas the IL-1R1 KO showed the least mortality (15.56%), though these differences were not statistically significant ($X^2(2)=4.86$, $p=0.088$). Although the following result did not show statistical significance, the IL-1Ra KO had the highest percentage of nonconvulsing animals (9.09%; $X^2(2)=2.64$, $p=0.122$). The IL-1R1 KO showed the highest percentage of animals with neuropathology after convulsion onset (75.90%), the IL-1Ra KO had the lowest percentage (41.87%), and the groups overall differed ($X^2(2)=13.232$, $p=0.00134$). The IL-1Ra KO mice had the highest percentage of convulsing animals with no pathology (15.70%), but the three strains were not significantly different on this measure ($X^2(2)=1.998$, $p=0.368$). Compared to the weight loss seen in the WT mice (Table 6), the IL-1R1 KO showed the greatest weight loss ($p<0.001$), followed by the IL-1Ra KO mice ($p<0.01$). The IL-1Ra KO group had the shortest convulsion onset time ($p<0.0001$) (Table 6).

Table 5. Physiological outcome data for the three mouse strains after GD exposure.
n, number of animals.

Mouse Strain	% Mortality (24 hrs)	% Non-Convulsion	% Pathogenic Convulsion	% No Pathogenic Convulsion
WT (n=67)	22.39	4.48	65.57	10.34
IL-1Ra KO (n=66)	33.33	9.09	41.87	15.70
IL-1R1 KO (n=45)	15.56	2.22	75.90	6.32

Table 6. Physiological outcome data for the three mouse strains after GD exposure that underwent behavioral studies.

Mouse Strain	$\Delta\%$ Weight 24 hrs after GD	Convulsion Onset Time (min)
WT (n=21)	-14.66 ± 0.34	4.15 ± 0.21
IL-1Ra KO (n=25)	-17.34 ± 1.98 **	2.89 ± 0.18 ***
IL-1R1 KO (n=13)	-18.44 ± 0.94 ***	4.38 ± 0.33

Represents significant differences compared to WT, (** $p < 0.01$, *** $p < 0.001$). n, number of animals. min, minutes.

Neuropathology

Injury to the various brain regions of interest is reflected in the H&E stained coronal sections (Fig. 10) that show injured and healthy tissue post exposure. Neuropathology results were compared between WT and the KO groups at 24 hrs and 18 days post exposure in six regions of the brain: piriform cortex (Fig. 11A), amygdala (Fig. 11B), thalamus (Fig. 11C), and the CA1 (Fig. 11D), CA2/3 (Fig. 11E), and CA4 (Fig. 11F) regions of the hippocampus. In the piriform cortex (Fig. 11A), the WT control strain developed severe neuropathology by 24 hrs that became worse over 18 days. The IL-1R1 KO strain had less brain damage at 24 hrs compared to WT controls that was not sustained up to 18 days. There was no significant difference in neuropathology for the IL-1Ra KO strain compared to WT controls at 24 hrs or 18 days, although neuropathology scores did significantly increase over time. In the amygdala (Fig. 11B), the WT had a significant reduction in neuropathology scores from 24 hrs to 18 days. The IL-1Ra KO had a similar though non-significant reduction. The IL-1R1 KO group again showed less brain damage at 24 hrs compared to the WT, but was not significantly different at 18 days. In the thalamus (Fig. 11C), the WT showed severe damage at 24 hrs and moderate injury at 18 days, whereas both KO strains showed moderate injury at 24 hrs that became severe injury at 18 days. Compared to the WT at 18 days, the IL-1Ra KO showed significantly more damage in the

thalamus. In the CA1 region of the hippocampus (Fig. 11D), all mouse strains showed severe injury at 24 hrs with no change at 18 days post exposure. The CA2/3 (Fig. 11E) was only mildly injured for the WT and IL-1R1 KO mouse strains at 24 hrs, though the IL-1Ra KO had a significantly higher neuropathology score than the WT. At 18 days, the WT and IL-1R1 KO showed reduced damage, though not significantly, whereas the IL-1Ra KO showed no such recovery. Finally, in the CA4 region of the hippocampus (Fig. 11F), the WT and IL-1Ra KO showed moderate and severe injury, respectively at 24 hrs, whereas the IL-1R1 KO had significantly less injury than the WT. All mouse strains showed severe injury at 18 days post exposure in the CA4 region of the hippocampus.

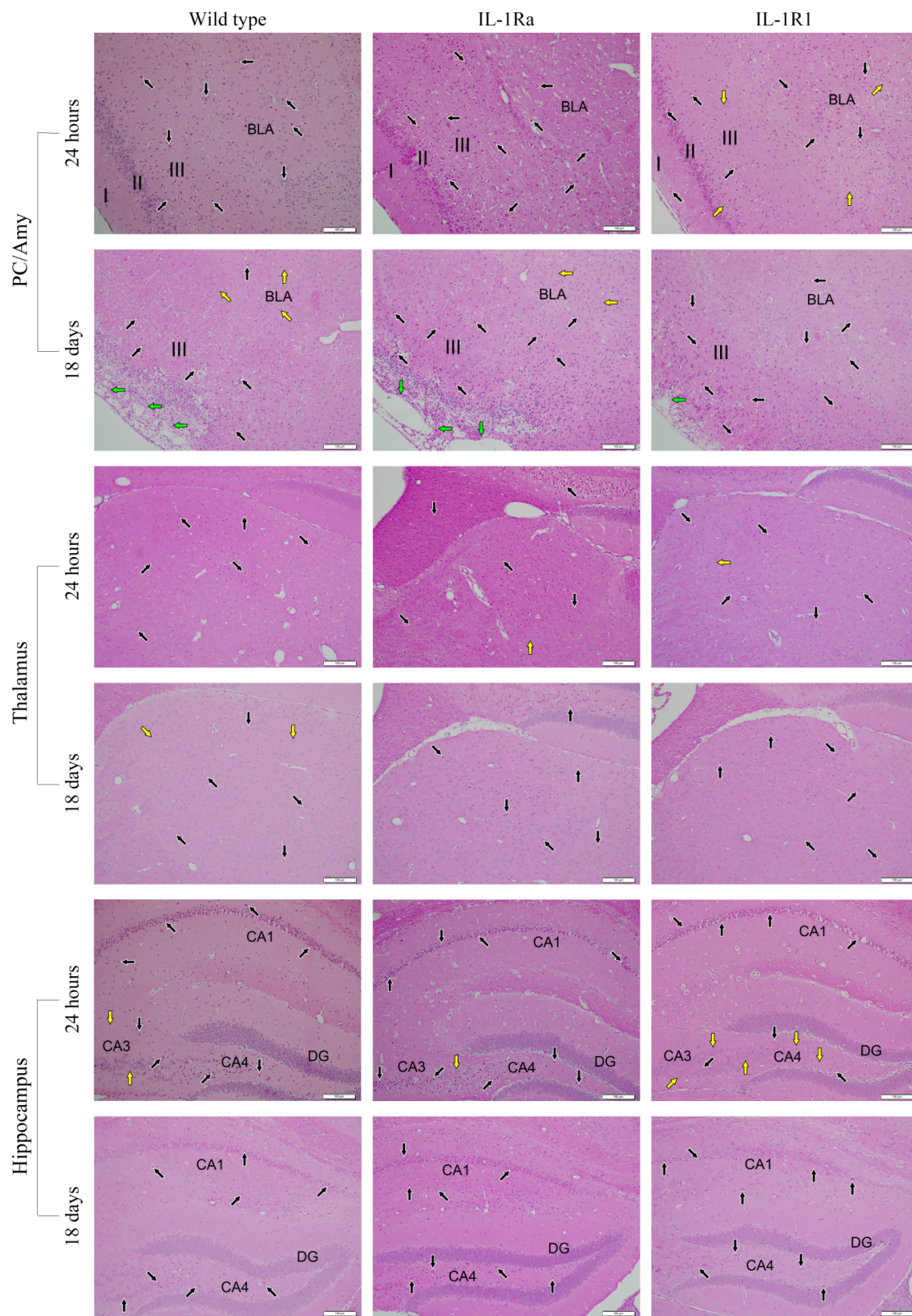


Fig. 10. H&E coronal sections depicting neuropathology 24 hrs and 18 days after GD-induced SE.

H&E stained coronal sections (10x images, scale: 100 μ m) demonstrating neuropathology 24 hrs and 18 days after seizurogenic exposure to GD in WT, IL-1Ra, and IL-1R1 KO mice. The black arrows indicate eosinophilic, dying neurons and cellular debris, yellow arrows indicate healthy neurons, and green arrows show lesion formation. PC/Amy, Piriform Cortex/Amygdala. BLA, basolateral amygdala.

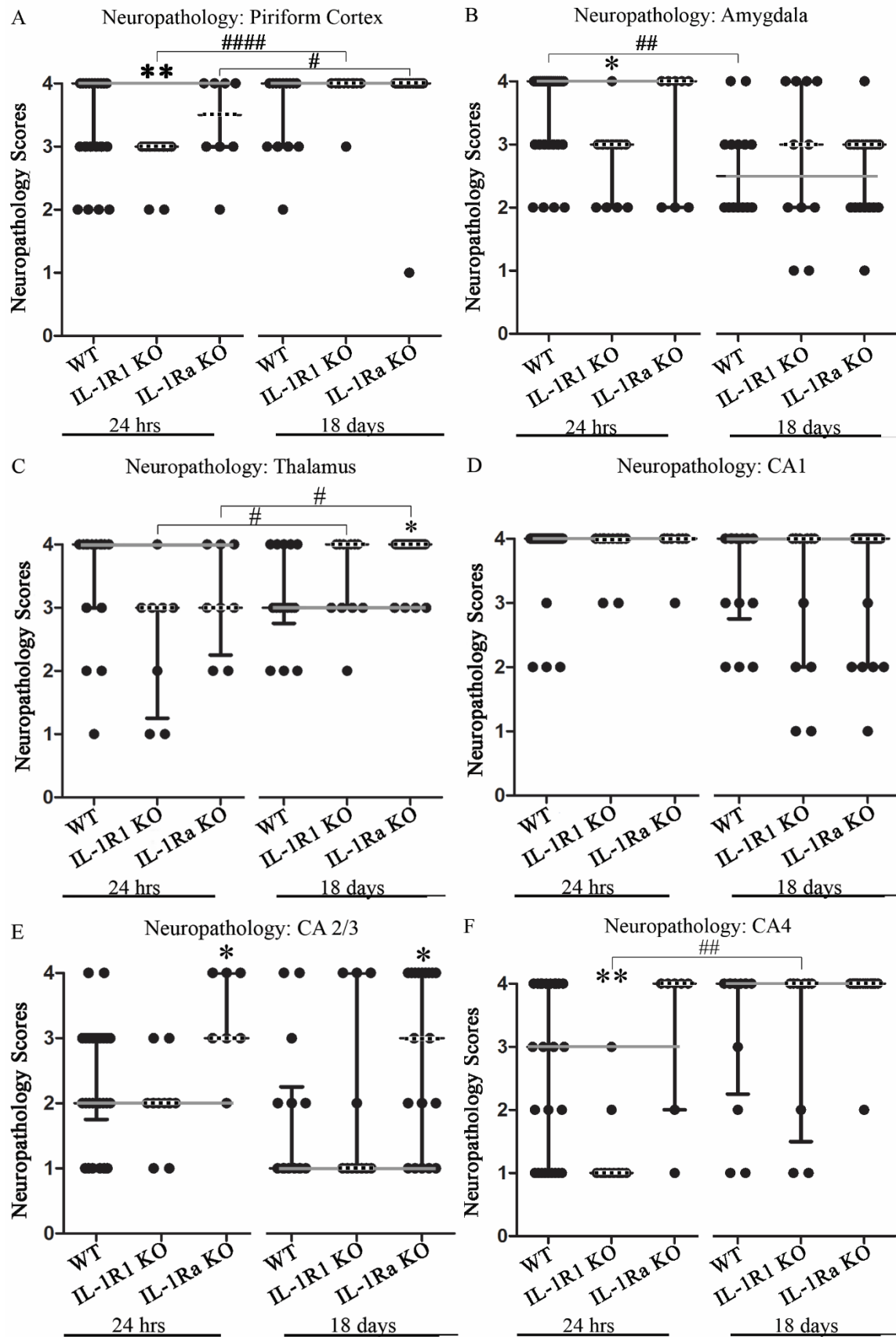


Fig. 11. Neuropathology scores 24 hrs and 18 days after GD exposure in the WT, IL-1Ra KO, and IL-1R1 KO mouse strains.

The solid line represents the score of the WT strain, and the dashed lines the score of the KO mouse strains; statistical differences within strain between 24 hrs and 18 days are represented by # (# $p < 0.05$; ## $p < 0.01$; #### $p < 0.0001$); differences between the IL-1Ra and IL-1R1 KO strains compared to the WT strain at 24 hrs and 18 days, respectively, are represented by * (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

Open Field

For total distance traveled, a significant treatment difference ($F(1,32) = 14.13, p < 0.001$) was found between the exposed WT group and SAL control, where the exposed group traveled farther on days 7 ($p < 0.05$) and 11 ($p < 0.001$, Fig. 12A). There was a significant interaction ($F(1,34) = 4.296, p < 0.05$) and treatment difference ($F(1,34) = 4.332, p < 0.05$) for the exposed IL-1Ra KO compared to its respective control, where the exposed group traveled farther on day 11 ($p < 0.05$, Fig. 12B). No significant difference in total distance traveled was found for the exposed IL-1R1 KO compared to the IL-1R1 KO SAL control (Fig. 12C), nor was there a significant difference between the exposed groups for total distance traveled (Fig. 12D).

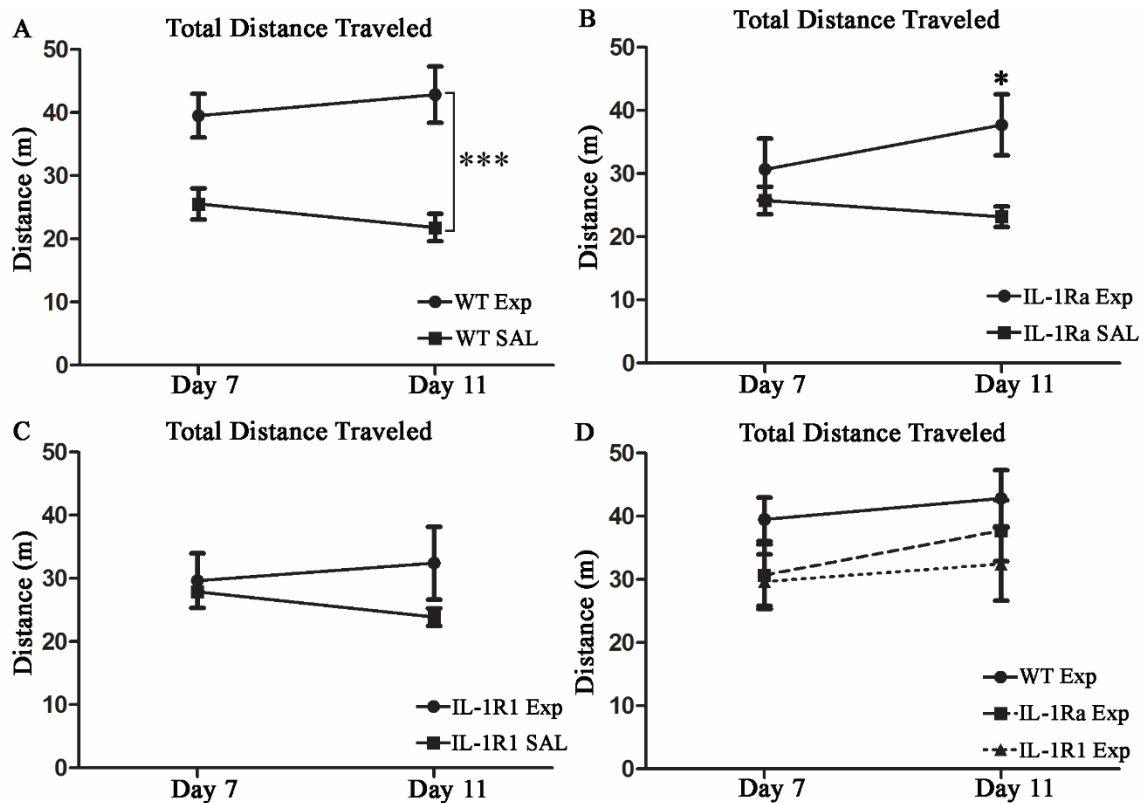


Fig. 12. Total distance traveled in the Open Field as a measure of activity.

The exposed WT and IL-1Ra KO mouse strains traveled farther in the Open Field compared to their SAL controls, showing hyperactive behavior after GD exposure (A, B). No significant difference in total distance traveled was found between the IL-1R1 KO and its SAL control or between the exposed mouse strains (D). * represents significant differences between the exposed groups and their respective SAL controls (* $p < 0.05$, *** $p < 0.001$)

Zero Maze

Previous research has reported that the relationship between exploratory activity and anxious behavior is not well defined, and that locomotor activity is a poor measure of anxiety (63). More specific anxiety-based tests such as the Zero Maze can be applied to address this issue (28; 117). In the Zero Maze, there was no significant difference in the time spent in the closed quadrants between the exposed WT group and its SAL control (Fig. 13A), the exposed IL-1Ra KO group and its SAL control (Fig. 13B) or the exposed IL-1R1 KO group and its SAL control (Fig. 13C). There was also no significant difference in time spent in the closed quadrants

when the exposed KO groups were compared to the exposed WT group (Fig. 13D). However, the primary latency to exit the closed quadrant was dramatically affected by GD exposure (Fig. 14), where GD-treated mice all took a longer time to leave the closed quadrant they began testing in versus their respective controls. A Kruskal-Wallis ANOVA on ranks was performed. Comparing the time to emerge for the six treatment groups, there was a significant difference observed ($H(5) = 42.343$, $p < 0.0001$). Subsequent pairwise comparisons (Dunn's Method) indicated the latency to exit the closed quadrant was earlier for the mice that were not exposed to GD (median exits in seconds: WT VC = 2.1, IL-1Ra KO VC = 3.4, and IL-1R1 KO VC = 3.3 seconds) compared to the groups of mice that sustained GD exposure (medians for WT exposed = 6.4, IL-1Ra KO exposed = 15.6, and IL-1R1 KO exposed = 9.1). Dunn's tests indicated the exposed and non-exposed WT ($p < 0.05$) and the exposed and non-exposed IL-1Ra KO ($p < 0.001$) groups were significantly different from one other.

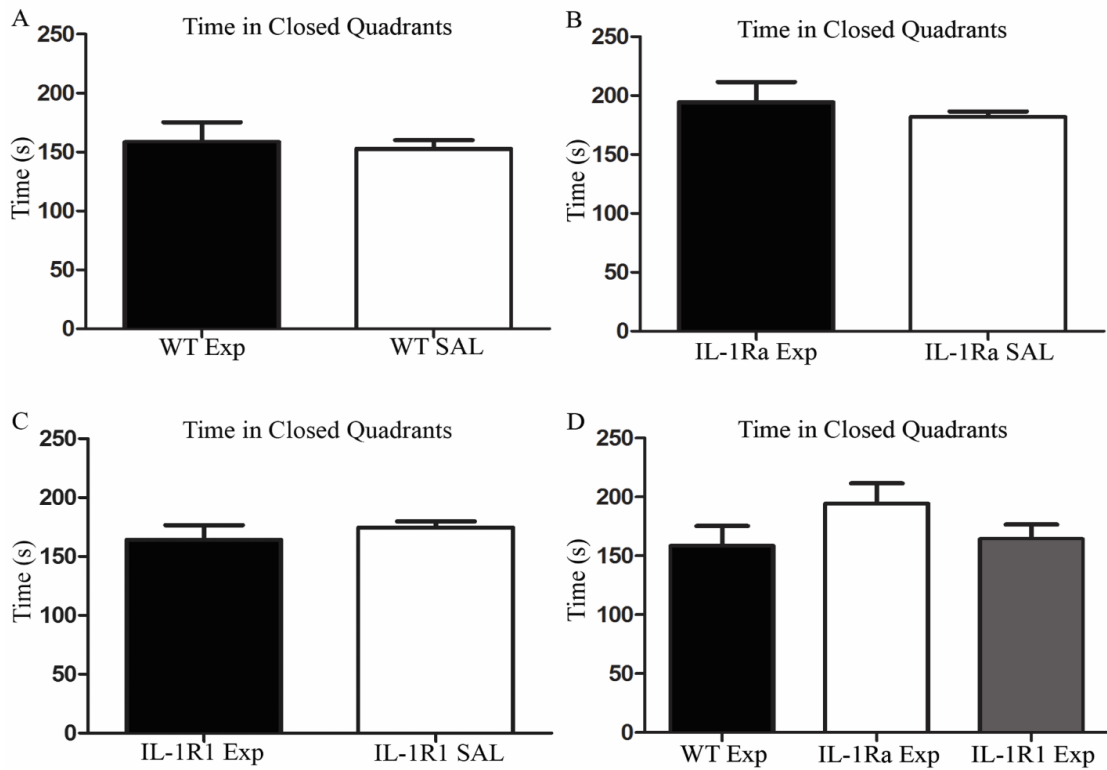


Fig. 13. Time spent in the closed quadrants of the Zero Maze.

No significant differences were found between the exposed strains and their respective controls for time spent in the closed quadrants (A, B, C). No difference in time spent in the closed quadrants was found between the exposed groups (D).

Median Time (seconds) to Emerge from the Dark Chamber of Zero Maze

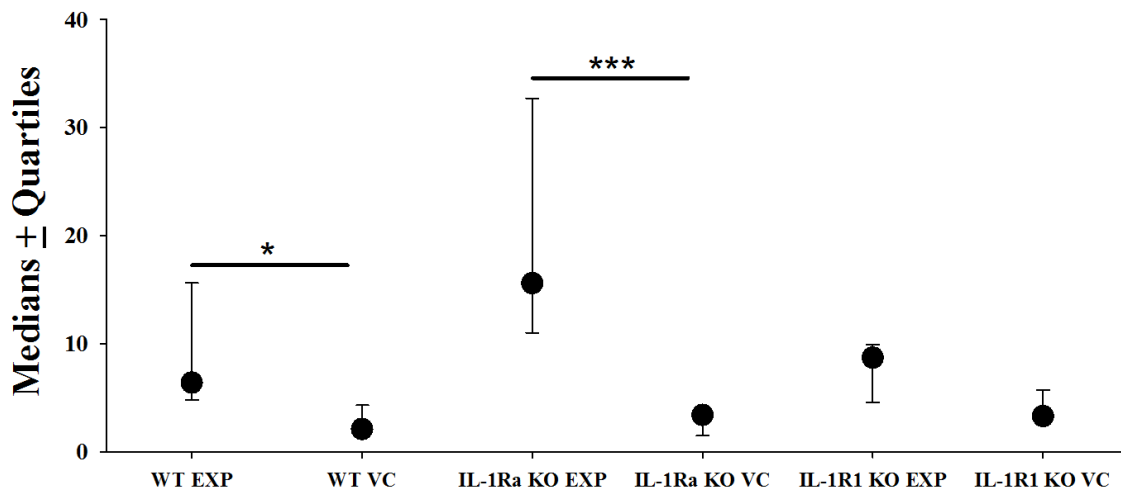


Fig. 14. Latency to first exit from the closed quadrant of the Zero Maze.

Significant differences were found between the WT and IL-1Ra KO exposed strains compared to their respective controls for latency to exit the closed quadrant where testing began. The WT and IL-1Ra KO SAL groups had a shorter latency to exit the closed quadrant compared to their respective GD-exposed groups. * represents significant differences between the exposed groups and their respective SAL controls (* $p < 0.05$, *** $p < 0.001$)

Barnes Maze

The Barnes Maze was used to measure spatial learning and memory after GD exposure. There was a significant treatment difference ($F(1,90) = 17.89$, $p < 0.001$) and a significant time difference ($F(3,90) = 33.54$, $p < 0.0001$) for escape latency between the exposed WT and SAL groups, defined as the amount of time it took for animals to find and enter the escape hole. Exposed animals escaped faster on days 1 ($p < 0.0001$), 2 ($p < 0.001$), 3 and 4 ($p < 0.01$) compared to the controls (Fig. 15A). A significant interaction ($F(3,114) = 5.406$, $p < 0.05$) and a significant difference in time ($F(3,114) = 5.406$, $p < 0.05$) was found between the exposed IL-1Ra KO and its SAL control, where the exposed escaped faster on day 1 versus the control ($p < 0.01$, Fig. 15B). Although a significant difference in time ($F(3,78) = 28.51$, $p < 0.0001$) was found between the exposed IL-1R1 KO and its SAL control (Fig. 15C), no significance was found as a function of GD exposure. When comparing the exposed KO strains to the exposed WT group, a significant interaction ($F(6,141) = 4.370$, $p < 0.01$) and a significant difference in time ($F(3,141) = 30.66$, $p < 0.0001$) was found with no post hoc significance (Fig. 15D). However, a pattern of behavior was seen here, where unlike the IL-1Ra KO, the WT and IL-1R1 KO groups escaped faster over time (Fig. 15D).

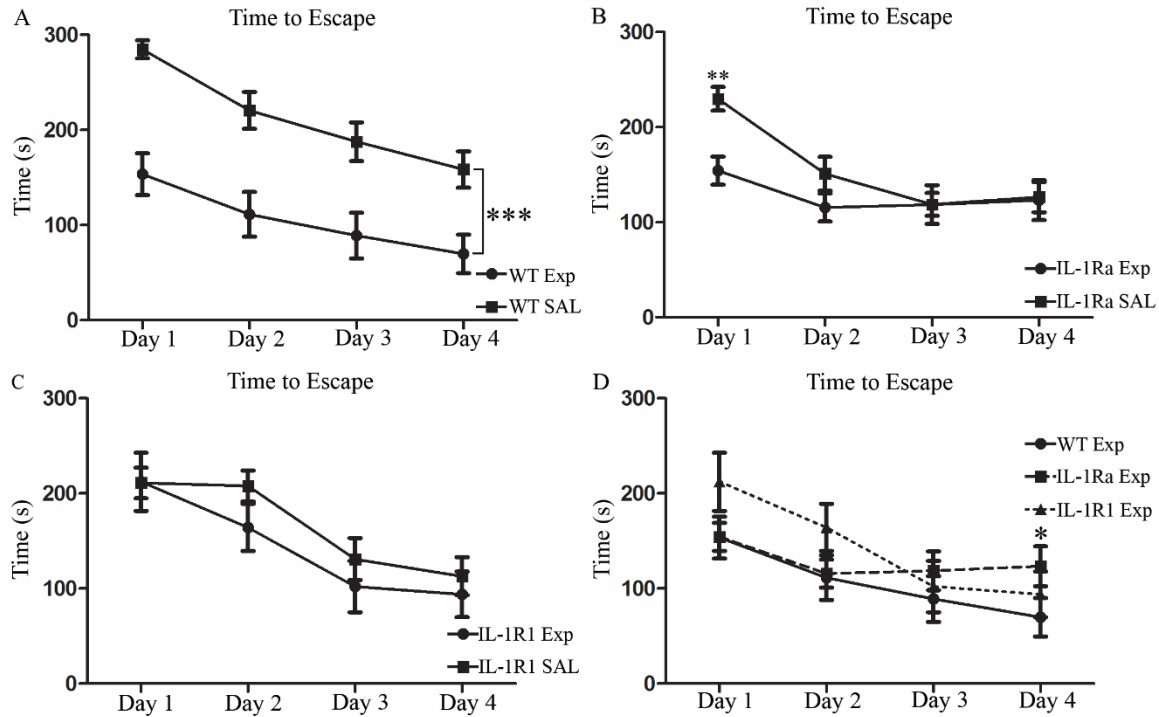


Fig. 15. Escape Latency in the Barnes Maze.

The WT exposed group escaped faster than the controls in the Barnes Maze on days 1 to 4 (A). The exposed IL-1Ra KO escaped faster on day 1 only compared to its respective control (B). No significant difference in time to escape was found between the exposed IL-1R1 KO group and its control (C) or between the exposed KO strains compared to the exposed WT group (D). * represents significant differences between the exposed groups and their respective SAL controls (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Primary latency was measured, which is defined as the amount of time an animal took to locate the hole (i.e. perform an initial nose poke) without entering the hole. A significant interaction ($F(3,87) = 4.833$, $p < 0.01$) and a significant difference in time ($F(3,87) = 26.90$, $p < 0.0001$) was found for the WT exposed and SAL control mice, where the control mice had a shorter primary latency to nose poke the escape hole versus the exposed WT mice on day 1 ($p < 0.05$, Fig. 16A). When the exposed IL-1Ra KO was compared to its SAL control mice, a significant difference in treatment ($F(1,111) = 9.735$, $p < 0.01$) was found where the SAL control mice had a faster primary latency on days 3 ($p < 0.01$) and 4 ($p < 0.05$, Fig. 16B). When the

exposed IL-1R1 KO was compared to its SAL control, a significant interaction $F(3,78) = 5.257$, $p < 0.01$), a significant treatment difference ($F(1,78) = 8.686$, $p < 0.05$), and a significant difference in time ($F(3,78) = 8.292$, $p < 0.05$) was found, where the SAL control had a faster primary latency on day 1 ($p < 0.001$) compared to the exposed group (Fig. 16C). A significant interaction $F(6, 141) = 4.392$, $p < 0.001$), a significant treatment difference ($F(2,141) = 4.809$, $p < 0.05$), and a significant time difference ($F(3,141) = 10.30$, $p < 0.0001$) was found between the exposed KO strains and WT group (Fig. 16D). Here, the WT had a faster primary latency than the IL-1R1 KO on day 1 ($\$ \$ p < 0.01$) and the IL-1Ra KO on day 3 ($* p < 0.05$).

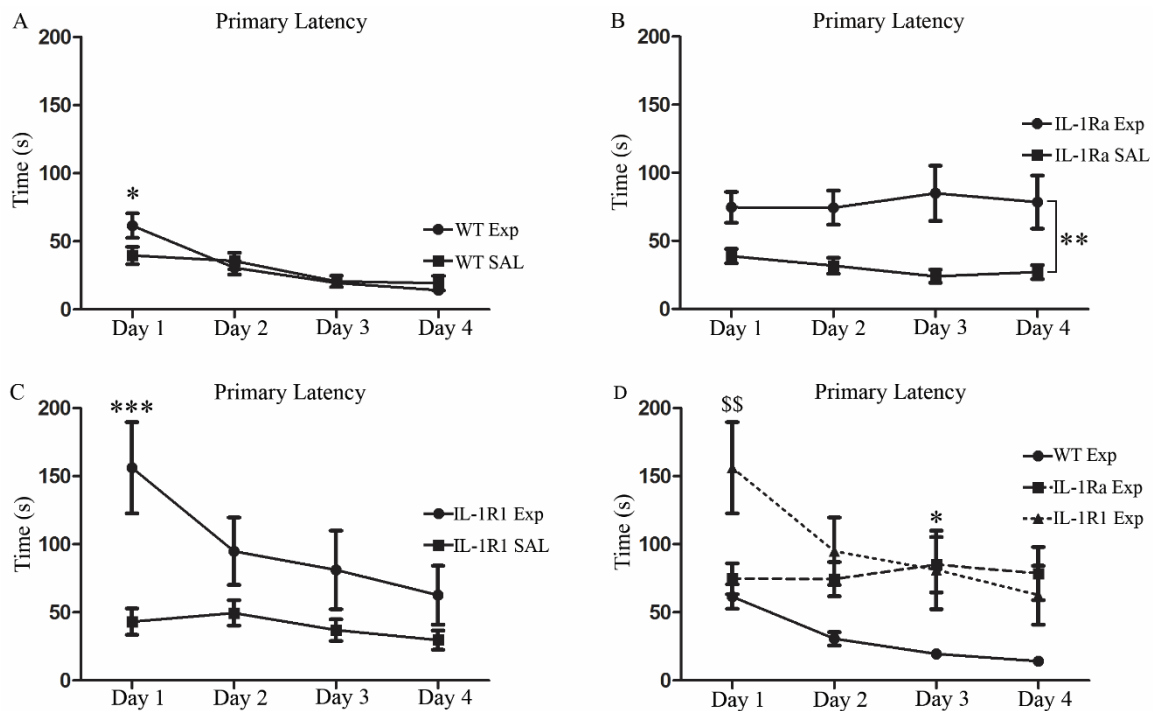


Fig. 16. Primary Latency to locate the escape hole in the Barnes Maze.

The WT SAL group had a shorter primary latency to find the escape hole compared to the exposed WT on Day 1 (A). The IL-1Ra KO SAL control had a shorter primary latency on days 3 and 4 compared to the IL-1Ra KO exposed group (B). The IL-1R1 KO SAL group had a shorter primary latency on day 1 compared to the exposed group (C). The exposed WT had a shorter primary latency on day 1 compared to the exposed IL-1R1 KO group and day 3 compared to the exposed IL-1Ra KO group. In Figs. A-C, * represents significant differences between the exposed groups and their respective SAL controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In Figure D, \$ refers to the difference in primary

latency between the exposed IL-1R1 KO group and the WT group (\$\$ $p < 0.01$); * refers to the difference in primary latency between the exposed IL-1Ra KO group and the WT group (* $p < 0.05$).

The number of errors committed was used as an additional measure of learning behavior in the Barnes Maze. There was a significant interaction ($F(3,87) = 10.49$, $p < 0.0001$), treatment difference ($F(1,87) = 7.224$, $p < 0.05$), and a difference in time ($F(3,87) = 33.35$, $p < 0.0001$), where the WT SAL control committed more errors on day 1 compared to the exposed WT ($p < 0.0001$, Fig. 17A). There was also a significant interaction ($F(3,111) = 6.514$, $p < 0.001$), treatment difference ($F(1,111) = 23.50$, $p < 0.0001$), and time difference ($F(3,111) = 15.54$, $p < 0.0001$), where the IL-1Ra KO SAL group committed more errors on days 1 ($p < 0.0001$), 2, and 4 ($p < 0.05$) versus the exposed IL-1Ra KO (Fig. 17B). Significant treatment main effect differences ($F(1,78) = 8.014$, $p < 0.01$), and a significant time difference ($F(3,78) = 11.69$, $p < 0.0001$) were found between the exposed IL-1R1 KO group and its respective control, but no post hoc differences were found for specific days between the groups (Fig. 17C). Finally, a significant difference in time ($F(3,141) = 10.78$, $p < 0.0001$) was found between the exposed WT and exposed KO mouse strains, indicating a general reduction in number of errors for all groups over days, but there were no strain differences (Fig. 17D).

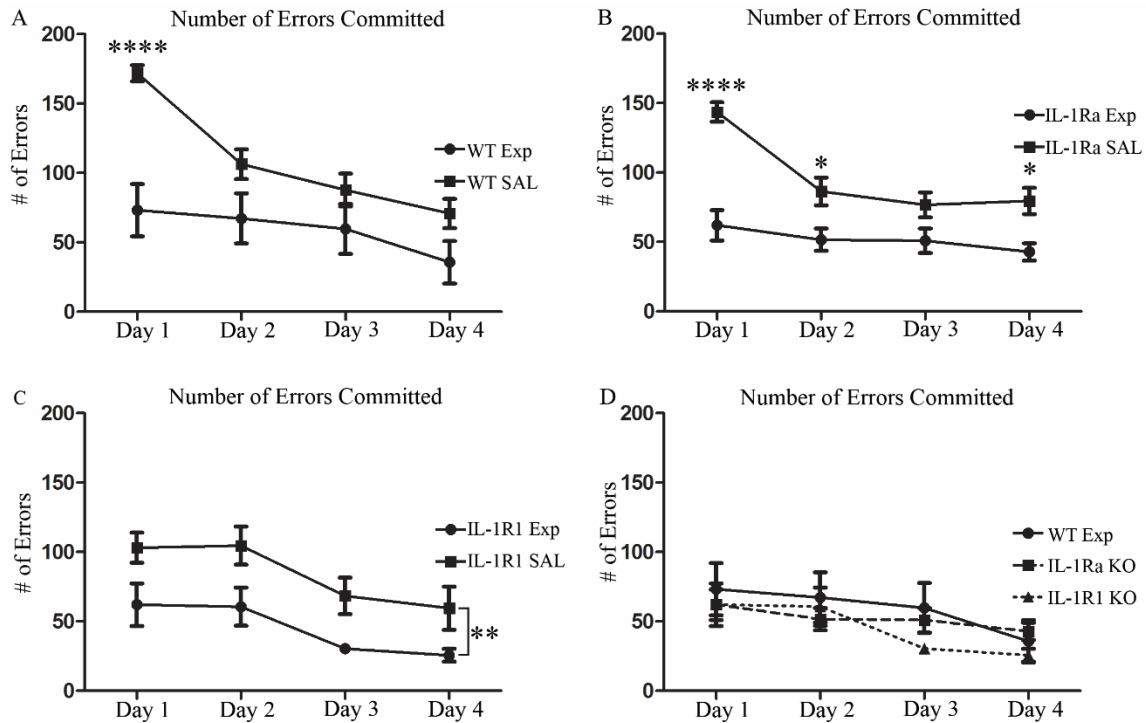


Fig. 17. The number of errors committed in the Barnes Maze.

The WT SAL control committed more errors than the exposed WT on day 1 (A). The IL-1Ra KO SAL control committed more errors on days 1, 2, and 4 compared to the exposed IL-1Ra KO (B). No significant difference in errors committed was found between the exposed IL-1R1 KO and its respective SAL control (C) or between the exposed WT compared to the exposed KO strains (D). * represents significant differences between the exposed groups and their respective SAL controls (* $p < 0.05$, **** $p < 0.0001$)

DISCUSSION.

Although it is known that nerve agent exposure leads to brain damage and changes in behavior, this study is the first to analyze the effect of GD exposure on physiology, behavioral changes, and brain damage in WT mice and KO mice with altered IL-1 signaling pathways.

Many differing physiological reactions following GD exposure were observed between the WT controls and the two KO strains. Although not significant, it was surprising to observe that the IL-1R1 and IL-1Ra KO strains had a greater weight loss at 24 hrs compared to the exposed WT group. These differences were not the result of differential reactions to handling,

injection, and stress as no significant difference was observed between WT controls and either KO strain for weight loss after SAL injection. IL-1Ra KO mice generally have a lower body weight and are smaller (50). The lean phenotype characterizing the IL-1Ra KO is attributed to increased sympathetic tone, characterized by higher body temperatures, more energy usage, and catecholamine and insulin production (128). Because these animals were smaller and, therefore, weighed less before GD exposure, the percentage of weight loss at 24 hrs was expected to be greater. Although the IL-1R1 KO does not sustain the characteristics of the IL-1Ra KO, this strain also exhibited significant weight loss compared to exposed WT mice. Previous research has shown that early weight loss correlates with neuronal degeneration in the hippocampus after GD exposure in mice (39). However, in the current study, the IL-1R1 KO showed less brain damage at 24 hrs compared to the WT in all regions but the CA1, where all strains were severely injured (Fig. 11D). Therefore, the absence of IL-1R1 has an effect on weight loss after GD exposure, but the reason is unknown. Although the IL-1R1 is missing, this KO strain has another receptor, the IL-1R3, that IL-1 β can bind to in order to initiate the neuroinflammatory process (91). Although this receptor was recently discovered and no established link to weight loss has been associated with the activation of this receptor, it is possible that it contributes to the weight loss occurring after seizure onset in the IL-1R1 KO strain because IL-1 β upregulation can induce a sickness effect (10).

Another interesting physiological outcome was the latency to the initiation of convulsions. The onset of convulsions after GD exposure occurred significantly faster for the IL-1Ra KO mice compared to the exposed WT animals, whereas no difference was observed between the IL-1R1 KO and WT group. Previous work in rats exposed to intrahippocampal injection of IL-1 β has shown that this cytokine produces a pro-convulsant response (120). The

stimulatory effect of IL-1 β and GD on seizure activity, in the presence of a functional loss of IL-1Ra (33; 54; 135) may explain why the IL-1Ra KO convulses faster than the WT control.

Neuropathology was observed in WT controls and was consistent with previous work in mouse GD models (21; 24; 25; 39). In this study, neuropathology scores were significantly different between the three strains investigated at 24 hrs. WT mice had moderate to severe neuropathology in five of six brain regions investigated at 24 hrs (Fig. 11). Similar results were seen for the IL-1Ra KO mice, although the CA2/3 also showed significantly more damage than WT controls possibly due to high concentrations of IL-1R1 in the hippocampus (120). The IL-1R1 KO strain, however, showed significantly less damage in three of the six regions investigated (piriform cortex, amygdala, and CA4) compared to WT controls. Previous work in IL-1R1 KO mice exposed to ischemic insult has shown a reduced infarct volume 24 hrs after injury in the cerebral cortex (9; 116). This suggests that IL-1 signaling plays a role in the progression of acute neuropathology following convulsive GD exposure and that inhibition of the IL-1 signaling pathway may explain the reduced damage seen in this study at 24 hrs after injury. These results are consistent with studies using IL-1Ra KO mice in other CNS injury models such as ischemia, stroke, and β -amyloid induced damage, where these animals have higher numbers of activated microglia and greater neurodegeneration compared to WT controls (27; 65; 86). Endogenous IL-1Ra inhibits the IL-1R1, the functional receptor for IL-1 (5; 122), thus reducing the proinflammatory cascade and reducing neuropathology. Although seizures also increase endogenous levels of IL-1Ra, upregulation of the inhibitor is not adequate to reduce the overwhelming increase in IL-1 after injury (32; 87; 122). In this model, upregulation of IL-1 likely helps initiate and maintain seizure activity by enhancing glutamatergic neurotransmission (120; 122) and, therefore, neuronal cell death via excitotoxicity (6; 74).

In WT mice, few changes were observed from 24 hrs to 18 days after GD exposure except for a significant decline in neuropathology scores in the amygdala (Fig. 11B). Moderate/severe damage was still evident in the piriform cortex, thalamus, CA1, and CA4 region of the hippocampus, and minimal/mild in the amygdala and CA2/3 region of the hippocampus. Similarly, degenerating eosinophilic neurons are found in the CA2/3 region of the hippocampus, the dentate gyrus, and thalamus in rats from days one to 30 after exposure to a convulsive dose of GD (23; 74). These data show that in this model of GD-induced SE, brain injury is acute and continued up to at least 18 days. These data also show that, at least in the amygdala, recovery is part of the natural progression of this injury mechanism and is consistent with previous findings (25). While IL-1 signaling inhibition resulted in less damage at 24 hrs, these benefits are not evident at 18 days as there were no significant differences between the WT and IL-1R1 KO strains. In fact, neuropathology scores significantly increased from 24 hrs to 18 days in the IL-1R1 KO in the piriform cortex, thalamus, and CA4 (Figs. 11A, 11C, 11D). These results suggest that the absence of IL-1R1 may attenuate the acute brain damage, but does not prevent it from progressing through 18 days. Exposed IL-1Ra KO mice had moderate to severe injury in all brain regions studied at 24 hrs, with some regions receiving significantly higher neuropathology scores at 18 days (Fig. 11). These data indicate that IL-1 signaling inhibition can reduce acute but not chronic brain injury. It is possible that the inhibition of IL-1 signaling after GD exposure is neuroprotective early, but the presence of IL-1 regulates IL-6 and TNF α expression, which have pro-inflammatory properties (59; 61). In fact, previous research in GD-exposed mice has shown the most intense microglia and astrocyte activation occurs between three and seven days after injury (6; 22). This inflammatory response participates in cell regeneration after brain

injury, while at the same time resulting in secondary brain damage (24; 33; 39). This study shows that the absence of IL-1 signaling does not prevent this brain damage over time.

The results of the current study exemplify the fine balance of IL-1 signaling and the dual role IL-1 can play after injury. This has been well established in previous research, where seizure activity induced by kainic acid or bicuculline in mice has been shown to trigger IL-1 release and prolong hippocampal seizures (120; 122). Although seizure activity induces IL-1Ra upregulation, it is increased in the CNS to a lower extent, and therefore, is less effective in terminating seizure activity. This was shown when control mice and IL-1R1 KO mice were exposed to bicuculline and treated with an intracerebral injection of human recombinant IL-1Ra (122). Unlike the control mice, the IL-1R1 KO was not protected from continued seizures because the drug could not bind to the missing IL-1R1, indicating the tight regulation of the IL-1 signaling pathway (99; 100; 110; 122; 133).

The amygdala and hippocampus are the primary brain regions that affect behavioral change with other parts of the limbic system including the thalamus and piriform cortex also affecting memory processes. This study and others have shown profound injury in these regions (22; 24; 25; 39), and GD-induced injury can affect behavioral performance (24; 71). This study used the Open Field to evaluate general activity, the Zero Maze for evaluating anxiety, and the Barnes Maze to test spatial learning and memory. In the Open Field, a pattern developed among the WT and KO mouse groups, where the exposed animals traveled further in the region outside the center of the Open Field compared to their individual SAL controls. Although distance traveled is a measure of exploratory behavior (130), these GD-exposed animals showed consistent increased hyperactivity compared to the controls, characterized by increased movement in a novel environment and movement near the edges of the Open Field (13; 106).

Previous work in rats exposed to a high dose of GD have shown that the animals tend to run around the walls of the apparatus when introduced to the Open Field, indicating hyperactive behavior (93). In this study, there was no difference in the total distance traveled outside center between the exposed WT or KO mouse groups, indicating that the absence of IL-1Ra or IL-1R1 does not affect the hyperactivity resulting from GD exposure.

The Zero Maze was used to assess anxiety-like behavior, where the time spent in the closed quadrants and the primary latency to exit the initial closed quadrant were used as measures of anxiety (52). Although no significant differences for time in the closed quadrants were found between each strain and its respective control, all strains of mice that were exposed took a longer time to initially emerge from the closed quadrant, suggesting overall, that GD exposure alters anxiety responses to a novel task. Furthermore, previous work has been performed with IL-1R1 KO mice in the Elevated Plus Maze (EPM), the predecessor of the Zero Maze that also tests for anxiety-like behavior (52). In one study, uninjured IL-1R1 KO mice spent more time in the open areas of the EPM compared to WT mice, indicating that the absence of IL-1R1 decreases anxiety (57). It has been reported that anxious behavior may be characterized by behavioral inhibition of mobility, where animals in the Zero Maze may stay in the dark and avoid moving into the open (75; 78). In the present study, the IL-1R1 KO showed reduced anxiety after GD exposure, and the IL-1Ra KO stayed in the initial closed quadrant the longest, indicating anxiety-like behavior. These results indicate that anxiety is influenced by the IL-1 signaling pathway.

The Barnes Maze was used to test spatial learning and memory in the WT and KO mouse strains. Surprisingly, exposed WT mice had a significantly shorter escape latencies than their SAL controls in the Barnes Maze at all time points investigated. In addition, even though the

escape latency of the exposed IL-1Ra and IL-1R1 KO mouse strains was not significantly different from their SAL controls, the SAL controls on average escaped faster as well. These results seem to contradict previous work, where GD-exposed mice were found to swim slower and travel a longer distance to the submerged platform in the Morris Water Maze (MWM), another test of spatial learning and memory, compared to SAL controls (21; 39). However, these discrepancies may be explained by a fundamental difference between the MWM and the Barnes Maze - the water in the MWM is a very aversive and stressful stimulus while the bright light of the Barnes Maze is much less aversive putting less pressure on the mice to escape (102). While the light might not be an aversive stimulus coaxing the SAL control animals to escape, the exposed animals may have a photophobic response to the lights making their escape times faster. Photophobia has been associated with inflammation and traumatic brain injury and may be active in this model as well (37; 115).

Because of the potential confounding of escape latency by light aversion and exploratory behavior, the primary latency was analyzed as an alternate metric of learning and memory (47; 81). There was a significant difference between the exposed IL-1Ra KO and its control to find the escape hole, where the SAL group found the hole faster on days 3 and 4 of testing (Fig. 16B). These results were unlike the exposed WT and IL-1R1 KO groups, where their SAL controls found the escape hole faster on day one only (Figs. 16A, 16C). This suggests slower spatial learning and memory when IL-1 signaling is unchecked in this model and may be related to the severity of CA2/3 injury (49; 108) in this strain compared to the WT and IL-1R1 KO strains.

Another measure of spatial learning and memory is the number of errors committed while attempting to find the escape hole. Again, as with escape latency, the SAL controls consistently had more errors than exposed mice regardless of strain or time (Figs. 17A, 17B, 17C). In the

absence of an aversive stimulus, the number of errors committed on the maze is interpreted as an indicator of exploratory behavior rather than cognitive impairment (40; 58; 102). These results indicate that the SAL controls were more exploratory than the exposed animals, and that the absence of IL-1R1 or IL-1Ra does not significantly affect exploratory behavior.

CONCLUSION.

This study showed that IL-1 signaling has an effect on neurodegeneration resulting from GD-induced SE. Whereas excessive IL-1 signaling after convulsive GD exposure results in brain damage and cognitive deficits, reducing IL-1 signaling can attenuate acute brain injury. Future studies will target the IL-1 signaling pathway with the human recombinant IL-1Ra (hr)-IL-1Ra (anakinra) as a means of decreasing brain damage and improving the behavioral consequences that result from nerve agent poisoning and long lasting seizure activity. Since there are no approved therapeutics targeting the neuroinflammatory response, secondary brain injury, and cognitive deficit resulting from GD-induced SE, this work will provide a means of attenuating the initial inflammatory response to reduce further damage and improve quality of life.

CHAPTER 4: Anakinra provides acute neuroprotection after soman induced status epilepticus in mice

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ABSTRACT.

Previous work has shown that the Interleukin-1 (IL-1) signaling pathway plays a significant role in the neurodegeneration and behavioral consequences that occur after soman (GD)-induced status epilepticus (SE) (Ferrara-Bowens, pending). Compared to wild type (WT) mice, IL-1 receptor knockout (IL-1R1 KO) mice exhibited less brain damage 24 hours after GD exposure and less anxiety-like behavior, while the IL-1 receptor antagonist knockout (IL-1Ra KO) mice exhibited anxiety-like behavior, impaired learning, and added neurodegeneration over time. Whereas the absence of IL-1R1 inhibits IL-1 signaling, the absence of the IL-1Ra promotes continuous IL-1 signaling, which may account for the latter results, suggesting an abatement of neuroinflammatory signaling is neuroprotective following GD. Regardless of strain, however, continued neurodegeneration was seen 18 days after GD exposure. Anakinra is the human recombinant IL-1Ra that has been shown to reduce seizure activity and mitigate the occurrence of SE in various models of CNS injury. However, it has never been used to treat GD-induced SE. Therefore, the purpose of this study was to determine the potential neuroprotective effect of anakinra by the evaluation of neurodegeneration, anxiety-like behavior, and cognitive function after GD exposure. Wild type (WT) mice exposed to GD were given a high dose of anakinra one hour after seizure onset, followed by repeated low doses up to 12 hours after seizure onset. Neurodegeneration in the piriform cortex, amygdala, thalamus, and hippocampus (CA1, CA2/3, CA4) was determined 24 hours after seizure onset, and 18 days after behavioral studies that included the Open Field and Zero Maze tasks to assess activity and anxiety, respectively, and the Barnes Maze to study spatial learning and memory. Results of this study showed that anakinra was neuroprotective 24 hours after seizure onset in the piriform cortex and amygdala, but did not improve anxiety-like behavior, cognitive function, or the central nervous system damage that is evident 18 days post exposure.

INTRODUCTION.

Exposure to soman (GD; pinacolyl methylphosphonofluoridate), a chemical warfare nerve agent (CWNA) and irreversible inhibitor of acetylcholinesterase (AChE), results in the accumulation of acetylcholine (ACh) in the synaptic cleft (26; 103). The consequences of ACh buildup include convulsions and status epilepticus (SE) leading to neurodegeneration and cognitive deficits in exposed rodents (26; 39). Previous work has reported that SE produces excitotoxic brain damage, that in turn induces a neuroinflammatory response characterized by upregulation of pro-inflammatory cytokines including IL-1 α and IL-1 β , collectively referred to as IL-1, leading to brain damage in the piriform cortex, amygdala, thalamus and CA1, CA2/3, and CA4 regions of the hippocampus (53; 54). Following this work, this group exposed wild type (WT) mice, IL-1Ra gene knockout (KO), and IL-1R1 KO mice to a convulsive dose of nerve agent, and found that the absence of IL-1R1 resulted in less damage in some brain regions, whereas the absence of IL-1Ra worsened the behavioral or neuropathological consequences of nerve agent exposure (Ferrara-Bowens, pending).

Anakinra is the human recombinant (hr)-IL-1Ra that is currently used as a treatment for rheumatoid arthritis, and in pre-clinical studies it was found to reduce peripheral inflammatory markers of stroke (43). Although anakinra is a 17 kDa protein, it has been reported to cross the blood brain barrier (BBB) after ischemic injury and mitigate brain damage after peripheral treatment (70). Anakinra has also been shown to attenuate seizure activity (122; 123). Endogenous IL-1Ra, which blocks the IL-1R1, is rapidly induced in the hippocampus after seizure onset. However, the imbalance of IL-1 signaling to inhibition maintains seizure activity (32; 87; 120; 122). Previous work by this group has shown the loss of IL-1Ra function in KO mice significantly hastens the onset of seizures after GD exposure (Ferrara-Bowens, pending).

When given by intracerebroventricular injection, anakinra has been shown to inhibit seizure activity induced by bicuculline, which promotes motor seizures without neurodegenerative effects (122). Therefore, not only does anakinra reduce seizure activity, but it has been reported to reduce the severity of SE (67).

The purpose of this study was to evaluate the potential neuroprotective effects of anakinra on neuropathology and behavioral impairments that occur after GD-induced convulsions in exposed WT mice. Following previous work, the Open Field and Zero Maze were used to evaluate exploratory behavior and anxiety-like behaviors, respectively, and the Barnes Maze was used to evaluate spatial learning and memory. GD exposure was followed by repeated peripheral treatments of anakinra up to 12 hours after convulsion onset. Although anakinra was found to reduce damage at 24 hours after exposure in the piriform cortex and amygdala, no improvement in hyperactivity, anxiety, or spatial learning and memory were found after anakinra treatment. There was also no improvement in neuropathology 18 days after GD exposure.

METHODS.

Animals

The model used for this study was adapted from previous work and is briefly explained (Ferrara-Bowens, pending). Male C57BL/6J mice (n=75) between eight and 10 weeks old were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were group housed in polycarbonate cages in a temperature- and humidity-controlled room (68-79°F, 30-70% humidity) for at least one week prior to experimentation. The animals were maintained on a 12-hour light-dark schedule (lights on at 0600) with ad libitum access to food and water. The experimental protocol was approved by the Animal Care and Use Committee at the United States

Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The animal care program at this Institute is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

GD Seizure Model and Anakinra treatment

Mice were randomly assigned to one of three treatment conditions: GD exposure with SAL treatment (n = 42), GD exposure with anakinra treatment (n = 18), or saline (SAL) with anakinra treatment (no GD, n = 15). The results from the wild type mice at 18 days in the accompanying paper (Ferrara-Bowens, pending) were also included here for comparison. On the day of exposure, all animals received an intraperitoneal (IP) pre-treatment of the oxime HI-6 dichloride (1-(((4-(aminocarbonyl)pyridinio) methoxy) methyl)-2-((hydroxymino)methyl)pyrdinium dichloride; BN44621, Starks Associates, Buffalo, NY; 50 mg/kg) in SAL five minutes prior to subcutaneous (SC) GD administration (147 μ g/kg). One hour after seizure onset, the animals in the anakinra groups were treated with a high dose of anakinra (Kineret, Ardmore, PA; 502 mg/kg, SC) in SAL, followed by a low dose (199 mg/kg SC) at four, eight, and 12 hours after seizure onset. Vehicle control animals received only HI-6 and SAL, followed by anakinra at the same doses and time points. GD (purity 98.8%) was obtained from the United States Army Medical Research Institute of Chemical Defense. Following GD exposure, the animals were observed for convulsion onset (defined as full body tremor) and monitored until they were returned to their home cages. Beginning the morning after exposure, animals were weighed and given wet mash to compensate for weight loss and prepare for behavioral testing. If the animals did not gain weight, they were given up to 0.2 mL

of SAL SC twice a day until they gained weight. Animals were transferred to the behavior room four days after exposure and were group housed and tested in the same room.

Behavior Testing

The behavioral testing used for this study was identical to the methods in the accompanying paper (Ferrara-Bowens, pending). Briefly, animals were acclimated to the testing environment for 10 minutes before behavioral testing began (48), and the behavioral apparatuses were cleaned with 70% ethanol between tests (55).

Open Field

On days seven and 11 post exposure, mice were placed in the Open Field to test for activity. The animals were placed in the center of the Open Field and monitored for 10 minutes with a camera linked to ANY-maze Behavioral Tracking Software (Stoelting, Wood Dale, IL). Presence in the center or outside center regions was determined by where the center of the animal was located (55). Total distance traveled was used to measure activity (13; 55; 90).

Zero Maze

On day nine post exposure, the animals were placed in a Zero Maze, and activity was used to evaluate anxiety-like behaviors (28; 52; 85; 117). The animals were placed in a closed quadrant of the maze and allowed to explore the maze for five minutes (52). Presence in each area of the maze was determined by where the center of the animal was located. The animals were monitored by a camera linked to ANY-maze software (Stoelting), which recorded the time spent in the closed quadrants and the latency to exit the initial closed quadrant where testing began (52; 85).

Barnes Maze

On days 14 to 17 after exposure, each animal underwent four trials on the Barnes Maze with 15 to 25 minutes between trials to determine spatial learning and memory (102). The animals were monitored by a camera linked to ANY-maze software (Stoelting), which recorded the latency to escape and the primary latency to head poke into the escape hole as measures of learning and memory, and the number of errors committed (defined as number of head pokes into the perimeter holes) as a measure of exploratory behavior (41; 85; 102).

Tissue Collection

The animals were euthanized at 24 hours (hrs) and 18 days to assess neuropathology. Briefly, the animals were anesthetized with sodium pentobarbital (75-100 mg/kg, IP), perfused with SAL for exsanguination followed by 4% paraformaldehyde (FD Neurotechnologies, Columbia, MD), and post fixed in 4% paraformaldehyde for up to 72 hrs before being paraffin processed. Serial sections (cut at 5 microns from Bregma -1.06mm to -1.34mm using a Leica RM225 microtome) were then stained with hematoxylin and eosin (H&E). The sections were scored by a board certified pathologist blinded to the experimental conditions. The extent of neuropathology in the piriform cortex, amygdala, thalamus, and hippocampal sections was evaluated in the following manner: 0: no damage; 1: minimal damage (1-10%); 2: mild damage (11-25%); 3: moderate damage (26-45%); 4: severe damage (>45%) (79). One section per brain region was scored according to the presence or lack of inflammation, dark neurons, and neuronal necrosis.

Statistical Analyses

Neuropathology data was expressed as the median $\pm$ the first and third quartile and analyzed with the Mann Whitney test. Open Field and Barnes Maze data were analyzed using a Repeated Measures analysis of variance (2-way ANOVA), with the factors, Treatment and Day.

When the interaction factor, treatment, or time was significant, Bonferroni post hoc comparisons were performed. A t-test or one way ANOVA followed by a post hoc Dunnett test were used to evaluate the Zero Maze data. Statistical tests and post hoc analyses were considered significant where $p < 0.05$.

RESULTS.

Neuropathology

Injury to the CNS is reflected in the H&E stained coronal sections (Fig. 18) that show injured tissue, healthy neurons, and brain lesions post exposure. Neuropathology results were compared between the WT mice exposed to GD that received SAL treatment at 1, 4, 8, and 12 hrs post exposure, and the anakinra-treated exposed animals at the same time points. The brain regions of interest included the piriform cortex (Fig. 19A), amygdala (Fig. 19B), thalamus (Fig. 19C), the CA1 (Fig. 19D), CA2/3 (Fig. 19E), and CA4 (Fig. 19F) regions of the hippocampus. The solid grey lines represent the median scores at 24 hrs and 18 days. Photomicrographs at 18 days after GD exposure with no SAL post treatment from the accompanying paper are included here for comparison (Fig 18), as no statistical significance was found between the GD exposed animals not given repeated post treatments of SAL, and the GD exposed animals repeatedly treated with SAL. Fig. 19A shows neuropathology in the piriform cortex of the GD exposed mice treated with SAL that was moderately damaged at 24 hrs unlike the severe damage at 18 days ($p < 0.05$). Although anakinra treatment resulted in only mild injury at 24 hrs, the reduction did not persist for 18 days ($p < 0.001$).

In the amygdala, GD exposed mice treated with SAL showed moderate injury unlike the mild to moderate injury seen at 18 days ($p < 0.05$, Fig. 19B). At 24 hrs after anakinra treatment, mild injury was sustained in the amygdala, unlike the GD exposed WT group treated with SAL

($p < 0.05$), but neuropathology was greater by the 18th day after GD exposure (Fig. 19B).

Although the results were not significant, GD exposed animals (regardless of post treatment) were moderately and severely damaged across time in the thalamus, and anakinra treatment was only neuroprotective at 24 hrs, not 18 days (Fig. 19C). No significant results were found in the hippocampal regions (Fig. 19D, 19E, 19F). Regardless of treatment, the CA1 region of the hippocampus sustained severe injury at 24 hrs and at 18 days (Fig. 19D). In the CA 2/3, mild injury was found in the GD exposed animals treated with SAL at 24 hrs compared to minimal injury at 18 days (Fig. 19E). Anakinra treatment resulted in mild injury at 24 hrs and 18 days post exposure (Fig. 19E). Finally, in the CA4 region of the hippocampus, moderate injury was found at 24 hrs in the GD exposed group treated with SAL and severe damage at 18 days, while damage was rated as severe at both times in the anakinra-treated groups (Fig. 19F).

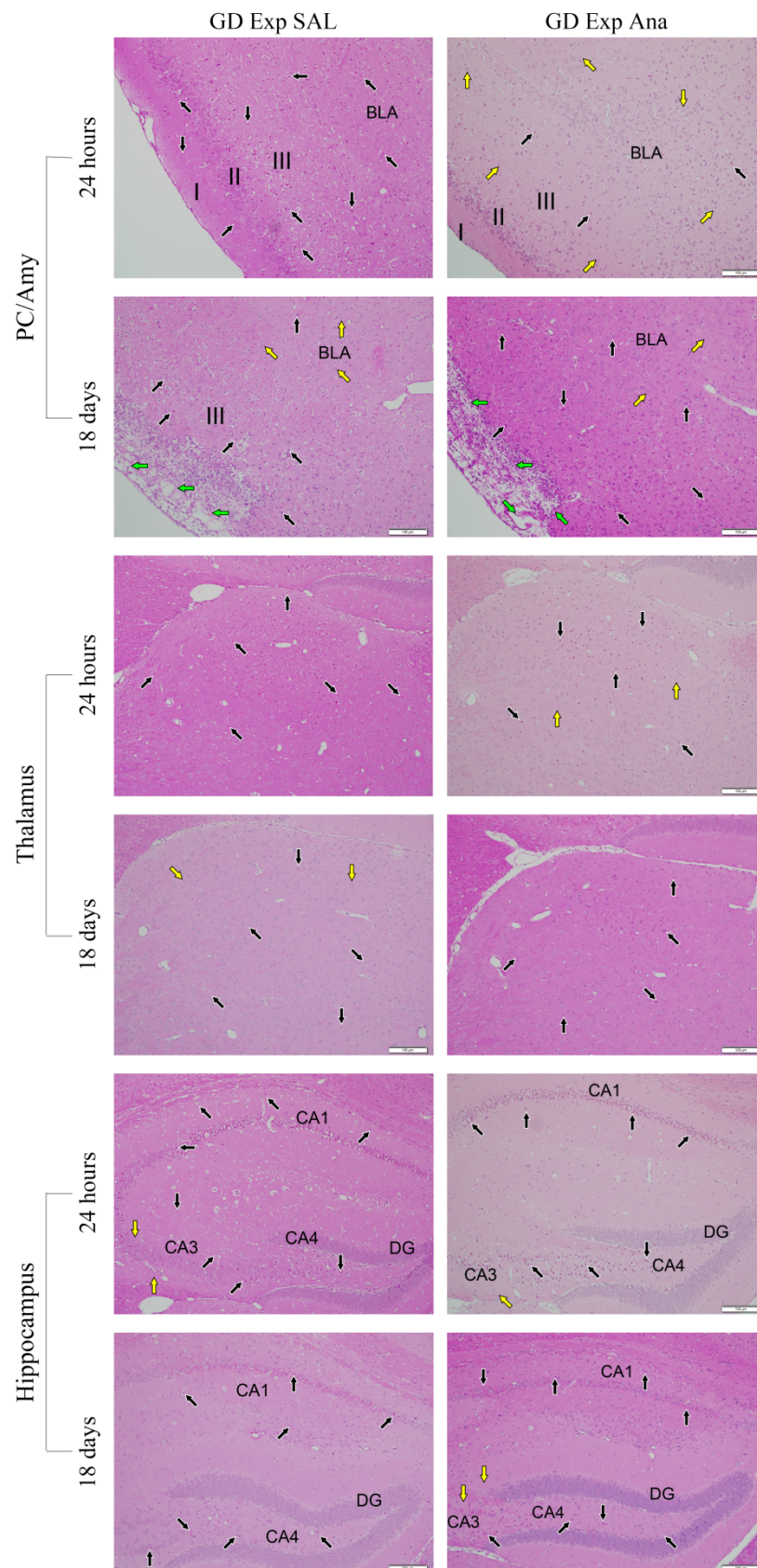


Fig. 18. H&E coronal sections depicting neuropathology 24 hrs and 18 days after GD-induced SE.

H&E stained coronal sections (10x images, bar shows 100 μ m) demonstrating neuropathology after seizurogenic exposure in untreated and anakinra-treated GD-exposed mice. The black arrows indicate eosinophilic, dying neurons and cellular debris; yellow arrows show healthy neurons, and green arrows indicate lesions after GD exposure. PC/Amy, Piriform Cortex/Amygdala. BLA, basolateral amygdala.

GD exposed groups at 24 hrs and 18 days are represented by * (* $p < 0.05$; *** $p < 0.001$).

Open Field

The Open Field was used to assess activity after GD exposure. For the total distance traveled in the Open Field, there was a significant interaction ($F(1,29) = 5.847$, $p < 0.05$) and a significant treatment difference ($F(1,29) = 34.46$, $p < 0.0001$) between GD exposed animals and their SAL controls, where the exposed animals traveled further in the Open Field compared to the controls on day 7 ($p < 0.05$) and day 11 ($p < 0.001$, Fig. 20A). There was a significant interaction ($F(1,30) = 7.695$, $p < 0.01$) and a significant treatment difference ($F(1,30) = 5.034$, $p < 0.05$) between the anakinra-treated exposed and treated SAL controls, where the exposed animals traveled further on day 11 ($p < 0.01$) versus the treated SAL group (Fig. 20B). Comparing the untreated exposed group to the anakinra-treated exposed group, no significant differences in distance traveled in the Open Field were found (Fig. 20C).

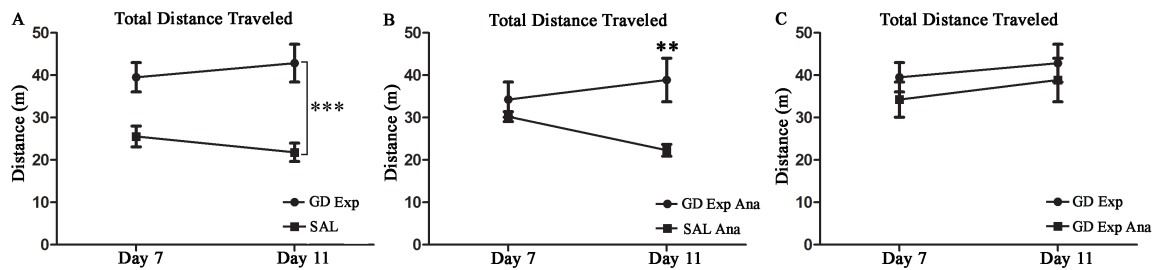


Fig. 20. Total distance traveled in the Open Field.

On days 7 and 11, the GD group traveled further in the Open Field versus the SAL control (A), and on day 11, the anakinra-treated exposed group traveled further on day 11 compared to the anakinra-treated SAL control (B). No significant difference in distance traveled was found between the untreated and anakinra-treated exposed groups (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Zero Maze

The Zero Maze was used to evaluate the impact of GD exposure on anxiety-like behavior (28; 117). For time spent in the closed quadrants, no significant differences were found between the untreated GD exposed group and the SAL control group (Fig. 21A) or between the the anakinra-treated exposed group and the anakinra-treated SAL group (Fig. 21B). When comparing the untreated exposed and GD exposed anakinra-treated groups, no significant difference in time spent in the closed quadrant was found (Fig. 21C).

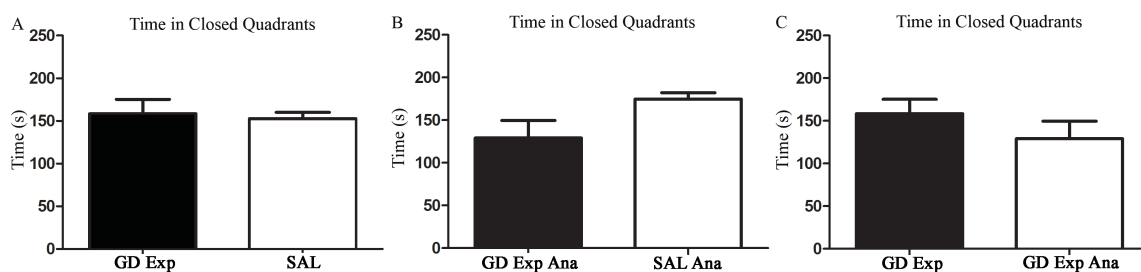


Fig. 21. Time in the closed quadrants of the Zero Maze.

There was no significant difference in time spent in the closed quadrants between the untreated GD exposed and SAL groups (A), the GD exposed group treated with anakinra and the anakinra-treated SAL group (B), or the untreated and anakinra-treated exposed groups (C).

A Kruskal-Wallis ANOVA on ranks showed that GD exposure altered the latency to exit the initial closed quadrant ($H(3)=19.704$, $p<0.001$). In mice exposed to GD, the latency to emerge from the closed quadrant was significantly greater than the time of emergence of the SAL controls ($Q=2.822$, $p<0.05$, Fig. 22). Likewise, the latency was longer in anakinra mice exposed to GD compared to unexposed anakinra-treated mice ($Q=3.160$, $p<0.01$, Fig. 22). However, in a direct comparison of GD mice that did not receive anakinra treatment versus GD mice receiving anakinra treatment, no significant difference was found ($Q=0.858$, $p>0.05$).

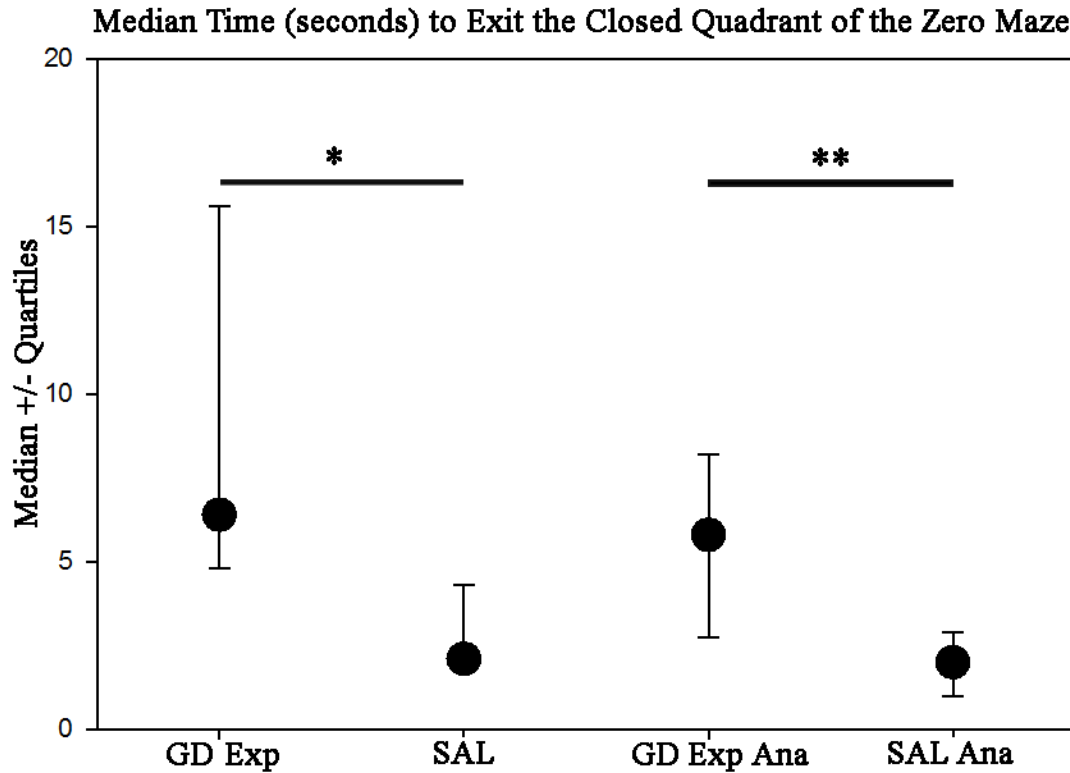


Fig. 22. Latency to exit the initial closed quadrant of the Zero Maze.

Compared to the GD exposed group, the SAL controls had a shorter latency to exit the closed quadrant where testing began. After anakinra treatment, the treated SAL controls had a shorter latency to exit the initial closed quadrant compared to the GD group treated with anakinra. * $p < 0.05$, ** $p < 0.01$

Barnes Maze

The Barnes Maze was used to test spatial learning and memory after GD exposure. There was a significant treatment difference ($F(1,90) = 17.89$, $p < 0.001$) and a significant time difference ($F(3,90) = 33.54$, $p < 0.0001$) between the WT exposed and the WT SAL groups for escape latency, defined as the amount of time it took for animals to find and enter the escape hole. Exposed animals escaped faster on days 1 ($p < 0.0001$), 2 ($p < 0.001$), 3 and 4 ($p < 0.01$) compared to the controls (Fig. 23A). There was also a significant treatment difference ($F(1,90) = 45.42$, $p < 0.0001$) and a significant difference in time ($F(3,90) = 19.59$, $p < 0.0001$) between the anakinra-treated exposed and the SAL group receiving anakinra, where the controls took a longer

time to escape on days 1 to 4 ($p < 0.0001$) compared to the exposed group (Fig. 23B). Although a significant difference in time ($F(3,93) = 25.47$, $p < 0.0001$) was found between the untreated exposed and anakinra-treated exposed groups, there was no significant difference in time to escape performance in mice with and without anakinra treatment (Fig. 23C).

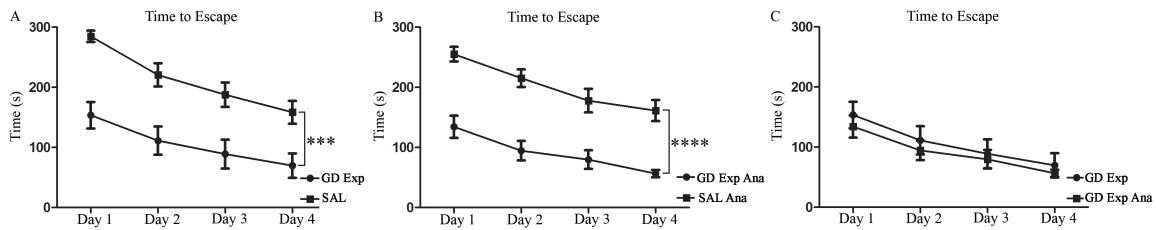


Fig. 23. Escape Latency in the Barnes Maze as a measure of learning and memory.

Compared to their respective controls, the untreated exposed and anakinra-treated exposed groups escaped faster in the Barnes Maze on days 1 to 4 (A, B). There was no significant difference in time to escape between the untreated exposed and anakinra-treated exposed groups (C). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Like previous work, primary latency (102) was used to measure learning and memory to account for the lack of aversive stimuli in the Barnes Maze. Comparing the untreated exposed and SAL groups, a significant interaction ($F(3,87) = 4.833$, $p < 0.01$) and a significant difference in time ($F(3,87) = 26.90$, $p < 0.0001$) were found where the control group located the escape hole faster than the exposed group on day 1 ($p < 0.05$, Fig. 24A). There was also a significant treatment difference ($F(1,90) = 5.285$, $p < 0.05$), and a significant difference in time ($F(3,90) = 4.941$, $p < 0.01$) between the anakinra-treated exposed group and the treated SAL control, where the treated controls found the escape hole faster, but no post hoc significance was found (Fig. 24B). Although a significant difference in time ($F(3,93) = 10.43$, $p < 0.0001$) was found between the untreated and anakinra-treated exposed groups, anakinra treatments did not result in a significant difference (Fig. 24C).

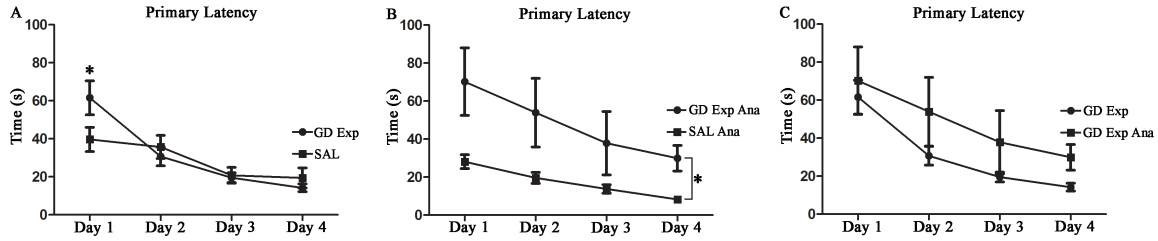


Fig. 24: Primary Latency to find the escape hole in the Barnes Maze.

The exposed WT mice located the escape hole faster on day 1 compared to the SAL group (A). No significant difference in primary latency to locate the escape hole was found between the anakinra-treated exposed and treated SAL group given anakinra (B). Finally, direct comparisons of the primary latencies of the exposed mice that received no treatment or anakinra showed no significant differences in primary latency (C). * $p < 0.05$

The number of errors committed was used as a measure of exploratory behavior in the Barnes Maze. Comparing the exposed WT to the SAL control, there was a significant interaction ($F(3,87) = 10.49$, $p < 0.0001$), a significant treatment difference ($F(1,87) = 7.224$, $p < 0.05$), and a significant difference in time ($F(3,87) = 33.35$, $p < 0.0001$), where the SAL control committed more errors on day 1 compared to the exposed group ($p < 0.0001$, Fig. 25A). There was also a significant interaction ($F(3,90) = 2.890$, $p < 0.05$), a significant treatment difference ($F(1,90) = 77.10$, $p < 0.0001$), and a significant difference in time ($F(3,90) = 21.71$, $p < 0.0001$) between the anakinra-treated exposed and treated SAL groups, where the SAL group committed more errors on days 1 to 4 ($p < 0.0001$) versus the exposed group (Fig. 25B). Although a significant interaction ($F(3,93) = 11.25$, $p < 0.0001$) occurred between the exposed mice and anakinra-treated exposed groups, no post hoc significance was found for number of errors committed (Fig. 25C).

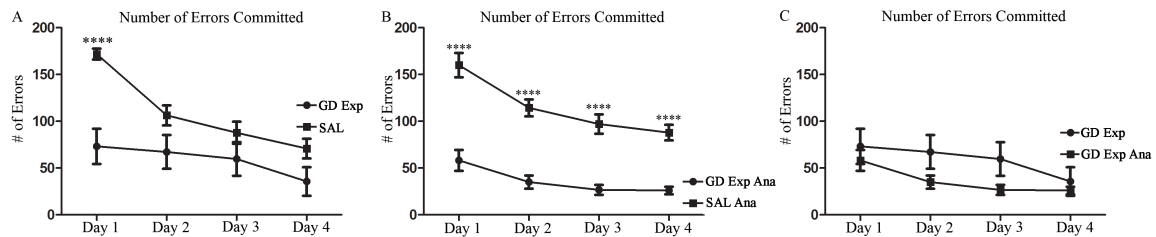


Fig. 25: The number of errors committed in the Barnes Maze.

The SAL control committed more errors on day 1 versus the exposed WT mice (A). The anakinra-treated SAL controls committed more errors on days 1 to 4 compared to the treated exposed group (B). No significant difference in errors committed was found between the WT exposed group and the anakinra-treated exposed group (C) ****
 $p < 0.0001$

DISCUSSION.

Recent work from this group employed KO mice to study the effect of the IL-1 signaling pathway on brain damage and behavior following GD-induced convulsions (Ferrara-Bowens, pending). It was discovered that the absence of the IL-1 receptor (IL-1R1) reduces anxiety-like behavior after GD exposure, whereas the absence of the IL-1 receptor antagonist (IL-1Ra) does not reduce hyperactivity or anxiety-like behavior after GD exposure, and slows down spatial learning and memory in these mice. Although reduced brain damage was seen 24 hrs after GD exposure in IL-1R1 KO mice, these animals were severely damaged by 18 days. The absence of IL-1Ra did not alter the severe damage to a different degree than what was seen in WT mice, and the IL-1Ra KO mice showed more severe damage in the brain 18 days after injury. The present study used anakinra to target the IL-1 signaling pathway in GD-exposed mice to determine if the neuropathological and behavioral consequences of the resulting SE can be mitigated.

As previously reported, all brain regions studied except the CA2/3 region of the hippocampus were moderately or severely injured 24 hrs and 18 days after GD exposure. With anakinra treatment, significant reductions in neuropathology were found 24 hrs after exposure in the piriform cortex and amygdala only, but that improvement was not maintained up to 18 days. There are several reasons to consider as to why anakinra was acutely protective. First, similar to IL-1 α and IL-1 β , anakinra crosses the BBB by a saturable process, but because of its size (17 kDa) only 0.33-0.65% enters the brain after peripheral administration at 100 mg/kg (46).

Anakinra's short half-life (43) requires repeated treatments to provide neuroprotection (101). Previous work has shown that when anakinra was given at 100 mg/kg immediately after middle cerebral artery occlusion in rats, then every 4 hrs up to 18 hrs post occlusion, then three times daily up to seven days after injury, there was a decrease in the number of necrotic neurons found in the treated versus untreated animals (44). Therefore, it is possible that repeated anakinra treatment past the 12 hr time point in this study could have provided greater and more sustained neuroprotection, but only with continued administration for perhaps days after GD exposure.

Previous research has also speculated that a dose of anakinra as high as 200 mg/kg may excessively block the IL-1R1 receptor (96). In the current study, the high dose (502 mg/kg) and low dose (199 mg/kg) of anakinra used were based on a pilot study in our lab, which tested various doses of anakinra ranging from 100 to 502 mg/kg after GD-induced convulsions. The chosen doses used in the current study were most protective in the piriform cortex, which was severely damaged at 24 hrs and 18 days post exposure. Although neuroprotection was seen in this study at 24 hrs with anakinra treatment, the high and low doses given may have saturated the IL-1R1 receptors so that further protection could not be warranted.

An alternative approach would be to administer anakinra with mini osmotic pumps. Previous work in rats with fluid percussion injury that were treated with anakinra via osmotic pump up to seven days after injury, showed less neuronal cell loss in the CA3 region of the hippocampus (101). Not only would osmotic treatment of anakinra provide consistent release of the drug, therefore maintaining a therapeutic dose, but the animals may be less stressed by repeated SC dosing of anakinra every four hours (64; 101). It has also been reported in previous ischemic research studies that delaying initial anakinra treatment to 30 minutes after injury did not reduce the infarct volume as seen with giving anakinra (100 mg/kg) immediately after injury

and repeatedly thereafter (70; 96). In the current study, the first dose of anakinra was only given one hour after convulsion onset. Further neuroprotection may be seen in future studies by treating with a high dose of anakinra immediately after GD exposure, followed by anakinra administration at a smaller dose via mini osmotic pump until the study endpoint (101).

Finally, anakinra was not found to alter hyperactivity in the Open Field or anxiety-like behavior in the Zero Maze that is observed in mice after GD exposure. Previous work by this group showed the same results for the untreated WT exposed group versus their respective controls. This study only looked at the piriform cortex, amygdala, thalamus, and hippocampus for neurodegenerative effects. Previous research has shown that lesions to the cerebral cortex disrupting the olfactory and entorhinal cortices can result in hyperactive behavior (13; 126). Although the current study did not analyze these specific brain regions, they are part of the limbic system, which is damaged after GD exposure (22; 24; 25; 39). Therefore, impairment in these additional brain regions may account for the hyperactivity seen. As for spatial learning and memory in the Barnes Maze, previous work from this group showed that when compared to the SAL controls, exposed WT mice escaped faster in the Barnes Maze, had a slower primary latency to find the escape hole, and committed less errors. The same results were found in this study after anakinra treatment. As indicated with the neuropathology results, it is possible that anakinra would have reduced anxiety-like behavior and improved spatial learning and memory if the treatment had been given consecutively via mini osmotic pump throughout the period of behavioral testing. Unlike the current study, previous work in rats with traumatic brain injury (TBI) treated systemically with anakinra via mini osmotic pump up to seven days after injury showed significant improvement in their performance in the MWM (101).

CONCLUSION.

Although results of this study indicate that anakinra did not improve behavioral activity or neuropathology 18 days after GD-induced convulsions, anakinra treatment provided some neuroprotection in the piriform cortex and amygdala after acute injury. These brain regions are damaged after GD exposure, and have the potential to be further protected with repeated anakinra treatment. This means that targeting the IL-1 signaling pathway may attenuate the damaging effects of GD-induced SE. Therefore, future work will incorporate continuous anakinra treatment to target the IL-1 signaling pathway to reduce neuroinflammation, and therefore, secondary brain injury, and improve neuropathological and cognitive function.

Chapter 5: DISCUSSION

These studies utilized wild type (WT), IL-1 receptor (IL-1R1) knockout (KO), and IL-1 receptor antagonist (IL-1Ra) KO mice to identify the role of Interleukin-1 (IL-1) signaling in neurodegeneration, neuroinflammation, and cognitive function after soman (GD)-induced status epilepticus (SE). The IL-1 signaling pathway was also targeted with a biologic drug, anakinra (human recombinant IL-1Ra), and its effect on neurodegeneration and behavioral activity was assessed.

Upon exposure of all three mouse strains to a convulsive dose of GD, the IL-1Ra KO group exhibited the earliest onset of convulsive activity. Previous work has found a connection between seizure onset and IL-1 β upregulation, where animals that do not seize show baseline levels of IL-1 β (113). Interestingly, it has been reported that circulating levels of IL-1Ra are higher in the periphery versus the brain as a result of inflammatory disease (122). However, without IL-1Ra, IL-1 signaling is constant in the IL-1Ra KO mice, as IL-1 is not inhibited from binding to the IL-1R1 (99; 100), which may explain the earlier convulsion onset after GD exposure in these mice. Although the mechanism of action is unknown, previous research has shown that seizure activity triggers IL-1 β upregulation, and the presence of IL-1 β maintains seizure activity by increasing glutamatergic function (120). Although seizure activity was not measured in the current study, IL-1 β was upregulated in the IL-1Ra KO mice after GD exposure. In fact, the early upregulation of IL-1 α in the IL-1Ra KO mice in the current study supports previous research indicating that IL-1 α is induced earlier than IL-1 β after CNS injury (66). Additionally, although endogenous IL-1Ra is induced in the hippocampus after seizure onset, it is produced to a similar or lesser magnitude than IL-1 β (122), and therefore, is ineffective at fully inhibiting IL-1 signaling. Overall, the combination of increased IL-1 expression due to

seizure activity, the absence of the IL-1Ra, and the effect of IL-1 on maintaining seizure activity may also help explain why the IL-1Ra KO animals had an earlier convulsion onset time compared to the WT mice exposed to GD.

After GD exposure, both KO mouse strains displayed a significant weight loss compared to the WT group. Compared to WT mice, IL-1Ra KO mice have a smaller phenotype, which is attributed to elevated body temperatures and greater energy usage in this strain (128). Because these animals weighed less than the WT strain before GD exposure, their percentage of weight loss at 24 hrs was expected to be greater. However, the IL-1R1 KO does not sustain the same physiological characteristics of the IL-1Ra KO. In the current study, the WT and IL-1R1 KO strains had comparable baseline weights before exposure. Previous research correlated higher weight loss after GD exposure with damage to the CA1 region of the hippocampus (39), but the current study showed severe damage to the CA1 in all three strains at 24 hrs and 18 days, which does not explain the greater weight loss in the IL-1R1 KO group. However, after central or peripheral administration, exposure to IL-1 β and TNF α has been reported to induce a sickness effect in mice characterized by fever (56) and anorexia (88). Furthermore, weight loss in IL-1R1 KO mice has been reported as early as six hours after peripheral LPS treatment, where TNF α may compensate the effect of IL-1 β on weight loss (10). In the current study, upregulation of TNF α was found at 24 hrs in the piriform cortex of IL-1R1 KO mice, which may play a role in the significant weight loss seen in this strain compared to the WT mice. The IL-1R1 KO has also been reported to have a functional IL-1R3 receptor that is present in the brain and actively binds IL-1 in IL-1R1 KO mice (91; 125). However, it was only recently discovered and has not been fully characterized. Like normal IL-1 signaling, IL-1 β binds to the IL-1R3 to induce transcription. Although the current study did not evaluate IL-1R3, if it is active in IL-1R1

KO mice, it is possible that IL-1 β is still functional in these mice and may play a role in 24 hr weight loss.

One limitation in studying neuropathology is the ceiling effect with the highest score of 4. Although this number encompasses greater than 45% damage to the brain regions of interest, some regions may have worse damage than others. This is especially important in comparing the injured WT and IL-1Ra KO mouse strains because the IL-1Ra KO is expected to have worse damage than the WT due to the missing IL-1Ra inhibitor. Despite this limitation, there are correlations in the results from the neuropathology and multiplex studies. Compared to the GD-exposed WT group, the IL-1Ra KO mice showed significantly worse brain injury in the CA2/3 region of the hippocampus at 24 hrs. At this time point, the IL-1Ra KO mice also had increased IL-1 β and TNF α expression in the hippocampus that was not seen in the WT. This result may be due to the fact that IL-1 signaling is constant in the IL-1Ra KO mice, whereas the WT mice have IL-1Ra to inhibit the signaling pathway. Therefore, the upregulation of IL-1 β and TNF α in the IL-1Ra KO may have contributed to the increased neuropathology in this study since these cytokines are pro-inflammatory (36; 59). IL-1 β and TNF α have also been found to work together to activate nitric oxide synthase (iNOS) expression, which contributes to neuropathology after CNS injury (32; 131). Although iNOS expression was not studied in the current work, the fact that IL-1 β and TNF α expression were upregulated supports a possible role for iNOS in the resulting neuropathology. In the IL-1R1 KO strain, neuroprotection was seen at 24 hrs in the piriform cortex, amygdala, and CA4 region of the hippocampus compared to the GD-exposed WT group. Unlike the WT, the IL-1R1 KO mice showed an increase in IL-6 in the piriform cortex and a decrease in IL-1 α and TNF α in the amygdala, which may account for the neuroprotection seen. Although seizure activity was not analyzed in this study, SE is the

contributing factor to primary damage after GD exposure (17). IL-6 has anti-convulsant properties (61), and since it was upregulated at 24 hrs after GD exposure in this study, it may have contributed to less seizure activity in the IL-1R1 KO and therefore, less brain damage at this time point.

By 18 days after GD exposure, the WT group showed reduced brain injury in the amygdala, thalamus, and CA2/3 region of the hippocampus. Previous research in mice exposed to GD has reported an increase in neural progenitor proliferation in the subventricular zone and the subgranular zone of the dentate gyrus as early as days three and eight post exposure, lasting up to one month after exposure (22). Other forms of CNS injury, including ischemia (136), seizures (83), and traumatic brain injury (29) have reported increases in neural progenitors after injury as a natural physiological process of repair (22). Additionally, activated astroglia produce pro-inflammatory cytokines such as IL-6, IL-1 β , and TNF α (62) that may activate neurogenesis (22). In the current study, the exposed WT group showed an upregulation of IL-6 in the piriform cortex, thalamus, and hippocampus early after exposure. Although it cannot be determined from the current work whether the same upregulation of cytokines was seen at 18 days, it can be speculated that neurogenesis may have been part of the recovery process 18 days post exposure.

By 18 days, injury in the IL-1Ra KO became significantly worse in the piriform cortex and thalamus, which may be attributed to the consistent IL-1 signaling, and therefore, consistent upregulation of pro-inflammatory cytokines in this KO group. Primary brain damage resulting from GD-induced SE initiates neuroinflammation that contributes to secondary brain damage days to months after initial exposure (9). In this study, the IL-1Ra KO group showed upregulation of IL-1 α , IL-1 β , IL-6, and TNF α at 24 hrs, which may have contributed to the neuropathology seen at 18 days. Additionally, spontaneous recurrent seizures (SRS) may have

contributed to further brain damage at 18 days. This study did not measure SRS, but it has been reported to be correlated with severe damage in the CA1 region of the hippocampus (31). Also, the presence of IL-1 β maintains seizure activity (120), and although it is unknown whether IL-1 β was upregulated past 24 hrs in the IL-1Ra KO mice, its possible presence could have contributed to SRS in these animals. Since all three strains in this study showed severe damage in the CA1 region of the hippocampus and SRS may have occurred when the animals were not under observation, it is possible that SRS may have contributed to the neuropathology seen in the IL-1Ra KO at 18 days.

Surprisingly, the IL-1R1 KO group showed more severe neuropathology in the piriform cortex, thalamus, and CA4 region of the hippocampus at 18 days post exposure, even though the absence of the receptor should attenuate IL-1 signaling, and therefore, secondary brain damage. This could also be due to SRS since the CA1 region was severely damaged (31) or the presence of the newly identified IL-1R3 that is active in IL-1R1 KO mice (91; 125). The activation of the IL-1R3 induces IL-1 signaling, which signals a pro-inflammatory response by upregulating IL-6 and TNF α expression (91). Similar to the IL-1Ra KO, the induced cytokine expression may contribute to secondary brain damage and neuropathology 18 days after exposure. Furthermore, GD exposure also compromises the BBB, and the thalamus is vulnerable to BBB breakdown (16), which may explain the damage to the thalamus seen in IL-1R1 KO mice. Finally, damage to the hippocampus may be due to paroxysmal influx from the piriform cortex, which has been reported to play a role in GD-induced seizure activity (103).

Previous research has reported that uninjured IL-1R1 KO mice travel increased distances in the Open Field compared to WT mice, and this increased distance traveled is a sign of exploratory behavior (78). Motor activity was not impaired in the current study, as each exposed

mouse strain traveled farther compared to their respective controls in the Open Field. Although it was not reported, each exposed strain traveled farther in the outside center region of the Open Field compared to their SAL controls, indicating hyperactive behavior (72). Since no significant difference in total distance traveled was found for the exposed IL-1Ra and IL-1R1 KO groups compared to the exposed WT mice, the results indicate that IL-1 signaling does not affect hyperactive behavior. Previous work indicates that lesions to the cortex connecting the olfactory cortex to the entorhinal cortex may increase distance traveled (13). Although these regions of the brain were not analyzed in this study, it is possible that such damage resulted in the hyperactive behavior seen.

Previous work credits IL-1 with mediating the stress response, where uninjured IL-1R1 KO mice are less anxious in the Elevated Plus Maze (129) because the animals do not respond to an upregulation of IL-1 (78). When uninjured IL-1Ra KO mice were tested in the Open Field, they did not show motor impairment or emotional behavior discrepancies in the Open Field or Elevated Plus Maze, respectively (128; 129). Upon injury in the current study, an upregulation of IL-1 occurred for the WT and both KO mouse strains. Unlike the WT and IL-1Ra KO, the IL-1R1 KO showed decreased anxiety in the Zero Maze. The results of the current study show that anxiety results from GD-induced SE, and this anxiety-like behavior may be related to the IL-1 signaling pathway.

Surprisingly, all exposed mice in this study escaped faster in the Barnes Maze compared to their respective SAL controls. It is possible that the brightness of the lights may have caused a photophobic response in the exposed animals, regardless of strain. Photophobia is a sensitivity to light that is associated with inflammation and traumatic brain injury (37; 115). Since the only dark area of the Barnes Maze was the escape hole, a photophobic response would have promoted

escape behavior. There was no problem with spatial learning and memory for the WT and IL-1R1 KO groups, as they escaped faster over time. However, the IL-1Ra KO group did not perform faster over time in the Barnes Maze, which may be due to the moderate injury sustained in the CA2/3 region of the hippocampus that plays a role in spatial memory (49; 108). Unlike the IL-1Ra KO mice, the WT and IL-1R1 KO groups had minimal injury to this brain region and displayed functional spatial learning and memory.

Although anakinra was neuroprotective in the amygdala and piriform cortex at 24 hrs, it did not improve the hyperactivity or anxiety-like behavior after GD exposure, nor did it significantly improve spatial learning and memory in GD exposed mice. The negative results of the current study concerning anakinra do not imply that anakinra cannot improve the behavioral consequences of nerve agent exposure. Rather, the paradigm in which anakinra treatment was incorporated may not be ideal to study its effect on GD-induced SE. An alternative approach for future studies would be to utilize mini osmotic pumps and give lower doses of anakinra over the time period tested. This would not only prevent further stress on the animals as repeated injections would be prevented, but the optimal dose of anakinra would be maintained. Whether neuropathology would improve at 18 days is questionable. Although anakinra has been reported to cross the BBB (70) and mitigate seizure activity (122; 123), only 0.33-0.65% has been reported to enter the brain after peripheral administration of the drug at 100 mg/kg (46). Whether anakinra would reduce anxiety-like behavior is also questionable. Although anakinra is the human recombinant IL-1Ra and blocks IL-1 signaling, this study showed that IL-1 upregulation is still seen in GD-exposed WT mice treated with anakinra. Previous research has reported that IL-1 mediates the stress response (129), so that if IL-1 is present, anxiety-like behavior will still exist.

Interestingly, a correlation was found in the multiplex results and neuropathology after GD exposure and anakinra treatment. There was a decrease in the expression of IL-1 α 24 hrs after GD exposure in the piriform cortex (Fig. 6A) and amygdala (Fig. 6B) and less neuropathology in these brain regions (Fig. 19A, 19B) at the same time point. Since IL-1 α is pro-inflammatory and is upregulated early after CNS injury (66), it is possible that anakinra inhibited the IL-1R1 receptor, resulting in less brain damage early after GD exposure and inhibited IL-1 α upregulation. Although IL-1 β was upregulated at 24 hrs after GD exposure, which may have induced IL-6 expression (42; 76), there was still less brain damage in the amygdala and piriform cortex so that the inhibition of IL-1 α and the increase in IL-6 after anakinra treatment may help reduce brain damage after GD-induced injury.

There are some limitations to consider with the multiplex and anakinra studies. First, there was no way to measure the amount of anakinra entering the brain. There are ELISA kits available to measure human recombinant IL-1Ra, but the kits tested in our laboratory did not provide a quantitative assessment for how much of the drug entered the brain. We considered conducting mass spectrometry to measure the amount of anakinra entering the CNS, but do not have the capability to conduct these experiments in our laboratory. Therefore, although the neuropathology results showed a neuroprotective effect of anakinra 24 hrs after GD exposure, we do not know how much of the drug entered the CNS. Additionally, we do not know how much endogenous IL-1Ra versus anakinra provided the neuroprotection after GD exposure. Before conducting the multiplex study, our laboratory ran a pilot study, where ELISA assays were used to measure how much endogenous IL-1Ra is present in the IL-1Ra KO mice. We found IL-1Ra is present in this KO strain. This is because the IL-1Ra KO bred from the Jackson Laboratory is a functional KO where the gene is not fully removed in the breeding process. Upon further

investigation with the Jackson Laboratory, it was discovered that the concentration of IL-1Ra found in the IL-1Ra KO mice was not considered to be functional in blocking the IL-1R1. This means that the results of the multiplex assays in the current work is accurate, but there is still no way to quantitatively assess how much endogenous IL-1Ra and anakinra is present after GD exposure, and their respective neuroprotective effects.

Future studies may involve studying cytokine upregulation at the level of gene expression to get a better understanding of the role of IL-1 signaling in GD-induced SE. Additionally, other inflammatory cytokines and chemokines may be analyzed to further understand the effect of peripheral inflammation on central inflammation, and the role of IL-1 in this relationship. Seizure activity and SRS may be quantified to further understand the seizurogenic effect of GD-induced SE, and anakinra may be given via osmotic pump to study its effect on seizure activity, neuropathology, neuroinflammation, and behavior.

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