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Soman-induced toxicity, cholinesterase inhibition and neuropathology in adult male Göttingen minipigs[☆]

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ABSTRACT

Animal models are essential for evaluating the toxicity of chemical warfare nerve agents (CWNAs) to extrapolate to human risk and are necessary to evaluate the efficacy of medical countermeasures. The Göttingen minipig is increasingly used for toxicological studies because it has anatomical and physiological characteristics that are similar to those of humans. Our objective was to determine whether the minipig would be a useful large animal model to evaluate the toxic effects of soman (GD). We determined the intramuscular (IM) median lethal dose (LD_{50}) of GD in adult male Göttingen minipigs using an up-and-down dosing method. In addition to lethality estimates, we characterized the observable signs of toxicity, blood and tissue cholinesterase (ChE) activity and brain pathology following GD exposure. The 24 h LD_{50} of GD was estimated to be 4.7 μg/kg, with 95 % confidence limits of 3.6 and 6.3 μg/kg. As anticipated, GD inhibited ChE activity in blood and several tissues. Neurohistopathological analysis showed neurodegeneration and neuroinflammation in survivors exposed to 4.7 μg/kg of GD, including in the primary visual cortex and various thalamic nuclei. These findings suggest that the minipig will be a useful large animal model for assessing drugs to mitigate neuropathological effects of exposure to CWNAs.

1. Introduction

Chemical warfare nerve agents (CWNAs), such as soman (pinacolyl methylphosphonofluoridate; GD), are extremely lethal organophosphorus compounds (OPs) that produce neurotoxicity primarily through the irreversible inhibition of acetylcholinesterase (AChE), a functional cholinesterase (ChE) active at cholinergic synapses and neuromuscular junctions, by binding to its active site. The excessive accumulation of acetylcholine in the synaptic cleft results in the hyperactivity of central and peripheral cholinergic systems, which can lead to physiological signs such as secretions, tremor and muscle fasciculations, prolonged seizures (i.e., status epilepticus; SE) and, in severe cases, fatal respiratory and cardiovascular complications [reviewed in 33,22,77]. Exposure to CWNAs may result from destruction of munitions, terrorist attacks, warfare or via accidental exposures in laboratories or storage facilities, and thus is of concern to both military and civilian populations [reviewed in 77]. Efficacious medical countermeasures are needed to prevent and/or treat acute and long-term effects of CWNA exposure. Because of ethical concerns, the scientific community is unable to test novel medical countermeasures against CWNA exposure in human subjects and, therefore, relies on the development and use of appropriate pre-clinical animal models.

In the U.S., the Food and Drug Administration (FDA) Animal Rule is used to advance medical countermeasures against chemical, biological, radiological, and nuclear threats on the basis of observations from adequately designed and controlled animal efficacy studies [reviewed in 4]. This rule states that the effect of the drug being tested should be demonstrated in two animal models with a response comparable with

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humans or one well-characterized animal model. Rodents, such as mice, rats, and guinea pigs, are commonly used small animal models in pre-clinical research of medical countermeasures against the effects of CWNA exposure. Non-human primates have been used in various non-rodent large animal studies related to GD exposure, but the number of studies is limited. Petras [60] reported on widespread brain damage in cynomolgus monkeys that developed grand mal seizure, including damage to the optic nerve, thalamic regions, amygdala, and piriform cortex, among other regions. Blick et al. [13] used rhesus monkeys to compare the effects of GD and pyridostigmine, a reversible inhibitor of ChE, on performance in an equilibrium behavior test. They determined that GD was 100 times more behaviorally disruptive than the reversible pyridostigmine. Other studies include the use of cynomolgus monkeys to compare oximes as combination therapy with atropine and diazepam [36], or to compare centrally acting reversible AChE inhibitors for efficacy against GD exposure [27,53]. As the availability of non-human primates for research dwindles and the difficulties of using them in research increase (e.g., high cost, lengthy quarantine, slow throughput, and ethical concerns), it is critical for drug advancement to identify another large animal model that may predict CWNA response in humans.

The past few years have seen a significant increase in the use of the Göttingen minipig as a large animal alternative to non-human primates due to the similarities between swine anatomy and physiology and those of humans [17,76]. Some of the benefits of using minipigs in pre-clinical studies are (1) shorter gestation periods and inter-birth intervals, (2) larger litter size, (3) sexual maturity at 5 months of age, and (4) the availability of controlled breeding for research purposes. These reasons, combined with a lower occupational exposure risk than with other animal species that may carry the Herpes B virus, have been some of the driving factors behind the increased use of minipigs as alternatives to non-human primates.

Göttingen minipigs have been used in a variety of toxicological studies, as well as for neuropathological assessments following acute brain trauma. Previous research with CWNAs in the minipig assessed toxicity of subcutaneous (SC), intravenous (IV) and whole-body inhalation exposure to sarin and cyclosarin [31] and intramuscular (IM) VX [37], as well as the efficacy of medical countermeasures in increasing survival following whole-body exposure to sarin vapor [66,67]. Other toxicological studies with OPs, pulmonary agents (e.g., chlorine), and vesicants (e.g., lewisite and sulfur mustard) have also been performed using swine models [21]. In addition to toxicology studies, swine may be a useful model to evaluate electroencephalographic (EEG) changes and neuropathological effects of brain insult [10,14,45]. Swine have been successfully used to model blast-induced brain injury, demonstrating fiber degeneration, and an activated neuroinflammatory system following injury [10].

The present study is the first to characterize the toxicological effects of acute GD poisoning in the minipig including GD-induced lethality, observable signs of seizure, and seizure-associated neurodegeneration and neuroinflammatory responses. Previously, the median lethal dose of IV administration of GD was determined in anesthetized Yorkshire swine [50], while the current study was conducted in awake minipigs. It is important to quantitatively determine the toxicity of GD for the purpose of subsequently validating the minipig as a large-animal model that models toxicity in humans and is in alignment with the seizurogenic and neuropathological responses to GD poisoning that have been extensively reported in other models. The minipig may then be used for the pre-clinical evaluation of novel medical countermeasures against CWNA exposure in support of drug development under the FDA Animal Rule.

2. Materials and methods

2.1. Animals

Adult male Göttingen minipigs (n = 9), obtained from Marshall BioResources (North Rose, New York), were exposed to GD at 4.5–5 months of age to determine the LD_{50}; animals weighed an average of 10.4 kg (range 8.7–12.1 kg) at time of exposure. To provide additional information beyond the LD_{50} value, toxic signs and ChE activity in blood and tissue were measured, and in survivors (n = 3) neuropathology was assessed. For comparison, three control animals (no GD) were perfused with fixative for neuroanatomical assessments, and two control animals (not perfused) had tissue harvested for ChE assay at approximately 6 months of age to establish a control comparison. Minipigs were acclimated for at least 5 days prior to their use in experiments and were pair-housed except during feeding, experimental assessments, and for reasons of behavioral incompatibility. They had ad libitum access to water in fully AAALAC-accredited facilities under a 12 h:12 h light-dark cycle with lights on at 0600 h. The animals were fed a commercial pig chow twice daily and given fruit or vegetable enrichment each afternoon. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD; Aberdeen Proving Ground, MD), and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals [16], the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

2.2. Sling training

On the week prior to exposure, minipigs underwent basic training to prepare them for brief restraint (up to 5 min) in a sling to administer injections, based on the techniques described by Zeltner [79]. Training consisted of placing animals in a pig sling and providing positive reinforcement (e.g., stroking back, food/juice reward) to acclimate them to the sling. Initial sessions were brief (less than one minute), and time in the sling gradually lengthened to up to 5 min with pigs removed from the sling and returned to their home pen 1 min after showing docile behavior. On the last two days of the sling training week, animals were acclimated to the exposure pen (in separate room) for 10 min prior to restraining them for a few minutes in the sling in the exposure room.

2.3. GD exposure and observable toxicity

GD was obtained from the U.S. Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD). All primary nerve agent stocks utilized to formulate diluted solutions for experimental use were Chemical Agent Standard Analytical Reference Material certified at a purity of >95 %. Subsequent experimental diluted GD stocks were formulated gravimetrically, and concentrations were confirmed by USAMRICD chemists using GC/MS and NMR. Sterile saline (0.9 % NaCl; Hospira, Lake Forest, IL) was used to dilute the stock solutions.

On the day of exposure, one minipig was placed in the exposure pen and allowed to habituate for approximately 10 min prior to exposure. Following habituation, the minipig was placed and restrained in the sling. GD was administered (IM) in the left lateral back. Immediately following exposure, the minipig was returned to the exposure pen and continuously observed for toxic signs for at least 7 h following GD exposure. Noldus Observer (Noldus, Inc., Leesburg, VA) was used by an observer blind to GD dose to score behaviors to include point events or duration of behaviors. A detailed description of the observed toxic behaviors is shown in Table 1. Based on a scale from Bachiega et al. [8] and our observations in the minipig, scored behaviors that were relevant to observable signs of motor or behavioral seizure were classified in a modified Racine scale as follows: 0, no abnormality; 1, ataxia, mastication, salivation, fasciculation; 2, head tremors, head bobs; 3, limb clonus or tonus, body tremor; and 4, generalized clonic seizure/myoclonic jerks, convulsions, loss of posture.

Table 1
Definitions of Toxic Signs Observed in Minipigs Following Soman Exposure.

Stage 1	
Mastication	Continuous chewing
Salivation	An increase in the production of saliva.
Ataxia	Impaired motor activity characterized by loss of coordination and unsteady gait.
Fasciculation	Persistent and rapid spontaneous contractions of muscle fibers that do not result in purposeful movement; twitching [1, 12].
Stage 2	
Head Bobs	Continuous nodding/shaking of the head.
Head Tremor	Tremors localized in the head and neck.
Stage 3	
Body Tremor	Rhythmic or non-rhythmic oscillating muscular movements [1]. May be intermittent or constant.
Limb Clonus	Involuntary and rhythmic muscular contractions and relaxations that cause rapid movement and shaking of the limb [30].
Limb Tonus	Tensing of the muscles of the limb causing limbs to remain in a fixed position [1].
Stage 4	
Convulsions	Transient electrical dysrhythmias that manifest as disorganized limb movements [1].
Myoclonic Jerks	Brief shock-like muscle contractions that are single or slowly repetitive, erratic, or irregular.
Loss of Posture	A point event for the inability to maintain stance; falling over due to seizure.
Death	When the animal's heart is no longer beating.

2.4. LD_{50} determination

A modified up-and-down method was used to determine the 24 h LD_{50} of GD (IM) exposure using minipigs. The IM route of exposure was chosen as it is frequently used in the determination of LD_{50} in large animals (see Table 2). The up-and-down method for estimating the LD_{50} of an acutely toxic compound was first described by Dixon and Mood [20] and involves exposing animals one at a time in a staircase fashion. As also described by Rispin et al. [64], the first animal is exposed to a dose of the compound that is a step below the best estimate of the LD_{50} value. If that animal survives, the second animal is exposed to a higher dose of the compound. Otherwise, the second animal is exposed to a lower dose. The progression factor for dosing is equivalent to the antilog of the inverse of the best estimate for the slope of the dose-response curve. Dosing continues until one of the following stopping criteria has been met: 1) three consecutive animals survive at the upper bounds (2,000 mg/kg), 2) five reversals occur in any six consecutive animals tested or 3) at least four animals have followed the first reversal and the specified likelihood ratios exceed the critical value. Decisions about the GD dose administered to each minipig and when to stop the exposures were guided by the AOT425StatPgm program, developed by the U.S. Environmental Protection Agency (Washington, D.C.), using 20 μg/kg as the best estimate for the LD_{50} value and 8 as the best estimate of the slope of the dose-response curve.

2.5. Blood and tissue cholinesterase (ChE)

Blood was collected in the weeks prior to exposure from a subset of minipigs to establish baseline levels of ChE. Minipigs were briefly anesthetized with isoflurane (1–5 %; Baxter, Deerfield, IL), and ~1.5 mL of blood was drawn from the cranial vena cava and placed into three heparinized tubes. To verify GD-induced inhibition of ChE, blood was collected via cardiac puncture from animals that died on the day of exposure. In minipigs that survived to 24 h after exposure, as well as in control (No GD) animals, telazol (2–8.8 mg/kg, IM; Zoetis, Parsippany, NJ) and isoflurane (5%) were used to sedate animals prior to placing an intravenous (IV) catheter in the auricular ear vein to draw blood. Whole blood (WB) was then diluted 1:25 (v:v) in 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) solution. The remainder of the original WB sample was centrifuged for 5 min at 14,000 rpm using an Eppendorf 5424R centrifuge. Packed red blood cells (RBC) were then diluted 1:50

Table 2
Median Lethal Dose of Parenteral (IM, SC, IM, IV) GD in Multiple Species.

Species	Strain	Sex	Route	Time Point	LD_{50} (95% CI)	Reference
Guinea Pig	Dunkin-Hartley	Male	IM	Not specified	25 μg/kg (21–27)	[5]
Guinea Pig	Hartley	Female	SC	24 h	27.0 μg/kg (25.2–29.7)	[23]
Guinea Pig	Hartley	Male	SC	24 h	32.3 μg/kg (29.4–35.7)	[46]
Guinea Pig	Hartley	Male	SC	24 h	24.9 μg/kg (24.0–26.9)	[23]
Mouse	BCBAF1	Female	SC	24 h	156 μg/kg (146–166)	[11]
Mouse	ICR	Male	SC	24 h	125 μg/kg (115–138)	[46]
Mouse	C57Bl/6	Male	SC	24 h	83 (unspecified)	[15]
Mouse	Es1-/-	Male	SC	24 h	19.2 μg/kg (18.0–20.5)	[44]
Mouse	Es1-/-	Female (proestrus)	SC	24 h	19.8 μg/kg (17.7–22.1)	[35]
Mouse	Es1-/-	Female (estrus)	SC	24 h	23.6 μg/kg (20.8–26.7)	[35]
Mouse	Es1-/-	Female (diestrus)	SC	24 h	20.8 μg/kg (18.9–22.8)	[35]
NHP	African green	Male	IM	48 h	7.15 μg/kg (6.28–8.13)	[18]
NHP	Baboon	Both	IV	Not specified	6.65 μg/kg	[6]
NHP	Cynomolgus	Not specified	IM	24 h	3.77 μg/kg (3.47–4.09)	[3]
NHP	Marmoset	Both	SC	7 d	8 μg/kg	[19]
NHP	Rhesus	Both	IM	Not specified	9.5 μg/kg	[42]
NHP	Rhesus	Not specified	IM	24 h	7.57 μg/kg (6.49–8.81)	[2]
NHP	Rhesus	Not specified	IM	48 h	7.40 μg/kg (6.33–8.64)	[2]
NHP	Rhesus	Not specified	IM	5 d	6.57 μg/kg (5.83–7.39)	[2]
NHP	Rhesus	Both	SC	7 d	13.0 μg/kg (9.1–18.1)	[19]
Pig	Domestic	Male	IV	Not specified	6.0 μg/kg	[26]
Pig	Yorkshire	Male	IV	Not specified	4.6 μg/kg	[50]
Rabbit	New Zealand White	Male	SC	24 h	22.8 μg/kg (18.7–27.8)	[45]
Rat	Sprague-Dawley	Female	SC	24 h	74.0 μg/kg (54.5–101.0)	[78]
Rat	Sprague-Dawley	Male	SC	24 h	116 μg/kg (102–132)	[45]
Rat	Sprague-Dawley	Male	SC	24 h	98.4 μg/kg (90.5–107.0)	[78]
Rat	Wistar	Male	IM	24 h	69 μg/kg (63–75)	[51]
Rat	Wistar	Male	IP	24 h	117 μg/kg (100–126)	[51]
Rat	Wistar	Male	SC	24 h	120 μg/kg (103–139)	[51]
Rat	Albino [SD x WI]	Male	IM	24 h	87 μg/kg (78–96)	[74]

in 1% Triton X-100 solution. Diluted samples were stored at -80 ℃ until analysis.

Select tissues were also collected for analysis of ChE activity. Brainstem, skeletal muscle (diaphragm, tongue, and thigh), smooth muscle (bladder), and cardiac muscle (heart ventricle) were harvested from a subset of animals that died on the day of exposure. The same tissues were also collected from control (no GD) animals to serve as a baseline for tissue comparison. In animals that survived to 24 h after exposure, tissue samples from the urinary bladder and diaphragm were collected immediately prior to perfusion. Brainstem was diluted 1:20 with 1% Triton X-100 (Sigma-Aldrich), and peripheral tissues were diluted 1:5 with the same solution. Tissues were then homogenized and centrifuged in a Thermo Fisher Sorvall LYNX centrifuge at 17,000 rpm at 4 ℃ for 30 min. Supernatant was collected (~1 mL per sample) and stored at -80 ℃ until analysis.

A modified Ellman's assay was used to measure the ChE activity in the blood, brain, and peripheral tissues, as described in Shih, Kan [73] and Skovira et al. [75]. On the day of ChE analysis, the homogenized tissues, WB, and RBC were thawed and three replicate samples (7 μL, tissues; 10 μL, WB and RBC) were pipetted into a 96-well UV star microplate (Greiner, Longwood, FL). To each well containing tissue samples, 20 μL of deionized water was added, while 17 μL of deionized water was added to each WB and RBC samples. Following the addition of water, 200 μL of DTNB (0.424 M, pH 8.2; Thermo Scientific, Rockford, IL) was added to each sample well. Each microplate was then incubated for 10 min at 37 °C before being placed in the Spectramax Plus microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) where it was allowed to shake for 2 min. Immediately after, 30 μL of the substrate acetylthiocholine iodide (51.4 mM; Sigma-Aldrich) was added to each well. The samples were read at 412 nm (at 20 s intervals) for 3.5 min, and the activity (μmol/mL/min) was determined using Softmax Plus 4.3 LS software (Molecular Devices Corporation). A bicinchoninic acid (BCA) protein assay (Thermo Scientific) was run, following the manufacturer's protocol, in conjunction with the ChE assay to measure total protein concentrations in the brain and peripheral tissue. Results from the BCA assay were used to normalize ChE activity by the total amount of protein in each sample.

2.6. Brain tissue collection and processing

Following anesthesia with telazol and isoflurane as described above, animals that survived to 24 h after GD exposure and No GD controls were injected with a sodium pentobarbital solution (25−150 mg/kg, IV, Fatal Plus; Patterson Veterinary, Greeley, CO) and euthanized via exsanguination. Minipigs were transcardially perfused with heparinized saline (0.9 % saline, 1000 IU/L heparin; Fresenius Kabi, Lake Zurich, IL) followed by fixation with phosphate-buffered 4% paraformaldehyde (FD NeuroTechnologies, Inc. Columbia, MD). The brain was removed and post-fixed at 4 °C in 4% paraformaldehyde for 24 h. Brains from animals that died prior to 24 h after GD exposure were immediately removed and immersion-fixed in phosphate-buffered 4% paraformaldehyde for 7 days at 4 °C. Each brain was coronally divided into 8 blocks.

2.6.1. Frozen sections

Four out of 8 blocks (1, 3, 5, 7) of brains from perfused animals were cryoprotected with FD tissue cryoprotection solution (FD NeuroTechnologies, Inc.) for 96 h at 4 °C and then rapidly frozen in isopentane that had been pre-cooled to -70 °C. All frozen brain blocks were stored in a freezer at −80 °C before sectioning. Fifty μm sections were cut coronally from each block with a cryostat. Ten serial sections were collected separately in FD section storage solution (FD NeuroTechnologies, Inc.) and stored at −20 °C before further processing.

2.6.2. Paraffin sections

Four blocks (2, 4, 6, 8,) from perfused animals were prepared for paraffin embedding by dehydration through a graded series of ethanol and xylenes. Sections were coronally cut at 6 μm of thickness on a rotary microtome. Ten serial sections were mounted on 50 × 75 mm Superfrost Plus microscope slides (1 section per slide) and stored at room temperature before further processing.

2.6.3. Silver staining

For detecting neuronal degeneration, the 1 st and 5th sections from each of block were refixed in phosphate-buffered 4% paraformaldehyde (FD NeuroTechnologies, Inc.) at 4 °C for 7 days. Sections were then processed with FD NeuroSilver Kit II (FD NeuroTechnologies, Inc.) according to the manufacturer's instructions. Subsequently, all sections were mounted on microscope slides. After air drying, the sections were cleared in xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

2.6.4. Ionized calcium binding adaptor molecule 1 (Iba1) immunohistochemistry

The 2nd sections were processed for Iba1-immunohistochemistry. Briefly, after endogenous peroxidase activity was blocked with 0.6 % hydrogen peroxidase, free-floating sections were incubated in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 1% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), 0.3 % Triton X-100 (Sigma, St. Louis, MO), and polyclonal rabbit anti-Iba1 antibody (1:10,000, Wako Chemicals USA, Richmond, VA #019−19741) for 65 h at 4 °C. The sections were then incubated in PBS containing Triton-X, normal blocking serum, and biotinylated secondary antibody for 1 h at room temperature, and then in PBS containing avidin-biotinylated HRP complex for another 1 h using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Subsequently, the sections were incubated for 5 min in 0.05 M Tris buffer (pH 7.2) containing 0.03 % 3′,3′-diaminobenzidine (DAB) as a chromogen (Sigma-Aldrich, St. Louis, MO) and 0.0075 % hydrogen peroxide. All sections were rinsed in distilled water, mounted on slides and were counterstained with FD cresyl violet solution (FD NeuroTechnologies, Inc.). After dehydration in ethyl alcohol, sections were cleared in xylene and cover-slipped in Permount (Fisher Scientific).

2.7. Data analysis

The LD_{50} estimate was calculated using the AOT425StatPgm program, developed by the U.S. Environmental Protection Agency. For the Ellman's assay, ChE activity was initially expressed as μmol substrate hydrolyzed/min/g protein for brain stem and peripheral tissues, and as μmol substrate hydrolyzed/min/mL for blood. These values were then converted to a percentage of the control group's average ChE activity for each tissue type (mean ± SEM % of control value) and a percentage of the baseline average ChE activity for each blood sample (mean ± SEM % of baseline value). Neuropathology and observable signs of toxicity assessments are qualitative and descriptive in nature, and they were not subjected to formal inferential statistical analyses.

3. Results

3.1. Determination of median lethal dose (LD_{50}) of GD (IM)

To estimate an LD_{50} value, minipigs were exposed (IM) to 3.6−15 μg/kg GD using the up-and-down method. As shown in Table 3, all of the minipigs exposed to GD doses of 6.3 μg/kg and higher died. Exposure to 4.7 μg/kg GD elicited a partial response where one minipig died and two minipigs survived, and the one minipig exposed to 3.6 μg/kg GD survived. The 24 h LD_{50} of IM administration of GD in the adult male Göttingen minipig was determined to be 4.7 μg/kg with a 95 % confidence interval of 3.6 and 6.3 μg/kg.

Table 3
Latency (min) to Toxic Signs following Soman Exposure in Minipigs.

Dose (µg/kg)	3.6	4.7	4.7	4.7	6.3	6.3	8.4	11.2	15
Stage 1									
Mastication	3	3	4	4	4	4	3	2	12
Salivation	9	7	5	5	6	6	6	3	3
Ataxia	58	6	10	5	5	4	4	3	2
Fasciculation	2	2	4	2	6	4	4	3	2
Stage 2									
Head Bobs/Head Tremors	—	10	—	75	11	11	5	5	5
Stage 3									
Body Tremor	95	6	14	8	5	10	5	3	3
Limb Clonus or Tonus	—	9	—	10	6	6	6	4	4
Stage 4									
Convulsions	—	13	35	15	7	5	8	3	6
Generalized Clonic Seizures/Myoclonic Jerks	—	10	29	11	7	5	8	3	8
Loss of Posture	—	7	49	50	10	6	4	4	5
Death	—	720	——	—	108	130	33	45	34

3.2. Toxic signs and ChE inhibition following IM exposure to GD

A modified Racine scale [8] was used to categorize the severity of motor or behavioral seizure or other overt signs of toxicity; the onset of toxic signs for each dose is shown in Table 3. Mastication, salivation, muscle fasciculation, and ataxia, categorized as Stage 1, appeared within minutes of GD exposure irrespective of dose. Following Stage 1 signs, animals exposed to doses higher than 6.3 µg/kg and two out of three exposed to 4.7 µg/kg experienced continuous head bobbing, a Stage 2 toxic sign. We considered Stages 3 (whole-body tremors, and limb tonus or clonus) and 4 (myoclonic jerks, convulsions, and loss of posture) to be comprised of signs that are characteristic of severe seizure activity. All minipigs exposed to doses of 4.7 µg/kg or higher of GD exhibited convulsions, myoclonic jerks, and postural impairment, with higher doses tending to produce a more rapid onset.

All animals that died on the day of exposure developed Stages 1 through 4 of motor seizure to include convulsions, prior to death. Latency to death was dose-dependent, with doses ranging from 4.7-15 µg/kg (Fig. 1A). In the three animals that survived, the animal exposed to 3.6 µg/kg had Stage 1–3 toxic signs to include ataxia, fasciculations and tremor, whereas the two minipigs exposed to 4.7 µg/kg that survived reached Stage 4 motor seizure to include prolonged convulsions and/or myoclonic jerks of 1−2 h (Fig. 1B).

Changes in ChE activity in various tissues were assessed by collecting blood and tissue samples at the time of death following GD exposure. ChE activity in the terminal RBC and plasma sample is shown as a percent of baseline (Fig. 2A), while activity in the terminal organ samples is shown as a percent of control samples (Fig. 2B). As expected, exposure (IM) to GD produced an inhibition of ChE in blood and tissues.

3.3. Neuropathology and neuroinflammatory response

3.3.1. Silver staining for detection of neurodegeneration

Qualitative neuropathological assessments were performed in minipigs exposed to GD. Examination of silver-stained sections of the brains from the control minipigs did not reveal positively stained neurons, including cell bodies and processes in any brain regions examined (Fig. 3). However, numerous silver-stained neurons, indicating

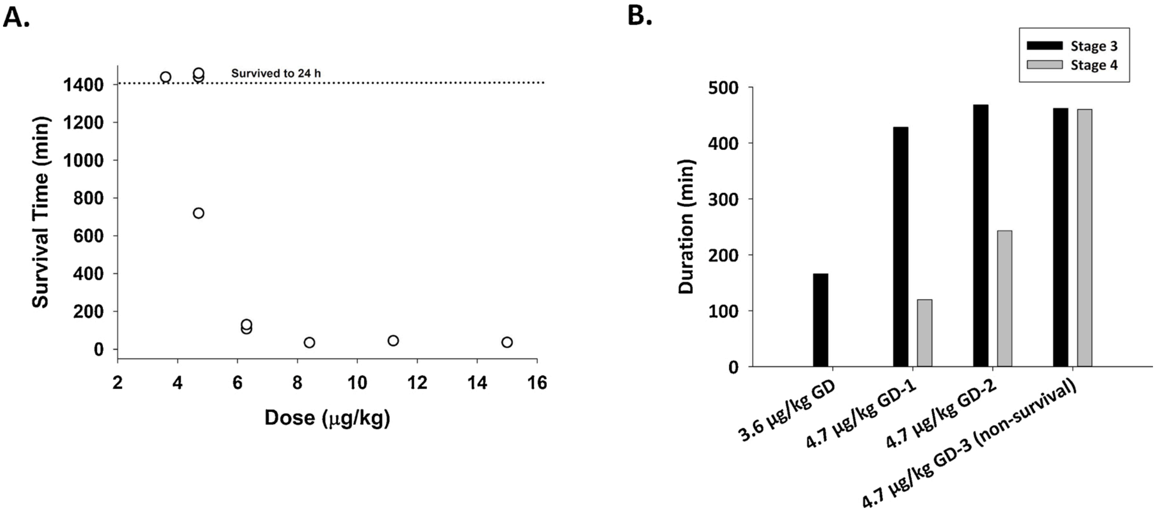

Fig. 1. Lethality and signs of toxicity following GD exposure in adult male Göttingen minipigs. Minipigs were exposed to GD (IM) via the up-and-down method and 24 h lethality and toxic signs were monitored. A) Survival latency in minipig exposed to a dose range of GD. Lethality occurred in a dose-dependent manner with death occurring within 33 min, at the earliest, in animals that were exposed to the highest dose of GD evaluated. The 24 h LD_{50} of GD (IM) was determined to be 4.7 µg/kg (with 95 % CI (3.58, 6.34)). (B) Signs of severe motor seizure manifested as body tremors/limb clonus or tonus (Stage 3 on modified Racine scale) and generalized clonic seizures/myoclonic jerks/loss of posture (Stage 4 on modified Racine scale). A dose of GD of 3.6 µg/kg did not elicit Stage 4 signs; the LD_{50} of GD (4.7 µg/kg) resulted in the death of an animal after prolonged Stage 3 and 4 seizure, while two other animals that survived spent less time in Stage 4.

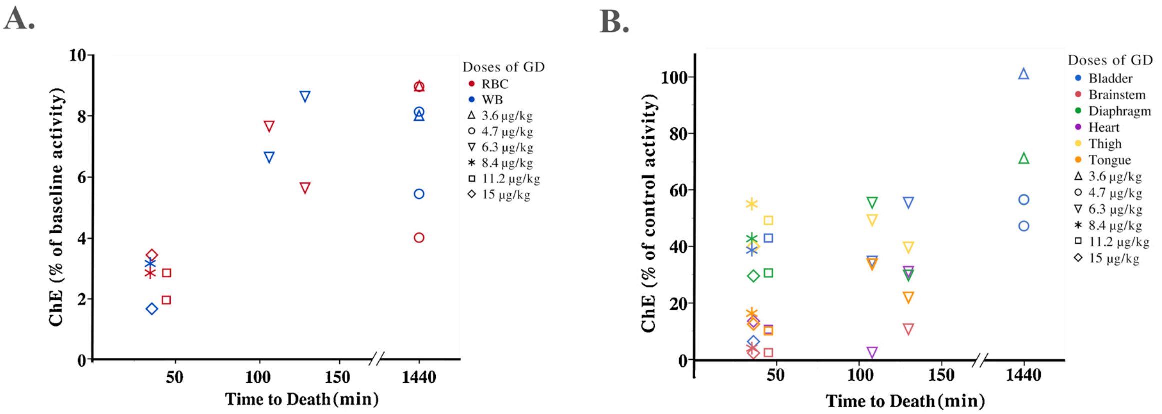

Fig. 2. Blood and tissue cholinesterase (ChE) activity following exposure to GD in adult male Göttingen minipigs. Minipigs were exposed IM to various doses of GD, and lethality was monitored. Animals that survived up to 24 h after exposure were anesthetized and transcardially perfused with saline and a fixative (time 1440 min), while animals that died prior to 24 h after exposure were not perfused. Diaphragm and bladder tissues were collected from perfused and non-perfused animals, while brainstem, heart, thigh, and tongue tissue were collected only from non-perfused animals. ChE activity was assessed using Ellman's assay, and results are shown for (A) red blood cells (RBC) and whole blood (WB) as percent average baseline activity, and (B) tissue as a percentage of control (No GD) animals. At time of death, GD exposure resulted in less than 10 % of baseline ChE activity in blood and less than 60 % of control ChE activity in the brainstem, heart, diaphragm, bladder, thigh, and tongue tissues.

undergoing degeneration, were detected in various brain regions in survivors at 24 h following exposure to 4.7 µg/kg GD. Degenerating neurons were consistently observed in the primary visual cortex and the lateral geniculate nucleus in the brains of the animals exposed to GD (4.7 µg/kg). Interestingly, degenerating neurons in the primary visual cortex were predominantly seen in layer IV, where pronounced degenerating axon terminals coexisted with dead neuronal perikarya. At the high magnification, silver-stained neurons in layer IV appeared to be mainly pyramidal neurons. A large number of degenerating neurons were also found in the ventral lateral thalamic nucleus, the ventral posterolateral thalamic nucleus, and ventral posteromedial thalamic nucleus in both hemispheres of the animals treated with a high dose of GD. In one animal with more prolonged seizure, degenerating neurons were also observed in other thalamic nuclei, including the anterodorsal thalamic nucleus, anteroventral thalamic nucleus, anteromedial thalamic nucleus, and pulvinar nuclear thalamic group. In addition, scattered degenerating neurons were noticed in other brain regions, such as the anterior cingulate cortex, motor and somatosensory cortices, perirhinal cortex, temporal cortex, amygdalopiriform transition area, claustrum, putamen, and globus pallidus.

3.3.2. *Fluoro-Jade B for detection of degenerating neurons*

Fluoro-Jade B staining has extensively been accepted as one of most reliable methods for the detection of neuronal death in the brain, especially for demonstration of neuronal perikarya undergoing degeneration. Examination of Fluoro-Jade B-stained sections of the brains from the control minipigs did not reveal positively stained neurons. However, numerous Fluoro-Jade B positive neurons were present in the regions where silver-stained neurons were observed. Thus, Fluoro-Jade B positive neurons were consistently found in layer IV of the visual cortex and in the lateral geniculate nucleus of the two surviving animals exposed to GD (4.7 µg/kg; Fig. 4). At the high magnification, many Fluoro-Jade B positive neurons appear to be shrunken, which is a characteristic of dead neurons. A large number of Fluoro-Jade B positive neurons were also detected in the thalamus of the surviving minipig exposed to GD that had more prolonged seizure. The distribution and locations of these degenerating neurons in the thalamus are very similar to those displayed with silver staining. Thus, they were mainly seen in the anterodorsal thalamic nucleus, the ventral lateral thalamic nucleus, the ventral posterolateral thalamic nucleus, the ventral posteromedial thalamic nucleus, the anteromedial thalamic nucleus, and the pulvinar nuclear thalamic group. In addition, scattered dead neurons were also noticed in other brain regions of this minipig, such as the anterior cingulate cortex, motor and somatosensory cortices, perirhinal cortex, temporal cortex, amygdalopiriform transition area, claustrum, putamen, and globus pallidus.

3.3.3. *Iba1 for detection of activated microglia*

Iba-1 has been extensively used as a specific marker for microglial cells (or microglia) in the brains of many species, including Göttingen minipigs [25]. Examination of Iba-1 immunostained sections from GD-exposed minipig brains revealed remarkable changes of microglial morphology. The most striking feature was the hypertrophy of microglia in the brain areas where neurodegeneration was observed. This was evident both in 50-µm cryostat sections and in 6-µm paraffin-embedded sections. The cell bodies of these immunoreactive microglia became substantially larger than normal cells either in adjacent areas that did not show neurodegeneration or in the homologous regions of control animals. In addition, the processes of these enlarged microglia were visibly hypertrophic and appeared to be shortened. Since the morphology of these cells resembled the classic reactive microglia, they will be referred to as reactive (or activated) microglia in the following.

Reactive microglia were observed in all brain regions where degenerating neurons were present in animals exposed to GD (4.7 µg/kg). Notably, numerous reactive microglia were consistently seen in layer IV of the visual cortex (Fig. 5D-F) and in the lateral geniculate nucleus (Fig. 5J-L) of GD-exposed animals, but not in the same regions from the controls (Fig. 5A-C and G–I, respectively). A large number of reactive microglia were also noticed in the thalamus of one GD-exposed animal. These hypertrophied microglia were mainly found in the anterodorsal thalamic nucleus, the ventral lateral thalamic nucleus, and the ventral posterolateral thalamic nucleus, the ventral posteromedial thalamic nucleus, the anteromedial thalamic nucleus, and the pulvinar nuclear thalamic group. In addition, reactive microglia were noticed in other brain regions as well, such as the anterior cingulate cortex, motor and somatosensory cortices, perirhinal cortex, temporal cortex, amygdalopiriform transition area, claustrum, putamen, and globus pallidus.

4. Discussion

The present study determined the toxicity of IM exposure to GD in the Göttingen minipig as a first step in exploring the relevance of this species as a large animal model for the evaluation of medical countermeasures against CWNA exposure. The up-and-down procedure was

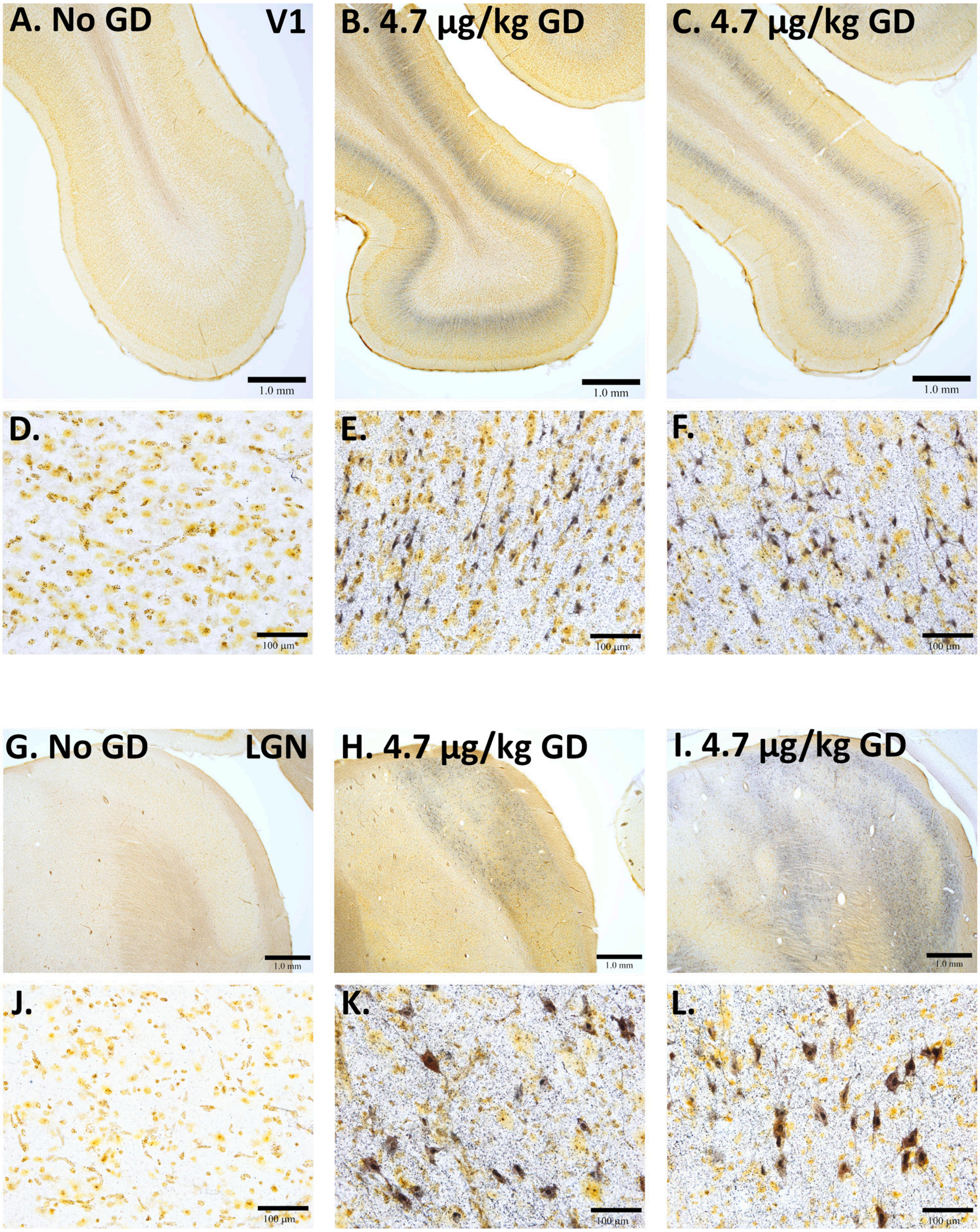

Fig. 3. Neuronal degeneration at 24 h following GD exposure in adult male Göttingen minipigs. Minipigs were exposed to GD (IM), and survival was monitored for up to 24 h. In surviving animals, brains were collected following anesthesia and transcardial perfusion and were processed with a silver stain to visualize neuronal degeneration. A, G: Representative images taken from the visual cortex (V1) and the lateral geniculate nucleus (LGN) of a brain from No GD control. D, J: High magnification of an area in A and G, respectively. B, C, H, I: Images taken from V1 (B, C) and LGN (H, I) of 2 minipigs exposed to 4.7 µg/kg of GD. E, F: High magnification of an area in B and C. K, L: High magnification of an area in H and I. Note silver-stained neuronal perikarya and processes, possibly axon terminals (black deposits) in layer IV of V1 (E, F) and LGN (K, L). 2x magnification (A, B, C, G, H, I); 40x magnification (D, E, F, J, K, L).

followed to estimate the 24 h LD_{50} of IM exposure to GD. The main benefit of the up-and-down method is the ability to determine the dose-lethality response of acute exposure to a toxicant with fewer test animals than would be needed if a sequential dosing step procedure were used. In comparison with rodent species, the minipig, with an estimated LD_{50} of 4.7 µg/kg, is more sensitive to the lethal effects of GD exposure and is more similar to the LD_{50} of non-human primates ranging from 3.77 to 9.5 µg/kg. Thus, the minipig may be a useful predictor of response to GD exposure in humans. A comprehensive list of LD_{50} data for GD, including other routes of administration, is shown in Table 2.

Differences in susceptibility to lethality as a result of exposure to GD between species may be the result of variability in the expression of

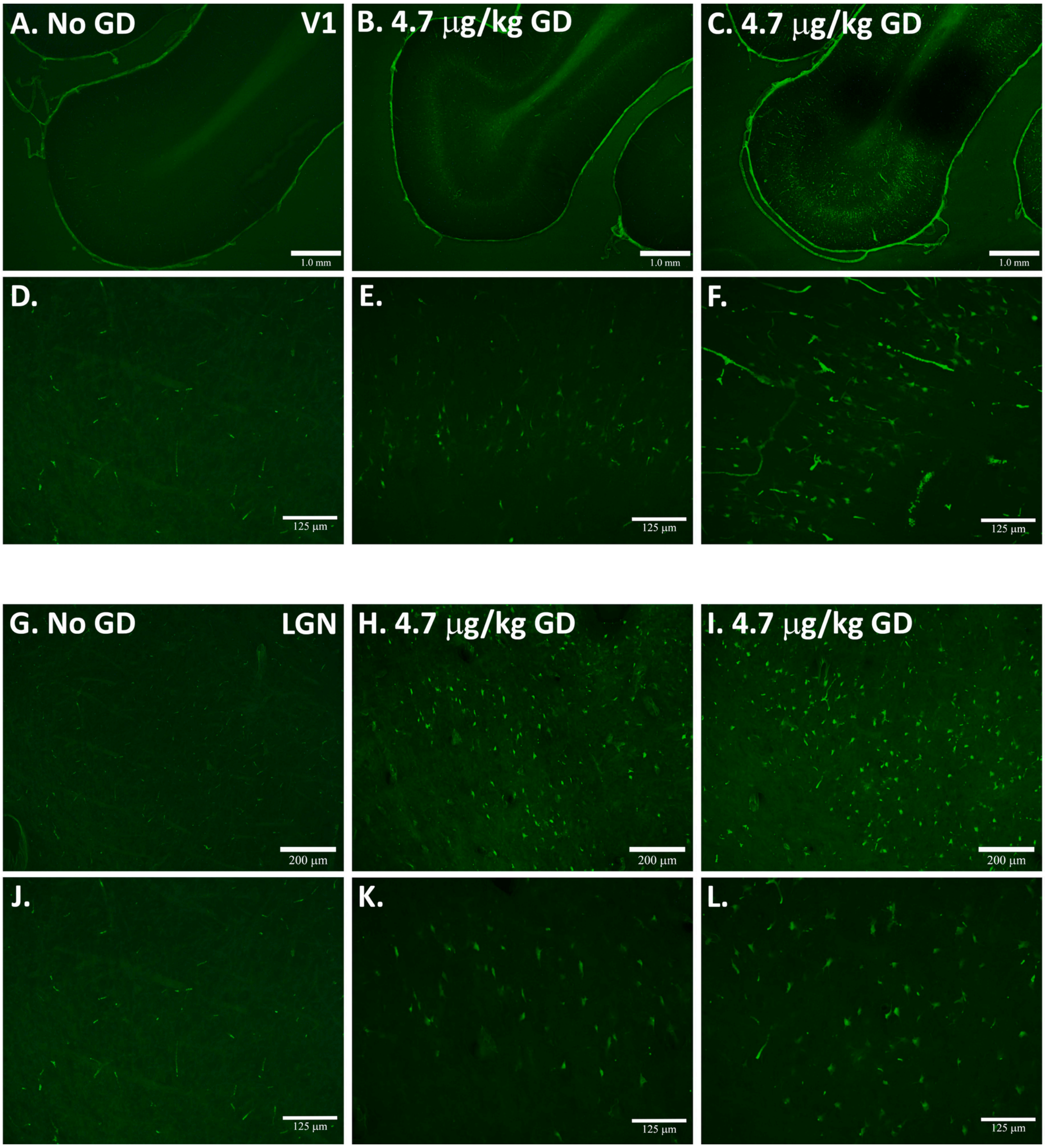

Fig. 4. Degeneration of neurons at 24 h following exposure to GD (IM) in adult male Göttingen minipigs. Minipigs were exposed to either saline (No GD) or GD, and survival was monitored for up to 24 h. In surviving animals, brains were collected following anesthesia and transcardial perfusion and were processed with Fluoro-Jade B stain to visualize degenerating neurons. A, G: Representative images taken from the visual cortex (V1) and the lateral geniculate nucleus (LGN) of a brain from No GD control. D, J: High magnification of an area in A and G. B, C, H, I: Images taken from V1 (B, C) and LGN (H, I) of minipigs exposed to 4.7 µg/kg of GD. E, F: High magnification of an area in B and C. K, L: High magnification of an area in H and I.

Note that Fluoro-Jade B-stained degenerating neurons in V1 (E, F) and LGN (H, I, K, L). 2x magnification (A, B, C), 10x magnification (G, H, I) and 20x magnification (D, E, F, J, K, L).

plasma ChE, which can act as endogenous scavengers of OP compounds. Rats and mice, contain plasma carboxylesterase activity, which, due to its role in the clearance of certain OP compounds [46], serves as an endogenous scavenger of GD, rendering the animals less susceptible to lower doses of GD, and the LD_{50} in rodents is magnitudes greater than the LD_{50} in non-human primates. In support of the role of carboxylesterase in GD toxicity, previous studies that pretreat various rodents with cresylbenzodioxaphosphorin oxide (CBDP) confirm a lower LD_{50} in those receiving the pretreatment compared to control animals [47], and our laboratory estimated the subcutaneous LD_{50} of GD in plasma carboxylesterase knockout mice to be 4-fold lower than wild-type C57BL/6 mice [35,44]. Humans, non-human primates, and swine have no detectable levels of plasma carboxylesterase [40], and thus, this enzyme does not contribute to differences in LD_{50} among these species. In contrast, butyrylcholinesterase levels in swine serum are lower than those in human plasma and rhesus macaque plasma (Peng et al. [58]), and systemic levels of AChE are lower in two species of pigs, Yorkshire and the Göttingen minipig, compared with those in humans and non-human primates [49]. Thus, the low expression of carboxylesterases in both swine and non-human primates likely makes these two species have similar susceptibility to GD lethality and renders them more optimal than rodents in studies of toxic effects of CWNAs.

Similarities in toxic signs between the minipig and other species following acute GD poisoning were observed. GD exposure in the

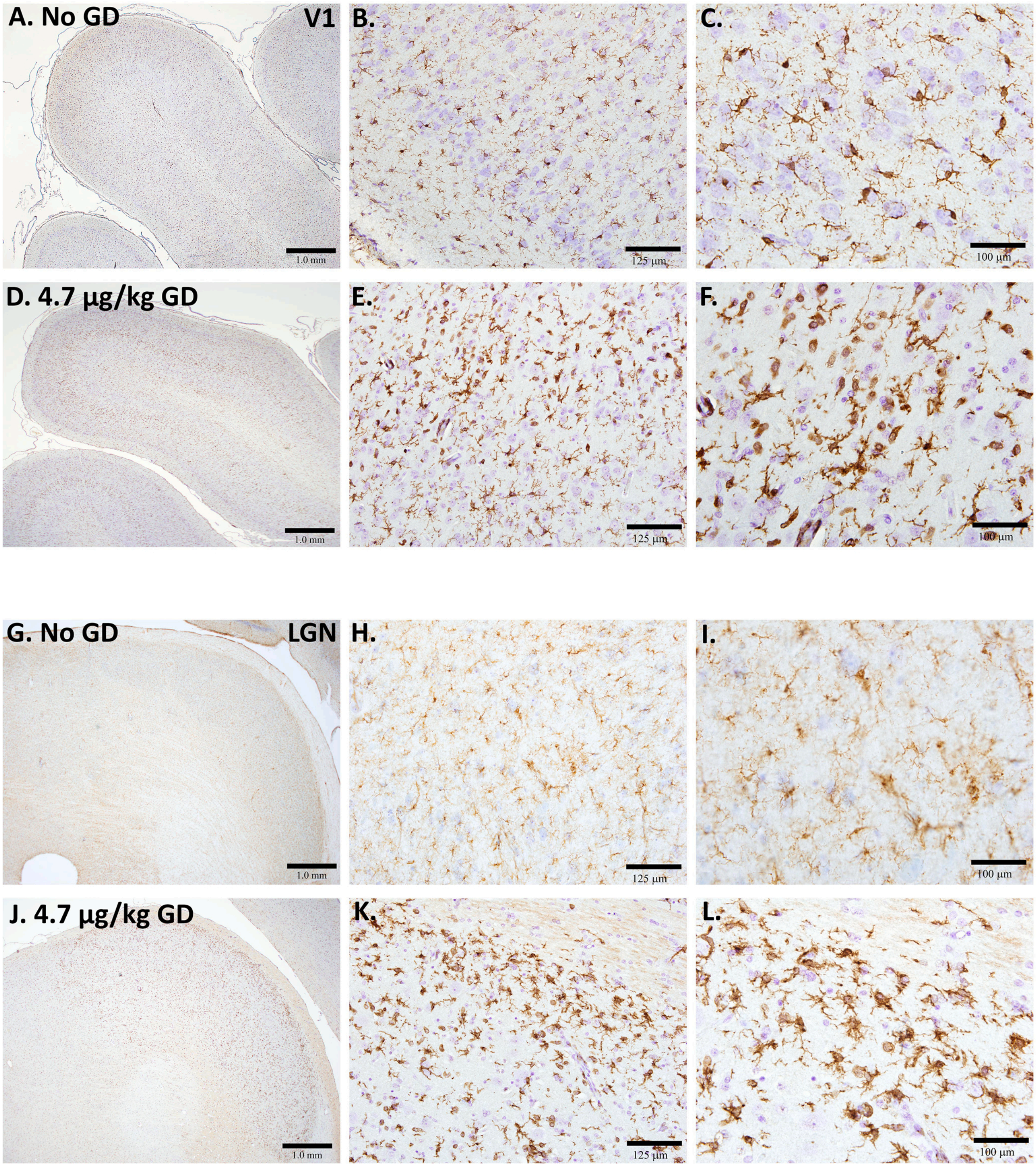

Fig. 5. Neuroinflammatory response at 24 h following exposure to GD (IM) in adult male Göttingen minipigs. Minipigs were exposed to either saline (No GD) or GD, and survival was monitored for up to 24 h. In surviving animals, brains were collected following anesthesia and transcardial perfusion, and all sections were immunohistochemically stained with an antibody against ionized calcium binding adaptor molecule 1 (Iba1) to visualize microglia (brown) and counterstained with cresyl violet (purple) to identify anatomical landmarks. A-F: Representative images taken from 6-μm paraffin sections cut through the visual cortex (V1) of a brain from No GD control (A-C) and a brain from minipig exposed to 4.7 μg/kg of GD (D-F). B, C: Higher magnifications of an area in A. E, F: Higher magnifications of an area in D. Note that Iba1-stained microglia in V1 from GD-exposed minipig brain (D-F) are remarkedly hypertrophied compared to those in V1 from No GD control (A-C). G, J: Images taken from 50-μm cryostat sections cut through the lateral geniculate nucleus (LGN) of a brain from No GD control (G), and a brain from minipig exposed to 4.7 μg/kg of GD (J). H, I: Higher magnifications of an area in G. K, L: Higher magnifications of an area in J. Note that hypertrophied Iba1-stained microglia in LGN from GD-exposed minipig brain (K, L). Images are at 2x (A, D, G, J), 20x (B, E, H, K), and 40x (C, F, I, L) magnification.

minipig led to a variety of cholinergic-induced signs of toxicity including salivation, ataxia, muscle fasciculation, tremor, dyspnea, limb tonus/clonus, myoclonic jerks, prostration and convulsions. Onset of toxicity occurred within minutes of exposure to the doses evaluated, with only the lowest dose tested (3.6 μg/kg) not resulting in stage 4 motor seizure. As anticipated, time to death was reduced with the administration of higher doses of GD. In the cynomolgus monkey, a non-human primate with an LD_{50} similar to that of the Göttingen minipig, IM exposure to GD followed by administration of standard medical countermeasures brings about similar acute signs of toxicity that include muscle fasciculation, tremors, clonic jerks, convulsions, respiratory disturbance, and prostration [36]. Similarly in rhesus monkeys, doses as low as 5 or 6 μg/kg induced tonic-clonic convulsions [41].

The characterization of ChE activity in blood as well as various tissue samples was also performed. We observed blood ChE activity to be less than 10 % of baseline at time of death or euthanasia (24 h after exposure) in all animals exposed to GD. These findings are similar to those observed in other species. Guinea pigs exposed SC to 1 LD_{50} of G- and V-agents have a rapid inhibition of ChE with whole blood inhibited to 5% of control within 10 min of exposure of 1 LD_{50} [73]. In rats, ChE activity remains largely inhibited at 24 h after GD exposure [34], which is similar to our current findings in that minipigs euthanized at 24 h after GD exposure had less than 10 % of baseline blood ChE activity. Also, the severity of signs of GD poisoning in rats is closely correlated with the level of ChE inhibition in blood during the first minutes following acute GD exposure [34]. In our study, GD-exposed minipigs developed signs of toxicity with all but one developing moderate to severe toxic signs. Inhibition of ChE activity in tissue was less than 60 % that of control animals with the exception of one animal exposed to the lowest dose (3.6 μg/kg) that displayed only mild toxic signs. These findings of greater inhibition in blood compared to tissue are in agreement with findings in guinea pigs exposed to 1 LD_{50} GD (SC), which show 20–40 % ChE activity in tissue including brain, diaphragm, heart and skeletal muscle compared to control animals. The *de novo* synthesis of AChE, which has been shown to occur at a rate of approximately 1% per 24 h in other models of GD exposure [29,72], is not believed to have accounted for the lower levels of inhibition observed in minipigs that were exposed to lower doses of GD. Rather, a dose-dependent effect of GD on AChE inhibition was demonstrated in the present study. In the event of exposure to a liquid CWNA (e.g., VX or other persistent agents), signs of toxicity may be delayed and monitoring ChE may be useful to guide therapeutic approach [reviewed in 77]. Although time course evaluation of ChE activity was beyond the scope of this study, the current findings also suggest that ChE assays may be useful in future studies in the minipig to assess the efficacy of medical countermeasures such as oximes on ChE activity.

In GD-exposed minipigs that presented with severe toxic signs following GD exposure, neuropathological injury was observed in brain regions that are consistently found to be damaged in other animal models of CWNA toxicity. In the rat model of GD-induced seizure, neuronal degeneration, visualized by silver staining, in the amygdala, piriform cortex, hippocampus, thalamic nuclei, perirhinal cortex, lateral geniculate nucleus (LGN), among others, occurs [52,68]. The control of seizure duration is a critical factor in determining the severity of neuropathological damage [48,65]. The extent of neuronal damage in GD-exposed mice also depends on the development and duration of convulsions, with affected regions that include the lateral septum, piriform cortex, CA1 and CA3 regions of the hippocampus, thalamus, and amygdala [9]. Cynomolgus monkeys exposed to GD that survived the seizure also have bilateral widespread loss of neurons in regions including the cerebral cortex, corpus callosum, basal ganglia, and thalamus, among others [60].

Novel to the current study is that GD exposure in minipigs that displayed prolonged motor seizure was associated with neuronal degeneration in the visual cortex and in the LGN, a signal relay center in the thalamus for visual pathway. In non-human primates, damage to the optic nerve was reported following GD exposure [59]. In rats, exposure to GD has also been demonstrated to result in damage to the LGN region [38,52]. Interestingly, epidemiological studies of sarin-exposed human victims report persistent visual impairment, yet animal models to date have not modelled these visual effects [32]. Damage to the visual system (optic tract, LGN, and optic nerve of the superior colliculus) is also observed in rodent blast models [28] as well as in humans exposed to blast [24]. In the latter study, soldiers that sustained mild traumatic brain injury had lower functional connectivity of four nodes within the visual system, which correlated with impairment on the Wechsler Adult Intelligence Scale (WAIS) digit-symbol coding task. More research is needed to determine the functional significance of GD-induced damage to the visual system in the minipig, if treatment with medical countermeasures prevents or reduces this damage, and whether this might be a useful model of long-term effects reported in humans exposed to nerve agents.

The pulvinar nuclear thalamic group is another region with strong connectivity to the visual cortex that showed degenerating neurons in one survivor to GD (4.7 μg/kg), demonstrated by silver staining and Fluoro-Jade B staining, as well as increased population of activated microglia. The neuropathological changes observed in the pulvinar nuclear thalamic group are consistent with a retrospective clinical study that found magnetic resonance imaging (MRI) abnormalities in this brain region in patients at 24 h after presenting with status epilepticus [57]. Degenerating neurons were also observed in other thalamic and cortical regions and in the amygdalopiriform transition area, which show similar damage in rodents following GD-induced seizure [7,44,68,69], but additional animals are needed to confirm this effect in the minipig following GD exposure.

Currently approved medical countermeasures against acute exposure to CWNAs include an oxime (e.g., 2-PAM, obidoxime, or HI-6) for reactivation of AChE, a muscarinic acetylcholine receptor antagonist (e.g., atropine) for amelioration of physiological manifestations from the cholinergic crisis, and a benzodiazepine (e.g., diazepam or midazolam) for control of seizure activity [reviewed in 22,33,56,63,77]. For severe cases, use of ventilation to restore oxygen is also critical [reviewed in 77]. Soman is one of the most difficult CWNAs to treat as soman-bound ChE complex ages very quickly (within minutes) and has poor response to current oxime therapy (e.g. 2-PAM approved for use in the USA) [71; reviewed in 63]. Moreover, when the benzodiazepine treatment is delayed to 40 min, refractoriness to treatment develops [70].

Additional medical countermeasures beyond traditional therapies are needed to better protect against CWNA-induced toxicity which require use of animal models that predict effects in humans. We and others have shown the benefits of adding antiglutamatergic and anticholinergic drugs as adjunct to other anti-seizure drugs against cholinergic-induced SE and neuropathology [43,54,55,61,62,68,69], but complete neuroprotection is not attained when treatment is delayed [43,61,62,68,69]. An effective prophylactic approach in animal models is the use of human butyrylcholinesterase as a stoichiometric bioscavenger of CWNA in the blood, which may also be effective as post-exposure treatment against persistent agents with delayed onset of action [reviewed in 39,63]. Others suggest that intravenous lipid emulsion may sequester highly lipophilic CWNAs such as VX and GD and rescue animals from poison [reviewed in 22] but more research is needed. These are a few examples and not intended as a thorough review of candidate therapeutics which is beyond the current scope. However, the search for more efficient therapies must continue, and our data in GD-exposed Göttingen minipigs suggest that this may be a useful large animal model for the advancement of novel oximes, anti-seizure drugs, bioscavengers, and neuroprotectants to counteract the toxic effects of CWNA exposure.

5. Conclusions

In summary, adult male Göttingen minipigs exposed to GD show similar toxic signs, cholinergic enzyme inhibition and brain pathology to those observed in rats and non-human primates, indicating that the minipig may be useful for the evaluation of medical countermeasures for efficacy against CWNA toxicity. The damage observed in the visual cortex and LGN is novel compared to what has been reported in other species following CWNA exposure and deserves further study. We, therefore, suggest that the minipig may be a useful large animal model, comparable to the non-human primate model, with predictive effects on seizure-induced brain pathology in humans and will be useful for drug advancement using the FDA Animal Rule.

CRediT authorship contribution statement

Lucille Lumley: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Fu Du:** Methodology, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Brenda Marrero-Rosado:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Michael Stone:** Investigation. **Zora-Maya Keith:** Investigation. **Caroline Schultz:** Investigation, Data curation. **Kimberly Whitten:** Investigation, Formal analysis, Supervision. **Katie Walker:** Investigation, Formal analysis. **Cindy Acon-Chen:** Investigation, Formal analysis. **Linnzi Wright:** Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Tsung-Ming Shih:** Methodology, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors have no conflicts of interest to report. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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