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Name of Journal: Elsevier NeuroToxicology 65 (2018) 28–37

Date of Publication from Ext Publisher: January 2018

Defence Research and Development Canada

External Literature (P)
DRDC-RDDC-2018-P018
February 2018

Canada

IMPORTANT INFORMATIVE STATEMENTS

NeuroToxicology 65 (2018) 28–37

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

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ARTICLE INFO

Article history:
Received 28 August 2017
Received in revised form 18 January 2018
Accepted 19 January 2018
Available online 31 January 2018

Keywords:
Soman
Nerve agents
Organophosphates
Seizures
Oxygenation
Cerebral blood flow

ABSTRACT

Nerve agents (NAs) are potent organophosphorus (OP) compounds with applications in chemical warfare. OP compounds act by inhibiting acetylcholinesterase (AChE). Soman (*O*-pinacolyl methylphosphonofluoridate) is one of the most potent NAs. It is well known that small doses of NAs can be lethal, and that even non-lethal exposure leads to long-term mental debilitation/neurological damage. However, the neuropathology following exposure to sub-lethal nerve agents is not well understood.

In this study, we examined changes in tissue oxygenation (pO_2) in the cortex and hippocampus after a sub-lethal dose of soman [80–90 μg/kg; subcutaneous]. pO_2 changes can provide information regarding oxygen delivery and utilization and may be indicative of a disruption in cerebral blood flow and/or metabolism. Changes in oxygenation were measured with chronically implanted oxygen sensors in awake and freely moving rats. Measurements were taken before, during, and after soman-induced convulsive seizures.

Soman exposure resulted in an immediate increase in pO_2 in the cortex, followed by an even greater increase that precedes the onset of soman-induced convulsive seizures. The rise in hippocampus pO_2 was delayed relative to the cortex, although the general pattern of brain oxygenation between these two regions was similar. After convulsive seizures began, pO_2 levels declined but usually remained hyperoxygenated. Following the decline in pO_2, low frequency cycles of large amplitude changes were observed in both the cortex and hippocampus. This pattern is consistent with recurring seizures.

Measuring real-time changes in brain pO_2 provides new information on the physiological status of the brain following soman exposure. These results highlight that the measurement of brain oxygenation could provide a sensitive marker of nerve agent exposure and serve as a biomarker for treatment studies.

1. Introduction

Nerve agents (NAs) are potent organophosphorus (OP) compounds with applications in chemical warfare and categorized as weapons of mass destruction. Less potent forms of OPs, including parathion and malathion, are commonly used as pesticides. NAs, which include soman, sarin, tabun, and VX are a significant and current threat to military and civilian populations, because of the potential to cause mass casualties (Ganesan et al., 2010). Despite a ban under the Chemical Weapons Convention Implementation Act, sarin was released in August of 2013 in Syria, killing 1400 people (Dolgin, 2013) and potentially exposing 1000′s to an acute dose.

It is clear that NAs are lethal, however, the pathophysiological neurological changes resulting from a sub-lethal exposure are not well characterized or understood. Repeated acute exposure to OP pesticides has been shown to affect information processing, verbal and visual attention, problem-solving, and motor dexterity (Dassanayake et al., 2007; Rosenstock et al., 1991; Steenland et al., 1994). Studies involving agricultural communities have shown that exposure to long-term low-levels of OP pesticide results in changes to neurological structures in developing children (Rauh et al., 2012). In addition, studies involving victims exposed to NAs have shown changes in regional white matter volume (Chao et al., 2011; Heaton et al., 2007; Yamasue et al., 2007) and grey matter volume (Chao et al., 2011, 2010). Victims of sarin exposure have reported visual, cognitive and motor dysfunction for up to 5 years after poisoning (Kawana et al., 2001). To better understand the neurological effects of NAs at a sub-lethal dose, we exposed rats to soman.

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https://doi.org/10.1016/j.neuro.2018.01.007
0161-813X/

Soman was selected for its fast action and complexity of treatment, due to the rapid aging of the agent-acetylcholinesterase (AChE) complex (Worek et al., 2004). Initial binding of soman to the AChE active site is a reversible process. However, after 2-2.5 min (Worek et al., 2004) soman undergoes a secondary reaction where the pinacolyl bond is cleaved and permanently binds to the AChE active site (Jokanovic, 2001; Zilker, 2005). Inhibition of AChE results in an accumulation of acetylcholine (ACh) in the synapse causing cholinergic hyperactivity, which leads to a wide range of symptoms, including lacrimation, fasciculation, paralysis, generalized convulsive seizures, and respiratory failure (McDonough and Shih, 1997). Soman also causes generalized convulsive seizures that can potentially develop into a condition known as *status epilepticus* (Lallement et al., 1998; Newmark, 2004; Petras, 1994; Shih and McDonough, 1997), which can last for hours (Koplovitz and Skvorak, 1998). *Status epilepticus* is a life threatening condition defined by seizures lasting more than 5 min or having multiple seizure episodes without recovering consciousness (Lowenstein et al., 1999).

Previous research with soman in animal models has shown seizures to be a contributing factor in neurological damage. In other studies, either through innate tolerance or anti-seizure medication, in the absence of convulsive seizures, no neurological degeneration was observed (Apland et al., 2010; Baille et al., 2005; Guo et al., 2015; Lallement et al., 1993; McDonough et al., 1987). When convulsive seizures were terminated within 20 min, minimal neuronal loss was seen (Baille et al., 2005; Lallement et al., 1993), indicating the presence and duration of seizures to be a critical factor in soman related neurological damage.

Seizures induced by soman (Shih and McDonough, 1997) cause neurological damage through excitotoxicity (Fujikawa et al., 2000; Lallement et al., 1993; Olney et al., 1974). The initiation of seizures is triggered by the excess accumulation of ACh within the synapses (Shih and McDonough, 1997), which in turn causes glutamatergic neurons to release glutamate activating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Intense activation of AMPA receptors is followed by the activation of *N*-methyl-D-aspartate (NMDA) receptor and enhanced calcium entry. The large influx of calcium results in neurons to be in a state of hyperexcitability, further facilitating the propagation and maintenance of seizures. The intense activation of NMDA receptors can activate a cell death pathway, leading to neuronal cell death (Fujikawa et al., 2000; Lallement et al., 1993).

Measurements of pO_2 provide information about metabolic imbalance in the brain, which in turn can lead to neuronal damage. By directly measuring pO_2, we can get an indication of the balance between oxygen delivery (cerebral blood flow or CBF) and cerebral metabolic rate of oxygen ($CMRO_2$). We developed a method to measure regional pO_2 in the brain using chronically implanted fiber-optic oxygen sensors in awake and freely moving rats (Ortiz-Prado et al., 2010). This method allows for the measurement of pO_2 without anesthesia or other stresses (handling or restraint) that can potentially alter related physiological responses.

The objective of this study was to measure pO_2 in the rat brain after soman-induced convulsive seizures. This paper is the first to measure pO_2 changes before, during, and after soman-induced convulsive seizures in awake and freely moving rats. Brain oxygenation was directly measured in the cortex and hippocampus, which are areas selected for their sensitivity to NA related neurological damage (Abdel-Rahman et al., 2002; Petras, 1994).

2. Materials and methods

2.1. Animals

Animal care protocols were approved by the University of Calgary Animal Care Committee and meet the Canadian Council of Animal Care (CCAC) guidelines. Twelve male Sprague-Dawley rats were obtained from Charles River Laboratories (Montréal, QC, Canada) weighing 200–350 g. Rats were housed in the University of Calgary Animal Care Facility (Calgary, AB, Canada) with a 12-h light/dark cycle. Each cage housed two rats until probe implantation. Rats were handled for an additional 2–3 days to acclimate to human touch. Surgical implantation of probes was completed following acclimation. Following the probe implantation, rats were housed in individual cages with access to food and water *ad libitum*. Rats were closely monitored on a daily basis by staff for general health status.

2.2. pO_2 probe implantation

Fiber-optic probes were implanted in the cortex and hippocampus via microsurgical methods. The fiber lengths were 4 mm for the hippocampus and 3 mm for the cortex. Animals were anesthetized with isoflurane via inhalation prior to surgery and maintained on 70% N_2, 30% O_2, and 2% isoflurane during the surgical procedure. Temperature and respiration rate were monitored and maintained over the course of implantation. Scalp was shaved and sterilized with iodine solution, a midline incision was made and the skin was retracted laterally to expose the skull. Holes were drilled through the skull at stereotaxic coordinates relative to the bregma. Cortex coordinates: +1 mm anterior/posterior, +1.5 mm medial/lateral, and −2 mm from the top of the skull. Hippocampus coordinates: −4 mm anterior/posterior, −3.5 mm medial/lateral, and −3 mm from the top of the skull. To further secure the probes, 3 additional holes were drilled and implanted with plastic screws. The probes and screws were secured with dental cement and molded into a head cap. The retracted skin was secured to the head cap with cyanoacrylate glue. Rats were administered buprenorphine (0.1 mg/kg; subcutaneous) post-surgery for analgesic control. Rats were monitored closely during surgical recovery to ensure the head cap was secure and discomfort was minimized. Rats were given two doses of buprenorphine (0.1 mg/kg) per day for up to three days post-surgery and were monitored minimum of twice daily for general health status and any signs of pain or stress.

2.3. Drugs

Soman (CAS 96-64-0) was diluted in isopropyl alcohol (Sigma-Aldrich, Millwaukee, WI, USA) and then sterile saline (0.9% NaCl, Baxter, Canada) to the maximum concentration required for the heaviest rat used on the day of exposure. A dose of 80–90 μg/kg was used in this study based on a pilot study that showed this dose consistently resulted in convulsive seizures. This dose is 0.72–0.82xLD50 based on a published LD50 of 110 μg/kg subcutaneous (Shih et al., 1990).

The oxime, HI-6 dimethanesulfonate (CAS 144252-71-1) was provided by the Defence Research and Development Canada (DRDC) Suffield Research Centre. A dose of 125 mg/kg was prepared in 0.9% sterile saline. The same dose of HI-6 was used pre- and post soman injection.

Atropine Methyl Nitrate (AMN) was purchased from Sigma Aldrich (Milwaukee, WI, USA). A dose of 20 mg/kg of AMN was prepared in 0.9% sterile saline. The same dose of AMN was used pre- and post soman injection.

2.4. Magnetic resonance imaging

Rats were imaged after probe implantation to confirm the relative probe location and check for intracranial bleeding 24 h after surgery. Imaging was done with a 9.4T MRI and a Bruker Avance console (Bruker Biospin GmbH, Rheinstetten, Germany).

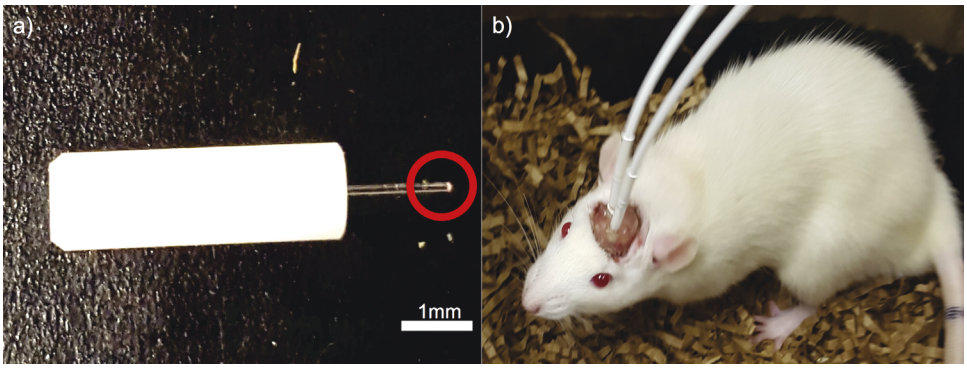

Fig. 1. Implantation of pO_2 probes in the rat cortex and hippocampus. **a**) Example probe. The red circle indicates the location of the fluorophore. **b**) Bilateral implantation of pO_2 probes in the cortex and hippocampus with the measurement leads attached. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Rats were secured in place with ear bars and ventilated with 70% N_2, 30% O_2, and 1.5–2.5% isoflurane. Body temperature (36.5 to 37 °C) and respiration rate (60–80 breaths/min) were monitored during imaging. Imaging was performed with a 20 mm surface coil using a rapid acquisition with relaxation enhancement (RARE) T_2-weighted sequence: TR = 2500 ms, TE = 36 ms, flip angle = 180°, RARE factor = 8, averages = 1, matrix = 384 × 384, 9 slices, and thickness of 0.5 mm. Total acquisition time was 20 min per rat.

2.5. Experimental design

Fiber-optic oxygen probes (Oxford Optronix, Oxford, UK) (Fig. 1a) were used to measure cortical and hippocampal pO_2. The oxygen probes utilize a platinum fluorophore embedded in silicone rubber at the tip of an optical fiber with an approximate diameter of 250 μm.

A short pulse of LED light (525 nm) is sent through a fiber-optic cable, which excites a platinum-based fluorophore at the end of fiber-optic oxygen probes, which emits at 650 nm. The platinum-based fluorophore is quenched by oxygen around the probe tip and the decay time is inversely proportional to oxygenation.

Each rat was implanted with 2 probes, one in the cortex and one in the hippocampus (implantation coordinates were standardized for all rats based on bregma). A structural MRI was obtained after surgery to confirm the location of the pO_2 probes and check for intracranial bleeding. Rats were monitored for 5–7 days prior to initial baseline measurement to allow for healing around the implant.

During pO_2 measurements, fiber-optic cables were attached to the implanted oxygen-sensing probes, which protrude from the implant (Fig. 1b). Animals were allowed to explore the measurement cage for 10 min before collection of pO_2 data at 1 Hz. The rats were awake and freely moving in an empty cage over the course of the measurement period. Fiber-optic cables were supported on a lab stand to minimize tension on the head cap.

Baseline pO_2 measurements were obtained for 15 min on 4 consecutive days (Fig. 2). The first measurement day was ignored in case of a training effect. A 15-min baseline was taken on the day of soman injection. During measurements, background stimuli (movement, noise) were kept to a consistent minimum. To reduce potential variability between rats, each rat was measured within 1 h at the same time of day. In addition, the first 5 min of each baseline measurement was discarded in case there was a handling effect causing stress. In total, we evaluated three baseline measurements of 10 min for each rat, which were averaged to one data point per baseline measurement.

Pretreatments of HI-6 dimethanesulfonate and atropine methyl nitrate (AMN) (125 mg/kg and 20 mg/kg; intraperitoneal) were co-administered 20–30 min before soman exposure to increase survival of rats (Fig. 2). HI-6 is an acetylcholinesterase reactivator (Jokanovic and Stojiljkovic, 2006) and has been shown to reduce mortality without effecting seizure occurrence (McDonough and Shih, 1993). AMN is an acetylcholine muscarinic receptor antagonist and reduces soman-induced peripheral symptoms. HI-6 and AMN do not readily cross the blood-brain barrier and therefore do not interfere with the central effect of soman (Shih et al., 1991). Pretreatment pO_2 values were measured 20 to 30 min after pretreatment injection until soman administration.

For soman injection, rats were temporarily disconnected from the Oxylite and anesthetized with 5% isoflurane for approximately 3–5 min. Anesthetized rats were moved into the fume hood and injected with soman (80–90 μg/kg; subcutaneously). The injection site was immediately scrubbed with Reactive Skin Decontamination Lotion (RSDL). Anesthesia was discontinued directly after

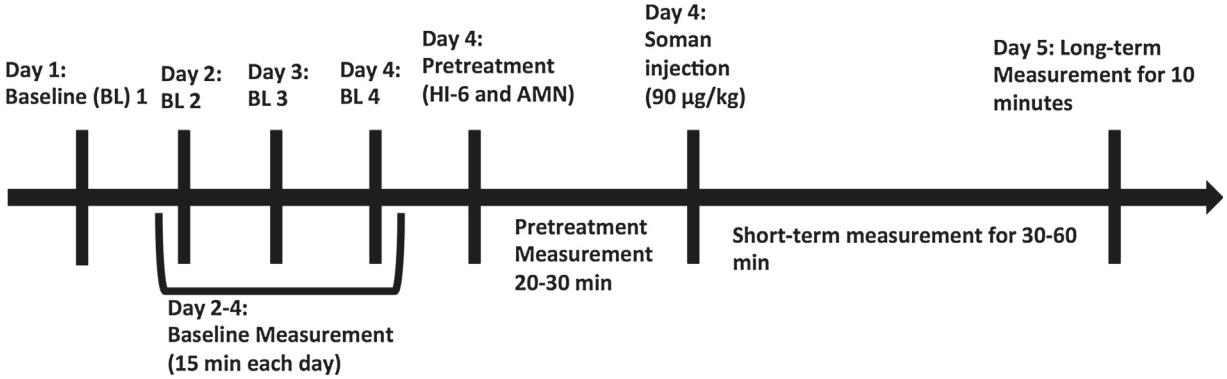

Fig. 2. Time course of the study depicting the treatment timing and measurement duration.

Table 1
Rating system to assess the severity of soman related symptoms. Suffield Rating Scale.

	Symptoms
Rating 1	Normal
Rating 2	One of the following signs: fasciculations, tremors, lacrimation, mouth movement, salivation, vocalizations, abnormal mobility, or abnormal responsiveness
Rating 3	2 or more of the above signs
Rating 4	Any of the above signs and partial paralysis (fore OR hind limbs affected)
Rating 5	Any of the above signs, full body paralysis (unable to right hind or front limbs), severely labored breathing, and/or seizures/convulsions
Rating 6	Unexpected Death

soman injection and rats were reconnected to the Oxylite and pO_2 measurements and video recordings were taken.

Short-term measurements were taken directly after soman injection for 30–60 min (Fig. 2). The first set of rats we measured was for 60 min. In all of these, the peak oxygenation occurred before 30 min. In order to increase our data collection throughput and reduce stress, we subsequently reduced the initial post soman recording to 30 min. Following short-term measurement, rats were transported into an empty cage to be continuously monitored and rated based on physical symptoms overnight (Table 1). To minimize mortality rate, additional treatment of AMN (20 mg/kg; i.p) was given every 20–30 min for up to three doses. Treatments were provided to minimize muscarinic effects of soman exposure.

A video camera was mounted on a stand to monitor behavioral seizure activity in the rats. Recordings began immediately following soman-injection and were stopped when pO_2 measurements were complete. The video recording was later analyzed to confirm the onset of soman-induced convulsive seizures in relation to pO_2 measurements. Observations of convulsions have been used to confirm the occurrence of seizures (Racine, 1972).

A final long-term measurement was taken for 15 min, 10–24 h after soman injection and rats were sacrificed immediately after (Fig. 2). To preserve brain tissue, the rats were perfused and fixed.

2.6. Histology

Histology was performed to determine exact probe location as MR images only provide a relative position. Rats were euthanized with Euthanyl (200 mg/kg i.p) for transcardial perfusion with 100 ml of 1X PBS, then 100 ml of 4% paraformaldehyde (4% PFA). Brains were carefully excised and fixed overnight in 4% PFA for up to 48 h. 4% PFA solution was replaced with 30% sucrose solution and remained in sucrose solution until sectioning. Tissue was embedded in optimal cutting temperature (OCT) compound and sectioned at 20 μm. Sections were stained with hematoxylin and eosin (H&E) to assess probe location.

2.7. Data analysis

Video recording of the pO_2 measurement after soman injection was reviewed for a rating 5 on the symptom severity (Table 1) to estimate the time point of onset of soman-induced convulsive seizures.

The total recording time was 140 min. Baseline pO_2 was measured on 3 consecutive days including the day of injection (30 min). Immediately following the last baseline measurement, pretreatment was injected and pO_2 was measured for a total of 20–30 min, but only the last 10 min were averaged. After soman

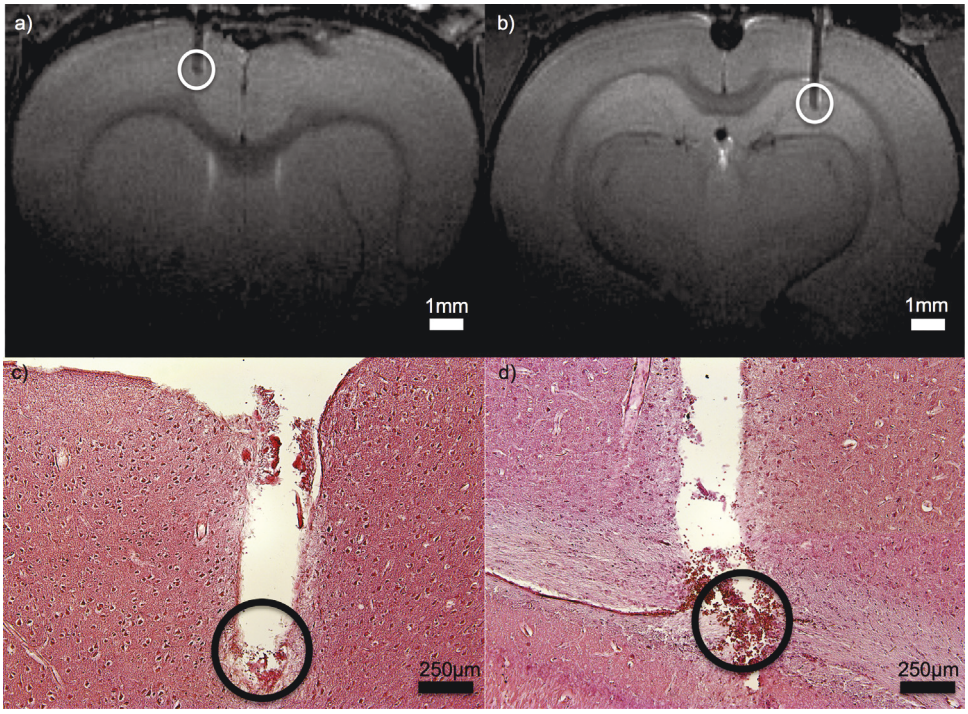

Fig. 3. Images showing the locations of the pO_2 probe tips in the brain. Example of a RARE T2-weighted image showing the relative location of the tips (white circles) in the **a**) cortex and **b**) hippocampus. H&E stained sections showing the exact location of the probe tip (black circles) in the **c**) cortex and **d**) hippocampus.

injection, pO_2 was recorded for 30–60 min and at peak, a total of 1-min was averaged for the short-term. The rat was disconnected from the Oxylite and placed in an empty cage until a final measurement at 10 to 24 h (long-term) for 10 min.

Heterogeneity in baseline oxygenation was expected between rats. Mean baseline and standard deviation was calculated using approximately 30-min of baseline pO_2 recordings on 3 consecutive days prior to pretreatment and soman injection. Any value above or below two standard deviations from the average baseline was defined as being significantly different from baseline.

A comparison between different time points was performed by averaging the pO_2 at each time point. All statistical analysis was performed using IBM SPSS Statistics. A one-way repeated measure analysis of variance (ANOVA) was used to compare the baseline, pretreatment, short-term, and long-term, with a Tukey post-hoc test to test for between group differences. If the F-test for normal variance was not significant, a non-parametric test (Friedman) was used. For the cortex pO_2, a Friedman test followed by a Wilcoxon rank sum test was used. The hippocampal pO_2 was further evaluated by a Tukey test. p-values $< .05$ were considered statistically significant.

The baseline pO_2 between the cortex and hippocampus was compared for each rat. In addition, the timing to peak oxygenation was compared between the cortex and hippocampus. A paired *t*-test was performed where a p-value $< .05$ was considered statistically significant.

3. Results

3.1. Behavioral effects of Soman

Soman induced convulsive seizures were assessed based on behaviour. Within 5 min after soman exposure, rats exhibited a symptom rating of 2 (Table 1; facial movement and salivation). Ten minutes following exposure, all rats exhibited signs rating of 5 (Table 1; convulsive seizures). After the convulsive seizures had terminated, rats exhibited a symptom rating of 4. Most had splayed hind limbs throughout the short-term measurement (within 30–60 min post-soman injection). Even at the time of euthanasia (after 10–24 h post-soman injection), most had rhythmic head movement. Three unexpected deaths occurred following soman exposure, one before the 1 h and two before the 24 h marks, most likely from respiratory difficulty due to reduced respiration and excessive secretions. However, all available data from these rats were included in the pO_2 analysis.

3.2. Imaging probe location

MRI showed that all probes were located in the region of interest (cortex and hippocampus). Representative sections of the cortex and hippocampus are shown in Fig. 3a and b, respectively. Histology confirms that the probes were located in the cortex and hippocampus (Fig. 3c and d).

3.3. Cortex pO_2 measurements

During baseline measurements in the cortex (n = 10), the baseline pO_2 remained relatively stable over time for each rat (Fig. 4a–j). Variation in the mean baseline pO_2 between rats ranged from 25.9 ± 4.2 mmHg to 45.5 ± 6.1 mmHg (mean ± SD) (Table 2) as expected (Johnson et al., 2016; Ortiz-Prado et al., 2010; Schilte et al., 2015). Mean cortical pO_2 from 10 rats was 32.4 ± 6.3 mmHg.

Cortical pO_2 during the 10 min of pretreatment is shown in Fig. 4a–j (yellow). During most of the pretreatment, the cortical pO_2 was within two standard deviations from the baseline in all rats (Fig. 4a–j).

Shortly after soman injection, there was a large increase in cortical pO_2 to above two standard deviations from the mean baseline in every rat. The largest increase was 43.8 mmHg (Fig. 4c, Table 2). Upon seizure onset (based on observed convulsions), pO_2 began to decline (arrows, Fig. 4a–j). 40–60 min post soman injection; pO_2 generally remained elevated over baseline (data not shown). Cortical pO_2 in two rats returned to near baseline values, within two standard deviations of the mean baseline (Fig. 4e and g) within 40 min. Two rats died unexpectedly and values are shown in Fig. 4d and f. Cyclical large amplitude changes in cortical pO_2 could be observed in every rat.

Long-term cortical pO_2 measurements (10–24 h post-soman injection) were variable between rats (n = 6/10). Due to the severity of symptoms, two rats were measured at 10 h and immediately

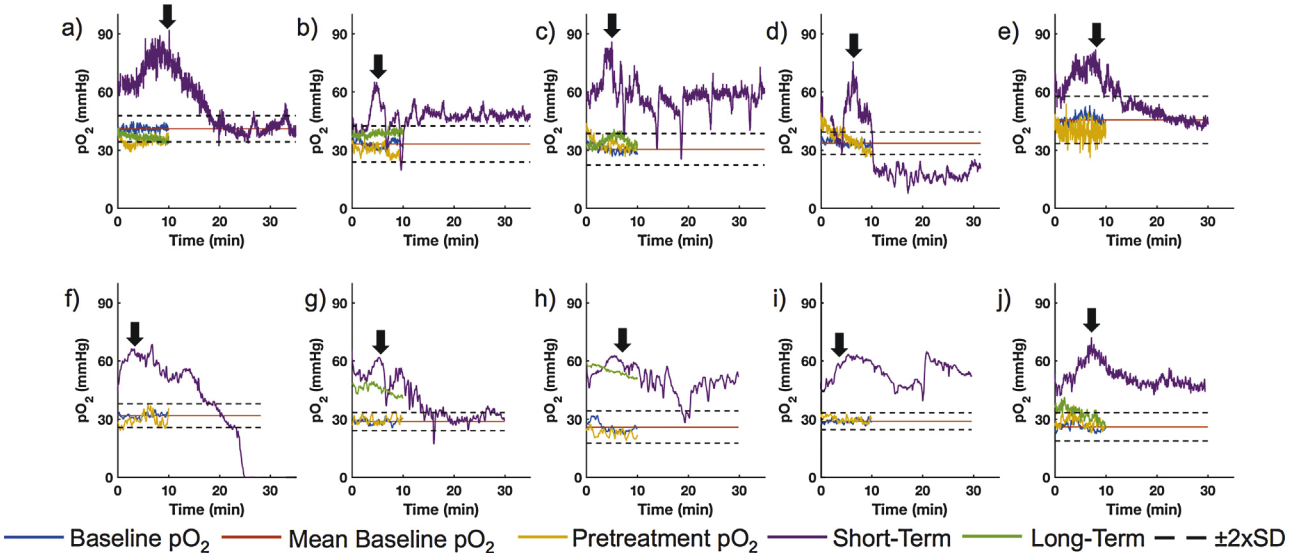

Fig. 4. The effect of soman injection on cortical pO_2. Plots of pO_2 data collected at 1 Hz from the cortex for 10 rats (**a**–**j**). Rats were awake and freely moving during recording. The dotted black lines indicate ±2xSD of the mean baseline. Time 0 for short-term starts within 1 min of soman injection. The other plots are aligned to 0 for ease of comparison. Arrows indicate the onset of soman-induced convulsive seizures. Short-term (within 40 min). Long-term (10 min of measurement between 10 and 12 h for **a**,**b** and 22–24 h post injection for **c**–**j**).

Table 2
Baseline and peak pO_2 in the cortex and hippocampus after soman injection. Each line represents a rat (n = 10). (Mean ± SD, mmHg).

Cortex			Hippocampus			Δ in Baselines
Baseline	Short Term Peak	Peak - Baseline	Baseline	Short Term Peak	Peak - Baseline	Cortex-hippocampus
40.9 ± 3.4	77.5 ± 4.2	37	20.2 ± 6.2	58.5 ± 4.0	38	21
33.1 ± 4.7	60.2 ± 2.7	27	17.9 ± 3.4	67.0 ± 3.2	49	15
30.4 ± 4.0	74.2 ± 7.7	44	13.6 ± 2.3	47.6 ± 1.6	34	17
33.3 ± 2.9	63.5 ± 4.8	30	15.9 ± 3.1	50.4 ± 6.8	35	18
45.5 ± 6.1	74.8 ± 3.4	29	34.1 ± 3.1	58.8 ± 5.0	25	11
31.7 ± 3.0	63.9 ± 3.8	32	22.6 ± 3.5	53.1 ± 5.1	31	9
28.7 ± 2.3	60.7 ± 0.9	32	21.1 ± 2.3	46.9 ± 1.2	26	8
25.8 ± 4.2	62.2 ± 0.4	36	16.9 ± 5.0	47.8 ± 0.3	31	9
28.8 ± 2.2	62.4 ± 0.7	34	25.4 ± 2.7	54.7 ± 0.6	29	3
26.0 ± 3.6	65.8 ± 2.4	40	24.6 ± 2.3	54.5 ± 0.8	30	1

Two animals were excluded from the hippocampus from cortical probes not working. Short-term peak data are calculated from the peak pO_2 pre-seizure ± 30 s. Baseline is calculated from three consecutive days including on the day of soman injection for a total of 30 min.

euthanized. Cortical pO_2 10 h post soman injection (n = 2/10) did not show any significant changes from the mean baseline (Fig. 4a and b). At 24 h (n = 4/10), two of the four rats had significantly elevated pO_2 with a decreasing trend (Fig. 4g and h), while the other two rats did not show a significant difference between baseline and 10 h post-soman injection pO_2 values (Fig. 4c and j).

Fig. 6a shows the change in the cortical pO_2 for each time point. When compared to the baseline (n = 10), the short-term (n = 10) exhibited higher pO_2 in the cortex (p = .005). No significance was found between the baseline and pretreatment (p = .285). Furthermore, the long-term (n = 6) showed an increase in cortical pO_2 values 10–24 h post-soman injection compared to the baseline, but this was not statistically significant (p = .075).

Although there are two outliers in the baseline group (Fig. 6a), pO_2 values for every rat followed the same general trend (Fig. 6b): Cortical pO_2 remained relatively stable between baseline and pretreatment, followed by a large increase in the short-term assessment. Long-term assessment had more variability as some rats had elevated pO_2, while others returned to or near baseline values.

3.4. Hippocampal pO_2 measurements

Within each rat, the baseline pO_2 remains relatively stable over time in the hippocampus (n = 12) (Fig. 5a–l). As with the cortex, variation between rats in baseline pO_2 ranged from 13.6 ± 2.3 mmHg to 34.1 ± 3.1 mmHg (mean ± SD) (Table 2). The combined average pO_2 in 12 rats was 21.1 ± 5.5 mmHg.

Measurements of hippocampal pO_2 had small variation within each rat (n = 12) and remained relatively stable (Fig. 5a–l). Most rats had pO_2 values that remained close to the baseline and within two standard deviations of the mean baseline. However, two had elevated pretreatment pO_2 values and at times above two standard deviations of the average baseline (Fig. 5d and l). The majority of pO_2, measured after pretreatment, did not show significant change compared to the mean baseline, as the pretreatment pO_2 remains within two standard deviations of the mean baseline. Between rats, there is some variability in the pretreatment pO_2 values, ranging from 16.0 ± 4.4 mmHg to 30.3 ± 2.6 mmHg (Table 2).

Immediately following soman injection, there is an elevation in the hippocampal pO_2 above two standard deviations from the mean baseline value in most rats (Fig. 5a–l). The largest increase in pO_2 was seen in Fig. 5b, and with a change of 49 mmHg (Table 2). After the onset of convulsive seizures, there was a delayed decrease in pO_2 in some rats (n = 4/12). However, the majority of rats' pO_2 continues to increase after seizure onset followed by a dip in pO_2 (n = 8/12). During the short-term (30–60 min post soman injection), we observed large variation in hippocampal pO_2 measurement (Fig. 5a–l). Shortly following the decrease, there was an increase in every rat above two standard deviations until the end of

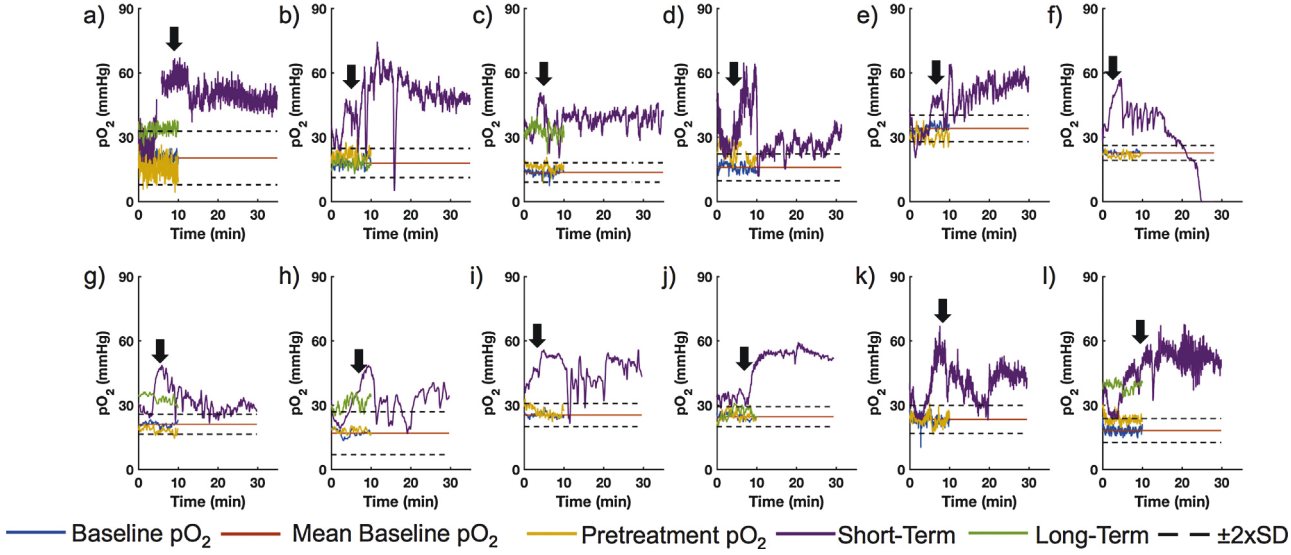

Fig. 5. The effect of soman injection on hippocampal pO_2. Plots of pO_2 data collected at 1 Hz from the hippocampus for 12 rats **(a–l)**. Rats were awake and freely moving during recording. The dotted black lines indicate ±2xSD of the mean baseline. Time 0 for short-term starts within 1 min of soman injection. The other plots are aligned to 0 for ease of comparison. Arrows indicate the onset of soman-induced convulsive seizures. Short-term (within 40 min). Long-term (10 min of measurement between 10 and 12 h for a,b and 22–24 h post injection for **c–l**).

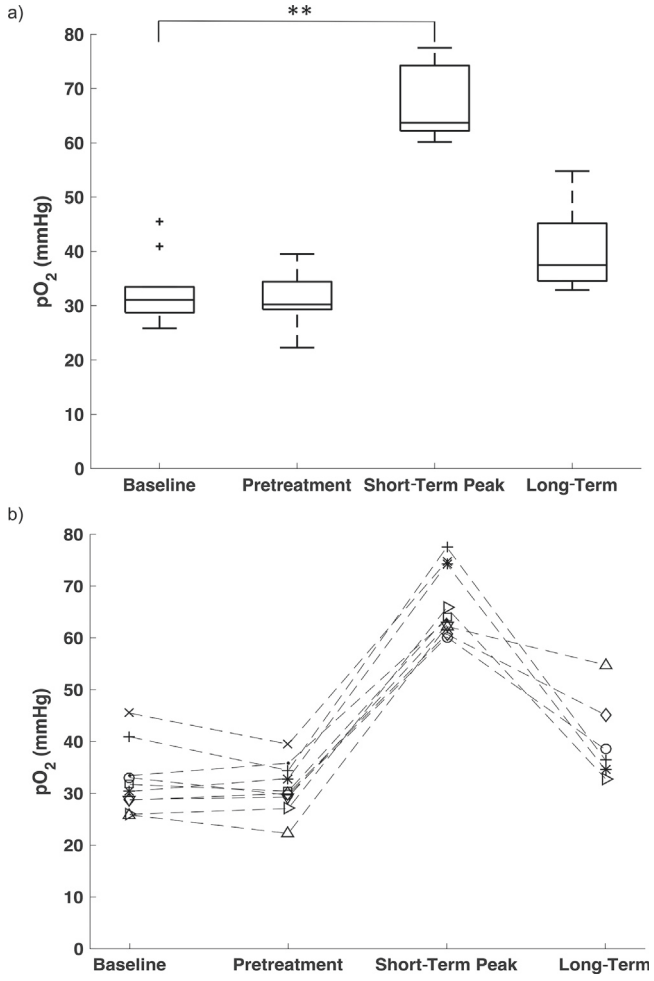

Fig. 6. Combined cortical pO_2 data **a**) grouped data showing the baseline, pretreatment, short-term peak and long-term data. Baseline (n = 10), pretreatment (n = 10) and long-term (n = 6) values were plotted using a 10 min mean. Short-term peak (n = 10) was the mean including 30 s before and after the peak pO_2 measurement that occurs near the onset of seizure. The black bars show the maximum and minimum. Middle black line indicates the median. Plus signs are outliers. $^{**}p \leq .01$ (Friedman test with Wilcoxon signed-rank test) **b**) Cortical oxygenation trend after each treatment. Each individual shape and line indicates one rat.

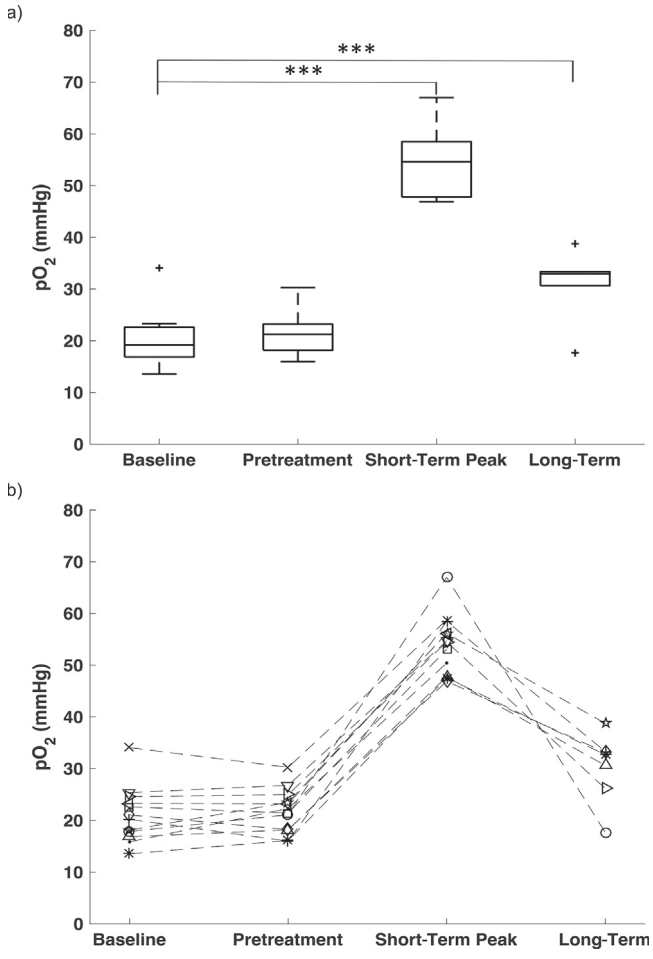

Fig. 7. Combined hippocampal pO_2 data **a**) grouped data showing the baseline, pretreatment, short-term peak and long-term data. Baseline (n = 12), pretreatment (n = 12) and long-term (n = 7) values were calculated from a mean of 10 min. Short-term peak (n = 12) was the mean including 30 s before and after the peak pO_2 measurement that occurs near the onset of seizure. The black bars show the maximum and minimum. Middle black lines indicate the median. Plus signs are outliers. $^{***}p \leq .001$ (ANOVA with Tukey-b post-hoc) **b**) Hippocampal oxygenation trend after each treatment. Each individual shape and line indicates one rat.

measurement except for one rat that died during the experiment (Fig. 5f). In every rat, we observed cyclical large amplitude changes in pO_2 within the hippocampus.

Hippocampal pO_2 measurement showed variation between rats during the long-term assessment. At 10 h (n = 2/12) after soman injection, one rat had significantly elevated hippocampal pO_2 above two standard deviations of the mean baseline (Fig. 5a), while another had long-term pO_2 near the baseline (Fig. 5b). At 24 h (n = 5/12), four had elevated long-term hippocampal pO_2 above +2xSD (Fig. 5c, g, h, and l) and one had pO_2 near the baseline level (Fig. 5j).

Hippocampal oxygenation shows a similar trend as the cortex. No significant difference was found between baseline and pretreatment pO_2 values (p = .985). There was a large increase in the short-term measurement (Fig. 7a). When baseline was compared to peak oxygenation during short-term assessment, short-term measurements were significantly elevated ($p < .001$). The long-term assessment (10–24 h) had significantly elevated hippocampal pO_2 compared to the baseline (p = .005).

Despite outliers in the baseline and long-term (n = 3), similar trends could be observed in every rat (Fig. 7b): Each rat had a similar baseline and pretreatment pO_2, followed by an increase during the short-term measurement. A decreasing trend can be observed in the long-term measurements, which were still elevated compared to the baseline.

3.5. Comparison between cortex and hippocampus

The time to peak pO_2 was compared between the cortex and the hippocampus. During baseline measurements, the cortex had a significantly higher pO_2 than the hippocampus ($p < .001$) (Table 2). The largest difference in pO_2 between the cortex and hippocampus baseline was 21 mmHg (Table 2). The peak pO_2 in the cortex occurred at a similar time to that of seizure onset (based on using behaviour as a marker for seizure onset). The peak pO_2 in the hippocampus was significantly later (n = 8/10) with the timings being 5.8 ± 1.5 min and 8.2 ± 3 min (mean ± SD, $p < .01$) for the cortex and hippocampus, respectively.

4. Discussion

Changes in cortical and hippocampal oxygenation after a convulsive dose of soman were measured using chronically implanted fiber optic oxygen probes. We used a novel method

to measure oxygenation *in vivo* without the need for anesthesia and restraints (Ortiz-Prado et al., 2010). Using this method, we demonstrated that 1) pO_2 increases in the cortex and the hippocampus shortly after injection of a convulsive dose of soman, until a maximum value was recorded prior to seizure onset; 2) after seizure onset, pO_2 declined but usually remained elevated above baseline for at least 60 min post soman exposure; 3) low frequency cycles of high amplitude changes in both cortical and hippocampal pO_2 were observed; and 4) pO_2 remained elevated after 10 to 24 h (long term) in the hippocampus but returned to baseline in the cortex.

4.1. Baseline pO_2 measurements

The baseline pO_2 measurements were variable as exemplified by the fact that the cortical pO_2 between rats ranged from 26 to 46 mmHg (Table 2). This was the first time that both hippocampal and cortical pO_2 were measured simultaneously in the same rat. Interestingly, the cortex pO_2 (32.4 ± 6.3 mmHg) was higher than the hippocampus (21.23 ± 6 mmHg). The values were within ranges reported previously for the cortex (30.2 ± 3.3 mmHg) (Ortiz-Prado et al., 2010) and hippocampus (18–30 mmHg) (Farrell et al., 2016). We expect some level of variability between rats as pO_2 values can change depending on conditions including stress level (Paisansathan et al., 2007), temperature (Gupta et al., 2002), and neuronal activity (Lindauer et al., 2003). Variation between subjects has been seen in other studies that measured brain pO_2 (Johnson et al., 2016; Ortiz-Prado et al., 2010; Schilte et al., 2015).

4.2. From soman exposure to seizure onset

Our results show that soman injection causes hyperoxygenation in the cortex and the hippocampus (Table 2). Soman inhibits AChE, which will cause an accumulation of ACh in the synapse, leading to sustained firing of cholinergic neurons and induce a state of hyperexcitability. Hyperexcited neurons have an increased $CMRO_2$ (Sheth et al., 2004). One would expect that if there is an increase in $CMRO_2$ there would also be an increase in CBF (Buxton et al., 1998) and pO_2 (Leniger-Follert, 1985; Offenhauser et al., 2005). Thus, this initial increase in pO_2 may reflect an increase in $CMRO_2$ caused by increased neuronal excitability.

There is an additional increase in pO_2 in the cortex (and often in the hippocampus), associated with the onset of soman related symptoms. This increase in pO_2 would be expected, as AChE becomes inhibited and more neurons are stimulated to a hyperexcitable state. As cholinergic neurons are stimulated, excitatory projection neurons such as glutamatergic neurons are recruited, $CMRO_2$ and CBF increase even more and pO_2 rises.

Although it is hypothetical, it may be interesting to keep in mind that increased metabolism can increase oxidative stress. This could cause membrane and mitochondrial damage (Baille et al., 2005). Oxidative stress has also been implicated in stimulating vasodilation through the effect of reactive oxygen and nitrogen species (Sobey et al., 1997). The vasodilatory effects will lead to an increase in CBF and pO_2, which may attribute to the secondary increase that is observed.

4.3. Onset of seizures may reduce oxygenation

The onset of soman-induced convulsive seizures correlates with a decrease in oxygenation in the cortex (although the pO_2 was still higher than "pre-soman"). We reason above that a hyperexcitable state would result in an elevated pO_2. However, seizures arise when neurons are in both a state of hyperexcitability and hypersynchrony (Fisher et al., 2005). This condition is highly metabolically demanding (Duffy et al., 1975). We know that there is an increase in CBF (Goldman et al., 1993; Shih and Scremin, 1992) and metabolism (McDonough et al., 1983; Shih and Scremin, 1992) after soman-induced convulsive seizures, but if the demand is too high for supply then we would expect a decline in pO_2. Such a decline with seizure has been termed the "epileptic dip" (Bahar et al., 2006; Suh et al., 2005). Similar decreases in cortical pO_2 at or near onset of seizures were reported in epilepsy patients, where an increase in cerebral oxygenation has preceded seizures (Moseley et al., 2012; Seyal, 2014; Slone et al., 2012; Zhao et al., 2007).

There may be more factors involved. There is evidence for abnormal vascular regulation in seizures (Farrell et al., 2016; Leal-Campanario et al., 2017; Zhao et al., 2011). Near the onset of seizures, vasoconstriction (Leal-Campanario et al., 2017; Zhao et al., 2011) would reduce CBF and lead to a decrease in pO_2 if $CMRO_2$ were to remain high.

Vasoconstriction can be long lasting. In a study using both electrically-induced and convulsant-induced seizures, both hypoxia and hypoperfusion were recorded following the termination of seizures (Farrell et al., 2016). In our study, we did not observe hypoxia following seizures. Except for the rats that unexpectedly died during pO_2 recording, pO_2 in the cortex and hippocampus never dropped below the severe hypoxic threshold as was reported in the previous study (where pO_2 regularly declined to <10 mmHg) (Farrell et al., 2016). This is likely due to a difference in the method of inducing seizures. Brief electrical stimulation in a non-epileptic animal results in a relatively short duration seizure and a rapid return to the non-epileptic state. Whereas following soman administration, the compound will continue to keep the brain in a hyperexcitable state and cause brain damage. We expect similar results to the chemically-induced seizure experiment once soman has been hydrolyzed and rats exhibit spontaneous recurrent seizures (de Araujo Furtado et al., 2010).

The decrease in hippocampal pO_2 after seizure onset was not as evident compared to the cortex. The secondary increase in the hippocampus did not reflect the time point when early soman related symptoms appeared. The temporal delay between the cortex and hippocampus pO_2 increase may be from a difference in neuronal innervation affecting vascular response. Areas surrounding a seizure focus showed a difference in vascular regulation (vasoconstriction and vasodilation) and metabolism (Zhao et al., 2011) suggests pO_2 changes depending on the onset site. The hippocampus receives projections from the medial septum and the entorhinal cortex. The medial septum is a speculated site for the generation of soman-induced convulsive seizures. The entorhinal cortex relays seizures that have been evoked from the area tempestas (Halonen et al., 1994), another speculated soman-induced seizure onset site.

4.4. Oscillating changes in oxygenation in the first hour

Oscillating changes in oxygenation after soman-induced convulsive seizures are similar to a *status epilepticus* model (Kreisman et al., 1983). In the cortex, following the initial gradual decline in pO_2 following the onset of seizures, large amplitude, low frequency cycles began to appear, indicating a non-steady state. The oscillating changes in pO_2 may reflect ongoing changes in vascular regulation during *status epilepticus*. When seizures stop momentarily, vasodilation allows reperfusion of the tissue and increases pO_2. At the onset of another seizure, vasoconstriction restricts blood flow.

In the hippocampus, the oscillating changes appear before the onset of seizures and become more distinct following onset. Cycling between vasoconstriction and vasodilation may be similar to the cortex. The difference in the oscillating pO_2 may be due to a regional difference in neuronal activity, changing the neurovascular response between the cortex and hippocampus (Shih and

Scremin, 1992). There was less increase in CBF relative to the increase in metabolism in the hippocampus compared to the cortex after soman-induced convulsive seizures (Shih and Scremin, 1992). The difference in the proportional uncoupling between the cortex and hippocampus may explain why there is no direct relationship in the oscillating changes in pO_2.

4.5. Potential link between oxygenation and pathology

Measurements at 10–24 h following soman exposure showed significantly elevated pO_2 in the hippocampus but not in the cortex. Elevated pO_2 indicates there may be changes in oxygen utilization likely due to neurodegeneration following soman-induced seizures. In rats that have been exposed to higher doses of soman (105–154 μg/kg; sc), neurodegeneration was histologically detected using Fluoro-Jade B (Apland et al., 2010; RamaRao et al., 2014) and H&E (Shih et al., 2003; Tryphonas and Clement, 1995) in the hippocampus. Although most studies use a higher dose than we have used, rats exposed to a similar dose (80 μg/kg; sc) showed neurodegeneration as early as 45 min after convulsive seizures in the hippocampus, amygdala, and piriform cortex (Myhrer et al., 2005). Studies have shown seizures lasting more than 40 min are an important factor in the development of neurodegeneration (Baille et al., 2005; Guo et al., 2015; Lallement et al., 1993; Myhrer et al., 2005). Histological damage in the hippocampus has also been observed in mice (172 μg/kg; sc) (Baille et al., 2005) and guinea pigs (26.6 μg/kg; sc) (Gullapalli et al., 2010) following soman-induced convulsive seizures. The sustained elevation of pO_2 over the 24 h post exposure may reflect a condition where oxygen utilization has been greatly reduced due to cell death in the hippocampus. In the cortex, the return to control pO_2 suggests that cell damage may not be as severe. This is consistent with previous reports arguing that the neocortex is less sensitive than the hippocampus to soman-related damage (Lemercier et al., 1983; Rossetti et al., 2012).

4.6. Future directions

In the future, it will be important to link the increase in pO_2 to soman-related cell death. This linkage would support the use of oxygen measurements during the acute phase post exposure as a biomarker of future neuropathological outcome. It would also be useful to study the mechanism behind the changes in oxygenation by correlating the changes with EEG and CBF. This will allow us to correlate neuronal activity and cerebral vascular regulation with pO_2 data. Furthermore, pO_2 measurements may be useful in treatment studies to provide a sensitive biomarker of whether metabolism and blood flow have been protected. The similarity of changes in pO_2 with that observed in animal models during *status epilepticus* supports further work studying anti-seizure treatment.

5. Conclusion

Measurements of brain oxygenation provide new information on changes in the physiological status of the brain over time following soman exposure. The temporal pO_2 profile support evidence that soman-induced seizures result in irregular vascular reactivity similar to that in other seizure models. Measurements of brain oxygenation could provide a sensitive marker of exposure and could be used as a biomarker for treatment studies.

Acknowledgements

This research was supported by the Department of National Defence, Canada.

We thank our colleagues from the Defence Research and Development Canada- Suffield Research Centre for providing the materials necessary for the research.

We thank Brain Canada Platform for a platform grant.

We thank Dr. Lia M. Hocke for assistance in creating the figures and comments that greatly improved the manuscript.

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Assessment of brain oxygenation imbalance following soman exposure in rats

4. AUTHORS (last name, followed by initials – ranks, titles, etc., not to be used)

Lee, K.; Bohnert, S.; Wu, Y.; Vair, C.; Mikler, J.; Campbell Teskey, G.; Dunn, J.F.

5. DATE OF PUBLICATION (Month and year of publication of document.)	6a. NO. OF PAGES (Total containing information, including Annexes, Appendices, etc.)	6b. NO. OF REFS (Total cited in document.)
February 2018	10	69

7. DESCRIPTIVE NOTES (The category of the document, e.g., technical report, technical note or memorandum. If appropriate, enter the type of report, e.g., interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

External Literature (P)

8. SPONSORING ACTIVITY (The name of the department project office or laboratory sponsoring the research and development – include address.)

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9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)	9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

10a. ORIGINATOR'S DOCUMENT NUMBER (The official document number by which the document is identified by the originating activity. This number must be unique to this document.)	10b. OTHER DOCUMENT NO(s). (Any other numbers which may be assigned this document either by the originator or by the sponsor.)
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Nerve agents (NAs) are potent organophosphorus (OP) compounds with applications in chemical warfare. OP compounds act by inhibiting acetylcholinesterase (AChE). Soman (O-pinacolyl methyl- phosphonofluoridate) is one of the most potent NAs. It is well known that small doses of NAs can be lethal, and that even non-lethal exposure leads to long-term mental debilitation/neurological damage. However, the neuropathology following exposure to sub-lethal nerve agents is not well understood. In this study, we examined changes in tissue oxygenation (pO^2) in the cortex and hippocampus after a sub-lethal dose of soman [80–90 mg/kg; subcutaneous]. pO^2 changes can provide information regarding oxygen delivery and utilization and may be indicative of a disruption in cerebral blood flow and/or metabolism. Changes in oxygenation were measured with chronically implanted oxygen sensors in awake and freely moving rats. Measurements were taken before, during, and after soman-induced convulsive seizures. Soman exposure resulted in an immediate increase in pO^2 in the cortex, followed by an even greater increase that precedes the onset of soman-induced convulsive seizures. The rise in hippocampus pO^2 was delayed relative to the cortex, although the general pattern of brain oxygenation between these two regions was similar. After convulsive seizures began, pO^2 levels declined but usually remained hyperoxygenated. Following the decline in pO^2, low frequency cycles of large amplitude changes were observed in both the cortex and hippocampus. This pattern is consistent with recurring seizures. Measuring real-time changes in brain pO^2 provides new information on the physiological status of the brain following soman exposure. These results highlight that the measurement of brain oxygenation could provide a sensitive marker of nerve agent exposure and serve as a biomarker for treatment studies.

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Soman, Nerve Agents, Seizures, Oxygenation, Metabolism, Cerebral Blood Flow, Organophosphates, Organophosphours Compounds