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The recovery of acetylcholinesterase activity and the progression of neuropathological and pathophysiological alterations in the rat basolateral amygdala after soman-induced status epilepticus: relation to anxiety-like behavior

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Abstract

Organophosphorus nerve agents are powerful neurotoxins that irreversibly inhibit acetylcholinesterase (AChE) activity. One of the consequences of AChE inhibition is the generation of seizures and status epilepticus (SE), which cause brain damage, resulting in long-term neurological and behavioral deficits. Increased anxiety is the most common behavioral abnormality after nerve agent exposure. This is not surprising considering that the amygdala, and the basolateral nucleus of the amygdala (BLA) in particular, plays a central role in anxiety, and this structure suffers severe damage by nerve agent-induced seizures. In the present study, we exposed male rats to lethal doses of the nerve agent soman, and determined the time course of recovery of AChE activity, along with the progression of neuropathological and pathophysiological alterations in the BLA, during a 30-day period after exposure. Measurements

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were taken at 24 hours, 7 days, 14 days, and 30 days after exposure, and at 14 and 30 days, anxiety-like behavior was also evaluated. We found that more than 90% of AChE is inhibited at the onset of SE, and AChE inhibition remains at this level 24 hours later, in the BLA, as well as in the hippocampus, piriform cortex, and prelimbic cortex, which we analyzed for comparison. AChE activity recovered by day 7 in the BLA and day 14 in the other three regions. Significant neuronal loss and neurodegeneration were present in the BLA at 24 hours and throughout the 30-day period. There was no significant loss of GABAergic interneurons in the BLA at 24 hours post-exposure. However, by day 7, the number of GABAergic interneurons in the BLA was reduced, and at 14 and 30 days after soman, the ratio of GABAergic interneurons to the total number of neurons was lower compared to controls. Anxiety-like behavior in the open-field and the acoustic startle response tests was increased at 14 and 30 days post-exposure. Accompanying pathophysiological alterations in the BLA – studied in *in vitro* brain slices – included a reduction in the amplitude of field potentials evoked by stimulation of the external capsule, along with prolongation of their time course and an increase in the paired-pulse ratio. Long-term potentiation was impaired at 24 hours, 7 days, and 14 days post-exposure. The loss of GABAergic interneurons in the BLA and the decreased interneuron to total number of neurons ratio may be the primary cause of the development of anxiety after nerve agent exposure.

Keywords

Soman; Status Epilepticus; Basolateral Amygdala; Acetylcholinesterase; Anxiety; Long-Term Potentiation

1. Introduction

Nerve agents are organophosphorus compounds that exert their toxic effects by rapidly and irreversibly inhibiting acetylcholinesterase (AChE) activity (Bajgar, 1997; Bajgar et al., 2008; Hajek et al., 2004; Shih et al., 2005; Sirin et al., 2012). In the brain, the resulting increase of acetylcholine and overstimulation of cholinergic receptors raises the excitation level to the point of seizure generation, and gives way to excessive activation of the glutamatergic system, which sustains and intensifies seizure activity (status epilepticus, SE), causing profound brain damage (McDonough and Shih, 1997; Petras, 1994). If death is prevented, long-term behavioral impairments may ensue due to brain pathologies (Coubard et al., 2008; Filliat et al., 2007).

Five years after the attacks with the nerve agent sarin in Matsumoto and Tokyo, individuals exposed to sarin reported persistent increases in symptoms that characterize anxiety disorders, including irritability and restlessness, avoidance of places that triggered recollection of the trauma, tension, and insomnia (Ohtani et al., 2004; Yanagisawa et al., 2006). Electrographic abnormalities indicative of epileptic activity were also present in exposed individuals (Murata et al., 1997; Nishiwaki et al., 2001; Yanagisawa et al., 2006). In animal models, exposure to nerve agents also results in long-term increases in anxiety and fear-like behaviors (Coubard et al., 2008; Filliat et al., 2007; Langston et al., 2012; Mamczarz et al., 2010; Moffett et al., 2011), as well as in the appearance of spontaneous recurrent seizures (de Araujo Furtado et al., 2010). The progression of pathological and pathophysiological alterations leading to these persistent behavioral and neurological

abnormalities has not yet been elucidated. A better understanding of these alterations and the time course of their occurrence may allow for the development of treatment interventions that will prevent or minimize long-term neurological and behavioral deficits.

The amygdala is well recognized for its central role in emotional behavior (Phelps and LeDoux, 2005), and the basolateral nucleus of the amygdala (BLA), in particular, is closely associated with the generation and expression of anxiety and fear (Davis et al., 1994; Etkin and Wager, 2007; LeDoux, 2003); a common feature of anxiety disorders is hyperexcitability in the BLA (Rauch et al., 2006; Villarreal and King, 2001). In addition, evidence points to the BLA as a key brain region for seizure initiation and propagation after nerve agent poisoning (McDonough et al., 1987), and we recently found that after exposure to the nerve agent soman, SE is induced only when AChE activity is sufficiently inhibited in the BLA (Prager et al., 2013). The BLA is also one of the most severely damaged regions after nerve agent-induced SE (Apland et al., 2010; Aroniadou-Anderjaska et al., 2009; Baille et al., 2005; Carpentier et al., 2000; Figueiredo et al., 2011b; Shih et al., 2003).

Since inhibition of AChE is the primary mechanism of nerve agent poisoning, and sustained cholinergic dysregulation of the BLA may alter its excitability contributing to behavioral abnormalities, in the present study we examined the time course of recovery of AChE activity in the BLA; for comparison with other brain regions that may play an important role in seizure generation and propagation after nerve agent exposure (Myhrer, 2007), we also measured AChE activity in the piriform cortex, hippocampus, and prelimbic cortex. In addition, we investigated the progression of neuronal loss and degeneration in the BLA, during a 30-day period after soman exposure. To gain insight into the impact of the neuropathology on emotional behavior, we tested for the presence of increased anxiety at 14 and 30 days after soman-induced SE, and correlated the behavioral observations with synaptic alterations in the BLA, at the same time points.

2. Materials and Methods

2.1 Animal model

Experiments were performed using 6-week old (150-200 g) male, Sprague-Dawley rats (Taconic Farms, Derwood, MD). Animals were individually housed in an environmentally controlled room (20-23°C, ~44% humidity, 12-h light/12-h dark cycle [350-400] lux, lights on at 6:00 am), with food (Harlan Teklad Global Diet 2018, 18% protein rodent diet; Harlan Laboratories; Indianapolis, IN) and water available *ad libitum*. Cages were cleaned weekly and animal handling was minimized to reduce animal stress (Prager et al., 2011). All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council), and were approved by the U.S. Army Medical Research Institute of Chemical Defense and the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committees.

2.2 Soman administration and drug treatment

Soman (pinacoyl methylphosphonofluoridate; obtained from Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, USA) was diluted in cold saline and

administered via a single subcutaneous injection (154 µg/kg), which, based on previous studies, is an approximate dose of $1.4 \times \text{LD}_{50}$ (Figueiredo et al., 2011a; Jimmerson et al., 1989). Following exposure to soman, rats were monitored for signs of seizure onset, and continuously rated for seizure severity according to the modified Racine Scale: Stage 0, no behavioral response; Stage 1, behavioral arrest, orofacial movements, chewing; Stage 2, head nodding/myoclonus; Stage 3, unilateral/bilateral forelimb clonus without rearing, straub tail, extended body posture; Stage 4, bilateral forelimb clonus plus rearing; Stage 5, rearing and falling; Stage 6, full tonic seizures (Racine et al., 1977; Racine, 1972). Twenty minutes after injection of soman, rats received an intramuscular (i.m.) injection of 2 mg/kg atropine sulfate (Sigma, St. Louis MO), a muscarinic receptor antagonist, in order to control the peripheral effects of soman and prevent death from respiratory suppression. One group of animals was sacrificed at the onset of Stage 3 seizures, and therefore did not receive atropine sulfate. Stage 3 behavioral seizures have previously been found to coincide with the initiation of electrographically monitored SE (Figueiredo et al., 2011a; Langston et al., 2012; Rossetti et al., 2012). The control groups received saline instead of soman and were injected with atropine.

2.3 Acetylcholinesterase activity assay

Total AChE activity was measured using a previously established spectrophotometric protocol (Ellman et al., 1961; Padilla et al., 1999), in animals randomly divided into control, seizure onset, 1-, 7-, 14-, and 30-day groups. Rats were anesthetized with 3-5% isoflurane and rapidly decapitated. The brain was removed and placed in ice-cold phosphate buffer (0.1 M, pH 8.0). Coronal brain slices (500 µm-thick) containing the prelimbic cortex (Bregma 5.16 mm to 2.52 mm), BLA (Bregma -2.28 mm to -3.72 mm), piriform cortex (Bregma -1.72 mm to -3.00 mm), and hippocampus (Bregma -2.28 mm to -4.68 mm) were cut using a vibratome (series 1000; Technical Products International, St. Louis, MO). Structures were isolated by hand and placed in an Eppendorf tube containing 1:20 Triton + phosphate buffer, homogenized for 10 seconds and centrifuged at 14,000g for 5 min. The supernatant was removed and placed into a separate container. On the day of sampling, a glutathione curve was made by adding glutathione (1 – 10 µL) to DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)] (up to 200 µL; Padilla et al., 1999). Glutathione supplies the sulfhydryl groups and is used to construct a standard curve. Tissue supernatants (10 µL per well) were added to either 10 µL eserine (an all-purpose cholinesterase inhibitor that inhibits both butyrylcholinesterase and acetylcholinesterase) or ethoprozone (a specific butyrylcholinesterase inhibitor), all in the presence of acetylthiocholine (5 µL) and 175 µL DTNB (all purchased from Sigma-Aldrich, St. Louis, MO). Samples were read by the Softmax Pro 5.2 kinetics every 20 sec for 4 min. The total butyrylcholinesterase inhibition was subtracted from the absorbance sample to provide a difference score, which was multiplied by the slope and intercept of the standard curve to provide a total concentration of AChE activity. AChE specific activity was calculated by dividing the total activity by the calculated protein concentration assayed by the Bradford method (see Bradford, 1976), using a protein assay dye reagent (Bio-Rad, Hercules CA).

2.4 Neuropathology experiments

2.4.1 Fixation and tissue processing—1-, 7-, 14-, and 30-days after SE, rats were deeply anesthetized with pentobarbital (75-100 mg/kg, i.p.) and transcardially perfused with PBS (100 mL) followed by 4% paraformaldehyde (200 mL). Brains were removed and post-fixed in 4% paraformaldehyde overnight at 4°C, then transferred to a solution of 30% sucrose in PBS for 72 hours, and frozen with dry ice before storage at -80°C until sectioning. A 1-in-5 series of sections containing the amygdala was cut at 40 µm on a sliding microtome (Leica Microsystems SM2000R). One series of sections was mounted on slides (Superfrost Plus, Daigger, Vernon Hills, IL) for Nissl staining with cresyl violet. Two adjacent series of sections were mounted on slides for Fluoro-Jade-C staining or were stored at -20°C in a cryoprotectant solution for GAD-67 immunohistochemistry (Figueiredo et al., 2011b).

2.4.2 Fluoro-Jade C (FJC) staining—FJC (Histo-Chem, Jefferson, AR) was used to identify irreversibly degenerating neurons in the BLA. 2D-sample quantitative assessments were made from 3 brain sections (both hemispheres) per animal from AP -2.00 mm to -3.96 mm. Mounted sections were treated and stained in a 0.0001% solution of FJC dissolved in 0.1% acetic acid for 10 min (Qashu et al., 2010). Mosaics were made of the BLA from each hemisphere through a 20× lens, using Stereo Investigator Virtual Tissue tools (6 × 7 square; MicroBrightField, Williston, VT). Cells were counted using ImageJ (NIH Image, Bethesda, MD) with the cell counter tool. The reported number of degenerating cells is the total number counted in the three sections, in both hemispheres.

2.4.3 GAD-67 Immunohistochemistry—Free-floating sections were collected from the cryoprotectant solution, washed three times for 5 min each in 0.1 M PBS, and then incubated in a blocking solution containing 10% normal goat serum (Millipore Bioscience Research Reagents, Temecula, CA) and 0.5% Triton X-100 in PBS, for 1 hour at room temperature (Figueiredo et al., 2011b). The sections were then incubated with mouse anti-GAD-67 serum (1:1000, MAB5406; Millipore Bioscience Research Reagents), 5% normal goat serum, 0.3% Triton X-100, and 1% bovine serum albumin, overnight at 4°C. After rinsing three times for 10 min each in 0.1% Triton X-100 in PBS, sections were incubated with Cy3-conjugated goat anti-mouse antibody (1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 0.0001% 4,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour at room temperature. Sections were given a final rinse in PBS for 10 min, then mounted on slides, air-dried for at least 30 min, and cover-slipped with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA).

2.4.4 Stereological quantification—Design-based stereology was used to determine total neuronal loss from Nissl-stained sections in the BLA, and interneuronal loss from GAD-67-immuno-stained sections in the BLA. Sections were viewed with a Zeiss (Oberkochen, Germany) Axioplan 2ie fluorescent microscope with a motorized stage, interfaced with a computer, running StereoInvestigator 8.0 (MicroBrightField, Williston, VT). The BLA was identified on slide-mounted sections under a 2.5× objective, based on the atlas of Paxinos and Watson (Paxinos and Watson, 2005). Sampling was performed

under a 63× oil immersion objective. Nissl-stained neurons were distinguished from glial cells by their larger size and pale nuclei surrounded by darkly-stained cytoplasm containing Nissl bodies. The total number of Nissl-stained and GAD-67-immunostained neurons was estimated using the optical fractionator probe along with the coefficient of error (CE). Nissl-stained neurons were calculated using Stereo Investigator 9.0. The CE was calculated by the software according to Gundersen ($m = 1$; Gundersen et al., 1999) and Schmitz-Hof (2nd estimation; Schmitz and Hof, 2000) equations.

A 1-in-5 series of sections (~7 sections per rat) was analyzed for Nissl-stained neurons in the BLA. The counting frame was $35 \times 35 \mu\text{m}$, the counting grid was $190 \times 190 \mu\text{m}$, and the dissector height was $12 \mu\text{m}$. Nuclei were counted when the cell body came into focus within the dissector, which was placed $2 \mu\text{m}$ below the section surface. An average of 235 neurons per rat was counted, and the average CE was 0.04 for both the Gundersen and Schmitz-Hof equations. Section thickness was measured at every counting site (averaging $20 \mu\text{m}$ for the BLA). For GABAergic interneurons immuno-stained with GAD-67, a 1-in-10 series of sections was analyzed (on average five sections). The counting frame was $60 \times 60 \mu\text{m}$, the counting grid was $100 \times 100 \mu\text{m}$, and the dissector height was $20 \mu\text{m}$. The average mounting section thickness of the BLA was $27.6 \mu\text{m}$. An average of 239 neurons per rat was counted in the BLA, and the average CE was 0.07 for both the Gundersen et al., and Schmitz-hof equations. Nuclei were counted when the top of the nucleus came into focus within the dissector, which was placed $2 \mu\text{m}$ below the section surface. Section thickness was measured at every fifth counting site.

2.5 Behavioral experiments

2.5.1 Open field test—Anxiety-like behavior was assessed using an open field apparatus ($40 \times 40 \times 20 \text{ cm}$ clear Plexiglas arena; Aroniadou-Anderjaska et al., 2012; Faraday et al., 2001). One day prior to testing, animals were acclimated to the apparatus for 20 min. On the test day, rats were placed in the center of the open field and activity was measured and recorded for 20 min using an Accuscan Electronics infrared photocell system (Accuscan Instruments Inc., Columbus, OH). Data were automatically collected and transmitted to a computer equipped with “Fusion” software (from Accuscan Electronics, Columbus, OH). Locomotion (distance traveled in cm) and time spent in the center of the open field were analyzed. Anxiety behavior was measured as the ratio of the time spent in the center over the total movement time, expressed as a percentage of the total movement time.

2.5.2 Acoustic startle response test—Acoustic startle response (ASR) testing was conducted with the use of the Med Associates Acoustic Response Test System (Med Associates, Georgia, VT), which consists of weight-sensitive platforms inside individual sound-attenuating chambers. Rats were individually placed in a ventilated holding cage, which is small enough to restrict extensive locomotion, but large enough to allow the subject to turn around and make other small movements (Aroniadou-Anderjaska et al., 2012). The chamber contained a ventilated fan to provide background noise. The cage was placed on a weight-sensitive platform; movements in response to stimuli were measured as a voltage change by a strain gauge inside each platform. Animals were acclimated to the apparatus in two sessions. A 3-min adaptation period was allowed, during which no startle stimuli were

presented. Startle stimuli consisted of 110 or 120 dB sound pressure level noise bursts of 20-ms duration. Each stimulus had a 2-ms rise and decay time, such that the onset and offset were abrupt, which is a primary requirement for startle (Faraday and Grunberg, 2000). Each trial type was presented eight times. Trial types were presented at random to avoid order effects and habituation, and inter-trial intervals ranged randomly from 15 to 25 s. Responses were recorded by an interfaced Pentium computer as the maximum response occurring during the no-stimulus periods, and during the startle period, and were assigned a value based on an arbitrary scale used by the software of the Test System.

2.6 Electrophysiological experiments

Animals were randomly divided into control, 1-, 7-, 14-, and 30-day groups and anesthetized with 3-5% isoflurane before decapitation. Coronal brain slices (400 μ m-thick) containing the amygdala (-2.64 to -3.36 from Bregma) were cut using a vibratome (series 1000; Technical Products International, St. Louis, MO), in ice-cold cutting solution consisting of (in mM): 115 sucrose, 70 NMDG, 1 KCl, 2 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 30 NaHCO₃. Slices were transferred to a holding chamber, at room temperature, in a bath solution containing (in mM): 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 21 NaHCO₃, 2 CaCl₂ 1.5 MgCl₂, and 11 D-glucose (all purchased from Sigma-Aldrich, St. Louis, MO). Recording solution was the same as the holding bath solution. All solutions were saturated with 95% O₂, 5% CO₂ to achieve a pH near 7.4. Field potential recordings were obtained in an interface-type chamber, maintained at 32~33°C, with a flow rate of the ACSF at ~1.5 mL/min. Field potentials were evoked by stimulation of the external capsule at 0.05 Hz with a bipolar concentric stimulating electrode made of tungsten (World Precision Instruments, Sarasota, FL). For LTP experiments, a 20 min baseline was recorded; stimulus intensity was adjusted to elicit a response of about 70 to 80% of the maximum amplitude. High frequency stimulation (HFS) consisted of 3 trains of pulses at 100 Hz, each train lasted 1 s, and the interval between trains was 20 sec. Following HFS, field potential recordings resumed with stimulation at 0.05 Hz. Recording glass pipettes were filled with ACSF, and had a resistance of approximately 5 M Ω . Signals were digitized using the pClamp 10.2 software (Molecular Devices, Union City, CA), analyzed using AxoGraph (AxoGraph X, Berkley, CA), and final presentation was prepared using GraphPad Prism (GraphPad Software, La Jolla, CA).

2.7 Statistical analysis

All data are expressed as mean $\pm$ standard error. Welch F ANOVA and a Games-Howell post hoc were used to compare the results from the analysis of AChE activity, and the stereological estimation of the total number of GAD-67-positive interneurons. Welch F ANOVA was used due to heterogeneity of variance. Statistical results derived with the use of the Welch F ANOVA were confirmed with a Kruskal-Wallis non-parametric test, which does not assume a normal distribution. One-way ANOVA with a Tukey post hoc test was used to compare the cell count from degenerating neurons, and to analyze the results from the behavioral tests. One-way ANOVA with a Dunnett's T post hoc test was used to compare the results from the stereological estimation of the total number of neurons and from the behavioral tests. Results from the electrophysiological experiments were analyzed using one-way ANOVA followed by a Dunnett's T post hoc test, paired-Student's t-test, or independent samples t-test. Statistical analyses were made using the software package

PAWS SPSS 20 (IBM, Armonk, NY, USA). Differences were considered significant when $p < 0.05$. Sample size “n” refers to the number of animals except for electrophysiology experiments where “n” refers to the number of slices.

3. Results

Soman was administered to 161 rats. The average latency to Stage 3 seizure onset (SE onset) was 8.7 ± 1.2 min. Of the surviving 104 rats that developed prolonged SE (see Aplan et al., 2012; Figueiredo et al., 2011a, 2011b), 51 rats were used to measure AChE activity, and 31 rats were used for electrophysiological experiments. From the remaining 22 soman-exposed rats, 5 rats were used for evaluation of neuropathology at 24-hours after soman exposure, 6 rats were used for evaluation of neuropathology at 7 days post-exposure, 5 rats were used for 14-day behavioral experiments followed by neuropathological studies, and 6 rats were used for 30-day behavioral tests followed by neuropathology assessments. Aged-matched rats that were not exposed to soman were used for control experiments.

3.1 Time course of recovery of AChE inhibition

The onset of SE induced by soman was associated with a dramatic reduction of AChE activity in the BLA ($91.9 \pm 4.0\%$), prelimbic cortex ($98.8 \pm 5.0\%$), piriform cortex ($99.2 \pm 8.3\%$), and hippocampus ($98.7 \pm 6.3\%$; all p 's < 0.001 ; Table 1 and Fig. 1). Twenty-four hours after SE, AChE activity was still reduced by at least 93% in all four brain regions (all p 's < 0.001). Seven days after SE, AChE activity in the BLA was no longer significantly lower than the control ($42.7 \pm 14.2\%$ lower; $p = 0.092$), while it remained significantly reduced in the prelimbic cortex ($52.1 \pm 10.3\%$ lower than control; $p = 0.001$), piriform cortex ($55.0 \pm 13.1\%$ lower; $p = 0.004$), and hippocampus ($46.6 \pm 11.7\%$ lower; $p = 0.009$). Within 14 days, AChE activity had recovered in all four brain regions; in the BLA, it was $116.3 \pm 18.8\%$ of the control activity, while in the prelimbic cortex, piriform cortex, and hippocampus it was $25.8 \pm 8.6\%$, $13.3 \pm 15.8\%$, and $15.1 \pm 14.5\%$ lower than the control values, respectively (all p 's > 0.05). Thirty days after SE, AChE activity was $118.8 \pm 10.7\%$ of control values in the BLA, while in the prelimbic cortex, piriform cortex, and hippocampus it was $144.8 \pm 18.4\%$, $147.4 \pm 18.2\%$, and $135.5 \pm 20.7\%$ of the control activity, respectively (all p 's > 0.05 ; Fig. 1; Table 1).

3.2 Time course of neurodegeneration and neuronal loss in the BLA

Twenty-four hours after exposure to soman, severe neurodegeneration was found in the BLA (Fig. 2). The number of degenerating neurons was 928 ± 153 at 24 hours, 481 ± 35 at 7 days ($p = 0.003$), 305 ± 77 at 14 days ($p < 0.001$), and 133 ± 17 at 30 days ($p < 0.001$; all p values are in comparison to the 24-hour group). The number of degenerating neurons at 30 days was also significantly lower when compared to the 7-day group ($p = 0.014$; Fig. 2).

Estimation of the total number of neurons in the BLA (Fig. 3A and B), using an unbiased stereological method in Nissl-stained sections, showed that animals exposed to soman had significantly fewer total number of neurons in the BLA at 24 hours ($71,003.5 \pm 1,198.1$), 7 days ($72,416.7 \pm 2,508.6$), 14 days ($72,499.4 \pm 1,713.8$) and 30-days ($71,412.1 \pm 2,731.8$) after exposure to soman, compared to the number of neurons in the control animals

(109,397.5 $\pm$ 2,719.5; all p 's < 0.05; Fig. 3C). There was no significant difference between the number of neurons in the BLA at the different time points after soman exposure.

Estimation of the total number of GABAergic interneurons in the BLA, using an unbiased stereological method on GAD-67-immunostained sections, showed that 24-hours after exposure, the number of interneurons in the soman group (10,539.1 $\pm$ 575.1; n = 5) did not differ significantly from the controls (13,107.4 $\pm$ 804.5; p = 0.129; n = 10; Fig. 4A, B). However, 7 days after soman exposure, the number of GABAergic interneurons in the soman-exposed group (7,131.3 $\pm$ 343.4; n = 6) was significantly lower than the number of GABAergic interneurons in the BLA of control animals (p < 0.001). No further loss of GABAergic interneurons was observed at 14 days (6,745.5 $\pm$ 315.4; n = 6), or 30 days (6,885.2 $\pm$ 195.4; n = 5) after SE, compared to the 7-day soman group (p > 0.05). The loss of GABAergic interneurons in the BLA was approximately 46%, 7-days after exposure. Moreover, the ratio of GABAergic interneurons to the total number of neurons in the BLA was altered after soman exposure. At 24 hours after SE, there was a significant increase in this ratio (p = 0.006). Seven days after exposure, the ratio of GABAergic interneurons to the total number of neurons was not significantly different from the control (p = 0.175), whereas it was significantly reduced at 14 days (p = 0.006) and 30 days (p = 0.030) after SE (Fig. 4C).

3.3 Anxiety-like behavior after soman exposure

The reduced ratio of the number of GABAergic interneurons to the total number of neurons in the BLA, on days 14 and 30 after soman-induced SE, suggests that GABAergic inhibition in the BLA may be impaired at these time points; the result could be a hyperexcitable amygdala, which can produce anxiety-related behavioral deficits. To determine if anxiety-like behavior was increased in the soman-exposed rats, we used the open field and the ASR tests. In the open field test, the more anxious an animal is, the less time it spends in the center of the open field (Pruet and Belzung, 2003). Compared to the time the control group spent in the center of the open field (14.2 $\pm$ 2.1% of the total movement time; n = 6), the soman-exposed rats spent significantly less time in the center, on day 14 post-exposure (7.6 $\pm$ 1.8% of the total movement time; n = 5, p = 0.046) and on day 30 post-exposure (5.3 $\pm$ 1.6% of the total movement time; n = 6, p = 0.004; Fig. 5A). The difference between the 14-day and 30-day groups was not statistically significant (p = 0.622). The total movement time did not differ significantly among the three groups (695 $\pm$ 48 s for the control group, 718 $\pm$ 51 s for the 14-day group, and 793 $\pm$ 38 s for the 30-day group).

In the ASR test, the more anxious and fearful an animal is, the larger the amplitude of the startle in response to the acoustic stimulus (Hitchcock and Davis, 1987; Li et al., 2009; Li et al., 1999). We measured the amplitude of the startle by averaging the amplitude of 8 trials, using two levels of acoustic stimuli (110 dB and 120 dB noise bursts). Compared to the amplitude of the ASR in the control group (11.5 $\pm$ 1.2 and 13.4 $\pm$ 2.2 in response to the 110 and 120 dB, respectively; n = 6), the ASR amplitude was increased in the soman-exposed rats, on day 14 (16.8 $\pm$ 1.9 in response to the 110 dB, p = 0.42, and 18.2 $\pm$ 1.1 in response to the 120 dB, p = 0.010; n = 5) and on day 30 after exposure (18.3 $\pm$ 2.2 in response to the 110 dB, p = 0.017, and 19.2 $\pm$ 0.9 in response to the 120 dB, p = 0.001; n = 6; Fig. 5B). There

was no significant difference in the amplitude of the ASR between the 14- and 30-day groups ($p > 0.05$).

3.4 Time course of pathophysiological alterations in the BLA

The observed neuronal damage in the BLA of the soman-exposed rats, and the accompanying behavioral impairments in anxiety-like behavior suggest that impairments were probably also present in BLA physiology. To gain some insight into the pathophysiological alterations in the BLA after soman exposure, we recorded BLA field potentials evoked by stimulation of the external capsule in brain slices obtained from soman-exposed rats, at 24 hours, 7 days, 14 days, and 30 days after soman administration.

Due to the non-laminar architecture of the amygdala, the activity of neuronal populations in this structure does not generate large extracellular electric dipoles, and, therefore, evoked field potentials have small amplitude. Nevertheless, field potentials of about 0.5 mV can be consistently evoked in the BLA, representing about 60 ~ 70% of the maximum evoked responses. In the soman-exposed rats, however, it was difficult to evoke field potentials, particularly at 24 hours and 7 days post-exposure. Stimulus intensities similar to those used in control slices would evoke field potentials in the BLA of soman-exposed rats that were about half the amplitude of the BLA field potentials in control rats. Notably, the BLA field potentials in the soman-exposed rats had a slow decay (Fig. 6), which is probably indicative of reduced inhibition. To better assess if inhibitory activity in the BLA was impaired after soman exposure, we compared responses to paired-pulse stimulation in soman-exposed versus control rats. In the slices from the control rats, the ratio of the amplitude of the response to the second pulse over the amplitude of the response to the first pulse (paired-pulse ratio, or PPR) was 0.89 ± 0.07 ($n = 9$). In the slices from soman-exposed rats, the PPR was significantly higher compared to the controls, at 24 hours ($PPR = 1.21 \pm 0.03$, $n = 9$; $p = 0.008$), 7 days ($PPR = 1.30 \pm 0.06$, $n = 9$; $p = 0.001$), 14 days ($PPR = 1.26 \pm 0.09$, $n = 10$; $p = 0.002$), and 30 days ($PPR = 1.23 \pm 0.03$, $n = 11$; $p = 0.005$) after soman administration (Fig. 6), suggesting that the inhibition which normally suppresses the amplitude of the synaptic response to the second stimulus pulse (Zinebi et al., 2001) and limits contribution of spiking activity to the field potentials was reduced.

A synaptic property that is considered to be the cellular mechanism underlying learning and memory processes is the long-term potentiation (LTP) of synaptic transmission (Malenka and Nicoll, 1999; Teyler and DiScenna, 1987). The amygdala plays a key role in fear-associated learning and memory formation (LeDoux, 2003; Pape and Pare, 2010). The BLA, in particular, mediates the formation of fear memory and may also be the site, or one of the sites, where fear memory is stored (Gale et al., 2004). Therefore, we examined if the capacity of neuronal synapses in the BLA to express LTP had been altered after soman exposure. Potentiation of the evoked field potentials was measured by averaging the amplitude of the responses from 50 to 60 min after HFS, and expressing it as a percentage of the baseline response. Compared to the percent change in age-matched control animals ($158.0 \pm 8.4\%$, from 0.48 ± 0.03 mV at baseline to 0.74 ± 0.04 mV at 50 to 60 min after HFS, $n = 7$), the percent change in the 24-hours post-exposure group ($112.3 \pm 5.24\%$, from 0.27 ± 0.01 mV to 0.30 ± 0.02 mV, $n = 7$; Fig. 7A), the 7-day post-exposure group ($122.3 \pm$

8.53%, from 0.27 ± 0.06 mV to 0.34 ± 0.08 mV, $n = 8$; Fig. 7B), and the 14-day post-exposure group ($126.9 \pm 10.6\%$, from 0.26 ± 0.02 mV to 0.35 ± 0.05 mV, $n = 10$; Fig. 7C) were significantly lower ($p < 0.05$). At 30 days post-exposure, the time course of LTP was slower compared to the control group (Fig. 7D), but the potentiation at 50 to 60 min post-HFS ($135.1 \pm 14.9\%$, from 0.40 ± 0.03 mV to 0.55 ± 0.07 mV, $n = 10$) was not significantly different from the controls ($p = 0.251$).

4. Discussion

After exposure of rats to lethal doses of soman, we found that more than 90% of AChE is inhibited at the onset of SE in brain regions that play a key role in seizure generation, and it takes up to two weeks for AChE activity to recover to normal levels. Significant neuronal loss and neurodegeneration was present in the BLA throughout the time period examined, from 24 hours to 30 days after soman exposure. Interestingly, at the 24-hour time point, there was no significant loss of GABAergic interneurons. However, by 7 days post-exposure, the number of GABAergic interneurons in the BLA was reduced, and by 14 and 30 days after soman, the ratio of GABAergic interneurons to the total number of neurons was lower compared to controls. At the same time points (14 and 30 days post-exposure), anxiety-like behavior was increased in the soman-exposed rats. Pathophysiological alterations accompanied the neuropathological damage and the behavioral deficits, namely a prolongation of evoked BLA field potentials and an increase in paired-pulse ratio, at all time points examined, as well as impaired synaptic plasticity at 24 hours, 7 days and 14 days post-exposure.

4.1 Inhibition and recovery of AChE

Nerve agents exert their toxic effects, including the induction of seizures, by inhibiting AChE (Bajgar, 2004; Harrison et al., 2004) via phosphorylation of the enzyme (Sirin et al., 2012). It has been previously observed that during nerve agent-induced convulsions, brain AChE is inhibited dramatically (Bajgar et al., 2008; Lallement et al., 1992). In the present study, we found that at the onset of SE after administration of soman, AChE has been inhibited by more than 90% in the BLA, piriform cortex, hippocampus, and prelimbic cortex, regions that play an important role in seizure generation by nerve agent exposure (Myhrer, 2007; Prager et al., 2013). It is noteworthy, in this regard, that a reduction of AChE activity of about 91% in the hippocampus and 87% in the piriform cortex after soman exposure is not accompanied by seizures if AChE in the BLA is not inhibited significantly (Prager et al., 2013).

Previous studies have examined AChE inhibition after administration of organophosphorus pesticides (Cardona et al., 2013; Kazi and Oommen, 2012; Kobayashi et al., 2007), or nerve agents (Clement et al., 1991; Grubic et al., 1981; Lintern et al., 1998), however, there have been no systematic studies of the time course of AChE inhibition and the recovery of its activity after exposure to lethal doses of a nerve agent that induces prolonged status epilepticus. Here, we found that AChE was still inhibited by more than 90% at 24 hours post-exposure (Fig. 1), when SE has stopped. This observation does not contradict the view that elevated acetylcholine (ACh) (due to inhibition of AChE) is the cause of seizures. First, it is well-understood that elevated ACh initiates seizures after nerve agent exposure, but

seizures are sustained and reinforced by glutamatergic mechanisms (Bodjarian et al., 1993; McDonough and Shih, 1993, 1997); therefore, the duration and intensity of SE is not related to elevated ACh. Second, it appears that persistent inhibition of AChE does not necessarily correlate with persistent elevation of ACh at cholinergic synapses. Thus, Lallement et al. (1992) measured extracellular ACh levels for 90 min after soman-induced SE, in rats, and found that ACh returned to baseline concentrations within 50 min in septohippocampal areas, and within 90 min in the amygdala. The return of ACh to control levels – at a time when AChE remained inhibited – was attributed to negative feedback of the released ACh onto cholinergic terminals via muscarinic autoreceptors, and to the decreased availability of choline (Lallement et al., 1992).

At 14 days after soman exposure, AChE had returned to control levels in the hippocampus, piriform, and prelimbic cortex, while in the BLA it recovered to nearly basal levels in 7 days. The faster time course of recovery in the BLA may relate to the exceptionally dense cholinergic innervation of this region (Mesulam et al., 1992) – also reflected in the high basal level of AChE (Ben-Ari et al., 1977; Prager et al., 2013) – which could result in a more efficient recovery of the enzyme. The recovery of AChE is probably the result of de novo AChE synthesis rather than dephosphorylation of the inhibited AChE, as the inhibition of AChE by nerve agents is irreversible, and the state of irreversibility is reached very fast after exposure to soman (Sirin et al., 2012). The consequences of the prolonged AChE inhibition after soman exposure can be expected to include abnormal fluctuations of ACh concentrations at cholinergic synapses, with possible short-term and long-term effects on cholinergic receptor activity and expression.

4.2 Acute and delayed neuropathology

Significant neuronal death had occurred by 24 hours, and the extent of neuronal loss did not increase over time, despite ongoing neurodegeneration. The initial neuronal death was probably a process of excitotoxicity-induced necrosis, whereas the slow death of the degenerating neurons may result from simultaneous influences and competition of survival- and death-inducing factors (Collombet, 2011; Fujikawa, 2005; Holmes, 2002). It has been shown previously that excitotoxic damage can induce either an immediate, necrotic death or a slow apoptotic death to the same neuronal population, where necrosis is associated with immediate loss of mitochondrial function, while apoptosis ensues in neurons that recover their mitochondrial function and energy levels, yet the excitotoxic insult has activated an endogenous cell death program (Ankarcrona et al., 1995). Our data suggest that BLA principal cells can undergo immediate, necrotic death (cell loss by 24 hours after exposure) or slow, apoptotic-like death (since neurodegeneration continues after the 24 hours). The relative stability of the level of neuronal loss from 24 hours to 30 days post-exposure, in the presence of degenerating neurons and loss of interneurons, may be explained by a concomitant process of neuronal regeneration (Collombet et al., 2006; Collombet et al., 2008).

As we have observed previously (Figueiredo et al., 2011b), at 24 hours post-exposure there was no loss of GABAergic interneurons, while by day 7, significant loss had occurred. Thus, GABAergic interneurons in the BLA appear to undergo only the apoptotic type of slow

death. This does not imply that they are less vulnerable, as the interneuron to total number of neurons ratio decreased over time. However, it implies that they are less susceptible to necrosis and more susceptible to apoptosis, for reasons that remain to be determined. Delayed death of GABAergic interneurons has also been observed after pilocarpine-induced SE, as the majority of chronically degenerating FJC-positive neurons exhibited GAD-immunoreactivity (Wang et al., 2008). One factor contributing to the death of GABAergic interneurons may involve NMDA receptor-mediated excitotoxicity due to a gradual upregulation of D-serine (Liu et al., 2009), a positive NMDA receptor modulator (Liu et al., 2009; Mothet et al., 2000; Wolosker, 2007).

By investigating the time course of neuronal loss beyond one week after soman exposure, in the present study, we found that although there was no significant further decrease of either the number of interneurons or the total number of neurons up to 30 days post-exposure, the small changes that occurred were sufficient to decrease the ratio of interneuron number to the total neuronal number. GABAergic interneurons account for only 15-20% of the neuronal population in the BLA (McDonald and Mascagni, 2002; Sah et al., 2003; Spanpanato et al., 2011) and tightly regulate principal cell excitability (Lang and Pare, 1998; Spanpanato et al., 2011; Woodruff and Sah, 2007). Therefore, the reduction in the interneuronal population by about 46% by day 7 after soman exposure and the subsequent decrease of the interneuronal to the total number of neurons ratio can be expected to have a significant impact on the excitability of the BLA network, contributing to the development of spontaneous recurrent seizures (de Araujo Furtado et al., 2010) and behavioral deficits.

4.3 Increased anxiety-like behavior and pathophysiological alterations

At 14 and 30 days after exposure to soman, the animals displayed increased anxiety-like behavior, assessed using the open field (Prut and Belzung, 2003) and the ASR (Hitchcock and Davis, 1987; Li et al., 2009; Li et al., 1999) tests. Increased anxiety has been observed previously after nerve agent exposure in animals (Coubard et al., 2008; Mamczarz et al., 2010) and humans. Some of the victims of the Tokyo sarin attacks, for example, presented posttraumatic stress disorder, five or seven years after the events (Hoffman et al., 2007). Hyperexcitability of the amygdala, and the BLA in particular, is a characteristic feature of anxiety-like behavior in animals (Bueno et al., 2005; Davis et al., 1994; Sanders et al., 1995) and anxiety disorders in humans (Koenigs and Grafman, 2009; Rauch et al., 2000; Villarreal and King, 2001). Therefore, the increased anxiety in animals and humans after nerve agent exposure is likely to be due to neuropathological and pathophysiological alterations that have increased the excitability of the BLA network.

A number of factors may have contributed to increased BLA excitability, including damage to afferents from other brain regions (such as the prefrontal cortex) that, normally, suppress activity and excitability of the BLA. Alterations in the cholinergic regulation of the BLA can potentially contribute to BLA hyperexcitability, despite that AChE has recovered at the time that increased anxiety was observed. For example, we have found that the net effect of the α_7 -containing nicotinic acetylcholine receptor (α_7 -nAChR) activation in the BLA is an increase in GABAergic inhibition (Pidoplichko et al., 2013). Since the activity and expression of the α_7 -nAChR is sensitive to ACh exposure (Giniatullin et al., 2005; Klein

and Yakel, 2006), desensitization or downregulation of these receptors could increase BLA excitability. Nevertheless, the present data suggest that the primary cause of an increased BLA excitability is probably the loss of GABAergic interneurons and the decreased ratio of the interneurons to the total number of neurons.

The pathophysiological significance of the interneuronal death was reflected in the increased duration of the field potentials evoked in the BLA and the increased PPR. In the soman-exposed rats, the field potentials were smaller and difficult to evoke, as it can be expected considering the significant neuropathological damage (neuronal loss and degeneration). At the same time, the decay of the field potentials was slower compared to controls; this suggests reduced GABAergic inhibition, resulting in the appearance of polysynaptic inputs and/or increased contribution of NMDA receptor-mediated synaptic activity to the evoked response. An increased PPR implies either that the probability of neurotransmitter release in response to the first pulse has been reduced, or that the evoked inhibition that normally suppresses the response to the second pulse is impaired. We do not know if there are alterations in the presynaptic release of glutamate, but considering the interneuronal loss and the prolongation of the field potentials, it is reasonable to suggest that the increased PPR reflects, at least in part, reduction of evoked GABAergic inhibition. Because the BLA does not have the discrete laminar architecture that other brain regions have, it cannot be ascertained if purely synaptic activity or population spikes contribute to the field potentials; the waveforms, however, suggest the presence of spiking activity in the evoked responses. When inhibition is reduced in the BLA, both the synaptic-(Zinebi et al., 2001) and the spiking component of the response to the second stimulus pulse can be expected to increase, producing an increased PPR.

The reduced inhibition may have facilitated the induction of LTP, despite the presence of severe neuronal damage. At 24 hours, 7 days, and 14 days after exposure, synaptic plasticity (as reflected in the synaptic capacity for LTP expression) was significantly impaired, but recovered by 30 days post-exposure. These results are consistent with previous behavioral data indicating severely impaired fear-conditioning at 8 days after soman exposure (Moffett et al., 2011). The recovery of synaptic plasticity over time after soman-induced SE could be related to synaptic re-organization (Mello et al., 1993; Sloviter, 1999).

The present study described the time course of AChE recovery in the brain, as well as neuropathological, behavioral, and pathophysiological alterations during 30 days after exposure of rats to soman, which corresponds, approximately, to 3 years of human life (Sengupta, 2011). We show that recovery of AChE is slow, which can have both acute and long-term consequences on the cholinergic modulation of neuronal excitability. The death of GABAergic interneurons is slow, which may provide an opportunity for pharmacological intervention to prevent it, if the mechanisms of this slow death become well-understood. Such intervention could have an impact in preventing long-term behavioral deficits, such as increased anxiety, which we found to persist at 30 days post-exposure. The apparent recovery of synaptic plasticity in the BLA by 30 days after exposure may suggest that at least certain aspects of fear-related learning may be normalized at this time, despite the persistence of increased BLA excitability, which may impact amygdala's modulation of cognitive processes.

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Highlights

Soman reduces brain AChE activity by more than 90% at the onset of status epilepticus

AChE activity recovers within 7-14 days after soman exposure

Severe neuronal loss and degeneration in the BLA by 24 hours after soman

Delayed death of GABAergic interneurons in the BLA

Persistent increase in anxiety and transient impairment in synaptic plasticity

Figure 1.

Time course of the recovery of AChE activity after soman-induced status epilepticus (SE). AChE activity was reduced by more than 90% in all four brain regions at the onset of SE ($n = 7$), as well as at 24 hours later ($n = 11$). At 7 days post-exposure ($n = 11$), AChE activity had recovered in the BLA, while at 14 days ($n = 10$) and 30 days ($n = 12$) post-exposure, it was not significantly different from the control levels in all 4 brain regions. $**p < 0.01$; $***p < 0.001$ compared to the controls (Welch F ANOVA with Games-Howell post hoc).

Figure 2.

Time course of neuronal degeneration in the BLA after soman-induced SE. **A, B.** The BLA region where neuronal degeneration was assessed. In **A**, the drawings are from Paxinos and Watson (2005), while in **B** the BLA is outlined on FJC-stained sections.

C. Representative photomicrographs of FJC-stained sections, at 24-hours, 7-, 14-, and 30-days after soman exposure. Rows correspond to the coordinates shown in **B**. Total magnification is 20 $\times$. Scale bar, 250 μ m. **D.** Quantitative assessment of the extent of neurodegeneration at 24-hours ($n = 4$), 7- ($n = 6$), 14- ($n = 4$), and 30 days ($n = 5$) after soman exposure. Values are mean $\pm$ SEM number of cells counted in the BLA from both hemispheres. ** $p < 0.01$, *** $p < 0.001$ (One-Way ANOVA with Tukey post hoc).

Figure 3.

Time course of neuronal loss in the BLA after soman-induced SE. **A.** Panoramic photomicrograph of a Nissl-stained section from half hemisphere, outlining the BLA where stereological analysis was performed (red highlight). **B.** Representative photomicrographs of Nissl-stained sections showing BLA cells from a control rat and from soman-exposed rats, analyzed at 24-hours, 7-, 14- and 30-days after exposure. Total magnification is 63× and scale bar is 50 μm. **C.** Group data of stereological estimation of the total number of Nissl-stained neurons in the BLA, expressed as percent of the control group (n = 11). Significant neuronal loss was present in rats at 24 hours (n = 5), 7 days (n = 6), 14 days (n = 5), and 30-days (n = 6) after soman exposure. There were no differences in the extent of neuronal loss among the 24-hour, 7-, 14-, and 30-day groups; *** $p < 0.001$ (One-Way ANOVA with Dunnett's T post hoc).

Figure 4.

Delayed loss of GABAergic interneurons in the BLA after soman-induced SE. **A.** Panoramic photomicrograph of an immunohistochemically-stained section for GAD-67 (BLA is outlined in white). **B.** Representative photomicrographs of GABAergic interneurons in the BLA from a control rat and from soman-exposed rats, analyzed at 24-hours, 7-, 14- and 30-days after exposure. Total magnification is 630×; scale bar, 50 μm. **C.** Group data showing the mean and standard error of the stereologically estimated total number of GAD-67-positive cells in the BLA. **D.** Group data showing the mean and standard error of the ratio of GABAergic interneurons to the total number of neurons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Welch F ANOVA with Games-Howell post hoc and one-way ANOVA with Dunnett's T post hoc).

Figure 5.

Exposure to soman causes long-lasting increases in anxiety-like behavior, as measured by the open field and acoustic startle response (ASR) tests. **A.** Soman-exposed rats spent significantly less time in the center of the open field, at 14 and 30 days after exposure, compared to control rats. **B.** Exposure to soman significantly increased the amplitude of the ASR to both the 110 and 120 dB startle stimuli, at 14 and 30 days after exposure, compared to controls. There were no significant differences between the 14- and 30-day groups. * $p < 0.05$, ** $p < 0.01$; $n = 17$ (One-Way ANOVA with Dunnett's T post hoc).

Figure 6.

Alterations in the BLA field potentials after soman-induced SE. **A to E** show representative field potentials evoked in the BLA by paired-pulse stimulation of the external capsule, from control rats ($n = 9$) and soman-exposed rats, at 24-hours ($n = 9$), 7 days ($n = 9$), 14 days ($n = 10$), and 30 days ($n = 11$) after exposure; each trace is an average of 10 to 15 sweeps. In the soman-exposed rats, higher stimulus intensities were necessary to evoke field potentials compared to controls, the duration of the field potentials was prolonged (notice the decay of the waveforms), and the paired-pulse ratio was significantly increased (**F**). $**p < 0.01$ (One-Way ANOVA with Dunnett's T post hoc).

Figure 7.

Effects of soman exposure on Long-Term Potentiation in the BLA. The plots show the time course of the changes in the amplitude of the field potentials after high-frequency stimulation (HFS). The amplitude of the 3 responses recorded in each min (stimulation at 0.05 Hz) was averaged, and each data point on the plots is the mean and standard error of these averages, from 7 to 10 slices (see sample sizes in the text). Traces over the plots are examples from a control rat and from soman-exposed rats studied at the indicated time-point after exposure; the superimposed field potentials are a baseline response and a response at 50 to 60 min after HFS (each trace is the average of 10 to 15 sweeps). Potentiation of the responses, measured at 50 to 60 min after HFS, was significantly lower – compared to the control group – at 24 hours, 7 days and 14 days post-exposure, but not at 30 days post-exposure.

Table 1
Reduction of AChE activity (in nmol/min/ng) and time course of recovery after soman-induced status epilepticus (SE)

Brain Region	Control	SE Onset	24-hours	7-days	14-days	30-days
Basolateral Amygdala	830.9 ± 33.6	68.8 ± 10.2	38.4 ± 6.1	488.2 ± 116.2	833.2 ± 114.2	1011.6 ± 84.2
Prelimbic Cortex	263.4 ± 13.2	3.0 ± 0.8	15.6 ± 4.3	130.2 ± 24.4	202.0 ± 19.1	393.9 ± 48.2
Periform Cortex	351.9 ± 29.1	2.8 ± 0.8	11.4 ± 2.4	158.3 ± 35.4	274.1 ± 39.4	518.8 ± 57.0
Hippocampus	311.3 ± 19.7	4.1 ± 0.3	23.2 ± 5.3	166.2 ± 30.5	231.8 ± 26.9	421.8 ± 61.3