Temporal Expression of Neuroinflammatory Markers and Brain Injury Following Acute Soman Poisoning

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ABSTRACT

Animals exposed to chemical nerve agent (i.e. soman, sarin, cyclosarin, tabun or VX) develop convulsive seizures that rapidly progress to status epilepticus and brain injury. Nerve agent-induced brain injury is caused primarily by the magnitude and duration of seizures, but inflammation may also play a role in the pathogenesis. Since a major goal of our neuroprotection program is to reduce nerve agent-induced brain damage, a more comprehensive survey of inflammatory factors in the brain is needed to accurately direct anti-inflammatory pharmacological intervention. The present study investigated the temporal expression of inflammatory mediators in the piriform cortex, correlated these findings with brain pathology and determined the anti-inflammatory therapeutic window for attenuation of neurodegeneration following acute nerve agent exposure. Male Sprague-Dawley rats were pretreated with oxime HI-6 30 min prior to soman challenge and treated with atropine methylnitrate one minute afterwards. At various time points (30 min, 1, 3, 6, 12, 24, 48 or 72 hr) after the onset of seizures, brains were processed for pathological and immunohistochemical studies or high throughput Luminex™ multiplex immunoassay. An extensive loss of microtubule-associated protein 2 (MAP-2) was observed in the piriform cortex beginning at 12 hr after the onset of seizures. At 24 hr after onset of seizures, MAP-2 immunoreactivity was completely lost in layer three of the piriform cortex. By Luminex™ immunoassay, the expression of several inflammatory mediators was also found to be time-dependent and parallel with severe brain injury. The expression of MIP-1α, TNFα and GRO KC was increased as early as one hour, and the expression of IL-1α, IL-1β and MCP-1 was elevated at 6 hr. Of these inflammatory markers, MIP-1α, TNFα, IL-1α, IL-1β and GRO KC expression levels peaked at 12 hr in the piriform cortex, coincident with a severe loss of MAP-2. These observations suggest a correlation between increasing neuroinflammation and brain damage following acute soman exposure. In addition, these results suggest that a therapeutic window for prophylactic anti-inflammatory treatment exists for at least 6 hr following soman exposure as evidenced by the less advanced state of MAP-2 pathology at this time point. Moreover, brain cytokines released into the blood or urine could be used clinically in the diagnosis, prognosis and therapeutic evaluation of brain injury induced by nerve agent.

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1.0 INTRODUCTION

Soman (pinacolylmethylphosphonofluoridate; O-1,2,2-trimethylpropylmethyl-phosphonofluoridate) is an organophosphonate nerve agent that causes convulsive seizures and death. The initiation of seizures is related to soman's ability to irreversibly inhibit acetylcholinesterase (AChE), leading to an excessive accumulation of the neurotransmitter acetylcholine (ACh) in neural and myoneural junctions (Shih, 1982; Lallement et al., 1992; McDonough and Shih, 1997). However, the secondary recruitment of the excitatory amino acid glutamate following the early phase of cholinergically induced hyperexcitation is believed to be the cause of severe brain injury after soman exposure (Wade et al., 1987; Lallement et al., 1992; McDonough and Shih, 1993; Solberg and Belkin, 1997). McDonough and Shih (1993) reported that anticholinergic drugs scopolamine and atropine failed to block soman-induced seizures when given 40 min after onset of soman-induced seizures, but MK-801 (dizocilpine), a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, was effective in terminating seizures induced by soman. In addition, MK-801 has been shown to arrest soman-induced seizures and reduced neuronal necrosis, further supporting a role of glutamate in soman poisoning (Braitman and Sparenborg, 1989; Sparenborg et al., 1992).

Nerve agent-induced neuropathology was first described by Petras (1981), who demonstrated profound axonal degeneration in the brains of rats poisoned with soman that survived for 15-28 days. Subsequently, a number of laboratories have confirmed that brain damage is a pathological characteristic of animals surviving an acute soman exposure (Lemercier et al., 1983; McLeod et al., 1984; McDonough et al., 1987; Ballough et al., 1995). Although the regional brain pathology has been defined by these investigators, a time-course of brain injury induced by soman poisoning has not been extensively studied.

The presence of reactive astrocytes, activation of microglial cells and release of inflammatory mediators, independently of the primary insult, may contribute to the progression of neuronal injury (Willard et al., 1999; Rizzi et al., 2003; Ravizza et al., 2005). It has been shown that activation of astrocytes and microglia is a pathological feature of soman-induced seizures (Zimmer et al., 1997). Although evidence of neuroinflammation has been found following chemical nerve agent exposure (Williams et al., 2003; Chapman et al., 2006), information on the extent and progression of the inflammatory process in the damaged areas of the brain is still limited. The present study was designed to evaluate the time course of brain injury progression and neuroinflammatory mediator expression following soman-induced seizures. The piriform cortex was used to study the progression of injury as it is a brain region that consistently showed damage in the rat exposed to soman.

2.0 MATERIALS AND METHODS

Male Sprague-Dawley rats (CRL: CD[SD]-BR), weighing 200-225 g, were used in the study. All animals were housed in individual cages on a standard 12/12 h light/dark cycle with free access to food and water. All experiments were conducted in compliance with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Representative animals were surgically implanted with cortical screw electrodes for monitoring the time of seizure initiation. The animals were allowed to recover from the surgery for 7 days before experimentation. On the day of the experiment, animals were randomly divided into control and experimental animals. Experimental animals were further divided into groups to be euthanized at different time points after the onset

of seizures (30 min, 1 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr). Animals that were implanted with an electrode were connected to EEG recording apparatus, and baseline EEG activity and behavior were monitored for 15 min; animals that did not have electrode implants remained in their cages. All animals were then pretreated with the oxime HI-6 (125 mg/kg, ip) 30 min prior to soman challenge (1.6 LD50 or180 μg/kg, sc). One minute after soman injection, animals were treated with atropine methyl nitrate (AMN) (2.0 mg/kg, im). HI-6 and AMN were used to decrease the mortality of soman-exposed animals (Shih et al., 1991). The concentration of soman was selected because it has been shown to produce seizures in 100% of the animals (Shih et al., 1991). Vehicle control animals received an equivalent volume of vehicle, HI-6 and AMN.

2.1 Brain Tissue Procurement

Animals were anesthetized with sodium pentobarbital (65 mg/kg, ip) at designated time points after seizure onset. For immunohistochemical studies, animals were transcardially perfused with 0.9% saline, followed by 10% phosphate buffered formalin (10% PBF). Following perfusion, brains were removed immediately from the skull and further fixed in 10% PBF at 4°C for 18 hr. Brains were then coronally cut into 3mm slices using a rat brain metrix (ASI Instrument, Warren, MI), paraffin processed and serially cut at 5 μm. Sections between bregma -2.30 mm and -3.80 mm, as described by Paxinos and Watson (Paxinos and Watson, 1998) were subjected to MAP-2 immunohistochemistry. For multiplex array immunoassay, brain tissue was processed as previously described (Johnson et al., 2005). Briefly, the piriform cortex was excised, rinsed with cold PBS, snap frozen in liquid nitrogen and homogenized in ice-cold triple detergent lysis buffer containing a Complete™ protease inhibitor cocktail (Roche Biochemicals; Indianapolis, IN).

2.2 Microwave-Assisted MAP-2 Immunohistochemistry

Sections were dewaxed in xylene and then hydrated in decreasing concentrations of ethyl alcohol to distilled water. To suppress endogenous peroxidase activity, sections were incubated in 5% hydrogen peroxide at room temperature for 20 min. Following thorough washing in running tap water (5 min), sections were rinsed in dH_2O and treated in the microwave (Kan et al., 2005). Briefly, sections were boiled 2 times (5 min each time) in 10 mM citric acid (Sigma-Aldrich, St Louis, MO; Lot 30H-0627) in a microwave (Pelco 3440 Max, 800 watts; Ted Pella, Inc, Redding, CA), and cooled at room temperature for 20 min prior to immunohistochemical staining.

Indirect immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method of Hsu and co-workers (Hsu et al., 1981). Following microwave antigen retrieval, brain sections were incubated in 5% horse serum for 30 min at 4°C to block tissue immunoglobulins that could react with secondary antibody. Sections were then sequentially incubated in MAP-2 monoclonal antibody (1:100; Clone AP-18; NeoMarkers, Fremont, CA) for 18 hr at 4°C, biotinylated secondary antibody for 1 hr at room temperature (Vector, Burlingame, CA) and ABC solution for 30 min at room temperature (Vector, Burlingame, CA). Negative control sections were simultaneously processed without either MAR or primary antibody to ensure specificity of MAP-2 immunostaining.

2.3 Image Analysis of MAP-2 Immunoreactivity

MAP-2 immunoreactivity was evaluated on a BX61 light microscope mounted with a DP-70 digital color camera (Olympus America Inc., Melville, NY). Brightfield images of MAP-2 immunostaining of control and soman-exposed animals were captured after the white-balanced function was performed. All images were stored in the computer as TIFF files at a magnification of 200X and resolution of 1039 X 1063 pixels. Each pixel was calibrated at 0.1024 μm^2.

Color digital images were converted to 8-bit grayscale images using Adobe® Photoshop 7.0 (Adobe System Incorporated, San Jose, CA). Grayscale images were then enhanced to render the areas of MAP-2 immunostaining clearly distinguishable by human eye from the background using Adobe® Photoshop 7.0 (Adobe System Incorporated, San Jose, CA).

Areas of MAP-2 immunostaining in the piriform cortex were quantified using the ImagePro Plus software (Version 6.0, Media Cybernetic, Silver Spring, MD). Using the segmentation function, the threshold value was automatically selected by the software. The threshold value is where pixels with grayscale values below the threshold are included in the measurement of areas of MAP-2 immunostaining and those above the threshold are excluded in the measurement. The summation of all the values of the pixels reflects the total areas of MAP-2 immunostaining on the image. The average of total areas of MAP-2 immunostaining per group (control, 1 hr, 3 hr, 6 hr 12, 24 hr, 48 hr or 72 hr) is calculated based on an N of six animals.

2.4 Multiplex Array Immunoassay

The rat cytokine multiplex immunoassay kit (LINCO Research, St. Charles, MO) was used to quantify the levels of cytokines in piriform cortex tissue lysates. Individual cytokine standard curves were generated using the supplied reference cytokine concentrations in duplicate. The rat cytokine multiplex immunoassay procedure was conducted according to the manufacturer's instructions and each sample was assayed in duplicate. The plate was read on a Luminex™ 100 Instrument (Bio-Rad Laboratories, Hercules, CA) and analyzed with BioRad software.

2.5 Statistical Analysis

The differences in the mean areas of MAP-2 immunoreactivity between control and experimental groups collected by imaging analysis were compared by one-way analysis of variance (ANOVA) followed by multiple comparisons using a Tukey's test. For the multiplex immunoassay, data were evaluated by a one-way ANOVA with a post-hoc Dunnet analysis. In all analysis, p-values less than or equal to 0.05 were interpreted as being statistically significant.

3.0 RESULTS

Following soman exposure, all animals developed behavioral convulsions and electroencephalographic seizures within 4-7 min. The first notable sign of soman intoxication was chewing, which was quickly followed by head bobbing, tremor and convulsive seizures. Excessive salivation developed in all animals within 1-2 min after seizure onset. Seizures eventually progressed into status epilepticus that usually persisted $\geq$ 8 hr.

Twenty-five percent (25%) of the animals dosed with soman died even with HI-6 and AMN as pretreatment and posttreatment, respectively. The time of death after the onset of seizures ranged from minutes to hours. Animals that died within minutes had massive hemorrhagic pulmonary edema. Surviving animals commonly lost significant amounts of body weight 24 hr (12%) and 48 (16%) following soman intoxication. During the first two days, animals were hand-fed wet mash and water fortified with table sugar to encourage feeding and drinking. Normal eating and drinking behavoir were noted at 72 hr, at which point animals regained weight back and continued to gain weight (Unpublished observation).

3.1 Temporal Progression of MAP-2 Immunoreactivity

The extent of soman-induced damage in the piriform cortex was evaluated using MAP-2 immunohistochemistry. The time-course of MAP-2 immunoreactivity is qualitatively illustrated in Figure 1. In control sections, MAP-2 immunoreactivity was mostly observed in dendritic processes and faintly stained in neurons (Figure 1A). At 30 min after the onset of seizures, no discernible changes in MAP-2 were detected (Data not shown). At 1 hr after seizure onset, loss of dendritic MAP-2 immunostaining was detected in layer III of the piriform cortex (Figures 1B). This loss of dendritic MAP-2 immunostaining expanded to layer II at 3 and 6 hr after seizure onset (Figures 1C and 1D). In addition, at these time points, degenerated neurons were strongly MAP-2 positive. At 12 hr after seizure onset, loss of MAP-2 immunoreactivity was clearly observed in layers II and III of the piriform cortex (Figure 1E). At 24 hr, lesions had an appearance similar to that caused by hypoxia/ischemia, which consisted of an inner necrotic core (layer III), completely devoid of MAP-2 immunostaining and outer penumbra (Layer I) (Figure 1F). At 48 and 72 hr after seizure onset, MAP-2 immunoreactivity continued to decline (Data not shown).

Quantitative assessment of the total areas occupied by MAP-2immunostaining is shown in Figure 2. The average total area of MAP-2 immunostaining was reduced by 20% as early as 1 hr after seizure onset. The percentages of reduction in the area of MAP-2 immunostaining at 3 hr and 6 hr were 14% and 16%, respectively. However, the differences in the total areas of MAP-2 immunoreactivity between these time points were not statistically significant ($P>0.05$). The largest reduction (65%) in the area of MAP-2 immunostaining was found beginning at 12 hr after seizure onset. At 24 hr, the area occupied by MAP-2 immunoreactivity was decreased by 83%. At 48 hr and 72 hr, the percentages of MAP-2 loss were not significant as compared to 24 hr (Data not shown).

3.2 Temporal Expression of Inflammatory Mediators

Concentrations of TNF-α increased in the piriform cortex (Fig. 3, gray line) becoming significant at 3 hr (11 ± 2 pg/ml), peaking at 12 hr (32 ± 15 pg/ml) and declining to non-significance by 48 hr after seizure onset compared to vehicle controls (0.2 ± 0.5 pg/ml). Concentrations of IL-1α and β significantly increase 12 hr after seizure onset. Increases in IL-1β concentration were much less robust in the piriform cortex (41 ± 10 pg/ml v. 16 ± 10 pg/ml in vehicle controls; Fig. 3, black line) compared to IL-1 α (205 ± 99 pg/ml v. 14 ± 7 pg/ml in vehicle control; Fig. 4, gray line). Concentrations of the chemokine MIP-1α (Fig. 4, black line) significantly increased at 3 hr (149 ± 34 pg/ml), peaked at 24 hr (200 ± 83 pg/ml) and remained significant through the study endpoint of 72 hr after seizure onset (139 ± 53 pg/ml) compared to vehicle controls (0 ± 0 pg/ml). Lastly, significant increases were observed for the chemokine GRO KC (Fig. 5) which peaked at 6 hr (1164 ± 477 pg/ml) and remained significantly elevated up to 24 hr (501 ± 431 pg/ml) after seizure onset compared to vehicle controls (49 ± 23 pg/ml).

4.0 DISCUSSION

All animals exposed to 1.6 LD50 of soman developed convulsive seizures as indicated by both behavioral signs and electrocortical activity. Behavioral signs of convulsion were similar to those described in experiments using the same dosing regimen (McDonough and Shih, 1993). All animals that died after soman-induced seizures had bloody, foamy nasal discharge. Necropsy of these animals showed various degrees of hemorrhagic pulmonary edema (HPE). The extent of HPE was generally severe in all lung lobes in animals

that died within the first 30 min after the onset of seizures. We concluded that respiratory failure due to the rapid development of HPE is a cause of death in soman-poisoned rats despite HI-6 and AMN treatments.

Loss of MAP-2 staining is widely used as a sensitive indicator of brain damage in various experimental conditions (Kitagawa et al., 1989; Matesic and Lin, 1994; Ballough et al., 1995; Posmantur et al., 1996; Folkerts et al., 1998). Our results show a time-course pattern of MAP-2 immunostaining. Although there was an initial decrease in MAP-2 immunoreactivity at 1 hr after the onset of seizures (-20%), the percentage of MAP-2 immunoreactivity reduction was less dramatic at 3 hr (-14%) and 6 hr (-16%). The reason for At these time points (3 and 6 hr), neuronal cell bodies exhibited an increase in MAP-2 immunostaining, which countered the reduction of MAP-2 immunoreactivity in the neuronal processes and accounts for the stabilization of total MAP-2 immunoreactivity in the sections. The increase in neuronal MAP-2 immunoreactivity may represent a compensatory response to facilitate recovery of neuronal injury induced by soman-induced seizures. However, loss of MAP-2 immunoreactivity found at 12 hr and longer surviving time points may reflect a failure of neurons to elicit a successful compensation, leading to brain injury. It is important to point out that MAP-2 loss is a result of neuronal degeneration and not due to the loss of immunoreactive antigenicity. It was found that the number of FJ B-positive neurons was increased in brain areas where MAP-2 immunoreactivity was decreased (Unpublished data).

Neuroinflammation has been observed in many models of CNS tissue injury and brain damage including brain trauma, stroke and excitotoxicity (Morganti-Kossmann et al., 2001, Allan and Rothwell, 2001; Frijns and Kappelle, 2002; Wang and Shuaib, 2002; Williams et al., 2007). Concurrently, upregulation of cytokines is also a common event following CNS injury and seizures (Wang and Shuaib, 2002; Lehtimaki et al., 2003; Shinoda et al., 2003; Williams et al., 2003) and their presence is toxic to neural tissues (Dommergues et al., 2000; Allan and Rothwell, 2001; Lenzlinger et al., 2001; Wang and Shuaib, 2002). Our results show a profound up-regulation of the cytokines TNF-α, IL-1α and β and the chemokines MIP-1α and GRO KC. These increases start as early as 3 hr after seizure onset but do not peak until 12 hr after seizure onset or later. Up-regulation of the chemokines MIP-1α and GRO KC is important in recruiting and confining leukocytes to sites of injury to further enhance the inflammatory response through microglial activation. Up-regulation of the acute phase response (APR) cytokines, the main amplifiers of the inflammatory process, correlates well with the pathology seen with MAP-2 loss in the piriform cortex. Though it is unknown whether this inflammation is a reaction to or the cause of this pathology, the potential neurotoxic properties of IL-1α/β and TNF-α suggest that inflammation likely plays some role in the progression of pathology (Viviani et al., 2004). In addition, these observations suggest that anti-inflammatory drugs may attenuate brain damage following soman-induced seizure.

In conclusion, we have shown that HPE contributes to rapid death in approximately 25% of soman-exposed animals despite antidote treatments to include HI-6 and AMN. MAP-2 is a sensitive marker for brain injury in the piriform cortex caused by soman-exposure that decreases substantially by 12 hr after seizure onset. Concomitantly, there is an increase in neurotoxic cytokines in the same region suggesting that inflammation may play a role in the pathology progression. These data suggest that anti-inflammatory therapeutics may be beneficial to attenuate the brain pathology associated with soman exposure and that the therapeutic window may be open as late as 6 hr after seizure onset, a time before the loss of MAP-2 is extensive and the expression of inflammatory mediators peaks.

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Figure 1: Temporal Alterations in MAP-2 Immunoreactivity in Piriform Cortex

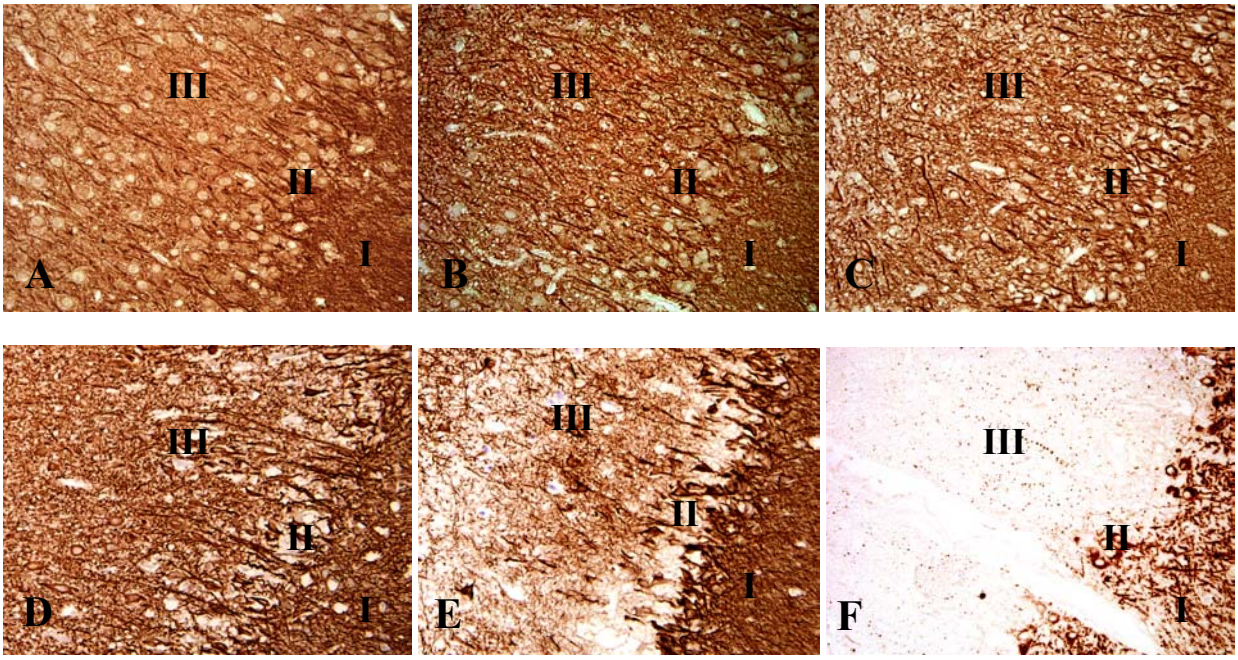

Figure 1. Time-course MAP-2 immuoreactivity in piriform cortex of vehicle control (A) and at 1 hr (B), 3 hr (C), 6 hr (D), 12 hr (E) and 24 hr (F) after soman-induced seizure onset. Vehicle control (A) sections show a typical pattern of MAP-2, predominantly in neuronal dendritic processes. At 1 hr after onset of seizures, there is a loss of MAP-2 immunoreactivity in layer III. At 3 and 6 hr, increase in neuronal MAP-2 staining in conjunction with loss of MAP-2 immunoreactivity is observed. At 12 hr, MAP-2 loss in layers II and III is pronounced and degenerated neurons are intensely labeled with MAP-2. By 24 hr, only scattered punctuate MAP-2 immunostaining remains in layer III and loss of MAP-2 immunoreactivity has spread to layer I.

Figure 2: Quantification of MAP-2 Immunoreactivity in Piriform Cortex

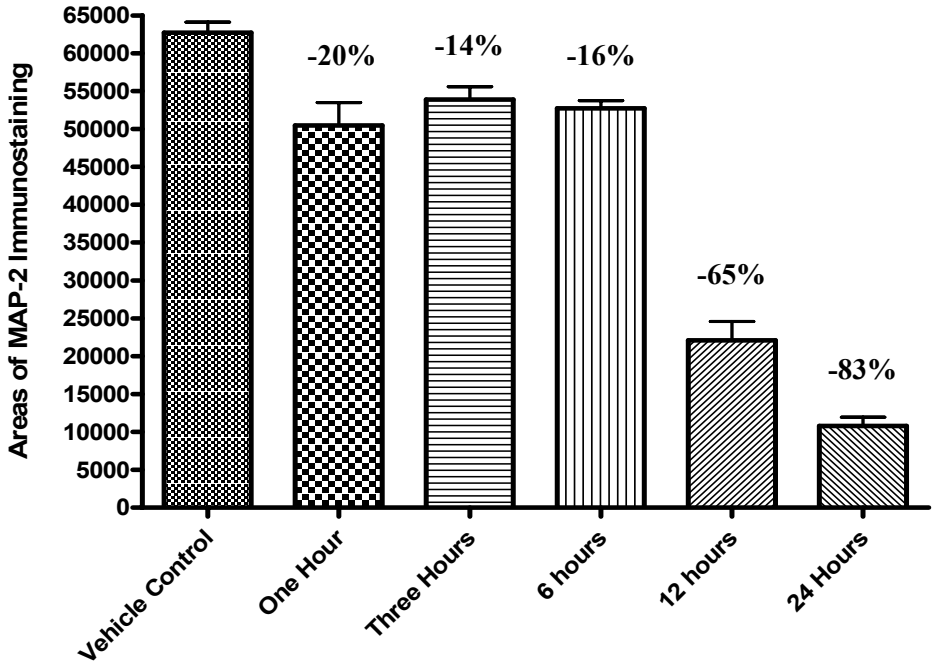

Figure 2. MAP-2 immunoreactivity in piriform cortex following soman-induced seizures. A significant reduction in MAP-2 immunoreactivity was observed as early as 1 hr after seizure onset ($p<0.01$). No further decrease in MAP-2 immunostaining was detected at 3 and 6 hr. The most significant decrease in MAP-2 immunoreactivity commenced at 12 hr ($p<0.001$). At 24 hr, MAP-2 immunoreactivity continued to decline ($p<0.001$).

Figure 3: Temporal Changes in Concentration of TNF-α and IL-1β in Piriform Cortex

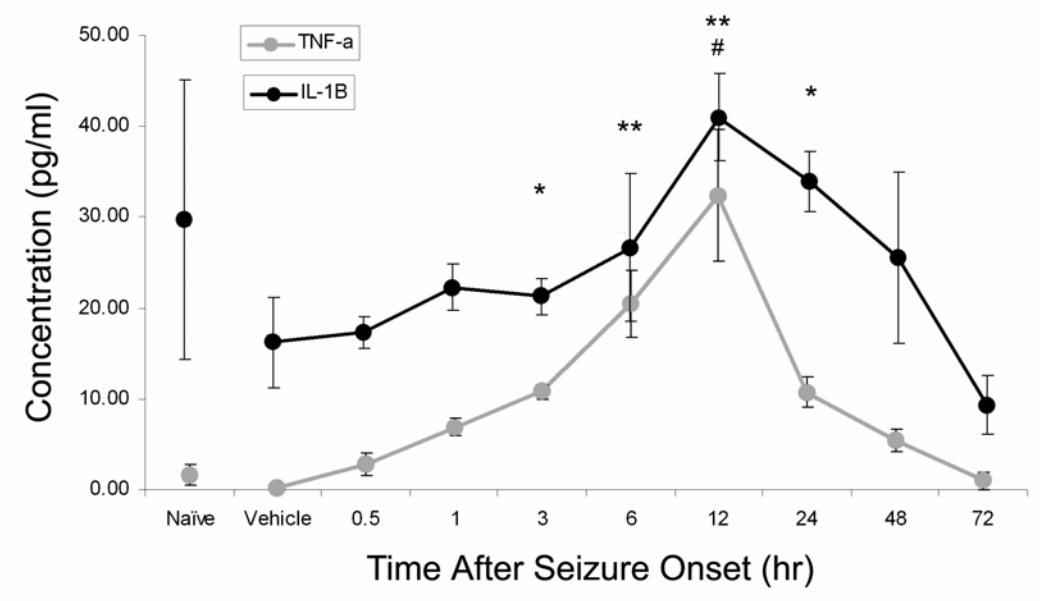

Figure 3: The concentration of TNF-α and IL-1β significantly increase in the piriform cortex following soman induced seizure. Multiplex immunoassay analysis in piriform cortex reveals significant increases in the acute phase response cytokines TNF-α (gray line) and IL-1β (black line). Data are given as pg/ml of tissue lysate; each group represents data from an $n \geq 5$ and is reported as mean ± STDEV. *$p<0.05$, ** $p<0.01$ versus vehicle control for TNF- α; # $p<0.05$ versus vehicle control for IL-1β.

Figure 4: Temporal Changes in Concentration of IL-1α and MIP-1α in Piriform Cortex

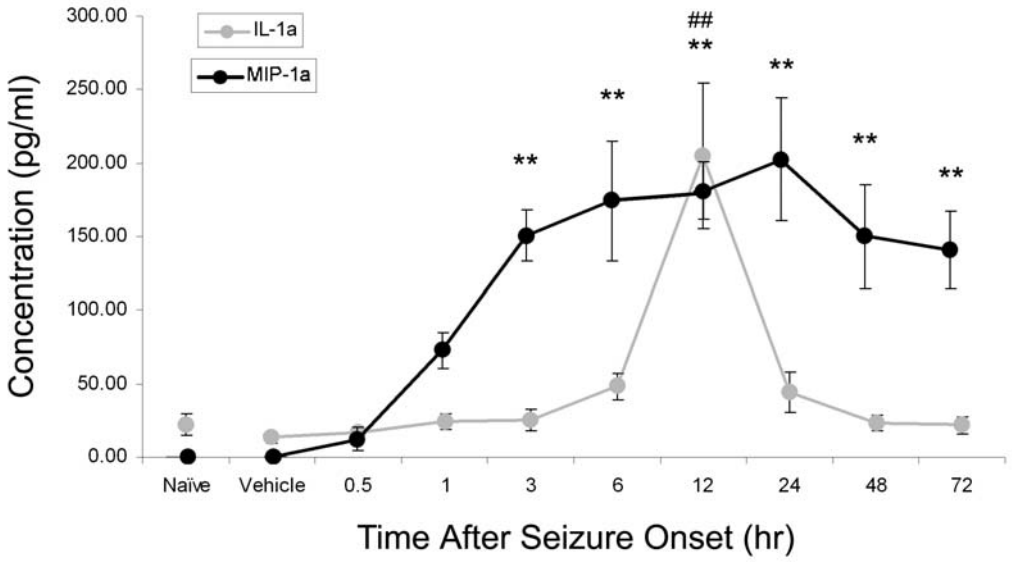

Figure 4: The concentration of MIP-1α and IL-1α significantly increase in the piriform cortex following soman induced seizure. Multiplex immunoassay analysis in piriform cortex reveals significant increases in the chemokine MIP-1α (black line) and the cytokine IL-1α (gray line). Data are given as pg/ml of tissue lysate; each group represents data from an $n \geq 5$ and is reported as mean ± STDEV. ** $p<0.01$ versus vehicle control for MIP-1α; ## $p<0.01$ versus vehicle control for IL-1α.

Figure 5: Temporal Changes in Concentration of GRO KC in Piriform Cortex

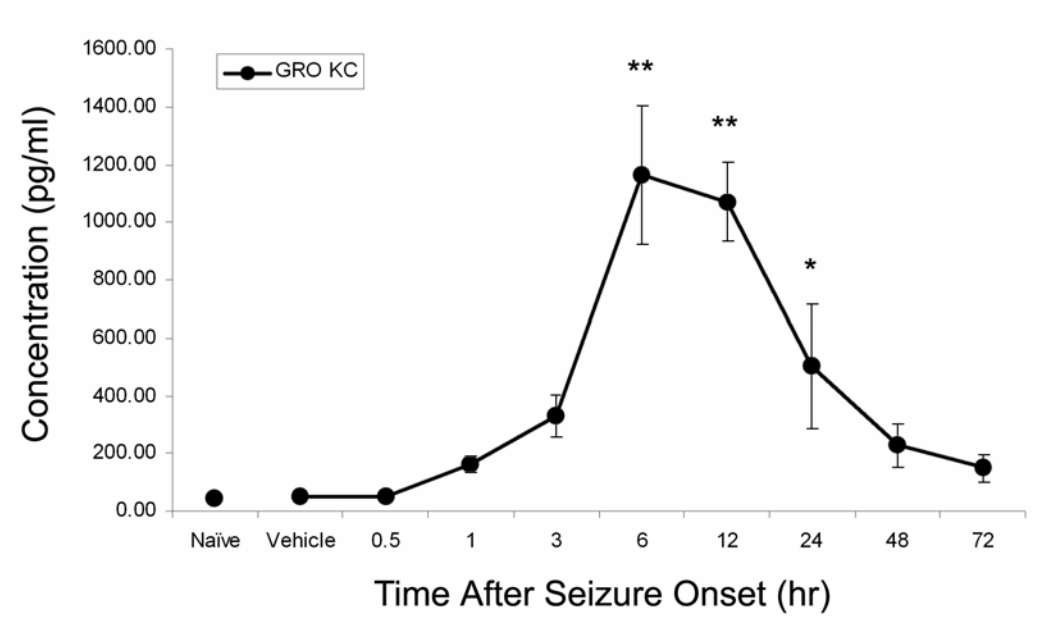

Figure 5: The concentration GRO KC significantly increases in the piriform cortex following soman induced seizure. Multiplex immunoassay analysis in piriform cortex reveals a significant increase in the chemokine GRO KC. Data are given as pg/ml of tissue lysate; each group represents data from an $n \geq 5$ and is reported as mean ± STDEV. *$p<0.05$, ** $p<0.01$ versus vehicle control.