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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 the nerve agent soman, at a dose that induce SE, 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 were taken at 24 h, 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 h 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 h and throughout the 30-day period. There was no significant loss of GABAergic interneurons in the BLA at 24 h 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 h, 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.

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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 × 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–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 s and centrifuged at 14,000 *g* 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 ethopropazine (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 s 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 h, 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 Fluro-Jade-C staining or were stored at −20 °C in a cryoprotectant solution for GAD-67 immunohistochemistry (Figueiredo et al., 2011b).