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Pathophysiological Mechanisms Underlying Increased Anxiety after Soman Exposure: Reduced GABAergic Inhibition in the Basolateral Amygdala

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Abstract

The recent sarin attack in Syria killed 1,429 people, including 426 children, and left countless more to deal with the health consequences of the exposure. Prior to the Syrian chemical assault, nerve agent attacks in Japan left many victims suffering from neuropsychiatric illnesses, particularly anxiety disorders, more than a decade later. Uncovering the neuro-pathophysiological mechanisms underlying the development of anxiety after nerve agent exposure is necessary for successful treatment. Anxiety is associated with hyperexcitability of the basolateral amygdala (BLA). The present study sought to determine the nature of the nerve agent-induced alterations in the BLA, which could explain the development of anxiety. Rats were exposed to soman, at a dose that induced prolonged status epilepticus. Twenty-four hours and 14-days after exposure, neurons from the BLA were recorded using whole-cell patch-clamp techniques. At both the 24 h and 14-day post-exposure time-points, the frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) in the BLA were reduced, along with reduction in the frequency but not amplitude of miniature IPSCs. In addition, activation of α_7 -nicotinic acetylcholine

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receptors, a cholinergic receptor that participates in the regulation of BLA excitability and is involved in anxiety, increased spontaneous excitatory postsynaptic currents (sEPSCs) in both soman-exposed rats and controls, but was less effective in increasing sIPSCs in soman-exposed rats. Despite the loss of both interneurons and principal cells after soman-induced status epilepticus, the frequency of sEPSCs was increased in the soman-exposed rats. Impaired function and cholinergic modulation of GABAergic inhibition in the BLA may underlie anxiety disorders that develop after nerve agent exposure.

Keywords

soman; basolateral amygdala; anxiety; GABA_A receptors; inhibition; alpha7 nicotinic receptors

1. Introduction

Many survivors of the August 2013 chemical nerve agent attack, which killed 1,429 Syrians including 426 children, will have to deal with the health consequences of the exposure (Dolgin, 2013). Nerve agents are organophosphorus compounds that irreversibly inhibit acetylcholinesterase (AChE). In the brain, accumulation of acetylcholine at cholinergic synapses and the resulting hyperstimulation of cholinergic receptors raise the excitation level to the point of seizure generation. If pharmacological intervention is not immediate, cholinergic receptor hyperstimulation gives way to excessive activation of the glutamatergic system, which sustains and intensifies seizure activity (status epilepticus, SE), causing profound brain damage (McDonough et al., 1995; McDonough and Shih, 1997; Prager et al., 2014; Shih et al., 2003) and long-term behavioral deficits (Collombet et al., 2008; Filliat et al., 2007).

Currently approved antidotes can be effective in suppressing seizure activity if given immediately after an exposure (Dolgin, 2013). However, civilians are unequipped to self-administer antidotes after an attack, and delays of at least 30 min may occur before emergency personnel can safely assess victims to provide initial treatments (Myhrer, 2010; Yanagisawa et al., 2006). Delaying treatment increases the incidence of neuropathology, as it has been observed in animal models of nerve agent exposure; seizures lasting 20 min cause brain damage in approximately 10% of animals, whereas 40 min of continuous seizures causes damage in approximately 79% of animals (McDonough et al., 1995). If seizures are not controlled, 98% of surviving animals suffer moderate to severe neuropathology, evident within 24 hours after nerve agent exposure (Figueiredo et al., 2011; McDonough et al., 1995; Prager et al., 2013, 2014), and go on to develop long-term neurological and behavioral impairments. Victims of the Tokyo and Matsumoto sarin attacks, for example, reported symptoms associated with anxiety disorders over a decade after the attacks (Hoffman et al., 2007; Ohtani et al., 2004). Similarly, animals display enduring anxiety and fear-like behaviors after exposure to soman (Coubard et al., 2008; Filliat et al., 2007; Langston et al., 2012; Moffett et al., 2011; Prager et al., 2014). The mechanisms underlying the development of anxiety after nerve agent exposure are unknown. Knowledge of these mechanisms is necessary for successfully treating or preventing the development of this behavioral impairment.

Anxiety disorders, including posttraumatic stress disorder (PTSD) – the most prevalent neuropsychiatric deficit present in the sarin attack victims in Japan (Yanagisawa et al., 2006) – are associated with amygdalar hyperactivity (Koenigs and Grafman, 2009) and volumetric loss (Rogers et al., 2009). The basolateral nucleus of the amygdala (BLA), in particular, is recognized for its central role in the generation and expression of anxiety and fear (Davis et al., 1994; Etkin and Wager, 2007; LeDoux, 2003; Phelps and LeDoux, 2005; Rauch et al., 2000; Shekhar et al., 2005; Stein and Stein, 2008). Because GABAergic inhibition in the BLA plays a key role in regulating network excitability (Lang and Pare, 1998; Spanpanato et al., 2011; Woodruff and Sah, 2007) and anxiety-like behavior (Truitt et al., 2009), we investigated alterations in GABAergic inhibitory activity in the rat BLA, 14 days after exposure to the nerve agent soman, a time-point when anxiety has already developed (Prager et al., 2014). To obtain an insight into the time course and the nature of the progression of these alterations, we also performed electrophysiological studies at 24 hours after exposure. In addition, we examined whether soman exposure alters the function of α_7 -nicotinic acetylcholine receptors (α_7 -nAChRs), which are involved in anxiety-like behavior (Feuerbach et al., 2009), and were recently shown to modulate GABAergic inhibition in the BLA (Pidoplichko et al., 2013). Our results revealed deficits in GABAergic inhibitory synaptic transmission, diminished α_7 -nAChR-mediated enhancement of GABAergic activity, and increased spontaneous excitatory activity in the BLA after soman exposure.

2. Materials and Methods

2.1 Animals

Experiments were performed using 27–30 day old (~75 g) male, Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA). 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 Experimental Procedures

2.2.1 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 (149 $\mu\text{g/kg}$, which is an approximate dose of $1.35 \times \text{LD}_{50}$; Figueiredo et al., 2011; Jimmerson et al., 1989), to 78 rats. Rats were monitored for signs of seizure onset (on the average, SE was initiated 5.76 ± 1.37 min after exposure), 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 (Apland et al., 2010; Racine et al., 1977; Racine, 1972). Seizures were monitored behaviorally; however, we have previously shown a good correlation between “behavioral seizures” and electrographic seizures (Figueiredo et al., 2011; Prager et al., 2013). In this manuscript, the term “seizures” used in describing the results refers to the behaviorally monitored seizures/convulsions. Rats were administered 2 mg/kg atropine sulfate (Sigma, St. Louis MO) at the onset of stage 3 seizures. Stage 3 seizures correspond to the initiation of SE (Figueiredo et al., 2011; Prager et al., 2014). Of the 78 animals exposed to soman, 69 animals developed SE, while 12 rats survived a prolonged (8 to 10 hours) SE. These 12 rats were randomly divided into either the 24-hour soman group or the 14-day soman group (n = 6 per group). The surviving rats were monitored and given lactated Ringer’s solution (5 ml), every 8 hours; this continued up to 3 days after exposure, at which time body weight started increasing. Control animals received atropine sulfate, and were injected with saline instead of soman.

2.2.2 Electrophysiological Experiments—Twenty-four hours or 14 days after soman exposure, animals were 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 (Leica VT 1200 S; Leica Microsystems, Buffalo Grove, IL), in ice-cold cutting solution consisting of (in mM): 115 sucrose, 70 NMDG, 1 KCl, 2 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 30 NaHCO₃. The slices were transferred to a holding chamber, at room temperature, in a bath solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 21 NaHCO₃, 2 CaCl₂, 1 MgCl₂, and 11 D-glucose. 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. The recording chamber (0.7 mL capacity) had continuously flowing ACSF (~6–8 mL/min) at temperature 32–33°C. The osmolarity of this external solution was adjusted to 325 mOsm with D-glucose.

Whole-cell recordings were obtained from neurons visualized under infrared light using Nomarski optics of an upright microscope (Zeiss Axioskop 2, Thornwood, NY) through a 40x water immersion objective, equipped with a CCD-100 camera (Dage-MTI, Michigan City, IN). The patch electrodes had resistances of 3.5–4.5 M Ω when filled with the internal solution (in mM): 60 CsCH₃SO₃, 60 KCH₃SO₃, 10 KCl, 10 EGTA, 10 HEPES, 5 Mg-ATP, 0.3 Na₃GTP (pH 7.2), 290 mOsm (Pidoplichko et al., 2013). When sIPSCs and sEPSCs were recorded simultaneously, the internal chloride concentration was 1 mM, and osmolarity was adjusted with potassium gluconate. Tight-seal (over 1 G Ω) whole-cell recordings were obtained from the cell body of pyramidal neurons in the BLA region and were identified on the basis of their electrophysiological properties (Park et al., 2007; Sah et al., 2003). Access resistance (15–24 M Ω) was regularly monitored during recordings, and cells were rejected if the resistance changed by more than 15% during the experiment. Ionic currents and action potentials were amplified and filtered (1 kHz) using the Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with a four-pole, low-pass Bessel filter, were digitally sampled (up to 2 kHz) using the pClamp 10.2 software (Molecular Devices, Sunnyvale, CA), and analyzed using the Mini Analysis program (Synaptosoft Inc., Fort Lee, NJ), Origin (OriginLab Corporation, Northampton, MA), or GraphPad Prism (GraphPad Software, La

Jolla, CA). In some experiments, the charge transferred by postsynaptic currents was calculated, using the Mini60 software by Synaptosoft (Synaptosoft Inc., Fort Lee, NJ).

Drugs used were as follows: bicuculline methiodide, a GABA_A receptor antagonist, atropine sulfate, a muscarinic AChR antagonist, dihydro- β -erythroidine (DH β E), an $\alpha_4\beta_2$ nicotinic receptor antagonist, choline chloride, an α_7 agonist, and CNQX, an AMPA/kainate receptor antagonist (all purchased from Sigma-Aldrich, St. Louis; MO). We also used D-AP5, an NMDA receptor antagonist, SCH50911, a GABA_B receptor antagonist, α -conotoxin Au1B, an $\alpha_3\beta_4$ nicotinic receptor antagonist, and LY 341495, a metabotropic glutamate group II/III receptor antagonist (all purchased from Tocris, Ellisville, MO).

2.3 Statistical Analysis

Statistical values are presented as mean $\pm$ standard error of the mean (SEM). Analyses were conducted using a one-way ANOVA followed by Tukey or Dunnett T post-hoc tests. Results were considered statistically significant when $P < 0.05$. Sample size “ n ” refers to the number of recorded neurons.

3. Results

Of the 78 animals exposed to soman, 69 animals developed SE, within 5 min after exposure. Many of these animals died within 10 to 20 min after the initiation of SE, while 12 rats survived a prolonged (8 to 10 hours) SE. These 12 rats were randomly divided into either the 24-hour soman group or the 14-day soman group ($n = 6$ per group). Age-matched rats that were not exposed to soman served as controls.

3.1 Diminished GABAergic Inhibition after Soman-Induced SE

To determine whether GABAergic inhibition was reduced after the prolonged SE induced by exposure to soman, we recorded GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) from principal neurons in the BLA, in the presence of α -conotoxin Au1B (1 μ M), DH β E (10 μ M), atropine sulfate (0.5 μ M), D-AP5 (50 μ M), CNQX (20 μ M), SCH50911 (10 μ M), and LY 341495 (3 μ M) at a holding potential of +30 mV. Principal neurons were identified based on their firing pattern in response to depolarizing current pulses in the current clamp mode, and the presence of the I_h current activated by hyperpolarizing voltage-steps, in the voltage-clamp mode (Aroniadou-Anderjaska et al., 2012; Park et al., 2007; Pidoplichko et al., 2013; Sah et al., 2003). The mean frequency of sIPSCs was reduced from 11.35 ± 0.61 Hz in the control rats ($n = 20$), to 6.63 ± 0.88 Hz ($n = 18$; $P < 0.001$) at 24 hours after soman exposure, and 7.78 ± 0.58 Hz ($n = 24$; $P = 0.001$) at 14 days after exposure, reflecting a $41.5 \pm 7.7\%$ and $32.3 \pm 5.2\%$ reduction at the 24 hour and the 14 day time points, respectively (Fig. 1A and B). The mean amplitude of sIPSCs in the soman-exposed rats was also reduced compared to controls (75.5 ± 9.2 pA), by $37.2 \pm 3.9\%$ (47.4 ± 2.9 pA; $P = 0.005$) at 24 hours, and $35.0 \pm 4.7\%$ (49.1 ± 3.5 pA; $P = 0.005$) at 14 days after exposure (Fig. 1A and C). There were no significant differences between soman-exposed rats and control rats in the rise time and the decay time constant of the sIPSCs. These results suggest a reduced inhibitory tone of BLA pyramidal cells at 24 hours and 14 days after SE.

To determine whether the reduction of sIPSCs was associated with a decreased responsiveness of postsynaptic GABA_A receptors or reduced presynaptic GABA release, we recorded action potential-independent, miniature IPSCs (mIPSCs) from BLA pyramidal neurons of soman-exposed and control rats. mIPSCs were recorded at a holding potential of +30 mV in the presence of α -conotoxin Au1B (1 μ M), DH β E (10 μ M), atropine sulfate (0.5 μ M), D-AP5 (50 μ M), CNQX (20 μ M), SCH50911 (10 μ M), LY 3414953 (3 μ M), and TTX (0.5 μ M). The mean frequency of mIPSCs was reduced from 7.41 ± 0.47 Hz in control rats ($n = 20$) to 3.19 ± 0.24 Hz ($n = 17$; $P < 0.001$) at 24 hours, and 4.75 ± 0.28 Hz ($n = 24$; $P < 0.001$) at 14 days after exposure, reflecting a $57.0 \pm 3.2\%$ and $36.0 \pm 3.8\%$ reduction at the two time points, respectively (Fig. 2A and B). There were no significant differences in mIPSC amplitude between the SE groups (24-hour, 34.9 ± 0.76 pA; 14 day, 37.6 ± 1.4 pA) and the controls (35.9 ± 1.3 pA; P 's > 0.05) (Fig. 2C). In addition, there were no significant differences between rats exposed to soman and control rats in the rise time and the decay time constant of the mIPSCs. Thus, the reduced frequency and amplitude of sIPSCs (Fig. 1) did not involve a decrease in the responsiveness of postsynaptic GABA_A receptors.

3.2 Impaired α_7 -nAChR Mediated Enhancement of Inhibitory Synaptic Transmission after Soman-Induced SE

The α_7 -nAChRs are expressed on both GABAergic interneurons and glutamatergic neurons in the BLA (Klein and Yakel, 2006; Pidoplichko et al., 2013); activation of these receptors enhance both spontaneous excitatory postsynaptic currents (sEPSCs) and sIPSCs, but the increase of sIPSCs is significantly greater than the increase of sEPSCs (Pidoplichko et al., 2013). To determine if the modulation of GABAergic transmission by α_7 -nAChRs had been altered in the soman-exposed rats, we bath applied choline chloride while recording from principal neurons, in the presence of α -conotoxin Au1B (1 μ M), DH β E (10 μ M), atropine sulfate (0.5 μ M), D-AP5 (50 μ M), CNQX (20 μ M), SCH50911 (10 μ M), and LY 3414953 (3 μ M), at a +30 mV holding potential. Choline chloride (5 mM) was bath-applied at a flow rate of ~ 8 ml/min. The effect was immediate and consisted of the transient appearance of a barrage of sIPSCs (Fig. 3A), as we have observed previously (Pidoplichko et al., 2013). To quantify the effects, we calculated the total charge transferred during choline application. The charge, in pico Coulombs (pC), was calculated as the area delimited by the inhibitory current and the baseline (Pidoplichko et al., 2013). The measurement of the charge transferred was made using a 30 second time window from the peak of the effect. In the control slices, choline application increased the charge transferred increased to $8,872.3 \pm 809.8$ pC (from a baseline of 224.4 ± 48.5 pC; $P < 0.001$; paired t-test; $n = 17$). Twenty-four hours and 14 days after soman exposure, the charge transferred in the presence of choline chloride increased from 58.4 ± 10.6 pC to $3,986.4 \pm 671.6$ pC ($n = 16$) and from 73.7 ± 10.1 pC to $6,262.2 \pm 684.3$ pC ($n = 22$; P 's < 0.001 ; paired t-test), at the two time points, respectively. The total charge transferred by GABA_A current in the presence of choline was significantly smaller in the 24-hour soman ($P < 0.001$) and the 14-day soman group ($P = 0.032$) compared to the controls (Fig. 3B). In addition, the mean amplitude of the sIPSCs recorded in the presence of choline was significantly reduced at 24 hours (95.7 ± 6.9 pA; $P = 0.037$) but not at 14 days (121.6 ± 7.8 pA; $P = 0.938$) after exposure, compared to controls (125.3 ± 8.2 pA) (Fig. 3C). These results suggest that the α_7 -nAChR mediated enhancement of GABAergic inhibition is impaired after soman exposure.

To determine if the α_7 -nAChR-mediated enhancement of excitatory activity was also impaired in the soman-exposed rats, we recorded sEPSCs and sIPSCs simultaneously from principal neurons in the presence of α -conotoxin Au1B (1 μ M), DH β E (10 μ M), atropine sulfate (0.5 μ M), D-AP5 (50 μ M), SCH50911 (10 μ M), and LY 3414953 (3 μ M), at a holding potential of -58 mV and internal chloride concentration of 1 mM. Bath application of choline chloride (5 mM) increased both sIPSCs and sEPSCs (Fig. 4). The current areas were analyzed for a time period of 5 seconds from the start of the response. The increase in charge transferred by sEPSCs in control slices (from 4.0 ± 0.6 pC to 15.5 ± 1.9 pC), was not significantly different from the increase in the 24-hour soman group (from 3.9 ± 0.5 pC to 14.3 ± 1.7 pC), or the 14-day soman group (from 3.1 ± 0.5 pC to 11.7 ± 1.4 pC; P 's > 0.05 , Fig. 4D). On the other hand, the charge transferred by sIPSCs in the presence of choline chloride was increased from 3.3 ± 0.7 pC to 137.9 ± 28.9 pC in the control rats ($n = 12$), from 3.0 ± 0.5 pC to 70.2 ± 10.5 pC in the 24 hour soman group ($n = 16$, $P = 0.017$ compared to controls), and from 1.1 ± 0.3 pC to 47.4 ± 7.3 pC in the 14 day soman group ($n = 14$, $P = 0.002$ compared to controls; Fig. 4E). Thus, SE induced by soman exposure does not impair the α_7 -nAChR-mediated enhancement of excitatory activity in the BLA.

3.3 Increased Spontaneous Excitatory Activity after Soman-Induced SE

After soman-induced SE, the BLA suffers significant loss of both principal cells and interneurons (Figueiredo et al., 2011; Prager et al., 2014). The loss of principal neurons could produce a reduction in tonic glutamatergic activity. However, this could be compensated by the concomitant loss of interneurons and the decreased tonic inhibition (Fig. 1 and 2). To determine whether there are alterations in the tonic glutamatergic activity in the BLA after soman exposure, we recorded sEPSCs from principal neurons in the presence of α -conotoxin Au1B (1 μ M), DH β E (10 μ M), atropine sulfate (0.5 μ M), D-AP5 (50 μ M), SCH50911 (10 μ M), and LY 3414953 (3 μ M), at a holding potential of -58 mV and internal chloride concentration of 1 mM. The mean frequency of sEPSCs was 6.3 ± 0.5 Hz in the control rats, 11.9 ± 1.3 Hz in the 24 hour soman group ($P < 0.001$ compared to controls), and 9.7 ± 1.6 Hz in the 14 day soman group ($P = 0.040$ compared to controls), reflecting a $87.7 \pm 21.0\%$ and $54.0 \pm 25.4\%$ increase after soman exposure, at 24 hours and 14 days, respectively (Fig. 5A). There were also differences between the control group and the soman-exposed rats in the amplitude of the sEPSCs. The mean amplitude of sEPSCs was 12.8 ± 0.3 pA in control rats ($n = 28$), 14.1 ± 0.6 pA in the 24 hour soman group ($n = 16$; $P = 0.172$) and 14.8 ± 1.0 pA in the 14 day soman group ($n = 14$; $P = 0.031$). The amplitude distribution in the three groups is shown in Fig. 5B.

4. Discussion

The most debilitating neuropsychiatric symptoms reported by victims of the Japan sarin attack, more than a decade later, were indicative of anxiety disorders, including PTSD (Yanagisawa et al., 2006). However, the pathophysiological mechanisms underlying these enduring emotional deficits have been unknown. This is the first study to provide evidence that anxiety disorders caused by nerve agent exposure are probably due to reduced GABAergic inhibition in the BLA and the increases in spontaneous excitatory activity. The observed decrease in spontaneous inhibitory synaptic transmission after soman exposure

was not due to postsynaptic alterations in GABA_A receptor function, as there was no significant decrease in the amplitude of GABA_A receptor-mediated mIPSCs. We also found that activation of α_7 -nAChRs by choline chloride was less effective in enhancing inhibitory activity in the soman-exposed rats compared to controls, while there was no significant difference between the groups in the enhancement of sEPSCs upon α_7 -nAChR activation. Spontaneous glutamatergic activity was enhanced in the BLA of the soman-exposed rats, which was probably a result of the decreased inhibitory tone. As anxiety disorders are associated with increased excitatory activity and excitability in the BLA (Etkin and Wager, 2007; Koenigs and Grafman, 2009; Phelps and LeDoux, 2005), the data presented here explain the development of anxiety after nerve agent exposure.

The presence of anxiety disorders that ensued in the Japan sarin-attack victims (Hoffman et al., 2007; Ohtani et al., 2004) was accompanied by reduced regional gray matter volumes in the amygdala (Rogers et al., 2009). Similar observations have been made in animals, where exposure to soman has caused amygdalar atrophy (Carpentier et al., 2008; Gullapalli et al., 2010) and enduring increases in anxiety- and fear-like behaviors (Coubard et al., 2008; Filliat et al., 2007; Langston et al., 2012; Moffett et al., 2011; Prager et al., 2014). The decreases in gray matter volume after nerve agent exposure probably reflect the death of principal neurons and GABAergic interneurons (Figueiredo et al., 2011; McDonough et al., 1995; Prager et al., 2014; Shih et al., 2003). GABAergic interneurons in the BLA account for approximately 15–20% of the total neuronal population (McDonald and Mascagni, 2002; Sah et al., 2003; Spanpanato et al., 2011), and tightly regulate principal neuron excitability (Lang and Pare, 1997; Pan et al., 2009; Popescu and Pare, 2011; Smith et al., 1998). Our results suggest that the dramatic reduction in spontaneous GABAergic activity after soman exposure, due to deficits in GABA release and/or the loss of GABAergic interneurons (Figueiredo et al., 2011; Prager et al., 2014), disrupts the capability of inhibitory inputs to regulate excitatory neurons in the BLA, causing a significant increase in glutamatergic activity; this can promote hyperactivity in the BLA network and ultimately result in the development of anxiety (Davis et al., 1994; Etkin and Wager, 2007; LeDoux, 2003; Rauch et al., 2006; Villarreal and King, 2001). In consistency with this view, it has been previously shown that damage to GABAergic interneurons in the BLA leads to anxiety-like behavior (Truitt et al., 2009).

Interestingly, after soman-induced SE, there is a significant loss of principal BLA neurons already at 24 hours after the exposure, but the number of GABAergic interneurons is not reduced at this early time point; when examined 7 days later, interneurons are also significantly reduced, and by 14 days after exposure, the ratio of the number of GABAergic interneurons to the total number of neurons in the BLA is significantly decreased (Prager et al., 2014). The results presented here indicate that tonic GABAergic activity is already dramatically decreased at 24 hours after exposure, despite that there is no evidence of interneuronal death at this time point, suggesting that the function of interneurons is already compromised. Considering that there is no significant downregulation or internalization of GABA_A receptors –as the stable amplitude of mIPSCs suggest– the reduced frequency and amplitude of sIPSCs and the reduced frequency of mIPSCs at 24 hours post-exposure would suggest dysfunction at the presynaptic GABAergic terminals, and perhaps deficits in quantal

release of GABA, as it has been observed in models of temporal lobe epilepsy (Hirsch et al., 1999).

Previous studies have shown that soman administered directly to brain slices (Wang et al., 2011), or epileptiform activity and SE induced by other mechanisms (Goodkin et al., 2008; Goodkin et al., 2005; Naylor et al., 2005) lead to internalization of GABA_A receptors in the hippocampus and reduced effectiveness of GABA agonists. However, recovery in the expression of GABA_A receptors has also been observed to occur rapidly upon cessation of SE (Naylor, 2010). The lack of a significant alteration in the amplitude of the mIPSCs, in the present study, may imply either that the BLA differs from the hippocampus in how the GABA_A receptors are affected by seizure activity, or that at the point of time we studied the pathophysiology of GABAergic transmission, postsynaptic changes in GABA_A receptors had been restored. Among other mechanisms, such a recovery could also involve movement of extrasynaptic GABA_A receptors into postsynaptic sites (Goodkin et al., 2005, 2008; Pavlov et al., 2011; Yu et al., 2013).

A number of modulatory systems are involved in regulating BLA excitability, mainly by affecting the excitability state of the principal cells and/or the GABAergic interneurons. The cholinergic input to the amygdala from the basal forebrain appears to be of major importance in this regard, as suggested by the remarkably dense cholinergic innervation of the BLA (Mesulam et al., 1992), its exceptionally high AChE activity (Ben-Ari et al., 1977; Prager et al., 2013; Prager et al., 2014), as well as the increase in anxiety-like behavior when the function of the basal forebrain is disrupted (Martinowich et al., 2012). The most well known behavioral evidence for the importance of the cholinergic system in the regulation of amygdalar function comes from the symptoms of Alzheimer's disease, where anxiety is prevalent (España et al., 2010; Ferretti et al., 2001). In the case of anxiety that develops after nerve agent exposure, its association with impairments in the cholinergic modulation of the BLA would not be surprising, particularly considering that inhibition of AChE is the primary effect of nerve agents, and AChE in the BLA remains inhibited for about a week (Prager et al., 2013); the resulting long-term derangements in cholinergic activity are likely to affect the function of the cholinergic system. From the different types of cholinergic receptors, the α_7 -nAChRs have been shown to play an anxiolytic role (Bencan and Levin, 2008; Feuerbach et al., 2009), and we recently demonstrated that in the rat BLA, α_7 -nAChR activation increases both sIPSCs and sEPSCs, but the net effect is a transient, dramatic increase of GABAergic activity (Pidoplichko et al., 2013). Here, we found that after exposure to soman, the α_7 -nAChR-mediated enhancement of inhibitory activity – but not the excitatory activity – is impaired. The most plausible explanation of these results is that the loss of principal BLA neurons in the soman-exposed rats (Prager et al., 2014) is not high enough to significantly impair the increase of excitatory activity upon α_7 -nAChR activation, while the 46% loss of interneurons (Prager et al., 2014) has more profound consequences, including an impaired enhancement of inhibitory activity when α_7 -nAChRs are activated in the BLA network. The involvement of other mechanisms, such as differential alterations in the function or density of α_7 -nAChRs on BLA interneurons versus principal cells, due to the prolonged inhibition of AChE (Prager et al., 2014) and the ensuing derangements in acetylcholine concentrations in the vicinity of the receptors, cannot be excluded. The

impaired modulation of GABAergic inhibition by α_7 -nAChRs after soman exposure may contribute to BLA hyperexcitability and the development of anxiety.

A major health concern for survivors of the Syrian nerve agent attack is the development of anxiety disorders. Even people who do not develop severe symptoms after the exposure may develop anxiety, as suggested by the high percentage (32–65%) of victims of the Japan sarin attacks, who went on to develop symptoms associated with PTSD (Hoffman et al., 2007; Ohtani et al., 2004), and suffered neuropathological alterations such as volumetric loss in the amygdala and cingulate cortex (Rogers et al., 2009). Thus far, no effective treatments against chronic neuropsychiatric and neurological symptoms caused by nerve agent exposure have been developed (Chen, 2012; Dolgin, 2013). Individuals with anxiety disorders are often treated with pharmacological agents that target the GABAergic system to enhance inhibitory transmission and thus reduce anxiety (Davidson et al., 2007; Ravindran and Stein, 2009). Epidemiological studies have shown that treatments targeting the GABAergic system have repeatedly failed to alleviate symptoms associated with PTSD (Braun et al., 1990; Cates et al., 2004; Davidson et al., 2007; Ravindran and Stein, 2009). While unclear why these drugs fail to mitigate the majority of symptoms associated with PTSD, our study suggests that a severely impaired inhibitory function in the BLA may be a reason for the lack of effectiveness of these compounds. Our results also suggest that targeting excitatory synaptic transmission may be a more direct and efficacious therapeutic strategy to reduce BLA hyperexcitability and the expression of anxiety in PTSD patients after nerve agent exposure. Following this strategy, a new class of therapeutic options can become available. For example, antagonists of kainate receptors containing the GluK1 subunit are not effective only against nerve agent-induced seizures and brain damage (Figueiredo et al., 2011), but they also have anxiolytic effects (Aroniadou-Anderjaska et al., 2012).

5. Conclusions

The present study showed that exposure to a nerve agent, at a dose that induces prolonged SE, damages the function of the GABAergic system in the BLA, resulting in significantly increased tonic excitatory activity. This is evidence of BLA hyperexcitability, which is a characteristic feature of anxiety disorders (Etkin and Wager, 2007; Rauch et al., 2000; Shekhar et al., 2005; Stein and Stein, 2008). These findings, therefore, suggest that the development of anxiety disorders after nerve agent exposure, as seen in the victims of the sarin attacks in Japan, is not necessarily due to the stress component of the experience, but rather it can be due to the damage of the GABAergic system by the seizures induced by the nerve agent. It is conceivable that any brain insult that produces long-term hyperexcitability in the BLA, by impairing GABAergic inhibition and/or by other mechanisms, can have the potential to culminate in development of anxiety. Our findings also suggest that because GABAergic inhibition in the BLA can be severely damaged after nerve agent exposure, a more efficacious approach to treating anxiety in exposure victims may be to suppress glutamatergic excitatory activity.

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Highlights

- Soman exposure reduces GABAergic inhibitory transmission in the basolateral amygdala
- The frequency but not amplitude of miniature IPSCs is also reduced
- Modulation of GABAergic synaptic transmission by α_7 -nAChRs is impaired
- Spontaneous excitatory transmission is increased in the basolateral amygdala
- These alterations are likely to explain the development of anxiety after exposure

Figure 1.

Exposure to soman reduces the frequency and amplitude of GABA_A receptor mediated spontaneous IPSCs. Recordings are from BLA principal cells at a holding potential of +30 mV and internal chloride concentration of 10 mM. **(A)** Representative traces of sIPSCs recorded from BLA pyramidal cells in slices obtained from control animals and from animals at 24 hours and 14 days after soman exposure. **(B)** Significant decreases in the frequency of sIPSCs was observed at 24 hours ($n = 18$; 6.63 ± 0.88 Hz; $P < 0.001$) and 14 days ($n = 24$; 7.78 ± 0.58 Hz; $P = 0.001$) after soman exposure compared to controls ($n = 20$; 11.35 ± 0.61 Hz). **(C)** Cumulative probability amplitude distributions of sIPSCs. The mean amplitude of sIPSCs was significantly reduced at 24 hours (47.39 ± 2.91 pA; $P = 0.005$) and 14 days (49.06 ± 3.52 pA; $P = 0.005$) after exposure compared to controls (75.51 ± 9.20 pA). *** $P < 0.001$ (One-way ANOVA with Tukey post hoc).

Figure 2.

Exposure to soman reduces the frequency but not the amplitude of GABA_A receptor mediated miniature IPSCs. Recordings are from BLA principal cells at a holding potential of +30 mV and internal chloride concentration of 10 mM. **(A)** Representative traces of mIPSCs recorded from BLA pyramidal cells, in slices obtained from control animals and from animals at 24 hours and 14 days after soman exposure. **(B)** A significantly lower frequency of mIPSCs was observed at 24 hours ($n = 17$; 3.19 ± 0.24 Hz; $P < 0.001$) and 14 days ($n = 24$; 4.75 ± 0.28 Hz; $P < 0.001$) after exposure, compared to controls ($n = 20$; 7.41 ± 0.47 Hz). **(C)** Cumulative probability amplitude distributions of mIPSCs. *** $P < 0.001$ (One-way ANOVA with Tukey post hoc).

Figure 3.

Exposure to soman impairs the α_7 -nAChR-mediated enhancement of spontaneous inhibitory synaptic transmission. Recordings are from BLA principal cells at a holding potential of +30 mV and internal chloride concentration of 10 mM. **(A)** Bath application of choline chloride (5 mM) increased the frequency and amplitude of sIPSCs in control slices, as well as in slices obtained from animals at 24 hours and 14 days after soman exposure. **(B)** The increase in the charge transferred by sIPSCs upon application of choline chloride was significantly lower at 24 hours after soman exposure ($n = 16$, $P < 0.001$) and 14 days after exposure ($n = 22$, $P = 0.032$) compared to the controls ($n = 17$). **(C)** Cumulative probability amplitude distributions of sIPSCs in the presence of choline. The mean amplitude of sIPSCs was significantly reduced at 24 hours ($P = 0.037$), but not at 14 days ($P = 0.938$) after exposure, compared to controls. * $P < 0.05$; *** $P < 0.001$ (One-way ANOVA with Dunnett T post hoc).

Figure 4.

The α_7 -nAChR-mediated enhancement of spontaneous excitatory synaptic transmission is not impaired after soman exposure. Recordings were obtained from principal neurons at a holding potential of -58 mV and internal chloride concentration of 1 mM. (A–C) Bath application of choline chloride (5 mM) induced a transient increase in the frequency and amplitude of both sIPSCs (outward currents) and sEPSCs (inward currents). (D) The charge transferred by sEPSCs during choline application was not significantly altered at 24 hours or 14 days after soman exposure compared to controls. (E) The charge transferred by sIPSCs was significantly reduced at 24 hours ($n = 16$; $P = 0.017$) and 14 days ($n = 14$; $P = 0.002$) after soman exposure compared to controls ($n = 12$). * $P < 0.05$; ** $P < 0.01$ (One-way ANOVA with Dunnett T post hoc).

Figure 5.

Spontaneous excitatory synaptic transmission is increased after soman exposure. Recordings were obtained from principal neurons at a holding potential of -58 mV and internal chloride concentration of 1 mM. **(A)** The frequency of sEPSCs was greater at 24 hours ($P < 0.001$; $n = 16$) and 14 days ($P = 0.04$; $n = 14$) after soman exposure, compared to controls ($n = 28$).

(B) Amplitude-frequency histograms showing the effects of soman on sEPSCs, at 24 hours ($n = 16$) and 14 days ($n = 14$) after exposure, compared to controls ($n = 28$). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (One-way ANOVA with Dunnett T post hoc).