NeuroToxicology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Early changes in M2 muscarinic acetylcholine receptors (mAChRs) induced by sarin intoxication may be linked to long lasting neurological effects

Zipora Pittel*, Shlomi Lazar, Rellie Gez, Shira Chapman

Department of Pharmacology, Israel Institute for Biological Research, P.O. Box 19, Ness Ziona, 74100, Israel

ARTICLE INFO

Article history:
Received 18 July 2017
Received in revised form 5 November 2017
Accepted 5 November 2017
Available online xxx

Keywords:
M2 muscarinic acetylcholine receptor
Sarin
Long lasting neurological effects
Organophosphate compounds
Brain damage

ABSTRACT

The effect of sarin on the binding parameters (KD & Bmax) of M2 muscarinic acetylcholine receptor (mAChR) was studied 24 h and 1 week post exposure. Male & female Sprague-Daweley rats were poisoned with 1XLD50 sarin (80 μg/kg, im) followed by treatment of trimedoxime bromide and atropine (7.5:5 mg/kg, im) 1 min later. Brains were removed and analyzed for M2 mAChR binding, using [^{3}H] AFDX384, an M2 selective antagonist. A significant increase in KD of M2 mAChR was found in the cortex 24 h post poisoning, displaying elevation from 4.65 ± 1.16 to 8.45 ± 1.06 nM and 5.24 ± 0.93 to 9.29 ± 1.56 nM in male and female rats, respectively. A rise in KD was also noted 1 week following exposure from 5.04 ± 1.20 to 11.75 ± 2.78 and from 5.37 ± 1.02 to 11.66 ± 1.73 nM, presenting an added increase of 51 and 40% (compared to 24 h) in males and females, respectively. Analysis of M2 receptor density (Bmax) revealed a significant reduction of 68% in males and insignificant reduction of 22% in females, 24 h after sarin exposure which was followed by 37% recovery in males and 100% recovery in females, 1 week later. These results indicate that sarin induces a long-term decreased affinity in M2 mAChR (elevated KDs) and a transient effect on the number of this receptor subtype (Bmax). We hypothesize that the reduced affinity of the M2 receptors (negative auto-regulatory receptors) may cause long-term brain deficits by impairing the normal regulation release of ACh into the synaptic cleft.

1. Introduction

Sarin, a chemical warfare of the organophosphate (OP) family known as a potent nerve agent, irreversibly inhibits the cholinesterase (ChE) enzyme (Kadar et al., 1995) and consequently prevents the hydrolysis of acetylcholine (ACh). Hence, ACh accumulates in the synapse and triggers the activation of the muscarinic and nicotinic ACh receptors. Additionally, sarin binds to the M2 muscarinic acetylcholine receptor (mAChR) in the brain (Bakry et al., 1988; Pittel et al., 2006) and in the heart (Silveira et al., 1990). Other OP compounds, such as soman, VX and echothiopate also bind M2 mAChR in the brain (Pittel et al., 2006). In addition, pesticides such as paraoxone and parathion were reported to bind mAChR in the brain (Abdalla et al., 1992; Jett et al., 1994). These findings support the notion that OP nerve agents bind and regulate muscarinic receptors. The M2 receptors function as negative auto-regulatory receptors and are activated when excess of ACh accumulates in the synaptic cleft, inhibiting additional release of the neurotransmitter. However, when sarin binds the M2 mAChR, it blocks the ability of the endogenous neurotransmitter, ACh, to bind the receptor and thereby neutralizes the negative feedback process, leading to an excess of ACh in the synaptic cleft.

Activation of the muscarinic and nicotinic receptors after OP poisoning quickly leads to toxic signs which include: diarrhea, excess mucus, sweating, tachycardia, hypertension, seizures, tremors and hyper-salivation (McDonough and Shih, 1997; Lallement et al., 1998; Bajgar, 2004). Severe toxic signs induce loss of consciousness and possible death due to depression of the respiratory system. Moreover, accumulation of ACh leads to excitation of other systems like the glutamatergic system causing convulsions and inflammation (Weissman and Raveh, 2008; McDonough and Shih, 1997; Lallement et al., 1998; Bajgar, 2004). Brain damage with neuronal degeneration was established in various animal models after OP exposure (McLeod et al., 1984; Carpentier et al., 1990, 2008; Clement and Broxup, 1993; Kadar

Abbreviations: ACh, acetylcholine; ChE, cholinesterase; GPCR, G-protein-coupled receptors; mAChR, muscarinic acetylcholine receptor; OP, organophosphate; TA, mixture of trimedoxime bromide and atropine; CIE, clathrin-independent endocytosis; CDE, clathrin-dependent endocytosis.

* Corresponding author.
E-mail addresses: zipip@iibr.gov.il, pitelzipi@hotmail.com (Z. Pittel).

https://doi.org/10.1016/j.neuro.2017.11.002
0161-813X/

et al., 1995; Baze, 1993; Dorandeu et al., 2005; Myhrer et al., 2007; Grauer et al., 2008; Lazar et al., 2016; Aroniadou-Anderjaska et al., 2009; De Araujo et al., 2010).

Soldiers exposed to sub-lethal doses of sarin and cyclosarin during the Gulf War and examined several years later showed neuronal malfunctions and behavioral disturbances (Proctor et al., 2006; Chao et al., 2010; Loh et al., 2010). MRI of their brains revealed structural and degenerative changes (Chao et al., 2011, 2014). In addition, decreased cognitive functions were found in subjects of the terror attacks in Japan long after the attack (Okumura et al., 2003) which deteriorated with time (Yokoyama, 2007; Tochigi et al., 2002; Miyaki et al., 2005).

The increased level of ACh, caused by sarin in the synaptic cleft is the main reason for those injuries (Fryer and Wils-Karp, 1991; Fryer et al., 2004; Lein and Fryer, 2005; Lee et al., 2004). In this regard the M2 mAChR is playing a pivotal role as a negative auto-receptor controlling the release of this neurotransmitter (Zhang et al., 2016; Santafe et al., 2006; Tomas et al., 2014; Nadal et al., 2016). We focused in this work on the early changes occurring in the M2 receptors after sarin poisoning, since immediate changes may lead to understanding of the causes for long term alterations. We followed the binding parameters of the M2 receptor by its ability to bind a ligand (characterized as KD) and by the number of these receptors in the cortex (characterized as Bmax). Since previous reports found gender differences in the toxicity of sarin (Smith et al., 2015; Wright et al., 2017), we have conducted this study in both, male and female rats.

2. Experimental procedures

2.1. Animals

All procedures in which animals were used, tested, or sacrificed were carried out in accordance with appropriate standards for animal use, and were in accordance with the Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC, 1996. The protocols were approved by the Animal Care and Use Committee of the Israel Institute for Biological Research. Male and female albino Sprague-Dawley (SD) rats weighing 300–350 g and 250–275 g, respectively, were purchased from Envigo RMS, Israel. Animals were housed on bedding in plastic (UTEMP) 3 per cage, in a controlled environment with a constant temperature of 21 ± 2 °C and 12 h light/dark cycle. Cages are made of polycarbonate, half transparent, temperature-resistant up 132 °C, floor area 0.8 m^2 enabling housing of 3 rats weighing up to 400 g/each (manufactured by Technoplast Industries, Israel). Food (Purina Chow rat) and water were available ad lib. Sarin-poisoned and non-poisoned rats were weighed before exposure and thereafter at day 1, 2, 4 and 7 (at 11 a.m.).

2.2. Materials

[^{3}H]AFDX384 (100–160 Ci/mmol) was purchased from Perkin Elmer (USA). Sarin (*O*-isopropyl methyl phosphonofluoridate) was supplied by the Organic Chemistry department of the Israel Institute for Biological research (IIBR), prepared according to the accepted synthetic procedure using methylphosphonodifluoride (Monard and Quinchon, 1961). Sarin was at least 96% pure, based on their ^{1}H and ^{31}P NMR. Net sarin was dissolved in propylene glycol and kept frozen (−20° C) till use. Fresh dilute sarin solutions were prepared in cold sterile saline (160 μg/ml) before administration to rats. Atropine sulfate and trimedoxime bromide (TMB4) were purchased from Sigma. Solutions of atropine (20 mg/ml) and TMB4 (30 mg/ml) were prepared in sterile cold saline. All ingredients for preparing Tris buffer were purchased from Sigma.

2.3. Sarin exposure

Male and female rats were poisoned by sarin (1XLD50: 80 μg/kg, intra muscular (im)) and then injected im with a mixture of 7.5 mg/kg TMB4 and 5 mg/kg atropine (TA) one min later. These doses of TA were previously verified as ensuring survival following sarin poisoning (Chapman et al., 2006, 2015). However, all the surviving rats exhibited convulsions, indicating that the central nervous system was not protected by TA. Rats were transferred to a separate cage immediately after the exposure and stayed there for 1 h for monitoring toxic signs before returning the rats to their original cages. Lethality rate was 17% in males within 24 h and 0% in females. Rats were sacrificed by decapitation 24 h and 7 days post sarin exposure and brains from poisoned and non-poisoned rats were taken for biochemical evaluation (see below).

2.4. Preparation of cortical membranes for binding assay

Twenty four hours and 7 days after sarin administration, rats were sacrificed by decapitation and their cortices were removed on ice. Cortical membranes were prepared as described previously by Pittel et al. (1990). Briefly, the cortex was homogenized using a glass homogenizer in 20 mM Tris buffer pH 7.4 (10% w/v) and centrifuged at 35,000*g* at 4 °C for 10 min. Then, the pellet was resuspended, frozen at −70° C, thawed at 4° C, re-centrifuged, suspended with Tris buffer (1:6 w/v) and kept frozen at −70° C until the binding assay was carried out. Membranes were thawed and diluted to give a final concentration of 25 mg tissue/ml in the reaction mixture.

2.5. Binding assays using [^{3}H]AFDX384

Binding assays were employed using the membranes as described above in Section 2.4. [^{3}H]AFDX384 was used at final concentrations of 0.1–10 nM (six concentrations for each cortex). The assay was carried out at a final volume of 0.5 ml in 20 mM Tris buffer pH 7.4 containing 1 mM EDTA and 2 mM $MnCl_2$ (Miller et al., 1991). The total and non-specific binding for each concentration was determined in absence or presence of 10 μM atropine (this high concentration of atropine was employed deliberately and intentionally in order to achieve non-specific binding for the Scatchard analysis, to obtain total displacement and thus to achieve high signal to noise ratio). The reaction mixture containing cortical membranes and the specified concentrations of the radioligand was incubated for 1 h at 25 °C. The incubation period was terminated by transferring the tubes to an ice bath and then the bound material was trapped on GF/B filter (purchased from Tamar, Jerusalem, Israel) using a Brandell Cell Harvester system (M-24R, MD, USA). The filter was pre-soaked in 0.5% polyethyleneimine for 1 h to prevent adsorption of the labeled ligand onto the glass fiber and thereby to reduce the non-specific binding. The filter was dried and punched into vials and 5 ml scintillation solution (Aqueous Fluid, Packard 299^{TM}) was added. Radioactivity was measured using the Scintillation Analyzer Tri-carb 1600 TR, Packard. GraphPad Prism 4.0 program was employed to obtain binding parameters (KD and Bmax values) exerted from linear regression curves (Scatchard curves).

2.6. Protein concentrations

Total protein concentration of the cortical isolates used in the binding assays were determined employing the Bradford method (Bradford, 1976). Bmax values obtained from Scatchard curves (see Section 2.5) were normalized to the protein amount found for each specific cortex and were given in units of fmoles/mg protein.

Please cite this article in press as: Z. Pittel, et al., Early changes in M2 muscarinic acetylcholine receptors (mAChRs) induced by sarin intoxication may be linked to long lasting neurological effects, Neurotoxicology (2017), https://doi.org/10.1016/j.neuro.2017.11.002