PORTLAND PRESS

Clinical Science

ACCEPTED MANUSCRIPT

Glufosinate aerogenic exposure induces glutamate and IL-1 receptors dependent lung inflammation.

Maillet I., Perche O., Pâris A., Richard O. , Gombault A. , Herzine A., Pichon J. , Huaux F. , Mortaud S., Ryffel B., Quesniaux VFJ., MontécotDubourg C

Glufosinate-ammonium (GLA), the active component of an herbicide, is known to cause neurotoxicity. GLA shares structural analogy with glutamate. It is a powerful inhibitor of glutamine synthetase (GS) and may bind to glutamate receptors. Since these potentials targets of GLA are present in lung and immune cells, we asked whether airway exposure to GLA may cause lung inflammation in mice. A single GLA exposure (1mg/kg) induced seizures and inflammatory cell recruitment in the broncho-alveolar space, increased myeloperoxidase (MPO), inducible NO synthase (iNOS), interstitial inflammation and disruption of alveolar septae within 6 to 24h. Interleukin 1β (IL-1β) was increased and lung inflammation depended on IL-1 receptor 1 (IL-1R1). We demonstrate that glutamate receptor pathway is central, since the N-methyl-D-aspartate (NMDA) receptor inhibitor MK-801 prevented GLA- 2 induced lung inflammation. Chronic exposure (0.2 mg/kg 3x per week for 4 weeks) caused moderate lung inflammation and enhanced airway hyperreactivity with significant increased airway resistance. In conclusion, GLA aerosol exposure causes glutamate signaling and IL-1R dependent pulmonary inflammation with airway hyperreactivity in mice.

Cite as Clinical Science (2016) DOI: 10.1042/CS20160530

Glufosinate aerogenic exposure induces glutamate and IL-1 receptors dependent lung inflammation.

Maillet I. [1,2], Perche O. [1,2,3], Pâris A. [1,2], Richard O. [1,2], Gombault A. [1,2] , Herzine A. [1,2] Pichon J. [1,2], Huaux F.[4], Mortaud S. [1,2], Ryffel B. [1,2], Quesniaux VFJ.[1,2], Montécot-Dubourg C. [1,2]

Affiliations

(1) UMR7355, CNRS, Orleans, France.
(2) INEM, Experimental and Molecular Immunology and Neurogenetics, University of Orleans, 3b rue de la Férollerie, 45071 Orleans Cedex 2, France.
(3) Genetics Department, Regional Hospital, 14 Avenue de l'Hôpital, 45100 Orleans, France.
(4) Louvain centre for Toxicology and Applied Pharmacology (LTAP), Institut de Recherche Expérimentale et Clinique (IREC) Avenue E. Mounier, 53 bte B1.52.12 B-1200-Bruxelles

Corresponding author

Montécot-Dubourg C UMR7355, INEM, CNRS and University of Orleans, 3B rue de la Ferollerie, F-45071 Orleans Cedex 2, France. Tel: 33 (0)2 38 25 79 30, Mail: celine.dubourg@cnrs-orleans.fr

Abstract

Glufosinate-ammonium (GLA), the active component of an herbicide, is known to cause neurotoxicity. GLA shares structural analogy with glutamate. It is a powerful inhibitor of glutamine synthetase (GS) and may bind to glutamate receptors. Since these potentials targets of GLA are present in lung and immune cells, we asked whether airway exposure to GLA may cause lung inflammation in mice. A single GLA exposure (1mg/kg) induced seizures and inflammatory cell recruitment in the broncho-alveolar space, increased myeloperoxidase (MPO), inducible NO synthase (iNOS), interstitial inflammation and disruption of alveolar septae within 6 to 24h. Interleukin 1β (IL-1β) was increased and lung inflammation depended on IL-1 receptor 1 (IL-1R1). We demonstrate that glutamate receptor pathway is central, since the N-methyl-D-aspartate (NMDA) receptor inhibitor MK-801 prevented GLA-

induced lung inflammation. Chronic exposure (0.2 mg/kg 3x per week for 4 weeks) caused moderate lung inflammation and enhanced airway hyperreactivity with significant increased airway resistance. In conclusion, GLA aerosol exposure causes glutamate signaling and IL-1R dependent pulmonary inflammation with airway hyperreactivity in mice.

Short title: Lung inflammation after aerosol glufosinate exposure

Keywords: pesticide, glufosinate ammonium, glutamate receptors, lung, inflammation, IL1β

Abbreviations list:

BAL: Broncho alveolar lavage, BALF: Broncho alveolar lavage fluid, CNS: central nervous system, EEG: Electroencephalogram, GLA: Glufosinate-ammonium, GS: Glutamine synthetase, IL : interleukin, IL-1R1 : IL-1 receptor 1, i.n. : Intranasal , iNOS: inducible NO synthase, i.p.: Intraperitoneally, KC: chemokine CXCL1, Mch: methacholine, MMP9 : Matrix metallopeptidase 9, MPO: myeloperoxidase, NMDAR : N-methyl-D-aspartate receptor, TIMP-1: metalloproteinase-1.

Introduction

Pesticides are highly reactive and exhibit undesirable effects on non-target organisms such as mammals including humans. Toxicity of pesticides in man is observed at low dose exposure and is a recognized occupational hazard in farm workers representing a high-risk group in terms of public health. Aerial spraying is a major source of exposure (1-3). A large number of epidemiological studies reported that long-term exposure to pesticides is associated with respiratory diseases (4, 5) and immunotoxicity (6, 7), including upper respiratory tract disorders such as rhinitis and lower airway disorders such as wheeze, asthma (1, 8) and airway obstruction (9). Experimental data are rather limited. In guinea pigs, organophosphate based insecticides cause airway hyperactivity (10). In mice, chronic exposure to glyphosate-rich air from farms, as well as to pure glyphosate induces type 2 airway inflammation associated with induction of IL-33 and TSLP (11).

Glufosinate-ammonium (GLA) is the active compound of a worldwide-used broad-spectrum herbicides commercially known as BASTA® or LIBERTY® (Bayer-Crop Science). It belongs to the organophosphorous family that inhibits irreversibly the activity of glutamine synthetase (GS) in plants, thereby blocking the synthesis of glutamine from glutamate and ammonia (12). Subsequent intracellular accumulation of ammonia causes tissue necrosis and death of the plant. GS is also present in almost every tissue in all animal species including mammals and particularly in lungs (13, 14). GLA is an analogue of glutamate, the most abundant and important excitatory neurotransmitter in the human central nervous system (CNS). Although widely found in the CNS, glutamate is also found in peripheral tissues (15-17). In particular, the lungs are among the organs outside the CNS where glutamate signaling plays a role both under physiological and pathologic conditions (18). Glutamate receptors have been found not only in the lung and the airways (19, 20), but also in immune cells (21, 22). Upon acute GLA poisoning, patients develop neurological symptoms (23-25), and respiratory dysfunction (26, 27). While neurological effects of GLA exposure are well documented (28-31), the effects on the respiratory system are largely unknown.

Here we investigated the pulmonary effects of intranasal (i.n.) GLA exposure and characterized mechanisms of GLA action on the lung. We first assessed the effects

of an acute GLA exposure (1mg/kg, i.n.) on lung inflammation and function. Then, we determined the role of glutamate receptors on these pulmonary effects by using specific antagonists and the role of interleukin (IL-1β) by using IL1R1 KO mice.

To mimic the chronic exposure of agricultural communities to aerosol pesticides, we next studied the consequences of long term airway exposure at low dose (i.e. 0.2 mg/kg 3x per week) over 4 weeks on lung inflammation and airway resistance using invasive plethysmography.

Materials and methods

Mice and *in vivo* protocols

C57BL/6 (B6) mice were bred in a specific pathogen-free animal facility at CNRS TAAM (UPS44, Orléans) and used at 8-10 weeks of age. Mice were maintained in a temperature-controlled (23°C) facility with a 12-hour light/dark cycle and were given free access to food and water. Experimental protocols complied with the French government's ethical and animal experiment regulations, Ethics CLE CCO 2015-1087. Mice deficient for IL-1R1 (32) were bred in the same animal facility.

Glufosinate ammonium (GLA) (PESTANAL®, Riedel-de Haën, Sigma-Aldrich, Isle d'Abeau, France) was given in 40 µL of saline solution by an intranasal administration (i.n.) to isoflurane anesthetized mice. Control mice received 40 µL saline vehicle. For acute studies, a single dose of GLA was administered at 1mg/kg and mouse behavior was observed during 6 h.

To exclude the potential link between seizure and lung inflammation, mice were treated with a convulsive dose (75 mg/kg) of GLA by intraperitoneal (i.p.) injection and lung inflammation assessed.

To address the role of glutamate receptors in lung response to GLA, MK-801 maleate, a potent selective, non-competitive NMDA receptor antagonist (Abcam, Paris,France; 1 mg/ kg, i.p.) or MPEP a potent and highly selective non-competitive antagonist at the mGlu5 receptor subtype (Tocris, Bio-Techne, Lille, France; 10 mg/ kg, i.p.) were administered 30 min prior GLA instillation.

For chronic studies, GLA was administered 3 times a week for 4 weeks at 0.2mg/kg i.n., a low dose avoiding the convulsions that are observed at higher doses.

Electroencephalogram recordings

Under isoflurane anesthesia (1.5%), mice (n=6) were implanted with three monopolar surface electrodes placed in the cranial bone. Two electrodes were set bilaterally over the parietal cortex and a ground electrode was placed over the frontal cortex. Electrodes were made of a tungsten wire (diameter 250 µm) soldered to a male connector (Wire-pro, Farnell, Villefranche sur Saône, France). They were inserted in the skull so that only the tip (0.5 mm) protruded onto tissue. The electrodes were glued to the skull with cyanoacrylate and dental cement. EEG was recorded on freely moving mice, after a recovery period of 7-10 days. EEG was monitored for a seven and half hours lasting period using data acquisition system (PowerLab 16/30, AD Instruments). The biological signals from the electrodes were amplified and filtered (0.1–50 Hz bandpass) using BioAmp amplifiers (AD Instruments). Then the EEG signal was digitized at a sampling rate of 1024. The digitized signals were displayed and stored on a computer. The EEG activity was analyzed with LabChart 7.2 software (AD Instruments). Mice behavior was observed during the same period of time.

Broncho alveolar lavage

Broncho alveolar lavage (BAL) was performed in CO_2 euthanized mice by 4 lavages with 500 µL saline solution via a cannula introduced into the trachea. BAL fluids (BALF) were centrifuged at 400 g for 10 min at 4°C, and the supernatants were stored at -20°C for ELISA analysis. Total cells counts were determined on the re-suspended cell pellet in 1ml saline and cytospins were prepared on glass slides (Thermo scientific, Waltham, USA) followed by a Diff-Quik staining (Merz & Dade A.G., Dudingen, Switzerland). Differential cell counts were performed with at least 400 cells per slide.

Lung histology

The left lobe of lung was fixed in 4% buffered formaldehyde, processed and paraffin embedded under standard conditions. Lung sections of 3 µm were stained with haematoxylin and eosin (H&E). The slides were blindly examined by using a

Leica microscope at 200X magnification (Leica, Solms, Germany). Cell infiltration and septae disruption were assessed by a semi-quantitative score (with increasing severity 0-5) by two independent observers.

Cytokines measurements

IL-1β, IL-6, chemokine CXCL1 (KC), Matrix metallopeptidase 9 (MMP9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) concentrations in BALF or lung homogenates were determined by enzyme-linked immunosorbent assay (ELISA, R&D Abingdon, UK) according to the manufacturer's recommendations. The lungs were homogenized for 1 minute using a Polytron homogenizer and the cell debris were removed by centrifugation at 10,000g for 10 minutes as reported (Gasse et al. 2007).
The extent of neutrophil accumulation in lung and BALF was quantified by myeloperoxidase (MPO) ELISA (R&D DY3667).

Glutamate glutamine measurements

Concentrations of glutamine and glutamate in lung tissue were determined using a glutamine/glutamate determination kit (GLN-1; Sigma-Aldrich) with slight modifications. After homogenization of lung tissue in acetate buffer (0.5M, pH 5.0), each sample was divided into two parts; part 1 was measured for glutaminase after transforming the glutamine into glutamate and thus obtaining the total glutamate concentration, part 2 was measured directly for the endogenous glutamate concentration. Samples were then dehydrogenized to α-ketoglutarate accompanied by reduction of NAD+ to NADH. The amount of NADH is proportional to the amount of glutamate and was measured using a spectrophotometer at 340 nm. A standard curve was determined for each experiment to calculate the concentration of glutamate in samples. Glutamine levels were calculated (part 1 minus part 2) and both glutamate and glutamine concentrations were normalized to total protein levels.

Glutamine synthetase activity

Glutamine synthetase (GS) activity was determined by Glutamine synthetase Microplate Assay Kit according to the manufacturer's recommendations (Ciohesion bioscience, UK).

Western blotting

Lung proteins were extracted as described (33). Briefly, lungs were homogenized in RIPA buffer (10mM Tris-HCl, pH 7.6; 1mM EDTA; NaCl 0.15mM; Igepal 1%; SDS 0.2%; supplemented with a cocktail of protease inhibitors (Pierce, Paris, France). Protein concentration was determined in the supernatant by BCA protein assay kit (Pierce, Paris, France). Protein samples (15µg) were run on SDS/PAGE gels (8-12%, w/v), transferred to a nitrocellulose membrane and probed with primary antibodies anti-GS (1:1000, G2781, Abcam, Paris,France), or anti-iNOS (1:1000, RB-9242, Applied Biosystems, ThermoFisher Scientific, Paris, France) and a secondary horseradish peroxidase-conjugated antibody (1:4000, W401B, Promega , Paris, France). Load of equal amounts of protein was checked by mouse anti-β actin antibody (1:4000, A2228, Sigma-Aldrich, Illkirch, France). The proteins of interest were revealed by chemiluminescence following addition of substrate to the membranes (reagent kit ECL ™ Western Blotting Detection Reagent, AmershamTM, GE Healthcare) and the immunopositive signals were recorded with the "Western Blotting Imager" PXi4 (Ozyme). Immunoreactive bands were quantified by using the Gene Tools® software. Western blots were performed in triplicate and representative results are shown in figures. Results are expressed as percentage of NaCl vehicle control.

Airway resistance assessed by invasive plethysmography

Measurements of dynamic lung resistance and compliance were performed by invasive plethysmography in response to the bronchoconstrictor methacholine on mice chronically exposed to GLA, using a FinePointe (Buxco, London, United Kingdom). Mice were anesthetized using 100µL/10g body weight of 2% xylazine (Rompun Bayer) and 10% ketamine (Imalgene; Merial, Toulouse, France), tracheotomized, paralyzed using D-tubocurarine (0.125%, Sigma), and immediately intubated with an 18-gauge catheter, followed by mechanical ventilation.

Respiratory frequency was set at 140 breaths per min with a tidal volume of 0.2 ml and a positive end-expiratory pressure of 2 ml H2O. Increasing concentrations of

methacholine (0-40 mg/ml PBS) were administered through intubation to induce bronchoconstriction at the rate of 20 puffs every 10 seconds, with each puff of aerosol delivery lasting 10 milliseconds, via a nebulizer aerosol system with a 2.5–4-mm-sized aerosol particle generated by a nebulizer head (Aeroneb; Aerogen, Galway, Ireland). Airway resistance (Ri) reflects bronchoconstriction and increases with methacholine exposure, while dynamic compliance (Cdyn) measures elastance upon methacholine exposure. Baseline resistance was restored before administering subsequent doses of methacholine.

Statistical analysis

All results are presented as mean ± SEM. Data involving more than two groups were analyzed by analysis of variance ($P > 0.05$, one-way ANOVA) followed by Bonferroni's Multiple Comparison Test. Student's t-test was used for comparisons involving two groups. For the histological scores, intergroup comparisons were performed using a non-parametric test (Mann–Whitney). For data from plethysmography experiments, statistical evaluation of differences between the experimental groups was determined by Mann-Witney "U" test. Statistical tests were made with the software Prism.

Results

Low dose intranasal GLA administration induces seizures via NMDA receptors activation in mice

The intranasal route is classically used to mimic aerogenic exposure in experimental murine models of acute lung inflammation or lung infection. Here, when exposing mice to low dose GLA (1mg/kg), we were surprised that mice developed seizures. The first behavioral changes appearing approximately 4h-4h30 after GLA exposure consisted of head shaking and myoclonic twitches of the body, followed by tonic-clonic seizures which appeared concomitantly with high amplitude and high frequency spikes on EEG recording (Figure 1 A). Epileptic activity was characterized by recurrent and intermittent seizure episodes whose mean duration was approximately 50 sec. Mice developed motor seizures with the same chronological events: retrograde movement, squatting, head nodding, forelimb contractions, rearing

and tail extension associated with hyperventilation and hypersalivation. During full motor seizures, mice finally developed: popping, running, falling with cycling movements of extremities. Interestingly epileptic seizures after intranasal instillation occurred at 1 mg//kg, while upon systemic i.p. injection the epileptogenic dose was 75 mg/kg (Figure 1B) as we have already demonstrated (30), indicating that the intranasal route has a higher epileptogenic potential. Among 10 mice tested, 8 displayed seizures activity after 1mg/kg GLA intranasal administration similar to the 9/10 mice that developed seizures after 75 mg/kg GLA intraperitoneal treatment (Figure 1B). The intensity of seizure was comparable between mice receiving GLA at 1mg/kg by intranasal route and mice receiving GLA at 75mg/kg by intraperitoneal route. The dose of GLA at 1mg/kg administrated intraperitoneally did not induced convulsions (0/6 mice treated).

This high sensitivity to low i.n. GLA exposure was mediated through glutamate NMDA receptors since none of ten mice pretreated with MK-801 developed GLA-induced seizures. In contrast, pretreatment with mGlu5 receptor subtype antagonist, MPEP yielded 7/10 mice with GLA-induced seizures.

Further, we addressed a possible link between GLA-induced seizures and inflammation in mice deficient for a master inflammatory pathway, IL-1R1. There was no difference of induction and intensity of seizures between IL1R1 KO mice and wild-type B6 control mice after GLA intranasal exposure at 1mg/kg.

Thus, GLA-induced seizures seem to be mediated through activation of a NMDA receptor, independent of IL-1R1 inflammatory pathway.

Intranasal GLA exposure induces lung inflammation

To investigate a potential inflammatory response in the lung, GLA was instilled by the intranasal route at 1mg/kg and the BALF was analyzed at 6h and 24h. There was a significant inflammation in the airways, largely accounted for by macrophages and neutrophils in mice treated with GLA 1 mg/kg at 6h, compared to saline controls, and both populations were still elevated at 24h (Figure 2A). Lymphocytes in BALF were delayed but significantly increased at 24h after GLA instillation. The MPO levels in BALF were increased at 6h after GLA instillation, consistent with the increased neutrophil counts in BALF. Moreover, IL-1β and TIMP1 concentrations in lung were essentially doubled at 24h after GLA instillation (Figure 2B). There was no significant

changes of pulmonary IL-6 and MMP9 levels and nor of KC in BALF (data not shown) after GLA exposure.

A significant increase in iNOS protein in the lung was induced at 6h and 24h after GLA instillation (Figure 2C). Microscopic analysis of lung sections revealed weak interstitial inflammation and some disruption of alveolar septae evoking some emphysema after GLA administration (Figure 2D).

GLA induced lung inflammation is not an indirect effect of convulsion.

We next verified that inflammation was due to a pulmonary effect of GLA and not to an indirect effect of GLA-induced convulsions (Figure 3). We compared the lung inflammatory cell recruitment in the airways after GLA administration given either by the intranasal (1 mg/kg) or intraperitoneal (75 mg/kg) route. Both GLA regimen induced convulsions, but only i.n. instillation caused significant macrophage, neutrophil and lymphocyte recruitment measured 24h after GLA exposure. Therefore the lung inflammation is not a consequence of a peripheral effect of convulsions but appears to be due to a direct effect of GLA on the airways.

GLA instillation perturbs glutamate homeostasis in lung

GLA is an analog of glutamate and a recognized inhibitor of glutamine synthetase (GS), an enzyme also present in lungs (34). Therefore we next investigated GS activity in the lung upon lung GLA exposure. A single dose of GLA at 1mg/Kg given to B6 mice by intranasal route induced a significant decrease in GS activity in the lung by *ca* 40% at 6h that returned to basal levels at 24h after GLA exposure. Moreover the enzymatic activity modification was associated with a significant GS protein upregulation by *ca* 50% and a significant increase of glutamate and glutamine concentrations in the lung 24h after GLA instillation.

GLA-induced lung inflammation depends on glutamate signaling

To address the role of glutamate receptors activation in GLA-induced lung inflammation we used the glutamate receptors antagonists, MK-801 and MPEP. MK-801 and MPEP significantly reduced the recruitment of total cells, macrophages

neutrophils and lymphocytes in BALF at 6h (Figure 5). The data strongly suggest that activation of glutamate receptors contributes to lung inflammation.

Inflammatory cell recruitment upon GLA exposure depends on IL-1R1 signaling

Since glutamate activates the NLPR3 inflammasome (35), we hypothesized a link between GLA, NLRP3 inflammasome activation, IL-1β pathway and lung inflammation. IL-1 is a potent inflammatory mediator involved in several lung inflammation conditions as seen in bleomycin induced inflammation and fibrosis (36) and we asked whether it might contribute to lung inflammation induced by GLA exposure. Therefore we asked whether IL-1β is involved in GLA-induced lung inflammation. Using IL-1R1 deficient mice we found a significant reduced neutrophil recruitment in BALF upon 1 mg/kg GLA instillation as compared to B6 mice (Figure 6A). Consistent with the reduced neutrophil recruitment in BALF, the concentration of the neutrophil attracting pro-inflammatory chemokine CXCL1 (KC) was significantly increased in WT, but not in IL-1R1KO mice 6h after GLA instillation (Figure 6B). No significant changes were found for IL-6, TIMP1, IL-1β, MMP9 and MPO levels between IL-1R1 deficient and wild-type mice 6h after GLA exposure (data not shown).

Chronic GLA exposure to very low, non-convulsive doses causes persistent lung inflammation

To assess the pulmonary effects upon chronic GLA exposure we first identified a dose regimen which did not cause seizures. GLA at 0.2mg/kg administered by instillation, 3 times a week during 4 weeks did not cause motor seizures. After 4 weeks under this exposure, GLA induced a significant increase of inflammatory cells in the airways, largely accounted for by macrophages, together with neutrophils, macrophages and lymphocytes (Figure 7A), similar to the acute response.

The MPO levels in BALF were significantly augmented after chronic GLA exposure consistent with the increased neutrophil counts in BALF. Moreover TIMP1 in lung was slightly but significantly increased after chronic GLA exposure. No significant changes were found for IL-1β, MMP9, KC levels (data not shown). Furthermore, iNOS and GS proteins in the lung were significantly increased by *ca* 50% after chronic GLA exposure (Figure 7B). The histological investigation of the lung tissue

upon chronic exposure revealed increased size of alveoli suggesting disruption of septae and incipient emphysema, with an increase of interstitial and peribronchial mononuclear cells (Figure 7C).

Chronic GLA exposure induces airway hyperreactivity

Airway hyperreactivity is a hallmark of allergic asthma and chemical irritation of the airways. Airway hyperreactivity was assessed by the responsive to the bronchoconstrictor methacholine using airways resistance (Ri) and dynamic compliance (Cdyn) of mice chronically exposed to GLA or saline using invasive plethysmography. Chronic GLA exposure to very low non-convulsive doses (0, 2 mg/kg) as above induced a significant increase of airway resistance and a declining trend in the dynamic compliance at the higher concentrations of methacholine (Figure 8). Increased hyperreactivity of the airways upon chronic GLA exposure is a significant finding suggesting potential enhanced bronchoconstrictive responses to other irritants including allergens.

Discussion

This is the first study demonstrating a significant airway inflammation upon GLA exposure. We demonstrate that lung inflammation is IL-1R1 signaling dependent and appears to be related to GLA-induced glutamate signaling, which likely activates the inflammasome and IL-1β dependent inflammation.

First, a single intranasal administration of GLA at 1mg/kg caused seizures in mice whereas intra-peritoneal administration at the same dose had no convulsive effect. A dose of 75mg/kg by intraperitoneal route is necessary to have a similar percentage of convulsive mice as with 1mg/kg by intranasal route. These results suggest that the intranasal exposure has higher epileptogenic potential. The olfactory system provides a direct link between the brain and the outside world, and it also has widespread connections with various brain regions, including the hippocampus. Nasal administration of a drug can result in significant delivery to the CNS, by effectively passing the blood brain barrier (37). Numerous publications confirm the efficacy of intranasal administration for the delivery of compounds to the CNS (37-39). Therefore it is conceivable that GLA accessed the CNS via the olfactory and trigeminal nerves

and the rostral migratory stream (37, 40, 41) and induced epileptiform activity through a glutamatergic neurotransmission activation (30, 42). We show that behavior modifications induced by intranasal GLA exposure can be prevented by pretreatment with MK-801, a NMDA-receptor antagonist, suggesting NMDA-receptor involvement in these epileptic seizures. These data are in line with our previous study showing that seizures induced by an intraperitoneal injection of GLA at 75 mg/kg were prevented by MK-801 pretreatment (30).

Importantly, a single intranasal administration of GLA at 1mg/kg induced pulmonary inflammation with recruitment of neutrophils and macrophages at 6h and lymphocytes at 24h. Lung inflammation is not a consequence of convulsions since an intraperitoneal injection of GLA (75mg/kg) that induced severe convulsions did not induce inflammatory cell recruitment to the airways. These results suggest that GLA acts directly on the airways and/or the immune system. Indeed, the potential targets of GLA, glutamine synthetase and glutamate receptors, are not only localized in the nervous system but also in lung and immune cells (13, 19, 21).
In agreement with Labow et al. (13) we detect active GS protein in lung. Further, GLA at 1mg/kg inhibited by 40% pulmonary GS activity 6 h after exposure, which in turn increased the concentration of its substrate, glutamate, in lung. In response to the inhibition of GS activity, GS protein levels were markedly upregulated and thus restored the overall pulmonary GS activity and increased glutamine concentration in lung 24h after GLA exposure. Indeed, the level of GS protein in the lung is partially governed by the intracellular glutamine concentration, a feedback regulatory mechanism (13, 43). It was demonstrated that the lung primarily contributes glutamine synthesized *de novo* from glutamate and ammonia and hence plays an important role in the maintenance of whole-body nitrogen and glutamine homeostasis (34).

Outside the central nervous system, the lung is among the organs where evidence exists for a role for glutamate signaling in physiology and pathologic states. Several recent reports describe the expression of ionotropic and metabotropic glutamate receptors in the lung and airways, as well as in immune cells (18, 19, 21, 44, 45) . Activation of glutamate receptors might be an important and previously unrecognized mechanism of the airways inflammation and hyperactivity (20, 46-48). In airways inflammation, glutamate plays a role from the first step of neutrophilic emigration, i.e.

neutrophil adhesion to pulmonary capillary endothelium by chemotaxism (44, 49). Glutamate-mediated increase in neutrophil cell migration is inhibited by class 1 metabotropic receptor antagonists (mGLUR) (44). Interestingly, activated polymorphonuclear leukocytes release glutamate, causing a decrease in endothelial barrier function via its action on endothelial expression of group I and III mGLuRs (47), paving the way for neutrophil trans-endothelial migration. We showed that MPEP, a class 1 mGLUR antagonist, significantly reduced the recruitment of total cells, neutrophils, lymphocytes and macrophages in BALF 6h after GLA exposure. These data suggest that GLA promotes neutrophil emigration into the lung by activation of class 1 mGLUR receptors. Therefore, it is possible that after being activated by glutamate or GLA, neutrophils further release glutamate to attract more neutrophils and/or other leukocytes forming an autocrine/paracrine loop.

The ionotropic glutamate NMDA receptors were recently found in all lung regions and in immune competent cells, including alveolar macrophages, lymphocytes, and neutrophils (19, 21, 22) and seem to play a critical role in lung diseases (16). In bleomycin-induced lung inflammation a selective release of endogenous glutamate was reported in lungs and memantine, a well-known NMDAR antagonist, attenuated lung inflammation (50). Moreover, it was demonstrated that GLA directly affects NMDAR (51). Therefore, we investigated the putative role of MK-801, a specific NMDAR antagonist, on GLA induced lung response. In the present study, we document that MK-801 prevents neutrophil, macrophage and lymphocyte recruitment in GLA-induced lung inflammation, suggesting a role of NMDAR activation in cell recruitment. In addition, it was demonstrated that activation of NMDAR subtypes in rat alveolar macrophages promotes NO secretion by increasing iNOS activity (45). In our conditions, intranasal exposure to GLA significantly increased iNOS protein in lung. Therefore GLA or glutamate may induce iNOS expression via NMDAR activation. Nevertheless, the exact mechanism of iNOS increased expression is not elucidated since iNOS can also be induced by inflammatory cytokines in phagocytic cells (macrophages, leukocytes). Indeed, we demonstrated that GLA induces IL-1β in lung 24h after a single intranasal exposure. In bleomycin-induced lung inflammation, blocking of NMDAR by memantine decreased the proinflammatory cytokines IL-1β and TNFα (50). In isolated hippocampus, glutamate stimulation increased NLRP3 expression accompanied by the up-regulation of cleaved caspase-1 expression and

enhanced of IL-1β secretion (35). Our results suggest that GLA might induce IL-1β secretion via NMDAR activation. Indeed, we hypothesize, as suggested by Li et al. (2015a), that glutamate could activate the NLRP3 signaling pathway via an increase of reactive oxidative species. In our conditions, GLA might induce IL-1β secretion and nitric oxide production through NMDA receptor activation.

The present study provides the first data documenting pulmonary modifications after aerogenic exposure to GLA, the active ingredient of some commercial herbicides. The effects of long-term exposure at low doses of GLA remain unknown as there is no specific epidemiological study on the effects of chronic exposure to this pesticide on lung. Indeed, pesticides may contribute to respiratory diseases among farmers (1, 5, 8), but data for individual chronic exposure to pesticides are limited to few studies (11). Therefore, we wondered if a chronic exposure at low doses of GLA perturbed the lung function too. We show that chronic GLA exposure at 0.2 mg/kg 3x per week over 4 weeks by intranasal instillation causes lung inflammation with disruption of alveolar septae and incipient emphysema with an increase of iNOS and TIMP-1 activity, the hallmark of evolution to pulmonary fibrosis. The mild emphysema observed after GLA exposure is likely due to the inflammatory response. However, nitric oxide production via iNOS expression, might contribute to the development of pulmonary emphysema since it has demonstrated that iNOS inhibition attenuates cigarette smoke or elastase induced pulmonary emphysema in mice (52, 53).

Moreover, chronic GLA exposure augmented airway hyperreactivity to methacholine with increased resistance and reduced compliance. Airway hyperreactivity induced by GLA exposure is likely linked to inflammation but might also be due to a direct effect of GLA on airway smooth muscle cells or nerves. Indeed, NMDA receptors are present in tracheal and lung smooth muscle cells and mediate contractile responses via glutamatergic mechanisms (46). According to the interesting theory of Hoang *et al*., airway hyperresponsiveness was considered as a “bronchial epilepsy” (20). This theory suggests that airway hyperresponsiveness occurs in particular as a result of hyperreactivity of glutamatergic receptors, mainly NMDAR in the airways. Indeed, it was demonstrated that NMDA administration caused an increase of tracheal smooth muscle response in ovalbumin-induced hyperreactivity to acetylcholine in guinea pigs (48, 54). In a similar way, GLA may act as an excitotoxin able to induce cerebral (30) and bronchial seizures.

In conclusion, this study, for the first time, provides evidence for the mechanism of glufosinate-induced occupational lung disease. Indeed our results demonstrate that GLA exposure by aerosol causes airway inflammation and hyperreactivity. Mechanistically GLA acts by interaction with glutamate signaling which induces the release for pro-inflammatory IL-1β leading to lung inflammation and injury. These data raises concerns on the potential risk of GLA as herbicide on public health.

Figure legends

Figure 1: Low dose intranasal GLA administration induces seizures via NMDA receptors activation in mice.

EEG recording of GLA-intranasal-treated mice before (A) and 6h after GLA instillation (1 mg/kg, i.n.; B). Cortical EEGs were recorded as described in materials and methods. Typical recording of one mouse representative of n=6 mice per group is presented (A).

Percentage of mice displaying seizures after NaCl or GLA treatment (B). A single low dose of GLA was given by the intranasal route (1mg/kg i.n.) or the intraperitoneal route (1mg/kg i.p. or 75mg/kg i.p.) to B6 mice. MK-801 (1 mg/ kg, i.p.) or MPEP (10 mg/ kg, i.p.) were injected 30 min prior GLA (1mg/kg i.n.) instillation in B6 mice. GLA was also given by the intranasal route (1mg/kg i.n.) to IL-1R1KO and B6 mice. Results are expressed as means ± SEM of n=10 mice per experimental group.

Figure 2: Intranasal GLA exposure induces lung inflammation

A single dose of GLA at 1mg/kg was given by intranasal route and the total and differential cell counts in BALF scored at 6h and 24h (n=12-15 per experimental group) (A). Neutrophil accumulation in BALF was measured by assaying myeloperoxidase (MPO) (n= 15-17 per experimental group) (B). IL-1β and TIMP-1 levels in lung were determined by ELISA (n= 12-15 per experimental group) (B). Inductible NOS protein levels were evaluated 6h or 24 hours after NaCl or GLA treatment (n= 6 per experimental group) (C). Representative micrographs (200X magnification) of lung sections stained with haematoxylin and eosin of a NaCl vehicle control mice and a GLA-treated mice 24h after exposure (D). Cell infiltration and septae disruption were assessed by a semi-quantitative score (0 - 5; n=4 per experimental group) (D). Results are expressed as means ± SEM, pooled from 2 experiments, *p<0.05, ** p<0.01, ***p<0.001 versus NaCl group. # p<0.05 versus GLA 6h group.

Figure 3: GLA induced lung inflammation is not an indirect effect of convulsion.

GLA was given to B6 mice by intranasal route (1mg/kg) or intraperitoneal route (75mg/kg) and the total and differential inflammation cell numbers in BALF were counted at 24h (n= 5-6 per experimental group).

Results are expressed as means ± SEM, ** p<0.01, ***p<0.001 versus NaCl group. # p<0.05, ##p<0.01 versus GLA i.n group.

Figure 4: GLA instillation perturbs glutamate homeostasis in lung.

The enzymatic activity, protein levels of glutamine synthetase and the concentrations of glutamate, glutamine concentrations were evaluated in the lung 6 h and 24h after a single intranasal dose of GLA at 1mg/kg in B6 mice by intranasal route. Results are expressed as means ± SEM of n= 6-9 per experimental group pooled from 2 independent experiments, *p<0.05, ** p<0.01, versus NaCl group, ##p<0.01 versus GLA 6h group.

Figure 5: GLA-induced airways inflammation depends on glutamate signaling.

The total and differential inflammatory cells were counted in BALF at 6h after GLA intranasal exposure (1mg/kg). MK-801 (1 mg/ kg, i.p.) or MPEP (10 mg/ kg, i.p.) were injected 30 min prior GLA instillation in B6 mice. Results are expressed as means ± SEM with n= 6-11 per experimental group pooled from 2 independent experiments, ** p<0.01, ***p<0.001 versus NaCl group. # p<0.05, ###p<0.001 versus GLA group.

Figure 6: Inflammatory cell recruitment upon GLA exposure depends on IL-1R1 signaling.

A single dose of GLA at 1mg/kg was given to B6 and IL-1R1 deficient mice by intranasal route and the total and differential cell numbers in BALF were counted at 6h after GLA intranasal exposure (A). KC levels in lung homogenates were determined by ELISA. Results are expressed as means ± SEM of n= 10 per experimental group pooled from 2 independent experiments, *p<0.05, ** p<0.01, ***p<0.001 versus NaCl group. ## p<0.01 versus B6 GLA group.

Figure 7: Chronic GLA exposure induces lung inflammation

GLA was administered 3 times weekly at 0.2mg/kg for 4 weeks to BL6 mice by intranasal route and the total and differential cell numbers in BALF were counted at 24h after the last instillation (n= 15-17 per experimental group, pooled from 2 independent experiments) (A). Neutrophil accumulation in BALF was also quantified by measuring myeloperoxidase (MPO) (n= 15-17 per experimental group pooled from 2 independent experiments). IL-1β and TIMP-1 levels in lung were determined by ELISA (n= 12-15 per experimental group) (A).
Glutamine synthetase and iNOS proteins levels were evaluated after NaCl or GLA chronic treatment (n= 6 per experimental group) (B). Representative micrographs (200X magnification) of lung sections stained with haematoxylin and eosin of a NaCl-treated mice and a GLA-treated mice 24h after exposure (C). Cell infiltration and septae disruption were assessed by a semi-quantitative score (0-5) treatment (n= 6-7 per experimental group) (C).

Figure 8: Chronic GLA exposure at low, non-convulsive doses increases airway hyperreactivity

Measurements of dynamic lung resistance (Ri) and compliance (Cdyn) in response to the bronchoconstrictor methacholine were performed on chronic-GLA-exposure mice using invasive plethysmography. Increasing concentrations of methacholine (Mch) (0–40 mg/ml) were administered at the rate of 20 puffs every 10 seconds. Ri and Cdyn were recorded as described in materials and methods. Results are expressed as means ± SEM of n= 5 per experimental group, ** $p<0.01$ versus NaCl group.

Declarations of interest and Funding information

The authors declare that they have no conflict of interest. We are grateful to the CNRS and the University of Orleans for their support. This work was also supported by FEDER project N° 2016-00110366.

References

1. Hoppin JA, Umbach DM, London SJ, Henneberger PK, Kullman GJ, Coble J, et al. Pesticide use and adult-onset asthma among male farmers in the Agricultural Health Study. The European respiratory journal. 2009 Dec;34(6):1296-303. PubMed PMID: 19541724. Pubmed Central PMCID: PMC2822385. Epub 2009/06/23. eng.
2. Nordgren TM, Bailey KL. Pulmonary health effects of agriculture. Current opinion in pulmonary medicine. 2016 Mar;22(2):144-9. PubMed PMID: 26761627. Pubmed Central PMCID: PMC4764055. Epub 2016/01/14. eng.
3. Proskocil BJ, Bruun DA, Garg JA, Villagomez CC, Jacoby DB, Lein PJ, et al. The influence of sensitization on mechanisms of organophosphorus pesticide-induced airway hyperreactivity. American journal of respiratory cell and molecular biology. 2015 Nov;53(5):738-47. PubMed PMID: 25897622. Pubmed Central PMCID: PMC4742952. Epub 2015/04/22. eng.
4. Amaral AF. Pesticides and asthma: challenges for epidemiology. Frontiers in public health. 2014;2:6. PubMed PMID: 24479117. Pubmed Central PMCID: PMC3901073. Epub 2014/01/31. eng.
5. Henneberger PK, Liang X, London SJ, Umbach DM, Sandler DP, Hoppin JA. Exacerbation of symptoms in agricultural pesticide applicators with asthma. International archives of occupational and environmental health. 2014 May;87(4):423-32. PubMed PMID: 23670403. Pubmed Central PMCID: PMC4089194. Epub 2013/05/15. eng.
6. Corsini E, Sokooti M, Galli CL, Moretto A, Colosio C. Pesticide induced immunotoxicity in humans: a comprehensive review of the existing evidence. Toxicology. 2013 May 10;307:123-35. PubMed PMID: 23116691. Epub 2012/11/03. eng.
7. Costa C, Rapisarda V, Catania S, Di Nola C, Ledda C, Fenga C. Cytokine patterns in greenhouse workers occupationally exposed to alpha-cypermethrin: an observational study. Environmental toxicology and pharmacology. 2013 Nov;36(3):796-800. PubMed PMID: 23958972. Epub 2013/08/21. eng.
8. Ndlovu V, Dalvie MA, Jeebhay MF. Asthma associated with pesticide exposure among women in rural Western Cape of South Africa. American journal of industrial medicine. 2014 Dec;57(12):1331-43. PubMed PMID: 25348901. Epub 2014/10/29. eng.
9. de Jong K, Boezen HM, Kromhout H, Vermeulen R, Postma DS, Vonk JM. Pesticides and other occupational exposures are associated with airway obstruction: the LifeLines cohort study. Occupational and environmental medicine. 2014 Feb;71(2):88-96. PubMed PMID: 24142985. Epub 2013/10/22. eng.
10. Proskocil BJ, Bruun DA, Lorton JK, Blensly KC, Jacoby DB, Lein PJ, et al. Antigen sensitization influences organophosphorus pesticide-induced airway hyperreactivity. Environmental health perspectives. 2008 Mar;116(3):381-8. PubMed PMID: 18335107. Pubmed Central PMCID: PMC2265045. Epub 2008/03/13. eng.
11. Kumar S, Khodoun M, Kettleson EM, McKnight C, Reponen T, Grinshpun SA, et al. Glyphosate-rich air samples induce IL-33, TSLP and generate IL-13 dependent airway inflammation. Toxicology. 2014 Nov 5;325:42-51. PubMed PMID: 25172162. Pubmed Central PMCID: PMC4195794. Epub 2014/08/31. eng.
12. Lea PJ, Joy KW, Ramos JL, Guerrero MG. The action of 2-amino-4-(methylphosphinyl)-butanoic acid (phosphinothricin) and its 2-oxo-derivative on the metabolism of cyanobacteria and higher plants. Phytochemistry. 1984;23(1):6.
13. Labow BI, Abcouwer SF, Lin CM, Souba WW. Glutamine synthetase expression in rat lung is regulated by protein stability. The American journal of physiology. 1998 Nov;275(5 Pt 1):L877-86. PubMed PMID: 9815104. Epub 1998/11/14. eng.
14. Meister A. Glutamine synthetase of mammals.In: Byer PD,editor. The Enzymes. New York: AcademicPress; 1974. p 699-754. . Boyer PD, editor1974. 699-754 p.
15. Boldyrev AA, Carpenter DO, Johnson P. Emerging evidence for a similar role of glutamate receptors in the nervous and immune systems. J Neurochem. 2005 Nov;95(4):913-8. PubMed PMID: 16271044. Epub 2005/11/08. eng.
16. Du J, Li XH, Li YJ. Glutamate in peripheral organs: Biology and pharmacology. European journal of pharmacology. 2016 May 7;784:42-8. PubMed PMID: 27164423. Epub 2016/05/11. Eng.

17. Hinoi E, Takarada T, Ueshima T, Tsuchihashi Y, Yoneda Y. Glutamate signaling in peripheral tissues. European journal of biochemistry / FEBS. 2004 Jan;271(1):1-13. PubMed PMID: 14686914. Epub 2003/12/23. eng.
18. Said SI, Berisha HI, Pakbaz H. N-methyl-D-aspartate receptors outside the central nervous system: activation causes acute lung injury that is mediated by nitric oxide synthesis and prevented by vasoactive intestinal peptide. Neuroscience. 1995 Apr;65(4):943-6. PubMed PMID: 7617171. Epub 1995/04/01. eng.
19. Dickman KG, Youssef JG, Mathew SM, Said SI. Ionotropic glutamate receptors in lungs and airways: molecular basis for glutamate toxicity. American journal of respiratory cell and molecular biology. 2004 Feb;30(2):139-44. PubMed PMID: 12855408. Epub 2003/07/12. eng.
20. Hoang BX, Levine SA, Graeme Shaw D, Pham P, Hoang C. Bronchial epilepsy or broncho-pulmonary hyper-excitability as a model of asthma pathogenesis. Medical hypotheses. 2006;67(5):1042-51. PubMed PMID: 16797869. Epub 2006/06/27. eng.
21. Boldyrev AA, Bryushkova EA, Vladychenskaya EA. NMDA receptors in immune competent cells. Biochemistry Biokhimiia. 2012 Feb;77(2):128-34. PubMed PMID: 22348471. Epub 2012/02/22. eng.
22. Mashkina AP, Cizkova D, Vanicky I, Boldyrev AA. NMDA receptors are expressed in lymphocytes activated both in vitro and in vivo. Cellular and molecular neurobiology. 2010 Aug;30(6):901-7. PubMed PMID: 20414717. Epub 2010/04/24. eng.
23. Park HY, Lee PH, Shin DH, Kim GW. Anterograde amnesia with hippocampal lesions following glufosinate intoxication. Neurology. 2006 Sep 12;67(5):914-5. PubMed PMID: 16966570.
24. Tanaka J, Yamashita M, Yamashita M, Matsuo H, Yamamoto T. Two cases of glufosinate poisoning with late onset convulsions. Vet Hum Toxicol. 1998 Aug;40(4):219-22. PubMed PMID: 9682408.
25. Watanabe T, Sano T. Neurological effects of glufosinate poisoning with a brief review. Hum Exp Toxicol. 1998 Jan;17(1):35-9. PubMed PMID: 9491336.
26. Mao YC, Hung DZ, Wu ML, Tsai WJ, Wang LM, Ger J, et al. Acute human glufosinate-containing herbicide poisoning. Clinical toxicology (Philadelphia, Pa). 2012 Jun;50(5):396-402. PubMed PMID: 22480254. Epub 2012/04/07. eng.
27. Inoue Y, Onodera M, Fujita Y, Fujino Y, Kikuchi S, Endo S. Factors associated with severe effects following acute glufosinate poisoning. Clinical toxicology (Philadelphia, Pa). 2013 Nov;51(9):846-9. PubMed PMID: 24044532. Epub 2013/09/21. eng.
28. Calas AG, Perche O, Richard O, Perche A, Paris A, Lauga F, et al. Characterization of seizures induced by acute exposure to an organophosphate herbicide, glufosinate-ammonium. Neuroreport. 2016 May 4;27(7):532-41. PubMed PMID: 27031874. Epub 2016/04/01. eng.
29. Calas AG, Richard O, Meme S, Beloeil JC, Doan BT, Gefflaut T, et al. Chronic exposure to glufosinate-ammonium induces spatial memory impairments, hippocampal MRI modifications and glutamine synthetase activation in mice. Neurotoxicology. 2008 Jul;29(4):740-7. PubMed PMID: 18562008.
30. Lapouble E, Montecot C, Sevestre A, Pichon J. Phosphinothricin induces epileptic activity via nitric oxide production through NMDA receptor activation in adult mice. Brain Res. 2002 Dec 6;957(1):46-52. PubMed PMID: 12443979.
31. Laugeray A, Herzine A, Perche O, Hebert B, Aguillon-Naury M, Richard O, et al. Pre- and postnatal exposure to low dose glufosinate ammonium induces autism-like phenotypes in mice. Frontiers in behavioral neuroscience. 2014;8:390. PubMed PMID: 25477793. Pubmed Central PMCID: PMC4238406. Epub 2014/12/06. eng.
32. Labow M, Shuster D, Zetterstrom M, Nunes P, Terry R, Cullinan EB, et al. Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. J Immunol. 1997 Sep 1;159(5):2452-61. PubMed PMID: 9278338.
33. Rossignol R, Ranchon-Cole I, Paris A, Herzine A, Perche A, Laurenceau D, et al. Visual sensorial impairments in neurodevelopmental disorders: evidence for a retinal phenotype in Fragile X

Syndrome. PloS one. 2014;9(8):e105996. PubMed PMID: 25153086. Pubmed Central PMCID: PMC4143372. Epub 2014/08/26. eng.
34. Cooper AJ, Freed BR. Metabolism of [13N]ammonia in rat lung. Neurochemistry international. 2005 Jul;47(1-2):103-18. PubMed PMID: 15923062. Epub 2005/06/01. eng.
35. Li Y, Li J, Li S, Li Y, Wang X, Liu B, et al. Curcumin attenuates glutamate neurotoxicity in the hippocampus by suppression of ER stress-associated TXNIP/NLRP3 inflammasome activation in a manner dependent on AMPK. Toxicology and applied pharmacology. 2015 Jul 1;286(1):53-63. PubMed PMID: 25791922. Epub 2015/03/21. eng.
36. Gasse P, Mary C, Guenon I, Noulin N, Charron S, Schnyder-Candrian S, et al. IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. The Journal of clinical investigation. 2007 Dec;117(12):3786-99. PubMed PMID: 17992263. Pubmed Central PMCID: PMC2066195. Epub 2007/11/10. eng.
37. Scranton RA, Fletcher L, Sprague S, Jimenez DF, Digicaylioglu M. The rostral migratory stream plays a key role in intranasal delivery of drugs into the CNS. PloS one. 2011;6(4):e18711. PubMed PMID: 21533252. Pubmed Central PMCID: PMC3076435. Epub 2011/05/03. eng.
38. Chen Z, Ljunggren HG, Bogdanovic N, Nennesmo I, Winblad B, Zhu J. Excitotoxic neurodegeneration induced by intranasal administration of kainic acid in C57BL/6 mice. Brain Res. 2002 Mar 29;931(2):135-45. PubMed PMID: 11897099.
39. Dhuria SV, Hanson LR, Frey WH, 2nd. Intranasal drug targeting of hypocretin-1 (orexin-A) to the central nervous system. Journal of pharmaceutical sciences. 2009 Jul;98(7):2501-15. PubMed PMID: 19025760. Epub 2008/11/26. eng.
40. Dhuria SV, Hanson LR, Frey WH, 2nd. Intranasal delivery to the central nervous system: mechanisms and experimental considerations. Journal of pharmaceutical sciences. 2010 Apr;99(4):1654-73. PubMed PMID: 19877171. Epub 2009/10/31. eng.
41. Johnson NJ, Hanson LR, Frey WH. Trigeminal pathways deliver a low molecular weight drug from the nose to the brain and orofacial structures. Molecular pharmaceutics. 2010 Jun 7;7(3):884-93. PubMed PMID: 20420446. Pubmed Central PMCID: PMC2892271. Epub 2010/04/28. eng.
42. Chen Z, Duan RS, Quezada HC, Mix E, Nennesmo I, Adem A, et al. Increased microglial activation and astrogliosis after intranasal administration of kainic acid in C57BL/6 mice. J Neurobiol. 2005 Feb 5;62(2):207-18. PubMed PMID: 15459893.
43. Labow BI, Souba WW, Abcouwer SF. Mechanisms governing the expression of the enzymes of glutamine metabolism--glutaminase and glutamine synthetase. The Journal of nutrition. 2001 Sep;131(9 Suppl):2467S-74S; discussion 86S-7S. PubMed PMID: 11533295. Epub 2001/09/05. eng.
44. Gupta R, Palchaudhuri S, Chattopadhyay D. Glutamate induces neutrophil cell migration by activating class I metabotropic glutamate receptors. Amino Acids. 2013 Feb;44(2):757-67. PubMed PMID: 22990843. Epub 2012/09/20. eng.
45. Shang LH, Luo ZQ, Deng XD, Wang MJ, Huang FR, Feng DD, et al. Expression of N-methyl-D-aspartate receptor and its effect on nitric oxide production of rat alveolar macrophages. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2010 Dec 15;23(4):327-31. PubMed PMID: 20884369. Epub 2010/10/05. eng.
46. Anaparti V, Ilarraza R, Orihara K, Stelmack GL, Ojo OO, Mahood TH, et al. NMDA receptors mediate contractile responses in human airway smooth muscle cells. American journal of physiology Lung cellular and molecular physiology. 2015 Jun 15;308(12):L1253-64. PubMed PMID: 25888577. Epub 2015/04/19. eng.
47. Collard CD, Park KA, Montalto MC, Alapati S, Buras JA, Stahl GL, et al. Neutrophil-derived glutamate regulates vascular endothelial barrier function. The Journal of biological chemistry. 2002 Apr 26;277(17):14801-11. PubMed PMID: 11847215. Epub 2002/02/16. eng.
48. Strapkova A, Antosova M. Glutamate receptors and the airways hyperreactivity. General physiology and biophysics. 2012 Mar;31(1):93-100. PubMed PMID: 22447835. Epub 2012/03/27. eng.
49. Zhou X, Dai Q, Huang X. Neutrophils in acute lung injury. Frontiers in bioscience (Landmark edition). 2012;17:2278-83. PubMed PMID: 22652778. Epub 2012/06/02. eng.

50. Li Y, Liu Y, Peng X, Liu W, Zhao F, Feng D, et al. NMDA Receptor Antagonist Attenuates Bleomycin-Induced Acute Lung Injury. PloS one. 2015;10(5):e0125873. PubMed PMID: 25942563. Pubmed Central PMCID: PMC4420245. Epub 2015/05/06. eng.
51. Lantz SR, Mack CM, Wallace K, Key EF, Shafer TJ, Casida JE. Glufosinate binds N-methyl-D-aspartate receptors and increases neuronal network activity in vitro. Neurotoxicology. 2014 Dec;45:38-47. PubMed PMID: 25268653. Epub 2014/10/01. eng.
52. Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel FC, et al. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. Cell. 2011 Oct 14;147(2):293-305. PubMed PMID: 22000010. Epub 2011/10/18. eng.
53. Lanzetti M, da Costa CA, Nesi RT, Barroso MV, Martins V, Victoni T, et al. Oxidative stress and nitrosative stress are involved in different stages of proteolytic pulmonary emphysema. Free radical biology & medicine. 2012 Dec 1;53(11):1993-2001. PubMed PMID: 23000243. Epub 2012/09/25. eng.
54. Antosova M, Strapkova A. Study of the interaction of glutamatergic and nitrergic signalling in conditions of the experimental airways hyperreactivity. Pharmacological reports : PR. 2013;65(3):650-7. PubMed PMID: 23950587. Epub 2013/08/21. eng.

Figure 1 :

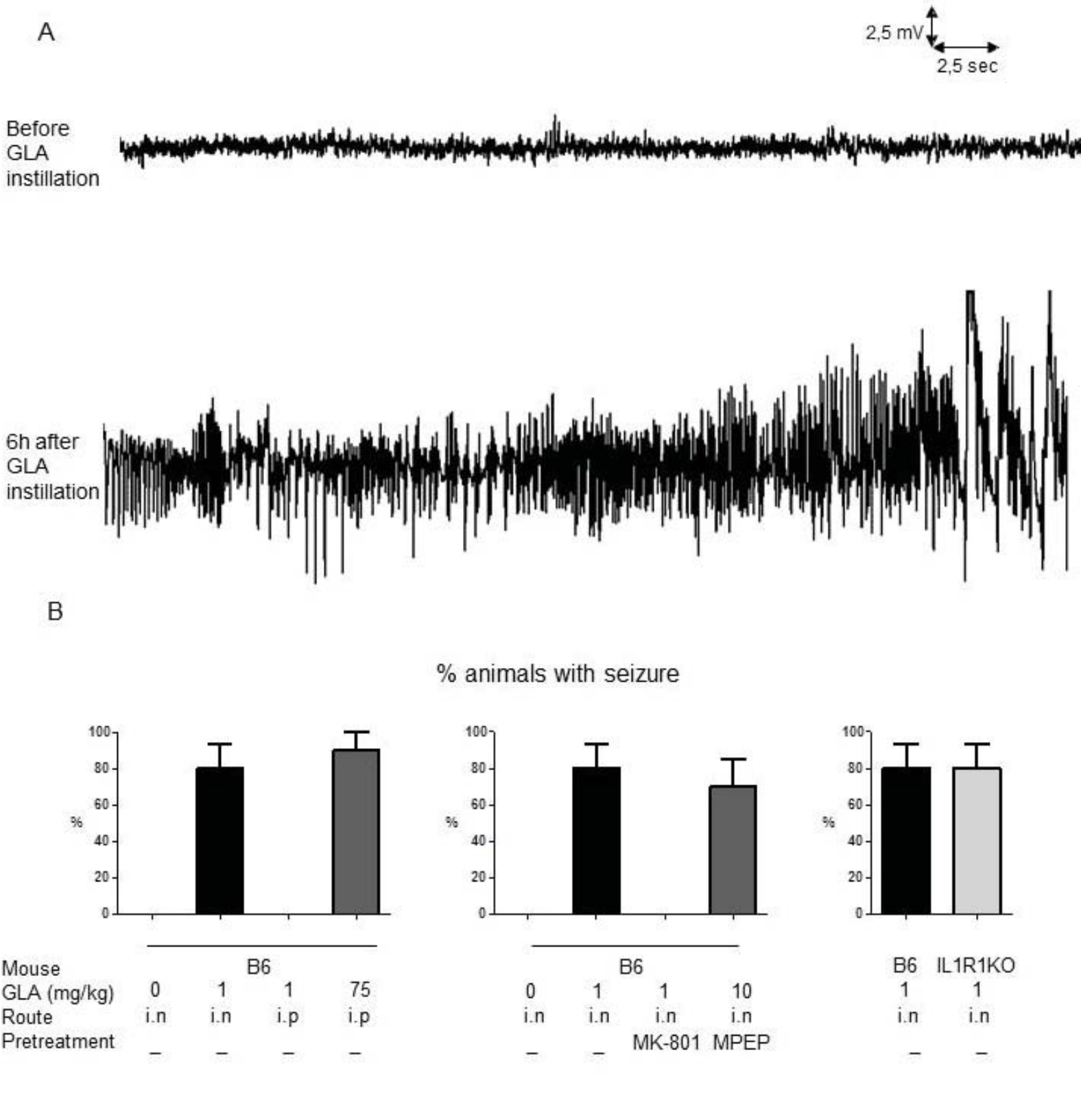
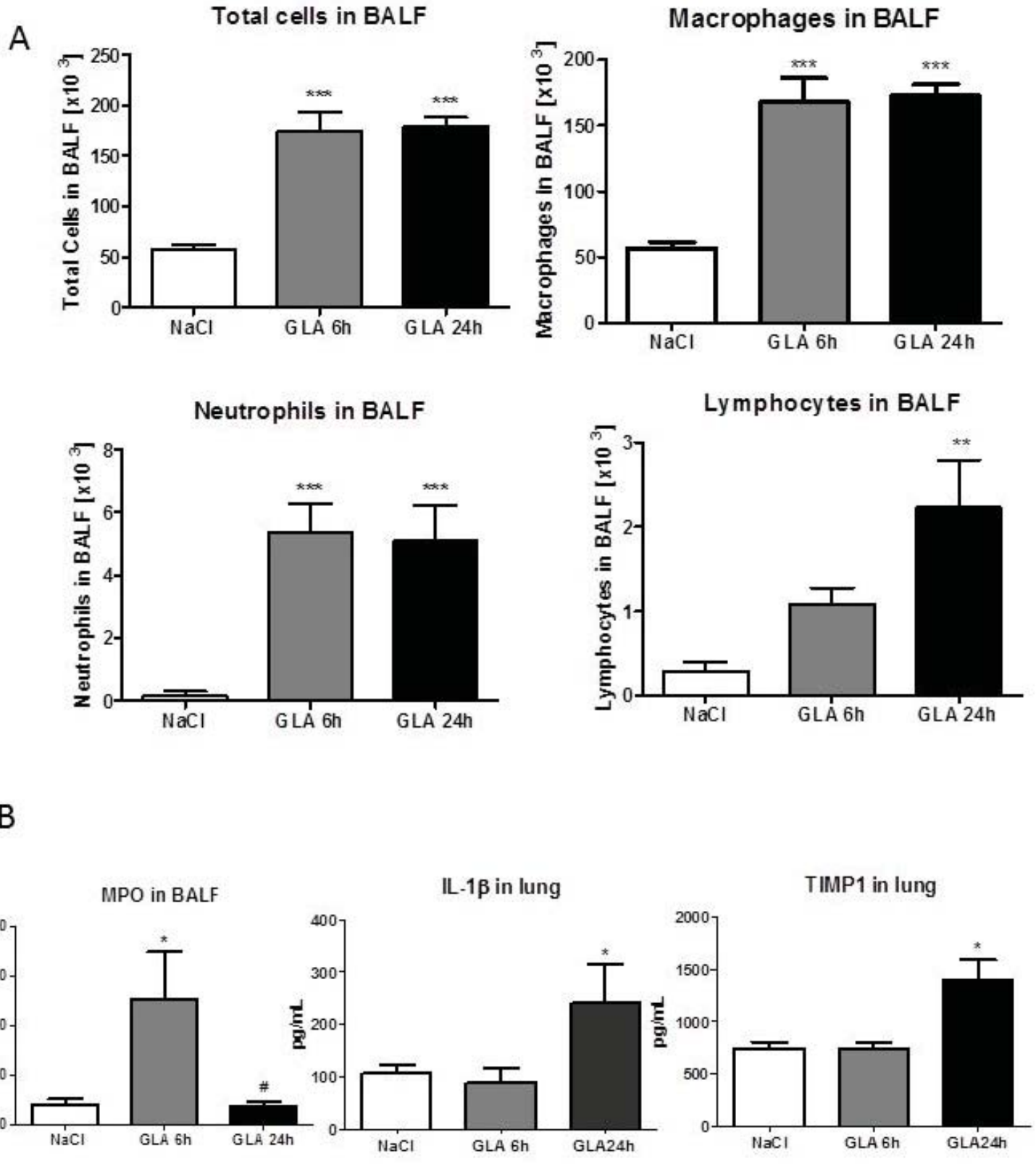
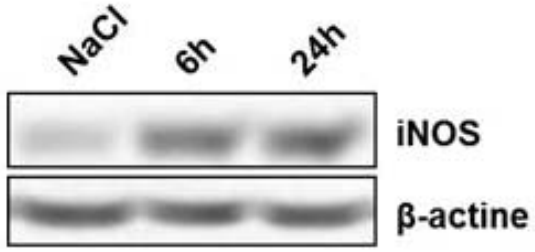
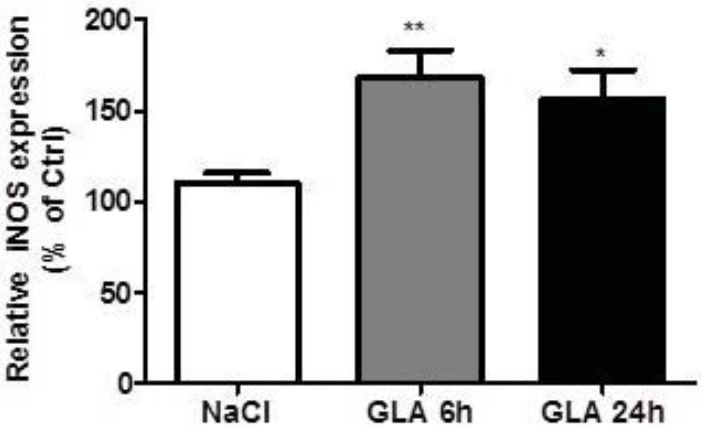

A
2,5 mV
2,5 sec
Before GLA instillation
6h after GLA instillation
B
% animals with seizure
100
80
60
40
20
0
%
Mouse
GLA (mg/kg)
Route
Pretreatment
B6
0
i.n
–
1
i.n
–
1
i.p
–
75
i.p
–
B6
0
i.n
–
1
i.n
–
1
i.n
MK-801
10
i.n
MPEP
B6
1
i.n
–
IL1R1KO
1
i.n
–

Figure 2:

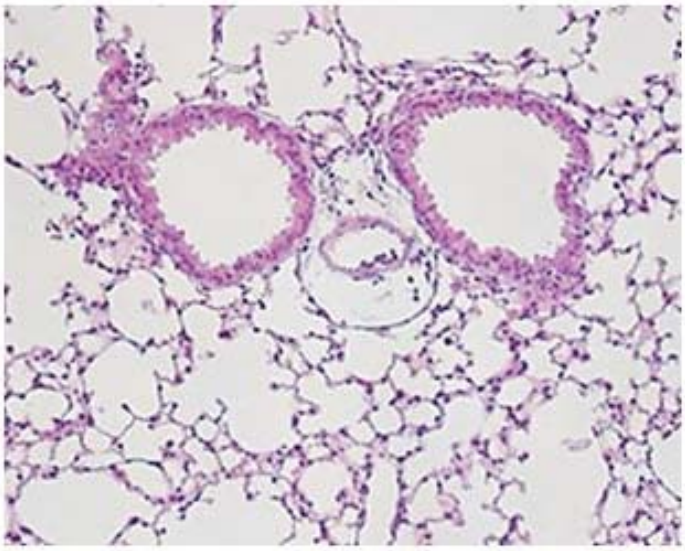

A
Total cells in BALF
Total Cells in BALF [x10 3]
250
200
150
100
50
0
***
***
NaCl
GLA 6h
GLA 24h
Macrophages in BALF
Macrophages in BALF [x10 3]
200
150
100
50
0
***
***
NaCl
GLA 6h
GLA 24h
Neutrophils in BALF
Neutrophils in BALF [x10 3]
8
6
4
2
0
***
***
NaCl
GLA 6h
GLA 24h
Lymphocytes in BALF
Lymphocytes in BALF [x10 3]
3
2
1
0
**
NaCl
GLA 6h
GLA 24h
B
MPO in BALF
pg/mL
4000
3000
2000
1000
0
*
#
NaCl
GLA 6h
GLA 24h
IL-1β in lung
pg/mL
400
300
200
100
0
*
NaCl
GLA 6h
GLA24h
TIMP1 in lung
pg/mL
2000
1500
1000
500
0
*
NaCl
GLA 6h
GLA24h

C

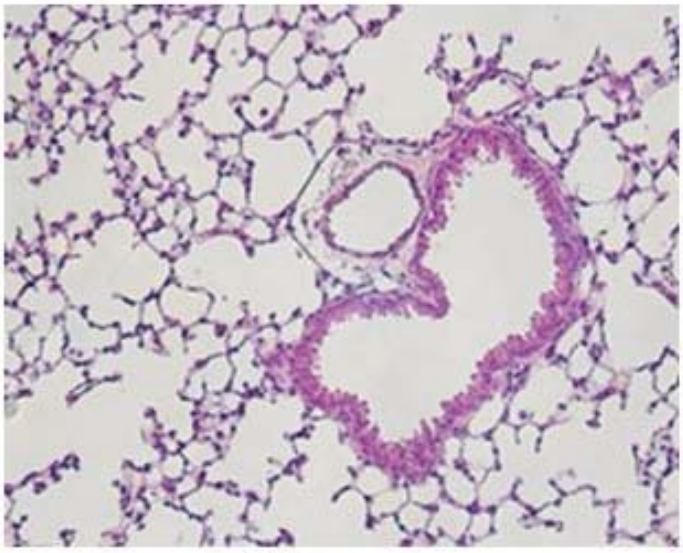

NaCl
6h
24h
iNOS
β-actine


Relative iNOS expression (% of Ctrl)
200
150
100
50
0
**
*
NaCl
GLA 6h
GLA 24h

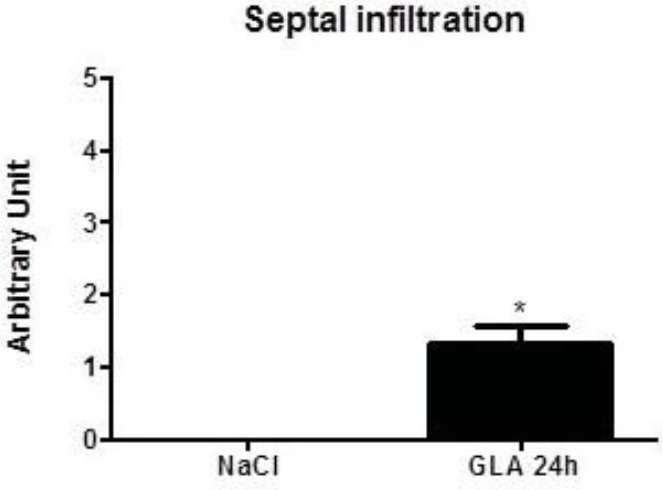
D



NaCl



GLA


Septal infiltration
Arbitrary Unit
5
4
3
2
1
0
*
NaCl
GLA 24h

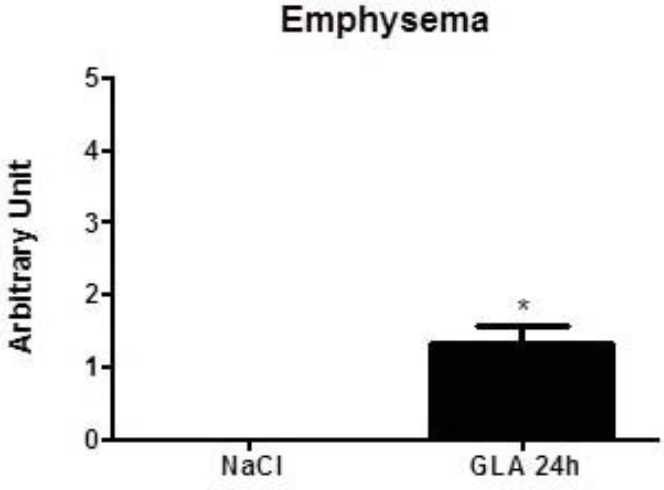
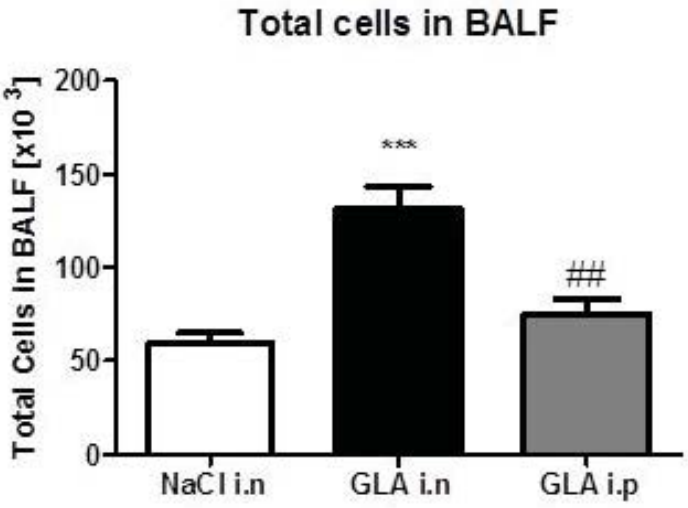
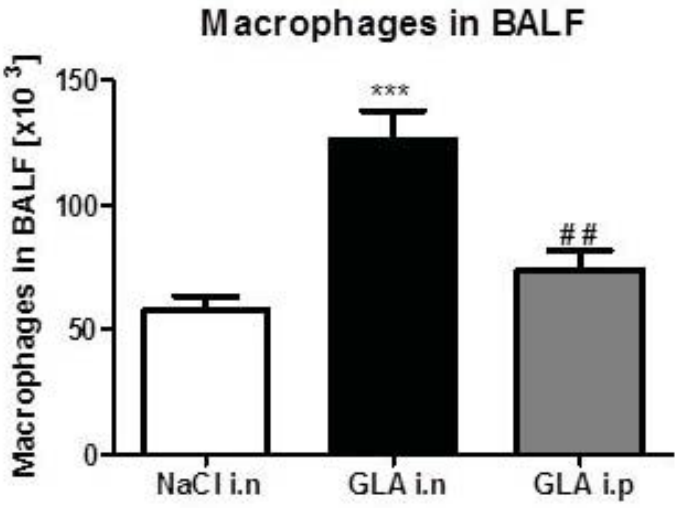
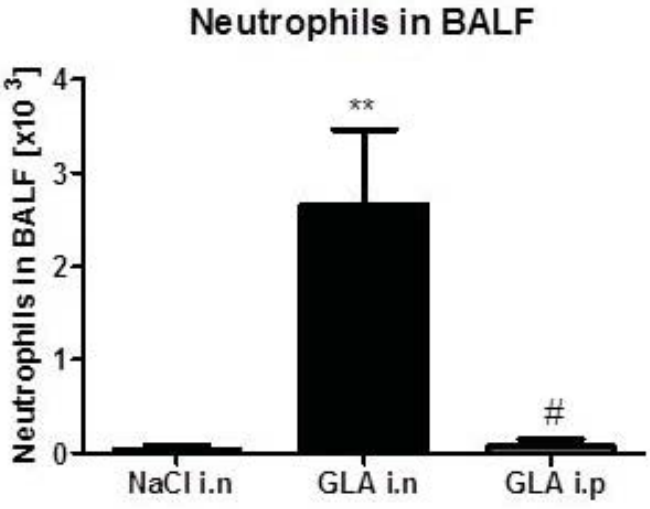

Emphysema
Arbitrary Unit
5
4
3
2
1
0
*
NaCl
GLA 24h

Figure 3:


Total cells in BALF
Total Cells In BALF [x10 3]
200
150
100
50
0
***
##
NaCl i.n
GLA i.n
GLA i.p


Macrophages in BALF
Macrophages In BALF [x10 3]
150
100
50
0
***
##
NaCl i.n
GLA i.n
GLA i.p


Neutrophils in BALF
Neutrophils In BALF [x10 3]
4
3
2
1
0
**
#
NaCl i.n
GLA i.n
GLA i.p

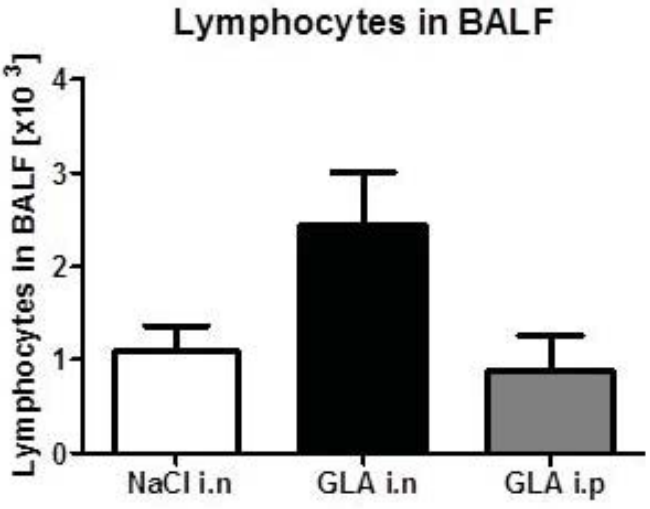
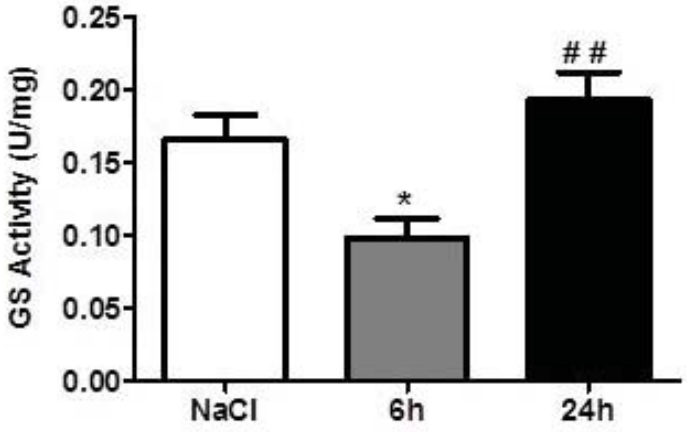
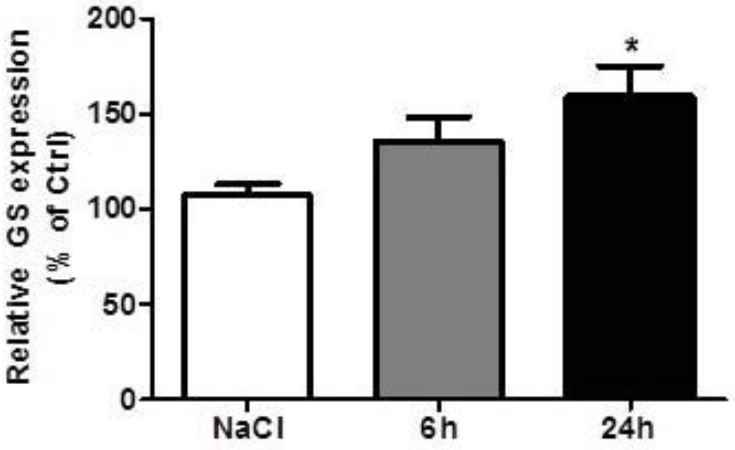
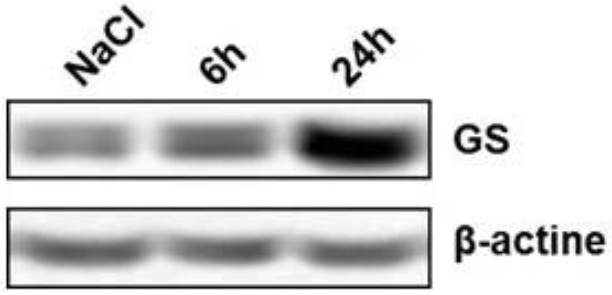
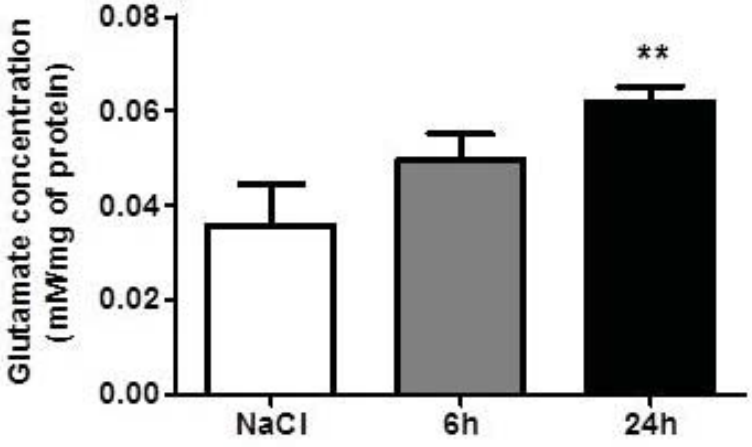

Lymphocytes in BALF
Lymphocytes In BALF [x10 3]
4
3
2
1
0
NaCl i.n
GLA i.n
GLA i.p

Figure 4 :


GS Activity (U/mg)
0.25
0.20
0.15
0.10
0.05
0.00
*
##
NaCl
6h
24h


Relative GS expression (% of Ctrl)
200
150
100
50
0
*
NaCl
6h
24h


NaCl
6h
24h
GS
β-actine


Glutamate concentration (mM/mg of protein)
0.08
0.06
0.04
0.02
0.00
**
NaCl
6h
24h

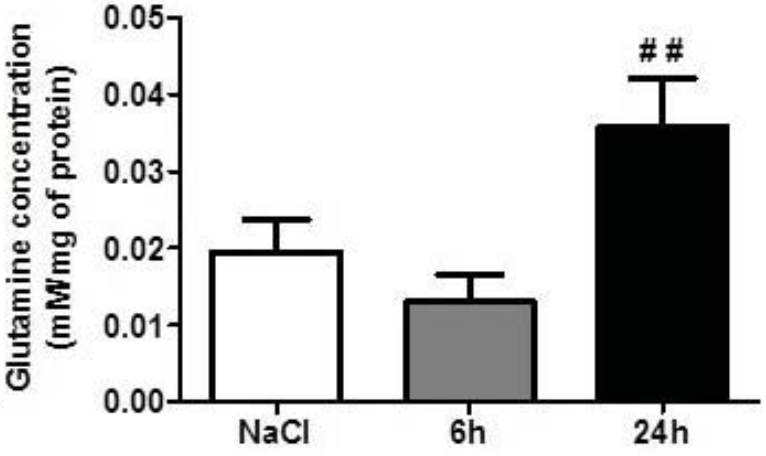

Glutamine concentration (mM/mg of protein)
0.05
0.04
0.03
0.02
0.01
0.00
##
NaCl
6h
24h

Figure 5

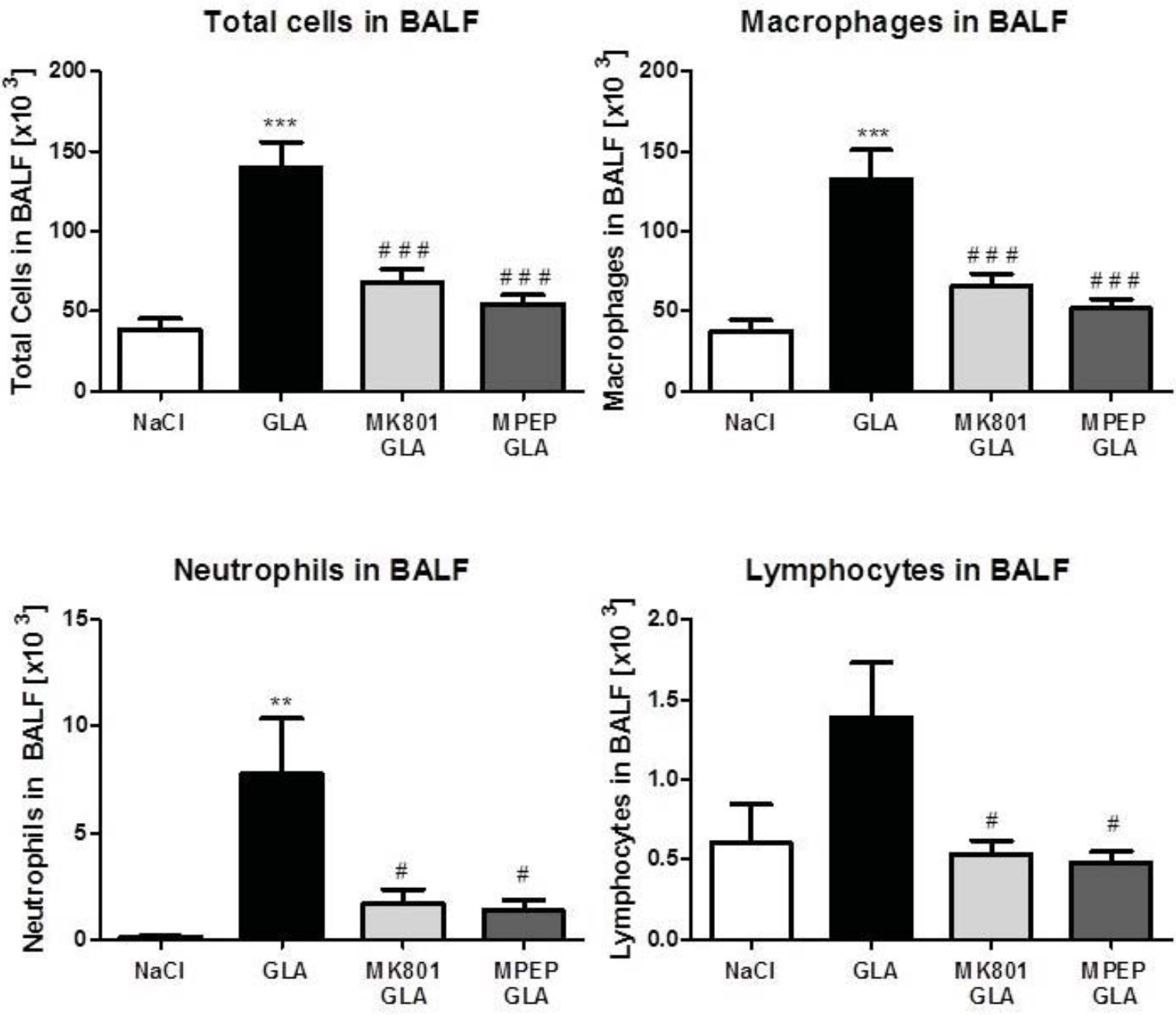

Total cells in BALF
Total Cells in BALF [x10 3]
200
150
100
50
0
***
###
###
NaCl
GLA
MK801 GLA
MPEP GLA
Macrophages in BALF
Macrophages in BALF [x10 3]
200
150
100
50
0
***
###
###
NaCl
GLA
MK801 GLA
MPEP GLA
Neutrophils in BALF
Neutrophils in BALF [x10 3]
15
10
5
0
**
#
#
NaCl
GLA
MK801 GLA
MPEP GLA
Lymphocytes in BALF
Lymphocytes in BALF [x10 3]
2.0
1.5
1.0
0.5
0.0
#
#
NaCl
GLA
MK801 GLA
MPEP GLA

Figure 6:

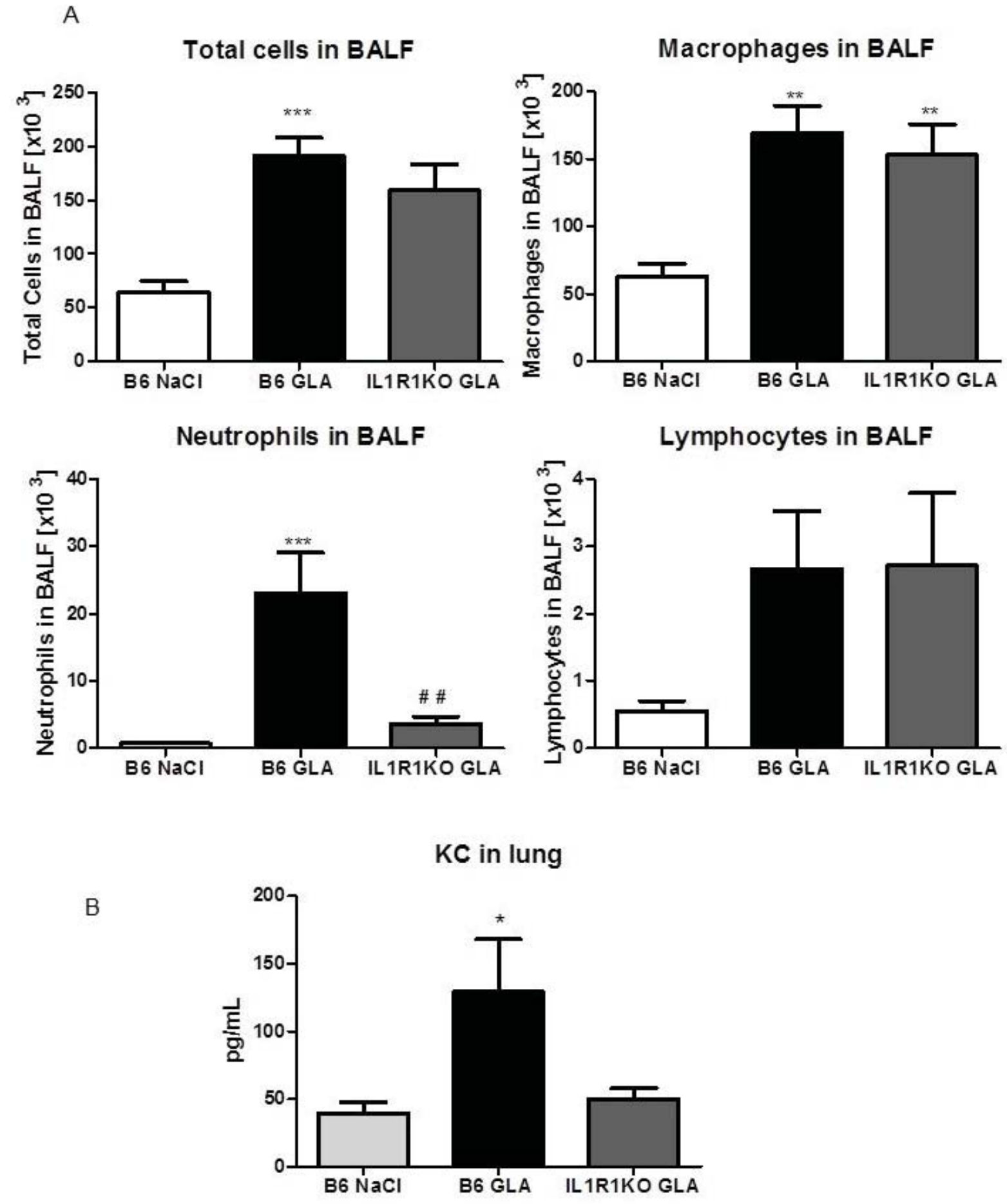
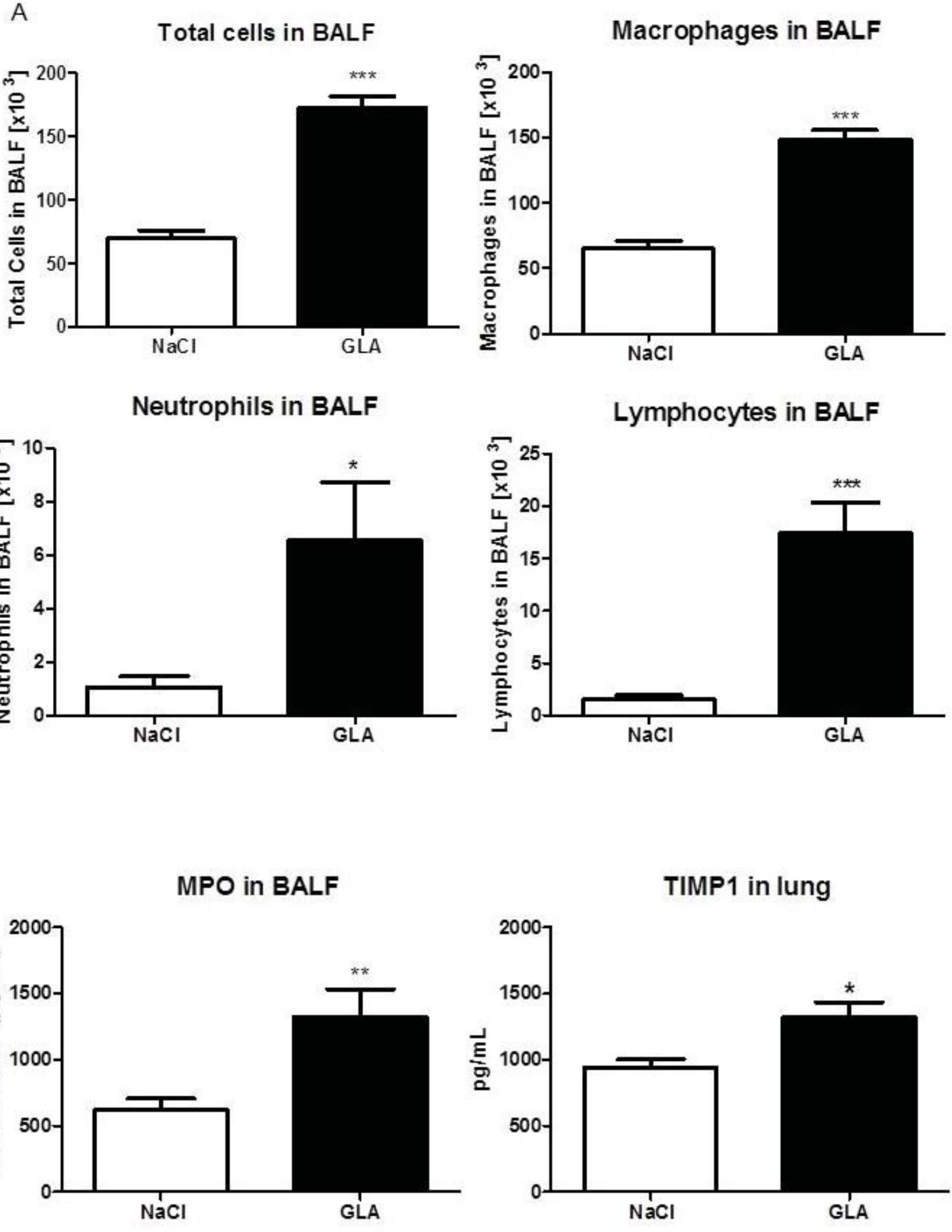
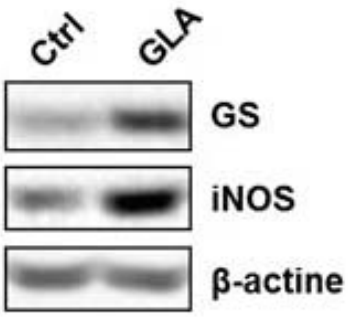
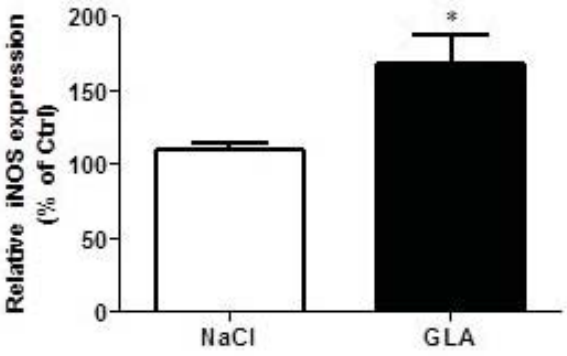

A
Total cells in BALF
Total Cells in BALF [x10 3]
250
200
150
100
50
0
***
B6 NaCl
B6 GLA
IL1R1KO GLA
Macrophages in BALF
Macrophages in BALF [x10 3]
200
150
100
50
0
**
**
B6 NaCl
B6 GLA
IL1R1KO GLA
Neutrophils in BALF
Neutrophils in BALF [x10 3]
40
30
20
10
0
***
# #
B6 NaCl
B6 GLA
IL1R1KO GLA
Lymphocytes in BALF
Lymphocytes in BALF [x10 3]
4
3
2
1
0
B6 NaCl
B6 GLA
IL1R1KO GLA
KC in lung
B
pg/mL
200
150
100
50
0
*
B6 NaCl
B6 GLA
IL1R1KO GLA

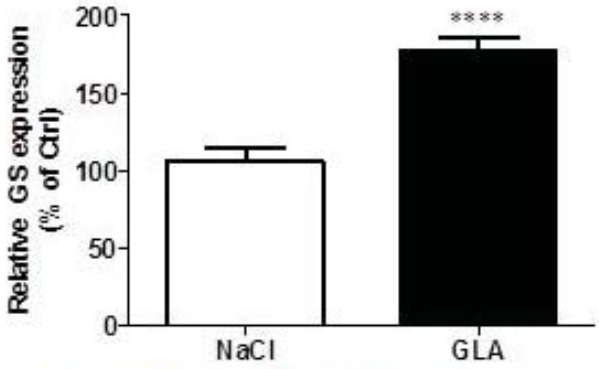
Figure 7:


A
Total cells in BALF
Total Cells in BALF [x10 3]
200
150
100
50
0
***
NaCl
GLA
Macrophages in BALF
Macrophages in BALF [x10 3]
200
150
100
50
0
***
NaCl
GLA
Neutrophils in BALF
Neutrophils in BALF [x10 3]
10
8
6
4
2
0
*
NaCl
GLA
Lymphocytes in BALF
Lymphocytes in BALF [x10 3]
25
20
15
10
5
0
***
NaCl
GLA
MPO in BALF
MPO in BALF (pg/mL)
2000
1500
1000
500
0
**
NaCl
GLA
TIMP1 in lung
pg/mL
2000
1500
1000
500
0
*
NaCl
GLA

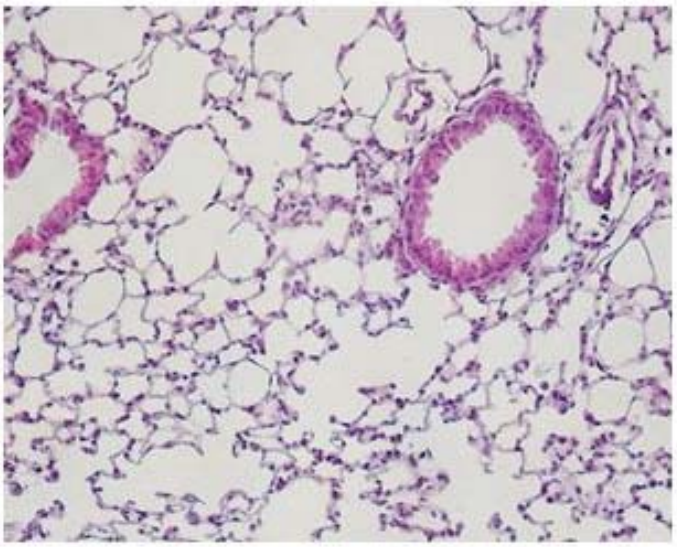
B



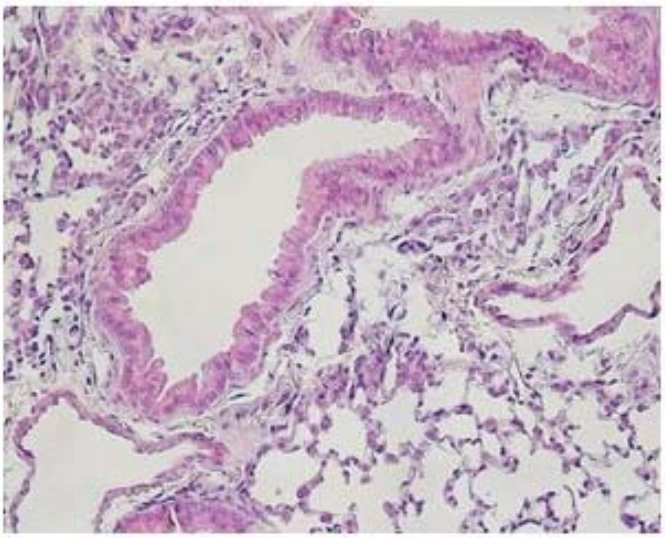




C

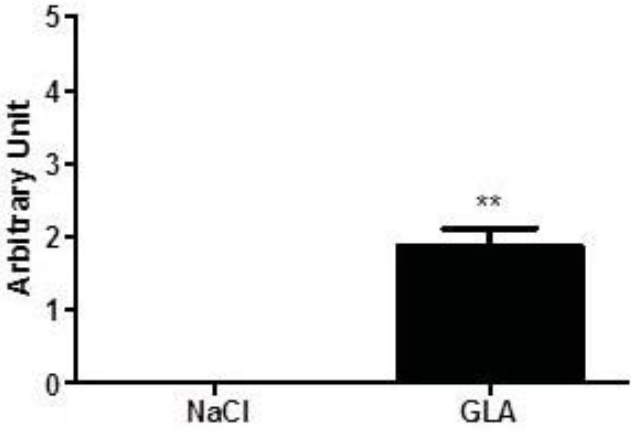


NaCl



GLA

Inflammation



Emphysema

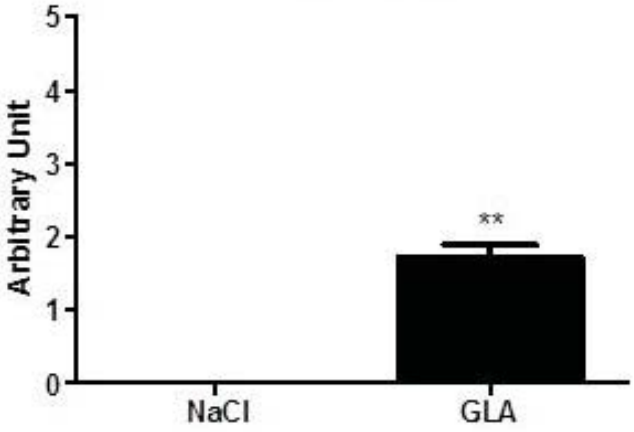
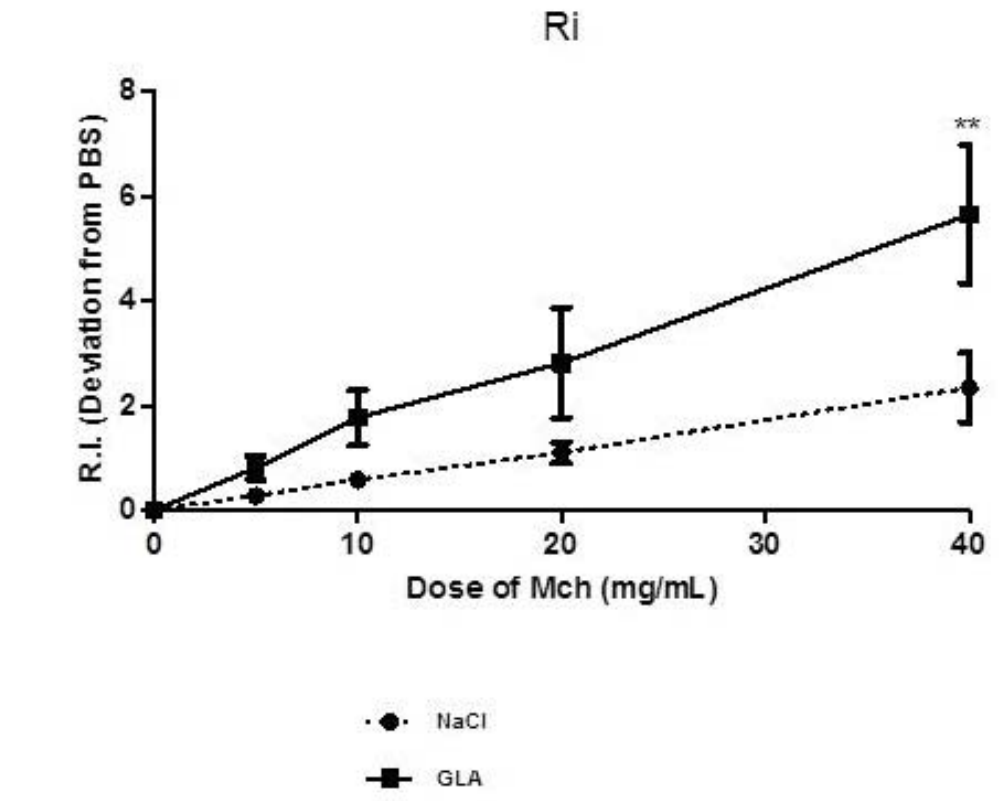
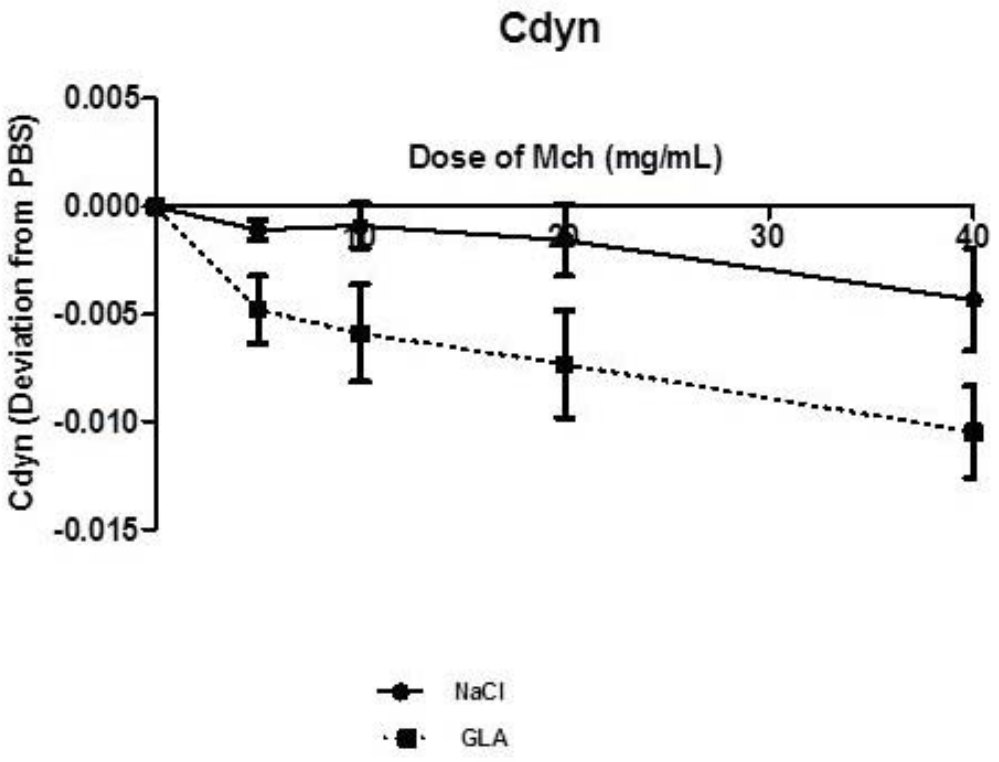


Figure 8:


Ri
R.I. (Deviation from PBS)
8
6
4
2
0
**
0
10
20
30
40
Dose of Mch (mg/mL)
NaCl
GLA


Cdyn
Cdyn (Deviation from PBS)
0.005
0.000
-0.005
-0.010
-0.015
Dose of Mch (mg/mL)
10
30
40
NaCl
GLA