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An Investigation of the Levels of Pro-Inflammation Cytokines in the Brain of Autoimmune Lewis Rats

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AN INVESTIGATION OF THE LEVELS OF PRO-INFLAMMATION CYTOKINES IN THE
BRAIN OF AUTOIMMUNE LEWIS RATS

by

HAN YANG

A THESIS

Presented to the Faculty of the University of the Incarnate Word
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DEDICATION

This thesis is dedicated to my family who has given me immeasurable courage and love.

AN INVESTIGATION OF THE LEVELS OF PRO-INFLAMMATION CYTOKINES IN THE BRAIN OF AUTOIMMUNE LEWIS RATS

Han Yang

University of the Incarnate Word, 2017

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, which can cause cartilage and bone damage, as well as disability. A reported symptom of RA includes tissue inflammation disease and cognitive dysfunction. This study aimed to compare the concentration of selected cytokines in the cerebral cortex, hippocampus, hypothalamus, amygdala, and cerebellum of Lewis rats. Age and sex-matched Lewis rats were separated into two groups: control and adjuvant-induced RA. Twenty-eight days after the adjuvant injection, groups were sacrificed and brains dissected using the stereotaxic atlas of Paxinos and Watson as a guide. Pro-inflammatory cytokines IL-1 β , IL-6, and IL-23, plus the anti-inflammatory IL-10 were measured by enzyme-linked immunosorbent assays (ELISA) in tissue homogenates. The results indicate that RA increased the concentration of pro-inflammatory cytokines and anti-inflammatory cytokine IL-10 in the amygdala and cerebellum, and IL-6 also increased in the cerebral cortex, hippocampus,

and hypothalamus. These data provide evidence that adjuvant-induced RA alters the inflammation status of these brain regions. The results are useful in the development of novel drug therapies for the millions of RA patients at risk of developing mood changes, memory loss, and other cognitive deficits.

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Human Autoimmunity Disorders

The immune system is an important defense mechanism in the mammalian body which defends against viruses, bacteria, or parasites. Autoimmune disorders occur when the body fails to tell the difference between self and non-self. When this happens, the self-immune system targets the wrong cell or tissue, and attacks itself, and the body makes antibodies that are directed towards the its own tissues. The autoantibodies attack the normal cells in error. Autoimmunity can be found in all vertebrates, but it is usually harmless (Mutsaers, 2016). The causes of autoimmune diseases are still largely unknown, which environment, gene, and immunity might be primary reasons to develop autoimmunity in recent studies (Kaczkowski, 2013). Common autoimmune diseases are lupus erythematosus, Sjögren's syndrome, and rheumatoid arthritis. Lupus erythematosus is a chronic systemic autoimmune disease, which is caused by an aberrant autoimmune response (Duarte, Couto, Ines, & Liang, 2011). It is regarded as a failure of the immune system to maintain tolerance to self-antigens (Singh et al., 2016). Lupus erythematosus can occur throughout the body, including joints, skin, brain, kidneys, blood cells, heart, and lungs. The most common and severe form is systemic lupus erythematosus. Sjögren's syndrome is a long-term autoimmune disease in which exocrine glands, particularly the lacrimal glands of the eyes, and salivary glands of the mouth, are damaged by the immune system. This leads to dry eyes and mouth. The disease affects mostly females. Also, Sjögren's syndrome can cause skin,

nasal and vaginal dryness, and may affect the body's other organs, such as kidney, blood vessels, lungs, liver, pancreas and brain. Patients may experience a secondary disease with typical symptoms associated with rheumatoid disease (Smolen, Aletaha, & McInnes, 2016).

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, which can cause cartilage and bone damage as well as disability (Moriyama et al., 2012). It usually leads to joint fever, swelling and pain. Pain and stiffness tend to worsen after rest. The most common affected area is the wrist and hand involving the same joint on both sides of the body. The disease may also affect other parts of the body. This can lead to low red blood cell count, plus lung and heart inflammation, and cognitive dysfunction. RA symptoms may also include fever and lack of vitality. The trigger of RA is still unknown, and highly activated macrophages are found in the RA patients, which cause pro-inflammatory cytokines (tumor necrosis factor- alpha, interleukin 1 beta, and interleukin 6) overproduction and leads to inflammation (Heo et al., 2017; Scott & Huizinga, 2010). This study used rheumatoid arthritis as an autoimmunity model.

Brain Regions and Cognition

Most autoimmune diseases can cause brain lesions, including RA (Ashraf et al., 2009; Diamond & Volpe, 2012; Koopman, 2001), and the effect of autoimmune diseases on the brain may cause greater damage to the patient. Cognitive dysfunction has been associated with autoimmune disorders; many autoimmune disorder patients have been detected with cognitive

impairment, which affects their learning, and memory (Adhikari, Piatti, & Luggen, 2011; Baptista et al., 2017). The limbic system includes several brain regions and involves in learning, memory, and emotional behavior functions (Barili, Zaccheo, & Amenta 1998). Therefore, detection of each brain region in the limbic system can help to better understand the relationship between cognitive dysfunction and autoimmune disorder. This experiment focused on five regions of the rat brain: cerebral cortex, cerebellum, hippocampus, hypothalamus, and amygdala. The cerebral cortex controls learning, language, emotions, somatic sensation, vision, and hearing, which links to limbic system. The cerebellum plays an important role in sensory perception, coordination, and motion control. The hippocampus, hypothalamus, and amygdala are part of the limbic system. The hippocampus mediates short-term memory, long-term memory, and spatial positioning. The hypothalamus plays a role in visceral and endocrine activity. The amygdala regulates visceral activity and produces emotions. All of these are important functions, and since autoimmune diseases cause brain damage (Ren & Torres, 2009), any changes can have a dramatic impact on individuals.

Cytokines and Inflammation

There are two categories of cytokines: one is pro-inflammatory cytokine, the other is anti-inflammatory cytokine. These cytokines are regulated to immune response. Microglia cells play a key role of brain inflammation by their production of cytokines. Pro-inflammatory cytokines

are produced by M1 microglia cells, and anti-inflammatory cytokines are produced by M2 microglia cells in the brain (Goldmann & Prinz, 2013). A number of pro-inflammatory cytokines play an important role in the development of RA. These include interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF- α). The high pro-inflammatory cytokine levels have been reported in RA patients (Kim & Moudgil, 2017).

IL-1 β is one of IL-1 family members, which is a pro-inflammatory cytokine that has been implicated in pain, inflammation and autoimmune condition (Steinman, 2008). IL-1 β can cause apoptosis, and many cells to secrete IL-1 β , such as, M1 microglia cell, macrophages, natural killer cells, and neutrophils. High concentrations of IL-1 β have been observed in acute and chronic neuroinflammation, such as brain ischemia, Alzheimer's disease and multiple sclerosis (MS) (Brosnan, Cannella, Battistini, & Raine, 1995).

IL-6 is a multifunctional cytokine, which can regulate the immune response, hematopoiesis, the acute phase response, and inflammation (Hirano, 1998). IL-6 can inhibit TNF- α and IL-1, and stimulate IL-1 β and IL-10 to anti-inflammation.

Cytokine synthesis inhibitory factor (CSIF) is the original name of IL-10, which was first documented to inhibit Th1 activation and cytokine production (Hofmann, Rösen-Wolff, Tsokos, & Hedrich, 2012). Thus, it has been characterized as an anti-inflammation cytokine. IL-10 is secreted by a wide variety of cell types, including macrophages and T cell subsets (Mannino,

2015). IL-10 reduces the production of proinflammatory cytokines by inhibiting the action of antigens present on T lymphocytes.

IL-23 is a heterodimeric cytokine composed of a p40 subunit shared with IL-12. They comprise p19 and p35 subunit (Murphy, 2003). IL-23 and IL-12 are important mediators of inflammation in the mouse models of inflammation (Tang, Chen, Qian, & Huang, 2012).

Due to the key role cytokines play in most autoimmune diseases, this study sought to investigate the inflammation and cytokine level profiles (IL-1 β , IL-6, IL-10, and IL-23) associated with autoimmunity in the brains of Lewis rats in which RA was experimentally induced versus control. The hypothesis is, when Lewis rats are challenged with *Mycobacterium tuberculosis* pro-inflammatory cytokines levels in the selected regions of the rat brain will increase, as anti-inflammatory cytokines levels decrease. By investigating the pro-inflammatory and anti-inflammatory cytokine profiles of animals with RA, a better understanding of the causes and extent of inflammation in the brain can be determined. In particular, the specific aims of this investigation were to:

- A) Development of Rheumatoid Arthritis in Lewis Rats. This would be achieved by injection of *Mycobacterium tuberculosis* adjuvant into Lewis rats' to induce rheumatoid arthritis conditions. Degree of inflammation would be recorded every 7 days for 28 days.
- B) Collect of serum samples to assess development of RA and inflammatory cytokines.

C) Detection of pro-inflammatory cytokines (IL-1 β , IL-6, and IL-23) and anti-inflammatory cytokines (IL-10) levels in all brain and serum samples for each control and treatment group by Enzyme Linked Immunosorbant Assay (ELISA), and comparison each cytokine level in different regions of brain.

Materials and Methods

Autoimmunity Rat Model

This animal protocol was approved by the University of Texas at San Antonio Institutional Animal Care and Use Committee, protocol number: #RA066-08/19. Three-month-old male Lewis rats (Charles River Laboratories, Wilmington, MA) were used as an autoimmune model. These rats are sensitive to the development of autoimmune diseases, including adjuvant-induced arthritis. There were two groups for this experiment, eight for control group, and eight for RA group, a total sixteen Lewis rats. The fur in the area around the base of the tail was clipped and 70% ethanol was used to disinfect the area to create a sterile field in preparation for the injection. This experiment spanned 28 days. The control group was injected with 100 μ l saline at day 1 and day 7. Rats in the RA group were injected once with 100 μ l of a reagent containing heat-killed and dried *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate, Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO) in the base of the tail at day 1, and injected with 100 μ l Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO) after 7 days.

Rats were observed daily for signs of pain, distress and abscess formation. If an abscess developed, distress, or pain became apparent, the rat would be euthanized. Monitoring of arthritis symptoms was done by measuring the hind paw volume by water displacement, using a rat plethysmometer on days 7, 14, 17, and 20 post-injections. A single boost of 100 µl of Freund's incomplete adjuvant was injected on day 7 to each rat in the treatment group. Arthritic intensity was scored based on physical examination of the four paws: 0 = no erythema or swelling, 1 = slight erythema or swelling of one toe or finger, 2 = erythema and swelling of more than one toe or finger, 3 = erythema and swelling of the ankle or wrist, and 4 = complete erythema and swelling of the toes or fingers and the ankle or wrist and an inability to bend the ankle or wrist (Hirano, 1994). The highest possible score was 16. Rats were sacrificed by guillotine, when a score was equal to or greater than 12. Remaining rats (control and treatment groups) were sacrificed at day 28 (Figure 1).

Serum and Brain Tissue Dissection

Once rats were sacrificed, a heart blood sample was collected and placed into heparin tubes. The blood samples were put in the centrifuge for 5 minutes to collect serum samples. Five regions of the rat brain (cerebral cortex, cerebellum, hippocampus, hypothalamus, and amygdala) from all rats of the control and treatment group were dissected. Paxinos and Watson Stereotaxic atlas (Paxinos & Watson, 2009) book was referred to determine the location of each part of brain

(130 mM NaCl, 30 mM Tris-base, 5 mM KCl, 2 mM MgCl₂, 1 mM EGTA). For brain tissue removal, rongeurs, scalpel, and a scooped spatula were used. After taking the brain, the brain was put in the brain buffer, all process was done in this buffer. Rat's brain was cut in half, and only the right side of brain was used in this experiment. Brain samples were placed in cryovial tubes, and temporarily stored in liquid nitrogen. All samples were stored in temperature -80 °C freezer.

Samples

All serum samples were diluted to 2-fold before measuring total protein. RIPA buffer with protease inhibitor buffer were prepared for sample dilution. The ratio was 1 protease inhibitor tablet (Sigma-Aldrich, St. Louis, MO), adding in 50ml RIPA buffer (Thermo Scientific, Carlsbad, CA). 1000 µl serum sample was mixed with 1000 µl RIPA with protease inhibitor buffer, which diluted to 2X serum sample. All brain samples were homogenized before measuring total protein. A 0.05g brain sample and 1000 µl RIPA with protease inhibitor buffer was added into the glass homogenizer, which made a 20X homogenized brain sample.

Bicinchronic Acid Assay (BCA)

The BCA assay (Thermo Scientific, Carlsbad, CA) is a biochemical assay using a colorimetric method to estimate protein concentration in a tissue sample. All samples were tested by the BCA Assay basis of the quantitation of total protein. Briefly, reagent A was mixed with reagent B at a ratio of 50:1, then 9µl of a sample was mixed with 260µl of the mixed AB reagent

in 96-well plate. The 96-well plate sample incubated 30 minutes at 37 °C temperature, and cooled to room temperature. The 96-well plate sample was read by the microplate reader. All samples were detected twice, $n = 3$. Each serum and brain region sample were made to the same protein concentration in control and RA group.

Enzyme-Linked Immunosorbent Assay

In this experiment, IL-1 β , IL-6, IL-10, and IL-23 were measured by sandwich enzyme-linked immunosorbent assay (ELISA [RayBiotech, Inc., Norcross, GA]) in each serum and brain sample. Using the BCA detected protein concentration samples measured each cytokine level. The RayBiotech ELISA kit information was used in the sandwich ELISA, the specific antibody was coated on the surface of a 96-well plate. A homogenized brain sample was placed into the 96-well plate, the antigen of the sample bound to the specific antibody. After washing, a specific antibody (primary antibody) which bound to the sample's antigen was placed on the 96-well plate. The remaining primary antibody was washed again. The primary antibody was bonded by an enzyme-linked secondary antibody. The plate was washed to remove the remaining enzyme-linked secondary antibody. This step was followed by adding a chemical to the 96-well plate, which caused a change color. The 96-well plate sample was detected by a microplate reader. The specific process:

1. 100 µl standard or sample added to precoated 96-well plates. Incubate 2.5 hours at room temperature.
2. 96-well plate washed 4 times with 300 µl prepared wash solution.
3. 100 µl prepared biotin antibody added to each well. Incubate 1 hour at room temperature.
4. 96-well plate washed 4 times with 300 µl prepared wash solution.
5. 100 µl prepared Streptavidin solution added. Incubate 45 minutes at room temperature.
6. 96-well plate washed 4 times with 300 µl prepared wash solution.
7. 100 µl TMB One-Step Substrate Reagent added to each well. Incubate 30 minutes at room temperature.
8. 50 µl Stop Solution added to each well. Read at 450 nm immediately.

Statistics

Comparison between control group and experimental. All group data was analyzed using the appropriate analysis of variance (2-way ANOVA). Differences between groups for all data was regarded as significant if $p < .05$.

Results

Rats Swelling

The rat plethysmometer data did not show significant differences between the control and RA group. The physical examination scoring observed swelling in the RA group. The RA

group rats recorded swelling in the paws, and the rats continued swelling at different days after injection (Figure 2, Figure 3). All control group rats did not have swelling or erythema in the toes or fingers, and all RA group rats were observed swelling in each paw.

Cytokine Levels

The serum cytokine levels were not successfully detected in any of the control and RA group samples. The concentrations of the cytokines IL-1 β , IL-6, IL-10, and IL-23 were measured in the cerebral cortex, hippocampus, hypothalamus, amygdala, and cerebellum regions of the brains of rats with adjuvant-induced rheumatoid arthritis.

In the cerebral cortex ($p < .05$, $n = 8$), IL-1 β content was 86.5 \pm 5.3 pg/ μ g protein in the control group, which decreased to 69.4 \pm 5.5 pg/ μ g protein in the RA group (Table 1, Figure 4). Similar results were obtained in the hippocampus where 113.9 \pm 8.9 pg/ μ g protein was measured in the control group, and 87.6 \pm 3.4 pg/ μ g protein in the RA group ($p < .05$, $n = 8$) (Table 1, Figure 5)

IL-1 β concentration increased in the hypothalamus of the RA group ($n = 8$), as compared to the control group 87.4 \pm 4.6 pg/ μ g protein in the control group, and 89.9 \pm 6.5 pg/ μ g protein in the RA group) (Table 1, Figure 6).

There was a significant increase ($p < .05$, $n = 8$) in the IL-1 β content in the adjuvant-induced RA amygdala, 68.7 \pm 6.3 pg/ μ g protein in the control group, and 91.4 \pm 5.9 pg/ μ g

protein in the RA group (Table 1, Figure 7). Additionally, IL-1 β significantly increased in adjuvant-induced RA cerebellum ($p < .05$, $n = 8$) 97.2 \pm 6.1 pg/ μ g protein in the control group, and 118.9 \pm 4.4 pg/ μ g protein in the RA group (Table 1, Figure 8).

The pro-inflammatory cytokine, IL-6 increased in all adjuvant-induced RA brain areas tested. In the cerebral cortex ($n = 8$), the IL-6 concentration increased from 22.5 \pm 3.1 pg/ μ g protein in the control group to 29.3 \pm 2.4 pg/ μ g protein in the RA group (Table 1, Figure 9). IL-6 significantly increased in the hippocampus ($p < .05$, $n = 8$) from 31.8 \pm 3.1 pg/ μ g protein in the control group to 47.1 \pm 3.7 pg/ μ g protein in the RA group (Table 1, Figure 10). In the hypothalamus ($p < .05$, $n = 8$), IL-6 increased from 20.5 \pm 3.0 pg/ μ g protein in the control group to 29.7 \pm 1.1 pg/ μ g protein in the RA group (Table 1, Figure 11). A larger IL-6 cytokine effect of RA was measured in the amygdala ($p < .005$, $n = 8$), where the concentration increased from 27.3 \pm 2.3 pg/ μ g protein in the control group to 51.7 \pm 2.9 pg/ μ g protein in the RA group (Table 1, Figure 12). Similarly, a significant effect ($p < .005$, $n = 8$) of RA was recorded in the cerebellum where the IL-6 level was 37.3 \pm 5.1 pg/ μ g protein in the control group, compared to 69.0 \pm 1.3 pg/ μ g protein in the RA group (Table 1, Figure 13).

The anti-inflammatory cytokine, IL-10, significantly decreased in the RA condition in the cerebral cortex ($p < .05$, $n = 8$). The IL-10 concentration was 65.7 \pm 9.3 pg/ μ g protein in the control group, and 35.9 \pm 1.7 pg/ μ g protein in the RA group (Table 1, Figure 14). Similarly, IL-

IL-10 significantly decreased in the hippocampus RA condition ($p < .05$, $n = 8$). The protein concentration was 106.3 ± 13.5 pg/ μ g protein in the control group, and 65.1 ± 7.4 pg/ μ g protein in the RA group (Table 1, Figure 15). In the hypothalamus ($p < .05$, $n = 8$) the IL-10 cytokine concentration was 78.1 ± 14.1 pg/ μ g protein in the control group, and 38.7 ± 1.9 pg/ μ g protein in the RA group (Table 1, Figure 16).

The IL-10 level in the adjuvant-induced RA amygdala ($n = 8$) increased, but this increase was not significant. The concentration in the control group was 70.6 ± 11.5 pg/ μ g protein in the control group, compared to 93.6 ± 19.3 pg/ μ g protein in the RA group (Table 1, Figure 17).

A significant IL-10 increase was recorded in cerebellum ($p < .05$, $n = 8$). The IL-10 cytokine concentration was 52.0 ± 14.7 pg/ μ g protein in the control group, and 90.4 ± 3.3 pg/ μ g protein in the RA group (Table 1, Figure 18).

The pro-inflammatory cytokine, IL-23 was not significantly changed in the RA condition in the cerebral cortex ($n = 8$). The IL-23 concentration was 1399.6 ± 172.1 pg/ μ g protein in the control group, and 1212.0 ± 147.8 pg/ μ g protein in the RA group (Table 1, Figure 19). A slight increase of IL-23 was found in the RA condition in the hippocampus ($n = 8$). The IL-23 cytokine concentration was 2000.8 ± 279.1 pg/ μ g protein in the control group, and 2053.1 ± 192.8 pg/ μ g protein in the RA group (Table 1, Figure 20). A slight decrease in IL-23 was measured in RA condition in hypothalamus ($n = 8$). The IL-23 concentration was 1062.9 ± 100.9 pg/ μ g

protein in the control group, and 860.5 +/- 47.1 pg/μg protein in the RA group (Table 1, Figure 21).

There was a significant IL-23 increase in the adjuvant-induced RA amygdala ($p < .05$, $n = 8$). The IL-23 cytokine concentration was 998.6 +/- 88.0 pg/μg protein in the control group, and 1396.3 +/- 106.1 pg/μg protein in the RA group (Table 1, Figure 22). The level of IL-23 also increased in the adjuvant-induced RA amygdala ($p < .005$, $n = 8$). The IL-23 concentration was 1429.9 +/- 45.7 pg/μg in the control group, and increased to 2037.2 +/- 172.7 pg/μg in the RA group (Table 1, Figure 23).

Discussion

Cognitive dysfunction is reported in autoimmune disorders in humans and rats (Meade et al., 2017; Shin, Katz, Wallhagen, & Julian, 2012). One of the objectives of this work was to investigate if cytokine inflammatory markers are altered in the RA condition. The pro-inflammatory and anti-inflammatory cytokine complex interactions play a role in regulating the inflammation status of the brain. The significant findings of this study are that the concentration of the pro-inflammatory cytokines IL-1β, IL-6, IL-23, and anti-inflammatory cytokine IL-10 increased in five regions of brain. The major findings of this study are that RA significantly increased the concentration of the pro-inflammatory cytokines IL-1β, IL-6 and IL-23 in the amygdala and cerebellum. Also, the content of the anti-inflammatory marker IL-10 significantly

increased in the frontal lobe of the cerebral cortex, hippocampus, and hypothalamus with a significant increase in the cerebellum.

Serum Cytokine Levels

The blood serum cytokine levels tested were not detected in this study. This may be due to technical difficulties in the initial blood collection. Also, a limitation of this study that regular blood draws were not collected throughout the 28 days to follow the development of RA in the animals.

Inflammation in the Cerebral Cortex

The significant decrease in IL-1 β , coupled with the significant increase in IL-10 indicates IL-10 is possibly inhibiting Th1 cell secretion of IL-1 (Tian, Li, Wang, & Zhou, 2014). The cytokines IL-6 and IL-23 are reported to be linked to RA. Although a slight increase in IL-6 and IL-23 was recorded, these results suggest both of these inflammatory markers do not play a significant role when compared to IL-1 β and IL-10 in the frontal lobe of the cerebral cortex.

Inflammation in the Hippocampus

The significant increase of IL-6 in the hippocampus supports the findings that IL-6 plays an important role in the inflammation response in RA, and in this brain region, may be the primary pro-inflammatory cytokine as compared to IL-23, which increased only slightly. Of note is that the significant increase in IL-10, as in the frontal lobe of the cerebral cortex, may be suppressing the production of IL-1 β , which significantly decreased.

Inflammation in the Hypothalamus

Similar to the hippocampus, RA resulted in significant increases in IL-6 and IL-10.

Therefore, it appears the pattern of IL-6-mediated inflammation continues in the hypothalamus.

Since this brain region connects the central nervous system to the endocrine system, further work is necessary to investigate what effect, if any, IL-6 plays in hormone secretion, particularly those that influence mood and other cognitive processes.

Inflammation in the Amygdala and Cerebellum

The most important findings of this study are related to the effect of RA in the amygdala and cerebellum. Of the inflammation markers investigated, both pro- and anti-inflammatory cytokine concentrations were significantly increased in these brain regions in the adjuvant-induced RA group. Autoimmune diseases, and specifically, RA causes T and B cells, synovial-like fibroblasts, and macrophages overproduction of pro-inflammatory cytokines. Investigators have reported IL-10 increases in the synovial fluid, and serum of RA patients, which suggests IL-10 suppresses inflammation, and triggers the production of autoantibodies (Katsikis, 1994; Lacki, Klama, Mackiewicz, Mackiewicz, & Müller, 1995). These investigators proposed IL-10 might have the ability to suppress pro-inflammatory cytokines and promotes the humoral autoimmune response. The results of this study support this hypothesis and extends it to the brain in adjuvant-induced RA rats. The amygdala plays an important role in the regulation of emotions

and the cerebellum modulates sensory perception. The data obtained in this study suggest that inflammation may be contributing factor to regional brain function which may lead to cognitive impairment.

Conclusion

There is no animal behavior monitoring and evaluation in this study, the present results show cytokine levels in five regions of adjuvant-induced RA Lewis rats' brain. The cytokine changes of adjuvant-induced RA models indicate inflammation in the five regions of the rat brain. The results obtained in this study might be useful in the development of novel drug therapies which targets the specific pro-inflammatory cytokine for RA patients with cognitive impairment.

Future Directions

Future studies will be directed to: Adjuvant-induced RA rats can separate into slight, moderate and severe groups by injecting different volume of adjuvant. After 28 days of the first injection day, all group rats will be sacrificed to detect each cytokine level in the serum and selected brain regions. Different swelling groups might cause different cytokine level changes and brain lesions. Also, the experiment would test cognitive functions of the animals as RA progresses over time. Another direction is using cell flow cytometry to count cytokine producing cells, microglial cells in the adjuvant-induced RA rat's brain, the result could be better

understanding of cytokine profiles in the brain of autoimmune patients.

Table 1.

Cytokine Levels in Five Regions of Normal and RA Induced Lewis Rat Brains

	IL-1 β		IL-6		IL-10		IL-23	
	<u>Control</u>	<u>RA</u>	<u>Control</u>	<u>RA</u>	<u>Control</u>	<u>RA</u>	<u>Control</u>	<u>RA</u>
Cerebral Cortex	86.5	69.4 *	22.5	29.3	65.7	35.9 *	1399.6	1212.0
Hippocampus	113.9	87.6 *	31.8	47.1 *	106.3	65.1 *	2000.8	2053.1
Hypothalamus	87.4	89.9	20.5	29.7 *	78.1	38.7 *	1062.9	860.5
Amygdala	68.7	91.4 *	27.3	51.7 **	91.1	181.5*	998.6	1396.3 *
Cerebellum	97.2	118.9 *	37.3	69.0 **	52.0	90.4 *	1429.9	2037.2 **

Note. Values represent the mean of eight determinations. Concentrations are expressed in pg/ μ g protein.

* denotes the values are significantly different at $p < .05$. ** denotes the values are significantly different at $p < .005$

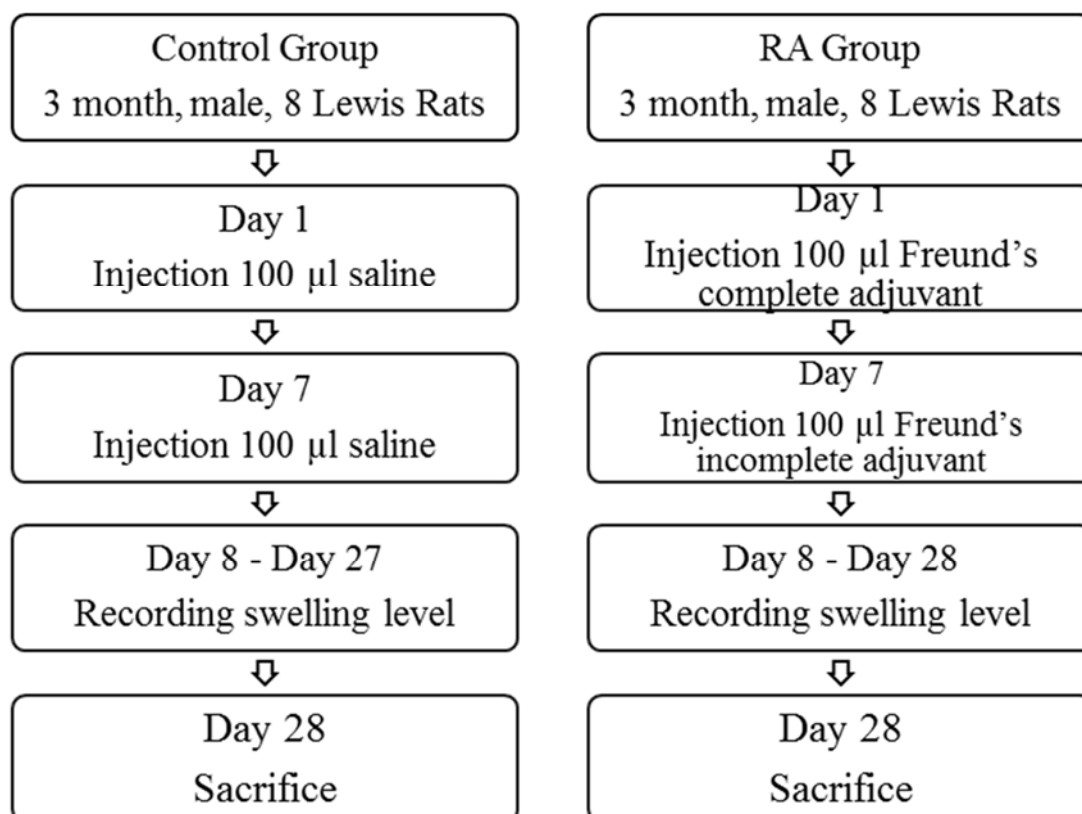


Figure 1. Animal model of adjuvant-induced rheumatoid arthritis (RA).



Figure 2. Control group rat's left back paw.

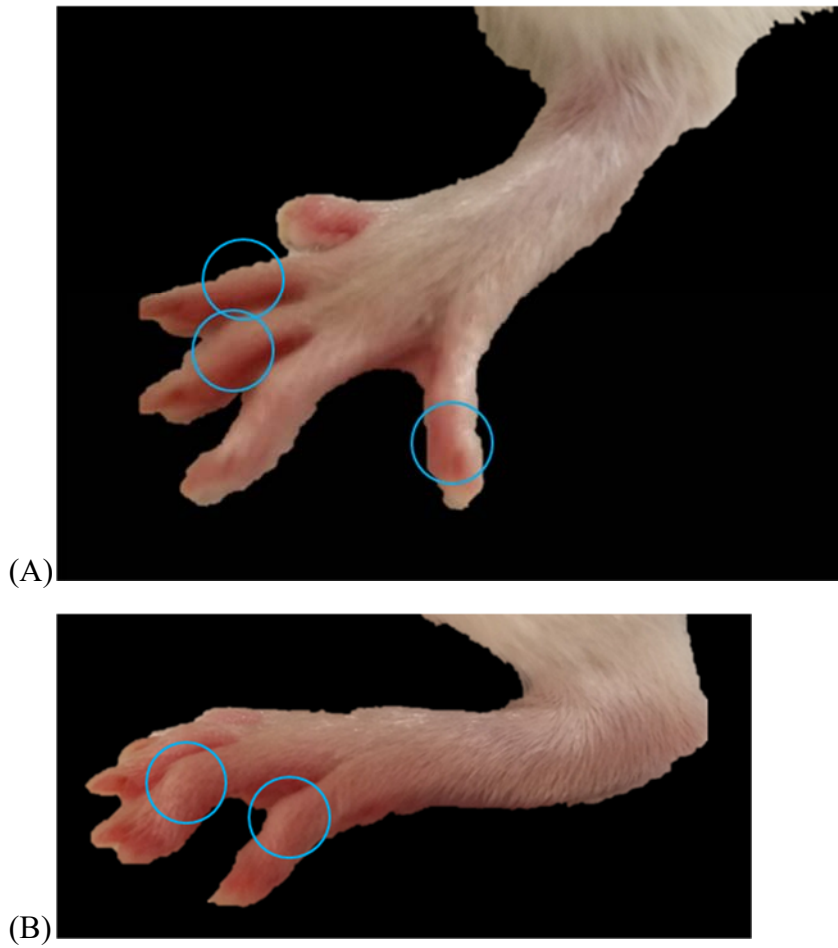


Figure 3. RA group rat's left back paw at different days. A) At day 14. Blue circles are swelling sites. B) At day 28. Blue circles are swelling sites. All pictures were taken from the same rat at different days.

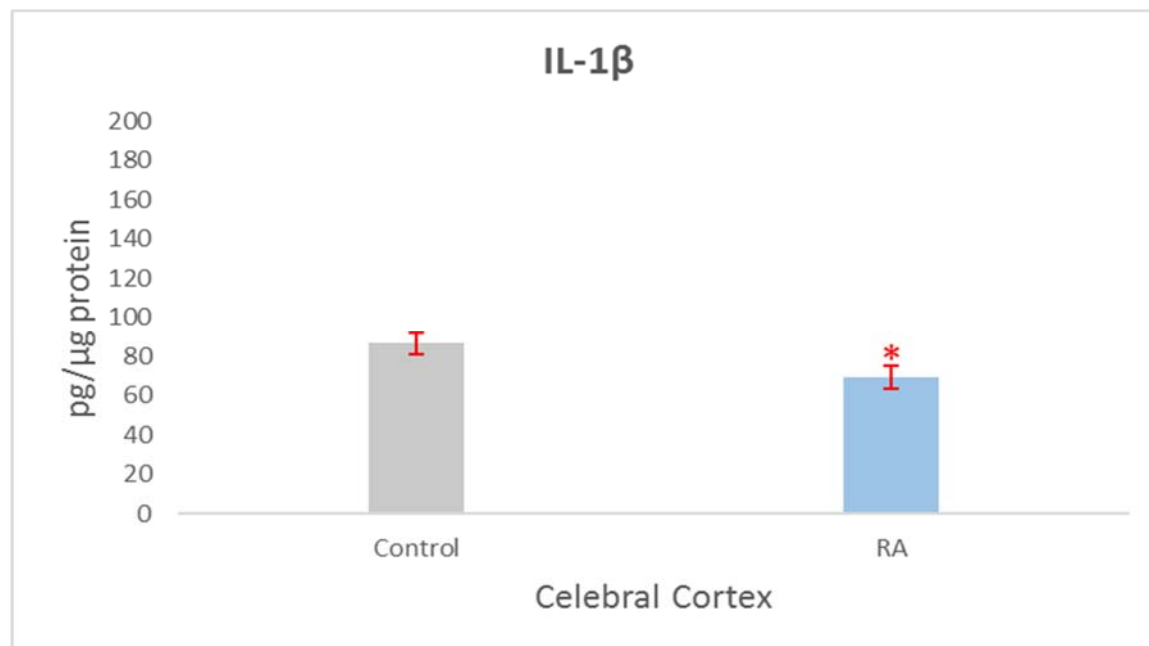


Figure 4. The inflammatory status of IL-1 β in Lewis rats' cerebral cortex. IL-1 β did not increase in cerebral cortex compared to control and RA groups ($p < .05$).

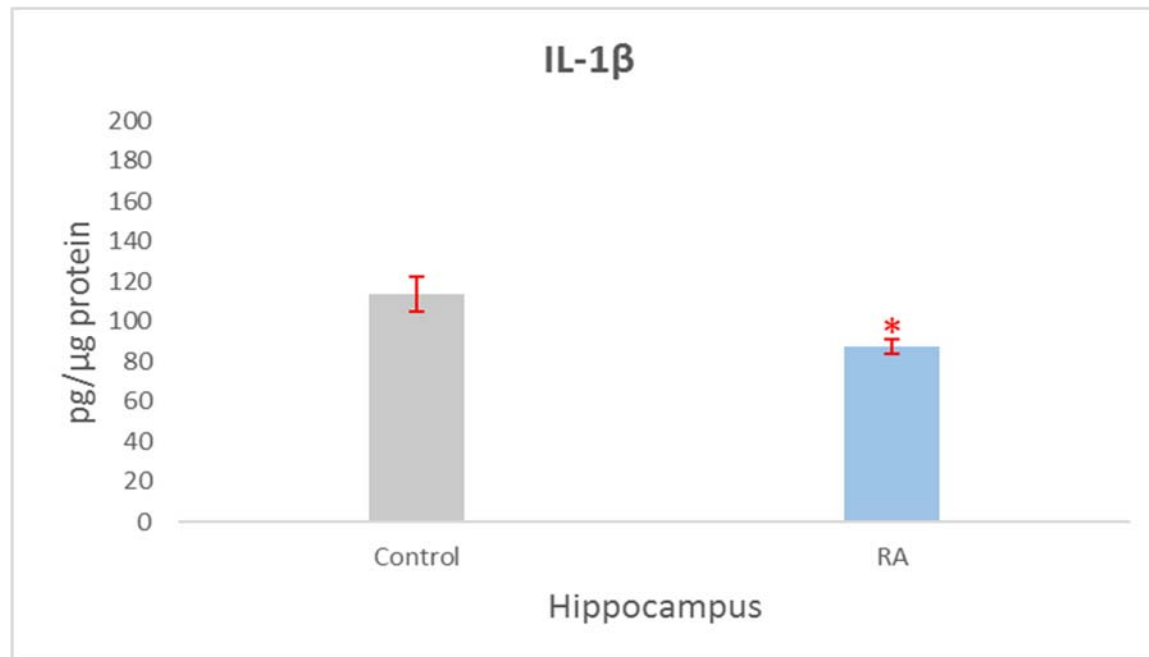


Figure 5. The inflammatory status of IL-1 β in Lewis rats' hippocampus. IL-1 β did not increase in hippocampus compared to control and RA groups ($p < .05$).

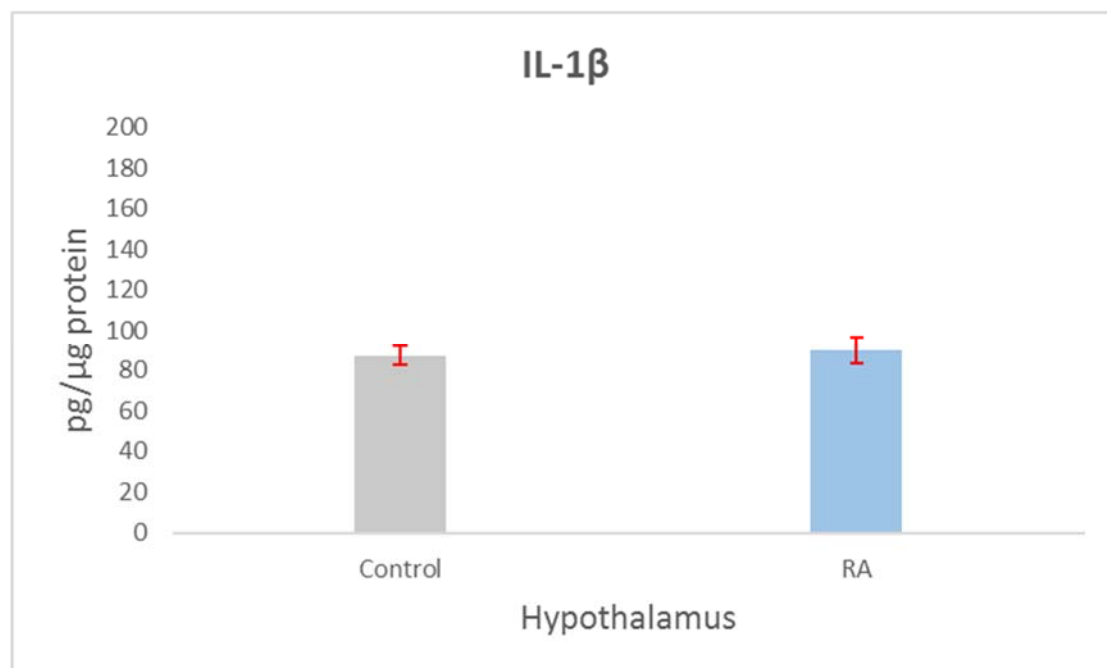


Figure 6. The inflammatory status of IL-1 β in Lewis rats' hypothalamus. IL-1 β had no significantly effects in hypothalamus compared to control and RA groups.

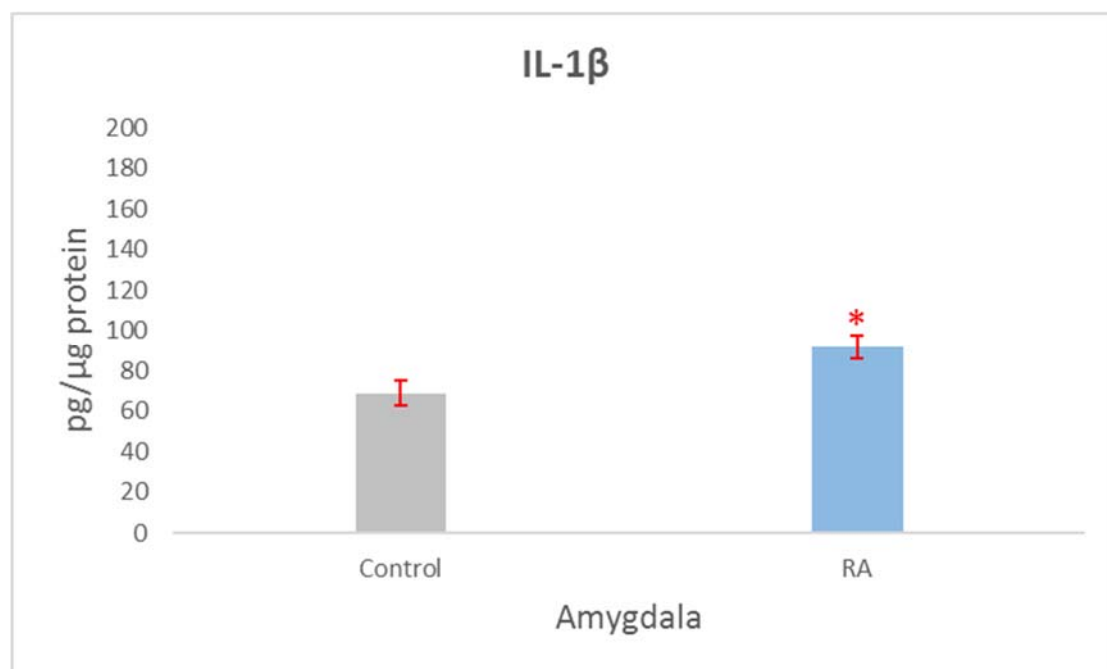


Figure 7. The inflammatory status of IL-1 β in Lewis rats' amygdala. IL-1 β significantly increased in the RA condition in amygdala region ($p < .05$).

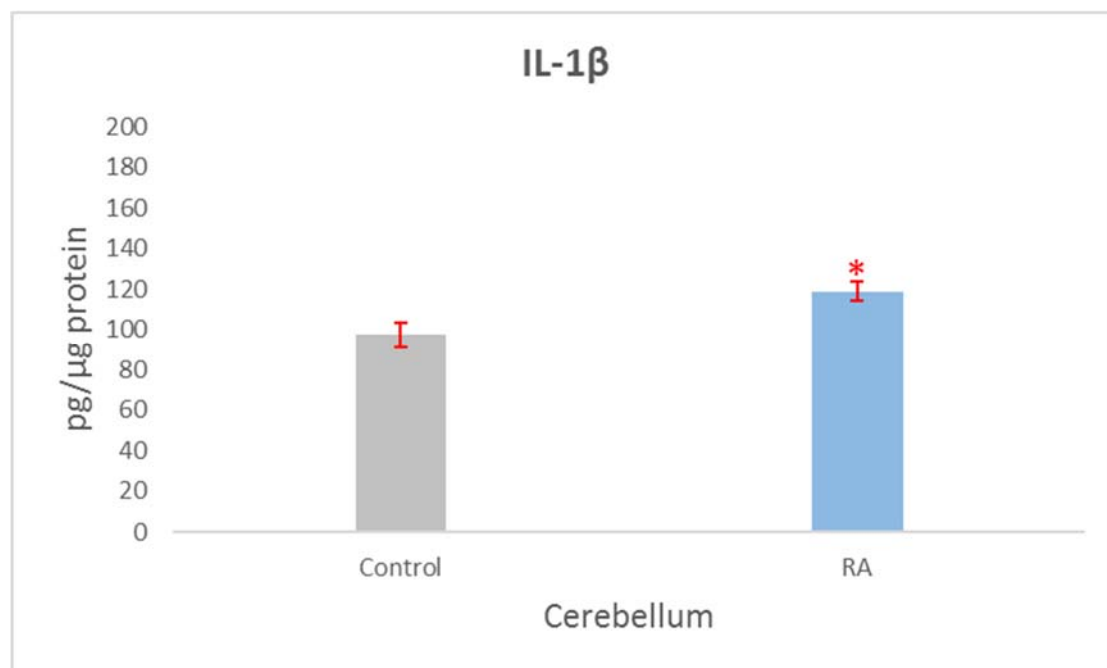


Figure 8. The inflammatory status of IL-1 β in Lewis rats' cerebellum. IL-1 β significantly increased in the RA condition in cerebellum region ($p < .05$).

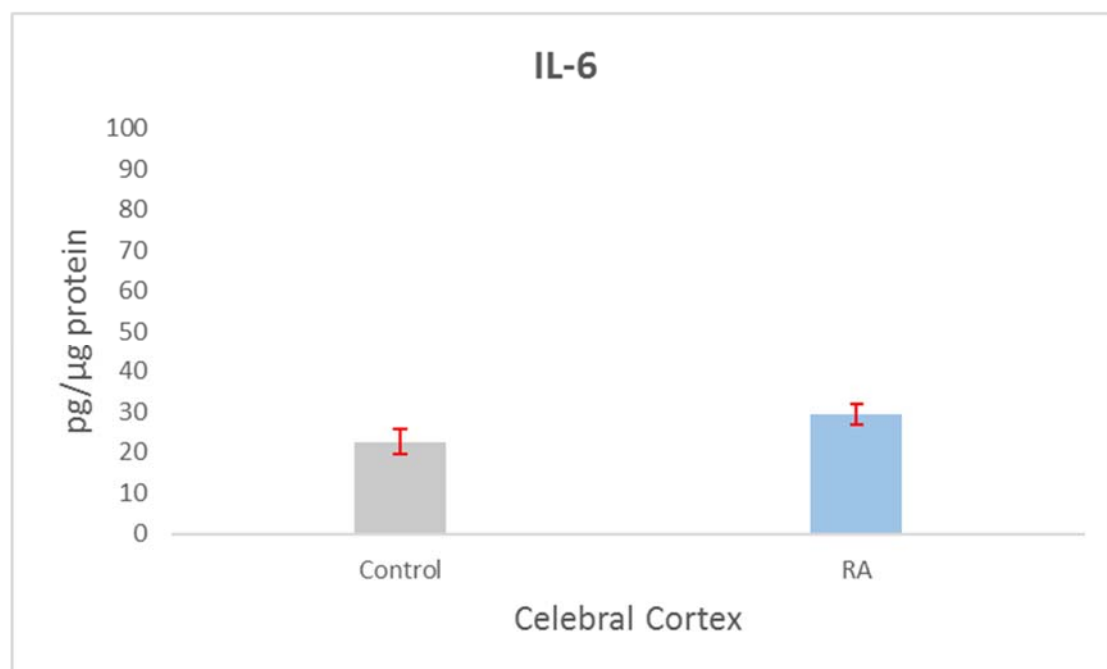


Figure 9. The inflammatory status of IL-6 in Lewis rats' cerebral cortex. IL-6 increased in the RA condition in cerebral cortex region but no significant effects.

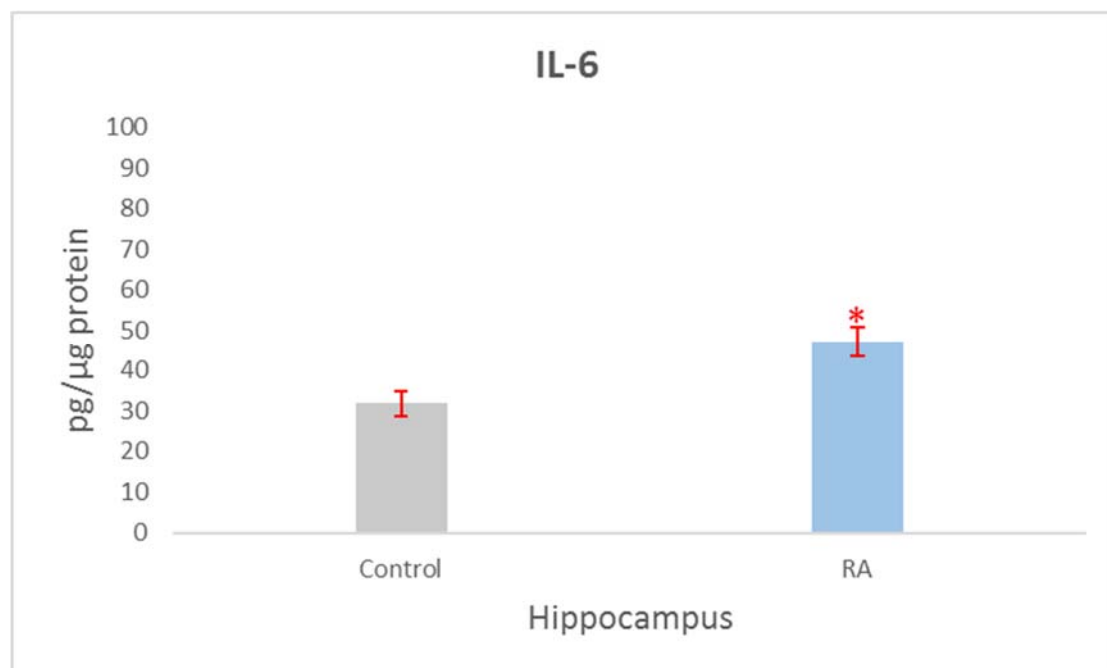


Figure 10. The inflammatory status of IL-6 in Lewis rats' hippocampus. IL-6 significantly increased in the RA condition in hippocampus region ($p < .05$).

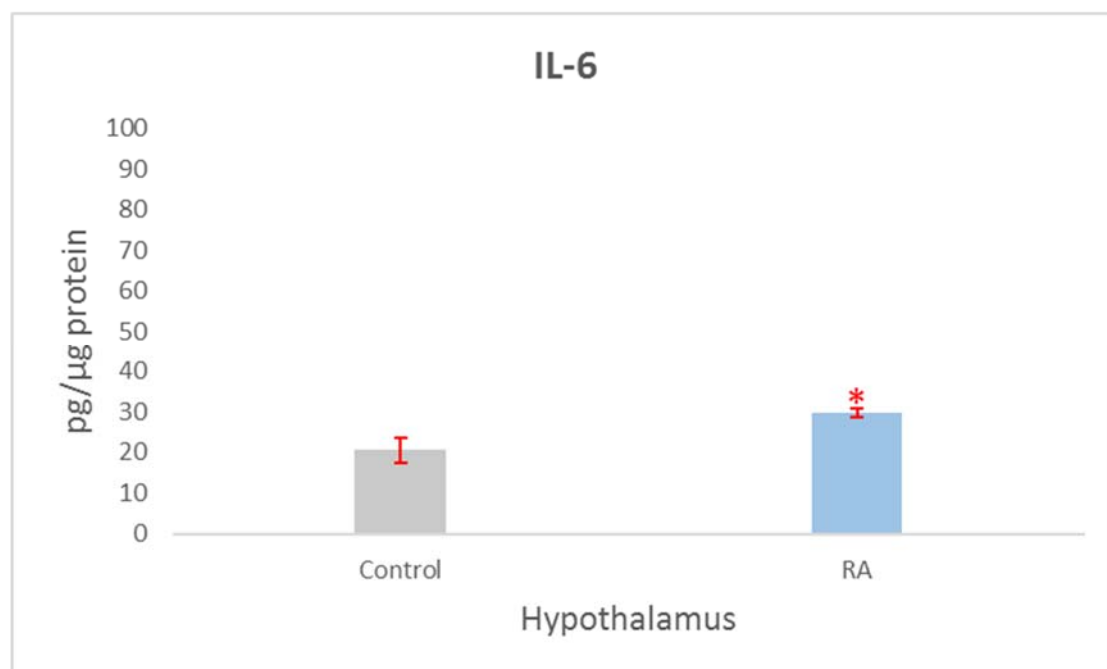


Figure 11. The inflammatory status of IL-6 in Lewis rats' hypothalamus. IL-6 significantly increased in the RA condition in hypothalamus region ($p < .05$).

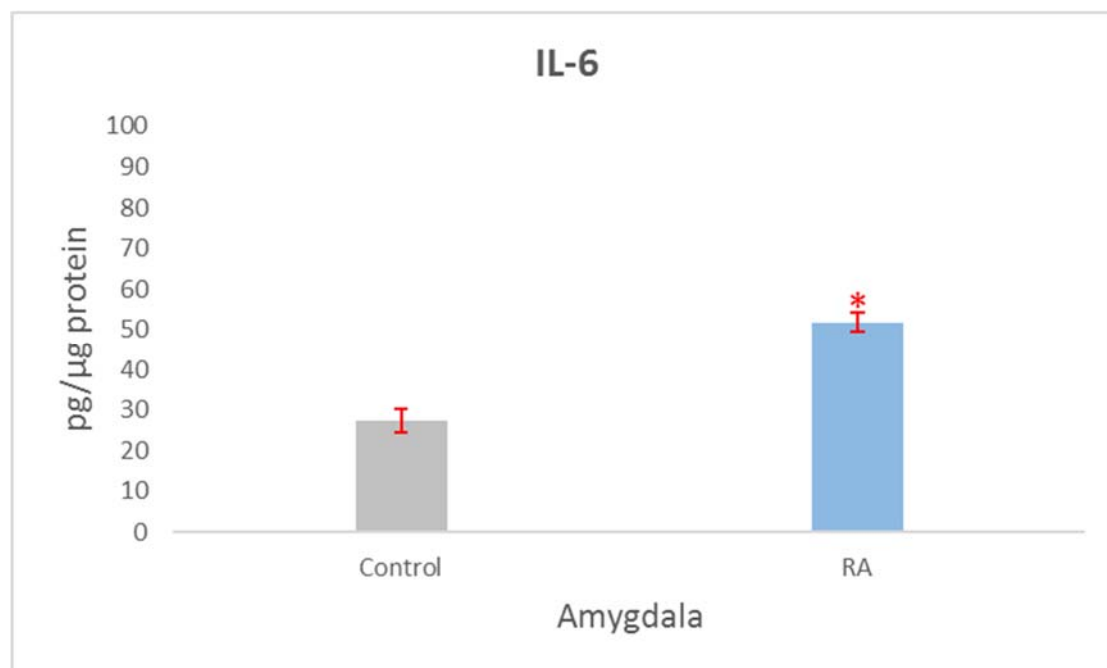


Figure 12. The inflammatory status of IL-6 in Lewis rats' amygdala. IL-6 significantly increased in the RA condition in amygdala region ($p < .005$).

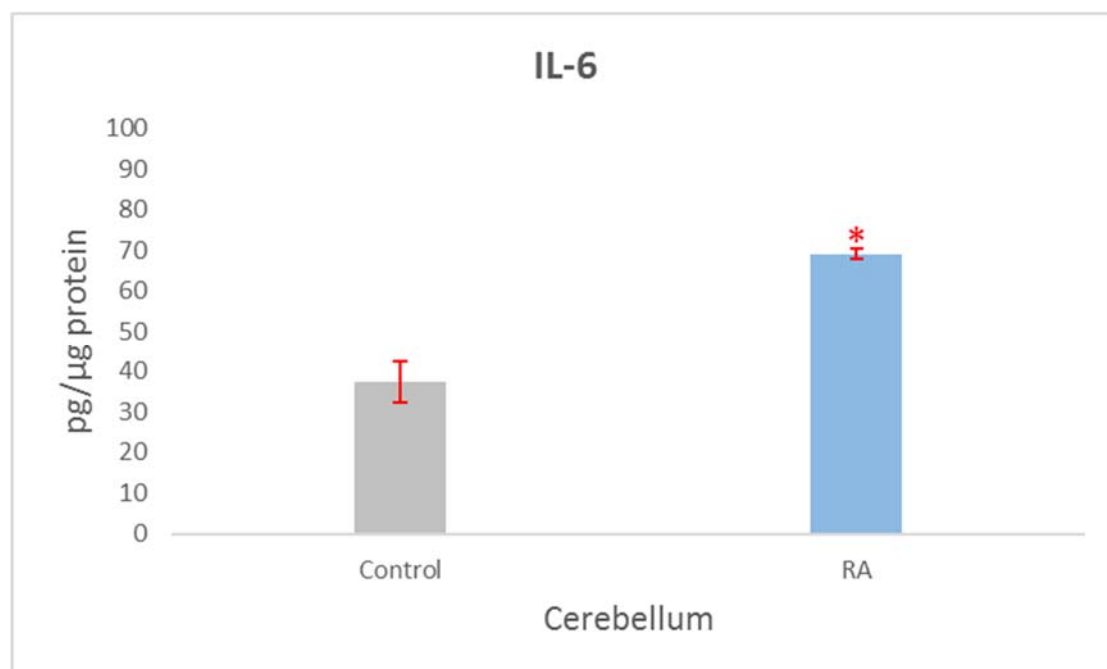


Figure 13. The inflammatory status of IL-6 in Lewis rats' cerebellum. IL-6 significantly increased in the RA condition in cerebellum region ($p < .005$).

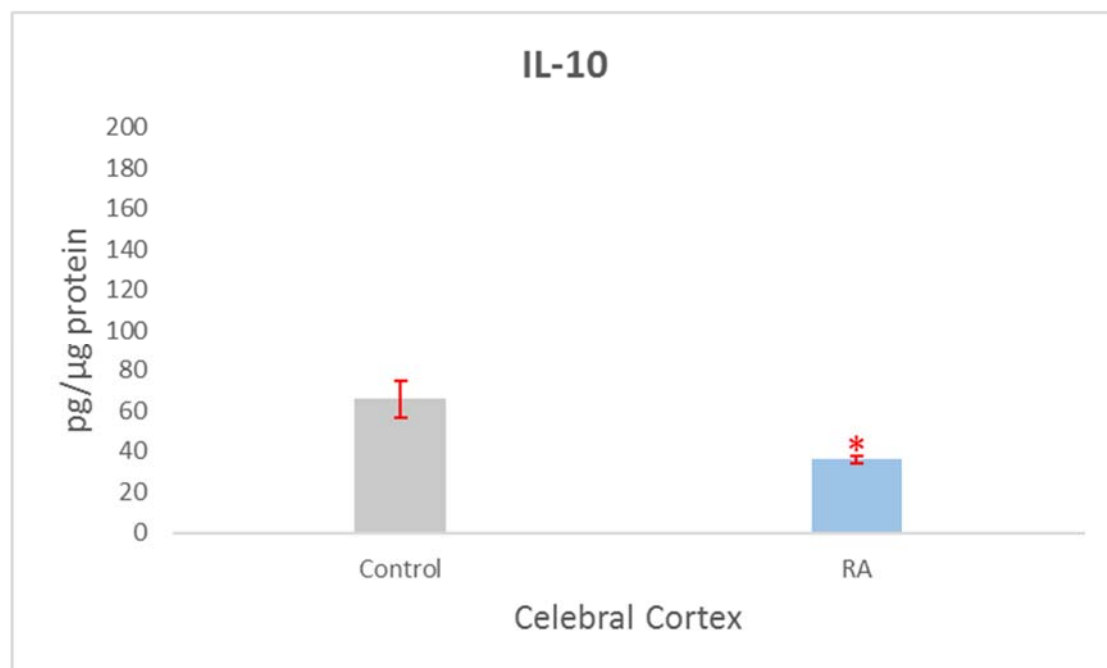


Figure 14. The inflammatory status of IL-10 in Lewis rats' cerebral cortex. IL-10 did not increase in cerebral cortex compared to control and RA groups ($p < .05$).

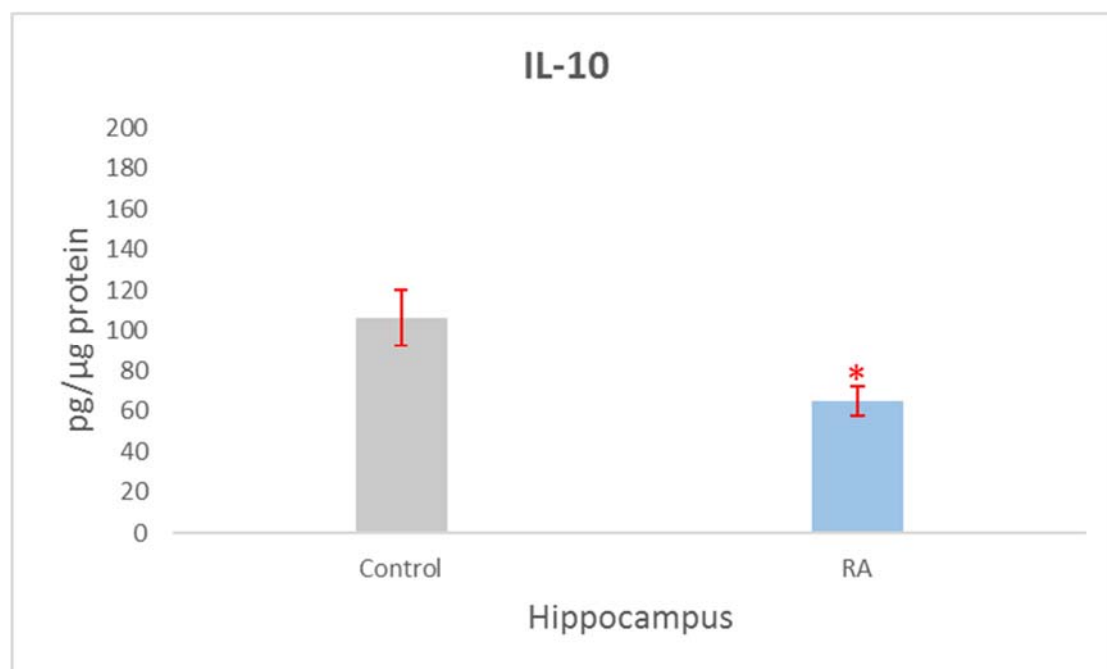


Figure 15. The inflammatory status of IL-10 in Lewis rats' hippocampus. IL-10 did not increase in hippocampus compared to control and RA groups ($p < .05$).

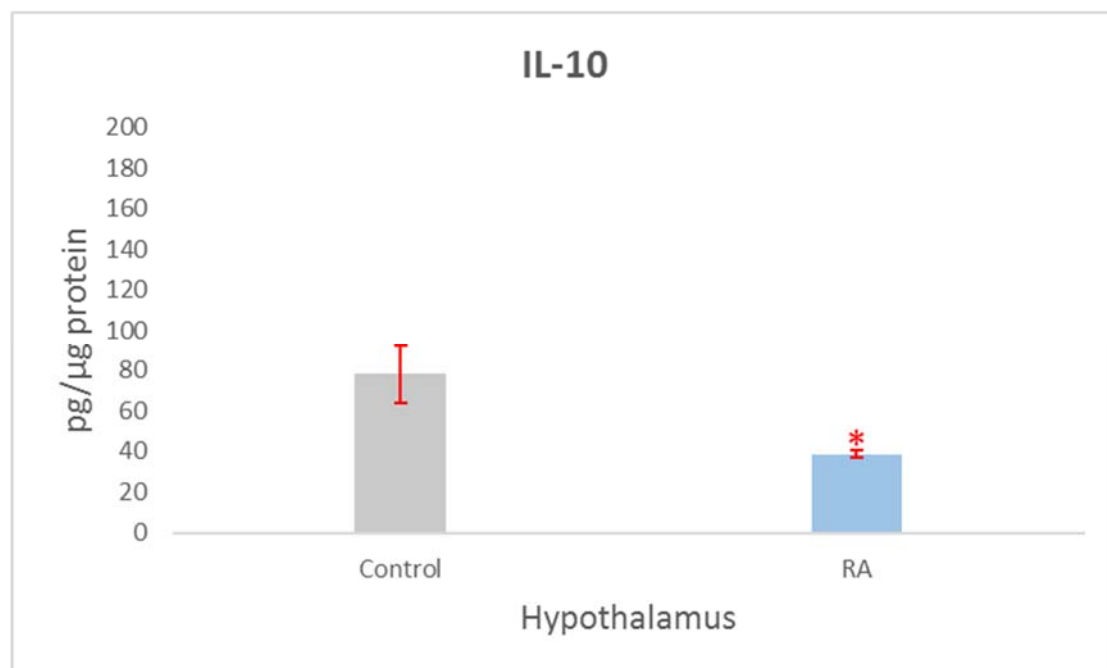


Figure 16. The inflammatory status of IL-10 in Lewis rats' hypothalamus. IL-10 did not increase in hypothalamus compared to control and RA groups ($p < .05$).

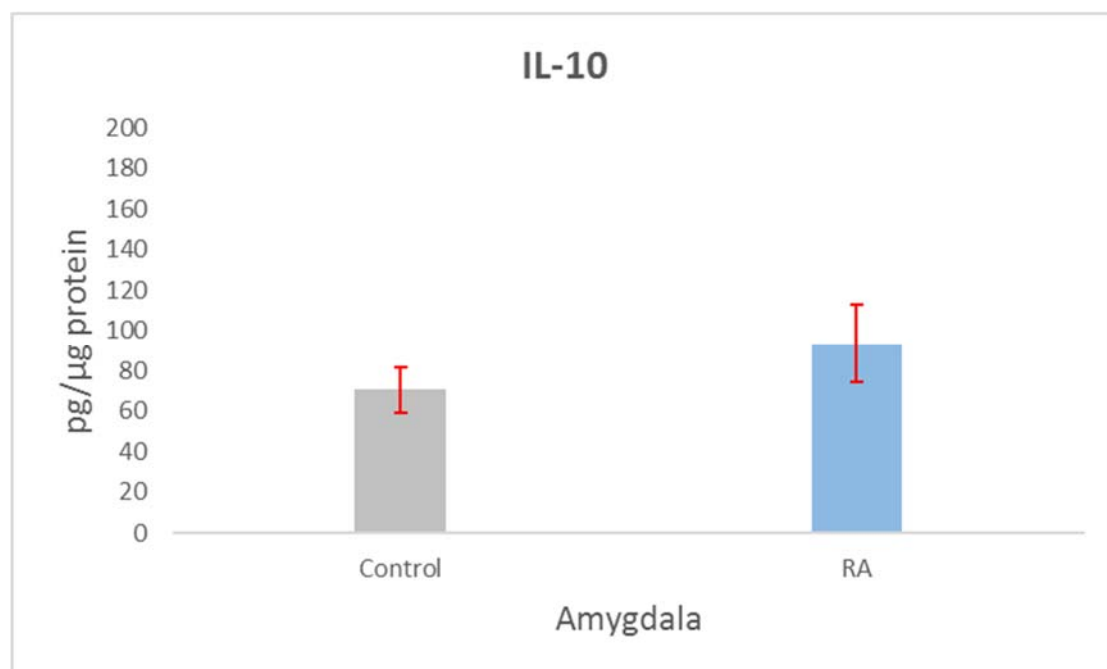


Figure 17. The inflammatory status of IL-10 in Lewis rats' amygdala. IL-10 increased in the RA condition in amygdala region but no significantly effects.

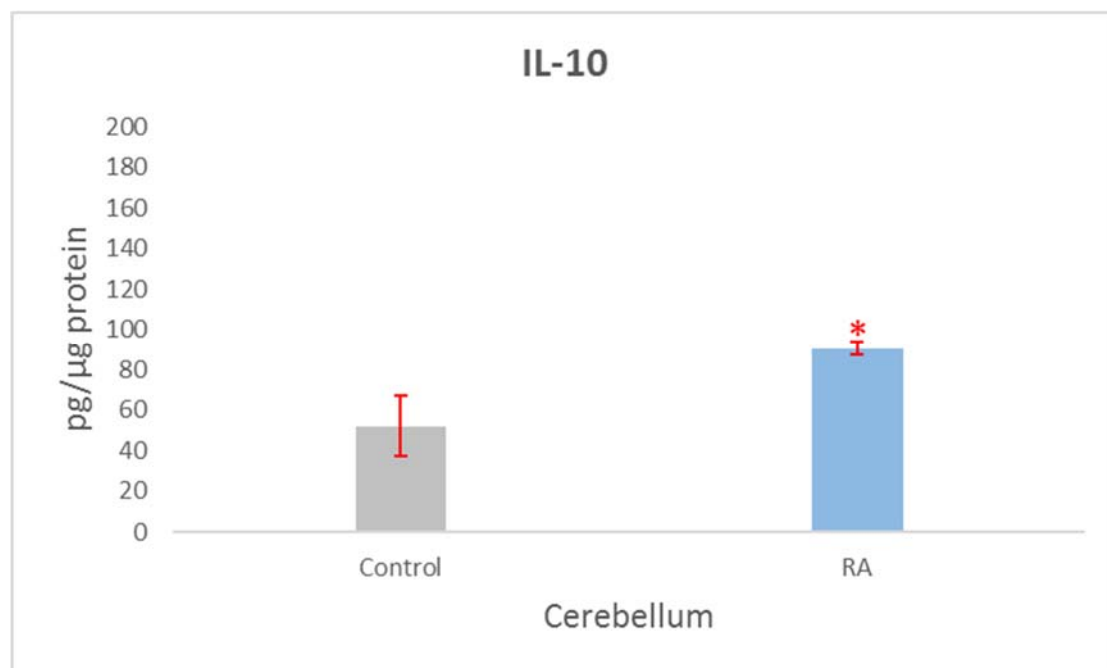


Figure 18. The inflammatory status of IL-10 in Lewis rats' cerebellum. IL-10 significantly increased in the RA condition in cerebellum region ($p < .005$).

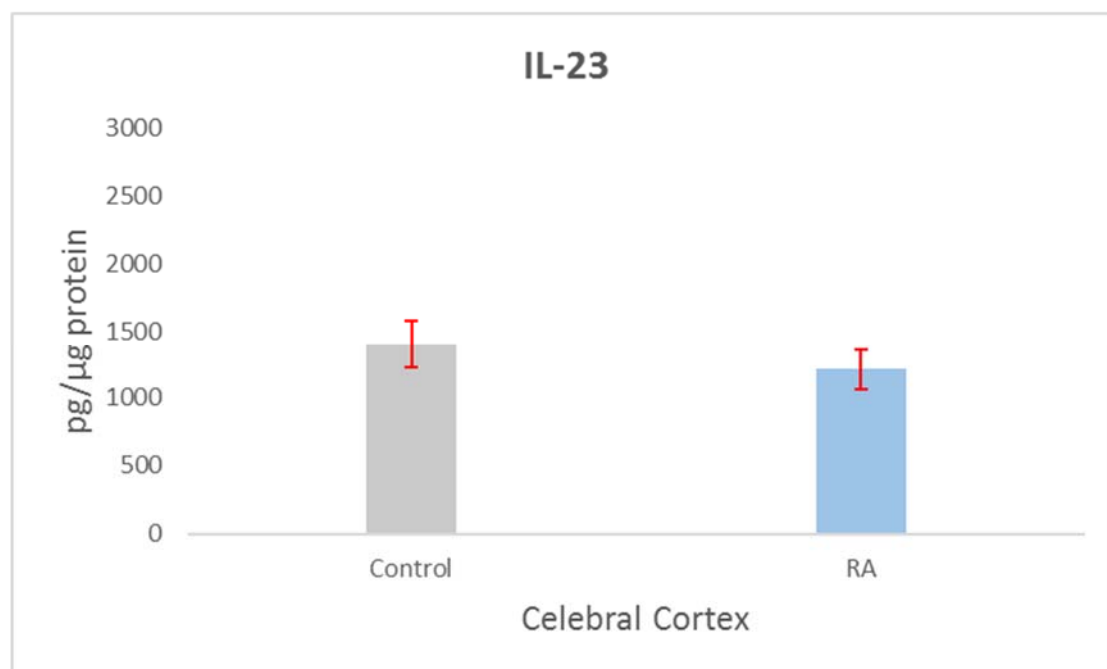


Figure 19. The inflammatory status of IL-23 in Lewis rats' cerebral cortex. IL-23 had no significantly effects in cerebral cortex compared to control and RA groups.

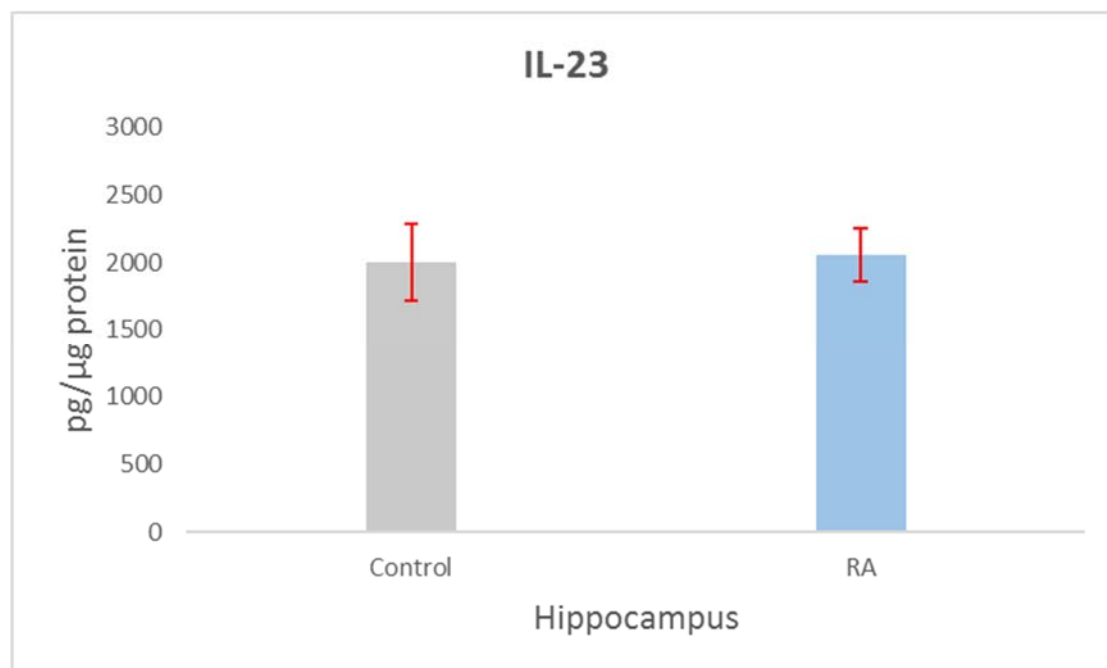


Figure 20. The inflammatory status of IL-23 in Lewis rats' hippocampus. IL-23 had no significantly effects in hippocampus compared to control and RA groups.

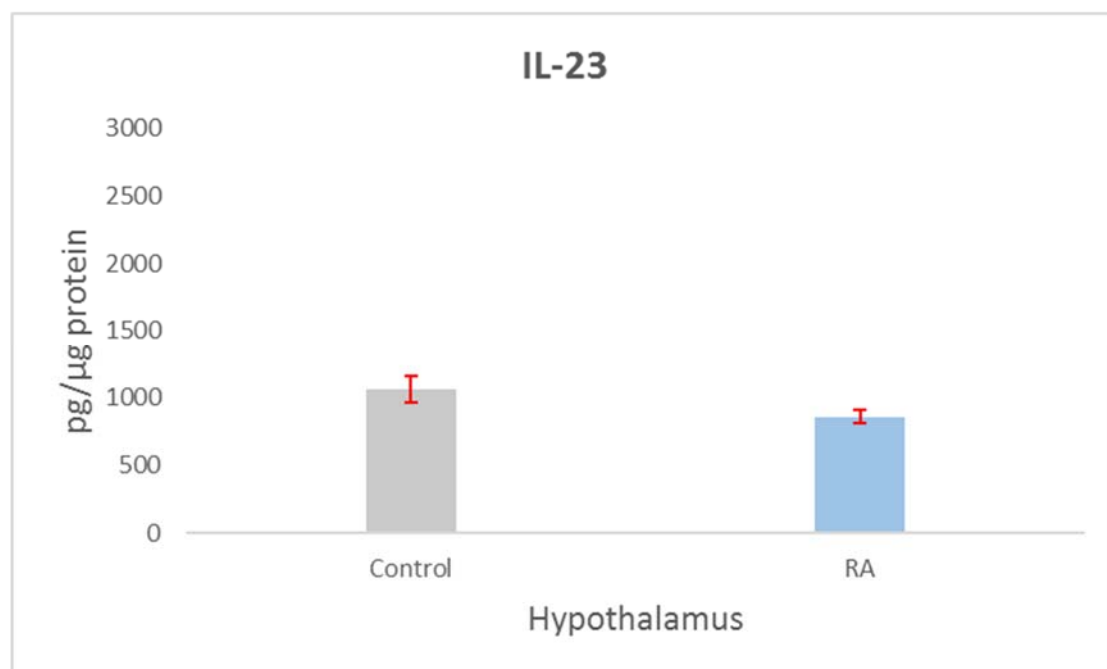


Figure 21. The inflammatory status of IL-23 in Lewis rats' hypothalamus. IL-23 had no significantly effects in hypothalamus compared to control and RA groups.

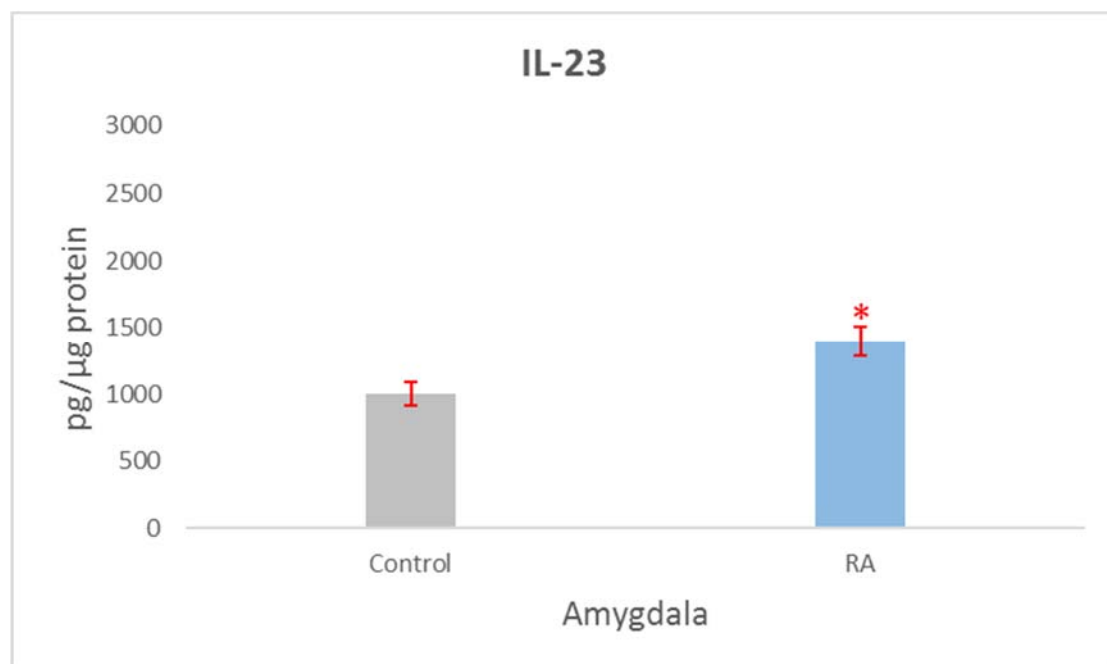


Figure 22. The inflammatory status of IL-23 in Lewis rats' amygdala. IL-23 significantly increased in the RA condition in amygdala region ($p < .05$).

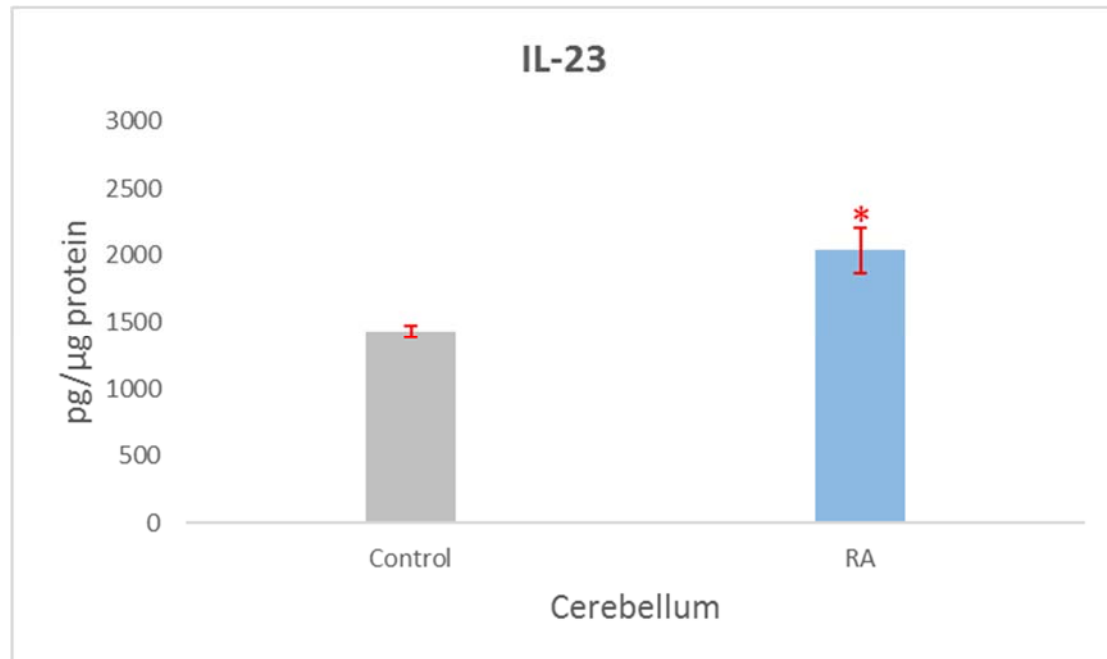


Figure 23. The inflammatory status of IL-23 in Lewis rats' cerebellum. IL-23 significantly increased in the RA condition in cerebellum region ($p < .005$).

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