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Dopamine Levels in the Brain of Rat Models of Human Rheumatoid Arthritis

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DOPAMINE LEVELS IN THE BRAIN OF RAT MODELS OF HUMAN RHEUMATOID ARTHRITIS

by

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Dedication

This thesis is dedicated to my mother, father, and best friend Marsha Watson. Their love, support, encouragement, and dedication to see me succeed gave me the strength to become who I am today.

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Research Focus. Rheumatoid arthritis is a chronic, debilitating, autoimmune disease that causes the destruction of bone tissue and the articular structures of joints. At least 30% of RA patient populations have cognitive impairment. Acidic dopamine (DA) is the principal neuroimmunotransmitter that links the central nervous system and peripheral nervous system together. The aim of the present study was to determine the levels of DA and its two acidic metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in arthritic induced rats, and whether their levels vary across four different parts of the brain: amygdala, front cerebral cortex, hippocampus, and cerebellum. Brain protein was also assessed.

Materials and Methods. 3-month old male Lewis Rats (n = 16) were randomized into either control (n = 10) or treatment (n = 6) groups. In the treatment group, arthritis was induced in the rats using Freund's Adjuvant and all rats were sacrificed on day 28. Dopamine, DOPAC, and HVA levels were quantified using High Performance Liquid Chromatography technique while proteins were quantified using Bicinchroninic Acid Protein Assay, in the four brain regions. Two-way ANOVA test was performed to determine whether brain regions, induce arthritis treatment or their interactions significantly influenced the levels of the analytes (at $p < .05$).

Research Results/Findings. Levels of brain protein (C-reactive protein) were elevated in arthritic rats across all brain regions ($p > .05$). Dopamine and DOPAC levels were lower in arthritic rats than

controls ($p > 0.05$). HVA levels were higher in arthritic rats compared to non-arthritic controls.

Conclusions from Research. The present study has demonstrated that C-reactive protein, dopamine, DOPAC and HVA are involved in the neurophysiology of arthritis. RA patients can benefit from treatment with dopamine agonists. However, more studies are warranted to determine the effect of DOPAC and HVA levels in the brain on dopamine utilization in arthritis.

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Context of This Study

Rheumatoid arthritis (RA) is a chronic, debilitating autoimmune disease that causes the destruction of bone tissue and the articular structures of joints. Thus, RA is characterized by infiltration of inflammatory immune cells, particularly, T-lymphocytes (T cells), macrophages, and neutrophils in joints. Activation of microphages immune cells results in the production of proinflammatory cytokine, particularly interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor-alpha (TNF-α), which are involved in the up-regulation of inflammatory reactions (Navegantes et al. 2017). This causes synovial inflammation, eventually leading to joint destruction and disability (Gorman & Cope, 2008; Tobon et al., 2010). While the etiology of RA is not well understood, it is thought to be a multifactorial disorder involving a complex interplay between genetic, (Kurkó et al., 2013; Okada et al., 2013;) and environmental factors, such as tobacco smoking (Tobon et al. 2010), bodily pain, decreased mobility, fatigue, stiffness, physical disability and the way RA patients have to cope with these problems during the activities of daily living, significantly affect their health-related quality of life (HR-QOL) (Matcham et al., 2014; Russell, 2008). There is some evidence that RA also impacts negatively on mental health (Matcham et al. 2014), indicating that there is a causal association between RA and psychiatric disorders (Nicassio, 2010). Psychological disorders in RA include depression, anxiety, emotional problems, and overall cognitive dysfunction a phenomenon referred to as "brain fog" (Covic et al., 2012; Dickens et al., 2001; Hewlett et al., 2005). Cognitive impairment in RA patient population is >30% (Joaquim et al., 2004; Shin et al., 2012;), with a majority exhibiting worse cognitive score in psychometric measures for verbal fluency and memory (Joaquim et al., 2004). In a longitudinal cohort study, Shin et al. (2012) demonstrated that a subgroup of RA patients with cognitive impairment exhibited significantly poor fine motor skills than the rest of RA patients. This implied that there should be a correlation between physical function and cognitive function in RA. Shin et al. (2013) demonstrated cognitive impairment was

significantly associated with greater functional limitations in RA patients, although only depression and fatigue (due to coping with functional limitations), were significantly associated with perceived cognitive dysfunction.

Psychological disorders like depression and anxiety in inflammation disorders are associated with chronic peripheral inflammation mediated by pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) (Süβ et al., 2015). Initially, cognitive dysfunction in RA patients was thought to be due the highly elevated systemic markers of inflammation, particularly C-reactive protein, which can potentially damage the integrity of blood-brain barrier thereby causing neuroinflammation (Eagan et al., 2012; Elwood et al., 2017). Neuroinflammation was thought to affect hippocampal plasticity, which triggers depression as repair response (Süβ et al., 2015; Wager-Smith et al. 2010). However, it has been demonstrated recently that hippocampal immunity, cellular plasticity and function are maintained despite severe peripheral autoimmune-mediated inflammation (Süβ et al. 2015). By this account, it can be inferred that there should be neuronal factors underlying psychological disorders in RA rather than systemic inflammations.

The neural activities in the brain are mediated by a wide repertoire of neurotransmitters in the brain. However, dopamine, gamma-Aminobutyric acid (GABA), and serotonin are among the few neurotransmitters frequently studied in relation to mental disorders due to their aggressive nature from neuropsychology and interdisciplinary perspectives (Narvaes, & Martins de Almeida, 2014). Acidic dopamine (DA) is the principal neurotransmitter involved in both the central nervous system (CNS) and peripheral nervous system (Basu & Dasgupta, 2000). It is one of the precursors to norepinephrine and epinephrine allowing it to be involved in the parasympathetic nervous system (PNS) as well. A decline in DA concentration or inhibition of its synthesis or metabolism rates in the CNS can result in impairment of critical neurologic functions, such as cognition, behavior and fine movements.

Surprisingly, apart from its principle neurotransmitter function in the CNS, DA also has some inductive effect on a multitude of T-cells and dendritic cells (DCs), and therefore, regarded as a neuroimmunotransmitter. The role of DA in the neural-immune communication has been demonstrated in some in vivo studies reviewed previously by Basu and Dasgupta (2000). The studies revealed that in vivo injury or stimulation of specific central dopaminergic (DA) system results in the suppression or enhancement of functional activities of the immune effector cells, especially T-cells and antigen-presenting DCs. While DCs expresses a complete machinery to synthesize and store dopamine, regulatory T-cells expresses dopamine receptors (DRs) and, therefore, have the ability to release, produce, and uptake dopamine (Pacheco et al. 2014). This underpins dopamine-mediated integration of the immune system to the CNS, where dopaminergic immune regulations could be the driving force during autoimmunity and associated inflammations (Pacheco et al. 2014). Thus, malfunctioned DRs on immune cells including T-cells could also be major cause of overall cognitive impairment. Impairment of the DRs implies a blockade of the interaction between T-cells and dendritic cells, and therefore, leading to impairment of dopaminergic immune regulations.

Monocyte-derived DCs produce DA and store it in vesicles. The interactions between T-cells and the DCs affect differentiation, polarization, and the secretion of pro-inflammatory cytokines (Levite, 2008). It has been shown (Levite, 2008) that high (10-4 - 10-3M) and medium levels (10-7 - 10-5 M) of DA are toxic to the T-cells and can result in unregulated stimulation. Through this, it can be inferred that DA affects a multitude of systems. It is necessary to understand and quantify the amounts present within these systems to link cognitive impairment to the body. However, the total amount DA in CNS includes its two acidic metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Thus, to better understand the amount of DA and its longevity in the brain, it is important to consider its principle acidic metabolites.

The purpose of study was to determine whether there are variations of DA concentration and its related catecholamines in rat models of RA compared to healthy controls. Different parts of the brain have different neurologic activities. The following amygdala, hypothalamus, front cerebral cortex, hippocampus, and cerebellum, which exhibit strong role in memory, balance, emotional stability, and overall cognition, were evaluated. The limbic system, which consists of the amygdala and hippocampus, was the main area of interest since this is where spatial reasoning, memories, and emotional behaviors are affected. The limbic system involves the dopaminergic mesolimbic pathway, which is made up of movements from the ventral tegmental area to the nucleus accumbens. This pathway along with the function of the two parts of interest has a role in motivation, and reward. A difference in catecholamines in any of the affected areas would imply that inflammation, and/or pain is not the only contributing source of cognitive impairment in patients with RA.

Materials and Methods

Animal Model of Human Rheumatoid Arthritis

Three-month old Male Lewis Rats (n=16) were purchased from Charles River Laboratories and maintained at the vivarium in the University of Texas at San Antonio (UTSA). The rats were maintained on a 12-hour light/12-hour dark cycle in a temperature and humidity controlled environment.

Adjuvant Induced Arthritis

The male rats were randomly assigned to one of two groups: control (non-arthritic), or the arthritic group. In the arthritic group, arthritis was induced by methods previously described by Tanimoto et al., (2015). Briefly, an intradermal injection of 0.01 ml of Freund's incomplete adjuvant (Sigma Aldrich™) was injected on the base of the tail (1 mg heat killed *Mycobacterium tuberculosis* H37Rak, suspended in 1.0 mL of liquid paraffin) at day one and day 7. Arthritis is usually present within 7 days after injection of the adjuvant. To confirm arthritis, erythema, swelling, and deformity

of the paw joints was scored by the scale shown in Table 1. With the highest possible score being 16, rats with a score > 12 were selected and sacrificed on 28. The schematic presentation of treated and control rat models is shown in Figure 1.

Table 1: Induced-arthritis scoring scale

Score	Indication
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
4	Complete erythema and swelling of the toes or fingers and the ankle or wrist and an inability to bend the ankle or wrist

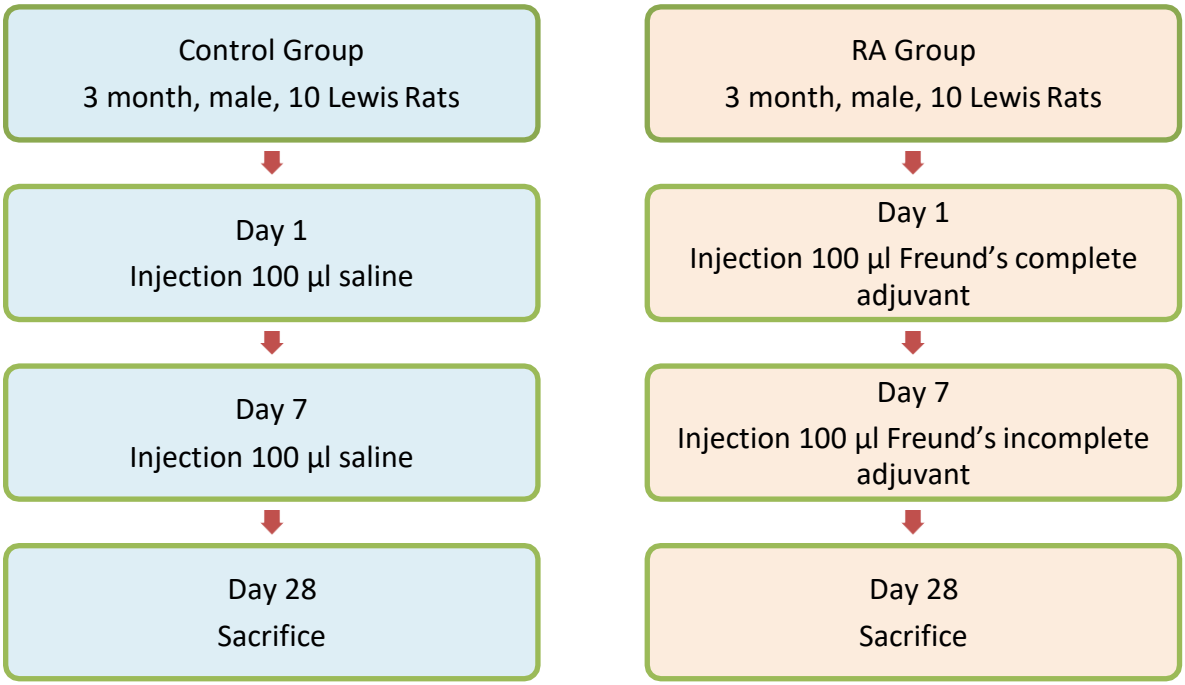

Figure 1: Rat model of adjuvant-induced rheumatoid arthritis (RA)

Brain Tissue Preparation

Once sacrificed by decapitation as mentioned above, five sections of the brain: the amygdala, hippocampus, hypothalamus, cerebellum, and front cerebral cortex, were removed using the

Stereotaxic Atlas of Paxinos and Watson as a guide. Additional organs of interest (lungs, heart, kidneys, and eyes) were also collected, removed, and immediately flash frozen in liquid nitrogen for transport to the University of the Incarnate Word (UIW). The organs were then stored at in the -80ºC UIW freezer for later analysis.

Bicinchroninic Acid (BCA) Protein Assay

Frozen brain tissues were homogenized using a stand homogenizer with 750µL of 0.2N perchloric acid buffer. The homogenized brain solutions (homogenates) were then centrifuged at 10,000 rpm, 4ºC for 10 minutes. The supernatant was transferred to a different tube and the pellet discarded. A Microplate BCA Protein Assay Kit, from Thermo Scientific™ was used to calculate the total amount of protein in a given sample of tissue homogenate. BCA protein assays were used a standardized curve to determine the amount of protein in each individual supernatant. The scheme of the standardized curve was as follows:

Table 2: Dilution scheme for standard test-tube procedure

Vial	Volume diluent(µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	66	200 of stock	1500
B	100	100 of stock	1000
C	100	100 of vial A dilution	750
D	100	100 of vial B dilution	500
E	100	100 of vial D dilution	250
F	100	100 of vial E dilution	125
G	100	100 of vial F dilution	62.5
H	100	0	0=Blank

Samples were then be diluted to achieve an overall R^2 value of 0.9 or higher. The scheme of the sample curve is shown in table 3.

Table 3: Dilution scheme for sample test tube procedure

Vial	Volume diluent (µL)	Volume and source of stock sample supernatant (µL)	Final sample concentration (%)
AS	0	21	100
BS	7	21	75
CS	21	21	50
DS	63	21	25

Once all standard and sample dilution vials were prepared, 9µL of brain samples and standards were loaded onto a microplate. 4µL of the compatibility reagent solution was then added to each well and incubated for 15 minutes. Then, 260µL of the working reagent solution prepared earlier (50:1 ratio of BCA working reagent A to BCA working reagent B provided by Thermo Scientific™) was added into each well and allowed to incubate for 30 minutes. A protein gradient then begun to form within the wells. Absorbance was then measured at 562nm using the Infinite 200 PRO Nano Quant Tecan Microplate Reader. Protein amounts were calculated by plotting the absorbance values read from the samples vs. the standardized curve, resulting in the protein concentration of each supernatant in µg/mL.

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was used for quantification of DA, HVA, and DOPAC in the brain of both control and arthritic rats. HPLC allows for the distinct separation of compounds by carrying the injected supernatant through a packed column (stationary phase) with a solvent (mobile phase). As the sample is passed through the column, it interacts between the two phases at a different rate. Analytes in the sample that have the least amount of interaction with the mobile phase, will elute the column sooner, and pass through the detector at different rates, resulting in varied retention times.

A modular design of the Agilent Infinity 1220 HPLC was used since it provides enough versatility and integration capabilities to overcome analytical challenges. 100μL of brain sample and standards were prepared and directly injected for analysis. A gradient elution table was used to ensure clean separation of peaks. Mobile phases consisted of ACN, H2O, and a buffer of 0.1 M trifluoroacetic acid (TFA). The mobile phase was used to bring the samples through a Zorbax SB-C18 reversed phased column (Agilent technology). Samples were read at a 250nm absorption wavelength. Each sample was injected in triplicates at a flow of 0.5μL per minute for ~10 minutes.

The area under each sample's analyte curve compared to that of the standards curve were used to determine the concentration of each analyte in ng of specific analyte/ mg of protein. Proper storage of the column and rinsing before and after each run was carried out using a solution of 50/50 methanol to water, to ensure that no carryover of any analyte peak was in sequential runs.

Statistical Analyses

The difference in levels of dopamine, DOPAC, HVA in the five different parts of the brain of arthritic groups versus non-arthritic controls were compared using t-test and two-way analysis of variance (two-way ANOVA) test. Differences between groups were considered to be statistically significant at a *P* value of <0.05. Statistical analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, Inc., San Diego, CA, USA, www.graphpad.com).

Results

Adjuvant-Induced Rheumatoid Arthritis

The levels of dopamine, DOPAC, HVA were measured in the cerebral cortex (CX), hippocampus (HIP), hypothalamus (HYP), amygdala (AMG), and cerebellum (CBL) regions of the brains of rats with adjuvant-induced rheumatoid arthritis and non-arthritic controls. However, due to lack of hypothalamus (HYP) brain tissue samples from the arthritic rats, tests for HYP were performed for controls only. Therefore, results for HYP were excluded from the analysis.

Protein Levels in Four Parts of the Brain

Total protein quantities in brain tissue homogenates were determined using the BCA protein assays. The protein quantities in the cerebellum, amygdala, hippocampus, and cerebral cortex were estimated for both arthritic (treated) and non-arthritic (control) Lewis rats (Fig. 1). From Figure 1 below, arthritic rats exhibited higher protein levels than non-arthritic rats in cerebellum, amygdala, and hippocampus but not in cerebral cortex. However, t-tests showed that between-group difference in protein concentration was not statistically significant for cerebellum (P=0.3414), amygdala (P=0.7618), hippocampus (P=0.7834) and cerebral cortex (p=0.7439). Two-way ANOVA test demonstrated that neither of the two independent variables (treatment/control and brain region) nor their interaction were statistically significant (p>0.05) in influencing the protein levels in the four parts of the brain (see Appendix A).

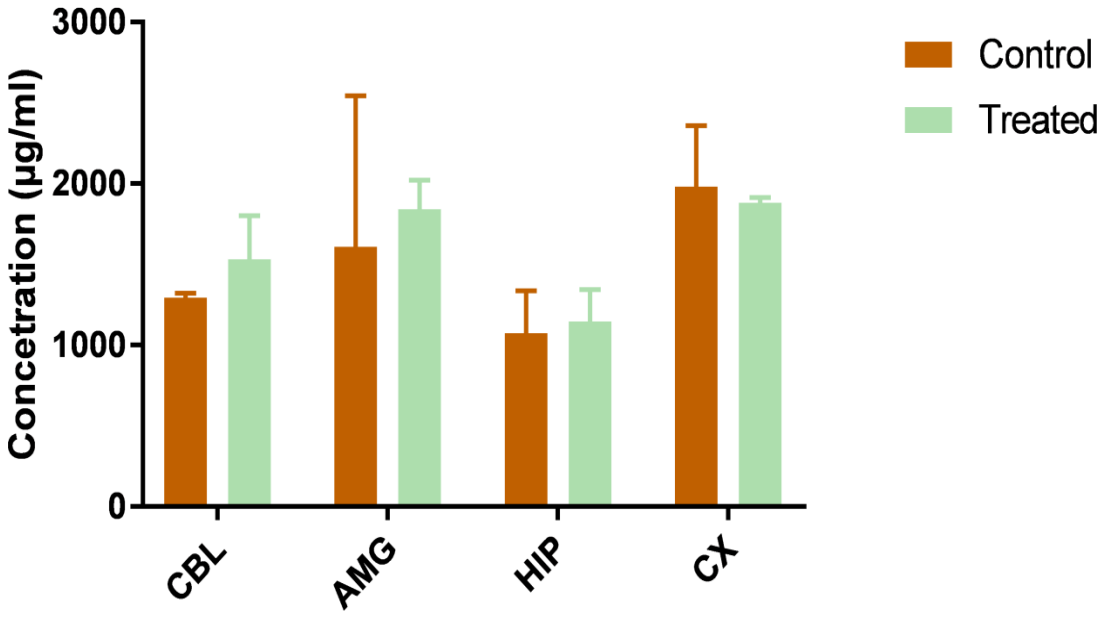

Figure 2: Total protein concentration (ug/ml) brains parts of arthritic (treated) versus non-arthritic (control) Lewis rats. The CBL, AMG, and HIP of the treated rats exhibited high protein levels than controls, while CX exhibited higher protein levels in controls than treated. The differences were not significant across all brain regions (p>0.05)

Dopamine Levels in Four Parts of the Brain

Dopamine concentrations in the four different parts of the brain from both arthritic (treated) and non-arthritic (control) Lewis rats were determined using the HPLC (Fig. 3). From Figure 3 below, arthritic rats exhibited lower dopamine concentration than non-arthritic rats across all the four parts of the brain. This was statistically significant in the cerebellum (P =0.0170) and cerebral cortex (0.0095), but not significant in amygdala P=0.2527) and hippocampus (P=0.7779). Two-way ANOVA test demonstrated that brain regions (cerebellum, amygdala, hippocampus and cerebral cortex) as an independent variable had statistically significant effect on the dopamine concentration (P=0.0131), but not its interactions (P=0.4002). On the other hand, treatment and control or their interactions did not seem to have any statistically significant effect on the distribution of dopamine across the four brain parts (p>0.05) (see Appendix B).

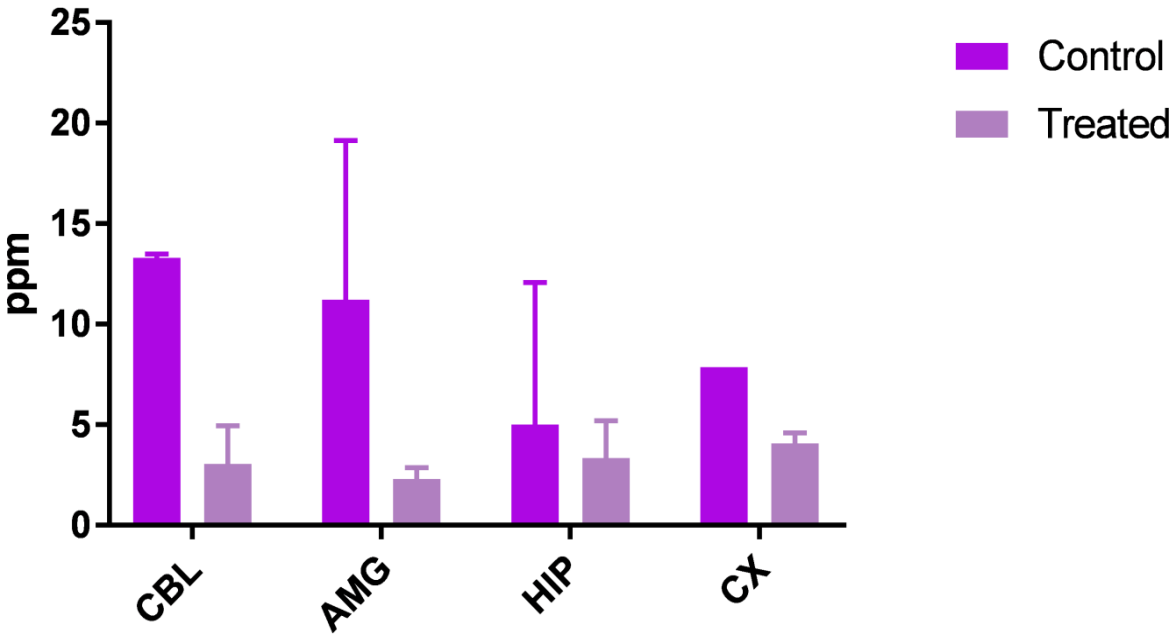

Figure 3: The levels of dopamine in CBL, AMG, HIP and CX. Treated (Arthritic) rats exhibited lower levels of dopamine than control rats, the difference being statistically significant for CBL (P =0.0170) and CX (0.0095) only.

DOPAC Levels in Four Parts of the Brain

Using HPLC, the concentration of DOPAC, a metabolite of dopamine, was determined in the four different parts of the brain of both arthritic (treated) and non-arthritic (control) Lewis rats (Fig. 4). From Figure 4 below, arthritic rats exhibited a lower DOPAC levels than non-arthritic rats in amygdala, though not statistically significant ($p=0.4314$). Both arthritic and non-arthritic Lewis rats, exhibited very low concentrations of DOPAC in hippocampus and cerebral cortex, with undetectable DOPAC observed in cerebellum of arthritic rats and hippocampus of non-arthritic rats. Two-way ANOVA test demonstrated that neither of the two independent variables (treatment/control and brain region) or their interaction were statistically significant ($p>0.05$) (see Appendix C).

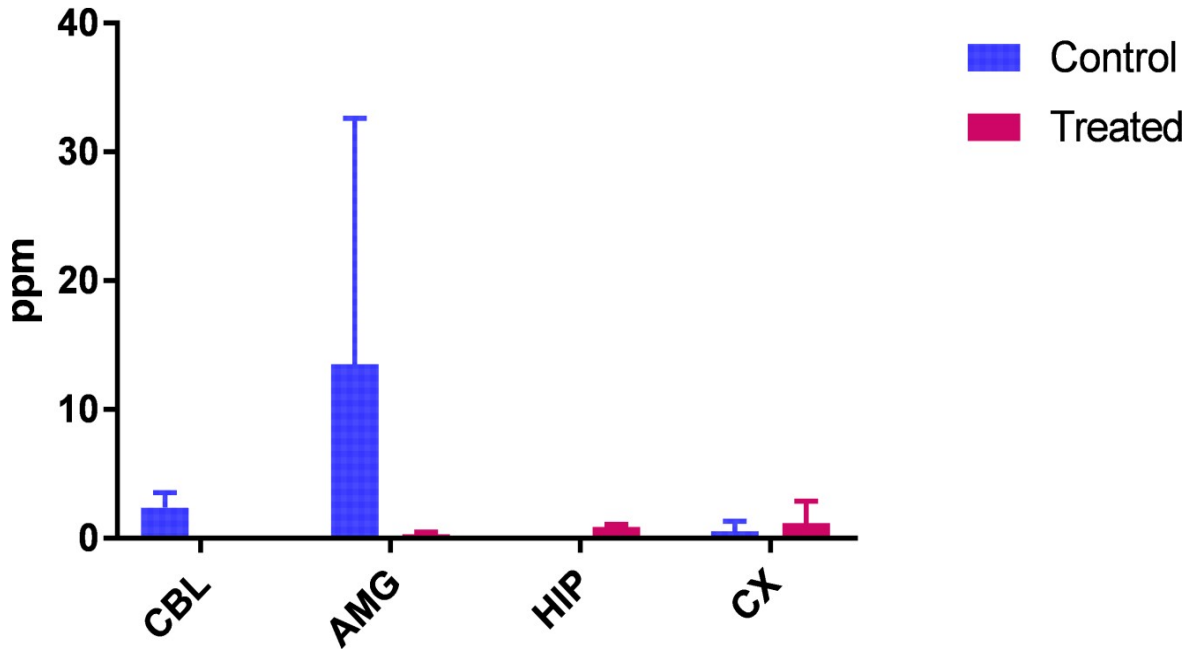

Figure 4: The levels of DOPAC in CBL, AMG, HIP and CX. DOPAC was undetectable in one control (HIP) and one treated brain part (CBL).

HVA Levels in Four Parts of the Brain

Using HPLC, the concentration of HVA, a metabolite of dopamine, was determined in four different parts of the brains in both arthritic (treated) and non-arthritic (control) Lewis rats (Fig. 4). From Figure 4 below, arthritic rats exhibited a significantly lower HVA levels than non-arthritic rats in cerebellum (p= 0.0414). On the other hand, arthritic rats exhibited a higher HVA levels than non-arthritic rats in hippocampus, though not statistically significant (p= 0.7625). Undetectable HVA were observed in amygdala and cerebral cortex of non-arthritic Lewis rats. Two-way ANOVA test demonstrated that only a single independent variable (treatment/control and brain region) and its interaction were statistically significant (P=0.0046; P=0.0463). On the other hand, the different regions of the brain did not seem to significantly influence HVA levels in both arthritic and non-arthritic rats (P=0.9601) (see Appendix D).

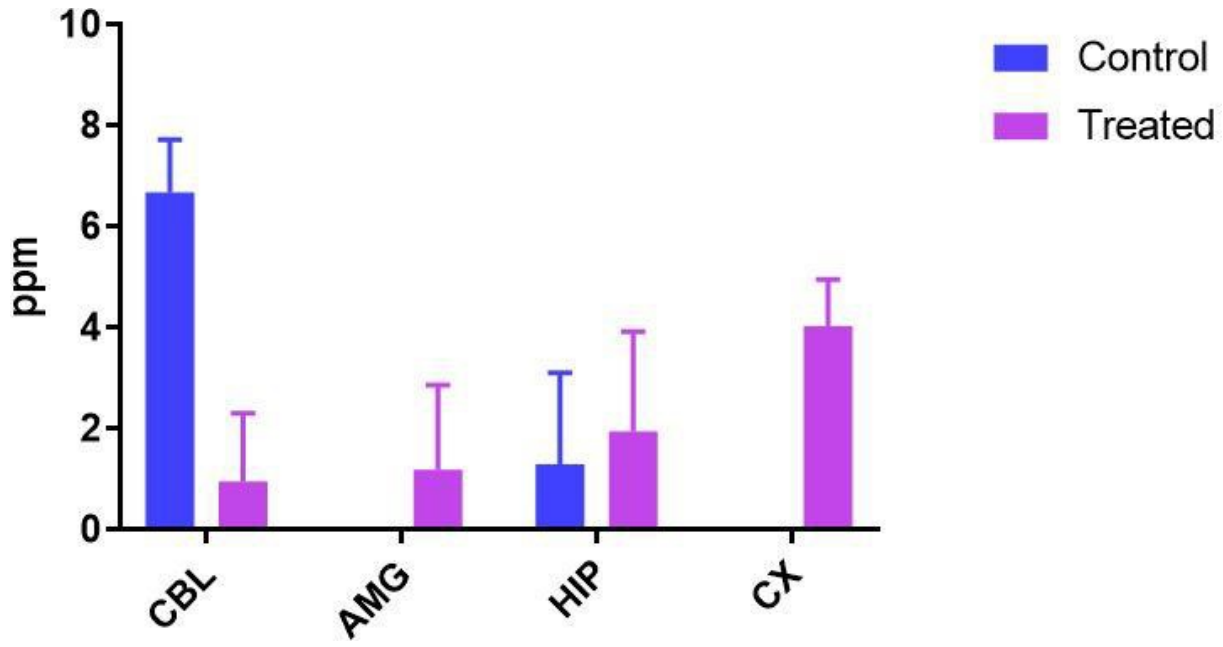

Figure 5: HVA levels in four parts of the brain. HVA was detected in all the four parts of the brain from treated rats. It was however, undetectable in AMG and CX of controls. HVA levels in CBL were significantly higher in controls but not in treated mice (p= 0.0414). In hip, HVA was higher in treated than controls, though not significant (p= 0.7625).

Discussion And Conclusion

More than 30% of populations of population with RA have cognitive impairments (Shin et al. 2012; Joaquim et al. 2004), with a significant subgroup of this RA patients exhibiting worse cognitive function in verbal fluency, memory (Joaquim et al. 2004) and poor fine motor skills (Shin et al. 2012). This study was based on the overarching hypothesis from the literature that acidic dopamine (DA), which is the principal neuroimmunotransmitter linking the immune system to the CNS and peripheral nervous system, its changes in the brain produces cognitive impairments in RA. Furthermore, this study was also guided by the hypothesis, C-reactive protein (CRP), which is a biomarker of cognitive impairment, should be elevated in the brains of RA patients with cognitive impairment (Eagan et al. 2012; Elwood et al. 2017). The present study demonstrated variations in the levels of both brain protein and DA with its two acidic metabolites (DOPAC and HVA) across four parts of the brain: AMG, CX, HIP, and CBL, which are mainly involved in cognition and motor function.

Brain Protein

The present study demonstrated elevated levels of total protein CBL, AMG, and HIP of arthritic than non-arthritic rats, while CX exhibited higher protein levels in non-arthritic than arthritic rats. Although not significant ($p>0.05$), the protein elevations in most of the brain regions of arthritic rats indicated that the proteins could be associated with cognitive impairment in RA. There several brain proteins involved in cognitive function or decline, including NPTX2—neuronal pentraxin 2 (Xiao et al. 2017), CRP (Eagan et al. 2012), albumin (Llewellyn et al. 2010). However, low levels of NPTX2 and albumin have been associated with cognitive impairment and neurological diseases, such as Alzheimer's Disease, in the elderly population (Llewellyn et al. 2010; Xiao et al. 2017). Although the present study did not characterize and identify the elevated protein components in the arthritic rats, CRP, was most likely to be involved (Eagan et al. 2012; Elwood et al. 2017).

CRP was elevated in the AMG, which is associated with fear, depression and anxiety disorder (Forster et al. 2012); CBL, which coordinates movement, motor learning or timing (Mauk et al. 2000); and HIP, which regulates emotions (Binder et al. 2012), but not in CX, which regulated high-level brain functions, such as memory, cognition, and perception. This demonstrates that CRP was largely elevated in the limbic system that regulates emotions and therefore, could be largely responsible for depression in RA. This was supported by findings from previous studies, which demonstrated that depression scores were slightly, but positively correlated with the CRP levels (r 0.46, $P < 0.001$) (Kojima et al. 2009), and that CRP was significant predictor of cognitive impairment in patients with RA (Shin et al. 2012). However, in the present study, CRP was not significantly elevated across the four brain regions of arthritic rats ($p>0.05$), indicating that it is not partly contributes to cognitive impairment. Therefore, there should be neuronal factors that also contribute to psychological disorders in RA rather in addition to systemic inflammation marker CRP.

Dopamine

Dopamine, a neurotransmitter in the brain that is important in regulating movement, emotional responses and sensations of pleasure and pain. It is critically regulating positive emotions and motivation (Schultz, 2002), and therefore, low levels in the brain could be involved in cognitive dysfunction in RA. Findings in the present study demonstrated that dopamine levels in the arthritic rats were generally low across all the four limbic brain regions analyzed, where dopamine levels were significantly low in CBL ($P =0.0170$) and CX (0.0095). There is a paucity of published data directly compares the level of dopamine in arthritic-induced rats or RA patients to healthy non-arthritic controls. However, low levels and altered dopamine neurotransmission has been observed in patients with fibromyalgia, a chronic rheumatic condition characterized by generalized pain, fatigue, and dysregulation of emotion. Pain in both fibromyalgia and RA are all attributed to increased levels

of proinflammatory cytokines. According to Miller et al. (2013), the levels and function of neurotransmitters (including dopamine) are broadly modulated by proinflammatory cytokines, at multiple levels. This is achieved by alteration of neurotransmitter synthesis, reuptake and release, via the activation of metabolic and epigenetic processes (Miller et al. 2013). Given that autoimmunity is coupled to CNS and PNS (Basu & Dasgupta, 2000), and the existence pathways linking systemic inflammation to dopaminergic reward system (Sturgeon et al. 2016), dopamine could be centrally involved cognitive impairment in autoimmune inflammatory diseases, including RA. According to Sturgeon et al. (2016), RA patients are likely to develop alteration in dopaminergic neurotransmission, and therefore, likely to develop cognitive and behavioral impairments.

This finding suggests that RA patients at increased risk of cognitive impairment can benefit from treatment with dopamine agonists (Eijsbouts et al. 1999). However, given that autoimmunity in RA is tightly coupled to CNS (Basu & Dasgupta, 2000), dopamine agonists is likely to suppress inflammatory response and improve quality of life. A previous case report (Erb et al. 2001), demonstrated that treating of unremitting RA with prolactin antagonist cabergoline resulted in a significant remission. D1-like dopamine receptor antagonist have been shown to ameliorated the severity of collagen-induced arthritis in mice, by promoting anti-inflammatory IFN-γ and inhibiting pro-inflammatory interleukin (IL)-17 production by T-cells (Nakashioya et al. 2011). Currently, the potential therapeutic benefits of dopamine-agonists are increasingly being studied for human RA.

DOPAC and HVA Levels

DOPAC and HVA are the acid metabolites of dopamine, and therefore, their levels in the brain should correlate with the utilization of dopamine in the brain. Findings in the present study, demonstrated very low levels of DOPAC in arthritic rats, with some brain parts being below detectable limits. However, levels HVA were higher in arthritic rats compare to non-arthritic controls. While low DOPAC levels as observed in arthritic rats indicates low utilization of dopamine

and low "turnover" of DA in the brain, this does not necessarily imply utilization of dopamine in the dopaminergic systems (Ebinger et al. 1987). An early *in vivo* study of dopamine release and metabolism in rat brain regions, demonstrated DOPAC/HVA ratio in varied between brain regions in accordance with whole tissue measurements. This is consistent with findings in the present *in vivo* study, which demonstrated varied DOPAC and HVA, levels of the difference regions of the arthritic rats.

Limitations of the Study and Future Perspective

The present *in vivo* study is the first to assess protein levels and utilization of neurotransmitters in dopaminergic systems in arthritic rats, to assess their roles in cognitive impairment in RA. Experimental materials were limited (arthritic rat brain samples) and therefore, it was not possible to evaluate the hypothalamus. Furthermore, the calibration standards for DOPAC and HVA appeared to be a bit higher, yet DOPAC and HVA are only available in smaller quantities than the parent neurotransmitter substance (dopamine). This could explain why some parts of the brain had undetectable levels of DOPAC and HVA. This warrants more *in vivo* studies to re-evaluate DOPAC and HVA in arthritic rats using calibration standards of lower concentration. Future, studies should focus on the potential therapeutic benefits of dopamine-agonists treating human RA and associated risk of cognitive impairment.

Conclusion

A significant subgroup of RA patients is likely to develop cognitive impairment. The present study has demonstrated that CRP, dopamine, DOPAC and HVA are involved the neuropathology and associated cognitive impairments in RA. Therefore, RA patients at increased risk of cognitive impairment can benefit from treatment with dopamine agonists. However, more studies are warranted to determine the effect of DOPAC and HVA levels in the brain on dopamine utilization in arthritic rats.

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Appendix A: Two-way ANOVA test of protein levels in four parts of the brain

Table Analyzed	Two-way ANOVA, not RM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	2.655	0.9156	ns	No	
Row Factor	53.28	0.0761	ns	No	
Column Factor	1.694	0.5872	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	77159	3	25720	F (3, 8) = 0.1671	P=0.9156
Row Factor	1548419	3	516140	F (3, 8) = 3.353	P=0.0761
Column Factor	49238	1	49238	F (1, 8) = 0.3198	P=0.5872
Residual	1231624	8	153953		
Number of missing values	0				

Appendix B: Two-way ANOVA test of dopamine levels in four parts of the brain

Table Analyzed	Two-way ANOVA, not RM				
Two-way ANOVA	Ordinary				
Alpha		0.05			
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction		14.09	0.4002	ns	No
Row Factor		9.424	0.556	ns	No
Column Factor		42.65	0.0131	*	Yes
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction		50.11	3	16.7	F (3, 8) = 1.11	P=0.4002
Row Factor		33.53	3	11.18	F (3, 8) = 0.7427	P=0.5560
Column Factor		151.7	1	151.7	F (1, 8) = 10.08	P=0.0131
Residual		120.4	8	15.05		
Number of missing values		0			

Appendix C: Two-way ANOVA test of DOPAC levels in four parts of the brain

Table Analyzed	Two-way ANOVA, not RM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	19.89	0.4609	ns	No	
Row Factor	16.86	0.525	ns	No	
Column Factor	7.455	0.3314	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	132	3	43.98	F (3, 8) = 0.9507	P=0.4609
Row Factor	111.9	3	37.29	F (3, 8) = 0.8059	P=0.5250
Column Factor	49.46	1	49.46	F (1, 8) = 1.069	P=0.3314
Residual	370.1	8	46.27		
Number of missing values	0				

Appendix D: Two-way ANOVA test of HVA levels in four parts of the brain

Table Analyzed	Two-way ANOVA, not RM				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	58.9	0.0046**		Yes	
Row Factor	25.15	0.0463*		Yes	
Column Factor	0.00532	0.9601ns		No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	50.71	3	16.9	F (3, 8) = 9.85	P=0.0046
Row Factor	21.65	3	7.216	F (3, 8) = 4.205	P=0.0463
Column Factor	0.004581	1	0.004581	F (1, 8) = 0.002669	P=0.9601
Residual	13.73	8	1.716		
Number of missing values	0				