PROGRESS REPORT

Committee for Aid and Education in Neurochemistry (CAEN)
CATEGORY 1A: Visit by the applicant to another laboratory

July, 2018

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Supervisor: Hugo Peluffo, PhD

1 - Project Title: Role of CD300f receptors in the behavioral and neurochemical modifications induced by chronic restraint stress: possible involvement of NLRP3 inflammasome

2 - Introduction:

Major depressive disorder (MDD) is a highly pervasive psychiatric condition, with as much as 350 million people affected worldwide [1]. Despite the significant social burden stemming from this disease, there still remain significant gaps in our scientific understanding of genesis and progression of MDD. The current diagnostic systems do not adequately reflect relevant neurobiological alterations to support the modified behavioral patterns found in patients [2]. Also, MDD and other psychiatric disorders have a multifactorial origin, involving dysfunction in multiple brain areas and different cell types along with alterations in several biochemical functions including immune system alterations [3]. Depressive symptoms are often associated with inflammatory diseases and peripheral increase on acute phase proteins, chemokines and inflammatory cytokines are frequently observed in MDD patients [4]. In some patients, the remission of clinical symptoms is accompanied by a normalization of inflammatory markers, while lack of response is associated with persistent elevated levels of inflammatory molecules [5]. The release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18) is controlled by pattern recognition receptors (PRRs) such as the Nod-like receptors (NLRs) [6]. These receptors are primarily expressed by immune cells and act through recognition of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) [7]. The activation of NLRs are involved in the assembly of cytosolic protein complexes known as inflammasomes. These multiprotein complexes are ultimately responsible for the activation of caspase-1, cleavage of precursor forms of pro-inflammatory IL-1β and IL-18 into their active forms, promoting innate immune responses associated with infection and inflammation [8]. The levels of caspase-1 and NLRP3 are increased in peripheral blood mononuclear cells of MDD patients when compared to non-depressed subjects. Besides that, inflammatory cytokines activated by the inflammasome complex, IL-1β and IL-18, were also elevated in the serum of MDD patients and positively correlated with the Beck Depression Inventory scores. These effects were reversed by treatment with classical antidepressants [9,10]. CD300 receptors compose a family of receptors that actuate in the tuning, directing or finishing immune active responses [11]. Among the CD300 receptor family there are the CD300f receptors that seem to act as anti-inflammatory regulators since their blockade can increase neuroinflammation [12-14]. Few studies have demonstrated the role of CD300f receptors in the CNS. It was observed that CD300f upregulation decreases the area of cerebral injury in mice [12]. Moreover, CD300f transfected astrocytes diminished the neuronal loss induced by Aβ peptide in co-culture [15]. However, the biological and pathological role of CD300f receptors in the control of inflammatory process associated with psychiatric disorders is less clear.

3 – Objectives and Results:

A) Establish the behavioral modifications induced by genetic deletion of CD300f receptor

Male and female adult (4-5 months old) and aged (18 months old) CD300f knockout (KO) and C57BL/6 wild-type (WT) mice were analyzed in different behavioral paradigms as shown in Figures 1, 2 and 3. Adult female CD300f KO mice presented significant increased immobility time in the tail suspension test (TST, Fig. 1B) and in the forced swimming test (FST, Fig. 1C), indicating depressive-like behavior when compared to WT mice. Additionally, CD300f KO female mice showed decreased grooming behavior (Fig. 1E) in the sucrose splash test (SST) demonstrating that these mice also present anhedonic-like behavior, a core symptom of MDD. No differences were observed in locomotor activity evaluated by the open field test (OFT, Fig. 1A). Then, to test whether the female CD300f KO mice displayed long-lasting and progressive depressive-like behavioral alterations, we exposed a different cohort of aged females (18 months old) to the previous behavioral test batteries. Aged CD300f KO female mice displayed a similar altered behavioral phenotype compatible with depressive and anhedonic-like symptoms, as observed in adult CD300f KO female mice.

Interestingly, the depressive-like symptoms were not observed in adult 4-5 months old males (Fig 2C-F). However, 4 months old male CD300f KO mice presented a protection against anxiety-like behavior, showing a tendency to spend more time in the center of the OFT arena (Fig. 3E) and remaining more time in the open arms of the elevated plus maze test (EPM, Fig. 3B). In addition, CD300f KO adult male mice did not demonstrate differences in the locomotor activity evaluated by the OFT (Fig. 3D). In order to strengthen these behavioral phenotype findings, we submitted these animals to the novelty suppressed feeding test (NSF) and again a protection against anxiety-like behavior was observed when male mice presented decreased latency to eat in a new environment (Fig. 3G).

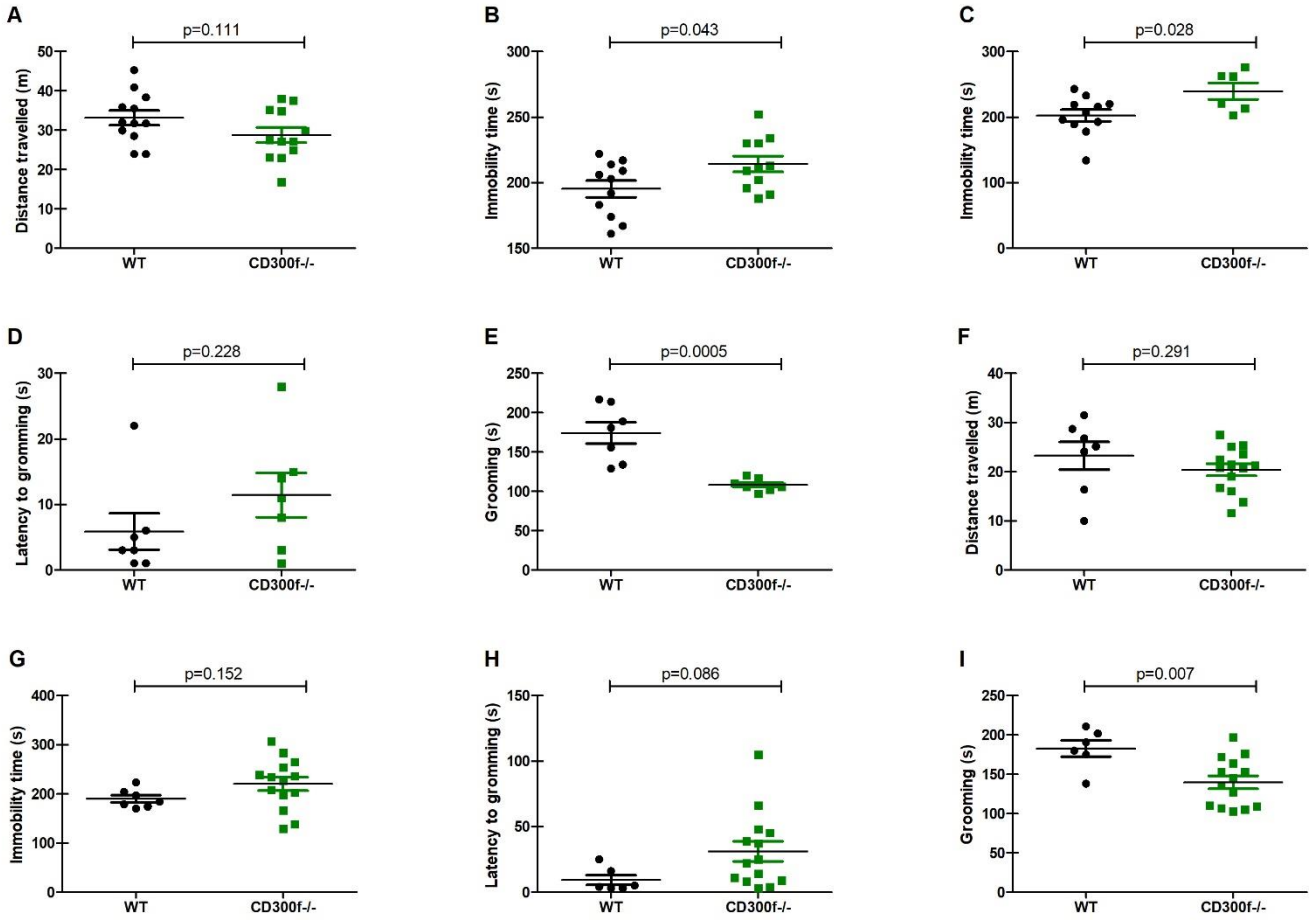


Figure 1. Behavior characterization of CD300f KO adult and old female mice. Adult (4-5 months) female mice were evaluated in the OFT and the total distance travelled was measured (meters) as an indication of mice spontaneous locomotor activity (A), the total immobility time in the TST (B) and the FST (C) was measured (seconds) to evaluate depressive-like behavior. The latency to start grooming behavior (seconds) in the SST was recorded to evaluate motivational and self-care behavior (D) and the total time in grooming behavior was recorded in order to evaluate hedonic-like behavior (E). The OFT (F), TST (G) and SST (H,I) were also performed in old (18 months) female mice. Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. OFT: Open field test; TST: Tail suspension test; FST: Forced Swimming test; SST: Sucrose splash test; CD300f-/-: CD300f knockout mice.

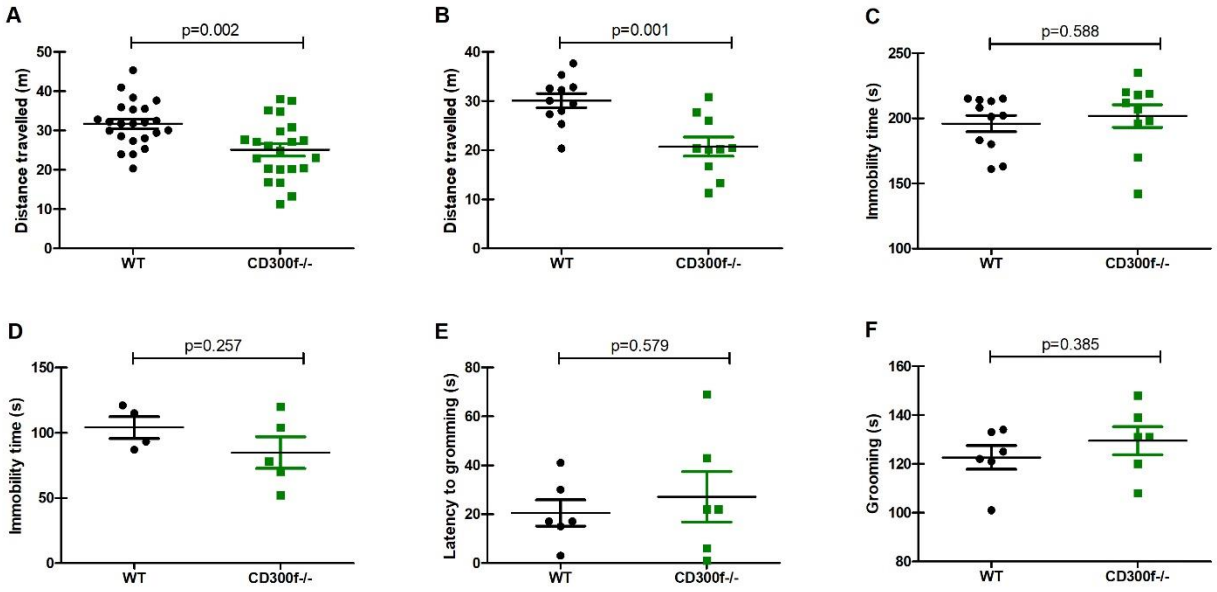


Figure 2. Behavior characterization of CD300f KO adult male mice. Male and Female adult mice (4-5 months) were evaluated as a unique group in the OFT and the total distance travelled was measured (meters) as indicative of mice spontaneous locomotor activity (A). In order to evaluate sex specificities, male mice was evaluated separately regarding the total distance travelled in the OFT (B), the total immobility time (seconds) in the TST (C) and in the FST (D) as an indicative of depressive-like behavior. The latency to start grooming behavior (seconds) in the SST was evaluated as an indication of motivational and self-care behavior (E) and the total time in grooming behavior was recorded to evaluate hedonic-like behavior (F). Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. OFT: Open field test; TST: Tail suspension test; FST: Forced Swimming test; SST: Sucrose splash test; CD300f-/-: CD300f knockout mice.

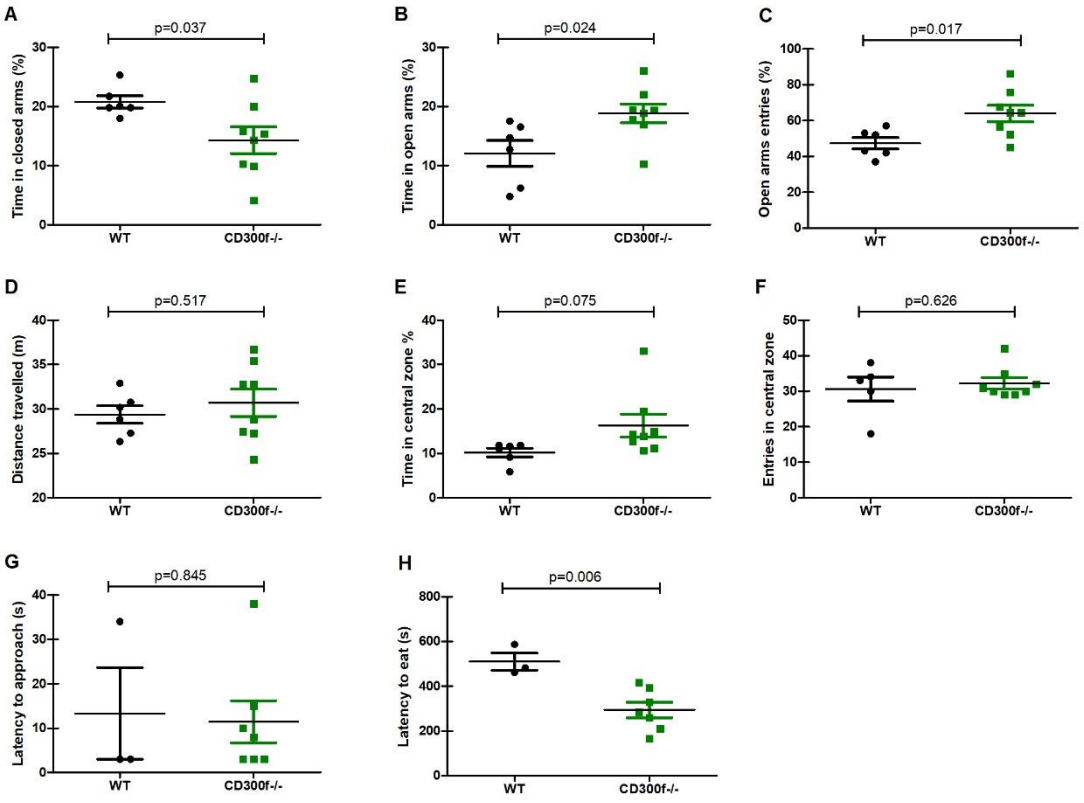


Figure 3. Anxiety-like behavior characterization of CD300f KO adult male mice. Male adult mice (4 months old) were evaluated in the EPM test and the time spent in the closed arms (A) and in the open arms (B) were recorded as a measure of anxiogenic-like and anxiolytic-like behavior, respectively. Number count of entries in the open arms was also recorded as a measure of anxiolytic-like behavior (C). In the OFT the total distance travelled was measured (meters) as indicative of mice spontaneous locomotor activity (D). The % of time spent in the center of the OFT (E) and the number of entries in the center (F) were used as an indicative of mice anxiolytic-like activity. Latency to approach (H) and to eat food (G) in a novel environment after being food deprived overnight were analyzed. Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. EPM: Elevated plus maze test; OFT: Open field test; NSF: Novelty suppressed feeding test; CD300f-/-: CD300f knockout mice.

To evaluate if the behavioral changes observed in female CD300f KO mice were associated with changes in monoaminergic neurotransmission, the levels of serotonin, dopamine and noradrenaline were analyzed in the mice hippocampus, an important brain area involved in the pathophysiology of MDD. This brain structure was dissected from saline perfused animals and the neurotransmitter levels measured by High Performance Liquid Chromatography (HPLC, Fig. 4). Interestingly, hippocampal noradrenaline was significantly decreased in CD300f KO female mice, while no other significant changes were observed in the levels of serotonin or dopamine. This results prompted us to evaluate whether 21 days of imipramine treatment (20 mg/kg v.o.), a tricycle antidepressant that acts augmenting monoamine levels, including noradrenaline, could ameliorate the depressive-like phenotype in adult female mice (Figure 5). However, imipramine treatment was not able to ameliorate the behavioral phenotype in most of the tests performed in adult female mice. Nevertheless, imipramine treatment increased CD300f KO time spent in the center of the OFT (Fig. 5B), suggesting a possible anxiolytic-like effect of imipramine in CD300f female mice, however this needs further investigation.

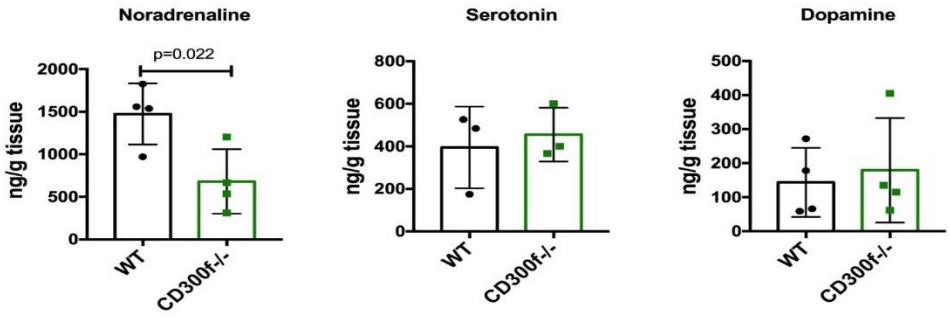


Figure 4. Female CD300f KO mice display reduced noradrenaline levels in the hippocampus. Female mice were perfused with saline and the hippocampus extracted for the analysis of the levels of different neurotransmitter levels by High Performance Liquid Chromatography (HPLC). Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. CD300f-/-: CD300f knockout mice.

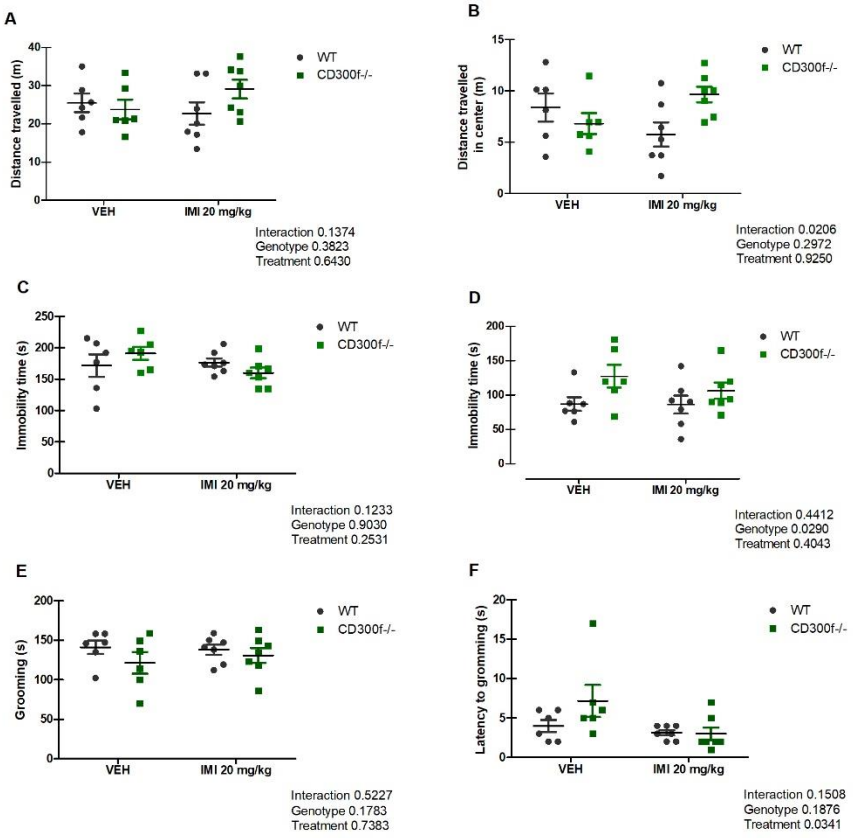


Figure 5. Behavioral evaluation of imipramine treatment in female CD300f KO mice. Adult (4 months old) female mice were treated with imipramine (20 mg/kg, v.o.) or vehicle (distilled water, v.o.) and their behavior were evaluated. The OFT was performed to determine the total distance travelled (meters) as an indication of mice spontaneous locomotor activity (A). Distance travelled in the center of the OFT were analyzed as an indicative of mice anxiolytic-like activity (B). The total immobility time in the TST (C) and the FST (D) was measured (seconds) to evaluate depressive-like behavior. The total time (seconds) in grooming behavior in the SST was recorded in order to evaluate hedonic-like behavior (E) and the latency to start grooming behavior in the SST was recorded to evaluate motivational and self-care behavior (F). Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. OFT: Open field test; TST: Tail suspension test; FST: Forced Swimming test; SST: Sucrose splash test; IMI: Imipramine; CD300f-/-: CD300f knockout mice.

B. Establish the behavioral modifications induced by chronic restraint stress of 21 days in CD300f KO mice

Considering the depressive and anhedonic-like phenotype found in CD300f KO female mice we asked whether these mice would be more susceptible to a chronic model of depression induced by 1h of daily restraint stress. Results are depicted in Figure 6 and show that stressed mice presented increased locomotor activity in the OFT (Fig. 6A). We could also observe that CD300f KO mice presented a tendency to increase immobility time in the FST (Fig. 6D), however, no effects of the stress model were observed neither in the FST nor in the TST (Fig. 6C). As expected, stress decreased the time of grooming in WT female mice at the SST, but no changes were observed in CD300f KO mice (Fig. 6.E).

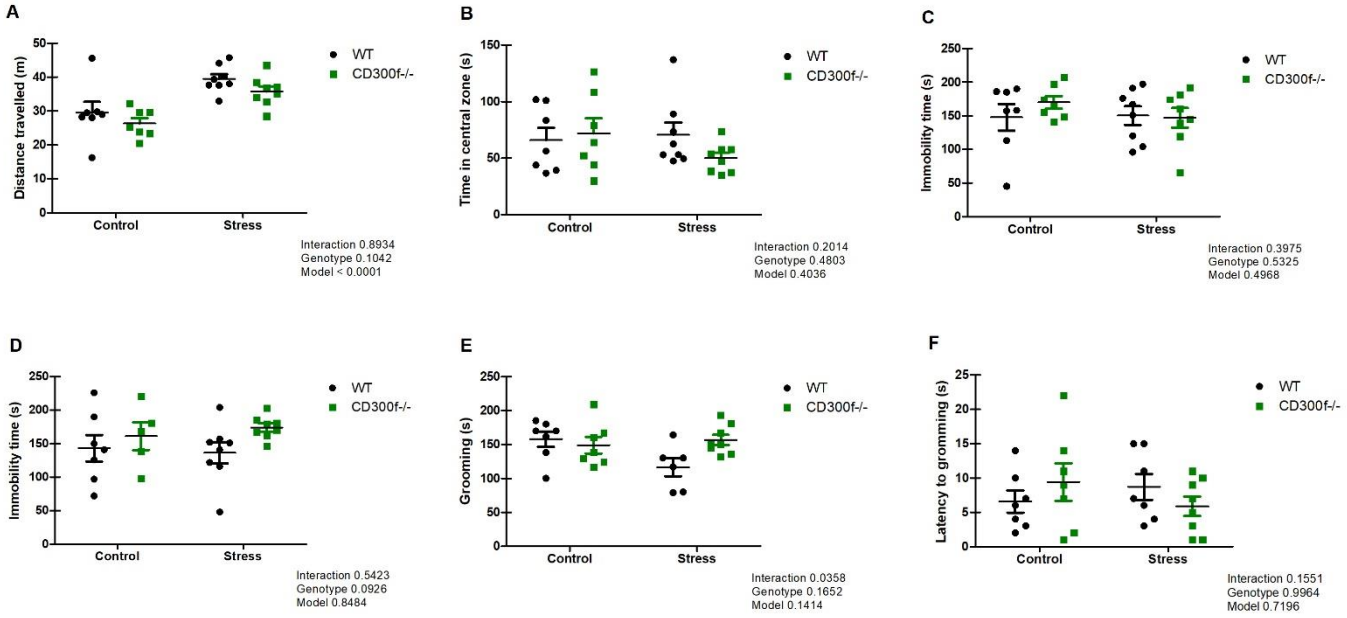


Figure 6. Behavior evaluation of CD300f KO female mice after chronic restraint stress. Adult (4 months old) female mice were evaluated in the OFT and the total distance travelled was measured (meters) as an indication of mice spontaneous locomotor activity (A), and time spent in the center of the OFT were analyzed as an indicative of mice anxiolytic-like activity (B). The total immobility time in the TST (C) and the FST (D) was measured (seconds) to evaluate depressive-like behavior. The total time (seconds) in grooming behavior in the SST was recorded to evaluate hedonic-like behavior (E) and the latency to start grooming behavior in the SST was evaluated as an indication of motivational and self-care behavior (F). Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. OFT: Open field test; TST: Tail suspension test; FST: Forced Swimming test; SST: Sucrose splash test; CD300f-/-: CD300f knockout mice.

C) Establish biochemical alterations induced by chronic stress in CD300f KO mice:

In order to evaluate whether CD300f KO could present changes in the microglial homeostatic phenotype, the expression of key proinflammatory and microglial signature genes in naïve WT and CD300f KO animals were evaluated and compared to a gene expression profile recently reported as damage associated microglia (DAM) signature genes [16]. Microglial RNA extracts from WT and CD300f KO mice were obtained from the total spinal cord and brain and further evaluated by qPCR. Results showed no significant alterations in the majority of the genes analyzed. From the homeostatic microglial signature markers, P2ry12 mRNA was increased and P2ry6 mRNA expression was reduced (Fig. 7A) which might suggest alterations in microglia phagocytic capacity associated to P2ry6 deficiency, but not associated to overt neuroinflammation. Regarding the set of genes known as DAM signature, CD300f KO mice showed decreased expression of Clec7a and Arg1, and increased expression of Lpl and Siglec1 (Fig. 7B). Moreover, no general increased inflammation was observed in the CD300f KO mice (Fig. 7D). Interestingly, only two cytokines genes, Il1rn and Il6, which were already reported to be associated with MDD [17,18] were upregulated (Fig. 7C).

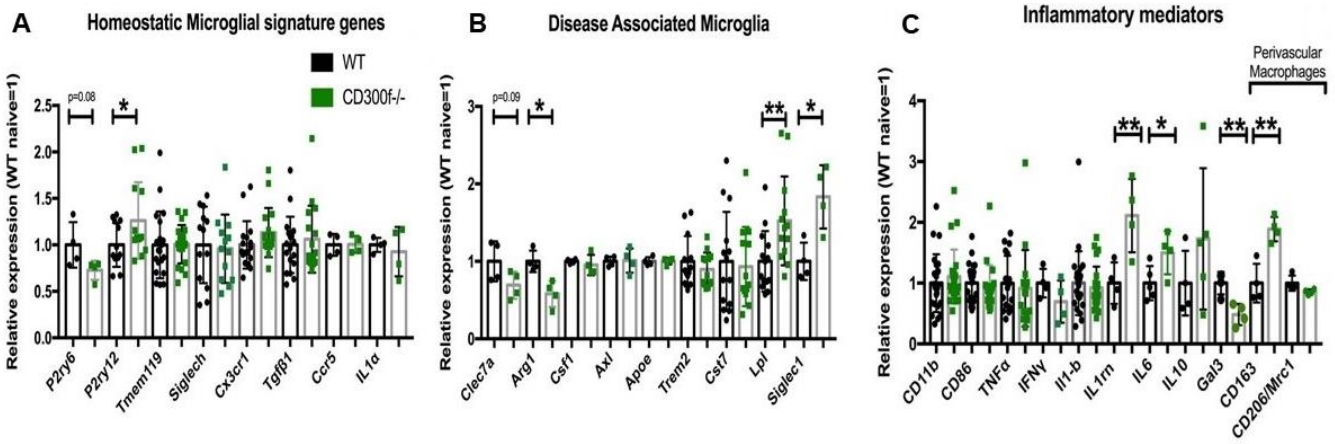


Figure 7. CD300f KO mice display subtle alterations in neuro-immune communication mediators and microglial phenotype. Central nervous system samples from saline perfused WT or CD300f KO animals were used to analyze gene expression by qPCR. Expression of selected genes of microglial homeostatic signature (A), DAM genes (B) and inflammatory mediators (C) were compared between CD300f KO and WT mice. Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. WT: Wild-type; DAM: Damage associated microglia; CD300f-/-: CD300f knockout mice.

Finally, one of our hypothesis was that depressive-like phenotype observed in CD300f KO female mice could be related to the presence of increased inflammation linked to the NLRP3 inflammasome activation and pro-inflammatory IL1β release. We evaluated the gene expression of NLRP3 in the total brain extract of CD300f KO mice and did not observe any significant alteration (Fig. 8A), moreover, no differences were also evidenced in hippocampal IL1β protein levels (Fig. 8B), suggesting that mice behavioral alterations observed with the lack of the CD300f gene might be related to another pathophysiological characteristic other than overall inflammatory profile and needs to be further uncovered.

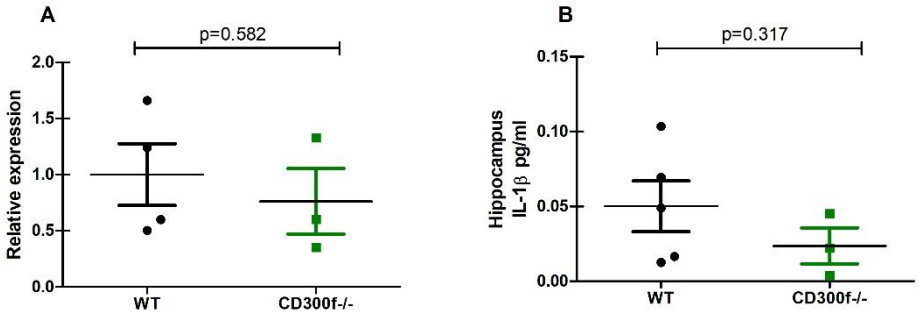


Figure 8. CD300f KO female mice do not display NLRP3 inflammasome alterations. Expression of NLRP3 gene was analyzed by qPCR from RNA isolated of mice total brain extract (A). Pro-inflammatory IL1β levels were analyzed in the hippocampus by ELISA (B). Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant. CD300f-/-: CD300f knockout mice.

4 – Effects of age and genotype on behavioral analysis:

We compared behaviorally animals from different ages, considering 3 months old female mice as the young group, 4 to 5 months old female mice as the adult group and 18 months old mice as the aged group. Figure 9A shows the two-way ANOVA analysis, demonstrating that CD300f KO female mice presents changes in their locomotor activity significantly dependent on age and genotype. Moreover, an interesting effect of age and genotype can be seen in the time spent in the central zone of the OFT as an indicative of anxiolytic-like behavior (Fig. 9B). The Bonferroni post-hoc analysis reveals that CD300f aged female mice remained more time in the center of the OFT when compared to the respective aged WT ($p<0.05$), suggesting that the lack of CD300f gene protects mice against anxiety. Another important result is depicted in figure 9D, which shows a significant statistical interaction between age and genotype for the time spent in grooming behavior in the SST. The Bonferroni post-hoc analysis demonstrated there is a significant decrease in grooming behavior of adult ($p<0.001$) and aged ($p<0.01$) CD300f KO female mice when compared to WT female mice.

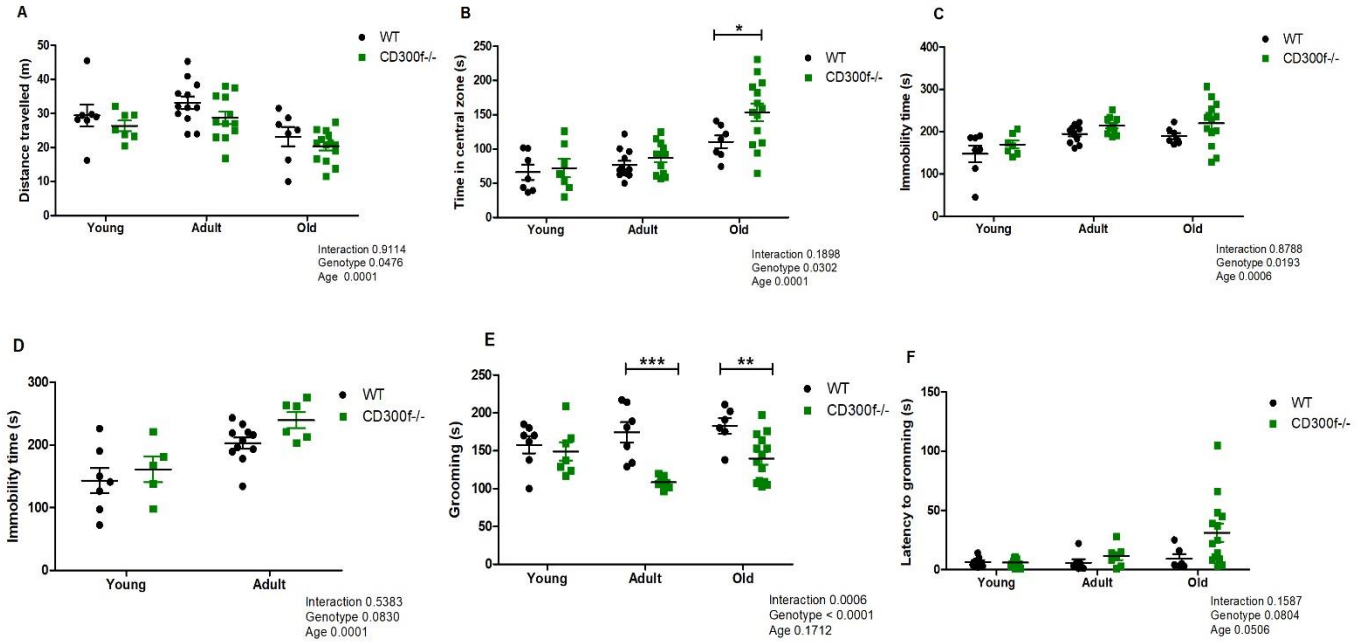


Figure 9. Female mice behavior according to age and genotype. Young (3 months old), Adult (4-5 months old) and aged (18 months old) female mice were evaluated in the OFT and the total distance travelled was measured (meters) as an indication of mice spontaneous locomotor activity (A), and time spent in the center of the OFT were analyzed as an indicative of mice anxiolytic-like activity (B). The total immobility time in the TST (C) and the FST (D) was measured (seconds) to evaluate depressive-like behavior. The total time (seconds) in grooming behavior in the SST was recorded to evaluate hedonic-like behavior (E) and the latency to start grooming behavior in the SST was evaluated as an indication of motivational and self-care behavior (F). Data are represented as mean ± S.E.M. and $p<0.05$ was considered statistically significant; * means $p<0.05$, ** means $p<0.01$ and *** means $p<0.001$ when compared to the respective WT group. OFT: Open field test; TST: Tail suspension test; FST: Forced Swimming test; SST: Sucrose splash test.

5 – Conclusions and future studies:

Conclusions:

Association of inflammation and MDD has been well documented both in animal models and clinical studies [19]. MDD frequently accompanies patients suffering from sustained systemic inflammation including autoimmune, cardiovascular and metabolic diseases [20,21]. Moreover, NLRP3 associated inflammation has also been strongly associated with MDD [10]. We hypothesized that the absence of a microglial restraining signal in the CD300f KO animals would induce a low-grade neuroinflammation that could lead to a depressive-like behavior. In this work we found that:

I. The absence of CD300f leads to depressive and anhedonic-like behaviors in adult female mice and that this phenotype persists with age. Interestingly male mice did not present depressive and anhedonic-like behaviors. On the other hand, male mice present a protective phenotype against anxiety that needs to be further investigated.

II. The lack of CD300f leads to decreased noradrenaline levels in adult female hippocampus, a fact that could be associated to their depressive and anhedonic-like behavior. We chronically treated mice with the tricycle antidepressant imipramine, although it was not able to improve CD300f KO behavior alterations and the use of a more specific compound that target the noradrenergic system will be required to confirm this possible association.

III. The performance of a chronic depressive model induced by daily restraint stress did not affect mice in the depressive and anhedonic-like behaviors. This might occur because mice have habituated to the stressor or it is possible that only a stronger stressor could exacerbate the behavioral alterations in CD300f KO female mice. Moreover, this protocol induced increased motor locomotion that was

observed in the OFT and this could be masking the observation of a depressive-like phenotype in the TST and FST. In this way, these mice need to be evaluated in another mice model of depression.

IV. CD300f KO mice present slightly alterations in the homeostatic and DAM gene expression profile compared to WT and no general increased inflammatory markers. Interestingly, two pro-inflammatory cytokine genes (Il1rn and il6) are increased in CD300f KO which might be associated with the depressive and anhedonic-like phenotype observed in these animals. Moreover, the decreased expression in P2ry6 (a homeostatic gene) could be associated to microglia altered phagocytic capacity, but needs further evaluations.

V. No activation was observed in the NLRP3 inflammasome suggesting that the behavioral alterations seen in these mice might be associated to other neuro-immune mechanisms.

VI. Anhedonic-like behavior can be observed in adult and aged mice, suggesting that the lack of CD300f receptor might be implicated in later behavioral alterations, however further investigations should be conducted to evaluate whether biochemical alterations start occurring during the development or later.

Future evaluations:

I. Perform an acute injection of lipopolysaccharide in CD300f KO female mice to evaluate depressive and anhedonic-like behavior under systemic inflammation;

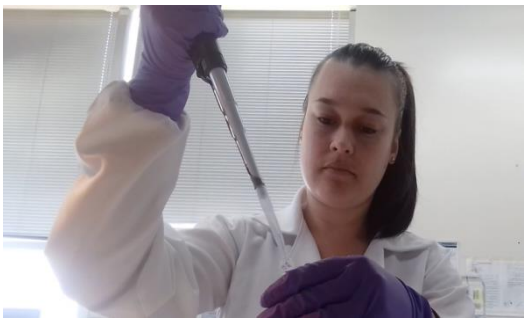
II. Treat mice with Bupropion, an inhibitor of norepinephrine and dopamine reuptake to evaluate its antidepressive-like effect in naïve CD300f female mice;

III. Characterize specific changes in the blood brain barrier of CD300f KO animals in the model of depression induced by lipopolysaccharide injection and in naïve conditions.

6 – Photography

6.1. Neuroinflammation and Gene Therapy Laboratory Staff / Institut Pasteur de Montevideo Staff

6.2. PhD Student Fernanda Neutzling Kaufmann performing animal behavior and biochemical analysis



7 - Important opportunities that emerged from this experience

A fruitful collaboration emerged from this work with Gabriele Ghisleni at Catholic University of Pelotas in Brazil in order to evaluate a polymorphism in the CD300f gene and the prevalence of psychiatric conditions (results in analysis). In addition it prompted us to a future project in the field of psychiatric disorders and neuroinflammation in collaboration with both groups. Additionally, an important publication will be produced from this results together with previous findings of the host laboratory that will be part of the thesis of the PhD student Fernanda Neutzling Kaufman and will be sooner submitted to Nature Communications.

In addition, despite the geographical proximity of Montevideo-UY (host laboratory) with Buenos Aires-AR, the PhD student was able to participate in Neurobiology Perspectives Symposium held in the *"Instituto de Investigación en Biomedicina de Buenos Aires CONICET – Instituto Partner de la Sociedad Max Planck"* on May, 16th to 17th and was able to participate in the Course and Symposium *"Trauma Encefálico: desde la investigación preclínica a la clínica"* held in the Institut Pasteur de Montevideo on April, 9th to 14th. The sum of these experiences was an immeasurable knowledge acquisition and exchange opportunity with many reputed researchers and students and it was a peculiar tool to improve the student's PhD thesis.

8 - ISN financial grant use

The grant received from ISN CAEN 1A was spend to contract the health insurance, to rent a place to live during 6 months and to buy the travel tickets (Brazil – Uruguay).

9 - References:

[1] Murray, C.J. et al. *JAMA* 2013, 310: 591-608. [2] Insel, T. et al. *Am. J Psychiatry* 2010, 167: 748-751. [3] Krishnan, V. and Nestler, E.J. *Nature* 2008, 455: 894-902. [4] Miller, et al. *Biol Psychiatry* 2009, 65: 732-741. [5] Lanquillon, et al. *Neuropsychopharmacology* 2000, 22: 370-379. [6] Kigerl, et al. *Exp Neurol* 2014, 258: 5-16. [7] Martinon, F. et al. *Nature* 2006, 440(7081): 237-241. [8] Singhal, G. et al. *Front Neurosci* 2014, 8: 315. [9] Alcocer-Gómez, et al., *Mol Neurobiol* 2016, 53: 4874-4882. [10] Kaufmann, F.N. et al. *Brain Behav Immun* 2017 64: 367-383. [11] Clark, G.J. et al. *Trends Immunol* 2009, 30: 209-217. [12] Peluffo, H. et al. *Brain Pathol* 2012, 22: 318-328. [13] Izawa, K. et al. *Immunity* 2012, 37: 827-839. [14] Xi, H. et al. *J Exp Med* 2010, 207: 7-16. [15] Lima, T.Z. et al. *Curr Alzheimer Res* 2017, 14: 778-783. [16] Keren-Shaul, H. et al. *Cell* 2017, 169: 1276-1290. [17] Maes, M. et al. *Cytokine* 1997, 9: 853-858. [18] Kohler, C.A. et al. *Acta Psychiatr Scand* 2017,135: 373-387. [19] Dantzer R, et al. *Nat Rev Neurosci* 2008, 9:46-56. [20] Katz PP, et al. *J Rheumatol* 1993, 20:790-796. [21] Zellweger MJ, et al. *Eur Heart J* 2004, 25:3-9.