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SERIES XXX

New insights into the communication between immune and central nervous system: novel strategies for the diagnosis and pharmacological treatment of ADHD and 22q11DS

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BACKGROUND

Mental disorders, such as Attention deficit hyperactivity disorder (ADHD), and 22q11 deletion syndrome (22q11DS) are both related to a dopaminergic system dysfunction. Several studies have shown that dopamine (DA) not only mediates interactions into the nervous system, but can also contribute to the modulation of immunity *via* receptors expressed in the immune cells (Nagakome et al., 2011; Nakano et al., 2011). Autoimmunity in the central nervous system has been suggested to play a pivotal role in the pathogenesis of neurodevelopmental disorders (ND) (Schwartz. 2013), but how an immune system alteration might lead to ADHD and participate to the psychiatric features of the 22q11DS is still unclear. The importance of immune system for life-long maintenance of the brain is demonstrated by its role in the formation of new neurons, support to normal cognitive performance and neurogenesis regulation. These evidences have broken the theory of the central nervous system (CNS) as immune privileged organ, indicating that peripheral immune cells can contribute to brain homeostasis and repair. In particular, it was demonstrated that circulating blood macrophages recruitment in brain parenchyma is needed for CNS health and homeostasis. The immune cells access to the CNS is regulated by choroid plexus (CP) activation, promoted by pro-inflammatory signals from both brain parenchyma or peripheral T cells accumulated in CP (Baruch et al., 2015).

Importantly, a possible role of the neuropeptide adrenomedullin (ADM) in ND has been suggested, since several studies reported a role of this peptide as a biomarker of different psychiatric disorders, such as Alzheimer (Fernandez et al., 2016), autism (Zoroglu et al., 2003), bipolar disorder (Akpinar et al., 2013), schizophrenia (Chia-Hsing Huang et al., 2004) and ADHD (Fernandez et al., 2008). ADM is a regulatory peptide involved in thymic immune tolerance mechanisms (Rulle et al., 2012; Castellani et al., 2016), which are suggested to be altered in ND.

On the basis of these considerations, in this study we investigated the involvement of immunological mechanisms and their dependence to ADM both in the periphery and in CNS in mouse models of ADHD and 22q11DS, in order to define whether an immunomodulatory approach can be useful in the management of these diseases.

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chapter 1

An intracellular adrenomedullin system reduces IL-6 release *via* a NF-kB-mediated, cAMP-independent transcriptional mechanism in rat thymic epithelial cells

ABSTRACT

Thymic epithelial cells (TECs) play a key role in the regulation of central immune tolerance by expressing autoantigens and eliminating self-reactive T cells. Our group has already demonstrated that adrenomedullin (ADM) and its co-receptor protein RAMP2 are located intracellularly in newborn human thymic epithelial cells (TECs). The main aim of this study was to investigate the potential modulating effect of ADM on the NF-kB pathway, which is involved, through the production of cytokines such as IL-6, in the maturation of T-lymphocytes and immunological tolerance. Our results show that, similarly to human newborn TECs, ADM is localized to the cytoplasm of adult rat TECs, and RAMP2 is expressed in the nucleus but not in the plasma membrane. Pre-treatment of TECs for 4 hours with ADM significantly reduced lipopolysaccharide (LPS)-induced release of IL-6 ($p<0.001$) and expression of the p65 subunit of NF-kB, while doubled the expression of IkBα ($p<0.001$), the physiological inhibitor of NF-kB nuclear translocation. These effects were not mediated by activation of the cAMP pathway, a signalling cascade that is rapidly activated by ADM in cells that express RAMP2 in the plasma membrane, but were the consequence of a reduction in the transcription of p65 ($p<0.001$) and an increase in the transcription of IkBα ($p<0.05$). Taken together, these results suggest that in rat TECs ADM reduces IL-6 secretion by modulating NF-kB genes transcription through an interaction with a receptor localized to the nucleus. This may partly explain the protective effects of ADM in autoimmune diseases and points to the ADM system of TECs as a novel potential target for immunomodulating drugs.

INTRODUCTION

1. ADM system.

ADM is a regulator peptide expressed and secreted in multiple tissues and organs, such as heart and blood vessels, kidneys, lungs, gastrointestinal tract, spleen, exocrine glands, brain, retinal pigment epithelium and pancreatic F-cells. It is synthesized and secreted abundantly by several cells of the cardiovascular system: endothelial cells, smooth muscle cells of the vessels and cardiomyocytes. In addition, the presence of ADM was discovered in the mastocytic granule of cardiac connective tissue (Belloni et al., 2006). It has been observed that ADM is expressed in rat thymus (Belloni et al., 2003) and, recently, the expression of ADM and its receptor proteins in the neonatal human thymus has been demonstrated (De Martin et al., 2014).

ADM has antimicrobial properties both against gram-positive and gram-negative bacteria (Allaker et al., 2006). Because of its anfipatic structure, it can enter into the bacterial membranes. The concentration of ADM needed to kill or inhibit bacterial growth is higher than the levels found in the circulatory system; however, in certain circumstances, such as sepsis, its plasma concentration increases. Finally, it also has protective anti-apoptotic action against oxidative stress and hypoxia (Oehler et al., 2001).

The adrenomedulin receptor is composed by two proteins: Calcitonin Receptor Like Receptor (CRLR), whose name derives from the strong homology with the amino acid sequence of the receptor for calcitonin, and the Receptor Activity Modyifing Protein 2 (RAMP2) (McLatchie et al., 1998) *(fig. 1)*. The RAMP2 protein is a membrane protein with a transmembrane domain that binds the CRLR on the cell surface to form the functional receptor for ADM. RAMP2 is expressed in several tissues as the lungs, heart, placenta, skeletal muscles, fatty tissue, thymus and pancreas.

A large number of studies showed that the binding of ADM to its receptor causes an increase in cAMP in various tissues and cells (Coppock et al., 1999). The initial mechanism of action of ADM involves the activation of G protein, adenyl cyclase and protein kinase A (PKA) (Coppock et al., 1996).

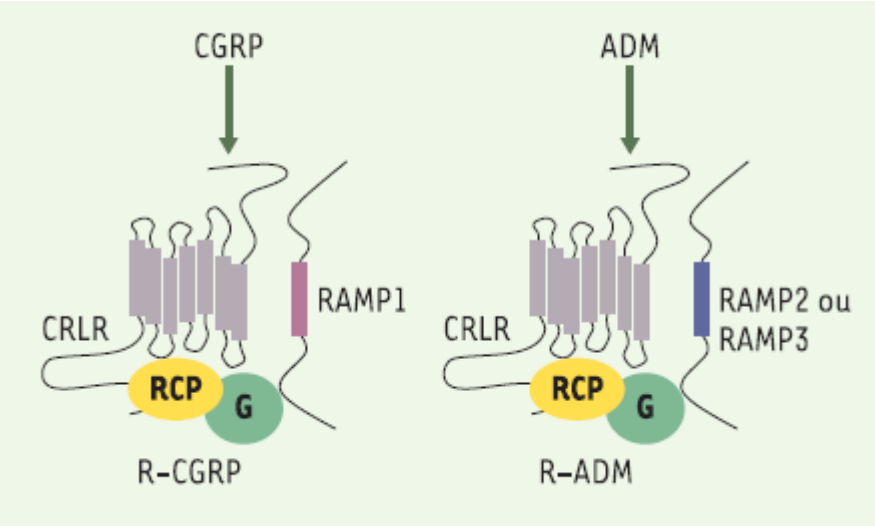

Figure 1 I ADM receptors. (Cueille et al,.2004).

2. ADM multifunctional activity.

ADM is a multifunctional peptide exerting, through an autocrine/paracrine action, multiple biological effects including blood pressure regulation and the potentiation of host defences against microbes. ADM has also been shown to exert a potent inhibitory effect on the release of cytokines, including IL-6, in LPS-stimulated microglial cells *in vitro* (Kato and Kitamura 2015), an observation consistent with its protective effect observed in such autoimmune diseases as multiple sclerosis (Pedreno et al., 2014) and rheumatoid arthritis (Gonzalez-Rey 2007), in which IL-6 plays a pivotal role (Caiello et al., 2014).

3. T lymphocytes maturation in thymus.

T lymphocytes develop from lymphoid progenitors located in the bone marrow, which are in common to B lymphocytes. Some of these progenitors migrate into the thymus, where they differentiate into T helper or cytotoxic lymphocytes; these cells then migrate into the bloodstream and to peripheral lymphoid organs (spleen, lymph nodes and lymphatic tissues). Thus, maturation of T cells occurs in discrete thymic regions and is driven by the interaction of thymocytes with specialized TECs (Anderson et al., 2012). These cells are present in both the cortex (cortical thymic epithelial cells, cTECs) and medulla (medullary thymic epithelial cells, mTECs) of thymus, and regulate immune tolerance by expressing auto antigens and eliminating self-reactive T cells (Alexandropoulos et al., 2012). In particular, mTECs ectopically express tissue-restricted antigens whose role is to delete autoreactive T cells which recognize antigens with high affinity (Klein et al., 2009). T cell maturation phases are characterized by changes of the T cells receptor (TCR) genes and expression and variations in the expression of surface proteins, such as CD3, CD8 and CD4. In the early phases of development, two distinct T cell lines are distinguished by two different types of TCR called α: β and γ: δ. Later, T α: β lymphocytes develop into two functionally distinct subtypes: CD4 and CD8 lymphocytes. This thymocyte development is characterized by three stages *(fig. 2):* these cells are initially classified as "double negative", since they do not express either CD4 or CD8. These cells are immature cortical thymocytes not yet expressing TCR complexes. The stimuli that drive proliferation and maturation of double-negative thymocytes are poorly known, although numerous data demonstrate the role of cytokines produced by non-lymphoid cells in thymus as growth factors for immature thymocytes (Gerondakis et al., 2014). The subsequent maturation stage leads the thymocytes to express both CD4 and CD8 receptors, thus becoming "double positive" thymocytes (CD4 + CD8 +), representing 80% of thymocytes present in an adult thymus (these cells are immature cortical timocytes in phase of active division or rearrangement of active TCR genes).

T cells that are unable to generate a functional TCR and therefore do not recognize the MHC complex (linked to self or non-self peptides) are eliminated by apoptosis through a process called positive selection, occurring with the help of cTEC expressing on their surface the MHC I or II complexes. During this process, mTEC eliminate all the T cells that would be unable to recognize the antigen in the periphery. The remaining T cells are exposed to peptides self-complexed with MHC, thereby becoming "single positive", following CD4 receptor expression (helper T cells, preferably binding to MHC II molecules) or CD8 receptor (cytotoxic T cells, which preferably bind to MHC I molecules) (Reyes

Garcia, Garcia Tamayo, 2012). At this stage, self-reactive cells are destroyed in the negative selection process, which ensures that the repertoire of mature T cells is tolerant of the autologous proteins present in thymus.

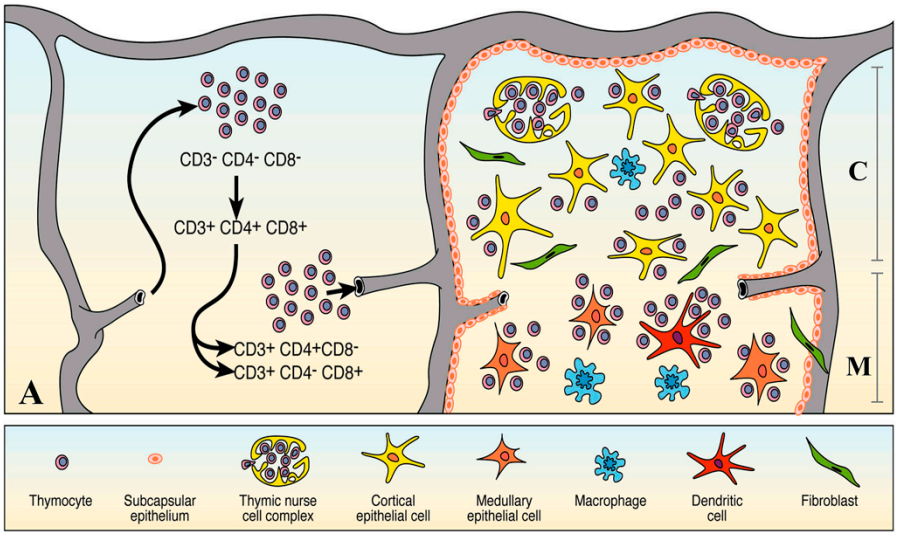

Figure 2 | Thymocytes maturation in the thymic microenvironment (Savino et al,.2006).

4. NF-kB pathway in T cell maturation

The generation of a specific T cell subset is regulated by unique patterns of gene expression, which are driven by numerous intracellular signalling networks (Rothenberg et al., 2011). In this context, the nuclear factor kappa B (NF-kB) pathway has recently been indicated as a fundamental contributor to the maturation of specific T cell subsets (see Gerondakis et al., 2014, and refs. therein) by promoting the synthesis and secretion of inflammatory cytokines (Takeuchi and Akira 2010) *(fig. 3)*. The NF-kB family of transcription factors consists of five DNA-binding members: c-Rel, RelB, p65, p50 and p52 that can assemble in homodimers or heterodimers with different gene-regulatory functions (Gilmore et al., 2006). Two different but interacting pathways are part of this signalling cascade, i.e.

the canonical and the non-canonical NF-kB pathways, which are activated by different extracellular signals (Hayden et al., 2008; Sun et al., 2012). In the canonical pathway, a dimeric factor consisting of the p50 and p65 subunits is kept inactive in the cytosol by a family of inhibitors, the IkB proteins, that mask the NF-kB nuclear localization sequence (Baldwin et al., 1996). Interleukin 6 (IL-6), which is produced in response to activation of the canonical NF-kB pathway, is a key pleiotropic cytokine involved in multiple biological processes including cell differentiation, proliferation, survival and apoptosis (reviewed in Rothaug et al., 2016). In thymus, IL-6 regulates many important physiological processes such as TEC growth (Meilin et al., 1995) and thymocyte adhesion (Ramarli et al., 1998), T-cell growth and differentiation, and the functional characteristics of thymic dendritic cells (Li et al., 2016). IL-6 also plays a role in pathological conditions, such as *myasthenia gravis,* in which its overproduction is considered responsible for the observed morphological and functional thymic abnormalities (Aricha et al., 2011).

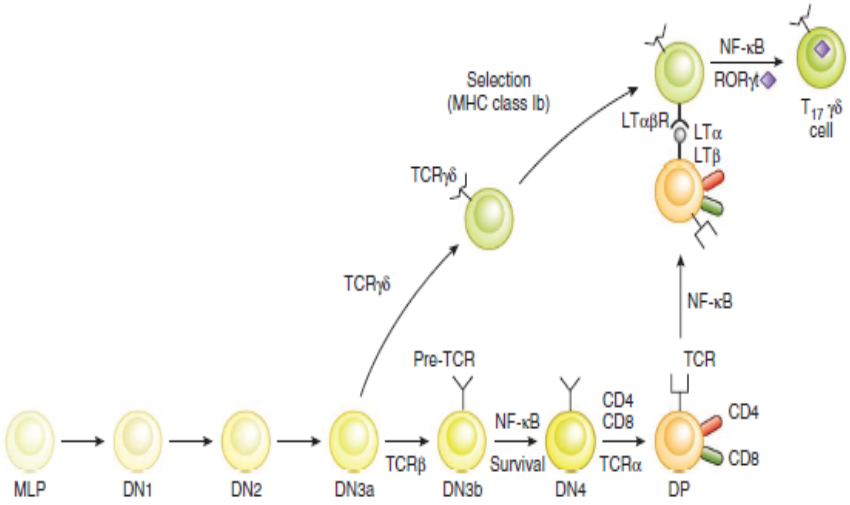

Figure 3 I NF-kB activity in the modulation of thymocytes maturation (Gerondakis et al,.2014).

AIMS

1. Evaluation of ADM system cellular localization in TECs of adult Wistar rats.

The first aim of this work was to examine the cellular localization of ADM and its receptor in TECs of adult Wistar rats to validate this animal model for the study of the ADM system and its function(s) in thymus.

2. Detection of ADM role in T cells maturation and self-tolerance.

The second aim of this study was to investigate the modulating effect of ADM on the NF-kB pathway, which is involved, in the maturation of T-lymphocytes and immunological tolerance, through the production of cytokines such as IL-6.

METHODS

1. Materials.

Minimum Dulbecco's Essential medium (DMEM), epidermal growth factor, hydrocortisone, cholera toxin, 40% acrylamide solution, sodium dodecyl sulfate (SDS), Tween 20, RNAse, and propidium iodide were obtained from Sigma-Aldrich Italy (Milan, Italy). Foetal bovine serum, glutamine and penicillin-streptomycin solutions were obtained from Gibco (Life Technologies Italia, Monza, Italy). Mouse monoclonal antibodies reactive against rat cytokeratins (Ck) 8/18, rat β-actin, rat GAPDH and rat lamin A/C, rabbit polyclonal antibody reactive against rat CRLR, rat ADM, rat p65, rat p50 and rat IkBα, and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibody directed against rat RAMP2 was purchased from Abcam (Cambridge, UK). Secondary antibodies conjugated with fluorescent dyes were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Goat anti-rabbit IgG coupled to 10 nm diameter gold particles and goat anti-mouse IgG coupled to 5 nm diameter gold particles were obtained from Sigma-Aldrich (St. Louis, MO, USA) and British BioCell International (Cardiff, UK), respectively.

2. Animals and treatments.

The procedures regarding animal care were in accordance with institutional guidelines that comply with the European Economic Community Council Directive 86/609 (OJ L 358, 1, Dec.12, 1987) and the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, 1985). The experimental design has been approved by the Ethics Committee of the University of Padova for the care and use of laboratory animals (Prot. N° 18758 – March 26, 2010).

Thymi were obtained from Wistar male adult rats sacrificed under sevoflurane anaesthesia (2.5% per litre of oxygen). The organs were rapidly removed, weighed and placed in a tube containing DMEM with a penicillin-streptomycin solution to obtain primary cultures of TECs.

3. Primary cultures of rat TECs.

TEC cultures were obtained from rat thymic fragments following the method already described by De Martin and colleagues *(De Martin et al., 2014)*. Briefly, small fragments of thymic tissue were anchored in cell culture flasks and cultured in TEC medium. Cultures were maintained in an incubator with a humidified atmosphere at 37°C and 5% CO_2 in air. At day 14, thymic fragments were removed from cell culture flasks, and adherent cells were passaged by tryptic digestion.

4. Immunofluorescence coupled with confocal microscopy.

RAMP2, CRLR, ADM, p50, p65 and IkBα expression and localization were evaluated in TECs (at passage 2-4). TECs were incubated with a mouse monoclonal antibody anti-RAMP2 (1:500) and a rabbit polyclonal antibody directed against either ADM or CRLR (both at 1:200). To analyze the activation of NF-kB, TECs were incubated 60 minutes at 37°C with a rabbit polyclonal anti-p65, anti-p50 or anti-IkBα antibody (1:200). Images of immunostained cells were acquired by means of a ×60 CFI Plan Apochromat Nikon objective with a Nikon C1 confocal microscope and analyzed with NIS Elements software (Nikon). The intensity of the fluorescent signal was quantified by means of ImageJ software. Each experiment was performed on TECs obtained from 5 different rats.

5. Immunogold labelling coupled with transmission electron microscopy (TEM).

Immunogold labelling assays were performed essentially as described by Calzia *et al (Calzia et al., 2014)*. After the preparation of the sections, cells were incubated with mouse monoclonal anti-RAMP2 or rabbit polyclonal anti-CRLR (both diluted 1:50) overnight at 4°C. Antibody binding was detected using a secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG, both diluted 1:100) coupled to either 10 nm diameter or 5 nm diameter gold particles. Images of stained sections were acquired by a FEI Tecnai G2 transmission electron microscope operating at 100 kV and analyzed by means of the TIA Fei software CAM. Corel Draw X3 was used for collection and typesetting of images. Control samples were obtained by omitting primary antibodies and resulted in absence of reactivity.

6. cAMP assay.

cAMP levels were determined by enzyme immunoassay (EIA) following the protocol proposed by the manufacturer (Cyclic AMP EIA Kit; Cayman Chemical Company, Ann Arbor, MI, USA). Experiments were performed in duplicate and repeated with cells obtained from 5 different animals.

7. IL-6 ELISA Assay.

TECs were stimulated to release inflammatory mediators in TEC medium added with 100 ng/ml of LPS (Von Patay et al., 1998), in the presence or absence of 10^{-7}M ADM. Briefly, cells were treated with 10^{-7}M ADM for 4 hours, 100 ng/ml LPS for 24 hours, 10^{-7}M ADM for 4 hours and then 100 ng/ml LPS for 24 hours, or 10^{-7}M ADM together with 10 µg/ml LPS for 24 hours. Cell supernatants were collected and stored at -20°C until the day of assay. IL-6 released into the culture medium was analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Antigenix America, Huntington Station, NY, USA). IL-6 concentration (pg/ml) in the medium was determined by reference to standard curves obtained with known amounts of this cytokine.

8. Determination of mRNA levels by real-time PCR.

In order to ascertain whether ADM influences the mRNA expressions of p50, p65, and IkBα genes, which belong to the NF-kB family, rat TECs were incubated with this peptide (10^{-7}M) for 4 hours at 37°C. At the end of the incubation period, TECs were scraped away from cell culture dishes and total RNA was extracted and purified by means of the SV Total RNA Isolation System (Promega Corporation, Madison, WI). Genomic DNA contamination was removed by means of a DNAse treatment. Integrity and quantity of RNA were evaluated by an RNA 6000 Nano assay in an Agilent BioAnalyser (Agilent Technologies Inc., Palo Alto, CA, USA). The relative expression of p50, p65, and IkBα was determined by real-time PCR (Eco™ Illumina, Real-Time PCR system, San Diego, CA, USA) using One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Shiga, Japan). PCR amplifications were tested for linearity and efficiency using standard curves obtained with serial dilution of cDNA; the specificity of amplification and absence of dimers were confirmed by melt-curve analysis. All genes were normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used in this study are listed in Table 1. Expression levels of p50, p65 and IkBα genes were calculated by the ΔΔCt method using the Eco™ Software v4.0.7.0. Modifications of mRNA levels were expressed as fold variation compared with that of untreated cells.

9. Statistical analysis.

Comparison of the experimental data obtained from control cell cultures and those treated with LPS and/or ADM was made by one-way analysis of variance (ANOVA). If differences were statistically significant ($\alpha = 0.05$), the analysis of variance was followed by the Dunnett (to compare different treatments with control) or the Newman-Keuls (to compare the different treatments) *post-hoc* test. For cAMP production experiments, comparison of the experimental data obtained from control cells and those treated with forskolin or different concentrations of ADM was made by the non-parametric Kruskal-Wallis test. $P<0.05$ was considered statistically significant. Data are presented as mean ± standard deviation.

RESULTS

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1. Localization of ADM and its receptor in rat TECs.

The cellular localization of ADM and its receptor proteins was investigated in cultured rat TECs by double immunofluorescence coupled to confocal microscopy and by immunogold labelling coupled to transmission electron microscopy. Preliminary experiments with an anti-cytokeratins 8/18 antibody allowed us to exclude fibroblast contamination of TEC cultures (data not shown). Figure 4 shows that ADM, RAMP2 and CRLR were all present in rat TECs. As previously observed in newborn human thymus, RAMP2 reactivity *(fig. 4A)* was only evident in the nucleus of TECs. CRLR, the other protein forming the functional ADM receptor, was found in the nucleus, cytoplasm and plasma membrane of TECs *(fig. 4B)*. ADM was localized exclusively to the cytoplasmic compartment, where it was evenly distributed *(fig. 4C)*.

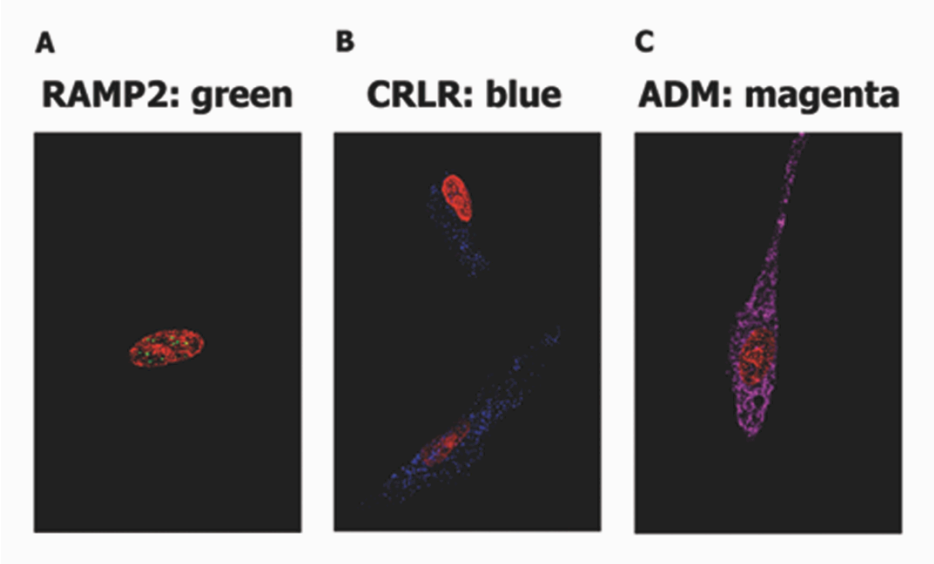


Fig 4 | Immunofluorescence staining of RAMP2, CRLR and ADM in cultured rat TECs. Immunofluorescence staining of TECs for RAMP2 (panel A, green fluorescence), CRLR (panel B, blue fluorescence) and ADM (panel C, magenta fluorescence). One representative image from five independent experiments is shown. Cell nuclei are stained red with propidium iodide.

Immunogold labelling coupled with TEM substantially confirmed the observations of immunofluorescence experiments, since RAMP2 immunopositivity was evident in the nucleus of TECs, particularly in the region close to the nuclear envelope *(fig. 5A)*, whereas CRLR was detected in the nuclear envelope, cisternae of endoplasmic reticulum, membrane of cytoplasmic vescicles, and plasma membrane *(fig. 5B)*.

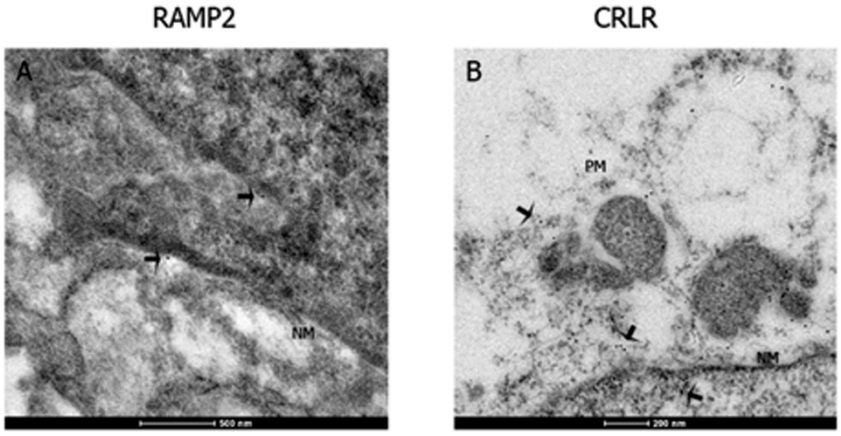


Fig 5 | Immunogold staining of CRLR and RAMP2 in cultured rat TECs. Immunogold staining (black dots indicated by arrows) of RAMP2 (panel A) and CRLR (panel B). PM: plasma membrane; NM: nuclear membrane. One representative image from five independent experiments is shown.

2. Evaluation of cAMP prduction in TECs treated with ADM.

The binding of ADM to its plasma membrane receptor causes the activation of adenylate cyclase and the consequent production of intracellular cAMP (Kuwasako et al., 2000). Accordingly, we evaluated cAMP production by rat TECs stimulated with ADM. As shown in *fig. 6*, treatment of TECs with increasing concentrations of ADM (10^{-9} to 10^{-7}) did not modify cAMP levels, whereas the adenylate cyclase stimulator forskolin, used as a positive control, induced a 100-fold increase ($p < 0.05$) of cAMP concentration.

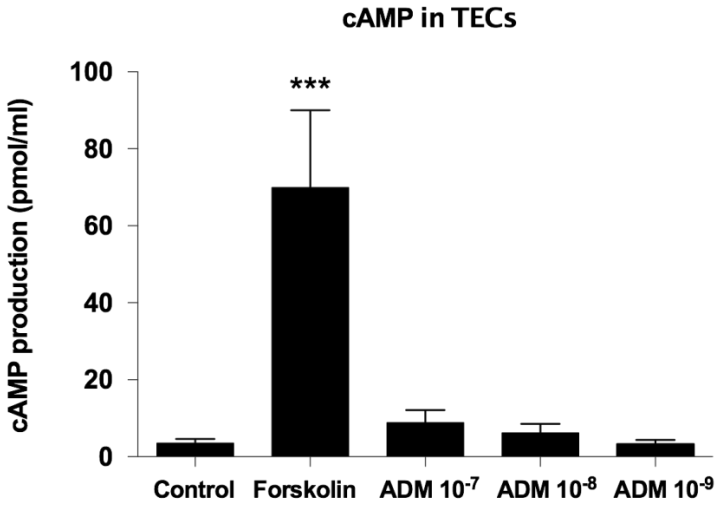


Fig 6 | cAMP production in rat thymic epithelial cells. cAMP levels in rat TECs after exposure to ADM or forskolin (FK). TECs were incubated with increasing concentrations of ADM (ranging from 10^{-9} to 10^{-7}). 10^{-4} FK was used as positive control. Values are means ± SD (n = 5). *P<0.001 *vs* control; Kruskal-Wallis test.

3. Effect of ADM on LPS-stimulated IL-6 release from TECs.

To ascertain whether ADM has any inhibitory effect on LPS-induced IL-6 production by TECs, as previously observed for microglia (Consonni et al., 2011), we performed an ELISA test on TECs incubated with LPS and/or ADM. As shown in *fig.7*, pre-treatment with 10^{-7} M ADM for 4 hours had no effect on the basal release of IL-6 from TECs. Treatment of TECs with LPS (10 µg/ml) caused a dramatic increase in IL-6 production that was significantly reduced ($p<0.001$) by pre-incubation for 4 hours with ADM. By contrast, LPS-induced IL-6 release was unaffected when ADM and LPS were simultaneously added to TECs.

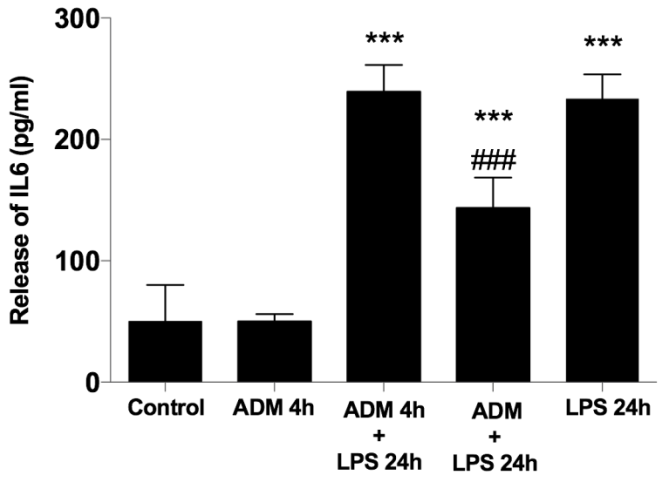
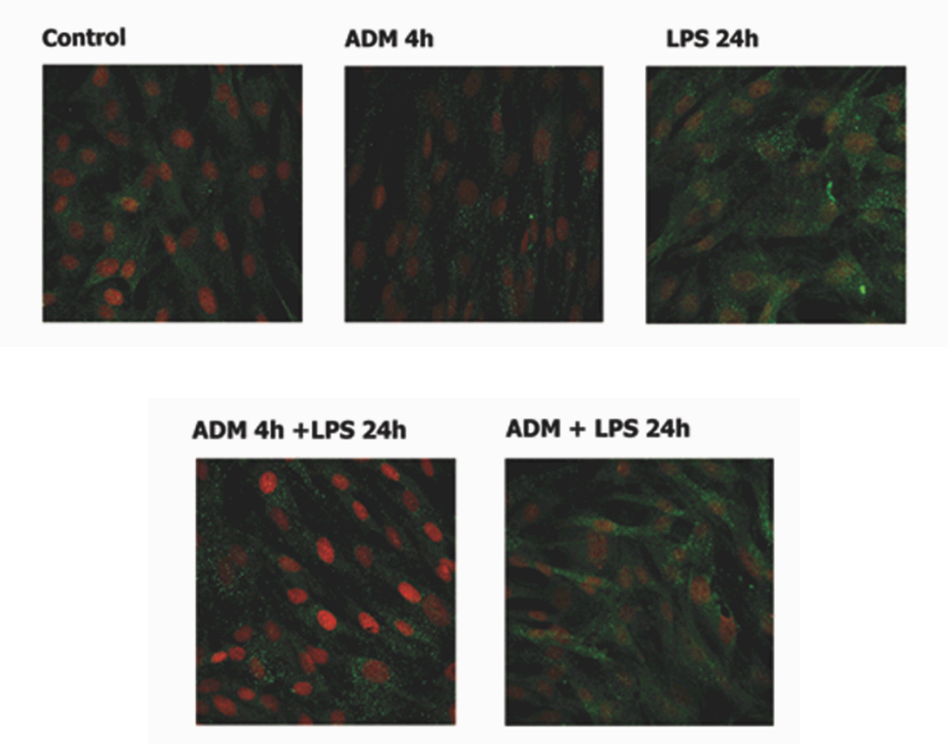


Fig 7 | IL-6 release from cultured rat TECs. IL-6 release by TECs after incubation in basal condition (control), with (from left to right): 10^{-7} ADM for 4 h, 10 µg/ml LPS for 24 h, 10^{-7} ADM for 4 h followed by 10 µg/ml LPS for 24 h, 10^{-7} ADM and 10 µg/ml LPS for 24 h. Values are means ± SD (n = 5). ***P<0.001 *vs* control, ###P<0.001 *vs* LPS-treated TECs; ANOVA followed by Newman-Keuls *post-hoc* test.

4. Effect of ADM on the canonical NF-kB pathway.

Since LPS increases IL-6 transcription *via* activation of the canonical NF-kB pathway (Brasier et al., 2010), we analyzed the expression of p65, p50 and IkBα in TECs incubated with LPS and/or ADM. *Fig. 8* clearly shows that treatment of TECs with ADM for 4 hours significantly lowered the basal expression of p65 ($p<0.05$), whereas incubation with LPS for 24 hours significantly increased its expression ($p<0.001$). ADM partially reduced the effect of LPS on p65 expression when added 4 hours before, but not when added at the same time as LPS. No significant differences in p50 expression were observed after treatment with ADM and/or LPS *(fig. 9)*.



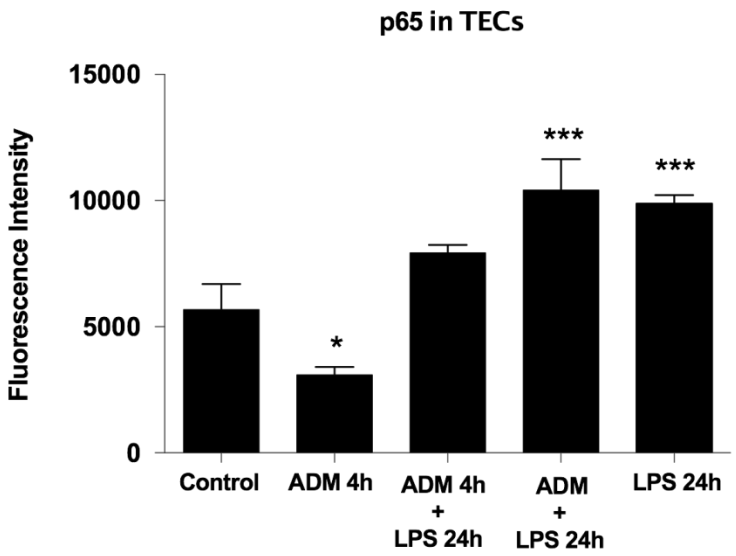
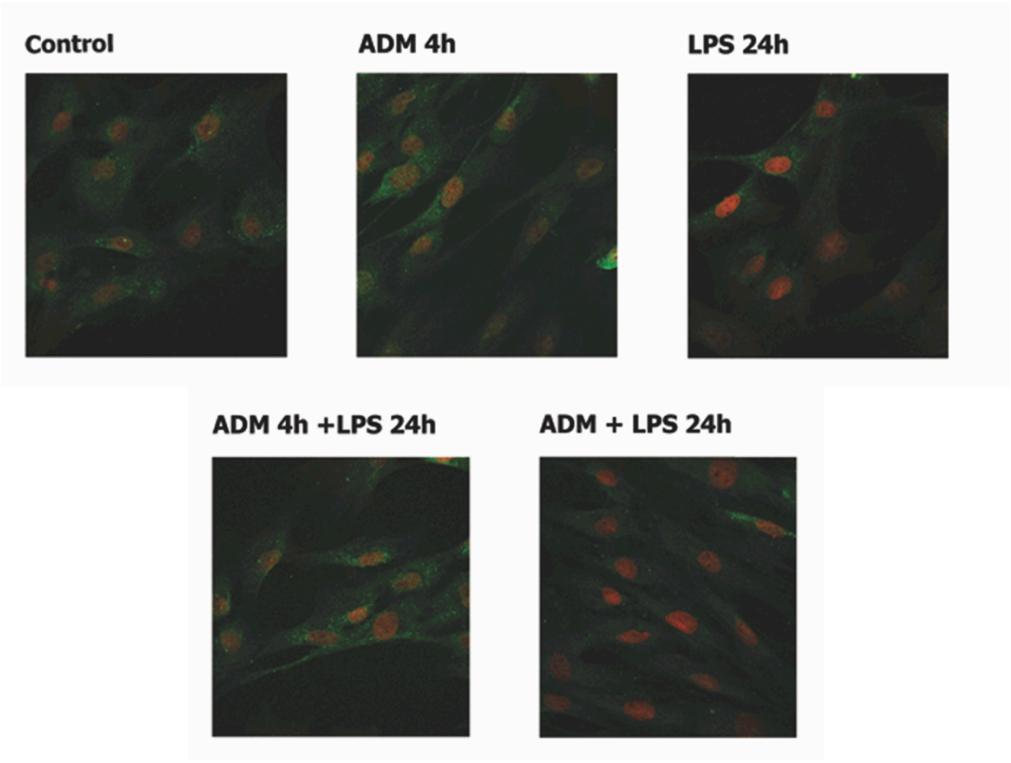


Fig. 8 | Immunofluorescence staining of p65 in cultured rat TECs. Immunostaining for the p65 subunit of NF-kB after incubation in basal condition (control), with (from left to right): 10^{-7} ADM for 4 h 10 µg/ml LPS for 24 h, 10^{-7} ADM for 4 h followed by 10 µg/ml LPS for 24 h, 10^{-7} ADM and 10 µg/ml LPS for 24 h. Values are means ± SD (n = 5). The histograms report the intensity of fluorescence (arbitrary units). *P<0.05; ***P<0.001 *vs* control; ANOVA followed by the Dunnett *post-hoc* test. Representative images from five independent experiments are shown. Cell nuclei are stained red with propidium iodide.



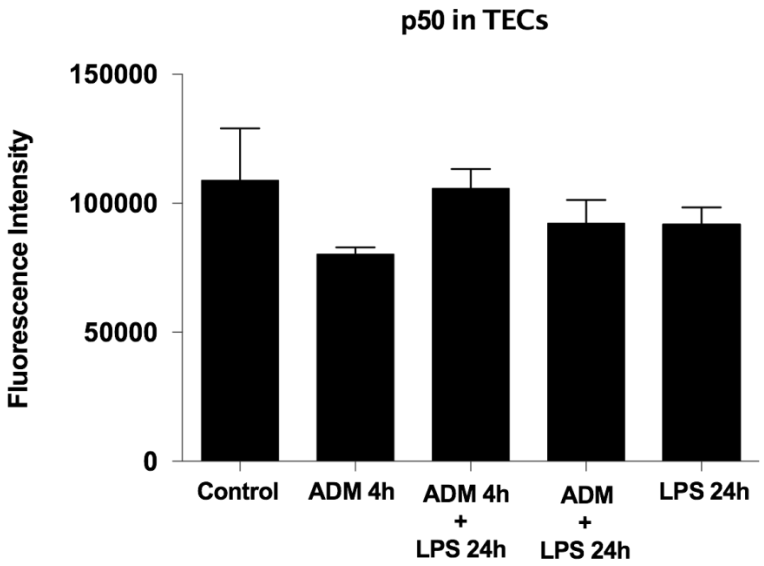


Fig. 9 | Immunofluorescence staining of p50 in cultured rat TECs. Immunostaining for the p50 subunit of NF-kB after incubation in basal condition (control), with (from left to right): 10^{-7} ADM for 4 h, 10 µg/ml LPS for 24 h, 10^{-7} ADM for 4 h followed by 10 µg/ml LPS for 24 h, 10^{-7} ADM and 10 µg/ml LPS for 24 h. The histograms report the intensity of fluorescence (arbitrary units). Values are means ± SD (n = 5). Representative images from five independent experiments are shown. Cell nuclei are stained red with propidium iodide.

A significant increase ($p<0.001$) in the expression of IkBα, a NF-kB inhibitor which prevents the nuclear translocation of p50 and p65, was observed after TEC pre-treatment with ADM for 4 hours followed by incubation with LPS *(fig. 10).* No effect on the expression of this protein was observed when TECs were incubated with ADM for 4 hours, LPS for 24 hours, or ADM plus LPS for 24 hours.

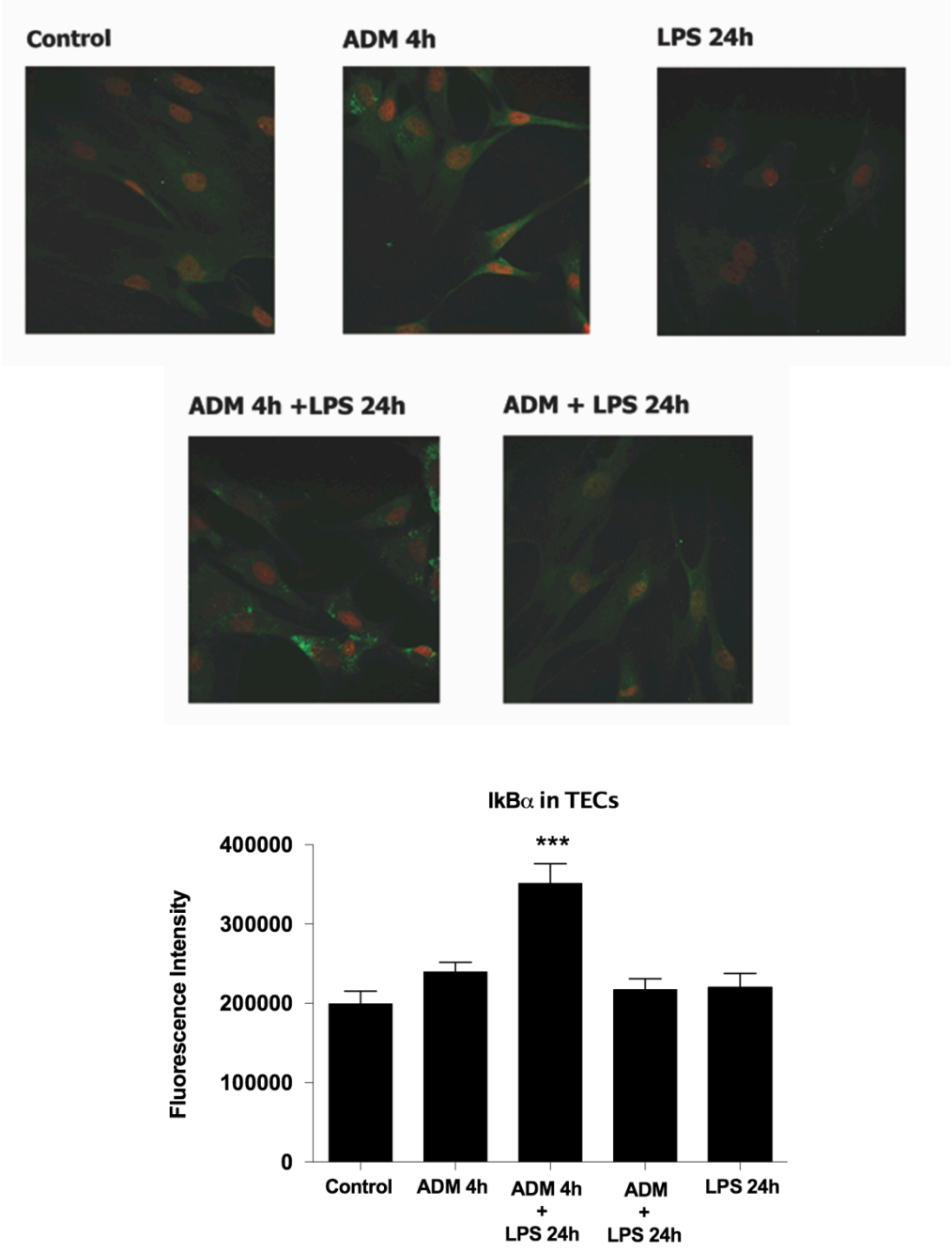


Fig. 10 | Immunofluorescence staining of IkBα in cultured rat TECs. Immunostaining for the IkBα inhibitor of NF-kB after incubation of TECs in basal condition (control), with (from left to right): 10^{-7} ADM for 4 h, 10 µg/ml LPS for 24 h, 10^{-7} ADM for 4 h followed by 10 µg/ml LPS for 24 h, 10^{-7} ADM and 10 µg/ml LPS for 24 h. Values are means ± SD (n = 5). The histograms report the intensity of fluorescence (arbitrary units). ***P<0.001 *vs* control; ANOVA followed by the Dunnett *post-hoc* test. Representative images from five independent experiments are shown. Cell nuclei are stained red with propidium iodide.

In order to assess whether ADM interacts with the canonical NF-kB pathway at the transcriptional or translational level, we measured the mRNA levels of p50, p65 and IkBα in TECs treated with ADM for 4 hours. In accordance with protein expression, the mRNA level of p65 was significantly decreased, whereas that of IkBα was significantly increased. No effect on p50 mRNA expression could be detected after a 4-hour incubation with ADM *(fig. 11)*.

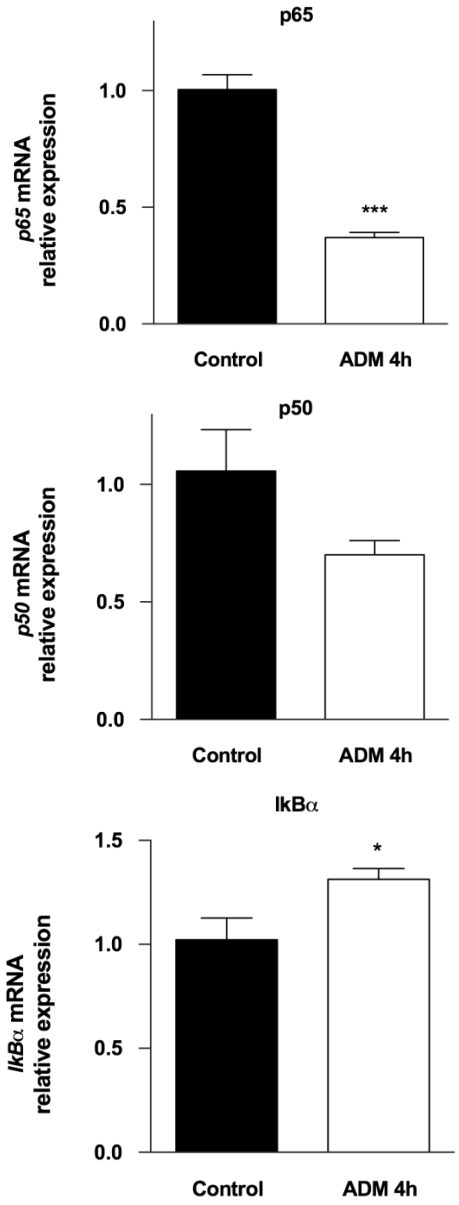


Fig. 11 | mRNA levels of p65, p50 and IkBα in cultured rat TECs. The experimental data for p65, p50 and IkBα mRNA levels in TECs treated with ADM for 4 hours are reported as fold variation compared with untreated cells. Results are means ± S.E. of data obtained from 4 different experiments preformed in triplicate per group. *P<0.05, ***P<0.001 vs. untreated cells; ANOVA followed by the Dunnett *post-hoc* test.

DISCUSSION

In a previous study (De Martin et al., 2014) we demonstrated that ADM and its receptor proteins CRLR and RAMP2 are expressed in newborn human thymus, essentially in the epithelial compartment. In human TECs, the functional ADM receptor, consisting of both CRLR and RAMP2, displays an intracellular localization, which we essentially observed also in the present study in rat cells. This peculiar localization was demonstrated by both confocal and electron microscopy, the latter providing a more detailed picture of RAMP2 distribution, prevalently evident in the nuclear envelope, not in the plasma membrane. This distribution is different from that observed in other cells, where the functional ADM receptor is localized to the plasma membrane (Kuwasako et al., 2000, Hay et al.,2003), pointing to a peculiar function of the ADM system in TECs. The results of this study have also shown that ADM regulates IL-6 secretion by TECs by modulating the transcription of genes belonging to the canonical NF-kB pathway, which is activated by various pro-inflammatory signals, such as pathogen-associated molecular patterns, including LPS (Kuwasako et al., 2000). In particular, we have demonstrated that ADM inhibits NF-kB activation as a consequence of both a decreased expression of its p65 subunit and an increased expression of its inhibitor protein, IkBα. These effects were not mediated by adenylate cyclase stimulation since, as previously observed in human TECs (De Martin et al., 2014), ADM failed to increase cAMP levels. A plausible explanation for the observation that the inhibitory effect on NF-kB activation could only be observed when ADM was added to TECs 4 hours before LPS is that the LPS effect on transcription is faster on that of ADM due to the different localizations of their receptors. The LPS effects are consequent to its interactions with the membrane Toll-like receptor 4 (Zhang et al., 2000), whereas ADM regulates p65 and IkBα expression by interacting with a receptor localized to the nucleus. This is in agreement with studies showing the presence of 7-transmembrane G-protein coupled receptors in cell nucleus, where they are involved in the modulation of various physiological processes, such as DNA synthesis and transcription (reviewed in Boivin et al., 2008). The finding that ADM stimulates a receptor localized to the nucleus of TECs implies that ADM is somehow transported across their plasma membrane. Further experiments are in progress in our laboratory to analyze the precise molecular mechanism by which ADM passes TECs membrane and interacts with its intracellularly located receptor modulating gene transcription.

The observation that ADM is able to inhibit IL-6 production by TECs may have clinical implications and partly explains the protective effect exerted by this peptide in

autoimmune diseases such as multiple sclerosis (Pedreno et al., 2014) and rheumatoid arthritis (Gonzales-Ray et al., 2007). In this regard, growing evidence indicates that IL-6 can influence regulatory T cells (Tregs) development and differentiation by acting on forkhead box P3 (Foxp3). In particular, a recent study has suggested that IL-6 can destabilize Foxp3 expression and increase the vulnerability of Tregs to loss of their identity (Feng et al.,2014). Since IL-6 plays a crucial role in the differentiation of Th17 (Singh et al., 2013; Acosta-Rodriguez et al., 2007), a T cell subset which produces IL-17 and contributes to inflammation and autoimmunity, and it has been shown that the inhibition of Th17 differentiation leads to an increase in phenotypic and functionally stable Tregs (Song et al., 2014), our observation that ADM reduces IL-6 production by TECs is also compatible with a participation of the ADM system in the regulation of Th17/Treg ratio.

In conclusion, we demonstrated that in rat TECs ADM reduces IL-6 release by inhibiting the canonical NF-kB pathway. This is due to an effect on NF-kB genes transcription consequent to an interaction with an intracellularly located receptor. This finding opens new avenues for the therapeutic management of central immune regulation defects, by restoring the correct Th17/Tregs ratio through the modulation of IL-6 production.

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chapter 2

Adrenomedullin and Regulatory T cells: effect on the cellular trafficking to CNS in an experimental model of ADHD

ABSTRACT

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder (ND) that affects a large percentage of the worldwide population. Autoimmunity in the central nervous system (CNS) has been suggested to play a pivotal role in the pathogenesis of ADHD, but whether an immune system alteration may be a direct cause of this disease remains to be demonstrated. Several studies indicated that adrenomedullin (ADM) can function as a biomarker involved in different psychiatric disorders, such as Alzheimer, autism, bipolar disorder, schizophrenia and ADHD. Since it is known that ADM plays a role in immunological tolerance and T cells maturation, the first aim of this study was to investigate whether the heterozygous deletion of dopamine (DA) transporter (DAT) gene (DAT+/- mice), which causes an ADHD-like phenotype in mice, also leads to difects of the ADM system in thymus and immunological tolerance.

Indeed, the pivotal role of Tregs for regulating immune cells trafficking through the Blood-Cerebrospinal Fluid Barrier (B-CSF-B) and Choroid Plexus (CP) have recently been demonstrated, as well as the importance of the afflux into the brain of other immune cells (peripheral monocytes/macrophages) for preserving CNS homeostasis. It has also been demonstrated that all these processes are dysregulated in different mental disorders. In particular, in several neurogical diseases, alterations of the accumulation of T cells have been observed in CP, together with a dysregulation of the inflammatory phenotype of microglia, which acts as modulator of the recruitment of the peripheral monocytes/macrophages into CNS, through the regulation of CP permeability.. This mechanism, that involves both periphery, brain and CP, has been described by the "protective autoimmunity" theory, postulated by Prof M. Schwartz (Weizmann Institute of Science, Rehovot, Israel). Therefore, the second aim of this study was to ascertain whether DAT+/- mice are characterized by alterations of peripheral Treg levels, microglia activation and B-CSF-B functionality in regulation of monocytes/macrophages recruitment.

Our results showed that the partial deletion of DAT significantly increases Tregs in thymus and spleen (30% increase compared to wild type (WT) ($p<0,05$), accompanied by a correlated ($p<0,001$) boost of ADM thymic expression (7 fold increase; $p<0,05$), and significantly reduces the activation of microglia in CNS (30% decrease of tspo expression, $p<0,05$). Accordingly, in these mice, an inhibition of Nf-kB activation was demonstrated (80% decrease of p65 nuclear translocation, $p<0,01$) in CP epithelial cells together with a drop in the mRNA levels of *crp, ifnγ, icam1, vcam1, cxcl10* and *ccl2* (50% decrease; $p<0,01$), all consistent with a reduced cell trafficking into the brain, which was also confirmed by the significant reduction of monocytes/macrophages accumulation in CNS (30% decrease; $p<0,05$).

In conclusion, our results demonstrate that alterations of the immune system are strongly present in this mouse model of ADHD, and suggest that the "protective autoimmunity theory" postulated for Alzheimer disease can be extended to this disorder. Moreover, these findings suggest immunomodulation as a novel approach for the pharmacological treatment of ADHD, to absolve neuroprotective effect and support CNS homeostasis just by modulating peripheral immunity, in particular Treg levels.

INTRODUCTION

1. Attention deficit hyperactivity disorder.

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by hyperactivity, impulsivity and poor levels of attention. Usually, it is diagnosed during childhood (6-7 years old), and is estimated to affect 5-8% of the worldwide population. ADHD is a chronic disorder, which ameliorates during adolescence and adulthood only in 45-50% of cases. Patients with ADHD may show, starting from childhood, hyperactivity that persists during adulthood. Cognitive deficits, especially of inattentive type, are another core and enduring feature of ADHD, even if the disease persists into adulthood only in approximately half of patients.

ADHD is more common in males compared to females, with a ratio of 2:1 in children and 1.6:1 in adults. The causes of ADHD onset are not yet fully known, but both genetic and environmental elements were identified as risk factors: a gene-gene or gene-environment interaction is considered to be the most likely susceptibility factor for the genesis of this disease.

The investigation to understand which genes are the most involved in the development of ADHD is focused on those related to the dopaminergic system, especially the ones encoding for DAT, dopamine D4 and D5 receptors and the catechol O-methyltransferase (COMT) enzyme. Recently, behavioural studies demonstrated the strongest involvement of DAT in ADHD onset (Mereu et al., 2017). In accordance, the drugs which are most commonly used for the treatment of this disease are methylphenidate and D-amphetamine, which have a specific action towards DAT, DA receptors (DAR) and pre-synaptic vecsicles.

The environmental factors most involved in the development of this disease are related to pregnancy, i.e. the exposure to toxic substances and infections of the urinary tract of the mother; indeed, the relevance of maternal immune activation (MIA) is known in predisposition of psychiatric disorders such as ADHD. The first trimester of pregnancy is a crucial period for the development of brain structures, and the action of toxic or pathogenic substances can lead to deficits in neurodevelopment and thus cause psychiatric illnesses such as ADHD. Other environmental factors are crucial during the first few years after birth: it has been observed that exposure to toxic substances and infections or autoimmune diseases increases the ADHD onset (Zhou et al., 2017).

2. DAT genetic alterations in ADHD.

DAT is a dopaminergic transporter of 620 amino acids which belongs to the SLC (Solute Carrier) family, expressed in the pre-synaptic neuronal membrane *(fig. 1)*. DAT uses the Na^+/Cl^- gradient to move the extracellular DA into the neurons. Its relapse activity affects the modulation of dopaminergic stimulation in the brain areas where is expressed, which are the nucleus accumbens, the olfactory tubercle, the hypothalamus, the prefrontal cortex (PFC), and in particular the striatum.
Structurally, DAT presents 12 transmembrane domains of about 20 α-helix amino acids, a hydrophobic loop protruding towards the extracellular portion with 2-4 sites of glycosylation and long cytoplasmic N and C terminals with different phosphorylation and ubiquitination sites *(fig. 2)* useful in the down-regulation process mediated by kinases that stimulate endocytosis of DAT. The consequent reduction of transporter expression in the neuronal surface leads to an increase in dopaminergic stimulation in the affected area (Vaughan et al., 2013).

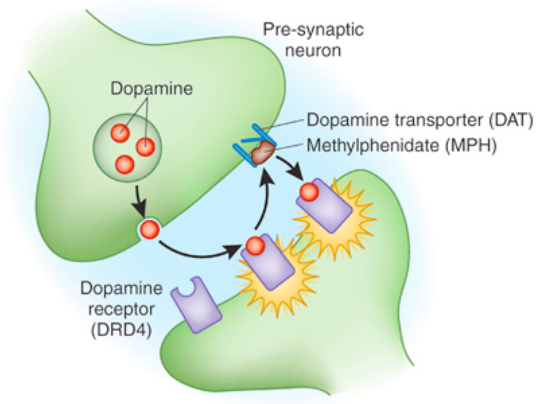

Figure 1 | DAT pre-synaptic activity.

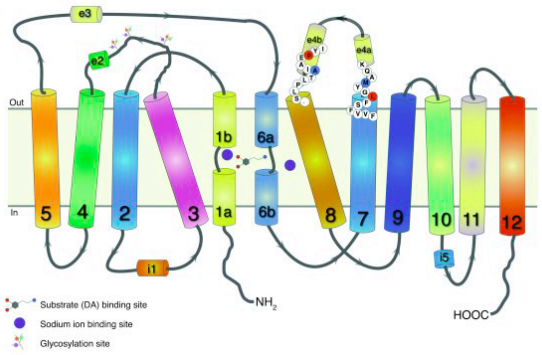

Figure 2 | DAT strutture in membrane.

3. Mouse model of ADHD, DAT+/- mice.

In this study, we used DAT hypo-functional mice (DAT+/-), which have been characterized by ADHD-relevant phenotypes including persistent hyperactivity, cognitive alterations in attentional- and impulsive-control with intact sensorimotor gating abilities, as well as behavioral amelioration after treatment with amphetamine (Mereu et al., 2017). We excluded DAT null mutant mice (DAT-/-) because these mice exhibit extreme phenotypes more similar to the dystonia-parkinsonism syndrome.
Furthermore, DAT-/- mice has to be obtained from DAT+/- or -/- mothers. DAT genetic modifications change maternal behavior (Soutschek et al., 2017), and in turn, this could influence the behavior of the offspring.

4. Immune system in mental disorders with deficits in dopaminergic transmission.

The role of the immune system in the pathogenesis of psychiatric disorders with alterations in dopaminergic transmission (such as schizophrenia or ADHD) is still unclear, but a correlation between psychiatric disorders and immune system disorders, particularly autoimmunity and mental diseases has been strongly suggested. Maternal infection during pregnancy is a risk factor for susceptibility to the development of autoimmune diseases and schizophrenia. Genome-wide association studies (GWAS) find a positive association between autoimmune diseases and mental disorders: in schizophrenic patients a 53% increase in incidence of autoimmune disease is estimated, and in patients with autoimmune disorders there is a greater risk of developing schizophrenia. Consequently, disorders of the dopaminergic transmission may be associated with a deficiency in the immune system involved in self-immunity. Schizophrenic patients also show a greater susceptibility to infections due to immune system changes (Benros et al., 2014).
The role of immune cells in psychiatric disorders and the consequent potential beneficial effect of immunomodulatory treatment was clearly demonstrated by studies conducted by the team of Professor Michal Schwartz. Their results revealed that immunodeficient mice show consistent impairment of cognitive abilities, reverted by immune restoration (by healthy lymph node lysates injection). It was also shown that vaccination of schizophrenic mice with immune cells reverts cognitive disabilities (Kipnis et al., 2004; Cardon et al., 2010).
Other evidence of the relationship between immune and psychiatric disorders has emerged

from studies finding genetic variations in major histocompatibility complex (MHC) in schizophrenia, thus correlating immune abnormalities to the development of mental illnesses (Elmer, McAllister 2012). Indeed, a recent publication unraveled the strong correlation between mental disorders and the allele C4A of complement component 4, present in MHC III (Sekar et al., 2016).
In addition, rats injected with LPS in the hippocampus showed behavioral alterations typical of schizophrenia with activation of the microglia and increase of pro-inflammatory factors, assuming a relationship between psychiatric infections and disorders (Zhu et al., 2014).
T cells, monocytes, dendritic cells, neutrophils and eosinophils express DAR on their cell surface, and the presence of dopaminergic innervations of the sympathetic nervous system in thymus, spleen and lymph nodes has been detected. This implies that DA may play a physiological role in regulating immune responses and a disorder in DA production may be involved in the pathogenesis of autoimmune diseases and cancer. Moreover, dendritic cells and T helper lymphocytes are able to produce, store and secrete DA (Pancheco et al., 2014).

5. Tregs role in immune tolerance process and thymic T cells maturation.

Potentially autoreactive T cells can develop because of the stochastic generation of diversity in the T cell repertoire. However, there are several central and peripheral mechanisms that maintain self-tolerance. These mechanisms include thymic clonal deletion, which is a major mechanism of tolerance induction that results in thymocytes expressing high-affinity self-reactive TCRs being physical eliminated. Tregs are specialized T cells which are able to suppress immune responses, and they acquire in thymus their phenotype and suppressive function (Cabarrocas et al., 2006).
Tregs represent between 2% and 10% of the peripheral CD4 T cells in mice and humans and express the interleukin 2 receptor's alpha subunit (CD25), the transcriptional factor forkhead box P3 (Foxp3) and the signal transducer and activator of transcription 5 (STAT5). These cells regulate the inflammatory response and play a key role in maintaining tolerance and immune homeostasis, by regulating the activation and expansion of T CD4+ and CD8+ cells, dendritic cells, granulocytes and macrophages, preventing autoimmunity (Rudensky et al., 2011).
Recent data suggest that Treg differentiation is promoted by interactions with thymic antigen-presenting cells (APCs) presenting self-peptide–MHC complexes. cTECs are involved in Tregs selection, and Tregs differentiation requires MHC class II expression. The

array of self-antigens (Ags) expressed by thymic APCs and exposed to Tregs, most notably by TECs, has been shown to include a variety of tissue-restricted proteins. This promiscuous expression of tissue-restricted self-Ags by thymic APCs is, at least partly, controlled by transcriptional regulators such as AIRE, and plays a role in clonal deletion (Cabarrocas et al., 2006).

As already specified, the main function of Treg in the thymus is to prevent the maturation of autoreactive T cells. It is clear that the correct Treg level is essential for health; whereas excessive Treg activity can lead to immunodeficiency, chronic infection and cancer, and too little Treg activity results in autoimmunity and immunopathology, and impairs the quality of pathogen-specific responses. These findings are highly relevant for developing effective strategies to manipulate Treg cell activity with the aim of promoting allograft tolerance and treat autoimmunity, chronic infection and cancer (Smigiel et al., 2014; Yuan et al., 2010).

6. Neuro-immunity in brain homeostasis: the "protective autoimmunity" theory.

The CNS is protected by means of two main barriers, i.e. the blood-brain barrier (BBB) and the B-CSF-B. The CNS has long been considered an immune privileged organ, from which circulating leukocytes were excluded, until new evidences have changed the current knowledge of the relationships between brain and immune system, with clinical implications to neurodevelopmental diseases such as ADHD. Prof. Schwartz and collaborators demonstrated that two types of immune cells, i.e. circulating blood macrophages and T-cells recognizing brain components, are needed for healing the damaged CNS; these findings were conceptualized as the "Protective autoimmunity" theory.

In almost all the diseases affecting CNS, the attempts to suppress systemic immunity as a way of mitigating brain inflammation were ineffective at best, or even harmful. It was showed that the immune system is pivotal for the life-long maintenance of the brain, enabling its proper function, as manifested by formation of new neurons or normal cognitive performance. Immune cells that recognize self-components of the CNS are pivotal for patrolling the healthy brain to identify any distressed situation and to resolve it before it develops into any disease, and to facilitate repair if pathology emerges.

However, although the immune system is not part of the of the brain, these studies proposed that it helps the optimal maintenance of the brain environment, as required for its complex and delicate activity. Immune cells are pivotal for CNS neuroprotection and

repair, but their spontaneous recruitment to the CNS is insufficient (Rapalino et al., 1998, Moalem et al., 1999); both circulating monocyte-derived macrophages, and helper T cells recognizing brain antigens were identified to have crucial and beneficial roles. The protective T cells were found to have specificity for self-brain antigens, leading Professor Schwartz's group to formulate the model of "Protective autoimmunity" (Schwartz, 2001). Over the years, this group found that this response involves a network of T cells including effector and memory T cells, and Tregs, whose role changes over time, and is temporally and spatially regulated (Raposo et al., 2014). They also showed that this T cell network, in the context of acute injuries, is involved in facilitating the recruitment of the anti-inflammatory macrophages to the CNS, which are able to support tissue repair (Schechter et al., 2009).

In an attempt to understand how T cells can support the brain even though they are excluded from direct interaction with the brain parenchyma, it was found that effector and regulatory CD4 T cells with specificity to CNS-antigens reside in the stroma of the brain's choroid plexus, and suggested the possibility that immune cells affect brain function from afar, via interactions with this compartment.

7. Communication between the brain and the circulation: choroid plexus as neuro-immunological interface.

The CNS barrier system includes the BBB, which is formed by tightly connected endothelium that surrounds parenchymal microvessels, and the B-CSF-B, the barrier properties of which are established by the CP, a highly vascularized epithelial monolayer that surrounds an inner stroma. While classically recognized for its role in CSF production, the CP is strategically positioned at the ventricles of the brain, to be exposed to brain-derived signals from CSF, and to peripheral signals from the circulation CP undergoes immunological changes which affect its epithelial tissue during aging or in pathological conditions (Baruch et al., 2013). It has been demonstrated that the blocking of IFN-I signaling within the aged brain can partially re-establish CP activity and also ameliorate cognitive deficits and induce hippocampal neurogenesis in aged mice. These findings suggested that CP immune-modulation can be evaluated as a novel approach for attenuating age-associated cognitive decline or other pathological conditions associated with IFN-I, often linked to cognitive dysfunction.

CP can function as a selective gateway for recruitment of monocyte-derived macrophages and T cells to the injured CNS parenchyma (Kunis et al., 2013; Shechter et al., 2013). Specifically, it was found that CP activity in supporting immune cell trafficking, depends on a local synergistic effect in the CP compartment between CNS-derived inflammatory signals and the immune cell-derived cytokine interferon-γ (Kunis et al., 2013), resulting in NFκB/p65-dependant CP gateway activity (Baruch et al., 2015).

Figure 3 illustrates the intracellular pathways physiologically mediated by TNFα. In the steady state, CP senses CSF-pro-inflammatory signals, derived from the brain parenchyma. Pro-inflammatory signals can be released by activated microglia, the CNS resident myeloid cells, which patrol the CNS tissue. TNFα, is sensed by the CP via the TNFα receptor (TNF-R). Upon TNFα stimulation, TNF-R signaling cascade is funneled through the NFκB pathway, into translocation of the p65 subunit to the nucleus, and this initiates a cellular response which leads to upregulation of IFNγ-R on CP epithelium, expression of the determinants of leukocyte trafficking and disruption of the epithelial tight junctions. IFNγ, secreted by CP stromal Th1 cells, acts in synergy with the NFκB signaling pathway, by inducing the expression of specific molecules such as ICAM-1, CCL2 and CXCL10 which activate leukocyte trafficking. Together, these events support CP-mediated leukocyte entry into the CNS (Baruch et al., 2015).

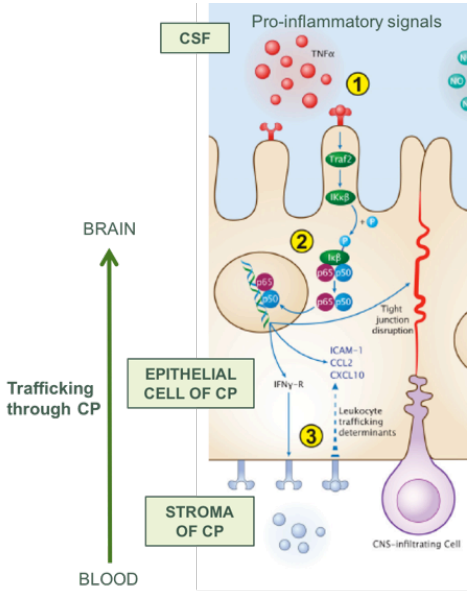

Figure 3 | Proposed model illustrating CP gateway for leukocyte trafficking.

The progression of AD or other neurodegenerative disease is associated with local suppression of CP activity in supporting immune cell trafficking to the CNS. Under these conditions, it was showed that breaking systemic immune tolerance, either by active vaccination approaches (Kunis et al., 2015) or by targeting regulatory T cells (Tregs) (Baruch et al., 2015), can augment CP-gateway activity for leukocyte trafficking. The activation of this immune-brain axis is followed by accumulation of immune-regulatory cells at cerebral sites of pathology, and in the case of AD transgenic mice, was associated with amyloid-β plaque clearance and mitigation of cognitive decline. Conversely, augmenting systemic immune suppression in AD mice was associated with pathology worsening of the disease symptoms. Collectively, these findings suggested that in chronic neurodegenerative diseases, systemic immune suppression interferes with the activity of the CP in orchestrating recruitment of leukocytes to the CNS, needed for coping with disease-escalating factors within the brain and with tissue remodeling.

The research field of neurodegenerative diseases in general, and Alzheimer's disease in particular, was dominated for the last two decades by clinical attempts to arrest the neuro-inflammatory response by administration of immune-suppressive and anti-inflammatory drugs; these attempts have generally failed. These findings suggest that in order to fight neuro-inflammation under chronic neurodegenerative conditions, systemic immunity should be boosted, rather than suppressed. Additionally, these findings introduce the idea that targeting the immune system rather than directly target specific disease escalating factors within the brain may be considered a brand new approach to fight AD. Such an approach, since recruited immune cells may display multiple functions, provides a comprehensive therapy, and is likely to be applicable to the diverse forms of AD and maybe other CNS diseases.

AIMS

ADM is a regulatory peptide involved in thymic immune tolerance mechanisms (Rulle et al., 2012; Castellani et al., 2016), which are suggested to be altered in different psychiatric diseases, such as Alzheimer (Fernandez et al., 2016), autism (Zoroglu et al., 2003), depression and bipolar disorder, (Akpinar et al., 2013) and schizophrenia (Huang et al., 2004).
The involvement of ADM in the ADHD aethiopathogenesis has been described (Fernandez et al., 2008), and a role for ADM in inhibiting the IL-6 release from microglia, the CNS resident myeloid cells responsible for monocytes/macrophages recruitment to the brain after immunological activation has also been outlined (Consonni et al., 2011). On the basis of these considerations, this study has the following aims.

1. Study of the ADM system involvement in thymic immune tolerance mechanisms in DAT+/- mice.

The first aim of this study was to identify the possible alterations in ADM system and Treg thymic levels in DAT+/- adult male mice, showing ADHD-relevant phenotype. Furthermore, we evaluated whether the ADM expression in thymus was correlated with foxp3 mRNA levels. We analysed also the Treg levels in spleen as index of peripheral immunity.

2. Neuro-immunological characterization of DAT+/- mice.

The second aim of this study was to investigate whether DAT+/- mice are characterized by alterations of activation of microglia by testing mRNA levels of inflammatory markers, such as *tspo, irf7, bdnf, cox2* and *tnfα*. Because of the effect of inflammatory activation of microglia in promotion of monocytes/macrophages recruitment in CNS, and its known beneficial role in psychiatric diseases as AD, we evaluated the monocytes/macrophages accumulation in brain by flow cytometry (FC) analysis. Moreover, in order to detect the involvement of B-CSF-B anomalies in ADHD mice models, we analysed the CP functionality studying different permeability markers in epithelial cells (*crp, icam1, vcam1, ikbα* and *p65*) and cytokines such as *cxcl10, ccl2* and *ifnγ,* known to be enhancers of CP-permeability.

METHODS

1. Animals.

Male wild type and DAT+/- C57/BL6 mice were breed and maintained in the Animal facility of the Department of Pharmaceutical and Pharmacological Sciences, University of Padua. All procedures were approved by the Italian Ministry of Health and Animal Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. Mice were identified by PCR analysis of tail DNA. Mice were group housed (two to four per cage) in a climate-controlled animal facility (22±2 °C) and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Thymi, spleens, brains and CPs were obtained from mice at PND>90 (adulthood).

2. Flow cytometry sample preparation and analysis.

Mice were sacrificed and tissues were extracted after PBS transcardial perfusion. Brains were removed and tissues were dissociated using the GentleMACS dissociator (Miltenyi Biotec) and myelin was eliminated using Percoll 40% solution. Thymi and spleens were mashed with the plunger of a syringe and spleens were then treated with ACK (ammonium chloride potassium)-lysing buffer to remove erythrocytes. All samples were filtered through a 70 mm nylon mesh, and blocked with anti-Fc CD16/32 (BD Biosciences). For Treg staining, an eBioscience FoxP3-staining buffer set (00-5523-00) was used. The following fluorochrome-labelled monoclonal antibodies were purchased from BD Pharmingen, BioLegend or eBiosciences, and used according to the manufacturer's protocols: v450-conjugated anti-CD4; PE-conjugated anti-CD25; FITC-conjugated anti-CD45; PE-Cy5-conjugated anti-TCRβ; APC-conjugated anti-FoxP3; PE–conjugated anti-CD11b; APC-conjugated anti-Ly6c. Cells were analyzed on a FACSAriaIII cytometer (BD Biosciences)

using FACS software. In each experiment, relevant negative control groups and single-stained samples for each tissue were used to identify the populations of interest. In sorting experiments, microglia were collected from whole brains into 350 µl of RNA lysis buffer. RNA was extracted from sorted cells, and mRNA of specific targets was measured as described below.

3. RNA purification, cDNA synthesis and quantitative real-time PCR analysis.

Total RNA of the sorted microglia was extracted using RNeasy Kit (QIAGEN) while total RNA of the thymus and CP (from all ventricles) was extracted using TRI Reagent (Euroclone). RNA integrity and quantity were determined by Nanodrop (Genova). The mRNA of thymic markers was measured using One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Shiga, Japan); while mRNA from microglia and CPs was converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression of specific mRNAs was determined by real-time PCR (EcoTM Illumina, Real-Time PCR system, San Diego, CA, USA) using Fast-SYBR PCR Master Mix (Applied Biosystems) and the genes expression levels were calculated by the ΔΔCt method using the EcoTM Software v4.0.7.0. Quantification reactions were performed in triplicate for each sample and the reaction thermic profile was: 95°C for 2m for DNA polymerase activation, 95°C for 5s, 60°C for 20s and 72°C for 15s (40 cycles) for PCR reaction, 95°C for 15s, 55°C for 15s, 95°C for 15s for dissociation. β-actin, peptidylprolyl isomerase A (*ppia*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was chosen as references (housekeeping) gene respectively for thymus, CP and sorted microglia. At the end of the assay, a melting curve was constructed to verify the specificity of the reaction.

The following primers were used:

Adm forward 5′-GAGGCAGAGGAACCCAAGAC-3′ and reverse 5′-ACCAGTTTATGAGCGGGCAA-3′ ;
Ramp2 forward 5′-CTCCCCTCCCTACCCACTTA-3′ and reverse 5′-CATTGTGTCGTGAGTCCCCT-3′ ;
Foxp3 forward 5′-ACTCGCATGTTCTGCCTACTT-3′ and reverse 5′-TCCACACTGCCTCCCTTCTC-3′ ;
Icam1 forward 5′-AGATCACATTCACGGTGCTGGCTA-3′ and reverse 5′-AGCTTTGGGATGGTAGCTGGAAGA-3′;
Vcam1 forward 5′-TGTGAAGGGATTAACGAGGCTGGA-3′ and reverse 5′-CCATGTTTCGGGCACATTTCCACA -3′;
Cxcl10 forward 5′-AACTGCATCCATATCGATGAC-3′ and reverse 5′-GTGGCAATGATCTCAACAC-3′;
Ccl2 forward 5′-CATCCACGTGTTGGCTCA-3′ and reverse 5′-GATCATCTTGCTGGTGAATGAGT-3′;
Crp forward 5′-CAGGAGCAGGACTCGTATG-3′ and reverse 5′-GCCCGCCAGTTCAAAACATT-3′;
Ifnγ forward 5′- TCAAGTGGCATAGATGTGGAAGAA -3′ and reverse 5′- TGGCTCTGCAGGATTTTCATG-3′ ;
Tspo forward 5′-TACTTTGTACGTGGCGAGGG-3′ and reverse 5′-ACCTCCCAGCTCTTTCCAGA-3′;
Irf7 forward 5′-CACCCCCATCTTCGACTTCA-3′ and reverse 5′-CCAAAACCCAGGTAGATGGTGTA-3′;
Bdnf forward 5′-GATGCTCAGCAGTCAAGTGCCTTT-3′ and reverse 5′-GACATGTTTGCGGCATCCAGGTAA-3′;
Cox2 forward 5′-GCTAGAAGTTGATAACCGAGTCG-3′ and reverse 5′-CTAGGGAGGGGACTGCTCAT-3′;
Tnfα forward 5′-GCCTCTTCTCATTCCTGCTT-3′ and reverse 5′-CTCCTCCACTTGGTGGTTTG-3′;
β-actin forward 5′-ATGTGGATCAGCAAGCAGGA-3′ and reverse 5′-AAGGGTGTAAAACGCAGCTCA-3′ ;
Ppia forward 5′-AGCATACAGGTCCTGGCATCTTGT-3′ and reverse 5′-CAAAGACCACATGCTTGCCATCCA-3′ ;
Gapdh forward 5′-TGGCAAAGTGGAGATTGTTGCC-3′ and reverse 5′-AAGATGGTGATGGGCTTCCCG-3′.

4. Immunohistochemistry, confocal microscopy and images analyses.

For tissue staining, mice were transcardially perfused with PBS prior to tissue excision and fixation. CP tissues were isolated under a dissecting microscope (Stemi DV4; Zeiss) from the lateral, third and fourth ventricles of the brain. For whole mount CP staining, tissues were fixed with 2.5% paraformaldehyde for 1 h at 4 °C, and subsequently transferred to PBS containing 0.05% sodium azide. Prior to staining, the dissected tissues were washed with PBS and blocked (20% horse serum, 0.3% Triton X-100 and PBS) for 1 h at room temperature. Whole mount staining with primary antibodies (in PBS containing 2% horse serum and 0.3% Triton X-100), or secondary antibodies, was performed for 1 h at room temperature. Each step was followed by three washes in PBS. The tissues were applied to slides, mounted with Mowiol (Sigma Aldrich) and sealed with cover-slips. The following primary antibodies were used: rabbit anti-p65 (1:200,Santa Cruz); and rabbit anti-Ikbα (1:100,Santa Cruz). The secondary antibody used was Alexa Fluor-conjugated donkey anti-rabbit (1:200; Jackson Immunoresearch). The slides were exposed to DAPI for nuclear staining (1:4000; Sigma Aldrich) during Mowiol fixation. Two negative controls were routinely used in immunostaining procedures, stained with isotype control antibody

followed by secondary antibody, or with the secondary antibody alone. Samples were imaged by confocal laser microscopy using a LSM 800 microscope (Carl Zeiss, Jena, Germany) equipped with a 63x, n.a. 1.4, oil immersion objective and ZEN 2.1 - blue edition- software (Carl Zeiss, Jena, Germany). To avoid emission crosstalk, each emission fluorescence was independently recorded with specific detector and optical cut-off filter, over the entire emission spectrum of related chromophores. The p65 nuclear translocation (colocalization with DAPI signal) and the fluorescent signal intensity of ikbα was quantified by means of Fiji software.

5. Statistical analysis.

The specific tests used to analyze each set of experiments are indicated in the figure legends. Data were analyzed using Student's t-test to compare between two groups, one-way ANOVA was used to compare several groups, followed by the Tukey's post hoc procedure for pairwise comparison of groups after the null hypothesis was rejected ($P<0.05$). In the graphs, y-axis error bars represent s.e.m. Statistical calculations were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

1. mRNA expression of adm system components and foxp3 in thymus; a correlation analysis in PND>90 male mice.

Thymus from adult mice were collected and RNA was extracted and prepared for RT-qPCR analysis. mRNA expression of *adm* and its receptor protein *ramp2* is significantly higher in the thymus of DAT+/- mice with respect to controls *(fig 4).* As stated in *chapter 1*, we demonstrated, by means of studies performed in rat TECs (Castellani et al., 2016), that the adm system may be involved in immunological tolerance by reducing mTEC IL-6 release. Indeed, the enhanced expression of the adm system found in DAT+/- mice thymus suggested a possible pivotal role for central immune tolerance in this pathological condition.

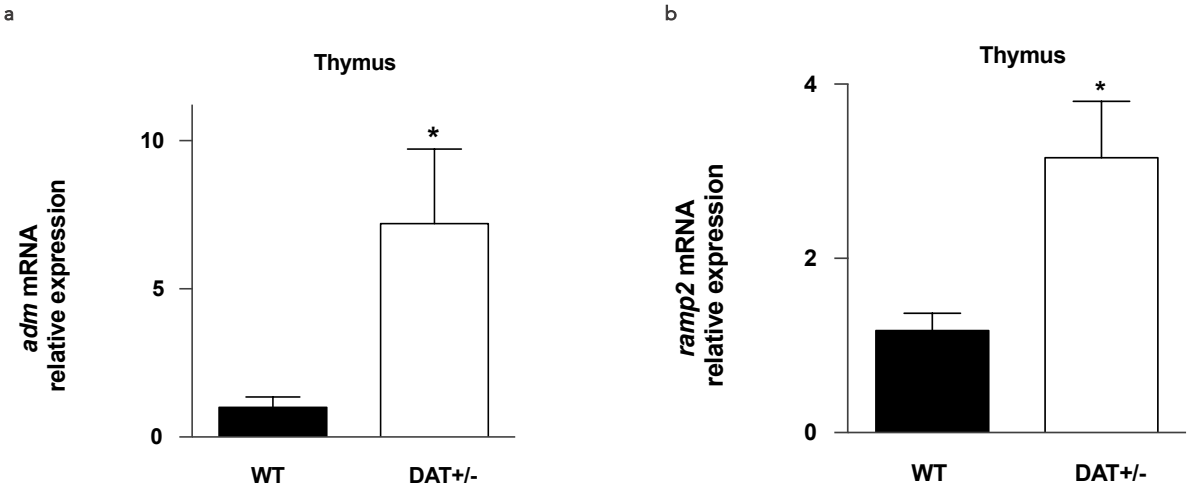

Figure 4 | Increase of ADM system expression in the thymus of male adult DAT+/- mice. mRNA expression of **(a)** *adm* and **(b)** its receptor *ramp2* was measured by RT-qPCR, in thymus isolated from adult (older then 90 days) mice. Data are shown as fold-change compared with WT mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

Moreover, *foxp3,* a nuclear receptor specific for Treg cells, shows a significant higher mRNA expression in the DAT+/- mice thymus *(fig 5).* This result seems to confirm the hypothesis postulated in our lab, that adm system is strongly associated with Treg maturation in thymus.

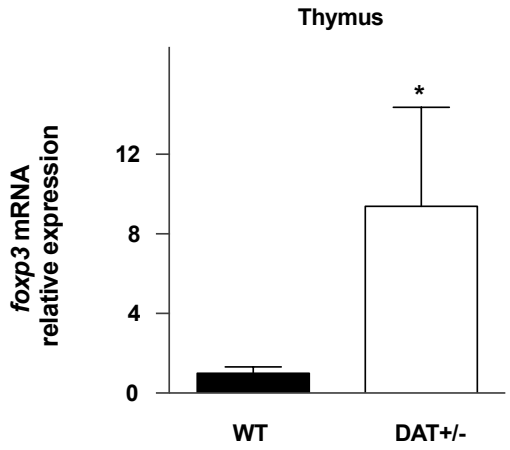

Figure 5 | Increase of foxp3 expression in the thymus of male adult DAT+/- mice. mRNA expression of *foxp3*, a nuclear receptor characteristic of Treg cells, was measured by RT-qPCR, in thymus isolated from adult (older then 90 days) mice. Data are shown as fold-change compared with WT mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

To investigate whether a correlation between *adm* and *foxp3* mRNA expression exists, the ΔCt between *adm/foxp3* and housekeeping β-actin Ct value were analyzed for both WT and DAT+/- mice. A significant positive correlation is evident between the mRNA thymic levels of these two markers (P<0.001) *(fig 6)*.

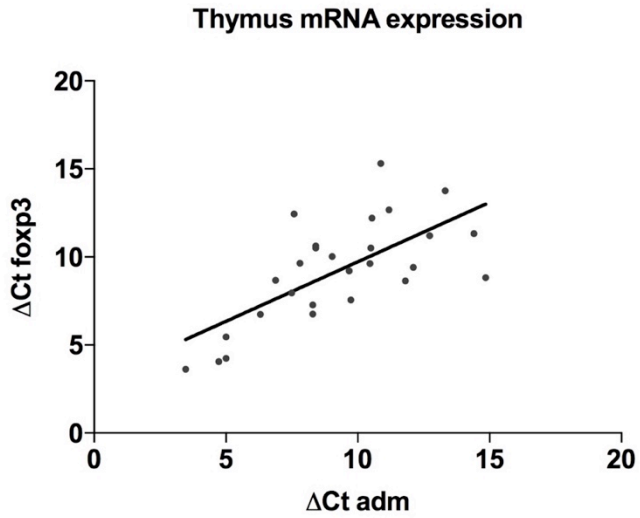

Figure 6 | The thymic expression of adm and foxp3 are significantly correlated. mRNA levels of adm and foxp3 in all samples analysed, presented in graph as ΔCq, are directly correlated: ***P<0.001; R pearson=0,686, R squared= 0,47, N of XY pairs=27.

2. Flow cytometry analysis of Treg in thymus and spleen.

Treg cells in thymus of adult mice were individuated as CD45+ CD4+ CD25+ Foxp3+ cells *(fig 7)*. TCRβ was not selected as a marker of thymic cells because of the complex expression of this protein during T cell maturation. Indeed, the TCR rearrangement takes place in thymus and causes the presence of not pure TCR positive or negative populations of cells. The FACS data confirm the increase of Tregs in thymus of DAT+/- observed by the real time PCR analysis of *foxp3*.

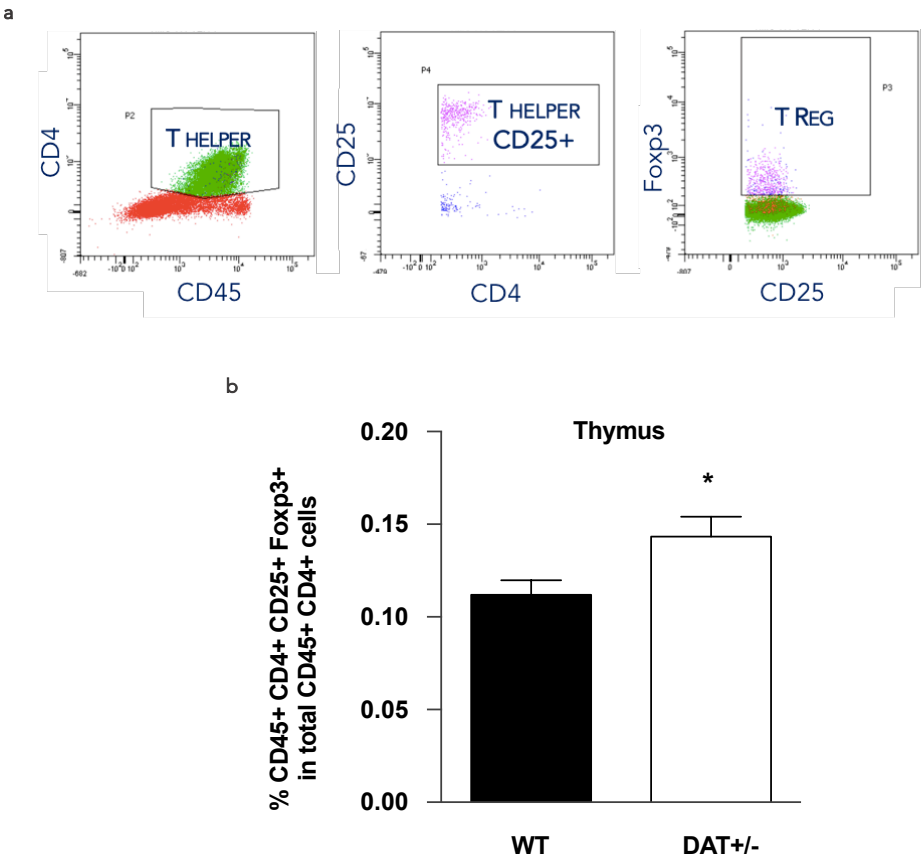

Figure 7 | Treg increase in thymus of adult DAT+/- mice. (a) Gating strategy and **(b)** flow cytometry analysis of T regulatory cells (intracellularly stained for foxp3) in thymus of adult DAT+/- mice and age-matched WT as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

Tregs in spleen were individuated as CD45+ TCRβ+ CD4+ CD25+ Foxp3+ cells *(fig 8)*. The results indicate an increase of Tregs in spleens of DAT+/- mice according with what was observed in thymus.

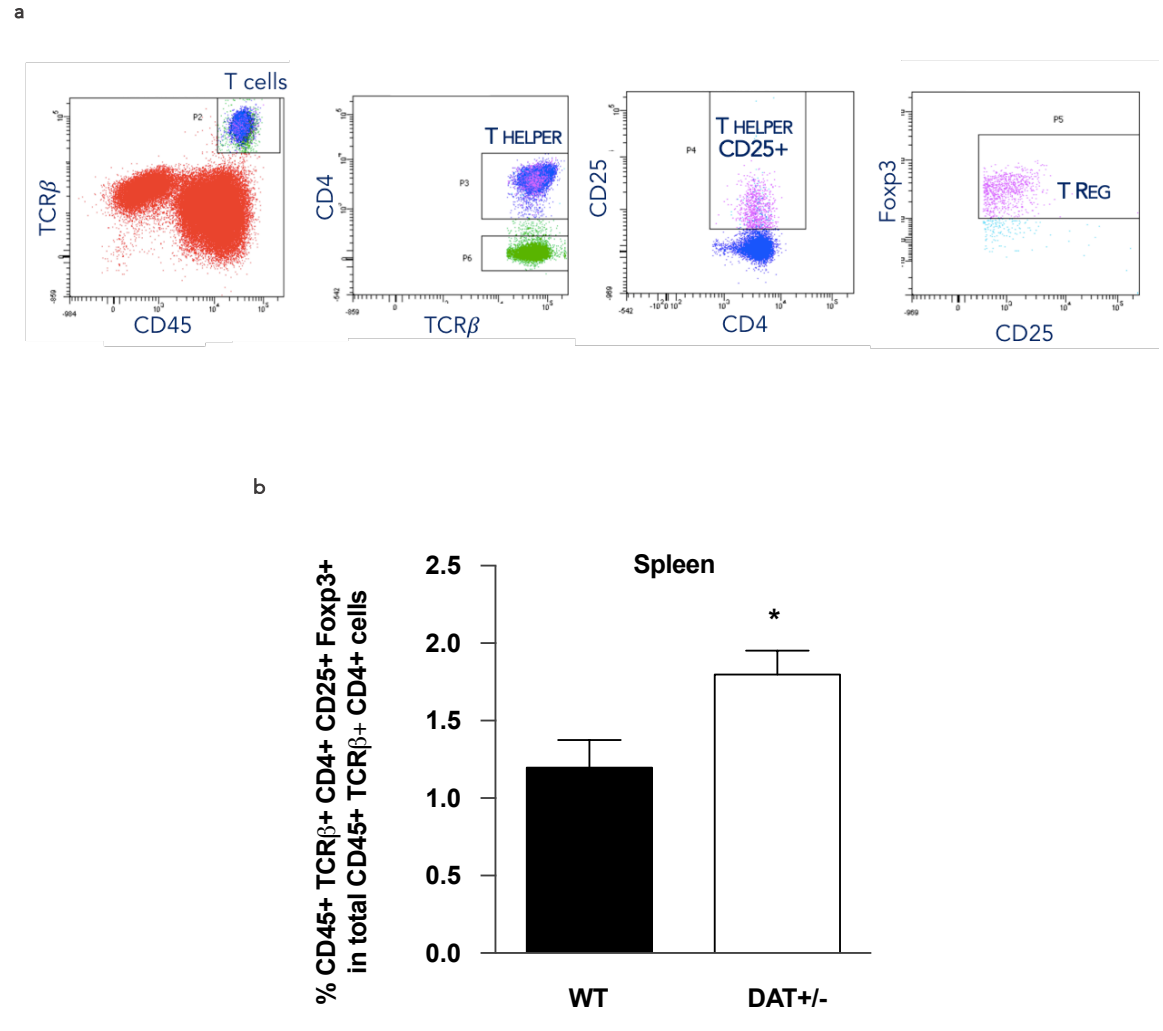

Figure 8 | Tregs increase in spleen of adult DAT+/- mice. (a) Gating strategy and **(b)** flow cytometry analysis of T regulatory cells (intracellularly stained for foxp3) in spleens of DAT+/- mice and age-matched WT as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

3. Flow cytometry analysis of microglia in brain of PND>90 male mice.

The count of microglia in brain was performed using the gating strategy shown below *(fig 9)*; microglia was individuated as CD11b+ CD45^{low} Ly6c- cells. The analysis was conducted in whole brain in order to individuate general abnormalities in microglia number in adult mice. The microglia levels in whole brain of WT and DAT+/- mice do not show evident changes.

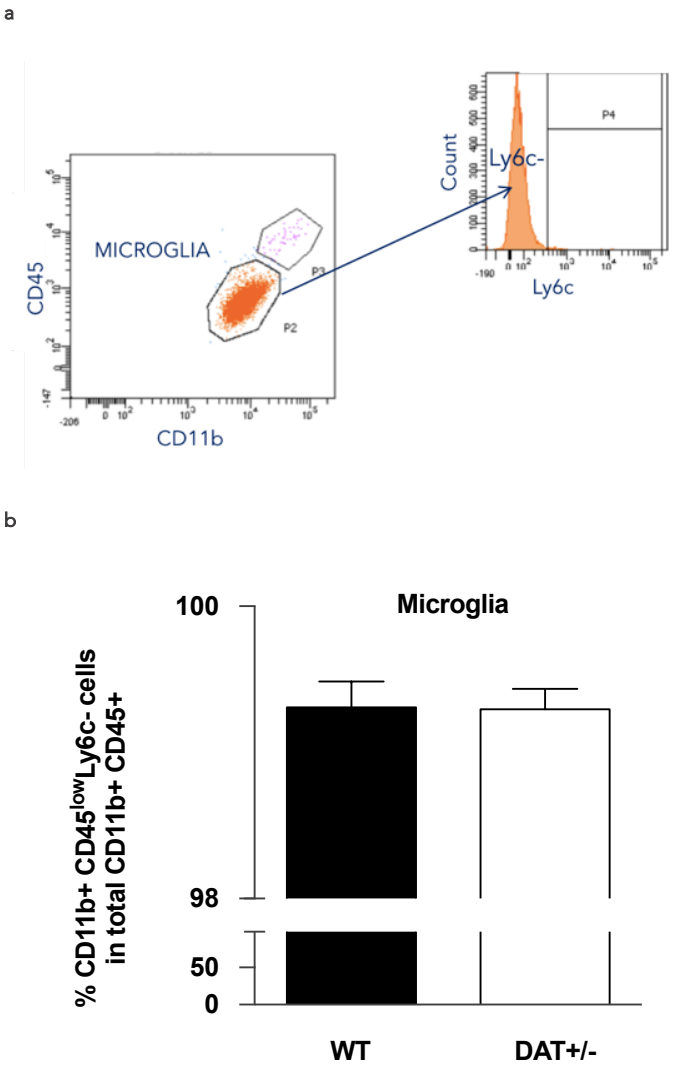

Figure 9 | No evident alterations in microglia levels in adult DAT+/- mice brain. (a) Gating strategy and **(b)** flow cytometry analysis of microglia in whole brain of DAT+/- PND>90 mice and age-matched WT as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.).

4. mRNA quantification of microglia inflammatory markers as activation index.

The sorted microglia from whole brain were collected in RLT buffer and prepared for RT-qPCR analysis. mRNA expression of *tspo*, activation marker, indicates that partial deletion of DAT gene causes a significant reduction of microglia activation in adult brain with respect to WT *(fig 10)*.

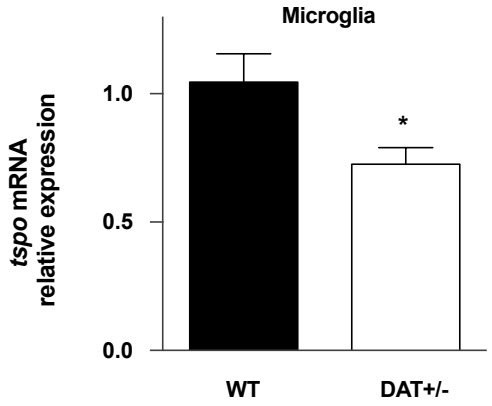

Figure 10 | Lower microglia activation in adult brain of DAT+/- mice. mRNA expression of *tspo*, a microglia activation marker, was measured by RT-qPCR, in microglia sorted from whole brain of adult mice. Data are shown as fold-change compared to WT mice as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

To characterize the inflammatory state of microglia in DAT+/- brains, we evaluated the mRNA expression of both M1 (pro-inflammatory) and M2 (anti-inflammatory) markers. As M1 markers *cox2* and *tnfα* were selected. F*igure 11* shows a significant decrease of mRNA expression of pro-inflammatory markers in in DAT+/- mice.

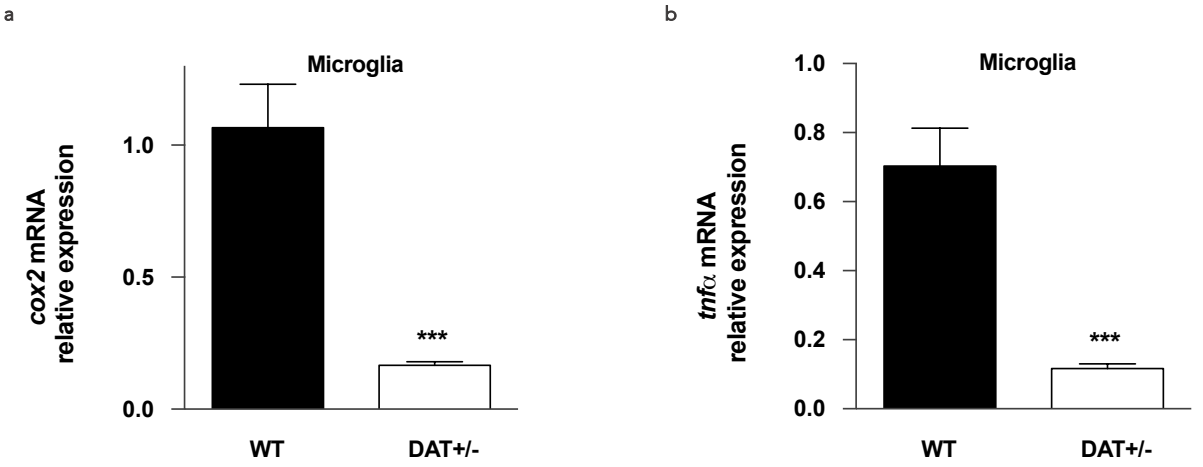

Figure 11 | mRNA expression of *cox2* and *tnfα*, M1 polarization markers, is significantly reduced in microglia of DAT+/- mice. mRNA expression of **(a)** *cox2* and **(b)** *tnfα*, was measured by RT-qPCR, in microglia sorted from whole brain of adult mice. Data are shown as fold-change compared to WT mice as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; ***P<0.001).

Irf7 and *bdnf* were chosen as M2 polarization markers; the mRNA quantification analysis revealed a decreased level of both anti-inflammatory markers in microglia of DAT+/- mice *(fig 12)*. These results suggest the correlation between the partial deletion of DAT gene and a lower activation of microglia, consistent in reduced M1 and M2 markers expression.

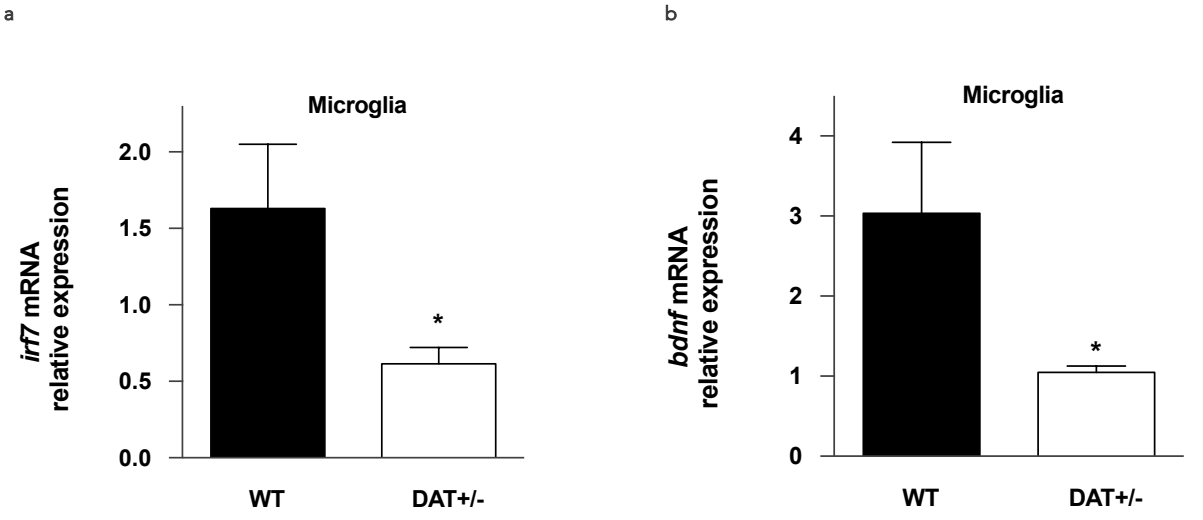

Figure 12 | DAT+/- mice microglia shows a lower expression of *irf7* and *bdnf*, M2 polarization markers. mRNA expression of **(a)** *irf7* and **(b)** *bdnf*, was measured by RT-qPCR, in microglia sorted from whole brain of adult mice. Data are shown as fold-change compared to WT mice as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

5. **B-CSF-B permeability evaluation by means of whole mount immunohistochemistry of CP from the fourth ventricle.**

To study B-CSF-B permeability, we analysed the activation of NF-kB pathway in CP epithelial cells; we evaluated the expression of the inhibitor IKBα and the translocation of the p65 subunit into the nucleus. DAPI was used as nuclear marker. *Figure 13* shows higher fluorescent intensity signal of IKBα in cytoplasm of DAT +/- mice CP epithelial cells compared to WT mice; this result indicates an inhibition of the Nf-kB pathway, due to the reduction of IKBα degradation.

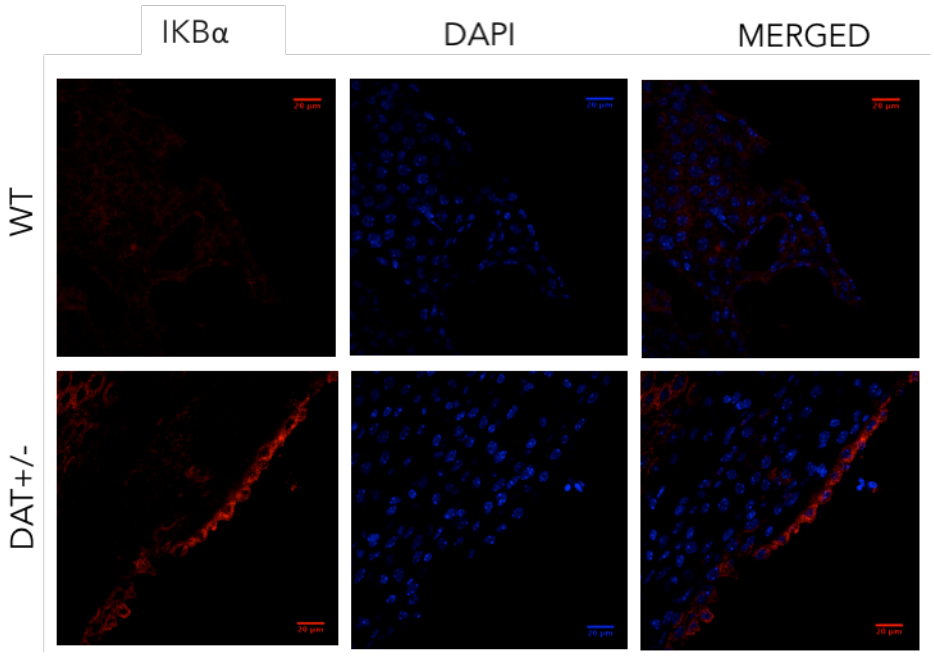

Figure 13 | IKBα is more expressed in CP epithelial cells of DAT+/- mice, suggesting a reduction of B-CSF-B permeability. Representative microscopic images of CPs from fourth ventricle of PND>90 DAT+/- mice and WT controls, immunostained for DAPI nuclear staining (blue) and the Nf-kB inhibitor, IKBα (red).

p65 staining is shown in *figure 14*; in DAT+/- mice, CP epithelial cells revealed a privileged cytoplasmic localization compared to WT, in which p65 was observed both in cytoplasm and nucleus. This distribution, together with IKBα higher expression in the cytoplasm, suggest an inhibition of Nf-kB pathway in CPs of DAT+/- animals, confirming a reduction of B-CSF-B permeability. To visualize the nuclear/cytoplasmic localization of p65 we graphed and compared the p65 and DAPI fluorescence profile on CP's epithelial cells diameter, showing lower concordance between the two signals in DAT+/- sample, which shows peaks of red (p65) in absence of the blue one (nucleus) *(fig 15)*. The colocalization between the p65 and DAPI signals is represented by the dot plots in *figure 16*, and summarized by the histogram below, which compares the Pearson's coefficients. The result shows negative Pearson's values for DAT+/- group, suggesting a low colocalization between these two signals, indicating a reduced Nf-kB activity, consistent in a lower B-CSF-B permeability.

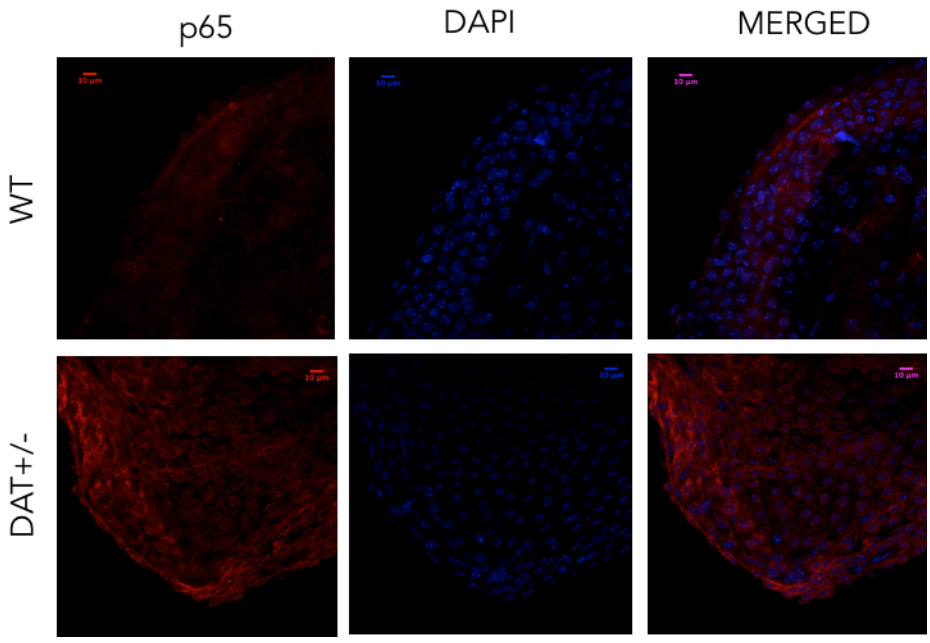

Figure 14 | p65 shows stronger cytoplasmatic localization in CP epithelial cells of DAT+/- mice compared to WT, suggesting a reduction of B-CSF-B permeability in deleted mice. Representative microscopic images of CPs from fourth ventricle of PND>90 DAT+/- mice and WT controls, immunostained for DAPI nuclear staining (blue) and the Nf-kB subunit, p65 (red).

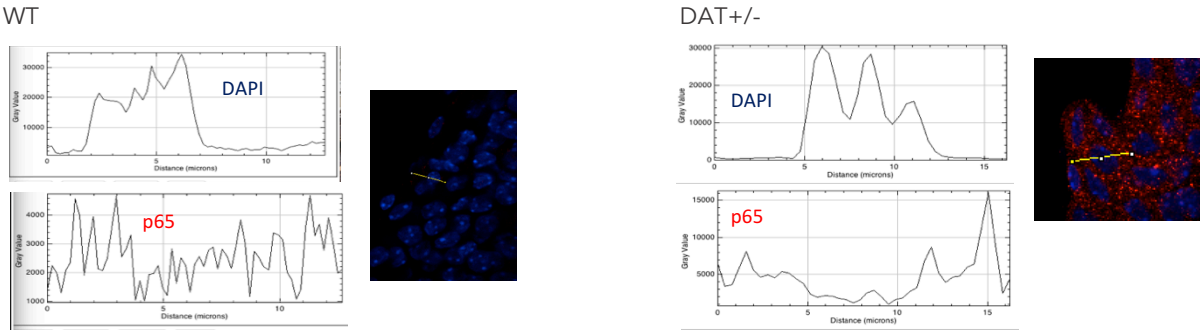

Figure 15 | p65 and DAPI plot profile analysis shows stronger cytoplasmatic localization of p65 signal in CP epithelial cells of DAT+/- mice compared to WT. Plot profile analysis and representative microscopic images of CPs from fourth ventricle of PND>90 DAT+/- mice and WT controls with the indication of cellular diameter selected for analysis.

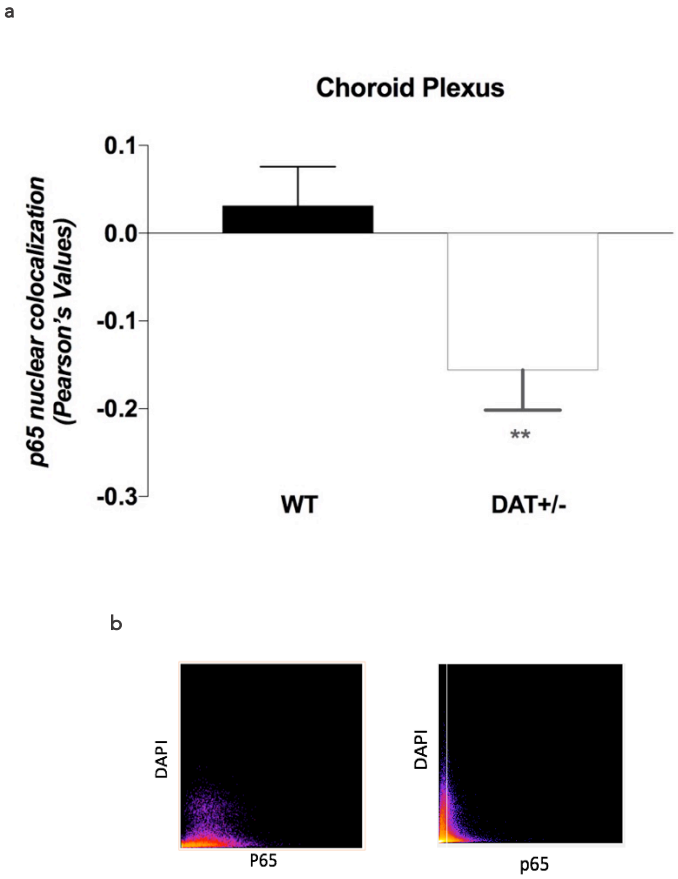

Figure 16 | Reduction of p65 and DAPI fluorescence colocalization in CP epithelial cells of DAT+/- mice compared to WT. Dot plots and summarizing histogram which compares Pearson's coefficients as colocalization index between p65 and DAPI. Data are shown as Pearson's values (0-1) compared to WT mice as controls (n=3 per group; Student's t-test. Error bars represent mean±s.e.m.; **P<0.01).

6. mRNA expression analysis of CP from all brain ventricles.

In order to evaluate B-CSF-B permeability, the expressions of *crp*, *ifnγ*, *icam1*, *vcam1*, *cxcl10* and *ccl2* were measured as indicators of CP activation *(fig 17)*. Our results confirm inhibition reduction of CP activity in DAT+/- mice with respect to WT mice, previously demonstrated by IHC.

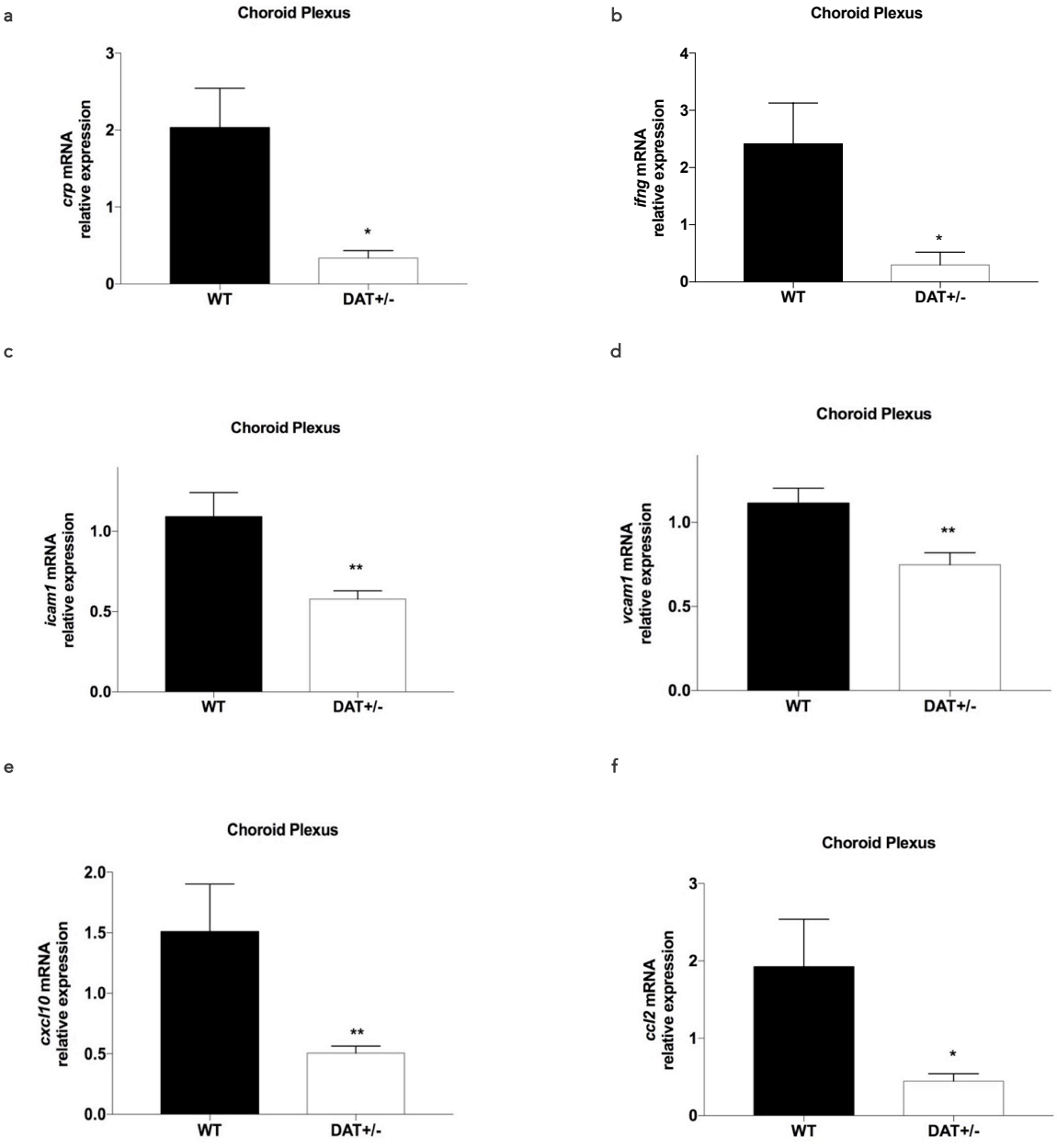

Figure 17 | Lower mRNA expression of B-CSF-B activation markers in CP of DAT+/- mice. mRNA expression of **(a)** *crp*, **(b)** *ifnγ*, **(c)** *icam1*, **(d)** *vcam1*, **(e)** *cxcl10* and **(f)** *ccl2* was measured by RT-qPCR, in CP from all the ventricles of PND>90 mice. Data are shown as fold-change compared with WT mice as controls (n=6 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01).

7. Flow cytometry analysis of Macrophages accumulation in brain.

The count of macrophages in brain of PND>90 mice was performed using the gating strategy shown below *(fig 18)*; macrophages were individuated as the CD11b+ CD45^{high} Ly6c+ population of cells. The macrophages level in whole brain of DAT+/- mice was significantly lower compared to WT. This finding indicates a reduced recruitment of peripheral myeloid cells in DAT+/- mice, probably induced by microglia lower activation.

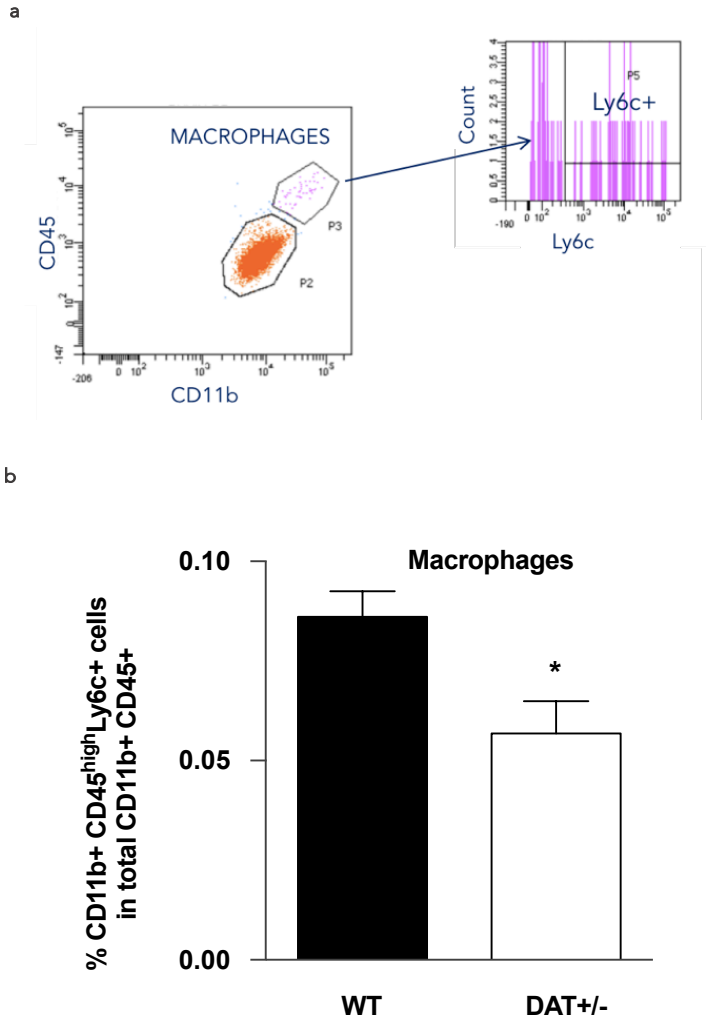

Figure 18 | Reduction of macrophages accumulation in brain of adult DAT+/- mice. (a) Gating strategy and **(b)** flow cytometry analysis of monocytes/macrophages in whole brain of DAT+/- PND>90 mice and age-matched WT as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

DISCUSSION

The aim of this project was to unravel the role of ADM and immune system in modulating CNS repair, protection and homeostasis in a mouse model of ADHD. There are growing evidences that immune cells play a protective role in CNS diseases, and immune system might be involved in brain functional integrity, since it has been demonstrated that the immune system participates in brain plasticity, with positive effects on adult neurogenesis, and spatial learning and memory (Kipnis et al., 2004, Ziv et al., 2006).

To our knowledge, few evidences are known about autoimmunity phenomena in ADHD patients. However, DAT is expressed in rat thymus and spleen, suggesting that DA may contribute to neuroimmune modulation in the lymphoid microenvironment. A differential activity of DA in primary and secondary immune organs, such as maturation and selection of lymphocytes and activation of immune responses in the spleen was not excluded (Mignini et al., 2006). Some clinical studies have also suggest alterations in circulating anti-DAT autoantibodies in ADHD patients, suggesting the presence of deficits in immunological tolerance, which needs to be further characterized (Giana et al., 2015).

The data collected in this study demonstrate the presence of immune system alterations in this model, which are strongly correlated to alterations of ADM expression in thymus *(fig. 6*, mRNA thymic expression of *adm* is correlated with the expression of specific Treg nuclear factor, *foxp3* $p<0,001$). Our immunological analysis demonstrates an increase of both thymic and circulating Treg levels in DAT+/- mice *(fig. 7, 8*, $p<0,05$), accompained by an increase of the ADM system expression in thymus *(fig. 4,* $p<0,05$). Based on the evidences about ADM activity in TEC regulation and thymocytes maturation published by our team (Castellani et al., 2016), we hypothesize a pivotal role for ADM in thymic microenvironment modulation, Treg maturation and immune tolerance. Since DATis expressed in the thymus (Mignini et al., 2006), the partial gene deletion in DAT+/- mice could affect the correct mechanism of self tolerance induction; the details of which are not jet understood.

Our findings clearly demonstrate a deficit in the trafficking of cells to the CNS in DAT+/- mice; indeed, in these animals, a lower microglia activation in brain parenchyma was found, consistent with the reduced expression of *tspo* (fig. 10, $p<0,05$), *cox2, tnfα (fig. 11;* $p<0,001$), *irf7* and *bdnf (fig.12*; $p<0,05$); this suggests an alteration of inflammatory signal release from CNS. Our data also show a correspondent reduction of monocytes/macrophages recruitment in the CNS in DAT+/- mice *(fig. 18*; $p<0,05$).

Because of the hypothesized ADM role in circulating Treg modulation, and the confirmed pivotal effect of Treg in inhibition of immune cells CP trafficking (Baruch et al., 2015), we decided to ascertain whether in DAT+/- mice modification of CP permeability are present. In this study we have shown that the observed reduction in CP trafficking is accompanied

by a reduced nuclear expression of the Nf-kB p65 subunit *(fig. 14,15,16;* p<0,01), in accordance with an increased level of IkBα. These data clearly suggest a reduced Nf-kB activity, consistent with an inhibition of CP trafficking. To confirm the observed B-CSF-B permeability reduction, we also analysed the mRNA expression of the adhesion proteins *icam1* and *vcam1,* and cytokines, chemokines and other markers such as *cxcl10, ccl2*, *ifnγ* and *crp* (by RT-qPCR), whose expression is dependent on the activation of the barrier. We have observed a significant reduction of mRNA expression of all these permeability markers *(fig. 17,* p<0,01), thereby confirming the inhibition of CP activity in DAT+/- mice.

In conclusion, ADHD is a neurodevelopmental disease carachterized by alterations in immune system and ADM thymic expression. In particular, in DAT+/- mice ADM thymic expression increases, and this is accompanied by a stronger peripheral immune tolerance activity and a lower CNS immune response. This increase of Treg in periphery could directly affect the B-CSF-B permeability, by inhibiting the CP. The effect of this mechanism is a lower recruitment of monocytes/macrophages to CNS, which is known to be essential for brain homeostasis in several pathological conditions, such as AD or spinal cord injury. Circulating monocytes/macrophages were found to be necessary also for the control of mental stress and depression (Lewitus et al., 2009).

Brain has long been considereded as an immune privileged organ and there was a general consensus that any immune activity found in the brain was thought to be a clear sign of disease, and should be eliminated. In fact, almost every CNS disease is associated with local inflammation and efforts were made to reduce inflammation in the brain through systemic administration of anti-inflammatory drugs without beneficial effects. As an evidence, this anti-inflammatory approach failed in most cases.

The evidences found in our study can, at least in part, explain the fail of this approch. In fact, we have found a reduction of Treg in DAT+/- mice, suggesting that immune system, that appears abnormally regulated, might be considered at least one of the targets useful to treat neurodevelopmental disease, thus indicating the need to evaluate an immunomodulatory approch to obtain more effective interventions.

It was shown that breaking systemic immune tolerance, either by active vaccination approaches (Kunis et al., 2015) or by targeting Tregs (Baruch et al., 2015), can augment CP-gateway activity for leukocyte trafficking. As previously explained, this causes an increase of the recruitment of monocytes/macropaghes from periphery, that produce beneficial effects in brain homeostasis.

As AD can be treated reducing Treg levels, and this improves the behavioural performances and reducing the hippocampus amyloid deposition (Baruch et al., 2015), we can hypothesize to use such a treatment in ADHD, in order to ameliorate the symptoms. Further *in vivo* studies are currently in progress to validate this approach.

Our findings indicate ADM as a possible biomarker useful for the clinical diagnosis of ADHD. Furthermore, since ADM is implicated in Treg development, targeting the ADM system rapresents another therapeutical option for the treatment of this disease. In conclusion, the modulation of the immune may be an innovative strategy for the pharmacological treatment of neurodevelopmental disorders, such as ADHD.

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chapter 3

Perinatal intranasal oxytocin rescues aberrant developmental trajectories in a mouse model of 22q11.2 microdeletion modulating the blood-cerebrospinal fluid-barrier permeability

ABSTRACT

Immunity and genetic vulnerability are suggested to play a pivotal role in the pathogenesis of several psychiatric disorders. However, the genetic influence in neuro-immunology and the consequent development of aberrant behaviors are unclear. Similarly, it is unknown if any therapeutical intervention might prevent an aberrant genetic-immunity interplay before the establishment of the altered developemtal trajectories. We first established that psychiatric-relevant behavioral alterations following 22q11.2 hemideletion in mice were evident as early as PND 10. The hemizygous genetic deletion in the 22q11.2 locus (22q11DS) is characterized in humans by developmental social and intellectual disabilities, high prevalence of attention deficit hyperactivity disorder (ADHD; ≈37%) and autism during childhood and schizophrenia (≈41%) in adulthood, making it one of the major known genetic vulnerability factor for psychiatric disorders. Notably, parallel cortical thinning and immunodeficits were particularly evident at the periadolescent/adolescent periods. Next we assessed the possible impact of preventive intervention with perinatal intransal administration of oxytocin, knowing its potential translational impact and the absence of side effects related to this treatment. Intranasal oxytocin (from post natal day-PND- 1 to PND 7, once a day) could modulate brain barrier permeability and immune cell trafficking to the CNS. These last experiments were performed since the pivotal role of both activated microglia in CNS and regulatory T cells (Treg) in the periphery in modulating immune cells trafficking through the Blood-Cerebrospinal Fluid-Barrier (B-CSF-B) have recently been demonstrated, together with the importance of peripheral monocytes/macrophages afflux into the brain in preserving CNS homeostasis. Indeed, it has been demonstrated that all these processes are impaired in different mental disorders. We demonstrated that oxytocin treatment increases microglia activation in LgDel/+ mice, and decreases macrophages and T cells accumulation in the brain. These results, together with the observed lower B-CSF-B activity and the higher Treg cells in spleen, indicate that oxytocin blocks immune cell trafficking through choroid plexus. Interestingly, these effects are evident not only at PND 10 but also during adolescence (PND 35), indicating that this treatment is able to balance macrophages accumulation in the brain, which had already been found to be excessive in perivascular areas in 22q11DS patients. The mechanism by which oxytocin regulates microglia and Treg cells remains to be clarified.

INTRODUCTION

1. 22q11 Deletion Syndrome.

22q11 Deletion Syndrome (22q11DS) is a complex disorder associated with different physical abnormalities, such as heart defects, cleft palate, gastrointestinal alterations, skeletal abnormalities (DiGeorge et al., 1965; McLean-Tooke et al., 2007). In addition to these defects, 22q11DS patients show absence or hypoplasia of the thymus and parathyroid glands. The 22q11 deletion is also one of the most significant genetic risks for autism, schizophrenia, and other cortical circuit disorders thought to arise during development (Meechan et al. 2011). Cognitive disabilities are common in 22q11DS; however, this disturb affects also social skills and executive functions (Jonas et al., 2014; Schneider et al., 2014) *(fig 1)*.

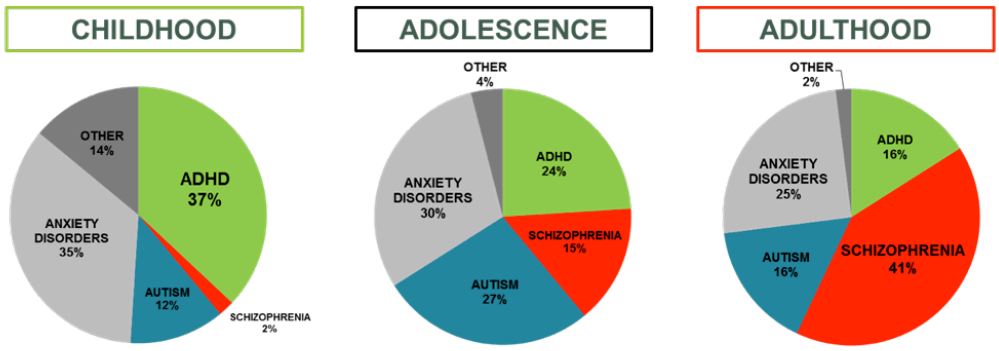


Fig 1 | Psychiatric disorders associated with 22q11DS diagnosed during neurodevelopmental stages (Jonas et al., 2014).

Brain imaging of 22q11DS patients shows consistent anatomical defects, including reduced cortical gray matter and *post mortem* analyses reveal the presence of pathological cells, associated with developmental defects including periventricular heteropias (Meechan et al., 2009). Most 22q11DS patients are hemizygous for a 3 Mb region on human chromosome

22 (HSA22q11) *(fig 2)* while others have a smaller 1.5 Mb deletion (Morrow et al., 1995; Carlson et al., 1997). These observations suggested that haploinsufficiency of one or more genes on human chromosome 22 is responsible for its aetiology.

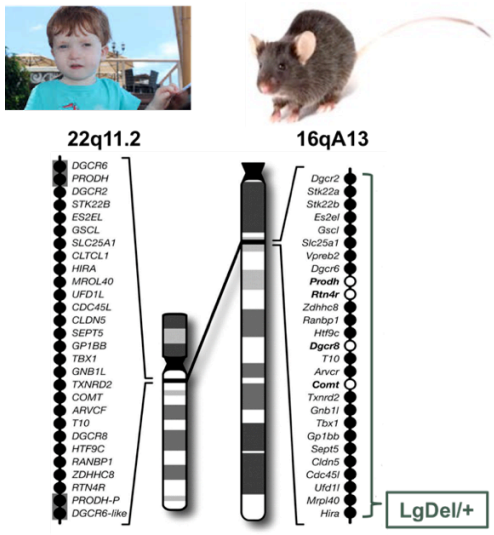


Fig 2 I Map of a part of human chromosome 22q11 (HSA22q11) and its corresponding region on mouse chromosome 16 (MMU16) (Gogos et al., 2009).

2. Mouse model of 22q11DS, behavioral deficits in Lgdel/+ mice.

2a. Mouse model of 22q11DS.

The mice used in this study were generated and characterized by Gogos and colleagues; these animals (Lgdel/+ mice) present a hemizygous deletion from *Dgcr2* to *Hira*, a region containing 24 genes with a length of 1.5 Mb *(fig 2)*. These authors have shown that nearly 50% of these mice exhibit conotruncal anomalies and this deletion leads also to significant perinatal lethality (Gogos et al., 2009). It was found that 22q11 deletion compromises specific cortical neural stem cells, basal progenitors, and alters frequency and distribution of cortical projection neurons and GABAergic interneurons. These phenotypes suggest a link between a genomic lesion, altered cortical development, and subsequent changes in cortical circuitry that likely intensify the risk for behavioral disorders in 22q11DS patients (Meechan et al., 2009).

2b. Preliminary behavioral characterization of LgDel/+ mice.

The first step of this study was performed by Doctor Francesco Papaleo's group in pre-adolescent (PND 14-19), adolescent (PND 28-45) and adult (PND 90-120) LgDel/+ mice with the aim to individuate the presence of behavioral alterations associated to this genetic condition. A brief summary of the behavioral tasks results indicating the presence of evident social alterations in LgDel/+ mice is shown in *figure 3*.

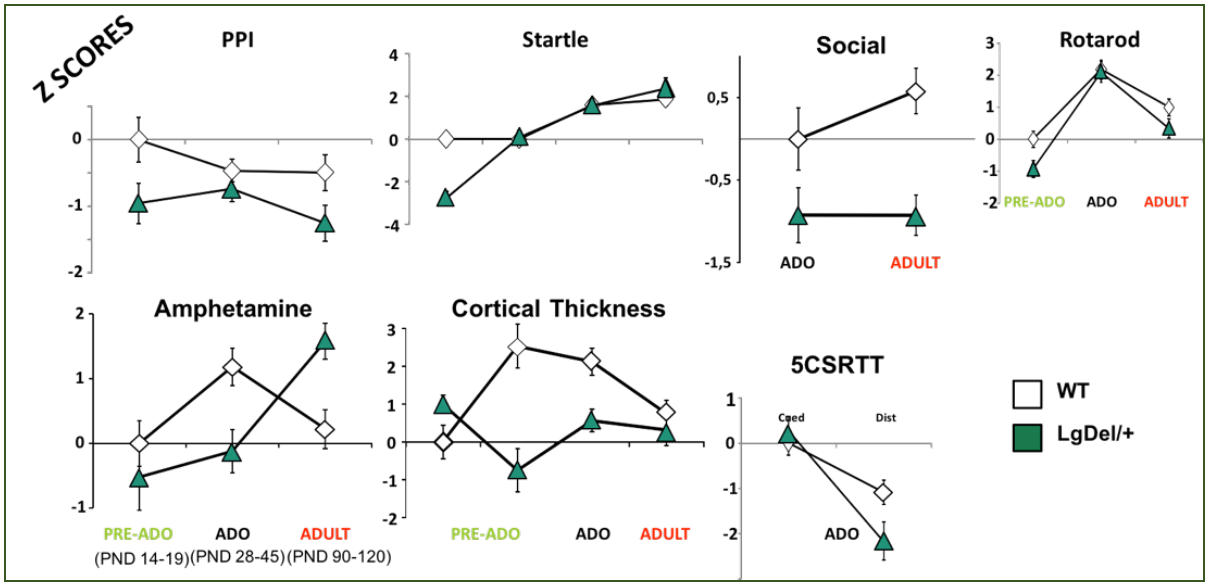


Fig 3 | Behavioral tasks in WT and LgDel/+ mice.

3. Oxytocin perinatal treatment in 22q11DS.

Oxytocin and its receptors are expressed in brain areas known to be involved in the symptoms of schizophrenia. An anti-psychotic role for oxytocin, when administered intravenously or intranasally, has already been demonstrated. Indeed, recent studies in which this peptide was administered intranasally to schizophrenic patients have confirmed the hypothesis that oxytocin can reduce the symptoms of this disease (Feifel et al., 2010). The intranasal treatment allows the overstep of the blood-brain-barrier (BBB) and its delivery from the nose to the CNS occurs within minutes along both the olfactory and trigeminal neural pathways. Intranasal delivery occurs by extracellular routes and does not require the drug binding to any receptor or axonal transport. This route of administration also targets the nasal associated lymphatic tissues (NALT) and deep cervical lymph nodes. In addition, intranasal administered therapeutics are observed at high levels both in blood vessel and in cerebrospinal fluid (CSF) (Dal Monte et al., 2014). In this study, an intranasal oxytocin administration was performed to newborn mice (from PND 1 to PND 7) since some evidences reported the beneficial effect of this treatment in patients with 22q11DS (fig 4) (Feifel et al., 2010).

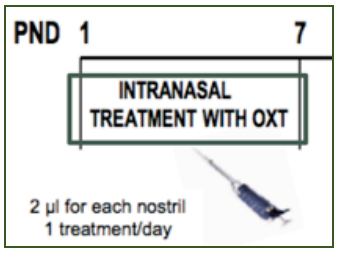


Fig 4 | Perinatal oxytocin treatment performed from PND 1 to PND 7 in mice.

During this study, it was demonstrated that the administration of oxytocin rescues social deficits in adolescent and adult LgDel/+ mice. This treatment shows opposed results in WT adult mice *(fig 5)*.

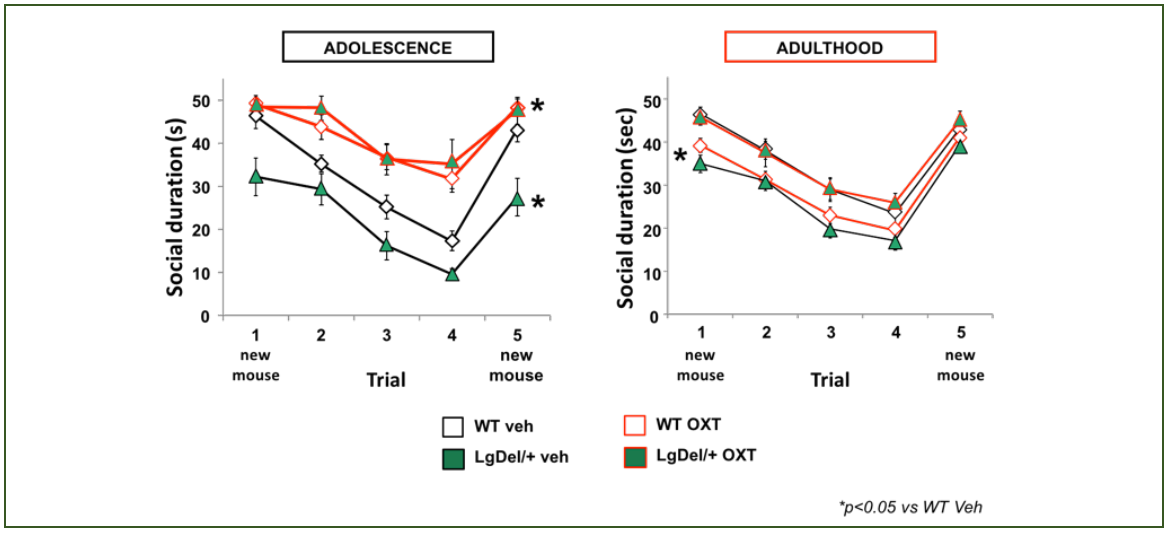


Fig 5 | Perinatal oxytocin rescues social deficits in LgDel/+ adolescent and adult mice.

It has recently been demonstrated that oxytocin promotes the development of thymus, performs immune surveillance, strengthens immune defense, and maintains immune homeostasis (Li et al., 2017). The possible action of oxytocin in modulating the immunity in CNS and its involvement in regulating the immune activation in psychiatric disorders remain to be investigated. The expression of oxytocin receptors has been noticed not only in T cells, but also in microglia (Ndiaye et al., 2008), the CNS resident immune cells, suggesting a direct effect of oxytocin in modulating neuro-immunity.

4. Neuro-immunity and regulation of B-CSF-B permeability.

4a. Neuro-immunity.

The role of innate and adaptive immune systems in the regulation of recruitment, activation, differentiation and homeostasis of immune cells has already been described (Meyer et al., 2011). Prof. Michal Schwartz and her collaborators demonstrated that the immune system is pivotal for life-long maintenance of the brain by contributing to the

formation of new neurons, the mantainment of normal cognitive performance and the regulation of neurogenesis. These evidences have broken the theory of the CNS as immune privileged organ, indicating that peripheral immune cells can contribute to the CNS homeostasis and repair. In particular, this group demonstrated that two types of immune cells, i.e. circulating blood macrophages and T-cells, are needed for CNS health and homeostasis.

4b. Choroid plexus.

Peripheral cells recruitment to CNS can occurs through changes in BBB and B-CSF-B permeability, which are consequences of the activation of CP. CP is strategically positioned at the brain ventricles in order to be exposed to brain-derived signals from CSF, which can be released by microglia, and to peripheral signals from the circulation. This tissue is defined as neuro-immunological interface and regulates the immune cells afflux to the CSF as a gate, which can be opened or closed. Thus, it was shown that the B-CSF-B is not a "true" fixed barrier that blocks immune cell entry, but rather a selective and educative gate that enables selective cell access, depending on CNS needs. The B-CSF-B is a unique barrier in the CNS as it is composed of epithelial cell tight junctions and fenestrated endothelial cells, rather than endothelial tight junctions (Shechter et al., 2013; Kunis et al., 2013). Between epithelial and fenestrated endothelial cells there is a stroma, where CD4 T cells from periphery can be accumulated; here, cytokines released by T cells regulate CP permeability. It has been demonstrated that an increased accumulation of Treg, Foxp3 expressing cells, causes CP inactivation, a process which is involved in Alzheimer disease (Kunis et al., 2013, Baruch et al., 2015). Approximately 15% of CD4 cells in the naive CP are IFNγ-producing Th1 cells, and their activity in this compartment was found to be crucial for the activation of the CP as a gate for leukocyte trafficking to the CNS, by inducing the expression of trafficking molecules (Kunis et al., 2013). The B-CSF-B trafficking is regulated by the expression of adhesion proteins, such as ICAM1 or VCAM1, and the intervention of cytokines or chemotactic mediators (CCL2, CXCL10 and IFNγ produced by Th1 cells). Altered stroma accumulation of specific T cells subsets have been noticed in several mental disorders *(fig 6)*. Interestingly, recent findings suggested that the immune-modulation of CP can be a novel approach for attenuating cognitive dysfunction (Baruch et al., 2015).

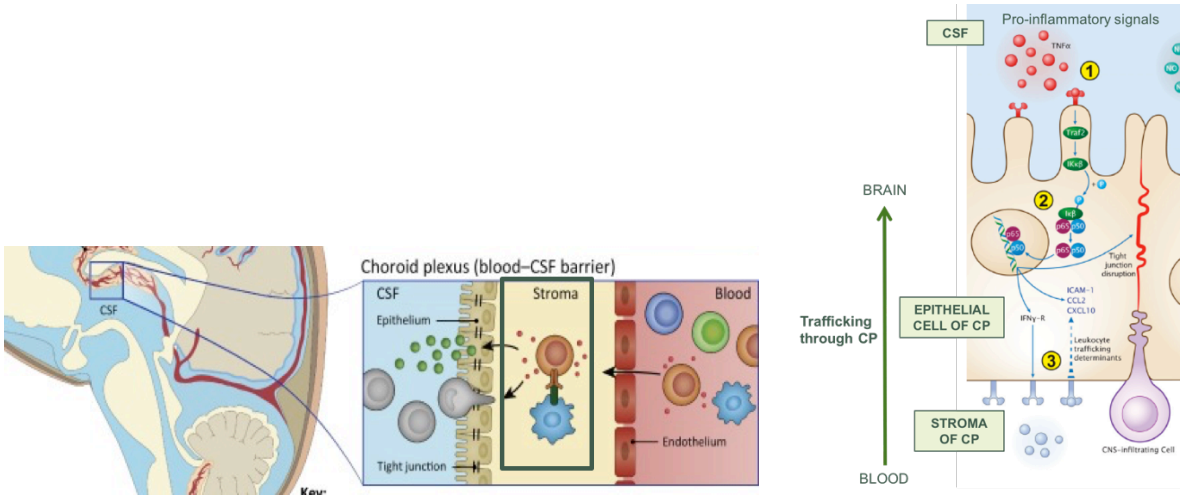


Fig 6 | CP structure and function. Description of CP trafficking (Baruch et al., 2015).

4c. Microglia.

Microglia are the resident myeloid cells of the CNS that control brain in early development and contribute to its homeostasis throughout life. Contrary to previous beliefs, microglia and macrophages do not share the same origin; microglia arise from the yolk sac, while macrophages from the bone marrow, and microglia cannot be replaced by macrophages even though both these cells absolve the phagocytic function. Microglia phagocytic activity has been demonstrated to be essential for the clearance of brain accumulated debris. Indeed, in mice in which microglia (and macrophages) have been genetically removed, postnatal brain development is severely perturbed, strongly supporting a role for microglia in the homeostasis of brain development and function (Schwartz et al., 2013). Microglia modulate synaptic transmission, formation and elimination, and shape embryonic and postnatal brain circuits. However, many of these processes, such as synaptic pruning and neuronal maturation, peak in mice during the first week after birth, a period that may be important to understanding the circuits and aetiology of many neurodevelopmental diseases. Perturbation of the microglia environment during development may alter the strict timing of developmental programs and lead to misplaced expression of gene pathways, such as inflammation, that disrupt neuronal development and lead to brain disorders at later stages in life. For example, prenatal infection has been correlated with an increased risk of schizophrenia and autism in both mouse and human offspring. The precise effects of perturbations on development are highly dependent on the timing of infection, suggesting the interference with specific processes (Matcovitch-Natan et al., 2016).

The classical paradigm distinguished ramified and amoeboid phenotypes; the first is also called M0-like or inactive type of microglia, while the second one is the active form, which expresses pro (M1-like) or anti-inflammatory (M2-like) markers. The activation of microglia is characterized by the expression of translocator protein 18kDa (TSPO) (Kim et al., 2015); the M1-like type presents inducible nitric oxide synthase (iNos), cyclooxygenase 2 (Cox2) and tumor necrosis factor alpha (Tnfα) expression, while M2-kile form shows high level of interferon regulatory factor 7 (Irf7), arginase 1 (Arg1) and brain-derived neurotrophic factor (Bdnf) (Cohen et al., 2014). The M2-like phenotype is linked with amelioration of psychiatric symptoms, while M1-like type increase corresponds to cognitive deficits aggravation *(fig 7)* (Nakagawa et al., 2014). Anyway, more recent findings suggest the need of redesigning the microglia's phenotypes classification indicating the existence of intermediate type of microglia expressing both pro and anti-inflammatory markers (Wlodarczyk et al., 2015). However, the potential effect of microglia activation and inflammatory cytokines release in CSF in order to modulate the CP permeability and immune cells recruitment to the CNS need further investigations. It is known that pro-inflammatory signals from the brain can induce immune cells trafficking through CP and accumulation in CNS, while anti-inflammatory mediators can block this process. The role of microglia in neurogenesis and synaptic pruning process, both compromised in 22q11DS, has been fully described, observing that M2-like phenotype is strongly related with induction of Bdnf production, a protein involved both in neurogenesis and dendritic spines modulation (Kowianski et al., 2017, Greenberg et al., 2009). A recent study revealed the microglia involvement in the production of the complement C4 protein, a mediator responsible of synaptic pruning process, which exists in two isoforms, C4a or C4b. This study correlated the C4a high expression with the excessive synaptic elimination occurring in schizophrenic patients during adolescence (Sekar et al., 2016).

The strong relationship between pruning and neurogenesis processes was confirmed; a study inhibited apoptotic cell clearance, which led to accumulation of apoptotic cell debris, and a marked reduction in neuronal differentiation and survival, indeed suggesting that an inability to clear cellular debris in brain actively neurogenic areas, such as sub-ventricular zone of the lateral ventricles and the sub-granular zone of the hippocampal dentate gyrus, impairs neurogenesis (Lu et al., 2011).

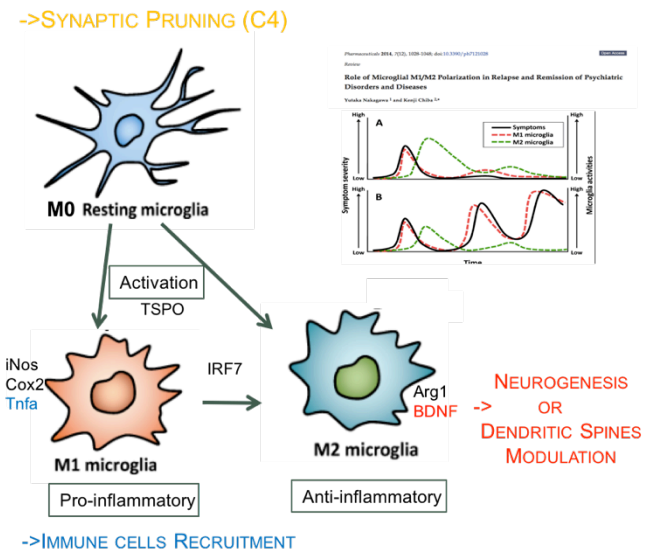


Fig 7 | Microglia different phenotypes and relation with psychiatric symptoms (Nakagawa et al., 2014).

4d. Monocytes/macrophages from periphery.

Monocyte-derived macrophages recruited to the CNS display a role that is no redundant with that of the microglia in resolving the inflammatory response (Shechter et al., 2009). Infiltration of circulating macrophages after CNS injury has been generally assumed to reflect mechanical damage to the parenchymal BBB. Several studies have shown parenchymal infiltration of macrophages when the BBB is resealed. In addition, blood-derived macrophages contribute to CNS repair, in part by displaying the resolving M2 phenotype. Conversely, boosting normal macrophages recruitment results in improved recovery. These findings indicate the essential role of a correct balance of macrophages afflux in CNS in order to ensure the proper homeostasis. The phenotype of macrophages, derived from circulating monocytes homing to injured tissues reflects two phases; the initial phase is characterized by monocyte-macrophages, corresponding to the ''classically activated'' (M1-like) cells, which have been shown to possess pro-inflammatory, phagocytic, and proteolytic functions, essential for damaged tissue digestion and debris removal. The second phase is associated with monocyte-macrophages, ''alternatively activated'' (M2-like) macrophages, which are anti-inflammatory in nature and are involved in tissue regeneration, growth, angiogenesis, and matrix deposition, thereby supporting tissue remodeling. It is still unclear whether the distinct macrophage populations are an outcome of monocyte recruitment in two waves or *in situ* phenotypic conversion of the already recruited cells. Phenotypic analysis revealed that monocyte-derived macrophages

expressing proinflammatory activity (M1-like) population appears earlier and its presence is transient (Shechter et al., 2013). After a CNS insult, the majority of macrophagic recruited cells arrived with a delay relative to the initial injury-associated breach of the BBB. Importantly, in the absence of injury, blood-derived macrophages are excluded from the healthy host brain (Shechter et al., 2009). However, macrophages parenchymal infiltration can occur in response of BBB and B-CSF-B permeability alterations and after microglia pro-inflammatory activation *(fig 8)*. Interesting abundant perivascular macrophages accumulations was found in post-mortem adult brain of 22q11DS patients, suggesting excessive immune cells recruitment in this pathology (Kiehl et al., 2008).

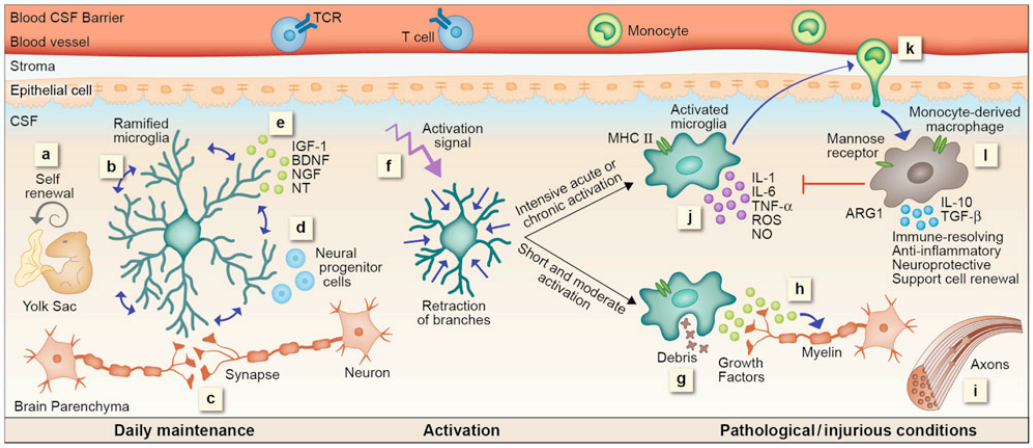


Fig 8 | Microglia different phenotypes and monocytes/macrophages recruitment through B-CSF-B (London et al., 2013).

4e. T cells from periphery.

The role of peripheral T cells in CNS homeostasis was confirmed by studies in which T cells deficient mice were found to exhibit reduced spatial learning and memory capabilities, which can be reversed by immune reconstitution. These publications, which suggested the need of immune system regulation to maintain brain plasticity, do not contradict other results, suggesting that severe inflammation impairs neurogenesis. Importantly, the capability of immune cells to regulate healthy brain parenchyma homeostasis even from afar has been described. Different outcomes depending on the type of immune activation have also been characterized . A direct correlation exists between changing in circulating immunity and in the cytokine milieu of local effectors in the CP stroma, which in turn affects CP function. Immunological dysregulation in CP stroma involves the secretion of specific factors to the CSF, that might interfere with brain function. However, the CP may be

inactivated because of either a deficiency in Th1 cells, elevated levels of circulating suppressor immune cells, such as Foxp3 regulatory T cells, or lack of pro-inflammatory signals from the parenchyma. Indeed, anti-inflammatory signals from brain microglia or Treg accumulation in CP lead to a reduction of CP permeability; this fact could have different implications, depending on the nature of the CNS disorder (Baruch et al., 2015). These findings indicate that peripheral T cells levels can be considered an indicator of CP proper activation and function as a gate for recruiting immune cells. The current understanding suggests that CP modulation can be evaluated as a novel therapeutic approach for psychiatric diseases; although the target is common to all pathological conditions, the specific approach will differ depending on the nature of the CP dysfunction, and whether such dysfunction is an outcome of the circulating immune milieu, or the CSF signaling (Schwartz et al., 2013).

5. Immune system alterations in 22q11DS.

5a. Peripheral immunity in 22q11DS.

The deficit in thymic function found in 22q11DS can result in a deficiency of T cells and can be evaluated clinically by measuring the proportion of CD4+ lymphocytes. The confirmation of these evidence is the recurrent infections and high risk for autoimmune disorders typical of 22q11DS patients (McLean-Tooke et al., 2007). To determine whether these features are also present in the Lgdel/+ mice, some authors analyzed E18.5 embryos for thymic hypo- or aplasia as well as T cell immune defects. All of the 24 embryos (7 Lgdel/+ embryos and 17 WT embryos) analyzed had an intact and normal sized thymus. Flow cytometry analysis of the T cell populations in the thymus was performed using CD3, CD4, and CD8 markers and no significant differences were found in Lgdel/+ mice compared to their WT littermates (Gogos et al., 2009).

5b. Prenatal/perinatal inflammation as vulnerability factor for schizophrenia.

A theory postulating the correlation between latent or persistent inflammation and deficit in brain barriers permeability has been described by Meyer and colleagues (2011). Indeed, the brain is highly vulnerable to environmental insults during early development. Therefore, immunological processes targeting the developing brain can be expected to have long-

lasting impact on brain and behavioral functions throughout the life span. An association between prenatal/perinatal infection and neurodevelopmental disorders has been demonstrated, describing in details the impact of prenatal maternal infection on the development of childhood and adult neuropsychiatric disorders, particularly schizophrenia and autism. Furthermore, pro-inflammatory cytokines are essential for facilitating the brain barrier permeability, and promoting the release of complement system components. It was found that adding an anti-inflammatory agent to standard antipsychotic medication seems to be superior to the antipsychotic treatment alone in the management of schizophrenia, especially when such anti-inflammatory therapy is initiated in the early phases of the disease (Meyer et al., 2011).

5c. Immunity in CNS, immune cells recruitment abnormalities and microglia involvement in neurogenesis and synaptic pruning process in 22q11DS.

Interestingly, in post-mortem adult brain of 22q11DS patients abundant perivascular macrophages accumulations were found, suggesting an excessive immune cell recruitment in this disease (Kiehl et al., 2008). Published data about microglia phenotype and activation in 22q11DS are controversial but it the involvement of microglia inflammatory state and this syndrome is known. A direct effect on the modulation of neurogenesis by microglia has been demonstrated and it is clear that neurogenesis is compromised in 22q11DS. Indeed, a role of five 22q11DS deleted genes (*Ranbp1*, *Cdc45l*, *Hira*, *Ufd1l*, and *Sept5*) in cortical neurogenesis process is known, in particular, LgDel/+ mice are characterized by an alteration of the cortical expression of cell cycle genes leading to proliferation abnormalities of cortical VZ/SVZ basal progenitor (Meechan et al., 2009). The synaptic pruning process is also dysregulated in 22q11DS, and this is strongly related with immune system and microglia activation. In particular, it is excessive during adolescence, causing high loss of synapsis. Recently, some authors have found that the specific cause of this deficit is the higher expression of the C4a isoform of the complement system in schizophrenia, produced by microglia (Sekar et al., 2016).

5d. Immunity and cognitive symptoms; immunomodulatory treatment in schizophrenia.

The role of immune cells in schizophrenia and the consequent potential beneficial effect of immune-modulatory treatment was demonstrated by Professor Michal Schwartz team. Their

studies, based on the use of behavioral tasks, revealed that SCID mice present consistent impairment of cognitive abilities, reverted by immune cell regulation; the injection of lymph node lysates from WT mice permits evident improvement of cognitive abilities. It was also shown that immune cell vaccination reverts the cognitive disabilities of mouse models of schizophrenia (Kipnis et al., 2004; Cardon et al., 2010).

AIMS

1. Neuro-immmunological characterization of Lgdel/+ mice.

The first aim of this study was the characterization of immune cells in the CNS of LgDel/+ mice, in order to ascertain the presence of alterations in their activation and accumulation in CNS and of inflammatory signals from the brain of the 22q11DS animal model. In details, we analyzed the Immune-Brain Axis activity, studying microglia inflammatory phenotype and accumulation of macrophages and T cells in the brain, evaluating the trafficking through B-CSF-B and CP activation.

2. Detection of immunomodulatory effect of Oxytocin.

The second aim of this study was the evaluation of the effects of oxytocin perinatal treatment in the modulation of B-CSF-B permeability and immune cell recruitment in the brain, with the final purpose of proposing this treatment as an efficient immune-modulatory therapy to prevent psychiatric symptoms of 22q11DS.

METHODS

1. Animals.

Both male and female wild type and LgDel/+ C57/BL6 mice were breed and maintained in the Animal House of the Department of Neuroscience and Brain Technologies of Italian Institute of Technology (iit) in Genoa. All the procedures involving animals were approved by the Italian Ministry of Health and Animal Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. Mouse genotypes were identified by PCR analysis of tail DNA. Mice were group housed (two to four per cage) in a climate-controlled animal facility (22±2 °C) and maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Brains, PFCs, CPs and spleens were obtained from mice at PND 10 and at PND 35. All biological analyses were performed after a behavioral characterization (during adolescence and adulthood) of mice according with the experimental design shown below *(fig 9)*.

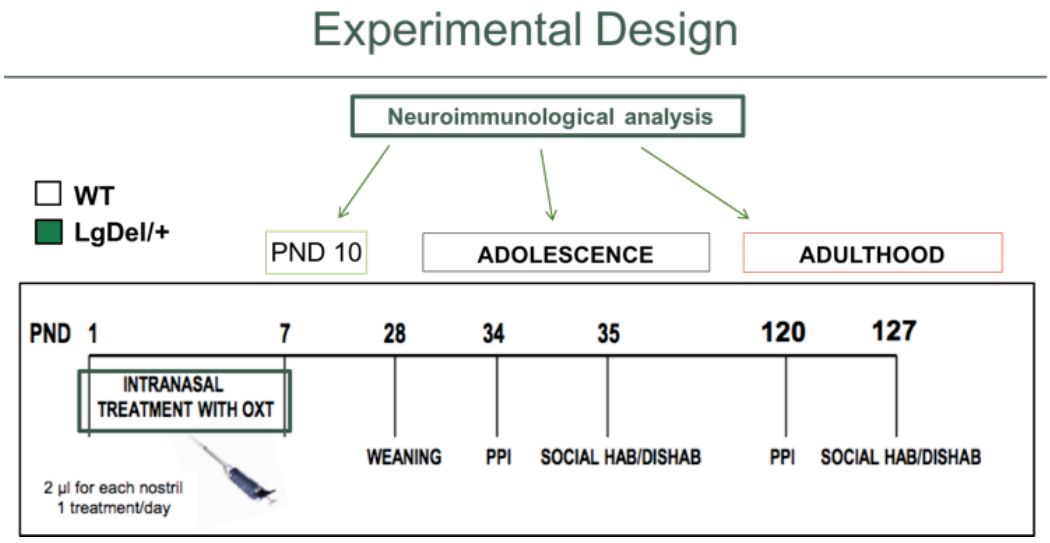


Fig 9 I Experimental design.

2. *In vivo* intranasal oxytocin administration.

Wild type and LgDel/+ mice were treated with oxytocin administered intranasally (0,25 μg, 2 μl each nostril) from postnatal day (PND) 1 once a day for 7 days. Physiological Saline Solution (vehicle) was used as control.

3. Flow cytometry sample preparation and analysis.

Mice were sacrificed and tissues were extracted after PBS transcardial perfusion. Brains and pooled PFCs were removed and tissues were dissociated using the GentleMACS dissociator (Miltenyi Biotec) and myelin was eliminated using Percoll 40% solution. CP tissues were isolated from the lateral, third and fourth ventricles of the brain under a dissecting microscope (Stemi DV4; Zeiss) in PBS, incubated at 37 °C for 45 min in PBS (with Ca^{2+} /Mg^{2+}) containing 400 U ml collagenase type IV (Worthington Biochemical Corporation), pooled and then manually homogenized by pipetting. Spleens were mashed with the plunger of a syringe and treated with ACK (ammonium chloride potassium)-lysing buffer to remove erythrocytes. All samples were filtered through a 70 mm nylon mesh, and blocked with anti-Fc CD16/32 (BD Biosciences). For Treg staining, an eBioscience FoxP3-staining buffer set (00-5523-00) was used. The following fluorochrome-labelled monoclonal antibodies were purchased from BD Pharmingen, BioLegend or eBiosciences, and used according to the manufacturer's protocols: v450-conjugated anti-CD4; PE-conjugated anti-CD25; FITC-conjugated anti-CD45; PE-Cy5-conjugated anti-TCRβ; APC-conjugated anti-FoxP3; PE–conjugated anti-CD11b; APC-conjugated anti-Ly6c. Cells were analyzed on a FACSAriaIII cytometer (BD Biosciences) using FACS software. In each experiment, relevant negative control groups and single-stained samples for each tissue were used to identify the populations of interest and to exclude other populations. In sorting experiments, microglia were collected from whole brains or pooled PFCs into 350 μl of RNA lysis buffer. RNA was extracted from sorted cells, and mRNA of specific targets was measured as described below.

4. RNA purification, cDNA synthesis and quantitative real-time PCR analysis.

Total RNA of the sorted microglia was extracted using RNeasy Kit (QIAGEN) while total RNA of the CP (from all ventricles) was extracted using TRI Reagent (Euroclone). RNA integrity and quantity were determined by Nanodrop (Genova). mRNA (1 μg) was converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression of specific mRNAs was determined by real-time PCR (EcoTM Illumina, Real-Time PCR system, San Diego, CA, USA) using Fast-SYBR PCR Master Mix (Applied Biosystems) and the genes expression levels were calculated by the ΔΔCt method using the EcoTM Software v4.0.7.0. Quantification reactions were performed in triplicate for each sample and the reaction thermic profile was: 95°C for 2m for DNA polymerase activation, 95°C for 5s, 60°C for 20s and 72°C for 15s (40 cycles) for PCR reaction, 95°C for 15s, 55°C for 15s, 95°C for 15s for dissociation. Peptidylprolyl isomerase A (*ppia*) and Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were chosen as references (housekeeping) genes for CP and sorted microglia, respectively. At the end of the assay, a melting curve was constructed to verify the specificity of the reaction. To determine the expression levels of *ifnγ* in CP, cDNA was pre-amplified for 14 PCR cycles, according to the manufacturer's protocol (PreAmp Master Mix Kit; Applied Biosystems), thereby increasing the sensitivity of the subsequent real-time PCR reaction. The TaqMan Assays-on-Demand probes Mm02342430_g1 (primer-limited) (*ppia*) and Mm01168134_m1 (*ifnγ*) were used.

For other genes examined, the following primers were used:

Ppia forward 5′-AGCATACAGGTCCTGGCATCTTGT-3′ and reverse 5′-CAAAGACCACATGCTTGCCATCCA-3′ ;
Gapdh forward 5′-TGGCAAAGTGGAGATTGTTGCC-3′ and reverse 5′-AAGATGGTGATGGGCTTCCCG-3′;
Icam1 forward 5′-AGATCACATTCACGGTGCTGGCTA-3′ and reverse 5′-AGCTTTGGGATGGTAGCTGGAAGA-3′;
Vcam1 forward 5′-TGTGAAGGGATTAACGAGGCTGGA-3′ and reverse 5′-CCATGTTTCGGGCACATTTCCACA -3′;
Cxcl10 forward 5′-AACTGCATCCATATCGATGAC-3′ and reverse 5′-GTGGCAATGATCTCAACAC-3′;
Ccl2 forward 5′-CATCCACGTGTTGGCTCA-3′ and reverse 5′-GATCATCTTGCTGGTGAATGAGT-3′;
Tspo forward 5′-TACTTTGTACGTGGCGAGGG-3′ and reverse 5′-ACCTCCCAGCTCTTTCCAGA-3′;
Arg1 forward 5′-TCACCTGAGCTTTGATGTCG-3′ and reverse 5′-CTGAAAGGAGCCCTGTCTTG-3′;
Irf7 forward 5′-CACCCCCATCTTCGACTTCA-3′ and reverse 5′-CCAAAACCCAGGTAGATGGTGTA-3′;
Bdnf forward 5′-GATGCTCAGCAGTCAAGTGCCTTT-3′ and reverse 5′-GACATGTTTGCGGCATCCAGGTAA-3′;
Inos forward 5′-CAAGCACCTTGGAAGAGGAG-3′ and reverse 5′-AAGGCCAAACACAGCATACC-3′;
Cox2 forward 5′-GCTAGAAGTTGATAACCGAGTCG-3′ and reverse 5′-CTAGGGAGGGGACTGCTCAT-3′;
Tnfα forward 5′-GCCTCTTCTCATTCCTGCTT-3′ and reverse 5′-CTCCTCCACTTGGTGGTTTG-3′;
Ki67 forward 5′-GTCTGATGTTAGGTGTTTGAGG-3′ and reverse 5′-CTTGTTCTTAACTTCTTGGTGC-3′;
C4a forward 5′-AAAGGTCCGGGACATGGT-3′ and reverse 5′-GCTTTTGGACAGGCTCACAC-3′;
C4b forward 5′-GCAGGAGGAAGACTTGATAGA-3′ and reverse 5′-ACAGACCTTTGCTTTTGGAC-3′.

5. Immunohistochemistry, confocal microscopy and images analyses.

For tissue staining, mice were transcardially perfused with PBS prior to tissue excision and fixation. CP tissues were isolated under a dissecting microscope (Stemi DV4; Zeiss) from the lateral, third and fourth ventricles of the brain. For whole mount CP staining, tissues were fixed with 2.5% paraformaldehyde for 1 h at 4 °C, and subsequently transferred to PBS containing 0.05% sodium azide. Prior to staining, the dissected tissues were washed with PBS and blocked (20% horse serum, 0.3% Triton X-100 and PBS) for 1 h at room temperature. Whole mount staining with primary antibodies (in PBS containing 2% horse serum and 0.3% Triton X-100), or secondary antibodies, was performed for 1 h at room temperature. Each step was followed by three washes in PBS. The tissues were applied to slides, mounted with Mowiol (Sigma Aldrich) and sealed with cover-slips. The following primary antibodies were used: rat anti-ICAM-1 (1:200, AB2213); and rabbit anti-Claudin-1 (1:100, Invitrogen, 519000). Secondary antibodies included: Cy2 and Cy5-conjugated donkey anti-rabbit/rat antibodies (1:200; from Jackson Immunoresearch). The slides were exposed to DAPI nuclear staining (1:4000; Sigma Aldrich) during Mowiol fixation. Two negative controls were routinely used in immunostaining procedures, staining with isotype control antibody followed by secondary antibody, or staining with the secondary antibody alone. Samples were imaged by confocal laser microscopy using a LSM 800 microscope (Carl Zeiss, Jena, Germany) equipped with a 63x, n.a. 1.4, oil immersion objective and ZEN 2.1 - blue edition- software (Carl Zeiss, Jena, Germany). To avoid emission crosstalk, each emission fluorescence was recorded independently with specific detector and optical cut-off filter, over the entire emission spectrum of related chromophores. The fluorescent signal intensity of ICAM1 and Claudin1 was quantified by means of Fiji software.

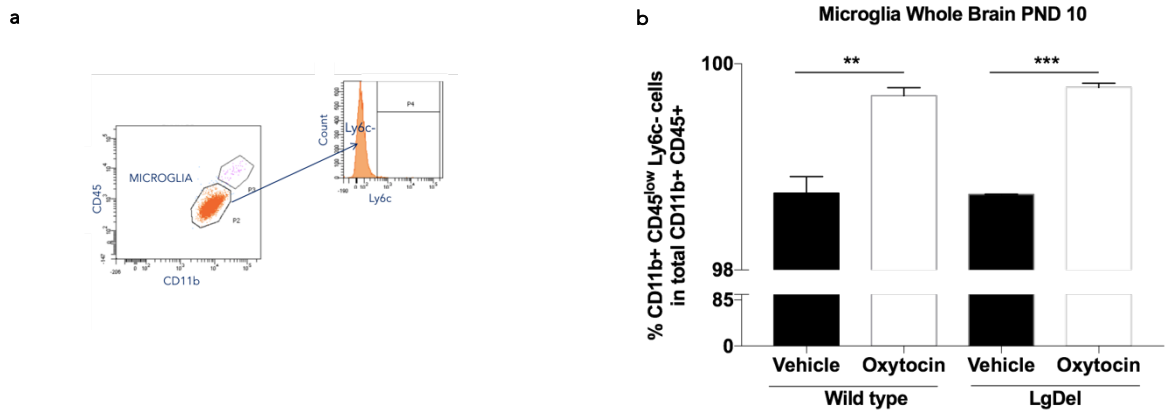
6. Statistical analysis.

The specific tests used to analyze each set of experiments are indicated in the figure legends. Data were analyzed using Student's t-test to compare two groups, whereas one-way ANOVA was used to compare several groups, followed by the Tukey's *post hoc* test for pairwise comparison of groups when the null hypothesis was rejected ($P<0.05$). Results are presented as mean ± s.e.m. In the graphs, y-axis error bars represent s.e.m. Statistical calculations were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

1. Flow cytometry analysis of microglia in brain of PND 10 and PND 35 mice.

The count of microglia in brain of PND 10 and PND 35 mice was performed using the gating strategy shown below; microglia was identified as the CD11b+ CD45^{low} Ly6c- cell population *(fig 10a)*. The analysis was conducted in whole brain for PND 10 mice in order to individuate general abnormalities in microglia number during this neurodevelopmental stage. The small dimension of brains at PND 10 is the reason why we did not focalize our tests in a particular brain region at this stage. The analyses on PND 35 mice were performed in PFC (the brain region more implicated in 22q11DS), pooled in groups of three or four, and in the rest of the brain as control. The microglia levels in whole brain of PND 10 mice were significantly increased after the oxytocin perinatal treatment both in WT and LgDel/+ mice. The analysis in PND 35 mice indicate that the amount of microglia in PFC is increased by the oxytocin treatment in LgDel/+ mice but not in WT; this trend is analogous to the results in the rest of the brain *(fig 10 b, c, d)*.



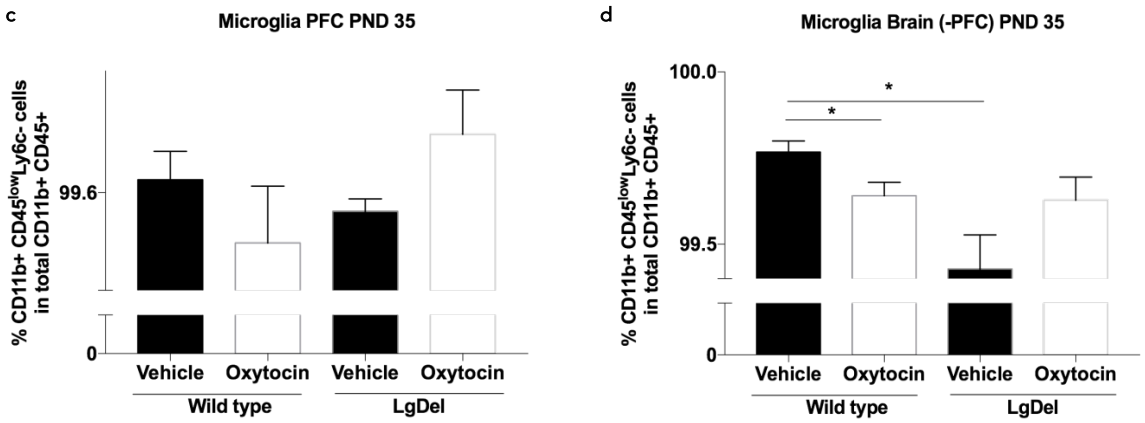


Fig 10 | Perinatal oxytocin administration increases microglia in whole brain of PND 10 mice and in PFC of LgDel/+ PND 35 mice. (a) Gating strategy and flow cytometry analysis of microglia in whole brain of **(b)** PND 10 and **(c, d)** PFC (and whole brain without PFC as controls) of PND 35 mice. Age-matched WT treated with oxytocin or vehicle were used as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01; ***P<0.001).

2. Ki67 expression in PND 10 mice whole brain sorted microglia as index of cellular proliferation.

Ki67 mRNA quantification in sorted microglia was useful to identify the rise of microglia enhancement in samples of treated WT and LgDel/+ mice. this experiment was performed in order to ascertain whether microglia gain was due to increased cellular migration or proliferation. ki67 induces cellular proliferation and is a validated marker of proliferating microglia or macrophages (*fig 11).*

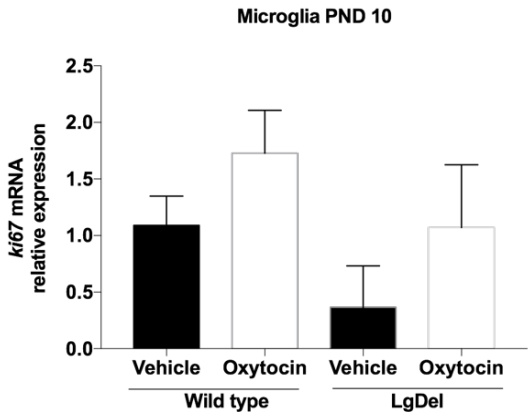


Fig 11 | Perinatal oxytocin induces microglia proliferation in whole brain of PND 10 mice. mRNA expression of *ki67* was measured by RT-qPCR, in microglia sorted from whole brain of PND 10 mice. Data are shown as fold-change compared with WT vehicle mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m).

3. mRNA quantification of microglia inflammatory markers as indicator of activation.

The sorted microglia were collected in RLT buffer and prepared for RT-qPCR analysis. F*igure 12* shown the data about PND 10 mice. mRNA expression of *tspo* indicates that the oxytocin perinatal treatment significantly activates the microglia; it is also evident that, in absence of treatment, LgDel/+ mice have less activated microglia than WT mice. *Irf7* mRNA expression is induced by the oxytocin administration both in LgDel/+ and WT mice, indicating an activated state and a switch from pro-inflammatory to anti-inflammatory phenotype both in LgDel/+ and WT mice.

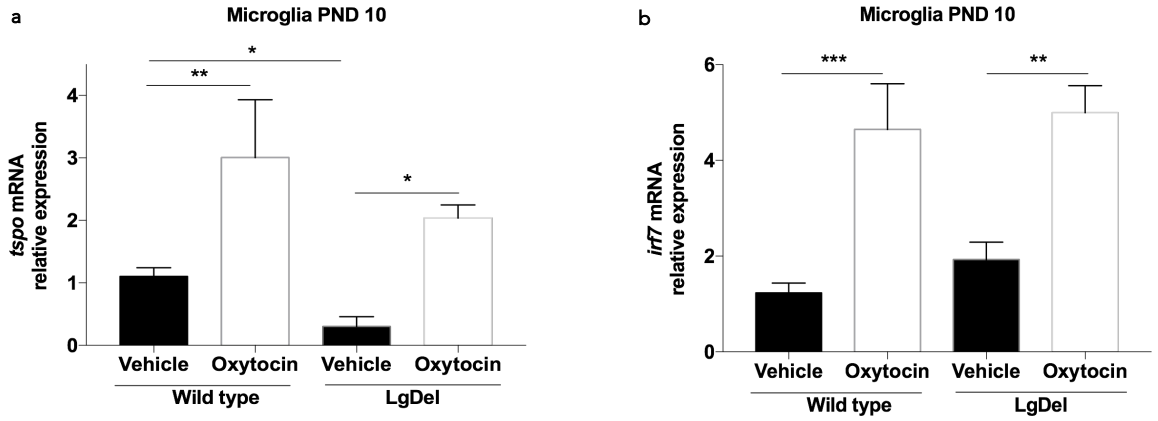
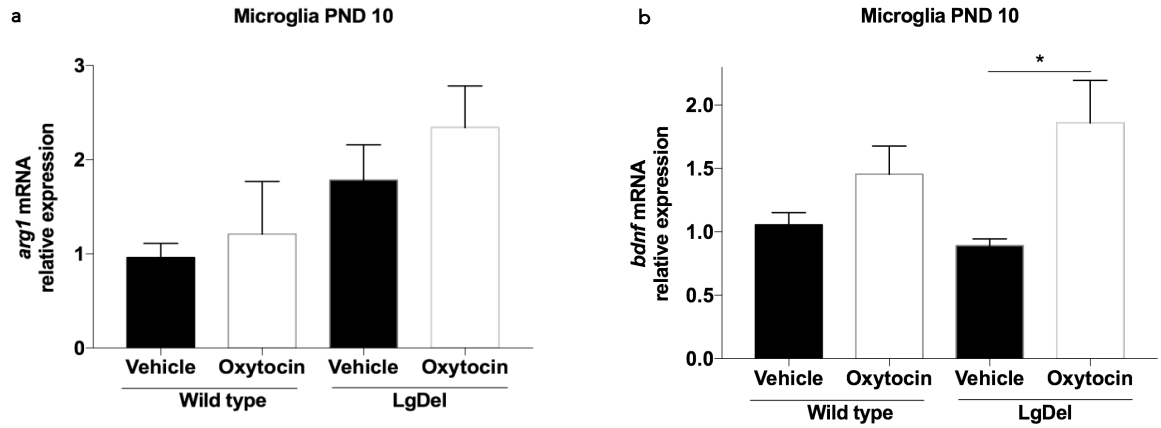


Fig 12 | Perinatal oxytocin promotes the activation of microglia of whole brain in PND 10. mRNA expression of **(a)** *tspo* and **(b)** *irf7* was measured by RT-qPCR, in microglia sorted from whole brain of PND 10 mice. Data are shown as fold-change compared with WT vehicle mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01; ***P<0.001).

Arg1 and *bdnf* mRNA expressions are higher in oxytocin treated mice, indicating an anti-inflammatory activated state of microglia both in LgDel/+ and WT mice. *Inos, cox2* and *tnfα* mRNA expressions are higher in LgDel/+ mice treated with oxytocin; indicating that the treatment induces the expression of pro-inflammatory markers in microglia in LgDel/+ but not in WT mice *(fig 13)*. These data suggest that this treatment promotes the activation of an intermedium phenotype of microglia, characterized by the simultaneous induction of pro and anti-inflammatory mediators only in LgDel/+ mice.



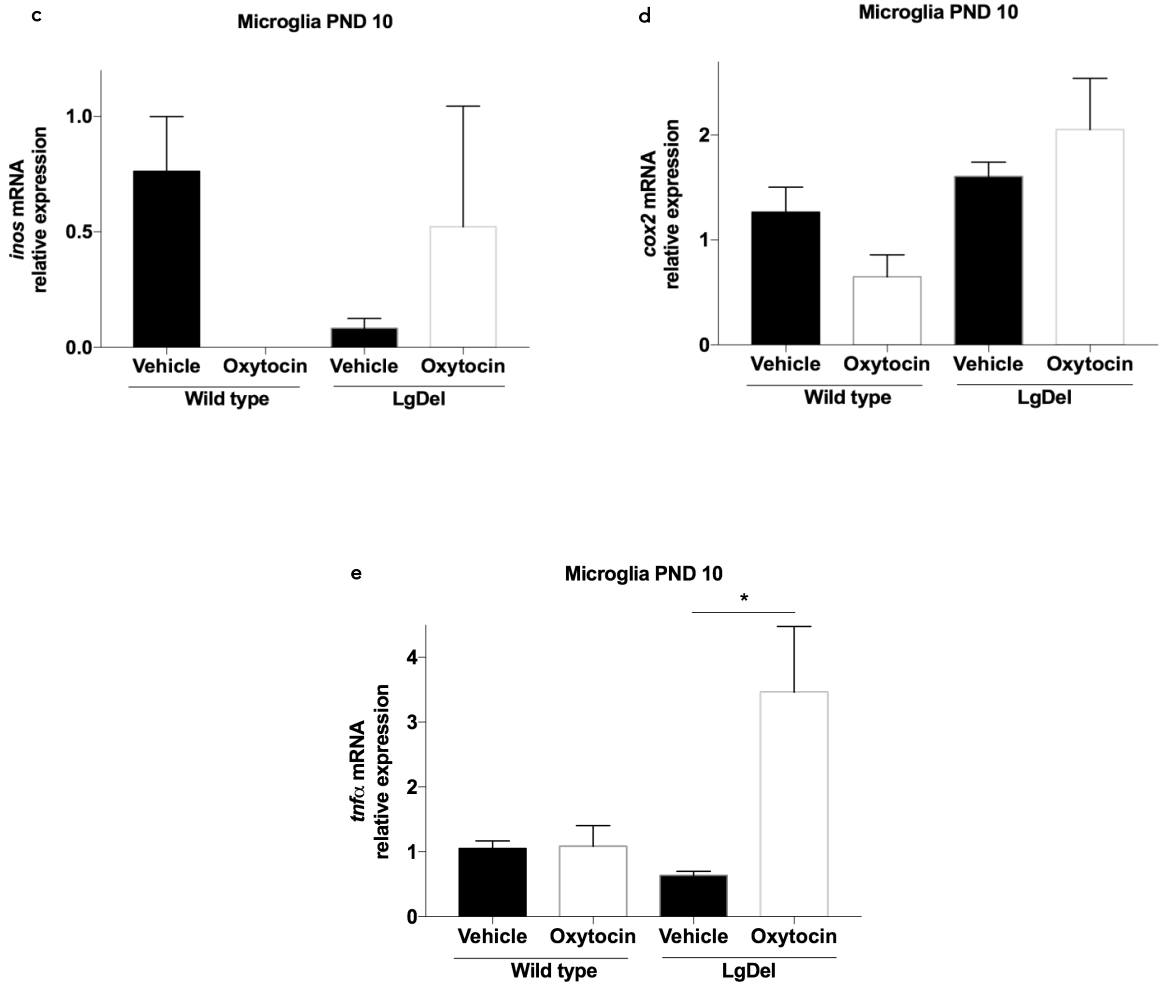
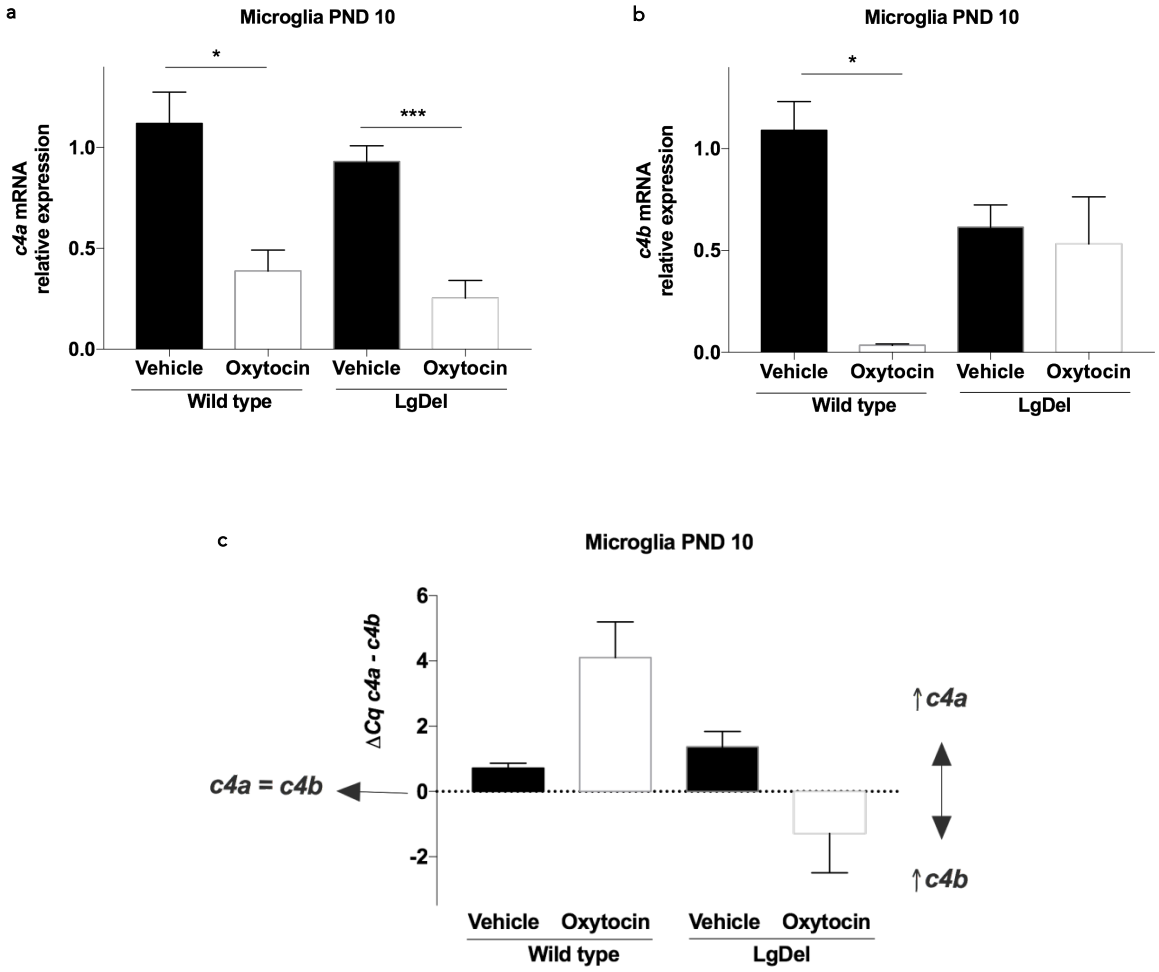


Fig 13 | Perinatal oxytocin has different effect in microglia of whole brain in WT and LgDel/+ PND 10 mice: M1 and M2 related genes are expressed simultaneously in LgDel/+ mice. mRNA expression of **(a)** *arg1*, **(b)** *bdnf*, **(c)** *inos*, **(d)** *cox2*, **(e)** *tnfα* was measured by RT-qPCR, in microglia sorted from whole brain of PND 10 mice. Data are shown as fold-change compared with WT vehicle mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

4. mRNA expression of complement 4 protein isoforms C4a and C4b in microglia as index of synaptic pruning process.

Perinatal oxytocin administration reduces both *C4a* and *C4b* mRNA expression in microglia of WT mice. This treatment decreases selectively *C4a* isoform in LgDel/+ mice, but doesn't affect the *C4b* expression *(fig 14)*. The graph below shows the difference between *C4a* and *C4b* expression for each sample; it is clear that the *C4a* isoform is downregulated by oxytocin specifically in LgDel/+ mice. Interestingly, the reduction of C4a isoform is found also in PFC in adolescent mice, and this age represents the more crucial neurodevelopmental stage for synapsis pruning process, which is compromised in schizophrenia (Sekar et al., 2016).



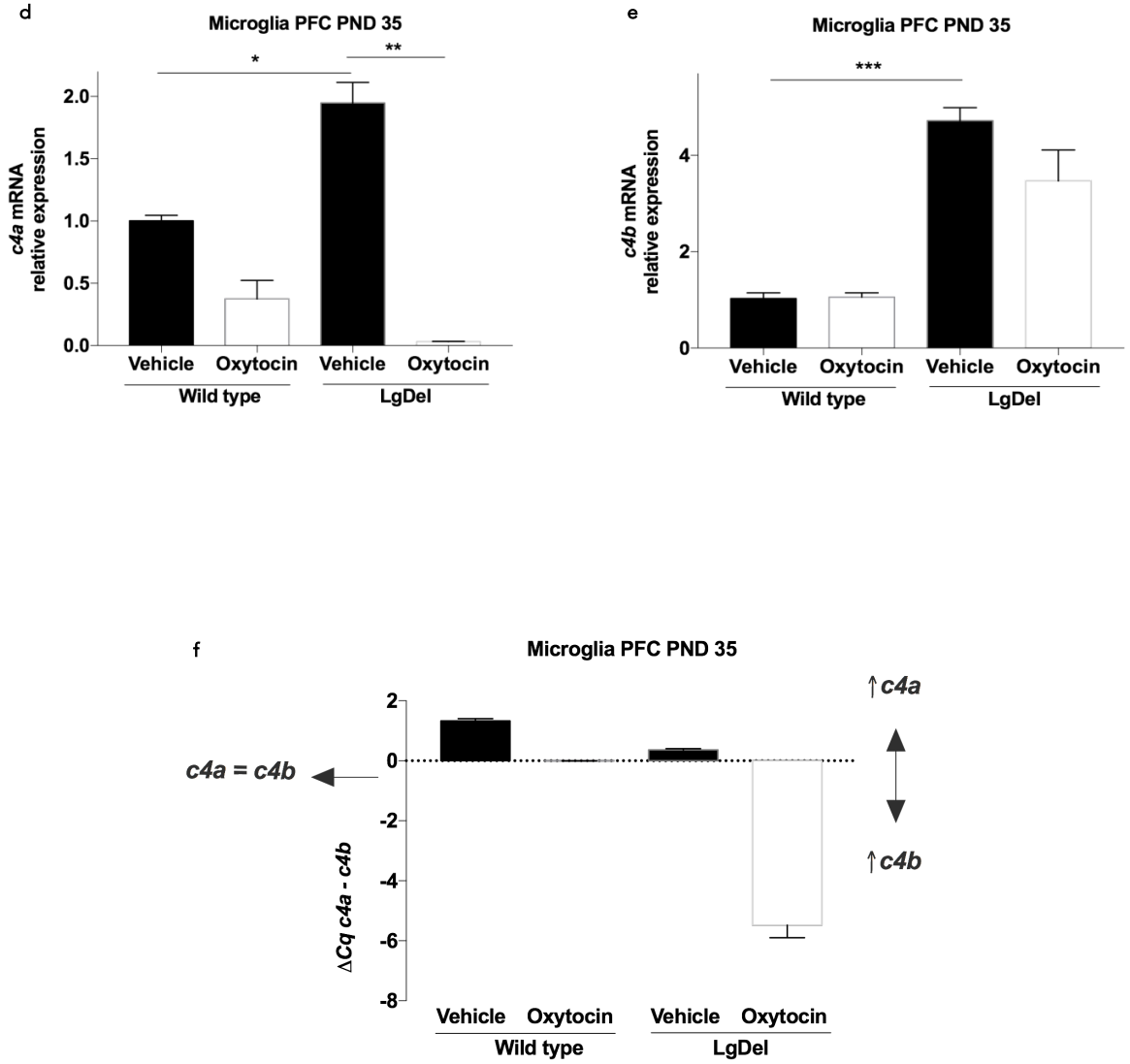


Fig 14 | Oxytocin reduced selectively the C4a isoform in PND 10 and PND 35 LgDel/+ mice. mRNA expression of *C4a* and *C4b* was measured by RT-qPCR, in microglia sorted from whole brain of **(a, b)** PND 10 mice and **(d, e)** PFC of PND 35 mice. **c** and **f** represent the Δ between *C4a* and *C4b* expression in each sample. Data are shown as fold-change compared with WT vehicle mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.1; ***P<0.001).

5. Flow cytometry analysis of macrophages accumulation in brain of PND 10 and PND 35 mice.

The count of macrophages in the brain of PND 10 and PND 35 mice was performed using the gating strategy shown below *(fig 15);* macrophages were individuated as the CD11b+ CD45^{high} Ly6c+ cells. The macrophages in whole brain of PND 10 and in PFC of PND 35 mice were significantly lower after the oxytocin perinatal treatment both in WT and LgDel/+ mice. The analyses of brains (without PFC) of PND 35 mice indicate an opposed trend, characterized by higher levels of infiltrated cells after the oxytocin treatment. This suggests that the abnormalities revealed in the whole brain at PND 10 are typical of PFC in later neurodevelopmental stages.

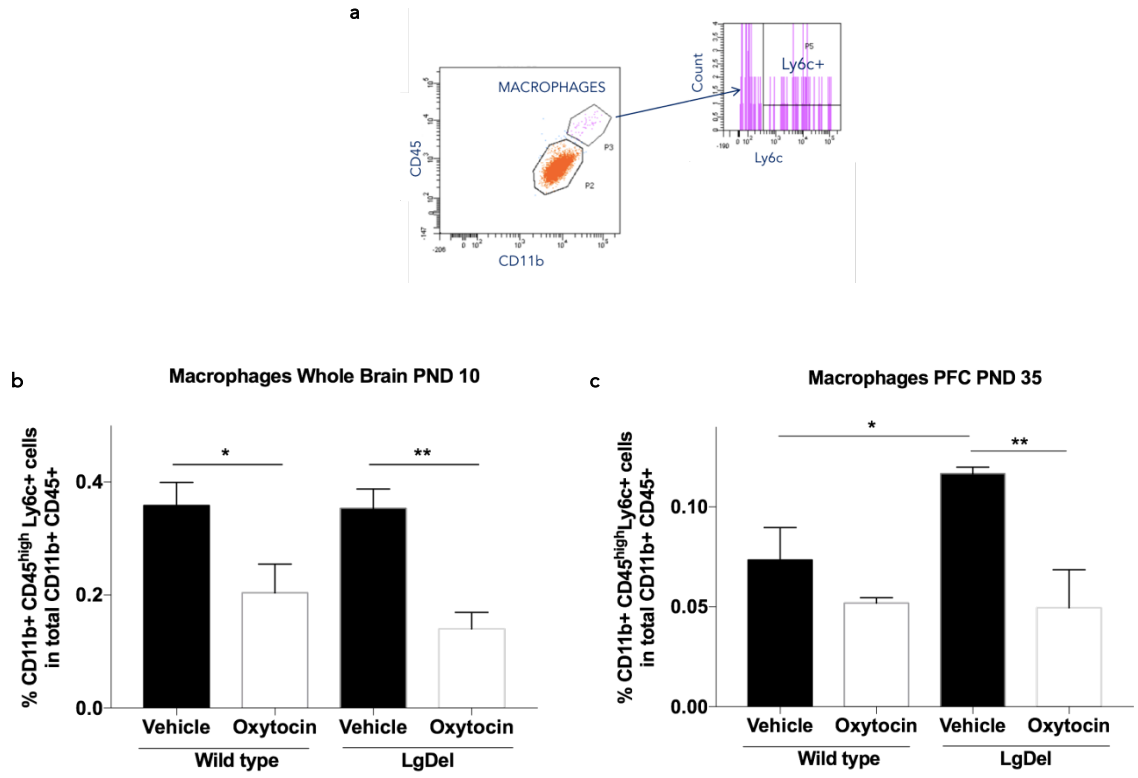
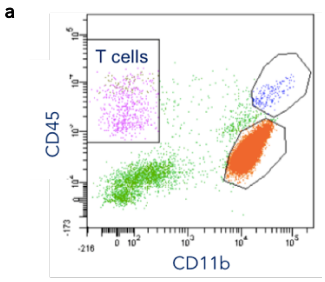
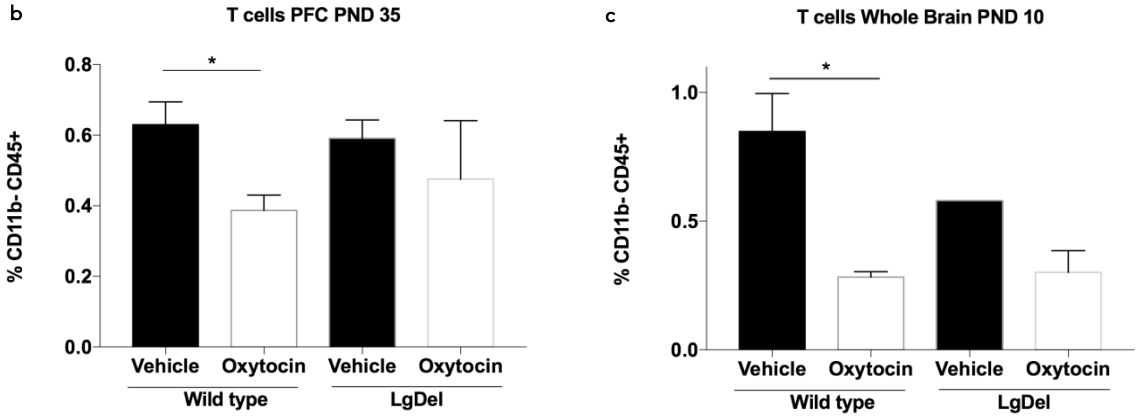


Fig 15 | Perinatal oxytocin reduces macrophages accumulation in whole brain of PND 10 mice and in PFC of LgDel/+ PND 35 mice. (a) Gating strategy and flow cytometry analysis of monocytes/macrophages in **(b)** whole brain of PND 10 and **(c)** PFC of PND 35 mice. Age-matched WT treated with oxytocin or vehicle were used as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01).

6. Flow cytometry analysis of T cells accumulation in brain of PND 10 and PND 35 mice.

T cells in the brain of PND 10 and PND 35 mice were identified as CD11b- CD45+ positive cells *(fig 16)*. Their levels in whole brain of PND 10 and in PFC of PND 35 mice were lower after the oxytocin perinatal treatment both in WT and LgDel/+ mice. T helper and T cytotoxic cells identified on the basis of CD4 expression; our results indicate lower expression of these infiltrated cells after oxytocin treatment in whole brain of PND 10 and in PFC of PND 35 mice. The analyses in brains (without PFC) of PND 35 mice do not reveal the analogous trend, indicating that these feature are typical of PFC and other brain regions.





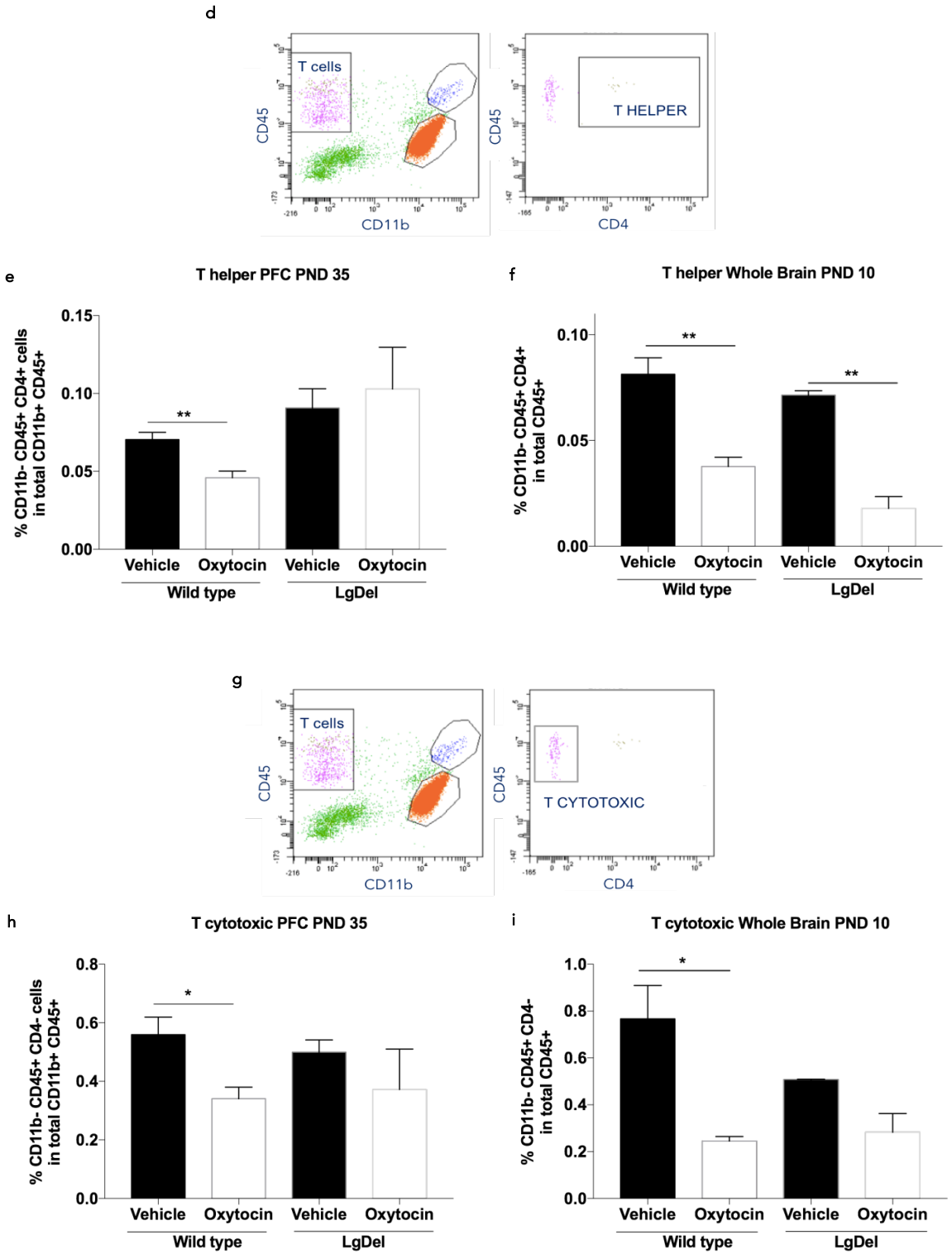


Fig 16 | Oxytocin reduces T cells accumulation in brain at PND 10 and the same effect is evident in PFC. (a, d, g) Gating strategy and **(b, c, e, f, h, i)** flow cytometry analysis of T CD11b-CD45+, T helper CD11b-CD45+CD4+, and T cytotoxic CD11b-CD45+CD4- cells in whole brain of PND 10 and PFC of PND 35 mice. Age-matched WT treated with oxytocin or vehicle were used as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01; ***P<0.001).

7. B-CSF-B activity evaluation using immunohistochemistry whole mount technique of CP from the fourth ventricle at PND 10.

For the staining of CP DAPI was used as nuclear marker, Claudin1 as epithelial cells marker, ICAM1 as an index of CP activation. F*igure 17* shows lower fluorescent intensity signal for ICAM1 in LgDel/+ mice compared to WT mice. A lower ICAM1 expression in LgDel/+ and WT mice is also evident after the treatment, indicating the capability of oxytocin in reducing cells trafficking through CP of the fourth ventricle at PND 10.

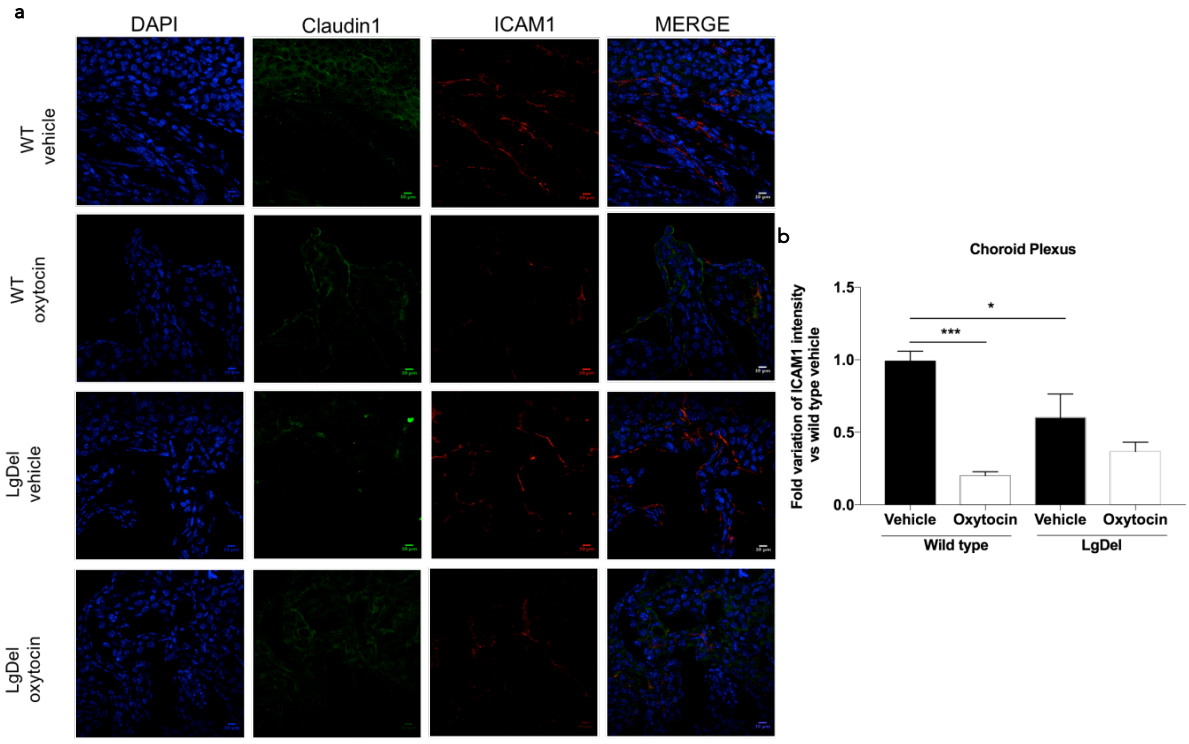


Fig 17 I Oxytocin reduces the expression of ICAM1 in CP, reducing the immune cells trafficking in PND 10 mice. (a) Representative microscopic images of CPs from fourth ventricle of PND 10 LgDel/+ mice and WT controls treated with oxytocin or vehicle, immunostained for the epithelial tight junction molecule Claudin-1 (green), DAPI nuclear staining (blue) and the integrin ligand, ICAM1 (red). **(b)** Representative quantification of ICAM1 immunoreactivity (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; ***P<0.001).

8. mRNA expression analysis of CP from all ventricles at PND 10.

For the analysis of CP activity, the expression of *icam1*, *vcam1*, *cxcl10*, *ccl2* and *ifnγ* was measured. The results shown in *figure 18* indicate lower activation of CP in LgDel/+ mice after the treatment at PND 10, but not in WT mice.

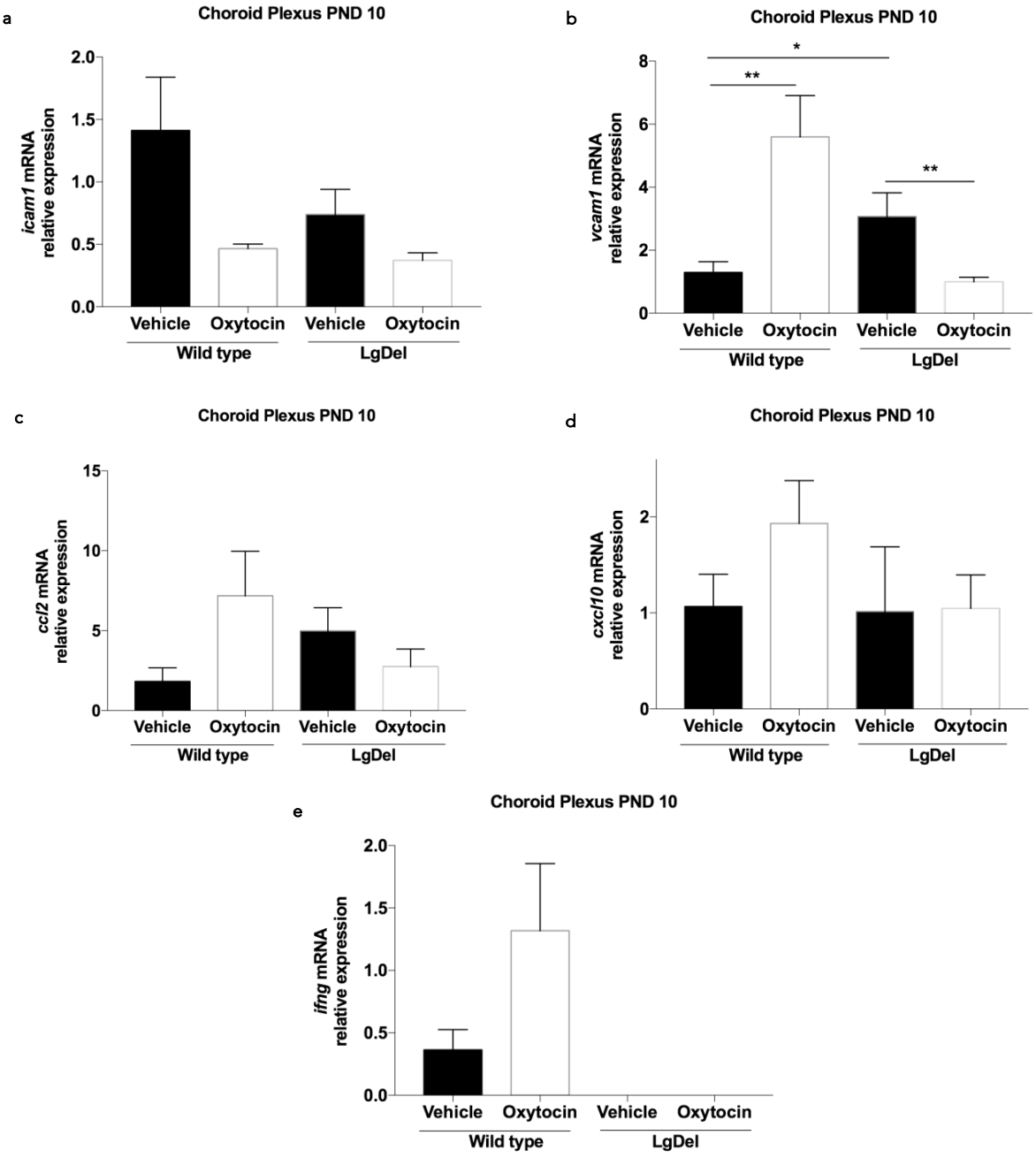


Fig 18 | Oxytocin reduces the trafficking through CP in PND 10 LgDel/+ mice. mRNA expression of **(a)** *icam1*, **(b)** *vcam1*, **(c)** *ccl2*, **(d)** *cxcl10* and **(e)** *ifnγ* was measured by RT-qPCR, in CP from all the ventricles of PND 10 mice. Data are shown as fold-change compared with WT vehicle mice as controls (n=5 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01).

9. Flow cytometry analysis of T cells accumulation in CP of PND 10 mice.

T helper cells in CP of PND 10 mice were individuated as CD45+ CD4+ population *(fig 19)*. The results indicate higher T helper cells accumulation exclusively in CP stroma of LgDel/+ treated mice.

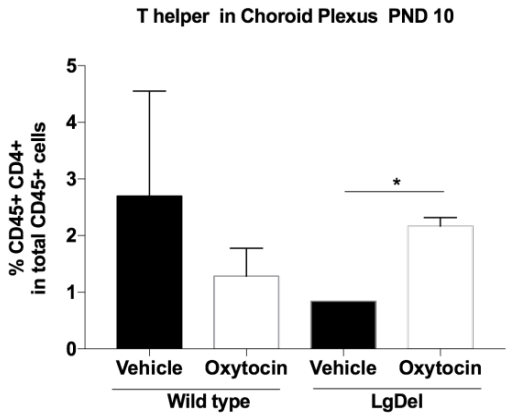


Fig 19 l: **Oxytocin induces T helper accumulation in CP in PND 10 LgDel/+ mice.** Flow cytometry analysis of CD4 T helper cells in CPs of PND 10 and PND 35 LgDel/+ mice treated with oxytocin or vehicle and age-matched WT as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05).

10. Flow cytometry analysis of Treg cells accumulation in spleen of PND 10 mice.

Treg cells in spleen of PND 10 and PND 35 mice were individuated as CD45+ TCRb+ CD4+ CD25+ Foxp3+ population *(fig 20)*. The results indicate lower Treg level in spleen of LgDel/+ mice, confirming their already known immunodeficiency (McLean-Tooke et al., 2007). The perinatal oxytocin administration increases Tregs at PND 10 and this effect is maintained also in adolescence in LgDel/+ mice. Curiously, the treatment in WT mice has opposed effect in Treg levels modulation ad it is not conserved during lifespan.

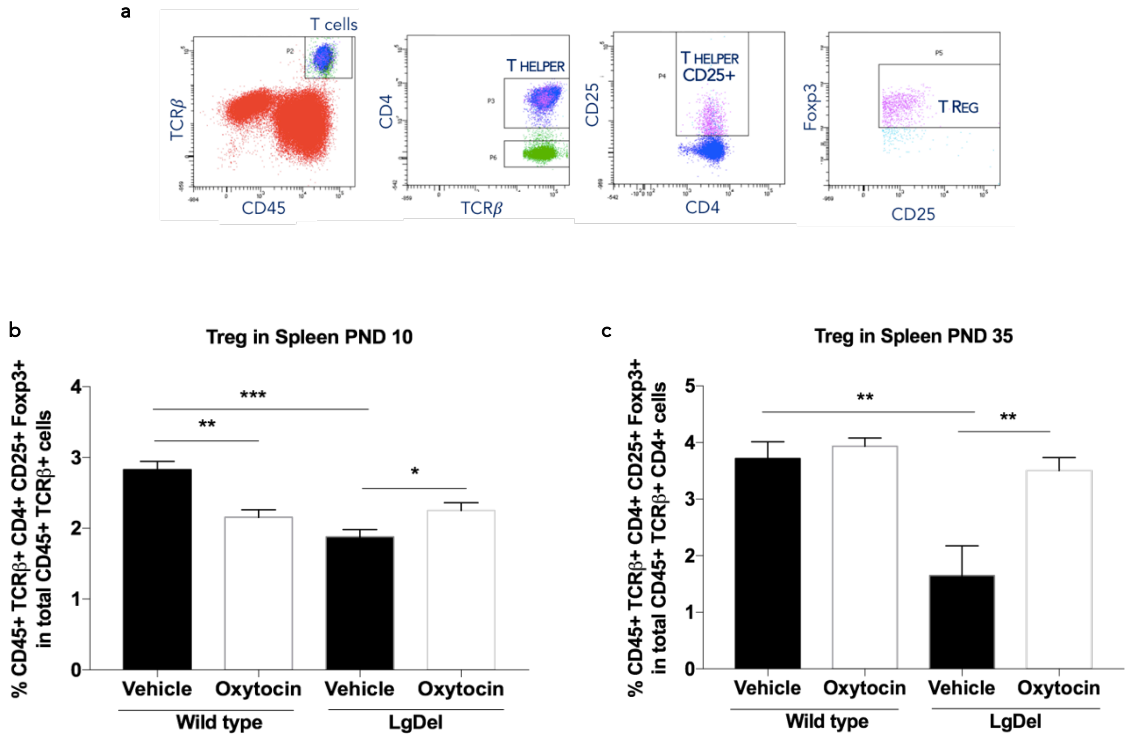


Fig 20 | Tregs reduction in PND 10 and PND 35 LgDel/+ mice; oxytocin supports self-tolerance in LgDel/+ mice inducing an increase of peripheral Treg. Gating strategy and flow cytometry analysis of T regulatory cells (intracellularly stained for Foxp3) in spleens of PND 10 and PND 35 LgDel/+ mice treated with oxytocin or vehicle and age-matched WT as controls (n=4 per group; Student's t-test. Error bars represent mean±s.e.m.; *P<0.05; **P<0.01; ***P<0.001).

DISCUSSION

The data collected in this study confirmed a direct involvement of the immune system in 22q11DS. In particular, neuro-immunological analyses conducted in LgDel/+ mice underline clear abnormalities in immune cells trafficking to the CNS. Our results also suggest the potential immunomodulatory activity of an intranasal oxytocin treatment, which was able to preserving the integrity of B-CSF-B permeability and prevent excessive immune cell infiltration in CNS during perinatal period, a neurodevelopmental stage crucial for the future development of schizophrenia and autism. Indeed, the perivascular accumulation of macrophages has been observed in post-mortem 22q11DS patients brain (Kiehl et al., 2008); and this indicates excessive immune cells trafficking, probably caused by BBB permeability deficit. As previously explained, maternal or perinatal infection have also been indicated as possible risk factors for neurodevelopmental disorders, due to the associated high production of pro-inflammatory cytokines. They can enhance BBB permeability, and the excessive cellular trafficking through BBB in early neurodevelopmental stages, causes an increased contact between the peripheral immune system and CNS-antigens, resulting in affection of self-tolerance mechanisms, which are developed during the first phases of life. Consequently, a release of the components of the complement system occurs, together with the impairment of neurogenesis process, homeostasis and brain circuits formation (Meyer et al., 2011).

Our results revealed the capability of perinatal administration of oxytocin in B-CSF-B modulation; this can be considered a compensatory activity able to reduce the excessive immune cell infiltration typical of 22q11DS, which was previously described and also confirmed by our data, showing the increase of macrophages ($p<0,05$) in PFC during adolescence (*fig 15*). The first experiments were performed at PND 10 because, in previous studies from professor Schwartz lab, the duration of CP activity perturbation, caused by different treatments, was detectable until 7 days after the last administration. In our study the treatment with oxytocin was administrated from PND 1 to PND 7, once a day, intranasally, since this administration route permits to bypass the BBB and obtain high drug concentration both in blood and CSF (Dal Monte et al., 2014). These findings suggest a stronger involvement of B-CSF-B, than to BBB, in response to intranasal oxytocin administration. The second end point was fixed at PND 35 (adolescent mice) in order to detect if immune perturbations, revealed at PND 10, could cause consequences during later neurodevelopmental stages. It is known that BBB alterations in early neurodevelopmental stages could lead to neuro-inflammation, altered levels of immune

cells in CNS, homeostasis dysregulation, disrupted neurogenesis and behavioral deficits. These data explain the reason why adding an anti-inflammatory drug to antipsychotics seems to be enhance its efficacy, especially when the first drug is started in the early phases of schizophrenia(Meyer et al., 2011).

As previously explained, these tests were conducted in whole brain for PND 10 mice in order to individuate general abnormalities in microglia number during this neurodevelopmental stage. The small dimension of brains at PND 10 addressed us to not focalize our tests in a particular brain region at this stage. The tests at PND 35 were instead performed in microglia sorted from PFC (the brain region most implicated in 22q11DS), pooled in groups of three or four, and in the rest of the brain was used as control. In detail, the comparison between LgDel/+ and WT mice revealed only few differences in immunity, consistent in lower *tspo* expression in microglia at PND 10 (*fig 12*) in 22q11DS mouse model, indicating a lower immune activation at early neurodevelopmental stage and reduced level of ICAM1 in CP at PND 10 (*fig 17*). Taken together, our data lead us to hypothesized that ICAM1 downregulation in CP of LgDel/+ is a protective process in pathological conditions, with the aim of compensating the excessive BBB trafficking characteristic in 22q11DS. In addition, an increased number of macrophages, particularly at PND 35, and not at PND 10, in the brain of LgDel/- mice was confirmed, as already reported in literature (Kiehl et al., 2008) (*fig 15)*. However, the most significant alteration between WT and LgDel/+ mice is shown in *figure 20:* Treg levels in spleen of LgDel/+ mice are significantly lower than in WT both at PND 10 and 35. This result confirms the immunodeficiency reported in literature, described as thymic hypoplasia and lower self-tolerance mechanisms efficacy, known to be driven by Treg (McLean-Tooke 2007), and shows that peripheral immune alterations are maintained for lifetime.

Data relative to several parameters show analogue outcomes in WT and LgDel/+ animals after oxytocin treatment. In WT and LgDel/+ PND 10 mice, oxytocin increases the number of microglia in whole brain; whether this was due to more extensive proliferation or massive cellular migration was evaluated by measuring *ki67* mRNA. (*fig 11)*. In a previous study, an increased number of microglia and perivascular macrophages in CNS of HIV-associated Encephalopathy (HIVE), a disorder associated with neurological deficits and immune system abnormalities, was found. In this study, the authors tested ki67 cellular expression and they excluded the proliferation as a reason for microglia and macrophages accumulation, postulating the hypothesis of higher cellular migration to the brain in pathological conditions (Fischer-Smith et al., 2004). Instead, our results indicate higher *ki67* expression in microglia of PND 10 treated groups, which show also raised microglia amount in brain, suggesting that cellular accumulation is the result of higher microglia proliferation induced by oxytocin. The analysis of PFC at PND 35 shows that in both WT and LgDel/+

mice the altered level of microglia detected at PND 10 is not conserved, demonstrating that the treatment has transient effect on microglia number regulation, detectable few days after the last administration. The FACS analysis of whole brain at PND 10 and PFC at PND 35 of both WT and LgDel/+ mice indicates a reduction of immune cells in CNS in treated mice. The cells measured by flow cytometer were monocytes/macrophages, total T cells and both T helper and T cytotoxic cells. The reduction of immune cells infiltration, in both the considered ages (*fig 15,16)*, suggests that the perinatal treatment can be efficient in blocking brain barriers excessive permeability, reducing this known risk factor for psychiatric diseases. This is a brand new evidence which gives new insights to explain the not yet clarified mechanism of action of oxytocin, known to be able to rescue social deficits in mice (*fig 5)* and to ameliorate the conditions of 22q11DS patients. The early treatment with oxytocin is more effective than a later administration (Guastella et al., 2010), because of the crucial involvement of brain barriers permeability in induction of a correct brain plasticity and homeostasis properly in perinatal stage. Since it is well known that oxytocin receptors are expressed in T cells and microglia (Ndiaye et al., 2008), this peptide plays a role in the regulation and activity of the immune system.

Interestingly, results about ICAM1 expression in CP of PND 10 mice show lower level of this adhesion protein in LgDel/+ mice compared to WT, but also a further reduction after oxytocin treatment in both WT and LgDel/+ mice. As our data revealed, the small reduction of ICAM1 in LgDel/+ mice is not able to block the accumulation of immune cells in CNS that, instead, is impaired in perinatal treated mice, at least of the LgDel/+ genotype. In fact, the PND 10 mRNA analyses of *icam1, vcam1, cxcl10, ccl2, ifnγ* essentially confirm lower B-CSF-B permeability in LgDel/+ treated with oxytocin mice compared to LgDel/+ treated with vehicle. The same analysis, relative to WT mice, shows not univocal results, and this is in agreement with the not prevedible oxytocin-induced effects found in WT animals in behavioral studies. CP was also studied by FACS and the analysis revealed a T cells accumulation only in the stroma of LgDel/+ oxytocin treated mice. The subtype of T cell involved in this process has not yet been identified; the CP cells were stained with Treg specific markers but the flow cytometry reading gave no detectable levels of expression as experimental outcome (data not shown). However, the hypothesis of Treg accumulation in CP of LgDel/+ oxytocin treated mice is the most probable because of the Treg peripheral increase demonstrated not only in PND 35 but also in PND 10 mice (*fig 20)*. The reason of failed CP Treg staining may be due to the low amount of tissue, even thou the attempt to pool CPs from two mice together. In the future, it will be important to correctly identify the T cell subtype present in the CP of LgDel/+ treated mice, trying to pool higher number of CPs for each group, in order to better delineate the oxytocin mechanism of action. Circulating Treg number variations in LgDel/+ treated mice, as already explained, suggest

the capability of oxytocin of increasing the Treg spleen level; it indicates the effect of intranasal administration of oxytocin, directly or not, in modulating also circulating cells and not only CNS microglia. The ability of circulating Treg in reducing B-CSF-B permeability is known (Baruch et al., 2015), and it remains to be clarified how spleen Treg levels variations are induced by oxytocin. Certainly, the strong increase of Treg number found in LgDel/+ treated group during adolescence and not only few days after treatment (PND 10), confirms the consistent effect of oxytocin on the modulation of immune system starting from early developmental stages until later phases of life. As expected, in WT mice the treatment did not cause the same outcomes revealed in LgDel/+ mice, according with other evidences found during our studies.

As oxytocin showed a clear effect in modulation of circulating T cells levels specifically in 22q11DS mouse model, the administration of this peptide also caused CNS immune cells phenotype alterations, not necessarily analogous for LgDel/+ and WT mice. In detail, the experiments were conducted in whole brain sorted microglia and mRNA expression analysis was performed at PND 10 to detect microglia inflammatory phenotype changes induced by oxytocin intranasal administration. The results show a higher microglia activation in both LgDel/+ and WT mice after oxytocin treatment, indicated by higher *tspo* expression (*fig 12)*. Interestingly, *irf7* expression analysis presents an analogous trend, suggesting the prevalence of M2-like phenotype of microglia. These results agree with anti-inflammatory markers mRNA expression analysis (*fig 13);* in particular, *bdnf* rise in the microglia of the LgDel/+ oxytocin treated group, indicates a possible involvement of *bdnf*-driven neurogenesis, process which should be evaluated in the future in our mice. Indeed, the role of *bdnf* in both dendritic spines modulation and in neurogenesis, process known to be dysregulated in LgDel/+ mice, had clearly been outlined (Kowianski et al., 2017; Greenberg et al., 2009). M1-like phenotype markers *inos, cox2* and *tnfα* analysis showed no correspondence in WT and LgDel/+ treated groups; a reduction of pro-inflammatory mediators was found in WT mice after oxytocin administration, while LgDel/+ mice presented an opposed response after the treatment, with higher M1-like phenotype markers levels (*fig 13)*. These findings suggest a pure anti-inflammatory activation, consistent in M1-like phenotype polarization of microglia, in WT mice in response to the treatment with oxytocin; the release of this kind of mediators is related with the reduction of CNS immune cells infiltration, confirmed by FACS analysis. Activation of microglia with increased expression of M1/M2 related genes was found in whole brain microglia from LgDel/+ mice at PND 10; the correct meaning of this peculiar microglial activated state needs to be better investigate with future experiments. These outcomes can be interpreted as the result of the coexistence of two different subpopulations of microglia (one expressing M1 and one M2 markers) at the same time or otherwise, as arise of a new type

of microglia activated type, expressing both M1 and M2 markers. The higher expression of *irf7* in microglia of treated mice, suggests that a phenotypic switch, from M1 to M2-like state, is happening at PND 10 (*fig 12)*. Probably the treatment activates, in different way and/or timing, the microglia in WT or LgDel/+ mice, but it promotes as final result anti-inflammatory cytokines secretion in both groups. This is confirmed by the other results obtained for oxytocin treated groups, which clearly demonstrated the reduction of immune cells trafficking and accumulation in CNS after oxytocin administration. Real time PCR analysis revealed a significant increase of *bdnf* expression in treated LgDel/+ mice, according with the enhanced levels of M2-like mediators and *irf7 (fig 12 and13);* this should indicate an increased neurogenesis activity, found to be compromised in early neurodevelopmental stages in 22q11DS. The microglia mRNA analysis of C4a and C4b isoforms of C4 protein of complement system revealed lower expression of C4a and strongest reduction of C4b in WT oxytocin treated mice compared to WT vehicle, revealing that this treatment is able to reduce the synaptic pruning process, in which C4a and C4b are closely involved. A recent study identified the C4a role from early neurodevelopmental stages in promoting excessive synaptic elimination in adolescence in schizophrenic patients (Sekar et al., 2016). Interestingly, the treatment in LgDel/+ mice caused the selective reduction of C4a isoform and not evident changes in C4b expression both at PND 10 and 35; because of the strongest involvement of C4a in synaptic pruning induction in 22q11DS, these findings suggest that oxytocin might be useful to revert these specific LgDel/+ mice anomalies. Sorprendently, no differences were found between WT and LgDel/+ vehicle at PND 10; however, the C4a/b expression is significantly higher at PND 35, and this confirms other groups that found an excessive synaptic elimination during adolescence of schizophrenic patients. The pruning process starts to be excessive in schizophrenia only during adolescence, even though the genome alterations are present from the first phases of life. In this context, our results suggest the efficacy of oxytocin perinatal treatment in regulate this process; it whether there are some alterations, as expected, during adolescence in LgDel/+ mice remains to be clarified.

In conclusion, the data collected in this study suggest a direct involvement of the immune system in LgDel/+ mice and the possibility to ameliorate the 22q11DS symptoms by means of an immunomodulatory approach, based on the oxytocin intranasal administration, which is able to regulate microglia inflammatory state, immune cells afflux to the brain through B-CSF-B, synaptic pruning process and probably neurogenesis. Taken together, our results indicate that oxytocin modulate B-CSF-B function; this can be considered a compensatory activity which reduces the excessive immune cells infiltration typical of 22q11DS, by inducing an anti-inflammatory response in CNS and mitigating the peripheral immune alteration.

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CONCLUDING REMARKS

Several published observations indicate a dysregulation of immune system as an etiological determinant of major psychiatric disorders. Maternal immune activation (MIA) *via* infection during pregnancy was demonstrated to be sufficient to impart lifelong neuro-pathology (Estes et al., 2016). Perinatal inflammation and both post-acute latent and persistent neuro-inflammation were found to be associated to mental disorders, such as schizophrenia and autism. Therefore, an excessive immune activation in early neurodevelopmental stages has been associated to defects in the permeability of the barriers between CNS and the peripheral blood, (Meyer et al., 2011).

Taken together, our results have shown the impairment of B-CSF-B permeability, peripheral immune tolerance process, and further, recruitment and accumulation of immune cells to the CNS in mouse models of ADHD and 22q11DS; interestingly our studies revealed also the efficacy of oxytocin perinatal treatment to revert 22q11DS-related behavioral and neuro-immunological deficits. Thus, our data obtained in the ADHD mouse model indicated a clear involvement of an altered immunological tolerance in the etiology of this disease. Basing on the evidences about ADM system role in thymic microenvironment modulation and Treg maturation (Castellani et al., 2016), we investigated the ADM system expression in thymus of these mice. Our results confirmed the potential contribute of ADM to the ADHD-related dysregulation of thymic functionality and Treg homeostasis, underlining the promising role of this peptide as a diagnostic and pharmacological target. It is worth to be noticed that this peptide has successfully been tested in animal studies for its immune regulatory activity in different experimental models of autoimmune disease (Gonzales-Rey et al., 2007; Pedreno et al., 2014; Nagata et al., 2017) and its inhibitor ADM22-52 was tested for its activity against leukemic cells (Di Liddo et al., 2016).

In conclusion, our studies revealed the impairment of specific neuro-immunological mechanisms in ADHD and 22q11DS mouse models, suggesting that the immune system might become a target for more effective interventions based on the immunomodulatory approach.

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