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Olfactory bulbectomy in mice triggers transient and long-lasting behavioral impairments and biochemical hippocampal disturbances

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ABSTRACT

Major depressive disorder (MDD) is a neuropsychiatric disease that is associated with profound disturbances in affected individuals. Elucidating the pathophysiology of MDD has been frustratingly slow, especially concerning the neurochemical events and brain regions associated with disease progression. Thus, we evaluated the time-course (up to 8 weeks) behavioral and biochemical effects in mice that underwent to a bilateral olfactory bulbectomy (OBX), which is used to modeling depressive-like behavior in rodents. Similar to the symptoms in patients with MDD, OBX induced long-lasting (*e.g.*, impairment of habituation to novelty, hyperactivity and an anxiety-like phenotype) and transient (*e.g.*, loss of self-care and motivational behavior) behavioral effects. Moreover, OBX temporarily impaired hippocampal synaptosomal mitochondria, in a manner that would be associated with hippocampal-related synaptotoxicity. Finally, long-lasting pro-oxidative (*i.e.*, increased levels of reactive oxygen species and nitric oxide and decreased glutathione levels) and pro-inflammatory (*i.e.*, increased levels of pro-inflammatory cytokines IL-1, IL-6, TNF-α and decreased anti-inflammatory cytokine IL-10 levels) effects were induced in the hippocampus by OBX. Additionally, these parameters were transiently affected in the posterior and frontal cortices. This study is the first to suggest that the transient and long-lasting behavioral effects from OBX strongly correlate with mitochondrial, oxidative and inflammatory parameters in the hippocampus; furthermore, these effects show a weak correlation with these parameters in the cortex. Our findings highlight the underlying mechanisms involved in the biochemical time course of events related to depressive behavior.

1. Introduction

Major depressive disorder (MDD) is a chronic and heterogeneous neuropsychiatric disease with a variable course and extremely high worldwide prevalence and incidence (Belmaker and Agam, 2008; Mann, 2005; Vos et al., 2012). This disorder is characterized by profound disturbances in emotional regulation, motivation, social cognition and other systemic physiological aspects that result in a poor quality of life and disability (Belmaker and Agam, 2008; Black et al., 2016). The treatment for depressive patients commonly includes a combination of psychotherapy and pharmacotherapy (Karyotaki et al., 2016); however, despite the recent advances in antidepressive drug development, more than 30% of patients do not benefit from conventional antidepressant treatments and remain with persistent symptomatology that leads to a chronic disease state (Balestri et al., 2016; Berton and Nestler, 2006). Progress in understanding the pathophysiology of major depression has been frustratingly slow (Berton and Nestler, 2006; Kim et al., 2016). Impairments in cognitive functioning (Black et al., 2016; Bora et al., 2013) and evidence of neurodegenerative symptomatology in patients with MDD (Hurley and Tizabi, 2013; Kim et al., 2016) highlight the importance of identifying the molecular pathways that contribute to the progressive nature of this disorder. The pathogenesis and temporal course of the disorder is complex and variable; thus, modeling human depressive abnormalities in animals is extremely challenging but could significantly contribute to a better understanding of the mechanisms associated with the disease (Nestler and Hyman, 2010).

Abbreviations: MDD, major depressive disorder; OBX, olfactory bulbectomy; CNS, central nervous system; OFT, open field test; i.p., intraperitoneal; ΔΨ, mitochondrial membrane potential; FL1-H, mitochondrial mass fluorescence intensity; FL3-H, mitochondrial membrane potential fluorescence intensity; ROS, reactive oxygen species; GSH, glutathione.

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In this context, the bilateral olfactory bulbectomy (OBX) has garnered attention as an animal model of depression (Hendriksen et al., 2015; Kelly et al., 1997; Song and Leonard, 2005). This model is based on the hypothesis that removal of the olfactory bulbs, which are part of the limbic system, affects their extensive efferent neuronal networks and disturbs the connection and function of the whole limbic system (Song and Leonard, 2005). The limbic circuit is essential for the maintenance of mood, emotional and memory components of behavior; thus, OBX induces depressive-like behaviors (Czeh et al., 2015; Hendriksen et al., 2015). Prominent behavioral changes that resemble the symptomatology observed in MDD patients (Hendriksen et al., 2015; Kelly et al., 1997; Song and Leonard, 2005) are apparent in the OBX animal model of depression, including anhedonia (Freitas et al., 2012) (*e.g.*, an impairment in self-care and motivational behavior), increased sensitivity to stressful environments (Hendriksen et al., 2015; Song and Leonard, 2005; Zueger et al., 2005) (*e.g.*, hyperactivity in the open field test), enhanced irritability (Song and Leonard, 2005) (*e.g.*, increased murecidal behavior and territorial aggression), and memory and cognition impairments (Holubova et al., 2016) (*e.g.*, deficits in the passive avoidance test and Morris water maze). Moreover, anatomical, cellular and biochemical changes similar to those observed in MDD patients were found in the central nervous system (CNS) of rodents that underwent an OBX, including a reduction in hippocampal volume (Wrynn et al., 2000), changes in synaptic strength (Czeh et al., 2015), impairments in mitochondrial metabolism (Rinwa et al., 2013), increased oxidative/nitrosative stress and inflammatory markers (Holzmann et al., 2015; Yang et al., 2014), and enhanced cell death (Gomez-Climent et al., 2011; Jarosik et al., 2007). Importantly, the chronic treatment of animals with antidepressants reverses the behavioral phenotypes and anatomical, cellular and biochemical changes induced by OBX (Freitas et al., 2012; Hendriksen et al., 2015; Song and Leonard, 2005). These data support the use of OBX as an important animal model to investigate the pathophysiology of MDD.

Similar to many other psychiatric disorders, the neurochemical mechanisms involved in the progression of MDD remain elusive. The time course of changes in the brain that accompany long-lasting depressive behaviors in patients is unclear. However, parallel to the progress made in the depression field, substantial data presented in the literature show that neuroinflammation plays an important role in MDD (Barnes et al., 2016; Maes et al., 2011a). Patients with major depression exhibit all of the cardinal features of inflammatory response in peripheral blood and in cerebrospinal fluid (CSF), including increased expression of pro-inflammatory cytokines, as well as their receptors in brain tissue (post mortem). It is, accompanied by a significant imbalance in the redox homeostasis, leading to a high functional damage in intracellular signaling molecules, and could influence the neuronal, astrocytic and microglial homeostasis, contributing to the neurodegenerative processes presented in the MDD (Barnes et al., 2016; Haroon and Miller, 2017; Maes et al., 2011a,b; Miller and Raison, 2016). Interestingly, OBX appears to be suitable animal model to explore the brain mechanisms associated with chronic depressive behaviors. Indeed, the OBX-induced disruption of neuronal connections between the olfactory bulbs and other brain regions resembles the neurodegenerative events in patients with MDD (Hendriksen et al., 2015). The majority of OBX studies focused mainly in two different time points (2 and/or 4 weeks after OBX surgery). Thus, there is lack of information on longer time course of the behavioral and neurochemical changes induced by OBX. To identify the putative pathways that contribute to the progression of MDD, we evaluated for 8 weeks the effects of OBX in mice by assessing behavioral patterns (*i.e.*, hyperactivity, habituation to novelty and anhedonia) and neurochemical parameters (*i.e.*, brain mitochondrial, oxidative, nitrosative and inflammatory markers) in MDD-related brain areas (*i.e.*, hippocampus, posterior cortex and frontal cortex).

2. Material and methods

2.1. Animals

Male C57BL/6 mice (45–50 days old, 20–25 g) were obtained from Fundação Estadual de Produção e Pesquisa do Rio Grande do Sul, Porto Alegre, Brazil. Animals were housed 5 per cage and housed in a room under a 12-h/12-h light/dark cycle with a controlled temperature (22 ± 1 °C) and *ad libitum* access to food and water. The cages were placed in the experimental room 24 h prior to behavioral tests, for acclimatization. All experiments were completed between 2:00 and 6:00 pm. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee (project number 24577). All efforts were made to minimize animal suffering and the number of animals used in the experiments.

2.2. Experimental schedule

To evaluate the long-term behavioral and neurochemical changes in an OBX model of depression, we designed 3 experimental schedules according to the time after surgery: 2 weeks (2W), 4 weeks (4W) and 8 weeks (8W). Naïve animals underwent an open field test (OFT) 1 day before the OBX (OFT1) to verify their baseline exploratory activity and discard any animals with behavioral abnormalities. Next, the animals were assigned to the Sham (mice that underwent the surgical procedure, but bulbs were left intact) or OBX (mice that underwent OBX) group.

The experimental scheme for the animals evaluated for 2 weeks after surgery (2W) is depicted in Fig. 1A. Accordingly, 2 weeks after surgery, the mice underwent a second OFT (OFT2). Two hours later, the mice underwent the splash test. The mice were anesthetized and euthanized the following day, and brain samples were collected. Fig. 1B shows the experimental schedule for animals evaluated for 4 weeks after surgery (4W). Two weeks after surgery, the mice underwent OFT2. A third OFT was completed 4 weeks after surgery (OFT3). The mice were submitted to the splash test 2 h after OFT3. On the following day, the mice were anesthetized and euthanized for sample collection. Fig. 1C shows the experimental schedule for animals evaluated for 8 weeks after surgery (8W). The schedule was similar to the 4W group, except OFT3 was performed 8 weeks (instead of 4 weeks) after surgery.

2.3. Bilateral olfactory bulbectomy (OBX)

2.3.1. Surgical procedure

The bilateral OBX was performed as previously described (Freitas et al., 2012) with minor modifications. Briefly, mice were anaesthetized via an intraperitoneal (i.p.) injection of xylazine (6 mg/kg) and ketamine (100 mg/kg) diluted in saline. The head was shaved and a burr hole (approximately 2 mm in diameter) was made in the skull above the olfactory bulbs 4 mm rostral to bregma. Both olfactory bulbs were then dissected with surgical micro scissors and removed by suction with a glass Pasteur pipette. Animals were excluded from the study if the bulbs were not completely removed or the frontal cortex was injured (Freitas et al., 2012).

2.4. Behavioral tests

2.4.1. Open field test (OFT)

The OFT was used as previously described (Zueger et al., 2005) to investigate locomotor/exploratory activity, habituation and anxiety. Mice were placed near the sidewall in a gray wooden box (50 × 50 × 50 cm, length × width × height) with a 200 lx white light intensity and then recorded individually for 10 min with a video-camera (positioned above and at *ca.* 90° to the square arena) that was connected to a monitor. The behavioral performance of mice was analyzed using the AnyMaze®

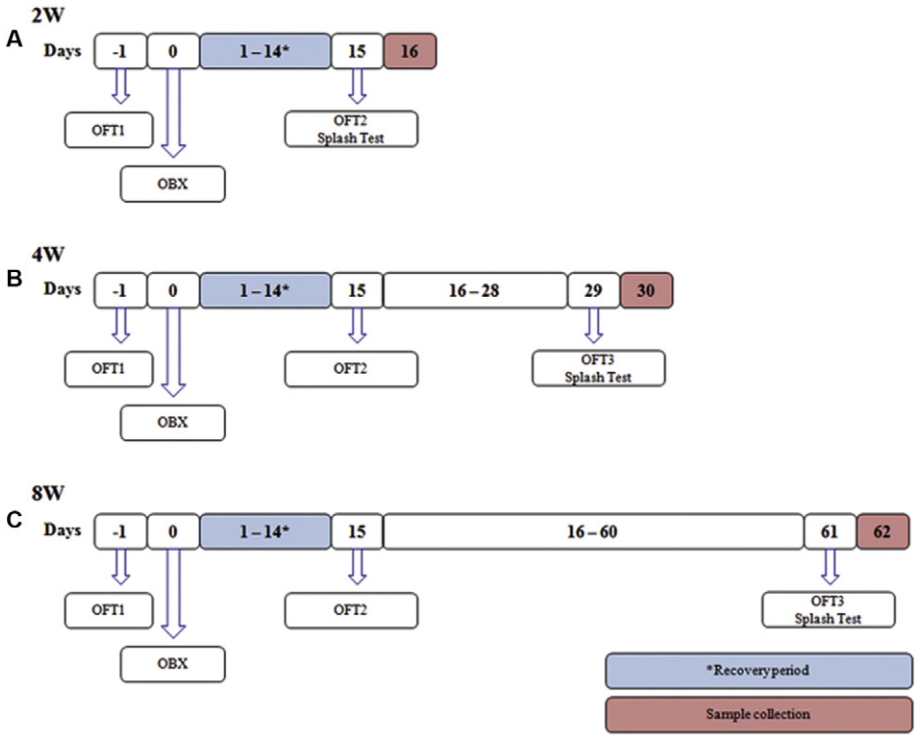

Fig. 1. Study protocols. The experimental schedule for mice consisted of 3 time points: 2 (2W), 4 (4W) and 8 (8W) weeks after the Sham or OBX surgery. Mice were euthanized on day 16 (2W), 30 (4W) or 62 (8W). Brain structures were dissected for immediate analysis (flow cytometry) or stored at −80 °C for subsequent biochemical analyses of redox and inflammatory parameters. OFT1: 1st open field exposure – all groups were tested; OFT2: 2nd open field exposure – all groups were tested; OFT3: 3rd open field exposure – only the 4W and 8W groups were tested.

software (Stoelting Co., Wood Dale, IL). Multiple parameters were determined: i) the total distance traveled in the first 3 min was used to measure habituation to novelty; ii) the decrease in the distance traveled during the 1st to 3rd minute of the test was used to measure short-term habituation to novelty; iii) total time spent immobile during the first 3 min (the minimum duration of an immobile episode was set at 5 s); iv) the distance traveled during the last 7 min of the test was used to measure locomotor/exploratory activity; v) the total time spent immobile during the last 7 min; and vi) the time and the % of distance traveled in the center zone was used to evaluate their anxiety-related phenotype. The apparatus was cleaned with 70% alcohol and dried after each test.

2.4.2. Splash test

The splash test was used to evaluate the loss of self-care and motivational behavior in mice (Freitas et al., 2012). A 10% sucrose solution was sprayed on the dorsal coat of mice. The sprayer delivered a fixed volume of 0.2 mL (each mouse received 3 sprays). As the viscosity of the sucrose solution dirties the mouse fur, the animals initiate the grooming behavior, which was considered as an index of self-cleanness. Thus, based in these natural rodent behavioral response, it is possible to infer that a decrease in grooming behavior reflect a loss of motivational and self-care behavior, which is strongly related to an anhedonic-like effect. The grooming time (a grooming episode was defined as a mouse response, such as licking, scratching or face-washing) during the first 5 min after application of the sucrose solution was recorded.

2.5. Neurochemical assays

Before the perfusion, mice were anesthetized via an intraperitoneal (i.p.) injection of xylazine (6 mg/kg) and ketamine (100 mg/kg) diluted in saline, transcardially perfused with PBS. Next, the brains were removed, and the hippocampus, posterior cortex and frontal cortex were dissected. Brain stereotaxic coordinates were obtained from Paxinos and Franklin (2004): i) frontal cortex, 3.14 mm until 1.32 mm from bregma; ii) posterior cortex (considered the total brain cortex excluding the frontal cortex), 1.34 mm until 4.04 mm from bregma. The samples were immediately processed for flow cytometry or frozen at −80 °C for other biochemical evaluations.

2.5.1. Flow cytometry

The mitochondrial mass and membrane potential ($\Delta\Psi$) were determined in synaptosomal-enriched preparations using flow cytometry (Becton Dickinson BD FACS Calibur cytometer). Mitochondrial mass (FL1-H) was detected with MitoTracker® Green FM (Life Technologies) labeling, and the mitochondrial membrane potential - $\Delta\Psi$ (FL3-H) was detected with Mitotracker™ Red detection.

2.5.2. Synaptosomal preparations

Synaptosomal preparations were obtained as previously described (Almeida et al., 2016) with minor modifications. Briefly, tissue samples were homogenized (manual small capacity Teflon/glass homogenizer in 10 × volume/weight) in 10 mM Tris buffer (pH = 7.4) containing 0.32 M sucrose, 1 mM EDTA and 0.25 mM DTT. The homogenates were centrifuged in microfuge tubes (1.5 mL per tube) at 1000*g* for 10 min at 4 °C using a fixed-angle rotor. The resulting supernatant was centrifuged at 11,000*g* for 20 min at 4 °C using the same rotor. The synaptosomal-enriched pellet was then washed twice with HBSS (pH = 7.4) by centrifugation at 16,000*g* for 10min at 4 °C to remove excess sucrose. The synaptosomal preparation was incubated with MitoTracker™ Green FM and Red FM dyes (100 nM each) for 45 min at 37 °C. The mean fluorescence intensity of FL1-H and FL3-H was used to estimate mitochondrial mass and $\Delta\Psi$, respectively. The emission of fluorescence was measured using green (FL1-H; 530 nm/30) and red (FL3-H; 670 nm long pass) bandpass filters with a FACSCalibur platform and CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA). Data from 40,000 events from synaptosomal preparation were acquired for FL1-H and FL3-H using forward scatter (FSC) and side

scatter (SSC) parameters with linear and log scales. All analyses were performed using Flow Jo software 7.6.3 (Treestar, Ashland, OR).

2.5.3. Estimation of redox homeostasis

2.5.3.1. Intracellular levels of reactive oxygen species (ROS). Tissue samples were homogenized in phosphate-KCl (20 mM/140 mM) buffer, pH = 7.4, and centrifuged at 1,000*g* for 5 min at 4 °C. An aliquot of the supernatant was used to evaluate DCFH-DA oxidation. DCFH oxidation was used to measure intracellular ROS levels. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) is hydrolyzed by intracellular esterases to produce dichlorofluorescin (DCFH). This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by cellular oxidants. The fluorescence intensity was determined at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) (Perez-Severiano et al., 2004).

2.5.3.2. Nitrite levels. Nitrite levels were determined by measuring the nitrite levels (a stable oxidation product of NO) in tissue homogenates using the Griess reaction. The Griess reagent was a 1:1 mixture of 1% (w/v) sulphanilamide in 2.5% (w/v) phosphoric acid and 0.1% (w/v) *N*-(1-napththyl) ethylene diamine dihydrochloride in deionized water. Briefly, the tissue was homogenized in phosphate-KCl (20 mM/140 mM) buffer, pH = 7.4 and centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was deproteinized with 20 μL TCA 25%, centrifuged at 2000*g* for 10 min at 4 °C and immediately neutralized with 2 M potassium bicarbonate. After this procedure, the Griess reagent was added directly to the neutralized sample. The sample was then incubated in the dark for 15 min at 22 °C (Hansel et al., 2014). Samples were analyzed at 550 nm on a microplate spectrophotometer. Nitrite concentrations were calculated using a standard curve, and the results are expressed as percentages relative to the control conditions.

2.5.3.3. Glutathione (GSH) levels. GSH levels were assessed as previously described (Hansel et al., 2014). The tissues were homogenized in a phosphate-KCl (20 mM/140 mM) buffer, pH 7.4, containing 5 mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The tissue homogenates were centrifuged at 1000*g* for 5 min at 4 °C. The supernatant was mixed with *o*-phthaldialdehyde (1 mg/mL methanol) and incubated at 22 °C for 15 min. The fluorescence intensity was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. A calibration curve was created with standard GSH solutions. GSH concentrations were calculated as nmol/mg protein.

2.5.4. Inflammatory cytokine levels

The samples were homogenized in a PBS/Tris-HCl/SDS 5% solution, pH 7.4, and centrifuged at 5000*g* for 10 min at 4 °C. Commercial enzyme-linked immunosorbent assay (ELISA) kits for rat IL-1, IL-6, TNF-α and IL-10 were used according to the manufacturer's instructions (eBIOSCIENCE, San Diego, CA, USA). Briefly, 96-well microplates were incubated with the primary antibody at 4 °C overnight, washed and then blocked at room temperature for 1 h. The cytokine standards, calibrators, and samples were added to the plate in triplicate and incubated at room temperature for 2 h. After washing, the secondary antibody conjugated with avidin-horseradish peroxidase was added, and the plate was incubated at room temperature for 1 h. After this procedure, the samples were washed and a tetramethylbenzidine (TMB) chromogen was added. The reaction was allowed to proceed for 15 min. The enzyme reaction was stopped by adding 1 M phosphoric acid (stop solution). The absorbance was measured at 450 nm. The results for the tissue sample are expressed as picograms per milligram of proteins.

2.5.5. Protein determination

Protein content was measured using the Pierce BCA® protein kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin as a standard.

2.6. Statistical analysis

A two-way ANOVA followed by Bonferroni post hoc test was used to analyze the effect of OBX surgery on behavioral and biochemical parameters and the time course changes induced by surgery in the Sham and OBX groups [factors: (1: surgery) Sham *versus* OBX within post-surgery time points and (2: time) comparison of different time points (naïve, 2W, 4W and 8W) within each group (Sham/OBX)]. Correlations among the grooming time in the Splash test and the first 3 min of distance travelled in the OFT, the last 7 min of distance travelled in the OFT and time in the center zone in the OFT and the correlation between the mitochondrial synaptosomal analysis with first 3 min of distance travelled in the OFT, the last 7 min of distance travelled in the OFT were analyzed by Pearson's correlation. The strength of the correlation was described using the guide suggested by Evans in 1996. Correlations were considered statistically significant at $r \geq 0.60$. All statistical procedures were carried out using Graph Pad Prism (Graph Pad Software, version 5, San Diego, CA, USA).

3. Results

Fig. 2A and B shows the minute-by-minute distance traveled for mice from the naïve, Sham and OBX groups (see ESM_1 for a representative supplementary video).

During the first 3 min of the OFT, comparisons of naïve mice with the Sham groups (2W, 4W and 8W) revealed that repeated exposure to the OFT induced significant decreases in both, the distance traveled (Fig. 2C; $F(3,172) = 6.275$, $p < 0.01$), and in the difference in distance traveled between the 1st and 3rd minutes of testing (Fig. 2D; $F(3,172) = 5.078$, $p < 0.01$), accompanied by a significant increase in the time spent immobile (Fig. 2E; $F(3,172) = 4.380$, $p < 0.01$), demonstrating that the Sham groups presented a strong habituation to novelty in OFT. However, OBX mice presented a significant long-lasting impairment in the habituation to novelty compared with the naïve group, as evidenced by no decrease in the distance traveled during the first 3 min (Fig. 2B; $F(3,172) = 1.940$, $p = 0.4134$) and no change in the distance traveled between the 1st and 3rd minute of testing (Fig. 2C; $F(3,172) = 0.9134$, $p = 0.9328$) or the time spent immobile (Fig. 2E; $F(3,172) = 1.831$, $p = 0.4134$). Additionally, comparing the OBX mice with their respective Sham groups from the 2nd week until the 8th weeks after surgery, a significant increase in the distance traveled (Fig. 2C; $F(1,172) = 106.2$, $p < 0.0001$) and in the difference in distance traveled between the 1st and 3rd minutes of testing (Fig. 2D; $F(1,172) = 18.80$, $p < 0.0001$), accompanied by a significant decrease in the time spent immobile (Fig. 2E; $F(1,172) = 57.09$, $p < 0.0001$) were observed.

Finally, during the last 7 min of testing, comparing the OBX mice with their naïve group a significant increase in the distance travelled was observed only in the 2W and 4W groups (Fig. 2F; $F(3,172) = 5.482$, $p < 0.01$). Furthermore, a long-lasting hyperactivity was observed in the OBX groups compared with their respective sham groups, as evidenced by a significant increase in the distance traveled (Fig. 2F; $F(1,172) = 51.27$, $p < 0.0001$). Interestingly, the Sham animals from the 4W and 8W groups but not the 2W group showed a significantly longer immobility time compared with their naïve counterparts (Fig. 2G; $F(3,172) = 4.503$, $p < 0.01$).

OBX caused a long-lasting anxiogenic effect, since the OBX mice presented a significant decreased in % of distanced traveled in the center zone in the 2W, 4W and 8W groups compared with the naïve group (Fig. 3A, $F(3,172) = 5.209$, $p < 0.01$). Similarly, comparing the OBX mice with their respective Sham groups a significant decreased in % of distanced traveled in the center zone in the 2W and 4W were observed

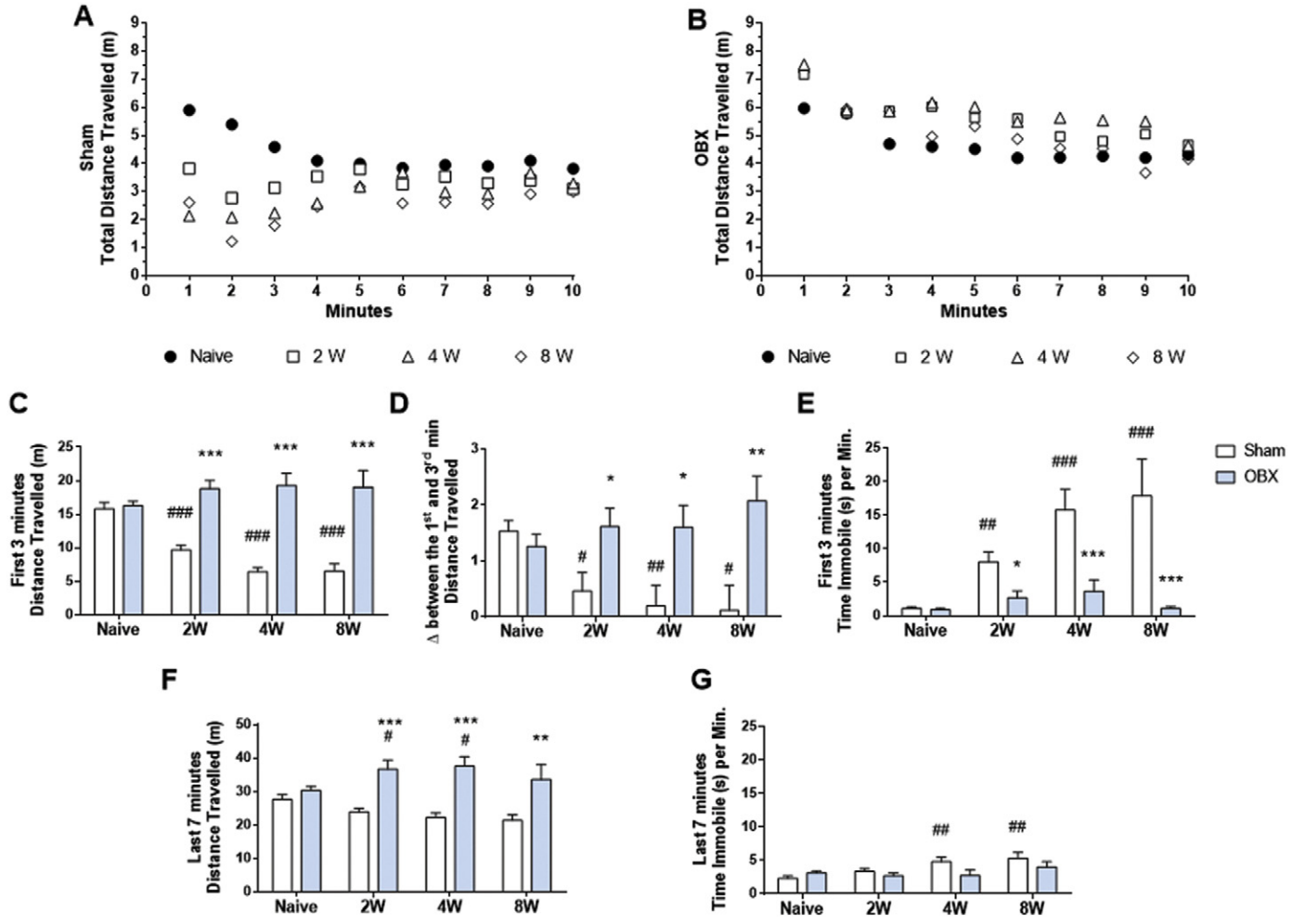

Fig. 2. Effect of OBX on habituation to novelty and locomotor activity in the OFT. A minute-by-minute analysis of the locomotor activity of Sham (A) and OBX (B) groups during the OFT. Each point represents the mean of the group. The effect of time and surgery on the distance traveled during the first 3 min of testing (C), the change in the distance traveled between the 1st and 3rd minute of testing (D), the time spent immobile during the first 3 min of testing (E), the distance traveled during the last 7 min of testing (F) and the time spent immobile during the last 7 min of testing (G). Each column represents the mean ± S.E.M. Data were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the respective Sham group (surgery effect); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the respective naïve group (time effect) (n = 15 animals/group).

(Fig. 3A, $F(1,172) = 15.32$, $p < 0.0001$). Finally, OBX mice also showed a decrease in the time spent in the center zone compared with the naïve group (Fig. 3B; $F(3,172) = 5.598$, $p < 0.001$) and with the 2W and 4W Sham groups (Fig. 3B; $F(1,172) = 20.29$, $p < 0.0001$).

The splash test was used to measure anhedonic-like behavior (*i.e.*, hedonic state, the ability to gain pleasure and motivational behavior). The transient anhedonic-like behavior induced by OBX was evidenced by a decrease in grooming time at 2 and 4 weeks; however, this effect was not present at 8 weeks (Fig. 4; $F(1,54) = 18.87$, $p < 0.0001$). There were transient correlations between grooming time and two OFT parameters, including the distance traveled during the first 3 min of testing (ESM_2A) and the distance traveled during the last 7 min of testing (ESM_2B). There were long-lasting correlations between grooming time and the time spent in the center zone of the arena (ESM_2C).

Synaptosomal preparations from hippocampus showed a transient impairment in mitochondrial parameters, including a decrease in mitochondrial mass (Fig. 5A; $F(1,30) = 13.85$, $p = 0.001$) and $\Delta\Psi$ (Fig. 5B; $F(1,30) = 18.53$, $p < 0.001$), in the 2W and 4W OBX groups but not the 8W group. There were only transient correlations between the distance travelled in the first 3 min and the distance travelled in the last 7 min with synaptosomal mitochondrial $\Delta\Psi$ (ESM_3A and B, respectively). However, it is important to mention that no significant differences were observed in the mitochondrial mass or $\Delta\Psi$ from whole-cell preparations of the hippocampus, posterior cortex and frontal cortex (data not shown).

Evaluation of the events from positively stained mitochondria (Mito+) in the hippocampal synaptosomal preparation revealed that OBX had no effect on mitochondrial mass (Mito+) (Fig. 5C; $F(1,30) = 0.0194$, $p = 0.8901$), and a transient effect on mitochondrial $\Delta\Psi$ (Mito+), as evidenced by a decrease in mitochondrial $\Delta\Psi$ from the 2W and 4W groups but not the 8W group (Fig. 5D; $F(1,30) = 9.076$, $p < 0.01$). Additionally, there were only transient negative correlations between the distance traveled during the first 3 min and the distance traveled during the last 7 min *versus* the synaptosomal positively stained with mitochondria in mitochondrial $\Delta\Psi$ parameter (ESM_3C and D, respectively).

In the hippocampus, OBX induced a long-lasting effect on redox homeostasis. The OBX groups showed a significant increase in DCFH and nitrite levels (Fig. 6A; $F(1,24) = 58.069$, $p < 0.0001$, and B; $F(1,24) = 26.68$, $p < 0.0001$) and a significant decrease in GSH levels (Fig. 6C; $F(1,24) = 87.55$, $p < 0.0001$) compared with their respective Sham groups.

In the posterior cortex, OBX induced a transient imbalance in redox homeostasis. A significant increase in DCFH levels was observed in the 2W and 4W OBX groups but not the 8W OBX group (Fig. 6D; $F(1,24) = 46.02$, $p < 0.0001$). A significant increase in nitrite levels was observed in the 2W OBX group but not the 4W and 8W OBX groups

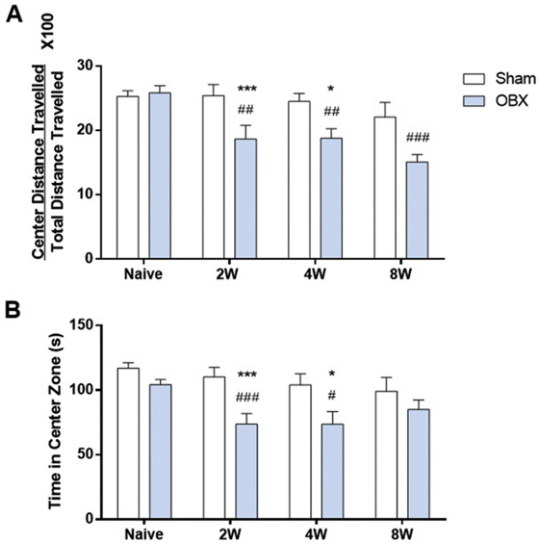

Fig. 3. The effect of OBX on anxiety-related behavior in the OFT. The effect of time and surgery on the time spent in the center zone (A) and the percentage of distance traveled in the center (B) by the Sham and OBX groups. Each column represents the mean ± S.E.M. Data were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the respective Sham group (surgery effect); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the respective naïve group (time effect) (n = 15 animals/group).

(Fig. 6E; $F(1,24) = 2.595$, $p = 0.1188$). A significant decrease in GSH levels was observed in the 2W and 4W OBX groups but not the 8W OBX group (Fig. 6F; $F(1,24) = 43.69$, $p < 0.0001$). In the frontal cortex, a transient disruption in redox homeostasis was evidenced by the significant increase in DCFH levels (Fig. 6G; $F(1,24) = 10.24$, $p < 0.01$) and significant decrease in GSH levels in the 2W OBX group but not the 4W and 8W OBX groups (Fig. 6I; $F(1,24) = 5.832$, $p < 0.05$).

There were only long-lasting correlations between the distance traveled during the first 3 min in OFT *versus* the imbalance in hippocampal redox homeostasis (intracellular ROS and GSH levels) (ESM_4A and C), and a transient correlation between the distance traveled during the first 3 min in OFT *versus* nitrite levels intracellular levels (ESM_4B).

OBX caused a long-lasting inflammatory response in hippocampus, as evidenced by a significant increase in the hippocampal pro-inflammatory cytokines IL-1 (Fig. 7A; $F(1,24) = 97.09$, $p < 0.0001$), IL-6 (Fig. 7B; $F(1,24) = 94.39$, $p < 0.0001$) and TNF-α (Fig. 7C; $F(1,24) = 83.56$, $p < 0.0001$) and a significant decrease in the anti-inflammatory cytokine IL-10 (Fig. 7D; $F(1,24) = 54.66$, $p < 0.0001$).

In the posterior cortex, OBX caused a mild but long-lasting inflammatory response. The OBX groups showed a significant increase in IL-1 (Fig. 7E; $F(1,24) = 30.57$, $p < 0.0001$) and a transient significant increase in TNF-α (Fig. 7G; $F(1,24) = 0.1359$, $p = 0.7151$). In the frontal cortex, OBX caused a permanent significant increase in IL-1 (Fig. 7I; $F(1,24) = 43.85$, $p < 0.0001$) and IL-6 (Fig. 7J; $F(1,24) = 35.29$, $p < 0.0001$), a transient significant increase in TNF-α (Fig. 7K; $F(1,24) = 34.03$, $p < 0.0001$) and a transient significant decrease in IL-10 (Fig. 7L; $F(1,24) = 33.60$, $p < 0.0001$).

There was only a long-lasting correlation between the distance traveled during the first 3 min of testing *versus* the hippocampal inflammatory response (*i.e.*, IL-1, IL-6, TNF-α and IL-10 release) (ESM_5A–D).

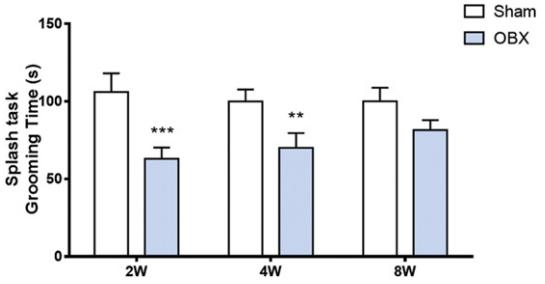

Fig. 4. The effect of OBX on self-care and motivational behavior. The effect of time and surgery on grooming time in the splash test for Sham and OBX mice. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the respective Sham group (n = 8–12 animals/group).

4. Discussion

We are the first to describe the transient and long-lasting effects (up to 8 weeks) of the OBX mouse model of depression. We observed a long-lasting impairment in the habituation to novelty, hyperactivity and anxiety-like phenotype in the OFT and a transient loss of self-care and motivational behavior in the splash test. The neurochemical analysis revealed that the hippocampus was the most affected brain structure compared with the posterior and frontal cortices. We observed multiple neurochemical changes in OBX mice: i) specific and transient impairment in synaptosomal (not in whole-cell) mitochondria mass and $\Delta\Psi$, which may be associated with hippocampal-related synaptotoxicity; and ii) long-lasting hippocampal imbalance in redox and inflammatory homeostasis. Our findings are strengthened by the presence of significant correlations between the behavioral and neurochemical parameters. Considering that the physiopathology of MDD and necessity for the novel therapeutics drugs remain under investigation our data highlight promising future targets for the depression field.

4.1. OBX induced long-lasting behavioral changes: potential translational relevance

The classical and the most widely accepted behavioral pattern in the OBX model of depression is the remarkable increase in locomotor/exploratory activity during the OFT (Czeh et al., 2015; Hendriksen et al., 2015; Kelly et al., 1997; Song and Leonard, 2005). Here, for the first time, the hyperactivity in OFT was evident for up to 8 weeks after OBX. OFT is a relevant tool for assessing behavioral disturbances in rodents (Gonzales et al., 2015; Padilla et al., 2010), and there is a wide diversity of symptoms present in mood disorders (Belmaker and Agam, 2008; Mann, 2005). However, there is lack of studies on the time-course of OBX-induced OFT behavioral changes (Mucignat-Caretta et al., 2006). To address this knowledge gap, we explored the long-term behavioral patterns of OBX.

Mice typically exhibit less exploratory behavior during the first few minutes of testing in a familiar open field arena (Almeida et al., 2010; Padilla et al., 2010). This parameter is a measure of habituation to a novel environment. Here, we observed a normal habituation performance by Sham animals (*i.e.*, a decrease in the total distance traveled, a lack of change in the distance traveled between the 1st and 3rd minute of testing, and an increase in immobility during the first 3 min of testing). In contrast, OBX mice showed long-lasting impairments and did not habituate to the open field up to 8 weeks post-surgery. OBX mice also showed chronic hyperactivity. Interestingly, all of these observations can be compared with clinical features that demonstrate a remarked cognitive decline in depressed patients (Cobb et al., 2016; Schmaal et al., 2016), which is predominantly diagnosed by strong declarative memory deficits (Bora et al., 2013; Papakostas, 2014; Vythilingam et al., 2004) rather than psychomotor agitation (Papakostas, 2014), a less frequent symptom of MDD. Considering that persistent cognitive decline is observed in MDD patients, the OBX model has good face (symptomatic homology) and constructs validity (theoretical rationale) to modeling the disturbances presented in depressive patients.

Another important aspect of mood disorders is the comorbidity between anxiety and MDD (Hofmeijer-Sevink et al., 2012; Stein and Sareen, 2015). Depression ranks among the top most frequent co-existing disorders with anxiety (Stein and Sareen, 2015). The

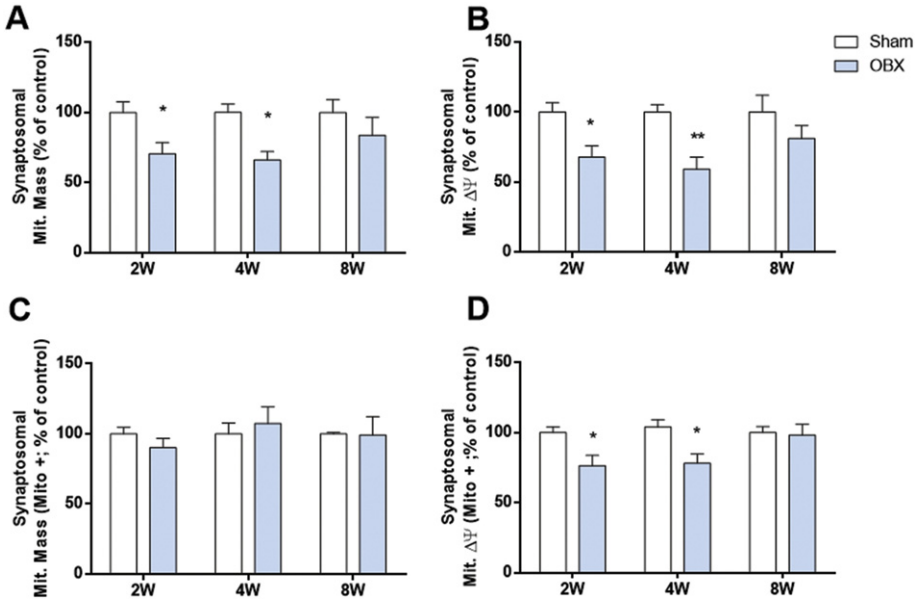

Fig. 5. The effect of OBX on the mass and ΔΨ of hippocampal synaptic mitochondria. The effects of OBX on hippocampal mitochondrial mass (A) and ΔΨ (B) and the mitochondrial mass (C) and ΔΨ (D) of only viable mitochondria (Mito+) from synaptosomal-enriched preparations. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p < 0.05 and **p < 0.01 compared to the respective sham group (n = 6 animals/group).

co-existence of these disorders increases the tendency toward chronicity and severity (Balestri et al., 2016), suggesting that they may also share common pathophysiological mechanisms. The OFT is associated with increased stress and/or anxiety, which explains OBX-related hyperactivity (Song and Leonard, 2005). We demonstrated long-lasting anxiety-like behavior, including decreased exploration of the center zone of the OFT arena. Considering that hyperactivity is a putative sign of agitation-like behavior in anxious patients (Gupta et al., 2014) and that the comorbidity of anxiety disorder and MDD increases the chronicity of the disease (van Loo et al., 2014), we postulate that the long-lasting impairment in habituation to novelty and anxious phenotype induced by OBX is sustained by the interaction between anxious and depressive behaviors. Importantly, other behavioral tests in rodents can be used to demonstrate depression-related phenotypes, such as object recognition, y-maze, passive avoidance,

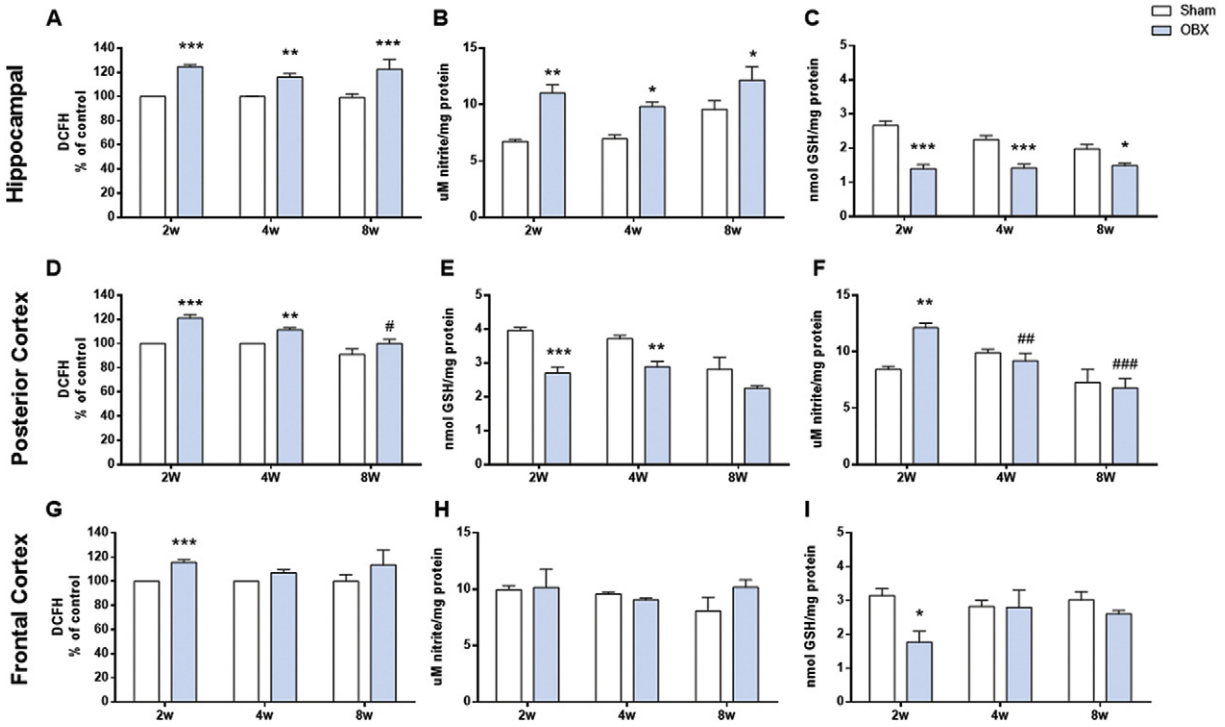

Fig. 6. The effect of OBX on redox homeostasis. Effects of OBX on the levels of DCFH (A), (D) and (G) in hippocampus, posterior cortex and frontal cortex, respectively; of nitrite levels (B), (E) and (H) in hippocampus, posterior cortex and frontal cortex, respectively; and of GSH (C), (F) and (I) in hippocampus, posterior cortex and frontal cortex, respectively. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the respective Sham group (surgery effect); #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to the respective OBX 2W group (time effect) (n = 5 animals/group).

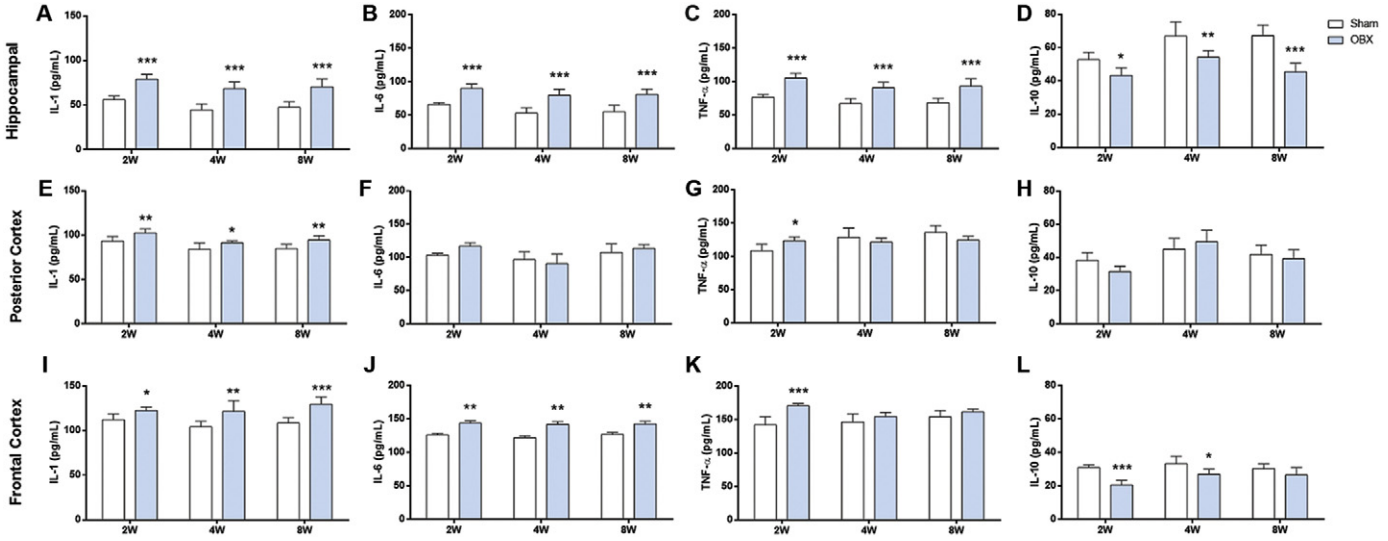

Fig. 7. The effect of OBX on inflammatory parameters. Effects of OBX on IL-1 (A), (E) and (I) in hippocampus, posterior cortex and frontal cortex, respectively; on IL-6 (B), (F) and (J) in hippocampus, posterior cortex and frontal cortex, respectively; on TNF-α (C), (G) and (K) in hippocampus, posterior cortex and frontal cortex, respectively; and on IL-10 (D), (H) and (L) in hippocampus, posterior cortex and frontal cortex, respectively. Data are reported as the mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc tests. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to their respective Sham group (n = 5 animals/group).

forced swim, tail suspension, light-dark and elevated plus maze tests (Gupta et al., 2014; Han et al., 2008; Nakagawasai et al., 2016; Song and Leonard, 2005).

According to the DSM-5, a formal MDD diagnosis is characterized by a persistent depressive mood or anhedonia, which is one of the cardinal signs of depression in humans (Lally et al., 2014; Taylor, 2014). Furthermore, several studies indicate that the measurement of anhedonia in MDD patients is a complex process that encompasses aspects of personality, learning and biases (Rizvi et al., 2016). We are the first to demonstrate that OBX induced the transient loss of self-care and motivational behavior for up to 4 weeks after surgery. In contrast, previous studies suggested that anhedonic-like behaviors are not clearly observed in the OBX model of depression (Czeh et al., 2015). Significant correlations between anhedonic-like behavior, habituation to novelty and anxiety in the OFT strengthen the face and construct validity of the OBX model of depression (Czeh et al., 2015).

4.2. OBX triggered transient hippocampal mitochondrial impairments and long-lasting imbalances in redox homeostasis and the inflammatory response of the hippocampus

Several studies demonstrated that mitochondrial cytopathies are a key feature in MDD (Aguiar et al., 2014; Scaglia, 2010). The mechanisms driving the observed changes in the OBX model were shown to include alterations in mitochondrial metabolism (Rinwa et al., 2013). Here, we observed that OBX profoundly affected hippocampal presynaptic mitochondria, indicating a remarkable specificity of OBX effects on presynaptic components, with no OBX-related effects on the mitochondrial parameters of whole-cell preparations from hippocampus, posterior and frontal cortices brain regions (data no shown).

Presynaptic mitochondria play important roles in synaptic transmission, plasticity and organization, including the movement of vesicles and calcium buffering (Mattson et al., 2008; Nicholls et al., 2015); thus, we conducted mitochondrial analyses of well-established preparations, including synaptosome-enriched preparations, to explore the putative specificity of mitochondrial parameters susceptible to OBX. Mitochondria from hippocampal synaptosome-enriched preparations uniquely showed a transient decrease in mitochondrial mass and $\Delta\Psi$. Positively-stained mitochondria (Mito+) also showed a transient decrease in the mitochondrial $\Delta\Psi$, highlighting the specific loss of hippocampal synaptic mitochondrial functionality in the OBX model of depression. The current results suggest that in addition to the decrease in synaptic mitochondria, there is also a decrease in mitochondrial functionality in the presynaptic terminals 2 weeks and 4 weeks after OBX. Interestingly, the OBX-induced effects on synaptic mitochondria were reversed by 8 weeks post-surgery. We observed significant negative correlations between mitochondria functionality ($\Delta\Psi$) *versus* the total distance traveled during the first 3 min of the OFT (habituation to novelty) and the distance traveled during the last 7 min of the OFT (hyperactivity) in the 2W and 4W groups, suggesting that the observed mitochondrial dysfunction in hippocampal synaptosomal-enriched preparation may contribute to synaptotoxicity-related effect. Mitochondria in the synaptosomal preparation (presynaptic terminals) exhibited a significant lower content of electron transport components, which could lead to an increased susceptibility to neurodegenerative dysfunction and synaptotoxicity (Nicholls et al., 2015; Picard and McEwen, 2014). Mitochondria are strongly involved in neuroplasticity/synaptogenesis (Cheng et al., 2010; Picard and McEwen, 2014) and play a key role in regulating synaptic transmission, cognition and aging (Nicholls et al., 2015; Picard and McEwen, 2014); therefore, the transient impairment in synaptic mitochondria homeostasis may contribute to the behavioral disturbances observed in the OBX model of depression. Since, the impairment of the high metabolic requirement in presynaptic terminals was previously associated with changes in synaptic strength and/or loss of spine density in the limbic areas of OBX animals (Czeh et al., 2015), this data could suggest a close link between the transient mitochondrial changes and anhedonic-like behaviors.

The hippocampal selectivity of the OBX-induced mitochondrial alterations is in accordance with the structural modifications in specific brain regions that resulted in deficits in hippocampus-dependent learning and memory in OBX mice (Hendriksen et al., 2015). Some authors postulate that the cognitive phenotype induced by OBX (loss of spatial memory), accompanied by increased brain levels of tau-protein hyperphosphorylation, could be associated as a model for Alzheimer´s disease (Bobkova et al., 2014; Hu et al., 2012). Although both pathologies (Alzheimer's disease and MDD) have a chronic effect on cognitive performance, the neurotoxicity in animal models of Alzheimer's involves b-amyloid peptide deposition (Crimins et al., 2013; Ferreira et al., 2015) and/or the abnormal phosphorylation of the microtubule-associated protein tau (Pooler et al., 2014). Although the precise synaptotoxic form of tau remains unclear, several studies suggest that aggregated tau may from primary synaptotoxic insults (Kopeikina et

al., 2012; Pooler et al., 2014; Spires-Jones et al., 2011). Notably, evidence suggests that tau regulates neuronal signal transduction by influencing the targeting and function of synaptic mitochondria; indeed, tau can bind to kinesin and compete with other cargo, which inhibits mitochondrial transport to the soma, axon, and pre-synaptic boutons (Pooler et al., 2014). At this time, although our results demonstrated the long-lasting effects on memory-related parameters (habituation to novelty), the transient neurochemical changes observed suggest that the OBX model of depression leads to a transient synaptotoxicity-related effect, which differs from the synaptotoxicity verified in Alzheimer's disease.

Several human and experimental studies, including meta-analyses (Black et al., 2015), suggested that an imbalance in several redox parameters contributed to the pathogenesis of MDD (Black et al., 2015; Hurley and Tizabi, 2013; Moylan et al., 2014; Yang et al., 2014). Here, our data show that OBX increased the production of ROS, nitrite levels and altered antioxidant defenses (*e.g.*, GSH), particularly in the hippocampus, for up to 8 weeks. We postulate that these effects lead to dysfunction in intracellular signaling contributing to the hippocampal synaptotoxicity (Pooler et al., 2014). Indeed, the disruption of redox homeostasis is strongly associated with mitochondrial damage (Moylan et al., 2014) and may contribute to the transient mitochondrial $\Delta\Psi$ impairment demonstrated in our study. Moreover, OBX effects on intracellular ROS and nitrite levels in the posterior and frontal cortices were transient.

Several lines of evidence suggest that pro-inflammatory cytokines are produced in response to oxidative stress (Moylan et al., 2014) and play a critical role in the pathogenesis of MDD. Many studies have demonstrated that pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α, are elevated in the serum and CNS of patients with MDD (Hurley and Tizabi, 2013). These data are reinforced by recent work showing that inflammation elicits symptoms of anhedonia (Swardfager et al., 2016). Our temporal analysis revealed an increase in the levels of pro-inflammatory cytokines and a decrease in an anti-inflammatory cytokine in the hippocampus; furthermore, the majority of these changes were long-lasting. Previous studies showed that the inflammatory and redox state are intimately linked in the cell (Moylan et al., 2014); thus, our findings of GSH depletion, the main neuronal antioxidant defense, and increased pro-inflammatory cytokines are in accordance with the presence of a pro-oxidative state in the hippocampus. Additionally, *in vitro* and *in vivo* studies have shown that increases in pro-inflammatory cytokines can alter synaptic plasticity (Hurley and Tizabi, 2013). Thus, the pronounced and long-lasting redox imbalance and pro-inflammatory response displayed in hippocampus of OBX mice presented in our study could strongly influence the increased mitochondrial dysfunction observed at the presynaptic terminals.

Interestingly, the hippocampus redox status and cytokines levels significantly correlates with the distance traveled in the OFT, predominantly during the first 3 min of the test. These data suggest an association between long-lasting behavioral changes and the disruption of redox homeostasis and enhancement of the inflammatory response. Additionally, considering that the hippocampus is intimately involved in emotional and spatial/topographical memory, our data reinforce the importance of separately evaluating OFT behavioral parameters and suggest that habituation to novelty in the OFT is an essential behavioral abnormality caused by OBX in mice.

Previous studies have shown that the olfactory bulbs have defined regions that communicate via neurotransmitters and projections to the amygdala, hippocampus, posterior piriform cortex and entorhinal cortex (Song and Leonard, 2005). This description of anatomical connections among the main olfactory bulbs and other brain regions reinforces our data because the most pronounced neurochemical changes (mitochondrial mass and $\Delta\Psi$, redox imbalance and pro-inflammatory cytokines) that we observed over the time occurred in the hippocampus and, to a lesser extent, in the posterior and frontal cortices. In consonance with these results, and considering the hippocampus as an important area of the brain involved in behavioral, emotional and physiological processing, important studies highlight that OBX reduce cell proliferation in the dentate gyrus, stimulates neuronal hypotrophy in CA1 pyramidal neurons, impairs the long-term potentiation in the CA1 and CA3 subregions, and decrease markers of cellular and synaptic plasticity in the hippocampus (Morales-Medina et al., 2017). Thus, the retrograde, anterograde and transneuronal degeneration may have occurred after the OBX surgery, leading to changes in the entire brain; however, some regions were more affected than others, such as hippocampus.

5. Conclusion

Therefore, we postulate that OBX surgery induces a number of maladaptive consequences mainly in the hippocampus in a transient or long-lasting manner: (i) changes in hippocampal anatomical structure, (ii) transient hippocampal-related synaptotoxicity, (iii) long-lasting increases in ROS and pro-inflammatory cytokines, (iv) decreased hippocampal synaptic plasticity, (v) reduced hippocampal cellular resilience and (vi) impairment of hippocampal-dependent behavioral performance. Based on the aforementioned evidence, the structural changes in the hippocampus are consistent with the pronounced anxiety and depressive-like behavior in OBX mice. So, our results provide additional characterization of the OBX model in mice and create new perspectives for the depression field, including future pharmacological studies, and potential targets for antidepressant drugs.

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Author contributions

Roberto F Almeida was responsible for the design, acquisition, analysis, interpretation, drafting, and approval of the final version of the manuscript. Marcelo Ganzella, Daniele G Machado, Samanta O Loureiro, André Quincozes-Santos and Letícia F Pettenuzzo were responsible for acquisition, analysis, interpretation, and approval of the final version of the manuscript. Douglas Leffa, Marta F Duarte and Tiago Duarte were responsible for the acquisition of some data displayed in the manuscript. Diogo O Souza was responsible for the design, interpretation, drafting, critical revision, and approval of the final version of the manuscript.

Compliance with ethical standards

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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