Sex Differences in the Brain-Blood Barrier in Rats Exposed to Early life Stress and the Treatment with Antidepressants and Psychobiotic

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Research Article

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Abstract

Major depressive disorder is a debilitating mental disorder. Although the etiology is not fully understood, the impairment to the blood-brain barrier (BBB) integrity may be involved. Maternal deprivation was performed in the first 10 postnatal days for 3h/day. Male and female rats were divided into control and maternal deprivation. Maternal deprivation animals were subdivided and received treatment with saline, escitalopram, ketamine, or probiotic. The integrity of BBB was evaluated in the prefrontal cortex and hippocampus at postnatal days 11, 21, 41, and 61. Maternal deprivation caused BBB breakdown in the prefrontal cortex and hippocampus in female and male rats in all ages evaluated, except in the prefrontal cortex of females at postnatal day 41. In females, escitalopram, ketamine, and probiotic reversed BBB breakdown in all ages evaluated, except probiotic at postnatal day 21 (prefrontal cortex), and ketamine at postnatal days 21 and 41 (hippocampus). In males, escitalopram, ketamine, and probiotic reversed BBB breakdown in the prefrontal cortex in all ages evaluated, except escitalopram at postnatal days 41 and 61. In the hippocampus of males, BBB damage was reversed by escitalopram at postnatal day 21 and ketamine at postnatal day 41. Treatment with escitalopram, ketamine, or probiotics can prevent changes in the BBB integrity, depending on the age and sex of the animal. Clinically it is important to evaluate different treatments depending on age and sex.

1. Introduction

Major depressive disorder is a debilitating mental disorder (American Psychiatry Association 2013). The pharmacotherapy for this disorder is based on the monoaminergic theory, which proposes a deficiency of serotonin, dopamine, and noradrenaline on the synaptic cleft. Treatment options include selective serotonin reuptake inhibitors, such as escitalopram. Although they are the first-line antidepressant medication class, they can produce significant side-effects and have a modest rate of efficacy (Malhi and Mann 2018).

Despite advances in the understanding of the pathophysiology of major depressive disorder, no single mechanism can explain all facets of this disorder. Some of the factors that seem to be involved are changes in the glutamatergic system (Sanacora et al. 2012), increase in oxidative stress (Czarny et al. 2018), neuroinflammation (Réus et al. 2019; Beurel et al. 2020), and impairment to the blood-brain barrier (BBB) integrity.

It is worth mentioning that several factors can overlap to increase major depressive disorder vulnerability. For example, mechanisms involved in the glial cells changes are associated with impairment of the BBB integrity (Haruwaka et al. 2019) and glutamatergic system dysregulation (Réus et al. 2015a). The neurotransmitter glutamate in high levels overstimulates the N-methyl-D-aspartate receptors, which in turn produce reactive oxygen species, proinflammatory cytokines, and can activate cell death pathways (Sanacora et al. 2012; Réus et al. 2015a). Ketamine, an antagonist of the N-methyl-D-aspartate receptor, emerges as a drug with effective and fast antidepressant action. Ketamine stimulates the regulation of

synaptic function and plasticity (Duman et al. 2012), reduces proinflammatory cytokines and oxidative stress (Réus et al. 2015a), and changes the gut microbiota diversity (Getachew et al. 2018).

The microbiota-gut-brain axis is a bidirectional communication system that can influence several neurological diseases (Dinan and Cryan 2017; Kelly et al. 2019). Notably, depressive individuals have significant changes in the gut-microbiota composition (Sanada et al. 2020). On the other hand, probiotic (live bacteria that, when ingested in appropriate amounts, confer beneficial effects on the health of the host) treatment can reduce depressive symptoms (Sanada et al. 2020).

Stress, is another factor that has an important impact on the development of major depressive disorder, especially stress exposition in childhood (Łosiak et al. 2019). To study the effects of early life stress in animals, the maternal deprivation protocol is widely used. Maternal deprivation in rodents induces depressive-like behavior, anhedonia, neuroinflammation, oxidative stress, and gut-microbiota changes (Réus et al. 2017; Rincel et al. 2019). However, these effects are seen to be different depending on developmental stages and sex (Réus et al. 2019; Giridharan et al. 2019).

Although the relationship between changes in the microbiota, inflammation, BBB dysfunction, microglial activation, and depressive symptoms is known, little is acknowledged about such changes throughout development. Thus, this study aimed to investigate the effects of the treatment with probiotic, ketamine, and escitalopram on the BBB integrity during different phases of development (infancy, adolescence, and adult life) of male and female rats exposed to maternal deprivation.

2. Material And Methods

2.1 Animals

Female Wistar rats with 3 months of age and weighing 250–280 g were obtained from the breeding colony of Universidade do Extremo Sul Catarinense (UNESC, Criciúma, SC, Brazil) and were housed for one week in the presence of males for mating purposes. At the end of 7 days, the pregnant rats were housed individually with ad libitum access to food and water until the birth of the pups and their identification. All mothers and pups were kept on a 12-hour light / dark cycle (06:00 a.m. to 06:00 p.m.) at a temperature of 23 ± 1°C. One day after birthing occurred, the maternal deprivation protocol was applied to a percental of male and female pups from days 1–10 after birth (deprived); other males and females were used as controls (non-deprived). All experimental procedures that involved animals were performed in accordance with the NIH Guide for the Care and Usage of Laboratory Animals, within the Brazilian Society for Neuroscience and Behavior recommendations for animal care. The experimental protocol was approved by the ethics committee from UNESC under protocol number: 032/2019-1.

2.2 Maternal deprivation

The deprivation protocol consisted of removing the mother from the residence box and taking her to another room. The pups were maintained in their home cage (grouped in the nest in the presence of

maternal odour). The pups were deprived of the mother for 3 hours per day during the first 10 days. We prefer this protocol because it does not require the manipulation of the pups (Ignácio et al. 2017; Réus et al. 2017). At the end of each daily deprivation session, the mothers were returned to their home boxes; this procedure was carried out during the light part of the cycle, between 8:00 a.m. and 12:00 p.m. The control rats (non-deprived) remained in their resident boxes together with their mothers throughout the experiment.

2.3 Experimental design and treatments

After maternal deprivation protocol pups were divided into new experimental groups: 1) non-deprived (control); 2) deprived + saline; 3) deprived + escitalopram; 4) deprived + ketamine; and 5) deprived + probiotic (Fig. 1). Individual groups of rats (male and female) were evaluated at different periods of development after postnatal days 11 (groups 1 and 2), 21, 41 and 61 (groups 1–5) (n = 06 animals/group for each stage of development: n = 06 for males and n = 06 for female). In the different stages of development and the different experimental groups, BBB integrity was evaluated as described in the methods section. The 11 days group was euthanized without receiving treatment. The 21 days group received the treatment at postnatal days 11–20, the 41 days group received the treatment at postnatal days 11–40. The 61 days group received the treatment at postnatal days 11–60. Escitalopram was administered orally at a dose of 10 mg/kg once daily. The probiotic *Bifidobacterium infantis* was administered orally in the dose $1x10^{10}$ CFU diluted in 100 mL of water once a day. Ketamine was administered intraperitoneally at a dose of 15 mg/kg twice a week. At the end of the treatment, the hippocampus and prefrontal cortex were used for the analysis of BBB integrity.

2.4 BBB

The BBB integrity was investigated using Evan's blue dye extravasations (Smith and Hall 1996). One hour before euthanization, 1% of 1 mL of Evan's blue dye was injected into the femoral vein. The chest was subsequently opened and transcardially perfused with 200 mL of saline through the left ventricle at 100 mmHg pressure until color-less perfusion fluid was obtained from the right atrium. The samples were weighed and placed in a 50% trichloroacetic solution. Following homogenization and centrifugation (for 20 min at 10,000 rpm), the extracted dye was diluted with ethanol (1:3), and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) using a luminescence spectrophotometer (Hitachi 650 − 40, Tokyo, Japan). Calculations were based on the external standard with the same solvent. The tissue containing Evan's blue dye was quantified with a standard linear line derived from known amounts of the dye and was expressed per gram of tissue (Smith and Hall 1996). BBB permeability was measured at 11, 21, 41, and 61 days after maternal deprivation.

2.5 Statistical analysis

Statistical analyzes were evaluated using SPSS Statistics 21.0 Software. Data from postnatal day 11 were evaluated according to the Student’s t-test for independent samples and are expressed as the mean ± standard error of the mean (S.E.M.). The other postnatal day analyses were performed by one-way ANOVA followed by Tukey post-hoc and are expressed as the mean ± standard error of the mean.

Differences between sex and groups interaction were determined by two-way ANOVA. Statistical significance was considered for p values less than 0.05.

3. Results

In the present study, we examined the permeability of the BBB by measuring the cerebral content of Evan's Blue. Figure 2 shows that the maternal deprivation caused BBB breakdown in the prefrontal cortex and hippocampus in female rats (prefrontal cortex t = -2.573, df = 10, p = 0.028; Hippocampus t = -3.753, df = 5.242, p = 0.012; Fig. 2a) and male rats (prefrontal cortex t = -3.359, df = 5.552, p = 0.017; Hippocampus t = -4.450, df = 8.559, p = 0.002; Fig. 2b) at postnatal day 11. Two-way ANOVA did not revealed differences for sex and groups interaction on BBB at postnatal day 11 in the prefrontal cortex (F_{1-20} = 0.317; p = 0.579) and hippocampus (F_{1-20} = 3.868; p = 0.06).

Figure 3 illustrates the effects of antidepressants (escitalopram and ketamine) and probiotic treatments on the integrity of the BBB in the prefrontal cortex and hippocampus at postnatal day 21. The treatments with escitalopram and ketamine decreased BBB permeability of females in the prefrontal cortex (F_{4-19} = 9.220, p < 0.0001), and escitalopram and probiotic in the hippocampus (F_{4-19} = 17.910, p < 0,0001). In males (Fig. 3b), all treatments were effective in reverse BBB breakdown in the prefrontal cortex after maternal deprivation (F_{4-20} = 18.706, p < 0.0001). In the hippocampus only escitalopram was effective (F_{4-20} = 12.480, p < 0.0001). Two-way ANOVA revealed differences for sex and groups interaction on BBB integrity in the hippocampus (F_{4-40} = 4.495, p = 0.004). Effects were observed for sex in the control, and probiotic and ketamine treatments. In the prefrontal cortex there was no differences for sex and groups interaction on BBB at postnatal day 21 (F_{4-40} = 1.684, p = 0.172).

At postnatal day 41 female rats (Fig. 4a), escitalopram and probiotic treatments were able to reverse BBB damage in the hippocampus (F_{4-19} = 8.739, p < 0.0001. Figure 4a). In males (Fig. 4b), the administration of probiotic and ketamine decreased BBB permeability in the prefrontal cortex (F_{4-17} = 5.448, p = 0.005), and only ketamine in the hippocampus (F_{4-18} = 7.303, p = 0.001). Two-way ANOVA did not revealed differences for sex and groups interaction on BBB at postnatal day 41 in the prefrontal cortex (F_{4-37} = 2.325; p = 0.074) and hippocampus (F_{4-37} = 1.278; p = 0.296).

Figure 5a shows that the increase in the integrity of the BBB in the prefrontal cortex (F_{4-20} = 9.281, p < 0.0001) and hippocampus (F_{4-20} = 119.503, p < 0.0001) induced by maternal deprivation were reversed with all treatments in the female at postnatal day 61. In males (Fig. 5b), the administration of probiotic and ketamine decreased BBB permeability after maternal deprivation in the prefrontal cortex (F_{4-20} = 12.197, p < 0,0001), but in the hippocampus of males at postnatal day 61 the treatments were not effective. Two-way ANOVA revealed differences for sex and groups interaction on BBB integrity in the prefrontal cortex (F_{4-40} = 5.442, p = 0.001) and hippocampus (F_{4-40} = 50.032, p < 0.0001). Effects were observed for sex in the control and maternal deprivation in the prefrontal cortex and in the probiotic treatment in the hippocampus.

4. Discussion

In the present study, it was evidenced that maternal deprivation protocol induced dysfunction of the BBB (in the prefrontal cortex and hippocampus) in both sexes and all ages evaluated, except in prefrontal cortex of females at postnatal day 41. We analyzed these two brain regions because the prefrontal cortex and hippocampus are two of the brain regions most involved in the pathophysiology of depression and which also seem to influence according to sex (Malhi and Mann 2018; LeGates et al. 2019).

The BBB provides a stable environment for all neural functions, besides being important for brain nutrition, and protecting of the brain from neurotoxins. Thus, BBB dysregulation may be associated with several neurological diseases (Abbott et al. 2010). Reviews of clinical and preclinical studies suggest that there is an association between neurovascular unit dysfunction, BBB hyperpermeability, and major depressive disorder, and that oxidative stress and inflammation is correlated with BBB dysfunction (Najjar et al. 2013; Kealy et al. 2020). It is worth mentioning that individuals with major depressive disorder have an increase in oxidative stress markers (Black et al. 2015) and inflammatory cytokines (Dowlati et al. 2010).

A good deal of data has established that early life stress is associated with depressive symptoms in humans (Chapman et al. 2004; Heim and Binder 2012) and depressive-like behavior in animals (Réus et al. 2011, 2017). There is also evidence to demonstrate that the maternal deprivation protocol is capable of inducing an increase in inflammatory cytokines in brain tissues and serum (Réus et al. 2017), and oxidative stress markers (Réus et al. 2015a), besides increase microglial activation and generate atrophy of astrocytes (Réus et al. 2019). These changes could justify the increase in BBB permeability observed in the present study. This is in line with another preclinical study that showed the relationship between BBB and early life stress (Gómez-González and Escobar 2009). In this study, maternal deprivation protocol increased Evans blue entry to the neocortex, hippocampus, diencephalon, basal ganglia, olfactory bulb, brain stem, cerebellum, and spinal cord at postnatal day 10, but not at postnatal day 20 or postnatal day 30. It is worth noting that in this study, males and females were evaluated together (Gómez-González and Escobar 2009). It is worth mentioning that others stress protocols can change BBB integrity (Sántha et al. 2016; Menard et al. 2017; Caron et al. 2018). Menard et al. (2017) evidenced that chronic social stress altered BBB integrity through downregulation of the tight junction protein claudin-5 in the nucleus accumbens, which, combined with stress-induced recruitment of peripheral immune signals, resulted in increased BBB permeability, the passage of blood circulating proteins such as IL-6, and the development of depression-like behaviors (Menard et al. 2017).

In the present study, the impact of three types of treatments on BBB integrity was also assessed. Regarding escitalopram, it was observed that in females, whenever the maternal deprivation protocol increased BBB permeability, the antidepressant was able to reverse this change (at all ages evaluated and in both brain regions). However, in males, escitalopram only reversed the increase in BBB permeability (in the prefrontal cortex and hippocampus) at postnatal day 21. This suggests that escitalopram was more effective in females than in male rats.

As far as we know, this was the first study that investigated the effect of escitalopram on BBB integrity. On the other hand, there is a study that evidenced that BBB dysfunction results in poorer less response to escitalopram treatment (Jha et al. 2019). It is worth mentioning that escitalopram was able to reverse the depressive-like behavior and the gut inflammation induced by a colitis protocol in ovariectomized rats, suggesting a potential anti-inflammatory effect of escitalopram (Abdo et al. 2019). Besides, a clinical study demonstrated that the treatment with SSRIs, including escitalopram, was able to decrease oxidative stress index, total oxidant status, and increase total antioxidant capacity in patients with depression (Cumurcu et al. 2009). In line with this, a recent preclinical study evidenced that escitalopram can change the expression and methylation level of genes involved in the oxidative and nitrosative stress in the hippocampus, amygdala, cerebral cortex, and blood of rats exposed to chronic mild stress (Wigner et al. 2021). This antioxidant and anti-inflammatory action could, at least in part, justify the results found in the present study. Interestingly, a clinical study with individual with post-stroke depressive symptoms evidenced that treatment responses of escitalopram tended to be more pronounced in the female group, suggesting different responses according to sex, going according to the data observed in the present study (Lee et al. 2020). Moreover, after review 15 randomized, placebo-controlled trials, Khan et al. (2005) observed that women had a significantly greater response than men to SSRI antidepressants (Khan et al. 2005). It is worth noting that there are sex differences in the pharmacokinetics and pharmacodynamics of antidepressants. For instance, women can absorb more efficiently SSRI and have a greater lipophilic antidepressant (e.g., escitalopram) distribution (Bigos et al. 2009). These differences can occur due to physiological changes, as well as hormonal changes, and differences in synaptic transmission between the sexes (Bigos et al. 2009; LeGates et al. 2019).

Regarding ketamine, it was able to reverse the change in BBB in females at postnatal day 21 (only in the prefrontal cortex) and postnatal day 61 (prefrontal cortex and hippocampus). In males, a beneficial effect of ketamine in the prefrontal cortex was observed in the three evaluated moments (postnatal day 21, 41, and 61), and in the hippocampus at postnatal day 41. Consistent with these data, literature findings show that ketamine can protect against bradykinin-induced breakage of the BBB (Chen et al. 2016). Previous data from our group also demonstrated that a single dose of ketamine (administered in postnatal day 46) in male rats submitted to the maternal deprivation protocol was able to decrease the oxidative stress induced by this protocol at postnatal day 60, especially in the hippocampus (Réus et al. 2015a). In the present study, the main results of ketamine in male rats were observed in the prefrontal cortex, however, it is worth mentioning that the treatment protocol with ketamine was different in these two studies. Literature data also show that ketamine has an anti-inflammatory effect (Nowak et al. 2019), and that, at least part of this action, can occur through interaction with gut bacteria (Getachew et al. 2018). In addition, maternal deprivation protocol induced an increase in pro-inflammatory cytokines in serum and cerebrospinal fluid, and on the other hand, ketamine treatment reduced the levels of these cytokines in deprived rats (Réus et al. 2015b).

Concerning the effects of probiotics, it was observed that in females, it reversed the change in the BBB of the hippocampus at all ages evaluated and reversed the change in the BBB of the prefrontal cortex at

postnatal day 61. In males, the probiotic reversed the changes in the BBB in the prefrontal cortex at all ages, without any effect on the hippocampus.

A growing body of evidence has suggested that lipopolysaccharide (LPS) induces neuroinflammation and BBB dysfunction (Sumi et al. 2010; Yang et al. 2019). LPS is a structural component of the outer membrane of Gram-negative bacteria and can induce systemic inflammation (Lu et al. 2008). *Lactobacillus* spp., and *Bifidobacterium* spp. are the most promising probiotic species that can modulate the gut microbiota, and perform several health benefits through various mechanisms of action (Azad et al. 2018; Khalesi et al. 2019). Here, it is worth noting the probiotics' ability to decrease inflammation. A meta-analysis of randomized clinical trials evidenced that probiotic supplementation reduces serum concentrations of pro-inflammatory cytokines (Milajerdi et al. 2020). Interestingly, chronic treatment with *Bifidobacterium infantis* reverted the increase in pro-inflammatory cytokine in rats subjected to maternal separation (Desbonnet et al. 2010).

Another important point to be mentioned is that preclinical evidence suggests that gut microbiota influences BBB permeability (Braniste et al. 2014). Probiotic treatment (composed of *Bifidobacterium lactis*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus acidophilus)* significantly attenuated BBB injury, inhibited neuroinflammation, reduced oxidative DNA damage in the brain, and decreased plasma LPS in aged mice (Yang et al. 2020). Furthermore, as recently revised, gut microbiota-derived metabolites also influence BBB integrity (Parker et al. 2020). However, as far as we know, this was the first study that investigated the effects of probiotics on the BBB integrity in a maternal deprivation protocol.

In summary, the present study demonstrated that early life stress, induced by maternal deprivation protocol, can lead to long-term BBB integrity changes in male and female rats. Moreover, treatment with escitalopram, ketamine, or probiotics can prevent some of these changes, depending on the age and sex of the animal. Noteworthy female rats at postnatal day 61 were the ones that most responded to all treatments in the two brain regions evaluated, suggesting that a more chronic treatment will bring more effective results. Thus, this study contributes to the understanding of the possible changes that early life stress can cause and possible treatments that could reverse these changes.

DECLARATIONS OF INTEREST

None

Declarations

DECLARATIONS OF INTEREST

None

AUTHOR CONTRIBUTIONS

All authors participated in the design and interpretation of the studies, analyzed the data and review of the manuscript; LAB performed maternal deprivation protocol. ASC, LAB, MEMB, ABM, LRM, NMA, NSM, CMG, CVB performed drug administration the sample collecting, NMA, BFL, AC, JSG, and TB did BBB preparing and analysis. GZR and JSJ performed statistical analysis and figures. MEMB, LM, ASC, and GZR wrote the manuscript. JQ reviewed the manuscript. GZR and ASC did the design of experiment and reviewed the manuscript.

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Figures

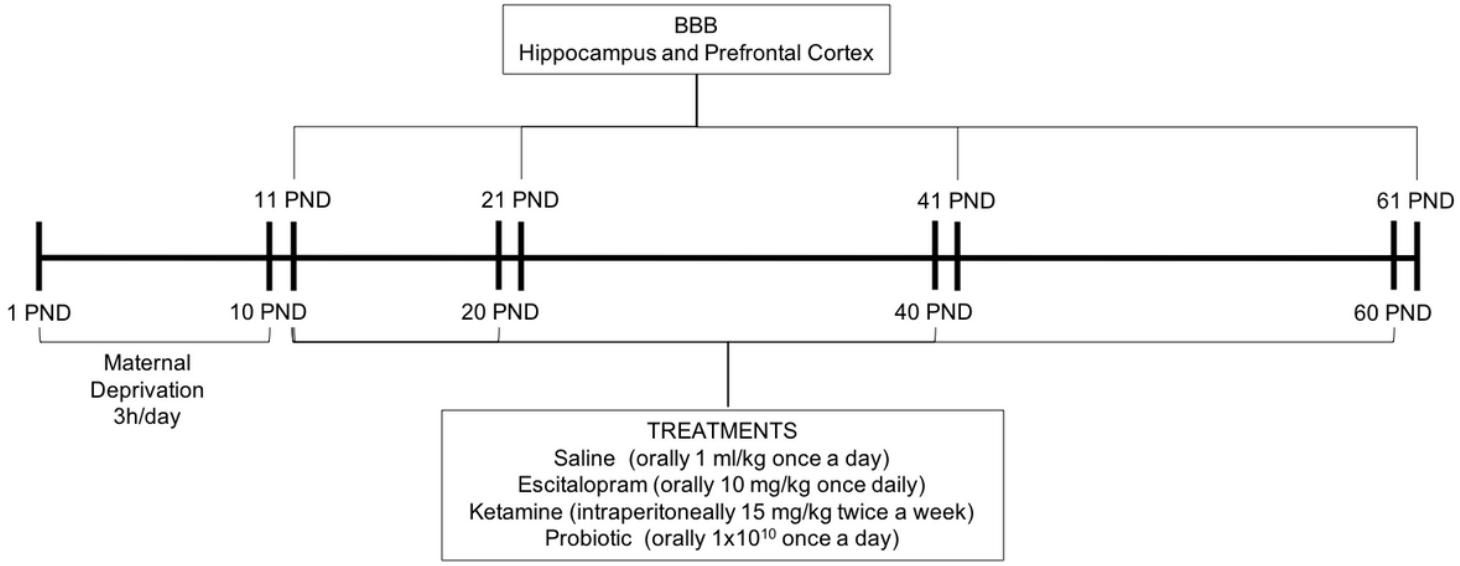

Figure 1

Schematic representation of timeline and experimental design. Part of the rats was submitted to the maternal deprivation 3h/day. After, the animals were divided into new experimental groups (male and female): 1) non-deprived (control); 2) deprived + saline; 3) deprived + escitalopram; 4) deprived + ketamine; and 5) deprived + probiotic. Escitalopram was administered orally at a dose of 10 mg/kg once daily. The probiotic Bifidobacterium infantis was administered orally in the dose 1x1010 diluted in 100 mL of water once a day. Saline was administered orally 1 mL/kg once a day. Ketamine was administered intraperitoneally at a dose of 15 mg/kg twice a week. Individual groups were evaluated at different periods of development: the 11 postnatal day (PND) group was euthanized without receiving treatment, the 21 days group received the treatment on 11-20 PND, the 41 days group, 11-40 PND, the 61 days group on 11-60 PND. blood-brain barrier (BBB) permeability was measured at 11, 21, 41, and 61 days after maternal deprivation

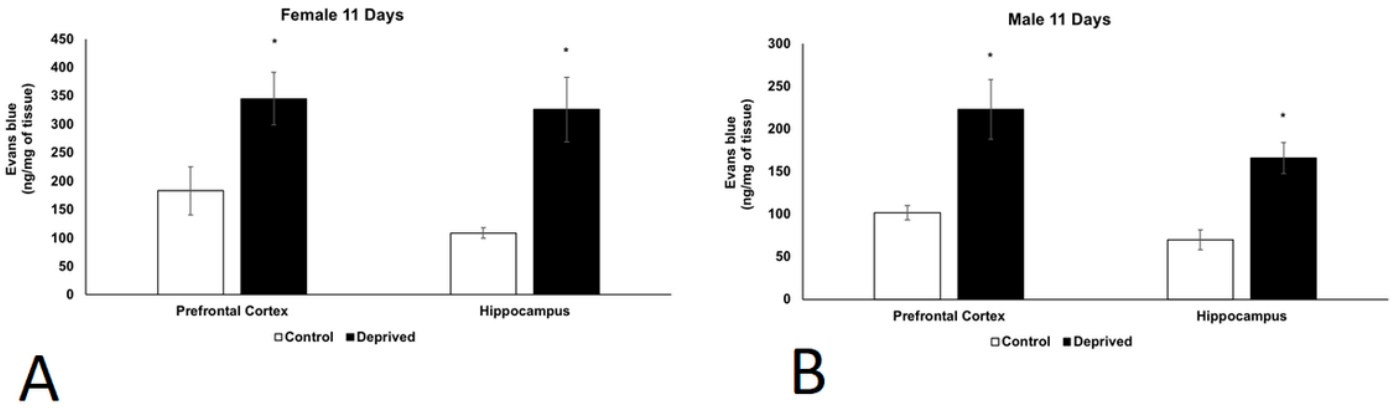

Figure 2

The integrity of the blood-brain barrier (BBB) was investigated using Evan's blue dye extravasation in the prefrontal cortex and hippocampus of female (a) and male (b) Wistar rats after at postnatal day 11 following maternal deprivation. The integrity of BBB was assessed by fluorescence and determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer. Results are shown as ng/mg tissue. Figure shows the mean ± S.E.M. of 6 animals in each group. *p <0.05 vs. Control, according to Student t-test

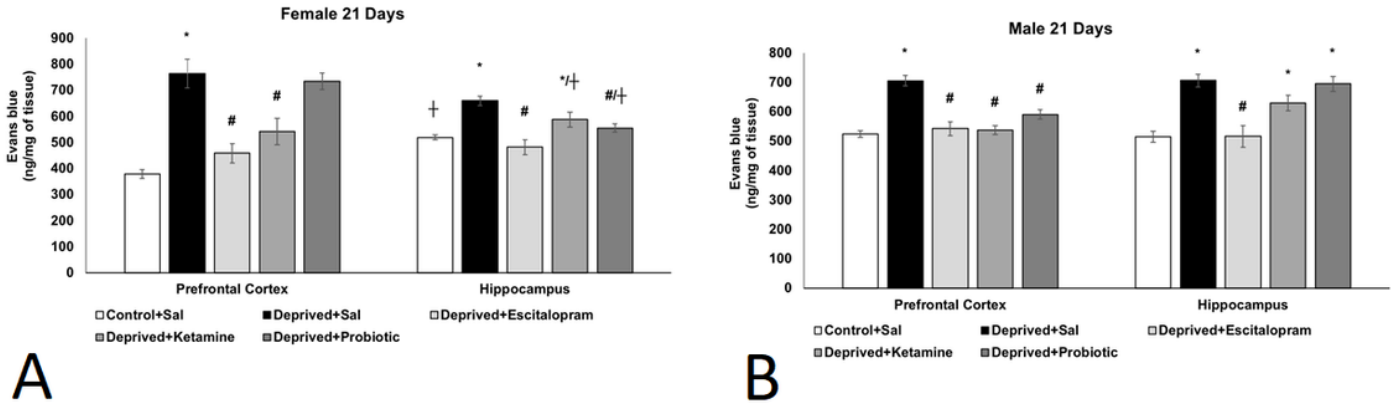

Figure 3

The integrity of the blood-brain barrier (BBB) was investigated using Evan's blue dye extravasation in the prefrontal cortex and hippocampus of female (a) and male (b) Wistar rats after at postnatal day 21 following maternal deprivation and treatment with escitalopram, probiotic, and ketamine. The integrity of BBB was assessed by fluorescence and determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer. Results are shown as ng/mg tissue. Figure shows the mean ± S.E.M. of 6 animals in each group. *p <0.05 vs. Control + Saline #p <0.05 vs. Deprived + Saline, according to one-way ANOVA followed by Tukey's post-hoc test. $p<0.05 vs. sex and groups interaction according to two-way ANOVA

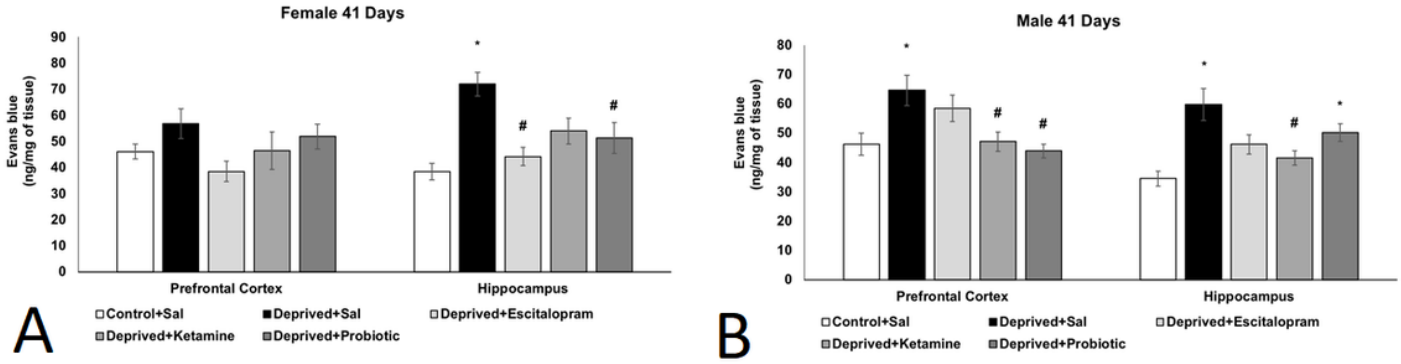

Figure 4

The integrity of the blood-brain barrier (BBB) was investigated using Evan's blue dye extravasation in the prefrontal cortex and hippocampus of female (a) and male (b) Wistar rats after at postnatal day 41 following maternal deprivation and treatment with escitalopram, probiotic, and ketamine. The integrity of BBB was assessed by fluorescence and determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer. Results are shown as ng/mg tissue. Figure shows the mean ± S.E.M. of 6 animals in each group. *p <0.05 vs. Control + Saline #p <0.05 vs. Deprived + Saline, according to one-way ANOVA followed by Tukey's post-hoc test

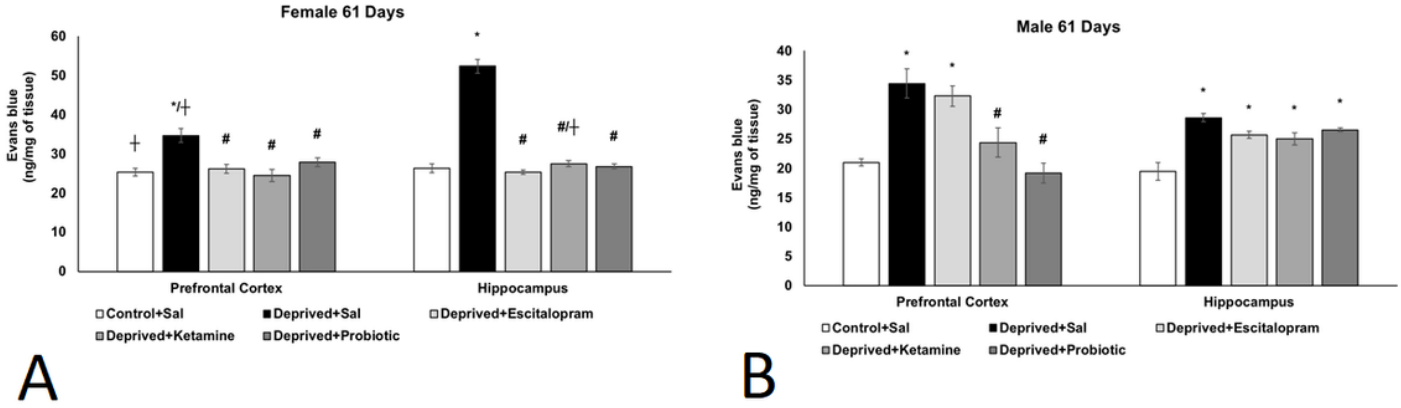

Figure 5

The integrity of the blood-brain barrier (BBB) was investigated using Evan's blue dye extravasation in the prefrontal cortex and hippocampus of female (a) and male (b) Wistar rats after at postnatal day 61 following maternal deprivation and treatment with escitalopram, probiotic, and ketamine. The integrity of BBB was assessed by fluorescence and determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer. Results are shown as ng/mg tissue. Figure shows the mean ± S.E.M. of 6 animals in each group. *p <0.05 vs. Control + Saline #p <0.05 vs. Deprived + Saline, according to one-way ANOVA followed by Tukey's post-hoc test. $p<0.05 vs. sex and groups interaction according to two-way ANOVA