Investigation of Blood-Brain Barrier Disruption in an Animal Model of Mania Induced by d-Amphetamine

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Research

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Abstract

Background: Bipolar disorder (BD) is a recurrent chronic psychiatric disorder. Evidence indicates that many individuals with BD exhibit high serum levels of inflammation and oxidative stress markers, which are further associated with mood symptoms and cognitive dysfunction. Due to the crosstalk between the periphery and central nervous system in BD, the disruption of the blood-brain barrier (BBB) has been proposed as a key mechanism of the pathophysiology of the disorder. This study aimed to investigate claudin-5 expression – a major protein of the BBB – in the brain of an animal model of mania induced by d-amphetamine (AMPH) and evaluate the effects of treatment with lithium.

Results: AMPH-injected animals exhibited increased overall activity in the open field test. In the serum, TBARS levels were augmented in the lithium-treated groups, regardless of AMPH injection, while TNF α was not detected. In the brain, TBARS and TNF α did not differ between groups but were positively and strongly correlated in the ST of AMPH-injected rats. Contrary to the primary hypothesis, AMPH and lithium injections did not affect brain claudin-5 protein levels.

Conclusions: This is one of the first attempts to investigate the effects of AMPH on BBB integrity. Although no evidence of BBB disruption was found in the current study, our results provide evidence and rationale for future research to elucidate the importance of such alteration in BD.

1. Background

Bipolar disorder (BD) is a recurrent chronic and disabling disorder characterized by fluctuations in mood, energy, and functioning (American Psychiatric Association, 2013). Specifically, mood episodes include mania, hypomania, and alternating episodes of depression (Grande et al., 2016). Although no specific biomarker has been identified, individuals with BD present with increased peripheral levels of inflammatory and oxidative stress markers accompanied by altered levels of neurotrophic factors (van den Ameele et al., 2020, 2017; Fernandes et al., 2011; Ghafouri-Fard et al., 2019; Kim et al., 2007; Petersen et al., 2021). Hence, BD is associated with a chronic low-grade inflammatory state, which seems to be coordinated with mood symptoms and cognitive deficits during the cour

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The crosstalk between the periphery and the central nervous system (CNS) has implicated the blood-brain barrier (BBB) dysfunction in the pathophysiology of BD (Patel and Frey, 2015). The BBB consists of endothelial cells of the brain capillaries, astrocyte end-feet, and pericytes (Ballabh et al., 2004). The presence of tight and adherens junctions between the endothelial cells contributes to the tightness of the BBB and its selective function (Banks, 2015). As most blood-borne substances are not allowed to enter the brain under physiological conditions, BBB integrity is essential for CNS homeostasis (Segarra et al., 2021). Evidence indicates that inflammatory cytokines such as tumour necrosis factor (TNF) α and interferon (IFN) γ (Capaldo and Nusrat, 2009), and oxidative stress (Sajja et al., 2016) are detrimental and contribute to BBB disruption. Recently, extensive BBB leakage has been associated with a more severe and chronic course of BD (Kamintsky et al., 2019). Still, research on the potential involvement of the BBB in the pathophysiology of BD is very scant.

In preclinical research, amphetamine (AMPH) has been used to resemble some aspects of the manic episode of BD in rodents (Sharma et al., 2016). Besides hyperlocomotion, AMPH-injected animals present with increased levels of inflammation (e.g., interleukin (IL)-6, TNF α) and oxidative stress (e.g., lipid peroxidation and protein carbonylation) in the periphery and CNS (Frey et al., 2006a; Gubert et al., 2016). Moreover, it is suggested that lithium can prevent and reverse most of these alterations induced by AMPH and its analogues (Frey et al., 2006b; Macêdo et al., 2013; Valvassori et al., 2015). Considering that both inflammation and oxidative stress increase BBB permeability, we aimed to investigate if BBB disruption is observed in an AMPH-induced animal model of mania. Also, we sought to evaluate whether treatment with lithium reverses any BBB damage induced by AMPH. To date, only a few studies have evaluated, to some extent, BBB disruption in an animal model of mania (Valvassori et al., 2015) or other psychostimulants-induced models (Northrop and Yamamoto, 2012), while none has investigated a specific marker, such as claudin-5.

2. Methods

2.1. Animals

Male Wistar rats (n=24, 2-month-old, 220-310 g) were purchased from Charles Rivers Laboratories (Massachusetts, USA). Animals were housed (n=2 per cage) at standard room temperature and 12-h inverse light/dark cycle, with free access to food and water. This study was approved by the institutional ethics committee from McMaster University (Animal Research Ethics Board, project #140828), and all experimental procedures were performed following national (Canadian Council on Animal Care, 2020) and international (National Research Council, US, 2011) ethical standards.

2.2. Treatment groups

First, animals were allocated in two groups. From day 1 to 14, rats were injected with AMPH (dextroamphetamine, 2 mg/kg i.p. diluted in saline solution 0.9%, at 1 mL/kg; SmithKline Beecham, Brentford, UK) or saline (SAL, 1 mL/kg i.p.) once a day. Then, animals were divided into two subgroups. From day 8 to 14, animals were treated lithium chloride (LI, 47.5 mg/kg i.p. diluted in saline solution 0.9%,

at 1 mL/kg; Sigma-Aldrich Corp., St Louis, USA) or saline (1 mL/kg i.p.) twice a day. This protocol has been previously described as a reversal model of mania (Frey et al., 2006a; Frey et al., 2006b; Valvassori et al., 2015). The animals were divided in four treatment groups including (1) SAL+SAL (n=6), (2) AMPH+SAL (n=6), (3) SAL+LI (n=6) and (4) AMPH+LI (n=6).

2.3. Open field

On day 14, spontaneous locomotor behaviour was evaluated 2 h after the last injection of AMPH using the open field. The apparatus consisted of a 60x40 cm open field with 50-cm-high walls and divided into 12 equal rectangles. Each animal was placed in the center of the open field and allowed to explore the apparatus for 5 min. All sessions were recorded using a webcam (C270 HD, Logitech), and a video tracking system was used to analyze the number of crossings, distance travelled (m), average speed (m/s) and time spent in the periphery (s).

2.4. Euthanasia and sample collection

After the behavioural task, rats were anesthetized with isoflurane (4-5% with oxygen at 1-2 L/min) and decapitated, and the brain was removed as quickly as possible. The right and left prefrontal cortex (PFC), hippocampus (HIP) and striatum (ST) were dissected and snap frozen in dry ice. The troncular blood was also collected and centrifuged (2057*g*, 10 min, at room temperature). Finally, brain structures and serum were stored at -80°C until sample preparation.

2.5. Sample preparation

Right- and left-brain structures were prepared differently for biochemical analyses and western blot. Brain tissue was homogenized using phosphate-buffered saline (PBS) for biochemical assays or RIPA buffer 1x (20-188, Merck Millipore) for western blot at 1:4 (w/v). Both lysis buffers were prepared with EDTA-free protease inhibitor cocktail (11836170001, Roche) according to the manufacturer's instructions. Then, samples were centrifuged (10,000*g*, 5 min, 5°C), and supernatants were collected and kept at -80°C until further analysis.

2.6. Biochemical assays

2.6.1. Total protein

The total protein was determined in the brain structures using DC Protein Assay Reagent (5000116, BioRad) according to the manufacturer's instructions. Briefly, samples were diluted 1:21 (v/v) in PBS and, after the addition of proper reagents, the total protein content in each sample was quantified in a spectrophotometer at 750 nm.

2.6.2. TNF α

TNF α levels were quantified using a sandwich ELISA kit (KRC3011, Invitrogen™) following the manufacturer's instructions. Serum samples were not diluted, while all brain structures homogenates were diluted at a 1:10 (v/v) ratio.

2.6.3. Thiobarbituric acid reactive substances (TBARS)

TBARS levels were detected and quantified using a colorimetric assay kit (KGE013, R&D Systems) according to the manufacturer's instructions. Serum samples were diluted at 1:2 (v/v) ratio, PFC and ST homogenates were diluted at 1:20 (v/v) and HIP at 1:20 (v/v) ratios.

2.7. Western blot

Briefly, homogenates samples of PFC, HIP, and ST were further diluted 1:1 (v/v) in 2x Laemmli sample buffer (1610737, BioRad Laboratories, Inc). After that, 20 μ g of each sample was loaded in 4-20% pre-cast and stain-free mini gels (4568094, BioRad Laboratories, Inc). A protein marker (1610373, BioRad Laboratories, Inc) was also loaded in the gels. Electrophoresis was performed at 100 V for 1 h 15 min. The protein transfer was performed using the Trans-Blot® Turbo™ Transfer System (BioRad Laboratories, Inc) to an LF-PVDF membrane (0.45 μ m pore size, BioRad Laboratories, Inc) at 25 V for 7 min. The total protein and transfer were verified using the ChemiDoc™ Imaging Systems (BioRad Laboratories, Inc); no specific staining was used. Blocking was performed using skim milk powder 5% (diluted in TBS-T) for 1h at room temperature. Primary (Claudin 5 Monoclonal Antibody, 35-2500, Thermo Fisher) and secondary (Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L), 115-035-003, Jackson Immuno Research) antibodies were diluted in TBS-T at 1:500 and 1:10,000, respectively. β -actin was the loading control (β -Actin Loading Control Monoclonal Antibody, MA5-15739, Thermo Fisher; same secondary antibody as previous), and antibodies were diluted in TBS-T at 1:10,000. Clarity Western ECL Substrate (1705060, BioRad Laboratories, Inc) was used for detection. Total protein was used for loading control as described previously (Taylor et al., 2013), and claudin-5 levels were normalized by the control group levels (i.e., SAL+SAL).

2.8. Statistical analysis

Shapiro-Wilk and Levene's tests were used to evaluate the normality of distribution and homogeneity of variance, respectively. Secondly, two-way ANOVA was performed, considering model (saline and AMPH) and treatment (saline and lithium) as independent factors, followed by Tukey posthoc analysis, if ANOVA is significant. Outliers were identified using the Grubbs' test ($\alpha=0.05$) and excluded from the analysis (n=5). All $p<0.05$ were considered statistically significant.

3. Results

3.1. AMPH induced hyperactivity

AMPH-injected rats presented overall hyperactivity in the open field, which was indicated by an increased number of crossings, average speed, and distance travelled (Figure 1, A-C). Two-way ANOVA indicated a main effect of the model for all variables, with no main effects for treatment and interaction of factors (Table 1). Also, no differences were observed for time spent in the periphery (Figure 1D, Table 1).

3.2. Effect of AMPH on serum and brain levels of TNF α and TBARS

In the serum, TNF α levels were below the limit of detection across all groups. In the brain regions analyzed, AMPH did not induce inflammation dependent on TNF α levels. Consequently, no effect of lithium was observed. Two-way ANOVA did not indicate main effects of model, treatment, and interaction for these variables (Figure 2A-D, Table 2).

TBARS levels in the serum and brain structures remained unchanged following AMPH injection (Figure 2E-H). However, lithium treatment increased lipid peroxidation in the serum, which was indicated by treatment effect, but no main effects for model and interaction of factors were observed following two-way ANOVA (Figure 2E, Table 2). In the brain, there were no main effects of model, treatment, and interaction (Table 2).

Interestingly, a significant strong positive correlation was observed between TNF α and TBARS levels in the ST of the AMPH+SAL ($r=0.87$, $p<0.001$) and AMPH+LI ($r=0.96$, $p=0.002$) groups. This correlation was weaker in the SAL+SAL group ($r=0.80$, $p=0.056$) and was negative in SAL+LI rats ($r=-0.89$, $p=0.017$) – possibly due to the effect of lithium on TBARS levels.

3.3. Claudin-5 protein levels in the PFC, HIP and ST of AMPH-injected rats

Contrary to our hypothesis, the levels of claudin-5 in the brain remained unchanged following AMPH and lithium injection. Two-way ANOVA did not indicate main effects for the model, treatment, or interaction (Figure 2I-L, Table 2).

4. Discussion

To our knowledge, this is the first study that investigated BBB disruption in an animal model of mania induced by AMPH. Corroborating with previous studies, AMPH-injected rats exhibited hyperactivity, which was determined by an increased frequency of crossings and distance travelled. Although our model showed face validity, no changes in peripheral and CNS levels of TNF α and TBARS were observed following AMPH and lithium injection. Consequently, protein levels of claudin-5, the most enriched tight junction protein in the BBB, also remained unchanged in the brain regions analyzed.

Psychostimulant-induced animal model of mania, such as the AMPH model, is frequently used to investigate biological mechanisms and alterations that have already been described in BD (Kara and Einat, 2013; Sharma et al., 2016). Clinical studies often report an increase in inflammatory and oxidative stress parameters (Rowland et al., 2018), and most preclinical evidence is consistent with such findings.

For instance, Valvassori et al. (2015) have shown that AMPH injections resulted in a pro-inflammatory effect. Injected rats presented with an augment of IL-4, IL-6, IL-10, and TNF α in the PFC, ST, and serum, which were restored to control levels following treatment with lithium. However, the authors did not find significant alterations in the HIP and cerebrospinal fluid (CSF). Other psychostimulant drugs, such as methylphenidate and methamphetamine, also seem to increase inflammatory markers in the HIP of rats (Beirami et al., 2017; Motaghinejad et al., 2017). However, no changes in TNF α levels were found in the serum and brain regions analyzed in our model. Still, no differences in inflammatory cytokines levels – such as TNF α , IL-1 β , and IL-10 – have been previously described in rodents injected with AMPH or its derivatives (Bristot et al., 2019; Gubert et al., 2016).

Classically, AMPH is responsible for enhancing dopamine (DA) release by inhibiting its reuptake, promoting reverse transport of DA into the synaptic cleft independent of stimulus and releasing DA from synaptic vesicles in the cytoplasm (Calipari and Ferris, 2013). These are the primary mechanisms involved in AMPH-induced hyperactivity and its neurotoxic effects (Valvassori et al., 2021). If not stored in synaptic vesicles, cytoplasmatic DA has a highly autooxidative capacity that can impair mitochondrial function and increase oxidative stress resulting in cell death (Brown and Yamamoto, 2003; Yamamoto and Bankson, 2005). However, in the present study, lipid peroxidation was not augmented following AMPH injection, given by TBARS levels. TBARS levels were higher in the ST, a brain region with many dopaminergic projections, but no statistical difference between groups was found. Still, a strong positive correlation between TBARS and TNF α levels was observed in this same brain region in AMPH-injected groups, regardless of treatment with lithium. Overall, findings regarding oxidative stress in AMPH models vary in the literature. More acute protocols or higher doses seem to be more likely to promote such alteration in the CNS (Frey et al., 2006; Gomes et al., 2017; Gubert et al., 2016). Also, higher levels of TBARS were observed in the serum of lithium-treated animals, independent of AMPH injection. Lithium *per se* is known to be nephrotoxic (Carter et al., 2013), and increased levels of TBARS in the kidney have already been described in rats (Davis et al., 2018; Ossani et al., 2019).

Oxidative stress is an important promoter and product of the inflammatory response (Biswas, 2016), and it is supposed to underly AMPH-induced inflammation. As oxidative stress and inflammation are predictive of promoting BBB disruption and increasing its permeability (Patel and Frey, 2015), we hypothesized that AMPH would exert a deleterious effect in the BBB by downregulating claudin-5 expression. However, no significant changes were observed on claudin-5 levels in the PFC, ST and HIP of rats after AMPH and lithium injections. A previous study showing increased levels of inflammatory cytokines in the brain parenchyma but no alteration in the CSF suggested that AMPH-injected rats might not present with disrupted BBB (Valvassori et al., 2015). However, *in vivo* and *in vitro* studies have already reported that methamphetamine and other psychostimulant drugs can disturb BBB integrity (Kousik et al., 2012; Northrop and Yamamoto, 2012).

Besides inflammation and oxidative stress, associated mechanisms have been proposed to underlie BBB disruption. For instance, the activation of inflammatory pathways, such as nuclear factor kappa B (NF κ B), can result in the amplification of a large array of genes involved in inflammation, including matrix

metalloproteinases (MMPs) (Hurtado-Alvarado et al., 2016). MMPs are enzymes that degrade tight junctions, such as claudin-5, which are essential for maintaining BBB properties (Rempe et al., 2016). Methamphetamine has already been described to upregulate MMPs expression (Mizoguchi et al., 2007). Additionally, NF κ B pathway activation following TNF α and IL-15 signalling may also be responsible for the downregulating the expression of tight junction proteins (e.g., claudin-2) in vitro (Stone et al., 2011). Therefore, it is relevant to investigate other pathways in BBB disruption and their potential association with BD and other psychiatric disorders.

Although BBB disruption has been implicated in the pathophysiology of psychiatric disorders (including BD; Greene et al., 2020), clinical and preclinical evidence is still scarce. A recent imaging study has shown that, among individuals with BD, only a sub-group exhibited an extensive BBB leakage that significantly differed from controls (Kamintsky et al., 2019). Interestingly, this sub-group of patients have a more chronic course of BD, with more severe symptoms of depression and anxiety. Other studies have evaluated biomarkers in the CSF and described higher levels of catecholamine and serotonin metabolites and inflammatory markers, such as IL-8, in individuals with BD (Isgren et al., 2015; Knorr et al., 2018). Furthermore, increased IL-8 levels in the CSF were associated with lithium treatment. Since its discovery, lithium has remained the first-line therapeutic choice for BD treatment (Yatham et al., 2018), but its effects on inflammation have yet to be fully elucidated. During euthymia, individuals with BD treated with lithium exhibited increased levels of TNF α and IL-4 compared to unmedicated patients (Guloksuz et al., 2010), which has also been described in vitro (Liu et al., 2011); but there is also evidence to show otherwise (Fernandes et al., 2019; Knijff et al., 2007). It is worth mentioning that augmented peripheral TNF α levels were further associated with poor response to lithium treatment in BD (Guloksuz et al., 2012).

Despite its novelty, some limitations should be addressed in our study. First, we did not explore more dynamic markers of the interface integrity between the blood, brain, and CSF, such as Evans Blue, which could be helpful as a first screening. Second, plasmatic levels of lithium were not assessed, but it has been described that therapeutic levels are reached following this protocol (Frey et al., 2006). Third, while only a few parameters were analyzed, their relevance to BD and the rationale for their role in the BBB disruption have been discussed. Although an ideal animal model for BD has not been developed, AMPH injection in rodents remains an established animal model of mania with good construct, face and predictive validity (Sharma et al., 2016). It should be noted that the latter has been questioned (Lan and Einat, 2019). Although this model may not mimic the vast complexity of BD pathophysiology, it would be advantageous to identify novel animal models that allow the evaluation of BBB disruption.

In summary, in one of the first attempts to investigate the effects of AMPH on BBB integrity, we did not find evidence that AMPH or lithium impact brain levels of claudin-5. It is only recently that clinical research has provided BBB disruption as a marker of progression in BD (Kamintsky et al., 2019). Still, our results provide evidence and rationale for future research to establish the best approach to model and better understand this relatively novel pathophysiological mechanism implicated in BD.

Abbreviations

AMPH	amphetamine
BBB	blood-brain barrier
BD	bipolar disorder
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dopamine
HIP	hippocampus
IFN	interferon
IL	interleukin
LI	lithium
MMP	matrix metalloproteinases
NFκB	nuclear factor kappa B
PBS	phosphate-buffered saline
PFC	prefrontal cortex
SAL	saline
ST	striatum
TBARS	thiobarbituric acid reactive substances
TNF	tumour necrosis factor

Declarations

Ethics approval

This study was approved by the institutional ethics committee from McMaster University (Animal Research Ethics Board, project #140828).

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author (BF) on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LPG, FK, RM and BNF designed and wrote the protocol. LPG, BWA, DW and WM performed the treatment, behavioural assessment, sample collection and biochemical analyses. LPG, ARR and BNF performed the statistical analysis. LPG wrote the manuscript draft. All authors contributed and approved the final article.

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Tables

Table 1. Results of two-way ANOVA for behavioural assessment.

Dependent variable	Effects	F-value	df	p-value
<i>Number of Crossing</i>	Model	7.30	1,20	0.014
	Treatment	0.10	1,20	0.761
	Interaction	0.48	1,20	0.495
<i>Speed (m/s)</i>	Model	7.23	1,20	0.014
	Treatment	0.59	1,20	0.451
	Interaction	0.04	1,20	0.850
<i>Distance (m)</i>	Model	8.35	1,20	0.009
	Treatment	0.84	1,20	0.371
	Interaction	0.02	1,20	0.885
<i>Time in the periphery (s)</i>	Model	1.09	1,20	0.309
	Treatment	1.31	1,20	0.266
	Interaction	3.31	1,20	0.084

Abbreviations: df, degrees of freedom.

Table 2. Results of two-way ANOVA for biochemical analyses.

Dependent variable	Effects	F-value	df	p-value
<i>PFC TNFα</i>	Model	0.01	1,20	0.960
	Treatment	0.04	1,20	0.837
	Interaction	0.13	1,20	0.727
<i>HIP TNFα[#]</i>	Model	0.42	1,19	0.523
	Treatment	0.88	1,19	0.361
	Interaction	2.23	1,19	0.152
<i>ST TNFα</i>	Model	0.08	1,20	0.786
	Treatment	0.17	1,20	0.683
	Interaction	0.53	1,20	0.474
<i>Serum TBARS</i>	Model	0.10	1,20	0.753
	Treatment	7.97	1,20	0.010
	Interaction	0.03	1,20	0.865
<i>PFC TBARS</i>	Model	0.09	1,20	0.770
	Treatment	0.97	1,20	0.335
	Interaction	0.04	1,20	0.846
<i>HIP TBARS[#]</i>	Model	0.09	1,19	0.763
	Treatment	0.04	1,19	0.854
	Interaction	0.15	1,19	0.705
<i>ST TBARS</i>	Model	0.32	1,20	0.579
	Treatment	0.01	1,20	0.917
	Interaction	1.02	1,20	0.325
<i>PFC Claudin-5</i>	Model	0.16	1,20	0.694
	Treatment	0.06	1,20	0.807
	Interaction	0.79	1,20	0.385
<i>HIP Claudin-5</i>	Model	2.02	1,20	0.171
	Treatment	2.21	1,20	0.153
	Interaction	0.01	1,20	0.929

<i>ST Claudin-5</i>	Model	0.81	1,20	0.379
	Treatment	0.81	1,20	0.379
	Interaction	0.34	1,20	0.568

Abbreviations: df, degrees of freedom; HIP, hippocampus; PFC, prefrontal cortex; ST, striatum; TBARS, thiobarbituric acid reactive substances; TNF α , tumor necrosis factor α . #AMPH+LI, n=5 for these variables.

Figures

Figure 1

Effects of AMPH and lithium in locomotor behaviour. A-C. AMPH increased the frequency of crossing, average speed, and distance travelled in the open field (*main effect of model, $p<0.005$). D. Time spent in the periphery was similar among groups. Two-way ANOVA, data expressed by mean $\pm$ SEM.

Figure 2

TNF α , TBARS and claudin-5 levels in the serum and brain. A. TNF α was not detected in the serum of the animals. B-D. AMPH and lithium injections did not change TNF α levels in the PFC, HIP and ST. E. AMPH did not change lipid peroxidation levels, an increase of serum TBARS was observed in lithium-treated rats (*main effect of treatment, $p=0.009$). F-H. TBARS levels in the PFC, HIP and ST were similar across groups. I-L. Claudin-5 protein levels in the PFC, HIP and ST did not differ following AMPH and lithium injections. A band corresponding to claudin-5 was observed at 15-20 kDa, and β -actin was found at 37-50 kDa. Two-way ANOVA, data expressed by mean $\pm$ SEM.