Early-life stress effects on BDNF DNA methylation in first-episode psychosis and in rats reared in isolation

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Published version

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Progress in Neuropsychopharmacology & Biological Psychiatry

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--Manuscript Draft--

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	Samia Joca
	Paulo Rossi-Menezes
	Adrian Heald
	Paulo Louzada-Jr
	Caroline Dalton
	Cristina Del-Ben
	Gavin Reynolds
Abstract:	Stressful events during early-life are risk factors for psychiatric disorders. Brain-derived neurotrophic factor (BDNF) is implicated in psychosis pathophysiology and deficits in BDNF mRNA in animal models of psychiatric disease are reported. DNA methylation can control gene expression and may be influenced by environmental factors such as early-life stress. We investigated BDNF methylation in first-episode psychosis (FEP) patients (n=58), their unaffected siblings (n=29) and community-based controls (n=59), each of whom completed the Childhood Trauma Questionnaire (CTQ); BDNF methylation was also tested in male Wistar rats housed isolated or grouped from weaning. DNA was extracted from human blood and rat brain (prefrontal cortex and hippocampus), bisulphite-converted and the methylation of equivalent sequences within BDNF exon IV determined by pyrosequencing. BDNF methylation did not differ significantly between diagnostic groups; however, individuals who had experienced trauma presented higher levels of methylation. We found association between the mean BDNF methylation and total CTQ score in FEP, as well as between individual CpG sites and subtypes of trauma. No significant correlations were found for controls or siblings with child trauma. These results were independent of age, gender, body mass index, BDNF genotype or LINE-1 , a measure of global methylation, which showed no significant association with trauma. Isolation rearing resulted in increased bdnf methylation in both brain regions compared to group-housed animals, a correlate of previously reported changes in gene expression. Our results suggest that childhood maltreatment may result in increased BDNF methylation, providing a mechanism underlying the association between early-life stress and psychosis.
Suggested Reviewers:	Kyla Pennington KPennington@lincoln.ac.uk
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	Sintia Belangero sinbelangero@gmail.com
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Response to Reviewers:	We thanks all reviewers for the constructive comments and for the chance to revise and improve our manuscript. We have provided a poin-by-point response, an updated version of our manuscript and supplementary material, and we hope that now our manuscript meet all their requirements. Reviewer #1: This manuscript includes two approaches for exploring the role of childhood trauma on BDNF methylation. First, a clinical study included first-episode psychosis (FEP) patients (n=58), their unaffected siblings (n=29) and community-based controls (n=59), each of whom completed the Childhood Trauma Questionnaire (CTQ). Second, an animal study in male Wistar rats housed isolated or grouped also assessed BDNF methylation in the hippocampus. They found increased BDNF methylation in people with a history of childhood trauma (independent of the diagnosis) and in rats with social isolation. In a stratified analysis by diagnosis, CTQ scores were associated with greater methylation in FEP patients but not in siblings or healthy controls (although the group of siblings was smaller, and the proportion of childhood trauma in these groups was lower too). The manuscript is well-written and the results are interesting. I enclose several comments that might be addressed to improve the quality of the manuscript: 1. The Introduction could be improved by being more specific on the relationship between stress, epigenetics and BDNF, as well as the effects of early life stress on BDNF expression in the hippocampus (which has been quite studied in animal models [Duman's lab among other authors] and even in patients with first episode psychosis [studies by Carmine Pariante and Valeria Mondelli]). For instance, the second paragraph of the introduction is quite vague, particularly the use of the terms 'hormones' or 'drugs'. I suggest to be more specific and cite HPA axis hormones, as well as the relationship between stress hormones and BDNF. I suggest to cite some studies on first episode psychosis that have explored the relationship between BDNF, childhood trauma and hippocampal volumes (Mondelli et al. 2011. Journal of Clinical Psychiatry. 72(12): 1677-1684; Aas et al., 2013. Progress in Neuropsychopharmacology and Biological Psychiatry. 2013. 46: 181-188). We have improved our introduction as suggested. 2. In the Methods, I suggest to briefly explain the meaning of the open field in animal research in terms of behavioural analysis. I am not sure why did the authors decide to choose this experimental design in line with their hypothesis. I recommend to justify in the methods the selection of this experimental procedure. The required information is now added to the methods section in the updated version of our manuscript. 3. The results of BDNF methylation (Figure 2) include all participants (patients, siblings, controls). Although the authors already mentioned that they did not found a significant interaction between diagnostic group and childhood trauma for mean BDNF methylation, I think that a stratified subanalysis by diagnostic group could be placed in the supplementary material (one figure for each subgroup). This might be considered exploratory, but it would help the reader to see whether the methylation follows the same pattern in all three groups. I know that the proportion of people with history of childhood trauma is lower for the healthy control group, and that the number of siblings is also small, but the inclusion of an additional figure with these analyses would be helpful. We have added the subanalysis suggested and the figures related to it in our updated version of the manuscript. 4. In line with the previous comments, Figure 3 represents the relationship between CTQ and BDNF methylation in FEP patients. I suggest to include additional figures as supplementary material for the other two diagnostic groups. We have added the details of statistical correlations analysis for the other groups as well as the figures related to it in our updated version of the manuscript. 5. In the Discussion section, I suggest to discuss a little bit more previous studies dealing with the role of BDNF and childhood trauma in clinical populations (first episode psychosis or schizophrenia). You can cite previous studies already mentioned

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Statistical analysis has been carefully done with all information given in methods and results. Therefore, I have some minor comments or advices for ameliorating the paper.

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Participants: In the line 52, where you mention SCID you should also give a full name for DSM-IV.
We described it in our methods.
Why did you use SCID and not SCID-5?
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Table 5: BDNF rs6265 is usually reported with alleles A and G in the literature. My advice would be to label the genotypes AA, GA and GG to avoid confusion. I am also worried about the fact that when you compared the distribution of BDNF rs6265 genotypes between 3 analyzed 0 TT genotype carriers in two groups and 1 in the third group. I would suggest combining TT with CT carriers and repeating the analysis (X2 test).
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Line 224: Please replace the uppercase letter with the lowercase in the word WITHIN: "Additionally, within each group…"
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Reviewer #3: In this manuscript the authors investigate Bdnf methylation in the periphery of adult psychosis patients and in the brain of rats isolated post-weaning. The human component of this manuscript is interesting, and the combination of data on genotype, methylation, psychosis, and childhood trauma is not necessarily novel but could be useful for the field if this part of the study was presented alone in a brief communication style.

Major comments:

1. Peripheral studies of Bdnf methylation have been common in studies of psychiatric disease and this only adds minimally to the current knowledge base.
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Cover Letter

6th August 2020

Dear Editor-in-Chief: Louis Gendron,

We are submitting our manuscript entitled "**Early-life stress effects on BDNF DNA methylation in first-episode psychosis and in rats reared in isolation**" as an *Original Article* to be considered for publication in *Progress in Neuro-Psychopharmacology & Biological Psychiatry.*

In this study, we analysed the *BDNF* DNA methylation changes in humans in first-episode psychosis, compared to their unaffected siblings and community-based controls and the effects of childhood trauma experiences. In parallel, we analysed equivalent changes in brain areas of rats reared in isolation. The most exciting and interesting result was that *BDNF* methylation changes were more linked to childhood trauma than with psychosis, and this effect was also seen in important areas of the rat brain (hippocampus and prefrontal cortex).

We believe that these results are very important to share with the scientific community and will bring new insights regarding *BDNF* methylation changes triggered by environmental factors in psychosis as well as in its animal model.

We assure that this manuscript has not been submitted elsewhere and that all authors checked and agreed with the version submitted.

Thanks for considering our manuscript for publication in *Progress in Neuro-Psychopharmacology & Biological Psychiatry,*

Best Regards,

Helene A. Fachim,
Postdoctoral Research Scientist
Salford Royal NHS Foundation Trust
Salford-UK
Helene.fachim@manchester.ac.uk

Cover Letter

26th October 2020

Dear Editor: Nela Pivac,

Thanks for the opportunity to revise our manuscript. We have made the changes as the reviewers suggested along the main file and provided the point by point response to the reviewer's comments. We are submitting an updated version of our manuscript with and additional supplementary file as requested by the reviewer 1. It is important to point out that reviewer 3 shows little understanding of the topic, and perhaps further comment from him may not be helpful.

We hope you agree that our manuscript is now suitable for publication in *Progress in Neuro-Psychopharmacology & Biological Psychiatry.*

Thanks once again for considering our manuscript for publication in *Progress in Neuro-Psychopharmacology & Biological Psychiatry,*

Best Regards,

Helene A. Fachim,
Postdoctoral Research Scientist
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Helene.fachim@manchester.ac.uk

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Highlights

- Stressfull events in early-life can modify the epigenome
- Early-life stress is a risk factor for psychiatric disorders
- BDNF is a key mediator of neural plasticity in brain areas has been associated with both early-life events and psychosis
- Environmental influences shape genomic expression through epigenetic mechanisms
- BDNF methylation changes could be a biomarker for early life adversity and hence for adult psychiatric illness

ABSTRACT

Stressful events during early-life are risk factors for psychiatric disorders. Brain-derived neurotrophic factor (BDNF) is implicated in psychosis pathophysiology and deficits in *BDNF* mRNA in animal models of psychiatric disease are reported. DNA methylation can control gene expression and may be influenced by environmental factors such as early-life stress. We investigated *BDNF* methylation in first-episode psychosis (FEP) patients (n=58), their unaffected siblings (n=29) and community-based controls (n=59), each of whom completed the Childhood Trauma Questionnaire (CTQ); *BDNF* methylation was also tested in male Wistar rats housed isolated or grouped from weaning. DNA was extracted from human blood and rat brain (prefrontal cortex and hippocampus), bisulphite-converted and the methylation of equivalent sequences within *BDNF* exon IV determined by pyrosequencing. *BDNF* methylation did not differ significantly between diagnostic groups; however, individuals who had experienced trauma presented higher levels of methylation. We found association between the mean *BDNF* methylation and total CTQ score in FEP, as well as between individual CpG sites and subtypes of trauma. No significant correlations were found for controls or siblings with child trauma. These results were independent of age, gender, body mass index, BDNF genotype or *LINE-1*, a measure of global methylation, which showed no significant association with trauma. Isolation rearing resulted in increased *BDNF* methylation in both brain regions compared to group-housed animals, a correlate of previously reported changes in gene expression. Our results suggest that childhood maltreatment may result in increased *BDNF* methylation, providing a mechanism underlying the association between early-life stress and psychosis.

Early-life stress effects on *BDNF* DNA methylation in first-episode psychosis and in rats reared in isolation

Helene A. Fachim [1,3,9], Fabiana Corsi-Zuelli [1], Camila M. Loureiro [2,3], Sri-arun Iamjan [3,4,5], Rosana Shuhama [1], Samia - Joca [6,7], Paulo Rossi Menezes [8], Adrian Heald [9], Paulo Louzada-Junior [2], Caroline F. Dalton [3], Cristina Marta Del-Ben [1*]and Gavin P. Reynolds [3*].

* These authors contributed equally to this paper.

[1] Department of Neuroscience and Behaviour, Ribeirão Preto Medical School, University of São Paulo, Brazil.

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[4] Division of Anatomy, Department of Biomedical Sciences, Faculty of Allied Health Sciences, Burapha University, Thailand

[5] Faculty of Medical Science, Centre of Excellence in Medical Biotechnology, Naresuan University, Phitsanulok, Thailand

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*Correspondent Author: Helene.fachim@manchester.ac.uk

1. Introduction

Childhood trauma, including physical, sexual, emotional abuse and neglect, are events known to compromise neural structure and function. This can result in increased susceptibility to developing cognitive deficits and psychiatric illness including schizophrenia, major depression and bipolar disorder (1–3) . It is already known from clinical and experimental studies that the prefrontal cortex (PFC) and hippocampus play a crucial role in the cognitive deficits and aberrant emotional behaviours originating from early-life adversity (4,5). Brain-derived neurotrophic factor (BDNF) is a neurotrophin that regulates synaptic transmission and plasticity (6), and it has a role in proliferation, differentiation, survival, and death of neuronal and non-neuronal cells. BDNF is also a key mediator of neural plasticity in both PFC and hippocampus and its decrease in the brain has been associated with both adverse early-life events (7–11) and schizophrenia (12).

BDNF gene regulation appears to be "stressor specific"; it was previously demonstrated in animal models that the BDNF regulation can be modulated by early life stress (13).

Environmental influences, including nutrition, maternal care and behaviour, hypothalamus-pituitary-axis (HPA) hormonal variations~~es~~, drug treatments~~s~~, and early-life experiences (14), shape genomic expression through epigenetic mechanisms (15). Furthermore, some studies have shown that childhood abuse has a long-lasting effect on both the HPA axis and BDNF ~~-~~in the brain (16) and blood (17,18,20). DNA methylation is one epigenetic mechanism that can inhibit or enhance gene transcription by, for example, modifying transcription factor (TF) binding sites within the promoter region of the gene (19).

Exposure to stress and maternal neglect in early life can disrupt epigenetic programming in the brain (21), with lasting consequences for brain gene expression and behaviour. This evidence is primarily derived from animal studies, with limited evidence in

humans (15) due to the inaccessibility of the target brain tissues. However, emerging evidence suggests that epigenetic biomarkers in peripheral tissues may be used to predict disease phenotypes in humans (22). Among the molecular alterations seen in schizophrenia, a decrease in *BDNF* mRNA was previously demonstrated in the brain in both animal models of psychosis (23–25) and human disease in post-mortem samples (26,27).

It has previously been suggested, from studies of the effects of an environmental toxin, that *BDNF* methylation changes could be a biomarker for early-life adversity, and hence, for adult psychiatric illness (21). Furthermore, *BDNF* methylation has also been proposed as an epigenetic mechanism underlying changes in gene expression and behaviour incited by early-life stress in rodents (28). We investigated if *BDNF* DNA methylation changes would be present in the peripheral blood of patients after a first-episode of psychosis (FEP) compared with their unaffected siblings and community-based controls and if childhood trauma could be a possible environmental factor associated with such DNA methylation changes among the three groups. We also studied the possible influence of the *BDNF* single nucleotide polymorphism (SNP) rs6265 (Val/Met), which has been associated with psychosis (29–31), on DNA methylation and its interaction with trauma. Furthermore, we investigated potential *BDNF* methylation changes in the PFC and hippocampus in a pre-clinical model of schizophrenia induced by early-life stress, namely rats reared in social isolation after weaning compared with group-housed animals. We hypothesised that early-life stress would be associated with changes in *BDNF* methylation in the clinical samples as well as in this animal model.

2. Methods

2.1. Participants

This study belongs to the epidemiological research "Schizophrenia and Other Psychosis Translational Research: Environment and Molecular Biology" referred to as STREAM (32), which is part of the international consortium European Network of National Schizophrenia Networks Studying Gene-Environment Interactions (EU-GEI; http://www.eu-gei.eu/) (33).

For the current study, we retrieved a subsample from the epidemiological study selecting FEP patients with the diagnosis of schizophreniform disorder or schizophrenia, bipolar disorder or depression with psychotic symptoms, confirmed by the Structural Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (SCID) (34,35). In total, we included 60 individuals with FEP, 60 age- and sex- matched community-based controls, and 30 unaffected siblings of patients, as described previously (36,37). All participants were between the ages of 16 and 64 years and from the catchment area of Ribeirão Preto, São Paulo, Brazil (38). We excluded patients with psychotic symptoms due to other medical conditions or associated with substance intoxication/withdrawal. The details of inclusion and exclusion criteria followed those of other EU-GEI studies (38,39).

The unaffected siblings of patients were included to represent a high-risk sample, which would serve as an intermediate group between patients and controls. They were asked to volunteer to join the study following agreement by the patient to the invitation and if they had no life-time history of psychotic symptoms.

The recruitment of controls, followed the population distribution of the Ribeirão Preto catchment area,~~,~~ as defined by the 2010 Brazilian Official Census Bureau (Instituto Brasileiro de Geografia e Estatística, IBGE). Controls had a lifelong absence of psychotic symptoms.

The study was approved by the local Ethics committee (process number 15280/2011), and all participants gave written informed consent.

2.2. Stress measurements

As previously described (40), we assessed the history of childhood maltreatment in our sample using the Childhood Trauma Questionnaire (CTQ) (41,42). The CTQ short form is a self-reporting questionnaire consisting of 25 items rated on a 5-point Likert scale (1 = never true; 5 = very often true) to assess separately the exposure to sexual, physical and emotional abuse, and physical and emotional neglect. The sum of values of the five sub-scales generates the CTQ total score, which ranges from 25 to 125 points. In addition, 4 cut-off scores are provided for each scale: none to low; low to moderate; moderate to severe and severe to extreme. Subjects who scored at or above the "moderate to severe" cut-off scores (≥ 13 for emotional abuse; ≥ 10 for physical abuse; ≥ 8 for sexual abuse; ≥ 15 for emotional neglect; and ≥ 10 for physical neglect) on at least one of the five subscales of the CTQ were defined as a "maltreated" group (42).

2.3. Animals and housing

The animal experiments were carried out as described in an earlier publication (43). Isolation rearing from weaning is a well-recognised animal model used to study psychosis as the isolation period results in behavioural, molecular and neurochemical changes which are similar to those found in schizophrenia (44–47). Male Wistar rat pups were purchased from the animal facility of the University of São Paulo (Ribeirão Preto) and transported with their mothers (6 pups per mother), on the day of birth, to the animal house associated to the Laboratory of Neuropsychopharmacology. They remained with their mother until weaning (21 days) in a temperature-controlled room (23 ± 1°C) on a 12:12 h light/dark cycle (lights on from 06:30 to 18:30), with free access to food and water, and were handled one to three times a week. Handling consisted of suspending the rats by the tail and moving them to a clean cage (approximately 5 seconds); the same person performed all animal handling. At weaning, when

the pups weighed 40 grams, they were allocated randomly to one of the two conditions for 10 weeks: (1) grouped (n = 10), housed (3 or 4 per cage) and handled three times a week; (2) isolated (n = 10), housed individually and handled once a week for cleaning purposes only. Animals (grouped or isolated) were housed in 48.5 cm × 25.8 cm × 15.6 cm plastic cages and could see, hear and smell the other animals.

The experiments were carried out according to the Council for Control of Animal Experimentation (CONCEA), and all efforts were made to minimize animal suffering. This study was approved by the local ethical committee (024/2016).

The Open Field test was performed in order to confirm the effectiveness of the isolation rearing protocol. Hyperlocomotion measured by the Open Field test is a reliable, consistent and easily-measured behavioural change seen in this animal model, characterized by the lack of normal habituation following placement in a novel arena (44).

The behavioural analysis was performed in the open field, exposing the animals to a squared arena (dimensions 40 cm x 72 cm x 72 cm) over 20 minutes, divided into four time bins (0-5; 5-10; 10-15; 15-20 min), and square crossings evaluated in the periphery and centre (horizontal exploration), as well as elevations (vertical exploration) as described before (43).

2.4. DNA extraction, Bisulphite Conversion and Pyrosequencing

Genomic DNA from human (blood) and rat (PFC and hippocampus) was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), and was bisulphite-modified to convert unmethylated cytosine residues to uracil using the EpiTec Fast DNA Bisulphite Kit (Qiagen) with a calculated mean conversion of 99%. We identified equivalent DNA sequences for both species (**Fig. 1**), within the exon IV region of the *BDNF* gene as previously studied by Kundakovic et al. (2015), and developed a pyrosequencing method for determination of methylation at each CpG site following bisulphite reaction (4 CpGs in Human and 3 CpGs in

rats). The respective sequences for each species were amplified by PCR using primers (Eurofins MWG Operon), including a biotinylated reverse primer. PCR reactions, amplification conditions and the methylation profile were carried out according to our previous studies (37,48,49).

PyroMark Q24 CpG *LINE-1* sequence-based pyrosequencing was used to quantify methylation at four CpG sites in positions 331 to 318 of *LINE-1* in humans (GenBank accession number X58075) (Qiagen), and to design equivalent primers for *LINE-1* in rats, we used Basic Local Alignment Search Tool (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi), to find the most similar genomic region between species.

Pyrosequencing setup and data reading were conducted by PyroMark Q24 2.0.6.20 software (UK). Samples underwent PCR and pyrosequencing in duplicate; any inconsistencies between samples were resolved following further repetition. Two samples from controls, one from siblings and one from FEP were excluded due to failure of PCR amplification. The primers for humans and rats are showed in **Table 1**.

2.3. Genotyping

We genotyped the SNP rs6265 for *BDNF* in all participants (60 FEP, 60 community-based controls and 30 unaffected siblings) using TaqMan® SNP Genotyping Assay C__11592758_10. Genotyping reactions were carried out in MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode 0.1 mL (REF: 4346906), each containing: 5µl of TaqMan® GTpress™ Master Mix 2X (Applied Biosystems), 0.25µL TaqMan® genotyping assay mix (20X), 2 µl DNA and 2.5µL of DNAse-free water.

The reactions were done according to the following thermocycling program (StepOnePlus™), stage 1: pre-PCR reading at 60°C for 1 minute; stage 2: holding of DNA polymerase activation at 95 °C for 20 seconds; stage 3: 40 cycles of denaturation at 95°C for 3

seconds and annealing at 60°C for 20 seconds; stage 4: 10 cycles of post-PCR reading of denature at 95°C for 3 seconds and annealing at 60°C for 20 seconds. The allelic discrimination analyses were performed in the Taqman® Genotype (Software Real Time PCR Systems Version 2.0 - Applied Biosystems®, 2007) program, with the specific algorithm compatible with the results generated by the equipment for the polymorphism.

2.4. Statistical Analysis

All the analyses were done using SPSS 20.0 (IBM Corp: Armonk, NY, USA). For the clinical sample, demographic and clinical data were analysed using descriptive statistics. Data were checked for normality using the Kolmorogov-Smirnov test.

We firstly tested the overall association of diagnostic groups and history of childhood trauma with *BDNF* methylation. Because our dependent variable (*BDNF* methylation) was not normally distributed we used the generalized linear model with gamma family and log link function and Bonferroni corrections with statistical significance set at $p<0.05$ (Wald Chi Square, χ^2), adjusting for the effects of age and gender, body mass index (BMI), and *LINE-1* (a measure of global methylation). We included *BDNF* methylation levels at CpG1, CpG2, CpG3 and CpG4 as well as the mean methylation as the dependent variables, and childhood trauma (yes or no), diagnostic groups (first-episode psychosis (FEP) patients, unaffected siblings, and community-based controls), sex, and childhood trauma by diagnostic groups as the independent variables, while adjusting for the effects of age, BMI and *LINE-1* methylation. Among the whole sample and for each subgroup, correlations between methylation and CTQ scores, and methylation and CTQ subtypes were tested by Spearman's correlation. Genotype rs6265 frequency differences for *BDNF* gene were compared among FEP patients, siblings and controls using Fisher's exact test and Chi-square test. We also analysed the genotype effects on methylation and association with trauma using the generalized linear model with gamma family

and log link function and Bonferroni corrections with statistical significance set at $p<0.05$ (Wald Chi Square, χ^2) controlling for age, gender, *LINE-1* and BMI.

For the isolation rearing study in rats, between-group differences were evaluated using the Student's t test and associations between methylation and behaviour were tested by Pearson's correlation.

3. Results

3.1.Socio-demographic and clinical characteristics of the sample

The socio-demographic and clinical characteristics of all the groups included in this study are shown in **Table 2**. The categories of duration of untreated psychosis (DUP) followed the same categorisation as published in our previous study (36).

3.2. Childhood maltreatment in FEP, unaffected siblings and community-based controls

Our final sample was composed by 58 FEP patients (28 without and 30 with childhood trauma experiences), 29 unaffected siblings (21 without and 8 with childhood trauma) and 59 community-based controls (52 without and 7 with childhood trauma experiences). The distribution of severity of trauma subtypes (sexual, physical and emotional abuse, and physical and emotional neglect) are described for each group in **Table 3**. Chi-square analysis shows childhood trauma to be significantly different between groups (51% in FEP, 27% in siblings and 12% in controls, $\chi^2=18.329$, $p<0.0001$), reflecting greater proportions in the FEP patients and their relatives.

3.3. BDNF methylation levels and childhood maltreatment in FEP, unaffected siblings and community-based controls

Considering only the diagnostic groups, we found no significant differences in mean methylation of *BDNF* (group factor Wald $\chi^2_{(2)}$=2.702; p=0.259). Among the four individual CpG sites, a significant group difference was found for CpG3 (CpG3: Wald $\chi^2_{(2)}$=6.710, p=0.035), but not for the remaining CpG sites (CpG1: Wald $\chi^2_{(2)}$=2.784; p=0.249; CpG2: Wald $\chi^2_{(2)}$=0.674, p=0.714;~~;~~ CpG4: Wald $\chi^2_{(2)}$=1.989, p=0.370). *BDNF* methylation was not significantly related to age, BMI or sex in the sample.

When considering childhood trauma exposure independent of diagnosis, we found that those individuals who had experienced childhood trauma presented higher levels of methylation than those without childhood trauma (*BDNF* mean methylation: Wald $\chi^2_{(2)}$=7.863, p=0.005). This reflected significant changes in all four CpG sites studied (CpG1: Wald $\chi^2_{(2)}$=7.318, p=0.007; CpG2: Wald $\chi^2_{(2)}$=8.507, p=0.004; CpG3: Wald $\chi^2_{(2)}$=6.159, p=0.013; CpG4: Wald $\chi^2_{(2)}$=4.359, p=0.037), as shown in **Fig. 2.** These results were essentially unchanged after including mean *LINE-1* methylation as covariate, with significantly higher methylation remaining in individuals who experienced trauma for CpG1, 2 and 3 (CpG1: Wald $\chi^2_{(2)}$=5.729, p=0.017; CpG2: Wald $\chi^2_{(2)}$=7.804, p=0.005; CpG3: Wald $\chi^2_{(2)}$=5.055, p=0.025; CpG4: Wald $\chi^2_{(2)}$=2.882, p=0.090), all results were already adjusted for sex, age and BMI. Our results also showed the absence of a significant interaction between diagnostic group and childhood trauma for mean *BDNF* methylation, nor any correlation between *BDNF* methylation and *LINE-1* methylation.

In a subanalysis stratified by diagnostic group, we found a strong association between *BDNF* mean methylation and occurrence of childhood trauma in FEP patients, also for each of the individual CpGs (**Suppl. 1A**). We did not seen any association for Controls and *BDNF* mean methylation, however we found significance between CpG1 methylation and trauma but no association for the remaining CpG sites in the Control group (**Suppl. 1B**). And considering only Siblings we did not find association between *BDNF* mean methylation and trauma, neither

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for any of the specific CpGs analysed (**Suppl. 1C**). The statistic results for these analyses are described on the **Supplementary Table1**.

Exploring the relationship between CTQ scores and *BDNF* methylation among the whole sample, we observed no significant correlation between total CTQ score and mean *BDNF* methylation (rho=0.060, p=0.410), although significant correlation was found with CpG2 (rho=0.203, p=0.014). Among the five subscores contributing to CTQ, these effects were seen between CpG2 in relation to three of five subscores, with significant correlations of CpG2 with emotional abuse (rho=0.236, p=0.004) sexual abuse (rho=0.188, p=0.023) and physical neglect (rho=0.190, p=0.021). These results appeared to be driven primarily by effects in the FEP subjects; within the sample group, no such significant correlations were identified in the control and/or sibling subjects while strong correlations were observed in the FEP group between total CTQ score and *BDNF* mean methylation (rho=0.430, p=0.001) (**Figure 3**), an effect also seen between specific CpGs and CTQ subscores specifically in the FEP sample **(Table 4)**, but not in siblings (rho=-0.187, p=0.332; **Suppl.2A**) or controls (rho=-0.200, p=0.123; **Suppl.2B**). ~~Significant correlations were found between CpG1 and CTQ total (rho=0.395, p=0.002), emotional abuse (rho=0.428, p=0.001), physical abuse (rho=0.263, p=0.046, and emotional neglect (rho=0.309, p=0.018); CpG2 and CTQ total (rho=0.467, p<0.0001) significant also in all five categories: emotional abuse (rho=0.507, p<0.0001), physical abuse (rho=0.321, p=0.014), sexual abuse (rho=0.286, p=0.030), emotional neglect (rho=0.327, p=0.012) and physical neglect (rho=0.284, p=0.030); CpG3 and CTQ total (rho=0.347, p=0.008) and emotional abuse (rho=0.348, p=0.007), sexual abuse (rho=0.304, p=0.020) and physical neglect (rho=0.276, p=0.036); and CpG4 CTQ total (rho=0.368, p=0.004), emotional abuse (rho=0.378, p=0.003, and emotional neglect (rho=0.313, p=0.017).~~

3.4. BDNF rs6265 genotype distribution and associations with trauma and DNA methylation

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Genotype frequencies of *BDNF* gene were in Hardy-Weinberg equilibrium for FEP patients, siblings and control groups (χ^2=2.46, p=0.652), also considering groups stratified by diagnosis (FEP: χ^2=0.373, p=0.830; Siblings: χ^2=0.429, p=0.807; Controls: χ^2=0.383, p=0.536). The frequency distribution of rs6265 is showed in **Table 5**. No significant association with sample subgroup is apparent (p>0.05).

We found no significant association of genotype with mean *BDNF* methylation (Wald $\chi^2_{(2)}$=0.159, p=0.690) or with individual CpG sites, nor was there interaction of genotype by trauma with *BDNF* methylation (Wald $\chi^2_{(2)}$=0.392, p=0.531).

3.5. Isolation rearing from weaning and ~~BDNF~~ bdnf methylation

Isolation rearing resulted in changes including increased locomotor activity in the open field (previously reported in ref. (43)). Isolation-reared animals exhibited greater methylation at CpG1 of ~~*BDNF*~~ *bdnf* in PFC (t=-2.13 p=0.046) and at CpG1 and 2 in hippocampus (CpG1: t=-2.20, p=0.007; CpG2: t=-2.64 p=0.016) compared to the group-housed animals **(Fig. 4)**.

4. Discussion

The present study showed that *BDNF* methylation did not significantly differ among FEP patients, their unaffected siblings and community-based controls; however, greater DNA methylation at CpG1, 2 and 3 was present in individuals who experienced childhood trauma, regardless of diagnostic group. Additionally, w~~W~~ithin each group, we found that FEP had positive correlations between all CpGs analysed and the total CTQ score as well as with the specific subtypes of emotional abuse and neglect, but such correlations were not found in siblings or controls. These results were independent of *LINE-1* methylation, as a measure of global DNA methylation, age, gender, BMI or *BDNF* genotype. Animal data provided

additional support, since increased ~~*BDNF*~~ *bdnf* methylation was observed in two brain regions implicated in psychosis, the PFC and hippocampus, of rats reared in isolation.

BDNF gene expression is altered in many psychiatric disorders, and consequently, the DNA methylation status has been investigated in several previous studies, albeit with inconsistent results, some showing association with psychosis (50–53), and others failing to find such associations (54,55). Our results reveal an interesting trait that may explain these inconsistencies, with *BDNF* methylation being linked to psychosis only in the presence of early-life adversity. Although there is previous evidence supporting the link between *BDNF* methylation and early-life adversity targeting psychosis (21,56–60), none investigated all four factors (*BDNF* methylation, trauma, FEP and genotype) together. Meta-analysis has shown an association of *BDNF* rs6265 with several psychiatric disorders, including schizophrenia (61). Others have previously shown an influence of this SNP in gene-environment interactions with childhood sexual abuse on depressive symptoms (62), although we did not find any such genotype association in our relatively small sample.

Since BDNF has a functional role in cerebral regions involved with emotional and behavioural regulation (61), and knowing that life stressors can modulate the methylome (56,63–65), the evidence supports the correlations we found between the CTQ subtypes and total scores with *BDNF* hypermethylation. Some evidence from animal studies have shown that *BDNF* methylation can be modified by negative early-life stressors (28,66), and increased ~~*BDNF*~~ *bdnf* exon IV methylation was previously associated with female maltreated rats (66). Additionally, recent studies in humans demonstrated an interaction between higher *BDNF* methylation and a greater history of childhood trauma in major depressive disorder patients (67,68), and an association was seen between maternal trauma and *BDNF* methylation in the newborn (69). A recent review (60) summarized studies showing evidence of childhood trauma and *BDNF* DNA methylation in different situations. Increased methylation was associated with

bulimia nervosa (57), patients exposed to psychotherapy intervention also demonstrated higher methylation levels compared to controls and childhood abuse severity predicted higher levels of *BDNF* methylation pre-therapy (58), and depressive symptoms in monozygotic twins were associated with *BDNF* methylation (70).

We have demonstrated ~~*BDNF*~~ *bdnf* methylation changes in two important brain regions of rats reared in isolation from weaning. The results from this animal model are consistent with our human findings representing a translational change in this gene as a result of early-life stress. The increased open field exploratory activity previously reported in these animals (43) is a hallmark behavioural change observed in isolation-reared rats that reflects increased vulnerability to psychiatric disorders related to early-life stress exposure, such as schizophrenia (71). Epigenetic modification of ~~*BDNF*~~ *bdnf* has been reported in prenatally-stressed mice modelling psychosis (72). Altogether, this evidence further supports the influence of early-life adversity in modifying the ~~*BDNF*~~ *bdnf* epigenetic profile.

Recent studies support the hypothesis that BDNF may create an important link between stress and mental illness. Stress is a well-established environmental risk factor triggering mental disorders, presumably due to impaired BDNF signalling, with the participation of other neurobiological mechanisms (73). It has been consistently demonstrated that exposure to a variety of stress models impairs BDNF expression in different cortical and limbic brain areas (74–76). For instance, rats subjected to chronic unpredictable mild stress show a significant decrease in BDNF hippocampal levels (77). Additionally, rats submitted to olfactory bulbectomy, modelling depression/anxiety, also had diminished concentrations of BDNF in the medial PFC accompanied by hyperlocomotion, both features being reversed by deep brain stimulation (78). Regarding rats undergoing isolation rearing from weaning, the majority of the findings using this animal model reported decreased *BDNF* mRNA and/or protein expression

in the hippocampus, while no change or increased levels after re-socialization have been reported in PFC (76).

To investigate the *BDNF* methylation profile in human and rat samples, we chose equivalent gene regions in both species in exon IV that included a cAMP-response element (CRE)-binding protein (CREB) TF binding site. As *BDNF* transcription is CREB dependent (79), the changes in methylation seen in our results may underlie the protein and gene expression *BDNF* alterations reported before in psychosis (80–87) and in its animal models (24,88). Methylation in this DNA sequence have been shown to affect CREB binding and consequent *BDNF* transcription (79,89). It is notable that the greatest effects of early-life trauma on methylation appear to be in CpG2 in the human samples, and CpG1 in the rat. These two methylation sites are equivalent and within the methylation-sensitive CREB binding sequence (figure 1).

Therefore, although we were unable to determine ~~*BDNF*~~ *bdnf* mRNA and protein in our samples, the increased methylation in the *BDNF* gene may represent an important epigenetic mechanism underlying reduced BDNF protein in brain regions important for emotional regulation throughout brain development, as earlier reported (24,74). However, since increased DNA methylation does not always reflect decreased gene expression, additional studies are necessary to confirm our hypothesis.

The human sample was limited in size, particularly in the small number of unaffected siblings included in this study, reflecting the difficulty in recruiting siblings. We relied on self-reporting to obtain CTQ data which may be open to systematic bias between the sample groups. In addition, our patients were not drug naïve, which could influence methylation levels, however, inclusion of the duration of treatment as a covariate did not qualitatively influence our results. This study is cross-sectional, which limits the possibility to infer causal pathways

between childhood trauma, *BDNF* methylation and psychosis risk. The sample size was not powered for a genetic association study, and hence, we cannot rule out a weak association of rs6265 with psychosis. Nevertheless, we can conclude that the effect of genotype relative to the influence of DNA methylation is small.

5. Conclusion

Altogether, our findings reinforce the hypothesis that DNA methylation may be a possible mechanism underlying the association between childhood maltreatment and ~~psychosis~~mental health difficulties in adult life, supporting the proposed gene-environment interaction model of psychosis (64). Considering that *BDNF* methylation has been associated with both childhood maltreatment and psychosis, replication and confirmation of our findings would indicate the value of BDNF expression or *BDNF* methylation as potential targets for amelioration of the psychiatric consequences of early-life trauma.

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Figure 1.

Figure 2.

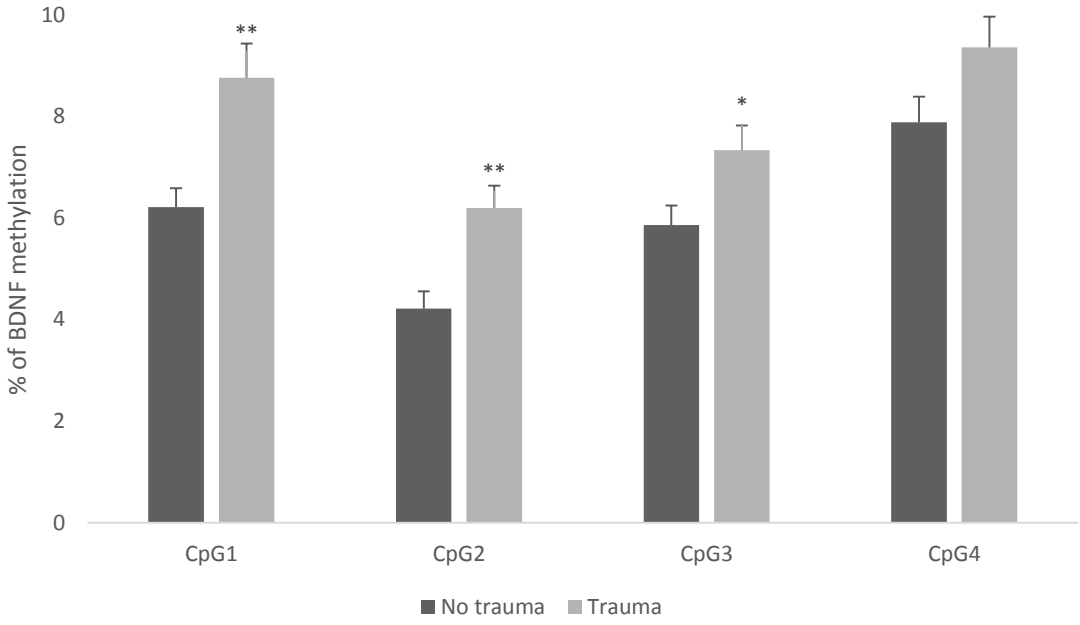
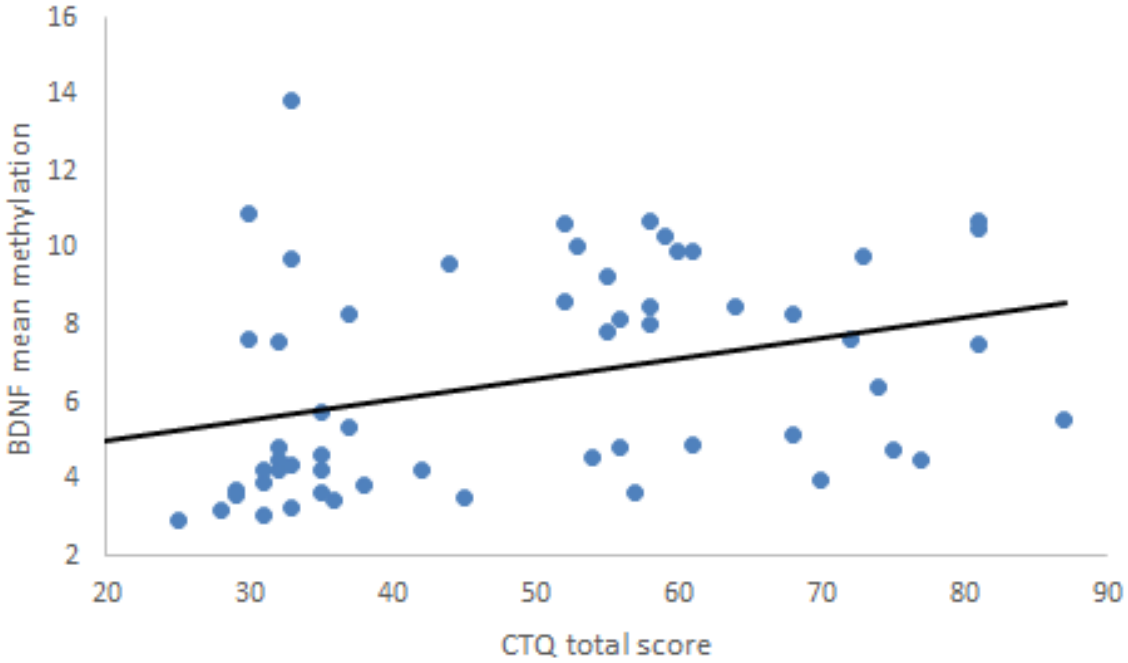


Figure 3.



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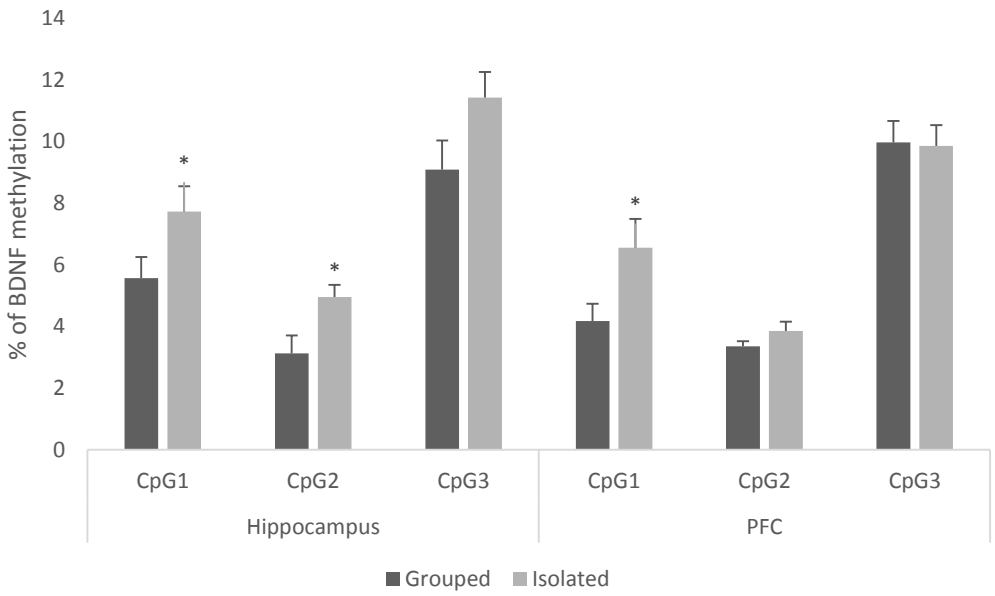

Table

Conflict of interest

We disclosure that the work described has not been published previously and it is not under consideration for publication elsewhere. We also declare that its publication is approved by all authors, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

All the authors declare no conflict of interest.

Ethical Statement

Hereby, I Helene Fachim, consciously assure that for the manuscript "***Early-life stress effects on BDNF DNA methylation in first-episode psychosis and in rats reared in isolation***" the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

I agree with all the above statements and declare that this submission follows the policies of ***Progress in Neuro-Psychopharmacology and Biological Psychiatry*** as outlined in the Guide for Authors and in the Ethical Statement.

Date: 30.07.2020

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Author Statement

HAF, FCZ, CML were involved in the Conceptualization; Data curation and Formal analysis; HAF was responsible for Funding acquisition and Investigation; HAF, CML, FCZ, RS and SAI participated of the Methodology; CMDB, PRM, PLJ, CFD SRJ, AH and GPR were responsible for the Project administration, Resources and Supervision; all the authors were involved in Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Figures Legends updated