Epigenetic-mediated N -methyl-D-aspartate receptor changes in the brain of isolated reared rats

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Abstract

Aims: We investigated: *Grin1, Grin2a, Grin2b* DNA methylation; ~~and~~ NR1 and NR2 mRNA/protein in the prefrontal cortex (PFC) and hippocampus ~~(HIPPO)~~ of male Wistar rats exposed to isolation rearing. **Materials & methods:** Animals were kept isolated or grouped (n=10/group) ~~housed~~ from weaning for 10 weeks. Tissues were dissected for RNA/DNA extraction and NMDAR subunits were analysed using qRT-PCR, ELISA and pyrosequencing. **Results:** Isolated-reared animals had: decreased mRNA in PFC for all markers; increased NR1 protein ~~levels~~ in hippocampus ~~HIPPO~~; hypermethylation of *Grin1* in PFC and *Grin2b* in hippocampus, ~~HIPPO~~ compared to grouped ~~housed~~ rats. Associations between mRNA/protein and DNA methylation were found ~~in~~ for both brain areas. **Conclusions:** This study ~~supports~~ indicates that ~~changes in~~ epigenetic DNA methylation may underlie NMDAR mRNA/protein expression alterations caused by isolation rearing.

Key words: Glutamate receptor; Early stress; Isolation rearing from weaning; Hippocampus; Gene expression; Prefrontal cortex; Protein expression; NMDAR; DNA methylation; Schizophrenia

Introduction

Interactions between biological and environmental factors are thought to be responsible for the development of schizophrenia, with early life adversity ~~as~~ a potent risk factor [1,2]. In this context, social isolation rearing from weaning is considered a valid animal model of schizophrenia [3–5] in inducing behavioural changes that are associated with the human condition and are sensitive to antipsychotic medication [3,5–7]. The N-methyl-d-aspartate receptor (NMDAR) plays an important role in neurodevelopment [8] and its hypofunction ~~may~~ is ~~are~~ thought to underlie the core symptoms of schizophrenia [9]. NMDARs are heteromeric receptors composed of NR1 (encoded by *GRIN1*, humans; *Grin1*, rodents) and NR2 subunits encoded by four distinct subtypes (*GRIN2A-D*, humans; *Grin2a-d*, rodents) [10]. The presence of both subunits is mandatory for the activity ~~e~~ of NMDAR ion channels that only open in the presence of both glycine and L-glutamate [11,12]. ~~Additionally, the~~The NR1 subunit is associated with regulatory processes controlling the structure and function of synapses [13]; NR2A and B are essential for synaptic plasticity [14] and NR2B is particularly important in working memory [15]. These subunits are good candidates for studying

the neurobiology of schizophrenia, considering that perturbation in NMDAR functioning can disrupt neural excitation ~~and~~ contributinge to altered brain function, especially in this disorder, ~~since~~ with several genetic findings ~~have~~ indicatinged the involvement of *GRIN2A* and *GRIN2B* in schizophrenia [16–18].

~~Together with the glutamatergic system, the GABAergic system also plays a central role in the neurobiology of schizophrenia [19]~~ The neuronal glutamatergic system has a strong interrelationship with GABAergic neurons, which provide inhibitory control of glutamatergic activity [19,20]. Moreover, glutamatergic activity drives GABAergic function since NMDARs are expressed on GABAergic interneurons, particularly the subtype containing the calcium binding protein parvalbumin (PV) in early stages of development [21,22]. Disruptions in this neurocircuit lead to disinhibition of the midbrain dopaminergic system, which plays a central role in the neurobiology of schizophrenia [23,24]. There is a reduced expression of genes associated with GABA neurons, such as glutamic acid decarboxylase (*GAD*), reelin (*RELN*) and ~~parvalbumin (~~*PVALB*, in the brains of schizophrenia patients [25–28]. Decreased *PVALB* is the most replicated finding reported in both schizophrenia *post-mortem* brain as well as in animal models of the disorder [27,29–31]; this finding may relate directly to the hyperfunction of dopamine in the disease [32]. The decreased PV-positive (PV+) interneurons result in imbalanced excitatory and inhibitory input [33,34], and consequent disruption of glutamatergic function, especially via NMDARs [35,36].

A variety of animal models have demonstrated an association between NMDAR subunits and schizophrenia. Genetic animal models that use NR1 knockdown and NR2A knockout have shown an association between reduced NMDAR activity and schizophrenia-like behaviours [37–39]. Social isolation in rodents has been shown to increase NR2 mRNA expression in the prefrontal cortex (PFC) and hippocampus ~~(HIPPO)~~ [40] and to decrease NR1 subunit protein in the PFC [41]. Additionally, evidence indicates~~d~~ that the administration of phencyclidine (PCP), an NMDAR antagonist, replicates ~~certain~~ some features of schizophrenia as negative ~~symptoms~~ and cognitive symptoms ~~deficits of schizophrenia~~ [42,43], related to ~~as~~ a functional consequence of ~~neuronal~~PFC and hippocampus ~~dysregulation~~ dysfunctions ~~in key brain areas such as the (HIPPO) and PFC~~ [42]. Recent evidence has demonstrated that epigenetic regulation, including that of NMDARs, may have a role in schizophrenia, suggesting that changes in DNA methylation may be responsible for deficiencies in

both GABAergic and glutamatergic neurotransmission [44–46]. This includes a reduced DNA methylation of the *Grin2b* promoter in both a neurodevelopmental animal models of schizophrenia [47] and in patients in their first episode of psychosis [48].

Although previous studies have shown abnormalities in the glutamatergic system in animal models of schizophrenia, it is not known if there are equivalent mRNA/protein alterations associated with DNA methylation changes in the brains of rats reared in isolation. Therefore, we evaluated mRNA expression of NMDAR genes (*Grin1*, *Grin2a,* and *Grin2b*), NR1 and NR2 protein expressions and DNA methylation of *Grin1* and *Grin2b* in two brain areas (PFC and hippocampus ~~HIPPO~~) of rats undergoing social isolation rearing. Furthermore, because the *PVALB* deficit is the most consistent finding across animal models and schizophrenia itself, we also evaluated the expression of *Pvalb*~~*V*~~ and other related GABAergic genes (*Reln*~~*EL*~~ and ~~*GAD1*~~*Gad1*) in the brain of rats undergoing social isolation rearing as a validation of this animal model. We hypothesized that isolation rearing would reduce the expression of NMDAR subunits at both mRNA and protein levels due to changes in DNA methylation.

Materials & methods

Behavioural testing: Open Field Test in isolation reared rats

Male Wistar rats were obtained from the Central Vivarium of the University of São Paulo, campus of Ribeirão Preto, Brazil. The animals (10/group) were brought to the vivarium of the Laboratory of Pharmacology and kept isolated from weaning (21 days after birth) or in groups of 3-4/cage (41 x 34 x 16 cm), during 10 days, under standard conditions: temperature (23~~0~~4 ± 1~~0~~°C), light cycle (lights on from 6:00 a.m. to 6:00 p.m.), free access to food (Rats and Mice Nutrition, Agromix, Brazil) and water. The welfare of the animals was assessed daily. The cages and bedding were changed every 2 days, as well as food and water replacement. Animals were randomly assigned to the different experimental groups and experiments were conducted from 6:30 a.m. to 6:30 p.m.~~, with randomization of treatment conditions along the day.~~ All procedures were developed in accordance with Brazilian Council for Animal Experimentation (CONCEA), and all efforts were made to minimize animal suffering. After this period, both groups were exposed to the open field test to assess locomotion, were sacrificed and DNA and RNA extracted from the PFC and hippocampus ~~HIPPO~~, as previously described [49].

Gene and protein expressions

DNA and RNA extracts of the PFC and hippocampus ~~HIPPO~~ were obtained by using the All prep DNA/RNA mini kit (Qiagen, Valencia). The mRNA expression of glutamatergic genes (*Grin1*, *Grin2a* and *Grin2b*) and GABAergic genes (*Pvalb*~~*V*~~, *Gad*~~*AD*~~*1* and *Reln*~~*EL*~~) were conducted by Real-Time quantitative PCR (qPCR) using β-actin (~~*ACTB*~~*Actb*) as a reference gene, and thermal cycling conditions as previously published [49], using the following hydrolysis probes (TaqMan assays): *Grin1* Rat: Rn01436034_m1, *Grin2a* Rat: Rn00561341_m1, *Grin2b* Rat: Rn00680474_m1, ~~*ACTB*~~ *Actb*Rat: Rn00667869_m1, *Gad*~~*AD*~~*1* Rat: Rn00690300_m1, ~~*REL*~~ *Reln* Rat: Rn00589609_m1 and ~~*PV*~~ *Pvalb* Rat: Rn00574541_m1. Gene expression was quantified using the Comparative Ct Method (ΔΔCt Method), using ~~*ACTB*~~ *Actb* as the endogenous (housekeeping) control gene as it showed to be stable across our samples. In relation to gene expression, we followed the manufacturer's instructions (Allprep DNA/RNA mini kit, QIAGEN) using 30 mg of tissue.

For the NR1 and NR2 protein assays, tissues were weighed and then homogenized in PBS buffer (1 mL of PBS per 100 mg of tissue), centrifuged (1 min, 8000 rpm) and the supernatant collected and stored frozen at -80 ºC until analysis.

Quantitative determination of NR1 and NR2 was performed by ELISA according to the manufacturer's instructions *(My BioSource, San Diego*, CA, USA). For the NR1 assay, the detection range was 0.5-10 ng/ml, the sensitivity was less than 0.1 ng/ml, and the coefficient of variation was <10% for intra- and inter-assays. For the NR2 assay, the detection range was 31.2-2000 pg/ml, the sensitivity was less than 18.75 pg/ml, and the coefficient of variation was <8% for intra-assay and <10% for inter-assay. The total protein concentration for each area (PFC and hippocampus~~HIPPO~~) was performed using the biuret method (Piotrowski's test) (Labtest Diagnóstica, Lagoa Santa, MG, Brazil).

DNA extraction, Bisulphite treatment and Pyrosequencing

For DNA methylation experiments, we used DNA prepared as described above and the quantification and purity of DNA/RNA were performed by Nanodrop™ 2000 UV spectrophotometer. The concentrations were adjusted according to the following steps. cDNA reverse transcription for RNA and bisulphite conversion for DNA.

Genomic DNA was extracted from all rat samples using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA/USA), 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%. DNA sequences for each gene were identified in the 5' region that contains likely transcription factor (TF) binding sequences for rats that we identified using ALLGEN-PROMO (http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), and a pyrosequencing method was developed for the determination of methylation at the CpG sites within those sequences following bisulphite reaction. The results were compared to methylation of ~~*LINE*~~*Line-1*, a measure of global methylation.

PCR reactions were carried out with 20 ng bisulphite-converted DNA using the PyroMark PCR Kit in a final volume of 25 µl containing 12.5 µl 1x PyroMark PCR Master Mix, 2.5 µl 1x CoralLoad Concentrate, 1 µl of each primer in a final concentration of 0.05 µM, 8 µl RNase-free water. Amplification conditions were as follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 56°C for 30 s (except for *LINE-1*: 52°C for 30s) and 72°C for 30 s, finally, 72°C for 10 min. Methylation status in the promoter sequence of the target genes was determined with a PyroMark Q24 pyrosequencer (Qiagen UK) using 15–20 µl PCR product and a sequencing primer.

Pyrosequencing setup and data reading were conducted by PyroMark Q24 2.0.6.20 software. We analysed samples in duplicate in both experiments, PCR and pyrosequencing, and any inconsistencies were resolved following further repetition. All the primers are listed i~~o~~n **Table 1**.

Statistical analysis

All results are expressed as the mean and standard error of the mean~~s~~ (SEM) and were analysed using SPSS 20 (IBM Corp: Armonk, NY, USA). The behavioural data analyses were done using the EthoLog 2.2 software [50] and were analysed with repeated measures ANOVA with Bonferroni *post-hoc* test, as described previously [49]. However, as the molecular data ~~was~~ were not normally distributed ~~and~~ we used the Mann-Whitney U test to investigate mRNA/protein expressions and the DNA methylation changes between the two groups (isolated and grouped), for the two brain regions (PFC and hippocampus~~HIPPO~~) under consideration.

Correlations between mRNA/protein expressions and DNA methylation were analysed by the Spearman correlation coefficient (rho). Qualitatively, we considered significant values of rho higher than 0.35. Furthermore, we removed rats that presented values clearly outside the bulk of the data after the descriptive statistics

using SPSS analysis identified outliers for each data set (Analysis – Descriptive statistics – explore – extreme values). The outlier criteria in SPSS consisted ~~in~~ of 1.5 x Interquartile range [51]. Values of $p<0.05$ were considered significant for two-tailed tests.

Results

Open Field test, Locomotion activity

The isolation-reared animals demonstrated hyperlocomotion in the two first time bins in the periphery of the arena when compared to the grouped [0-5 min: $F_{(1,15)}=6.209$, $p=0.025$; 5-10 min: $F_{(1,15)}=14.272$, $p=0.002$], as well as~~,~~ at the centre of the arena during 5-10 min [$F_{(1,15)}=6.452$, $p=0.023$]. These data have been previously published [49].

Gene expression of brain tissues

The RT-qPCR showed that ~~*ACTB*~~ *Actb* was expressed at a stable level across all the samples for both groups (PFC: U=22, p=0.060; hippocampus ~~HIPPO~~: U=34, p=0.369) and therefore was used to normalise the data. **Figure 1A** shows decreased expression of *Grin1* (0.6-fold), *Grin2a* (0.7-fold) and *Grin2b* (1.0-fold) in the PFC of isolated animals when compared to grouped (U=22, p=0.034; U=23, p=0.041; U=19, p=0.019); while no significant changes were found in the hippocampus~~HIPPO~~ (*Grin1*: U=32, p=0.174; *Grin2a*: U=42, p=0.545 and *Grin2b*: U=36, p=0.290).

In **Figure 1B**, we demonstrated the decreased mRNA expression of ~~*PV*~~ *Pvalb* (1.3-fold) ~~*GAD1*~~ *Gad1* (0.9-fold) and ~~*REL*~~ *Reln* (2.1-fold) in the PFC of isolated animals when compared to grouped (*Pvalb*: U=18, p=0.027; *Gad1*: U=14, p=0.019; *Reln*: U=16, p=0.031~~, respectively~~)~~,~~ ~~while no~~No ~~significant changes~~differences were found in the hippocampus ~~HIPPO~~ (*Pvalb*: U=39, p=0.624; *Gad1*: U=41, p=0.744; *Reln*: U=31, p=0.253~~, respectively~~). We excluded outlier values for ~~*GAD1*~~ *Gad1* and ~~*REL*~~ *Reln* in the PFC for one group-housed rat.

NR1 and NR2 protein expression of brain tissues

Isolation-reared rats showed increased NR1 concentrations in the hippocamps ~~HIPPO~~ when compared with grouped (U=8, p=0.001). However, NR1 concentrations in the PFC of isolation-reared rats did not differ from group-housed animals (U=39, p=0.406) (**Figure 2A**). Regarding NR2 protein expression, there were no significant differences

between the groups either in the PFC or in the hippocampus ~~HIPPO~~ (U=36, p=0.462; U=32, p=0.174, respectively) (**Figure 2B**).

DNA methylation of NMDAR subunit genes

DNA rat samples successfully underwent bisulphite conversion, PCR and pyrosequencing to determine methylation in the glutamate (*Grin1* and *Grin2b*) and ~~*LINE*~~*Line-1* sequences. All samples demonstrated single PCR bands with no evidence of DNA degradation.

Regarding ~~*LINE*~~*Line-1,* no significant difference was found between the groups in mean levels of methylation (PFC: U=35, p=0.414; hippocampus ~~HIPPO~~: U=33, p=0.199) (**Figure 3**).

In glutamatergic genes, *Grin1* showed higher methylation at CpG5 in the PFC (U=18, p=0.047) of rats reared in isolation when compared to controls, while no differences were found in the hippocampus ~~HIPPO~~ in any CpG (**Figure 4A**). We also found hypermethylation in *Grin2b* in the hippocampus ~~HIPPO~~ at CpG4 in isolated rats compared to grouped (U=15, p=0.024), shown in **Figure 4B**. In this assay, some animals were excluded by outlier criteria mentioned previously (one isolated and one grouped in the PFC of *Grin1* at CpG5; one grouped and one isolated in the hippocampus ~~HIPPO~~ of *Grin2b* at CpG4).

Correlations among mRNA, protein and DNA methylation in isolated and grouped rats

Negative correlations between DNA methylation and mRNA/protein levels of NMDAR subunits

We found that isolated and grouped animals presented a negative correlation between *Grin1* mRNA and *Grin1* methylation levels at CpG5 in the PFC (rho: -0.488; p=0.040, **Figure 5A**). Moreover, isolated rats presented a negative correlation between *Grin2b* methylation at CpG4 and NR2 protein levels in the hippocampus ~~HIPPO~~ (rho: -0.800; p=0.010, **Figure 5B**). We did not find any significant associations between behavioural changes and molecular alterations.

Positive correlations between mRNA of glutamatergic and GABAergic markers in the PFC

We found the following positive correlations: (A) mRNA of *Grin1* and *Pvalb* ~~*V*~~(rho: 0.563; p=0.012); (B) mRNA of *Grin1* and ~~*GAD1*~~*Gad1*(rho: 0.754; p<0.001); (C)

mRNA of *Grin1* and ~~*REL*~~*Reln* (rho: 0.663; p=0.005); (D) mRNA of *Grin2a* and ~~*PV*~~*Pvalb* (rho: 0.482; p=0.036); (E) mRNA of *Grin2b* and ~~*PV*~~*Pvalb* (rho: 0.646; p=0.00); and (F) mRNA of *Grin2b* and ~~*REL*~~*Reln* (rho: 0.501; p=0.034) HIPPO (**Figure 6**). ~~These associations are demonstrated in the PFC of isolated and grouped rats (**Figures 6A-F**). All the other~~ Ssignificant correlations are showed in **Table S1**.

Discussion

NMDAR subunits alterations in isolation rearing

We found that rats undergoing social isolation rearing from weaning showed NR1 and NR2 changes in ~~at both the~~ both mRNA and protein, as well as at the DNA methylation level; the results indicate ~~alterations, in the NR1 and NR2 NMDAR subunits, indicating~~ that DNA hypermethylation may be ~~as~~ a potential mechanism underlying the changes seen in protein and gene expression of NMDAR subunits. In addition, we demonstrated that isolation-reared animals had robust alterations in multiple indicators of glutamatergic and GABAergic neuronal function in the hippocampus ~~HIPPO~~ and PFC, in line with evidence describing dysfunctional NMDAR signalling in schizophrenia.

Firstly, isolated rats had an overall reduction of mRNA expression in the PFC ~~of~~ for all NMDARs subunits analysed, similar to previous studies that showed decreased NR1 mRNA expression in the striatum and PFC [52,53]. Accordingly, a downregulation of NR2A mRNA in the PFC of rats after isolation rearing has been reported [40], although opposite results were found in the same brain area by another group [54].

As glutamate is a key mediator of synaptic plasticity, these results indicate a glutamatergic dysfunction that likely affects synaptic plasticity in the PFC as a consequence of the social isolation rearing regime. This may be associated with NMDAR dysfunction and an imbalance between excitatory and inhibitory circuits, notably in the PFC [55]. Indeed, previous studies indicated that NMDAR subunits, mainly NR1 and NR2A-B, are involved in the early stages of brain development [22,56]. It is relevant in this respect that an abnormal glutamatergic system in the PFC may underlie the cognitive impairments and memory deficits present in schizophrenia [57,58], which is also in accordance with the hypofrontality already described in this animal model [59], based on an impairment of neuronal transmission and synaptic connectivity [60,61].

Secondly, consistent with our findings of decreased ~~*PV*~~*Pvalb*, ~~*GAD1*~~ *Gad1* and ~~*REL*~~ *Reln* expression in the PFC of isolated reared rats, other studies have shown downregulation of *PVALB*, *GAD1* and *RELN* mRNA in the PFC of schizophrenia patients [28,62–64] as well as in animal models of the disorder [63,65], implicating neurodevelopmental impairments of synaptic function and plasticity, and cognition [66]. Given that the hypofunction of NMDARs on GABAergic interneurons results in a decreased activity of this system [67], our results suggest that the reduced *Pvalb* mRNA expression reflects an indirect reduction of GABA neuron activity driven by dysfunctional NMDARs.

Thirdly, our findings showed increased NR1 protein levels in the hippocampus ~~HIPPO~~ of isolated rats, similar to a previous study showing increased NR1 following five weeks of social isolation, although not reaching statistical significance [53]. On the other hand, NR1 protein levels were reported to be significantly reduced in the PFC in chronic isolation-reared rats [41,53], similarly to our results demonstrating lower NR1 concentrations in the same area, but without achieving significant differences. The increased NR1 in the hippocampus ~~HIPPO~~ found in our study may reflect the dysfunction in the PFC as well as the NMDAR activation in response to the chronic stress from social isolation rearing [68]. In addition, several direct and indirect anatomical pathways link the hippocampus ~~HIPPO~~ and the PFC [69–71] and interactions between hippocampus and cortical regions ~~those two brain areas~~ have long been known to play a central role in behavioural and cognitive functions [72,73], as already previously demonstrated in the post-mortem temporal cortex of schizophrenia patients [74].

In relation to epigenetic markers, isolated animals did not show any significant difference in ~~*LINE*~~*Line-1* methylation, a global measure of DNA methylation. However, we found significantly greater methylation of *Grin1* and *Grin2b*, providing a potential mechanism underlying the NMDAR impairments discussed previously. Thus, the *Grin1* and *Grin2b* hypermethylation do not reflect effects on global methylation, but instead, may represent gene-specific results of social isolation rearing, equivalent to previous alterations already showed in schizophrenia patients [75].

We identified *Grin1* hypermethylation at CpG5 in the PFC of isolated rats. At CpG5 are situated binding sites for two TFs with promoter activity, the specificity protein 1 transcription factor (Sp1) and the CCAAT/Enhancer Binding Protein β (C/EBPβ). However, C/EBPβ activity is reportedly not altered by CpG methylation

[76]. Sp1 ~~is~~ has a dual activity as ~~an important~~ TF promoter TF ~~that can~~ activatinge or repressing transcription in response to physiological and pathological stimuli [77–79]. Sp1 also has a direct role in transcriptional activation and ~~is involved for~~in the ~~initiation~~ initial process of gene expression [80] ~~and~~ bindings with high affinity to GC-rich motifs to regulate the genes ~~expression of a large number of genes~~ involved in ~~a variety of processes such as~~ cell growth, apoptosis, differentiation and immune responses [78,81,82]. Hence, our findings suggest that ~~DNA~~ *Grin1* hypermethylation ~~of~~ ~~*Grin1*~~ following early life stress may interfere with Sp1 binding site and thereby bring about a reduction of *Grin1* mRNA expression.

MoreoverRegarding ~~*Grin2b*,~~ isolated rats presented *Grin2b* hypermethylation at CpG4 of the gene promoter in the hippocampus ~~HIPPO~~, which haswhere ~~. CpG4 is found within a sequence with~~ binding sites for ~~several TFs, among them the~~ Pax family TFs (Pax 5, 6 and 9a-b) are located. The Pax family ~~is important~~has an important role in the specification of tissues during early animal development ~~for the specification of tissues~~ [83,84] via a regulatory function on the gene expression [85]. Considering that isolation rearing is an early life stressor, it seems likely that DNA methylation may contribute to the disruptions seen in the adult life of these rats.

Finally, we found negative correlations between *Grin1* and *Grin2b* methylation~~of *Grin1* and *Grin2b*~~, and respectively *Grin1* mRNA and NR2 protein levels. ~~The~~ Our results indicate that ~~higher levels of~~hyper-methylation levels are associated with reduced gene/protein expressions, supporting our hypothesis that ~~variation in~~ DNA methylation changes ~~is a~~may be a potential mechanism influencing NMDAR protein and ~~gene~~ mRNA expressions. In addition, we also found positive correlations between glutamatergic (mRNA of *Grin1*, *Grin2a* and *Grin2b* and NR2 protein) and GABAergic (mRNA of ~~*PV*~~*Pvalb*, ~~*GAD1*~~ *Gad1* and ~~*REL*~~*Reln*) markers, consistent with previous evidence that NMDARs are particularly found on GABAergic neurons [86,87].

In conclusion, our study showed that DNA methylation ~~might be~~is associated with gene/protein expression of NMDAR subunits in isolation-reared rats. Given that social isolation from weaning characterises a chronic early life stress model, the observed alterations in methylation could result from this period of stress, leading afterwards to disruptions in glutamatergic and GABAergic neurotransmission, resulting in an imbalance in the excitatory/inhibitory tone equivalent to that seen in schizophrenia.

Strengths and Limitations

The most important aspect of this study was to integrate and correlate observations of methylation, gene and protein expression in a range of relevant markers of NMDAR and GABAergic function in a valid animal model of schizophrenia. In order to improve the role glutamatergic system played in the onset of schizophrenia, we first tested the specific hypothesis (for methylation-protein/mRNA correlations) and after we did the secondary exploratory analysis for the other correlations between glutamatergic and GABAergic markers ~~to~~ that may link these changes with schizophrenia.

In this study, we did not measure ~~the~~ all four of the distinct subtypes of NR2 (NR2a-d); this could underlie the lack of ~~that could explain the non-~~significant results in relation to NR2 protein expression between the groups. However, we investigated only NR2 subunit in this animal model of schizophrenia, because in our previous finding, we found low NR2 plasma concentrations in first-episode psychosis patients compared to unaffected siblings and community-based controls [88]. In the present study, we found increased methylation levels at the *Grin2b* gene and, consistently, low expression of this subunit at the gene level. However, contrary to our expectations, no differences were found at the protein level for this subunit, in any of the brain sites investigated. This may reflect the fact that we were only able to assess the total NR2 protein expression rather than the protein subunits. Discrimination between the NR2 subunits is essential for determining the decreased NMDAR activity, considering that the two NR2a-b subunits have different properties in relation to NMDAR function [14,89]. It has been shown that maturation of brain circuits occurs subsequent to the switch of NR2b to NR2a during critical periods of the development [14,58,90]. Thus, the lack of significant differences at the NR2 protein levels in our study should be interpreted with caution and it is important that future studies consider the analyses of NR2a and NR2b subunits separately.

We used hyperlocomotion as a proxy for validation of the model, given that this alteration is the most consistent behavioural change observed [3]; however, we did not include additional experiments related to other disturbances in behavioural domains associated with the isolation-rearing model and relevant to the symptoms of schizophrenia. In addition, our data did not ~~present~~ demonstrate a normal distribution and we used the non-parametric tests; however, our sample size is similar and provides a small variance that reduces the chances of our results as false positives.

Conclusions

Our study reinforces the validity of social isolation rearing after weaning in modelling aspects of schizophrenia, highlighting the glutamatergic and GABAergic disturbances in the disease. We also provide evidence in support of the hypothesis that the NMDAR hypermethylation found in the brain tissues may underlie the NMDAR mRNA/protein expression alterations caused by early isolation. These results highlight the importance of the environment during development as a contributor to behavioural and neurochemical changes during adulthood. In conclusion, our study contributes to the identification of epigenetic mechanisms involved in the neuropathophysiology of schizophrenia, which may provide new approaches for pharmacotherapy as well as identifying biological factors that could improve early diagnosis and intervention.

Summary points

- NMDAR methylation changes found in the brain tissues may underlie the NMDAR mRNA/protein expression alterations caused by the isolation period.
- Early social isolation induces epigenetic modifications in the NMDA receptor subunits.
- Our data support the validity of social isolation after weaning in modeling aspects of schizophrenia, highlighting changes in the glutamatergic and GABAergic systems commonly seen in schizophrenia.
- Our study also reinforces the strong correlations between glutamatergic and GABAergic genes that are involved in schizophrenia.
- ~~Changes in DNA methylation may be a plausible mechanism underlying the gene/protein expression alterations of NMDARs subunits after isolation rearing in rats.~~
- Our findings may contribute to understanding the pathophysiological consequences of decreased NMDAR subunits expression in schizophrenia.
- This study contributes to the identification of epigenetic mechanisms involved in the neuropathophysiology of schizophrenia, which may lead to new pharmacotherapeutic strategies.
- In our study, the period of social isolation from weaning may characterise a chronic early life stress model that induced the alterations in methylation, resulting in an imbalance in the excitatory/inhibitory tone equivalent to that seen in schizophrenia.

- Our findings highlight the importance of the environment during development as a contributor to behavioural and neurochemical changes in adulthood.

Future Perspective

Even though NMDARs are well characterized and much is known about its implication in schizophrenia pathogenesis, the role of epigenetic mechanisms in its dysregulation is still unclear. The results presented in this paper pave the way for further studies and highlight a possible epigenetic mechanism whereby early life adversities contribute to dysregulation in the glutamatergic system, more specifically in the hypofunction of NMDARs and~~,~~ their ~~impact~~ effect on GABAergic function~~ and subsequent disinhibition of dopaminergic neurons in the midbrain~~. The glutamatergic and GABAergic epigenetic dysregulations ~~of the glutamatergic and GABAergic neurotransmitter systems~~ observed in ~~this~~ our study have important translational ~~value~~ utility/merit not only for schizophrenia, but also for a host of psychiatric disorders associated with exposure to environmental adversities. Future research should investigate the association between DNA methylation and early life stress in pharmacological models of schizophrenia, and test correlations between blood and brain biological markers. Finally, the results observed offer mechanistic pathways for translation in clinical settings, including the identification of more vulnerable populations exposed to early-life adversities and the screening of more specific pharmacological tools for these subgroups.

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Author contributions

CML, HAF, PL-Jr and GPR conceived the study. PRM, CFD and CMD-B contributed to the study design. PRM, CMD-B and PL-Jr obtained funding. RS obtained ethical approval. CML, HAF, FC-Z and SJ managed the behavioural and molecular analysis. CML, HAF, FC-Z and RS analysed the data. All authors collaborated in the interpretation of the data. CML wrote the first draft of the manuscript. HAF, FC-Z, RS, SJ, CFD, CMD-B, PL-Jr and GPR critically revised the manuscript. All the authors approved the final version of the manuscript.

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Conflict of Interest

The authors report no biomedical financial interests or potential conflicts of interest.

Ethical conduct of research statement

This study was approved by the local Ethics committee (024/2016) and the experiments were carried out according to the Brazilian Society of Neuroscience and Behaviour guidelines (NIH Publications No. 8023, revised 1978).

Figure Legends

Figure 1. Effects of rearing condition (isolated vs. grouped) on glutamatergic and GABAergic markers gene expression in the PFC and hippocampus ~~HIPPO~~ of rats. The figures show the mean fold change ± SEM of *Grin1*, *Grin2a*, *Grin2b*, *Pvalb~~V~~*, *Gad~~AD~~1* and *Reln~~EL~~* mRNA levels using the housekeeping gene β-actin (*~~ACTB~~Actb*) as reference. Glutamatergic and GABAergic markers mRNA expression were measured by qRT-PCR. (A) Isolated rats presented decreased expression of *Grin1*, *Grin2a* and *Grin2b* mRNA in the PFC when compared to grouped (p=0.034; p=0.041; p=0.019) respectively, while no statistical differences were found in the hippocampus ~~HIPPO~~ (*Grin1*: p=0.174; *Grin2a*: p=0.545 and *Grin2b*: p=0.290) of these animals. (B) Isolated animals also showed decreased expression of *Pvalb~~V~~*, *Gad~~AD~~1* and *Reln~~EL~~* in the PFC when compared to grouped (p=0.027; p=0.019; p=0.031, respectively), while no significant changes were found in the hippocampus ~~HIPPO~~ (p=0.624; p=0.744; p=0.253, respectively). *p<0.05; Mann-Whitney U test.

Figure 2. Effects of rearing condition (isolated vs. grouped) on glutamatergic markers protein expression in the PFC and hippocampus ~~HIPPO~~ of rats. The figures show the mean ± SEM of NR1 (ng/mg) and NR2 (pg/mg) proteins. Glutamatergic markers protein expression was measured by ELISA test. Isolated rats showed increased protein expression of NR1 subunit in the hippocampus ~~HIPPO~~ when compared to grouped (p=0.001); while, no statistical differences were observed in the PFC (p=0.406). In relation to NR2 subunit protein, no differences were observed in the PFC (p=0.462) and hippocampus ~~HIPPO~~ (p=0.174) of these animals. *p<0.05; Mann-Whitney U test.

Figure 3. Effects of rearing condition (isolated vs. grouped) on *~~LINE~~Line-1* methylation in the PFC, hippocampus ~~HIPPO~~ and peripheral blood of rats. The figure shows the mean of percentage of methylation in *~~LINE~~Line-1*. No statistical differences were observed between the groups in relation to PFC (p=0.414) and

hippocampus ~~HIPPO~~ (p=0.199). The ~~*LINE*~~*Line-1* DNA methylation was measured by Pyrosequencing. Mann-Whitney U test.

Figure 4. Effects of rearing condition (isolated vs. grouped) on DNA methylation of *Grin1* and *Grin2b* in the PFC and hippocampus ~~HIPPO~~ of rats. The figure shows the mean ± SEM of percentage of methylation in *Grin1* and *Grin2b* in the PFC and hippocampus ~~HIPPO~~ of rats reared in isolation or grouped. Glutamatergic markers DNA methylation was measured by Pyrosequencing. Increased DNA methylation of *Grin1* at CpG5 (p=0.047) were found in the PFC of isolated-reared rats (A) and increased methylation of *Grin2b* at CpG4 were found in the hippocampus ~~HIPPO~~ of isolated animals (B) when compared to grouped (p=0.024). *p<0.05; Mann-Whitney U test.

Figure 5. Correlations between DNA methylation in the brain tissue and gene/protein levels in the PFC and hippocampus ~~HIPPO~~ of isolated and grouped animals: (A) All rats presented a negative correlation between *Grin1* methylation at CpG5 and *Grin1* mRNA levels in the PFC (rho: -0.488; p=0.040). (B) Isolated rats presented a negative correlation between *Grin2b* methylation at CpG4 and NR2 protein levels in the hippocampus ~~HIPPO~~ (rho: -0.800; p=0.010; Spearman correlation).

Figure 6. Correlations between mRNA of glutamatergic and GABAergic genes in the brain tissues of isolated and grouped animals: All rats presented positive correlations between (A) *Grin1* and *Pvalb~~V~~* mRNA levels in the PFC (rho: 0.563; p=0.012); (B) *Grin1* and *Gad~~AD~~1* mRNA levels in the PFC (rho: 0.754; p<0.001); (C) *Grin1* and *Reln~~EL~~* mRNA levels in the PFC (rho: 0.633; p=0.005); (D) *Grin2a* and *Pvalb~~V~~* mRNA levels in the PFC (rho: 0.482; p=0.036); (E) *Grin2b* and *Pvalb~~V~~* mRNA levels in the PFC (rho: 0.646; p=0.003; and (F) *Grin2b* and *Reln~~EL~~* mRNA levels in the PFC (rho: 0.501; p=0.034; Spearman correlation).

Table 1. List of Forward (F) and biotinylated Reverse (R) primers used in PCR reactions, and Sequencing (Seq) primers for pyrosequencing

Gene	Rats

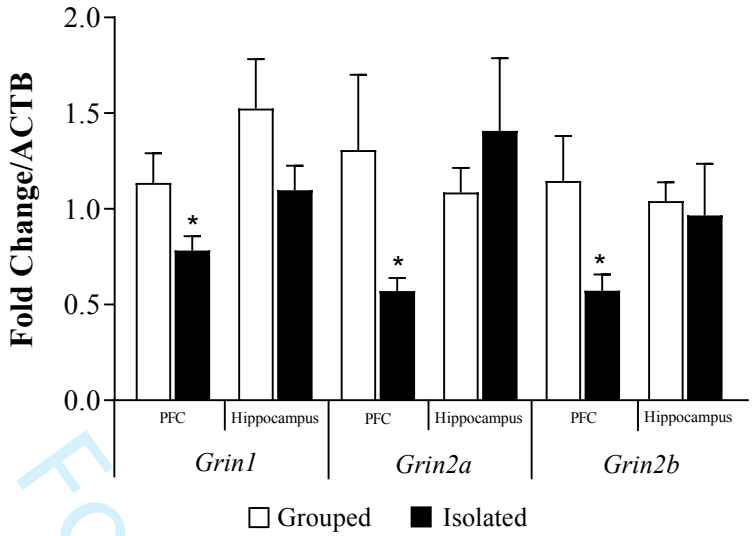
~~LINE~~Line-1	F 5'TTGTTGTAAGAAAGTTGTTTGGTGAGTT3'
	R 5'ACCTCAAAAATACCCACCTAACC3'
	Seq 5'GGTGAGTTTGGGATA3'
Grin1	F 5'TTGGGTTTGTGGGTGATAGAAG3'
	R 5'ACCTACTAACATTCCCCCTACTTTTTTCCT3'
	Seq 5'ATGTTGAAGATTTTGGGGT3'
Grin2a	F 5'TGGCCTCAGTGACAAGAAGTTC3'
	R 5'AGACGGCTGCGTCATAGATGAA3'
	Seq 5'AGAAGAATGGATTTTTTTTA3'
Grin2b	F 5'TTGGGTGTGAGATTTAAATTAAGATTAG3'
	R 5'AAAATAAAAAAAAACCTTCCTTTCTCAA3'
	Seq 5'AGATTAGGATTTTTGATGTT3'

808

809 **Table S1.** Correlations between glutamatergic and GABAergic markers in isolated
810 animals

Figure 1.

(A)



(B)

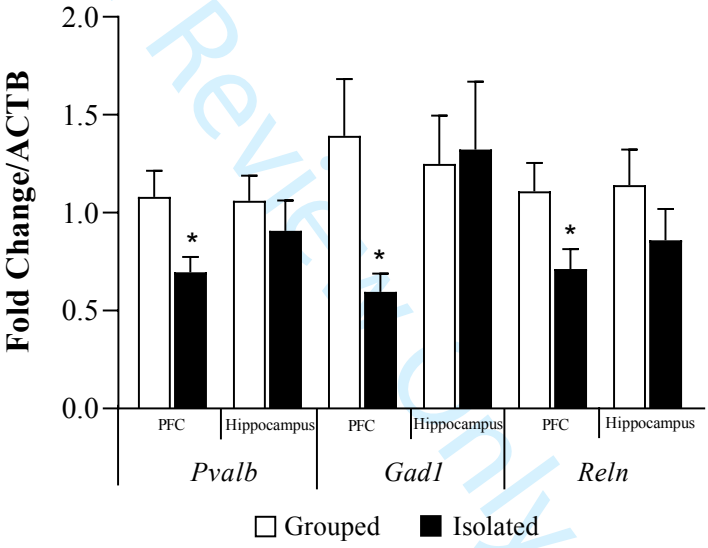


Figure 2.

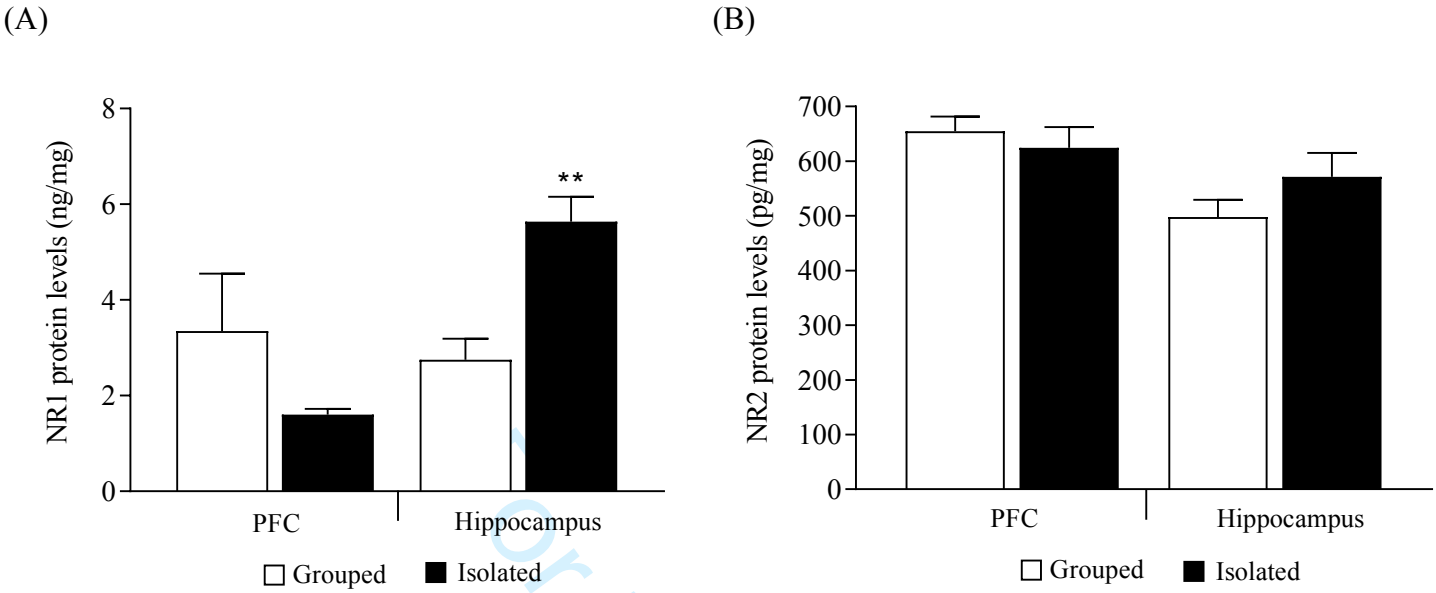


Figure 3.

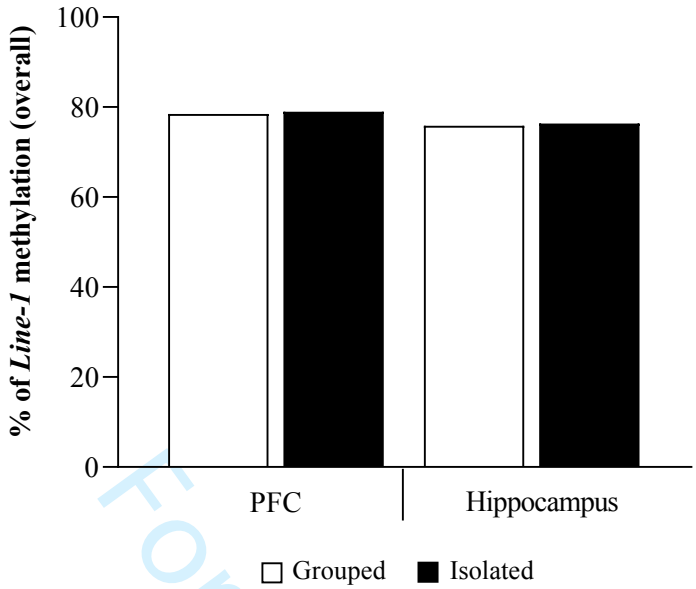
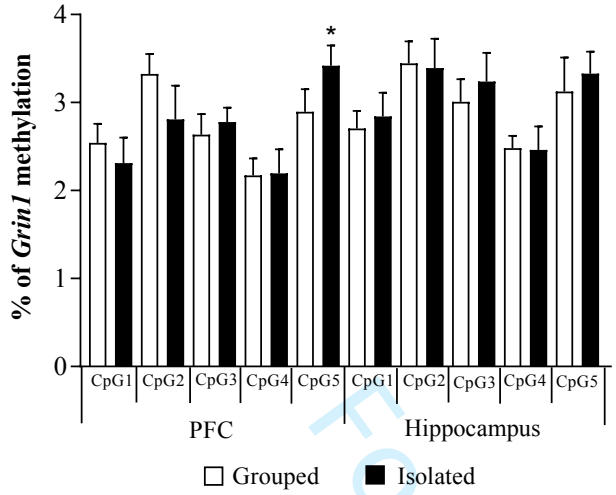


Figure 4.

(A)



(B)

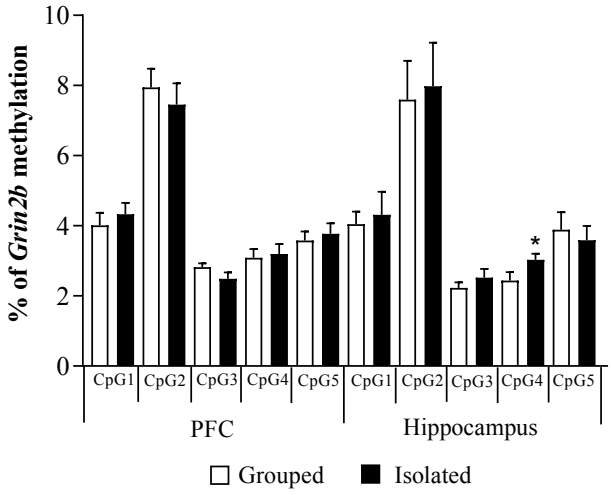
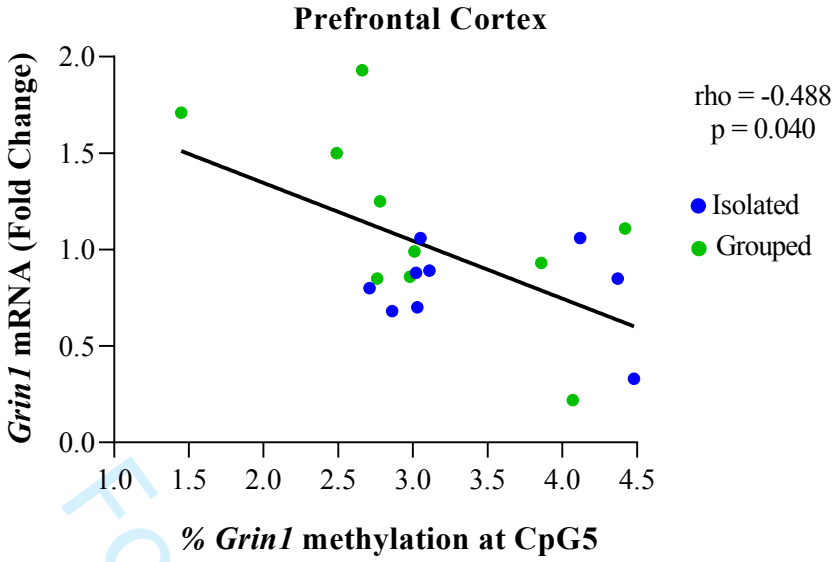


Figure 5.

(A)



(B)

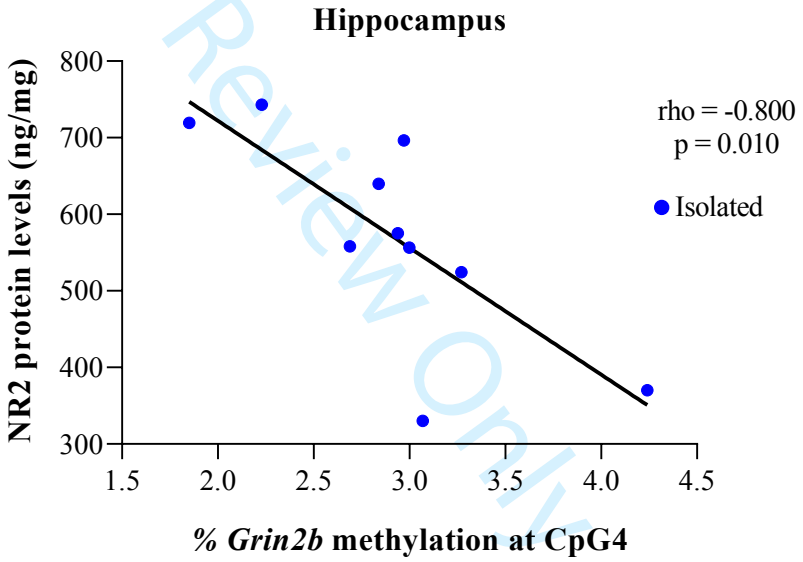
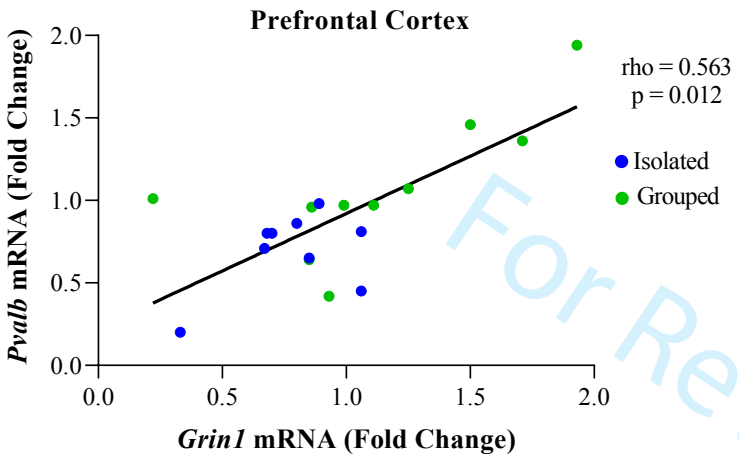
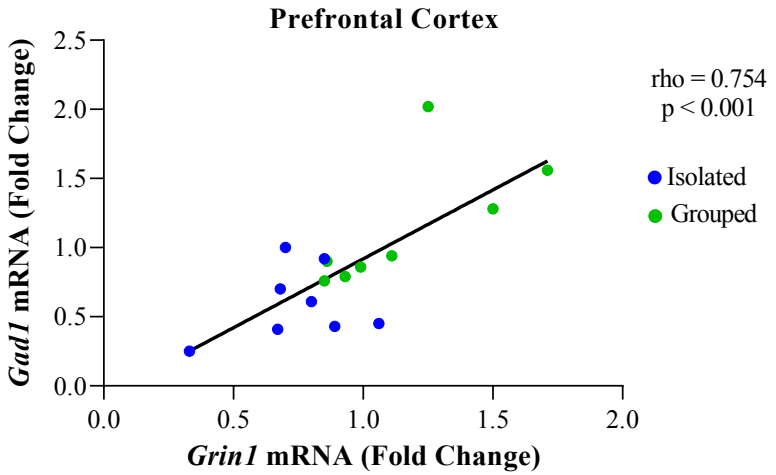


Figure 6.

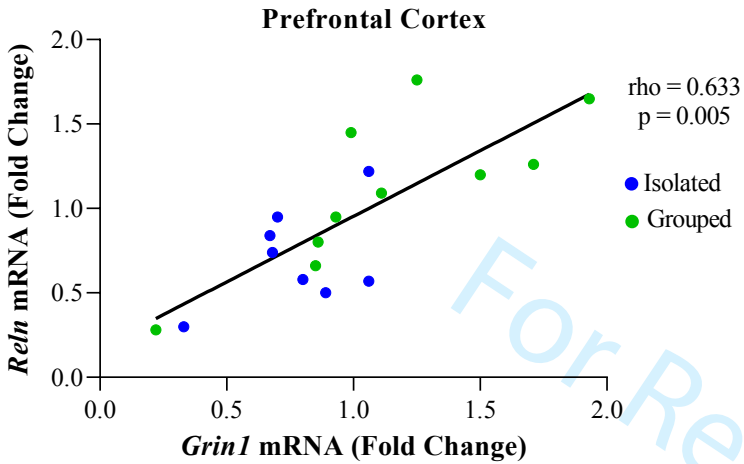
(A)



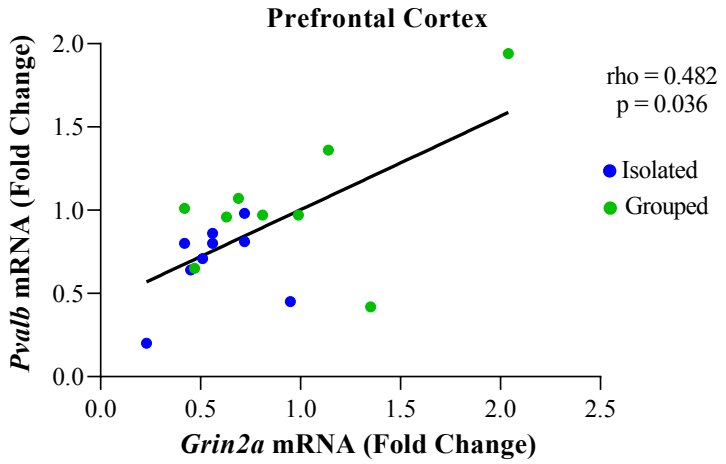
(B)



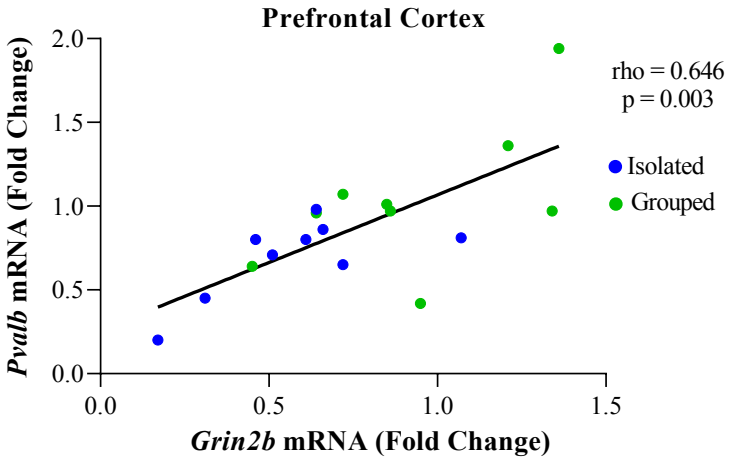
(C)



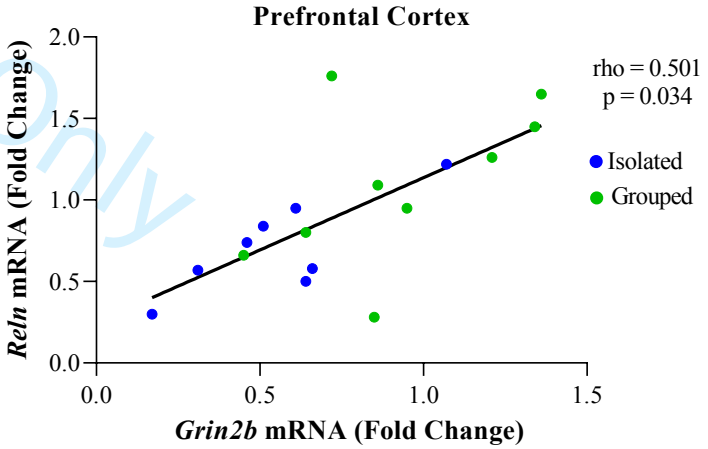
(D)



(E)



(F)



	Grin1 PFC	Grin2a Hippocampus	Grin2a PFC	Grin2b Hippocampus	Grin2b PFC	Pvalb Hippocampus
Grin1 Hippocampus	.045 .850	,812** .000	.011 .965	.250 .289	-.054 .821	.454 .051
Grin1 PFC		-.036 .880	,863** .000	.111 .640	,656** .002	-.116 .637
Grin2a Hippocampus			-.012 .960	,547* .012	-.220 .352	,523* .022
Grin2a PFC				.235 .319	,693** .001	.116 .637
Grin2b Hippocampus					.051 .830	,537* .018
Grin2b PFC						-.049 .842
Pvalb Hippocampus						

Pvalb PFC	Gad1 Hippocampus	Gad1 PFC	Reln Hippocampus	Reln PFC	Grin1 CpG1 Hippocampus	Grin1 CpG2 Hippocampus
.081	,854**	.018	,642**	-.184	-.114	-.177
.743	.000	.943	.003	.450	.631	.454
,563*	.125	,791**	.270	,688**	.042	.078
.012	.611	.000	.263	.001	.860	.743
.005	,747**	-.002	,637**	-.233	-.189	-.245
.983	.000	.994	.003	.336	.425	.298
,482*	.100	,607**	.260	,561*	.211	.173
.036	.684	.006	.283	.012	.373	.466
-.116	.225	.121	.211	-.084	-.058	-.239
.637	.355	.622	.387	.732	.808	.310
,693**	.168	,628**	.116	,674**	.270	.257
.001	.491	.004	.637	.002	.250	.274
-.071	,514*	-.356	,514*	-,492*	.074	.044
.779	.024	.147	.024	.038	.764	.858
Pvalb PFC	.377	,535*	.449	.428	.272	.268
	.123	.018	.062	.067	.260	.267
	Gad1 Hippocampus	.102	,814**	-.040	-.167	-.184
		.687	.000	.874	.495	.450
		Gad1 PFC	.098	,865**	.091	.130
			.699	.000	.710	.596
			Reln Hippocampus	-.121	-.189	-.221
				.633	.439	.363
				Reln PFC	.091	.177
					.710	.468
					Grin1 CpG1 Hippocampus	,892**
						.000
						Grin1 CpG2 Hippocampus

Grin1 CpG3 Hippocampus	Grin1 CpG4 Hippocampus	Grin1 CpG5 Hippocampus	Grin1 CpG1 PFC	Grin1 CpG2 PFC	Grin1 CpG3 PFC	Grin1 CpG4 PFC
-.092	-.196	.096	-.004	-.051	.268	.408
.700	.409	.686	.989	.836	.267	.083
.005	.175	.204	.018	.248	-.014	-.082
.985	.462	.389	.943	.305	.955	.740
-.194	-.099	-.012	-.111	-.108	.079	.274
.412	.679	.960	.652	.660	.748	.257
.160	.245	.361	-.133	.133	.049	.018
.500	.297	.118	.586	.586	.842	.943
-.201	-.094	-.214	-.061	-.009	-.082	.211
.396	.693	.364	.803	.972	.737	.387
.183	.061	.384	.167	.320	-.168	-.052
.441	.799	.094	.495	.181	.491	.833
.196	-.028	.052	-.003	-.147	.385	,491*
.420	.909	.833	.990	.562	.115	.038
.134	.075	.262	-.065	.090	-.034	-.140
.584	.759	.278	.798	.723	.893	.578
-.111	-.327	.031	.040	-.050	.125	.258
.652	.171	.901	.874	.845	.622	.301
-.201	-.029	.122	.096	.152	-.240	-.318
.409	.906	.619	.705	.548	.336	.198
-.002	-.176	.184	-.071	-.121	.197	.236
.994	.470	.450	.779	.633	.433	.345
-.209	-.039	.059	.150	.182	-.375	-,494*
.391	.875	.811	.553	.470	.126	.037
,601**	.396	.427	-.197	-.241	-.243	-.138
.005	.084	.061	.420	.321	.316	.574
,726**	,497*	,489*	-.111	-.185	-.172	-.278
.000	.026	.029	.652	.448	.482	.249
Grin1 CpG3 Hippocampus	.436	,515*	-.011	-.012	.054	-.032
	.055	.020	.963	.960	.825	.895
	Grin1 CpG4 Hippocampus	,518*	-.260	.086	.225	-.043
		.019	.283	.726	.355	.861
		Grin1 CpG5 Hippocampus	-.238	-.144	-.014	.045
			.327	.558	.955	.856
			Grin1 CpG1 PFC	,729**	.205	.390
				.000	.399	.099
				Grin1 CpG2 PFC	.308	,495*
					.199	.031

Grin1 CpG3 PFC	,633** .004
Grin1 CpG4 PFC	

Grin1 CpG5 PFC	Grin2b CpG1 Hippocampus	Grin2b CpG2 Hippocampus	Grin2b CpG3 Hippocampus	Grin2b CpG4 Hippocampus	Grin2b CpG5 Hippocampus	Grin2b CpG1 PFC
.012	-.056	-.080	-.057	.245	-.046	.219
.960	.816	.738	.811	.328	.848	.369
-.400	.251	.074	.111	.005	.246	.106
.090	.286	.758	.640	.984	.296	.665
-.081	-.120	-.090	.077	.133	-.008	.226
.743	.613	.705	.748	.598	.972	.351
-.321	.326	.295	.188	.046	.321	.011
.180	.160	.207	.427	.855	.167	.966
.016	-.146	-.238	.102	-.119	.070	.330
.949	.539	.313	.668	.639	.769	.168
-.342	.417	.233	.220	-.094	.436	-.135
.152	.068	.323	.352	.711	.055	.581
.059	-.153	.093	.039	-.179	-.036	.073
.817	.533	.705	.875	.492	.884	.773
-,575*	.289	.158	.242	.029	.303	-.188
.013	.229	.519	.318	.911	.208	.455
-.193	-.084	-.168	-.061	-.059	-.057	.131
.443	.732	.491	.803	.823	.817	.604
-,517*	.275	-.207	.126	.037	.295	.287
.028	.254	.395	.606	.889	.220	.248
-.362	-.130	-.075	.054	-.311	-.039	.118
.140	.596	.759	.825	.224	.872	.642
-.381	.360	-.128	.123	.022	.334	.103
.119	.130	.601	.616	.933	.162	.683
-.086	,655**	.406	,493*	.391	,609**	-.168
.726	.002	.076	.027	.108	.004	.491
-.077	,701**	,465*	,502*	.467	,608**	-.282
.753	.001	.039	.024	.050	.004	.243
.243	.426	,496*	.356	.214	.351	-.431
.316	.061	.026	.124	.394	.129	.066
-.009	,500*	,741**	.384	.412	,468*	-.366
.972	.025	.000	.095	.089	.037	.123
-.242	,585**	,748**	,453*	.285	,583**	-.351
.318	.007	.000	.045	.252	.007	.141
.179	.119	-.202	.167	-.331	.141	,554*
.464	.627	.408	.495	.195	.564	.014
.224	.081	.115	-.018	-.091	.083	.405
.357	.742	.639	.940	.729	.734	.086

.135	-.093	.239	-.098	.103	-.191	.204
.581	.705	.325	.689	.694	.433	.403
.208	-.112	.255	-.037	-.005	-.069	.448
.393	.647	.291	.881	.985	.778	.055
Grin1 CpG5 PFC	-.032	-.063	.009	.199	-.148	.023
	.898	.797	.972	.445	.545	.926
	Grin2b CpG1 Hippocampus	,517*	,845**	,503*	,945**	-.035
		.020	.000	.034	.000	.887
		Grin2b CpG2 Hippocampus	.364	.449	,496*	-.418
			.115	.062	.026	.075
			Grin2b CpG3 Hippocampus	.176	,908**	.022
				.484	.000	.929
				Grin2b CpG4 Hippocampus	.352	-.083
					.152	.751
					Grin2b CpG5 Hippocampus	.002
						.994
						Grin2b CpG1 PFC

Grin2b CpG2 PFC	Grin2b CpG3 PFC	Grin2b CpG4 PFC	Grin2b CpG5 PFC	NR1 Hippocampus	NR1 PFC	NR2 Hippocampus
.153	.091	.042	.283	.320	.183	.113
.533	.710	.864	.241	.169	.441	.636
,502*	,723**	.324	.251	-.310	-.162	-.227
.029	.000	.176	.300	.184	.494	.336
.249	.157	.026	.255	.232	.086	.161
.304	.521	.915	.293	.326	.719	.498
.328	,677**	.147	.124	-.185	-.068	-.382
.170	.001	.549	.613	.435	.774	.097
.204	.044	0.000	.053	-.275	-.234	.021
.403	.858	1.000	.830	.240	.321	.930
.105	.399	.061	.175	-,465*	.099	-.179
.668	.091	.805	.474	.039	.679	.450
-.199	-.034	-,508*	-.068	-.035	.440	.035
.428	.893	.031	.788	.887	.060	.887
.059	,649**	-.109	.023	-.270	.161	-.298
.817	.004	.666	.929	.263	.509	.215
.125	.133	-.232	.218	.014	.334	.193
.622	.598	.353	.385	.955	.162	.429
.395	.427	.374	.142	-.326	-.215	-.237
.104	.077	.127	.575	.173	.377	.329
.234	.314	-.205	.240	.054	.291	.061
.349	.204	.416	.338	.825	.226	.803
.209	.351	.394	.013	-.377	-.054	-.144
.404	.153	.105	.958	.111	.825	.557
-,469*	.105	-.088	-.261	.071	-.029	-.371
.043	.668	.719	.281	.765	.905	.107
-.416	.149	-.209	-.306	.089	.099	-.266
.077	.542	.391	.203	.710	.679	.257
-.233	.109	-.335	-.121	.296	.015	-.091
.338	.657	.161	.621	.206	.950	.703
-.037	,464*	.005	-.250	-.070	.200	-,473*
.881	.046	.983	.301	.769	.398	.035
-.156	.163	-.062	.134	.193	.239	-.194
.523	.504	.801	.583	.416	.310	.412
.311	-.020	.338	.411	-.179	.147	.335
.196	.935	.157	.080	.464	.547	.161
,611**	.365	,490*	,521*	-.357	.135	.044
.005	.125	.033	.022	.133	.581	.858

.219	.297	-.180	-.040	.205	.312	-.279
.367	.217	.461	.872	.399	.194	.247
.307	.096	.120	,562*	.026	.220	.010
.201	.697	.625	.012	.915	.365	.969
-.035	-.180	.176	-.065	.175	-.025	-.056
.887	.461	.472	.791	.473	.920	.819
-.137	.378	.122	-.117	.075	.029	-.238
.576	.110	.619	.634	.753	.905	.313
-.154	.359	-.097	.058	.105	.344	-.253
.528	.131	.694	.814	.659	.137	.283
-.158	.321	.038	-.110	.093	-.026	-.111
.519	.180	.878	.655	.696	.912	.640
-.199	.287	.109	-.162	.356	-.092	-.309
.445	.264	.677	.534	.147	.717	.213
-.133	.357	.136	-.034	-.025	.007	-.130
.589	.134	.578	.891	.917	.977	.585
,503*	.061	,506*	.385	-.105	-.102	-.011
.028	.805	.027	.104	.668	.678	.966
Grin2b CpG2 PFC	.447	.450	,633**	-.074	-.297	.075
	Grin2b CpG3 PFC	.053	.004	.764	.218	.759
		.253	.059	-.121	.070	-,478*
		Grin2b CpG4 PFC	.297	.811	.621	.775	.039
			.440	-.154	-.101	-.167
			Grin2b CpG5 PFC	.059	.530	.679	.495
				-.027	-.121	.410
				.912	.621	.081
				NR1 Hippocampus	-.253	.182
					.282	.443
					NR1 PFC	-.219
						.354
						NR2 Hippocampus

NR2 PFC
.123
.605
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NR2 PFC