

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

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**Epigenetic-mediated NMDA receptor changes in the brain of isolated reared rats**

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## NMDAR alterations in isolated reared rats

1 **Abstract**

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

14

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

18

19 **Introduction**

20 Interactions between biological and environmental factors are thought to be responsible  
21 for the development of schizophrenia, with early life adversity ~~as~~ a potent risk factor  
22 [1,2]. In this context, social isolation rearing from weaning is considered a valid animal  
23 model of schizophrenia [3–5] in inducing behavioural changes that are associated with  
24 the human condition and are sensitive to antipsychotic medication [3,5–7]. The N-  
25 methyl-d-aspartate receptor (NMDAR) plays an important role in neurodevelopment  
26 [8] and its hypofunction ~~may is are thought to~~ underlie the core symptoms of  
27 schizophrenia [9]. NMDARs are heteromeric receptors composed of NR1 (encoded by  
28 *GRIN1*, humans; *Grin1*, rodents) and NR2 subunits encoded by four distinct subtypes  
29 (*GRIN2A-D*, humans; *Grin2a-d*, rodents) [10]. The presence of both subunits is  
30 mandatory for the activity of NMDAR ion channels that only open in the presence of  
31 both glycine and L-glutamate [11,12]. ~~Additionally, the~~ The NR1 subunit is associated  
32 with regulatory processes controlling the structure and function of synapses [13];  
33 NR2A and B are essential for synaptic plasticity [14] and NR2B is particularly  
34 important in working memory [15]. These subunits are good candidates for studying

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

39 ~~Together with the glutamatergic system, the GABAergic system also plays a~~  
40 ~~central role in the neurobiology of schizophrenia [19]. The neuronal glutamatergic~~  
41 ~~system has a strong interrelationship with GABAergic neurons, which provide~~  
42 ~~inhibitory control of glutamatergic activity [19,20]. Moreover, glutamatergic activity~~  
43 ~~drives GABAergic function since NMDARs are expressed on GABAergic~~  
44 ~~interneurons, particularly the subtype containing the calcium binding protein~~  
45 ~~parvalbumin (PV) in early stages of development [21,22]. Disruptions in this~~  
46 ~~neurocircuit lead to disinhibition of the midbrain dopaminergic system, which plays a~~  
47 ~~central role in the neurobiology of schizophrenia [23,24].~~ There is a reduced expression  
48 of genes associated with GABA neurons, such as glutamic acid decarboxylase (*GAD*),  
49 reelin (*RELN*) and ~~parvalbumin~~ (*PVALB*), in the brains of schizophrenia patients [25–  
50 28]. Decreased *PVALB* is the most replicated finding reported in both schizophrenia  
51 *post-mortem* brain as well as in animal models of the disorder [27,29–31]; this finding  
52 may relate directly to the hyperfunction of dopamine in the disease [32]. The decreased  
53 PV-positive (PV+) interneurons result in imbalanced excitatory and inhibitory input  
54 [33,34], and consequent disruption of glutamatergic function, especially via NMDARs  
55 [35,36].

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

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69 both GABAergic and glutamatergic neurotransmission [44–46]. This includes a  
70 reduced DNA methylation of the *Grin2b* promoter in both a neurodevelopmental  
71 animal models of schizophrenia [47] and in patients in their first episode of psychosis  
72 [48].

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

## 86 **Materials & methods**

### 87 *Behavioural testing: Open Field Test in isolation reared rats*

88 Male Wistar rats were obtained from the Central Vivarium of the University of São  
89 Paulo, campus of Ribeirão Preto, Brazil. The animals (10/group) were brought to the  
90 vivarium of the Laboratory of Pharmacology and kept isolated from weaning (21 days  
91 after birth) or in groups of 3-4/cage (41 x 34 x 16 cm), during 10 days, under standard  
92 conditions: temperature ( $23.4 \pm 1.0^\circ\text{C}$ ), light cycle (lights on from 6:00 a.m. to 6:00  
93 p.m.), free access to food (Rats and Mice Nutrition, Agromix, Brazil) and water. The  
94 welfare of the animals was assessed daily. The cages and bedding were changed every  
95 2 days, as well as food and water replacement. Animals were randomly assigned to the  
96 different experimental groups and experiments were conducted from 6:30 a.m. to 6:30  
97 p.m., ~~with randomization of treatment conditions along the day~~. All procedures were  
98 developed in accordance with Brazilian Council for Animal Experimentation  
99 (CONCEA), and all efforts were made to minimize animal suffering. After this period,  
100 both groups were exposed to the open field test to assess locomotion, were sacrificed  
101 and DNA and RNA extracted from the PFC and hippocampus–HPPΘ, as previously  
102 described [49].

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103 *Gene and protein expressions*

104 DNA and RNA extracts of the PFC and hippocampus HPPPO were obtained by using  
105 the All prep DNA/RNA mini kit (Qiagen, Valencia). The mRNA expression of  
106 glutamatergic genes (*Grin1*, *Grin2a* and *Grin2b*) and GABAergic genes (*Pvalb*~~V~~,  
107 *GadAD1* and *Reln*~~EL~~) were conducted by Real-Time quantitative PCR (qPCR) using  
108  $\beta$ -actin (*ACTB*~~Actb~~) as a reference gene, and thermal cycling conditions as previously  
109 published [49], using the following hydrolysis probes (TaqMan assays): *Grin1* Rat:  
110 Rn01436034\_m1, *Grin2a* Rat: Rn00561341\_m1, *Grin2b* Rat: Rn00680474\_m1, *ACTB*  
111 *Actb* Rat: Rn00667869\_m1, *GadAD1* Rat: Rn00690300\_m1, *REL*~~—Reln~~ Rat:  
112 Rn00589609\_m1 and *PV*~~Pvalb~~ Rat: Rn00574541\_m1. Gene expression was quantified  
113 using the Comparative Ct Method ( $\Delta\Delta$ Ct Method), using *ACTB*~~Actb~~ as the endogenous  
114 (housekeeping) control gene as it showed to be stable across our samples. In relation to  
115 gene expression, we followed the manufacturer's instructions (Allprep DNA/RNA mini  
116 kit, QIAGEN) using 30 mg of tissue.

117 For the NR1 and NR2 protein assays, tissues were weighed and then  
118 homogenized in PBS buffer (1 mL of PBS per 100 mg of tissue), centrifuged (1 min,  
119 8000 rpm) and the supernatant collected and stored frozen at -80 °C until analysis.

120 Quantitative determination of NR1 and NR2 was performed by ELISA  
121 according to the manufacturer's instructions (*My BioSource, San Diego, CA, USA*).  
122 For the NR1 assay, the detection range was 0.5-10 ng/ml, the sensitivity was less than  
123 0.1 ng/ml, and the coefficient of variation was <10% for intra- and inter-assays. For the  
124 NR2 assay, the detection range was 31.2-2000 pg/ml, the sensitivity was less than 18.75  
125 pg/ml, and the coefficient of variation was <8% for intra-assay and <10% for inter-  
126 assay. The total protein concentration for each area (PFC and hippocampus HPPPO) was  
127 performed using the biuret method (Piotrowski's test) (Labtest Diagnóstica, Lagoa  
128 Santa, MG, Brazil).

129

130 *DNA extraction, Bisulphite treatment and Pyrosequencing*

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

135 Genomic DNA was extracted from all rat samples using the AllPrep DNA/RNA Mini  
136 Kit (Qiagen, Valencia, CA/USA), and was bisulphite-modified to convert

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137 unmethylated cytosine residues to uracil using the EpiTec Fast DNA Bisulphite Kit  
138 (Qiagen) with a calculated mean conversion of 99%. DNA sequences for each gene  
139 were identified in the 5' region that contains likely transcription factor (TF) binding  
140 sequences for rats that we identified using ALLGEN-PROMO  
141 ([http://algggen.lsi.upc.es/cgibin/promo\\_v3/promo/promoinit.cgi?dirDB=TF\\_8.3](http://algggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)), and a  
142 pyrosequencing method was developed for the determination of methylation at the CpG  
143 sites within those sequences following bisulphite reaction. The results were compared  
144 to methylation of LINE1, a measure of global methylation.

145 PCR reactions were carried out with 20 ng bisulphite-converted DNA using the  
146 PyroMark PCR Kit in a final volume of 25 µl containing 12.5 µl 1x PyroMark PCR  
147 Master Mix, 2.5 µl 1x CoralLoad Concentrate, 1 µl of each primer in a final  
148 concentration of 0.05 µM, 8 µl RNase-free water. Amplification conditions were as  
149 follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 56°C for 30 s (except for *LINE-*  
150 *1*: 52°C for 30s) and 72°C for 30 s, finally, 72°C for 10 min. Methylation status in the  
151 promoter sequence of the target genes was determined with a PyroMark Q24  
152 pyrosequencer (Qiagen UK) using 15–20 µl PCR product and a sequencing primer.

153 Pyrosequencing setup and data reading were conducted by PyroMark Q24  
154 2.0.6.20 software. We analysed samples in duplicate in both experiments, PCR and  
155 pyrosequencing, and any inconsistencies were resolved following further repetition. All  
156 the primers are listed in **Table 1**.

157

### 158 *Statistical analysis*

159 All results are expressed as the mean and standard error of the means (SEM) and were  
160 analysed using SPSS 20 (IBM Corp: Armonk, NY, USA). The behavioural data  
161 analyses were done using the EthoLog 2.2 software [50] and were analysed with  
162 repeated measures ANOVA with Bonferroni *post-hoc* test, as described previously  
163 [49]. However, as the molecular data was/were not normally distributed ~~and~~ we used  
164 the Mann-Whitney U test to investigate mRNA/protein expressions and the DNA  
165 methylation changes between the two groups (isolated and grouped), for the two brain  
166 regions (PFC and hippocampus~~HIPPO~~) under consideration.

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

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

175

## 176 Results

### 177 *Open Field test, Locomotion activity*

178 The isolation-reared animals demonstrated hyperlocomotion in the two first time bins  
179 in the periphery of the arena when compared to the grouped [0-5 min:  $F_{(1,15)}=6.209$ ,  
180  $p=0.025$ ; 5-10 min:  $F_{(1,15)}=14.272$ ,  $p=0.002$ ], as well as, at the centre of the arena during  
181 5-10 min [ $F_{(1,15)}=6.452$ ,  $p=0.023$ ]. These data have been previously published [49].

182

### 183 *Gene expression of brain tissues*

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

191 In **Figure 1B**, we demonstrated the decreased mRNA expression of ~~PV-Pvalb~~  
192 (1.3-fold) ~~GAD1-Gad1~~ (0.9-fold) and ~~REL-ReIn~~ (2.1-fold) in the PFC of isolated  
193 animals when compared to grouped (*Pvalb*:  $U=18$ ,  $p=0.027$ ; *Gad1*:  $U=14$ ,  $p=0.019$ ;  
194 *Reln*:  $U=16$ ,  $p=0.031$ , respectively), while no significant changes differences were  
195 found in the ~~hippocampus HIPPO~~ (*Pvalb*:  $U=39$ ,  $p=0.624$ ; *Gad1*:  $U=41$ ,  $p=0.744$ ; *Reln*:  
196  $U=31$ ,  $p=0.253$ , respectively). We excluded outlier values for ~~GAD1-Gad1~~ and ~~REL~~  
197 ~~Reln~~ in the PFC for one group-housed rat.

198

### 199 *NR1 and NR2 protein expression of brain tissues*

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



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

206

#### 207 DNA methylation of NMDAR subunit genes

208 DNA rat samples successfully underwent bisulphite conversion, PCR and  
 209 pyrosequencing to determine methylation in the glutamate (*Grin1* and *Grin2b*) and  
 210 LINE1 sequences. All samples demonstrated single PCR bands with no evidence  
 211 of DNA degradation.

212 Regarding LINE1, no significant difference was found between the groups  
 213 in mean levels of methylation (PFC: U=35, p=0.414; hippocampus HPPQ: U=33,  
 214 p=0.199) (**Figure 3**).

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

223

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

#### 225 Negative correlations between DNA methylation and mRNA/protein levels of 226 NMDAR subunits

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

233

#### 234 Positive correlations between mRNA of glutamatergic and GABAergic markers 235 in the PFC

236 We found the following positive correlations: (A) mRNA of *Grin1* and *Pvalb* (rho:  
 237 0.563; p=0.012); (B) mRNA of *Grin1* and *GAD1* (rho: 0.754; p<0.001); (C)

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238 mRNA of *Grin1* and *RELReIn* ( $\rho: 0.663$ ;  $p=0.005$ ); (D) mRNA of *Grin2a* and  
239 *PVPvalb* ( $\rho: 0.482$ ;  $p=0.036$ ); (E) mRNA of *Grin2b* and *PVPvalb* ( $\rho: 0.646$ ;  
240  $p=0.00$ ); and (F) mRNA of *Grin2b* and *RELReIn* ( $\rho: 0.501$ ;  $p=0.034$ ) HIPPO (**Figure**  
241 **6**). ~~These associations are demonstrated in the PFC of isolated and grouped rats~~  
242 ~~(Figures 6A-F). All the other S~~ignificant correlations are showed in **Table S1**.

243

244 **Discussion**245 *NMDAR subunits alterations in isolation rearing*

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

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

260 As glutamate is a key mediator of synaptic plasticity, these results indicate a  
261 glutamatergic dysfunction that likely affects synaptic plasticity in the PFC as a  
262 consequence of the social isolation rearing regime. This may be associated with  
263 NMDAR dysfunction and an imbalance between excitatory and inhibitory circuits,  
264 notably in the PFC [55]. Indeed, previous studies indicated that NMDAR subunits,  
265 mainly NR1 and NR2A-B, are involved in the early stages of brain development  
266 [22,56]. It is relevant in this respect that an abnormal glutamatergic system in the PFC  
267 may underlie the cognitive impairments and memory deficits present in schizophrenia  
268 [57,58], which is also in accordance with the hypofrontality already described in this  
269 animal model [59], based on an impairment of neuronal transmission and synaptic  
270 connectivity [60,61].

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271 Secondly, consistent with our findings of decreased *PVPvalb*, *GAD1-Gad1* and  
272 *REL-Reln* expression in the PFC of isolated reared rats, other studies have shown  
273 downregulation of *PVALB*, *GAD1* and *RELN* mRNA in the PFC of schizophrenia  
274 patients [28,62–64] as well as in animal models of the disorder [63,65], implicating  
275 neurodevelopmental impairments of synaptic function and plasticity, and cognition  
276 [66]. Given that the hypofunction of NMDARs on GABAergic interneurons results in  
277 a decreased activity of this system [67], our results suggest that the reduced *Pvalb*  
278 mRNA expression reflects an indirect reduction of GABA neuron activity driven by  
279 dysfunctional NMDARs.

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

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

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

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305 [76]. Sp1 ~~is has a dual activity as an important TF promoter TF that can activate~~ or  
306 ~~repressing~~ transcription in response to physiological and pathological stimuli [77–79].  
307 Sp1 ~~also~~ has a direct role in transcriptional activation and ~~is involved for in~~ the ~~initiation~~  
308 ~~initial process~~ of gene expression [80] ~~and bindings~~ with high affinity to GC-rich motifs  
309 to regulate the ~~genes expression of a large number of genes~~ involved in a variety of  
310 ~~processes such as~~ cell growth, apoptosis, differentiation and immune responses  
311 [78,81,82]. Hence, our findings suggest that ~~DNA-Grin1~~ hypermethylation ~~of Grin1~~  
312 following early life stress may interfere with Sp1 binding ~~site~~ and thereby bring about  
313 a reduction of *Grin1* mRNA expression.

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

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

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

### 339 **Strengths and Limitations**

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

347 In this study, we did not measure ~~the all~~ four of the distinct subtypes of NR2  
348 (NR2a-d); this could underlie the lack of that could explain the non-significant results  
349 in relation to NR2 protein expression between the groups. However, we investigated  
350 only NR2 subunit in this animal model of schizophrenia, because in our previous  
351 finding, we found low NR2 plasma concentrations in first-episode psychosis patients  
352 compared to unaffected siblings and community-based controls [88]. In the present  
353 study, we found increased methylation levels at the *Grin2b* gene and, consistently, low  
354 expression of this subunit at the gene level. However, contrary to our expectations, no  
355 differences were found at the protein level for this subunit, in any of the brain sites  
356 investigated. This may reflect the fact that we were only able to assess the total NR2  
357 protein expression rather than the protein subunits. Discrimination between the NR2  
358 subunits is essential for determining the decreased NMDAR activity, considering that  
359 the two NR2a-b subunits have different properties in relation to NMDAR function  
360 [14,89]. It has been shown that maturation of brain circuits occurs subsequent to the  
361 switch of NR2b to NR2a during critical periods of the development [14,58,90]. Thus,  
362 the lack of significant differences at the NR2 protein levels in our study should be  
363 interpreted with caution and it is important that future studies consider the analyses of  
364 NR2a and NR2b subunits separately.

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

372

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373 **Conclusions**

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

384

385 **Summary points**

386 • NMDAR methylation changes found in the brain tissues may underlie the  
387 NMDAR mRNA/protein expression alterations caused by the isolation period.

388 • Early social isolation induces epigenetic modifications in the NMDA  
389 receptor subunits.

390 • Our data support the validity of social isolation after weaning in modeling  
391 aspects of schizophrenia, highlighting changes in the glutamatergic and GABAergic  
392 systems commonly seen in schizophrenia.

393 • Our study also reinforces the strong correlations between glutamatergic and  
394 GABAergic genes that are involved in schizophrenia.

395 ~~• Changes in DNA methylation may be a plausible mechanism underlying the~~  
396 ~~gene/protein expression alterations of NMDARs subunits after isolation rearing in rats.~~

397 • Our findings may contribute to understanding the pathophysiological  
398 consequences of decreased NMDAR subunits expression in schizophrenia.

399 • This study contributes to the identification of epigenetic mechanisms  
400 involved in the neuropathophysiology of schizophrenia, which may lead to new  
401 pharmacotherapeutic strategies.

402 • In our study, the period of social isolation from weaning may characterise a  
403 chronic early life stress model that induced the alterations in methylation, resulting in  
404 an imbalance in the excitatory/inhibitory tone equivalent to that seen in schizophrenia.

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

407

408 **Future Perspective**

409 Even though NMDARs are well characterized and much is known about its implication  
410 in schizophrenia pathogenesis, the role of epigenetic mechanisms in its dysregulation  
411 is still unclear. The results presented in this paper pave the way for further studies and  
412 highlight a possible epigenetic mechanism whereby early life adversities contribute to  
413 dysregulation in the glutamatergic system, more specifically in the hypofunction of  
414 NMDARs and, their impact-effect on GABAergic function and subsequent  
415 disinhibition of dopaminergic neurons in the midbrain. The glutamatergic and  
416 GABAergic epigenetic dysregulations of the glutamatergic and GABAergic  
417 neurotransmitter systems observed in this-our study have important translational value  
418 addition not only for schizophrenia, but also for a host of psychiatric disorders  
419 associated with exposure to environmental adversities. Future research should  
420 investigate the association between DNA methylation and early life stress in  
421 pharmacological models of schizophrenia, and test correlations between blood and  
422 brain biological markers. Finally, the results observed offer mechanistic pathways for  
423 translation in clinical settings, including the identification of more vulnerable  
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426

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711

712 **Author contributions**

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

720

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731

732 **Disclosures**

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738

739 **Conflict of Interest**

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

741

742 **Ethical conduct of research statement**



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

746

#### 747 **Figure Legends**

748

#### 749 **Figure 1. Effects of rearing condition (isolated vs. grouped) on glutamatergic and** 750 **GABAergic markers gene expression in the PFC and hippocampus HIPP0 of rats.**

751 The figures show the mean fold change  $\pm$  SEM of *Grin1*, *Grin2a*, *Grin2b*, *Pvalb~~V~~*,  
 752 *GadAD1* and *Reln~~EL~~* mRNA levels using the housekeeping gene  $\beta$ -actin (*ACTBActb*)  
 753 as reference. Glutamatergic and GABAergic markers mRNA expression were  
 754 measured by qRT-PCR. (A) Isolated rats presented decreased expression of *Grin1*,  
 755 *Grin2a* and *Grin2b* mRNA in the PFC when compared to grouped (p=0.034; p=0.041;  
 756 p=0.019) respectively, while no statistical differences were found in the hippocampus  
 757 HIPP0 (*Grin1*: p=0.174; *Grin2a*: p=0.545 and *Grin2b*: p=0.290) of these animals. (B)  
 758 Isolated animals also showed decreased expression of *Pvalb~~V~~*, *GadAD1* and *Reln~~EL~~* in  
 759 the PFC when compared to grouped (p=0.027; p=0.019; p=0.031, respectively), while  
 760 no significant changes were found in the hippocampus HIPP0 (p=0.624; p=0.744;  
 761 p=0.253, respectively). \*p<0.05; Mann-Whitney U test.

762

#### 763 **Figure 2. Effects of rearing condition (isolated vs. grouped) on glutamatergic** 764 **markers protein expression in the PFC and hippocampus HIPP0 of rats.** The

765 figures show the mean  $\pm$  SEM of NR1 (ng/mg) and NR2 (pg/mg) proteins.  
 766 Glutamatergic markers protein expression was measured by ELISA test. Isolated rats  
 767 showed increased protein expression of NR1 subunit in the hippocampus HIPP0-when  
 768 compared to grouped (p=0.001); while, no statistical differences were observed in the  
 769 PFC (p=0.406). In relation to NR2 subunit protein, no differences were observed in the  
 770 PFC (p=0.462) and hippocampus HIPP0 (p=0.174) of these animals. \*p<0.05; Mann-  
 771 Whitney U test.

772

#### 773 **Figure 3. Effects of rearing condition (isolated vs. grouped) on LINELine-1** 774 **methylation in the PFC, hippocampus HIPP0 and peripheral blood of rats.** The

775 figure shows the mean of percentage of methylation in LINELine-1. No statistical  
 776 differences were observed between the groups in relation to PFC (p=0.414) and

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777 hippocampus HPPPO (p=0.199). The LINE1 DNA methylation was measured by  
 778 Pyrosequencing. Mann-Whitney U test.

779

780 **Figure 4. Effects of rearing condition (isolated vs. grouped) on DNA methylation**

781 **of *Grin1* and *Grin2b* in the PFC and hippocampus HPPPO of rats.** The figure shows  
 782 the mean  $\pm$  SEM of percentage of methylation in *Grin1* and *Grin2b* in the PFC and  
 783 hippocampus HPPPO of rats reared in isolation or grouped. Glutamatergic markers  
 784 DNA methylation was measured by Pyrosequencing. Increased DNA methylation of  
 785 *Grin1* at CpG5 (p=0.047) were found in the PFC of isolated-reared rats (A) and  
 786 increased methylation of *Grin2b* at CpG4 were found in the hippocampus HPPPO of  
 787 isolated animals (B) when compared to grouped (p=0.024). \*p<0.05; Mann-Whitney U  
 788 test.

789

790 **Figure 5. Correlations between DNA methylation in the brain tissue and**

791 **gene/protein levels in the PFC and hippocampus HPPPO of isolated and grouped**  
 792 **animals:** (A) All rats presented a negative correlation between *Grin1* methylation at  
 793 CpG5 and *Grin1* mRNA levels in the PFC (rho: -0.488; p=0.040). (B) Isolated rats  
 794 presented a negative correlation between *Grin2b* methylation at CpG4 and NR2 protein  
 795 levels in the hippocampus HPPPO (rho: -0.800; p=0.010; Spearman correlation).

796

797 **Figure 6. Correlations between mRNA of glutamatergic and GABAergic genes in**

798 **the brain tissues of isolated and grouped animals:** All rats presented positive  
 799 correlations between (A) *Grin1* and *Pvalb* mRNA levels in the PFC (rho: 0.563;  
 800 p=0.012); (B) *Grin1* and *Gad67* mRNA levels in the PFC (rho: 0.754; p<0.001); (C)  
 801 *Grin1* and *Reln* mRNA levels in the PFC (rho: 0.633; p=0.005); (D) *Grin2a* and  
 802 *Pvalb* mRNA levels in the PFC (rho: 0.482; p=0.036); (E) *Grin2b* and *Pvalb* mRNA  
 803 levels in the PFC (rho: 0.646; p=0.003; and (F) *Grin2b* and *Reln* mRNA levels in  
 804 the PFC (rho: 0.501; p=0.034; Spearman correlation).

805

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

Gene	Rats
------	------

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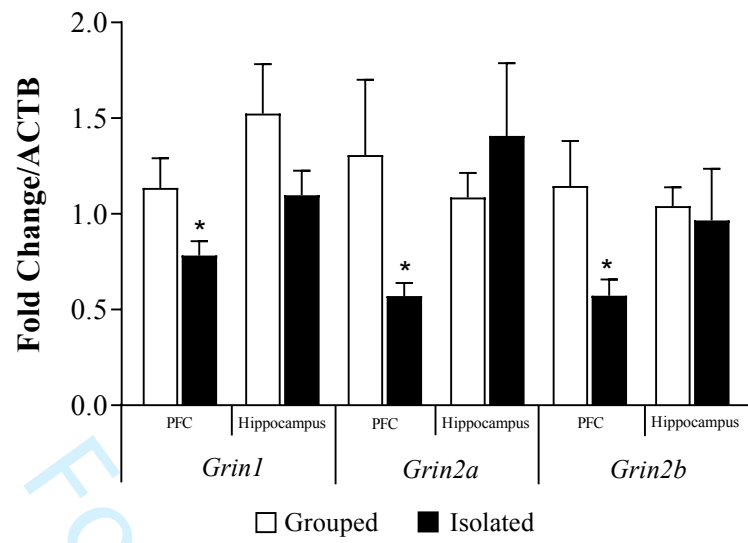
	F 5'TTGTTGTAAGAAAGTTGTTTGGTGAGTT3'
<i>LINE1</i>	R 5'ACCTCAAAAATACCCACCTAACCC3'
	Seq 5'GGTGAGTTTGGGATA3'
	F 5'TTGGGTTTGTGGGTGATAGAAG3'
<i>Grin1</i>	R 5'ACCTACTAACATTCCCCCTACTTTTTTCCT3'
	Seq 5'ATGTTGAAGATTTTGGGGT3'
	F 5'TGGCCTCAGTGACAAGAAGTTC3'
<i>Grin2a</i>	R 5'AGACGGCTGCGTCATAGATGAA3'
	Seq 5'AGAAGAATGGATTTTTTTTA3'
	F 5'TTGGGTGTGAGATTTAAATTAAGATTAG3'
<i>Grin2b</i>	R 5'AAAATAAAAAAAAAACCTTCTCTCAA3'
	Seq 5'AGATTAGGATTTTGGATGTT3'

808

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

Figure 1.

(A)



(B)

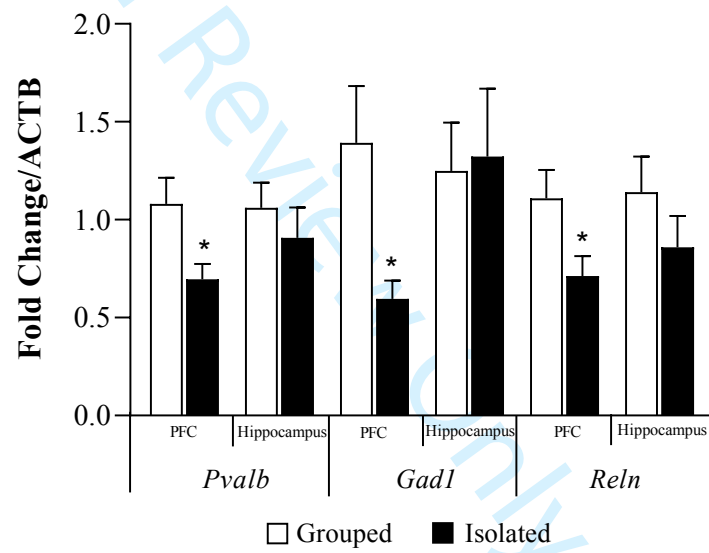
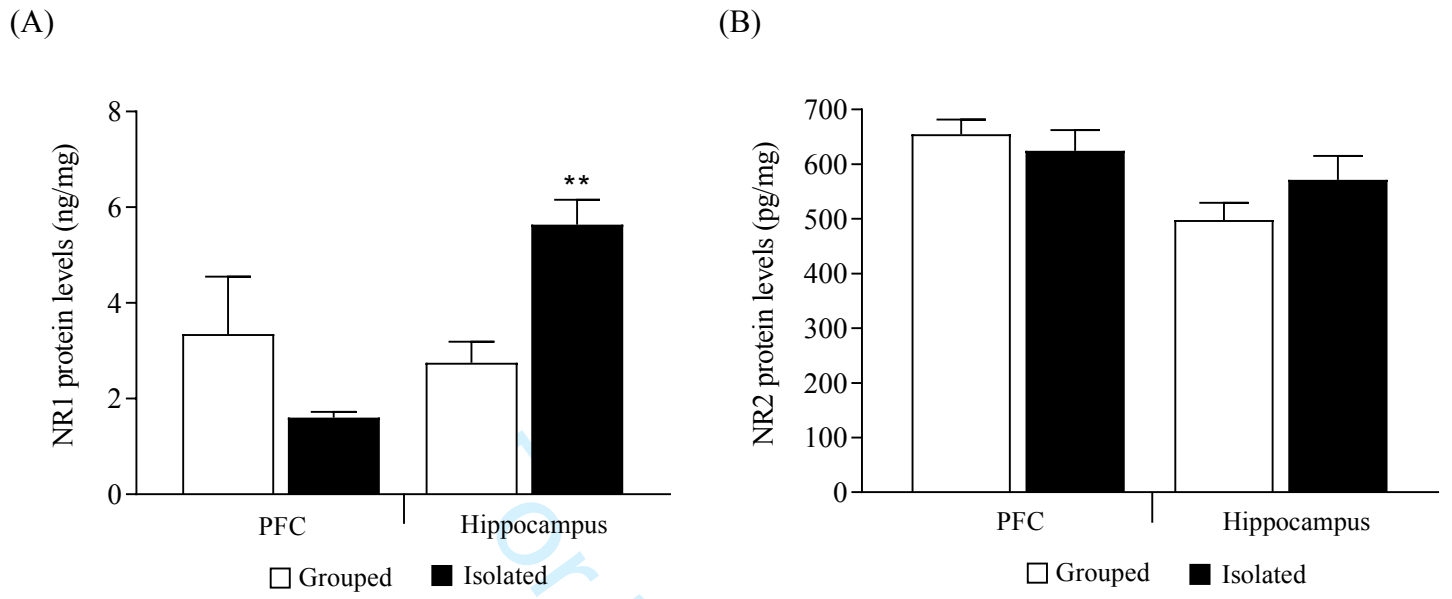


Figure 2.



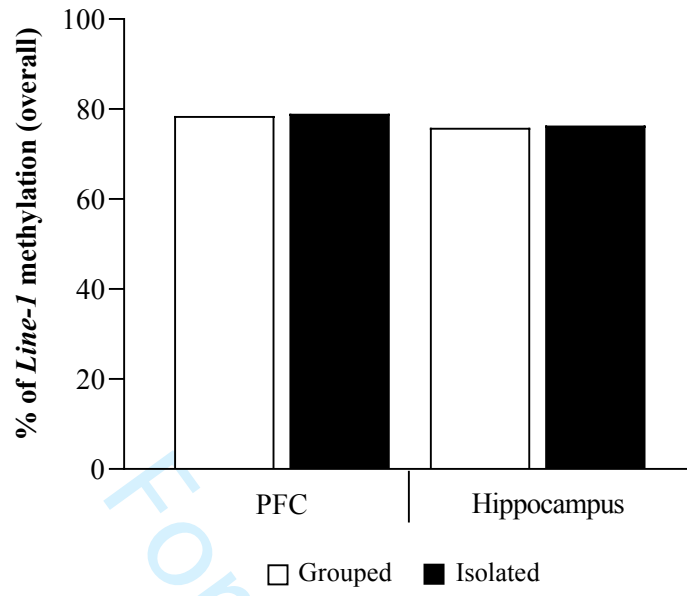
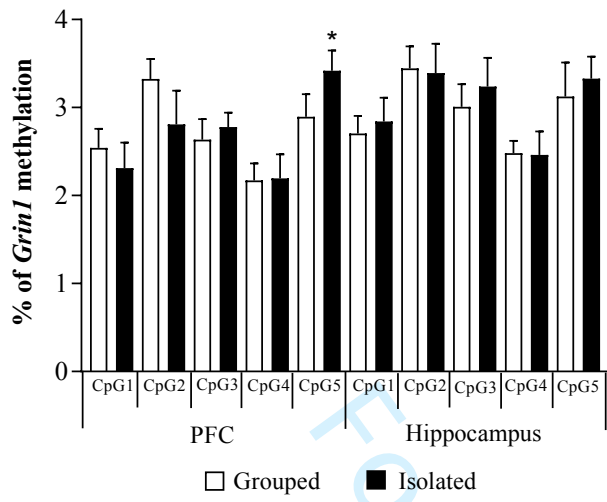
**Figure 3.**

Figure 4.

(A)



(B)

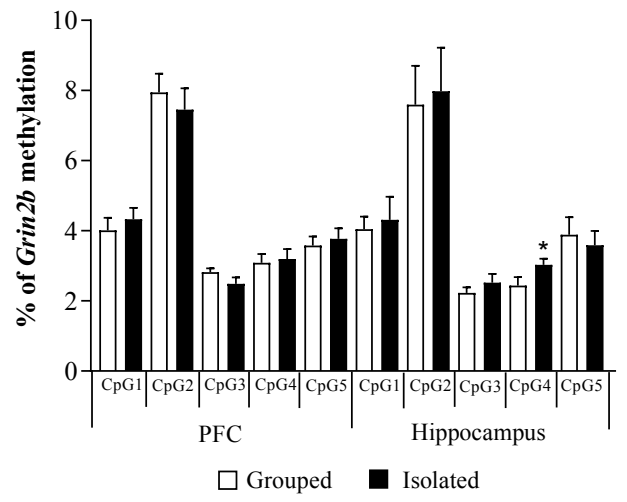
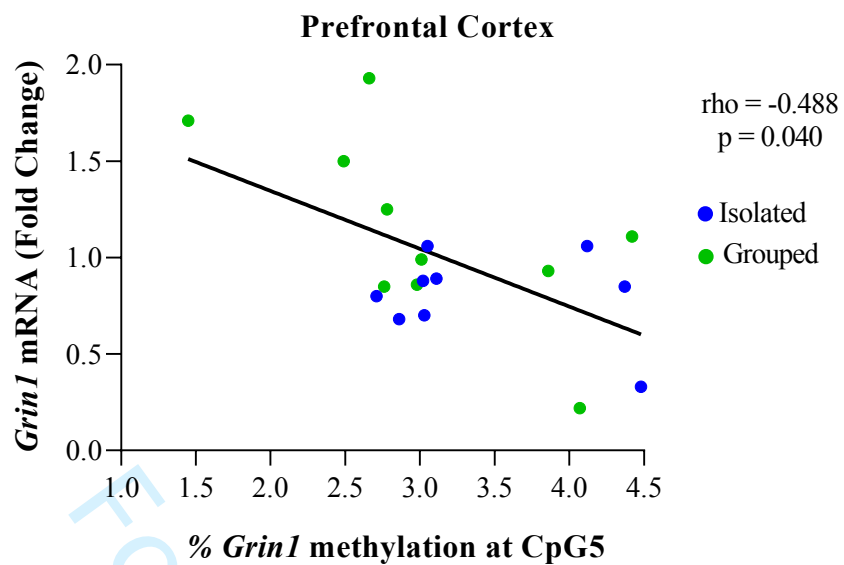


Figure 5.

(A)



(B)

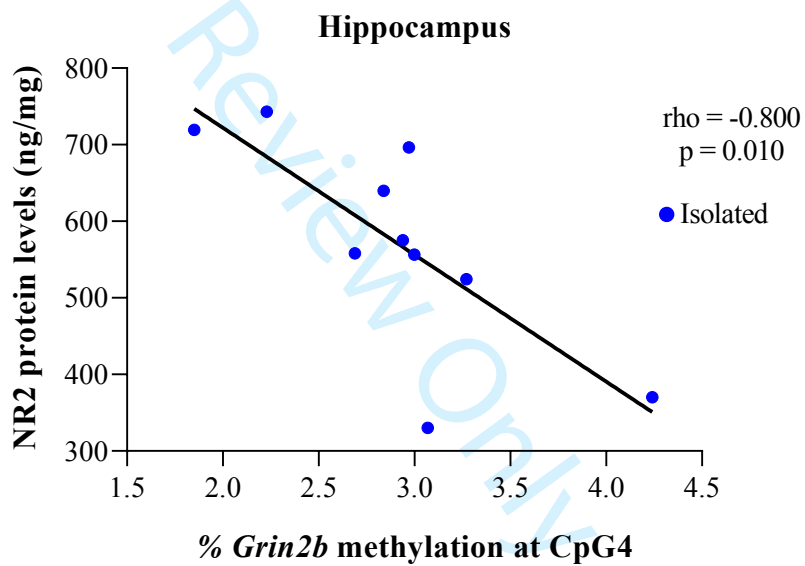
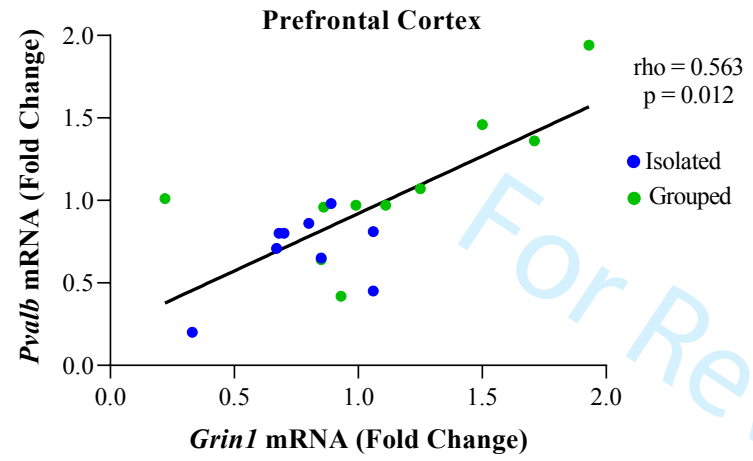


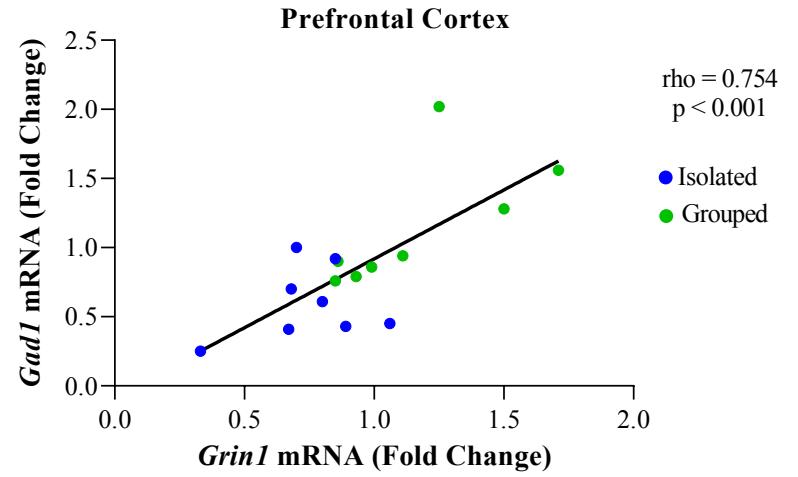


Figure 6.

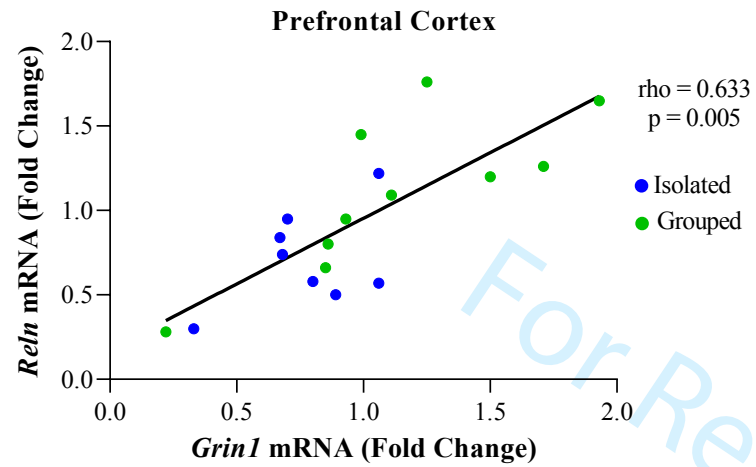
(A)



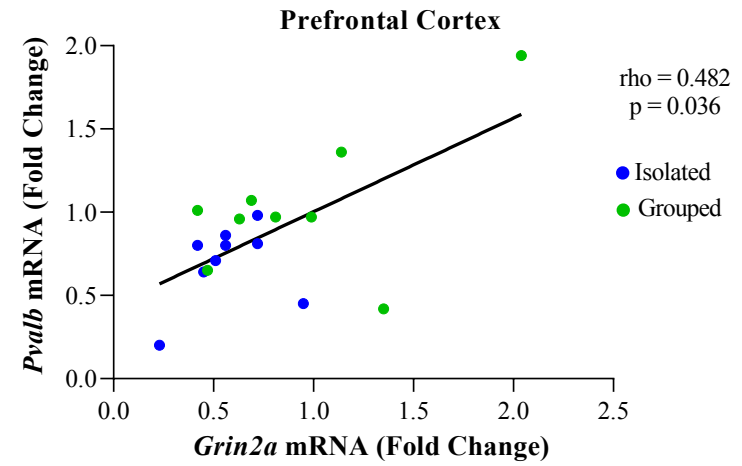
(B)



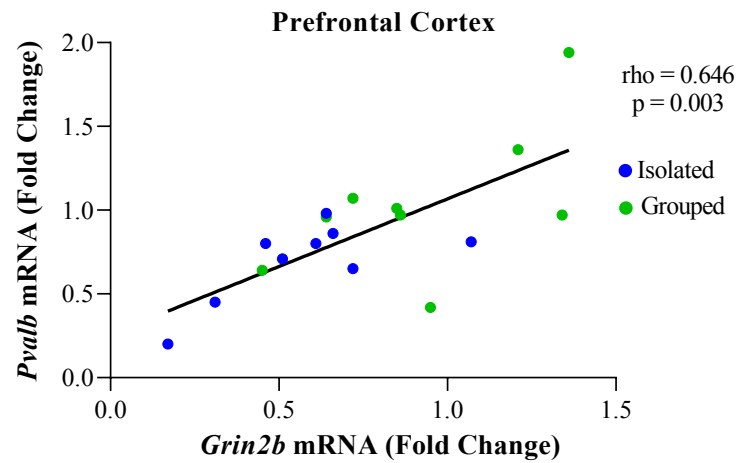
(C)



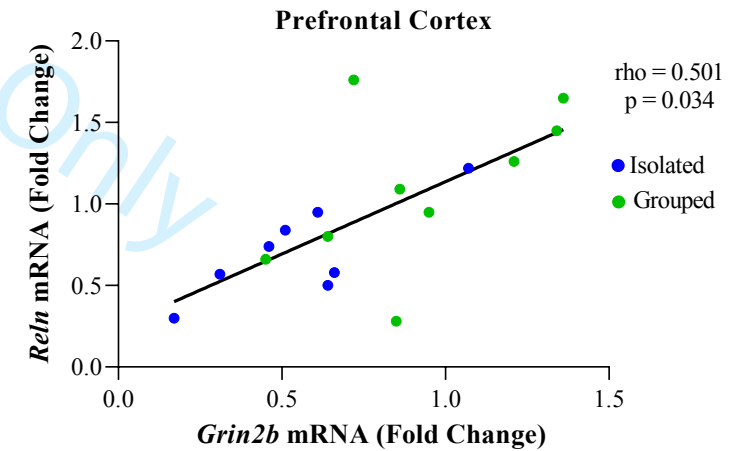
(D)



(E)



(F)



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

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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

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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	

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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							

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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
	.055	.053	.004	.764	.218	.759
	Grin2b CpG3 PFC	.253	.059	-.121	.070	-.478*
		.297	.811	.621	.775	.039
		Grin2b CpG4 PFC	.440	-.154	-.101	-.167
			.059	.530	.679	.495
			Grin2b CpG5 PFC	-.027	-.121	.410
				.912	.621	.081
				NR1	-.253	.182
				Hippocampus	.282	.443
						-.219
					NR1 PFC	.354
						NR2
						Hippocampus

NR2 PFC

.123  
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For Review Only

-068  
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For Review Only

NR2 PFC