*Article*

# Folic Acid and Vitamin B12 Prevent Deleterious Effects of Rotenone on Object Novelty Recognition Memory and *Kynu* Expression in an Animal Model of Parkinson's Disease.

**Gabriela Canalli Kretzschmar [1,†], Adriano D. S. Targa [2,†], Sheila Coelho Soares-Lima [3], Priscila I. dos Santos [1], Lais S. Rodrigues [2], Daniel A. Macedo [2], Luis Felipe Ribeiro Pinto [3], Marcelo M. S. Lima [2] and Angelica Beate Winter Boldt [1,*]**

[1] Laboratory of Human Molecular Genetics, Postgraduate Program in Genetics, Department of Genetics, Federal University of Paraná (UFPR), Centro Politécnico, Jardim das Américas, Curitiba 81531-990, PR, Brazil
[2] Laboratory of Neurophysiology, Department of Physiology, Federal University of Paraná (UFPR), Centro Politécnico, Jardim das Américas, Curitiba 81531-990, PR, Brazil
[3] Molecular Carcinogenesis Program, National Cancer Institute, Research Coordination, Rio de Janeiro 20231-050, RJ, Brazil
* Correspondence: angelicaboldt@gmail.com; Tel.: +55-(41)-3361-1553
† These authors contributed equally to this work.

**Supplementary Table S1—Primers for *Kynu* Pyrosequencing.**

| Primer | 5'- 3' Sequence | Nt | Tm ºC | %GC |
|---|---|---|---|---|
| Forward | AAGAGTTGGAAGAGGTTGTTAGT | 23 | 60.8 | 39.1 |
| Reverse -biotinylated | CTCCACCCTATAAAAAATTTACATATCAAC | 30 | 59.1 | 30 |
| Sequencing | GGTTGTTAGTAGAGTTAGAT | 20 | 45.4 | 35 |

**Sequence with marked primers**

```
     AAGAGTTGGA AGAGGTTGTT AGT
     |||||||||| |||||||||| |||
                GGTTGTT AGTAGAGTTA GAT
                ||||||| |||||||||| |||
271 AAGAGTTGGA AGAGGTTGTT AGTAGAGTTA GATGTTTGGT AAGTTGGGGT GGGGATGGGG GAGGTGTYGT ATTTTGTTTA GTTTTTGTTG
                                                                      cg1
361 GTGGTTAGAA ATTTGTAATT YGATTTAATT GGGTGGGAGG AATTAAAGTT GATATGTAAA TTTTTTATAG GGTGGAG 438
                          cg2                          ||| |||||||||| |||||||||| |||||||
                                                       CAA CTATACATTT AAAAAATATC CCACCTC
```

The primers were designed using the PyroMark Assay Design 2.0 (Qiagen), considering the promotor sequence of the *Kynu* gene in the RGSC 6.0/rn6 rat genome. The software provides a reliability score for the generated primers, and the highest option was chosen (93%). The analyzed CpG sites are represented in red. The *cg2* site is orthologous to *cg15836722* in the *KYNU* human promoter and is the closest CpG to the transcription start site.

**Supplementary Table S2—Pyrosequencing PCR Protocol.**

| Reagents | [Initial] | [Final] |
|---|---|---|
| RNase-Free Water | 0 | 0 |
| PyroMark PCR Master Mix, 2x | 2 | 1 |
| CoralLoad Buffer, 10x | 10 | 1 |
| MgCl2 (mM) | 25 | 2 |
| Primer set (nmol) | 2.54 | 0.2 |
| DNA converted with bisulfite (ng/uL) | 20 | |
| **Total** | | |
| **Expected products** | | |
| ***Kynu*** | 167 bp | |

We used the PyroMark PCR kit (Qiagen) and evaluated the quality of PCR amplification in 1.5% agarose gel electrophoresis.

**Supplementary Table S3—Primers for *Kynu* Expression Analysis.**

| Gene | Primer | 5'-3'Sequence | Nt | Tm°C | %CG | bp |
|---|---|---|---|---|---|---|
| *Kynu* | F | TCTGTGACAAGCGAGAACCA | 20 | 59.25 | 50 | 113 |
| | R | TGTAGAGTCGAGTATGGCAGTAAG | 24 | 59.42 | 45.83 | |
| *Actb** | F | TGTCACCAACTGGGACGATA | 20 | 58.37 | 50 | 166 |
| | R | GGGGTGTTGAAAGGTCTCAAA | 21 | 58.34 | 47.62 | |
| *Hprt1** | F | GCAGACTTTGCTTTCCTTGG | 20 | 57.57 | 50 | 81 |
| | R | CGAGAGGTCCTTTTCACCAG | 20 | 57.91 | 55 | |

* Primer sequences already described by Elfving et al., (2019).

**Supplementary Table S4—qPCR Protocol.**

| Reagents | [Initial] | [Final] |
|---|---|---|
| Rnase-Free Water | 0 | 0 |
| GO Taq Master Mix, 2x | 2 | 1 |
| Primer Forward (uM) | 10 | 10 |
| Primer Reverse (uM) | 10 | 10 |
| cDNA (ng/uL) | 12.5 | 12.5 |

**Supplementary Figure S1—*Kynu* Methylation Levels of *cg1* and *cg2*.**

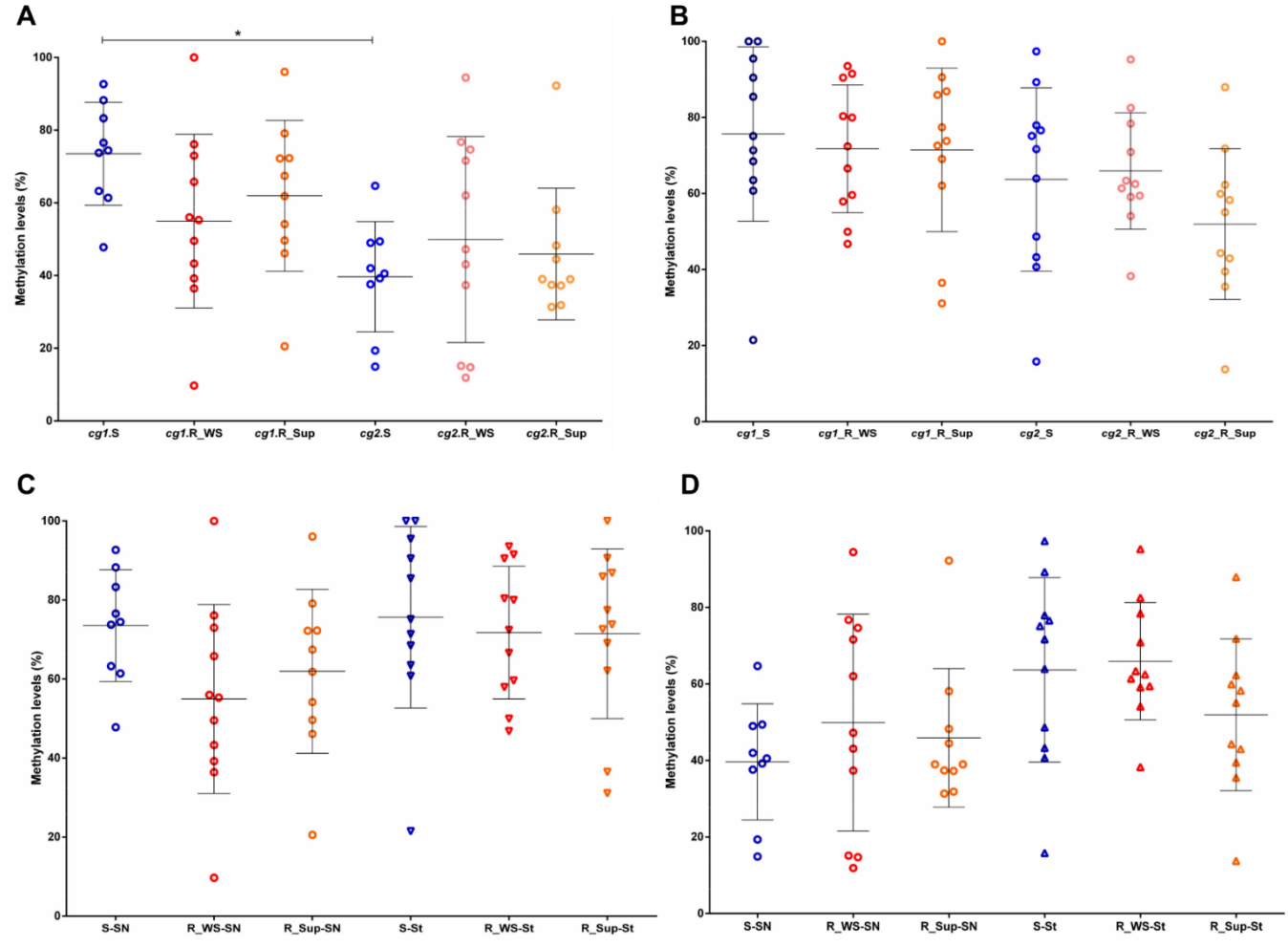


**Figure S1.** Methylation levels of *cg1* and *cg2* in the analyzed groups in (**A**) substantia nigra and (**B**) striatum; dark color = *cg1*; light color = *cg2*; (**C**) Methylation levels for *cg1* between analyzed groups and different brain regions; (**D**) Methylation levels for *cg2* between studied groups and different brain regions; S = sham; R_WS = rotenone without supplementation; R_Sup = rotenone supplemented. In B and C, the circles represent substantia nigra, and the triangles represent the striatum region. No significant differences were found between the groups in B, C, and D. * $p <$ .01. Data are the mean with SD.

**Supplementary Figure S2—Differences in Methylation Levels Between the Substantia Nigra and the Striatum.**

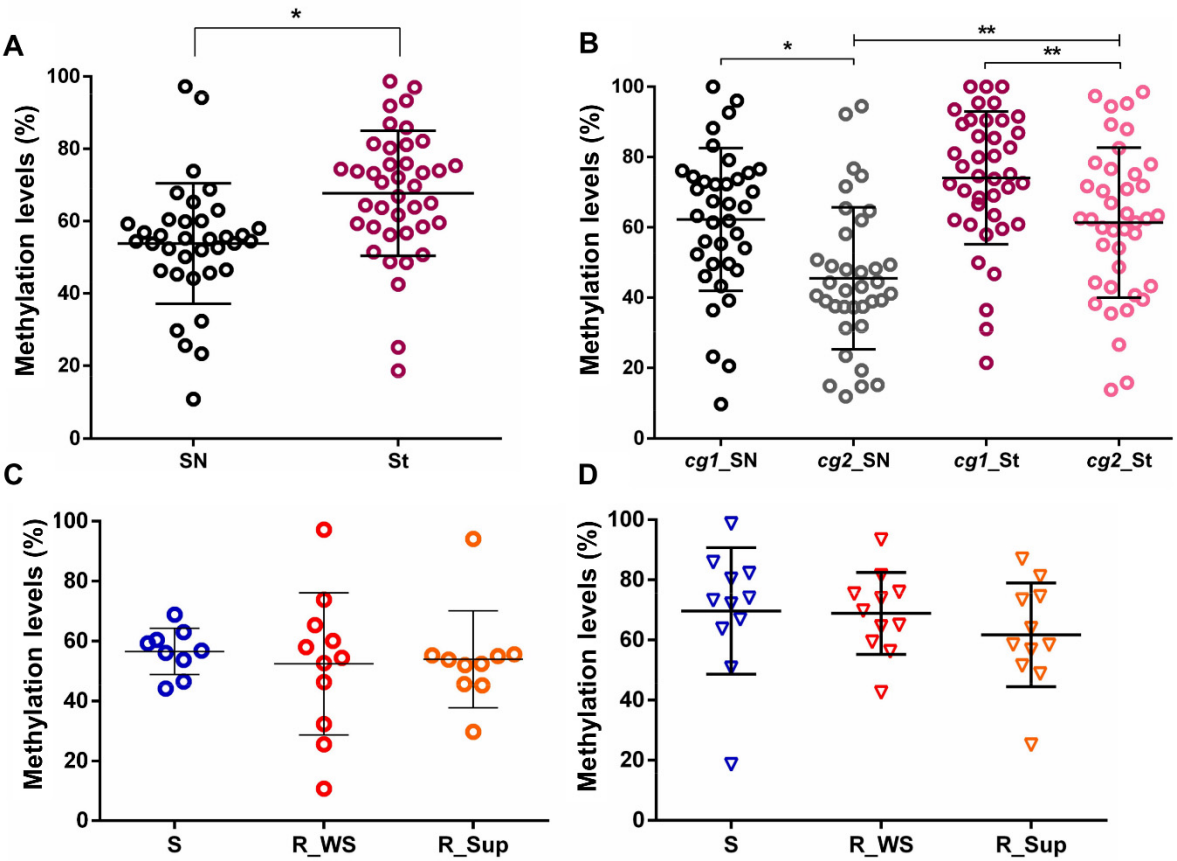


**Figure S2.** (**A**) Methylation mean (*cg1* and *cg2*) between the substantia nigra (SN) and the striatum (St). The methylation levels are higher in St (mean = 67.69 ± 17.31, n = 40) than SN (mean = 53.81 ± 16.66, n = 36). (**B**) Differences in methylation levels of two CpG sites analyzed between SN and St. *cg1* (SN: mean = 62.23 ± 20.34, n = 37; St: mean = 74.05 ± 18.86, n = 40) showed a higher methylation level than *cg2* (SN: mean = 45.51 ± 20.21, n = 36; St: = 61.32 ± 21.35, n = 40) in both brain regions. (**C**) Methylation mean (*cg1* and *cg2*) between the groups in SN. S = sham (mean = 56.58, 68.84 ± 44.20, n = 9); R_WS = rotenone without supplementation (mean = 52.44, 97.24 ± 10.82, n = 11); R_Sup = rotenone supplemented (mean = 53.93, 94.16 ± 29.79, n = 10). (**D**) Methylation mean (*cg1* and *cg2*) between the groups in St. S: mean = 69.67, 98.68 ± 18.66, n = 11; R_WS: = mean = 68.84, 93.38 ± 42.54, n = 11; R_Sup: mean = 61.70, 86.94 ± 25.14, n = 11. * $p < 0.01$; ** $p < 0.05$. Data are the mean with SD.

**Supplementary Figure S3—Correlation Between *Kynu* Gene Expression and Methylation Levels.**

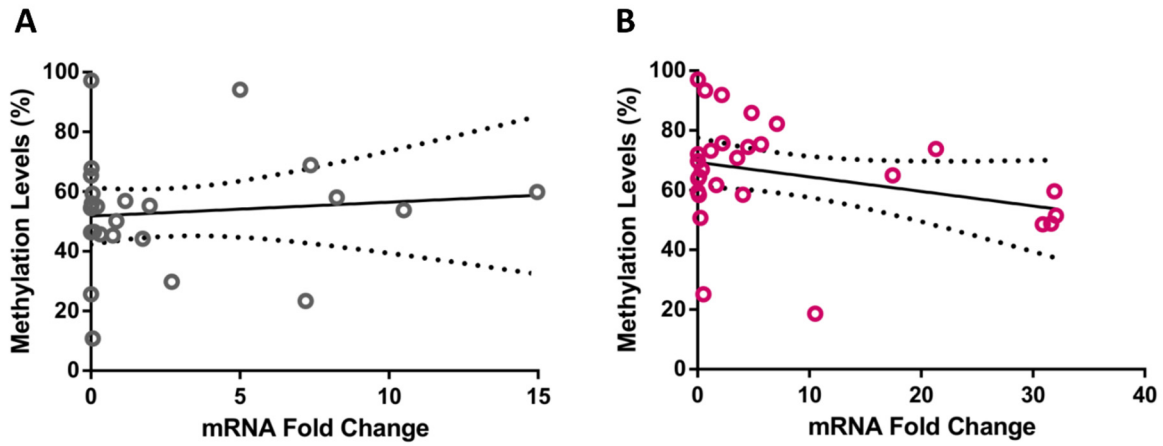


**Figure S3.**Absence of correlation between the expression of the *Kynu* gene and the CpG sites analyzed in (**A**) the substantia nigra (r = .09, p>.5, n = 25) and (**B**) the striatum (r = -0.31, p>.1, n = 28).

**Supplementary Figure S4—*Kynu* Expression in the Substantia Nigra and the Striatum.**

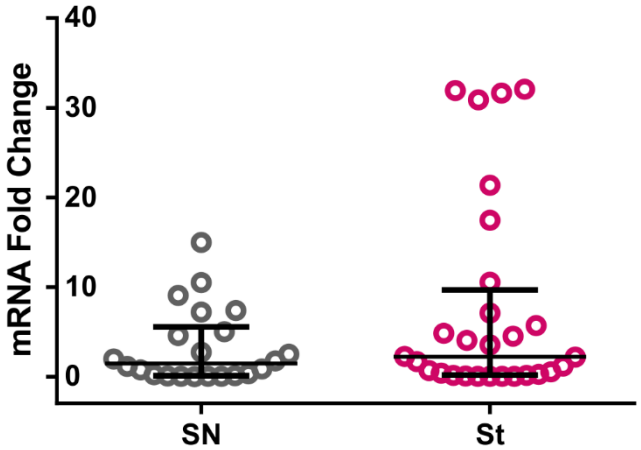


**Figure S4.**There were no significant changes in *Kynu* expression between the substantia nigra (median: 1.14, min: 0.004, max: 14.97, n = 22) and striatum (median: 2.21, min: 0.02, max: 32.06, n = 28). Data are the median with interquartile range. Min = minimum; Max = maximum.