

1 **Title:** Region-specific microRNA alterations in marmosets carrying SLC6A4 polymorphisms
2 are associated with anxiety-like behavior.

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30 **Running title:** Marmoset SLC6A4 polymorphisms, anxiety and region-specific miRNAs
31

32 **ABSTRACT**

33 Background

34 Psychiatric diseases such as depression and anxiety are multifactorial conditions, highly
35 prevalent in western societies. Human studies have identified a number of high-risk genetic
36 variants for these diseases. Among them, polymorphisms in the promoter region of the
37 serotonin transporter gene (*SLC6A4*) have attracted much attention. However, due to the
38 paucity of experimental models, molecular alterations induced by these genetic variants and
39 how they correlate to behavioral deficits have not been examined. In this regard, marmosets
40 have emerged as a powerful model in translational neuroscience to investigate molecular
41 underpinnings of complex behaviors.

42 Methods

43 Here, we took advantage of naturally occurring genetic polymorphisms in
44 marmoset *SLC6A4* gene that have been linked to anxiety-like behaviors. Using FACS-sorting,
45 we profiled microRNA contents in different brain regions of genotyped and behaviorally-
46 phenotyped marmosets.

47 Findings

48 We revealed that marmosets bearing different *SLC6A4* variants exhibit distinct microRNAs
49 signatures in a region of the prefrontal cortex whose activity has been consistently altered in
50 patients with depression/anxiety. We also identified Deleted in Colorectal Cancer (DCC), a
51 gene previously linked to these diseases, as a downstream target of the differently
52 expressed microRNAs. Significantly, we showed that levels of both microRNAs and DCC in
53 this region were highly correlated to anxiety-like behaviors.

54 Interpretation

55 Our findings establish links between genetic variants, molecular modifications in specific
56 cortical regions and complex behavioral responses, providing new insights into gene-
57 behavior relationships underlying human psychopathology.

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60 Fondation de France as well as the Wellcome Trust

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64 **Keywords:** anxiety, depression, non-human primates, marmoset, microRNA, ventro-medial
65 prefrontal cortex, DCC

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70 **RESEARCH IN CONTEXT**

71

72 Evidence before this study

73 Stress-related disorders such as depression or anxiety are a highly prevalent condition with
74 an important socioeconomic impact. Multiple human studies have highlighted: i) a
75 correlation between genetic factors (e.g. polymorphisms in the serotonin transporter gene,
76 SLC6A4) and the risk of suffering from diseases; and ii) the dysfunction of precise regions of
77 the prefrontal cortex in these patients. However, investigating how genetic factors impinge
78 on vulnerable emotional networks has proven to be challenging be due to the lack of
79 appropriate animal models. Recent work has suggested that epigenetic mechanisms such as
80 microRNA would play a pivotal role in mediating the effects of genetic variations on the
81 susceptibility to stress-related disorders.

82

83 Added value of this study

84 Here, we showed that marmosets bearing different SLC6A4 polymorphisms show distinct
85 microRNA signatures in specific areas of the prefrontal cortex, namely in area 32. These
86 specific signatures resulted in a differential regulation of Deleted in Colon Carcinoma (DCC),
87 a gene previously linked to multiple psychiatric diseases. Importantly, levels of DCC and
88 some microRNAs expressed differently depending on the genetic variant, are correlated to
89 the anxiety behavior in response to an ambiguous threaten. Our results provide therefore
90 evidence of how epigenetic factors acting downstream of genetic variation might impinge on
91 particular neuronal circuits on area 32 and fine-tune the expression of key genes such as
92 DCC.

93

94 Implications of all available evidences
95 Our study together with human and non-human primate work suggest that genetic
96 variation, although present in all cells, might selectively affect specific neuronal circuits in
97 vulnerable brain regions via microRNAs (and likely other epigenetic mechanisms). Using
98 novel technologies to manipulate such epigenetic factors might provide more direct links
99 between genetic polymorphisms, epigenetic modulation and vulnerability to psychiatric
100 diseases. This will be of particular interest for human health as we might uncover novel
101 strategies to prevent such conditions.

102

103 **INTRODUCTION**

104

105 Stress-related disorders such as depression and anxiety are common, highly debilitating and
106 burdensome conditions whose incidence has dramatically increased during the COVID-19
107 pandemic^{1, 2}. Despite multiple therapeutic options, a large proportion of patients show no
108 clinical improvement after treatment. Indeed, only about one third of depressed patients
109 respond to serotonin reuptake inhibitors (SSRIs), the most widely prescribed antidepressants
110 (ADs)^{3, 4}.

111

112 The pathophysiology of psychiatric diseases is complex. Nonetheless, twin and, more
113 recently, large-scale genomic studies have demonstrated the substantial influence of genetic
114 variation on the risk for multiple psychiatric disorders^{5, 6}. Among genetic variants,
115 polymorphisms in *SLC6A4*, the gene encoding the serotonin transporter, are of particular
116 interest⁷.

117

118 The *SLC6A4* gene is located in human chromosome 17q11-12. Although variants have been
119 found across the gene, most studies have focused on the so-called serotonin-transporter
120 linked promoter region (5-HTTLPR)^{7, 8}. This region is around 1,5 kb upstream of the first
121 exon and contains a variable number of repeats. Importantly, this particular arrangement of
122 *SLC6A4* promoter is conserved in primates but not in rodents (Suppl. Fig. 1a). A short allele
123 containing 14 repeats (5-HTTLPR S) and a long allele (5-HTTLPR L) comprising 16 repeats
124 have been widely documented. Most studies have reported that 5-HTTLPR S results in lower
125 mRNA and protein levels of the serotonin transporter and, as a consequence, a reduction of
126 circulating serotonin (from the synapse to the presynaptic terminal)^{8, 9}. Multiple studies

127 have linked these polymorphisms with depression/anxiety¹⁰⁻¹². Later work supported the
128 interaction between stress, a risk factor for developing psychiatric conditions, and SLC6A4
129 polymorphisms in depression^{13, 14}. Serotonin neurotransmission is particularly prominent in
130 the regions of the ventro-medial prefrontal cortex (vmPFC) that are dysregulated in patients
131^{15, 16}. Finally, these polymorphisms have also been associated with poor treatment
132 response to ADs^{17, 18}.

133

134 In support of human findings, several groups have found an association between SLC6A4
135 polymorphisms in other primates and phenotypes relevant to psychiatric symptoms¹⁹⁻²².
136 We focused our work on the common marmoset, *Callithrix jacchus*, which has emerged as a
137 reference non-human primate model in modern neuroscience^{23, 24}. Rather than a difference
138 in repeat number, however, marmoset polymorphisms are discrete sequence substitutions
139 in the promoter region²¹. Importantly, Santangelo et al. found two predominant variants,
140 AC/C/G and CT/T/C, in captivity as well as in the wild. Similar to humans, one of those
141 variants (AC/C/G) resulted in reduced mRNA expression²¹. Marmosets carrying two alleles
142 of this variant exhibited an anxious-like behavior when confronted with a human intruder
143 standing in front of their cage, a poor response to citalopram and altered serotonin receptor
144 2a binding and mRNA levels in emotion-related brain regions^{21, 25}. Together these
145 observations support the notion that SLC6A4 promoter structure as well as its genetic
146 variants are functionally relevant for psychiatric conditions across multiple primate species.

147

148 Although 5-HTTLPR polymorphisms influence serotonin transporter levels^{7, 9, 26}, the
149 mechanisms by which these genetic variants increase the risk of psychopathology are
150 currently unknown. In this regard, microRNAs (miRNAs), a class of short (20-25 nt) non-

151 coding RNAs acting as posttranscriptional repressors of gene expression, are attractive
152 candidates. On one hand, their regulatory potential is vast as most protein coding genes are
153 computationally predicted to be miRNAs targets ²⁷. On the other hand, previous work has
154 shown selectivity in the relationship between miRNAs and stress-related disorders as well as
155 therapeutic responses ²⁸⁻³². Finally, the investigation of miRNAs in psychiatric disorders has
156 gained momentum as accumulating evidence indicates that miRNAs could potentially be
157 used as biomarkers ^{33, 34}.

158

159 In order to provide insight into the mechanisms of gene-behavior interactions in
160 primates, we determine whether the distinct *SLC6A4* haplotypes related to trait-like anxiety
161 in marmosets are associated with region-specific differences in miRNA expression within the
162 vmPFC. Not only do we reveal such region-specific miRNA associations related to
163 the *SLC6A4* variants but we also identify Deleted in Colorectal Cancer (DCC) ³⁵, the cognate
164 receptor of Netrin-1 and a gene previously implicated in affective disorders, as a
165 downstream target of the differently expressed miRNA networks. Such changes are highly
166 correlated with individual behavioral anxiety-like responses. They underscore the intimate
167 link between genetic variants, molecular differences among vmPFC areas and complex
168 behavioral outcomes, of major relevance for our understanding of human psychopathology.

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170

171

172 **METHODS**

173

174 *Subjects and genotyping*

175 Marmosets were bred onsite at the Innes Marmoset Colony (Behavioral and Clinical
176 Neuroscience Institute, University of Cambridge) and housed as male-female pairs (males
177 were vasectomized). Temperature (24 °C) and humidity (55%) conditions were controlled,
178 and a dawn/dusk-like 12-h period was maintained. They were provided with a balanced diet
179 and water ad libitum. All procedures were carried out in accordance with the UK Animals
180 (Scientific Procedures) Act 1986 as amended in 2012, under project licences 80/2225 and
181 70/7618. In addition, the University of Cambridge Animal Welfare and Ethical Review Body
182 (AWERB) provided ethical approval of the project licence and its amendments, as well as
183 individual studies and procedures via delegation of authorization to the NACWO for
184 individual study plans.

185

186 For this study, 6 adult male common marmosets, *Callithrix jacchus*, (26 ± 2 mo, 413 ± 17 g)
187 balanced for SLC6A4 genotype (Suppl. table 1) were used. Genotyping was carried out
188 following the protocol described in ²¹. Primers used for sequencing can be found in Suppl.
189 Table 2. We followed ARRIVE guidelines for this study.

190

191 *Behavioral testing and quantification*

192 All the behavioral data in this study were collected and reported previously ^{21, 25, 36}. All the
193 behavioral procedures have been extensively described in the same references as well as in
194 the Supplementary Materials section. Analyzed behaviors are summarized in Suppl. Table 3.

195

196 *Sample preparation*

197 At the end of the study, animals were premedicated with ketamine hydrochloride (20
198 mg/kg) before being euthanized with pentobarbital sodium (Dolethal; 1 mL of a 200-mg/mL
199 solution; Merial Animal Health). Brains were dissected, frozen using liquid nitrogen, and then
200 sliced in a cryostat at $-20\text{ }^{\circ}\text{C}$ to 200- μm -thick sections. Tissue samples for each target region
201 were excised using punches of 1.0 and 1.5-mm radius. Eight punches per target region were
202 used in this study (4 from the right hemisphere and 4 from the left hemisphere). Area 17 can
203 be easily identified as it lies around the calcarine sulcus. Area 25 and 32 are located in
204 regions where there are clear 'easy-to-see' landmarks to guide punching from frozen
205 sections and we took punches in the rostro-caudal centre of both regions, avoiding 'difficult-
206 to-see' boundaries. Area 25 primarily lies at the level of, and caudal to, the onset of the
207 lateral ventricles and the genu of the corpus callosum and so we took punches from eight,
208 200 μm sections, starting at the section in which the onset of the lateral ventricles,
209 bilaterally, could be discerned (see Suppl. Fig. 1c). Area 32, on the other hand, is a relatively
210 large area extending across much of the medial wall of the frontal lobes in front of the genu
211 of the corpus callosum. We started our punches for area 32, 600 μm in front of the lateral
212 ventricles (three 200 μm sections more rostral than the start of the lateral ventricles and area
213 25) and then took punches from the next 8 sections, moving rostrally. The top of the punch
214 for area 32 on the medial wall was aligned with the dorso-medial apex of the underlying
215 white matter (see Suppl. Fig. 1c). Our punches targeted the dorsal part of this region starting
216 behind the frontal pole (using the shape of the underlying white matter - elongated triangle -
217 to guide our selection of the most rostral section and then taking punches from the next 8
218 sections.

219

220

221 *Nuclei isolation and sorting*

222 8 punches/area/animal were used. Nuclei extraction protocol was adapted from ³⁷. All steps
223 were performed at 4 °C or on ice. Tissues were homogenized in nuclei isolation buffer (0.32
224 M Sucrose, 10 mM HEPES pH 8.0, 5 mM CaCl₂, 3 mM Mg(CH₃COO)₂, 0.1 mM EDTA, 1 mM
225 DTT, 0.1% Triton X-100) with a 2 mL Dounce homogenizer by 10 gentle strokes with each
226 pestle and filtered through a 40 µm strainer. After centrifugation, nuclei pellets were
227 resuspended in 1 ml PBS-RI (PBS, 50 U/mL Rnase-OUT Recombinant Ribonuclease Inhibitor
228 (Invitrogen), 1 mM DTT) and fixed by the addition of 3 ml PBS 1.33% paraformaldehyde
229 (Electron Microscopy Sciences) for 30 min on ice. Fixed nuclei were spun down, washed with
230 1 ml PBS 0.1% triton-X-100, pelleted again and resuspended at 10⁶ nuclei per ml in
231 stain/wash buffer (PBS-RI, 0.5% BSA, 0.1% Triton-X-100) containing 2 µg/mL anti-NeuN-
232 alexa-488 antibody (Millipore, MAB377X) and 1 µg/mL Hoechst 33342 (Molecular Probes).
233 After 30 min incubation on ice protected from light, nuclei were washed with 2 mL
234 stain/wash buffer and spun down. Finally, stained nuclei were resuspended in 1 mL PBS-RI
235 0.5% BSA and filtered again through a 40 µm strainer. Nuclei suspensions were maintained
236 on ice protected from light until sorting.

237 Sorting of nuclei was achieved with a MoFlo Astrios EQ Cell sorter (Beckman Coulter). After
238 positive selection of intact Hoechst-positive nuclei and doublets exclusion, all NeuN-positive
239 (NeuN⁺) and NeuN-negative (NeuN⁻) nuclei were separately isolated. Sorted nuclei were
240 collected in refrigerated 2 mL microtubes containing 0.5 ml PBS-RI 0.5% BSA. Finally, nuclei
241 were spun down, supernatants eliminated and pellets were conserved at -80°C until RNA
242 extraction.

243

244 *RNA extraction and reverse transcription*

245 Total RNAs (small and large RNAs) were extracted in one fraction with miRNeasy FFPE kit
246 (Qiagen) following manufacturer's protocol with minor changes. Briefly, nuclei pellets were
247 lysed in 150 μ L PKD buffer and 10 μ L proteinase K for 15 min at 56°C, then immediately
248 incubated at 80°C for 15 min in order to reverse formaldehyde modification of nucleic acids
249 and then immediately incubated 3 min on ice. After centrifugation, supernatants were
250 transferred in new 2 mL microtubes and remaining DNA was degraded during a 30 min
251 incubation with DNase Booster Buffer and DNase I. Addition of RBC buffer and ethanol
252 allowed RNA binding to MiniElute spin columns. After washing steps, pure RNAs were eluted
253 with 20 μ L of RNase-free water. Total RNA concentrations were determined with a
254 Nanodrop spectrophotometer (Fisher Scientific).

255

256 *miRNA reverse transcription and quantification*

257 miRNAs were specifically reverse transcribed with TaqMan Advanced miRNA cDNA Synthesis
258 Kit (Applied Biosystems). Depending on RNA concentration, 10 ng or 2 μ L total RNA were
259 used as starting material for each poly(A) tailing reaction, followed by adaptor ligation and
260 reverse transcription. We chose not to perform the last pre-amplification reaction in order
261 to avoid eventual amplification bias.

262 The expression level of 752 miRNAs was screened by real-time PCR with TaqMan Advanced
263 miRNA Human A and B Cards (Applied Biosystems A31805). cDNAs were diluted 1:10 with
264 0.1X TE buffer, then mixed with water and TaqMan Fast Advanced Master Mix 2X (Applied
265 Biosystems) and 100 μ L of this mix was loaded in each fill reservoir of two array cards. Real-
266 time PCR reactions were run on a QuantStudio 7 Flex Real-Time PCR System (Applied
267 Biosystems).

268

269 *mRNA reverse transcription and quantification*

270 40 ng total RNAs were reverse transcribed with SuperScript IV Reverse Transcriptase
271 (Invitrogen) and random hexamers in 30 μ L total reaction volumes. cDNAs were diluted with
272 water and 266 pg of cDNA was used in each 20 μ L-PCR reaction in 96-well plates. Gene
273 expression was quantified by real-time PCR with marmoset specific TaqMan Gene
274 Expression Assays (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied
275 Biosystems) on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems).

276

277 *Data analysis*

278 Data were revised and analyzed using ThermoFisher Scientific Digital Science online tools
279 (thermofisher.com/fr/en/home/digital-science.html). Relative quantification was performed
280 with the Δ Ct method.

281 368 miRNAs were robustly amplified in more than 75% of the samples and were considered
282 for subsequent analysis. From them, 103 were shared by the NeuN⁺ and NeuN⁻ fractions. Δ Ct
283 values were obtained by global normalization method. qPCR results were first normalized
284 (using global mean normalization method) and then transformed to relative expression
285 levels via the $2^{-\Delta\text{Ct}}$ equation.

286 Four references genes were used as endogenous control genes (POLR2A, TBP, HPRT1, PGK1).
287 Δ Ct values were obtained by subtracting the mean Ct value of these 4 control genes to the
288 Ct value of each target gene.

289

290 To obtain potential mRNA targets, we applied miRNet algorithm³⁸. Using as input the 6
291 miRNAs found to be differentially expressed across genotypes in area 32, we obtained a list

292 of potential targets (546 mRNAs). We refined our list to 25 targets using the following
293 criteria: i) the potential targets have been confirmed using an alternative prediction
294 algorithm (Target Scan); and ii) miRNAs binding sequences show evolutionary conservation
295 (present in humans and macaques). Among them, 15 mRNAs contained target sequences for
296 3 miRNAs (high probability targets) and another 10 only 1 putative binding site (low
297 probability targets). As a control, we also selected 10 reference genes bearing no binding
298 sequence for any of the 6 miRNAs.

299

300 For behavioral experiments, Exploratory Factor Analysis (EFA) in HI test or snake model tests
301 were extracted as previously described ³⁶.

302

303 *Statistics*

304 All values were represented with the mean \pm SEM unless indicated. Statistical analyses were
305 performed using XLStat (PCA), GraphPad 7.0 (ANOVA, correlation analysis and post-hoc
306 tests) and R (PCA and regression analysis). A significance threshold of $p < 0,05$ was used in all
307 experiments.

308

309 No previous marmoset study has analysed microRNA expression in relation to this
310 polymorphism , therefore a robust a priori analysis of sample size based on previous effect
311 sizes was not possible. However, our previous work on 5HT2A receptor binding relating to
312 this SLC6A4 mutation ²⁵ showed an effect size of cohen's $d=5.22519$ with a sample size of 3
313 computed within G*Power with an $\alpha=0.05$ and power=80%. We tested the normal
314 distribution of our data set using the Shapiro-Wilks and Kolmogorov-Smirnov tests in our

315 entire dataset (6 animals, 3 regions and 2 fractions). All data were normally distributed and
316 variance was similar between groups, supporting the use of parametric statistics.

317

318 For all PCA analysis, we used Pearson correlation and n standardization. For the
319 discrimination of NeuN⁺ and NeuN⁻ fractions, we considered the expression levels of 103
320 shared miRNAs in both fractions. Although each fraction expresses 225-250 miRNAs, we
321 displayed in all main figures PCA analysis obtained from the common pool of 103 miRNAs. A
322 similar result was obtained when considering 192 miRNAs expressed in NeuN⁺ nuclei (Suppl.
323 Fig. 1f). Apart from the first two, none of the remaining principal components (we analyzed
324 15) shows any correlation to the cell fraction, region or genotype. The list of the 103 miRNAs
325 is shown in Suppl. Table 4.

326

327 For the analysis of miRNAs differences linked to SLC6A4 variants, we first performed a 2-way
328 ANOVA on the relative expression levels of the 103 miRNAs shared by NeuN⁺ and NeuN⁻
329 fractions in each cortical region. To limit type II error intrinsic to multiple comparison
330 corrections, we reduced the dimensionality of our dataset to the most relevant miRNAs
331 raised by our PCA analysis. Thus, we selected 15 miRNAs showing the strongest contribution
332 to PC2 (the main dimension associated to the genetic polymorphisms in area 32) and 10
333 from the PC1. Although representing a small fraction of the analyzed miRNAs, these 25
334 miRNAs contribute to almost 40% and 30% of PC2 and PC1, respectively. We compared the
335 expression levels of these 25 miRNAs in area 32 and 25 using 1-way ANOVA and Bonferroni's
336 post-hoc test to adjust for multiple comparisons (AC/C/G versus CT/T/C). The list of these
337 miRNAs as well as the adjusted p values are presented in Table 1.

338

339 For the analysis of downstream targets, we analyzed the relative abundance of the mRNAs in
340 area 25 and 32 using 2-way ANOVA followed by Tukey test.

341

342 Linear regression and correlations were calculated using the Pearson correlation coefficient
343 with 2-tailed analysis. We used scatter plots to visualize the data and the residuals to verify
344 the linearity of our data. In addition, we performed a runs test for deviation from linearity
345 and none of our data show any significant deviation from linearity. Individual p values were
346 adjusted for multiple comparison using the Holm-Sidak correction (null hypothesis slope is
347 not different from 0). A summary of all statistical findings is presented in the supplementary
348 material.

349

350 *Role of Funders*

351

352 Funders had no role in study design, data collection or analysis, interpretation, or writing of
353 report.

354

355 **RESULTS**

356

357 *microRNA profiling in the marmoset cortex discriminates between NeuN⁺ and NeuN⁻ cells*

358 *across cortical areas*

359

360 In order to investigate whether previously described genetic variants in marmoset 5-HTTLPR

361 are associated with differences in miRNAs expression that could be linked to behavioral

362 responses, we first validated an experimental approach previously applied to human

363 samples³⁹ (Fig. 1a). Brains from genotyped and behaviorally phenotyped marmosets were

364 sliced and punches from selected brain regions (primary visual cortex: area 17 and two

365 discrete regions within the vmPFC: area 25 and 32) were harvested. After nuclear isolation,

366 samples were FACS sorted into NeuN⁺ and NeuN⁻ nuclei (Suppl. Fig. 1b) and RNA extracted

367 from each fraction. As expected, NeuN⁺ nuclei are enriched in neuron-specific markers

368 (*Grm7*, *Gabra1*, *Camk2*) and deprived almost entirely of glial-associated genes (astrocyte,

369 oligodendrocytes and microglia markers, Fig. 1b and Suppl. Fig. 1d). In contrast, NeuN⁻ nuclei

370 express strong levels of astrocytes (*Gfap*, *Aldh1l1* or *Slc1a3*), oligodendrocytes (*Klk6*, *Plp1*,

371 *Cnscr3*) and microglial genes (*Aif1*) (Fig. 1B and Suppl. Fig. 1d). We also observe a low

372 expression of neuronal genes in this fraction as it is known that a subset of neurons of the

373 primate cortex are NeuN⁻³⁹. Similar profiles were obtained in samples from different cortical

374 regions (area 17, 25 and 32) indicating that FACS sorting is a reliable method to enrich

375 neuron nuclei from different marmoset cortical areas for transcriptomic analysis.

376

377 Although miRNA expression in different brain cell types remains largely unexplored, we

378 hypothesize that, given the differences in cell composition, miRNAs signatures present in

379 NeuN⁺ and NeuN⁻ populations should be dramatically distinct. Using miRNA quantitative

380 PCR, we profiled 754 miRNAs in our samples and found that more than 100 miRNAs were
381 shared by both subpopulations. Focusing on this common pool of miRNAs, we performed a
382 principal component analysis (PCA) in NeuN⁺ and NeuN⁻ nuclei coming from 6 different
383 marmosets. As shown in Fig. 1c, NeuN⁺ and NeuN⁻ samples formed separated clusters across
384 the major PC1 axis confirming that, even considering only those miRNAs whose expression is
385 shared, miRNAs profiles readily distinguish both fractions.

386

387 Recent work revealed important regional differences in gene expression across the
388 marmoset cortex⁴⁰. We next investigated whether, similarly, miRNA profiling might be
389 sensitive enough and detect such regional variations. For that purpose, we examined 3
390 cortical areas; on one hand, we profiled the primary visual cortex (corresponding to
391 Brodmann area 17) as an example of sensory region endowed with specific cytoarchitectonic
392 and functional features (e.g. expanded layer IV, strong myelination, major inputs from the
393 thalamus). On the other hand, we considered two high-order association areas within the
394 vmPFC (Brodmann area 25 and 32) whose activity has been shown to be consistently
395 dysregulated in affective disorders⁴¹⁻⁴³. A clear segregation between sensory and
396 association areas in terms of miRNAs signature was reconstructed using PCA in NeuN⁺ nuclei
397 (Fig. 1d). Whilst samples from the visual cortex clustered together on one side of the PC1
398 axis, the two vmPFC regions appeared intermingled on the other side of the PC1. Such
399 regional pattern cannot be observed in the NeuN⁻ fraction (Suppl. Fig. 1e) suggesting that
400 anatomo-molecular differences largely arise from neurons. Interestingly, expanding the
401 number of miRNAs in the NeuN⁺ fraction for PCA (to 192 miRNAs showing the most robust
402 expression in NeuN⁺ nuclei) did not affect the regional distribution (Suppl. Fig 1e) indicating
403 that these additional miRNAs are similarly expressed across different brain areas.

404

405 Finally, we found that miR-195-5p, miR-221-3p, miR-222-3p and miR-497-5p (Suppl. Fig. 1g)
406 are differentially expressed in the visual cortex compared to the vmPFC (miR-195, $p < 0.0001$
407 vs BA25 and $p = 0.0006$ vs BA32; miR-221, $p = 0.0001$ vs BA25 and $p < 0.0001$ vs BA32; miR-222,
408 $p < 0.0001$ vs BA25 and $p = 0.0002$ vs BA32; miR-497, $p = 0.0003$ vs BA25 and $p = 0.0003$ vs BA32;
409 1-way ANOVA followed by Bonferroni's test). Together, these findings support the notion
410 that miRNAs profiling is a powerful method to uncover molecular differences in the brain.

411

412 *microRNA profiling uncovers region-specific molecular differences in marmosets bearing*
413 *different SLC6A4 variants/haplotypes*

414

415 Since half of the 6 marmosets analyzed here bear each of the two most frequent SLC6A4
416 haplotypes (AC/C/G versus CT/T/C), we next sought to determine whether miRNA profiling
417 could unveil molecular differences related to those polymorphisms. Fig. 2a depicts the PCA
418 analysis in the NeuN+ fraction according to the genetic variant of each animal as well as the
419 anatomical region. While there is no obvious link in area 17 or 25, each haplotype
420 segregated into two independent clusters across the PC2 axis in area 32. These results
421 suggest that this region might be specifically affected by SLC6A4 polymorphisms. In order to
422 confirm this genetic effect, we carried out a 2-way ANOVA on the miRNA expression levels in
423 each region. In line with the results of PCA, we observed no influence of genotype on the
424 miRNA expression neither in area 17 ($F(1, 4) = 1.973$; $p = 0.2328$; 2-way ANOVA) nor in area 25
425 ($F(1, 4) = 3.244$; $p = 0.146$; 2-way ANOVA). In contrast, in area 32 we found a significant effect
426 of the genotype ($F(1, 4) = 20.27$; $p = 0.0108$; 2-way ANOVA) but no interaction between
427 genotype x miRNA ($F(102, 408) = 1,071$, $p = 0.3174$; 2-way ANOVA).

428

429 To determine which miRNAs are significantly and specifically modified in area 32
430 (differentially expressed miRNAs, DEmiRs), we focused on the 25 miRNAs that showed the
431 strongest contribution to the first two components in the PCA (Table 1, see also materials).
432 We found that 6 out these 25 miRNAs exhibited differential expression in area 32 but not in
433 the closely related area 25 (miR-9-5p, $p=0.0475$; miR-125a-5p, $p=0.0013$; miR-125b-5p,
434 $p=0.0196$; miR-190a-5p, $p=0.0032$ miR-525-3p, $p=0.0019$ and let-7d-5p, $p=0.0208$, 1-way
435 ANOVA followed by Bonferroni's test) (Fig. 2b).

436

437 To further confirm the specificity of area 32 miRNA changes, we examined whether SLC6A4
438 polymorphisms impacted the expression of miRNAs in the visual cortex. As shown in Suppl.
439 Fig 2a, miR-195, miR-221, miR-222 and miR-497, although being differentially expressed in
440 this area, showed no difference across the genotypes either in the visual cortex or in the
441 vmPFC. Similarly, levels of miR-9-5p, miR-125a-5p, miR-125b-5p, miR-190a-5p, miR-525-3p
442 and let-7d-5p also exhibited a similar expression pattern in animals bearing the two variants
443 (Suppl. Fig 2b). Overall, our observations confirm that miRNAs could reliably uncover
444 molecular differences in the marmoset cortex and indicate that *SLC6A4* polymorphisms
445 selectively impinge on miRNA signatures in area 32.

446

447 *Genotype-specific changes of DCC expression in area 32*

448 miRNAs regulate gene expression post-transcriptionally. We reasoned that differences in
449 expression of miRNAs in area 32 would result in significant changes in downstream target
450 transcripts. To identify those targets and thus further validate our miRNAs signatures, we
451 carried out a network analysis of the area 32 differently expressed miRNAs using miRNet 38

452 (Fig 3a). Using additional criteria (see Material and Methods), we selected 25 target genes
453 (10 low probability targets and 15 high probability targets) as well as 10 control transcripts
454 for further expression analysis in area 25 and 32. We found no difference across haplotypes
455 in the expression of any low probability target or reference targets in area 32 (Suppl. Fig 3).
456 Among the 15 high probability targets (Suppl. Fig 3), only DCC was found to be differentially
457 expressed depending on SLC6A4 polymorphisms (Fig 3b, $p=0.0407$, 2-way ANOVA followed
458 by Tukey test, $F(3, 8) = 0,07356$). Although the DCC transcript contains putative sequences
459 for miR-9-5p, let7-5p and miR-190, the observed downregulation was moderate, in
460 agreement with the contention that miRNAs fine-tune gene expression^{44, 45}. Since miRNAs
461 repress gene expression, levels of DCC and those of miR-9-5p, let7-5p and miR-190 miRNAs
462 were regulated in the opposite direction. Thus, CT/T/C marmosets show reduced DCC and
463 high levels of those miRNAs compared to AC/C/G animals (Fig. 2b). Finally, we observed no
464 significant different level of expression in those transcripts including DCC in area 25 (Fig. 3b
465 and Suppl. Fig 3) arguing again for the anatomical specificity of molecular repertoire
466 associated with SLC6A4 polymorphisms.

467

468 *Molecular differences in area 32 correlate with behavioral response to uncertain threat*

469 It has been previously shown that SLC6A4 polymorphisms strongly influence anxiety-like
470 behavior in response to uncertainty in the human intruder (HI) test but do not alter evoked
471 fear-like behavior in the more certain context of the snake test (ST)^{21, 46}. Using EFA, a recent
472 study demonstrated that a single factor in the EFA explained behavior on the HI
473 test whereas two factors were necessary to describe behaviors elicited on the ST³⁶.

474

475 We reasoned that, if relevant, the molecular differences identified in area 32 might correlate
476 with behavioral responses in the anxiety-related HI-test but not the fear-related ST. We
477 therefore performed a correlation analysis on the levels of miRNAs differently regulated
478 with the EFA score for HI-test. We observed a negative correlation in area 32 (Fig. 4), with R^2
479 coefficient ranging from 0.68 to 0.94. A significant association was observed for two of them
480 (miR-525-3p and let-7d-5p) after correcting for multiple comparisons. Remarkably, DCC
481 contents also showed a significant but, in this case, positive correlation to the behavioral
482 score ($R^2=0.8994$). In sharp contrast, levels of the same miRNAs and DCC in area 25 exhibited
483 no correlation with the HI test EFA (Fig. 4) arguing for the specificity of our findings.
484 Moreover, behavioral specificity was indicated by the finding that the two behavioral scores
485 in the ST were not correlated with any of these miRNAs in area 32 (Suppl. Fig 4), altogether
486 supporting the notion that the molecular alterations we found in area 32 may be selectively
487 related to the differential behavioral response to uncertain threat in marmosets bearing the
488 different *SLC6A4* variants.

489

490

491

492 **DISCUSSION**

493 Here, we setup a FACS-based approach never applied before in non-human primates to
494 assess miRNA expression in different cell subsets of genotyped and behaviorally phenotyped
495 animals. Our results show that: i) miRNAs are dramatically different across cell types and
496 cortical regions; ii) SLC6A4 polymorphisms are correlated with miRNA signatures in area
497 32 of vmPFC; and iii) levels of specific miRNAs as well as of the target gene, DCC, correlate
498 with anxiety-like behavior in response to uncertain threat in an intruder test.

499

500 Research in biological and molecular psychiatry is confronted by the enormous differences
501 between the human brain and those of experimental animals most commonly used (mice,
502 fish or fly). Non-human primates, especially marmosets, might represent a more appropriate
503 model. First, in terms of genome, primates are evolutionary closer to humans and share
504 most of the coding and non-coding sequences. Thus, 5-HTTLPR genomic arrangement is
505 highly conserved in primates but does not exist in other vertebrates such as mouse or
506 zebrafish. Similar to that reported in humans^{7, 9}, naturally occurring polymorphisms in the
507 marmoset SLC6A4 promoter region are linked to anxious-like behaviors^{21, 36} highlighting the
508 conservation of genomic structure and function. Second, monkey brain anatomy is
509 more similar to humans than that found in these other species, especially in those areas
510 related to affective disorders^{43, 47, 48}. Finally, in molecular terms, it has been shown that the
511 repertoire of non-coding RNAs (ncRNAs) has expanded across evolution, and multiple clinical
512 studies support the idea that primate-restricted ncRNAs contribute to psychiatric conditions
513 including depression/anxiety^{30, 49}. Our findings in non-human primates provide additional
514 evidence to support this notion. Thus, miR-525b-3p, one of the miRNAs whose levels
515 in vmPFC area 32 are correlated with SLC6A4 genetic variants, is an evolutionary recent

516 miRNA as shown by the exact sequence conservation between human, gorilla, and
517 chimpanzee (Suppl. Fig 5a). A less conserved sequence is found in other catarrhine primates
518 (orangutan, baboon and macaques) and New World monkeys (marmosets and squirrel
519 monkeys) but not in prosimians. Similarly, miR-190-5p binding sequences in DCC transcript
520 are conserved across primates but mutated in rodents such as rats or guinea pig (Suppl. Fig
521 5b). As such, our study is a proof of principle investigation highlighting the enormous
522 potential of carrying out molecular investigations on non-human primates that, followed up
523 by experimental manipulations (e.g. using last generation viral vectors ⁵⁰), will facilitate the
524 establishment of causal links of how genetic variants in SLC6A4 might affect miRNAs.

525

526 In light of our results, an important mechanistic question is how genetic variants in SLC6A4
527 affect miRNAs. Considerable evidence suggests that miRNAs have a pivotal role in conferring
528 robustness to biological processes ^{51, 52} and thus modulate gene x environment
529 interactions ^{53, 54}. In the brain, this refers to the ability to maintain a function in spite of
530 genetic or environmental fluctuations. An appealing hypothesis is that, in response to
531 genetic variation (e.g. 5-HTTLPR S allele in humans or AC/C/G in marmosets), miRNAs might
532 provide a molecular mechanism to limit the functional impact of enhanced sensitivity to
533 negative environmental events. Our results suggest that this might only occur in specific
534 networks particularly vulnerable to negative environmental influences such as those
535 involving vmPFC area 32. Area 32 is a key brain region involved in decision making in
536 conflict environments as shown by studies of approach-avoidance decision making in
537 primates ^{55, 56}. This area along with dorsolateral prefrontal cortex, with which it shares bi-
538 directional connections, is dysregulated in depression ⁵⁷. Interestingly, a recent human study
539 using PET scan suggested marked regional differences in the expression of key elements of

540 serotonin neurotransmission within the vmPFC¹⁵. According to this work, area 32 showed
541 considerably lower levels of the serotonin transporter than area 25 providing a rationale for
542 the differential impact of the genetic polymorphisms on those two regions.

543

544 Our work has potential important implications for affective disorders. miRNAs are short
545 molecules endowed with an enormous therapeutic potential^{58, 59}. Accumulating evidence
546 also indicates that miRNAs could be useful biomarkers in psychiatry as multiple studies have
547 demonstrated that blood levels faithfully translate contents of miRNAs in the brain^{33, 34}.

548 Here, we have identified a set of miRNAs whose levels are correlated to different behavioral
549 outcomes in response to uncertainty measured by the HI test, a paradigm ethologically
550 relevant for the investigation of stress-related disorders⁶⁰. Previous work has pointed out
551 miR-218 as an important contributor to stress-related disorders in humans and rodent
552 models^{32, 44, 45, 61} through DCC. We could not observe any difference in miR-218 associated
553 to SLC6A4 variants (not shown). This apparent discrepancy might be simply reflecting the
554 fact that marmosets carrying the AC/C/G polymorphism, although exhibiting high-anxiety
555 trait, are far from an overt pathological condition. Alternatively, our data support miRNAs
556 signatures (representing precise levels of multiple miRNAs) rather than discrete miRNAs as
557 contributing to the fine-tuning of DCC (and other key molecular players), behavior and
558 adaptive responses to ambiguous threatens.

559

560 In this regard, it is now well established that mutations in DCC are strongly linked to human
561 pathology^{35, 62}. Since DCC is the cognate receptor for Netrin-1, an important guidance
562 cue for developing axons⁶³, its role in brain wiring has been widely documented⁶⁴.

563 Accordingly, loss-of-function mutations in DCC result in severe neurodevelopmental

564 disorders involving different degrees of disorganization of axonal tracts^{65, 66}. In the adult
565 brain, DCC may regulate synaptic functions, including synaptic plasticity⁶⁷. Increasing
566 evidence highlights the link between DCC and psychiatric conditions with adolescence
567 and adult onset^{68, 69}), especially depression^{32, 70}. Furthermore, a recent genome-wide study
568 on eight major psychiatric disorders identified DCC as the only genetic locus whose variants
569 are associated with all eight disorders⁵. This is consistent with recent work indicating that
570 DCC might be essential not only to the early organization but also the remodeling of critical
571 circuits in the prefrontal cortex⁷¹⁻⁷³. Our work provides additional insight into the role of
572 DCC in the activity of these circuits and behavioral outcomes, potentially involving
573 interactions between genotypic and phenotypic variation. In addition, our finding that
574 genetic variants in *SLC6A4* are correlated with a differential expression of miRNAs and,
575 ultimately, in DCC further supports the central position of DCC in the genetic networks of
576 psychiatric disorders.

577

578 It is important to note that this study is endowed with two methodological limitations. First,
579 it is based on a reduced number of samples and only includes male marmosets (although
580 females are twice as likely as men to develop MDD and other stress-related disorders⁷⁴).
581 Second, RNA quantification from bulk tissue provides poor cellular resolution. To circumvent
582 these issues, a larger marmoset cohort with no gender bias and the application of last-
583 generation transcriptomic techniques such as single-cell RNA will be required to provide
584 further confirmation of our findings. Finally, molecular profiling of other high-order cognitive
585 areas of the prefrontal cortex (such as dorsolateral prefrontal and orbitofrontal cortex) is
586 necessary to validate the specificity of the *SLC6A4* polymorphisms on emotional circuits of
587 area 32.

589 **CONTRIBUTORS**

590 ACR, AMS and EG conceived and designed the project. AMS performed the behavioral
591 testing and sample preparation. NP performed miRNA and gene expression experiments. NP,
592 AMS, DB and EG analyzed the data. ACR provided contribution to the interpretation of data.
593 AMS, ACR and EG wrote the manuscript. NP, ACR, AMS and EG discussed the results,
594 reviewed and edited the final manuscript. Verified underlying data: NP, ACR, AMS, EG. All
595 authors read and approved the final version of the manuscript.

596

597 **DATA SHARING STATEMENT**

598 The main data supporting the results of this study are available within the paper and its
599 Supplementary materials. They have also been deposited on Mendeley
600 ([doi:10.17632/dng88rcmfg.1](https://doi.org/10.17632/dng88rcmfg.1)).

601

602 **DECLARATION OF INTEREST**

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

604

605

606

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614

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781

782

783 **FIGURE LEGENDS**

784

785 *Figure 1. Schematic representation of experimental protocol and validation steps.*

786 a) Experimental protocol includes the genotypic and phenotypic characterization of the
787 marmosets. After sacrifice, brains were frozen and sliced without fixation. RNA was
788 extracted from punches of 3 different cortical regions. Samples were previously submitted
789 to nuclear isolation, NeuN staining and FACS sorting.

790 b) Expression of neuronal (top panel), astrocytic (middle panel) and oligodendrocytic
791 (bottom panels) markers in NeuN⁺ and NeuN⁻ fractions confirms the efficiency of the FACS
792 sorting strategy. Statistical analysis using 2-way ANOVA revealed a significant effect of the
793 cell type on the expression of these markers ($F(1,299)=192,1$, $p<0,0001$).

794 c) miRNA profiling enables differentiation of NeuN⁺ and NeuN⁻ subsets. Using PCA on 103
795 shared miRNAs, NeuN⁺ and NeuN⁻ nuclei clearly segregate across the PC1 axis.

796 d) Discrimination of regional differences based on miRNAs levels. PCA analysis on the NeuN⁺
797 fraction clearly distinguishes the visual cortex (positive values) from the highly associative
798 areas of the vmPFC (negative values).

799

800 *Figure 2. SLC6A4 polymorphisms (AC/C/G and CT/T/C) alter miRNA signature in area 32.*

801 a) PCA analysis on miRNAs levels in NeuN⁺ nuclei shows genotype-linked differences in area
802 32.

803 b) miRNAs differentially expressed in area 32 in AC/C/G and CT/T/C marmosets (One way
804 ANOVA followed by Bonferroni's correction for multiple comparisons, * $p<0.05$).

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808 *Figure 3. Target mRNAs differently expressed in area 32.*

809 a) Network analysis using miRNAs differently expressed in area 32.

810 b) Changes in DCC expression in area 32 are related to SLC6A4 variant. DCC was found to be
811 significantly decreased in area 32 of marmosets bearing CT/T/C haplotype (2-way ANOVA
812 followed by Tukey's correction for multiple comparisons, * $p < 0.05$.)

813

814 *Figure 4. Correlation between miRNAs and DCC levels in area 32 (left panels) or area 25 (right*
815 *panels) and behavioral response in the human intruder test. Individual p values are adjusted*
816 *for multiple comparison using the Holm-Sidak correction.*

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| miRNA | Area 25 (AC vs CT) | Area 32 (AC vs CT) |
|--------------|--------------------|--------------------|
| | Adjusted p value | Adjusted p value |
| miR-628-3p | >0.999 | >0.999 |
| miR-645 | 0.4508 | >0.999 |
| miR-129-1-3p | 0.2080 | 0.6278 |
| miR-144-3p | >0.999 | >0.999 |
| miR-497-5p | 0.9585 | 0.6780 |
| miR-195-5p | >0.999 | 0.6839 |
| miR-196a-5p | 0.4076 | 0.5621 |
| miR-1260a | 0.1224 | 0.5981 |
| miR-125b-5p | 0.4031 | 0.0196 |
| miR-551a-5p | 0.4356 | >0.999 |
| miR-9-5p | 0.9891 | 0.0475 |
| miR-200a-3p | >0.999 | 0.1737 |
| let-7d-5p | >0.999 | 0.0208 |
| miR-26a-5p | >0.999 | 0.4908 |
| miR-190a-5p | >0.999 | 0.0032 |
| miR-29a-3p | 0.9459 | 0.0688 |
| let-7a-5p | >0.999 | 0.1181 |
| miR-133a-5p | >0.999 | 0.8187 |
| miR-124-3p | 0.3564 | 0.1234 |
| miR-378a-3p | >0.999 | 0.0554 |
| miR-376a-3p | 0.5647 | >0.999 |
| miR-320a | 0.9693 | >0.999 |
| miR-525-3p | 0.3089 | 0.0019 |
| miR-125a-5p | >0.999 | 0.0013 |
| miR-181c-5p | 0.8613 | >0.999 |

822 *Statistical analysis of expression levels of the top 25 miRNAs from PCA (One-way ANOVA*
823 *adjusted for multiple comparison with Bonferroni's correction). The first 10 miRNAs*
824 *correspond to PC1 (gray shading) and the last 15 to PC2 (no shading).*

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