Region-specific microRNA alterations in marmosets carrying SLC6A4 polymorphisms are associated with anxiety-like behavior

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

ABSTRACT

Background

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

Methods

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

Findings

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

Interpretation

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

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

RESEARCH IN CONTEXT

Evidence before this study

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

Added value of this study

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

Implications of all available evidences

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

INTRODUCTION

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

The pathophysiology of psychiatric diseases is complex. Nonetheless, twin and, more recently, large-scale genomic studies have demonstrated the substantial influence of genetic variation on the risk for multiple psychiatric disorders [5, 6]. Among genetic variants, polymorphisms in *SLC6A4*, the gene encoding the serotonin transporter, are of particular interest [7].

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

have linked these polymorphisms with depression/anxiety [10–12]. Later work supported the interaction between stress, a risk factor for developing psychiatric conditions, and SLC6A4 polymorphisms in depression [13, 14]. Serotonin neurotransmission is particularly prominent in the regions of the ventro-medial prefrontal cortex (vmPFC) that are dysregulated in patients [15, 16]. Finally, these polymorphisms have also been associated with poor treatment response to ADs [17, 18].

In support of human findings, several groups have found an association between SLC6A4 polymorphisms in other primates and phenotypes relevant to psychiatric symptoms [19–22]. We focused our work on the common marmoset, *Callithrix jacchus*, which has emerged as a reference non-human primate model in modern neuroscience [23, 24]. Rather than a difference in repeat number, however, marmoset polymorphisms are discrete sequence substitutions in the promoter region [21]. Importantly, Santangelo et al. found two predominant variants, AC/C/G and CT/T/C, in captivity as well as in the wild. Similar to humans, one of those variants (AC/C/G) resulted in reduced mRNA expression [21]. Marmosets carrying two alleles of this variant exhibited an anxious-like behavior when confronted with a human intruder standing in front of their cage, a poor response to citalopram and altered serotonin receptor 2a binding and mRNA levels in emotion-related brain regions [21, 25]. Together these observations support the notion that SLC6A4 promoter structure as well as its genetic variants are functionally relevant for psychiatric conditions across multiple primate species.

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

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

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

METHODS

Subjects and genotyping

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

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

Behavioral testing and quantification

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

Sample preparation

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

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 $CaCl_2$, 3 mM $Mg(CH_3COO)_2$, 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 10^6 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

RNA extraction and reverse transcription

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

miRNA reverse transcription and quantification

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

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

mRNA reverse transcription and quantification

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

Data analysis

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

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

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

To obtain potential mRNA targets, we applied miRNet algorithm [38]. Using as input the 6 miRNAs found to be differentially expressed across genotypes in area 32, we obtained a list

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

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

Statistics

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

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

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

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

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

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

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

Role of Funders

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

RESULTS

microRNA profiling in the marmoset cortex discriminates between NeuN$^+$ and NeuN$^-$ cells across cortical areas

In order to investigate whether previously described genetic variants in marmoset 5-HTTLPR are associated with differences in miRNAs expression that could be linked to behavioral responses, we first validated an experimental approach previously applied to human samples [39] (Fig. 1a). Brains from genotyped and behaviorally phenotyped marmosets were sliced and punches from selected brain regions (primary visual cortex: area 17 and two discrete regions within the vmPFC: area 25 and 32) were harvested. After nuclear isolation, samples were FACS sorted into NeuN$^+$ and NeuN$^-$ nuclei (Suppl. Fig. 1b) and RNA extracted from each fraction. As expected, NeuN$^+$ nuclei are enriched in neuron-specific markers (Grm7, Gabra1, Camk2) and deprived almost entirely of glial-associated genes (astrocyte, oligodendrocytes and microglia markers, Fig. 1b and Suppl. Fig. 1d). In contrast, NeuN$^-$ nuclei express strong levels of astrocytes (Gfap, Aldh1l1 or Slc1a3), oligodendrocytes (Klk6, Plp1, Cnskr3) and microglial genes (Aif1) (Fig. 1B and Suppl. Fig. 1d). We also observe a low expression of neuronal genes in this fraction as it is known that a subset of neurons of the primate cortex are NeuN$^-$ [39]. Similar profiles were obtained in samples from different cortical regions (area 17, 25 and 32) indicating that FACS sorting is a reliable method to enrich neuron nuclei from different marmoset cortical areas for transcriptomic analysis.

Although miRNA expression in different brain cell types remains largely unexplored, we hypothesize that, given the differences in cell composition, miRNAs signatures present in NeuN$^+$ and NeuN$^-$ populations should be dramatically distinct. Using miRNA quantitative

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

Recent work revealed important regional differences in gene expression across the marmoset cortex [40]. We next investigated whether, similarly, miRNA profiling might be sensitive enough and detect such regional variations. For that purpose, we examined 3 cortical areas; on one hand, we profiled the primary visual cortex (corresponding to Brodmann area 17) as an example of sensory region endowed with specific cytoarchitectonic and functional features (e.g. expanded layer IV, strong myelination, major inputs from the thalamus). On the other hand, we considered two high-order association areas within the vmPFC (Brodmann area 25 and 32) whose activity has been shown to be consistently dysregulated in affective disorders [41–43]. A clear segregation between sensory and association areas in terms of miRNAs signature was reconstructed using PCA in $NeuN^+$ nuclei (Fig. 1d). Whilst samples from the visual cortex clustered together on one side of the PC1 axis, the two vmPFC regions appeared intermingled on the other side of the PC1. Such regional pattern cannot be observed in the $NeuN^-$ fraction (Suppl. Fig. 1e) suggesting that anatomo-molecular differences largely arise from neurons. Interestingly, expanding the number of miRNAs in the $NeuN^+$ fraction for PCA (to 192 miRNAs showing the most robust expression in $NeuN^+$ nuclei) did not affect the regional distribution (Suppl. Fig 1e) indicating that these additional miRNAs are similarly expressed across different brain areas.

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

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

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

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

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

Genotype-specific changes of DCC expression in area 32

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

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

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

It has been previously shown that *SLC6A4* polymorphisms strongly influence anxiety-like behavior in response to uncertainty in the human intruder (HI) test but do not alter evoked fear-like behavior in the more certain context of the snake test (ST) [21, 46]. Using EFA, a recent study demonstrated that a single factor in the EFA explained behavior on the HI test whereas two factors were necessary to describe behaviors elicited on the ST [36].

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

DISCUSSION

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

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

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

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

serotonin neurotransmission within the vmPFC [15]. According to this work, area 32 showed considerably lower levels of the serotonin transporter than area 25 providing a rationale for the differential impact of the genetic polymorphisms on those two regions.

Our work has potential important implications for affective disorders. miRNAs are short molecules endowed with an enormous therapeutic potential [58, 59]. Accumulating evidence also indicates that miRNAs could be useful biomarkers in psychiatry as multiple studies have demonstrated that blood levels faithfully translate contents of miRNAs in the brain [33, 34]. Here, we have identified a set of miRNAs whose levels are correlated to different behavioral outcomes in response to uncertainty measured by the HI test, a paradigm ethologically relevant for the investigation of stress-related disorders [60]. Previous work has pointed out miR-218 as an important contributor to stress-related disorders in humans and rodent models [32, 44, 45, 61] through DCC. We could not observe any difference in miR-218 associated to SLC6A4 variants (not shown). This apparent discrepancy might be simply reflecting the fact that marmosets carrying the AC/C/G polymorphism, although exhibiting high-anxiety trait, are far from an overt pathological condition. Alternatively, our data support miRNAs signatures (representing precise levels of multiple miRNAs) rather than discrete miRNAs as contributing to the fine-tuning of DCC (and other key molecular players), behavior and adaptive responses to ambiguous threatens.

In this regard, it is now well established that mutations in DCC are strongly linked to human pathology [35, 62]. Since DCC is the cognate receptor for Netrin-1, an important guidance cue for developing axons [63], its role in brain wiring has been widely documented [64]. Accordingly, loss-of-function mutations in DCC result in severe neurodevelopmental

disorders involving different degrees of disorganization of axonal tracts [65, 66]. In the adult brain, DCC may regulate synaptic functions, including synaptic plasticity [67]. Increasing evidence highlights the link between DCC and psychiatric conditions with adolescence and adult onset [68, 69]), especially depression [32, 70]. Furthermore, a recent genome-wide study on eight major psychiatric disorders identified DCC as the only genetic locus whose variants are associated with all eight disorders [5]. This is consistent with recent work indicating that DCC might be essential not only to the early organization but also the remodeling of critical circuits in the prefrontal cortex [71–73]. Our work provides additional insight into the role of DCC in the activity of these circuits and behavioral outcomes, potentially involving interactions between genotypic and phenotypic variation. In addition, our finding that genetic variants in *SLC6A4* are correlated with a differential expression of miRNAs and, ultimately, in DCC further supports the central position of DCC in the genetic networks of psychiatric disorders.

It is important to note that this study is endowed with two methodological limitations. First, it is based on a reduced number of samples and only includes male marmosets (although females are twice as likely as men to develop MDD and other stress-related disorders [74]). Second, RNA quantification from bulk tissue provides poor cellular resolution. To circumvent these issues, a larger marmoset cohort with no gender bias and the application of last-generation transcriptomic techniques such as single-cell RNA will be required to provide further confirmation of our findings. Finally, molecular profiling of other high-order cognitive areas of the prefrontal cortex (such as dorsolateral prefrontal and orbitofrontal cortex) is necessary to validate the specificity of the SLC6A4 polymorphisms on emotional circuits of area 32.

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CONTRIBUTORS

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

DATA SHARING STATEMENT

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

DECLARATION OF INTEREST

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

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FIGURE LEGENDS

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

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

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

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

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

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

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

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

Figure 3. Target mRNAs differently expressed in area 32.

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

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

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

820 *Table 1.*

miRNA	Area 25 (AC vs CT) Adjusted p value	Area 32 (AC vs CT) 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

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