Title: microRNA profiling uncovers region-specific molecular correlates of threat responses in a marmoset model of anxiety

Authors:, Natalia Popa[1], Angela C. Roberts[2], Andrea M. Santangelo[2,‡] and Eduardo Gascon [1,‡, *]

[1] Aix Marseille Univ, CNRS, INT, Inst Neurosci Timone, Marseille, France

[2] Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom

[‡] Co-senior authors

*Corresponding author: Eduardo Gascon, Institut de Neurosciences de la Timone, Aix-Marseille Université; CNRS UMR7289; 27, Boulevard Jean Moulin, 13005 Marseille (France); E-mail: eduardo.gascon-gonzalo@univ-amu.fr

Running title: cortical miRNAs in marmoset threat responses

ABSTRACT

Background

Neuroimaging studies have consistently reported that stress-related disorders such as depression and anxiety impinge on the activity of emotion regulation networks, namely in the ventromedial prefrontal cortex (vmPFC). This circuitry is known to be extensively modulated by serotonin and it has been long shown that genetic polymorphisms in the serotonin transporter gene (*SLC6A4*) are linked to anxiety and depression. vmPFC encompasses different brain regions in terms of cytoarchitecture, activity and connectivity. However, molecular heterogeneity, including microRNAs, within the vmPFC and how these differences affect emotion regulation and behavior have not been elucidated.

Methods

Here, we took advantage of recently described polymorphisms in marmoset *SLC6A4* gene linked to altered threat responses. Using FACS-sorted cells from different brain regions of genotyped marmosets, we tested the hypothesis that specific molecular changes in the form of microRNA's in precise regions of the vmPFC underlie these behavioral differences.

Results

We showed that microRNA profiling is a good means to assess molecular heterogeneity across cell subsets ($NeuN^+$ versus $NeuN^-$ cells) or cortical regions (visual cortex versus vmPFC) in the primate cortex. More importantly, we provided evidence that marmosets bearing different *SLC6A4* polymorphisms exhibit distinct miRNAs signatures specifically in vmPFC area 32 neurons, but not in the closely

related vmPFC area 25 neurons. Finally, levels of these miRNAs were highly correlated to an anxiety-like score in a test of uncertain threat.

Conclusions

These data demonstrate that molecular changes within area 32 likely underlie the differential anxiety-like responses associated with *SLC6A4* polymorphisms.

Keywords: miRNA, marmoset, Brodmann area 25 and 32, threat, serotonin transporter polymorphisms

INTRODUCTION

Anxiety is a core symptom of multiple psychiatric disorders including depression and schizophrenia. Unravelling the neurobiological substrates of anxious behaviors represent therefore a major issue in biological psychiatry. It is widely accepted that anxiety stems from the evaluation of cost/benefits and/or the subsequent decision-making process [1–3], functions that are highly dependent upon the prefrontal cortex (PFC). In particular, human neuroimaging studies have linked vmPFC activity in cost-benefit decisions [4–6] and in emotional responses under conflict situations [7, 8]. Similarly, whilst decision variables appear widely represented across the frontal cortex of macaque monkeys [9, 10] neurons in the vmPFC have been specifically identified to encode the gain or cost associated with expected outcomes [9, 11–13] as well as their emotional valence [14, 15]. Not surprisingly then, altered processing and deficiencies in vmPFC circuitry have been implicated in the enhanced negative emotion typical of anxiety and affective disorders [16, 17]. Activity in the vmPFC has also been shown not only to predict treatment efficacy but its normalization parallels remission [18, 19] .

Of particular relevance to the vmPFC's involvement in the etiology and treatment of anxiety are its particularly high levels of the serotonin transporter, compared to the rest of the PFC [20]. Selective serotonin reuptake inhibitors that target the transporter are the first line treatment for anxiety and depression [21] and genetic variants in the *SLC6A4* gene (encoding the serotonin transporter) [22] or HTR1A [23, 24] are linked to a higher risk of anxiety and depression. Thus, understanding the cellular mechanisms by which serotonin regulates vmPFC function will help in the quest for

more rational therapeutic targets in anxiety treatment. MicroRNAs (miRNAs) are attractive candidates as they have been shown to modulate such regulatory neurotransmitters and signaling cascades and to be de-regulated in patients with affective disorders (for review see [25, 26]). They are a class of short (20-25 nt) non-coding RNAs and one of the best-studied posttranscriptional modifiers of gene expression [27, 28]. miRNAs repress expression of messenger RNAs (mRNAs) containing complementary sequences and have been involved in multiple pathological processes, including psychiatric diseases [29, 30]. In particular, the investigation of miRNAs in psychiatric disorders has gained momentum as accumulating evidences indicate that miRNAs could be potentially used as biomarkers and/or therapies [31, 32].

To determine whether miRNAs can provide insight into the mechanism by which alterations in the serotonin transporter can modulate vmPFC function, of relevance to our understanding of anxiety, the present study investigated miRNAs within the vmPFC of marmoset monkeys. The common marmoset, *Callithrix jacchus*, has emerged as a reference model in modern neuroscience. Compared to rodents, marmosets are closer to humans, not only in their behavioral repertoire and brain organization, including vmPFC, but also in molecular terms [33, 34]. Recent studies in this species have thus shown that *SLC6A4* polymorphisms affect the regulation of behaviors elicited by threat in the human intruder paradigm, the response to antidepressants and the neurochemical balance in brain areas implicated in emotional processing [35–38]. In addition, recent studies have begun to reveal distinct functional units within the vmPFC [39, 40]. It is important to recognize that the vmPFC encompasses distinct regions with marked differences in cytoarchitecture, cellular

composition, connectivity patterns and function [16, 41–45]. Here, we used marmosets carrying distinct *SLC6A4* haplotypes as the experimental model to dissect out molecular (namely in miRNAs) alterations linked to trait-like anxiety, within distinct regions of vmPFC.

A better understanding of molecular heterogeneity within the vmPFC should contribute to uncovering their functional divisions and thus provide novel insights into psychiatric disorders related to dysfunction within this region.

Methods and Materials

A more detailed description of methods is provided in the Supplementary File

Subjects

For this study 6 adult male common marmosets, Callithrix jacchus, (26 ± 2 mo, 413 ± 17 g) balanced for *SLC6A4* genotype were used in this study. Marmosets were bred onsite at the Innes Marmoset Colony (Behavioral and Clinical Neuroscience Institute) and housed in pairs. This research has been approved by the University of Cambridge Animal Welfare and Ethical Review Body.

Behavioral testing

The human intruder (HI) test involves measuring the animal's behavioral response to an unfamiliar human, the "human intruder," who stands in front of the animal's home-cage and maintains eye contact with the animal. The procedure for the HI test is based on the method used by [37]. An experimenter (unfamiliar to the animal) wearing a realistic latex human mask (Greyland Film, UK) and standard lab attire stood 40 cm from the cage and maintained eye contact with the subject for 2 min (intruder phase). Recording continued for a further 5 min after the intruder left (recovery phase).

The model snake test involves recording the animal's behavioral response to a rubber snake which acts as an inherent predatory stimulus, provoking an innate fear response [46]. The procedure of the model snake test is based on the methods in [47].

Genotyping

Genomic DNA (gDNA) was extracted from hair follicles of the back of the animals using the QIAamp DNA Micro kit for forensic casework samples (Qiagen). Primers and PCR conditions are described in Supplementary Methods.

Sample preparation

At the end of the study, animals were premedicated with ketamine hydrochloride before being euthanized with pentobarbital sodium (Dolethal; 1 mL of a 200-mg/mL solution; Merial Animal Health). Brain were dissected, frozen using liquid nitrogen, and then sliced in a cryostat at −20 °C to 200-µm-thick sections. Eight punches (1-1.5 mm) per target region were used in this study.

Nuclei isolation and sorting

Nuclei extraction protocol was adapted from [48] (see supplementary Materials and Methods). All steps were performed at 4 °C or on ice. Fixed nuclei were spun down, washed with 1 ml PBS 0.1% triton-X-100, pelleted again and resuspended at 10^6 nuclei per ml in stain/wash buffer (PBS-RI, 0.5% BSA, 0.1% Triton-X-100) containing 2 µg/ml anti-NeuN-alexa-488 antibody (Millipore, MAB377X) and 1 µg/ml Hoechst 33342 (Molecular Probes). Stained nuclei were resuspended in 1 ml PBS-RI 0.5% BSA and filtered again through a 40 µm strainer.

Sorting of nuclei was achieved with a MoFlo Astrios EQ Cell sorter (Beckman Coulter). Sorted nuclei were collected in refrigerated 2 ml microtubes containing 0.5 ml PBS-RI 0.5% BSA. Finally, nuclei were spun down and pellets conserved at -80°C until RNA extraction.

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 minutes at 56°C, then immediately incubated at 80°C for 15 minutes in order to reverse formaldehyde modification of nucleic acids and then immediately incubated 3 minutes on ice. After centrifugation, supernatants were transferred in new 2 ml microtubes and remaining DNA was degraded during a 30 minutes incubation with DNase Booster Buffer and DNase I.

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 of 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). 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. Relative quantification was performed with the ΔΔCt method. 368 miRNAs were robustly amplified and were considered for subsequent analysis. qPCR results were first normalized (using global mean normalization method) and then transformed to relative expression levels via the 2^{-DCt} equation. Four references genes were used as endogenous control (POLR2A, TBP, HPRT1, PGK1) for target analysis.

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

Statistics

Statistical differences between two groups were analyzed with Student's t tests. Correlations were calculated using the Pearson correlation coefficient with 2-tailed analysis. Otherwise, 1- or 2-way analyses of variance were performed. No statistical methods were used to determine the sample sizes, but the number of experimental subjects is similar to sample sizes routinely used in our laboratory and in the field for similar experiments. All data were normally distributed and variance was not

significantly different between groups, supporting the use of parametric statistics. Statistical differences were set to $p<.05$ (GraphPad 7, XLStat or R).

RESULTS

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

In order to investigate whether miRNAs signatures could be linked to behavioral responses, we first validated an experimental approach previously applied to human samples [49] (Fig. 1A). Brains from genotyped and behaviorally phenotyped marmosets were sliced and punches from selected brain regions were harvested. After nuclear isolation, samples were FACS sorted into NeuN$^+$ and NeuN$^-$ cells (Suppl. Fig. 1A) and RNA extracted from each fraction. As expected, NeuN$^+$ cells 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. 1B). In contrast, NeuN$^-$ cells express strong levels of astrocytes (Gfap, Aldh1l1 or Slc1a3), oligodendrocytes (Klk6, Plp1, Cnskr3) and microglial markers (Aif1) (Fig. 1B and Suppl. Fig. 1B). 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$^-$ [49]. 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 neurons 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

almost 100 miRNAs were consistently expressed in both subpopulations. Using these miRNAs, we performed a principal component analysis (PCA) in $NeuN^+$ and $NeuN^-$ nuclei coming from 3 brain regions and 6 different marmosets. As shown in Fig. 1C, $NeuN^+$ and $NeuN^-$ samples formed separated clustered across the PC1 axis confirming that, even considering only those miRNAs whose expression is shared, miRNAs profiles readily distinguish both fractions.

Recent work [50] revealed important regional differences in gene expression across the marmoset cortex. 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 specialized 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 deregulated in affective disorders [7, 8]. PCA on miRNA levels (154 miRNAs that are consistently detected in all $NeuN^+$ samples) from these different areas enabled reconstruction of such regional pattern in $NeuN^+$ cells (Fig. 1D) but not in $NeuN^-$ cells (Suppl. Fig. 1C). Samples from the visual cortex clustered together on one side of the PC1 axis indicating a clear segregation between sensory and association areas in terms of miRNAs signature. In contrast, the two regions of the PFC appeared intermingled. Accordingly, a number of miRNAs (Suppl. Fig. 1D) are differentially expressed in the visual cortex. Again, such regional pattern cannot be observed in the $NeuN^-$ fraction (Suppl. Fig. 1C) suggesting that anatomo-molecular differences largely arise from

neurons. 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

Genetic polymorphisms in the marmoset *SLC6A4* promoter region have been linked to high trait anxiety [37]. Since primate vmPFC has been widely involved in emotional processing [41], we determined whether miRNA profiling could unveil molecular differences in marmosets bearing the two most frequent *SLC6A4* haplotypes (AC/C/G versus CT/T/C) described previously. For that purpose, we analyzed miRNAs contents in 3 marmosets for each variant. As depicted in Fig. 2A, PCA on $NeuN^+$ neurons showed two independent clusters in area 32 corresponding to each genotype suggesting that this region might be specifically affected by *SLC6A4* polymorphisms. No such pattern was observed either in the primary visual cortex or in area 25 arguing against a broad cortical effect of the polymorphism. In order to confirm this genetic effect, we carried out a 2-way ANOVA on the miRNA levels in each region. We found that area 32 exhibited a significant effect of genotype on the miRNA expression ($F(1, 4)=17.06$; $p=0.0145$) whereas this was not observed in areas 17 ($F(1, 4)= 2.419$; $p=0.1949$) and 25 ($F(1, 4)=0.1311$; $p=0.1311$).

To determine which miRNAs are significantly and specifically deregulated in area 32 (differentially expressed miRNAs, DEmiRs), we took into account our results from the PCA and considered a list of 20 miRNAs that contributed the most to the first two

PCs (Table 1). miRNAs that exhibited differential expression in area 32 but not in BA17 and 25 are shown in Fig. 2B.

To confirm the specificity of this observations, we also analyzed neuronal miRNAs the expression of which is associated with visual cortex. Although a number of miRNAs (miR-195, miR-221, miR-222 and miR-497) were found to be differentially expressed in the visual cortex compared to area 25 and 32 (Suppl. Fig. 2), none of the BA17 DEmiRs showed any difference across the genotypes suggesting that there exist both region-specific miRNAs and miRNAs altered in precise regions in a genotype-dependent manner. Overall, our observations confirm that miRNAs could reliably uncover molecular differences in the marmoset cortex and indicate *SLC6A4* polymorphisms selectively alter miRNA signatures in area 32.

Specific changes in target genes linked to genotype in area 32

miRNAs regulate gene expression post-transcriptionally. We reasoned that miRNAs alterations 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 deregulated miRNAs using miRNet [51]. We restricted our analysis to the most likely candidates and focused on genes bearing binding sequences for, at least, three of the miRNAs differentially expressed in area 32 across *SLC6A4* variants. More than 30 genes fulfilled our criteria (Fig. 3A) and we then quantified those predicted targets showing the strongest brain expression (15 targets). Note, we also measured in the same samples, the levels of 15 control genes, exhibiting similar brain enrichment but encompassing no binding sequences for BA32-specific miRNAs. Among the

candidate genes, only Map2k7 was found to be differentially expressed in area 32 across *SLC6A4* genotypes. Map2k7 is a member of JNK kinases and its transcript contains 5 putative sequences for miR-125a-5p and 125b-5p, 2 for miR-9-5p and one for let7-5p. Interestingly, Map2k7 levels were reduced in CT/T/C marmosets and inversely correlated to the levels of BA32-specific miRNAs (higher in CT/T/C animals, Fig. 2B). Despite the number of miRNA sequences present in Map2k7 mRNA, the observed downregulation was moderate, in agreement with the contention that miRNAs fine-tune gene expression [52, 53]. Finally, we observed no reduction of Map2k7 in area 25 (Suppl. Fig. 3) and no alteration of the reference transcripts in either BA25 or 32. Overall, our findings argue again for the anatomical specificity of molecular alterations associated to *SLC6A4* polymorphisms.

microRNA levels of DEmiRs 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 test (HI-test), but do not alter evoked fear-like behavior in the more certain context of the snake test (ST) [35, 37]. Using exploratory factor analysis (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 alterations identified in area 32 might correlate with behavioral responses in the HI-test but not the snake test. We therefore performed a linear regression analysis on the levels of miRNAs

deregulated with the EFA score for HI-test. We observed a significant negative correlation for four miRNAs in area 32 (Fig. 4A), showing a determination coefficient R^2 ranging from .45 to .94; miR-525-3p showed the strongest association with an R^2=.94 (p=.0013), followed by miR-125b-5p (R^2=.78; p=.0197), let-7d-5p (R^2=.69; p=.0393) and miR-125a-5p (R^2=.68; p=.0407). Remarkably, levels of the same miRNAs but in area 25 exhibited no significant correlation with the HI test EFA (Fig. 4) supporting the specificity of our findings. Moreover, the two behavioral scores in the ST were also not correlated with any of these miRNAs (Suppl. Fig. 4) supporting the notion that the molecular alterations we found in area 32 are connected to the differential behavioral response to uncertain threat in marmosets bearing the different *SLC6A4* variants.

DISCUSSION

Using a novel approach to investigate miRNA signatures, we show here specific molecular alterations underlying the anxiety-related *SLC6A4* polymorphisms within distinct regions of the primate vmPFC. Focusing on miRNAs in areas 32 and 25, with primary visual area 17 acting as a control region, we show that: i) miRNAs repertoire is dramatically different across cell types and cortical regions; ii) *SLC6A4* polymorphisms impinge selectively on miRNA signature in area 32; and iii) miRNA levels specifically in area 32 correlate with anxiety-like behavior in response to an uncertain threat. Together, these results support the notion that genetic differences might critically alter molecular pathways within specific cortical regions and that these changes perturb the behavioral repertoire.

The paucity of appropriate experimental models has precluded anatomical and molecular investigation of vmPFC in anxiety-like behaviors. Rodents have been extensively used in a variety of threat paradigms. Nonetheless, vmPFC parcellation appears markedly different from that seen in humans whilst there is far greater comparability between non-human primates and humans [41]. Although both peri- and subgenual cortex have been consistently implicated in threat processing and stress-related disorders, it has been difficult to establish their functional differences. A recent study [54] used a common approach-avoidance task in macaques and humans (both healthy controls and depressed patients) to compare fMRI data. Interestingly, this work highlighted a shared primate network for aversive behavior deregulated in patients involving perigenual (but not subgenual cingulate cortex). In the marmoset, previous work has clearly demonstrated the functional differences

between marmoset area 25 and area 32 in threat reactivity, not only in the extinction of Pavlovian conditioned threat responses [55] but also in approach-avoidance decision making [44]. In addition, overactivation of area 25 specifically evokes anxiety-like behavior in response to uncertainty evoked in the HI threat test [40]. However, the involvement of area 32 has not been addressed yet in this paradigm. In light of our findings and the mentioned work, future experiments using opto/chemogenetics should be carried out in marmosets carrying the distinct haplotypes to precisely delineate the contribution of area 32 in the HI test.

From a molecular perspective, recent data using single-cell RNA sequencing have provided experimental evidences of the existence of unique neuronal types and primate-specific genetic programs [50, 56]. In the same line, the repertoire of non-coding RNAs (ncRNAs) has expanded across evolution and multiple studies support the notion that primate-restricted ncRNAs contribute to psychiatric conditions including depression [57, 58]. Building on these findings, our results reveal a primate-specific miRNA, miR-525-3p, the levels of which are best correlated with uncertainty-evoked anxiety-like, as distinct from direct, threat-evoked, fear-like behavior. miR-525 is an evolutionary recent miRNA as shown by the exact sequence conservation between human, gorilla, and chimpanzee. A less conserved sequence is found in other old-world (orangutan, baboon and macaques) and new-world monkeys (marmosets and squirrel monkeys) but not in prosimians (mouse lemur) suggesting that a common miR-525 ancestor appeared about 40 million years ago (Suppl. Fig. 5A). Interestingly, it has also been shown that sequences of ancient miRNAs such as miR-9 or miR-125 have a strong evolutionary tendency for conservation [59] whereas the mRNAs regulated by these miRNAs are submitted to substantial evolutionary

turnover [60, 61]. Recent data on two human ancestors (*H. Sapiens denisova* and *neanderthalensis*) suggest that this process might have enabled further refinement of gene regulatory networks being therefore instrumental in human brain evolution [62]. In support of this, we found that MAP2K7 target sequences for miR-9 have been conserved across vertebrates whereas those for miR-125 or let-7 show poor conservation arguing for species-specific regulation (Suppl. Fig. 5B). Together, these results highlight the importance of molecular investigations in the brains of primates that might uncover evolutionary recent molecular pathways of utmost interest for biological psychiatry. It is important to note though that this study only included male marmosets whilst evidence shows that females are twice as likely as males to develop major depression and other stress-related disorders [63–65]. Thus, future studies should assess whether the observed region-specific changes in miRNAs are also present in females.

Our study provides evidence supporting that miRNA alterations in area 32 affect the expression of target genes. Thus, animals bearing the CT/T/C variant show an increase of miRNAs regulating Map2k7, whose levels are accordingly downregulated in area 32 but not in area 25. It is worth highlighting that, rather than switching on and off gene expression, changes in brain miRNAs lead to modest reductions of target mRNAs [66, 67]. Nonetheless, miRNA-dependent fine-tuning of specific targets has been consistently involved in the physiopathology of stress-related disorders [68–70]. Map2k7 is a member of JNK kinases, a family of proteins known to be activated in the brain by stress [71]. Although Map2k7 mutations have been linked to schizophrenia [72], recent data in mice suggest that JNK kinase might be more broadly implicated in anxiety [73, 74]. miRNA signatures from area 32 might therefore

help identifying potential downstream effectors/signaling pathways relevant for anxiety. A caveat of the present study is the poor cellular resolution intrinsic to mRNA quantification from bulk tissue. Applying novel techniques such as single-cell RNA should circumvent this limitation and provide more detailed transcriptomic data in upcoming work.

Investigating the reactivity of marmosets to direct/immediate and more uncertain/ambiguous threat as revealed by responsivity to a rubber snake and an unknown human provides experimental settings in which to model different aspects of anxiety-like and fear-like behaviors which likely engage distinct neural networks and psychological processes. In humans, reactivity to immediate threat has been shown to engage dorsal cingulate and brainstem circuitry, whilst reactivity to more ambiguous, potentially distal threat has been shown to engage higher-order prefrontal brain regions [75]. Our finding here that molecular differences between individuals bearing specific *SLC6A4* variants are specifically associated with area 32 and are related to reactivity to uncertain, but not certain threat, is consistent with this. Given the known involvement of serotonin in brain development [76] future studies should determine the relationship of such variants within the developing brain, in particular, the process by which area 32 integrates into prefrontal executive networks. This is highly relevant to our understanding of the onset of psychiatric disorders since the majority begin during adolescence [77, 78], a period in which miRNAs changes might significantly contribute to brain vulnerability [52, 53].

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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 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.

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

FIGURE LEGENDS

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

A) Experimental protocol included the genotypic and phenotypic characterization of the marmosets. After sacrifice, brains were frozen and sliced without fixation. RNA was extracted from punches of different cortical regions. Samples were subsequently 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 confirmed the efficiency of the FACS sorting strategy.

C) PCA analysis on the levels of 92 miRNAs expressed in both $NeuN^+$ and $NeuN^-$ nuclei demonstrated that miRNAs profiles clearly differentiate both fractions.

D) PCA analysis on miRNAs level in $NeuN^+$ fraction enabled regional discrimination. Samples from the visual cortex clearly clustered apart from those of the vmPFC which, in turn, are intermingled.

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

A) PCA analysis on miRNAs level in $NeuN^+$ nuclei showed genotypic differences only 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 test for multiple comparisons, * $p<.05$).

Figure 3. Target mRNAs in area 32.

A) Network analysis using miRNAs deregulated in area 32.

B) Expression of target mRNAs identified by network analysis in area 32. Using as reference the AC/C/G genotype (red dotted line), we measured the abundance of 15 potential targets as well as 15 reference genes. Only Map2k7 was found to be differentially expressed in area 32 (two-tailed unpaired t-test, * p<.05, t-test)

Figure 4. Correlation between miRNA levels in area 32 (upper panels) or 25 (lower panels) and behavioral response in the human intruder test.

Table 1. Statistical analysis of expression levels of top 25 miRNAs from PCA (One-way ANOVA adjusted for multiple comparison with Bonferroni's correction).

miRNA	Area 25 (AC vs CT) Adjusted p value	Area 32 (AC vs CT) Adjusted p value
let-7a-5p	>0.999	0.1181
let-7d-5p	>0.999	**0.0208**
miR-9-5p	0.9891	**0.0475**
miR-26a-5p	>0.999	0.4908
miR-100-5p	0.2004	0.1486
miR-124-3p	0.3564	0.1234
miR-125a-5p	>0.999	**0.0013**
miR-125b-5p	0.4031	**0.0196**
miR-129-1-3p	0.2080	0.6278
miR-133a-5p	>0.999	0.8187
miR-144-3p	>0.999	>0.999
miR-190a-5p	>0.999	**0.0032**
miR-195-5p	>0.999	0.6839
miR-200a-3p	>0.999	0.1737
miR-221-3p	>0.999	>0.999
miR-222-3p	>0.999	0.4192
miR-302b-3p	0.2303	0.5073
miR-320a	0.9693	>0.999
miR-376a-3p	0.5647	>0.999
miR-378a-3p	>0.999	0.0554

miR-495-3p	>0.999	>0.999
miR-497-5p	0.9585	0.6780
miR-525-3p	0.3089	**0.0019**
miR-628-3p	>0.999	>0.999
miR-645	0.4508	>0.999

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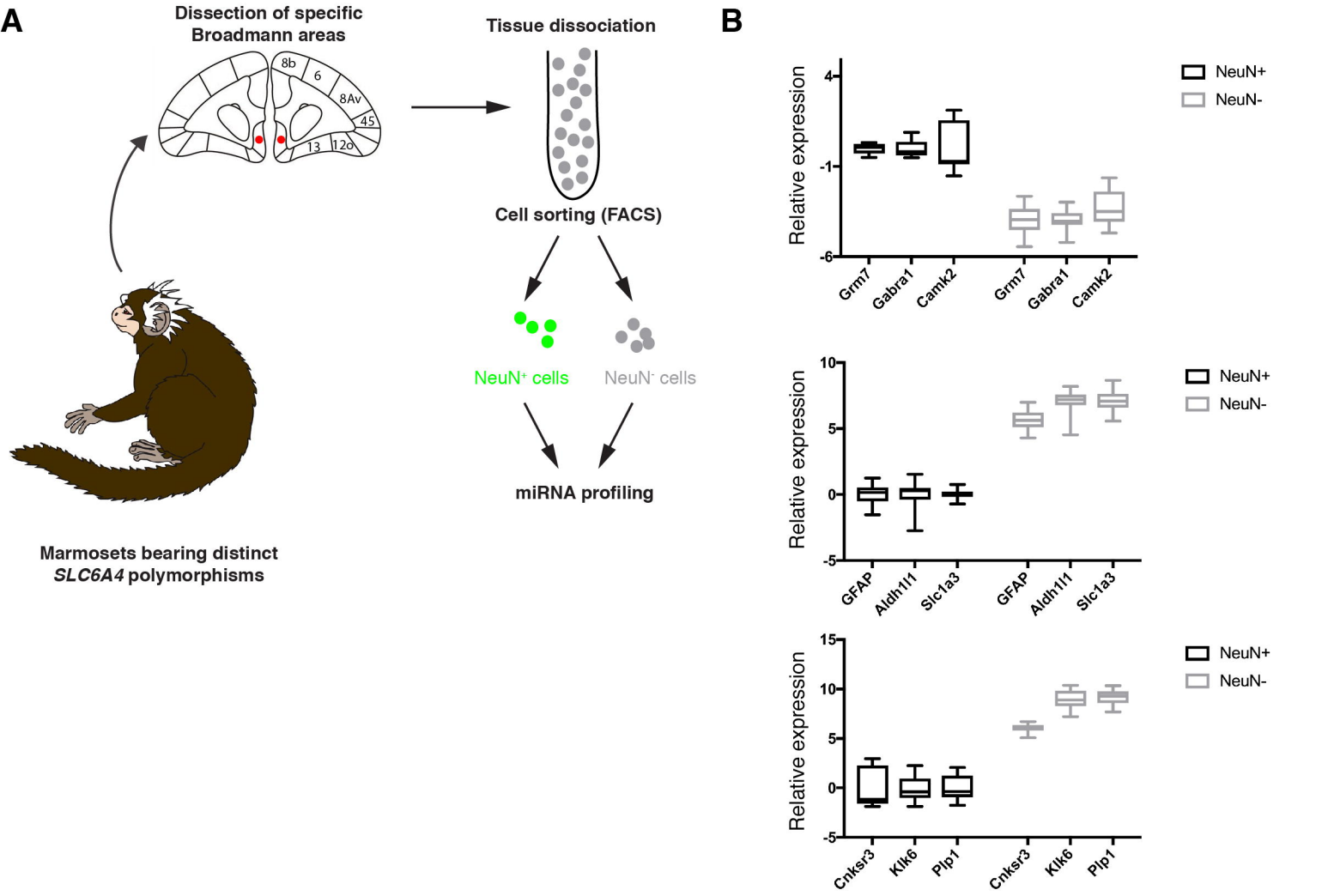

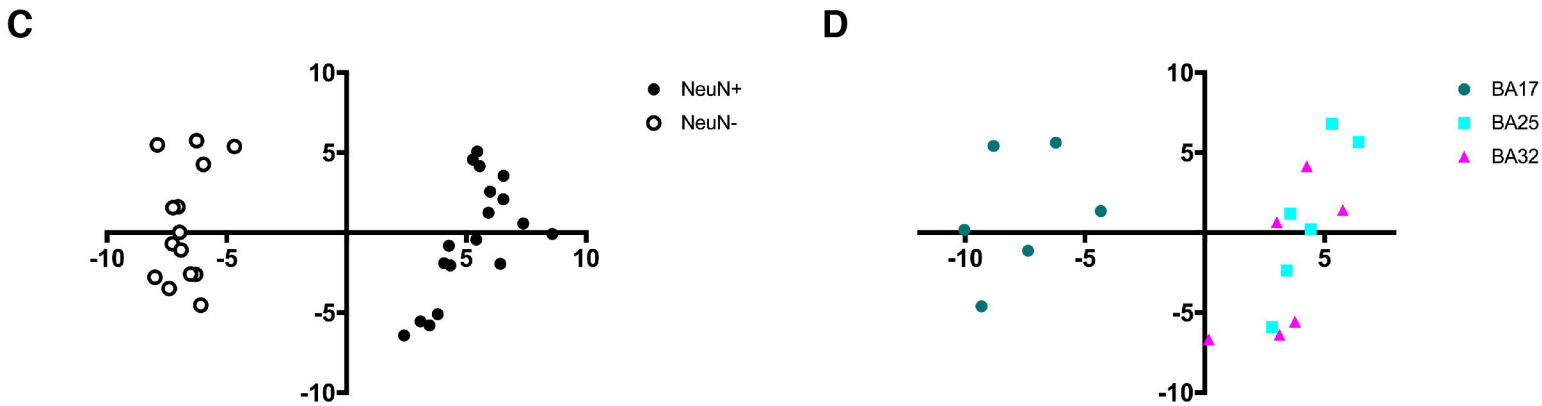

Figure 1. Schematic representation of experimental protocol and validation steps.
A) Experimental protocol include the genotypic and phenotypic characterization of the marmosets. After sacrifice, brains were frozen and sliced without fixation. RNA was extracted from punches of different cortical regions. 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.
C) PCA analysis on the levels of 92 miRNAs expressed in both NeuN$^+$ and NeuN$^-$ nuclei demonstrate that miRNAs profiles clearly differentiate both fractions.
D) PCA analysis on miRNAs level in NeuN$^+$ fraction enables regional discrimination. Samples from the visual clearly cluster apart from those of the vmPFC which, in turn, are intermingled.

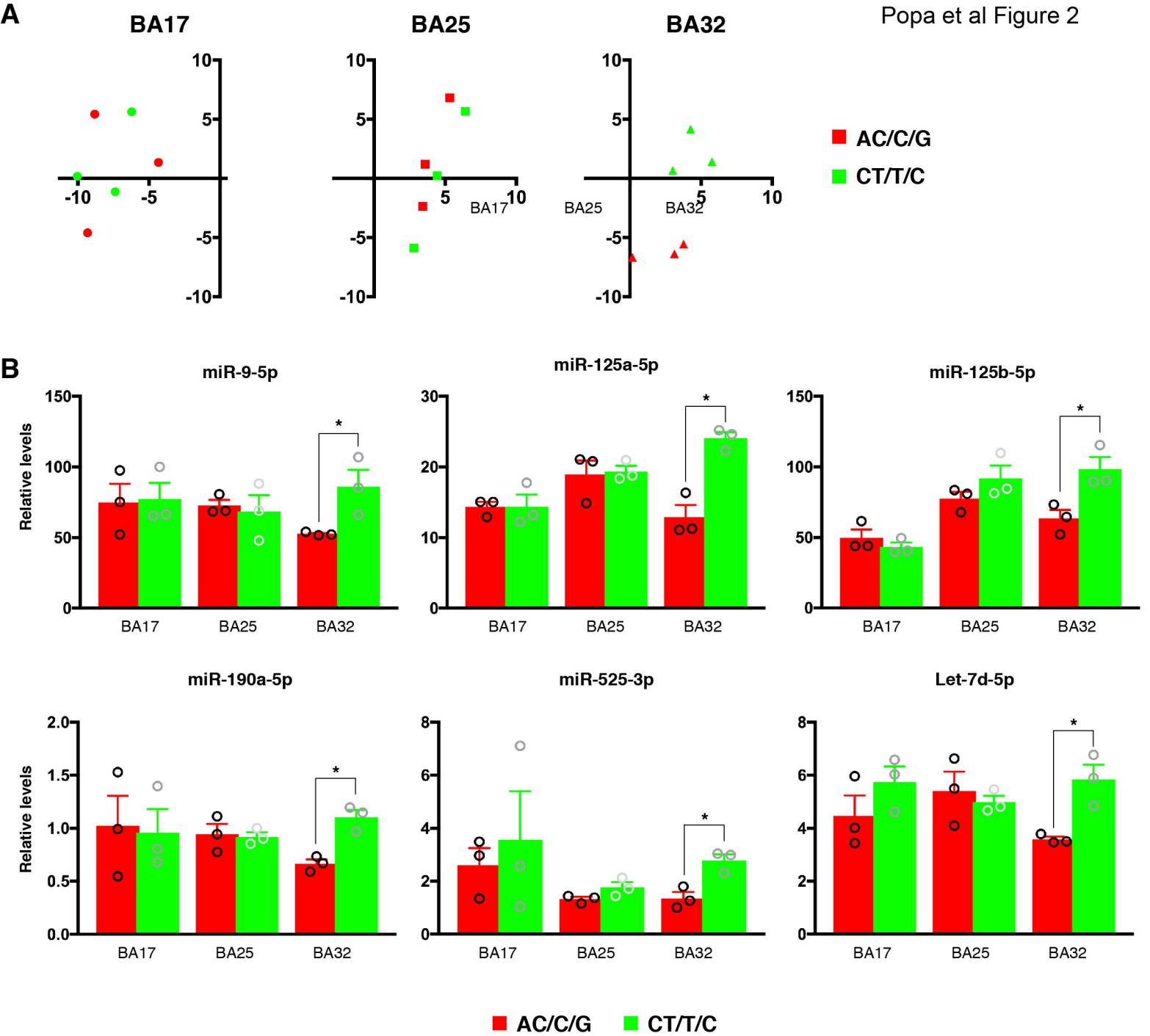

Figure 2. Slc6a4 polymorphisms (AC/C/G and CT/T/C) alter miRNA signature in area 32.
A) PCA analysis on miRNAs level in NeuN^{+} nuclei shows genotypic differences only in area 32.
B) Top miRNAs differentially expressed in area 32 in AC/C/G and CT/T/C marmosets (One way ANOVA fo llowed by Bonferroni's test for multiple comparisons, * $p<0.05$).

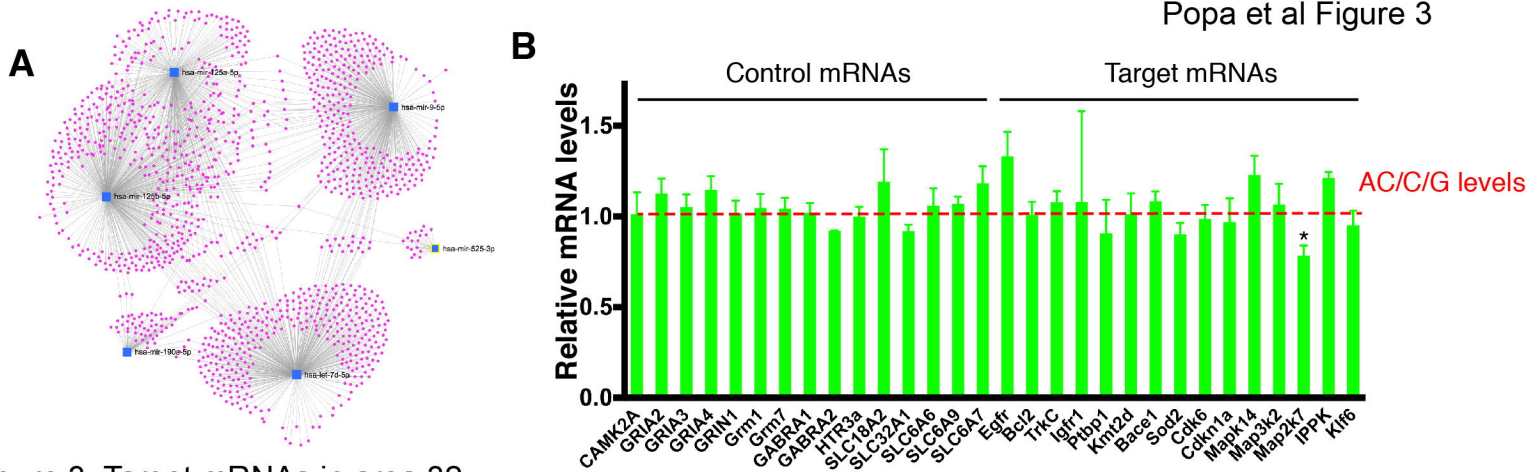

Figure 3. Target mRNAs in area 32.

A) Network analysis using miRNAs deregulated in area 32.

B) Expression of target mRNAs identified by network analysis in area 32. Using as reference the AC/C/G geno type (red dotted line), we measured the abundance of 15 potential targets as well as 15 reference genes. Only Map2k7 was found to be differentially expressed in area 32 (* $p<0.05$, t-test)

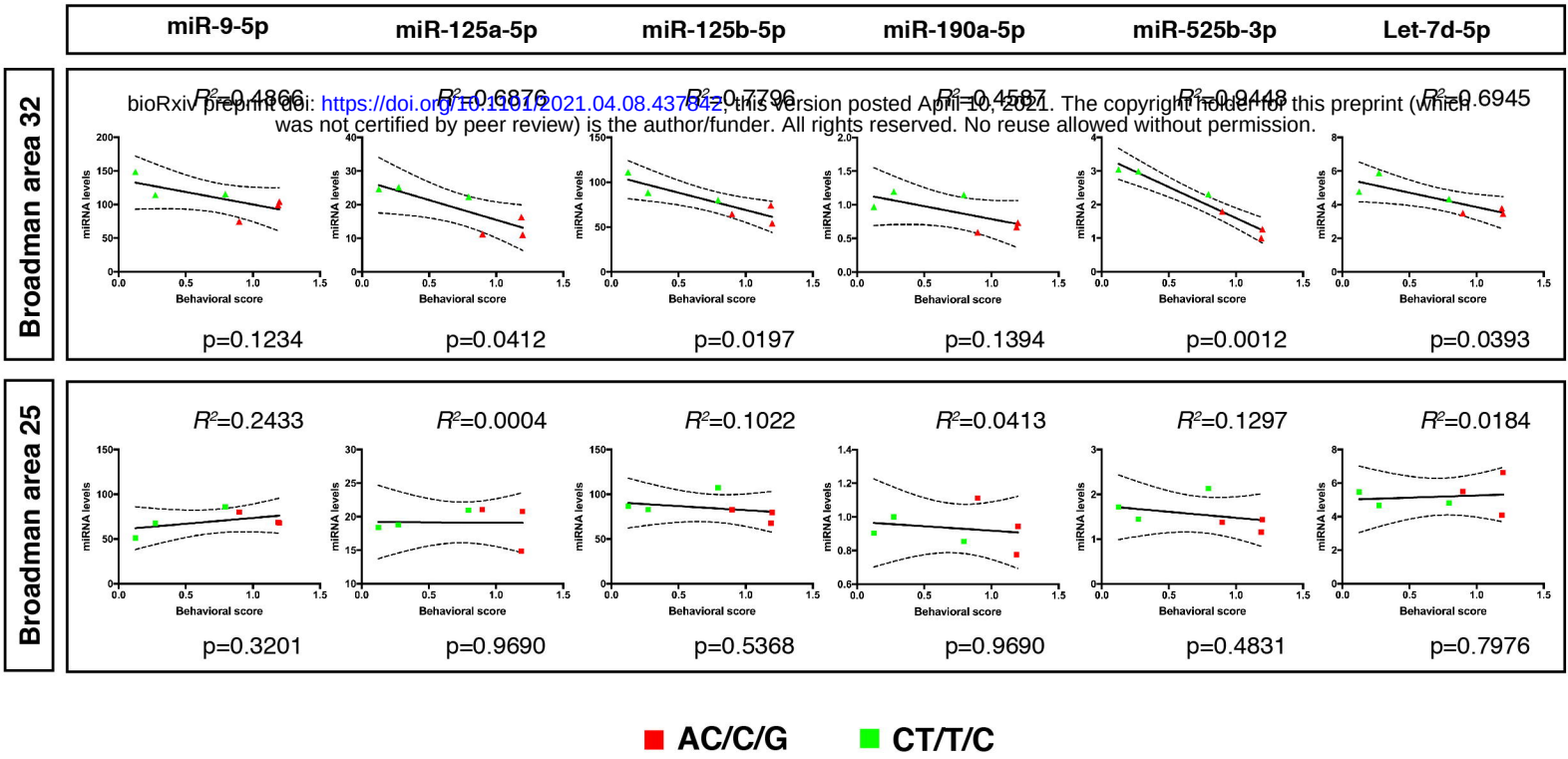

Figure 4. Correlation between miRNA levels in area 32 (upper panels) or 25 (lower panels) and behavioral response in the human intruder test.