Identification of a miRNAs signature associated with exposure to stress early in life and enhanced vulnerability for schizophrenia: New insights for the key role of miR-125b-1-3p in neurodevelopmental processes

Nadia Cattane [a], Cristina Mora [a], Nicola Lopizzo [a], Alessandra Borsini [b], Carlo Maj [c], Laura Pedrini [d], Roberta Rossi [d], Marco Andrea Riva [e], Carmine Maria Pariante [b], Annamaria Cattaneo [a,b,*]

[a] Biological Psychiatry Unit, IRCCS Fatebenefratelli San Giovanni di Dio, via Pilastroni 4, Brescia, Italy
[b] Stress, Psychiatry and Immunology Laboratory, Department of Psychological Medicine, Institute of Psychiatry, King's College London, 125 Coldharbour Lane, SE5 9NU London, UK
[c] Institute for Genomic Statistics and Bioinformatics, University Hospital, Bonn, Germany
[d] Psychiatry Unit, IRCCS Fatebenefratelli San Giovanni di Dio, via Pilastroni 4, Brescia, Italy
[e] Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy

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ABSTRACT

Epidemiological and clinical studies have provided evidence for a role of both genetic and environmental factors, such as stressful experiences early in life, in the pathogenesis of Schizophrenia (SZ) and microRNAs (miRNAs) have been suggested to play a key role in the interplay between the environment and our genome.

In this study, we conducted a miRNOme analysis in different samples (blood of adult subjects exposed to childhood trauma, brain (hippocampus) of rats exposed to prenatal stress and human hippocampal progenitor cells treated with cortisol) and we identified miR-125b-1-3p as a down-regulated miRNA in all the three datasets. Interestingly, a significant down-regulation was observed also in SZ patients exposed to childhood trauma. To investigate the biological systems targeted by miR-125b-1-3p and also involved in the effects of stress, we combined the list of biological pathways modulated by predicted and validated target genes of miR-125b-1-3p, with the biological systems significantly regulated by cortisol in the in vitro model. We found, as common pathways, the CXCR4 signaling, the G-alpha signaling, and the P2Y Purigenic Receptor Signaling Pathway, which are all involved in neurodevelopmental processes.

Our data, obtained from the combining of miRNAs datasets across different tissues and species, identified miR-125b-1-3p as a key marker associated with the long-term effects of stress early in life and also with the enhanced vulnerability of developing SZ. The identification of such a miRNA biomarker could allow the early detection of vulnerable subjects for SZ and could provide the basis for the development of preventive therapeutic strategies.

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1. Introduction

Schizophrenia (SZ) is a chronic, severe disabling neurodevelopmental disorder that affects about 1% of the general population (Jeffries et al., 2016). While the exact causes of SZ remain unknown, family, twin and adoption studies have provided strong evidence for a role of both genetic and environmental factors, in particular stressful and traumatic experiences early in life, in contributing to the complex etiology of the illness (Howes and McCutcheon, 2017; Narahari et al., 2017). This is also in line with clinical studies, indicating that adverse and traumatic experiences during childhood represent one of the most important clinical risk factor for the development of psychopathology, including SZ, later in life (Howes and McCutcheon, 2017). For example, in a prospective study, a large cohort of adolescents and adults who have been sexually abused before the age of 16 years old showed a 2-fold increased risk for a psychotic disorder and a 2.6-fold increased risk for SZ (Cutajar et al., 2010). Moreover, several meta-analyses have provided robust evidence for an association between childhood trauma and psychosis (Matheson et al., 2013; Varese et al., 2012). For example, Varese and collaborators showed that childhood trauma experiences increased the odds ratio for the development of a psychotic disorder by a factor of 3 (Varese et al., 2012) and the meta-analyses conducted by Matheson and colleagues indicated that childhood adversity had a medium to large effect with an odds ratio of 3.6 of increasing the risk for SZ (Matheson et al., 2013).

Several studies have proposed that gene-environment (GXE) interactions may underlie the association between exposures to childhood trauma and SZ. Indeed, environmental factors early in life can induce, through epigenetic mechanisms, changes in the levels of key genes that might increase the risk of developing psychotic disorders in adulthood (Stankiewicz et al., 2013). Importantly, a defective epigenome, characterized by altered miRNAs expression or epimutations in the form of aberrant DNA methylation and histone modifications have been reported in patients affected by SZ (Swathy and Banerjee, 2017).

Epigenetic mechanisms include histone modifications, DNA methylation and post-transcriptional regulation by non-coding RNAs such as microRNAs (miRNAs). MiRNAs are a subset of endogenous, small (about 22 nucleotides) non-coding RNA molecules that can repress gene expression post-transcriptionally (Issler and Chen, 2015; Rupaimoole and Slack, 2017). Collectively, miRNAs are predicted to regulate more than half of all protein-coding genes and to affect many cellular processes and functions both in health and disease conditions. Indeed, the miRNA binding, primarily to the 3′ UTR of mRNAs, leads to mRNA destabilization or translational repression, ultimately resulting in reduced protein levels of the miRNA-target genes (Bartel, 2004; Gulyaeva and Kushlinskiy, 2016). MiRNAs regulation of gene expression by targeting the sequences outside the 3′ UTR of target mRNAs has also been reported (Catalanotto et al., 2016).

Peripheral blood, due to its simple and non-invasive accessibility, is widely used for the searching of biomarkers and, among the different kinds of blood molecules, a recent meta-analysis has proposed that blood-derived miRNAs might be useful biomarkers in psychiatric disorders both for diagnosis as well as for treatment response prediction (Issler and Chen, 2015; Luoni and Riva, 2016), also in the context of SZ (Liu et al., 2017).

Indeed, multiple studies have identified specific miRNAs signatures associated with SZ in peripheral blood and some of these molecules have been also validated for their functional implication (Gardiner et al., 2012; Lai et al., 2011; Wang et al., 2014). In particular, Gardiner and collaborators investigated the expression profiles of miRNAs in peripheral blood mononuclear cells (PBMCs) of SZ patients and controls and they identified 83 miRNAs, which were significantly downregulated in SZ patients, including a large subgroup of miRNAs transcribed from a single imprinted locus at the maternally expressed DLK1-DIO3 region on chromosome 14q32 (Gardiner et al., 2012). Similarly, Lai and colleagues identified a signature of 7 miRNAs in a cohort of SZ patients and controls, which included the up-regulated miR-34a, miR-449a, miR-564, miR-548d, miR-572 and miR-652, and the downregulated miR-432. These results were subsequently validated in a second cohort of SZ patients and controls and the expression levels of a number of these miRNAs correlated with negative symptoms, neurocognitive dysfunction and mismatched negativity performances of SZ patients (Lai et al., 2011). More recently, Jeffries and collaborators compared baseline miRNAs profiles in subjects at high risk for psychosis (SZ or a related disorder) with those who were not at risk, by performing small RNA sequencing on RNA isolated from circulating immune cells. While no single miRNA exhibits a statistically significant predictive power, the authors found that a sum of five abundantly expressed miRNAs (miR-941, miR-103a-3p, miR-199a-3p, miR-92a-3p, and miR-31-5p) produced a risk classifier that survived randomization testing (Jeffries et al., 2016).

However, all the above-mentioned studies have mainly investigated the alterations in miRNAs expression that could represent peripheral signatures associated with the pathology or with specific clinical features, but none of these studies have focused the attention on miRNAs signatures associated with stress early in life that could in turn enhance the vulnerability of developing SZ later on.

Therefore, the aim of the present study was to identify, by using a hypothesis-free approach, a specific miRNAs signature associated with stress exposure early in life, which could also enhance the vulnerability of developing SZ. In order to reach this aim, we merged the miRNOme profiles obtained in blood samples of adult control subjects exposed to childhood trauma with those obtained from the brain (hippocampus) of adult rats exposed to prenatal stress (PNS) and from human hippocampal progenitor stem cells treated with cortisol, in order to identify common miRNAs associated with stress exposure. Subsequently, to evaluate whether this miRNA could also enhance the vulnerability of developing stress-related disorders, such as SZ, we analyzed this stress-related miRNAs signature in a clinical sample of controls and SZ patients, where a small subgroup was characterized for traumatic experiences during childhood.

The identification of such a miRNAs signature could allow to better understand the mechanisms targeted by exposure to stress early in life that, in turn, could underlie the enhanced risk of developing SZ, and could allow an early identification of vulnerable subjects, providing the basis for the development of preventive therapeutic strategies.

2. Materials and methods

2.1. Human samples

2.1.1. Clinical sample of adult control subjects characterized for childhood trauma (cohort 1)

MiRNAs expression analyses were performed in peripheral blood obtained from a clinical sample of adult control subjects (n = 52 samples), who were characterized for childhood trauma by the administration of the CECA-Q scale (Bifulco et al., 2005). MiRNOme analyses have been performed in a subsample of 32 subjects (11/32 subjects reported a history of traumatic experiences in childhood) and the validation analyses in the entire datasets (n = 52 subjects; 21/52 subjects reported a history of childhood trauma). Individuals presenting a history of neurological or psychiatric diseases, prior traumatic brain injury, or mental retardation (IQ < 70) were excluded from the study. The absence of psychiatric disorders was ascertained via the Mini International Neuropsychiatric Interview (M.I.N.I. Plus) (Sheehan et al., 1998) and the Structured Clinical Interview for DSM disorders (SCID-II) (Spitzer et al., 1992), to exclude any psychiatric disorders in Axis I and II.

These control subjects were recruited within several projects running in our Institute, which have been approved by the local Ethic Committee and written informed consent was obtained by participants after receiving a complete description of the study.

Subjects exposed to childhood trauma versus subjects non-exposed were not different in age (mean age ± SEM: 48.5 ± 3.1 years and 50.1 ± 2.5 years, respectively) and gender (12 males and 9 females and 18 males and 13 females, respectively, p-value >0.05).

2.1.2. Clinical sample of controls and patients with schizophrenia (cohort 2)

The expression analyses of miR-125b-1-3p have been performed in a group of 17 controls (9 males and 8 females; mean age ± SEM: 46.5 ± 2.3 years) and 32 patients (18 males and 14 females; mean age ± SEM: 48.1 ± 1.1 years) with a diagnosis of SZ, where a small subgroup (n = 6) reported a history of childhood trauma and was available and recruited in our Psychiatric Institute as a part of several projects.

Control Group: adult subjects mentally and physically healthy with no neurological or psychiatric disorder history in their first-degree relatives, with no psychiatric disorders in Axis I and II.

Patient Group: In-Patients with a diagnosis of SZ, which was determined using the Structured Clinical Interview for DSM-IV (Association, 2000), were in treatment with atypical antipsychotics for more than three years and none of the patients was in the acute phase of illness. Information regarding gender and age was recorded for all the patients and controls and the two groups were not different for age (mean age ± SEM: 48.1 ± 1.1 years and 46.5 ± 2.3 years, respectively) and gender (18 males and 14 females and 9 males and 8 females respectively, p > 0.05).

Within the patients, only in a small subgroup (n = 12) we have collected information on childhood trauma history by using the CECA-Q

scale (Bifulco et al., 2005), and this group was not different in term of severity of illness as compared to the other patients (Personal and Social Functioning Scale (FPS) ± SEM: 49.2 ± 13.61 in patients with childhood trauma history and 46.5 ± 11.25 in patients with no childhood trauma history or information). Patients with organic psychosis and/or learning disabilities were excluded from the study.

The study was approved by the local Ethics Committee and written informed consent was obtained from all participants. All the samples were stored at the IRCCS Fatebenefratelli Institute in Brescia (Italy).

2.1.3. Blood samples collection

Peripheral venous blood samples were collected from all the participants, both patients and controls, in PAXGene tubes in the morning, after an overnight fast. The tubes were kept at room temperature for 2 h, then frozen at −20 °C for 24 h and finally moved to a −80 °C freezer until their processing for mRNA and miRNAs extraction, according to the manufacturer's instructions.

2.2. Cell culture

The immortalized multipotent, human hippocampal progenitor cell line, HPC0A07/03C (propriety of ReNeuron), was used for the experiments. Because these cells are grown in a tightly-controlled experimental environment, this model allows to overcome the unavoidable variability of clinical samples and to reproduce data from human brain cells, inaccessible in patients. As we have previously described (Anacker et al., 2013a; Anacker et al., 2013b), HPC0A07/03C cells can proliferate indefinitely in the presence of epidermal growth factor (EGF), fibroblast growth factor (bFGF) and 4-hydroxytamoxifen (4-OHT), whereas differentiation is induced by the removal of these molecules.

For this experiment, HPC0A07/03C cells were treated with the stress hormone cortisol (100 μM) or vehicle for 3 days during the proliferation stage (to mimic the early stress exposure). Five biological replicates were performed for each condition (cortisol or vehicle).

2.3. Prenatal stress model

Prenatal Stress (PNS) procedure was performed as already published (Cattaneo et al., 2018; Luoni et al., 2016). Briefly, pregnant dams, during the last week of gestation, were restrained in a transparent Plexiglas cylinder, under bright light, for 45 min, three times a day for 1 week. Control pregnant females were left undisturbed in their home cages. Male offspring from control and PNS groups was sacrificed at postnatal day (PND) 62 (early adulthood) for whole dissection of the hippocampus. Rat handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (D.L. 116/92), in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. RNA samples from the hippocampi of animals exposed or not to PNS ($n = 10$ per group) were used for the microarray assays and for the expression analyses of miR-125b-1-3p.

2.4. RNA isolation and microarray analyses

Total RNA, including miRNAs, was isolated according to the manufacturer's protocols from human peripheral blood using the PaxGene Blood miRNA Kit (Qiagen, Hilden, Germany), from HPC0A07/03C cells using the AllPrep DNA/RNA/miRNA kit (Qiagen, Hilden, Germany) and from rat brains using PureZol RNA isolation reagents (Bio-Rad Laboratories, Hercules, CA, USA). RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.4.1. MicroRNA microarray analyses

A total amount of 250–500 ng of total RNA from each sample was processed with the FlashTag Biotin HSR RNA Labeling kit (Thermofisher, Waltham, MA, USA) and subsequently hybridized onto the GeneChip miRNA 4.1 Arrays (Thermofisher, Waltham, MA, USA), which cover 30,424 total mature miRNA probe sets, including 2578 human mature miRNAs annotated in miRBase version 20 (online miRNA database, http://www.mirbase. org). Washing/staining and scanning procedures were respectively conducted on the Fluidics Station 450 and the GeneChip Scanner 3000 7G of a GeneAtlas instrument (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions.

2.4.2. Whole transcriptome analyses

Transcriptome analyses were performed in HPC0A07/03C cells using the HuGene 2.1st Array strips, which cover 31,650 coding transcripts. Briefly, 250 ng of total RNA were used to synthesize second strand cDNA with the GeneChip® WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA, USA). Subsequently, purified cDNA was fragmented and 5.5 μg of fragmented cDNA were labeled and hybridized onto the HuGene2.1 Array strips. The reactions of hybridization, fluidics and imaging were performed on the Affymetrix Gene Atlas instrument, according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA).

2.5. Real-time PCR validation analyses

The expression levels of miR-125b-1-3p were analyzed in human peripheral blood (clinical cohorts), in the hippocampi of animals (adult male rats exposed or not to PNS), as well as in HPC0A07/03C cells (treated or not with cortisol 100 μM) by Real Time PCR using the CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA) and the TaqMan MicroRNA Assays (Thermofisher, Waltham, MA, USA), following the manufacturer's instructions. The relative expression of miR-125b-1-3p was normalized to the levels of three housekeeping genes RNU44, RNU48 and RNU49 in humans, and to the levels of U6 and U87 in rats. All the reactions were performed in triplicates.

The expression levels of the candidate genes Signal Transducer And Activator Of Transcription 1 (STAT1), Forkhead Box O1 (FoxO1), Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1), and of the housekeeping genes Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Actin Beta (ACTB) were evaluated by using TaqMan Assays (Thermofisher, Waltham, MA, USA) on the CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions.

The Pfaffl Method was used to determine the relative expression values of miR-125b-1-3p and of genes of interest (Pfaffl, 2001).

2.6. Statistical and bioinformatics analyses

2.6.1. Microarray data

Raw microarray data (.CEL files) were imported and the data analyses of the miRNA arrays (quality controls and statistical analyses) were performed using Partek Genomics Suite 6.6 software (Partek, St. Louis, MO, USA). Principal-component analysis (PCA) was carried out to identify possible outliers and major effects in the data. All the Affymetrix GeneChip miRNA arrays provide comprehensive miRNAs coverage for multiple organisms including human, mouse, rat, canine and monkey on a single chip. Thus, only miRNAs from humans were filtered from the dataset and, subsequently, statistical analysis for the evaluation of differences in miRNAs levels was performed by Analysis of Variance Test (ANOVA).

2.6.2. Gene-targeting prediction and validation analyses

The gene-targeting analysis for predicted and validated genes of miR-125b-1-3p was performed by using miRWalk2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) (Dweep and Gretz, 2015), a comprehensive database that provides predicted as well as

validated miRNA binding site information. To make any possible infer-
ence and to obtain a comparative view, miRWalk automatically com-
bines and integrates the identified miRNA binding sites with the
results obtained from other established miRNA-target prediction data-
bases (miRWalk, miRanda and Targetscan).

We integrated target gene lists with genome-wide association study
(GWAS) SZ significant loci to investigate potential correlations with SZ
genetic susceptibility. We also considered tissue specific cis-eQTL
models (http://gusevlab.org/projects/fusion/) to identify possible
genes with a different genetic regulation of gene expression that could
be in line with the gene expression modulation potentially exerted by
miR-125b-1-3p. Moreover, we searched for miRNAs expression
datasets within Gene Expression Omnibus repository (GEO, https://
www.cbi.nlm.nih.gov/geo/) to evaluate potential alterations of miR-
125b-1-3p expression in SZ.

2.6.3. Pathway and network analyses

The identification of potentially altered molecular pathways as
targeted by the differentially modulated miRNAs in the three different
datasets was performed with DIANA-miRPath (v.3) tool web-server
(Vlachos et al., 2012). The "Core Analysis" function included in IPA (In-
genuity System Inc., USA http://www.ingenuity.com) was used to un-
derstand the data in the context of biological processes, pathways and
networks associated with the experimental system. Kyoto Encyclopedia
of Genes and Genomes (KEGG) pathway database (https://www.
genome.jp/kegg/pathway.html) was used to investigate the molecular
interaction, reaction and relation networks for several biological sys-
tems. STRING v10.5 database (https://string-db.org/) (Szklarczyk et al.,
2017) was used to evaluate physical and/or functional interactions be-
tween proteins encoded by genes identified by gene expression analysis
and/or whose transcripts were targets of miR-125b-1-3p.

2.6.4. Real time PCR analyses

Normal distribution of data was evaluated through the Shapiro-Wilk
test. One-way ANOVA was used to analyze the differences between
groups, and the chi-squared test was used to evaluate categorical vari-
ables. Data are expressed as mean $\pm$ SEM and were performed using
the Statistical Package for the Social Sciences (SPSS) software, Version
24.0.

3. Results

3.1. MiRNAs signature associated with childhood trauma

Using a miRNome approach, we investigated all the expressed
miRNAs in blood samples of adult subjects characterized for childhood
trauma events ($n = 32$ subjects, 11/32 reported a history of childhood
trauma). By applying a cut-off of Fold Change (FC) $\pm$ 1.2 and p-value
<0.05, we identified a list of 80 mature miRNAs differentially expressed
in subjects exposed to childhood trauma versus those who had not been
exposed. Among them, 57 miRNAs were up-regulated, whereas 23 were
down-regulated (the entire list of the 80 significant miRNAs is provided
in Supplementary Table 1).

We then performed a pathway analysis using Diana Tool software
(miRPath v.3) on the list of 80 differentially modulated miRNAs, to inves-
tigate possible biological systems altered in association with childhood
trauma and targeted by the significantly modulated miRNAs. We identi-
fied 53 statistically significant biological pathways (p-value < 0.05) in
association with childhood trauma exposure and among the most signif-
icant ones, we found pathways involved in: i) *neurodevelopment* (i.e. FoxO
signaling pathway, p-value = 3.24 E-04; GABAergic synapse, p-value =
3.31 E-04; Hippo signaling pathway, p-value = 7.51 E-04; Cholinergic
synapse, p-value = 1.06 E-03; Axon guidance, p-value = 3.46 E-03;
mTOR signaling pathway, p-value = 6.28 E-03; Glutamatergic synapse,
p-value = 1.03 E-02); ii) *inflammation* (i.e. TGF-beta signaling pathway,
p-value = 1.06 E-03; T cell receptor signaling pathway, p-value = 7.53

E-03) and iii) *intracellular transduction signaling* (i.e. PI3K-Akt signaling
pathway, p-value = 1.07 E-04; Ras signaling pathway, p-value = 2.55
E-04; Rap1 signaling pathway, p-value = 1.62 E-02; cAMP signaling path-
way, p-value = 3.14 E-02; MAPK signaling pathway, p-value = 3.16 E-
02). The complete list of the 53 pathways significantly modulated in asso-
ciation with childhood trauma exposure is reported in Supplementary
Table 2.

3.2. MiRNAs signature in an animal model of prenatal stress (PNS)

In order to investigate a miRNAs signature associated with the ef-
fects of early life stressful events also on brain functions, we performed
a miRNome analysis in the hippocampus of adult rats (PND 62) that had
been exposed or not to PNS. By using a cut-off of FC $\pm$ 1.2 and p-value <
0.05, we identified 57 differentially expressed mature miRNAs, 1 up-
regulated and 56 down-regulated (See Supplementary Table 3). Follow-
ing the same experimental approach, we conducted a pathway analysis
using Diana Tool software (miRPath v.3), as we wanted to investigate
the biological systems long-lasting modulated by PNS.

Interestingly, we identified a list of 39 statistically significant path-
ways (see Supplementary Table 4) mainly involved: i) in *inflammatory/
immune system response*, including TGF-beta signaling pathway (p-
value = 3.49 E-05), T cell receptor signaling pathway (p-value = 1.72
E-02), TNF signaling pathway (p-value = 1.72 E-02); ii) in
neurodevelopment, such as Hippo signaling pathway (p-value = 1.25 E-
03), Synaptic vesicle cycle (p-value = 3.59 E-03), Wnt signaling pathway
(p-value = 7.21 E-03), FoxO signaling pathway (p-value = 7.32 E-03),
mTOR signaling pathway (p-value = 1.99 E-02) and Neurotrophin sig-
naling pathway (p-value = 2.12 E-02), and iii) in *intracellular signal
transduction* (i.e. MAPK signaling pathway (p-value = 3.33 E-04),
cGMP-PKG signaling pathway (p-value = 1.72 E-02), Rap1 signaling
pathway (p-value = 1.72 E-02) and Ras signaling pathway (p-value =
2.65 E-02).

3.3. MiRNAs signature associated with stress exposure in the "in vitro" model

We used our in vitro model, represented by HPC0A07/03C cells
treated with vehicle or cortisol (100 μM) to mimic the stress condition,
to better dissect the pool of miRNAs and their related processes that
were targeted by stress. By using the same cut-off (FC $\pm$ 1.2 and p-
value < 0.05) used in the other models, we identified 208 mature
miRNAs (137 miRNAs were up-regulated, whereas 71 were down-
regulated), which were differentially expressed in cells treated with
cortisol as compared to vehicle (see Supplementary Table 5 for the en-
tire list of 208 modulated miRNAs).

For pathway analyses, as the number of input genes can affect en-
richment results (Huang da et al., 2009), we applied a more stringent
cut-off (FC $\pm$ 1.5 and p-value < 0.05) to obtain a comparable input size
for pathway enrichment analysis among the three datasets. We obtained
a list of 69 mature miRNAs (59 up-regulated and 10 down-regulated, see
Supplementary Table 6) and the pathway analysis identified 47 statisti-
cally significant biological processes (p-value < 0.05) that we listed in
Supplementary Table 7. Among the most significant pathways, we
found several biological processes involved in: i) *neurodevelopment*,
such as Hippo signaling pathway (p-value = 9.36 E-06), FoxO signaling
pathway (p-value = 9.89 E-06), Axon guidance (p-value = 6.04 E-05),
mTOR signaling pathway (p-value = 3.40 E-03), Glutamatergic synapse
(p-value = 2.20 E-02), Wnt signaling pathway (p-value = 3.88 E-02); ii)
biological processes involved in *inflammation*, such as TGF-beta signaling
pathway (p-value = 8.74 E-07) and Inflammatory mediator regulation
of TRP channels (p-value = 2.39 E-02); and iii) biological systems in-
volved in the *intracellular signal transduction*, including Ras signaling
pathway (p-value = 9.89 E-06), AMPK signaling pathway (p-value =
1.79 E-03), PI3K-Akt signaling pathway (p-value = 5.77 E-03), MAPK

signaling pathway (*p*-value = 1.02 E-02) and Rap1 signaling pathway (*p*-value = 4.82 E-02).

3.4. Common miRNAs signature associated with the effect of early life stress exposure

As we were interested in identifying a miRNAs signature associated with stress exposure across different models and tissues, we intersected the list of miRNAs significantly modulated in the three datasets (peripheral blood of controls characterized for childhood trauma, hippocampus of rats exposed or not to PNS and HPC0A07/03C cells treated with cortisol or vehicle) by using the Venn Diagram (Venny 2.1.0 - BioinfoGP – CSIC http://bioinfogp.cnb.csic.es/tools/venny/index.html) (see Fig. 1).

Interestingly, we identified one miRNA, miR-125b-1-3p, as the only common miRNA modulated in the same direction (down-regulation) in all the three datasets (FC = −1.27, *p*-value = 0.013 in controls exposed to childhood trauma versus non-exposed subjects; FC = −1.39, *p*-value = 0.027 in the hippocampus of PNS rats versus non-exposed animals; FC = −1.57, *p*-value = 0.013 in HPC0A07/03C cells treated with cortisol versus vehicle).

We also validated the expression levels of miR-125b-1-3p in the three datasets by looking specifically at its expression levels and our results confirmed the miRNA microarray data, suggesting miR-125b-1-3p as a possible biomarker associated with stress exposure. In particular, we observed a down-regulation of miR-125b-1-3p levels of about 30–40% in all the cohorts, with an overall correlation with the microarray FC data of the 93% (FC = −1.32, *p*-value = 0.041 in blood samples of subjects exposed to childhood trauma as compared to non-exposed individuals; FC = −1.45, *p*-value = 0.011 in PNS rats as compared to their matched controls; FC = −1.52, *p*-value = 0.008 in HPC0A07/03C cells treated with cortisol as compared to vehicle).
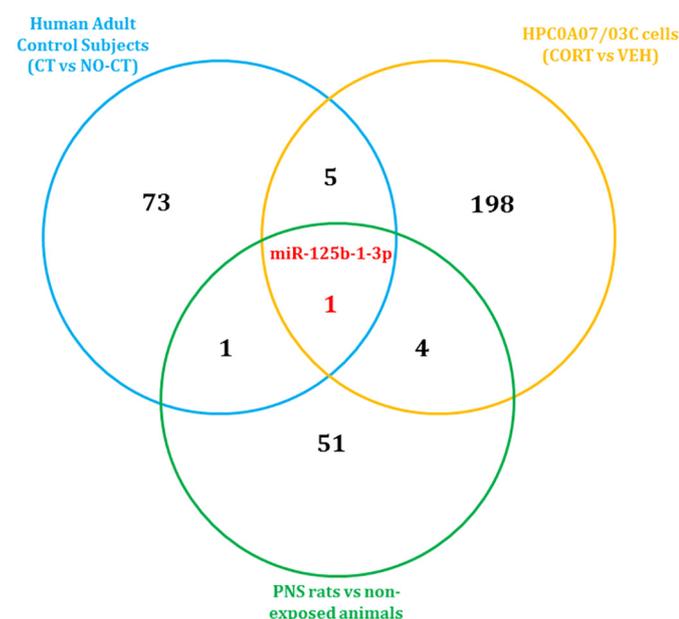

Fig. 1. The Venn diagrams represents the overlap between differentially expressed miRNAs obtained in three different datasets: i) peripheral blood of controls characterized for childhood trauma vs controls non-exposed to traumatic events; ii) HPC0A07/03C cells treated with cortisol vs vehicle; iii) hippocampus of PNS rats vs non-exposed rats. The intersection, showing 1 overlapping miRNA (miR-125b-1-3p), represents the miRNAs signature associated with the vulnerability to stress.

3.5. MiR-125b-1-3p downregulation is associated with an increased vulnerability of developing schizophrenia in association with a history of childhood trauma

With the aim to evaluate whether miR-125b-1-3p, which resulted to be involved in the long-lasting effects of stress, could enhance the risk of developing SZ, we analyzed its expression levels in blood samples from controls and SZ patients (where a small subgroup was characterized for childhood trauma experiences). Although we identified a down-regulation of this miRNA levels in the group of SZ patients (where no information on childhood trauma was reported), this did not reach the significance (FC = −1.19, *p*-value = 0.659). However, a significant down-regulation of the miR-125b-1-3p was observed in the blood samples of SZ patients who reported a childhood trauma history (FC = −4.76, F = 5.005, *p*-value = 0.029), supporting the hypothesis that this miRNA could enhance the risk of SZ, especially in those subjects exposed to childhood trauma.

3.6. Biological systems regulated by miR-125b-1-3p predicted and validated genes

Since miR-125b-1-3p resulted to be a key miRNA involved in the long-term effect of stress and was also associated with enhanced vulnerability for SZ, we decided to investigate the biological processes and signaling potentially targeted by this miRNA.

First, we ran a gene-targeting analysis using miRWalk2.0 database to identify predicted or validated target genes of miR-125b-1-3p. We obtained a list of 196 predicted genes (see Supplementary Table 8) and a list of 12 validated genes, which included NR3C1, Zinc Finger Protein 682 (ZNF682), WD Repeat Domain 12 (WDR12), Cerebral Dopamine Neurotrophic Factor (CDNF), Bone Morphogenetic Protein Receptor Type 2 (BMPR2), Cytochrome C Oxidase Assembly Factor (COXO20), Sphingosine-1-Phosphate Receptor 1 (S1PR1), Tumor Associated Calcium Signal Transducer 2 (TACSTD2), ADP-Ribosyltransferase 4 (ART4), Prion Protein (PRNP), Superoxide Dismutase 2 (SOD2), and Insulin Like Growth Factor 1 Receptor (IGF1R).

We then ran a pathway analysis gathering both predicted and validated target genes (208 genes), and we identified 38 statistically significant pathways potentially targeted by the miR-125b-1-3p (see Table 1). Among them, we found several pathways involved in the *inflammatory and immune response* (i.e. CCR3 Signaling in Eosinophils, CCR5 Signaling in Macrophages, CXCR4 Signaling, Role of NFAT in Regulation of the Immune Response, IL-8 Signaling, TGF-β Signaling), and in *neurodevelopment* (i.e. Sphingomyelin metabolism, Gαq and Gαi signaling, CXCR4 signaling, P2Y Purigenic Receptor Signaling Pathway).

We also looked at the mRNA levels of a few genes targeted by miR-125b-1-3p and/or involved in these pathways, including NR3C1, FoxO1, STAT1, GNG4, GNG7, PRKCA, PRKCQ and DIRAS3 in response to cortisol treatment in our in vitro model. We found a significant decrease in the NR3C1 levels, and a significant up-regulation in the expression levels of FoxO1, whereas we observed only a trend of increase in the mRNA levels of GNG4, GNG7, PRKCA, PRKCQ and DIRAS3 (data not shown).

With the aim to prove the real involvement of some of these biological processes in the effects of stress via the modulation of miR-125b-1-3p, we ran a whole transcriptome study followed by a pathway analysis in the in vitro model represented by HPC0A07/03C cells treated with cortisol (100 μM) or vehicle.

Cortisol induced the modulation of 1145 differentially expressed genes (FC ± 1.2; *p*-value < 0.05), which are involved in 60 biological pathways (see Supplementary Table 9). Among the most significant ones, we found several pathways related to i) *inflammation*, such as the IL-7, IL-1 and IL-3 signaling, the chemokine signaling and the CXCR4 signaling, and ii) *neurodevelopment*, such as the WNT/β catenin signaling, the CXCR4 signaling, the Axonal Guidance signaling, the sphingosine-1-phosphate signaling, the Gαi signaling and the P2Y Purigenic Receptor Signaling Pathway.

Table 1
38 statistically significant biological pathways (*p*-value <0.05) modulated by both predicted and validated target genes of miR-125b-1-3p.

	Ingenuity canonical pathways	-log (P-value)	P-value	Ratio
1	Phospholipase C signaling	3.52	0.00030	3.72E-02
2	Superoxide radicals degradation	2.68	0.00209	2.5E-01
3	Sphingomyelin metabolism	2.68	0.00209	2.5E-01
4	Role of NFAT in cardiac hypertrophy	2.48	0.00331	3.2E-02
5	fMLP signaling in neutrophils	2.33	0.00468	4.07E-02
6	CCR3 signaling in eosinophils	2.23	0.00589	3.85E-02
7	Prolactin signaling	2.21	0.00617	4.82E-02
8	UVC-induced MAPK signaling	2.20	0.00631	6.98E-02
9	α-Adrenergic signaling	2.14	0.00724	4.6E-02
10	Apoptosis signaling	2.08	0.00832	4.44E-02
11	Factors promoting cardiogenesis in vertebrates	2.05	0.00891	4.35E-02
12	Thrombin signaling	2.02	0.00955	2.94E-02
13	CCR5 signaling in macrophages	2.00	0.01000	4.21E-02
14	Cholecystokinin/gastrin-mediated signaling	1.91	0.01230	3.96E-02
15	Cardiomyocyte differentiation via BMP receptors	1.88	0.01318	1.00E-01
16	Gαq signaling	1.85	0.01413	3.11E-02
17	Glioblastoma multiforme signaling	1.84	0.01445	3.09E-02
18	CXCR4 signaling	1.81	0.01549	3.03E-02
19	GPCR-mediated nutrient sensing in enteroendocrine cells	1.76	0.01738	3.57E-02
20	Tec kinase signaling	1.76	0.01738	2.94E-02
21	G beta gamma signaling	1.74	0.01820	3.51E-02
22	RhoGDI signaling	1.69	0.02042	2.82E-02
23	Gαi signaling	1.66	0.02188	3.33E-02
24	Huntington's disease signaling	1.63	0.02344	2.4E-02
25	Role of NFAT in regulation of the immune response	1.61	0.02455	2.69E-02
26	Protein kinase A signaling	1.59	0.02570	2.00E-02
27	Production of nitric oxide and reactive oxygen species in macrophages	1.54	0.02884	2.58E-02
28	IL-8 signaling	1.52	0.03020	2.54E-02
29	P2Y purigenic receptor signaling pathway	1.51	0.03090	2.99E-02
30	G protein signaling mediated by tubby	1.49	0.03236	6.25E-02
31	Androgen signaling	1.48	0.03311	2.92E-02
32	Macropinocytosis signaling	1.46	0.03467	3.7E-02
33	Breast cancer regulation by stathmin1	1.45	0.03548	2.44E-02
34	Cardiac β-adrenergic signaling	1.44	0.03631	2.84E-02
35	Growth hormone signaling	1.41	0.03890	3.53E-02
36	NF-κB activation by viruses	1.38	0.04169	3.45E-02
37	TGF-β signaling	1.38	0.04169	3.45E-02
38	Mechanisms of viral exit from host cells	1.3	0.05012	4.88E-02

Interestingly, seven biological pathways, which were significantly modulated by cortisol in our in vitro model, were also potentially targeted by miR-125b-1-3p. These systems included: i) Androgen Signaling, ii) CXCR4 Signaling, iii) Factors Promoting Cardiogenesis in Vertebrates, iv) Gαi Signaling, v) Huntington's Disease Signaling, vi) P2Y Purigenic Receptor Signaling Pathway, and vii) Role of NFAT in Cardiac Hypertrophy (see Table 2). Among these seven common pathways, the CXCR4 signaling, the Gαi Signaling and the P2Y Purigenic Receptor Signaling Pathway (see Supplementary Figs. 1, 2, 3), have been described to be involved in neurodevelopment and in the inflammatory/immune response, also in the context of SZ (Borgmann-Winter et al., 2016; Krugel, 2016; Meechan et al., 2012; Toritsuka et al., 2013; Urs et al., 2017).

We also noticed that these three pathways had in common several genes, represented by guanine nucleotide-binding regulatory proteins (G-proteins) subunits, namely GNG4, GNG7, GNG3, GNAI3, where GNG4 and GNG7 are predicted target genes of the miR-125b-1-3p (See Supplementary Figs. 1, 2, 3). Moreover, by investigating the molecular interaction and relation networks of these biological systems (https://www.genome.jp/kegg/pathway.html), it appeared that the activation of the intracellular transduction signaling required the activation of these G-proteins, suggesting GNG4, GNG7, GNG3 and GNAI3 as "hub-genes" within these pathways, able to drive the modulation of the signaling.

We then specifically looked, within the transcriptome profile coming from cell treated with cortisol or vehicle, at those genes significantly modulated by cortisol and belonging to the CXCR4 signaling (PIK3C2B, CXCR4, PIK3R1, MAPK8, GNG3, GNB4, GNAI3, JUN, RND3, GNAO1, PLCB1, PLCB3, ARHGEF11, ELMO1, PRKD3) and to the P2Y Purigenic Receptor Signaling Pathway (GNAI3, PIK3C2B, GNB4, JUN, PIK3R1, PRKAR2A, PRKACA, PLCB3, PLCB1, GNG3, PRKD3). Although we expected to observe an up-regulation of these genes as they are also potentially targeted by miR-125b-1-3p, which was down-regulated by stress, from the transcriptome and IPA pathway analyses we observed that most of them were down-regulated by cortisol. However, it is important to note that these cortisol-down-regulated genes within these pathways are not target genes of the miR-125b-1-3p. Conversely, another set of genes which are targeted by miR-125b-1-3p and belong to the same pathways (namely GNG4, PRKCQ, DIRAS3, GNG7 and PRKCA within the CXCR4 signaling; GNG4, PRKCQ, GNG7 and PRKCA within the P2Y Purigenic Receptor Signaling Pathway) showed an activated z score and an overall increase of their gene expression, although with a smaller effect (FC = 1.1, *p*-value = 0.273 for GNG4; FC = 1.1, p-value = 0.858 for PRKCQ; FC = 1.1, p-value = 0.442 for GNG7; FC = 1.2, p-value = 0.007 for PRKCA; FC = 1.1, p-value = 0.217 for DIRAS3).

In addition, we used the functional protein association networks (https://string-db.org/) to better investigate the interactions between sets of genes targeted by miR-125b-1-3p, which were up-regulated, and genes modulated by cortisol which were down-regulated (see Figs. 2, 3, 4). As we can see in Figs. 2 and 4, PIK3C2B (which is down-regulated by cortisol) interacts with PRKCQ (a predicted target gene of miR-125b-1-3p which is slightly up-regulated) exerting an inhibitory effect. In our context, the down-regulation of PIK3C2B (FC = −1.40, *p*-value = 0.018) could lead to a lack of inhibition and thus to an activation of PRKCQ and its downstream signaling.

3.7. Bioinformatics analyses of miR-125b-1-3p

In order to evaluate a possible involvement of miR-125b-1-3p in SZ, we investigated whether the potential regulation of gene expression exerted by miR-125b-1-3p could be correlated with SZ genetic susceptibility by considering genome wide association data from the Psychiatric Genomic Consortium (PGC, http://www.med.unc.edu/pgc). Moreover, we investigated whether a down-regulation of miR-125b-1-3p could be in line with SZ expression alterations by evaluating cis-eQTL models of gene expression (http://gusevlab.org/projects/fusion/). We also queried Gene Expression Omnibus (GEO, https://www.cbi.nlm.nih.gov/geo/) for miRNAs analysis in SZ to check for potential alterations of miR-125b-1-3p expression in association with SZ.

By assessing PGC genetic data for SZ association, we found that the locus of miR-125b-1-3p does not lie within one of the 108 significant genome-wide significant loci (Schizophrenia Working Group of the Psychiatric Genomics, 2014); however, it is relatively close (~1.4 Mb and ~2.6 Mb) to two of the significant loci within chromosome 11 at a distance compatible with Topological Associating Domain (TAD) size. TADs are regions of the genome with enriched genomic loci interactions (e.g., enhancers) which span from several hundred Kb to a few Mb (Dixon et al., 2012) that can have correlations phenotype associations (Yan et al., 2017). Noteworthy, one of these two SZ related loci contains the neurogranin gene (NRGN) that has been found to be associated with SZ both from genetic (Wen et al., 2016) and gene expression studies (Ohi et al., 2013).

We have also looked at the predicted target genes of miR-125b-1-3p, and we found that, four of them, namely BCL11B, CREB3L1, HCN1 and YPEL3 are located within the SZ significant loci (Schizophrenia Working Group of the Psychiatric Genomics, 2014). This suggests that, based on genetic data, miR-125b-1-3p can potentially induce alterations in the expression of a panel of its genes, which in turn may contribute to the pathogenesis of SZ.

Table 2

7 biological systems significantly modulated by cortisol treatment and by predicted and validated target genes of miR-125b-1-3p.

Ingenuity canonical pathways	CORT vs VEH (p-value < 0.05, FC ± 1.2)		MiR-125b-1-3p predicted and validated target genes	
	P-value	Genes	P-value	Genes
Androgen signaling	0.046	GNAI3, GNB4, JUN, CACNG4, SRY, GNAO1, CACNG5, PRKAR2A, PRKACA, GNG3, PRKD3	0.033	GNG4, PRKCQ, GNG7, PRKCA
CXCR4 signaling	0.008	PIK3C2B, CXCR4, PIK3R1, MAPK8, GNG3, GNB4, GNAI3, JUN, RND3, GNAO1, PLCB1, PLCB3, ARHGEF11, ELMO1, PRKD3	0.015	GNG4, PRKCQ, DIRAS3, GNG7, PRKCA
Factors promoting cardiogenesis in vertebrates	0.023	TGFBR2, WNT3, SMO, TGFB3, DKK1, SMAD5, FZD2, PRKD3, ACVR2A	0.009	PRKCQ, BMPR2, MEF2C, PRKCA
Gαi signaling	0.045	ADRA2B, GNAI3, GNB4, CNR1, CHRM4, PRKAR2A, PRKACA, HTR1F, GNG3, AGTR2	0.022	GNG4, SSTR3, S1PR1, GNG7
Huntington's disease signaling	0.035	CAPN5, PIK3C2B, UBB, CASP3, SGK1, PIK3R1, MAPK8, GNG3, RCOR2, DNM1, BCL2L1, GNB4, CASP6, JUN, CASQ1, PLCB3, PLCB1, PRKD3	0.023	GNG4, PRKCQ, IGF1R, GNG7, PRKCA, HDAC5
P2Y purigenic receptor signaling pathway	0.041	GNAI3, PIK3C2B, GNB4, JUN, PIK3R1, PRKAR2A, PRKACA, PLCB3, PLCB1, GNG3, PRKD3	0.031	GNG4, PRKCQ, GNG7, PRKCA
Role of NFAT in cardiac hypertrophy	0.011	MAP2K6, PIK3C2B, CACNG4, PIK3R1, PRKAR2A, MAPK8, SLC8A3, GNG3, TGFBR2, GNB4, GNAI3, CAMK2D, CACNG5, PRKACA, TGFB3, PLCB3, PLCB1, PRKD3	0.003	GNG4, PRKCQ, IGF1R, MEF2C, GNG7, PRKCA, HDAC5

Moreover, with the aim to further investigate the impact of a down-regulation of miR-125b-1-3p on the vulnerability of developing SZ, we looked at the results coming from Transcription Wide Association Study (TWAS) for SZ (http://twas-hub.org/). TWAS are gene association methods estimating whether a different gene expression regulation (e.g., up or down-regulation) could be expected for the analyzed phenotype based on GWAS associations. This can be done through the imputation of the genetic component of gene expression using tissue specific cis-eQTL models (Gusev et al., 2016). By considering gene expression regulation in blood and brain, we found that, among the predicted target genes of miR-125b-1-3p, three genes (GLYCTK, KIFC2 and RAKBP1) were found as significant up-regulated TWAS genes. Specifically, an up-regulation of GLYCTK and KIFC2 expression is expected to occur in the brain, whereas an upregulation of RALBP1 is expected to be present in the blood of SZ patients when compared to controls.

4. Discussion

In the current study, by using a miRNOme approach across different species and tissues, we identified the miR-125b-1-3p as a miRNA associated with the long-lasting effect of stress early in life and with an enhanced vulnerability of developing SZ, especially in association with traumatic experiences early in life.

We have initially identified a specific miRNAs signature associated with stress exposure in different species and tissues, which were: i) peripheral blood of human controls exposed to childhood trauma as compared to controls who did not experience such stressful events; ii) hippocampus of PNS rats as compared with non-exposed animals; iii) HPC0A07/03C cells treated with cortisol during proliferation as compared to vehicle. Subsequently, a pathway analysis performed individually on the three lists of miRNAs differentially affected by stress revealed the modulation of several common biological systems, including those associated with *neurodevelopment*, such as Forkhead Box O (FoxO) and Mammalian Target Of Rapamycin (mTOR) signaling pathways, with *immunity and inflammation*, such as the TGF-β signaling, and with the *intracellular signal transduction*, such as Mitogen-Activated Protein Kinase (MAPK), Ras-Related Protein Rap-1A (Rap1) and Ras signaling pathways. All these findings are in line with literature data (Abbott et al., 2018; Cattane et al., 2018; Malarbi et al., 2017) and suggest that stressful experiences, especially early in life, can affect
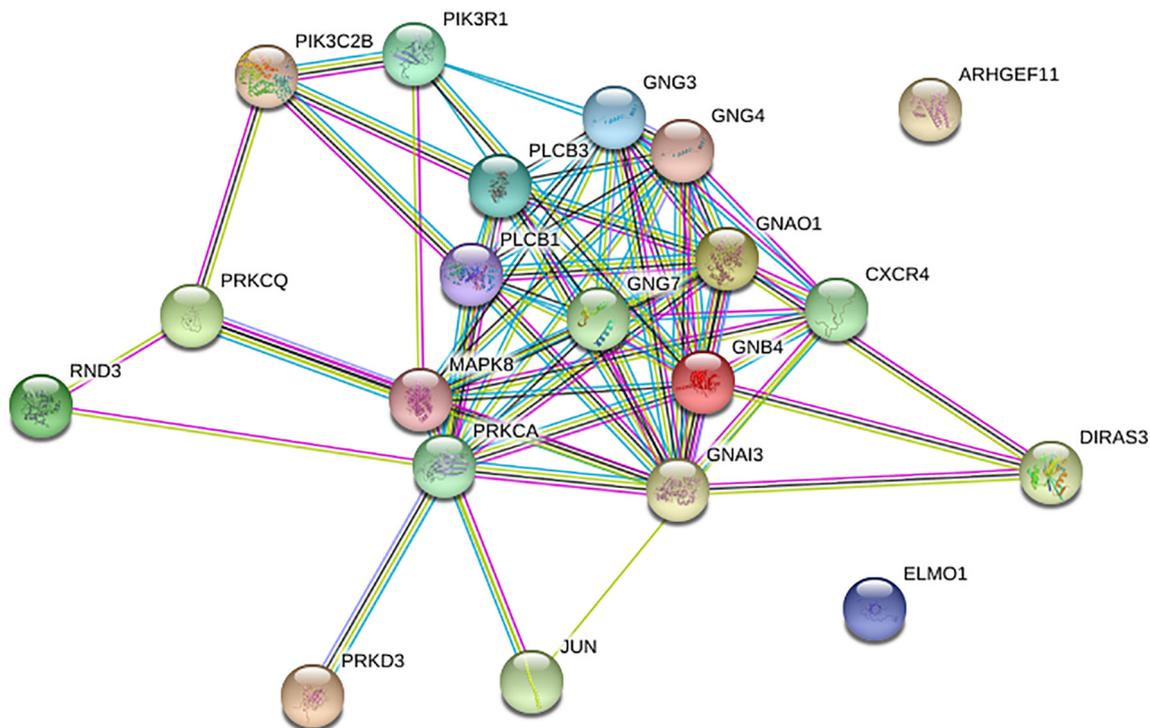

Fig. 2. CXCR4 signaling pathway protein-protein interactions between genes targeted by miR-125b-1-3p and genes modulated by stress (STRING data).
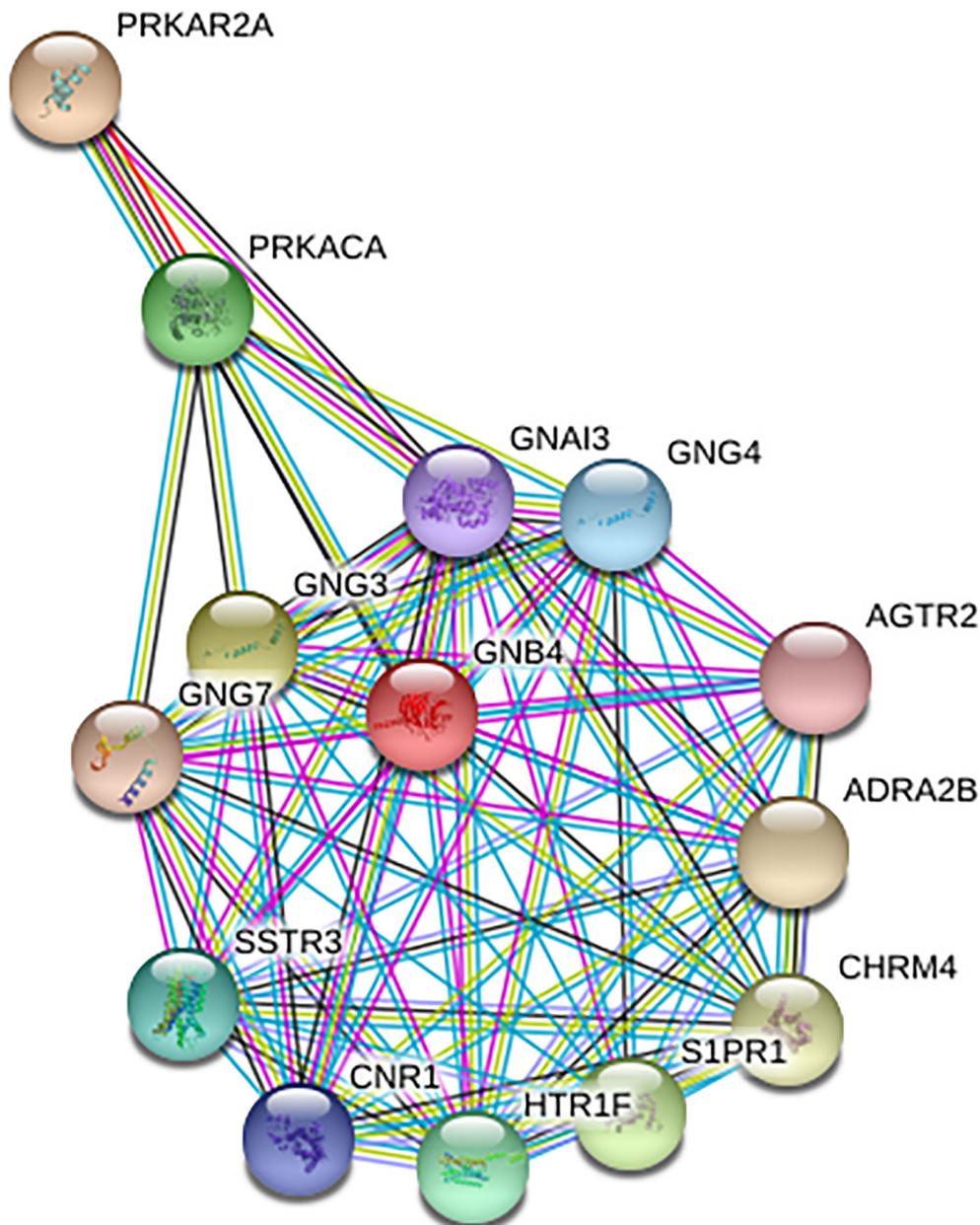
Fig. 3. Gαi signaling pathway protein-protein interactions between genes targeted by miR-125b-1-3p and genes modulated by stress (STRING data).

neurodevelopmental and inflammatory-related systems, rendering stressed individuals more vulnerable to a further second stressful challenge (Debost et al., 2017; Giovanoli et al., 2013) and thus more susceptible to develop a stress-related psychiatric disorder. Moreover, in our recent paper, we have demonstrated that FoxO1 is a novel potential candidate gene able to mediate the effect of stress early in life on the future vulnerability of developing a psychopathology (Cattaneo et al., 2018).

We were then interested in identifying a common miRNAs signature associated with stress exposure, and by combining the lists of miRNAs differentially modulated in the three datasets i) blood samples of adult subjects exposed to childhood trauma, ii) brain of animals exposed to PNS and iii) HPC0A07/03C cells treated with cortisol, we found the miR-125b-1-3p as the only miRNA modulated in the same direction (down-regulation) in all the three lists, an effect that was also confirmed by Real Time PCR.

We have also analyzed the expression levels of the miR-125b-1-3p in a group of controls and in SZ patients, where a small sample was characterized for a history of childhood trauma. MiR-125b-1-3p was significantly downregulated only in the group of SZ patients who reported traumatic experiences in childhood although a trend was observed in the entire group of SZ patients. The downregulation of the miR-125b-1-3p is in line with a study conducted by Zhang and collaborators, (GSE54578, https://www.cbi.nlm.nih.gov/geo/) where gene expression analyses were performed in blood samples of 15 early-onset SZ patients as compared to 15 controls and, despite miR-125b-1-3p was not significantly differently expressed in cases versus controls, a trend versus a downregulation was reported (Zhang et al., 2015).

Overall, these data suggest that miR-125b-1-3p may be involved in the mechanisms associated with the early exposure to stress and, through its long-lasting effects, could promote the future development of SZ, especially in those subjects who have been exposed to childhood trauma.

MiR-125b consists of three homologs, miR-125a, miR-125b-1 and miR-125b-2 and the members of this family play crucial roles in many different cellular processes such as cell differentiation, proliferation
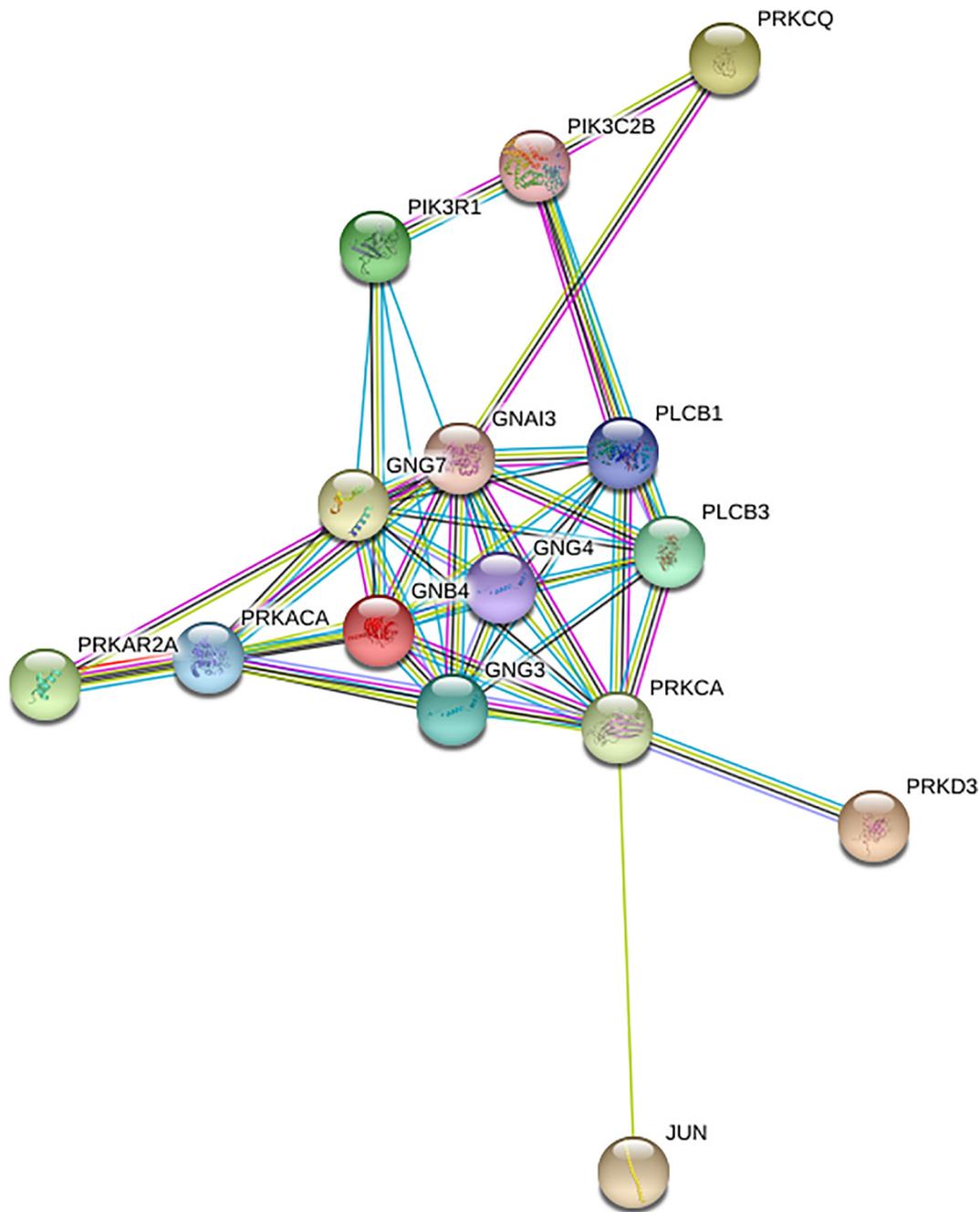
Fig. 4. P2Y purigenic receptor signaling pathway protein-protein interactions between genes targeted by miR-125b-1-3p and genes modulated by stress (STRING data).

and apoptosis via their target genes (Sun et al., 2013). MiR-125b belongs to phylogenetically high conserved tricistrons of miRNA genes on chromosomes 21 and 11 and, specifically, miR-125b-1 is encoded by the miR-100/let-7a-2/miR-125b-1 cluster from a locus on chromosome 11q23 (Emmrich et al., 2014).

Interestingly, although the locus of miR-125b-1-3p does not lie within one of the 108 significant genome-wide significant loci (Schizophrenia Working Group of the Psychiatric Genomics, 2014), it is relatively close (~1.4 Mb and ~2.6 Mb) to two of them, which contain the neurogranin gene (NRGN). This is a postsynaptic protein kinase substrate that is abundantly expressed in structures involved in learning and memory, such as dendritic spines and CA1 pyramidal neurons of the hippocampus (Smith et al., 2011) and that has been associated with SZ both from genetic (Stefansson et al., 2009; Wen et al., 2016) and gene expression studies (Ohi et al., 2013). Moreover, we found that four target genes of miR-125b-1-3p, namely BCL11B, CREB3L1,

HCN1 and YPEL3 are located within the SZ significant loci, suggesting that, based on genetic data, miR-125b-1-3p could potentially induce alterations in the expression of a panel of genes, including miR-125b-1-3p target genes, which in turn, may contribute to the pathogenesis of SZ.

All these findings suggest miR-125b-1-3p as a key player in the mechanisms underlying stress vulnerability, and support a possible involvement of this miRNA in the development of SZ. This is in line with epidemiological and experimental findings that have widely indicated early life adversities as key players in increasing the risk of developing SZ (Ryan and Saffery, 2014). The specific hypothesis that a preexisting vulnerability and further external stressors may interact in contributing to the pathogenesis of SZ was suggested for the first time over half a century ago, and then was confirmed by subsequent several studies (Davis et al., 2016; Howes and McCutcheon, 2017; Jones and Fernyhough, 2007). For example, a longitudinal 10-year prospective cohort study of 3021 adolescents and young adults showed that

experiences of childhood trauma and recent life events (namely the second challenge) are strongly correlated and interact additively in increasing the risk of psychosis (Lataster et al., 2012). Moreover, several meta-analyses have provided robust evidence for an association between childhood trauma and SZ (Matheson et al., 2013; Varese et al., 2012).

To better understand the possible role of miR-125b-1-3p in the functional mechanisms underlying the long-lasting effects of stress and the enhanced vulnerability for SZ, in our study we have also performed a gene-targeting analysis to investigate genes and pathways that were potentially targeted by this miRNA. In total, the miR-125b-1-3p has 196 potential predicted target genes and 12 validated target genes. Of note, among the validated targets, we found the NR3C1 gene, which is involved in the mechanisms of stress response, is affected by stress early in life and it has been found altered in SZ (Farrell et al., 2018; Kundakovic and Jaric, 2017; Schechter et al., 2015). We also found that other validated target genes, SOD1 and IGF1R, have been associated with SZ (Coughlin et al., 2017; Gunnell et al., 2007) and that the predicted genes BCL11B, CREB3L1, HCN1 and YPEL3 are located within the SZ significant loci.

A pathway analysis run on the total number of genes targeted by miR-125b-1-3p indicated that several biological systems involved in the inflammatory and immune response (i.e. CCR3 Signaling in Eosinophils, CCR5 Signaling in Macrophages, CXCR4 Signaling, Role of NFAT in Regulation of the Immune Response, IL-8 Signaling, TGF-β Signaling) and in neurodevelopment (i.e. Gαi Signaling, CXCR4 Signaling and P2Y Purigenic Receptor Signaling Pathway) were potentially targeted by miR-125b-1-3p.

Interestingly, with the aim to prove the real involvement of some of these biological processes in the effects of stress via the modulation of miR-125b-1-3p, we overlapped the list of pathways potentially targeted by miR-125b-1-3p with pathways experimentally and significantly modulated by cortisol in our in vitro model. We found seven common pathways, such as the Chemokine receptor 4 (CXCR4) signaling, the Gαi Signaling, and the P2Y Purigenic Receptor Signaling Pathway, which are all involved in neurodevelopment (Jiang and Bajpayee, 2009; Mayhew et al., 2018; Zhu and Murakami, 2012) and have been also described in contributing to the pathogenesis of several brain disorders (Bonham et al., 2018; Del Puerto et al., 2013). Of note, all these pathways require the activation of G-proteins and their receptors (GPCRs), and indeed we found that, within the CXCR4 signaling, the Gαi Signaling, and the P2Y Purigenic Receptor Signaling, several genes encoding for G proteins such as GNG4, GNG7, GNG3, GNAI3 are "hub-genes" able to drive the activation of these signaling.

G-proteins are identified by their Gα subunits. Gαi family is the largest and most diverse family, which has been detected in most types of cells (Syrovatkina et al., 2016). Many physiological functions, including those related to neurodevelopment, have been observed in G-proteins subunits knockout mice and have been described for several G-protein signaling pathways. For example, an ablation of several Gα subunits directly within the Central Nervous System (CNS) results in neuronal ectopia of the cerebral and cerebellar cortices, indicating that G-proteins are required for proper positioning of migrating cortical plate neurons and Purkinje cells during brain development (Syrovatkina et al., 2016). Moreover, they have been suggested to regulate the neurochemistry of SZ and the mechanism of action of certain antipsychotic medications (Sealfon and Gonzalez-Maeso, 2008). Based on this, it is possible that an alteration of this signaling may directly contribute to the onset of SZ.

The CXCR4 signaling is another well-known pathway involved in neurodevelopment, also in the context of SZ (Meechan et al., 2012; Toritsuka et al., 2013). Indeed, the CXCR4 signaling is crucial for interneuron and dentate gyrus development and several studies have suggested that this pathway may represent a common downstream mediator involved in the pathophysiology of SZ, that frequently shows interneuron abnormalities (Toritsuka et al., 2013). The CXCR4 signaling

belongs to the pathways regulated by chemokines, a large family of small secreted polypeptides, whose signals are transduced by 7-transmembrane GPCRs. The first evidence that chemokines were required for the proper development of the nervous system emerged several years ago, when it was found that the chemokine CXCL12 and its receptor CXCR4 regulate cerebellar granule cell development (Zhu and Murakami, 2012). By generating and analyzing CXCR4 knockout mice, it has been also demonstrated an involvement of CXCL12/CXCR4 in several processes occurring during the normal development of the brain, including neuronal migration, axon guidance, and regulation of neural stem/progenitor cells (Zhu and Murakami, 2012). Based on this, it is possible that an alteration of this signaling may contribute in enhancing the risk of SZ, above all in those subjects exposed to traumatic and stressful events.

The signal transduction pathway activated by P2Y receptors has been also described to modulate several neurobiological processes, including neuroinflammation, synaptic plasticity, and energy homeostasis in communicating neurons and glia (Lindberg et al., 2015). In addition, the purinergic signaling can elicit synaptic currents and induce the release of neurotransmitters, such as glutamate (Mayhew et al., 2018). P2Y receptors are 7-transmembrane GPCRs and bind purines. The two principle purines, ATP and adenosine, act as excitatory and inhibitory neurotransmitters, respectively, and are ubiquitously expressed in the central and peripheral nervous systems (Van Kolen and Slegers, 2006). In physiological conditions, synaptically released ATP can bind to P2 receptors on the presynaptic membrane to modulate the release of glutamate from excitatory terminals. P2X receptor-activation facilitates glutamate release, whereas P2Y receptors inhibit vesicular glutamate transmission and release (Mayhew et al., 2018). Exposure to chronic stress has been demonstrated to modulate the purinergic system, and, as a consequence, to disrupt glutamatergic signaling. Moreover, increasing clinical evidence has shown the presence of a hypo-purinergic state in SZ patients (Krugel, 2016), with reduced purinergic receptors at both transcriptional and translational levels (Villar-Menendez et al., 2014). This is also in line with the neurodevelopmental hypothesis of SZ, suggesting that this disorder is characterized by an altered development of the CNS due to exposure to insults during the first period of life, leading to the manifestation of brain dysfunctions later on. Any environmental adversities such as stressful/traumatic experiences or exposure to inflammatory insults in utero that interfere with the CNS development can, therefore, potentially produce acute and/or long-lasting alterations in brain structure and function, contributing to the onset of SZ (Cattane et al., 2018; Davis et al., 2016). Importantly, whereas stress exposure in adult life induces changes on neuronal plasticity that are largely reversible, early life stress induces, via epigenetic mechanisms, changes in several neuronal processes that can persist over time and increases the vulnerability to the future development of several stress-related disorders, including SZ (Narahari et al., 2017). Based on this, it is possible that stressful and traumatic events may cause alterations in this signaling, and also promote a disruption in the glutamate neurotransmission. Overall, these alterations may contribute to enhance the risk of SZ in a subject who have experienced traumatic events during childhood.

According to well-established literature data, the miRNA-binding, primarily to the 3' UTR of mRNAs, leads to direct mRNA destabilization or translational repression, ultimately resulting in reduced levels of target genes (in term of mRNA or protein levels). Therefore, since we have found a decrease in miR-125b-1-3p levels following a stress exposure across different species and tissues, we expected to observe an upregulation of the target genes belonging to the CXCR4 signaling and to the P2Y Purigenic Receptor Signaling Pathway. Nevertheless, we found that most of these genes and the pathways as well were downregulated as consequence of stress mimicked by cortisol, in our in vitro model. However, we also found that the genes significantly modulated by cortisol by using specific cut-off of FC and p-value were not target genes of miR-125b-1-3p; conversely, when we specifically

looked at the miR-125b-1-3p target genes from the transcriptome of cells treated or not with cortisol, we found an overall increase of these miRNA target genes, although with a smaller effect that was not detectable by using the same cut-offs.

The presence of both up-regulated and down-regulated genes within the same signaling as consequence of the same condition could be explained, at least in part, by looking at the gene-gene interactions. Indeed, when we looked specifically at genes targeted by miR-125b-1-3p and modulated by cortisol belonging to the same pathway, we observed some interesting interactions. For example, we found a direct interaction between PIK3C2B, strongly downregulated by cortisol treatment, and PRKCQ, a predicted gene target of miR-125b-1-3p. In physiological condition, PIK3C2B acts by inhibiting PRKCQ. Thus, it is possible that in our in vitro model where cortisol treatment decreases PIK3C2B expression levels, there is a lack of ability of PIK3C2B to inhibit PRKCQ, which is indeed activated in this model. Therefore, this can lead to the activation of PRKCQ and its downstream intracellular transduction signaling and contribute to trigger the transcription of important genes at the end of the pathway.

Several limitations of this study should be acknowledged. First, the relatively small sample size of the human samples of SZ patients characterized for a history of childhood trauma may limit the statistical power; however, the validity of our data is strengthened by the fact that we compared and integrated data coming from human blood samples with data coming from an in vitro and an in vivo model.

Second, the measurement of the miR-125b-1-3p and several candidate genes expression levels in human peripheral blood of SZ patients could be confounded by antipsychotic medication and, third, the reported alterations of miR-125b-1-3p have been observed in human peripheral blood and it is currently not clear to what extent peripheral miRNA modifications could reflect alterations occurring in the human CNS. However, the alterations observed in the periphery might directly reflect brain modifications, since miRNAs can pass through membranes in free form or in microvesicles (Maffioletti et al., 2016; Narahari et al., 2017). Moreover, although peripheral and central changes could be different, our aim is mainly focused to identify some peripheral signature that somehow can be associated with a disease state or phenotype, even though they do not reflect what happens in the brain.

Overall, our findings, obtained from the combining of the miRNOme data coming from different datasets, have identified the miR-125b-1-3p as a key marker associated with the long-term effects of stress early in life and also with an enhanced vulnerability of developing SZ. The identification of such a miRNA signature could allow the early detection of vulnerable subjects and could provide the basis for the development of preventive therapeutic strategies. Moreover, miR-125b-1-3p targets several genes involved in different biological processes that are mainly related to neurodevelopment. Therefore, alterations in the correct formation of brain circuits, observed in SZ patients, could be due to, at least in part, the involvement of these pathways through the action of miR-125b-1-3p.

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Author disclosure

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