

## Long-term effects of stress early in life on microRNA-30a and its network: Preventive effects of lurasidone and potential implications for depression vulnerability

Annamaria Cattaneo<sup>a,b</sup>, Matthew Suderman<sup>c</sup>, Nadia Cattane<sup>b</sup>, Monica Mazzelli<sup>a,b</sup>, Veronica Begni<sup>a</sup>, Carlo Maj<sup>d</sup>, Ilari D'Aprile<sup>b</sup>, Carmine M. Pariante<sup>e</sup>, Alessia Luoni<sup>a</sup>, Alessandra Berry<sup>f</sup>, Katharina Wurst<sup>g</sup>, Leif Hommers<sup>g,h</sup>, Katharina Domschke<sup>i,j</sup>, Francesca Cirulli<sup>f</sup>, Moshe Szyf<sup>k</sup>, Andreas Menke<sup>g,h,j</sup>, Marco A. Riva<sup>a,\*</sup>

<sup>a</sup> Department of Pharmacological and Biomolecular Sciences, University of Milan, via Balzaretti 9, 20133, Milan, Italy

<sup>b</sup> Biological Psychiatry Unit, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia, Italy

<sup>c</sup> MRC Integrative Epidemiology Unit, School of Social and Community Medicine, University of Bristol, Bristol, BSB 1TH, UK

<sup>d</sup> Institute for Genomic Statistics and Bioinformatics, University Hospital, Bonn, Germany

<sup>e</sup> Stress, Psychiatry and Immunology Laboratory, Department of Psychological Medicine, Institute of Psychiatry, Psychology and Neuroscience, King's College, London, United Kingdom

<sup>f</sup> Section of Behavioural Neurosciences, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy

<sup>g</sup> Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital, Würzburg, Germany

<sup>h</sup> Interdisciplinary Center for Clinical Research, University Hospital of Würzburg, Germany

<sup>i</sup> Department of Psychiatry and Psychotherapy, Medical Center-University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

<sup>j</sup> Center for Basics in Neuromodulation, Faculty of Medicine, University of Freiburg, Freiburg, Germany

<sup>k</sup> Department of Pharmacology and Therapeutics, McGill University, 3655 Sir William Osler Promenade #1311, Montreal, Quebec, Canada, H3G 1Y6

### ARTICLE INFO

#### Keywords:

Prenatal stress  
DNA methylation  
miR-30a  
Neurotrophin signaling  
Axon guidance signaling

### ABSTRACT

Exposure to early life stress can interfere with neurodevelopmental trajectories to increase the vulnerability for psychiatric disorders later in life. With this respect, epigenetic mechanisms play a key role for the long-lasting changes in brain functions that may elicit and sustain psychopathologic outcomes.

Here, we investigated DNA methylation changes as possible epigenetic mechanism mediating the effect of prenatal stress (PNS), an experimental paradigm associated with behavioral and molecular alterations relevant for psychiatric disorders. We identified 138 genes as being differentially methylated in the prefrontal cortex (PFC) and in the hippocampus (HIP) of male and female adult rats exposed to PNS. Among these genes, miR-30a and Neurod1 emerged as potential players for the negative outcomes associated with PNS exposure. Indeed, in addition to showing consistent methylation differences in both brain regions and in both sexes, and interacting with each other, they are both involved in *Axon guidance* and *Neurotrophin signaling*, which are important to neurodevelopmental disorders. We also found a significant reduction in the expression of a panel of genes (CAMK2A, c-JUN, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1) that belong to these two biological pathways and are also validated targets of miR-30a, pointing to a down-regulation of these pathways as a consequence of PNS exposure. Interestingly, we also found that miR-30a levels were significantly upregulated in depressed patients exposed to childhood trauma, as compared to control individuals. Importantly, we also found that a sub-chronic treatment with the atypical antipsychotic drug, lurasidone, during adolescence was able to prevent the up-regulation of miR-30a and normalized the expression of its target genes in response to PNS exposure.

Our results demonstrate that miR-30a undergoes epigenetic changes following early life stress exposure and suggest that this miRNA could play a key role in producing broad and long-lasting alterations in neuroplasticity-related pathways, contributing to the etiology of psychiatric disorders.

\* Corresponding author. Department of Pharmacological and Biomolecular Sciences, University of Milan, Via Balzaretti, 9, 20133, Milano, Italy.

E-mail address: [m.riva@unimi.it](mailto:m.riva@unimi.it) (M.A. Riva).

<https://doi.org/10.1016/j.ynstr.2020.100271>

Received 24 August 2020; Received in revised form 5 November 2020; Accepted 16 November 2020

Available online 23 November 2020

2352-2895/© 2020 The Authors.

Published by Elsevier Inc.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Abbreviations

Prenatal Stress (PNS)	Mitogen-Activated Protein Kinase Kinase 2 (MAP2K2)
Hippocampus (HIP)	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA)
Prefrontal Cortex (PFC)	Phospholipase C Gamma 1 (PLCG1)
Hypothalamic-Pituitary-Adrenal (HPA) axis	Area Under the Curve (AUC)
Brain-Derived Neurotrophic Factor (BDNF)	Ingenuity Pathway Analysis (IPA)
Postnatal days (PNDs)	Transcriptome Wide Association Study (TWAS)
Hydroxyethylcellulose (HEC)	Transcription Start Site (TSS)
Methylated DNA immunoprecipitation (MeDIP)	Axl receptor tyrosine kinase ( <i>Axl</i> )
Childhood Trauma Questionnaire (CTQ)	microtubule-associated protein 7 ( <i>Map7</i> )
Mini International Neuropsychiatric Interview (M.I.N.I. Plus)	muscle RAS oncogene homolog ( <i>Mras</i> )
Beck Depression Inventory scale (BDI)	CCR4-NOT transcription complex, subunit 2 ( <i>Cnot2</i> )
Hamilton Depression Rating Scale (HDRS)	ciliary neurotrophic factor ( <i>Cntf</i> )
Montgomery-Asberg Depression Scale (MADRS)	microRNA-30a ( <i>mir-30a</i> )
Generalized Anxiety Disorder 7-item (GAD-7) scale	neuronal differentiation 1 ( <i>Neurod1</i> )
State-Trait Anxiety Inventory questionnaire (STAI)	NOP2/Sun domain family, member 6 ( <i>Nsun6</i> )
Patient Health Questionnaire (PHQ-9)	olfactory receptor 115 ( <i>Olr115</i> )
List of Threatening Experiences Questionnaire (LTE)	3-oxoacid CoA transferase 1 ( <i>Oxct1</i> )
Adverse Childhood Experience (ACE) Questionnaire	SET domain and mariner transposase fusion gene ( <i>Setmar</i> )
Coping Strategies Questionnaire (CSQ)	human induced pluripotent stem cells (hiPSCs)
Cognitive Emotion Regulation Questionnaire (CERQ)	neural progenitor cells (NPCs)
Calcium/Calmodulin Dependent Protein Kinase II Alpha (CAMK2A)	N-methyl-D-aspartate receptors (NMDARs)
Jun Proto-Oncogene, AP-1 Transcription Factor Subunit (c-JUN)	Neuroigin 1 (NLG1)
LIM Domain Kinase 1 (LIMK1)	quantitative Real-Time PCR (qRT-PCR)
Mitogen-Activated Protein Kinase Kinase 1 (MAP2K1)	NCBI Reference Sequence Database (RefSeq)
	NIH genetic sequence database (GenBank®)

## 1. Introduction

Exposure to early life stress represents a well-known risk factor for the development of psychiatric disorders later in life. Ultimate outcome will depend on additional factors, including timing and duration of both positive and negative life experiences (Herzog and Schmahl, 2018). There is growing evidence indicating that stress during early life alters the default program of brain maturation, leading to long-term functional changes in multiple brain structures (Bick and Nelson, 2016). Hence, it is important to grow our knowledge of the complex processes and mechanisms that shape neurodevelopmental trajectories which depend upon early events that may lead to persistent brain dysfunction. Epigenetic mechanisms, such as DNA methylation and microRNA-mediated post-transcriptional regulation, have been suggested to play a major role in sustaining the long-lasting effects of adverse events early in life in shaping the individual vulnerability to psychopathology (Bale, 2014; Luoni and Riva, 2016; Provencal and Binder, 2015).

Animal models are particularly useful to investigate molecular mechanisms modulated by stress and to characterize the potential psychopathologic implications. A well-established model of early life stress in rodents is represented by the prenatal stress (PNS) paradigm, where pregnant dams are exposed to restraint stress during the last week of gestation (Luoni et al., 2014a). The functional consequences of PNS exposure has been extensively reviewed in the last decades, showing that stressful experiences during gestation leads to major effects on adult behavior (Lupien et al., 2009; Weinstock, 2008, 2017). Indeed, we and others have shown that PNS rats show impaired learning and memory (Cattaneo et al., 2019), anxiety-like phenotypes (Weinstock, 2017) as well as depressive-like behaviors, including loss of copying in the forced swim stress (Weinstock, 2017). Similarly, exposure to stress during the perinatal period produces alterations of hippocampal structure and cognitive function, along with altered HPA axis reactivity and impaired emotional responses (Naninck et al., 2015, 2017; Krugers et al., 2017). Such evidence suggests that the vulnerable period to stress may span from late gestation to early postnatal life. PNS exposure produces alterations in different biological substrates known to represent

vulnerability elements for the development of psychiatric conditions, including, but not limited to, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, altered stress-responsiveness (Maccari et al., 2014) and deficits in neuronal plasticity (Fumagalli et al., 2007). We have previously demonstrated that the expression of the neurotrophin Brain-Derived Neurotrophic Factor (BDNF) is significantly reduced in the prefrontal cortex (PFC) of adult rats that were exposed to PNS *in utero*, an effect that became fully manifest after adolescence (Luoni et al., 2014a). Moreover, PNS rats show an impaired ability to cope with challenging stressful events at adulthood, through alterations in activity-dependent epigenetic mechanisms (Luoni et al., 2016a).

Several studies have demonstrated that an exposure to PNS produces long-term behavioral, cognitive and functional alterations in the adult offspring (Cattaneo et al., 2019; Gur et al., 2019; Panetta et al., 2017). For instance, in a recent study performed by our group (Cattaneo et al., 2019), we observed a significant disruption in the novel object recognition test both in male and female adult rats exposed to PNS, with a more pronounced effect in females. This cognitive dysfunction, originating from the PNS exposure, appeared to be the consequence of an inability to activate the proper transcriptional machinery required for the correct cognitive performances in relevant brain areas, such as the dorsal hippocampus, during a cognitive task (Cattaneo et al., 2019).

In the present study, we used a hypothesis-free genome-wide approach to investigate the role of DNA methylation in mediating the long-term effects of PNS exposure. We focused on the prefrontal cortex (PFC) and hippocampus (HIP), two brain regions that play a relevant role in key functional alterations associated with psychiatric disorders (Hao et al., 2020; Kolb et al., 2012; Levone et al., 2015; Paus et al., 2008). We hypothesized that differentially methylated regions, most likely responsible for mediating the effect of PNS, would have consistent methylation differences following PNS in both brain regions from male and female rats. We thus focused the attention on genes, which were modulated in the same direction in both brain regions and sexes, and we characterized their expression profile during postnatal maturation, in order to establish potential changes during developmental trajectories.

Moreover, we investigated whether sub-chronic treatment with the antipsychotic drug lurasidone, which is clinically approved for the treatment of schizophrenia and major depressive episodes in patients with bipolar disorder, given during the peripubertal period, could prevent or modulate some of the alterations caused by the PNS exposure. Finally, we investigated a cohort of controls and depressed patients all characterized for childhood trauma events to provide a test of the translation of our preclinical findings in a clinical setting.

## 2. Methods

### 2.1. Animals and experimental paradigms

Pregnant rats were randomly assigned to control (CTRL) or prenatal stress (PNS) conditions. The stress paradigm was carried out as previously described (Luoni et al., 2014a). Briefly, pregnant dams were restrained in a transparent Plexiglas cylinder, under bright light, for 45 min, three times a day during the last week of gestation. Control pregnant females were left undisturbed in their home cages. As a proxy of stress effectiveness during gestation, we have previously shown that the expression levels of the placental enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2), which allows for controlled fetal exposure to maternal glucocorticoids, was significantly reduced in dams exposed to prenatal stress (Panetta et al., 2017).

Offspring from the control and the PNS groups were sacrificed at different postnatal days (PNDs), namely at PND21, PND40 and PND62 for the dissection of the HIP and PFC. Specifically, the whole HIP comprises the ventral and the dorsal sub-regions, whereas the PFC comprises cingulate and infralimbic cortex. The whole genome methylation analyses were performed in the HIP and PFC of male and female offspring at PND62. Gene expression analyses were performed in animals of both sexes and in both brain regions at PND21, 40 and 62.

Based on the results that we obtained with the first PNS experiment, we decided to test whether a pharmacological treatment could prevent the effects caused by PNS exposure. Thus, a new batch of animals was prepared with the same experimental protocol. In this case, starting from PND35, a group of male offspring was treated for two weeks with the antipsychotic drug lurasidone (3 mg/kg in a 1% hydroxyethylcellulose, HEC, solution) or vehicle (HEC solution) by oral gavage in the amount of 1 ml/kg body weight. We selected the dose and route of delivery based on previous studies demonstrating the antidepressant and pro-cognitive actions of lurasidone (Ishiyama et al., 2007; Tarazi and Riva, 2013). Rats were sacrificed 2 weeks after the end of the treatment, which corresponds to PND62.

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. All efforts were made to minimize animal suffering and reduce the total number of animals used, while maintaining statistically valid group numbers.

### 2.2. Brain region dissection and DNA/RNA preparation

After decapitation, the prefrontal cortex (defined as Cg1, Cg3, and IL sub-regions corresponding to the plates 6–10 according to the atlas of Paxinos and Watson) was dissected from 2-mm-thick slices, whereas the hippocampus was dissected from the whole brain. The brain specimens were frozen on dry ice and stored at  $-80^{\circ}\text{C}$  until further analyses. HIP and PFC were dissected and immediately stored at  $-80^{\circ}\text{C}$ . The isolation of DNA and RNA from these brain regions was performed using AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Milan, Italy), in accordance with the instructions of the manufacturer.

### 2.3. Methylated DNA immunoprecipitation (MeDIP) analysis

MeDIP analyses were performed as previously described (Luoni et al., 2016b; Nieratschker et al., 2014). Briefly, DNA was quantified by fluorometric analysis (Qubit<sup>®</sup> 2.0 Fluorometer, Life Technologies), 2  $\mu\text{g}$  of DNA were sheared by sonication and methylated DNA was immunoprecipitated using 10  $\mu\text{g}$  of anti-5-methyl-cytosine (Eurogentec, Fremont, CA, USA). For each condition (both sexes and brain regions analyzed), DNA was extracted from 4 Ctrl and 4 PNS rats at PND62.

Genome-wide DNA methylation levels were measured using custom promoter tiling microarrays. All gene transcription start sites were tiled from 1000bp upstream to 200bp downstream with probes placed approximately every 100bp. Any genomic coordinates are given with respect to the rn4 (RGSC 3.4) rat genome assembly. All the steps of scanning and analysis were performed as previously described (Nieratschker et al., 2014).

### 2.4. Analysis of MiR-30 in clinical sample

MiR-30 expression levels were analyzed in peripheral blood samples from a clinical cohort represented by adult control subjects ( $n = 62$ ) and depressed patients ( $n = 79$ ), who have been all screened for exposure to childhood trauma through the administration of the Childhood Trauma Questionnaire (CTQ) (Bernstein et al., 1994). As we wanted to specifically evaluate the effects of childhood trauma on the modulation of MiR-30 levels, we focused our attention on the group of controls ( $n = 27$ ) who had never reported childhood trauma and on the group of subjects, both controls ( $n = 35$ ) and depressed patients ( $n = 79$ ), who experienced at least one traumatic event in childhood, among the five CTQ subscales (emotional abuse, physical abuse, sexual abuse, emotional neglect and physical neglect).

All subjects (controls and depressed patients) were recruited at the Dept. of Psychiatry, University of Würzburg (Germany). The recruitment was approved by the local Ethic Committee (no. 128/15 and no. 231/15), and a written informed consent was obtained by participants after receiving a complete description of the study.

Out of 62 control subjects, 35 reported a history of at least one traumatic experience in childhood (mean age  $\pm$  SEM:  $53.06 \pm 14.38$  years; 16 males and 19 females), whereas 27 subjects did not experience traumatic events in childhood (mean age  $\pm$  SEM:  $47.26 \pm 16.96$  years; 19 males and 8 females). Individuals with a history of neurological or psychiatric diseases, prior traumatic brain injury, or mental retardation 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 axis I and II psychiatric disorders, referred to all psychological diagnostic categories except mental retardation and personality disorder (axis I) or to personality disorders and mental retardation (axis II), respectively.

Clinical diagnosis of depression in patients (mean age  $\pm$  SEM:  $50.03 \pm 15.12$  years; 31 males and 48 females) was ascertained using the Structured Clinical Interview for DSM disorders (SCID-I). A history of neurological or other severe somatic diseases, prior traumatic brain injury, or mental retardation were exclusion criteria. All depressed patients reported at least one traumatic event in childhood.

Healthy controls not exposed to childhood trauma, healthy controls exposed to childhood trauma and depressed patients did not differ regarding age (all  $p > 0.05$ ).

Depressed Patients have also been clinically assessed by using different questionnaires: Beck Depression Inventory scale (BDI) (Beck et al., 1961), Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960), Montgomery-Asberg Depression Scale (MADRS) (Montgomery and Asberg, 1979), Generalized Anxiety Disorder 7-item (GAD-7) scale (Spitzer et al., 2006), State-Trait Anxiety Inventory questionnaire (STAI) (Spielberger, 1983), Patient Health Questionnaire (PHQ-9) (Spitzer et al., 1999), List of Threatening Experiences Questionnaire (LTE)

(Brugha and Cragg, 1990), Adverse Childhood Experience (ACE) Questionnaire (Anda et al., 2006), Coping Strategies Questionnaire (CSQ) (Rosenstiel and Keefe, 1983), Cognitive Emotion Regulation Questionnaire (CERQ) (Garnefski et al., 2007).

Blood samples have been collected fasting in all the subjects by using Paxgene Tubes and stored at  $-80$  until their processing for mRNA isolation and Real Time PCR.

## 2.5. Gene and miRNA expression analyses by Real Time PCR

After RNA isolation, the expression levels of miR-30a-5p, of Neurod1 and of the panel of miR-30a-5p target genes (Calcium/Calmodulin Dependent Protein Kinase II Alpha (CAMK2A), Jun Proto-Oncogene, AP-1 Transcription Factor Subunit (c-JUN), LIM Domain Kinase 1 (LIMK1), Mitogen-Activated Protein Kinase Kinase 1 (MAP2K1), MAP2K2, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) and Phospholipase C Gamma 1 (PLCG1)) have been analyzed in the HIP and/or in the PFC of rats exposed or not to PNS and sacrificed at PND21, 40 and 62. Moreover, the levels of these same genes and of the miR-30a-5p were analyzed in the PFC of animals exposed or not to PNS and receiving or not the antipsychotic drug lurasidone (3 mg/kg, by oral gavage) for 2 weeks starting from PND 35 and sacrificed 2 weeks after the end of the treatment, which corresponds to PND62.

The expression levels of Neurod1, CAMK2A, C-Jun, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1 were analyzed by using TaqMan Assays (ThermoFisher, Waltham, MA, USA), and the iTaq Universal Probes One-Step Kit (Bio-Rad Laboratories, Hercules, CA, USA) on the CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. All the reactions were performed in triplicates and the relative expression of all the genes were normalized to the levels of the housekeeping genes beta-Actin (b-Act).

In order to analyze the expression levels of miR-30a-5p in animal and human samples, the extracted total RNA, including miRNAs, was firstly reverse transcribed using the TaqMan MicroRNA RT Kit (ThermoFisher, Waltham, MA, USA) and, subsequently, the RT product was pre-amplified using TaqMan PreAmp Master Mix (ThermoFisher, Waltham, MA, US). At this point, the PreAmp product was diluted with 0.1XTE and then evaluated for the expression levels analysis of miR-30a-5p by Real Time PCR using the CFX384 instrument (Bio-Rad Laboratories, Hercules, CA, USA), the TaqMan MicroRNA Assays (ThermoFisher, Waltham, MA, USA) and the TaqMan Universal Master Mix, no AmpErase UNG (ThermoFisher, Waltham, MA, USA) following the manufacturer's instructions. The relative expression of miR-30a-5p was normalized to the levels of U87 in rats and to the levels of RNU24 in human blood samples. All the reactions were performed in triplicates. The Pfaffl Method was then used to determine the relative expression values of miR-30a-5p and of the Neurod1, CAMK2A, C-Jun, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1 genes (Pfaffl, 2001).

## 2.6. Statistical and bioinformatic analyses

### 2.6.1. Methylation analyses

Probe intensities from MeDIP microarrays were extracted from scan images using Agilent's Feature Extraction 9.5.3 Image Analysis Software. Extracted microarray intensities were processed and analyzed using the R software environment for statistical computing. Log-ratios of the bound (Cy5) and input (Cy3) microarray channel intensities were computed for each microarray. Microarrays were normalized to one another using quantile-normalization under the assumption that all samples had identical overall methylation levels. Estimates of DNA methylation levels from microarray probe intensities were obtained using a Bayesian deconvolution algorithm (Down et al., 2008). These estimates were used in figures to indicate overall methylation levels but were not used for detecting differential methylation. Differential methylation between PNS exposed and not exposed animals was determined in stages to ensure both statistical significance and biological

relevance. In the first stage, linear models and modified t-tests implemented in the 'limma' package of Bioconductor (Gentleman et al., 2004) were used to test methylation differences between sample groups at each microarray probe. A probe was classified as being differentially methylated if the resulting p-value was less than 0.05 (uncorrected for multiple testing), and the associated difference of means between the groups was at least 0.5. Given that the DNA samples were sonicated prior to hybridization, the assumption was made that probes within 500bp should show approximate agreement. Therefore, the genome was grouped into 1000bp regions and the significance of enrichment for high or low t-statistics of probes within each region (containing at least 1 probe) was calculated. Significance was determined using the Wilcoxon rank-sum test comparing t-statistics of the probes within the region against those of all the probes on the microarray and then adjusted to obtain false discovery rates for each region. A probe was classified as being differentially methylated if it satisfied each of the following criteria: 1) the significance of its t-statistic was a maximum of 0.05 and the difference of means between the groups was at least 0.5, and 2) it belonged to a region whose false discovery rate was a maximum of 0.2.

### 2.6.2. Gene expression and pathways analyses

All the gene expression data of miR-30a-5p and of the Neurod1, CAMK2A, C-Jun, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1 genes have been graphically expressed as means  $\pm$  SEM. The significant differences among the groups have been assessed with the Student's t-test and the statistical significance was set at  $p < 0.05$ .

Receiving operating curve analysis has been performed to evaluate the association of polygenic transcriptomic profiles derived by the linear combination of single gene expression level. The Area Under the Curve (AUC) was used to evaluate the performance of multi-gene classification models.

The list of miR-30a-5p target genes were obtained from the miR-Walk2.0 database ([http://zmf.umm.uni-heidelberg.de/apps/zmf/mi\\_rwalk2/](http://zmf.umm.uni-heidelberg.de/apps/zmf/mi_rwalk2/)) (Dweep and Gretz, 2015), a comprehensive database that provides predicted as well as validated miRNA binding site information. The identification of potentially altered molecular pathways as targeted by validated target genes of miR-30a-5p was performed with the "Core Analysis" function included in Ingenuity Pathway Analysis (IPA) (Ingenuity System Inc., USA <http://www.ingenuity.com>). Moreover, to identify pathways modulated by miR-30a-5p we have also interrogated mirPath v.3 (<http://snf-515788.vm.oceanos.grnet.gr>).

In order to evaluate the potential association between the differentially methylated and differentially expressed genes with psychiatric phenotypes in humans, we performed a Transcriptome Wide Association Study (TWAS), considering brain tissues (<http://twas-hub.org/>). These models integrate genome-wide association for a given phenotype with expression quantitative loci from gene-expression tissue database (<https://gtexportal.org/home/>) and common mind consortium (<https://www.nimhgenetics.org/resources/commonmind>) to test whether or not a differential tissue specific gene expression regulation can be expected according to the genetic signal.

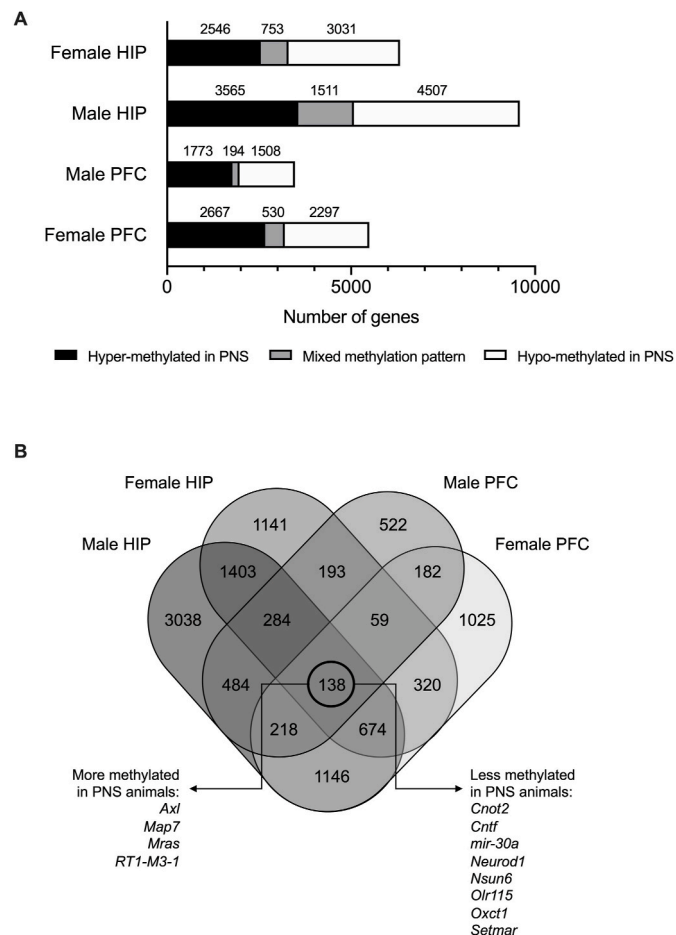
## 3. Results

### 3.1. Long-lasting DNA methylation alterations induced by PNS

In order to identify genes whose expression and function may be persistently altered as a consequence of PNS exposure, we performed genome-wide methylation analyses in the HIP and in the PFC of adult rats (PND62), both males and females, which were exposed (PNS) or not (CTRL) to maternal stress during the last week of gestation. We obtained four lists corresponding to the genes differentially methylated between CTRL and PNS animals in the two brain regions of male and female animals (FDR < 0.2). Since several probes can map different regions of a single gene, these lists include genes that were globally hypermethylated in PNS rats, genes that were hypo-methylated in PNS rats,

as well as genes showing a mixed methylation pattern (See Fig. 1A to visualize the number of genes differentially methylated across brain regions and groups).

Among the genes differentially methylated between CTRL and PNS rats, we found 893 genes, which were differentially methylated both in the HIP and PFC of both male and female rats. Considering the high number of potential candidates, we decided to restrict the list by filtering the data considering only differences that occurred around the transcription start site (TSS, between  $-1000$  and  $+500$  base pairs), which may identify the promoter region and therefore be more relevant loci for transcriptional regulation (Weber et al., 2007). We thus identified 138 genes (See Fig. 1B and Supplementary Table 1 for the entire list of genes) in common between HIP and PFC of male and female rats and, among them, we found 12 genes which were also significantly modulated in the same direction (See Fig. 1B). In particular, four genes were more methylated in PNS rats compared to CTRLs (*Axl* receptor tyrosine kinase (*Axl*), microtubule-associated protein 7 (*Map7*), muscle RAS oncogene homolog (*Mras*) and RT1-M3-1); another group of eight genes were less methylated in the PNS rats (CCR4-NOT transcription complex, subunit 2 (*Cnot2*), ciliary neurotrophic factor (*Cntf*), microRNA-30a (*mir-30a*), neuronal differentiation 1 (*Neurod1*), NOP2/Sun domain family, member 6 (*Nsun6*), olfactory receptor 115 (*Olr115*), 3-oxoacid CoA transferase 1 (*Oxct1*) and SET domain and mariner transposase



**Fig. 1.** Genome-wide methylation changes of adult rats (PND62) exposed to prenatal stress.

A) The stacked bar graph depicts the number of genes that were more (black) or less (white) methylated in the hippocampus (HIP) or prefrontal cortex (PFC) from male and female rats exposed to prenatal stress (PNS), or that showed a mixed methylation pattern (grey). B) Venn diagram showing the overlap of the number of differentially methylated genes, around the TSS (between  $-2000$  and  $+500$  base pairs) in HIP or PFC from male and female rats exposed to PNS.

fusion gene (*Setmar*)).

We then investigated potential interactions among these 12 genes by using i) miRWalk 2.0 Software to identify miRNAs target genes, ii) Ingenuity Software Pathway Analyses to establish pathways and networks and iii) by literature search. Only *mir-30a* and *Neurod1* were found to interact with each other.

Importantly, we found that *Neurod1* is a validated target gene of *mir-30a* (Beveridge et al., 2014; Liu et al., 2017), that both are involved in Neurotrophin/TRK signaling and in the Axonal Guidance signaling, two pathways closely related to neurodevelopment (Beveridge et al., 2014; Liu et al., 2017), and that both are associated with vulnerability to be diagnosed with psychotic spectrum disorders, including schizophrenia, schizoaffective disorder and major depression, which includes psychotic depression (Mellios et al., 2008; Perkins et al., 2007; Wan et al., 2015; Yu et al., 2014). Based on these three points, we decided to focus our attention on these two candidate genes for subsequent analyses.

### 3.2. Methylation pattern in the promoter region of *mir-30a* and *Neurod1* affects their long-term expression levels

In order to test the possibility that DNA methylation changes around the TSS may affect *mir-30a* expression levels, we investigated, in the same samples used for MeDIP-chip analysis, the expression levels of the two mature miRNA isoforms that originate from *mir-30a* biogenesis, namely *mir-30a-5p* and *mir-30a-3p*.

Consistent with a reduced DNA methylation in PNS rats, we found that the expression of *mir-30a-5p* was significantly up-regulated in the male PFC ( $+27%$ ,  $p < 0.05$ , Fig. 2A and B), as well as in the female and male HIP ( $+42%$ ,  $p < 0.05$  and  $+38%$ ,  $p < 0.05$ , respectively, Fig. 2E, F and 2G, 2H) of rats previously exposed to PNS. However, we did not detect any expression differences in the female PFC ( $p = 0.97$ , Fig. 2C and D) or in the expression of *mir-30a-3p* within the PFC and HIP of PNS male and female rats.

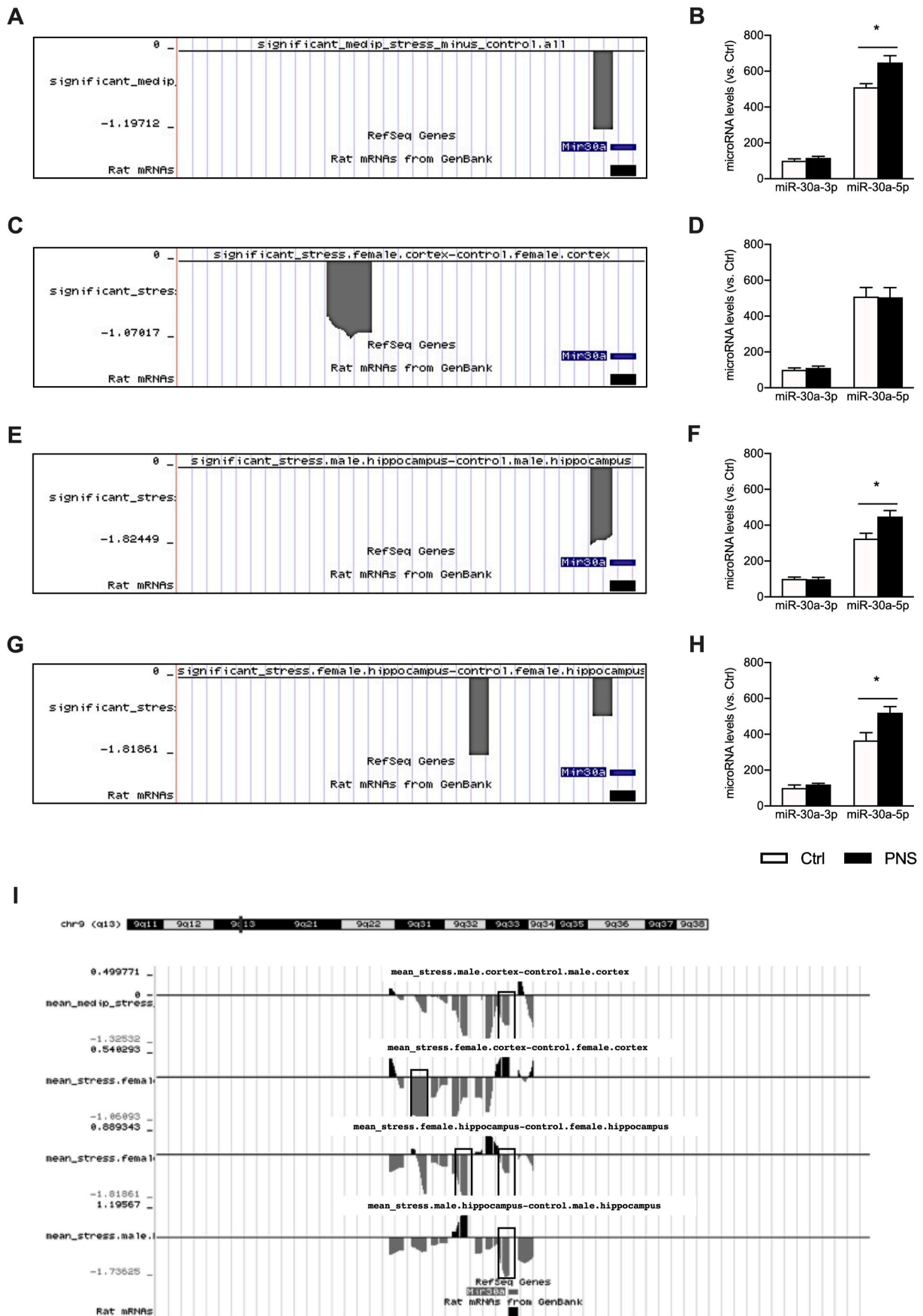
The failure to detect differences in *mir-30a-5p* or *mir-30a-3p* expression in female PFC may be due to the location of the differentially methylated region in female PFC. In all samples, except female PFC, the differentially methylated region is located immediately before the TSS (Fig. 2I, Supplementary Table 2), whereas in female PFC the differentially methylated region is located about 700 bp further upstream.

The failure to detect differences in *mir-30a-3p* expression is likely due to low expression levels; indeed, the expression of *mir-30a-3p* is about 3-5-fold lower than the levels of *mir-30a-5p* in both brain regions and sexes (see Fig. 2B, D for PFC and Fig. 2F, H for HIP).

We have also measured the mRNA levels of *Neurod1* in the same animals and we observed a significant decrease ( $-23%$ ,  $p < 0.05$ ) of *Neurod1* mRNA levels in the PFC of adult (PND62) PNS male rats. This decrease coincides with a decrease in *Neurod1* promoter methylation, contradicting the usual repressive relationship between DNA methylation and gene expression. However, *Neurod1* is a target gene of *mir-30a-5p* and the observed down-regulation in its mRNA levels may represent a consequence of *mir-30a-5p* up-regulation, rather than of changes in DNA methylation. Interestingly we found a significant negative correlation ( $p < 0.05$ ,  $r = -0.58$ ) between the modulation of *mir-30a-5p* and *Neurod1*, supporting the hypothesis that the effect observed on *Neurod1* is mainly driven by an upregulation of *mir-30a-5p*, rather than an effect of altered methylation".

### 3.3. *MiR-30a* and *Neurod1* expression levels during postnatal development

One important question related to the effects of PNS exposure is to establish to what extent the changes in gene expression observed at adulthood are preceded by significant alterations at earlier developmental stages. Hence, we investigated the expression of *mir-30a-5p* and *Neurod1* in the PFC of CTRL and PNS male rats at weaning (PND21) as



(caption on next page)

**Fig. 2.** Methylation status of the genomic location around *mir-30a* gene transcription start site and analysis of *miR-30a-5p* and *miR-30a-3p* expression levels in adult rats exposed to prenatal stress.

The panels on the left, obtained from UCSC genome browser, depict the regions around the TSS of *mir-30a* whose methylation status is significantly different as a consequence of PNS in the prefrontal cortex of male (A) and female (C) rats, as well as in the hippocampus of male (E) and female (G) rats. The bar graphs on the right show the results of qRT-PCR analysis for *miR-30a-5p* and *miR-30a-3p*, represented as percentage changes vs. *miR-30a-3p* levels in Ctrl, set at 100% (B, D, F, H). \* $p < 0.05$  (Student's *t*-test).

Panel (I) represents expanded views from the UCSC genome browser at the rat *mir-30a* gene location. Each of the four upper tracks depicts the average methylation probe fold difference (Log 2) expressed as the value in PNS rats minus Ctrl. Grey bars indicate probes more methylated in Ctrl rats, while black bars are probes more methylated in PNS rats. Starting from the top, the tracks correspond to the data obtained in male PFC, female PFC, female HIP and male HIP. Black empty rectangles highlight the locations at which the methylation difference between PNS and Ctrl rats resulted to be statistically significant. The two lower tracks show *mir-30a* gene location taken from NCBI Reference Sequence Database (RefSeq) and from NIH genetic sequence database (GenBank®).

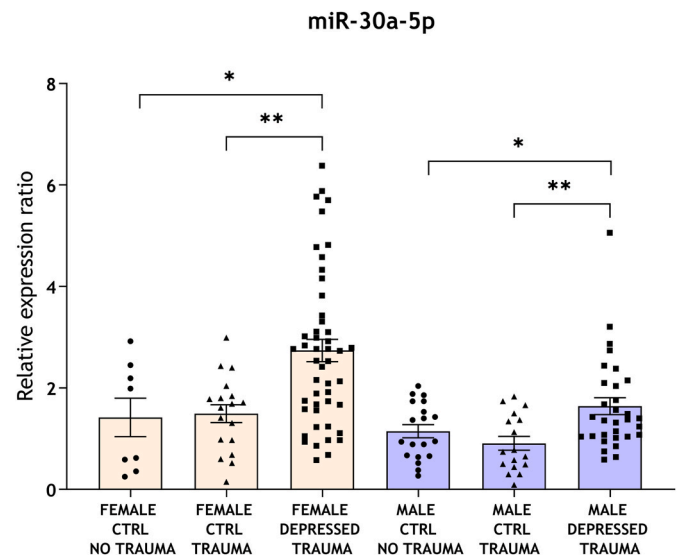
well as in the peripubertal period (PND40). As shown in Fig. 3A, *miR-30a-5p* levels show a transient down-regulation at PND21 (−38%,  $p < 0.01$ ), which was followed by a significant up-regulation at PND40 (+80%,  $p < 0.01$ ), an effect that was maintained at PND62 (+55%,  $p < 0.001$ ). On the other hand, the expression of *Neurod1* was not significantly modulated by PNS at PND21 or PND40 whereas, as already described, its mRNA levels were significantly down-regulated at early adulthood (PND62, −23%,  $p < 0.05$ ) (Fig. 3B).

### 3.4. *MiR-30a* expression levels in blood samples from controls and depressed patients

In order to establish whether the results obtained in the brain of PNS exposed animals could be observed also in humans, we measured *miR-30a-5p* expression in peripheral blood samples of a group of controls (CTRLs) and depressed patients, all characterized for exposure to childhood trauma. We observed a significant up-regulation (+76%,  $p < 0.001$ ) of *miR-30a-5p* levels in depressed patients who experienced childhood trauma as compared to the group of CTRLs who had never experienced traumatic events in childhood. Interestingly this up-regulation was more pronounced in the group of women as compared to males (+133% in depressed women with childhood trauma versus CTRL with no childhood trauma,  $p < 0.05$ ; +74% in depressed males with childhood trauma versus CTRL with no childhood trauma,  $p < 0.05$ ). No significant effect was observed in CTRL subjects who experienced trauma early in life, as compared to not-exposed CTRLs (−11%,  $p > 0.05$ ) (see Fig. 4).

### 3.5. Identification of biological systems targeted by *mir-30a-5p* and involved in the long-term effect of PNS

In order to identify the biological systems that may be affected as consequence of the altered methylation status and expression levels of

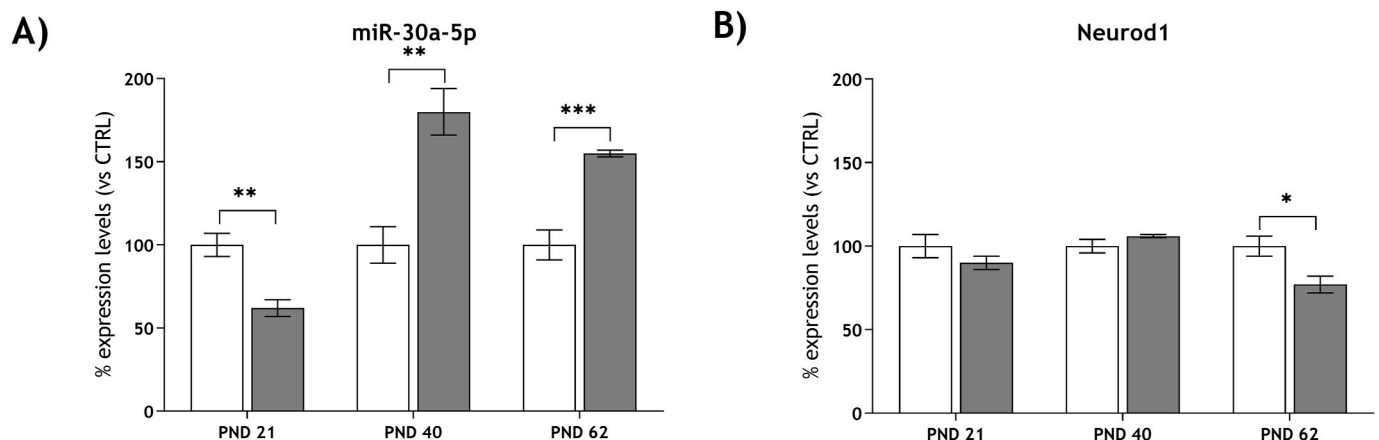


**Fig. 4.** Analysis of the *miR30a-5p* levels in blood samples of healthy controls and depressed subjects exposed to childhood trauma.

The data, expressed as the mean ± SEM, represent the Relative Expression Ratio of *miR-30a-5p*, normalized to RNU24 levels in control subjects, with or without childhood trauma, as compared to a cohort of depressed patients exposed to trauma. \*\*\* $p < 0.001$  vs CTRL not exposed to Childhood trauma.

*miR-30a-5p* in PNS rats we used two different approaches that we have then integrated.

As first approach we used the miRWalk2.0 database (Dweep and Gretz, 2015), a miRNA database that provides evidence for experimentally validated and predicted miRNA-target interactions. Since



**Fig. 3.** Time course analysis of *miR30a-5p* and *Neurod1* mRNA levels in the prefrontal cortex of male rats exposed to prenatal stress.

The expression levels of *miR30a-5p* (A) and *Neurod1* (B) were investigated at weaning (PND21), at peripuberty (PND40) and at adulthood (PND62). The data, expressed as the mean ± SEM of at least 5 independent determinations, represent the percentage of CTRL rats (not exposed to PNS) at each respective age (set at 100%). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. CTRL animals (Student's test).

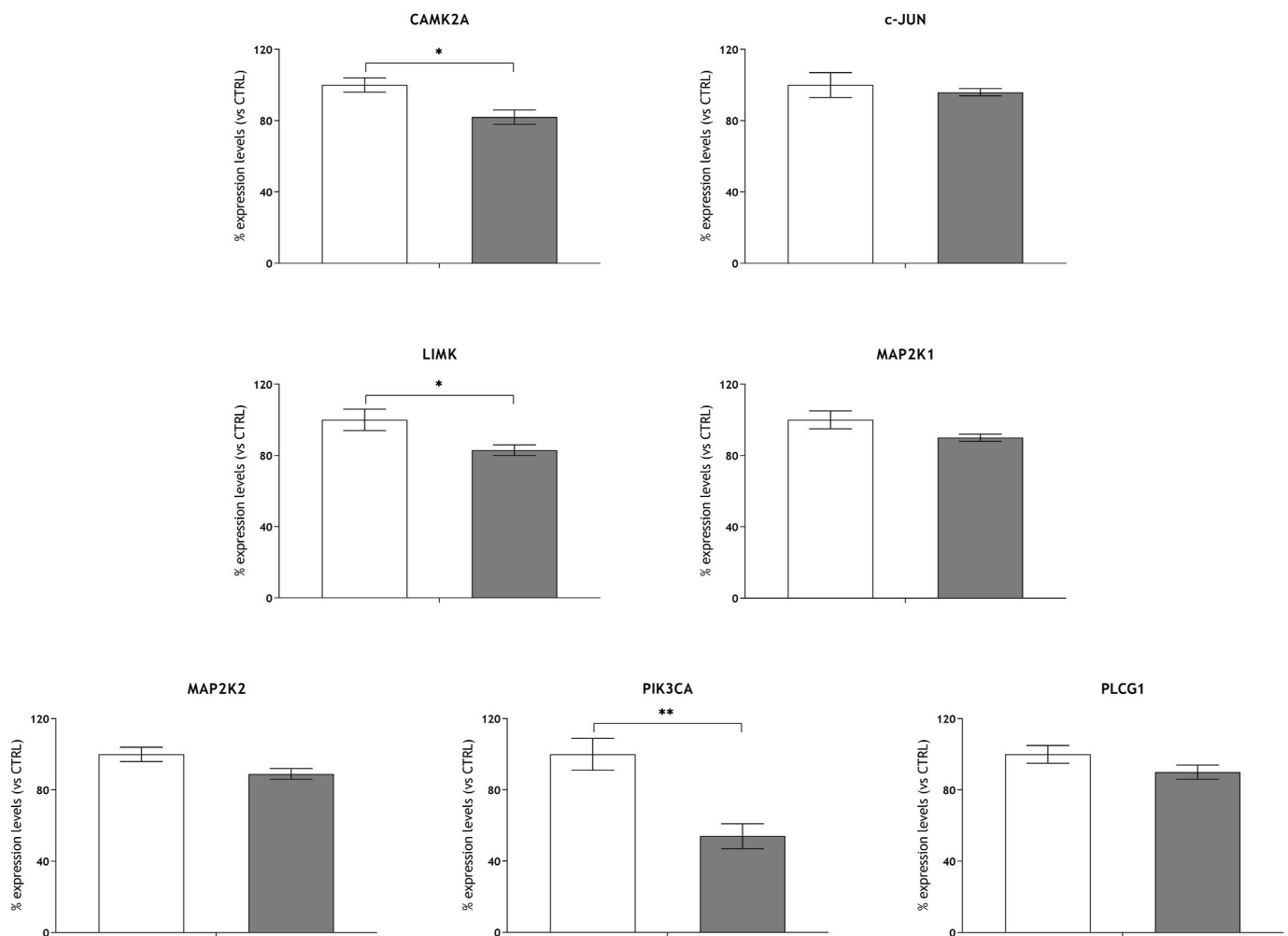
miR-30a-5p seed sequence has 100% homology between *Rattus Norvegicus* and *Homo sapiens* and the whole identity between the two sequences is of 97%, we accepted the list of validated targets of miR-30a-5p in both species to be the results of this analysis (the list of validated genes in humans and rodents is provided as [Supplementary Table 3](#)). We then used Ingenuity Pathway Analysis (IPA) (<http://www.ingenuity.com/products/ipa>) to identify pathways found to be significantly altered as a consequence of miR-30a-5p expression changes on its downstream validated targets. We identified in this way a list of 245 significant pathways.

As second approach we have interrogated mirPath v.3 (<http://nf-515788.vm.okeanos.grnet.gr>) to get a list of pathways that are modulated by our miR-30a-5p and we have identified 31 pathways with Axonal guidance and Neurotrophin signaling pathways in the 2nd and 17th position within the list.

We have then overlapped the list of 245 pathways with the 31 pathways to identify 11 pathways in common among the two approaches. These 11 pathways included: Axon guidance, B Cell Receptor Signaling, Amyotrophic lateral sclerosis (ALS), T cell receptor signaling pathway, Gap junction, Type II diabetes mellitus, PI3K-Akt signaling pathway, Prostate cancer, VEGF signaling pathway, Neurotrophin signaling pathway, Hypertrophic cardiomyopathy. Among them we selected the Neurotrophin signaling and Axonal guidance signaling, because are known to be affected by stress exposure so relevant for our experimental condition. The two pathways with the miR-30a-5p

targeted genes are shown in [Supplementary Figs. 1 and 2](#).

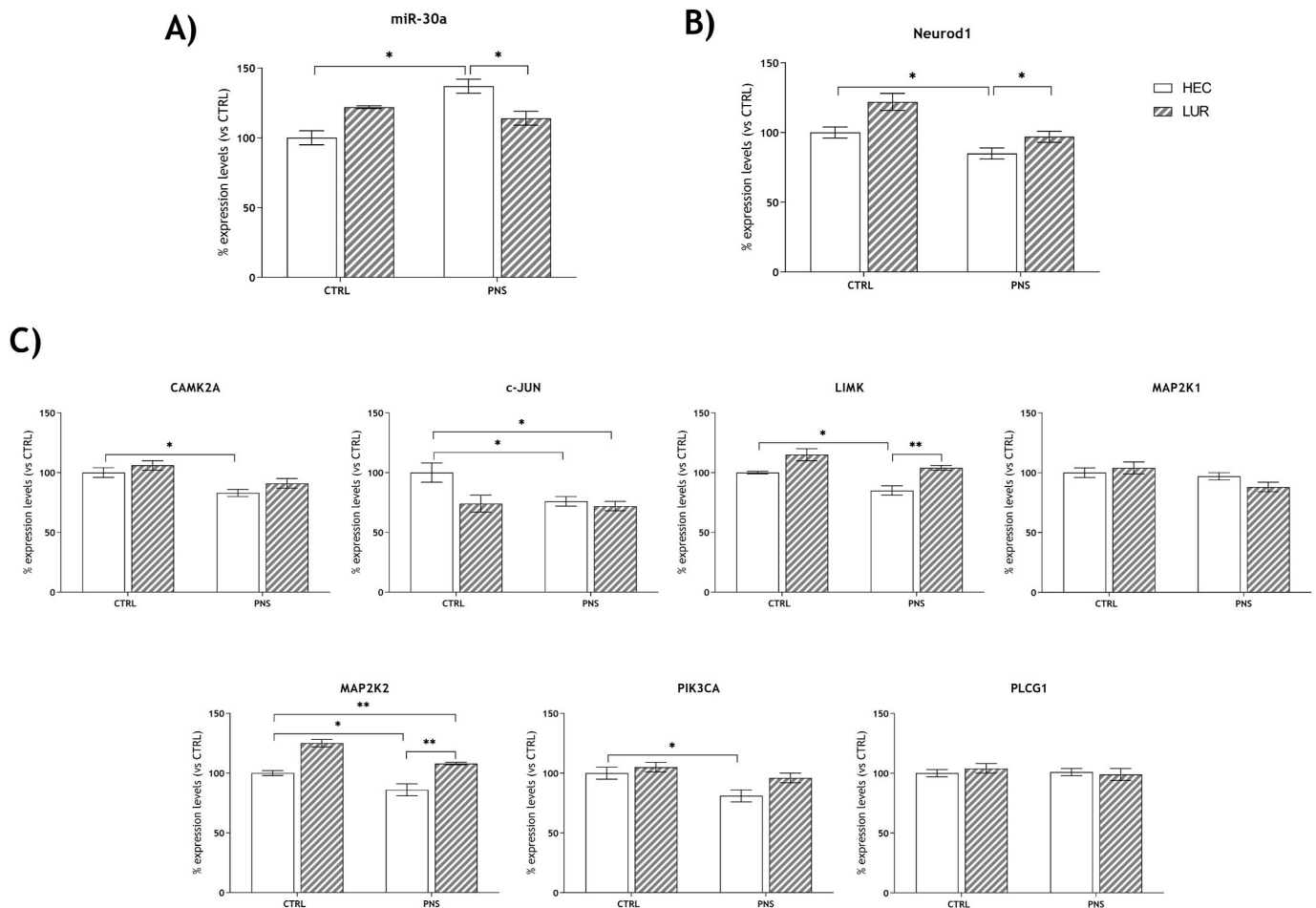
In order to determine whether PNS-induced changes of miR-30a-5p levels could have a significant impact on the modulation of biological processes involved in neurodevelopment, via *Neurotrophin/TRK signaling* and the *Axonal Guidance signaling*, we selected a panel of 7 genes (CAMK2A, c-JUN, LIMK1, MAP2K1, MAP2K2, PIK3CA, PLCG1) that are all involved in these two biological pathways and also validated target genes of miR-30a-5p as well as known to be important for neuroplasticity (Matsuo et al., 2009; Yamasaki et al., 2008; Ogundele and Lee, 2018; Fuchsova et al., 2015; Todorovski et al., 2015; Liu et al., 2016; Butler et al., 2016; Kordi-Tamandani and Mir, 2016). Hence, we analyzed their expression levels in the PFC of adult PNS rats, compared to controls. In line with the changes observed for miR-30a-5p, we found an overall reduction in the mRNA levels of all selected genes, although statistically significant only for CAM2KA (−18%,  $p < 0.05$ ), LIMK1 (−17%,  $p < 0.05$ ) and PIK3CA (−46%,  $p < 0.01$ ) (see [Fig. 5](#)). Interestingly, despite the limited differences in terms of individual expression, a simple logistic classification model based on the combined expression of CAMK2A, c-JUN, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1 reached an area under the curve of 0.87 in the analyzed dataset. Such a classification performance, though potentially inflated due to the absence of an independent testing cohort, suggests that PNS-induced alterations in the expression levels of CAMK2A, c-JUN, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1 can exert a cumulative effect, which produces an overall downregulation of the pathways modulated



**Fig. 5.** Analysis of the mRNA levels for genes that are target of miR30a-5p, as measured in the prefrontal cortex from male rats exposed to prenatal stress.

The data, expressed as the mean  $\pm$  SEM of at least 6 independent determinations, represent the percentage of CTRL rats (not exposed to PNS), set at 100%. \* $p < 0.05$  and \*\* $p < 0.01$  vs. CTRL animals (Student's test).





**Fig. 6.** Modulation of *Neurod1*, *miR-30a-5p* and its target genes in the prefrontal cortex of adult male rats exposed to prenatal stress and treated with lurasidone during peri puberty.

The mRNA levels of *miR-30a-5p* (A), *Neurod1* (B) and of the validated target genes for *miR-30a-5p* (C) were analyzed at PND62 in the PFC of male rats exposed to PNS and treated with vehicle, *Hydroxyethylcellulose* (HEC), or lurasidone during peri puberty (from PND35 to PND49), as compared to Ctrl animals. The data, expressed as percentage changes of CTRL rats set at 100%, are the mean  $\pm$  SEM of at least 6 independent determinations.

\* $p < 0.05$ ; \*\* $p < 0.01$  (Two-way ANOVA following Fisher's LSD post-hoc comparison).

by these genes. The biologically effect driven by *miR-30a-5p* can be associated with the combined effect exerted on the expression regulation of multiple genes rather than from a single causal-gene. Therefore, to test this hypothesis, we derived a prediction model of stress status based on CAMK2A, c-JUN, LIMK1, MAP2K1, MAP2K2, PIK3CA and PLCG1 expression levels. The developed logistic model is based on the linear additive effect of individual gene mRNA levels. A leave one-out approach was implemented to train the model while permutation-based tests were generated to compute sensitivity and specificity of the classification model. Despite an independent test cohort is required to corroborate the result, the area under the curve (AUC) of such a stress status classification model reached a value 0.875 (see Supplementary Fig. 3). These results are in line with a potential cumulative effect due to the combined gene expression downregulation of the analyzed *miR-30a-5p* target genes.

### 3.6. Lurasidone treatment during adolescence to counteract the effect of PNS on *miR-30a-5p* levels and on its target genes and signaling

Because of the long-lasting effects of PNS on *miR-30a-5p* upregulation, we wondered whether a preventive pharmacological intervention would have potential protective properties on such alterations. Thus, we performed another study with the exposure to PNS and, starting from PND35, we treated a group of PNS rats (or controls) with the

antipsychotic and mood-stabilizing drug lurasidone for 2 weeks. Animals were then sacrificed at PND62 in order to analyze the expression levels of *miR-30a-5p* and its target genes in the PFC of male animals.

First of all, as shown in Fig. 6A, we confirmed that PNS exposure produced a significant upregulation of *miR-30a-5p* levels (+37% vs. CTRL,  $p < 0.05$ ) and a significant downregulation of *Neurod1* (−15%,  $p < 0.05$ , see Fig. 6B) at PND62. Interestingly, both effects were prevented by lurasidone treatment during the peripubertal period (PND35), since the levels of *miR-30a-5p* and of *Neurod1* in PNS rats treated with lurasidone were not significantly different from CTRL animals (+14% vs. CTRL,  $p > 0.05$ ; −3% vs CTRL,  $p > 0.05$ ) (see Fig. 6A and B).

Next, we measured the expression levels of the 7 genes targeted by *miR-30a-5p* and involved in the *Neurotrophin/TRK* and *Axon guidance signaling* pathways. We confirmed the presence of an overall reduction in the selected genes as found in the first PNS experiment, with CAMK2A (−17%,  $p < 0.05$ ), LIMK1 (−15%,  $p < 0.05$ ), MAP2K2 (−14%,  $p < 0.05$ ), PIK3CA (−19%,  $p < 0.05$ ) statistically significant, an effect that was not observed in PNS rats treated with lurasidone, providing further support to the ability of this broadly effective drug to prevent the changes produced by this prenatal manipulation (See Fig. 6C).

## 4. Discussion

In this study, we used an unbiased genome-wide DNA methylation

analysis and we identified miR-30a as a potential mediator of the long-term changes caused by an exposure to early life stress. We found that the methylation status of the miR-30a gene is significantly reduced in the HIP and PFC of adult male and female rats whose mothers had been exposed to stress during the last week of gestation, as compared to controls. These changes are associated with an altered expression of its mature form, namely miR-30a-5p, and of its downstream target genes and signaling, some of which have previously been associated with the pathogenesis of psychiatric diseases, such as schizophrenia and major depression (Fan et al., 2018; Hagihara et al., 2016; Kordi-Tamandani and Mir, 2016; Liu et al., 2016, 2017; Mao et al., 2019; Ogundele and Lee, 2018; Yang et al., 2017). Interestingly, we also found that miR-30a-5p levels were significantly elevated in the blood of depressed patients who experienced traumatic events early in life, as compared to controls. Last, we showed that sub-chronic treatment during adolescence with the antipsychotic and mood stabilizing drug lurasidone was able to ameliorate the effect of PNS exposure on the alterations of miR-30a-5p and its related downstream targets.

There is now compelling evidence that altered developmental trajectories of brain structures and circuits, as a result of exposures to stressful events early in life (Bale et al., 2010; Lucassen et al., 2013), lead to an enhanced vulnerability for different psychiatric diseases at adulthood (Maccari et al., 2014). Epigenetic changes, such as DNA methylation, are considered crucial mechanisms leading to long-term dysfunction resulting from early life stress exposure, not only because they may determine persistent modifications in gene expression, but also through their interference with gene expression regulation (McGowan and Szyf, 2010; Provencal and Binder, 2015). Using an unbiased whole genome approach, we analyzed the methylation profiles of genes affected by PNS and identified a large number of differentially methylated genes in the PFC and HIP of rats exposed to stress during gestation, compared to non-exposed animals. When restricting the list of these genes to those whose methylation changes occurred around the TSS and that were in common across the two brain regions and sexes, we found 12 potential genes for mediating the long-term effects of stress exposure. Among them, miR-30a and Neurod1 were the only two genes interacting with each other (Liu et al., 2017), representing two interesting candidates as they have also been previously associated with the pathogenesis of psychiatric disorders (Liu et al., 2017; Mellios et al., 2008; Perkins et al., 2007; Wan et al., 2015).

We found that the hypo-methylated status of mir-30a in the HIP and PFC from PNS rats (males and females) was paralleled by a significant increase of miR-30a-5p expression levels, the most abundant among the mature forms of mir-30a (Mellios et al., 2008). The preferential loading of the strand into the miRISC complex is a mechanism that is thermodynamically regulated, though it also depends on the identity of the 5'-terminal nucleotides, with a higher degree of affinity between AGO proteins and U or A nucleotides (Frank et al., 2010). We hypothesized that this mechanism of strand selection may also apply to mir-30a, since miR-30a-3p possesses a C at its 5' end, while miR-30a-5p has a U. Our results show consistency between the DNA methylation status and miR-30a-5p expression levels in all conditions (different brain areas and sexes), with the exception of female PFC, a discrepancy that may be due to the fact that the reduced methylation in female PFC occurred at a different location, compared to male PFC and to male and female HIP. Of note, the expression of several members of the microRNA-30 family, including miR-30 b, miR-30 d, miR-30e and the one analyzed in the present study, miR-30a-5p, are also altered in the PFC of patients affected by schizophrenia, a disorder linked to early life stress exposure (Perkins et al., 2007).

Conversely, although a similar hypo-methylation status was observed for Neurod1, its mRNA levels were significantly reduced in the PFC of adult PNS rats, as compared to non-exposed animals. Since Neurod1 is a target of miR-30a-5p (Liu et al., 2017), we hypothesize that the decreased expression of Neurod1 may represent the consequence of the miR-30a-5p changes, rather than changes in DNA methylation.

Interestingly, a significant upregulation of miR-30a was found at PND40 and was maintained until PND62, suggesting that it may represent the consequence of an alteration in the developmental profile governing its expression. On the other hand, a significant down-regulation of Neurod1 was observed only at PND62, supporting our idea that lower levels of Neurod1 in early adulthood (PND62) could be the consequence of PNS-induced miR-30a alterations in adolescence. Neurod1 is a basic helix-loop-helix transcription factor that plays an important role during neuronal differentiation and it is required for neurogenesis as well as for migration of newborn neurons, by regulating the underlying transcriptional program. Interestingly, lower levels of Neurod1 expression levels were found in human induced pluripotent stem cells (hiPSCs) derived from hippocampal neural progenitor cells (NPCs) of schizophrenic patients as compared to those of controls (Yu et al., 2014). Moreover, an open target platform (<https://www.targetvalidation.org/>), a repository for gene prioritization based on multiple levels of information, showed a pivotal association for Neurod1 with schizophrenia and behavioral traits according to a text-mining literature analysis and animal models.

Indeed, to further investigate the potential role of the genes differentially regulated in terms of methylation and expression in association with PNS, we used public available resources to evaluate possible genetic associations between these genes and psychiatry diseases. Concerning the miR-30a-5p target genes that we selected, genome-wide significant association with psychiatric disorders were found for PLCG1 and PIK3CA. In particular, two variants within the PIK3CA genomic region have been associated with schizophrenia (rs9859557 (Li et al., 2017) and rs34796896 (Pardinas et al., 2018)), while the variant rs6065325 within PLCG1 has been associated with an enhanced risk for depression (Turley et al., 2018). Of particular interest, open target platform (<https://www.targetvalidation.org/>), a repository for gene prioritization based on multiple levels of information (e.g., genetic, expression, animal model, literature integration) showed a pivotal association for Neurod1 with schizophrenia and behavioral traits according to a text-mining literature analysis and animal models.

It has also been found that a single intraperitoneal injection of the mood stabilizer valproic acid into male rat pups at PND7, the age corresponding to the peak in brain growth and connections, is associated with a significant decrease of Neurod1 in the hippocampus and with the development of depressive and anxiety-like behaviors at late postnatal ages (Wang et al., 2016). On these bases, the reduced levels of Neurod1 in the brain of adult animals exposed to PNS may indicate the presence of a long-term disruption in key neurodevelopmental mechanisms that could underlie the development of altered behaviors as a consequence of adverse experiences early in life.

In line with the findings obtained in the brain of PNS exposed rats, we observed a significant increase in the expression levels of miR-30a-5p in depressed patients exposed to traumatic events early in life, as compared to CTRLs. These results corroborate previous data showing that traumatic and stressful events, especially when experienced during the perinatal period and childhood, significantly increase the risk for stress-related psychiatric disorders, such as depression, during adulthood (Kaczmarczyk et al., 2018; Menke et al., 2018; Stenz et al., 2018). Our cohort of depressed patients with a childhood trauma history is however represented by patients that are treated with antidepressant drugs, so we can't exclude an effect of antidepressant drugs on miR-30a-5p modulation, however, we suggest that, also in humans, early life stress may lead to neurodevelopmental changes with an increased risk for psychiatric disorders, also through the epigenetic regulation of miR-30a-5p expression.

Since each miRNA can regulate hundreds of genes, an important consequence for the changes observed after exposure to early life stress is represented by alterations of different interconnected pathways in multiple brain regions, which will eventually contribute to the vulnerability for a variety of neuropsychiatric disorders which are known to be related by a wide range of other data (Beveridge and Cairns, 2012; Luoni

and Riva, 2016). We found that miR-30a-5p is involved in the modulation of several brain-related mechanisms, particularly in the *Neurotrophin/TRK signaling* and *Axonal Guidance signaling*. In accordance with the up-regulation of miR-30a-5p levels in PNS rats, we found a significant decrease in the expression of CAMK2A, LIMK1 and PIK3CA, which belong to these pathways. CAMK2A belongs to a calcium-calmodulin-dependent kinases family, which plays a key role in the regulation of neuronal growth and synaptic functions. Previous studies have shown that genetic deletion of hippocampal CAMK2A in mice caused synaptic and behavioral defects associated with schizophrenia (Matsuo et al., 2009; Yamasaki et al., 2008). Moreover, since CAMK2A facilitates N-methyl-D-aspartate receptors (NMDARs), its reduced activity may contribute to the hypofunction of NMDAR that has been associated to the pathophysiology of this disorder (Ogundele and Lee, 2018).

Interestingly, a reduced expression of CAMK2A was also found in the hippocampus of depressed suicides, which provides further support to the role of this kinase in different psychiatric conditions (Fuchsova et al., 2015). Similarly, LIMK1 belongs to a family of serine/threonine protein kinases involved in neuronal development, synaptic function as well as learning and memory (Todorovski et al., 2015). In addition, LIMK1 has been identified as a key molecule in the postsynaptic mechanism by which Neuroligin 1 (NLG1), which has been associated with mental disorders such as schizophrenia, regulates synapse development and function (Liu et al., 2016). Last, the PIK3CA gene, a member of the phosphoinositide-3-kinases (PI3Ks) that encodes the alpha catalytic subunit of the PI3K enzyme, has been widely associated to neurodevelopmental disorders, including schizophrenia (Butler et al., 2016; Kordi-Tamandani and Mir, 2016).

All in all, we suggest that the alterations of miR-30a-5p levels produced by the adverse experience during gestation may contribute to the down-regulation of CAMK2A, LIMK1 and PIK3CA, which have been associated with schizophrenia and depression, leading to a deficit in neuroplasticity, a key mechanism for the susceptibility for psychiatric disorders.

Interestingly, miR-30a-5p exerts also a pronounced inhibitory effect on the neurotrophin Brain Derived Neurotrophic factor (BDNF) by binding to the long 3'-UTR of the neurotrophin (Darcq et al., 2015; Mellios et al., 2008). Indeed, it has been demonstrated that the over-expression of miR-30a-5p in the medial-PFC reduces BDNF expression and has functional effects (Darcq et al., 2015). In line with this, we have previously demonstrated that the levels of long 3'-UTR BDNF mRNA were significantly reduced in the PFC of rats exposed to stress during gestation (Luoni et al., 2014a). The association between miR-30a-5p and BDNF is particularly intriguing considering the role of the neurotrophin in different psychiatric conditions (Krishnan and Nestler, 2008). Indeed, a reduction of BDNF expression was found in brain specimen from depressed and schizophrenic subjects (Dwivedi, 2013; Hashimoto et al., 2005), an evidence supported by a number of preclinical studies that have demonstrated a strong link between BDNF and psychiatric disorders (Begni et al., 2017; Cattaneo et al., 2016).

The changes of miR-30a levels as a consequence of PNS exposure become manifest around peripuberty, suggesting that they may precede the functional and molecular alterations that take place at adulthood. Accordingly, therapeutic intervention during the peripubertal period may prove effective in counteracting some of the changes produced by early life stress. In line with this possibility, we show that sub-chronic treatment with the antipsychotic drug lurasidone, given during the peripubertal period, was able to prevent PNS-induced changes of miR-30a-5p levels as well as of its target genes involved in Neurotrophin/TRK and Axonal Guidance signaling, with a major effect on CAMK2A, LIMK1 and PIK3CA. Importantly, we have already demonstrated that a similar treatment is effective in preventing the reduction of BDNF levels caused by PNS exposure (Luoni et al., 2014a). Lurasidone is a multi-receptor modulator (Ishibashi et al., 2010) that, beyond its well-established antipsychotic activity, possesses pro-cognitive and

antidepressant efficacy, at clinical level and in different experimental models (Calabrese et al., 2020; Luoni et al., 2014b; Rossetti et al., 2018; Tarazi and Riva, 2013), which appears to rely on its ability to promote neuronal plasticity (Calabrese et al., 2020; Luoni et al., 2014a, 2014b; Rossetti et al., 2018; Tarazi and Riva, 2013). The present results confirm these findings and provide support to the potential of lurasidone in preventing the long-term negative effects of PNS exposure on neuroplasticity.

Some limitations related to our study have to be mentioned. Given the complexity of the experimental paradigm, we were not able to introduce a group of animals that underwent behavioral testing at adulthood, although we have recently demonstrated that, among other alterations, PNS rats show a significant impairment in working memory (Cattaneo et al., 2019). Secondly, we cannot exclude the possibility that changes in maternal care can contribute to the long-term effects observed following a gestational stress. Moreover, while our approach was useful to identify miR-30a, a number of other relevant genes may be regulated by PNS in a brain region- and sex-specific manner. While such analyses were not the purpose of the present study, they will provide additional information on the long-term functional impact of early life stress. Last, we are aware that additional experiments manipulating miR-30a-5p will be needed to establish the cause and effect relationship between this miRNA and the long-term consequences of early life stress exposure.

In conclusion, the present study provides further evidence that the long-term consequences of early life stress on adult pathological states are associated with significant changes in DNA methylation, which may affect relevant genes, including miRNAs, producing stable alterations as master regulators of gene expression. Indeed, miR-30a-5p regulates hundreds of downstream targets that, being involved in neurodevelopmental processes, may represent an important biological signature associated with the risk to develop psychiatric disorders as a consequence of the exposure to early life adversities.

#### Disclosure/conflict of interest

M.A.R. has received compensation as speaker/consultant from Angelini Pharma, Lundbeck, Otsuka, Recordati, Sumitomo Dainippon Pharma and Sunovion, and he has received research grants from Sumitomo Dainippon Pharma and Sunovion, although none of these had a role in the preparation and publication of this article. K.D. is a member of the Janssen Pharmaceuticals, Inc. Steering Committee Neurosciences. A.M. has received compensation as speaker from the health insurance company AOK, and the companies Servier, Mecice and Recordati. All the other authors have no disclosures.

#### Author contributions

A.C., V.B., A.L., A.B. and F.C. contributed to the experiments in the PNS model. A.C., Ma.S., V.B., A.L. and Mo.S. contributed to the whole-genome procedure and analysis. A.C., N.C., M.M. and I.D.A. performed gene expression analyses in the PNS model and in the clinical cohorts. C.M. contributed with statistical and bioinformatic analyses.

K.W., L.F., K.D. and A.M. recruited depressed patients and control subjects characterized for childhood trauma and collected peripheral blood samples.

A.C., N.C., M.M. and M.A.R. managed the literature searches. A.C., N.C., M.M. C.M. and A.L. contributed to the first draft of the manuscript, A.C., C.P., F.C. and A.M. contributed to the revision, and M.A.R. revised all the versions of the manuscript and approved the final one.

All the authors have contributed to and have approved the final manuscript.

#### Data availability

Data will be made available on request.

## Acknowledgments

This work was supported by an ERANET Neuron grant to M.S., M.A.R. and F.C. (EMBED), as well as by grants from the Italian Ministry of University and Research to M.A.R. (PRIN – grants number 2015SKN9YT and 2017AY8BP4 and PON “Ricerca e Innovazione” PerMedNet -project ARS01\_01226) and from Fondazione CARIPLO (grant number 2012–0503) to M.A.R..

A.C., N.C., M.M. and I.D.A. have been supported by the Italian Ministry of Health (Ricerca Corrente). We are also grateful to Sumitomo Dainippon Pharma Co. Ltd for the generous gift of Lurasidone. This work was also supported by the Interdisciplinary Center for Clinical Research, University of Würzburg research grants N-355 and N-258 to A.M. and L.H.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jynstr.2020.100271>.

## References

- Anda, R.F., Felitti, V.J., Bremner, J.D., Walker, J.D., Whitfield, C., Perry, B.D., Dube, S. R., Giles, W.H., 2006. The enduring effects of abuse and related adverse experiences in childhood. A convergence of evidence from neurobiology and epidemiology. *Eur. Arch. Psychiatr. Clin. Neurosci.* 256 (3), 174–186.
- Bale, T.L., 2014. Lifetime stress experience: transgenerational epigenetics and germ cell programming. *Dialogues Clin. Neurosci.* 16 (3), 297–305.
- Bale, T.L., Baram, T.Z., Brown, A.S., Goldstein, J.M., Insel, T.R., McCarthy, M.M., Nemeroff, C.B., Reyes, T.M., Simerly, R.B., Sussner, E.S., Nestler, E.J., 2010. Early life programming and neurodevelopmental disorders. *Biol. Psychiatr.* 68 (4), 314–319.
- Beck, A.T., Ward, C.H., Mendelson, M., Mock, J., Erbaugh, J., 1961. An inventory for measuring depression. *Arch. Gen. Psychiatr.* 5, 561–571.
- Begni, V., Riva, M.A., Cattaneo, A., 2017. Cellular and molecular mechanisms of the brain-derived neurotrophic factor in physiological and pathological conditions. *Clin. Sci.* 131 (2), 123–138.
- Bernstein, D.P., Fink, L., Handelsman, L., Foote, J., Lovejoy, M., Wenzel, K., et al., 1994. Initial reliability and validity of a new retrospective measure of child abuse and neglect. *Am. J. Psychiatr.* 151, 1132–1136.
- Beveridge, N.J., Cairns, M.J., 2012. MicroRNA dysregulation in schizophrenia. *Neurobiol. Dis.* 46 (2), 263–271.
- Beveridge, N.J., Santarelli, D.M., Wang, X., Tooney, P.A., Webster, M.J., Weickert, C.S., Cairns, M.J., 2014. Maturation of the human dorsolateral prefrontal cortex coincides with a dynamic shift in microRNA expression. *Schizophr. Bull.* 40 (2), 399–409.
- Bick, J., Nelson, C.A., 2016. Early adverse experiences and the developing brain. *Neuropsychopharmacology* 41 (1), 177–196.
- Brugha, T.S., Cragg, D., 1990. The List of Threatening Experiences: the reliability and validity of a brief life events questionnaire. *Acta Psychiatr. Scand.* 82 (1), 77–81.
- Butler, M.G., McGuire, A.B., Masoud, H., Manzardo, A.M., 2016. Currently recognized genes for schizophrenia: high-resolution chromosome ideogram representation. *Am J Med Genet B Neuropsychiatr Genet* 171B (2), 181–202.
- Calabrese, F., Brivio, P., Sbrini, G., Gruca, P., Lason, M., Litwa, E., Niemczyk, M., Papp, M., Riva, M.A., 2020. Effect of lurasidone treatment on chronic mild stress-induced behavioural deficits in male rats: the potential role of glucocorticoid receptor signalling. *J. Psychopharmacol.* 34 (4), 420–428.
- Cattaneo, A., Begni, V., Malpighi, C., Cattaneo, N., Luoni, A., Pariante, C., Riva, M.A., 2019. Transcriptional signatures of cognitive impairment in rat exposed to prenatal stress. *Mol. Neurobiol.* 56 (9), 6251–6260.
- Cattaneo, A., Cattaneo, N., Begni, V., Pariante, C.M., Riva, M.A., 2016. The human BDNF gene: peripheral gene expression and protein levels as biomarkers for psychiatric disorders. *Transl. Psychiatry* 6 (11), e958.
- Darcq, E., Warnault, V., Phamluong, K., Besserer, G.M., Liu, F., Ron, D., 2015. MicroRNA-30a-5p in the prefrontal cortex controls the transition from moderate to excessive alcohol consumption. *Mol. Psychiatr.* 20 (10), 1261.
- Down, T.A., Rakyan, V.K., Turner, D.J., Flicek, P., Li, H., Kulesha, E., Graf, S., Johnson, N., Herrero, J., Tomazou, E.M., Thorne, N.P., Backdahl, L., Herberth, M., Howe, K.L., Jackson, D.K., Miretti, M.M., Marioni, J.C., Birney, E., Hubbard, T.J., Durbin, R., Tavare, S., Beck, S., 2008. A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. *Nat. Biotechnol.* 26 (7), 779–785.
- Dweep, H., Gretz, N., 2015. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat. Methods* 12 (8), 697.
- Dwivedi, Y., 2013. Involvement of brain-derived neurotrophic factor in late-life depression. *Am. J. Geriatr. Psychiatr.* 21 (5), 433–449.
- Fan, C., Zhu, X., Song, Q., Wang, P., Liu, Z., Yu, S.Y., 2018. MiR-134 modulates chronic stress-induced structural plasticity and depression-like behaviors via downregulation of Limk1/cofilin signaling in rats. *Neuropharmacology* 131, 364–376.
- Frank, F., Sonenberg, N., Nagar, B., 2010. Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature* 465 (7299), 818–822.
- Fuchsova, B., Alvarez Julia, A., Rizavi, H.S., Frasch, A.C., Pandey, G.N., 2015. Altered expression of neuroplasticity-related genes in the brain of depressed suicides. *Neuroscience* 299, 1–17.
- Fumagalli, F., Molteni, R., Racagni, G., Riva, M.A., 2007. Stress during development: impact on neuroplasticity and relevance to psychopathology. *Prog. Neurobiol.* 81 (4), 197–217.
- Garnefski, N., Rief, C., Jellesma, F., Terwogt, M.M., Kraaij, V., 2007. Cognitive emotion regulation strategies and emotional problems in 9-11-year-old children: the development of an instrument. *Eur. Child Adolesc. Psychiatr.* 16 (1), 1–9.
- Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5 (10), R80.
- Gur, T.L., Palkar, A.V., Rajasekera, T., Allen, J., Niraula, A., Godbout, J., Bailey, M.T., 2019. Prenatal stress disrupts social behavior, cortical neurobiology and commensal microbes in adult male offspring. *Behav. Brain Res.* 359, 886–894.
- Hagihara, H., Horikawa, T., Nakamura, H.K., Umemori, J., Shoji, H., Kamitani, Y., Miyakawa, T., 2016. Circadian gene circuitry predicts hyperactive behavior in a mood disorder mouse model. *Cell Rep.* 14 (12), 2784–2796.
- Hamilton, M., 1960. A rating scale for depression. *J. Neurol. Neurosurg. Psychiatr.* 23, 56–62.
- Hao, Z.Y., Zhong, Y., Ma, Z.J., Xu, H.Z., Kong, J.Y., Wu, Z., Wu, Y., Li, J., Lu, X., Zhang, N., Wang, C., 2020. Abnormal resting-state functional connectivity of hippocampal subfields in patients with major depressive disorder. *BMC Psychiatr.* 20 (1), 71.
- Hashimoto, T., Bergen, S.E., Nguyen, Q.L., Xu, B., Monteggia, L.M., Pierri, J.N., Sun, Z., Sampson, A.R., Lewis, D.A., 2005. Relationship of brain-derived neurotrophic factor and its receptor TrkB to altered inhibitory prefrontal circuitry in schizophrenia. *J. Neurosci.* 25 (2), 372–383.
- Herzog, J.I., Schmahl, C., 2018. Adverse childhood experiences and the consequences on neurobiological, psychosocial, and somatic conditions across the lifespan. *Front. Psychiatr.* 9, 420.
- Ishibashi, T., Horisawa, T., Tokuda, K., Ishiyama, T., Ogasa, M., Tagashira, R., Matsumoto, K., Nishikawa, H., Ueda, Y., Toma, S., Oki, H., Tanno, N., Saji, I., Ito, A., Ohno, Y., Nakamura, M., 2010. Pharmacological profile of lurasidone, a novel antipsychotic agent with potent 5-hydroxytryptamine 7 (5-HT7) and 5-HT1A receptor activity. *J. Pharmacol. Exp. Therapeut.* 334 (1), 171–181.
- Ishiyama, T., Tokuda, K., Ishibashi, T., Ito, A., Toma, S., Ohno, Y., 2007. Lurasidone (SM-13496), a novel atypical antipsychotic drug, reverses MK-801-induced impairment of learning and memory in the rat passive-avoidance test. *Eur. J. Pharmacol.* 572 (2–3), 160–170.
- Kaczmarczyk, M., Wingenfeld, K., Kuehl, L.K., Otte, C., Hinkelmann, K., 2018. Childhood trauma and diagnosis of major depression: association with memory and executive function. *Psychiatr. Res.* 270, 880–886.
- Kolb, B., Mychasiuk, R., Muhammad, A., Li, Y., Frost, D.O., Gibb, R., 2012. Experience and the developing prefrontal cortex. *Proc. Natl. Acad. Sci. U. S. A.* 109 (Suppl. 2), 17186–17193.
- Kordi-Tamandani, D.M., Mir, A., 2016. Relationship between phosphoinositide-3-kinase genetic polymorphism and schizophrenia. *Nord. J. Psychiatr.* 70 (4), 272–275.
- Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. *Nature* 455 (7215), 894–902.
- Kruegers, H.J., Arp, J.M., Xiong, H., Kanatsou, S., Lesuis, S.L., Korosi, A., Joels, M., Lucassen, P.J., 2017. Early life adversity: lasting consequences for emotional learning. *Neurobiol. Stress.* 6, 14–21.
- Levone, B.R., Cryan, J.F., O'Leary, O.F., 2015. Role of adult hippocampal neurogenesis in stress resilience. *Neurobiology of stress* 1, 147–155.
- Li, Z., Chen, J., Yu, H., He, L., Xu, Y., Zhang, D., Yi, Q., Li, C., Li, X., Shen, J., Song, Z., Ji, W., Wang, M., Zhou, J., Chen, B., Liu, Y., Wang, J., Wang, P., Yang, P., Wang, Q., Feng, G., Liu, B., Sun, W., Li, B., He, G., Li, W., Wan, C., Xu, Q., Li, W., Wen, Z., Liu, K., Huang, F., Ji, J., Ripke, S., Yue, W., Sullivan, P.F., O'Donovan, M.C., Shi, Y., 2017. Genome-wide association analysis identifies 30 new susceptibility loci for schizophrenia. *Nat. Genet.* 49 (11), 1576–1583.
- Liu, A., Zhou, Z., Dang, R., Zhu, Y., Qi, J., He, G., Leung, C., Pak, D., Jia, Z., Xie, W., 2016. Neurotrophin 1 regulates spines and synaptic plasticity via LIMK1/cofilin-mediated actin reorganization. *J. Cell Biol.* 212 (4), 449–463.
- Liu, S., Zhang, F., Shugart, Y.Y., Yang, L., Li, X., Liu, Z., Sun, N., Yang, C., Guo, X., Shi, J., Wang, L., Cheng, L., Zhang, K., Yang, T., Xu, Y., 2017. The early growth response protein 1-miR-30a-5p-neurogenic differentiation factor 1 axis as a novel biomarker for schizophrenia diagnosis and treatment monitoring. *Transl. Psychiatry* 7 (1), e998.
- Lucassen, P.J., Naninck, E.F., van Goudoever, J.B., Fitzsimons, C., Joels, M., Korosi, A., 2013. Perinatal programming of adult hippocampal structure and function; emerging roles of stress, nutrition and epigenetics. *Trends Neurosci.* 36 (11), 621–631.
- Luoni, A., Berry, A., Calabrese, F., Capocchia, S., Bellisario, V., Gass, P., Cirulli, F., Riva, M.A., 2014a. Delayed BDNF alterations in the prefrontal cortex of rats exposed to prenatal stress: preventive effect of lurasidone treatment during adolescence. *Eur. Neuropsychopharmacol.* 24 (6), 986–995.
- Luoni, A., Berry, A., Raggi, C., Bellisario, V., Cirulli, F., Riva, M.A., 2016a. Sex-specific effects of prenatal stress on bdnf expression in response to an acute challenge in rats: a role for Gadd45beta. *Mol. Neurobiol.* 53 (10), 7037–7047.
- Luoni, A., Macchi, F., Papp, M., Molteni, R., Riva, M.A., 2014b. Lurasidone exerts antidepressant properties in the chronic mild stress model through the regulation of

- synaptic and neuroplastic mechanisms in the rat prefrontal cortex. *Int. J. Neuropsychopharmacol.* 18 (4).
- Luoni, A., Massart, R., Nieratschker, V., Nemoda, Z., Blasi, G., Gilles, M., Witt, S.H., Suderman, M.J., Suomi, S.J., Porcelli, A., Rizzo, G., Fazio, L., Torretta, S., Rampino, A., Berry, A., Gass, P., Cirulli, F., Rietschel, M., Bertolino, A., Deuschle, M., Szyf, M., Riva, M.A., 2016b. Ankyrin-3 as a molecular marker of early-life stress and vulnerability to psychiatric disorders. *Transl. Psychiatry* 6 (11), e943.
- Luoni, A., Riva, M.A., 2016. MicroRNAs and psychiatric disorders: from aetiology to treatment. *Pharmacol. Ther.* 167, 13–27.
- Lupien, S.J., McEwen, B.S., Gunnar, M.R., Heim, C., 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat. Rev. Neurosci.* 10, 434–445.
- Maccari, S., Krugers, H.J., Morley-Fletcher, S., Szyf, M., Brunton, P.J., 2014. The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. *J. Neuroendocrinol.* 26 (10), 707–723.
- Mao, R., Deng, R., Wei, Y., Han, L., Meng, Y., Xie, W., Jia, Z., 2019. LIMK1 and LIMK2 regulate cortical development through affecting neural progenitor cell proliferation and migration. *Mol. Brain* 12 (1), 67.
- Matsuo, N., Yamasaki, N., Ohira, K., Takao, K., Toyama, K., Eguchi, M., Yamaguchi, S., Miyakawa, T., 2009. Neural activity changes underlying the working memory deficit in alpha-CaMKII heterozygous knockout mice. *Front. Behav. Neurosci.* 3, 20.
- McGowan, P.O., Szyf, M., 2010. Environmental epigenomics: understanding the effects of parental care on the epigenome. *Essays Biochem.* 48 (1), 275–287.
- Mellios, N., Huang, H.S., Grigorenko, A., Rogae, E., Akbarian, S., 2008. A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. *Hum. Mol. Genet.* 17 (19), 3030–3042.
- Menke, A., Lehrieder, D., Fietz, J., Leistner, C., Wurst, C., Stonawski, S., Reitz, J., Lechner, K., Busch, Y., Weber, H., Deckert, J., Domschke, K., 2018. Childhood trauma dependent anxious depression sensitizes HPA axis function. *Psychoneuroendocrinology* 98, 22–29.
- Montgomery, S.A., Asberg, M., 1979. A new depression scale designed to be sensitive to change. *Br. J. Psychiatr.* : J. Ment. Sci. 134, 382–389.
- Naninck, E.F.G., Hoeijmakers, L., Kakava-Georgiadou, N., Meesters, A., Lasic, S.E., Lucassen, P.J., Korosi, A., 2015. Chronic early life stress alters developmental and adult neurogenesis and impairs cognitive function in mice. *Hippocampus* 25, 309–328.
- Naninck, E.F.G., Oosterink, J.E., Yam, K.Y., Vries, L.P. De, Schierbeek, H., Goudoever, J. B. Van, Verkaik-Schakel, R.N., Plantinga, J.A., Plosch, T., Lucassen, P.J., Korosi, A., 2017. Early micronutrient supplementation protects against early stress-induced cognitive impairments. *Faseb. J.* 31, 505–518.
- Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M.J., Krumm, B., Meier, S., Witt, S.H., Nothen, M.M., Suomi, S.J., Peus, V., Scharnholtz, B., Duka, H., Hohmeyer, C., Wolf, I.A., Cirulli, F., Gass, P., Sutterlin, M.W., Filsinger, B., Laucht, M., Riva, M.A., Rietschel, M., Deuschle, M., Szyf, M., 2014. MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. *Transl. Psychiatry* 4, e429.
- Ogundele, O.M., Lee, C.C., 2018. CaMKIIalpha expression in a mouse model of NMDAR hypofunction schizophrenia: putative roles for IGF-1R and TLR4. *Brain Res. Bull.* 137, 53–70.
- Panetta, P., Berry, A., Bellisario, V., Capoccia, S., Raggi, C., Luoni, A., Longo, L., Riva, M. A., Cirulli, F., 2017. Long-term sex-dependent vulnerability to metabolic challenges in prenatally stressed rats. *Front. Behav. Neurosci.* 11.
- Pardinas, A.F., Holmans, P., Pocklington, A.J., Escott-Price, V., Ripke, S., Carrera, N., Legge, S.E., Bishop, S., Cameron, D., Hamshire, M.L., Han, J., Hubbard, L., Lynham, A., Mantripragada, K., Rees, E., MacCabe, J.H., McCarrroll, S.A., Baune, B. T., Breen, G., Byrne, E.M., Dannowski, U., Eley, T.C., Hayward, C., Martin, N.G., McIntosh, A.M., Plomin, R., Porteous, D.J., Wray, N.R., Caballero, A., Geschwind, D. H., Huckins, L.M., Ruderfer, D.M., Santiago, E., Sklar, P., Stahl, E.A., Won, H., Agerbo, E., Als, T.D., Andreassen, O.A., Baekvad-Hansen, M., Mortensen, P.B., Pedersen, C.B., Borglum, A.D., Bybjerg-Grauholm, J., Djurovic, S., Durmishi, N., Pedersen, M.G., Golumbet, V., Grove, J., Hougaard, D.M., Mattheisen, M., Molden, E., Mors, O., Nordentoft, M., Pejovic-Milovancevic, M., Sigurdsson, E., Silagadze, T., Hansen, C.S., Stefansson, K., Stefansson, H., Steinberg, S., Tosato, S., Werge, T., Consortium, G., Consortium, C., Collier, D.A., Rujescu, D., Kirov, G., Owen, M.J., O'Donovan, M.C., Walters, J.T.R., 2018. Common schizophrenia alleles are enriched in mutation-intolerant genes and in regions under strong background selection. *Nat. Genet.* 50 (3), 381–389.
- Paus, T., Keshavan, M., Giedd, J.N., 2008. Why do many psychiatric disorders emerge during adolescence? *Nat. Rev. Neurosci.* 9 (12), 947–957.
- Perkins, D.O., Jeffries, C.D., Jarskog, L.F., Thomson, J.M., Woods, K., Newman, M.A., Parker, J.S., Jin, J., Hammond, S.M., 2007. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biol.* 8 (2), R27.
- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29 (9), e45.
- Provençal, N., Binder, E.B., 2015. The effects of early life stress on the epigenome: from the womb to adulthood and even before. *Exp. Neurol.* 268, 10–20.
- Rosenstiel, A.K., Keefe, F.J., 1983. The use of coping strategies in chronic low back pain patients: relationship to patient characteristics and current adjustment. *Pain* 17 (1), 33–44.
- Rossetti, A.C., Paladini, M.S., Colombo, M., Gruca, P., Lason-Tyburkiewicz, M., Tota-Glowczyk, K., Papp, M., Riva, M.A., Molteni, R., 2018. Chronic stress exposure reduces parvalbumin expression in the rat Hippocampus through an imbalance of redox mechanisms: restorative effect of the antipsychotic lurasidone. *Int. J. Neuropsychopharmacol.* 21 (9), 883–893.
- Sheehan, D.V., Lecrubier, Y., Sheehan, K.H., Amorim, P., Janavs, J., Weiller, E., Hergueta, T., Baker, R., Dunbar, G.C., 1998. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J. Clin. Psychiatr.* 59 (Suppl. 20), 22–33 quiz 34–57.
- Spielberger, C.D., 1983. Manual for the State-Trait Inventory STAI (Form Y). Mind Garden, Palo Alto, CA.
- Spitzer, R.L., Kroenke, K., Williams, J.B., Lowe, B., 2006. A brief measure for assessing generalized anxiety disorder: the GAD-7. *Arch. Intern. Med.* 166 (10), 1092–1097.
- Spitzer, R.L., Kroenke, K., Williams, J.B., 1999. Validation and utility of a self-report version of PRIME-MD: the PHQ primary care study. Primary care evaluation of mental disorders. Patient health questionnaire. *J. Am. Med. Assoc. JAMA, J. Am. Med. Assoc.* 282 (18), 1737–1744.
- Spitzer, R.L., Williams, J.B., Gibbon, M., First, M.B., 1992. The structured clinical interview for DSM-III-R (SCID). I: history, rationale, and description. *Arch. Gen. Psychiatr.* 49 (8), 624–629.
- Stenz, L., Schechter, D.S., Serpa, S.R., Paoloni-Giacobino, A., 2018. Intergenerational transmission of DNA methylation signatures associated with early life stress. *Curr. Genom.* 19 (8), 665–675.
- Tarazi, F.I., Riva, M.A., 2013. The preclinical profile of lurasidone: clinical relevance for the treatment of schizophrenia. *Expert Opin. Drug Discov.* 8 (10), 1297–1307.
- Todorovski, Z., Asrar, S., Liu, J., Saw, N.M., Joshi, K., Cortez, M.A., Snead 3rd, O.C., Xie, W., Jia, Z., 2015. LIMK1 regulates long-term memory and synaptic plasticity via the transcriptional factor CREB. *Mol. Cell Biol.* 35 (8), 1316–1328.
- Turley, P., Walters, R.K., Maghzi, O., Okbay, A., Lee, J.J., Fontana, M.A., Nguyen-Viet, T.A., Wedow, R., Zacher, M., Furlotte, N.A., andMe Research, T., Social Science Genetic Association, C., Magnusson, P., Oskarsson, S., Johannesson, M., Visscher, P. M., Laibson, D., Cesarini, D., Neale, B.M., Benjamin, D.J., 2018. Multi-trait analysis of genome-wide association summary statistics using MTAG. *Nat. Genet.* 50 (2), 229–237.
- Wan, Y., Liu, Y., Wang, X., Wu, J., Liu, K., Zhou, J., Liu, L., Zhang, C., 2015. Identification of differential microRNAs in cerebrospinal fluid and serum of patients with major depressive disorder. *PLoS One* 10 (3), e0121975.
- Wang, C.Y., Cheng, C.W., Wang, W.H., Chen, P.S., Tzeng, S.F., 2016. Postnatal stress induced by injection with valproate leads to developing emotional disorders along with molecular and cellular changes in the Hippocampus and amygdala. *Mol. Neurobiol.* 53 (10), 6774–6785.
- Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., Schubeler, D., 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat. Genet.* 39 (4), 457–466.
- Weinstock, M., 2017. Prenatal stressors in rodents: effects on behavior. *Neurobiol. Stress* 3, 3–13.
- Weinstock, M., 2008. The long-term behavioural consequences of prenatal stress. *Neurosci. Biobehav. Rev.* 32, 1073–1086.
- Yamasaki, N., Maekawa, M., Kobayashi, K., Kajii, Y., Maeda, J., Soma, M., Takao, K., Tanda, K., Ohira, K., Toyama, K., Kanzaki, K., Fukunaga, K., Sudo, Y., Ichinose, H., Ikeda, M., Iwata, N., Ozaki, N., Suzuki, H., Higuchi, M., Suhara, T., Yuasa, S., Miyakawa, T., 2008. Alpha-CaMKII deficiency causes immature dentate gyrus, a novel candidate endophenotype of psychiatric disorders. *Mol. Brain* 1, 6.
- Yang, Y.R., Jung, J.H., Kim, S.J., Hamada, K., Suzuki, A., Kim, H.J., Lee, J.H., Kwon, O. B., Lee, Y.K., Kim, J., Kim, E.K., Jang, H.J., Kang, D.S., Choi, J.S., Lee, C.J., Marshall, J., Koh, H.Y., Kim, C.J., Seok, H., Kim, S.H., Choi, J.H., Choi, Y.B., Cocco, L., Ryu, S.H., Kim, J.H., Suh, P.G., 2017. Forebrain-specific ablation of phospholipase Cgamma1 causes manic-like behavior. *Mol. Psychiatr.* 22 (10), 1473–1482.
- Yu, D.X., Di Giorgio, F.P., Yao, J., Marchetto, M.C., Brennand, K., Wright, R., Mei, A., McHenry, L., Lisuk, D., Grasmick, J.M., Silberman, P., Silberman, G., Jappelli, R., Gage, F.H., 2014. Modeling hippocampal neurogenesis using human pluripotent stem cells. *Stem Cell Reports* 2 (3), 295–310.