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Title: NURR1 and ERR1 modulate the expression of genes of a *DRD2* co-expression network enriched for schizophrenia risk

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Abstract

Multiple schizophrenia (SCZ) risk loci may be involved in gene co-regulation mechanisms, and analysis of co-expressed gene networks may help to clarify SCZ molecular basis. We have previously identified a dopamine D2 receptor (*DRD2*) co-expression module enriched for SCZ risk genes and associated with cognitive and neuroimaging phenotypes of SCZ, as well as with response to treatment with antipsychotics. Here we aimed to identify regulatory factors modulating this co-expression module and their relevance to SCZ.

We performed motif enrichment analysis to identify transcription factor (TF) binding sites in human promoters of genes co-expressed with *DRD2.* Then, we measured transcript levels of a group of these genes in primary mouse cortical neurons in basal conditions and upon overexpression and knockdown of predicted TFs. Finally, we analyzed expression levels of these TFs in dorsolateral prefrontal cortex (DLPFC) of SCZ patients.

Our *in silico* analysis revealed enrichment for NURR1 and ERR1 binding sites. In neuronal cultures, the expression of genes either relevant to SCZ risk (*Drd2, Gatad2a*, *Slc28a1*, *Cnr1*) or indexing co-expression in our module (*Btg4*, *Chit1*, *Osr1*, *Gpld1*) was significantly modified by gain and loss of Nurr1 and Err1. P*ostmortem* DLPFC expression data analysis showed decreased expression levels of NURR1 and ERR1 in patients with SCZ. For NURR1 such decreased expression is associated with treatment with antipsychotics.

Our results show that NURR1 and ERR1 modulate the transcription of *DRD2* co-expression partners and support the hypothesis that NURR1 is involved in the response to SCZ treatment.

Significance Statement

In the present study, we provide *in silico* and experimental evidence for a role of the TFs NURR1 and ERR1 in modulating the expression pattern of genes co-expressed with *DRD2* in

human DLPFC. Notably, genetic variations in these genes is associated with SCZ risk and behavioral and neuroimaging phenotypes of the disease, as well as with response to treatment. Furthermore, this study presents novel findings on a possible interplay between D2 receptor-mediated dopamine signaling involved in treatment with antipsychotics and the transcriptional regulation mechanisms exerted by NURR1. Our results suggest that co-expression and co-regulation mechanisms may help to explain some of the complex biology of genetic associations with SCZ.

Introduction

Schizophrenia (SCZ) is a chronic brain disorder associated with multiple genetic risk factors (Ripke et al., 2014). Several studies showed that risk genes for SCZ are co-regulated and operate in gene networks (Pergola et al., 2017; Chen et al., 2018; Gandal et al., 2018; Li et al., 2018; Meng et al., 2018; Pergola et al., 2019). The investigation of gene networks relevant to SCZ thus allows to identify molecular dynamics and mechanisms of co-regulation crucially involved in this brain disorder. An approach to characterize genetic networks is based on gene co-expression. Co-expression estimates the simultaneous transcription of genes and is often associated with shared gene functions and co-regulation (Serin et al., 2016; van Dam et al., 2018).

The largest genome wide association study on SCZ includes the *DRD2* locus (Ripke et al., 2014), coding for the dopamine D2 receptor. Genetic variation within *DRD2* is associated with working memory (WM) deficits and related prefrontal cortex (PFC) activity in SCZ (Zhang et al., 2007; Bertolino et al., 2010; Slifstein et al., 2015). Dopaminergic signaling is altered in SCZ (Abi-Dargham, 2014), and current available antipsychotic medications (APs) either hamper or modulate D2 receptors (Miller, 2009). Using a genome-wide Weighted Genes Co-expression Network Analysis approach, we have previously identified a set of co-expressed genes (module) comprising the *DRD2* transcript coding for the long isoform of the D2 receptor (D2L) in *postmortem* DLPFC of healthy individuals (Pergola et al., 2017). This module encompassed 85 genes and was significantly enriched for SCZ risk genes (Ripke et al., 2014). Furthermore, we computed a polygenic co-expression index (PCI) related to inter-individual variability of gene co-expression, which was associated with behavioral and neuroimaging phenotypes crucially associated with SCZ (i.e., WM performance and related brain activity) (Bertolino et al., 2006), as well as with response to treatment with APs (Pergola et al., 2017). Nevertheless, gene networks established by statistical correlation do not provide insight into the regulatory processes underpinning co-expression

(Parikshak et al., 2015). Several studies suggest that genes with strongly correlated mRNA expression profiles are more likely to be co-regulated by shared transcription factors (TFs) (Allocco et al., 2004; Serin et al., 2016). Interestingly, a proportion of genetic variability associated with SCZ involves genes coding for TFs, i.e. *TCF4*, *CTCF*, *EGR1*, *EP300, SREBF1* and *STAT6* (Ripke et al., 2014). Although this evidence supports the relevance of TFs in SCZ pathogenesis, little is known as to how the coordinated action of TFs reprograms gene expression. Our previous finding that a co-expression relationship occurs among *DRD2* and other genes in DLPFC suggests that correlation of expression profiles takes place at the transcriptional level, implicating that a relevant fraction of the genes associated with *DRD2* co-expression are target of the same TF(s) (Pergola et al., 2017). Since dysregulation of such TF(s) in the DLPFC of patients with SCZ may alter the expression patterns of *DRD2* and its co-expressed genes (Pergola et al., 2017; Selvaggi et al., 2018), we sought to investigate their role in the pathophysiology of SCZ and its pharmacological treatment.

Here, we performed motif enrichment analysis of TF binding sites located within the human promoter regions of the genes included in the *DRD2* co-expression module. Then, we performed validation experiments in mouse primary cortical neurons by measuring mRNA levels of *DRD2* co-expression module-selected genes upon gain (overexpression, OE) and loss (knockdown, KD) of function of candidate TFs. We found that two TFs, NURR1 (nuclear receptor-related 1, coded by the *NR4A2* gene) and ERR1 (estrogen-related receptor 1, coded by the *ESRRA* gene), are likely to bind promoters of our genes of interest. In mouse cortex, gain and loss of function of NURR1 and ERR1 is associated with expression changes of genes within the *DRD2* co-expression module, including *Drd2* long isoform. Importantly, we found that *NR4A2* and *ESRRA* expression in human DLPFC is associated with SCZ diagnosis, and the reduced expression observed in patients is associated to treatment with APs for *NR4A2*.

Materials and Methods

Experimental design and statistical analysis

In order to carry out the motif enrichment analysis in promoters of the *DRD2* co-expression gene set, we used the Pscan software (http://159.149.160.88/pscan/) (Zambelli et al., 2009). The software performed a series of z-tests, associating a *p*-value with each TF position frequency matrix included in the JASPAR database (Zambelli et al., 2009). Then, we removed from the Pscan list those TFs that were not expressed in human DLPFC, i.e., the brain area where the *DRD2* co-expression gene set was detected. To determine if TFs expression was significantly greater than zero with $\alpha = 0.05$, we performed one-sample t-tests in the same discovery dataset of the *DRD2* co-expression gene set (Colantuoni et al., 2011). Finally, we corrected the z-test *p*-values of TFs that were expressed in human DLPFC for False Discovery Rate (FDR $q < 0.05$).

Following the motif enrichment analysis, we performed validation experiment in mouse cortical neurons. We used the lentiviral infection to induce OE and KD of *Nr4a2* and *Esrra.* Then, to determine the efficacy of such procedures and the differences in target genes expression upon *Nr4a2* and *Esrra* OE/KD, we performed unpaired t-tests across experimental conditions (i.e. *Nr4a2* OE, *Nr4a2* KD, *Essra* OE, *Essra* KD, and respective control conditions), with $\alpha = 0.05$. Gene expression interquartile range (IQR) was used to detect outliers (Schwertman et al., 2004), defined as observations that fell below Q1 − 1.5 IQR or above Q3 + 1.5 IQR. Each experiment was repeated at least three times (biological replicates) and outliers removed where detected (see Extended Data Fig. 4-4 and 5-1). Cortical neurons cultures were prepared weekly from mice embryos ranging from N=5 to N=10.

We also aimed to estimate *NR4A2* and *ESRRA* differential expression in the DLPFC of SCZ versus Healthy Controls (HC). To this purpose, we used regression analysis. The same regression model was used to assess difference in *NR4A2* and *ESRRA* expression between SCZ who had *postmortem* positive toxicology testing for APs versus those who did not. Adjustments for genetic

stratification (10 genomic ancestries according to Pergola et al. (Pergola et al., 2019)), age, gender, RNA integrity, mitochondrial mapping rate, gene mapping rate and 9 qualitative surrogate variables, were made as suggested by Jaffe et al. as best practice (Jaffe et al., 2017). Data were analyzed by either Graphpad Prism v7 (GraphPad, San Diego, CA, USA) or R3.5.1.

Motif enrichment analysis

Among the five different region lengths to set for the analysis in the Pscan software web interface, we focused on the largest possible, i.e., 950 bp upstream and 50 bp downstream of the transcription start site (TSS) of genes included in the *DRD2* co-expression module, as we aimed to capture also regulatory elements potentially acting as enhancers or repressors proximal to promoters. The JASPAR database was used as a reference for TF position frequency matrices determination.

We replicated the motif enrichment analysis using the Genomatix MatInspector software (Cartharius et al., 2005). To this aim, we used the MatInspector default parameters, i.e., relative scores of matrix similarity > 0.80 and of Transcription Factor Binding Sites common to at least the 85% of the input sequences.

Selection of genes of interest

We tested a subset of eight genes from the 85 included in the *DRD2* co-expression gene set (Pergola et al., 2017). In particular, we selected the genes according to the following criteria: 1) genes previously associated with SCZ, such as *GATAD2A* (p= 3.63E-10), *SLC28A1* (p= 1.62E-11) (Ripke et al., 2014), and *CNR1* (Colizzi et al., 2015; Taurisano et al., 2016); 2) genes harboring single nucleotide polymorphisms (SNPs) previously included in the *DRD2*-related PCI (Pergola et al., 2017), and showing the highest affinity scores for binding to NURR1 and ERR1 according to Pscan prediction, such as *GPLD1, CHIT1, BTG4, OSR1*; 3) genes whose expression was conserved in the mouse brain. In case of multiple annotated transcripts, we designed primers annealing to exon

boundaries that are common to all isoforms in order to detect the total gene level expression, except for the *DRD2* gene, whose long and short isoforms (D2L and D2S, respectively), along with total *DRD2*, were detected. The rationale for this choice was to test whether the TF activity was responsible for the preferential expression of one of the two isoforms.

Primary neuronal cultures

Animal care and experimental procedures were carried out in accordance with the University of Trento ethical committee and were approved by the Italian Ministry of Health. Primary cortical neurons were obtained from C57BL/6J mouse embryos at day 15 (E15.5) as previously described (Tripathy et al., 2017). Briefly, cortices were isolated from the whole brain, dissociated in Papain solution (20 U Papain, 5mM EDTA and 30 mM Cysteine in 1x Eagles' Balanced Salt Solution (EBSS)) for 20 min at 37°C. After 3 min at 37°C with DNAse I, the dissociated tissues were centrifuged at 1000x g for 5 min. After removing the supernatant, the pellet was re-suspended in a solution containing Soybean Trypsin inhibitor and bovine serum albumin in EBSS to stop the digestion. Cells were pelleted by a centrifugation step of 1000x g for 10 min. Finally, the cells were plated on Poly-D-Lysine-coated plates in Neurobasal medium supplemented with B27, PenStrep (100 units), L-Glutamine (2mM) and AraC (100 µM). For cell maintenance, half of the medium was replaced with fresh medium every three days. For all the experiments, cells were analyzed at day in vitro (DIV) 8.

Plasmids

Mouse Lenti-ORF *Nr4a2* and *Esrra* viral vectors were purchased from Origene (Rockville, MD, USA). As a negative control, mCherry sequence was sub-cloned within the same Lentiviral vector backbone (mCherry Vector). Mouse 4unique 29mer shRNA constructs in Lentiviral GFP vector for *Nr4a2* and *Esrra*, were used for KD experiments, with the relative scrambled negative control (Scramble) (Origene).

223

224 **Cell lines**

225 Human embryonic kidney (HEK) 293T cells were used to package lentiviral vectors for
226 overexpression (OE) and knockdown (KD) of *Nr4a2* and *Esrra*. Cells were maintained in DMEM
227 High Glucose supplemented with 10% fetal bovine serum, L-Glutamine (2 mM) and PenStrep
228 (1%).

229

230 **Lentiviral vector packaging and titer**

231 PCMV-dR8.91 (Delta 8.9) plasmid containing *gag*, *pol* and *rev* genes and VSV-G envelope
232 plasmid for lentiviral vector packaging were co-transfected along with Lenti-ORF or Lenti-shRNAs
233 and their control vectors in HEK293T cells using the calcium phosphate method (Kingston et al.,
234 2001). Viral particles were then quantified as described by Pizzato and colleagues (Pizzato et al.,
235 2009).

236

237 ***Nr4a2* and *Esrra* overexpression/knockdown**

238 DIV1 cortical neurons were infected with Lenti-ORF *Nr4a2* and *Esrra* viral particles or
239 mCherry Vector for OE experiments, and with shRNA constructs in Lentiviral GFP vector or
240 Scramble for KD experiments four hours after plating, at a multiplicity of infection (MOI) of 0.125.
241 The next day, culture medium was entirely replaced.

242

243 **MTT cell viability assay**

244 Cortical neurons were plated on 24-wells at a concentration of $5x10^5$ cells/ml. At the day of
245 samples collection, PBS-solved MTT reagent (Sigma-Aldrich, Saint Louis, MO, USA) was added
246 to the culture medium to a final concentration of 0.5 mg/ml and incubated for 1 hour. At the end of
247 the incubation period, the medium was removed, and the converted dye solubilized with DMSO

(Sigma-Aldrich). Cell viability was measured as fold change in absorbance at 570 nm with background subtraction at 630-690 nm.

Immunoblot analysis

Cortical neurons were plated on 10 cm dishes ($6x10^6$ cells/dish), washed in ice-cold PBS and lysed in ice-cold RIPA buffer (150 mMNaCl, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 2 mM EDTA pH8, 1% Na-deoxycholate, 0.5% TritonX-100, 0.1% SDS) in presence of a protease inhibitor cocktail. The lysates were collected, sonicated, and clarified by centrifugation at 4 °C at 16000x g. Protein extracts were quantified by a BCA assay method (ThermoFisher Scientific). Lysates were denatured at 95 °C in 5X sample buffer (0.5 M Tris-HCL, pH = 6.8, 10% SDS, 25% glycerol, 0.5% bromophenol blue, 0.5% β-mercaptoethanol), separated on SDS-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in 5% dry milk in 0.02 M Tris/HCl pH = 7.5, 137 mMNaCl, and 0.1% (v/v) Tween-20 for 1 h at room temperature. Antibodies against Nurr1 (Abcam, Cambridge, UK, ab41917), Err1 (Abcam, ab16363) and β-Tubulin (Sigma-Aldrich, T7816) were diluted 1:1000, 1:500 and 1:5000, respectively. Proteins were detected using the Odyssey infrared imaging system (LI- COR Biosciences, Lincoln, NE, USA).

RNA extraction and Real Time PCR

Cortical neurons were plated on 6-wells at a concentration of $5x10^5$ cells/ml. At DIV8 cells were lysed in 500 µl of TRIzol® Reagent (ThermoFisher Scientific, Massachusetts, MA, USA). Then, RNA was extracted following the TRIzol® manufacturer's protocol with minor modifications. RNA quantification was performed by reading absorbance at 260 nm, using NanoDrop 2000 (ThermoFisher Scientific). One µg of total RNA was reverse transcribed into cDNA using the iScript RT Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primers for Real Time PCR were designed using the IDT Primer Quest tool. Oligonucleotides for this experiment were synthesized by Eurofins Scientific (Luxembourg) (see Fig. 4-1 of Extended

Data for complete sequences and assay efficiency). Standard curves for each primer pair revealed efficiency close to 100%. Real Time PCR was performed using a CFX 96 Real Time System (Bio-Rad Laboratories), in a final volume of 20 µl, using the iTaq SYBR Green Supermix (Bio-Rad Laboratories), and with 0.5 mM primers. PCR protocol was performed as follows: 95 °C for 30 sec, 40 cycles at 95 °C for 5 sec, and 60 °C for 30 sec. Melting curve analysis allowed to confirm specificity of the amplified products (Bustin et al., 2009). Mean Ct values of genes of interest were normalized to those of *Actin Beta* (*ACTB*) as the reference gene. These normalized values were analyzed through the comparative Ct Method for the relative quantification of targets, as previously reported (Pergola et al., 2016).

_NR4A2_ and _ESRRA_ mRNA expression in human postmortem brain tissue

We investigated *NR4A2* and *ESRRA* mRNA expression in human *postmortem* DLPFC specimens in two large RNA-seq datasets comparing patients with SCZ to HC, both males and females. These datasets included one from the Lieber Institute for Brain Development (LIBD, "discovery" dataset, polyA+ protocol, N=155 SCZ cases and N=196 controls) and one from the CommonMind Consortium (CMC, "replication" dataset, RiboZero protocol, N=159 patients and N=172 controls). Detailed sample description has been reported elsewhere (Pergola et al., 2019). Within the SCZ group in the LIBD sample, we tested whether *NR4A2* expression levels were different between patients on APs at the moment of death compared with patients who were free from treatment with APs. AP positivity was assessed with toxicological tests performed on frozen *postmortem* tissue long after death and recorded as a binary yes/no variable.

Results

Promoters of the *DRD2* co-expression gene set are enriched for ERR1 and NURR1 binding sites

Using the Pscan software to perform motif enrichment analysis on the upstream regions of all the genes included in the *DRD2* co-expression module, we retrieved 263 position frequency matrices from the JASPAR database, each one representing a specific TF sequence binding motif. According to *postmortem* brain expression data collected in the 249 PFC brain tissues of the BrainCloud dataset, 32% of the matrices were referred to TFs expressed in human PFC as assessed with a one-sample t-test (Fig. 1-1, Extended Data). Two sequence motifs were significantly enriched within the promoter regions of genes included in the *DRD2* co-expression gene set. Such motifs were recognized by ERR1 (Estrogen related receptor 1, coded by the *ESRRA* gene) and NURR1 (Nuclear receptor related 1 protein, coded by the *NR4A2* gene) TFs (FDR-adjusted p=0.02 and p=0.03 for ERR1 and NURR1 matrices, respectively) (Fig. 1). All predicted binding sequences identified by Pscan matched ERR1 and NURR1 binding motifs with a fitting greater than 75% and 85%, respectively (Fig.1-2 and 1-3, Extended Data). These results were confirmed using another prediction tool, the Genomatix MatInspector software (ERR1: uncorrected $p=1.2\text{x}10^{-16}$; NURR1: uncorrected $p=4.5\text{x}10^{-32}$) (Fig. 1-4, Extended Data).

According to two independent prediction tools, ERR1 and NURR1 transcription factors were likely to regulate the expression of genes in the *DRD2* co-expression module.

Gain or loss of *Nr4a2* and *Esrra* expression do not affect the viability of primary cortical neurons

To investigate whether NURR1 or ERR1 regulate the expression of genes in the *DRD2* co-expression module as predicted, we tested the effect of gain and loss of function of the two TFs in mouse primary cortical neurons. First, we verified the mRNA and protein expression levels of

NURR1 in neurons transduced with lentiviruses that either overexpressed (OE) or knocked down (KD) the TF gene. Upon OE, we obtained a 4-fold and increase of *Nr4a2* mRNA (t_6=3.06, p=0.02, unpaired t-test; fig. 2A) and 3-fold increase of protein levels compared to control samples (t_4=3.165, p=0.03, unpaired t-test; fig. 2B; fig. 2-1, Extended Data). With KD, *Nr4a2* mRNA transcript levels were decreased by 75% (t_8=4.232, p=0.003, unpaired t-test; fig. 2C) and protein levels by 50% compared to the control condition (t_4=15.02, p=0.0001, unpaired t-test; fig. 2D; fig. 2-1, Extended Data). Similarly, the transcript levels of *Esrra* mRNA were enhanced by 20-fold (t_6=2.785, p=0.03, unpaired t-test; fig. 3A) and protein levels by 1.5-fold compared to the control condition (t_4=9.192, p=0.0008, unpaired t-test; fig. 3B; fig. 3-1, Extended Data). On the other hand, KD of *Esrra* resulted in 60% decrease in mRNA transcript levels (t_4=4.75, p=0.009, unpaired t-test; fig. 3C) and 50% decrease in protein levels (t_4=3.123, p=0.03, unpaired t-test; fig 3D; fig. 3-1, Extended Data). In order to exclude any possible toxic effect due to lentiviral infection and/or aberrant expression of the two TFs, we used a cell viability assay. *Nr4a2* and *Esrra* OE and KD mediated by lentiviral transduction did not induce cellular death on neuronal cultures in any of our experimental conditions compared to both cells transduced with lentiviruses expressing scramble RNA **or** mCherry as well as non-infected neurons (WT) (Fig. 2E and 2F; Fig. 3E and 3F). Taken together, these results show that modulation of *Nr4a2* and *Esrra* expression in primary neurons is not associated with overt toxic effects.

***Nr4a2* and *Esrra* modulation alters the expression of target genes within the *Drd2* co-expression module**

In order to assess whether *Nr4a2* and *Esrra* OE and KD modulate expression of our genes of interest, we measured their transcript levels in basal conditions and upon OE and KD of the two TFs. We first evaluated the effect of *Nr4a2* and *Esrra* OE in primary neurons (Fig. 4; see Fig. 4-4 of Extended Data for graph with all data points and outliers plotted). Increased levels of *Nr4a2* were associated with a 2-fold up-regulation of *Gpld1* (t_8=2.302, p=0.04, unpaired t-test), as well as with a

50% down-regulation of *Cnr1* (t_6=7.787, p=0.0002, unpaired t-test) and *Chit1* (t_6=5.49, p=0.002, unpaired t-test) expression levels compared to the control condition (Fig. 4A). *Esrra* OE was associated with down-regulation of D2L (50%; t_6=8.251, p=0.0002, unpaired t-test), *Slc28a1* (60%; t_4=3.839, p=0.02, unpaired t-test), and *Osr1* (80%; t_4=7.505, p=0.002, unpaired t-test) compared to the control condition, and no change in the expression of *Cnr1*, *Gatad2a*, *Gpld1*, *Btg4* and *Chit1* was observed under these experimental conditions (Fig. 4B). These results indicate that gain of Nurr1 function suppressed the expression of *Cnr1* and *Chit1* gene and enhanced that of *Gpld1.* On the other hand, gain of Err1 function suppressed *Slc28a1* and *Osr1* gene expression, along with D2L. This analysis suggests that specific genes within the *DRD2* co-expression module associated with the risk for SCZ are negatively regulated in conditions in which the function of two key TFs is aberrantly enhanced.

We next assess the effect of the two endogenous TFs by gene KD (Fig. 5A, see Fig. 5-1 of Extended Data for graph with all data points and outliers plotted). *Nr4a2* KD was associated with down-regulation of *Gpld1* (50%; t_6=3.265, p=0.02, unpaired t-test), D2L (60%; t_6=3.859, p=0.008, unpaired t-test), *Drd2* total gene levels (50%; t_6=6.886; p= 0.0004), and *Gatad2a* (75%; t_8=9.385, p=1.36076E-05, unpaired t-test), and with a 2-fold up-regulation of *Cnr1* (t_6=3.044, p=0.02, unpaired t-test), and 8-fold up-regulation of *Slc28a1* (t_4=10.3, p=0.0005, unpaired t-test). D2S transcript levels were not affected by *Nr4a2* KD (Fig 5A). On the other hand, *Esrra* KD was associated with a 1.6-fold up-regulation of *Drd2* total gene levels (t_4=37.61, p= 3E-06), and down-regulation of D2S (55%; t_6=2.745, p=0.03, unpaired t-test), *Cnr1* (55%; t_6=2.851, p=0.03, unpaired t-test), and *Slc28a1* (50%; t_6=4.104, p=0.006, unpaired t-test) compared to the control condition (Fig. 5B). Altogether, six and five out of the eight examined genes displayed significant expression profile changes upon modulation of expression of *Nr4a2* and *Esrra*, respectively.

We also sought to verify whether the results described above were consistent with the co-expression analysis of our previous work (Pergola et al., 2017) (see Fig. 4-2 and 4-3, Extended Data).

These results support the idea that these two TFs control the expression of the *DRD2* co-expression module associated with the risk for SCZ.

_NR4A2_ and _ESRRA_ expression is reduced in patients with SCZ

Next, we set out to determine the potential role of NURR1 and ERR1 for the pathophysiology of SCZ and its contribution to pharmacological treatment. Therefore, we compared *NR4A2* and *ESRRA* expression in the DLPFC of SCZ patients and HC using RNA-seq data from *postmortem* DLPFC specimens of the LIBD (discovery set) and CMC samples (replication set). We found that *NR4A2* and *ESRRA* expression levels were significantly reduced in patients in the discovery sample (*NR4A2*: t=-2.8, p=0.0048; *ESRRA*: t=-2.5, p=0.013; multiple regressions). In the replication sample only *NR4A2* was significantly reduced (t=-1.9, one-tailed p=0.032, multiple regression), but not *ESSRA* (t=-1.0, one-tailed p=0.32; multiple regression). In order to investigate whether the replicated difference between patients and control subjects was associated with treatment with APs, we analyzed the LIBD sample by comparing patients who were being treated with APs (N=90) versus those who were not (N=50) using a regression model. We found that patients treated with APs have reduced *NR4A2* expression (t=-2.46, p=0.015, multiple regression) compared with those who were not. Moreover, *NR4A2* expression levels in controls did not differ from those detected in SCZ patients that were negative at toxicological tests for APs treatment (t=-0.9, p=0.4, multiple regression).

Discussion

In a previous study (Pergola et al., 2017), we investigated how prefrontal gene co-expression of *DRD2* is associated with clinical and system-level phenotypes, i.e., WM performance and related brain activity. Using a network approach, we identified co-expression *trans*-eQTLs to predict gene co-expression, and identified a co-expression gene set in which other genes associated with SCZ risk converged, such as *CNR1* (Colizzi et al., 2015), *GATAD2A* and *SLC28A1* (Ripke et al., 2014). We found that individuals with increased simultaneous expression of the *DRD2* gene set manifested greater PFC activity as well as less efficient WM processing, along with greater clinical improvement in response to D2 antagonist antipsychotic drugs.

In the current study, we sought to extend our understanding of the biological mechanisms underpinning co-expression of these genes and their relevance to SCZ and its pharmacological treatment. We tested the hypothesis that the identified *DRD2* co-expression gene set is associated with transcription regulation processes mediated by one or more shared TFs. According to two independent bioinformatics tools, we found that NURR1 and ERR1 are potential transcriptional regulators of the *DRD2* co-expression gene set. To confirm these findings and provide further evidence of the role of NURR1 and ERR1 in the regulation of *DRD2* co-expression module, we performed biological validation experiments in mouse primary neuronal cultures. We tested the effect of gain and loss of function of NURR1 and ERR1 on the expression of selected genes belonging to the identified *DRD2* co-expression module. The results were consistent with the bioinformatic prediction and support a model whereby NURR1 and ERR1 are key regulators of the *DRD2*-related network genes.

More in detail, *Nr4a2* OE resulted in reduced *Cnr1* and increased *Gpld1* expression, and *Nr4a2* KD had the opposite effect on the expression of these two genes. Such a strong relationship between NURR1 and *Cnr1* and *Gpld1* levels suggests that these two genes are directly regulated by NURR1. *CNR1* codes for the cannabinoid receptor 1 (CB1), which is involved in brain functions

such as emotional responses, motivated behavior (Mouslech and Valla, 2009), cognitive processing and motor control (Ruiz-Contreras et al., 2013; Ruiz-Contreras et al., 2014). Endocannabinoids are CB1 partial agonists. Cannabis use and abuse contribute to increase the risk for SCZ (Murray et al., 2007), and genetic variation within the CB1 receptor has been associated with cognitive and imaging endophenotypes of SCZ (Colizzi et al., 2015; Taurisano et al., 2016). Conversely, *GPLD1* involvement in SCZ pathophysiology has not been explored so far. *GPLD1* encodes an enzyme with phosphatidylinositol-glycan-specific phospholipase D activity, which degrades the glycosylphosphatidylinositol anchor that tethers proteins to the extracellular face of plasma membranes, thereby releasing the target protein from the plasma membrane. A recent study has listed *GPLD1* among the genes differentially expressed in blood samples of patients with psychotic disorder (English et al., 2018). *Nr4a2* KD was associated with altered expression of *Gatad2a*, *Slc28a1* and *Drd2* (total gene level and long isoform). Thus, expression of six out of eight genes was modulated by *Nr4a2* levels, supporting the view that NURR1 regulates transcriptional processes of genes in the *DRD2* co-expression network acting as enhancer or repressor. Concerning *Gatad2a*, *Slc28a1* and D2L expression, we found association with *Nr4a2* KD, but not with OE. Moreover, three out of eight genes were down-regulated upon *Esrra* OE, and three upon *Esrra* KD, with no evident correlation between the effects of OE and KD. We obtained a 4-fold and 20-fold increase in *Nr4a2* and *Esrra* expression levels, respectively. It is possible that the levels of expression of endogenous genes are sufficient to regulate the transcription of the target genes, resulting in no further change in target gene expression upon OE. Rather, suppression of expression of the endogenous TFs resulted in major changes in the transcriptional regulation of expression of the target genes. ERR1 as well as NURR1 are known to act in molecular complexes with transcriptional co-activators or co-repressors (Sacchetti et al., 2001; Huss et al., 2015). The involvement of co-activators and co-repressors may induce the same effect on target genes since the TF may act in couple with a co-activator and in turn with a co-repressor.

Concerning *DRD2* at total gene level expression, we found downregulation and upregulation in the *Nr4a2* and *Esrra* KD conditions, respectively. These findings are in line with those obtained for D2L upon *Nr4a2* KD, while D2S changed neither upon KD nor upon OE. The picture is not quite as clear for *Esrra,* since we observed downregulation of D2L upon OE and of D2S upon KD. Our findings are consistent with the fact that the co-expression gene set included selectively the long isoform D2L and could be explained by mechanisms of TF-mediated pre-mRNA splicing. Such mechanisms are usually exerted by controlling transcription elongation rates of RNA polymerase II (Brzyzek and Swiezewski, 2015), or the recruiting of splicing factors to pre-mRNA (Auboeuf et al., 2005; Pandit et al., 2008). Consistently, the STRING database shows that NURR1 and ERR1 interact with mediator 1 (MED1), a co-activator that functions as a bridge to convey information from regulatory proteins to the RNA polymerase II transcription machinery (Szklarczyk et al., 2015). In addition, NURR1 has been found to interact with splicing factor proline- and glutamine-rich (SFPQ) (Jacobs et al., 2009). Thus, our results are consistent with a possible role of NURR1 and ERR1 in mediating pre-mRNA splicing of *DRD2* gene by interacting with the transcriptional and the RNA processing machineries.

NURR1 and ERR1 are nuclear receptors with a well-characterized crystal structure, even though their natural ligands are unknown (Giguere et al., 1988; Wang et al., 2003; Huss et al., 2015). *NR4A2* is expressed in dopaminergic neurons of *substantia nigra*, ventral tegmental area, PFC, hippocampus, and other brain regions (Xiao et al., 1996; Zetterstrom et al., 1996b; Zetterstrom et al., 1996a), where it is involved in development, differentiation, maintenance and survival processes. This TF regulates the transcription of genes crucially contributing to dopamine signaling, such as *DRD2*, tyrosine hydroxylase (*TH*), vesicular monoamine transporter (*VMAT2*), tyrosine kinase receptor (*RET)*, and dopamine transporter (*DAT*) (Wallen et al., 2001). *NR4A2*-deficient mice have symptoms associated with dysfunction in dopamine neurotransmission, such as hyperactivity in novel environments, abnormal retention of emotional memory and increased locomotor response to stress (Rojas et al., 2007; Vuillermot et al., 2011). Indeed, a recent study

472 confirmed that *NR4A2* has a pivotal role in neurodevelopment (Li et al., 2018) and association
473 between *NR4A2* genetic variation and diagnosis of SCZ has been reported (Liu et al., 2015).
474 Altogether, these results are consistent with NURR1 implication in SCZ.

475 *ESRRA* is highly expressed in tissues with high-energy demands, such as brain, heart and
476 skeletal muscle, where it acts as energy sensor to control cellular adaptation to energy demand and
477 stress (Heard et al., 2000). It has been implicated in the regulation of biological processes of critical
478 relevance to neuronal function (Ranhotra, 2010), and risk for psychiatric disorders (Cui et al.,
479 2013). Interestingly, a recent study showed that *ESRRA* is a target of the SCZ-associated
480 microRNA-137 (de Sena Cortabitarte et al., 2018).

481 In the current study, we also analyzed *NR4A2* and *ESRRA* expression in *postmortem* PFC
482 specimens from SCZ patients and HC. We found significant reduction of *NR4A2* expression in the
483 PFC of patients with SCZ compared to HC, whereas there is no clear relationship between *ESRRA*
484 expression and SCZ. Differences between the two groups in *NR4A2* expression are associated with
485 treatment with APs in patients. Considering that toxicology was performed at the moment of death,
486 we cannot assume that patients who were "AP-negative" had never been treated with APs for their
487 whole life. This is a limitation of the toxicological testing approach when clinical data are lacking.
488 However, these results are suggestive and worth further exploration, since some studies have
489 pointed to a cross talk occurring between NURR1 and D2 receptor, showing that D2 receptor
490 functions as a potent NURR1 activator (Tseng et al., 2000; Kim et al., 2006). If confirmed, this
491 evidence would suggest that APs – by blocking the D2 receptor – modulate the expression of
492 NURR1, which in turn regulates the co-expression of the whole gene set of *DRD2* partners.
493 Therefore, our results may suggest another molecular mechanism by which APs exert their clinical
494 effect.

495 A limitation of the current study is the lack of a clear evidence demonstrating NURR1 and
496 ERR1 physical binding to regulatory sequences of the genes investigated in the present study.

497 Moreover, we focused only on *cis*-acting regulatory elements, hence potentially missing *trans*-
498 acting regulators, including co-activators and co-repressors other than NURR1 and ERR1. This
499 limitation calls for further studies that might help the understanding of these mechanisms of
500 regulation.
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663

664 **Figure legends**

665

666 **Figure 1. Enrichment analysis of transcription factor binding sites (TFBSs) in the**
667 **promoter regions of genes belonging to the *DRD2*-related co-expression network.** Putative
668 TFBSs were evaluated for enrichment using the Pscan software with False Discovery Rate (FDR)
669 correction for multiple testing. Shown on the *x* axis are all transcription factor motifs reported by
670 the JASPAR database and referred to TFs expressed in human PFC. Shown on the *y* axis is the *p*-
671 value of the enrichment analysis expressed as $-\log_{10}$ (*p*-value). The red line indicates FDR
672 correction threshold. NURR1 and ERR1 were significantly enriched (p=0.00076 and p=0.00023,
673 respectively). TFBS sequence motifs for NURR1 and for ERR1 are illustrated with the height of
674 each base, indicating the probability of the presence of the corresponding base at the designated
675 position.

676

677 **Figure 2. *Nr4a2* overexpression (OE) and knockdown (KD) efficiency and toxicity in**
678 **mouse cortical neurons.** Real Time PCR (A, C) and Western blotting (B, D) analyses in primary
679 mouse cortical neurons were performed to verify the efficiency of *Nr4a2* OE (*Nr4a2* OE) and KD
680 (*Nr4a2* KD) with respect to the control conditions (mCherry Vector and Scramble, respectively).
681 Neuronal viability was assessed by MTT assay (E, F). All results are the mean±SEM of three
682 independent experiments (*p<0.05; ** 0.01 <p< 0.0; *** p< 0.001).

683

684 **Figure 3. *Esrra* overexpression (OE) and knockdown (KD) efficiency and toxicity in**
685 **mouse cortical neurons.** Real Time PCR (A, C) and Western blotting analysis (B, D) were
686 performed to verify the efficiency of *Esrra* OE (*Esrra* OE) and of KD (*Esrra* KD) with respect to
687 the control conditions (mCherry Vector and Scramble, respectively). Neuronal viability was also

688 determined by MTT assay in all the experimental condition (E, F). All results are the mean±SEM of
689 three independent experiments (*p< 0.05; ** 0.01 <p< 0.0; *** p< 0.001).
690
691 **Figure 4**. **Nurr1 and Err1 OE induces expression changes in target genes of the *DRD2***
692 **– related co-expression module.** Real Time PCR analysis of the indicated genes in primary mouse
693 cortical neurons upon *Nr4a2* OE (A) and *Esrra* OE (B) normalized to the control condition (dashed
694 line). All results are expressed as the mean±SEM of at four independent experiments (*p< 0.05; **
695 0.01 <p< 0.0; ***p< 0.001).
696
697 **Figure 5**. **Nurr1 and Err1 KD modulates the expression of target genes of the *DRD2* –**
698 **related co-expression module.** Real Time PCR analysis of the indicated genes in primary mouse
699 cortical neurons upon *Nr4a2* KD (A) and *Esrra* KD (B) normalized to the control condition (dashed
700 line). All results are expressed as the mean±SEM of four independent experiments (*p< 0.05; **
701 0.01 <p< 0.0; ***p< 0.001).
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709 **Extended data legends**

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711 **Figure 1-1. Human TFs matrices retrieved from the JASPAR database through the Pscan**
712 **software.** This table lists 85 of the 263 matrices retrieved from the Pscan software tool and referred
713 to TFs that are expressed in the human PFC. More matrices can be associated with the same TF,
714 since one TF can recognize different DNA sequences. For each TF is showed the *p*-value of the z-
715 test performed by the Pscan software.

716

717 **Figure 1-2. Detailed results of the TFBS enrichment analysis by the Pscan software on the**
718 ***DRD2* co-expression gene network: ERR1.** The table lists, for each gene examined, the best
719 matching oligo containing the TFBS recognized by ERR1, as well as a score (from 0 to 1)
720 indicating how well the oligo fits ERR1 matrix, and the position of the predicted TFBS with respect
721 to the annotated TSS.

722

723 **Figure 1-3. Detailed results of the TFBS enrichment analysis by the Pscan software on the**
724 ***DRD2* co-expression gene network: NURR1.** The table lists, for each gene examined, the best
725 matching oligo containing the TFBS recognized by NURR1, as well as a score (from 0 to 1)
726 indicating how well the oligo fits NURR1 matrix, and the position of the predicted TFBS with
727 respect to the annotated TSS.

728

729 **Figure 1-4. Confirmation of TFBS enrichment analysis using GenomatixMatInspector**
730 **software.** The table reports the results of the enrichment analysis performed by the Genomatix
731 MatInspector software in the 85 promoters of genes belonging to the *DRD2* co-expression module.
732 The *p*-value of the enrichment analysis as well as the number of network genes in which the TFBS
733 was identified are also reported. Each matrix is defined as a matrix family, where similar matrices
734 are grouped together, in order to eliminate redundant matches.

Figure 2-1. Nurr1 full length western blots. Nurr1 full length western blots in OE (left) and KD (right) conditions compared to the control.

Figure 3-1. Err1 full length western blots. Err1 full length western blots in OE (left) and KD (right) conditions compared to the control.

Figure 4-1. Primers sequences and efficiency. This table shows primers used to amplify transcripts of the genes of interest in *Nr4a2* and *Esrra* OE/KD experiments in mouse cortical neurons and their efficiency.

Figure 4-2. *NR4A2* expression correlation with genes of the *Drd2* co-expression module in human DLPFC. This table shows Spearman's Rank correlations between *NR4A2* expression levels and the expression of genes we analyzed from the *DRD2* co-expression module in DLPFC. False Discovery Rate (FDR) correction for multiple comparisons was used to adjust the *p*-values of Spearman's Rank correlations (all adjusted $p<0.05$) in case of multiple probes for the same target gene. All significant correlations in red.

Figure 4-3. *ESRRA* expression correlation with genes of the *Drd2* co-expression module in human DLPFC. This table shows Spearman's Rank correlations between *ESRRA* expression levels and the expression of genes we analyzed from the *DRD2* co-expression module in DLPFC. False Discovery Rate (FDR) correction for multiple comparisons was used to adjust the *p*-values of Spearman's Rank correlations (all adjusted $p<0.05$) in case of multiple probes for the same target gene. All significant correlations in red.

Figure 4-4. Nurr1 and Err1 OE induces expression changes in target genes of the *DRD2* – related co-expression module: detailed plot. Real Time PCR analysis of the indicated genes in

primary mouse cortical neurons upon *Nr4a2* OE (A) and *Esrra* OE (B) normalized to the control condition (dashed line). All data points are plotted. Red dots (outliers) were excluded from statistical analyses. Results are expressed as the mean±SEM (*p<0.05; ** 0.01 <p< 0.0; ***p< 0.001).

Figure 5-1. Nurr1 and Err1 KD modulates the expression of target genes of the *DRD2* – related co-expression module: detailed plot. Real Time PCR analysis of the indicated genes in primary mouse cortical neurons upon *Nr4a2* KD (A) and *Esrra* KD (B) normalized to the control condition (dashed line). All data points are plotted. Red dots (outliers) were excluded from statistical analyses. Results are expressed as the mean±SEM (*p< 0.05; ** 0.01 <p< 0.0; ***p< 0.001).