Title: Sex-related perturbations in schizophrenia and bipolar disorder brains reflect microRNA-mediated cholinergic/neurokine interactions

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One Sentence Summary: **Sex-related perturbations in schizophrenia and bipolar disorder patient brain transcripts converge onto modified microRNA regulators of neurokine-gp130 pro-cholinergic signaling under circadian modulation.**

Summary: Recent reports revealed massive overlaps between the molecular pathologies of schizophrenia (SCZ) and bipolar disorder (BD), but did not address the prominent clinical differences between men and women patients with these syndromes and the corresponding neurotransmitter and transcript regulators of their biological features. Here, we report sex-specific divergence of central cholinergic transcript networks in large patient brain datasets. Connectome analyses based on single cell- and small RNA-sequencing highlighted the gp130-family neurokine pathway controllers of immune functions, the LHX6 and LHX8 transcription factors that can drive neuronal cholinergic differentiation, the circadian regulators CLOCK and RORA, and the microRNA hsa-miR-125, -520, and -1275 families. Moreover, exposing the female human-originated LA-N-2 neuronal cells to the neurokine *'ciliary neurotrophic factor'* induced the transcriptional network surrounding acetylcholine (ACh) function, including the choline acetyltransferase (ChAT) and its intronic vesicular transporter (vAChT), the high affinity choline transporter (HACU, aka SLC5A7), muscarinic and nicotinic ACh receptors and the ACh hydrolyzing enzyme acetylcholinesterase (AChE), which we showed to be suppressed by miR-125b-5p. Independent patient datasets implicated miR-regulated cholinergic-neurokine interactions in SCZ and BD in the sex-related and circadian control of cholinergic neurons, opening new venues for seeking sex-related biomarkers and therapeutic targets, also in other transmitter systems and diseases.

Keywords: Schizophrenia, Bipolar Disorder, connectomics, microRNA, transcription factors, cholinergic systems, RNA-sequencing, circadian regulation, sexual dimorphism, network analysis

[Main Text:]

Introduction

Recent large-scale genomic *(1)* and transcriptomic *(2)* analyses uncovered previously unknown magnitudes of correlation and overlap (71%) between disease-modified transcripts in SCZ and BD

brains. This indicated a shared SCZ/BD *spectrum*, but left the causes for the widely divergent sex-specific manifestations of these clinical pathologies largely unclear. Compared to women, men present a higher prevalence of SCZ (odds ratio (OR) = 1,4), a ten years earlier mean age of highest disease risk (15-25 vs. 25-35 years of age) and a worse prognosis *(3)*. In comparison, BD incidence is not dissimilar in men and women, although women are 80-90% of "BD Rapid Cyclers" with a particularly bad prognosis, and major depressive disorder (MDD) which is a prerequisite for BD diagnosis affects women more often (OR = 2) *(4)*. Both diseases involve premorbid cognitive impairments and reduced intelligence, but those are more frequent and severe in SCZ than in BD *(5)*, compatible with its original description as "dementia praecox" (premature dementia) early in the 20th century *(6)*. Specifically, cognitive deficits in SCZ and BD have both been associated with cholinergic dysfunctions *(7, 8)* and with the sum of anticholinergic medications *(9, 10)*, and a polymorphism in the α5 nicotinic ACh receptor associates with both SCZ and smoking in humans, and with parallel manifestations in engineered male mice *(11)* and rats *(12)*. Also, male SCZ patients self-administer more nicotine by smoking than females (7.2 vs 3.3 weighted average OR *(13)* and life time prevalence 90%), attributing particular importance to the central cholinergic systems for the sex-specific aspects of SCZ/BD *(14)*.

The genomic origin of SCZ and BD is highly complex. Genome-wide association studies (GWAS) have identified several genotype markers distinguishing the (sub-) phenotypes of BD and SCZ *(15)* in genes encoding receptors (e.g. for ACh, dopamine or glutamate), scaffolding proteins (e.g. DISC1, "disrupted in schizophrenia"), transcription factors (TFs), microRNAs (miRs), or non-coding genomic regions without known function *(16–18)*, but the latter were insignificant, possibly indicating that they exert minor effects or do not change expression levels, but rather modify the genomic impact on secondary gene products *(19)*. To address these multiple differences, we focused on perturbed cholinergic transcripts, TFs and miRs in SCZ and BD as genes of interest (GoIs) that may affect neuroanatomy, cognition and saliency *(20)*.

Notably, 3D models of cholinergic cortical interneurons *(21)* differ from those of cholinergic projection neurons in the basal forebrain (nuclei Ch1-Ch4) that depend on trophic support from their target neurons by retrograde supply of nerve growth factor (NGF) *(22, 23)*. We hypothesized that cortical interneurons might be influenced by "neurokines", brain-specific cytokines which share common second messenger pathways and transduce signals through dimeric proteins of one or both transmembrane neurokine receptors, the gp130 co-receptor, also known as Il6st (IL-6 signal transducer) and the LIF-receptor (Lifr). Gp130-family cytokines unite properties of neurotrophic (e.g. on dopaminergic neurons) and immunogenic nature, both prominent facets in the molecular etiology of SCZ and BD *(18)* as well as in cholinergic signaling *(24)*. Neurokines can activate JAK1/2, TYK2 and STAT1/3/5A/5B *(25)*, affecting neurotransmission as well as immunity. They include Interleukin 6 (IL-6) and the *'ciliary neurotrophic factor' (*CNTF) *(26–31)*, whose receptors are soluble, secreted proteins, which bind the dimeric receptors (gp130 and Lifr) from the extracellular space *(32)*. However, to the best of our knowledge, their roles in SCZ/BD brains were not yet studied.

SCZ/BD hallmarks also involve circadian perturbations. The cholinergic-catecholaminergic imbalance in BD *(8)* follows variable transcriptionally-regulated rhythms (e.g. CLOCK, ARNTL, RORA), and affected individuals exhibit decreased REM latency (the duration from onset of sleep to the first rapid eye movement phase) and increased vulnerability for disease that can be modulated by muscarinic agonists/antagonists *(33)*. Correspondingly, the muscarinic M1/M3 receptor genes are essential for REM sleep *(34)*, and sleep deprivation exerts short-term

antidepressant effects *(35)*, reduced brain ACh levels and vast expressional changes in basal forebrain cholinergic neurons *(36)*. Inversely, cholinergic activation may improve cognition *(37–39)* and mood *(40)*, but provoked schizotypic behavior in Alzheimer's disease patients *(41)*. Therefore, we further pursued circadian influences on the SCZ/BD expression patterns of cholinergic transcripts.

To interrogate the combined influence and circadian regulation of cholinergic regulating neurokines, TFs and miRs in SCZ/BD men and women, we explored the transcriptomic *differences* between SCZ and BD brains and identified sex-related inflammation, immunity, development and differentiation modifications, in spite of cholinergic neurons' paucity in the cortex *(42)*. Single cell sequencing ascertained co-expression of the corresponding genes in cortical tissues and proposed a trophic role for neurokine signalling, especially IL-6, in cortical cholinergic systems while implicating inter-related cholinergic and neurokine systems in SCZ and BD with specific TFs, miRs and circadian regulators. We experimentally validated these findings in a female human-originated, neurokine-dependent cellular model of cholinergic neurodevelopment and in additional transcriptomic test datasets from patient brains.

Results

The massive transcriptomic overlaps between cortical SCZ/BD transcriptomes raised the question of how those reflect the clinical *differences* between SCZ/BD men and women patients and the neurotransmitter regulators of their circadian features. Transcript clustering *(43)* is often employed in transcriptomic analyses to reduce complexity. However, clustering strategies attribute most significance to signals with the highest log-fold differences and lowest p-values, which does not agree with a polygenic model where each component contributes a small effect. Rather, we performed an un-biased gene ontology (GO) analysis on meta-analyzed patient brain transcriptomes, and then defined an objective set of "genes of interest" (GoI) to seek specific cholinergic perturbations. This approach enables, on one set of data (i.e., arrays or RNA-sequencing), as many analyses as there are meaningful gene sets, and has the added benefit of reducing the burden of correction for multiple testing, deepening the coverage of perturbed genes for the GoI.

SCZ/BD transcriptomes show sex-related immunity and neurotransmission perturbations

To identify transcripts distinguishing between diseased men and women, we first studied the entire deposited array data of brain transcriptomes (139 SCZ and 82 BD brains with matched controls; Supplementary Figure 1). Supporting recent reports *(2)*, we found highly correlated SCZ/BD expression beta values (Spearman's rho = 0.7100, $p < 0.001$). However, segregated male/female data presented somewhat lower correlations than sex-independent data (F: 0.6150, $p < 0.001$, M: 0.5783, $p < 0.001$). We then tracked the most discriminating molecular pathways exhibiting the largest difference in Spearman's ranks between SCZ and BD, and examined their GO enrichments (Figure 1). In the sex-independent analysis, GO enrichment of the top 100 diverging genes showed numerous terms connected to inflammation and immunity ("acute inflammatory response", P = 0.003, "cellular response to cytokine stimulus", P = 0.01) as well as association to developmental processes and response to organophosphates (Supplementary File 1). Organophosphates primarily target cholinergic proteins *(44)*, indicating involvement of cholinergic pathways in these differences.

Next, we extended the datasets by the factor sex, calculating expression beta values for every one of the four possible groups (SCZ males, SCZ females, BD males, BD females) against their respective controls and subjected the most diverging beta values in any meaningful combination to GO enrichment analysis (Figure 1). The results indicated a larger divergence between sexes in SCZ than in BD: gene sets *elevated* in SCZ male or female brains showed no overlapping GO terms (Figure 1A, Supplementary File 1), but the top 100 ranked genes elevated in BD male or female brains showed large GO term overlap, particularly in inflammatory components (Figure 1B). Of interest, specific components of neurokine signalling *(25)* were elevated in both males (IL-6, P = 0.007) and females (JAK/STAT, P = 0.01) with BD.

Intriguingly, we found SCZ/BD overlapping GO terms for genes elevated in male, but not female patients. Males with either disease showed elevated inflammation- and immunity-related genes (Figure 1C), also implicating cholinergic regulation which can interfere with inflammation *(45)*. In contrast, genes elevated in female patients with *BD* were enriched in terms associated with CNS function or development (Figure 1D), but enrichment analysis of female-elevated *SCZ* genes yielded no CNS-relevant terms. GO terms pertaining to immune processes were very specific, many referring to single mechanistic components (e.g., IL-6), whereas terms regarding neuronal processes failed to implicate specific neurotransmitters. GO analysis hence supported the

hypothesis of shared molecular patterns *(2)* between BD and SCZ, even for diverging transcripts, *but only in males* (Figure 1 C-D, Supplementary File 1).

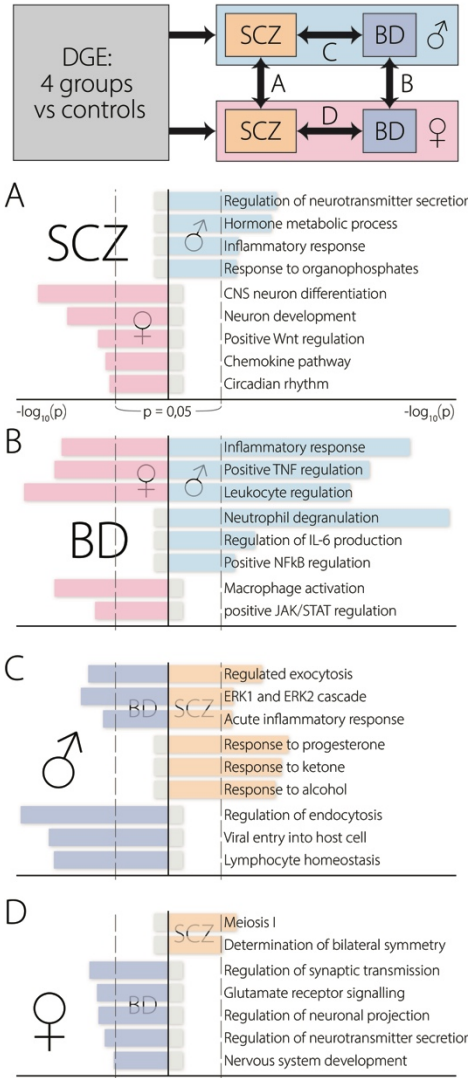

Fig. 1. **Diverging brain transcriptomes in males and females with SCZ/BD**. Differential gene expression (DGE) transcriptome datasets were dually compared: "SCZ vs BD" or "male vs female". GO enrichment of the top 100 distinguishing genes in one dimension was compared to the other, for each pair of combinations. (A) SCZ-elevated genes diverge between males and females. (B) BD-elevated genes share immunological ontology in both males and females. (C) Male-elevated genes share immunological ontology in BD and SCZ. (D) Female-elevated genes *diverge* between SCZ and BD.

One single column, half page depiction

Cholinergic networks predict distinct brain region-related effects on cognitive features

Circos plot analysis of curated functional and anatomical research (Figure 2) and 3D tracing experiments (Supplementary Videos 1-3) demonstrated a wide-spread influence of cholinergic systems on both rudimentary and higher cognitive processes throughout the entire mammalian

brain *(46, 47, 56–58, 48–55)*. However, trophic factor dependency has only been proven for basal forebrain projection neurons Ch1-Ch4, leaving many open questions about the nature of cholinergic interneurons in the striatum and cortex *(23)*.

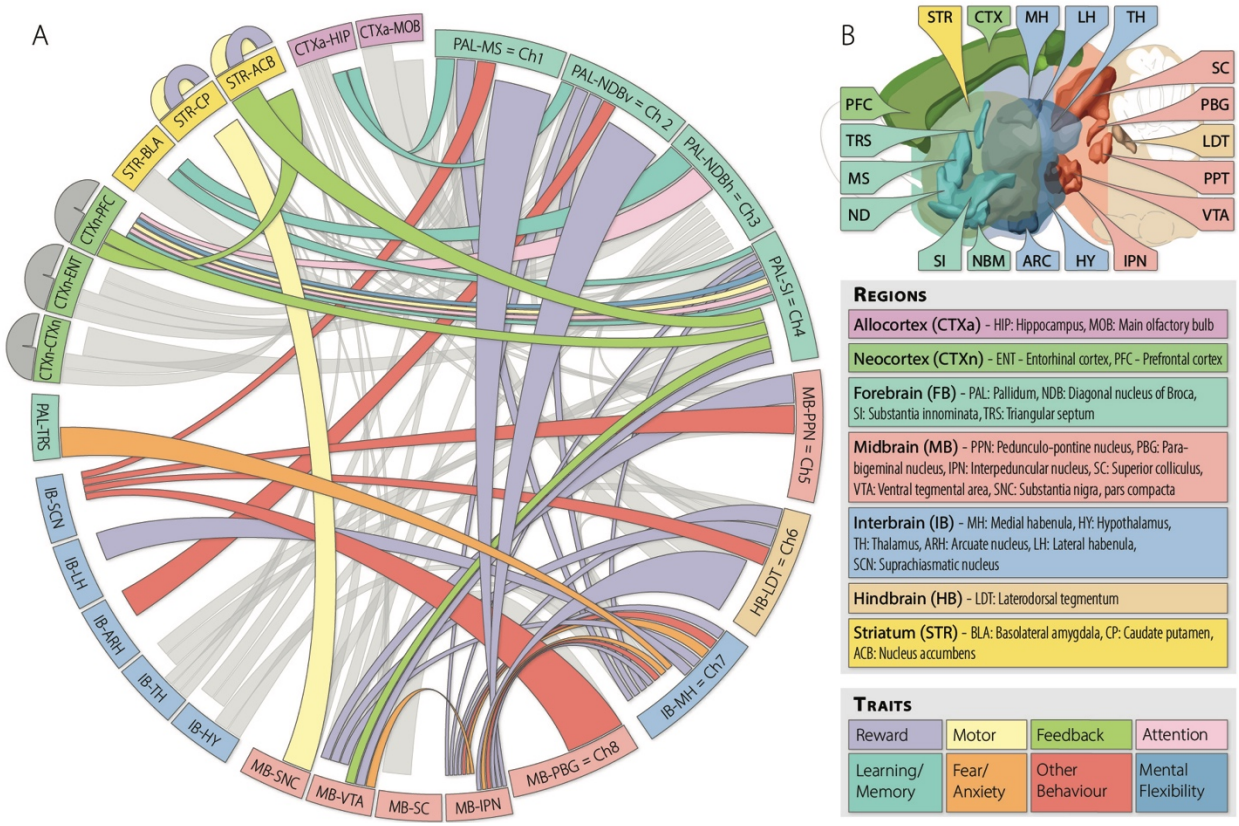

Fig. 2. **The complexity of cholinergic networks in the mammalian brain**. (**A**) Circos overview demonstrating the wide-spread interactions of the mammalian brain's cholinergic systems with physiologic and cognitive processes. Projection origin denoted by closeness of connector and ideogram (first half clockwise), projection termini by spacing between connector and ideogram (second half clockwise). Cholinergic neurons have been shown in nuclei Ch1-Ch8 (smaller right half of ideogram) and interneurons in the striatum and cortex (outside of ideogram). Functional traits indicated by color of connectors. *(46, 47, 56–58, 48–55)* (**B**) Brain regions and projection-trait legend for **A**.

Single cell sequencing indicates neurokine influence on cortical cholinergic neurons

The predicted connection between cholinergic and trophic factor systems can only be pathologically relevant if the key elements of both pathways coexist in the same cell. However, patient tissues are almost exclusively collected from brain regions (cortex, hippocampus, seldom striatum) where cholinergic cells are vastly underrepresented *(42)*, which complicates the direct retrieval of information on cholinergic processes from total transcriptomes. To examine trophic influences, we hence turned to web-available single cell sequencing datasets *(59–62)* and identified putative cholinergic cells in these datasets as those expressing the cholinergic biomarkers choline acetyl transferase (ChAT) and/or the vesicular ACh transporter vAChT. Notably, most of these cells or clusters expressed the neuronal marker RBFOX3 (aka Neu-N), but not the microglial marker AIF1. Oligodendrocyte and astrocyte markers OLIG1 and GFAP were both detected in a minority of samples, indicating sparse cholinergic functions in non-neuronal cells. In both mouse and human brains, the identified cells co-expressed the BDNF receptor Ntrk2, the low-affinity neurotrophin receptor Ngfr (aka p75), and the two transmembrane neurokine receptor proteins, gp130 (aka Il6st) and Lifr (Figure 3 A-D). However, the cortical neurons lacked the high-affinity receptor for NGF, Ntrk1, but appeared to possess the molecular machinery to process neurokine signalling *(22)*, distinguishing them from basal forebrain cholinergic projection neurons.

TFs exhibit particularly high complexity in the central nervous system *(63)*, together with short and long non-coding RNAs (ncRNAs), regulatory elements whose expression is modified in mental diseases *(18)*. The best studied ncRNA species are miRs, small (18-22 bases) single-stranded RNAs which interfere with translation of transcribed mRNA via guiding an RNA-induced silencing complex (RISC) to the mRNA through sequence complementarity followed by inhibition and/or degradation of those mRNAs. By targeting multiple genes, miRs can exert complex contextual regulation in a temporal and/or spatial fashion *(64)*, retaining homeostasis, which is paramount for nervous system health *(65)*, or leading to nervous system disease when disturbed *(66, 67)*. Hsa-miR-137, -335, and -10a are among the factors most tightly linked to SCZ in the literature *(19, 68, 69)*, but miR regulation of the cholinergic network is likely broader and partially primate-specific *(70)*.

To pursue the regulatory elements of coding and non-coding nature which control cholinergic reactions, we further employed random permutation targeting analyses of TF-gene and miR-gene interactions in a graph database specifically constructed from comprehensive state-of-the-art data (*miRNet*, see Methods). This identified 288 miR candidates (out of 2588) and 18 TFs (out of 618). Intriguingly, we found that the indicated TFs may be regulated by a subset of the identified miRs, both conserved and primate-specific, indicating nested layers of regulation (Figure 3E, Supplementary File 2). Together, these analyses called for experimental validation of the indicated regulatory pathways linking TFs, neurokine-cholinergic signalling and miRs.

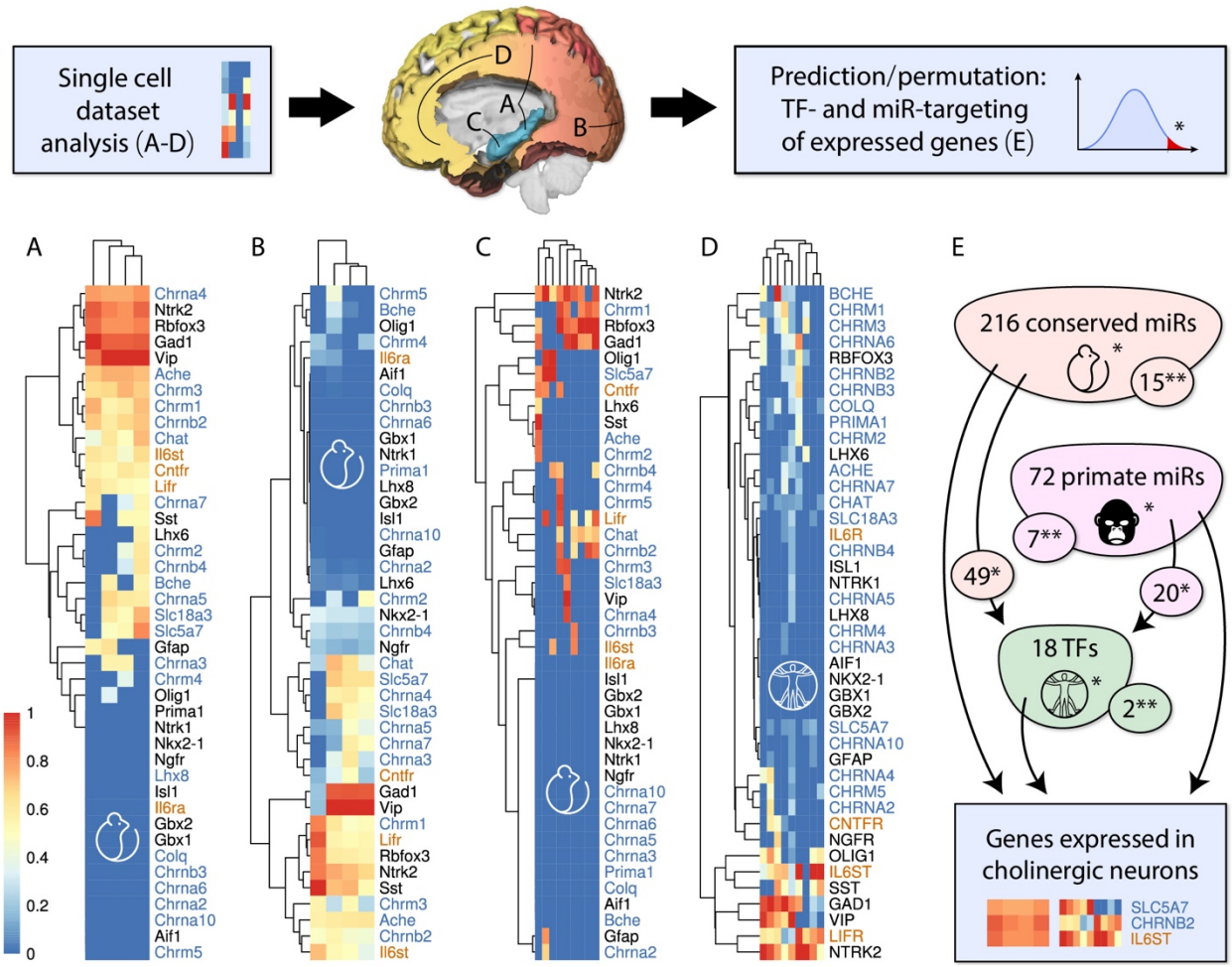

Fig. 3. **Single cell sequencing of cortical cells expressing ChAT/VAChT and targeting analysis of expressed transcripts**. Transcript profiles indicate neurokine functions in cholinergic neueons (A-D). Expression values were normalized between 0 and 1 for each data set. The order of genes in each heatmap is a result of the clustering algorithm and does not reflect level differences. Columns represent individual samples from original data (column names in Supplementary Figure 2). (**A**) Clustered single cell sequences of transgenic mouse somatosensory cortex and hippocampus *(59)*; (**B**) Clustered single cell sequences from transgenic mouse visual cortex *(60)*; (**C**) Single nucleus sequencing of adult mouse hippocampus *(61)*; (**D**) Single cell sequencing of human developing neocortex *(62)*. Cholinergic genes denoted in blue, neurokine receptors in orange. (**E**) Targeting relationships (arrows) indicating a nested regulation of miRs and TFs towards the genes expressed in neurons identified as cholinergic (**A-D**). Permutation analysis of miR- and TF-targeting data of genes expressed in cholinergic cells (via *miRNet*) identified putative cholinergic/neurokine co-regulators (* $p < 0.05$, ** $p < 0.001$). Implicated TFs are regulated by a subset of miRs targeting cholinergic genes (see Supplementary File 2 for details).

Cell culture tests identify miR regulators linking neurokines with cholinergic systems

Identifying cholinergic-regulating miR transcripts in homogenized patient brain samples is challenging due to their tissue-specific and low level expression *(71)*. To pursue sex-specific links of cholinergic reactions with neurokine signals, we exposed cultured female human-originated LA-N-2 cholinergic-like neuronal cells to 4.4 nM (100 ng/ml) CNTF and used small RNA-sequencing to identify the affected miRs following exposure to this neurokine (Figure 4A). Notably, pico- to nanomolar concentrations of CNTF, well within its physiological range *(72)*, elevated the cholinergic marker genes ChAT ($P_{Day2} = 0.022$, $P_{Day4} < 0.001$) and vAChT ($P_{Day2} = 0.002$, $P_{Day4} < 0.001$). ChAT elevation was visible at low concentrations and significant at medium concentrations (Figure 4B). Also, the impact of 440 pM appeared to peak at or before 48 h, whereas 4.4 nM elicited a dramatic long-term response. At 4.4 nM CNTF, 232 miRs showed rapid yet long-lasting expression changes along the examined time course (Figure 4C, Supplementary File 3). The 162 long-term perturbed miRs included 32 conserved and 6 primate-specific miRs overlapping those miRs predicted based on single cell sequencing (see Figure 3E and Figure 4D). The miRs differentially expressed during CNTF exposure of LA-N-2 cells showed an "immediate early" / "long term" dichotomy, and included 26 and 16 miRs that have formerly been associated with SCZ *(73)* and BD *(74)*, respectively (Figure 4E), all of which were evolutionarily conserved in all mammals. Of those, miR-17, -29c, -30d, and -212 were included in all of the sets. Moreover, GO enrichment of the most prominent 217 targets (via *miRNet*) of the long term-perturbed miRs revealed deep involvement in neuronal processes pertinent to our questions (Figure 4F, Supplementary File 1). These experimental results supported the inherent connections between SCZ and BD, cortical cholinergic systems (as indicated by single-cell sequencing) and neurokines.

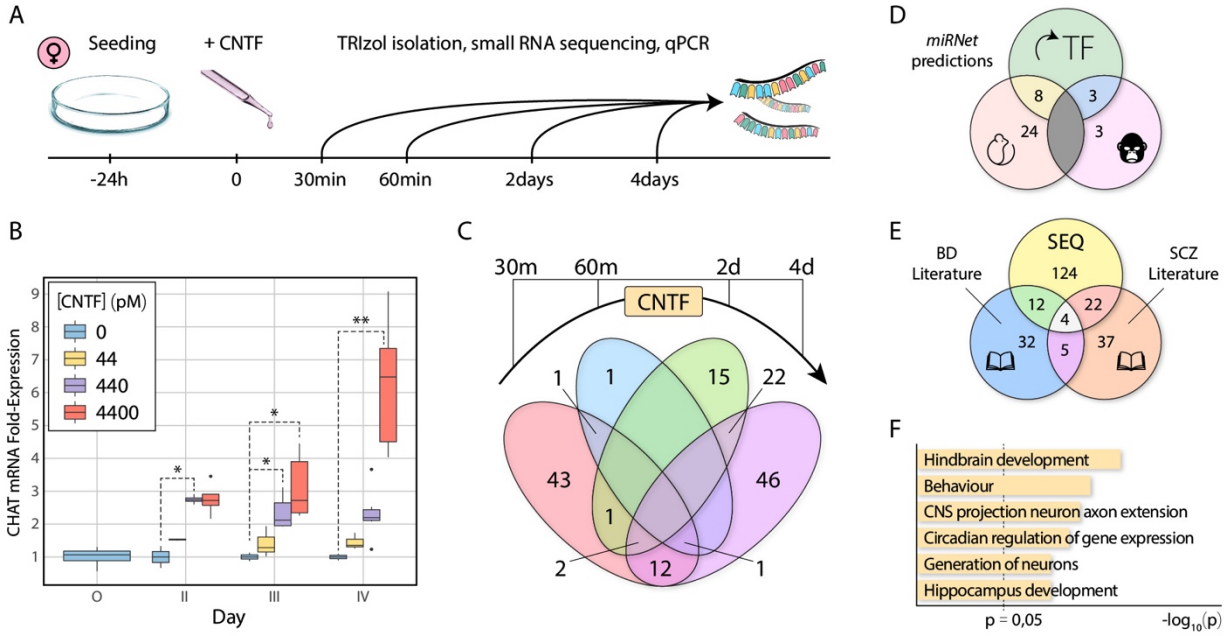

Fig. 4. **CNTF induction of cholinergic miRs in female-originated LA-N-2 cells.** (**A**) CNTF differentiation of LA-N-2, timeline. (**B**) Time-dose-curves of CHAT mRNA expression during differentiation with CNTF. (**C**) Venn diagram of miRs differentially expressed during exposure of LA-N-2 cells to 4,4 nM CNTF. Red, blue, green and purple denote 30 min, 60 min, 2 days and 4 days. (**D**) Conserved and primate miRs found as differentially

expressed by RNA-sequencing which overlapped predictions from single cell profiles (compare to Figure 3E). (**E**) Long-term perturbed miRs (2 and 4 days) overlap with known BD- and SCZ-relevant miRs *(73, 74)*. (**F**) GO enrichment analysis demonstrates that genes most targeted by long-term perturbed miRs regulate CNS processes.

Cholinergic/neurokine analysis reveals perturbed SCZ/BD genes

The deposited array data of SCZ/BD patient brains showed differentially expressed cholinergic/neurokine transcripts (workflow in Figure 5A). Of the 547 interrogated GoI, 251 showed sex-dependent differential expression ($p < 0.05$) in BD, SCZ or both; 48 were differentially regulated in both diseases, and 160 were TFs (Figure 5B, Supplementary File 4). The TFs with highest mean specific expression were LHX6, LHX5, ASCL1, and LHX8, known to be actively involved in cholinergic differentiation *(75, 76)* and pertinent to SCZ *(77)*. Of the 18 TFs predicted by single cell RNA-sequencing permutation analysis to regulate cholinergic pathways, 7 were perturbed in this analysis (LHX8, KLF4, TFAP4, SMAD2, HLX, IRF7, ARHGEF12, ordered by decreasing mean activity). Network analysis (via *miRNet*) of perturbed genes and their TF-based interactions *(63)* showed high interconnectedness of the pertinent TFs, with highest degrees found in cholinergic (LHX), general development/differentiation (SP1, POU, FOX, EGR, FEV), and circadian functions (RORA) (*Figure 5*B).

The GoI-specific differentially expressed genes (DEGs) showed similar gender-related distinctions as the genome-wide GO analysis: in BD, female data highlighted 28% vs 13% of 183 DEG fractions compared to males, whereas in SCZ, male data showed 43% vs 1% of 95 DEGs compared to females (Supplementary Figure 3). Central cholinergic, neurokine or circadian regulated genes (e.g. LHX TFs, HACU, AChE, CHRNA7, IL6, CLOCK and RORA) were detected sex-independently, indicating global impact, whereas many other genes could only be discovered in sex-specific datasets. Of the discriminating genes in each disease, 123 have already been reported (Supplementary File 4) in the context of BD (36%) and SCZ (51%). However, neither the identity of genes formerly reported to be involved in SCZ/BD nor their numbers correlated with the measured log-fold expression differences in patients (19%, Kendall's tau, $p < 0.001$ BD, $p < 0.05$ SCZ, Supplementary Figure 4).

To examine the potential gender-related differences in miR regulation of these transcripts, we analyzed targeting relationships of CNTF-induced miRs towards the sex-independent and the male- and female-specific DEGs. Although the patient brains showed somewhat more male- than female-specific DEGs (74 vs 58), the CNTF-induced miRs in the cultured cells dominantly targeted female-specific DEGs (69% compared to 31%, Figure 5C, Supplementary File 3), compatible with the female origin of LA-N-2 cells. That some disease modified neurokine-cholinergic transcripts in SCZ and BD brains may be caused by sex-related miR changes may indicate single cell ncRNA sequencing on patients' tissues for experimental validation of these observations.

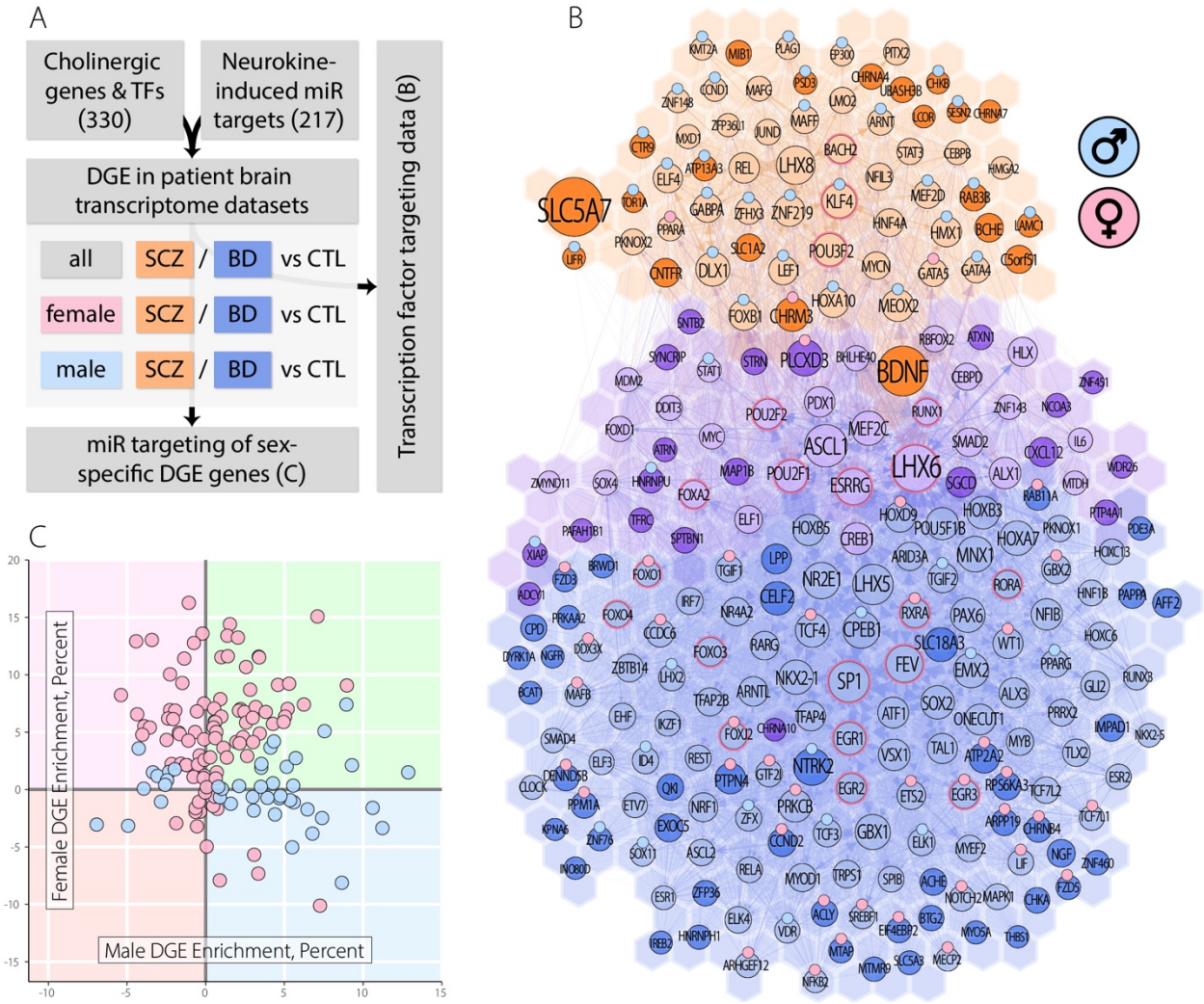

Fig. 5. **Sex-specific DGE, network, and miR-targeting analyses of cholinergic GoI in patient brain transcriptomes**. (**A**) Analysis workflow. (**B**) Perturbed SCZ/BD brain transcripts show overlapping TF regulation. Blue, orange and purple nodes denote BD, SCZ and overlapping transcripts. Lighter shades are TFs (targeting after *(63)*). The layout was generated as a force-directed network, such that nodes with high connectivity cluster together. Edge weight and target node size (reflecting expression levels in CNS tissue *(63)*) correspond to TF activity towards each gene. Note male-specific genes in SCZ and female-specific genes in BD (genes discovered only in sex-specific datasets, marked by pictograms for male/female). Hub genes showing highest degrees of connectedness are exclusively TFs (in order): SP1, LHX6, ESRRG, FEV, POU family, KLF4, FOX family, EGR family, RXRA, and circadian RORA (marked in red), with the cholinergic LHX6 showing highest transcriptional activity. (**C**) Enrichment of miRs from CNTF-differentiation targeting male- and female-specific DEGs of BD and SCZ patients shows higher female involvement. Each point represents one miR, colors indicate sex with higher absolute enrichment.

Small RNAs are perturbed in SCZ *(73)* and BD *(74)*, predicting impairment of their ability to target multiple genes and pathways *(78)*. To zoom into the association of SCZ/BD miR regulators with neurokine/cholinergic pathways, we performed permutation targeting analysis (via *miRNet*) by combining BD- or SCZ-miRs *(73, 74)* with neurokine or cholinergic pathways, which in all cases yielded -0- p-values with binomial 95% confidence intervals between 0 and < 0.001 (Supplementary Figure 5). To determine the impact of neurokine-regulated miRs on these pathways, we constructed the targeting network of DGE miRs induced by CNTF signaling *in vitro* with the pathway genes (complete results in Supplementary File 3, Supplementary Figure 6), and included circadian TFs found in our DGE analysis as indicated in our prior analyses (see Figs. 1/5): CLOCK, ARNTL (aka BMAL1), and RORA. Multiple miR families emerged as potential links between disease-relevant neurokines and cholinergic genes, with several components of both pathways being targeted. MiR families with highest association counts included the evolutionarily conserved hsa-miR-125, -129, -30, and the primate-specific hsa-miR-320, -520, and -1275. In this network, the top SCZ risk hsa-miR-137 *(68)*, which is involved in AMPA-mediated glutamatergic transmission *(79)*, showed high closeness to these miRs, and the circadian regulator CLOCK emerged as pivotal hub with the highest centrality (Supplementary Figure 6). This analysis indicated that cholinergic and neurokine pathway genes which were perturbed in patient brains of both diseases share common miR regulators.

miR regulators link cholinergic and receptive neuron functions

In silico network analysis of the comprehensive network of cholinergic/neurokine miRs identified the evolutionarily conserved miR-125a/b-5p and -129-5p, and the primate-specific miR-520g and -1275 as linking cholinergic with neurokine regulation by targeting nicotinic/muscarinic and neurokine receptors, transcriptional regulators of cholinergic differentiation and circadian rhythm, AChE/BChE and the AChE-linked protein PRiMA (Figure 6A). Of those miRs, hsa-miR-125b-5p displayed more targeting relationships with cholinergic/neurokine genes than other miRs and targets several inflammation-related neurokine pathway genes (miRTarBase accessions; IL6: MIRT022105, IL6R: MIRT006844, JAK2: MIRT734987, LIF: MIRT001037, LIFR: MIRT732494, STAT3: MIRT005006), and other inflammatory pathways (e.g. TNF: MIRT733472, IRF4: MIRT004534). Additionally, hsa-miR-125b-5p harbors a seed sequence predicting the nicotinic α4β2 receptor and AChE as targets. To test the capacity of hsa-miR-125b-5p to impact cholinergic signaling, we performed a cell culture luciferase assay, cell death and endogenous enzyme suppression tests on the secreted AChE protein (Figure 6 B-D). This analysis revealed functional suppression of AChE by miR-125b-5p with similar efficacy to that of the validated miR-132 compared to a scrambled control *(80, 81)*. Briefly, lentiviral infection of miR-132 and -125b led to equally efficient suppression of luciferase activity in 293T cells stably transfected with a plasmid expressing luciferase fused to the AChE mRNA 3'-UTR (one-way ANOVA: $P = 0.004$, Figure 6B). Furthermore, miR-125b-5p suppressed the endogenous AChE hydrolytic activity in human monocyte-like U-937 cells (one-way ANOVA: $P = 0.005$, Figure 6C). Also, co-transfection of miR-125b-5p and a vector expressing a cytotoxic sensor fused to AChE 3'-UTR resulted in cell survival with similar efficacy to that of the positive miR-132-3p control ($n = 3$, Figure 6D), further indicating physical binding of miR-125b-5p to the AChE 3'-UTR.

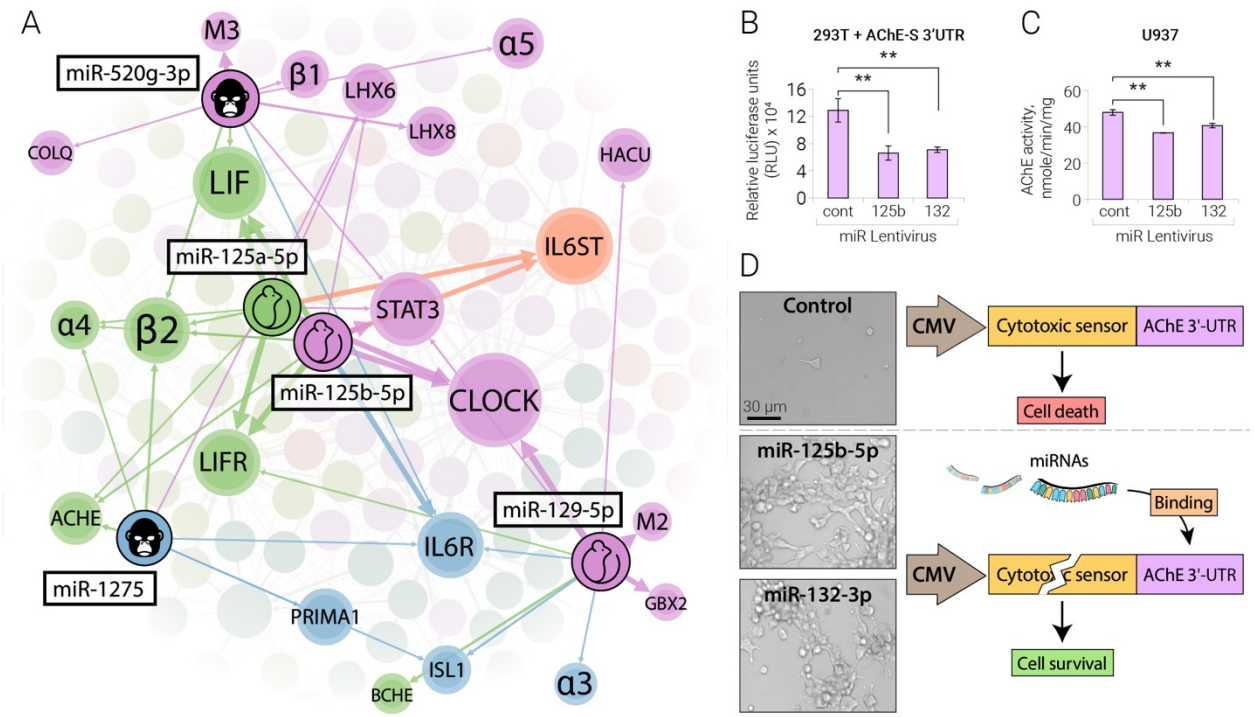

Fig. 6. **Experimental validation of AChE targeting by Hsa-miR-125b.** (**A**) The 5 most central cholinergic/neurokine miRs, evolutionarily conserved miR-125a/b-5p, -129-5p, and primate-specific miR-520g and -1275 pose an interface of cholinergic and neurokine regulation by targeting of the nicotinic/muscarinic and neurokine receptors, transcriptional regulators of cholinergic differentiation and circadian rhythm, AChE/BChE and the AChE linker protein PRiMA. The green module reflects cholinoceptive functions (e.g. via the α4β2 receptor and AChE); the purple module indicates association with functions of cholinergic neurons (e.g. via the M2 auto-receptors, HACU, and LHX TFs; Supplementary Figure 6). Node size corresponds to node degree, thicker edges indicate experimentally validated connections. (**B-D**) Validation experiments of AChE targeting by miR-125b-5p, with miR-132-3p as a positive control. (**B**) Lentiviral expression of miRs-132 and -125b suppresses luciferase fused to the 3'-UTR of AChE. (**C**) Lentiviral expression of miR-132 and miR-125b suppresses the endogenous AChE hydrolytic activity of U937 cells with similar efficacy. (**D**) Life/death assay of HEK-293T cells carrying the AChE 3'-UTR fused to a cytotoxic sensor, and infected with miR-125b-5p, -132-3p, or control lentiviruses. Cells survive in case of binding of miRs -132-3p and -125-5p to the 3'-UTR.

To co-function as predicted, cholinergic/neurokine transcripts should be disease-affected and co-regulated in time and space (Figure 7 A-D presents this pathway schematically). To seek these aspects in cholinergic/neurokine interactions, we studied these genes in additional web-available human RNA sequencing datasets. Post-mortem profiling of brain tissues from three psychiatric disorders (SCZ, BD, MDD) *(82)* confirmed perturbation of the neurokines IL-6 and LIF as well as cholinergic genes and the pivotal TF EGR1 (Supplementary File 7), supporting reports of decreasing transcriptomic severity from SCZ/BD to MDD compared to control brains *(2)*. In RNA sequencing of 600 prefrontal cortices of SCZ patients and controls, IL6ST (aka gp130), and the host gene for the IL6ST-targeting hsa-miR-335, were among the top 50 imprinted genes *(19)*. While the data used in that study yields surprisingly little DEGs, it discovered significant

depression of the CNTFR and cholinergic receptors, accompanied by an elevation of CLOCK and RORA *(19)*.

Circadian patterns of gene expression in mental disease patients and controls *(83)* revealed correlation between the levels of cholinergic/neurokine transcripts and the time of day in several brain regions. To study the intra-daily chronological interactions of the examined transcripts, we sought stable relationships between peak expression hours of cholinergic/neurokine genes relative to sunrise by performing permutation analysis. Similar to the miR-connectivity results, CLOCK emerged as the principal regulator of genes in our set of interest, showing 17 significant ($P < 0.05$) circadian interactions with other genes in the set, including HACU, nicotinic and muscarinic receptors, and several TFs (complete data and P-Values in Supplementary File 6). Additionally, muscarinic receptors (M2, M4, and M5) correlated with IL6ST, CHAT, and HACU expression, whereas nicotinic and muscarinic receptors were largely non-connected; rather, nicotinic subunits correlated with LIFR and neurotrophins (Figure 7E). The networks generated from these associative groups and our CNTF-induced miRs revealed the pivotal players in cholinergic/neurokine circadian homeostasis (Figure 7F). CLOCK and RORA were associated with both nicotinic and muscarinic genes, but their regulation by miRs presented quantitative differences. Notably, the 24 genes associated with nicotinic receptors were under putative control of 21 miRs (0.88 miRs/gene) with predicted or validated targeting of more than 2 transcripts in each set. In comparison, the 7 muscarinic-associated transcripts associated with 24 miRs (3.43 miRs/gene). Unlike most nicotinic receptors, the M2 and M4 muscarinic receptors are often expressed as auto-receptors on cholinergic neurons, indicating that the identified muscarinic-targeting miRs in our CNTF-induced cells may regulate cholinergic neurons.

Although miR targeting of CHAT mRNA itself was minute, CHAT expression correlated with both nicotinic and muscarinic receptors. Also, SLC18A3 (aka vAChT) and AChE expression were correlated, indicating a relationship of vesicular packaging and degradation of ACh, but neither was correlated to ChAT, in spite of the genomic location of vAChT within the first exon of ChAT. This in turn supports an additional layer of mRNA regulation, possibly by miRs *(20)*. In evolutionary terms, primate-specific miRs emerged as the only major factor differentiating higher from lower mammals (all other participating transcripts were conserved in all mammals), pointing at a potentially superior control of cognitive development in primate species (Supplementary File 3). We also noted brain region differences: similar to whole-genome analyses *(19)*, the prefrontal cortex (PFC) did not show grave transcriptome perturbations of a cholinergic nature. However, more caudal parts, such as the anterior cingulate cortex (ACC), presented larger cholinergic perturbations (Supplementary File 7). Also, patient brain samples *(82)* (e.g. nucleus accumbens, ACC) as well as patient-derived iPSCs *(84)* showed significant differences in neurokine/cholinergic perturbations between males and females. Specifically, neurokine-related transcripts were down-regulated and nicotinic and muscarinic receptors up-regulated in the prefrontal and anterior cingulate cortices and nucleus accumbens of SCZ patients and in SCZ patients' iPSCs. However, these changes were largely inverse in males and females in the same datasets (Supplementary File 7). Further, even when transcript levels showed no difference (likely due to small group sizes), transctriptomes presented high isoform variability of IL6ST (aka gp130) between the 10 brain regions analyzed in 10 individuals *(85)*. Finally, a knockdown of CLOCK in *in-vitro* differentiated human neurons *(86)* caused parallel neurokine receptor decreases and cholinergic transcript perturbation (Supplementary File 7), supporting a neurokine-mediated circadian induction. Thus, the cholinergic/neurokine-related molecular pathology of SCZ and BD showed context-dependent modifications based on sex, brain region, and time of day.

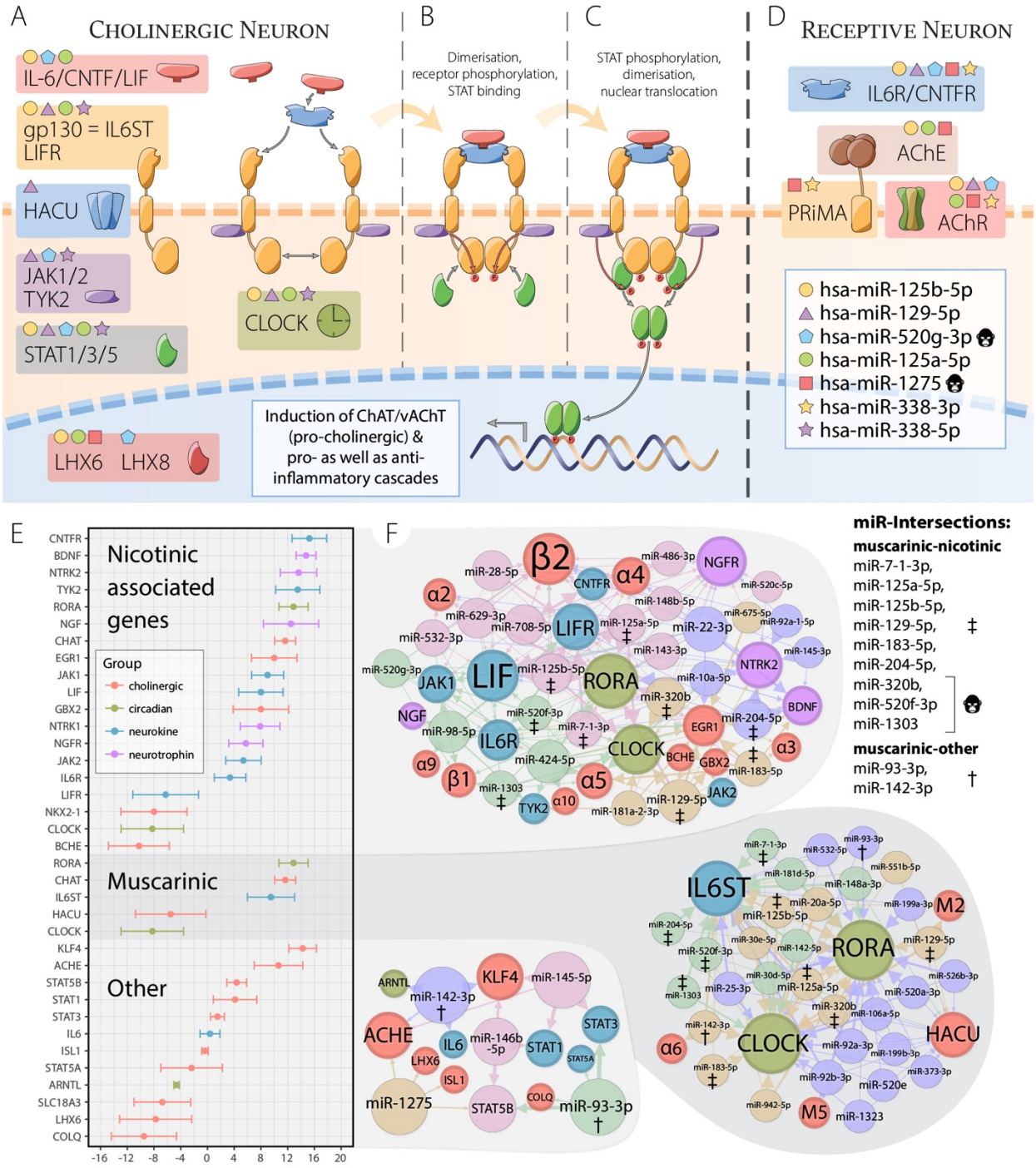

Fig. 7. **miR-regulation of neurokine effects in cholinergic cells by time of day. (A-D)** Gp130-family neurokine, cholinergic, and circadian signaling pathways are controlled by hsa-miR-125, -129, -520, 1275, and -338 family members. Colored symbols denote predicted/validated miR

targeting of individual genes, including overlapping (miR-125, -520) and diverging (miR-338) target profiles. MiR-338-3p (yellow star) and -5p (purple star) show diversified target complementarity, suggesting cell type-specific behavior, whereas the -5p variants of miR-125a and miR-125b share most of their targets. Primate-specific (cartoon) miR-families 520 and 1275 (among others) may confer superior regulatory capacities. **(E)** Circadian expression of cholinergic/neurokine genes, adjusted to sunrise time (= 0) in 4 brain regions (PFC, ACC, NAC, HC) of 55 healthy controls *(83)*. Displayed are the mean and standard error of peak hours of monophasic expression cycles. Genes are grouped by their circadian association (constant lag across brain regions, $p < 0.05$) with nicotinic (top, 24 genes, 21 miRs) or muscarinic (middle, 7 genes, 24 miRs) receptors, and the remaining genes (bottom, 11 genes, 5 miRs). ACh receptor genes themselves were omitted. **(F)** Force-directed networks generated (as in Fig. 6) from these genes and long-term CNTF-perturbed miRs targeting at least 3 genes of each group ($p < 0.05$) support the central roles of CLOCK, neurokines, and the noted miR-families. Node size corresponds to connectivity. Node colour: genes with dark border, coloured as in **E**; miRs coloured by associative network module.

Discussion

By addressing the transcriptomic *differences*, rather than the overlaps, between SCZ and BD we identified inflammation, immunity, development and differentiation as the leading sex-related distinctions between male and female cortical transcripts. GO terms pertaining to immunological processes were highly specific, but neurotransmitter-related terms were of general nature, possibly reflecting a failure of traditional transcriptomic analyses to characterize quantitatively inferior cell types due to transcriptomic "dilution" in cortical tissues. Specifically, individual types of cholinergic neurons are quantitatively inferior to supporting cells in the cortex *(42)*. However, single cell sequencing of cortical tissues ascertained co-expression of the corresponding genes. This approach identified a feasible trophic role for neurokine signalling in cortical cholinergic systems, especially for IL-6, distinguishing these cortical neurons from the NGF-dependent basal forebrain population. Further, permutation analyses pointed at a nested inter-relation of the cholinergic and neurokine systems in SCZ/BD brains with both TFs and miR regulators. To experimentally challenge these predictions, we established a female human-originated and neurokine-dependent cellular model of cholinergic neurodevelopment, validated miR-125-5p suppression of AChE and interrogated additional male and female-derived transcriptomic test datasets from patient brains and iPSCs.

Focusing on a specific subset of genes further enabled us to manually correct annotation errors, which in a whole-genome analysis would have led to loss of information on, e.g., the nicotinic α7 and stress-responding M1 cholinergic receptors. The combined data supported sex-dependent molecular pathology of SCZ and BD and identified neurokine-gp130 signalling as a prominent neurokine input to its pro-cholinergic sex-related aspects, deepening and extending the view of cholinergic control over neuroinflammation into its psychiatric realm. Our tests also implicated several novel factors pivotal for control of cholinergic function in BD and SCZ pathology (e.g., miR-125-5p and its AChE target). In the studied arrays, BD patient brains showed slightly reduced IL-6 levels, whereas in SCZ they were elevated. This is compatible with the opposing roles proposed for interactions of brain neurokine systems with direct nicotinic signalling *(80)*.

In the NGS test set data, IL-6 and LIF levels were *reduced* in the PFC and ACC of female SCZ patients, but *elevated* in male patients. The DGE cortical cholinergic SCZ and BD transcripts substantiated the sex-related impact of neuroinflammation on these diseases and corresponded more prominently to previously reported disease-related processes than those cholinergic genes which did not emerge in our approach. The poor correlation of former reports with absolute log-fold changes in patient brains substantiates the notion that highest log-fold changes, which often coincide with the smallest p-values, may be biomedically irrelevant in a polygenic context. The host gene for hsa-miR-335-3p and its target gene, IL6ST (aka gp130), have recently been identified among the top 50 unperturbed imprinted genes in RNA-sequencing of nearly 600 prefrontal cortex samples of SCZ patients and controls *(19)*. That these transcripts are unperturbed in patients' brains, yet possess heritable, sex-specific epigenetic attributes, underlines the importance of alternative methods to identify pivotal targets for therapeutic development.

Using our neurotransmission-focused approach, we identified a comprehensive network (for limitations, see Methods) of cholinergic/neurokine-related SCZ/BD perturbed genes and their governing TFs and miRs. This further indicated a central role for the neurokine transmembrane receptors gp130 and Lifr, whose transcript levels were not perturbed, in the cholinergic/neurokine interface. Basal neurokine levels in the brain are comparatively low (Supplementary Videos 4-6), and current methods of measurement do not allow an analysis of short-term fluctuations in alive

human subjects. However, CNTF influences behaviour through its impact on cholinergic neurons in the arcuate nucleus *(31)*. Paracrine control of cholinergic neurons by neurokine-affected supporting cells such as glia or astrocytes is also physiologically feasible, pending further analyses. The neuroinflammatory aspect of SCZ/BD supports temporary overstimulation via excessive IL-6 as well as other neurokine signals due to disturbed homeostasis. Compatible with our previous findings *(87)*, we found a paramount role of CLOCK in this regulatory network, indicating circadian regulation of neurokine control over cholinergic signalling.

MiRs, similar to polygenic disease loci, exert combinatorial effects of individual, low-impact components, which can be more adequately represented by a network approach *(88)*. Two of the top five network miRs (miR-520g, -1275) are primate-specific, supporting the notion that primates gained an additional layer of cholinergic/neurokine regulation by miRs *(70)*, and calling for caution when using rodent experimental models. The evolutionarily conserved miR-132-3p, through its suppression of AChE, interrupts the cholinergic modulation of acute stress *(65)*, immune response *(80)* and metabolic activities *(81)* and leads to a transient increase of IL-6, which can be mitigated by nicotine *(80)*. However, miR-125-5p but not miR-132-3p may target the α4β2 nicotinic receptor subunits, such that its perturbation could convey an additional impact. Additionally, miR-125-5p can influence inflammatory processes directly by targeting 5-lipoxygenase *(89)*, and indirectly via regulation of epigenetic controllers *(90)*. Moreover, miR-125b-5p is directly induced by the Vitamin D-receptor *(91)*, and decreased Vitamin D levels are thought to be a risk factor for SCZ/BD development *(92)*. MiR-125-5p hence possesses both cholinergic and several other features potentially relevant to SCZ/BD pathology. In comparison, a recent analysis of a complete miR-124 knockout in human iPSCs *(93)* found extensive subsequent transcriptional perturbations, in STAT5B among others, and a shift from glutamate to ACh in the resulting functional neurons. Although the authors did not adress this cholinergic aspect, their data indicates an anti-cholinergic role of miR-124, which is correspondingly down-regulated in LA-N-2 after neurokine induction.

At present, treating cognitive malfunctioning through stimulation of the cholinergic system is limited to AChE inhibitors and nicotinic/muscarinic agonists *(37)*. Stimulations of nicotinic (e.g. α4β2, the α5-subunit *(11)* and α7) and muscarinic (e.g. M1) receptors *(94)* display a therapy-limiting lack of specificity for individual receptors/subunits, and neurokine-based interventions have repeatedly failed due to intolerable side effects, even when applied directly to the brain *(23)*. In comparison, antisense oligonucleotide therapeutics can simultaneously target multiple disease-relevant genes, be it as mimic/antagonist of an existing miR, or a synthetic oligonucleotide aptamer engineered for a specific target profile. Moreover, peripherally acting oligonucleotides may modify cholinergic activities and send a vagus-mediated message to the brain, such that the success of oligonucleotide therapy will depend on calibrating its impact on target genes and achieving a positive balance between pathway and off-target effects *(64)*. For example, miR-338 family members completely diverge in their neurokine/cholinergic profiles, allowing for differential control of two groups of genes (and, possibly, cell type specificity), whereas miR-125 family members demonstrate a larger overlap in the functional features of their targets. Therapy with miR-like molecules can also potentially ameliorate tachyphylactic effects by targeting a gene and its regulatory elements at the same time (essentially via feed-forward-loops *(95)*). Interrogating databases with lists of selected genes may hence aid in selecting particular miRs as lead therapeutic targets and excluding others, while paying special attention to the primate-specific miRs *(70)*.

Finally, sexual dimorphisms play an important role in many afflictions of the human brain, and insight gained from animal models, particularly rodents, may represent only part of the complexity involved. Recent advances involve evaluating the benefit of sex-specific therapeutic adjustments in cholinergic medication, at least for Alzheimer's disease (reviewed in *(14)*), but the molecular examination of cholinergic systems (e.g., co-expression of sex hormone receptors) is limited to the basal forebrain Ch1-4 nuclei. While some of the datasets covered in the present study allow sex-specific analyses, many questions concerning gender differences remain unaddressed and warrant extensive future studies. Additionally, our study paves the way for investigating diverse processes by replacing the gene set and repeating the analysis, e.g. with dopaminergic pathways or immunity-related genes. High quality datasets large enough to allow statistical analyses can further enable the extension of this approach to other brain regions and other psychiatric and non-psychiatric mental disorders such as Alzheimer's and Parkinson's diseases.

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Materials and Methods

Methodological outline

Focusing on a well-defined set of genes aims to avoid some of the data loss that occurs when patient tissues consisting of multiple cell types are homogenized for analysis. This is particularly relevant for cortical cholinergic interneurons, where the cell type of interest is numerically inferior, and transcriptomic data can be "diluted" by the other, more prominent cell types, such as glia or astrocytes. The consequent increase in detection threshold can be re-lowered by reducing the number of genes tested. Analysis of single cell datasets can ascertain that the genes analysed are representative and fairly unique to the cell type in question, as was the case for cholinergic markers

in our study, and utmost care should be taken for any gene set of interest and any kind of tissue subjected to this kind of analysis.

Our approach is based on the availability of suitable amounts of patient data in web-available form, combined with a standardised pipeline of statistical pre-processing to equilibrate individual statistical influences. Regardless of how patient data and the subset of genes of interest are selected, the reduction in number of analysed genes has to be performed after application of the linear model to the (batch- and covariate-corrected) data to avoid interference with correct linear regression. Once the expression data (be it as array- or sequencing-derived data) has been corrected for batch effects and covariates, and outliers have been removed, it can be analysed with a suitable DGE algorithm. The resulting, individual DGE datasets should ideally converge on similar logFC values, but can also show controversy between individual experiments, which can result from a multitude of factors from biological variety to sampling procedures or exact tissue composition, all of which have to be interpreted at the discretion of the scientist.

Small RNA sequencing of human cholinergic neurons

We employed LA-N-2 *(96)* cells as our main experimental model system. These cells respond to differentiation by several neurokines (CNTF, leukaemia-inhibiting factor (LIF), interleukin 6 (IL-6)) by cholinergic differentiation corresponding with elevation of choline acetyltransferase (ChAT), the central cholinergic marker (mRNA, protein, and activity) as well as its intronic vesicular ACh-transporter gene, vAChT (aka SLC5A7). RNA was extracted in biological triplicates using TRIzol as described *(81)* before and after differentiation with CNTF, at time points 30 and 60 min, and 2 and 4 days (vs matched control cultures). Small RNA sequencing was performed using Illumina NextSeq according to the manufacturer´s instructions, after cDNA library preparation as described *(66)*. Sequenced reads were aligned to miRBase v21 sequences via miRExpress, and to the whole human genome (GRCh38) via bowtie2 and BWA (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml, http://bio-bwa.sourceforge.net/). Differential expression was determined via R/DESeq2 *(97)*. Only miRs found by miRExpress as well as one of the whole-genome approaches to be differentially expressed between condition and control samples were included in the interrogation.

LA-N-2 quantative real-time PCR

For characterization of differentiation, mRNA levels of cholinergic markers ChAT and vAChT were determined by qPCR after two and four days of differentiation with 1, 10, and 100 ng/ml CNTF. RNA was extracted with TRIzol in biological triplicates (controls: 4 samples) as described *(81)* and analysed on a BioRad CFX96 real-time PCR cycler using PowerUp SYBR Green Master Mix (Applied Biosystems) in technical duplicate. Primers were designed using primer3 and are as follows (5' to 3'): ChAT [FW: CAC TTG GTG TCT GAG CA, RV: AGT TTC TGC TGC AGG GTC TC], ACTB (housekeeping) [FW: GCT GTA TTC CCC TCC ATC GT, RV: CTT CTC CAT GTC GTC CCA GT]; additional primers were ordered from BioRad, Germany: vAChT [PrimePCR "qHsaCED0047922"], RPLP0 (housekeeping) [PrimePCR "qHsaCED0038653"]. Data were analysed with BioRad CFX manager and expression values exported for statistical testing. Statistical information (Welch two-sample t-test): Chat, Day 2, 10 ng/ml, t = -3.2436, df = 4.9872; 100 ng/ml, t = -2.349, df = 4.1296; Day 3, 10 ng/ml, t = -2.8481, df = 6.9658; 100 ng/ml, t = -6.3786, df = 3.3998; Day 4, 100 ng/ml, t = -9.0836, df = 6.9835. vAChT, Day 2, t = 5.9222, df = 5.3619; Day 4, t = 7.1016, df = 4.8784.

Whole transcriptome construction and meta-analysis

To apply differential gene expression (DGE) analysis to gene ontology (GO) enrichment of DGE results, we downloaded several cortical datasets in raw format (Affymetrix) from NCBI GEO: GSE35978 *(98)* (SCZ & BD), GSE53987 *(99)* (SCZ & BD), GSE12649 *(100)* (SCZ & BD), GSE17612 *(101)* (SCZ), GSE21138 *(102)* (SCZ), GSE5392 *(103)* (BD) and processed them by state-of-the-art procedures, analogous to Gandal et al *(2)*, to generate transcriptional disease profiles (Supplementary Figure 1). The following R packages (Bioconductor) were used according to the developer's instructions: Raw data read, RMA normalization, RNA degradation: affy *(104)*. Batch correction (as per chip scan date) involved: sva (Combat) *(105)*. Outliers were removed as described *(2)*. The array probes were annotated via ENSEMBL gene ID, database version v75, to ensure congruency with prior analyses, using biomaRt *(106)*, and were collapsed via the collapseRows function of WGCNA *(43)*. Prior to application of the generalized linear mixed model (nlme *(107)*), datasets were rebalanced and regressed to correct for technical and biological covariate influences.

Meta-analysis on all genes present in all datasets (12,391 genes in total) employed a generalized linear mixed model to account for variation per dataset and individual, yielding $\log_2$-fold change (logFC) values for each gene between control and disease, which were correlated between SCZ and BD using Spearman's method *(108)*. To determine significance, the meta-analysis process was repeated 10,000 times with randomized case/control status, forming a permutation null distribution of the individual correlation coefficients (Supplementary Figure 7). Of note, several cholinergic and neurokine genes were missing from the whole-genome meta-analysis because of annotation deficits (CHRNA7, CHRM1, LHX8, CHKB, PRIMA1, CNTF), and, at this stage, could not be easily re-introduced.

Gene ontology (GO) enrichment analyses

To focus on the differences, as opposed to the similarities, between BD and SCZ patient brain transcriptomes, we performed GO enrichment analysis on the genes showing the highest rank differences between datasets (Supplementary Figure 8), using the R package topGO *(109)*. Briefly, we evaluated (smaller) groups of genes for enrichment against a (bigger) background of genes for presence in individual GO terms exceeding statistical estimates. The background comprised of the first 2000 genes according to the applied rank system, computed as a function of Spearman's rank differences of regression beta values (logFC) between the two compared groups, either as absolute values or "as is" (elevation in one group as opposed to the other). The target genes of the analysis were defined to be the 100 top ranked genes (top 5% of background), unless otherwise stated. Statistically significant GO results were compiled and curated for CNS-relevant terms.

miR-gene-TF-targeting: miRNet

To address miR-mRNA targeting relations, we developed an integrated miR-targeting graph database (*'miRNet'*) out of publicly available validated and predicted data, implementing a scoring system from 10 leading prediction algorithms *(110)* based on their statistical performance at the whole-genome level. For simplification, targeting data was cumulated by summing the amount of positive "hits" of prediction algorithms (1 point) and positive experimental validations (10,5 points) of the targeting relationship, yielding a targeting score between 0 and 20,5. We found a score of > 5 to be sufficient for a good balance between type I and II errors. Analyses performed

using this database were implemented in R using the 'RNeo4j' package. To include TF regulatory processes in the analysis, we used bioinformatically processed "cap analysis of gene expression" (CAGE *(111)*), focusing on brain tissues *(63)*, and included the resulting tissue-specific transcriptional activities in the database (Supplementary Figure 9).

Limitations

Through recent methodical and bioinformatical advances, targeting data of transcription factors and miRs has become comprehensive. The limitations of these "complete" datasets derive from the methods used to accumulate raw data. For TF-targeting, CAGE 5' peaks were analysed towards their correlation with gene expression in all available tissues. A cholinergic example of limitations derived from this data is the CHAT gene, which does not provide a measurable CAGE peak, leading to non-representation in the targeting dataset. In those cases, targeting data acquired through conventional methods have to be substituted, but this cannot easily be done for all affected genes, and is also not comprehensive. In the case of miR-targeting, validated interactions are based on experimental work mainly performed on rodents, leading to a research bias towards evolutionarily conserved miRs. Primate-specific miRs therefore are underrepresented, at least in validated data.

Permutation targeting analyses

In the current state of comprehensive data on miR-gene (and TF-gene) targeting, no statements with absolute certainty can be made. Thus, an approach which considers relative measures is preferable. For this reason, whenever whole-genome/whole-miRnome targeting was concerned, we employed random permutation of the prediction dataset or each single predicted miR against a randomized background of the same size as the original set. The resulting null distribution yields a basis for determination of a false discovery rate.

Neurokine-induced miRs and the cholinergic/neurokine pathway

Gene targets of the 162 long-term differentially regulated miRs following CNTF exposure were determined by *miRNet* query (targeting score > 5). Notably, these genes were limited to the cholinergic and neurokine pathways perturbed in patient brain transcriptomes, with an additional inclusion of the major molecular circadian TFs, CLOCK and ARNTL. The resulting network and individual miR-family-subnetworks were plotted using a force-directed layout (Force Atlas 2) in gephi. Network modularization utilized the approach proposed by Blondel et al *(112)*, yielding 4 communities with a modularity of 0.405 at a resolution of 1.2.

Sex influence on transcriptomic differences

Studying web-available datasets of non-degenerative mental disease patients revealed that SCZ and BD datasets possessed sufficient numbers to allow sex-discriminative meta-analysis of statistical significance. Hence, we repeated the above steps for the individual subsets of male and female patients, with the sole change of eliminating the covariate regression for sex, as this would preclude further analysis of this variable, in 4 distinct GO enrichment group comparisons between SCZ-elevated, BD-elevated, male-elevated, and female-elevated genes.

Single cell sequencing data set analysis and permutation

We analysed 4 web-available datasets of brain single cell gene expression *(59–62)* for neurokine signaling transcripts in cholinergic neurons, identified by their expression of the ACh-synthesizing enzyme choline acetyltransferase (ChAT), and its embedded gene encoding the vesicular acetylcholine transporter SLC18A3, also known as vAChT. Raw gene expression data was normalized and then clustered and plotted via R/pheatmap *(113)*. The genes expressed in more than one sample per dataset were enriched for targeting of TFs and miRs by random permutation analysis (via *miRNet*), with 10 000 permutations for conserved and primate miR-target relationships, and human CNS TF interactions.

Subset analyses

Cholinergic genes, transcription factor and neurokine analyses

To follow our cholinergic interest, we used a recent review *(20)* to define a core set of genes. Via *miRNet*, we identified 267 brain-expressed "cholinergic" TFs (Supplementary File 5) and their CNS transcriptional activity towards each targeted cholinergic gene, resulting in a set of 330 genes. Upon confirmation of broad neurokine receptor expression in cholinergic neurons, we determined the genes most likely to be affected by the miRs induced by CNTF in LA-N-2. The top 1% most targeted genes (217 genes) showed significant GO enrichment of neuron and brain development/differentiation pathways, and were included in the neurokine-related gene set (Supplementary File 5).

Execution of subset analyses

To separately analyse male and female data in the original datasets, we repeated the sex-independent analyses of the identified 547 genes (330 cholinergic and 217 neurokine-related) using the limma *(114)* pipeline. Pre-processing was identical to the whole-transcriptome approach, and dataset reduction involved restricting the output table (*topTable* function) to the studied genes. This further allowed controlling of missing genes of interest by manually solving problems of annotation, resulting in full coverage of core cholinergic genes. The network of differentially regulated genes and TFs was plotted via a force-directed layout in gephi (Force Atlas 2 *(115)*). To account for sex-related differences in miR targeting, we performed enrichment analysis of miR-targeted sex-specific sets of perturbed genes via *miRNet* by comparing the size of target sets of DEGs in male and female cortices with sex-independent DEGs for each miR separately.

Disease-relevant miRs targeting cholinergic/neurokine pathways

To test for the relevance of our genes of interest to SCZ and BD pathology, we performed random permutation testing of miR targeting (via our miRNet database) of the two pathways (cholinergic/neurokine) of curated lists of BD- *(74)* and SCZ- *(73)* relevant miRs (117 in BD, 124 in SCZ, Supplementary Table 1) against all annotated miRs (miRBase v21, www.mirbase.org), again with a minimum score of > 5. Targeting scores were summed for all targeting relationships, and were each repeated 50 000 times to yield a null distribution for statistical testing (Supplementary Figure 5). Since the p-values were calculated as zero, we computed their binomial 95% confidence intervals. To determine overlap with the cell culture experiment, we intersected meta-analysed literature datasets with the miRs discovered in our differentiation experiment.

Validation of the cholinergic involvement of miR-125b-5p

To test binding of hsa-miR-125b-5p to the acetylcholinesterase (AChE) 3'-UTR, we performed vector-based assays via suppression of a cytotoxic sensor as well as of Renilla luciferase, both fused to the AChE-3'-UTR, essentially as previously described *(116)*. To show effects of changes in this miR's levels on real-life protein activities, we performed an AChE hydrolytic activity assay before and after transfection of hsa-miR-125b-5p via a lentiviral vector, as recently described *(81)*. In all cases, hsa-miR-132-3p served as a positive control. Statistical details: Figure 6B, n=4, f-ratio value 9.19882, df between treatments 2, within treatments 9; Figure 6C, control n= 5, 125b n=4, 132 n=3, f-ratio value 11.59814, df between treatments 2, within treatments 8.

RNA sequencing data set analysis

Data sets were collected from GEO: GSE80655 *(82)*, GSE106589 *(84)*, GSE68559 *(85)*, GSE96659 *(86)*. Data of DLPFC sequencing of 600 SCZ patients and controls was obtained from the Common Mind Consortium (http:// www.synapse.org/CMC). Raw count data was corrected for covariates where applicable and analysed with R/limma *(114)* and R/DESeq2 *(97)*.

Analysis of circadian gene expression

Data was downloaded from NCBI GEO (GSE45642 *(83)*) and imported to R. Expression peak times for the genes of interest were extracted for the anterior cingulate cortex, dorsolateral prefrontal cortex, hippocampus, and nucleus accumbens. For each gene, mean and standard error across these regions were computed. For each combination of any two genes of interest, their mean lag in hours across the four tissues, and the standard error of this lag, were computed and compared to a null distribution of 10 000 iterations of random partner genes from the original data set, generating permutation P-values for their standard error. Gene pairs with consistent lag with a standard error false discovery rate below 5% were considered as circadially connected. Gene-miR networks were generated via *miRNet* in the same manner as before.

Data and materials availability: LA-N-2 sequencing data are available on NCBI GEO (GSE120520). All code of bioinformatics analyses is available at https://bitbucket.org/rndm4000/sex-related-perturbations-in-schizophrenia-and-bipolar (private repository, please contact for login).

Supplementary Materials

CAPTIONS FOR INTERNAL REFERENCING

Supplementary Figure 1

Fig. S1. Workflow meta-analysis GO enrichment, related to Figure 1

Supplementary Figure 2

Fig. S2. Single-cell heatmaps including sample name columns, related to Figure 3

Supplementary Figure 3

Fig. S3. Enrichment of GoI-specific DEGs in males and females, related to Figure 5

Supplementary Figure 4

Fig. S4. Correlation of log-fold-changes of SCZ/BD DEGs with their literature mentions, related to Figure 5

Supplementary Figure 5

Fig. S5. Permutation null distributions of literature miR targeting of cholinergic/neurokine genes, related to Figure 6

Supplementary Figure 6

Fig. S6. Complete network of CNTF-induced miRs targeting SCZ/BD-perturbed cholinergic/neurokine genes, related to Figure 6

Supplementary Figure 7

Fig. S7. Permutation null distributions of sex-specific meta-analysis, related to Methods - Whole transcriptome meta-analysis

Supplementary Figure 8

Fig. S8. Rank differences of DEGs from meta-analysis between any two compared conditions, related to Methods - Whole transcriptome meta-analysis

Supplementary Figure 9

Fig. S9. Transcriptional activities of TFs in single CNS tissues as derived from Marbach et al *(63)*, related to Methods - miR-gene-TF targeting, Figure 5

Supplementary Table 1

Table S1. Disease-relevant miRs in SCZ/BD from literature, related to Figure 4

Supplementary File 1

Data file S1. Tables of GO terms from unbiased meta-analysis rank difference enrichments, related to Figure 1

Supplementary File 2

Data file S2. Tables of miRs and TFs identified from single cell seq targeting permutation analysis, related to Figure 3

Supplementary File 3

Data file S3. Tables of miRs identified by small RNA sequencing in CNTF-differentiation of LA-N-2 and their sex-dependent enrichments in subset DEGs, related to Figure 4

Supplementary File 4

Data file S4. Tables of perturbed genes in SCZ/BD identified by subset-specific analysis and their literature associations, related to Figure 5

Supplementary File 5

Data file S5. List of cholinergic genes, TFs targeting cholinergic genes, and genes targeted by CNTF-induced miRs in LA-N-2 (gene subsets of interest), related to Figures 4, 5

Supplementary File 6

Data file S6. Circadian associations of genes of interest, related to Figure 7

Supplementary File 7

Data File S7. Web-available NGS dataset GoI DGE results, related to Figure 7, Discussion

Supplementary Video 1

Movie S1. Septohippocampal projections, related to Figure 2

Supplementary Video 2

Movie S2. Cholinergic projections from basal forebrain to hippocampus and cortex, related to Figure 2

Supplementary Video 3

Movie S3. Cholinergic projections from medial habenula to interpeduncular nucleus, related to Figure 2

Supplementary Video 4

Movie S4. NGF-related transcript levels, related to Figure3/Discussion

Supplementary Video 5

Movie S5. BDNF-related transcript levels, related to Figure3/Discussion

Supplementary Video 6

Movie S6. gp130-related transcript levels, related to Figure3/Discussion