Circular RNA circCCNT2 is upregulated in the anterior cingulate cortex of individuals with bipolar disorder

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INTRODUCTION

Bipolar disorder (BD) is a debilitating and chronic psychiatric disorder characterized by cyclic mood episodes, including mania, hypomania, and depression [1]. BD affects approximately 1% of the general population and increases the risk of suicide [2–4]. It is unlikely that a single etiological factor is associated with BD as several environmental, genetic, epigenetic, and transcriptomic factors appear to play important roles in the BD pathology. While there has been some previous work investigating the role of non-coding RNAs in BD, circular RNAs (circRNAs), an intriguing new class of noncoding RNAs, have remained understudied [5, 6].

Circularized RNAs were first observed by electron microscopy in the early 1980s [7–9]. With the development of RNA sequencing, researchers observed strange scrambled exon orders starting in the early 1990s [10–18]. Thus began the infancy of circRNA research, however, it was not until the implementation of massive parallel sequencing and modern computation capabilities that circRNAs were properly characterized [19–21]. CircRNAs are a category of long noncoding RNAs and make up a diverse and abundant collection of circularized RNA transcripts formed through a process known as back-splicing, where linear RNA loops in on itself and the 5′ and 3′ ends are covalently joined [22, 23]. CircRNAs are generally composed of one to five exons primarily arising from protein-coding genes; however, intronic regions can also be incorporated into the circularized transcript [24, 25]. Interestingly, circRNAs are temporally regulated and show tissue-specific expression patterns, with enrichment in brain tissue, making them an exceptionally interesting category of RNA to investigate in the context of psychiatric disorders [26–28]. While their function is not yet fully understood, some circRNAs have shown the ability to sponge microRNAs or RNA-binding proteins (RBPs)/transcription factors by containing complementary microRNA sequences or sequence motifs for protein binding [29–33]. That said, much has still to be discovered on the function of the large majority of circRNAs as there is no unifying mechanism of action which applies to all circRNAs.

Recent work has shown that circRNA dysregulation is associated with psychiatric disorders, making them an exciting new avenue...
of investigation for molecular psychiatry research [31, 33–35]. Using RNA sequencing followed by bioinformatic identification of back-spliced reads allows a non-biased approach to identify and profile global circRNA expression. In this study, we identified differential expression of circRNAs between individuals with BD and psychiatrically healthy controls in post-mortem human anterior cingulate cortex (ACC) (Brodman area 24). Differentially expressed circRNAs from RNA sequencing were validated and further interrogated in an external replication cohort followed by an investigation in vitro.

METHODS AND MATERIALS

Human post-mortem ACC

Discovery cohort. Post-mortem human brain samples from ACC were obtained from the Douglas-Bell Canada Brain Bank (Douglas Mental Health University Institute, Montreal, Quebec, Canada) [5]. All individuals included in this study were of French–Canadian origin and psychological autopsies were performed, as previously described by Dumais et al. [36], on both cases and controls using clinical information ascertained by structured interviews and interpreted using best-consensus methods by a panel of clinicians in order to elicit diagnoses based on DSM-IV criteria. A total of 26 brain samples (13 individuals with BD and 13 psychiatrically healthy controls) were included in this study. Cases included individuals who had a diagnosis of BD type I or type II. The control group had no history of schizophrenia, including suicidal behavior, major mood or psychotic disorders, or subjects in the control group died suddenly by accidental causes or myocardial infarction. This study was approved by the Douglas Hospital Research Ethics Board, and written informed consent was obtained from the next-of-kin for each subject through an agreement with the Quebec Coroner’s Office.

Library Preparation and RNA sequencing. Library preparation and RNA sequencing were conducted as described by Cruceau et al. [5]. Briefly, total RNA was extracted from brain tissue using the RNAeasy kit (Qiagen). Ribosome depleted RNA was used to construct libraries using TruSeq dUTP degradation-based directional protocol (Illumina). All sequencing was carried out at the Genome Quebec Innovation Center using the Illumina HiSeq 2000 platform.

Bioinformatics analysis: Illumina RNA-seq reads were pre-processed with Flexbar 3 for quality clipping and sequencing adapter removal [37]. We used Bowtie2 and human rRNA reference transcripts as described by R. Lin et al. [42]. In short, each of the 31 B-LCL samples (19 cases and 12 controls) was split into two equal aliquots and cultured in separate flasks; one with culture media containing 1 mM of lithium chloride (Li) and one without for seven days (Fig. 3A). B-LCLs were pelleted via centrifugation for total RNA extraction using miRNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. Total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco) following the manufacturer’s protocol with random hexamer priming. To measure RNA expression, we used custom-designed probes (IDT) with PowerUp SYBR Green Master Mix (ThermoFisher) as described above. GAPDH and 18S rRNA was used for the normalization of circCCNT2.

Prediction algorithms for circCCNT2 interactions and functional outcomes

Algorithms within circAtlas, circBank, and circular RNA Interactome were used to determine miRNA or RBPs that interact with circCCNT2 [43–45]. STRING v11 was used to determine protein interaction networks and functional enrichment analysis for circCCNT2 interacting RBPs [46]. miRwalk 2.0 was used to determine gene targets for circCCNT2 interacting miRNAs [47, 48]. Gene ontology enrichment analysis was used for predicted miRNA gene targets [49, 50]. Algorithms within circAtlas and circBank were used to identify open reading frames (ORFs) and to quantify circular RNAs relative to their host gene expression. In this study, we identified open reading frames (ORFs) in circCCNT2 with random hexamers. cDNA was then used together with a custom TaqMan probe (Supplementary Table 1), which spans the back-splice junction of circCCNT2, and TaqMan Universal PCR Master Mix (ThermoFisher Scientific) for RT-qPCR. RT-qPCR quantification was performed using the following formula: relative value = 2^(ΔΔCt) for RT-qPCR. RT-qPCR quantification was performed using the following formula: relative value = 2^(ΔΔCt) for RT-qPCR. RT-qPCR quantification was performed using the following formula: relative value = 2^(ΔΔCt) for RT-qPCR.

Circulation confirmation

CircCCNT2 primers were run on an agarose gel and were sequence-validated. Moreover, resistance to RNase R and reduced abundance in oligo-dT reverse-transcribed cDNA was tested.

Sanger sequencing. Approximately, 50 ng of cDNA was PCR amplified (95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min) using custom-designed primers for circCCNT2 followed by gel electrophoresis for product purification. The expected band product (180nt) was cutout for gel purification (NEB gel purification kit). Sequencing was performed at Genome Quebec Innovation Center on a 3730xl DNA analyzer platform (Applied Biosystem). The same forward and reverse primers used for qPCR were used for Sanger sequencing (Supplementary Table 1).

B lymphoblastoid cohort

B lymphoblastoid cell lines (B-LCL) were collected and immortalized from peripheral blood samples of BD patients and healthy controls as described by Squassina et al. [42]. In short, each of the 31 B-LCL samples (19 cases and 12 controls) was split into two equal aliquots and cultured in separate flasks; one with culture media containing 1 mM of lithium chloride (Li) and one without for seven days (Fig. 3A). B-LCLs were pelleted via centrifugation for total RNA extraction using miRNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. Total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco) following the manufacturer’s protocol with random hexamer priming. To measure RNA expression, we used custom-designed probes (IDT) with PowerUp SYBR Green Master Mix (ThermoFisher) as described above. GAPDH and 18S rRNA was used for the normalization of circCCNT2.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 22.0 and GraphPad Prism 9. Student two-tailed t tests were used to assess expression changes between BD and control. A two-way mixed ANOVA test was performed with Bonferroni correction to assess group, Li treatment, and group by Li treatment interaction effects for the B-LCL dataset.

RESULTS

circRNAs associated with bipolar disorder in human post-mortem ACC

To explore the role of circRNAs in BD, we used RNA sequencing data previously generated by our laboratory from human post-mortem ACC samples comprising 13 individuals with BD and healthy controls (24 individuals with BD and 27 psychiatrically healthy controls).

Expression changes were calculated using the absolute quantitation standard curve method, with GAPDH used as endogenous control.

Replication cohort. Post-mortem human ACC samples were obtained from the Stanley Medical Research Institute as described by Zimmerman et al. [34]. A detailed description of inclusion/exclusion criteria for donors with BD and controls can be found at Torrey et al. [41]. Here, we used a subset of the cohort described by Zimmerman et al. [20], which includes a total of 51 brain samples (24 individuals with BD and 27 psychiatrically healthy controls).

TaqMan RT-qPCR quantification: Reverse transcription was performed using the SuperScript IV First-Strand Synthesis System (ThermoFisher Scientific) with random hexamers. cDNA was then used together with a custom TaqMan probe (Supplementary Table 1), which spans the
circCCNT2 is altered in B-LCL cells of individuals with BD and by lithium treatment

Lithium treatment is a first-line prophylactic treatment for BD [55–57]. We next sought to see if lithium influences the expression of circCCNT2. B Lymphoblastoid cells (B-LCLs) were collected from 19 bipolar cases and 12 healthy controls. Each cell line was divided in half and treated with lithium or left untreated (Fig. 3A). CircCCNT2 expression showed a significant main effect of group (F(1, 29) = 30.171, p = 0.00000645), treatment (F(1, 29) = 4.596, p = 0.0410), and a significant interaction of group by treatment (F(1, 29) = 6.131, p = 0.0190) (Fig. 3B–D). Similar to our results in the brain, we observed an upregulation of circCCNT2 in individuals with BD (Fig. 3B). Interestingly, lithium treatment reversed the upregulation seen in the BD group and downregulated circCCNT2 expression (Fig. 3C). Furthermore, the expression of circCCNT2 was specifically reduced in BD B-LCLs treated with lithium (F(1, 18) = 11.740, p = 0.006), but not in control B-LCLs treated with lithium (F(1, 11) = 0.666, p = 1.000) (Fig. 3D). This indicates that the significant main effect of lithium treatment was predominantly driven by the BD group.

Functional significance of circCCNT2

CircRNAs have functional biological significance and regulatory roles through interaction with miRNA, RNA-binding proteins (RBPs), or translation into peptides [32, 58, 59]. To assess circCCNT2’s ability to sequester RBPs we utilized circAtlas and found at least one binding site for 26 unique RBPs. Using STRING, we discovered these 26 RBPs are enriched for terms related to post-transcriptional regulation of both pre-mRNA and mRNA (Supplementary Fig. 4A and Supplementary Table 3). Using three different miRNA prediction platforms, miR-877-5p was consistently predicted to bind to circCCNT2 [43–45]. Gene ontology (GO) analysis of miR-877-5p targets showed cellular component terms related to synapse formation (Supplementary Table 4). Using the circCCNT2 sequence we did not identify any ORFs or presence of IRE elements indicating a low probability of
| circRNA name | CircRNA ID | Host gene description | Genomic location | Strand | BSJ | Size | p-Value | Adjusted p-value | Fold change (%) |
|--------------|------------|-----------------------|------------------|--------|-----|------|---------|-----------------|-----------------|
| circCCNT2    | hsa_circ_0056537 | Cyclin T2            | chr2:134936841–134942674 | +     | exon5–exon3 | 253nt | 0.00006 | 0.09035 | 83              |
| circCLOCK    | hsa_circ_0126631 | Clock circadian regulator | chr4:55479553–55482828 | –     | exon8–exon5 | 391nt | 0.00021 | 0.09362 | 82              |
| circRERE     | hsa_circ_0002158 | Arginine-glutamic acid dipeptide repeats | chr1:8541214–8557523 | –     | exon8–exon6 | 308nt | 0.00013 | 0.09035 | 66              |
| circSGMS1    | hsa_circ_0093713 | Sphingomyelin synthase 1 | chr10:50433476–50519921 | –     | exon6–exon3 | 357nt | 0.0001  | 0.09035 | 43              |
| circPTPN4    | hsa_circ_0117151 | Protein tyrosine phosphatase non-receptor type 4 | chr2:119809837–119900806 | +     | exon10–exon2 | 781nt | 0.00008 | 0.09035 | 36              |
| circUBR5     | N/A         | Ubiquitin protein ligase E3 component N-recognin 5 | chr8:102271134–102272772 | –     | exon49–exon48 | 356nt | 0.00009 | 0.09035 | –21             |
| circGLRB     | hsa_circ_0125612 | Glycine receptor beta | chr4:157120556–157153010 | +     | exon9–exon3 | 1075nt | 0.00003 | 0.09035 | –38             |
| circCYFIP2   | hsa_circ_0074763 | Cytoplasmic FMR1 interacting protein 2 | chr5:157294783–157304366 | +     | exon7–exon4 | 588nt | 0.00023 | 0.09362 | –45             |
| circSHC3     | hsa_circ_0003708 | SHC adaptor protein 3 | chr5:89037993–89046994 | –     | exon11–exon8 | 694nt | 0.00014 | 0.09035 | –51             |
| circLRBA     | hsa_circ_0071174 | LPS responsive beige-like anchor protein | chr4:150735258–150808398 | –     | exon36–exon32 | 449nt | 0.00013 | 0.09035 | –55             |

*aCircRNA expression quantification followed by differential expression analysis and fold change filtering identified ten candidate circRNAs (BA: Brodmann's Area).
*bIn this study circRNAs were named as their host gene with “circ” prefix.
*circRNA ID as listed in circBase. circUBR5 did not have a known circRNA ID and is believed to be a newly identified splice variant.
*dGenomic location is outlined as follows: chromosome#:start-end for GRch38/hg38 build.
*eBSJ: back splice junction. This column indicates between which two exons the back splice event occurred.
*fSize of the mature circRNA splice variant in nucleotides (nt).
*gNominal p-values were adjusted for false discovery rate (FDR) correction; significant at an FDR of <0.1.
circADAM22 were signiﬁcantly upregulated in BD in three independent cohorts and two tissue types. Furthermore, circCCNT2 is downregulated in BD. In our ACC dataset, we were not able to replicate previous ﬁndings. However, these studies investigated different brain regions which could point to brain region-speciﬁc regulation of circRNAs in BD. Another study investigating peripheral samples of BD patients and healthy controls identiﬁed 33 circRNAs as nominally differentially expressed between groups [61]. Our study was the ﬁrst to point to circCCNT2 as possibly implicated in BD.

RBP s that are predicted to interact with circCCNT2 show enrichment for RNA regulatory proteins. This suggests that the altered circCCNT2 expression in BD could play a role in the regulation of differentially expressed genes associated with BD. Moreover, Cruceanu et al. [5] did not ﬁnd CCNT2 to be differentially expressed between BD and controls, in the same cohort used in this study, giving evidence that circCCNT2 has speciﬁc roles in BD independent of its linear host gene counterpart. We also identiﬁed miR-877-5p binding sites on circCCNT2. Interestingly, miR-877-5p and miR-877-3p have been reported to interact with several other circRNAs in relation to cancer progression [62–64]. However, this relationship has not previously been reported in the context of any neuropsychiatric disorders. When we performed enrichment analysis on predicted targets of miR-877-5p, we observed several cellular component terms related to neuronal synapses. We also observed molecular function terms related to ion binding and hydrolase and biological processes terms such as neurogenesis and cell signaling. Together, this gives the possibility that the predicted circCCNT2/miR-877-5p interaction might regulate genes involved in modulating neuronal signaling in BD and lithium treatment and may also be involved in lithium metabolism. This possible interaction of circCCNT2 with RBPs and miRNAs may be an interesting avenue for future work in BD research.

Our study has many advantages including the use of RNA sequencing as a discovery method to proﬁle global circRNA expression, the use of three independent cohorts and two tissue types to replicate our ﬁndings, and the use of multiple measuring techniques and other assays to conﬁdently assess the levels of speciﬁc circularized RNAs. However, there are several limitations to this study. The sample sizes of our cohorts are relatively small, but we were able to identify circCCNT2 and replicate it in an independent sample. Although we are able to conﬁrm the circular structure of circCCNT2 and its association to BD, the function of circCCNT2 remains unknown. Much more detailed analyses exploring the potential mechanism of action for circCCNT2 are needed to understand its role in BD pathology and lithium response. Finally, the relationship between the expression of peripheral and central circRNAs is unclear. However, given the consistency of dysregulation between the peripheral and central tissues, it is possible that circRNAs may cross the blood–brain barrier.
Fig. 3 Replication and effects of lithium treatment on circCCNT2 expression. A Schematic diagram of the B-LCL cohort. B Expression of circCCNT2 in B-LCL cells collected from individuals with bipolar disorder (BD) and controls (CTL). Showing the main effect of the group from a two-way mixed ANOVA. C Expression of circCCNT2 in B-LCL cells that were treated and not treated with lithium. Showing the main effect of treatment from a two-way mixed ANOVA. D Expression of circCCNT2 showing the decomposition of the significant interaction of group by treatment from a two-way mixed ANOVA. Lithium treatment specifically reduces circCCNT2 expression in individuals with bipolar disorder with no effect in control subjects. All bar graphs represent the mean expression of circCCNT2; each dot represents a single data point; error bars represent SEM; *<0.05, **<0.01, ***<0.001, n.s. not significant.

barrier. For example, it has been shown that circRNAs are packaged into exosomes, or other extracellular vesicles, which have been shown to cross the blood–brain barrier [65–71]. Altogether, our study is the first to demonstrate that circCCNT2 is associated with BD and lithium treatment and has predicted functional implications related to neuronal functioning.

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ACKNOWLEDGEMENTS
G.T. holds a Canada Research Chair (Tier 1) and is supported by grants from the Canadian Institute of Health Research (CIHR) (FDN148374, FRN 141899, ENP161427), and by the Fonds de recherche du Québec—Santé (FRQS) (EGM141899) through the Quebec Network on Suicide, Mood Disorders, and Related Disorders. This work was supported by the Canadian Institute of Health Research (CIHR) grants FDN148374, FRN 141899, ENP161427, and by the Fonds de recherche du Québec—Santé (FRQS) (EGM141899) through the Quebec Network on Suicide, Mood Disorders, and Related Disorders. The authors have nothing to disclose.
AUTHOR CONTRIBUTIONS
R.L., J.P.L., C.C., and G.T. designed the study. R.L., L.M.F., and G.T. wrote the paper. C.C. performed sample processing and sequencing for the discovery human ACC cohort. C.D. performed bioinformatic identification, quantification, and differential analysis of circRNAs from RNA sequencing. N.M. and C.P. obtained and provided the replication human brain cohort data. C.C. and A.S. provided B-LCL cell cultures. R.L. performed validation RT-qPCRs, RNase R assays, RT-qPCR from B-LCL cells, and statistical analysis.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41398-021-01746-4.

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