miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment

Juan Pablo Lopez¹, Raymond Lim², Cristiana Cruceanu¹, Liam Crapper¹, Caroline Fasano³, Benoit Labonte¹, Gilles Maussion¹, Jennie P Yang¹, Volodymyr Yenko¹, Erika Vigneault³, Salah El Mestikawy³, Naguib Mechawar¹, Paul Pavlidis² & Gustavo Turecki¹

Major depressive disorder (MDD) is a prevalent mood disorder that is associated with differential prefrontal brain expression patterns¹. Treatment of MDD includes a variety of biopsychosocial approaches. In medical practice, antidepressant drugs are the most common treatment for depressive episodes, and they are among the most prescribed medications in North America²,³. Although antidepressants are clearly effective, particularly for moderate to severe depressive episodes, there is variability in how individuals respond to antidepressant treatment. Failure to respond has individual, economic and social consequences for patients and their families⁴. Several lines of evidence demonstrate that genes are regulated through the activity of microRNAs (miRNAs), which act as fine-tuners and on-off switches of gene expression⁵–⁷. These results suggest that miR-1202 is associated with the pathophysiology of depression and is a potential target for new antidepressant treatments.

We assessed miRNA expression in the ventrolateral prefrontal cortex (PFC) of individuals with depression compared to psychiatrically healthy controls (Supplementary Table 1). Statistical analysis, using Benjamini-Hochberg correction for multiple testing and corrected P values <0.05, identified miR-1202 as the most dysregulated miRNA, with decreased expression in depressed brains (Fig. 1a), findings that we validated by quantitative RT-PCR (qRT-PCR) (Fig. 1b,c). According to miRBase⁸, miR-1202 has very little evolutionary conservation.
genes (Fig. 1g). Additionally, we found an increase of GRM4 protein expression levels that correlated with the increased mRNA levels (Supplementary Fig. 2). GRM4 is expressed throughout the brain and is localized pre- and postsynaptically, where it modulates glutamatergic, dopaminergic, GABAergic and serotonergic neurotransmission. In recent years, GRM4 has been implicated in the regulation of anxiety-related behaviors and it is seen as an attractive target for drug discovery and development.

To test the external validity of our findings, we measured the expression of miR-1202 and GRM4 in a larger, independent sample composed of PFC tissue from control subjects and individuals with depression. Additionally, we included a third group, individuals with depression who had a history of antidepressant use and positive antidepressant toxicity at the time of death (Supplementary Table 3). In line with previous findings, miR-1202 expression was decreased in subjects with depression as compared to controls. Notably, we also found a difference in miR-1202 expression between subjects with depression depending on whether they had a history of antidepressant use (Fig. 1h). Consistent with our previous findings, GRM4 was upregulated in depressed brains, whereas there was no difference in GRM4 expression between controls and individuals with depression and antidepressant history. Nevertheless, subjects with depression showed differential expression of GRM4 dependent on antidepressant history (Fig. 1i). Furthermore, we observed a negative correlation between miR-1202 and GRM4 expression levels (Fig. 1j). Finally, in order to explore the effects of anxiety, we investigated PFC tissue from individuals who at the time of their death met criteria for a major anxiety disorder and were of similar age to the individuals with MDD and control subjects. However, the vast majority of these individuals died by suicide, and, as expected, they also met criteria for MDD. Our results showed differential expression of miR-1202 and GRM4 in PFC tissue from these individuals as compared to controls, although the effects were stronger in the group of subjects with MDD alone (Supplementary Fig. 3). These findings suggest a relationship between miR-1202, GRM4, MDD and antidepressant treatment in humans.

To experimentally confirm the interaction between miR-1202 and GRM4, we performed functional experiments in human embryonic kidney cells (HEK 293). We selected HEK 293 cells after screening miR-1202 and GRM4 expression in six different cell lines (Supplementary Table 4). HEK 293 cells showed no endogenous expression of miR-1202 and relatively high levels of GRM4 and thus were an ideal model for our tests. We examined whether...
overexpression of miR-1202 affects the expression of GRM4. We transfected cells with an miR-1202 mimic (Online Methods), a scramble control or a mock vehicle for 24 h. We found miR-1202 levels after transfection of miR-1202 mimic in HEK 293 cells but no effects on GRM4 expression after treatment with the scramble or vehicle controls (Fig. 2a). We then tested whether neutralizing miR-1202 affected GRM4 using miRNA target protectors (Online Methods). We treated cells with miR-1202 mimic and either a target protector or a scramble control for 24 h. We specifically designed the target protectors to interfere with the two predicted binding sites of miR-1202 in the 3' untranslated region of the GRM4 gene (Supplementary Fig. 4). Cotransfection of miR-1202 mimic with either target protector (TP1 or TP2) in HEK 293 cells reversed GRM4 levels to baseline (Fig. 2b). There were no effects on GRM4 expression levels after treatment with either target protector alone, scramble or vehicle controls (Supplementary Fig. 5). These results confirm an interaction between miR-1202 and GRM4.

Subsequently, we tested whether miR-1202 responds to changes in the expression of GRM4. We performed functional experiments using GRM4 agonist and antagonist in human neural progenitor cells (NPCs). Human NPCs showed relatively high levels of miR-1202 after 1 week of differentiation and expressed GRM4 (Supplementary Fig. 4 and Supplementary Fig. 6). We used the GRM4 agonist (2S)-2-amino-4-phosphonomobutanoic acid (L-AP4) and antagonist 2-methyl-2-phosphonoserine (MSOP) at nontoxic concentrations based on results from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Supplementary Fig. 7). NPCs were treated for 7 d with L-AP4, MSOP or a no-drug control. Treatment with L-AP4 reduced GRM4 expression, whereas MSOP treatment increased expression (Fig. 2c). Consistent with our hypothesis, miR-1202 expression was upregulated after 1-AP4 treatment and downregulated after MSOP treatment (Fig. 2d). These results suggest a bidirectional interaction between miR-1202 and GRM4.

There has been increased interest in the potential role of miRNAs as molecular intermediaries of therapeutic response. Given our postmortem results, we hypothesized that miR-1202 may mediate response to antidepressant treatment. We examined the effects of antidepressants on miR-1202 by treating NPCs with two commonly prescribed antidepressants, citalopram or imipramine, or a no-drug control. NPCs display a serotonergic phenotype (Supplementary Fig. 8a,b). We treated cells for 24 h (acute treatment) or for 15 d (chronic treatment) at nontoxic concentrations (Supplementary Fig. 9). We found no effect of acute treatment with either drug on miR-1202 or GRM4 expression (Fig. 2e,f), but we found an upregulation of miR-1202 after chronic treatment with either imipramine or citalopram (Fig. 2g). Chronic treatment with either antidepressant reduced GRM4 mRNA expression (Fig. 2h), findings that we also observed at the protein level using imaging and immunocytochemistry assays (Supplementary Fig. 10). We did not observe these effects when treating cells with medications that do not have direct effects on serotonin or the serotonin transporter (SERT) (lithium and valproate). Furthermore, through knockdown experiments, we found that the increase in miR-1202 expression after chronic treatment with citalopram or imipramine is dependent on SERT and the reuptake blockade needed for antidepressant action (Supplementary Fig. 11a–g). Finally, to investigate whether the effects of chronic treatment were due to a global miRNA dysregulation, we measured the expression levels of several miRNAs known to be ubiquitously expressed, but we found no differences in the expression of any of these miRNAs after chronic antidepressant treatment (Supplementary Fig. 12). These findings indicate a relationship between miR-1202 and antidepressant treatment response.

On the basis of our in vitro results, we explored the regulation of miR-1202 in patients with MDD according to antidepressant treatment. We measured blood levels of miR-1202 in treatment-naïve patients with MDD and psychiatrically healthy control subjects (Supplementary Table 5). We treated patients with citalopram as previously reported 23 (Fig. 3a). In line with previous findings, we found a decrease of miR-1202 expression in patients with depression (Fig. 3b). We classified subjects with depression into remitters and nonresponders on the basis of changes in their Hamilton Rating Scale for Depression (HAM-D) scores. Notably, a comparison...
between groups revealed that the miR-1202 dysregulation, at baseline, was driven by the remitter group. There was a decrease in miR-1202 expression in the remitter group as compared to the other groups but no difference in its expression between nonresponders and controls (Fig. 3c). Moreover, the remitter group showed increased miR-1202 levels after 8 weeks of treatment (Fig. 3d), whereas we found no difference in miR-1202 levels in nonresponders or controls (Fig. 3e,f). Furthermore, change in depression severity negatively correlated with change in miR-1202 expression (Fig. 3g). These findings confirm a relationship between peripheral miR-1202 expression and citalopram treatment response in patients with MDD.

To our knowledge, this is the first study to report, in humans, a consistent miRNA dysregulation in postmortem brain tissue and blood samples from individuals with MDD. Additionally, and most notably, our results suggest the possibility that changes in miR-1202 expression levels could predict citalopram treatment response. These results may have implications in the search for biomarkers and predictors of treatment response in MDD and suggest that miR-1202 could be potentially used as a biomarker of treatment prediction or response. Although it is unclear how miRNA levels in the periphery and the brain interact during antidepressant treatment, it can be hypothesized that miRNAs may actively cross the blood–brain barrier

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data were deposited in the Gene Expression Omnibus database with accession code GSE58105.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
We are grateful for the contributions made by the families consenting to donate brain tissue to the Douglas–Bell Canada Brain Bank. We thank A. Ryan and C. Ernst at McGill University for their contributions of chick brain tissue and human NPCs, respectively. This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR) (2008#190734 and 2013#311113), as well as support from the Fonds de recherche du Québec Santé (FRQS) through its network program, Quebec Network on Suicide, Mood Disorders and Related Disorders (RQSHA). J.P.L. received doctoral funding awards from FRQS and CIHR. G.T. is an FRQS chercheur national.

AUTHOR CONTRIBUTIONS
J.P.L. was involved in conducting and coordinating all aspects of the research, including testing feasibility and planning the experiments, processing human and animal tissues, validating results, executing most molecular and cellular experiments, analyzing data, and interpreting and preparing the manuscript. R.L. and P.P. were responsible for bioinformatics and statistical analysis of the miRNA microarray data. C.C. planned and carried out antidepressant treatment of human NPCs and screening for cytotoxic effects. L.C. performed the agonist and antagonist treatment of human NPCs. C.C., L.C. and G.M. were responsible for the maintenance of human NPCs and screening for cytotoxic effects. L.C. performed the agonist and antagonist treatment of human NPCs. J.P.L. and V.Y. conducted the experiments involving overexpression and neutralization of miR-1202 effects on HEK 293 cells. B.L. and N.M. participated in the design of the study and interpretation of the data. G.T. conceived, supported and neutralized cell lines. C.F., E.V. and S.E.M. were responsible for the maintenance of human NPCs and knockdown cell lines. C.C., L.C. and G.M. were responsible for the maintenance of human NPCs and knockdown cell lines. C.C., L.C. and G.M. were responsible for the maintenance of human NPCs and knockdown cell lines. C.C., L.C. and G.M. were responsible for the maintenance of human NPCs and knockdown cell lines.


**ONLINE METHODS**

**Human samples.** Postmortem prefrontal cortex (BA44) brain tissue was obtained in collaboration with the Quebec Coroner’s Office and the Suicide section of the Douglas-Bell Canada Brain Bank (Douglas Mental Health University Institute, Montreal, Quebec, Canada). A total of 104 brain samples were included in the present study. All individuals were of French-Canadian origin, a homogeneous population with a well-documented founder effect, and were matched for refrigeration delay, age and pH. Refrigeration delay refers to the difference between the estimated time of death (determined by the pathologist through external body examination details) and the time at which the body was refrigerated. Psychological autopsies were performed postmortem on both cases and controls by a panel of psychiatrists, and diagnoses were assigned based on DSM-IV criteria. The control group had no history of suicidal behavior or major mood or psychotic disorders. Subjects in the control group died by accidental (sudden death) causes. Commercially available human miRNA samples from heart (cat. HR-801-SR), intestine (cat. HR-306-SR), kidney (cat. HR-901-SR), liver (cat. HR-314-SR), lung (cat. HR-601-SR), skin (cat. HR-101-SR), spleen (cat. HR-701-SR) and stomach (cat. 302-SR) were obtained from AMS Biotechnology (Lake Forest, CA). Ethical approval for this study was obtained from The Institutional Review Board of the Douglas Mental Health University Institute, and written informed consent was obtained from the family of each deceased subject before inclusion in the study.

**Human blood samples.** Patients were ascertained at a community outpatient clinic at the Douglas Mental Health University Institute. Subjects were excluded from the study if they had comorbidity with other major psychiatric disorders, if they had positive tests for illicit drugs at any point during the study or if they had general medical illnesses. Subjects with MDD were untreated patients with a diagnosis of MDD without psychotic features, according to the Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were also excluded if they had a history of antidepressant treatment. All subjects included in the study provided informed consent, and the project was approved by The Institutional Review Board of the Douglas Mental Health University Institute.

**Animal brain samples.** Commercially available brain miRNA samples from cynomolgus (cat. KR-201-SR) and rhesus (cat. UR-201-SR) monkeys were obtained from AMS Biotechnology (Lake Forest, CA). Mouse and rat brains were dissected in house and obtained from Charles River (Canada).

**Samples processing.** Total RNA (including miRNA fraction) was isolated from frozen brain and blood samples using the mirEasy Mini Kit protocol (Qiagen, Canada) with no modifications. Blood samples were collected in PAXgene blood RNA tubes (PreAnalytix, Switzerland). PAXgene tubes were frozen using a sequential freezing process. RNA and miRNA yield and quality were determined using the Nanodrop 1000 (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA), respectively.

**miRNA microarray and data analysis.** Expression levels of 866 human and 89 human viral microRNAs (miRNA Sanger Base release 12.0) were studied using the Human miRNA microarray kit (V2) (Agilent Technologies, USA). We used the AgiMicroRNA package in Bioconductor to read the miRNA data. The robust multivariate average algorithm, developed for Affymetrix arrays, was used to summarize the results. Data were normalized using the quantile method, and miRNAs flagged as absent were later removed. We applied standard linear regression techniques in conjunction with surrogate variable analysis (SVA), which attempts to capture the heterogeneity involved in a gene expression study. We fitted models using the Limma Bioconductor package for linear regression. To select a model, we fitted a number of different linear models that had been augmented with surrogate variables to each gene and scored each model fit using the Akaike information criterion (AIC). AIC measures the goodness of fit while penalizing for greater number of terms in the model. Finally, we chose the model that had the highest number of best AIC scores.

**qRT-PCR.** Total miRNA was reverse transcribed using M-MLV reverse transcriptase (Gibco) and oligo(dT)16 primers (Invitrogen). miRNA was reverse transcribed using TaqMan RT-PCR microRNA assays (Applied Biosystems) according to the manufacturer’s instructions. Real-time PCR reactions were run in quadruplets using the ABI 7900HT Fast Real-Time PCR System, and data were collected using the Sequence Detection System (SDS) software (Applied Biosystems). To measure miRNA expression, we used miRNA TaqMan probes, considered to be the gold standard for miRNA quantification. Expression levels were calculated using the Absolute Quantitation (AQ) standard curve method, with β-actin and GAPDH used as endogenous controls for miRNA quantification. Five endogenous controls were tested for miRNA quantification, U6, RNU6B, RNU44, U47 and RNU48, but only RNU6B was selected, as it showed expression levels that remained relatively constant with low variance and high abundance across samples tested. All primers used in the study can be found in Supplementary Table 6.

**Western blot analysis.** Human PFC samples were dissected taking care not to include white matter. Samples were homogenized by sonication in PBS containing protease inhibitors (Roche). Equal concentrations of protein (25 µg per lane) were separated by SDS-PAGE (NuPage Bis-Tris 4–12%) (Invitrogen) and transferred onto a nitrocellulose membrane. Membranes were incubated overnight with antibodies to GRM4 protein (dilution 1:100, Santa Cruz, USA, cat. no. sc-76485) or β-actin protein (dilution 1:20,000, Sigma, Canada, cat. no. A2066). Bound antibodies were detected with HRP 800 or ~700 (dilution 1:5,000) and analyzed with an infrared Odyssey detection system (LI-COR Biosciences, Lincoln, Nebraska, USA). All cases and controls were analyzed in the same experiment, and experiments were performed in triplicates.

**miRNA target prediction analysis and validation.** Gene targets of miR-1202 were predicted using five miRNA target prediction databases: miRWalk, microRNA.org, RNA22, RNA Hybrid and TargetScan. Only genes predicted by all 5 databases were chosen. We then crossreferenced these genes with our existing microarray expression libraries, and only genes that were expressed in human brain and upregulated in PFC of subjects with depression were selected for further validation.

**Cell culture.** Cell lines used in this study were obtained from the American Type Culture Collection (ATCC). Human embryonic kidney (HEK 293, cat. no. CRL1573), human neuroblastoma (Be2C, cat. no. CRL2268; SK-N-AS, cat. no. CRL2137; and SH-SY5Y, cat. no. CRL2266) and human glioblastoma (U-118 MG, cat. no. HTB-15) cells were used. Human neural progenitor cells (NPCs) derived from wild-type induced pluripotent stem cell (iPSC) line 8330-8 were provided by C. Ernst at McGill University. miRNA overexpression and neutralization of miR-1202. Human embryonic kidney cells (HEK 293) were cultured in DMEM supplemented with 10% FBS, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin (Invitrogen) in a 5% CO2 humidified incubator at 37 °C. For miR-1202 mimic treatments, cells were grown in the continuous presence of either 10 nM miR-1202 mimic, 10 nM miR-mimic scramble control or mock vehicle for 24 h. miRNA mimics are synthetic double-stranded RNAs that mimic naturally occurring miRNAs after transfection into cells. For miR-1202 target protector treatments, cells were transfected and grown in the continuous presence of either 10 nM miR-1202 mimic and 1 µM miR-1202 target protector, 10 nM miR-1202 mimic and 1 µM target protector scramble control, 1 µM target protector alone or mock vehicle for 24 h. Target protectors are single-stranded modified RNAs that interfere with the interaction of a specific miRNA with a single target while leaving the regulation of other targets of the same miRNA unaffected. All experiments were performed in triplicate.

**GRM4 agonist and antagonist treatment.** NPCs were maintained on culture plates coated with 200 µg ml−1 poly-l-ornithine hydrobromide (Sigma) and 5 mg ml−1 laminin (Sigma) in 70% DMEM (Invitrogen) 30% Ham’s F12 (Mediatech) with 1× penicillin-streptomycin (Invitrogen) and supplemented with B-27 (Invitrogen). During expansion, cells were grown in medium containing 20n g ml−1 of human EGF (Sigma), FGF (R&D Systems) and 5 µg ml−1...
heparin (Sigma). To induce neural differentiation, cells were allowed to reach 90% confluence before growth factors were removed.

We used the mGluR III agonist (2S)-α-amino-4-phosphonobutanoic acid (t-AP4) and antagonist 2-methyl-O-phosphonosorine (MSOP). Cells were screened for cytotoxic effects by measuring the activity of mitochondrial dehydrogenase using the MTT assay (Sigma-Aldrich) and showed no toxicity after 1 week of treatment with t-AP4 or MSOP. NPCs were treated for 7 d with 100 µM t-AP4, 150 µM MSOP or a no-drug control. All experiments were performed in triplicate.

Antidepressant treatment. Human NPCs were screened for cytotoxic effects using the MTT assay, and antidepressants were applied at nontoxic concentrations. Cells were grown in the continuous presence of either 50 µM citalopram hydrobromide, 12.5 µM imipramine hydrochloride or no-drug control for 24 h (acute) or 15 d (chronic). All experiments were performed in triplicate.

Generation of stable knockdown cell lines. All shRNAs used in this study were cloned into the pLKO.1 vector and packaged into lentivirus. NPCs were maintained at 50% confluence (500,000 cells) in a single well of a six-well plate and infected with 20 µl viral (titer = 1 × 10⁹) medium in 2 mL cell culture medium without penicillin-streptomycin. Plates were spun for 20 min at room temperature at 2,000 r.p.m. and then placed back in an incubator at 5% CO2 and 37 °C. Puromycin (0.8 µg ml⁻¹), resistance to which is produced by the pLKO.1 vector, was added to cultures 48 h after lentiviral infection, and this followed an initial medium change 24 h after infection. Three days after puromycin, penicillin-streptomycin and low-dose puromycin were maintained (0.2 µg ml⁻¹) until freezing down of cell culture stocks.

Immunocytochemistry. Cells were grown and differentiated on coverslips coated with 200 µg ml⁻¹ poly-l-ornithine and 5 mg ml⁻¹ laminin for 30 d. Cells were then fixed in 4% formaldehyde for 20 min at room temperature at 2,000 r.p.m. and then placed back in an incubator at 5% CO2 and 37 °C. Puromycin (0.8 µg ml⁻¹), resistance to which is produced by the pLKO.1 vector, was added to cultures 48 h after lentiviral infection, and this followed an initial medium change 24 h after infection. Three days after puromycin, penicillin-streptomycin and low-dose puromycin were maintained (0.2 µg ml⁻¹) until freezing down of cell culture stocks.

Cells were permeabilized with 0.25% Triton X-100, and nonspecific binding was blocked with gelatin (2 g l⁻¹). Cells were incubated overnight with primary antibodies: mouse monoclonal anti-α-tubulin antibody (1:5,000, cat. T5168, Sigma-Aldrich), mouse monoclonal anti-5-HT transporter (SERT) antibody (1:2,000, cat. MAB1564, Chemicon), mouse monoclonal anti-triptophane hydroxylase (TpoH) antibody (1:2,000, cat. T0678, Sigma-Aldrich), rabbit polyclonal anti-mGlu receptor-4 antibody (1:1,000, cat. 51-3100, Invitrogen) and rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:5,000, cat. MAB318, Chemicon). Tissue and cells were then incubated for 1 h with goat anti-mouse Alexa Fluor 488–conjugated secondary antibodies (1:2,000, cat. A11029, Molecular Probes Inc., USA) or goat anti-rabbit Alexa Fluor 488–conjugated secondary antibodies (1:2,000, cat. A11034, Molecular Probes Inc., USA), according to each experimental design. Brain slices and coverslips were then incubated with Hoescht 33342 nucleic acid stain (2 µg ml⁻¹) for 10 min and finally mounted with Fluoromount-G (SouthernBiotech, USA).

Imaging and analysis. Images were taken using a Zeiss Axio Observer.Z1 inverted fluorescence microscope with AxioCam MRm camera and ApoTome.2 attachment (Carl Zeiss, Canada). The same exposure time was used to capture the images comparing NPCs, controls and the effects of antidepressant treatment. Images were analyzed with ImageJ software (NIH). Quantification of GRM4 expression in NPCs treated with antidepressants was performed by subtracting, to each image, the average background intensity observed without primary antibody (No Ab). Cells were selected by applying the same threshold to each image. The average gray value per pixel was automatically measured in the subthreshold elements. Data were then presented as a percentage of the intensity in the control group.

Citalopram treatment. Patients were treated with citalopram, starting with an initial dose of 10 mg d⁻¹, which was titrated progressively to a maximum of 60 mg d⁻¹, and all final doses were within the therapeutic range. Assessments of depression severity were carried out at each time point using the 21-item Hamilton Rating Scale for Depression (HAM-D21). Treatment compliance was assessed using high-performance liquid chromatography at the end of the trial. All subjects showed detectable plasma citalopram levels, and we observed a correlation between citalopram dose and plasma concentration.

Statistical analyses. All numerical data are expressed as the mean ± s.e.m. Statistical differences among groups were analyzed by Student’s t-test, one-way ANOVA with post hoc correction and Pearson’s correlation coefficients. Statistical significance was calculated using GraphPad Prism5 and SPSS 20. P < 0.05 was considered statistically significant.