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miR-1202: A Primate Specific and Brain Enriched miRNA Involved in Major Depression and Antidepressant Treatment

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Abstract

Major depressive disorder (MDD), is a prevalent mood disorder that associates with differential prefrontal brain expression patterns[1]. Treatment of MDD includes a variety of biopsychosocial approaches, but in medical practice, antidepressant drugs are the most common treatment for depressive episodes, and not surprisingly, they are among the most prescribed medications in North America[2,3]. While they are clearly effective, particularly for moderate to severe depressive episodes, there is important variability in how individuals respond to antidepressant treatment. Failure to respond has important individual, economic and social consequences for patients and their families[4]. Several lines of evidence demonstrate that genes are regulated through the activity of microRNAs (miRNAs), which act as fine–tuners and on–off switches in gene expression patterns[5–7]. Here we report on complementary studies using postmortem human brain samples, cellular assays and samples from clinical trials of depressed patients, and show that miR-1202, a miRNA specific to primates and enriched in the human brain, is differentially expressed in depressed individuals. Additionally, miR-1202 regulates the expression of the Metabotropic Glutamate Receptor 4 (*GRM4*) gene and predicts antidepressant response at baseline. These

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Author Contributions

J.P.L was involved in conducting and coordination of all aspects of the research, including testing feasibility and planning the experiments, processing of human and animal tissues, validation of results, execution of most molecular and cellular experiments, data analysis, interpretation and preparation of the manuscript. R.L and P.P were responsible for bioinformatics and statistical analysis of the microRNA microarray data. C.C planned and carried out antidepressant treatment of human NPCs and screening for cytotoxic effects. L.C performed the agonist/antagonist treatment of human NPCs. C.C, L.C and G.M were responsible for the maintenance human NPCs and knock–down cell lines. C.F, E.V and S.E.M were responsible for immunocytochemistry, western blot and imaging analysis. J.P.Y and V.Y conducted the overexpression and neutralization of miR-1202 effects on HEK293 cells. B.L and N.M participated in the design of the study and interpretation of the data. G.T conceived, supported and designed this study, was responsible for overseeing the experiments, including all aspects of design, interpretation of data and preparation of the manuscript and figures. All authors discussed the results presented in the manuscript.

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results suggest that miR-1202 is associated with the pathophysiology of depression and is a potential target for novel antidepressant treatments.

We assessed miRNA expression in the ventrolateral prefrontal cortex (PFC) of depressed individuals, 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 which we validated by qRT–PCR (Fig. 1b–c). According to miRBase[8], miR-1202 has very little evolutionary conservation. To better characterize this miRNA, we first investigated *in silico* its expression and conservation/divergence among species. Using the UCSC Genome Browser, hg19 assembly[9,10], we investigated the conservation of miR-1202 in 100 animal genomes. While miRNAs are generally well conserved across species[7], miR-1202 is only present in humans and other primates (Supplementary Fig. 1). In contrast, we found that Let–7a1, a miRNA known to be well conserved[11], is coded in 95/100 genomes investigated. We subsequently investigated miR-1202 conservation experimentally, by measuring expression in brains of six representative animal species. Total miRNA was extracted from human, *cynomolgus* monkey, *rhesus* monkey, rat, mouse and chicken brains. We found higher expression of miR-1202 in human brain as compared to cynomolgus and rhesus monkey, and no detection in other species (Fig. 1d). To investigate tissue specificity, we measured miR-1202 levels in 10 human tissues. While some level of miR-1202 was detected in all tissues, expression was considerably enriched in the brain (Fig. 1e). These findings suggest that expression of miR-1202 may be important for cognitive processes that are unique to primates, and particularly, humans.

Gene targets of miR-1202 were predicted using five miRNA target prediction databases[12–16]. We only considered those predicted by all five databases, and then cross–referenced these predicted genes with mRNA microarray expression libraries from PFC from an overlapping sample[17–20]. As there is an expected inverse relationship between miRNAs and their mRNA targets, only genes expressed and up-regulated in the PFC of depressed subjects were selected (Supplementary Table. 5). Interestingly, all selected genes are linked with neurological processes associated with the pathogenesis of MDD. Next, we quantified the expression of the predicted gene targets of miR-1202, using qRT-PCR, in subjects previously used for microarray analysis. Five genes were up–regulated in the PFC of depressed subjects (Fig. 1f). MiR-1202 levels correlated negatively with the expression of the metabotropic glutamate receptor 4 (*GRM4*), but not with the other predicted genes (Fig. 1g). Additionally, we found an increase of GRM4 protein expression levels that was correlated with the increased mRNA levels (Supplementary Fig. 2). *GRM4* is expressed throughout the brain and is localized pre–and post–synaptically, where it modulates glutamatergic, as well as dopaminergic, GABAergic, and serotonergic neurotransmission[21]. In recent years it has been implicated in the regulation of anxiety–related behaviors[21–23] and it is seen as an attractive target for drug discovery and development[24].

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 controls and depressed

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individuals. Additionally, we included a third group of depressed individuals with history of antidepressant use and positive antidepressant toxicology (Supplementary Table. 2). In line with previous findings, miR-1202 was decreased in depressed subjects as compared to controls. Interestingly, we also found a difference in miR-1202 expression between depressed subjects with and without antidepressant history (Fig. 1h). Consistently, *GRM4* was up-regulated in depressed brains, whereas there was no difference between controls and depressed individuals with antidepressant history. Nevertheless, there was a difference in the expression of *GRM4* between depressed subjects with and without 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 from individuals who at the time of their death met criteria to a major anxiety disorder and were of similar age to our depressed cases and controls. However, as the vast majority of these individuals died by suicide, as expected they also met criteria for MDD. Our results show differential expression of miR-1202 and *GRM4* in PFC from these individuals as compared to controls, although the effects were stronger in the MDD group (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 (HEK293). We selected HEK293 cells after screening miR-1202 and *GRM4* expression in six different cell lines (Supplementary Table. 3). HEK293 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.* Cells were transfected with miR-1202 mimic, a scramble control, or a mock vehicle for 24 hrs. We found decreased *GRM4* levels after transfection of miR-1202 mimic in HEK293 cells (Fig. 2a), but no effects on *GRM4* expression after treatment with the scramble or vehicle controls. We then tested whether neutralizing miR-1202 affected *GRM4* using miRNA target protectors. Cells were treated with miR-1202 mimic, and either a target protector or a scramble control for 24 hrs. Target protectors were specifically designed to interfere with the two predicted binding sites of miR-1202 in the 3′UTR of the *GRM4* gene (Supplementary Fig. 4). Co–transfection of miR-1202 mimic with either target protector (TP1 or TP2) in HEK293 cells reversed *GRM4* levels to baseline (Fig. 2b). There were no effects on *GRM4* after treatment with either target protector without miR-1202 mimic, 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 *GRM4.* We performed functional experiments using a *GRM4* agonist and antagonist in human neural progenitor cells (NPCs). Human NPCs showed relatively high levels of miR-1202 after one week of differentiation and expressed *GRM4* (Supplementary Table. 3, Supplementary Fig. 7a). We used the *GRM4* agonist L–AP4 and antagonist MSOP at non–toxic concentrations based on results from the 3–(4, 5–dimethylthiazol–2–yl)–2, 5–diphenyltetrazolium bromide (MTT) assay (Supplementary Fig. 6). NPCs were treated for 7 days with L–AP4, MSOP or a no–drug control. Treatment with L–AP4 reduced *GRM4* expression, while MSOP treatment increased expression (Fig. 2c). Consistent with our hypothesis, miR-1202 expression was

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up–regulated after L–AP4 treatment and down–regulated after MSOP treatment (Fig. 2d). These results suggest a bi–directional 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 post–mortem 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, imipramine, or a no–drug control. NPCs display a serotonergic phenotype (Supplementary Fig. 7b–c). Cells were treated for 24 hrs (acute treatment) or for 15 days (chronic treatment) at non–toxic concentrations (Supplementary Fig. 8). We found no effect of acute treatment with either drug on miR-1202 or *GRM4* expression (Fig. 2e–f), but an up–regulation 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 which we also observed at the protein level using imaging and immunocytochemistry assays (Supplementary Fig. 9). These effects were not observed when treating cells with medications (lithium and valproate) that do not have direct effects on serotonin or the serotonin transporter (*SERT*). Furthermore, through knock–down experiments, we found that the increase of miR-1202 after chronic treatment with citalopram and imipramine is dependent on *SERT* and the reuptake blockade needed for antidepressant action (Supplementary Fig. 10a–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 no differences were found (Supplementary Fig. 11). These findings indicate a relationship between miR-1202 and antidepressant treatment response.

Based on our *in vitro* results, we explored the regulation of miR-1202 in MDD patients according to antidepressant treatment. We measured blood levels of miR-1202 in treatment–naïve MDD patients and psychiatrically healthy controls (Supplementary Table. 4). Patients were treated with citalopram as previously reported[25] (Fig. 3a). In line with previous findings, we found a decrease of miR-1202 in depressed patients (Fig. 3b). Depressed subjects were classified into remitters (REM) and non–responders (NRES), based on changes in Hamilton Rating Scale for Depression (HAM–D) scores. Interestingly, a comparison between groups revealed that the miR-1202 dysregulation, at baseline, was driven by the REM group. There was a difference in miR-1202 expression between REM and the other groups but no differences between NRES and controls (Fig. 3c). Moreover, the REM group showed increased miR-1202 levels after 8 weeks of treatment (Fig. 3d), whereas there were no differences in NRES or controls (Fig. 3e–f). Furthermore, change in depression severity was 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 MDD patients.

To our knowledge, this is the first study to report, in humans, a consistent miRNA dysregulation in postmortem brain tissue and blood samples from MDD individuals. Additionally, and most importantly, our results suggest that miR-1202 predicts citalopram treatment response. These results have important implications in the search of biomarkers and predictors of treatment response in MDD and suggest that miR-1202 could be

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potentially used as a biomarker of treatment 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[26]. Alternatively, changes observed in blood might reflect neuroendocrine or neuroimmune responses elicited by the brain. Indeed, several miRNAs appear to modulate both immune and neuronal processes and may mediate the interaction between these systems[27]. Our findings and results from others support the hypothesis that targeting miRNAs directly could be therapeutically beneficial.

Together, our results suggest that the dysregulation of miR-1202 in postmortem brain and peripheral blood is associated with the pathophysiology of MDD. Our results postulate miR-1202 as a biomarker of MDD and a predictor of antidepressant treatment response. This study highlights the role of miRNAs in neuropsychiatric disorders, and provides important steps in the development of early diagnostic tools, preventive strategies, and effective pharmacological treatment for mood disorders.

Methods

Human Samples

Post–mortem 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[28], 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 post–mortem 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. 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). Ethics approval for this study was obtained from the research ethics board at the Douglas University Mental Health University Institute.

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. MDD Subjects 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

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informed consent and the project was approved by the internal review board for 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 miRNeasy 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 Bioanalizer (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) was studied using the Human miRNA microarray kit (V2) (Agilent Technologies, USA). We used the AgiMicroRna package in Bioconductor to read the miRNA data[29]. The robust multiarray average algorithm, developed for Affimetrix arrays, was used to summarize the results. Data was 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[30]. We fitted models using the Limma Bioconductor package for linear regression[31]. 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 Akike information criterion (AIC)[32]. 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.

Quantitative Real–Time Polymerase Chain Reaction (qRT–PCR)

Total mRNA 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 was 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[33]. Expression levels were calculated using the Absolute Quantitation (AQ) standard curve method, with β–Actin and GAPDH used as endogenous controls for mRNA 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

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low variance and high abundance across samples tested. All primers used in the study can be found in Supplementary Table. 8.

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 (25μg per lane) were separated by SDS–PAGE (NuPage Bis–Tris 4–12%) (Invitrogen) and transferred onto a nitrocellulose membrane. Membranes were incubated overnight with *GRM4* antibodies (dilution 1:100, Santa Cruz, USA) or B–Actin antibodies (dilution 1:20,000, Sigma, Canada). Bound antibodies were detected with IRdye 800 or –700, (dilution 1:5000) and analyzed with an infra–red 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 cross–referenced these genes with our existing microarray expression libraries and only genes that were expressed in human brain and up–regulated in PFC of depressed subjects were selected for further validation.

Cell Culture

miRNA Overexpression and Neutralization of miR-1202—Human embryonic kidney cells (HEK293) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen) in a 5% CO_2 humidified incubator at 37°C. For miR-1202 mimic treatments, cells were grown in the continuous presence of either 10nM miR-1202 Mimic, 10nM miR–Mimic scramble control or mock vehicle for 24hrs. For miR-1202 target protector treatments, cells were co–transfected and grown in the continuous presence of either 10nM miR-1202 mimic and 1μM miR-1202 target protector, 10nM miR-1202 mimic and 1μM target protector scramble control, 1μM target protector alone or mock vehicle for 24hrs. All experiments were performed in triplicate.

GRM4 Agonist and Antagonist Treatment—Human neural progenitor cells (NPCs) derived from wild type induced pluripotent stem cell (iPSC) line 8330–8, were generously provided by Dr. Stephen Haggarty. NPCs were maintained on culture plates coated with 200 μg ml^{-1} Poly–L–ornithine hydrobromide (Sigma) and 5mg ml^{-1} laminin (sigma) in 70% DMEM (Invitrogen) 30% Hams F12 (Mediatech) with 1x penicillin/streptomycin (Invitrogen) and supplemented with B–27 (Invitrogen). During expansion cells were grown in media containing 20ng ml^{-1} of human EGF (Sigma), FGF (R&D Systems) and 5μg 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)–2–amino–4–phosphonobutanoic acid (L–AP4) and antagonist 2–Methyl–O–phosphonoserine (MSOP). Cells were screened for cytotoxic effects

by measuring the activity of mitochondrial dehydrogenase using the 3–(4, 5–dimethylthiazol–2–yl)–2, 5–diphenyltetrazolium bromide (MTT) assay (Sigma–Aldrich Co) and showed no toxicity after one week of treatment with L–AP4 or MSOP. NPCs were treated for 7 days with 100μM L–AP4, 150μM MSOP or a no–drug control. All experiments were performed in triplicate.

Antidepressants Treatment—Human NPCs were screened for cytotoxic effects using the MTT assay and antidepressants were applied at non–toxic concentrations. Cells were grown in the continuous presence of either 50μM citalopram hydrobromide, 12.5 μM imipramine hydrochloride or no–drug control for 24hrs (acute) or 15days (chronic). All experiments were performed in triplicate.

Generation of stable knock–down cell lines

All short–hairpin RNA used in this study were cloned into the pLKO.1 vector, and packaged into lentivirus. NPCs were maintained at 50% confluency (500 000 cells) in a single well of a six–well plate and infected with 20ul viral (titre =10e9) media in 2 mL cell culture media without Pen/Strep. Plates were spun for 20 minutes at room temperature at 2000RPM, then placed back in an incubator at 5% CO2 and 37C. Puromycin (0.8ul ml^{-1}), resistance to which is produced by the pLKO.1 vector, was added to cultures 48 hours after lentiviral infection and this followed an initial media change 24 hours after infection. Three days after puromycin, pen/strep and low dose puromycin were maintained (0.2 ul ml^{-1}) until freezing down of cell culture stocks.

Immunocytochemistry

Cells were grown and differentiated on coverslips coated with 200μg ml^{-1} poly–L–ornithine and 5mg ml^{-1} lamanin for 30 days. Cells were then fixed in 4% formaldehyde for 20 minutes, and stored in PBS. Membranes were permeabilized with 0.25% Triton X–100 and nonspecific binding was blocked with gelatin (2g l^{-1}). Cells were incubated overnight with primary antibodies: mouse monoclonal anti–alpha–tubulin antibody (1:5000, cat.T5168, Sigma–Aldrich), mouse monoclonal anti–5–HT transporter (SERT) antibody (1:2000, cat.MAB1564, Chemicon), mouse monoclonal anti–triptophane hydroxylase (TpOH) antibody (1:2000, cat.T0678, Sigma–Aldrich), rabbit polyclonal anti–mGlu–receptor–4 antibody (1:1000, cat.51–3100, Invitrogen), rabbit polyclonal anti–tyrosine hydroxylase (TH) antibody (1:5000, cat.MAB318, Chemicon). Tissue and cells were then incubated for 1 hour with goat anti–mouse Alexa–fluor 488 conjugated secondary antibodies (1:2000, cat.A11029, Molecular Probes Inc., USA) or goat anti–rabbit Alexa–fluor 488 conjugated secondary antibodies (1:2000, cat.A11034, Molecular Probes Inc., USA), according to each experimental design. Brain slices and coverslips were then incubated with Hoescht 33342 nucleic acid stain (2μg 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

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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 sub–threshold 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 10mg die, which was titrated progressively to a maximum of 60mg die 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 (HAMD–21). 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.

Data Analysis

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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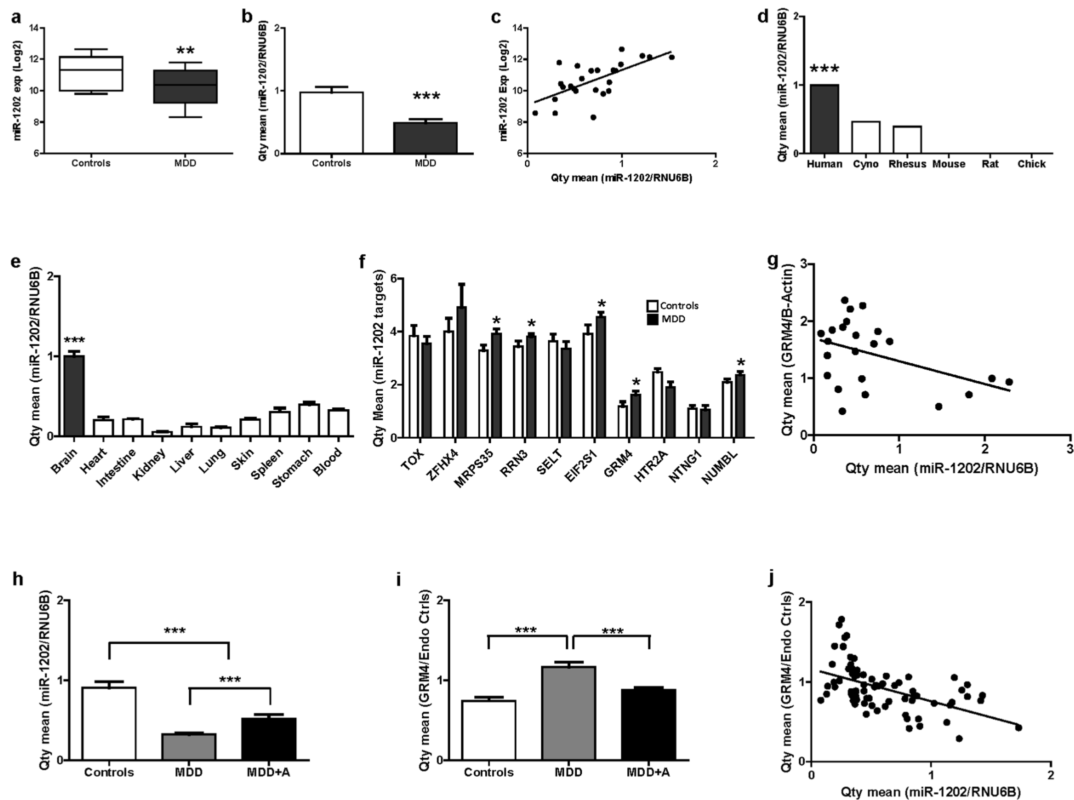

Figure 1.

Expression of miR-1202 (**a**) Boxplot showing BA44 microarray Log2 expression of miR-1202 in MDD subjects (n=14) and controls (n=11). (**b**) qRT–PCR validation (**c**) Correlation of microarray and qRT–PCR expression levels of miR-1202. (**d**) Expression of miR-1202 across animal brains. (**e**) Expression of miR-1202 across human tissues. (**f**) Expression of top ten genes predicted to target miR-1202 in brains of MDD subjects (n=14) and controls (n=11). (**g**) Correlation between *GRM4* and miR-1202 expression in PFC from MDD subjects and controls. (**h**) Expression of miR-1202 in a larger and independent sample. None of these subjects were used in the original microarray experiment. MDD (n=25), MDD+A (n=25) and controls (n=29). (**i**) Expression of *GRM4* in a larger and independent sample. MDD (n=25), MDD+A (n=25) and controls (n=29). (**j**) Correlation between miR-1202 and *GRM4* expression using a larger and independent sample. All numerical data are expressed as the mean ± s.e.m. Normality was assessed by Shapiro–Wilk normality tests, and statistical differences between groups were analyzed using Student's t–test (two–sided), One–Way ANOVA with post–hoc correction and Pearson's correlation coefficients. (MDD) Depressed suicide completers; (MDD+A) Depressed suicide completers with antidepressant history; (n) represents biological replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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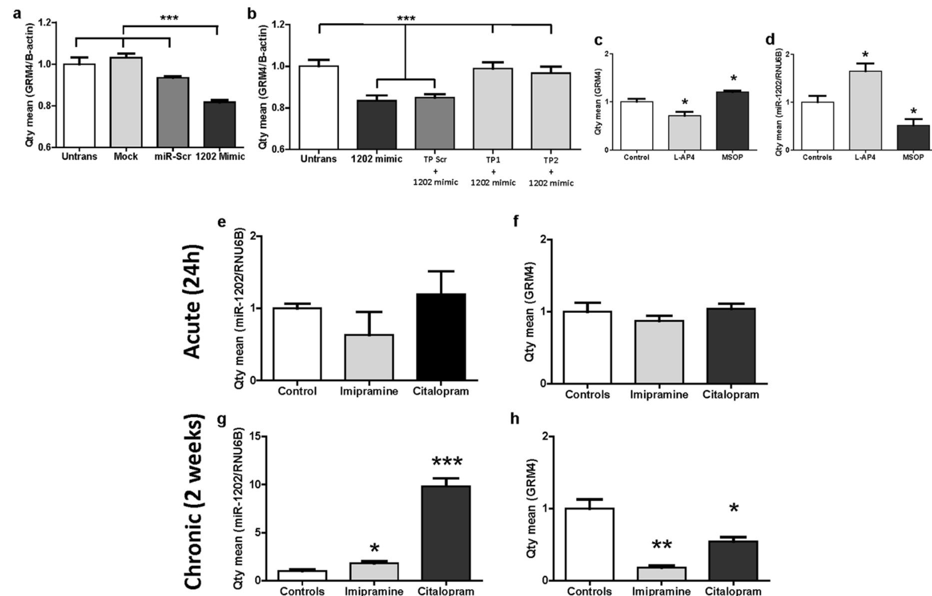

Figure 2.

Functional experiments – validation of miR-1202 and *GRM4* interaction (**a**) Overexpression of miR-1202. *GRM4* mRNA expression levels after transfection (24hrs) with miR-1202 mimic, miR–scramble control, mock vehicle or H2O control (untransfected) in HEK293 cells. (**b**) Neutralization of miR-1202. Expression levels of *GRM4* after co–transfection (48hrs) of miR-1202 mimic with target protectors (TP1 or TP2), target protector scramble control or H2O control (untransfected) in HEK293 cells. (**c**) Dysregulation of *GRM4* after chronic treatment (1 week) with agonist (L–AP4) and antagonist (MSOP) in human neural progenitor cells (NPCs) (**d**) Expression of miR-1202 after chronic treatment (1 week) with agonist (L–AP4) and antagonist (MSOP) in human NPCs. (**e–f**) Expression of miR-1202 and *GRM4* in human NPCs treated with Citalopram, Imipramine, or a no–drug control for 24hrs (acute treatment). (**g–h**) Expression of miR-1202 and *GRM4* in human NPCs treated with Citalopram, Imipramine, or a no–drug control for 2 weeks (chronic treatment). All numerical data are expressed as the mean ± s.e.m. Statistical differences between groups were analyzed using Student's t–test (two–sided), One–Way ANOVA with post–hoc correction. All experiments were performed in triplicates and (n) represents biological replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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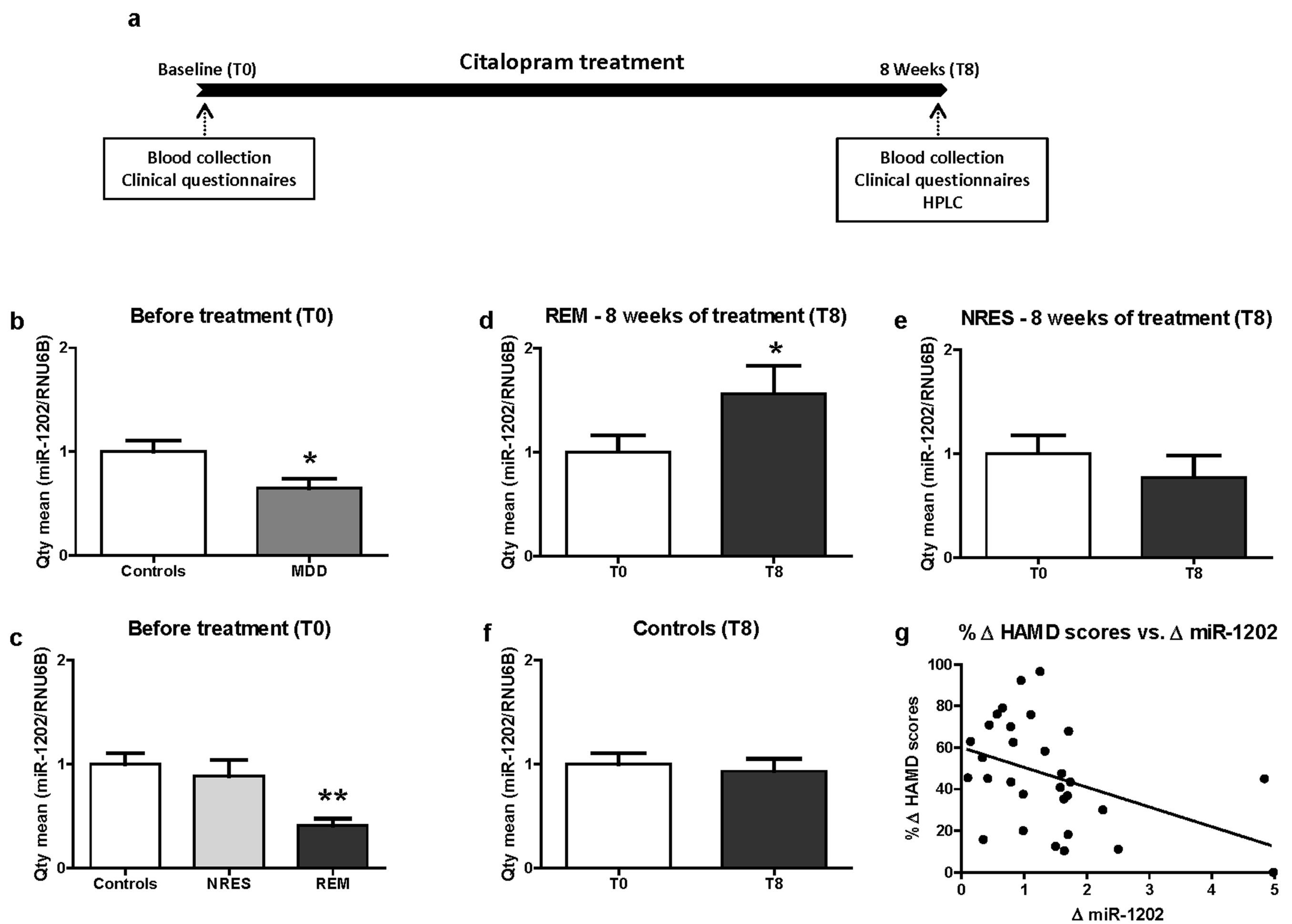

Figure 3.

Antidepressant Treatment in Humans (**a**) Timeline and steps taken within the 8–week citalopram trial, Clinical questionnaires: (HAMD–21) 21–item Hamilton Rating Scale for Depression; (SCID–I) Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders–fourth edition (DSM–IV) Axis I Disorders (**b**) Decreased expression of miR-1202 in MDD patients (n=32) as compared to controls (n=18) at baseline (T0). (**c**) Expression of miR-1202 in controls (n=18), non–responders (n=16) and remitters (n=16) at baseline (T0). (**d**) miR-1202 expression levels after 8 weeks of treatment in remitters, (**e**) non–responders (**f**) and controls. (**g**) Correlation between change in depression severity and change in miR-1202 expression. All numerical data are expressed as the mean ± s.e.m. Normality was assessed by Shapiro–Wilk normality tests, and statistical differences between groups were analyzed using Student's t–test (two–sided), One–Way ANOVA with post–hoc correction and Pearson's correlation coefficients. (MDD) Depressed patients; (REM) Remitters; (NRES) Non–responders; (HPLC) high–performance liquid chromatography; (n) represents biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.