miR-15a and miR-16 regulate serotonin transporter expression in human placental and rat brain raphe cells

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Abstract

The serotonin transporter (SERT) is a key regulatory molecule in serotonergic transmission implicated in numerous biological processes relevant to human disorders. Recently, it was shown that SERT expression is controlled by miR-16 in mouse brain. Here, we show that SERT expression is regulated additionally by miR-15a as well as miR-16 in human and rat tissues. This post-transcriptional regulation was observed and characterized in reporter assays and likewise when endogenous SERT expression was evaluated in human placental choriocarcinoma JAR cells and rat brain raphe RN46A cells – two cell lines that endogenously express SERT. Similar effects for miR-16 to those of miR-15a were found in both human and rat cell lines. The effects of miR-15a and miR-16 were comparable in extent to those originally reported for miR-16 in mice. These findings represent a novel layer of complexity for SERT expression regulation exerted by the miR-15a/16 cluster, whose genes are adjacently located at human chromosome 13q14.3.

Received 2 November 2011; Reviewed 12 December 2011; Revised 30 March 2012; Accepted 1 April 2012; First published online 8 May 2012

Key words: miR-15a, miR-16, serotonin transporter, SERT, SLC6A4.

Introduction

The serotonin transporter (SERT) gene, SLC6A4, maps to chromosome 17q11.2 and is composed of 14 exons spanning 40 kb. SERT is a 603 amino acid protein with 12 transmembrane domains that regulates neurotransmitter reuptake. In SLC6A4, allelic length variation in the SLC6A4 promoter region [i.e. serotonin transporter-linked polymorphic region (5-HTTLPR)] and modifying single-nucleotide polymorphisms (SNPs; rs25531 and rs25532), alternate promoters, differential splicing involving exons 1A, B and C, intronic and 3’ untranslated region (3’-UTR) variability, as well as different SNPs result in multiple variants that regulate gene expression in humans and other species (Murphy & Moya, 2011). Since the first report describing the SLC6A4 5-HTTLPR being associated with anxiety-related personality traits (Lesch et al. 1996), there has been a constantly growing database describing altered SERT expression and function as being associated with diseases (Caspi et al. 2003, 2010; Fan & Sklar, 2005; Hu et al. 2006; Mitchell et al. 2011; Wendland et al. 2008). In particular, the SLC6A4 5-HTTLPR and its other variants have been associated with a plethora of human disorders, such as anxiety spectrum disorders like obsessive–compulsive disorder, bipolar disorder, attention deficit hyperactivity disorder, autism, depression and neurodevelopmental and peripherally based disorders of cardiovascular, bone, gastrointestinal, endocrine and other systems (Berger et al. 2009; Hoffman et al. 2007; Murphy & Lesch, 2008; Murphy & Moya, 2011; Murphy et al. 2004a, b).

In contrast to the intensive study of 5’ and intronic SLC6A4 variants, genetic research focusing on the SLC6A4 3’-UTR is sparse. The 3’-UTR plays important roles in mRNA translation, localization and stability. Importantly, these processes have recently been shown to be modulated by microRNAs (miRNAs) that exert their action through imperfect complementary base-pairing with regions mainly located in the 3’-UTR...
of target mRNAs (Kosik, 2006; Millan, 2011), thought to regulate at least 20% of all genes (Xie et al. 2005). In a recent report, Baudry and colleagues demonstrated that SLC6A4 expression in the mouse is regulated by miR-16 by interacting with a miRNA-binding site in the SLC6A4 3'-UTR (Baudry et al. 2010).

We have examined putative miRNA binding sites in the human SLC6A4 3'-UTR by bioinformatic predictions and experimental validation. We show here that SLC6A4 expression is also post-transcriptionally regulated by miR-15a, as well as miR-16, in a human cell line and in a rat brain raphe cell line.

Materials and method

In silico analysis

We performed computational predictions of putative miRNA binding sites within the SLC6A4 3'-UTR. We selected a list of miRNA candidates that were predicted in at least two of three studied algorithms: PicTar (Krek et al. 2005), miRanda (John et al. 2004) and TargetScan (Lewis et al. 2005).

Gene reporter assays

The SLC6A4 3'-UTR (696 bp) was amplified from human genomic DNA obtained from 10 Caucasian controls (LaSalle et al. 2004; Wendland et al. 2008). A total of 30–50 ng of genomic DNA were amplified using final concentrations of 1× multiplex master mix (Qiagen, USA) and 0.5 μM each of primers F: CACACTCACCAGAGGAAA and R: CACAAATTGAGTGTGCTGCTGTGGCATCAA (Operon, USA) and cloned into pMIR vector (Ambion, USA) downstream to the Firefly luciferase gene to generate the reporter vector pMIR-5-HTTfull. A 70 bp DNA segment (GAGTAGAATTTGTT (Operon, USA). Thermocycling conditions were as follows: 15 min at 95°C; followed by 35 cycles of 95°C (10 s), 63°C (30 s) and 72°C (50 s); plus a final extension step at 72°C for 5 min. The amplicon was cloned into pCRII-TOPO vector (Invitrogen, USA) and then subcloned into pMR vector (Ambion, USA) downstream to the Firefly luciferase gene to generate the reporter vector pMIR-5-HTTfull. A 70 bp DNA segment (GAGTAGAATTTGTT (Operon, USA) and cloned into pMIR vector both in sense (pMIR-5HTTmiRsite) and antisense orientation (pMIR-5HTTmiRsite-inv), downstream to the Firefly luciferase gene. Site directed mutagenesis of pMIR-5HTTfull and pMIR-5HTTmiRsite was performed to eliminate the miRNA binding site by mutating the sequence ACCTTCTAATCCA to ACCTTAGGCC-TCCA and to generate pMIR-5HTTfull-MUT and pMIR-5HTTmiRsite-MUT, respectively using Quick-Change II XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA). Bidirectional sequencing was performed after every cloning step to confirm sequence specificity and lack of unwanted mutations.

Undifferentiated rat raphe medullary raphe cells (RN46A, a kind gift from Dr Scott R. Whittemore, University of Louisville) and human choriocarcinoma cells (JAR; ATCC, USA) grown under standard conditions were co-transfected with the reporter constructs plus pRL (Renilla luciferase; Promega, USA) and premiR-15a, premiR-16, antimiR-15a, antimiR-16 (p/ns PM10235, PM10339, AM10235, AM10339, AM17010, respectively, Ambion) or premiR-Neg (p/n AM17010; Life Technologies, USA) oligos using siPORT NeoFX transfection reagent (Ambion). We selected human JAR and rat RN46A cells for our in vitro assays since they both are well-characterized cell models where SERT is known to be endogenously expressed (Balkovetz et al. 1989; Bethea et al. 2003).

Twenty four hours after transfection, cells were harvested and luciferase activity was measured using Dual Luciferase Assay (Promega) following manufacturer’s protocol in a 20/20n luminometer (Turner Biosystems, USA). Renilla luminescence was used to correct for transfection efficiency variability. Each experiment consisted of transfecting reporter vectors 3–4 times in triplicate, for a total of 9–12 independent transfections. Empty vector control was used in every transfection experiment. Normalized ratios for the constructs were then analysed by one-way analysis of variance followed by the Newman-Keuls post-hoc test to compare all pair-wise group means (using Prism for Windows, USA).

Quantitative PCR (qPCR) experiments

For RNA extraction, we used the mirVana PARIS kit (Ambion) with the enrichment step for small RNA according to the manufacturer’s guide. Reverse transcription was performed using the ABI TaqMan microRNA RT kit (p/n 4366597; Applied Biosystems, USA) as per protocol’s instructions except that 2 ng small RNA instead of total RNA were used, and with 75 U instead of 50 U of RT enzyme per individual reaction. qPCR was performed using ABI TaqMan FAM assays specific for each miRNA together with assays for CANX, UBC and MDH1 as controls (Applied Biosystems). Thermocycling conditions were as follows: 95°C/10’00”; 40× (95°C→60/10’00”→acquire FAM); 10/∞.
To evaluate miR-15a and miR-16 effects on SERT protein levels, we used pre-designed lentiviral particles for stable overexpression (lentimiRs; System Biosciences, USA). JAR cells at 80% confluency were transduced with lentiviral particles. Protein determinations were made at days 2, 4 and 7 post-transduction, using SERT antibody (catalog no. SAB2500950; Sigma, USA) at 1:1000 dilution.

Results

Our computational search predicted miRNA binding sites in the human SLC6A4 gene structure and reporter vectors generated. The entire 3' untranslated region (3'-UTR; containing the miR-15a/miR-16 site and the two endogenous polyA signals) was cloned into pMIR vector to give pMIR-5HTTfull; a 70 bp region containing the miRNA binding site was cloned in sense to generate pMIR-5HTTmiRsite, whereas the same region cloned in antisense was used to generate pMIR-5HTTmiRsite-inv. Therefore, because of being co-expressed in the same cells, miR-15a and miR-16 can potentially regulate SLC6A4 expression. Sequence alignments of SLC6A4 3'-UTR with miR-15a and miR-16 are shown in Fig. 1b. Alignment of the putative miRNA seed region in human, rat and mouse is shown in Fig. 1c.

When transfected into RN46 cells, pMIR-5HTTfull resulted in a reduced luciferase expression to approximately 50% compared to empty vector (Fig. 2a). When pMIR-5HTTfull was co-transfected with synthetic precursors (premiRs) for each of the miRNA candidates into RN46A cells, we observed that both premiR-15a and premiR-16 further decreased luciferase expression (Fig. 2a). In addition, pMIR-5HTTfull expression was not affected by co-transfection with premiR-neg, a scrambled premiR oligo used as a negative control, as shown in Fig. 2a. The observed effects of miR-15a, miR-16 and miR-neg on
by a Newman-Keuls post-hoc One-way analysis of variance for all transfections followed are expressed relative to luciferase activity of empty vector. Data experiments where pMIR-5HTTfull-MUT was used as premiR-15a or premiR-16 or premiR-Neg. White bars indicate transfected alone or with increasing concentrations of premiR-15a, premiR-16, or premiR-Neg. The data represent means ± S.E.M. of 9–12 transfections.

pMIR-5HTTfull expression were identical when tested in JAR transfected cells, as shown in Fig. 2b.

Since the SLC6A4 3′-UTR contains endogenous polyadenylation signals that are cloned into the reporter construct, the observed differences in reporter expression (relative to empty vector control) might be attributable to a differential polyadenylation signal use. To overcome this potential artefact, we generated a small 70 bp region containing the predicted miRNA binding site and cloned it into pMIR to obtain the pMIR-5HTTmiRsite reporter vector, as shown in Fig. 1a. Co-transfection of pMIR-5HTTmiRsite reporter vector, as shown in Fig. 3a. Co-transfection of pMIR-5HTTmiRsite with premiR-15a or premiR-16 in JAR cells resulted again in decreased reporter expression (Fig. 3b) to a similar extent as that observed in pMIR-5HTTfull experiments. An identical effect was found on RN46 cells (Fig. 3a). miR-neg had no effect on pMIR-5HTTmiRsite expression in any cell line, as shown in Fig. 3a, b.

To confirm that SLC6A4 miRNA binding site interacts with endogenous miR-15a and miR-16, we antagonized their activity by using antimiRs. Upon co-transfection of pMIR-5HTTmiRsite with antimiR-15 or antimiR-16 in RN46 cells, the luciferase expression was de-repressed in a concentration dependent manner, confirming the interaction of endogenous miR-15a and miR-16 with the SLC6A4 3′-UTR (Fig. 3c). Identical responses were obtained in JAR cells (Fig. 3d).

To further validate the functionality of the miRNA binding site, we performed site-directed mutagenesis of the putative miRNA site to generate pMIR-5HTTmiRsite-MUT; this reporter vector was insensitive to miRNA-mediated repression by either endogenous miRNAs or exogenously applied premiRs in RN46A cells, as shown in Fig. 3a (white bars). As expected, the de-repressing effect of antimiR oligos was not observable when evaluated on pMIR-5HTTmiRsite-MUT expression (Fig. 3c, white bars). Essentially identical responses were obtained for pMIR-5HTTmiRsite-MUT when evaluated in JAR cells, as shown in Fig. 3b, d. Similar results were obtained when we performed the mutagenesis in pMIR-5HTTfull to generate pMIR-5HTTfull-MUT, which showed no difference in expression when compared to empty vector as well as it lacked miRNA-mediated effects when co-transfected with premiR-15a or premiR-16 (Fig. 3a, b, white bars).

An additional validation approach used was to clone the 70 bp region containing the miRNA site in inverted orientation to generate pMIR-5HTTmiRsite-inv. As shown in Fig. 3, pMIR-5HTTmiRsite-inv also lacked miRNA-mediated repression by either endogenous as well as exogenously applied premiRs (Fig. 3a, black bars) or antimiRs (Fig. 3c, black bars) at different concentrations in RN46A cells. As shown in Fig. 3b (black bars) and 3c (black bars), essentially identical responses were obtained for pMIR-5HTTmiRsite-inv when evaluated in JAR cells.

Similar experimental procedures using premiRs and antimiRs oligonucleotide transfections failed to detect any effect on endogenous SERT expression. Since miRNAs affect de novo protein synthesis, we used lentiviral-mediated miRNA expression to achieve sustained expression. Upon 4 d post-transduction with lentiviral particles coding for miR-15a, SERT protein levels were clearly knocked down compared to scrambled-lentivirus transduced JAR cells (Fig. 4a). Similarly, lentiviral overexpression of miR-16 also significantly reduced SERT protein. The SERT protein expression inhibition occurred in parallel with detectable, significant increases of miR-15a or miR-16, as shown in Fig. 4b, c.

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**Fig. 2.** Effect of miR-15a and miR-16 on luciferase activity of pMIR-5HTTfull (entire SLC6A4 3′UTR) measured in (a) RN46A cells and (b) JAR cells. pMIR-5HTTfull was transfected alone or with increasing concentrations of premiR-15a or premiR-16 or premiR-Neg. White bars indicate experiments where pMIR-5HTTfull-MUT was used as negative control (see Materials and method section). Data are expressed relative to luciferase activity of empty vector. One-way analysis of variance for all transfections followed by a Newman-Keuls post-hoc test. * and ** indicate significance p < 0.05, p < 0.01 and p < 0.001, respectively compared to pMIR-5HTTfull transfected alone (i.e. 0 nM, first bar to the left). Data represent means ± S.E.M. of 9–12 transfections.

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Our findings clearly indicate that miR-15a regulates SLC6A4 expression. In addition, we corroborated here the single previously published report of a miR-16 effect on SLC6A4 expression measured by reporter vector assays and quantification of radioligand binding sites in mouse brain (Baudry et al. 2010). Thus, these two complementary studies establish that miR-15a and miR-16 comprise an important novel coordinated regulatory mechanism controlling SLC6A4 expression.

miRNAs can regulate gene expression at the translational level through interacting with their target mRNAs, most commonly located in 3'-UTR regions. By manipulating miR-15a and miR-16 levels using precursor and antisense oligonucleotides, we demonstrated that these miRNA-mediated phenomena occurred when the SLC6A4 3'-UTR was cloned into a reporter vector (Fig. 2). Importantly, we obtained similar results with the entire SLC6A4 3'-UTR as well as with a small 70 bp region containing the predicted miRNA binding site (Fig. 3). The observed phenomena cannot be attributed to off-target effects, since premiR-Neg was devoid of activity on both reporter vectors and because mutation of the seed region the miRNA binding site abolished the miRNA:mRNA interaction in both pMIR-5HTTfull and pMIR-5HTTmiRsite with endogenous and exogenous miR-15a and miR-16. Therefore, the results shown are sequence-specific and attributable to the miRNA binding site validated here. Further, miR-15a and miR-16 were ineffective in pMIR-5HTTmiRsite-inv reporter vector and pMIR-5HTTmiRsite-MUT, where the short region containing the miRNA binding site was either cloned in reverse orientation or mutated to disrupt miRNA:mRNA hybridization (Fig. 3); similar results were found when mutating the miRNA site in the full length SLC6A4 3'-UTR in pMIR-5HTTfull-MUT (Fig. 2). The antagonism of endogenous miRNA activity by antimiRs was observable in both human JAR and rat RN46A cell lines, in agreement with the full sequence conservation of miR-15a and miR-16 between human, rat and mouse. As shown in Fig. 1, there is also conservation of the seed region of the

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**Discussion**

Our findings clearly indicate that miR-15a regulates SLC6A4 expression. In addition, we corroborated here the single previously published report of a miR-16 effect on SLC6A4 expression measured by reporter vector assays and quantification of radioligand binding sites in mouse brain (Baudry et al. 2010). Thus, these two complementary studies establish that miR-15a and miR-16 comprise an important novel coordinated regulatory mechanism controlling SLC6A4 expression.

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miRNA site in SLC6A4 3'-UTR characterized in this study. In addition, using lentiviral particles to obtain sustained miRNA overexpression, endogenous SERT protein levels were effectively reduced in human JAR cells (Fig. 4). Thus, our data strongly support that miR-15a, as well as miR-16, mediate SLC6A4 translational control in multiple species.

Alterations in SLC6A4 expression have been associated with anxiety and depression-related traits. In particular, the 5-HTTLPR and other variations have been associated with neuropsychiatric conditions such as bipolar disorder, depression, anxiety disorders, eating disorders and neurodegenerative disorders (Hoffman et al. 2007; Mitchell et al. 2011; Murphy & Moya, 2011; Murphy et al. 2004a, b). Therapeutic responses and side-effects following treatment have also been found to be associated with alterations in SERT expression (Murphy & Lesch, 2008; Murphy & Moya, 2011). The present results thus open a new avenue to unravel additional factors that regulate SLC6A4 expression beyond classical variations in the 5-HTTLPR plus rs25531 and rs25532, Stin2 and coding region (1425V, G56A) variants (Kilic et al. 2003; Prasad et al. 2005; Sutcliffe et al. 2005). Additionally, novel interactions between polymorphisms located in the 3'-UTR SLC6A4 region with other SLC6A4 variants as well as other genes observed to interact with SLC6A4 (ITGB3, BDNF) might be responsible for lack of replication in some negative human genetics association reports, as well as variants that may be present in miR-15a/miR-16 regulatory gene regions.

Both miR-15a and miR-16 are encoded by adjacent genes on chromosome 13q14.3 and represent a gene cluster located within the intron 6 of the DLEU2 gene (Calin et al. 2002, 2008; Lerner et al. 2009). Of interest, the miR-15a/miR-16 cluster has been implicated in several other mammalian cell processes, most notably haematopoiesis and its related cancers, such as chronic lymphocytic leukaemia, plus other types of cancers, and sometimes associated with down-regulation/deletion of BCL-2 or Myc and involving changes in cell survival, proliferation and migration (Bonci et al. 2008; Calin et al. 2002, 2008; Lerner et al. 2009). The miR-15a/miR-16 cluster also appears to participate in hierarchical regulatory processes such that its functions are sometimes altered by other miRNAs (e.g. miR-709; Tang et al. 2012).

When analysing the DNA sequence of the SLC6A4 3'-UTR amplicons generated from the human samples used here, we found two SNPs rs1042173 (T/G) and rs38130344 (T/G). We found two haplotypes that included these polymorphisms in the SLC6A4
3′-UTR: T-T and G-G (i.e. rs1042173 and rs38130344 were always in phase). Reporter vectors made with these two SLC6A4 3′-UTR molecular haplotypes resulted in no significant differences in regard to miR-15a or miR-16 translational repression (data not shown). This is in agreement with a previous report by others, showing that the reduced reporter expression of SLC6A4 3′-UTR was not affected by rs1042173, which lies in putative miR-545 binding site according to their predictions (Jensen et al. 2009). In their study, however, Jensen and colleagues focused rather on searching for polymorphisms that might affect miRNA-mediated regulation. Therefore, the putative miR-545 site within SLC6A4 3′-UTR was not validated in that report.

Our findings indicate that human and rat SLC6A4 expression is subject to post-transcriptional control by the miR-15a/16 gene cluster. Like many, if not all miRNAs, both miR-15 and miR-16 are predicted to bind to and regulate multiple gene targets. Intriguingly, we noticed during our bioinformatic search that several of the miR-15a and miR-16 predicted targets are indeed related to serotonergic transmission. Therefore, it is tempting to speculate that miR-15a and miR-16 can be key master regulators for multiple serotonergic pathway-related genes.

Serotonergic receptor genes have also been shown to exhibit miRNA-mediated expression control, such as HTR2A (Beveridge et al. 2010), HTR3E (Kapeller et al. 2008) and HRT1B (Jensen et al. 2009). Of interest, in the two latter reports, the functional polymorphisms within the miRNA sites were associated with disease: the HTR3E 3′-UTR functional variant was found to be associated with diarrhea predominant irritable bowel syndrome (Kapeller et al. 2008), while HRT1B 3′-UTR variant was found to be associated with aggressive behaviours (Jensen et al. 2009). In turn, serotonin may influence additional miRNAs, like miR-124 as shown in Aplysia (Rajasethupathy et al. 2009). The case of miR-448 is remarkable: the miR-448 gene is located in the fourth intron of the human HTR2C gene, which is located in Xq23. Both 5-HT2C receptors and miR-448 oppositely regulate adipocyte differentiation, opening exciting questions about feedback cycles impacting the role of serotonin in physiological processes such as obesity (Kinoshita et al. 2010).

Both serotonin and miRNAs have been implicated in multiple functions such as neuroplasticity, mood and cognition as well as in neuropsychiatric disorders such as schizophrenia, mood and anxiety disorders and drug addiction (Millan, 2011; Sethupathy & Collins, 2008). Additionally, serotonin is known to have a critical role in many major organ systems such as pulmonary, gastrointestinal, cardiovascular and genitourinary systems (for a review, see Berger et al. 2009). We hope our present results will contribute to refine the understanding of the serotonergic transmission control and provide a link to this fascinating novel layer of serotonergic and other gene expression control by miRNAs.

Acknowledgements

This research was supported by the Intramural Research Program of the National Institute of Mental Health, National Institutes of Health. The authors also thank James Nagle and Debbie Kauffman, from the NINDS DNA sequencing core facility, for outstanding technical assistance with sequencing the SLC6A4 3′-UTR and reporter constructs, and Dr Scott R. Whittemore, University of Louisville, for providing us with the RN46A cell line.

Statement of Interest

Jens R. Wendland is a full-time employee of F. Hoffmann-La Roche Ltd.

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Molecular Psychiatry 14, 381–389.


