The 5′ untranslated region of the serotonin receptor 2C pre-mRNA generates miRNAs and is expressed in non-neuronal cells

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Abstract The serotonin receptor 2C (HTR2C) gene encodes a G protein-coupled receptor that is exclusively expressed in neurons. Here, we report that the 5′ untranslated region of the receptor pre-mRNA as well as its hosted miRNAs is widely expressed in non-neuronal cell lines. Alternative splicing of HTR2C is regulated by MBII-52. MBII-52 and the neighboring MBII-85 cluster are absent in people with Prader–Willi syndrome, which likely causes the disease. We show that MBII-52 and MBII-85 increase expression of the HTR2C 5′ UTR and influence expression of the hosted miRNAs. The data indicate that the transcriptional unit expressing HTR2C is more complex than previously recognized and likely deregulated in Prader–Willi syndrome.

Keywords miRNA · Alternative splicing · snoRNA · Serotonin receptor

Introduction

The serotonin receptor 2C (HTR2C) is a G protein-coupled receptor located on the X chromosome. Binding studies indicate that the receptor is expressed exclusively in the brain, predominantly in neurons (Pompeiano et al. 1994). Mouse studies and the development of weight-loss drugs like Fen-Phen validated the serotonin receptor 2C (HTR2C) protein as an anti-obesity drug target (Miller 2005). In addition, HTR2C plays a central role in the regulation of mood and anxiety. It has also been implicated in depression, suicide, and schizophrenia (see Di Giovanni et al. 2011 for reviews). The HTR2C protein receptor is targeted by second generation anti-psychotics used for mania and depression, which block the receptor activity. One major side effect of these substances is weight gain (Lett et al. 2012), and there is genetic evidence that HTR2C is associated with the anti-psychotic-induced weight gain (Sicard et al. 2010). Direct investigation into the receptor function is limited, as it is expressed in neurons that are accessible only postmortem.

The receptor is encoded by a complex transcription unit spanning at least 326 kilobase pairs. Its pre-mRNA undergoes extensive processing that includes alternative splicing as well as editing of exon Vb, generating a total of 25 isoforms. Skipping of exon Vb generates a truncated protein isoform. Studies in transfected cells show that the truncated isoform forms a heterodimer with the full-length receptor, causing an entrapment in the endoplasmic reticulum and decrease of active cell surface receptor (Martin et al. 2012). The full-length protein isoforms are generated through editing and SNORD115 action. Central to the regulation of the HTR2C editing in exon Vb, is the formation of an extended stem-loop structure that is the substrate for RNA editing through ADAR2. The formation of the non-edited form is
promoted by the non-coding RNA SNORD115 (MBII-52 in mouse). SNORD115 is a C/D box snoRNA that is further processed into smaller RNAs (Kishore et al. 2010; Falaleeva and Stamm 2013). SNORD115 binds to the double-stranded region that is regulated in exon Vb (Kishore and Stamm 2006b). The mouse and human orthologues of SNORD115 are almost identical (>90% identity) and MBII-52 works on both human and mouse HTR2C reporter constructs (Kishore and Stamm 2006a). SNORD115 is absent in patients with Prader–Willi syndrome, and mouse models indicate that a deregulation of the serotonergic system contributes to the disease (Morabito et al. 2010; Doe et al. 2009).

In contrast to the well-studied coding region of the HTR2C receptor pre-mRNA, little is known of the extended 5′ untranslated region of the receptor pre-mRNA that contains two introns and three exons (Fig. 1a), which host miRNAs. Mouse models employed to study HTR2C function still contain the 5′ UTR and the hosted miRNAs (Tecott et al. 1995; Xu et al. 2008), and it is therefore possible that the mouse knockouts capture only the protein function of the HTR2C transcriptional unit, but not its function as a host of miRNAs.

miRNAs are 22nt long non-coding RNAs. The majority of miRNAs reside in pre-mRNA, both in the UTRs and in introns. In the nuclear pre-mRNA, miRNAs are part of extended double-strand structures forming pri-miRNAs that are recognized and cleaved by DROSHA/DGCR8, forming pre-miRNA that are exported into the cytosol. In the cytosol, DICER forms mature miRNAs that are loaded on argonaute proteins. miRNAs act in gene regulation by binding to mRNA, where they can block translation, cause RNA decay, RNA cleavage, as well as chromatin silencing (Esteller 2011).

Here, we report that the HTR2C gene expresses exons 1–3 and miRNAs located in intron 2 in non-neuronal cell lines, demonstrating that part of the HTR2C gene is expressed outside the brain and in non-neuronal cell types. The abundance of the encoded miRNAs is regulated by MBII-52 and MBII-85, two RNAs implicated in Prader–Willi syndrome. Since miRNAs encoded by HTR2C intron 2 are broadly expressed and detectable in lymphocytes, they contribute to the gene function in non-neuronal cells and could allow to monitor the response of the HTR2C gene to drug treatments.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Expression of miRNAs located in serotonin receptor intron 2 in different cell lines. **a** Schematic diagram of the serotonin receptor 2C pre-mRNA. **b–d** qRT-PCR showing amplification of miRNAs. The amount of miRNAs in human cortex tissue was set to one, and miRNA abundance in other cells was expressed as a fraction of the expression in cortex.
Materials and methods

RNase protection

HEK 293T cells were transiently transfected with plasmids coding for MBII-52 and MBII-85 snoRNA (mouse orthologous of SNORD115 and SNORD116 respectively). Total RNA was isolated from cells using Trizol LS reagent (Invitrogen) according to manufacturer protocol.

As a probe, we used a uniformly labeled RNA against mouse chr7:59,520,283–59,520,365 for MBII-52 and chr7:59,861,729–59,861,827 for MBII-85, as previously described (Shen et al. 2011).

RNAs from human cortex was isolated using the RNeasy Lipid Tissue Kit (Qiagen, Hilden) according to manufacturer protocol.

Cell culture

The HEK293, HeLa, and A549 cells were obtained from the American Type Culture Collection (ATCC). RH18 cells were obtained from St. Jude Children’s Research Hospital, and the primary human fibroblast cells were obtained from Coriell Institute (GM 00498D). All the cells were grown in the recommended medium containing 10 % fetal bovine serum (FBS) at 37 °C under 5 % CO2. Lymphocytes were collected from the blood of a healthy male donor and were purified using ACCUSPIN System-Histopaque-1077 (Nishimura et al. 2007).

RT-PCR

Sixty nanograms of total RNA (30 ng/μl), 5 pmol of reverse primer, and 40 U of SuperScript III reverse transcriptase (Invitrogen) were mixed in 5 μl of RT (reverse transcription) buffer. To reverse transcribe the RNA, the reaction was incubated at 55 °C for 50 min. Half of the RT reaction was used for cDNA amplification. The reaction was performed in 25 μl and contained 10 pmol of specific forward and reverse primers, 200 μM dNTPs, 1× Taq polymerase buffer, and 1 U of Taq DNA polymerase. The amplification was performed in an Eppendorf PCR System with the following conditions: initial denaturation for 10 min at 95 °C, 40 cycles for 15 s at 95 °C, and an extension of 1 min at 60 °C.

Brain tissues

Cortex tissues were obtained postmortem from three patients with Prader–Willi syndrome and three individuals as a control group. The tissue was obtained from the NICHID brain bank in Maryland. A detailed analysis of the tissues was performed in (Falaleeva et al. 2013).

TaqMan analysis

Thirty nanograms of total RNA, 1× RT primer, 15 mM dNTPs, 50 U of SuperScript III Reverse Transcriptase, 1× RT (Reverse Transcription) buffer, 4 U of RNase inhibitor were mixed in a 15 μl RT reaction. The reverse transcription was performed under the following condition: 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C.

One-fifth of the RT reaction was used for the qPCR. The reaction was performed in 20 μl and contained 1× TaqMan MicroRNA assay primer, 1× TaqMan universal PCR Master Mix no AmpErase UNG (Applied Biosystems). The amplification was carried out in a Stratagene Mx3000p thermocycler with the following conditions: initial denaturation for 10 min at 95 °C, 40 cycles for 15 s at 95 °C, and an extension of 1 min at 60 °C.

RNA isolation and deep sequencing

RNA was extracted from supraspliceosomes prepared from frozen HeLa cells (CILBIOTECH), as previously described (Azubel et al. 2006). The integrity of the RNA was evaluated by an Agilent 2100 bioanalyzer. For small RNA library construction, ∼10 mg of RNA was used, following the Illumina Directional mRNA-Seq Library Prep. (Pre-release Protocol) with the following changes: (1) The poly-A selection and fragmentation of mRNA steps were omitted; (2) To enrich for small RNAs, ethanol precipitation was used instead of column fractionation of the PNK-treated RNA. Adaptors were then ligated to the 5' and 3' ends of the RNA, and cDNA was prepared from the ligated RNA and amplified to prepare the sequencing library. The amplified sequences were purified by PAGE and sequences representing RNA smaller than 200 nt were extracted from the gel. The library was sequenced using the Genome AnalyzerHX System by Illumina. The sequencing data, after filtering the adaptors and low-quality sequences, were aligned to mirBase.

For RNase protection assays and miRNA analysis, RNA was isolated by Trizol LS reagent, and total RNA (including small RNAs and ribosomal RNAs) was used.

Search of MBII-85 and MBII-52 targets in the serotonin receptor 2C (HTR2C) pre-mRNA

The HTR2C pre-mRNA sequence was downloaded from ENSEMBL release 54. Subsequently, for each psnoRNA construct from MBII-52 and MBII-85, all the possible substrings with base pairing with any substring of the HTR2C genomic sequence were searched exhaustively. The following criteria were used as follows: (a) The substrings were
at least 15 nucleotides long; (b) Up to 2 mismatches were allowed; (c) Non-canonical G-U base-pairings were also considered.

Results

Detection of miRNAs located in the 5′ UTR by deep sequencing

During investigation of the splicing regulation of the serotonin receptor 2C, we performed deep sequencing of non-coding RNA from supraspliceosomes (Sperling et al. 2008) isolated from HeLa cells. The entire repertoire of nuclear pre-mRNAs are individually packaged in splicing active supraspliceosomes, that likely represent the in vivo composition of spliceosomes (Sperling et al. 2008). The sequencing data showed the expression of miR-764, miR-1260, miR-1912, and miR-1298. These four miRNAs are located in the 5′ UTR of the serotonin receptor 2C pre-mRNA, between exon 2 and 3 (Fig. 1a). miR-1298 was most abundant with 4217 reads, followed by miR-1264 with 2560 reads, miR-764 with 817 reads, and miR-1912 with 522 reads. miR-764, miR-1264, miR-1912, and miR-1298 are only found in the serotonin receptor 2C pre-mRNA. Since the expression of the serotonin receptor 2C is considered neuron-specific, these results were highly surprising.

Expression of miRNAs in cell lines

We next validated the deep-sequencing findings using qRT-PCR employing available TaqMan probes for miR-764, miR-1260, miR-1912, and miR-1298. These four miRNAs are located in the 5′ UTR of the serotonin receptor 2C pre-mRNA, between exon 2 and 3 (Fig. 1a). miR-1298 was most abundant with 4217 reads, followed by miR-1264 with 2560 reads, miR-764 with 817 reads, and miR-1912 with 522 reads. miR-764, miR-1264, miR-1912, and miR-1298 are only found in the serotonin receptor 2C pre-mRNA. Since the expression of the serotonin receptor 2C is considered neuron-specific, these results were highly surprising.
PWS subjects, where miR-764 is down and miR-1264 is upregulated.

Next, we determined the influence of MBII-52 and MBII-85 on the expression of the hosting intron using RT-PCR that amplifies the flanking exons 2–3. As shown in Fig. 4d (quantified in Fig. 4e), we found an upregulation of the exons caused by the overexpression of both psnoRNAs. Together, the data suggest an influence of MBII-52 and MBII-85 on the expression of miR-764 and miR-1264 and the hosting part of the gene.
Target sequences of MBII-52 and MBII-85 in the serotonin receptor pre-mRNA

We analyzed binding sites for MBII-52 and MBII-85 on the serotonin receptor pre-mRNA. Very little is known so far about how psnoRNAs bind to their targets. Accordingly, we performed an unbiased, exhaustive search of potential targets of MBII-52 and MBII-85 psnoRNA constructs on the serotonin receptor 2C (HTR2C) gene. We initially searched for at least 15 nt matches and allowed up to two mismatches between psnoRNA sequences and the serotonin receptor 2C pre-mRNA. As shown in Fig. 5, we detect binding sites for MBII-52 and MBII-85 on the receptor 2C pre-mRNA, flanking the pre-miRNA stem-loop structure. All putative binding sites are visualized on the UCSC browser, under http://regulatorygenomics.upf.edu/Projects/htr2c-targets.html.

As shown in Fig. 5a–d, most of these binding sites are in evolutionary conserved regions. Importantly, the binding sites are conserved between the mouse and human copies. However, there are numerous binding sites in the intron as well. Therefore, the exact mechanism remains to be determined.

Discussion

HTR2C gives rise to mRNA widespread expression

Unexpectedly, we found that intron 2 of the serotonin receptor 2C is expressed in all cell lines investigated, which is in contrast to the protein-coding region of the HTR2C gene. The most likely explanation is the presence of a...
transcriptional termination signal prior to exon 4 that is not used in neurons. In contrast to other cells, neurons will therefore express the full-length mRNA encoding the protein. This notion is supported by ESTs encompassing only the first three exons and by the observation that HT2R2C mRNA from brain has the predicted size of one long mRNA containing exons one to six. Furthermore, databases indicate the presence of CpG islands only in exon I, suggesting that there is only one transcriptional start site. The HT2R2C transcriptional unit is therefore more complex than previously thought.

The expression of HT2R2C intron 2 could have practical use, as it offers the possibility to monitor HT2R2C expression under various pharmacological treatments that target the encoded protein. Numerous gene systems show a regulation between the 3′ UTR or encoded proteins and gene expression. Given the pharmacological importance of HT2R2C, it will be interesting to investigate whether the miRNAs change during treatment with drugs that target HT2R2C protein.

Fig. 5 Bioinformatically predicted binding sites of MBII-52 and MBII-52 located next to miRNAs in intron 2. The top line shows a schematic representation of intron 2. Numbers indicate the coordinates in human genome built (Hg19). psnoRNA-binding sites are indicated by small boxes; square: MBII-85, oval: MBII-52. a-d Alignment and phylogenetic conservation of the binding sites. The human sequences closest to the mouse sequences are indicated with their copy numbers in Hg19 after SNORD115 and SNORD116 (shown in red) (color figure online)

miRNAs encoded by HTR2C have different regulation

The 5′ UTR of the HTR2C gene functions to host the miRNAs. Whereas intron 2 expression is comparable between cell lines, the miRNAs show differences in expression, which suggest a further regulation, either a cell-type-specific processing of the pre-miRNA or a cell-type-specific stability.

The function of these miRNAs and their regulation remains to be determined. Mir-764-5p promotes osteoblast differentiation as it translationally represses Hsc70-interacting protein/STIP1 homology and U-Box containing protein 1 (CHIP/STUB1), which promotes osteoblast differentiation (Guo et al. 2012). Although there are no experimentally validated targets for the other miRNAs, it is almost certain that they control other cellular processes, which adds to the function of the serotonin receptor 2C pre-mRNA. These functions were not captured by knock-out mice that removed only the coding region, but left the genomic region
encoding intron 2 intact (Tecott et al. 1995; Xu et al. 2008). The knock-out mice were mostly studied with regard to changes in food intake, and it will be interesting to analyze whether the intron 2-encoded miRNAs can modify the phenotype or are deregulated in these knock-out mice.

psnoRNAs from the MBII-52 and MBII-85 loci influence the expression of encoded miRNAs

Inclusion of the serotonin receptor’s alternative exon Vb is promoted by processed snoRNAs from the MBII-52 cluster (Kishore and Stamm 2006b; Kishore et al. 2010). We therefore tested whether the two psnoRNAs, MBII-52 and MBII-85, influence expression of miRNAs encoded in intron 2. Transfection studies showed that both psnoRNAs increase abundance of the exons flanking intron 2, suggesting an influence on the pre-mRNA. This finding is supported by the analysis of postmortem material, where we found less exon change to two/three expression in samples from PWS patients who do not express these psnoRNAs.

The effect of psnoRNAs on the encoded miRNAs is more complicated, as miR764 is promoted by MBII-52 and MBII-85, whereas miR1264 is repressed. This interdependency is reflected in samples from PWS subjects, but the mechanism for the different regulations is not clear. We also noticed that one patient (4805) expressed less mir-1264 than controls, for the different regulations is not clear. We also noticed that one patient (4805) expressed less mir-1264 than controls, for the different regulations is not clear. Nonetheless, our data show that a deregulation of microRNAs located in intron 2 of the serotonin receptor 2C likely contribute to the Prader–Willi syndrome phenotype.

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