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Research report

Identification of alternatively spliced multiple transcripts of 5-hydroxytryptamine receptor in mouse

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ABSTRACT

5-Hydroxytryptamine receptors (HTRs) are coded by seventeen different genes in mouse. One of them is *htr4* that codes for the HTR4 receptor, a G-protein coupled receptor containing seven transmembrane domains. In mouse, the gene is reported to contain 6 exons and 5 introns. Our present study reports the presence of four transcript variants of this gene encoding different N-termini. These transcripts are expressed in neuronal as well as non-neuronal tissues of mouse. We have identified five novel coding exons present at the 5′ end of the gene which splice with the published internal exon in an alternative manner making a total of five transcripts, four new transcript variants (T1, T2l, T2s and T3) and one published earlier. All five transcripts encoding different N-termini were expressed in mouse brain. It was interesting to note the expression of only T3 transcript that was also detected in heart muscle and is the only *htr4* transcript expressed in heart. For the first time a transcript of *htr4* gene was detected in the heart of the mouse which might help us to make use of small laboratory animals to study HTR4 in heart. As this transcript is unique to the heart it can serve as potential therapeutic target for various cardiovascular disorders and dysregulation of heart rate, atrial contraction and atrial relaxation. These variants display heterogeneous properties in terms of the presence of signal peptide, acetylation, phosphorylation and glycosylation. Thus alternative splicing of *htr4* producing heterogeneous N-termini increases the diversity of the receptor.

1. Introduction

5-Hydroxytryptamine receptor (HTR) more frequently called serotonin receptors bind to the neurotransmitter serotonin. HTR includes seven different receptor classes classified as HTR1-7. HTR3 is a ligand gated ion channel (LGIC) while the rest of the receptors belong to the G-protein coupled receptors family [31,33]. Seventeen different genes encode serotonin receptor family members. Out of these five of the genes belonging of HTR1 class contain a single exon thereby lacking alternatively spliced variants. Two genes encoding HTR5 contain two exons and do not report any alternative splicing events. However alternatively spliced variants have been reported for HTR2 [11,18,43], HTR3 [8], HTR4 [38,12,41,3,6,16], HTR6 [32] and HTR7 [15,23] in various organisms. *htr4* gene in mouse is found to undergo alternative splicing at its C-terminus to produce four variants. $HTR4_{(a)}$ and $HTR4_{(b)}$ are abundantly expressed in brain. $HTR4_{(e)}$ and $HTR4_{(f)}$ are myenteric plexus specific [12,24]. Even in other vertebrates, HTR4 is extensively spliced alternatively to generate several isoforms. In rats, four variants are reported $HTR_{(a)}$, $HTR_{(b)}$, $HTR_{(e)}$ and $HTR_{(c1)}$ exhibiting differential expression where $HTR4_{(a)}$ is expressed more in brain and dorsal root ganglion, $HTR4_{(b)}$ and $HTR_{(c1)}$ are abundant in gastrointestinal tract and $HTR_{(c1)}$ does not exhibit constitutive receptor activity [38]. In humans, there are eight alternatively spliced variants (a–g, and n) of HTR4 receptor which differ in their C-terminal tail length and structure, however the ninth variant contains an insertion of 14 amino acids in the second extracellular loop [12,41,3,6]. In porcine, eleven variants have been reported [12] which differ at the level of receptor palmitoylation [37,36], downstream coupling [39,34] receptor desensitization [35,29] and constitutive receptor activity [10].

HTR4 is involved in cognitive function and memory consolidation [42], enteric nervous system growth and maintenance [24], increasing heart rate, atrial contractile force, hastening atrial relaxation [2]. It is responsible for feeding [21] and stress responses [7]. HTR4 is expressed in a variety of tissues namely brain [13,14], heart [22,5], and intestine [26] where it performs diverse functions. This receptor serves as a potential target for the development of therapeutic agents involved in the treatment of numerous mental illness including Alzheimer's disease [27], feeding disorders such as anorexia nervosa [21], and stress related disorders such as anxiety [7] and depression [25]. *htr4* gene is located on chromosome

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number 18 and spans 145 kb. The gene encoding beta adrenergic (β_2-AR, *adrb2* gene) receptor is embedded within this gene. *htr4* and *adrb2* can interact at transcriptional as well as post transcriptional levels [19]. *adrb2* is intronless and spans 2.2 kb. These two receptors can form homodimers and heterodimers which can integrate serotonergic and nor-adrenergic signals. The expressions of both these receptors are interdependent at transcriptional level. Both HTR4 and β_2-AR are implicated in autism spectrum disorders, depression and Alzheimer's disease. The requirement for a homodimeric or heterodimeric receptor assembly by a cell might involve transcriptional regulation of the genes. This suggests that the N-terminal variants of the receptors produced through alternative splicing might be involved in facilitating their assembly. As *adbr2* is intronless the major site of regulation appears to be the N-terminal of HTR4. Alternative splicing provides transcriptional regulation through the use of new exons, exon skipping or alternate promoter usage. β_2-AR has been implicated in cardio inotropy. It is hypothesized that β_2-AR-coupled PI3K constrain increases in cardiac inotropy through cyclic adenosine monophosphate-dependent phosphodiesterase activation although the underlying mechanism remains elusive [17]. HTR4 receptors are present in human and porcine atrial myocytes while they are reported to be absent from the heart of small laboratory animals [12].

Although a number of C-terminal variants have been reported which display differential expression, no N-terminal variants has been demonstrated so far. Keeping in mind that the β_2-AR is located in the 5′ untranslated region (5′ UTR) of the *htr4* gene, we decided to study the nature of 5′ UTR of *htr4* gene and search for potential transcripts encoding N-terminal variants which might be expressed in brain and heart. Moreover, the restriction upon the use of small laboratory animals to study the functions of HTR4 in heart propelled us to take up the study of *htr4* transcripts in heart. Our study provides the first experimental evidence for the presence of four new transcripts encoding HTR4 variants differing at their 5′ ends from mouse brain. Five novel exons (N1, N2, N3, N4 and N5) splice with internal exons in different combinations to produce four transcript variants designated as T1, T2l, T2s and T3. N1 and N2 exons are located in the 5′ UTR of the gene while N3, N4 and N5 are located in the first intron of the gene. All the four transcripts are expressed in mouse brain. T3 transcript is unique being expressed in heart making it a good candidate for further studies which can help to understand the role of the receptor in heart. Further the expression of T3 transcript in the heart of the mouse removes constrain upon the use of small laboratory animals to study HTR4 functioning in heart. Moreover, amino acid sequences corresponding to the N-termini of the HTR4 in mouse, human, rat and porcine are comparable. The difference in the receptor majorly lies in their C-termini which are found to be divergent. A heart selective drug is achievable if the heart is found to express a transcript variant *htr4* that is unique to the heart. The existence of T3 transcript can aid in designing a specific drug targeting various cardiovascular disorders.

2. Materials and method

2.1. Materials

Mice (A/J) were bred in house according to the Institutional Animal Care and Use Committee and Guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare; National Institutes of Health). The total RNA extraction kit was purchased from iNtRON Biotechnology, Inc. (Gyeonggi-do, Korea). M-MuLV-Reverse Transcriptase, PCR nucleotide mix, 100 bp PCR DNA ladders were purchased from GenScript (USA). The TOPO-TA cloning kit II was obtained from Invitrogen Corp. (Carlsbad, CA). The Plasmid DNA miniprep kit and Qiaquick PCR gel purification kits were purchased from Qiagen, Inc. (Santa Clarita, CA). Primers were custom synthesized from MWG Biotech, Pvt. Ltd., India. All other chemicals used in the experiments were of molecular biology grade.

2.2. RNA preparation

Total cellular RNA of brain and heart of 2 months old mouse was prepared using the easy spin™ (DNA free) Total RNA Extraction Kit according to the manufacturer's instructions. The eluted RNA was quantitated spectrophotometrically and RNA integrity was checked by ethidium bromide staining on denaturing agarose gel electrophoresis.

2.3. Primers

Genomic sequence of *htr4* gene was downloaded from NCBI with accession number GenBank ID NM008313. Primers were designed using the downloaded sequence. The oligonucleotides primers were used are shown in Table 1.

2.4. 5′ RACE

In order to detect the presence of possible alternatively spliced transcript variants of *htr4* gene, 5′ rapid amplification of cDNA (5′ RACE) was performed using 2 μg of total RNA from brain and heart of mouse, 5′ RACE kit and *htr4* specific reverse primer (M5HTR4REV1 from the junction of exon 4 and exon 5). The amplification was done following the instructions with the kit. The amplified RACE product was fractionated by electrophoresis using 1.2% agarose gel. Several bands were excised from the gel, purified and subcloned into the TOPO vector and sequenced as described later.

2.5. Reverse transcriptase (RT)-PCR

The total isolated RNA was amplified using RT-PCR which led to the identification of several alternatively spliced transcript variants from both brain and heart of mouse. 2 μg of total RNA from both the tissues were primed with oligo $(dT)_{18}$ and single-stranded cDNA was synthesized using the RevertAid™ H-Minus Reverse Transcriptase at 43 °C for 1 h in a total volume 20 μl. 1 μl of the synthesized single-stranded cDNA was used for PCR amplification using appropriate sets of primers in a total volume of 50 μl employing touchdown PCR for 30 cycles. The cycle were performed as; denaturation at 94 °C for 4 min, 1 cycle; 93 °C, 30 s; annealing 66 °C, 30 s with a decrease of 0.3 °C per cycle, extension 72 °C for 45 s, final extension at 72 °C for 8 min. Final product was subjected to electrophoresis on a 1.2% agarose gel, stained with ethidium bromide and photographed on a UV transilluminator.

2.6. Semi-nested RT-PCR

To further confirm the results obtained after the first round of PCR, semi-nested PCR was performed. 1 μl of the RT-PCR product was further amplified for 30 cycles PCR reaction using a gene-specific reverse primer (from the junction of exon 4–exon 5) and first-exon-specific primer as down and upstream primers, respectively. This was followed by a second round of PCR utilizing 1 μl of the PCR product obtained above and same upstream primer while using a different downstream primer designed from the sequence located internally to the first reverse primer (from exon 4). The amplification was carried out for 30 cycles as above. 10 μl of the PCR product was then subjected to 1.2% (w/v) agarose gel electrophoresis, stained with ethidium bromide and photographed on a UV transilluminator.

2.7. Subcloning and sequencing of RT-PCR products

The resulting bands of RACE, RT-PCR and semi-nested PCR products were fractionated and excised from 1.2% agarose gel. These products were purified using PCR gel purification kit. The purified DNA was subcloned using the plasmid cloning vector. *Escherichia coli* JM109-competent cells were transformed. Transformed colonies were grown overnight at 37 °C and plasmid DNA was purified using plasmid purification kit. Plasmids containing the insert were sequenced in an automatic sequencer using either M13 forward or reverse primers [40].

2.8. Bioinformatics analysis

Homology and similarity searches of the obtained nucleotide sequences were performed using the BLASTN nonredundant database (http://www.ncbi.nlm.nih.gov/BLAST). Alignment analysis was carried out using the Gene stream Align tool (http://www2.igh.cnrs.fr/bin/align-guess.cgi) and ClustalW tool available at www.ebi.ac.uk/clustalw [1]. Various bioinformatics tools were further used to reach conclusive results. The gene coding for *htr4* along with 5′ UTR region in mouse was downloaded from Mouse Genome Informatics (MGI www.informatics.jax.org/). Primers specific to the new exons were designed using web-based Oligo Calc: Oligonucleotide properties calculator (www.basic.northwestern.edu/biotools/oligocalc.html). Some of the properties of the amino acid sequences coded by the new exons were analyzed using ExPASy tools (http://ca.expasy.org/).