



# Novel RNA Editing Sites of 5-HT<sub>2C</sub> Receptor Encoding the Third Intracellular Loop Domain in Undifferentiated NG108-15 Cells

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## Abstract

The 5 sites for RNA editing on 5-HT<sub>2C</sub> receptor are well known. On these sites, adenosine deaminases acting on RNA (ADARs) are involved in the reactions which convert adenine on RNA to inosine resulting that the inosine acts as guanine. Many reports exist about the physiological significance. In this article, novel 4 sites finding in the 3rd intracellular loop site of 5-HT<sub>2C</sub> using NG-108-15 cells were reported. There are 3 subtype of ADAR. The type-3 was not expressed in NG108-15 cells. Combining with previous report, the type-1 ADAR also seems to be involved in the novel RNA editings. Editing rates of 5 reported sites in 5-HT<sub>2C</sub> receptors change during neuronal development. Although the functional significance of newly finding 4 sites is still unknown, further studies will be continued in considering the relationship with neuronal development since these sites were found in undifferentiated neuronal cloned cells.

## Introduction

At least 13 kinds of subtypes of serotonin receptors have been reported [1]. Among those, 2C subtype (5HT<sub>2C</sub>CR) is unique as for followings reasons on molecular regulations [2]. The relations between the various psychological diseases have also been suggested [3]. It has been well known that RNA editing occurs at five extremely limited positions in 5HT<sub>2C</sub>CR where encodes the second intracellular loop domain. The RNA editing is the reaction on mRNA after transcription from genomic DNA. Adenosine (A) residues on the mRNA are deaminated by adenosine deaminase acting on RNA (ADAR) to inosine, where is recognized as guanine (G) during translation [4]. Although the RNA editing usually occurs in a non-coding region, in the case of 5HT<sub>2C</sub>CR, that is generated in coding regions resulting that the equivalent amino acid changes to produce proteins with different functions. Others of interesting facts at the area of RNA editing sites of 5HT<sub>2C</sub>CR are also reported; short variant and binding with small RNA, HBII-52 sno RNA [5]. The physiological significance of 5HT<sub>2C</sub>CR RNA editing has been well discussed, for examples on GTP-binding proteins activation abilities [6], on the relation with the depression [7], schizophrenia [8], Prader-Willi syndrome [9] and others, and so on [3]. The author also reported on some papers about the 5HT<sub>2C</sub>CR editing functions and still continued the studying. In the examination using the cloned culture neuronal cells NG108-15 cells, the editing rates are changed by drug-induced neuronal differentiation [10]. Same results are also obtained in primary cultured cells and in the neonatal/postnatal rats brains [11]. During the examinations to know the functional details in NG108-15 cells, it is a little bit hard to obtain the PCR product where stepped over the part of lacking in short variant. In process to examine the reason of the difficulty, four of novel editing sites on 5HT<sub>2C</sub>CR mRNA where are in code of third intracellular loop domain were found. There are reportedly 3 subtypes of ADAR that related to RNA editing [12]. So the RNA expression level of each subtype was also examined in this cells. Hypothesis of the functions of novel editing and the directionality of the future studies would be also discussed.

## Materials and Methods

### Cell cultures

Mouse neuroblastoma and rat glioma hybrid cell NG108-15 cell were continuously cultured by using our previously described methods with slight modifications [13]. Briefly, the cells were cultured in DMEM supplemented with 4% FBS, 100 μM hypoxanthine, 16 μM thymidine, 1 μM aminopterin, and 1 μg/ml minomycin. All cultures were maintained at 37°C under 10% CO<sub>2</sub>.

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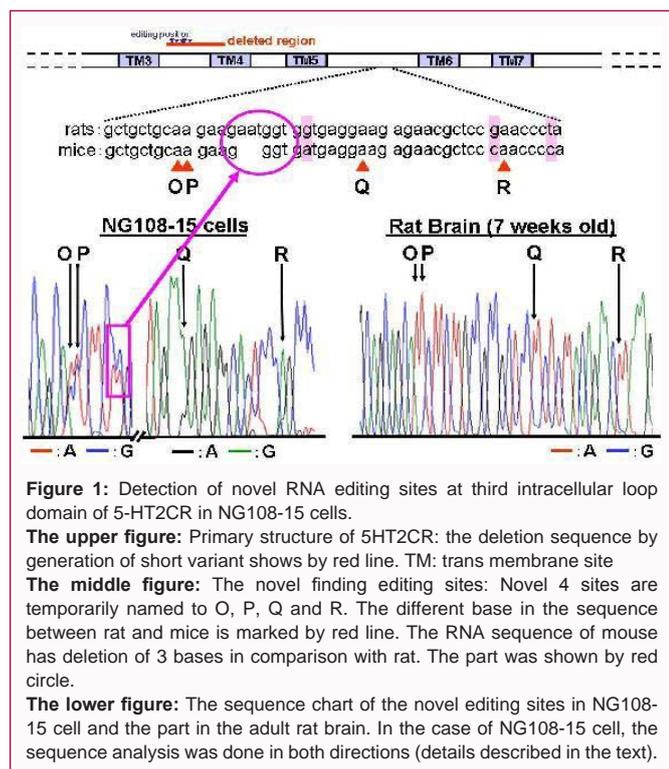
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**RNA isolation**

When NG108-15 cells reached confluent (normally for 5 or 6 days), the acidic phenol method was used to extract RNA. Immediately after remove the cultivation medium, 1 ml isolation solution (TRIsure: Nippon genetics, Tokyo, Japan) for 35 mm dish was directly apply to the culture dish and mildly mixed followed by stand for 1 min. After that the solution was collected into 1.5 ml tube. According to the instruction sheet, addition of CHCl3 followed by centrifugation treatment was repeated some times, and same volume of 2-propanol was applied to the finally obtained solution as an ethanol precipitation step. The resultant pellet after centrifugation was washed some times by 70% EtOH with caution not to be perfectly dried. The total RNA pellet was added the RNA-free water approximately (usually 20-30 ul) to make RNA solution. The concentration was measured by the absorbance of 260 nm.

**RT/PCR**

First strand cDNA was synthesized using 200 units of Reverscript IIL reverse transcriptase (Wako, Oosaka, Japan) incubated with 0.5 µg of total RNA and 0.5 µM oligo(dT) primer in a 20 µl mixture for 60 min at 42°C. Polymerase chain reaction was carried out in 10 µl reaction mixtures containing 1 µl of the first strand cDNA, 0.5 µl of sense and antisense primers, 250 µM dNTPs, and 2 units of Taq polymerase (Promega, Madison, WI, U.S.A.) with 2.5 mM MgCl2. Thermocycling was performed using the following protocol: 1) 72 °C for 3 min, 2) designated cycles of 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min, and 3) 72 °C for 10 min. The primers used in PCR are shown in (Table 1).

**Detection and Analysis of 5-HT2CR RNA Editing**

To detect the novel editing sites and to analyze the editing frequencies, a direct sequencing method with Tex-Red-labeled primer adapted to the PCR product was employed [11]. The amplified product was sequenced using an old type of gel electrophoresis

sequencer, SQ-5500 (Hitachi, Tokyo, Japan), as for advantage convenience. Mixed sequence signals (A and G) indicate RNA editing, and the editing frequency was determined by calculating the height of peak G per the total height of peaks A and G for each sample as previously reported [11]. This method is not suitable to analyze protein isoforms synthesized from edited genes but it is useful to determine overall frequencies of RNA editing occurring at specific positions of a RNA sequence. Therefore, this study has taken advantage of the direct sequencing method to examine the editing ratio as previously reported [11]. NG108-15 cell is a hybrid cell of mouse neuroblastoma and rat glioma. 5HT2CR mRNA of mouse has a consecutively omission of 3 bases for 1 protein in third intracellular loop site in compared with rat sequence (Figure 1). Therefore, the sequences are analyzed in both ways using the both forward and backward primers with Tex Red-labeled because reading shift will occur if we use one way sequencing. In addition, sequence differences in nature between rats and mice were also considered to analyze.

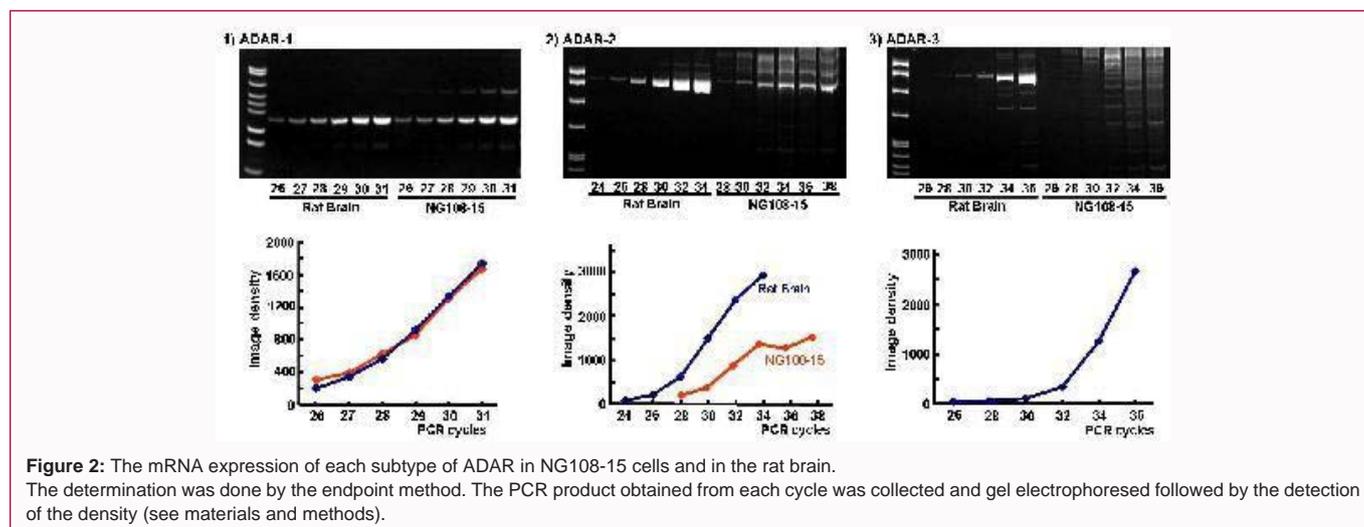
**Quantity of the ADAR mRNA expression**

The PCR product of each ADAR mRNA subtype were resolved with 6% polyacrylamide gel electrophoresis and stained with ethidium bromide. The bands were visualized under UV light and quantified using Densitograph software, version 4.0 (ATTO, Tokyo, Japan). RNA expression changes are usually detected by rel-time PCR method. But in this experiment, the gel electrophoresis method (endpoint detection) was used. The reason would be described in the discussion part.

**Results**

The newly finding editing sites in 5HT2CR was shown in (Figure 1). As previous knowing editing sites are called site A -E, novel 4 sites were temporarily named O, P, Q and R. Upper line of (Figure 1) shows the primary structure of 5HT2CR. The known 5 editing sites exist in the part encoding the second intracelluer loop domain. This part lacks when the short variant generated. The newly discovered 4 editing sites existed in the coding area of third intracellular domain in contiguity with each. Second line of (Figure 1) shows genomic sequence of rat's and mouse's 5-HT2CR and display O, P, Q, R part. In lower berth, the charts of the sequence were displayed; right side was provided from NG108-15, and left from rat. Site O and P were next to each other, and signal of G was almost mixed with A in half in the sequence chart of NG108-15 cell. Otherwise, only A signals were detected in rat at both sites.

The RNA sequence of mouse 5HT2CR has deletion of 3 bases in comparison with rat. The part was shown in a red box in (Figure 1). Because of that, the confusion of the sequence signals were generated when the sequence analyzed on one way direction. Since the sequence was analyzed from both sides, the colors of a displayed chart for A and G are different. About the Q and R site, although the detected base was only A at both sites in rat as same as the genome, there was considerably much G as resulting of RNA editing in NG108-15 cells. The editing rate in the part of the each O, P, Q, R in NG108-15 cells was calculated from the high peak of the chart; O: 40%, P: 46%, Q: 82%, and R: 81%. This calculated value was not the quantitatively numerical value for a real editing rate, but will not be so far apart. Adenosine deaminase (ADAR) is known as an enzyme in conjunction with this editing. There are three kinds of subtypes in ADAR [12]. Type-3 reportedly controls type-1 and type-2 restrainingly, but the details are unclear [12]. The author already reported type-1 and type-2 mRNA expression in the NG108-15 cell [14]. In this experiment, type-3 expression was



**Figure 2:** The mRNA expression of each subtype of ADAR in NG108-15 cells and in the rat brain. The determination was done by the endpoint method. The PCR product obtained from each cycle was collected and gel electrophoresed followed by the detection of the density (see materials and methods).

**Table 1:** The primer sets used in the RT-PCR.

Gene	Accession No.	Size	Forward	Backward
5HT2CR	NM008312	399	ggt cct atc cct gtg att gg	cac aaa gaa tac aat gcc aag g
ADAR1	AF052506	175	ccg aga tgt gac ctc agt gc	tct agt ggc ctc agg gac ag
ADAR2	NM_012894	532	cgt gat gat ctt gaa tga gc	aag ctc gag ctg agc gta ta
ADAR3	AF034837	498	ggc tga gca gta aav tca aa	tct tgg cct tct tct tgg tg

also determined, and expression differences of all types were also studied between in NG108-15 cells and in the rat brain. The endpoint detection method by gel electrophoresis was used for the detection (the reason that the real-time method was not usable was described in the discussion part). In type-1, the expression was almost same level in NG108-15 cells and the adult rat brain. Type-2 expression was extremely low in NG108-15 cells in compared with the rat brain. Although the highly expression was observed in the rat brain, very little or no expression was detected for type-3 in NG108-15 cells.

### Discussion

Although this article only shows the preliminary fact, the obtained results may have a future with fascinations. Further studies started from this result may give us interesting and important information about the physiological significance and the molecular functions of 5HT2CR RNA editing including about the pervious known 5 sites. 5HT2CR has an extremely interesting diversity of function and regulation [2]. Probably she has not only a receptor function but also some kinds of physiological functions, but these are still not yet assumed. It is well known that RNA editing occurs in the part encoding the second intracellular loop site on the 5HT2CR mRNA for a long time [15]. In addition, this time, four of novel editing sites were found in the part encoding the third intracellular loop domain. The novel editing could not be detected in at least adult rat brain. The author previously reported that editing rate dramatically changes by birth, especially at A and B sites [11]. Same changes of the editing rate was also observed in the primary cultured nerve cells during the cultivation period, maybe depending on neurite re-extension [11]. These results may propose the hypothesis that functional changes of 5HT2CR by editing have influences on the nerve development. The NG108-15 cell is blastoma cell, and it basically seems to be in non-differentiated state. The author previously reported that the rate at the known 5 editing sites changes by neuronal differentiation [16] with the mRNA expression changes of ADAR [14]. Since the novel editing

sites could not be detected in adult rat brain, the novel editing also may have some roles in neuronal differentiation steps. The author will perform the examinations about the points, by using the fetal brain, primary culture neron/glia and others in future. In addition, although the existence of 5HT2CR mRNA is relatively limited in the brain in adult animals, it can be interested whether the distribution involves the whole body at the fetus period. The enzyme in conjunction with RNA editing is ADAR. There are three subtypes in ADAR [12]. The drug treatment-induced neuronal differentiation changes the expression level of type-1 and type-2 ADAR in NG108-15 cells [14]. In this experiments, including type-3, the RNA expression levels in undifferentiated NG108-15cells compared with the adult rat brain were studied to know which subtype is involved in 5HT2CR editing, especially for novel editing.

As for determination method, gel electrophoresis method (endpoint detection) was adopted. The real-time PCR protocol, usually use as determination method, did not use in this experiment. When we use the real-time PCR, the expression of the target gene is necessary at least (the differences are only on the expression quantities). And the highly selectivity of primers to the target is also required, because only the double strand formation by PCR makes signals for determination. If the expression is extremely few or non, the endpoint detection method seems to be superior. There was no expression of type-3 in NG108-15 cells in this report. Therefore, primers showing high selectivity on the rat RNA detection could only show non-specific broad band, because there is not a target for NG108-15 cell. In addition, since the expression was very low, there are many bands including the non-specific bindings for detection of type-2 in NG108-15 sells. Under such situation, the real time PCR method cannot guarantee the quantifiability. The result of each subtype of ADAR mRNA expression in undifferentiated NG108-15cells by using the endpoint detection methods showed that 1) type-1 expression was almost same level in adult rat brain, 2) type-

2 expression was extremely low and 3) very little or no expression was observed for type-3. The type-1 probably contributes to RNA editing for both known 5 sites and novel 4 sites in undifferentiated NG108-15 cells, although the future further experiments are necessary. This article reported that the four of novel editing sites in 5HT2CR were found in NG108-15 cells. These editing sites may have a possibility to be related to three-dimensional placement of mRNA, because the susceptibility of the PCR might be related in process of the discovery. It is well known that the three-dimensional placement of the 5HT2CR mRNA remarkably varies among animals and the species [17]. The novel editing sites encodes the third intracellular domain may have a possibility to contact with the second intracellular loop sites domain which include the previously known 5 sites in cytoplasm.

This knowledge is only the fact to show "discovery of new 4 editing sites" in present, but this finding may be not only the fact but also to give us interesting and important information for study about the physiological significance of 5HT2CR RNA editing.

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