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Possible interaction of serotonin 2C receptor mRNA editing at C-site with expression of microtubule-associated protein 2 and neurite outgrowth in rat cultured cortical cells

Abstract

Editing of the mRNA of the serotonin 2C receptor (5-HT2CR) has been reported to be involved in mental disorders. In order to study the physiological role of this editing in neuronal functions, we previously reported changes in the levels of 5-HT2CR mRNA in the rat cerebral cortex during prenatal to postnatal development and in the primary cultured cortical cells. The results show that C-site editing is not stable for which seems to be reflected the environmental changes in neuron. In this report, individual difference in C-site editing pattern among experiments during the cultivation period in primary cultures neuronal cells was analyzed as well as mRNA expression of Microtubule Associated Protein 2 (MAP-2), a dendritic maker, and found a possibility of the relationship between C-site editing and MAP-2 mRNA expression. Although results in this article are in preliminary, this will be a starting point to know the functional significance of 5-HT2CR editing at C-site in neuron.

Keywords: RNA editing, serotonin 2C receptor, neuronal development, neurite outgrowth, neuronal activity, depression

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Introduction

The serotonin 2C receptor (5-HT2CR) is widely distributed in the central nervous system and participates in a variety of brain functions and mental diseases such as depression.[1] The 5-HT2CR mRNA, as well as genes encoding receptors such as the AMPA type glutamate receptor,[2] the potassium channel KCNA1[3] and $GABA_A$ receptor subunit α3[4] undergoes adenosine-to-inosine RNA editing[5] mediated by adenosine deaminases acting on RNA (ADARs).[6,7] Although RNA editing in non-coding regions is common to all types of animals,[8] theses receptors have been reported to undergo editing in their coding regions. However, the functional significance of 5-HT2CR mRNA editing remains to be clarified. There are five editing sites, named A, B, E (C'), C and D from the 5'- side, in 5-HT2CR mRNA.[9,10] All of the sites are located near each other in the region encoding the second intracellular loop of 5-HT2CR mRNA.[9,10] The isoforms of 5-HT2CR synthesized from the edited mRNA have a decreased ability to activate G protein, as compared to the unedited isoform. We previously reported the C-site editing give an unique character to membrane signal transduction through G protein by using the Xenopus oocyte system injected with mutated cRNAs in 5-HT2CR editing sites.[11]

Dramatic increase of the 5-HT2C mRNA editing ratio has been reported by us when it is compared between before and after birth in the rat cerebral cortex.[12] Previous our report show that the editing level in embryonic day 14 (E14) in the rat cerebral cortex is 4% and that increases to 100% in postnatal day 1 (PN1) at A-site. That at B-site also increased from 0% at E14 to 83% at postnatal day 7 (PN7). Interestingly, the editing efficacy at C-site increases gradually from 4% at E14 to 28% at postnatal day 3 (PN3) and then decreases to 6% at postnatal day 49 (PN49). These results may suggest that the editing at A- and B-sites may depend on the state of the neuron, but that at C-site may have reflection of the functional activity. Previous report also shows the same change has been mimicked in the primary cultured rat neuronal cells during the cultivation periods.[12] The A- and B-sites are almost perfectly edited from day 3, 6, 9, 12 and 15 of the cultivation days. Although the editing frequency at C-site gradually increased and then decreased when 6 experimental results were gathered, the difference among the values was seen in individual experiments. Then, I hypothesize that the mild changing in C-site may seem to be reflected the environment or regulation in neuron. In this study, individual difference in C-site editing pattern during the cultivation period in primary cultures neuronal cells was analyzed as well as mRNA expression of microtubule associated protein 2 (MAP-2), a dendritic maker. As a result, a possibility that the relationships between the neurite developments signal and the rising of C-site editing ratio was found, accidentally. Although results in this article remained in preliminary stage, further experiments starting from here will give us more interesting information.

Materials and methods

Animals

Pregnant Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan) and housed in a laboratory animal room maintained at 25±1°C with 65±5% humidity on a 12 hr light/dark cycle (lights on: 07:30 to 19:30). Rat embryos obtained on gestational days 14 and 19 (E14 and E19) and pups obtained on postnatal days 1, 3, 7 and 49 (PN1, PN3, PN7, and PN49) were used for the experiments. All experiments were conducted in accordance with Guiding Principles for Care and Use of Animals in the Field of

Physiological Science of the Physiological Society of Japan and had the approval of the Institutional Animal Use and Care Committee of University of Toyama.

Primary neuronal cell cultures

Cultures of cortical cells were prepared as previously described.[12] The cerebral cortices were dissected from E20 embryos and placed in Neuronbasal™ Medium supplemented with 12% horse serum, 0.6% glucose, 200 μM L-glutamine and 1μg/ml minomycin (named glu(+) medium). The meninges and superficial blood vessels were removed, and the cerebral cortex tissues were minced and incubated at 37 °C for 15 min in 2 ml of 0.05% trypsin solution (Gibco, USA; No.25300-054) to dissociate the cells. During incubation, the cell suspension was briefly shaken every 3 min. After the incubation, 4 ml of glu(+) medium was added to inhibit trypsin activity, and then the suspension was centrifuged at 800 rpm for 7 min. The pellet was suspended in 2 ml of PBS containing 100 U/ml of DNase I. The suspension was incubated for 15 min at 37°C with brief shaking every 3 min, followed by the addition of 4 ml of medium and centrifugation. The final pellet was re-suspended in 4 ml of glu(+) medium and dissociated by a special Pasteur pipette. The cell suspension was applied to a 70μm cell strainer (BD Falcon, USA) to isolate the individual cells. A small amount of the cell suspension was removed and stained with 0.2% trypan blue to determine cell viability and density. Cells were plated on 60-mm dishes precoated with 5μg/ml of poly D-Lysine (PDL: polymerization 30.000-70.000; Sigma, USA) dissolved in PBS at a density of $3.6x10^6$ cells/dish and maintained at 37°C in an atmosphere containing 10% CO_2. The medium was changed to serum-free glu(+) medium supplemented with B-27 (Gibco No.17504-44) after a 2-day cultivation.

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from primary cultures of cortical cells was extracted using Isogen® (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The RNA concentration was measured spectrophotometrically at 260nm (Beckman, USA). First-strand cDNA was synthesized using M-MLV Reverse Transcriptase® (Invitrogen, MD, USA) from 1.0μg of total RNA and 0.4μM random hexamer primers in a 20μl mixture. PCR was conducted in 20μl reaction mixtures containing 1μl of first strand cDNA, 1.0μM sense and antisense primers, 0.2mM dNTPs, 2.5mM $MgCl_2$, and 5 units of Taq polymerase (Promega, WI, U.S.A). Thermocycling was performed using the following protocol: (1) 94°C for 3min, (2) designated cycles of 94°C for 30 sec; 65°C for 1 min and 72°C for 1min; and then (3) 72°C for 5min, before cooling to 4°C. PCR products were electrophoresed on a 6% polyacrylamide gel and then stained with ethidium bromide. The bands were visualized under UV light and quantified using Densitograph (ATTO Co., Tokyo, Japan). Primers (Nippon Gene, Toyama, Japan) used in this experiments are listed in Table 1. To obtain the integrated value of MAP-2 mRNA expression in primary cultured cortical cells in Figure 2, expression levels of MAP-2 mRNA in each experiment were recalculated as a % of a "global value". The "global value" was the total intensity of MAP-2 mRNA bands at 5 time points in each experiment. Each image density value at a time point was divided by the corresponding "global value" to get the "% global value". The "% global values" obtained from 6 individual experiments at each time point was used to calculate the mean±SEM for Figure 2.

Table 1 Primer sequences used for RT-PCR and sequencing

Target gene	Gen bank accession no.	Primer sequence	Product size
5'HT2CR	NM_008312	5'atg tcc cta gcc att gct gat atg ctg gtg 5'atg cca cga agg acc cga tga gaa cga agt	391 (long) 296(short)
5'HT2CR (Tex Ref-labeled)	NM_008312	5'ata ttt gtg ccc cgt cgt ga	
MAP2	M21041	5'gtg act ttg gac aga tgg ct 5'tct gag cgg aag agc agt tt	607

Detection of RNA editing of 5-HT2CR

We used a direct sequencing method with a Tex-Red–labeled primer adapted to the PCR product. This method is easy to use and accurate in determining the overall frequency of RNA editing at the five sites of 5-HT2CR from the PCR product, although it is not suitable for analyse of the combination of RNA editing and protein isoforms. The Texas Red-labeled primer for the edited part is 5'-ata ttt gtg ccc cgt cgt ga -3'. This primer and a SQ-5500 sequencer (Hitachi, Tokyo, Japan) were used. PCR was performed to amplify the product containing the five edited sites of the mRNA. 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 (Figure 1).

Histological analysis of MAP-2 protein expression in cultured cell

Rat cortical cells were cultured in 8-well chamber slides (BD Biosciences, MA, USA) coated with 50μg/ml of PDL at a density of 1.45– 2.2 X 10^5cells/cm^2. After cultivation for determined periods, cells were fixed with 4% paraformaldehyde and then immunostained with a monoclonal antibody against MAP-2 (1:500) as a dendritic marker. Alexa Fluor 488-conjugated goat anti-mouse IgG (1:300) was used as a secondary antibody. The fluorescent images were captured by a fluorescence microscope (AX-80) at 2.26 mm X 1.70 mm, and four images were captured per treatment. The area of the part dyed with an antibody was measured by "Lane & Spot Analyzer Ver. 6.0" (ATTO Co., Tokyo, Japan). The protein expression levels are shown as a percentage of the level at day 3.

Statistical analysis

Statistical comparisons were performed using a two-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. The F- and P-values of each experiment compared with total are shown in Table 2.

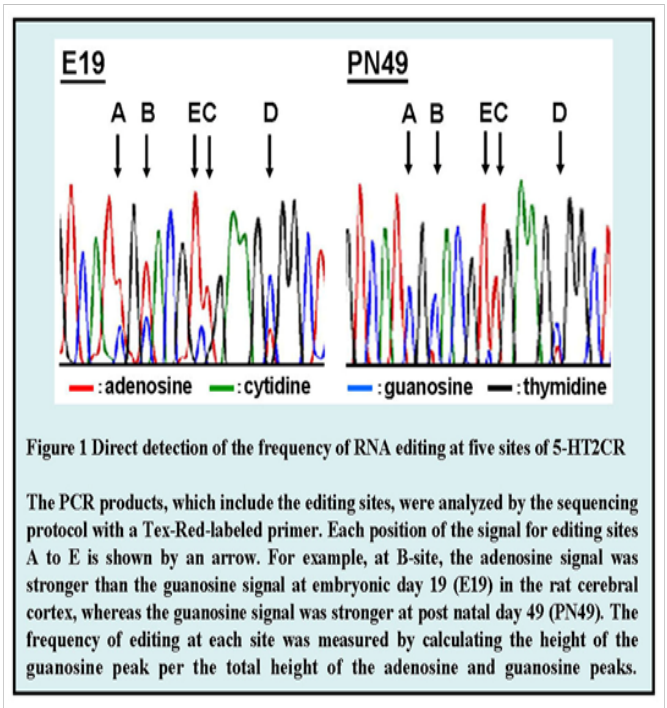

Figure 1 Direct detection of the frequency of RNA editing at five sites of 5-HT2CR

The PCR products, which include the editing sites, were analyzed by the sequencing protocol with a Tex-Red-labeled primer. Each position of the signal for editing sites A to E is shown by an arrow. For example, at B-site, the adenosine signal was stronger than the guanosine signal at embryonic day 19 (E19) in the rat cerebral cortex, whereas the guanosine signal was stronger at post natal day 49 (PN49). The frequency of editing at each site was measured by calculating the height of the guanosine peak per the total height of the adenosine and guanosine peaks.

Figure 1 Direct detection of the frequency of RNA editing at five sites of 5-HT2CR.

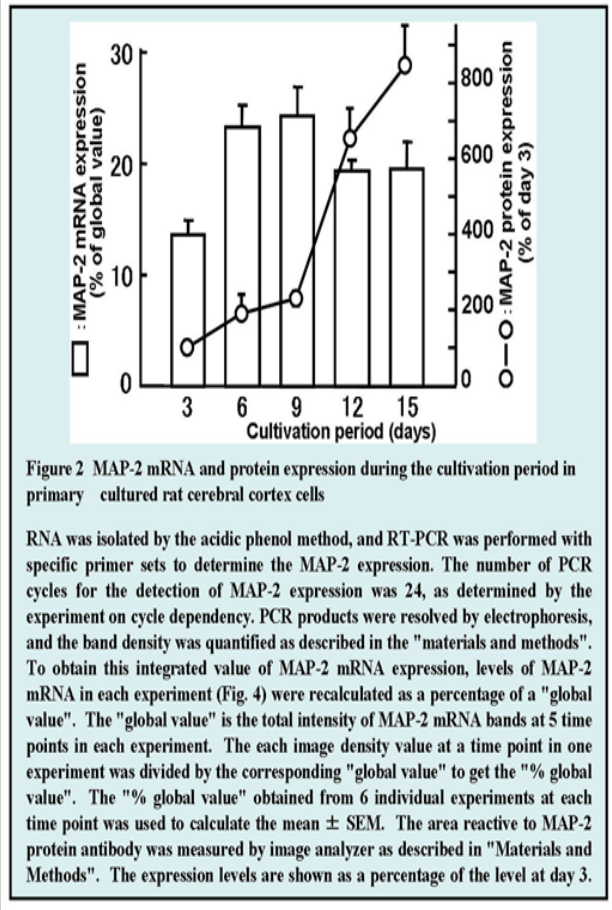

Figure 2 MAP-2 mRNA and protein expression during the cultivation period in primary cultured rat cerebral cortex cells

RNA was isolated by the acidic phenol method, and RT-PCR was performed with specific primer sets to determine the MAP-2 expression. The number of PCR cycles for the detection of MAP-2 expression was 24, as determined by the experiment on cycle dependency. PCR products were resolved by electrophoresis, and the band density was quantified as described in the "materials and methods". To obtain this integrated value of MAP-2 mRNA expression, levels of MAP-2 mRNA in each experiment (Fig. 4) were recalculated as a percentage of a "global value". The "global value" is the total intensity of MAP-2 mRNA bands at 5 time points in each experiment. The each image density value at a time point in one experiment was divided by the corresponding "global value" to get the "% global value". The "% global value" obtained from 6 individual experiments at each time point was used to calculate the mean ± SEM. The area reactive to MAP-2 protein antibody was measured by image analyzer as described in "Materials and Methods". The expression levels are shown as a percentage of the level at day 3.

Figure 2 MAP-2 mRNA and protein expression during the cultivation period in primary cultured rat cerebral cortex cell

Table 2 The values of statistical analysis for the results in Figures 3 and 4

	Frequencies of C-site editing		MAP2 mRNA	
	F value	P value	F value	P value
Exp. 1	F(4,100)=1.785	0.138	0.846	0.499
Exp. 2	F(4,101)=0.456	0.768	2.026	0.096
Exp. 3	F(4,104)=4.744	0.002*	4.127	0.004*
Exp. 4	F(4,114)=2.297	0.064	1.246	0.295
Exp. 5	F(4,110)=0.308	0.872	1.778	0.138
Exp. 6	F(4,111)=0.471	0.757	3.193	0.016

*Statistical comparisons in Figures 3 and 4 were performed using a two-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. The F- and P-values of each experiment were compared with the averaged pattern for all experiments.

Results

Morphological observations of primary cultured cortical cells showed that neurite outgrowth was activated at day 6 or 9 (data not shown). The expression of MAP-2 protein, a dendrite marker, was markedly enhanced at day 12 and 15 (Figure 2). The expression of MAP-2 mRNA was enhanced at day 6 and 9 prior to the increase in protein, and then decreased slightly to maintain a constant level of expression (Figure 2). Figure 3 shows individual frequencies of editing at C-site. The integrated pattern has a peak at day 6 or 9; a smooth increase followed by a smooth decrease (already reported in ref 12). In the case of individual patterns, experiments 1, 2, 4, 5 and 6 show almost the same patterns as the integrated type. However, that in experiment 3 differed from patterns than other experiments. Figure 4 shows expression changes of MAP-2 mRNA in same samples of Figure 3. The results seem to be shown that the experiment 3 was also different pattern in compared with others. The fluctuating pattern of C-site editing and the expression time cause of MAP-2 in experiment 3 differed from patterns in other experiments. Then, the significances of "time vs experiment interaction" were calculated between experiment 3 and the average pattern for all experiments (Table 2). Although other five experiments showed no significant, both C-site editing and MAP-2 expression in experiment 3 showed significant difference on "time vs experiment interaction" in compared with the average pattern for all experiments (Table 2).

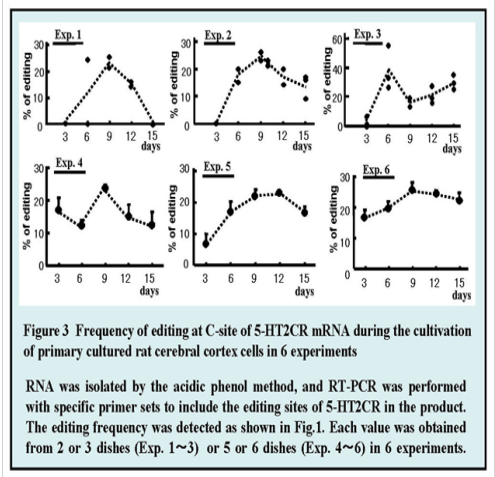

Figure 3 Frequency of editing at C-site of 5-HT2CR mRNA during the cultivation of primary cultured rat cerebral cortex cells in 6 experiments

RNA was isolated by the acidic phenol method, and RT-PCR was performed with specific primer sets to include the editing sites of 5-HT2CR in the product. The editing frequency was detected as shown in Fig.1. Each value was obtained from 2 or 3 dishes (Exp. 1~3) or 5 or 6 dishes (Exp. 4~6) in 6 experiments.

Figure 3 Frequency of editing at C-site of 5-HT2CR mRNA during the cultivation of primary cultured rat cerebral cortex cells in 6 experiments.

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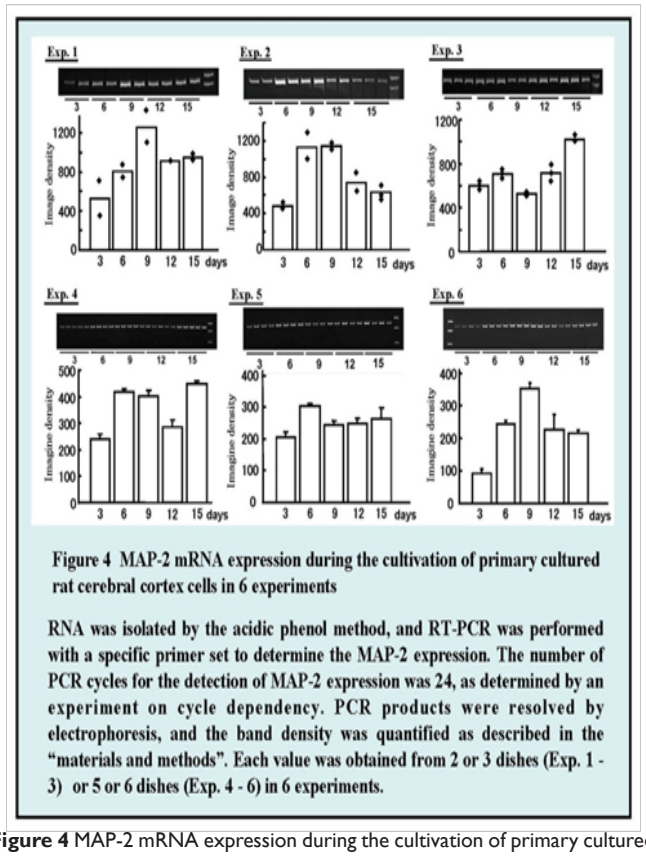

Figure 4 MAP-2 mRNA expression during the cultivation of primary cultured rat cerebral cortex cells in 6 experiments RNA was isolated by the acidic phenol method, and RT-PCR was performed with a specific primer set to determine the MAP-2 expression. The number of PCR cycles for the detection of MAP-2 expression was 24, as determined by an experiment on cycle dependency. PCR products were resolved by electrophoresis, and the band density was quantified as described in the "materials and methods". Each value was obtained from 2 or 3 dishes (Exp. 1〜3) or 5 or 6 dishes (Exp. 4〜6) in 6 experiments.

Discussion

This article may have an interest to show the possibility that changing of C-site editing has co-relation with the MAP-2 mRNA/protein expression and the related neuronal function such as neuronal differentiation and activity, although the number of experiment in this report is not enough and the result is still in the accidental territory. In other words, changes of the C-site editing may be delicately reflected with environmental changes in the neuron. There is no report about pharmacological regulation and physiological mechanism on C-site editing, except for related enzyme adenosine deaminases acting on RNA. If pharmacology and physiology of C-site editing are clarified, neuronal activities and neuronal differentiation may be regulated by some drugs which control the C-site editing to overcome the neuronal disease such as dementia and also depression. MAP-2 is as a dendritic marker of neurons.[13] We determined the levels of its mRNA and protein in the primary cultures, and compared the results with the frequencies of 5-HT2CR mRNA editing. The MAP-2 mRNA expression increased remarkably at an early stage of cultivation due to the necessity for protein synthesis, followed by the maintenance phase of the expression depending on the cell conditions. As we shown in Figures 3 & 4, the variation of the changing pattern in C-site editing induced variation in MAP-2 mRNA expression; the increase of editing induced enhancement expression of MAP-2 mRNA, suggesting that the editing is involved in the formation of the neuronal network and/or synapses. Author previously hypothesized that 5-HT2CR mRNA makes functional factors, other than receptors, that participate in new gene expression and that might be related to the anti-depressive effects of antidepressants.[14]

It has been suggested that RNA editing is involved in the generation of small RNA[10,15] which has been reported to be involved in cellular development and post-transcriptional regulation. The C-site editing may participate in neuronal re-generation through the generation of small RNA and/or some other functional RNA. Indeed, a short variant RNA, missing 95 nucleotides including RNA editing area of the full-length 5-HT2CR mRNA, has been reported.[16,17] Brain-specific small nuclear RNA HBII-52, which has a sequence complementary to the C-site region, but dose not include the A- and B-sites region, interferes with the RNA editing at C-site of 5-HT2CR.[18] The generation of small RNA or a control mechanism may play a role in C-site editing. Changes in C-site editing have been reported to have a relation with depressive disorder. It has been reported that the editing efficiency at C-site was increased in the postmortem brains of suicide victims with a history of major depression, as compared with that found in normal brains.[14] Increased editing at this site was also observed in the learned helplessness rat, an animal model relevant to depression.[19] Decreased editing at C-site has been found in normal mice treated with fluoxetine, an antidepressant.[14] Moreover, C-site editing is significantly decreased in the brains of mice treated to induce serotonin depletion.[20] The augmentation of C-site editing may be suggested to be involved in the pathological state of depression and the actions of antidepressants. A possibility of the relationship between C-site editing and MAP-2 mRNA expression was suggested in this report, although there is little number of experiment to convince the possibility and it is still in the accidental stage. To know further information about the subtle change in C-site editing and the neuronal function, small RNAs' modulation and interaction with newly founded editing site in 5-HT2CR mRNA[21] would also participate in. Discovery and development of drugs which controls the C-site editing directly will fascinate further research.

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Conflict of interest

The author declares no conflict of interest.

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