Functional amino acid residues in agonist binding and its linking to channel gating of 5-HT_{3A} receptor in anesthetic modulation

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Directed by Professor Kyeong Tae Min

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This certifies that the Doctoral Dissertation of Mi Kyeong Kim is approved.

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Functional amino acid residues in agonist binding and its linking to channel gating of 5-HT_{3A} receptor in anesthetic modulation

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The 5-hydroxytryptamine type 3 (5-HT_3) receptor is a member of the Cys-loop superfamily of ligand-gated ion channels (LGICs) and, like most LGIC receptors, is modulated by pharmacologically relevant concentrations of volatile anesthetics or n-alcohols. The goal of this study was to determine whether mutations of E106, F107 and R222 in the 5-HT_{3A} receptor may affect the anesthetic modulation of the positive modulators, halothane and desflurane, and the negative modulator, propofol.

The author expressed the wild-type and mutant receptors in *Xenopus Laevis* oocytes and assessed receptor function using two electrode voltage clamp techniques. E106D, F107Y, R222F, R222V mutant 5-HT_{3A} receptors were functionally expressed. The EC_{50} of 5-HT in wild-type, E106D, F107Y, and R222F mutant 5-HT_{3A} receptors was 1.29 ± 0.18, 1.41 ± 0.21, 7.47 ± 0.85, and 0.61 ± 0.16, respectively. The F107Y mutant 5-HT_{3A} receptor displayed decreased sensitivity to 5-HT compared to the wild type 5-HT_{3A} receptor, with an observed six-fold rightward shift ($P<0.05$). Hill coefficients for 5-HT were significantly decreased in E106D and R222F mutant 5-HT_{3A} receptors compared to the wild-type 5-HT_{3A} receptor. Desflurane and halothane

positively modulated both the wild-type and the F107Y mutant 5-HT_{3A} receptors, however the F107Y mutant 5-HT_{3A} receptor showed a greater enhancing modulation compared to the wild-type receptor. Meanwhile, the R222F and R222V mutant 5-HT_{3A} receptors lost positive modulation with 1 and 2 MAC of halothane. Most interestingly, positive modulation by desflurane and halothane was converted into negative modulation in E106D mutant 5-HT_{3A} receptor. However, propofol, which is a known negative modulator of the 5-HT_{3A} receptor, negatively modulated both the E106D mutant and the wild-type 5-HT_{3A} receptors. These results, conducted at the molecular level of the 5-HT_{3A} receptor, might indicate that anesthetic modulation in the 5-HT_{3A} receptor could be affected by mutations of the amino acid residues that are important not only for agonist binding and its linking to channel gating, but also for channel gating. The present study implicates that the amino acid residues known for agonist binding sites and linking site of agonist binding to channel gating might also have an important role for anesthetic modulation in the 5-HT_{3A} receptor.

Key words: Electrophysiology, 5-HT_{3A} receptor, 5-HT, propofol, site-directed single mutation, *Xenopus Laevis* Oocyte, volatile anesthetics.

Amino acid residues involved in agonist binding and linking agonist binding to channel gating, proximal to transmembrane domain of 5-HT_{3A} receptor in anesthetic modulation

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Ⅰ. INTRODUCTION

The 5-hydroxytryptamine type 3 (5-HT_3) receptor is a member of the Cys-loop superfamily of ligand-gated ion channels (LGICs) that includes the nicotinic acetylcholine, glycine, and [gamma]-amino butyric acid type A ($GABA_A$) receptors.[1] Among the five subunits (A-E) cloned to date, the 5-HT_{3A} and 5-HT_{3B} subunits have been demonstrated to have functional significance in the central and peripheral nervous systems. The 5-HT_{3B} subunit must be coexpressed with the 5-HT_{3A} subunit to be functional in the peripheral nervous system, whereas the 5-HT_{3A} subunit can form homomeric functional channels. These homomers are predominantly expressed in the central and peripheral nervous systems.[2-4]

Receptors in the LGIC superfamily are comprised of a pentameric arrangement, with each subunit containing a large extracellular N-terminal domain, four transmembrane domains (TM1-TM4), a large intracellular loop between TM3 and TM4, and an extracellular C-terminal domain.[5] The structure and function of LGICs is under intense investigation, but is still ambiguously defined. The ligand binding sites are thought to be located in

the extracellular N-terminal domain at the subunit-subunit interface, and the channel pore is believed to be formed by TM2.[5] LGIC activation involves neurotransmitter binding to the closed, resting state receptors, triggering a complex conformational change, which results in the channels opening. Many electrophysiological studies of volatile anesthetics have focused on their effects on LGICs. Like most LGIC receptors, the 5-HT_3 receptor is modulated by pharmacologically relevant concentrations of volatile anesthetics or n-alcohols.[6,7] Most volatile anesthetics, such as desflurane, isoflurane, halothane, enflurane, and methoxyflurane, potentiate the 5-HT_3 receptor at their human alveolar concentrations.[7-11] However, sevoflurane and two gaseous anesthetics (nitrous oxide, xenon) as well as intravenous anesthetics, such as pentobarbital and propofol, inhibit the 5-HT_{3A} receptor.[11-3] Studies of chimera or single residue mutagenic recombinant LGIC receptors suggest that the LGICs function was characterized by the N-terminal domain rather than the transmembrane and C-terminal domains and that the channel gating sites are located in the TM2.[14] The majority of the studies on the anesthetic modulation of LGICs have focused on amino acid residues in the TM2 that are associated with channel gating.[15-9] However, for the receptor to be activated, several amino acids in the area proximal to the TM1 are also important for agonist recognition (glutamate 106[21] and phenylalanine 107[20] in the N-terminal area) and the possible coupling between agonist binding and gating (arginine 222[22] in the pre-TM1 domain). In the chimeric receptor that is comprised of the N-terminal domain from the nACh alpha 7 receptor and the TM and C-terminal domains from the 5-HT_{3A} receptor, modulation by isoflurane was involved with the N-terminal domain of the receptor. This means that the anesthetic modulation in receptor may also be characterized by the N-terminal domain.[23] Considering that three amino acids proximal to the TM1 domain of the 5-HT_{3A} receptor are important for agonist binding (glutamate 106[21] and phenylalanine 107[20]) and coupling of agonist binding to

channel gating (arginine 222[22]), the anesthetic modulatory effect might be affected by the mutagenesis of these amino acid residues of the 5-HT_{3A} receptor. (Fig. 1, 2)

Therefore, this study was intended to reveal whether the mutations of glutamate 106, phenylalanine 107, and arginine 222 at the N-terminal and pre-TM1 domains may affect the anesthetic modulation of the positive modulators halothane and desflurane, and the negative modulator propofol in 5-HT_{3A} receptor expressed in *Xenopus Laevis* oocytes using two electrode voltage clamp techniques.

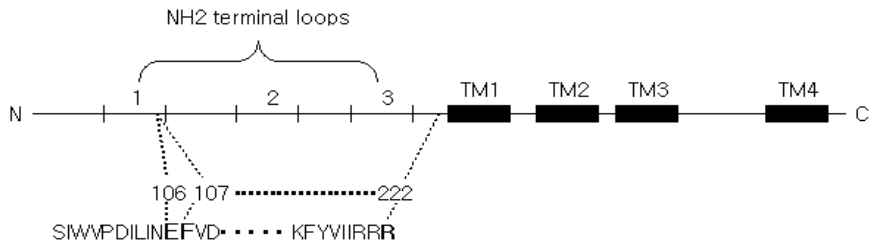

Fig. 1. Primary structure of 5-HT_{3A} receptor

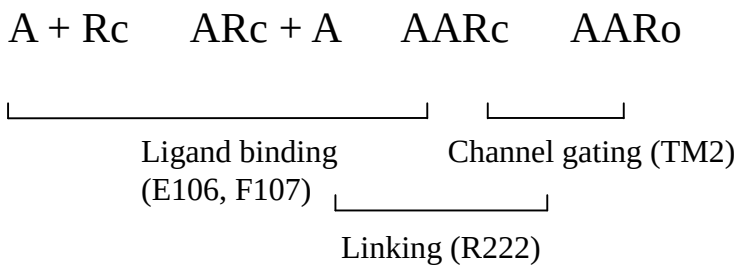

Fig 2. Simplified schematic for ligand binding and channel gating of LGICs with two agonist binding sites. Altering agonist binding, the open gating, or linking both might affect allosteric modulation of anesthetics in LGICs. A: agonist molecule bound, Rc: non-conducting, closed channel, Ro: conducting, open channel.

MATERIALS AND METHODS

1. Site-directed mutagenesis of the 5-HT_{3A} cDNA

cDNA isolated from the mouse 5-HT_{3A} receptor was generously provided by Dr. Jay Yang (Columbia University, USA). The author constructed seven kinds of mutant 5-HT_{3A} receptor. The following alterations were made to generate the mutant receptors: glutamate (E) 106 was substituted with aspartate (E106D) or tyrosine (E106Y), phenylalanine (F) 107 was substituted with tyrosine (F107Y) or serine (F107S), and arginine (R) 222 was substituted with proline (R222P), phenylalanine (R222F) or valine (R222V).

For site-directed mutagenesis, sense and antisense primer oligonucleotides used were as following;

E106D

Sense primer oligonucleotides:

5'CTGACATTCTCATCAATGACTTTGTGGACGTGGGG3'

Anti sense primer oligonucleotides:

5'CCCCACGTCCACAAAGTCATTGATGAGAATGTCAG3'

E106Y

Sense primer oligonucleotides:

5'TCCCTGACATTCTCATCAATTACTTTGTGGACGTGGGGAAG3'

Anti sense primer oligonucleotides:

5'CTTCCCCACGTCCACAAAGTAATTGATGAGAATGTCAGGGA3'

F107Y

Sense primer oligonucleotides:

5'TGACATTCTCATCAATGAGTACGTGGACGTGGGGAAGTC3'

Anti sense primer oligonucleotides:

5'GACTTCCCCACGTCCACGTACTCATTGATGAGAATGTCA3'

F107S

Sense primer oligonucleotides:

5’CCTGACATTCTCATCAATGAGAGCGTGGACGTGGGGAAGTC3’

Anti sense primer oligonucleotides:

5’GACTTCCCCACGTCCACGCTCTCATTGATGAGAATGTCAGG3’

R222P

Sense primer oligonucleotides:

5’CGTGATCATCCGCCGGCCACCTTTATTCTATGCAGTCAG3’

Anti sense primer oligonucleotides:

5’CTGACTGCATAGAATAAAGGTGGCCGGCGGATGATCACT3’

R222F

Sense primer oligonucleotides:

5’ACGTGATCATCCGCCGGTTCCCTTTATTCTATGCAGTCAGC3’

Anti sense primer oligonucleotides:

5’GCTGACTGCATAGAATAAAGGGAACCGGCGGATGATCACGT3’

R222V

Sense primer oligonucleotides:

5’ACGTGATCATCCGCCGGGTACCTTTATTCTATGCAGTCAGC3’

Anti sense primer oligonucleotides:

5’GCTGACTGCATAGAATAAAGGTACCCGGCGGATGATCACGT3’

The point mutation of the mouse 5-HT_{3A} receptor was created using a Quickchange site-directed mutagenesis kit (Stragene, La Jolla, CA, USA) using the 20-25bp oligonucleotide-sized primer via Generunner v3.02 (Hastings software Inc.). The successful incorporation of the mutation was verified by sequencing the clones using an automated DNA sequencer (Genetic Analyzer 3100, ABI, CA, USA).

2. Expression of 5-HT_{3A} receptors into *Xenopus laevis* oocytes

Wild-type and point mutant mouse 5-HT_{3A} receptor cDNAs were subcloned into a custom oocyte expression vector, pCR-Script SK(+), and

linearized by *Sal* Ⅰ digestion in order to prepare the template cDNA. cRNA was synthesized *in vitro* using T3 RNA polymerase (Message Machine, Ambion, TX, USA) following the manufacturer's recommended protocol.

All procedures for animal care and use were approved by the Yonsei University Committee on Animal Care. Frogs were anesthetized by cold-emersion anesthesia with 0.15% 3-*p*-aminobenzoic acid for 30 min. The ovarian lobes were removed through a small incision in the frog's abdomen and were placed in modified Barth's Solution (MBS) (88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 0.83 mM $MgSO_4$, 250 mM Na pyruvate, 5 mM HEPES; pH 7.4, 0.5 mM theophylline, 10 U/ml Penicillin, 10 U/ml streptomycin, 10 U/ml gentamycin). The ovarian lobes were manually dissected into clumps of 4 to 10 oocytes and then treated with 1.5 mg/mL collagenase IA in Ca^{2+} free frog Ringer's solution (120 mM NaCl, 2 mM KCl, 5 mM HEPES 5 mM; pH 7.4) for 30 min to remove the follicular-cell layer. After the oocytes were rinsed several times, approximately 50 ng of cRNA was injected into stage Ⅴ-Ⅵ oocytes with a microinjector (Nanojector, Drummond Scientific, Broomall, PA, USA). The oocytes were incubated in modified MBS (88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 0.83 mM $MgSO_4$, 250 mM Na pyruvate, 5 mM HEPES; pH 7.4, 0.5 mM theophylline, 10 U/ml Penicillin, 10 U/ml streptomycin, 10 U/ml gentamycin) at 18℃ for 48-96 hr. The incubation medium was changed daily.

3. Two electrode voltage clamp recording

After a 48-96 hour incubation period at 18℃, an oocyte was placed into a Plexiglas recording chamber in approximately 300 μL of volume and continuously perfused with 1.8 mM Ca^{2+} frog Ringer's solution (120 mM NaCl, 2 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2$; pH 7.4) at 3-7 mL/min.

The oocyte was penetrated with two glass electrodes with a resistance of 1-3 MΩ when filled with a 3 M KCl solution. Two electrode voltage clamp recordings at -50 mV were obtained with an Oocyte Clamp (OC 725C, Warner Instruments, Hamden, CT, USA).

After 5-HT dose response curves were taken from functionally expressed wild-type and mutant receptors, the effects of the clinical doses of anesthetics (1 and 2 MAC of halothane and desflurane; 1, 10 and 100 uM of propofol,) on 5-HT-mediated currents were compared under conditions in which 5-HT concentration evokes approximately 20% of the maximal peak current for each receptor that was used (EC_{20}).

The oocytes were preincubated with volatile anesthetics for 2 min prior to the application of 5-HT for 20-30s until the peak current was reached. Saturated solutions of volatile anesthetics were prepared by bubbling each agent with a microbubbler into a sealed bottle containing the recording solution at a gas flow rate of 200 mL/min for more than 30 min. Then, 100% O_2 was passed through the agent specific calibrated vaporizers. Propofol solutions were prepared daily from ethanol stock (1 M), which was diluted 1:10,000 (resulting in a 100 μM propofol solution) in frog Ringer's solution. This solution was serially diluted before use. The propofol was co-applied with the 5-HT.[13] Each experiment was preceded and followed by a control application of 5-HT, both to normalize data and to ensure the reversibility of any drug-induced current modulation. Cumulative desensitization was excluded by confirming that the control response (within 90% of recovery) was induced. For analysis, the average of these two measurements was used as the control. A 5-30 min recovery period was allowed after each application of the agonist (with or without anesthetic). Experiments were repeated in at least four oocytes.

The bath solution exchange time constant was approximately 0.5 s and orders of magnitude slower than the biochemical interactions between ligand

and receptors. The timing of the drug application and the current digitization were controlled by Clampex v 5.2 (Axon Instruments; Burlingame, Ca).

5-HT (serotonin), collagenase IA, and almost all of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Halothane was purchased from Sigma-Aldrich (St. Louis, MO, USA). Desflurane was purchased from Baxter Health Corporation (Il, USA).

4. Data Analysis

Peak currents induced by the drug applications were measured and concentration-response curves were fit (Sigmaplot v 7.0; SPSS Inc., CA) to the equation $I/ I_{max}=C^n/(C^n + EC_{50}{}^n)$, where I is the normalized peak current form serotonin, I_{max} is the maximal normalized peak current, C is the serotonin concentration, *n* is the Hill coefficient, and EC_{50} is the concentration at which the half-maximal peak current is induced.

The inhibitory or potentiating effects of the test drugs were presented as a percentage in comparison to the currents induced by serotonin at EC_{20}.

The values represented mean ± SEM. Statistical analysis was performed using ANOVA with a Tukey test for the multiple comparison and a Mann-Whitney U test when appropriate. $P<0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. Functional Characterization of the wild-type and mutant 5-HT_{3A} Receptors:

E106D, F107Y, R222F, and R222V mutant 5-HT_{3A} receptors were functionally expressed but E106Y, F107S, and R222P mutant 5-HT_{3A} receptors were not functionally expressed, even with a 1 mM 5-HT.

Figure 3 shows 5-HT concentration-response relationships for currents in wild-type and mutant 5-HT_{3A} receptors. The 5-HT EC_{50} values, Hill coefficients, and I_{max} obtained from the analyses of these concentration-response curves are listed in Table 1. The EC_{50} and Hill coefficients of 5-HT in wild-type, E106D, F107Y, and R222F mutant 5-HT_{3A} receptors were 1.29 ± 0.18 and 2.12 ± 0.08, 1.41 ± 0.21 and 1.22 ± 0.16, 7.47 ± 0.85 and 2.43 ± 0.17, and 0.61 ± 0.16 and 0.75 ± 0.06, respectively. The F107Y mutant receptor displayed a decreased sensitivity to 5-HT compared to the wild-type receptor, with an observed approximate six-fold rightward shift. Hill coefficients for 5-HT were significantly decreased in the E106D and R222F mutant receptors compared to the wild-type 5-HT_{3A} receptors.

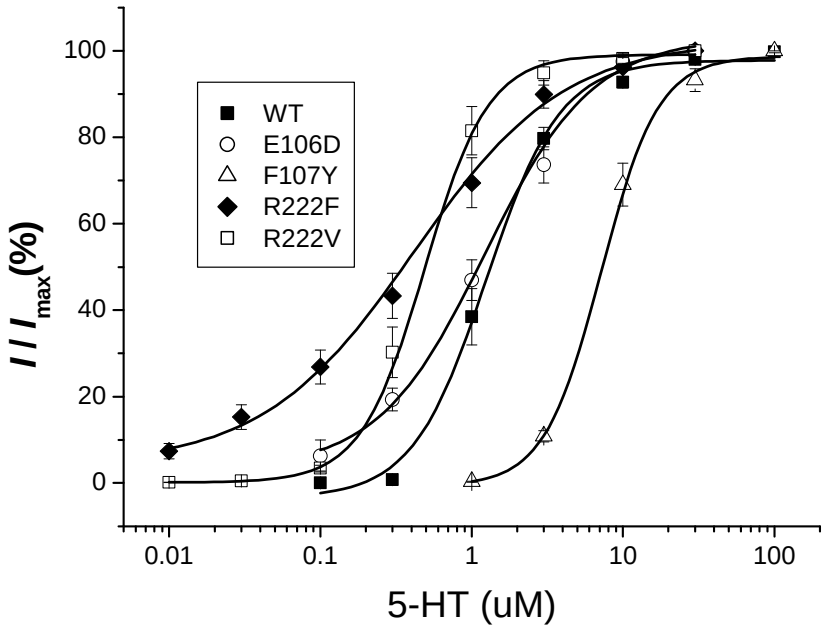

Fig.3. Concentration response curves for 5-HT in wild type (WT) and mutant 5-HT_{3A} receptors. The F107Y mutant 5-HT_{3A} receptor decreased the sensitivity to agonists. I and I_{max} are the current at a given 5-HT concentration and the maximal current, respectively. Each data point represents mean ± SEM from 5-8 cells.

Table 1. Summary of the Properties of the Wild Type (WT) and Mutant 5-HT_{3A} Receptors Expressed in *Xenopus Laevis* Oocytes

Receptor	5-HT EC_{50} (μM)	Hill coefficient	I_{max} (μA)
WT	1.29 ± 0.18	2.12 ± 0.08	3.84 ± 0.36
E106D	1.41 ± 0.21	1.22 ± 0.16*	1.05 ± 0.21
F107Y	7.47 ± 0.85*	2.43 ± 0.17	7.39 ± 1.51
R222F	0.61 ± 0.16	0.75 ± 0.06*	0.38 ± 0.09
R222V	0.50 ± 0.08	2.24 ± 0.19	3.51 ± 0.74

EC_{50}, Hill coefficient (n), and I_{max} of the 5-HT concentration-response curves for WT and various mutant receptors are listed, expressed as mean ± SEM of 4-8 oocytes. These values were obtained by fitting the data to the equation given in the section of "Experimental Procedures." The values for mutant receptor were compared with those of the WT receptor, and the statistical significance was calculated using ANOVA with Tukey test for multiple comparisons. * $p < 0.05$, compared with WT.

2. Modulation of 5-HT EC_{20}-induced currents by desflurane and halothane in wild-type and mutant 5-HT_{3A} receptors.

Neither desflurane nor halothane itself induced any current in wild-type and mutant receptors.

Current tracings, responses to 5-HT with and without anesthetics in wild-type and mutant 5-HT_{3A} receptors were shown in figure 4.

Figure 6 depicts the modulation of 5-HT EC_{20}-induced currents by desflurane and halothane. The F107Y mutant 5-HT_{3A} receptor increased the positive modulation by desflurane and halothane. The degree of positive modulation by desflurane was greater than the modulation by halothane. The R222F mutant 5-HT_{3A} receptor increased a positive modulation by 1 MAC of desflurane but decreased by 2 MAC of desflurane. The R222V mutant 5-HT_{3A} receptor decreased the positive modulation with 2 MAC of desflurane. Both the R222F and the R222V mutant 5-HT_{3A} receptors had little influence on the positive modulation by desflurane and halothane. In marked contrast to these results, the E106D mutant 5-HT_{3A} receptor was negatively modulated by desflurane and halothane. The degree of negative modulation by desflurane was greater than the modulation by halothane.

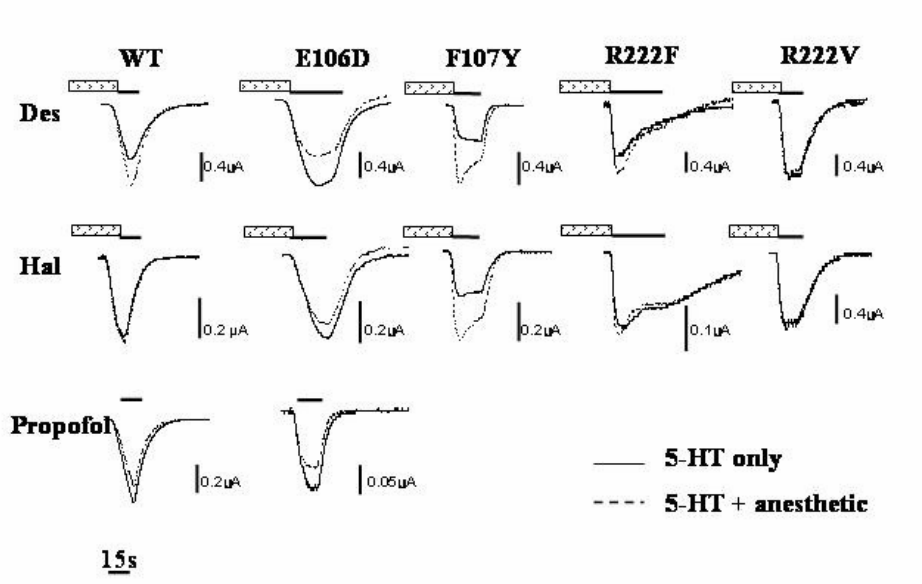

Fig.4. Representative current tracings, responses to 5-HT with and without anesthetics in wild-type and mutant 5-HT_{3A} receptors. Oocytes were first preincubated with volatile anesthetics for 2 min prior to application of 5-HT. Propofol was co-applied with 5-HT. Peak currents were recorded and compared to anesthetic-free controls.

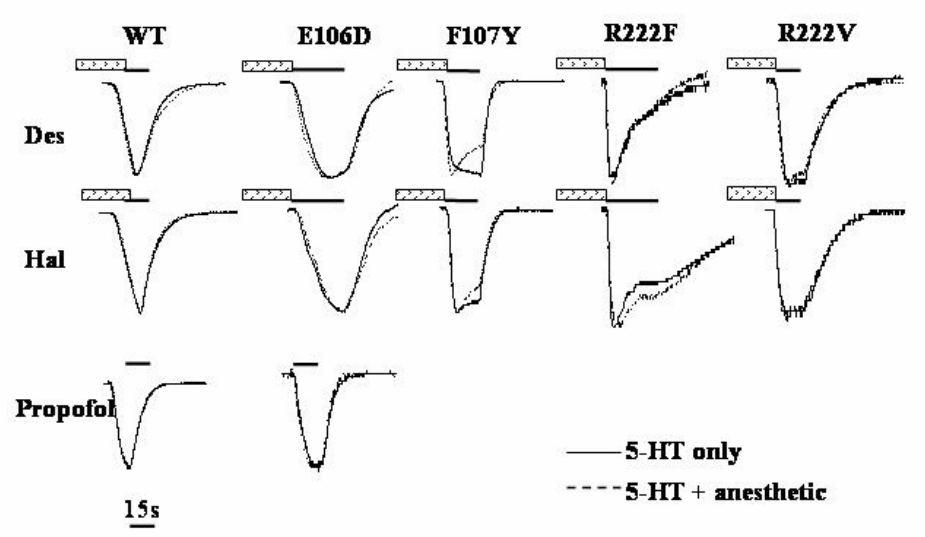

Fig.5. Normalized current tracings of figure 4 by peak currents responses to 5-HT with and without anesthetics in wild-type and mutant 5-HT_{3A} receptors.

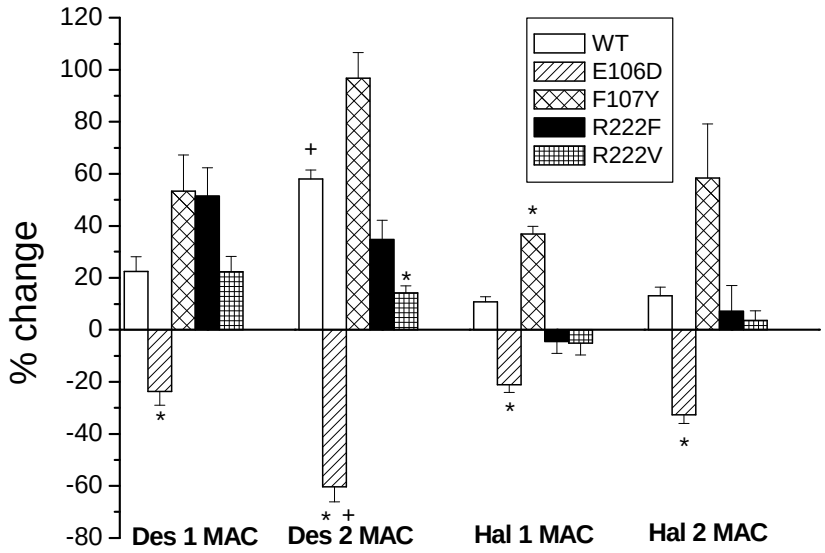

Fig.6. Effects of desflurane and halothane on 5-HT-induced currents in the wild-type and mutant 5-HT_{3A} receptors. In E106D mutant 5-HT_{3A} receptor, desflurane and halothane did not potentiate but inhibited the 5-HT-induced currents. Values are percent change of the control response without anesthetics. Negative numbers indicate percentage of inhibition. Data from more than 8 oocytes were expressed as mean ± SEM. *: $P < 0.05$ compared with the value of wild 5-HT_{3A} receptor. $^{+}$: $P < 0.05$ compared with the value of 1 MAC desflurane in same receptor.

3. Effect of inhibitory modulation of 5-HT EC_{20} induced currents by propofol in wild-type and E106D mutant 5-HT_{3A} receptors

Propofol itself did not induce any currents in wild-type and mutant 5-HT_{3A} receptors.

Propofol inhibited the 5-HT-induced currents in both wild-type and E106D mutant 5-HT_{3A} receptors (Fig. 7). The inhibitory effects of propofol were greater in E106D mutant 5-HT_{3A} receptors than in wild-type receptors at the 1, 10, and 100 μM concentrations of propofol.

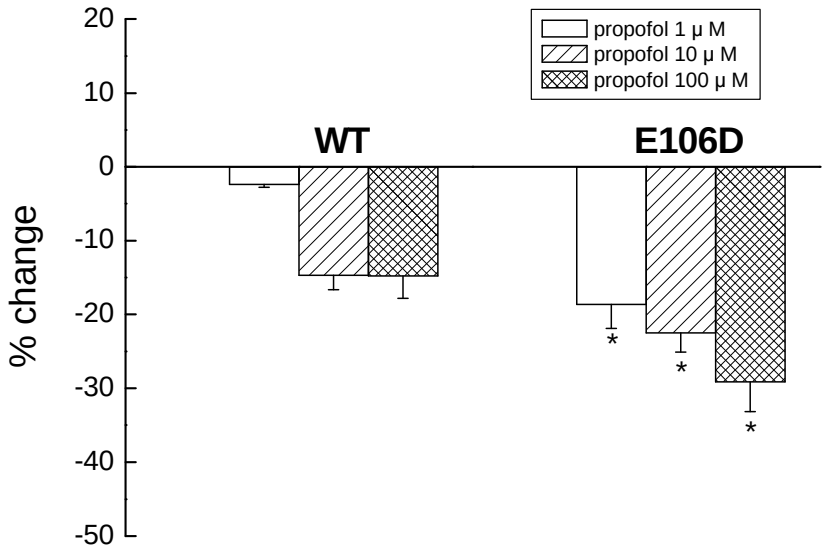

Fig. 7. Effects of propofol on 5-HT-induced currents in the wild-type and E106D mutant 5-HT_{3A} receptors. This study compared the effects of propofol at concentrations of 1, 10, and 100 μM on 5-HT (EC_{20})-induced currents. Propofol inhibited 5-HT-induced currents in both wild-type and E106D mutant 5-HT_{3A} receptors. Negative numbers indicate the percentage of inhibition. Data from more than five oocytes were expressed as the mean ± SEM. *: $P<0.05$ compared to the value of wild-type 5-HT_{3A} receptor at the same concentration of propofol.

Ⅳ. DISCUSSION

In present study, the author investigated the effects of a single mutation on three amino acid residues (E106, F107, R222), which are located in the proximal to the TM1 domain of 5-HT_{3A} receptors which are involved in agonist binding and linking of the agonist binding to the channel gating, on the modulation of the anesthetics in the 5-HT_{3A} receptor.

LGICs seem to be the target receptors of anesthetic action. Most studies on the mechanism of anesthetics are conducted using the nACh receptor or $GABA_A$ receptors. Along with understanding the anesthetic mechanism via the LGICs, studies on anesthetics-induced side effects such as postoperative nausea and vomiting have focused on the 5-HT_{3A} receptor. LGIC member subunits share significant sequence homology and consist of a large, extracellular N-terminal domain, four transmembrane α-helical segments, and an intracellular component.[22,24,25] Many studies have identified amino acid residues that are critical for LGIC assembly, agonist affinity, and conductance.[5,22,26] From these studies, extracellular N-terminal domain is responsible for agonist binding, the TM2 domain lines the channel pore and forms the channel gate, and the pre-TM1 region as well as the TM2-TM3 loop of the 5-HT_{3A} receptor are implicated in the coupling process between agonist binding and channel gating.[22,25] Furthermore, studies have suggested that the anesthetic binding site of LGICs exists in the transmembrane domain.[27-30] At present, many studies have found anesthetic modulation in the anesthetic or alcohol binding pocket located in TM2 and/or TM3.[15-18] According to these studies, anesthetics and alcohols concentrate in the water-filled protein clefts, altering the flexibility of the protein, and hence changing protein function. Miyazawa *et al*. reported that the L257 residue of the α subunit of the *Torpedo* nACh receptor (analogous to S267 in the α1

glycine receptor and L270 in the 5-HT_{3A} receptor) faces away from the pore and towards the other three α-helical transmembrane domains, presumably forming part of a water-filled cavity in which anesthetics can bind.[28] Mihic *et al*. demonstrated that mutations of two amino acids, serine 267 in the TM2 and alanine 288 in the TM3 blocked alcohol and anesthetic enhancement of glycine receptor-mediated currents.[19] In addition, mutations of leucine 293 or isoleucine 294 in the TM2 of the 5-HT_{3A} receptor alter alcohol modulatory actions.[17,18] Moreover, phenylalanine 269 and leucine 270 in the TM2 markedly affected alcohol and anesthetic enhancement of 5-HT_{3A} receptor function.[15] Mutation of a single amino acid in the extracellular TM2 domain induces resistance to ketamine inhibition in the α7 nicotinic receptor as well as a sensitivity to inhibition in the 5-HT_{3A} receptor.[16] For LGIC activation, the signal transduction travels from the agonist binding site to a conformational change in the channel. However, few studies have focused on the functional role of amino acid residues located proximally to the TM domain at the time of anesthetic modulation, even though these sites are involved in the initial process of the signal transduction pathway. The author have raised the question that mutation of the single amino acid residues might affect the anesthetic modulation in the 5-HT_{3A} receptor, in particular, we focused on the mutation of E106[21] and F107[20], which are thought to be important for agonist binding and R222[22], which is involved in the linking of the agonist binding to channel gating.

The author constructed seven individual mutants. Of these mutants, the E106D, F107Y, R222F, and R222V mutant 5-HT_{3A} receptors were functionally expressed but the E106Y, F107S, and R222P mutant 5-HT_{3A} receptors were not functionally expressed. The functional characteristics of expressed wild-type, E106D, F107Y, and R222F mutant 5-HT_{3A} receptors, were comparable to the characteristics described in previous studies.[20-2,31] In

contrast, the F107Y mutant 5-HT_{3A} receptors displayed decreased sensitivity to 5-HT compared to the wild-type 5-HT_{3A} receptor, with an observed six-fold rightward shift ($P<0.05$). While the Hill coefficient of 2.12 ± 0.08 in the wild-type receptor indicated positive cooperativity of the agonist activation, both E106D and R222F mutant 5-HT_{3A} receptors lost a positive cooperativeness (E106D, 1.22 ± 0.16; R222F, 0.75 ± 0.06 vs. wild-type, 2.12 ± 0.08, $P<0.05$). Although the functional characteristics of the R222V mutant 5-HT_{3A} receptor were not compared to others, the R222V mutant certainly constructed the functional channel to the agonist, 5-HT.

To find the anesthetic modulation effect in wild-type 5-HT_{3A} receptor and various mutant 5-HT_{3A} receptors, the author preapplied the saturated volatile anesthetics for 2 minutes with 1 and 2 MAC of the positive modulators halothane and desflurane to the 5-HT_{3A} receptor, before applying 5-HT (EC_{20} taken from each dose response plot) (Fig. 6). Stevens *et al*. noted that anesthetic modulation in the 5-HT_{3A} receptor exhibits a dependence on molecular volume and volatile anesthetics except when sevoflurane enhanced the 5-HT induced currents.[7] In their study, desflurane and halothane enhanced modulation in wild-type 5-HT_{3A} receptor, a result that is similar to this study, although the degrees of enhancing the 5-HT induced currents were greater in the study by Stevens *et al*. than this study. This difference could be explained by the fact that Stevens *et al*. used an EC_{10} concentration of 5-HT while this study used an EC_{20} concentration of 5-HT. I also only pre-applied the volatile anesthetic before applying the 5-HT so that the remaining anesthetic concentrations in the measuring bath must be less than the one used by Stevens *et al*. Desflurane and halothane showed positive modulation in both the wild-type and F107Y mutant 5-HT_{3A} receptors and the F107Y mutant 5-HT_{3A} receptor showed a greater enhancing modulation compared to the wild-type receptor with 1 MAC of halothane.

Meanwhile, the R222F and R222V mutant 5-HT_{3A} receptor lost positive modulation with 1 and 2 MAC of halothane. Most interestingly, positive molulation by desflurane and halothane was converted into negative modulation in E106D mutant 5-HT_{3A} receptors. The direction of modulation by volatile anesthetics displayed differently between the mutagenesis of agonist binding sites, glutamate 106 and its adjacent phenylalanine 107. In particular, the mutation of glutamate to aspartate resulted in shortening the methylene group without altering its polarity. The author looked for any directional changes of modulation by the known negative modulator of the 5-HT_{3A} receptor, propofol. Like the volatile anesthetic, propofol showed a negative modulation with the E106D mutant and wild-type 5-HT_{3A} receptors (Fig. 7). Interestingly, the shortening of the methylene group in the side chain of leucine by mutation to valine in the TM2 domain lost the positive modulation of the volatile anesthetic.[15] However, it is probably not rational that a simple shortening of the methylene group in the amino acid residues of the agonist binding site or the channel pore site determines the anesthetic modulatory direction.

Although this study has the limitation of deriving kinetic informations from constructed receptors, normalized current tracings of 5-HT induced in the presence or absence of volatile anesthetic as shown in Figure 5 displayed identical current shapes in all of the constructed receptors except for the F107Y mutant 5-HT_{3A} receptor. The F107Y mutant 5-HT_{3A} receptor exhibited rapid desensitization during 5-HT application in the presence of volatile anesthetics. On the contrary, 5-HT induced gating of the F107Y mutant 5-HT_{3A} receptor expressed in HEK 293 cells did not display rapid desensitization even in 10 mM concentrations of 5-HT.[20]

The anesthetic effect on 5-HT_{3A} receptors may not contribute to the establishment of an anesthetized state but may be related to anesthetic-induced nausea and vomiting. In *in vitro* studies most volatile anesthetics,

such as enflurane, halothane, and isoflurane, enhanced the 5-HT-induced current in the wild type 5-HT_{3A} receptor by a varying degrees, but sevoflurane or propofol inhibited this current.[7-11,13] These findings were also noted in clinical studies. Philip *et al*. showed that the incidence of postoperative nausea and vomiting after ambulatory anesthesia was lower in the sevoflurane group than in the isoflurane group. Moreover, Raeder *et al*.[33] reported that the incidence of postoperative nausea and vomiting was decreased following the use of propofol rather than desflurane after laparoscopic cholecystectomy. 5-HT_{3A} receptors in the postrema area of the brain is believed to be associated with anesthetic-associated nausea and vomiting .[34]

At present, several subtypes of the 5-HT_3 receptor were identified and the recombinant 5-HT_{3A} receptor showed most of the native 5-HT_3 receptor properties. Nevertheless, whether native 5-HT_3 receptors in different brain areas are constituted by homomeric 5-HT_{3A} pentamer or heteromeric pentamers derived from a combination of 5-HT_{3A} and 5-HT_{3B} or other subtypes is not completely clear. Therefore, mechanisms of anesthetic modulation in the 5-HT3 receptor seem to be multiple and complex. This was evident from the fact that modulation by volatile anesthetic was decreased in the 5-$HT3_{AB}$ receptor compared to the 5-HT_{3A} receptor. Incorporation of the 5-HT_{3B} receptor might alter the anesthetic binding site or the allosteric interaction between anesthetic binding and channel opening.[35] Furthermore, the additive effect of halothane and ethanol on the 5-HT_3 receptor suggests that these compounds have different targeting sites within the 5-HT_3 receptor.[9] However, at the molecular level of the 5-HT_3 receptor, anesthetic modulating sites are much more complex. In this study, which was conducted at the molecular level of the 5-HT_{3A} receptor, anesthetic modulation of the 5-HT_{3A} receptor was also affected by a single mutagenesis of the agonist binding sites (E106 and F107) or the site that is presumed to be the coupling site (R222) between agonist binding and channel gating as well

as a of channel pore sites or the anesthetic binding pocket in LGICs. [10,15,17-9,25,27,36]

The underlying mechanism of how anesthetics interact with the LGICs is assumed to be allosteric.[37] As shown in the simplified kinetic scheme involving the anesthetic modulation mechanism (Fig. 1 and 2), allosteric modulation of anesthetics in 5-HT_{3A} receptors might be achieved by altering agonist binding, the channel gating, linking both, or desensitization.

In summary, this study has shown that mutation of glutamate 106 to aspartate in the 5-HT_{3A} receptor changed the direction of desflurane and halothane modulation from positive to negative and potentiated the negative modulation of propofol. Mutations of phenylalanine 107 to tyrosine potentiated volatile anesthetic modulation, while mutation of arginine 222 to phenylalanine or valine lessened desflurane modulation, or even lost halothane modulation. These findings, conducted at the molecular level of the 5-HT_{3A} receptor, might indicate that anesthetic modulation in 5-HT_{3A} receptor could be affected by the mutation of amino acid residues important for agonist binding and linking of the agonist binding to the channel gating as well as channel gating.

Ⅴ. CONCLUSION

In conclusion, amino acid residues important for agonist binding as well as for linking of agonist binding to channel gating might also have an important role for the anesthetic modulation in 5-HT_{3A} receptor.

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< ABSTRACT (IN KOREAN)>

세로토닌 3형 수용체의 신호전달에 중요한 아미노산들이 마취제 조절작용에 미치는 영향

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김미경

세로토닌 3형 ($5\text{-}HT_3$) 수용체는 ligand-gated ion channels (LGICs) superfamily에 속하며, 다른 대부분의 LGICs에서와 마찬가지로 임상적인 농도의 흡입마취제 및 n-alcohols은 $5\text{-}HT_3$ 수용체의 작용을 조절(modulation)한다. $5\text{-}HT_3$ 수용체에 작용물질(agonist)이 결합하면 수용체의 통로가 열리게 되는 일련의 신호전달과정을 통하여 이 수용체는 작동하게 되는데, 마취제의 조절작용의 기전에 수용체의 통로에 위치한 단백질이 중요한 역할을 하고 있음이 알려졌다. 그러나 마취제의 조절작용에는 수용체의 작용물질이 결합하는 부위와 수용체의 통로를 열림을 매개하는 부위의 단백질도 중요한 역할을 할 것이라는 생각되지만, 지금까지 이에 대한 연구는 거의 이루어 지지 않았다. 따라서 이 연구에서는 $5\text{-}HT_3$ 수용체에 작용물질이 결합하고 이 결합과 통로의 열림을 매개하는 것으로 알려진 단백질인 E106, F107 혹은 R222 아미노산을 다른 아미노산으로 치환하여 만든 유전자 재조합 $5\text{-}HT_3$ 수용체를 통하여 흡입마취제인 halothane과 desflurane 그리고 정맥마취제인 propofol이

이 수용체에 대한 조절작용의 기전에 있어서 이들 아미노산의 역할을 알아보고자 하였다.

Wild-type과 치환된 유전자 재조합 5-HT_3 수용체를 *Xenopus Laevis* oocytes에서 발현시켰고, 수용체의 반응은 two electrode voltage clamp techniques를 이용한 전기 생리 실험으로 관찰하였다. E106D, F107Y, R222F, R222V 유전자 재조합 5-HT_{3A} 수용체들이 기능적으로 발현되었다. E106D, F107Y, R222F 유전자 재조합 5-HT_{3A} 수용체들의 EC_{50}은 각각 1.29 ± 0.18, 1.41 ± 0.21, 7.47 ± 0.85, 그리고 0.61 ± 0.16 uM이었다. 치환된 유전자 재조합 수용체들 중, F107Y 유전자 재조합 5-HT_{3A} 수용체는 wild-type에 비해 5-HT에 대한 감수성이 6배 정도 감소하였다 ($P<0.05$). Hill coefficients는 wild-type 수용체에 비해 E106D와 R222F 수용체에서 유의하게 감소하였다. 이들 수용체에 대한 마취제의 조절작용을 관찰한 실험에서 desflurane과 halothane은 wild-type과 F107Y 유전자 재조합 5-HT_{3A} 수용체를 항진시켰다. 이 중 1 MAC의 halothane에 대한 F107Y 유전자 재조합 5-HT_3 receptor의 항진작용은 wild-type과 비교해서 유의하게 증가되었다. 반면에 R222F와 R222V 유전자 재조합 5-HT_3 receptor는 1, 2 MAC의 halothane 모두에서 항진작용이 소실되었다. 이들 수용체에 대한 마취제의 조절작용 중 가장 주목할 것은 E106D 유전자 재조합 5-HT_3 수용체에서 desflurane과 halothane에 의한 항진작용이 억제작용으로 변화되었다는 것이다. 하지만 wild-type 5-HT_{3A} 수용체를 억제하는 것으로 알려진 propofol은 E106D 유전자 재조합 수용체와 wild-type 5-HT_{3A} 수용체 모두를 억제했다. 5-HT_{3A} 수용체에서 통로의 열림에 중요한 역할을 담당하는 것으로 알려진 부위의 단백질 뿐만 아니라 결합물질이 결합하고 이 결합과 통로의

열림을 매개하는 것으로 알려진 아미노산도 5-HT_{3A} 수용체에 대한 마취제의 조절작용에 중요한 역할을 하는 것으로 생각된다.

핵심되는 말: Electrophysiology, 5-HT_{3A} receptor, 5-HT, propofol, site-directed single mutation, *Xenopus Laevis* Oocyte, volatile anesthetics.