Insertion of an Epitope Tag in the $5HT_{2C}$-Receptor

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KEYWORDS. Receptor, tag, $5HT_{2C}$-receptor

BRIEF. The insertion of a tag into the $5HT_{2C}$-receptor to purify it in large quantities for future studies to be done on the different disorders and functions associated with it.

ABSTRACT. A cell receptor is a membrane protein that is involved in communication between different cells. This study focuses on the one type of serotonin, chemical messenger sent by neurons to communicate between brain cells, the $5HT_{2C}$-receptor. This receptor is important because it is closely associated with obesity, anxiety, sleep disorders, movement, and appetite. Because there are not good tools for studying this receptor, it is difficult to purify. The goal of this research was to purify this receptor in large quantities to allow for future studies to be done on many different disorders and functions associated with it. The purification method used here was to insert a tag into the $5HT_{2C}$-receptor. To confirm that the tagged receptor was functioning just like a $5HT_{2C}$-receptor without a tag, functionality tests were performed. The tag was successfully inserted into the receptor. Future studies are required to determine if the tagged receptor functions identical to the untagged receptor and if it can be obtained and purified in large quantities. This would lead to studies of many disorders and functions associated with this receptor.

INTRODUCTION.

A cell receptor allows cells in the body to communicate with each other. It is necessary for a cell receptor to function normally, which means the signal must be received by the receptor, travel through the receptor, and be transmitted to the cell normally. The $5HT_{2C}$-receptor, is a serotonin cell receptor that plays a major role in different functions of the brain and it facilitates many different behaviors, including reward behavior, appetite, locomotion, energy balance, and sexual behavior [1]. It is vital to understand functionality of this receptor because it has been shown, when deregulated, to be closely associated with obesity, epilepsy, anxiety, sleep disorders, and motor dysfunction [2]. However, previous data has suggested a disparity between the expression of the $5HT_{2C}$-receptor RNA and $5HT_{2C}$-receptor protein [3]. Unfortunately, there are not any specific antibodies that are capable of clearly detecting this receptor that can be used for localization of the protein. In addition, the lack of specific antibodies has also prevented purification of the receptor in order to clearly identify its binding partners.

One approach that we have used for characterizing the expression of the receptor is to genetically engineer the receptor by inserting a specific type of tag, a Strep-tag (*Strep* II) and histidine tag (*Strep*/6xHis). This specific tag was chosen because *Strep* II binds to strep-tactin with high affinity, which allows the tag to bind to the receptor. The 6xHis tag binds to Cobalt or Nickel containing resins with high affinity, which allows the receptor to be purified

If the *Strep*/6xHis tag could be inserted into the receptor in such a way that it does not disrupt the function of the receptor, then it could be inserted into the genome of a mouse in place of the normal receptor gene. Mice with this altered receptor "knocked in" to their genome would express the tagged receptor and allow characterization of its expression and easy purification using biochemical tools that bind to the tags with very high affinity and specificity.

The goal of this project was to create a receptor with such a tag inserted. It was hypothesized that the *Strep*/6xHis tag could be inserted into the receptor in the way that it would function and be expressed identically to the native, untagged receptor. Consequently it could be used in a knock-in mouse model, which is integral for future studies of $5HT_{2C}$. This research along with future studies could lead to understanding of many disorders and functions the $5HT_{2C}$-receptor is associated with and lead to pharmacological studies.

MATERIALS AND METHODS.

Tag Creation and Insertion.

Two tags were inserted into the cDNA of the $5HT_{2C}$ receptor. Each tag was a combination of a Strep-tag sequence followed by six histidines. The first insertion site was at nucleotide position 140 (referred to as N-term tag) and the other was at nucleotide position 1406 (referred to as C-term tag) in the $5HT_{2C}$-receptor cDNA sequence. For each tag, two primers were designed that aligned with the cDNA of the $5HT_{2C}$-receptor (panel 1 of Fig. 1). For the N-term tag, the primers were:

5' - GTT TTG TAC CCC GTC CGG GAA TTG GTG ATG GTG ATG GTG ATG GC - 3' and 5' - CTC CGA TGG TGG ACG CTT GTT TTG GAG CCA CCC CCA GTT CGA GAA G - 3'

and for the C-term tag the primers were:

5' - CCG TCG ACT GCA GAA TTA CAC ACT GTG ATG GTG ATG GTG ATG GC – 3' and 5' - CCT CTAA ATG TGG TCA GCG AGA GGA TTT GGA TTT GGA ACC CCC AGT TCG AG - 3'.

Using each primer pair, a Polymerase Chain Reaction (PCR) was carried out with Dream Taq Polymerase (Fermentas) at 95°C for 2 min, then 34 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, and was completed with 72°C for 5 min. (panel 2 of Fig. 1) This created a megaprimer, a large primer that could be used in site-directed mutagenesis (panel 3 of Fig. 1). To insert the mega primer into the receptor cDNA, a second PCR was run using the Quick Change II site directed mutagenesis kit (Stratagene), as recommended by the manufacturer. The reaction was run using the megaprimer and the plasmid containing the $5HT_{2C}$-receptor cDNA as template at 95°C for 30 sec then 5 cycles of 95°C for 30 sec, 52°C for 1 min, 68°C for 1 min, followed by 13 cycles of 95°C for 30 sec, 55°C for 1 min, 68°C for 1 min, inserting the megaprimer containing the tag within the cDNA of the $5HT_{2C}$-receptor at the desired location. DpnI was used to digest the parent strands without the megaprimer (in panel 4 of Fig. 1) and the products were transferred into DH5α. DNA plasmid was extracted from individual colonies and sequenced to confirm that the tag had been successfully inserted within the $5HT_{2C}$-receptor.

Cell culture.

Human Embryonic Kidney (HEK) 293 cells were cultured in Delbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. The cells were grown on 10 cm tissue culture plates.

Transfection.

For the expression of the tagged $5HT_{2C}$-receptor, the plasmid containing tagged cDNA of the $5HT_{2C}$-receptor, under control of the constitutive CMV promoter, was transfected into HEK 293 cells. X-tremeGENE reagent: pEGFP-C1= 2:1 (2μl reagent: 1μg of plasmid) was used to transfect the cells as recommended by manufacturer (Ambion).

Reverse transcription-PCR.

RNA was extracted from transfected HEK 293 cells with Tri Reagent (Ambion) as recommended. 1 μg of RNA was then subjected to Reverse-Transcription and PCR (RT-PCR). The reverse transcription was run at 25°C for 10 min, 37°C for 1hr, 85°C for 5 min followed by a PCR at 95°C for 5 min, then 34 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, and was completed with 72°C for 10 min.

Western blotting.

Transfected cells were collected and lysed in RIPA buffer (Tris-HCL,pH 7.5, 50mM; NaCl 150mM; SDS 0.1%; EDTA 1mM; Sodium Deoxycholate 1%; NP40 1%). Protein was separated by SDS-PAGE, and transferred to nitrocellulose. The blot was then blocked with PBS containing 0.3% fat-free milk. The receptor was detected with mouse anti-2c antibody (3:1000) and fluorescently labeled goat anti-mouse antibody (1:10,000). The protein was then visualized on the Odyssey (Licor) imaging system.

RESULTS.

Strep/6xHis Tag Insertion Locations.

Regions of a protein that are similar across the species, or conserved, are of the high interest; therefore, the areas with low conservation as optimal locations for the placement of the tag were selected. Accordingly, protein sequences of $5HT_{2C}$-receptor of mice, rats, dogs, rhesus monkeys, humans, and chimpanzees were aligned in order to find areas of low conservation (panel 1 and 2 of Fig. 1S). The locations for the *Strep*/6xHis tag were deemed the best, based on low conservation at cDNA nucleotide position 140, near the N-terminus in an extracellular domain of the receptor, and at nucleotide position 1406, near the C-terminus within an intracellular domain.

Creating a Megaprimer.

To insert the large *Strep*/6xHis tag into the receptor, a Megaprimer was created. The sequence of the tag (Fig. 2S) was flanked by regions homologous to the $5HT_{2C}$-receptor at the site of insertion. A PCR reaction was run using the cDNA of the receptor as template (panel 2 of Fig. 1).

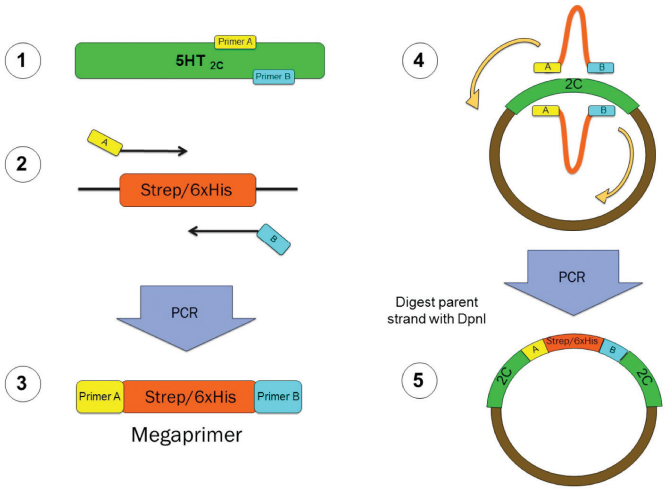

Figure 1. Flow chart of the methods for inserting the 6xHis/Strep tag. Panel 1. The $5HT_{2C}$-receptor cDNA with two primers. Panel 2. The primers were set-up flanking the 6xHis/Strep tag in preparation for PCR. PCR was run to create Megaprimer Panel 3. Megaprimer with the tag inserted between the two primers. Panel 4. Megaprimer, with two original primers, was realigned along $5HT_{2C}$-receptor cDNA. PCR was run and DpnI was used to digest the parent strands of the $5HT_{2C}$-receptor cDNA. Panel 5. The $5HT_{2C}$-receptor cDNA with the tag inserted.

Insertion of Tag into $5HT_{2C}$ Receptor.

The megaprimer was then used in site-directed mutagenesis reaction to insert the tag at the desired location by PCR based methods. The correct insertion of the tag was confirmed by sequencing the resulting plasmid. Figure 2 shows the sequence of the tag within the $5HT_{2C}$-receptor sequence at both locations. The nucleotide sequence of the plasmid in the region of the tag matched exactly with the template cDNA, containing the tag in the proper reading frame proves that the correct tag was inserted at the desired location.

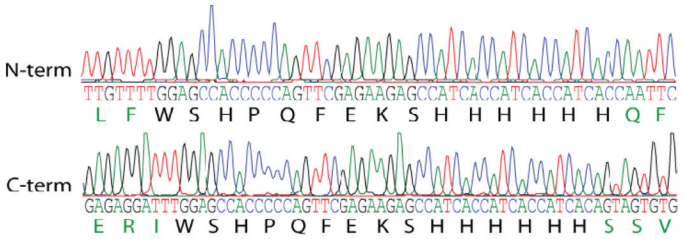

Figure 2. A sequence trace of the *Strep*/6xHis tag inserted into the cDNA of the $5HT_{2C}$-receptor. The amino acid sequence of the tag is shown in black and the sequence of the receptor is shown in green.

Analyzing the Expression of the Tagged $5HT_{2C}$-receptor.

The expression of the tagged receptor in mammalian cells was evaluated by transfecting the construct into HEK 293 cells. Two days after transfection, RNA was collected to determine if the mRNA was produced. A reverse transcription-PCR reaction was run to determine if the tagged $5HT_{2C}$-receptor mRNA was present (Fig. 2). The gel electrophoresis of the PCR reaction is shown in panel 1 of Figure 3. There was a single, distinct band in the reaction with reverse transcriptase, which suggests that the mRNA for the $5HT_{2C}$-receptor with the tag at the C-terminus was expressed. Cells transfected with GFP and a reaction lacking reverse transcriptase served as negative controls. Results for the gel electrophoresis of the PCR for the N-terminus were not possible to obtain due to time constraints.

To determine whether the protein was expressed, HEK 293 cells were transfected with the constructs or a plasmid expressing GFP, as a negative control, and the cells were lysed and the expression of the protein analyzed by Western blot with an antibody directed against $5HT_{2C}$ (Fig. 2). As shown in panel 2 of Figure 3, expression of the N-terminally and C-terminally tagged receptor was detected, indicating that insertion of the tags did not disrupt expression of the protein.

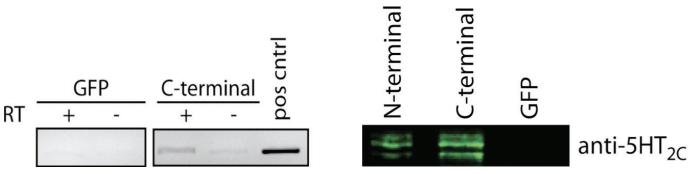

Figure 3. The tagged receptor was expressed by mammalian cells. (Panel 1.) Gel electrophoresis of an RT-PCR reaction demonstrating mRNA expression of the $5HT_{2C}$-receptor with the tag at the C-terminus. (Panel 2.) Western blot of HEK 293 cells transfected with the plasmid containing the receptor with the C-terminal tag, the N-terminal tag or GFP, as a control.

Proving the Hypothesis.

The data shown in these studies prove that the *Strep*/6xHis tag can be successfully inserted into the $5HT_{2C}$-receptor. These results suggest that the tagged $5HT_{2C}$-receptor correctly expresses the mRNA for the C-terminally tagged receptor and the protein for the receptor with the tag at the C-terminus and N-terminus in a mammalian cell, further proving a component of the hypothesis.

DISCUSSION.

The $5HT_{2C}$-receptor has proven difficult to fully characterize because there is a lack of biochemical and immunological tools, which prevents purification of the receptor and analysis of spatial expression patterns by immunohistochemistry. Therefore, a strategy was designed to insert a tag that will not disrupt function, but allow biochemical purification and detection. Results here suggest that the *Strep*/6xHis tag has been successfully inserted in the receptor, such that protein and RNA is expressed. Future studies must be done to determine

if the tagged $5HT_{2C}$-receptor is fully functional and if the tag is accessible for biochemical purification.

Future tests must be conducted to determine if the tagged $5HT_{2C}$-receptor functions identical the untagged $5HT_{2C}$-receptor. These functionality tests will include: further analysis of the protein and RNA expression of the $5HT_{2C}$-receptor to ensure both are being expressed properly (Fig. 2), ligand binding tests to determine if the ligand is binding properly, and a PI hydrolysis test to determine if the PI is functioning properly. If all of these tests prove that the tagged $5HT_{2C}$-receptor is functioning identical to the untagged $5HT_{2C}$-receptor, then a knock-in mouse model can be applied to determine if the tagged $5HT_{2C}$-receptor is functioning exactly like the untagged $5HT_{2C}$-receptor in a mammal. If the $5HT_{2C}$-receptor does function normally in the mouse model, then it can be purified in large quantities, which will allow for future pharmacological and disorder oriented studies to be done with the $5HT_{2C}$-receptor.

CONCLUSION.

The $5HT_{2C}$-receptor has been successfully tagged in two locations, near the N-terminus and C-terminus. The tagged $5HT_{2C}$-receptor expresses protein identical to the untagged $5HT_{2C}$-receptor at both locations and it expresses RNA identical to the untagged 5HT2C-receptor at C-term location. It can be inferred that because the tagged receptor at the N-term expresses protein normally, it would express RNA normally; however, this can only be confirmed by conducting an RT-PCR to determine if this is a reasonable conclusion. The conclusion is supported by the found results and enhances other receptor-based studies [4].

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SUPPORTING INFORMATION.

Figure S1. Portion of the protein sequence where the N-term, nucleotide 180, tag (1) and the C-term, nucleotide 1406, tag (2) was inserted

Figure S2. The Strep/6xHis tag sequence

Figure S3. A sequence trace of the Strep/6xHis tag inserted into the cDNA of the $5HT_{2C}$-receptor

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