

Short communication

## Solubilization of human serotonin<sub>1A</sub> receptors expressed in neuronal cells

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### Abstract

The serotonin<sub>1A</sub> receptor is an important member of the G-protein coupled receptor family, and is involved in a variety of cognitive, behavioral, and developmental functions. None of the subtypes of G-protein coupled serotonin receptors have yet been purified to homogeneity from natural sources. We report here, for the first time, the solubilization of human serotonin<sub>1A</sub> receptors stably expressed in neuronal (HN2) cells. Importantly, ligand binding assay shows that the serotonin<sub>1A</sub> receptor solubilized this way is functionally active. The effective solubilization of the serotonin<sub>1A</sub> receptor from neuronal cells represents an important step toward the purification of the receptor in native-like membrane environment.

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**Keywords:** Solubilization; Serotonin<sub>1A</sub> receptors; CHAPS; Neuronal cells; 8-OH-DPAT; Native environment

### 1. Introduction

The G-protein coupled receptors (GPCRs) are the largest class of molecules involved in signal transduction across membranes (Pierce et al., 2002), and represent a major fraction of membrane proteins. GPCRs are prototypical members of the family of seven trans-membrane domain proteins and constitute ~2% of the human genome (Fredriksson et al., 2003). They respond to a diverse variety of ligands and mediate multiple physiological processes and have therefore emerged as

major targets for the development of novel drug candidates in all clinical areas (Insel et al., 2007). The serotonin<sub>1A</sub> (also referred to as 5-HT<sub>1A</sub>) receptor is an important neurotransmitter receptor and is a representative member of the large family of GPCRs. It is the most extensively studied of the serotonin receptors for a number of reasons. The serotonin<sub>1A</sub> receptor is involved in the generation and modulation of a variety of cognitive, behavioral, and developmental functions (for a recent review, see Pucadyil et al., 2005). The serotonin<sub>1A</sub> receptor agonists and antagonists represent major classes of molecules with potential therapeutic effects in anxiety- or stress-related disorders. Interestingly, mice lacking the serotonin<sub>1A</sub> receptor exhibit enhanced anxiety-related behavior (Julius, 1998) and represent an important animal model for genetic vulnerability to conditions such as anxiety disorders and aggression (Toth, 2003).

Membrane protein purification represents an area of considerable challenge in contemporary membrane biol-

**Abbreviations:** 5-HT<sub>1A</sub> receptor, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; BCA, bicinchoninic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; GPCR, G-protein coupled receptor; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride

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ogy. Studies carried out on purified and reconstituted membrane receptors have considerably advanced our knowledge of the molecular aspects of receptor function (Gether, 2000). In this context, it is noteworthy that none of the subtypes of G-protein coupled serotonin receptors have yet been purified to homogeneity from natural sources. An essential criterion for purification of an integral membrane protein is that the protein must be carefully removed from the native membrane and individually dispersed in solution. This process is known as solubilization and is most effectively accomplished using amphiphilic detergents (Garavito and Ferguson-Miller, 2001; Kalipatnapu and Chattopadhyay, 2005). Solubilization of a membrane protein is a process in which the proteins and lipids that are held together in the native membrane are suitably dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in the formation of small clusters of lipid and protein that remain dissolved in the aqueous solution. Effective solubilization and purification of G-protein coupled receptors in a functionally active form represent important steps in understanding structure–function relationship and pharmacological characterization of a specific receptor. Yet, solubilization of a membrane protein with retention of activity poses a formidable challenge since many detergents irreversibly denature membrane proteins (Garavito and Ferguson-Miller, 2001). This is the main reason for the rather modest list of membrane proteins which have been solubilized with retention of function.

Mammalian cells in culture heterologously expressing membrane receptors represent convenient systems to address problems in receptor biology due to higher expression levels of the receptors (Tate and Grisshammer, 1996). An important consideration in such expression systems is selecting a cell type which is derived from the tissue of natural occurrence of the receptor. This is particularly true for receptors of neural origin since the membrane lipid composition of cells in the nervous system is unique and displays remarkable diversity (Sastry, 1985). Lipids found in neuronal membranes are often necessary for maintaining the structure and function of neuronal receptors. Keeping this in mind, we earlier reported the pharmacological and functional characterization of the human serotonin<sub>1A</sub> receptor stably expressed in HN2 cells (Paila and Chattopadhyay, 2006), which are a hybrid cell line between hippocampal cells and mouse neuroblastoma. Our results showed that the human serotonin<sub>1A</sub> receptor expressed in HN2 cells displays characteristic features found in the native receptor isolated from bovine hippocampus and represents a realistic model system for the receptor. In this paper, we

report the solubilization of serotonin<sub>1A</sub> receptors stably expressed in HN2 cells using the zwitterionic detergent CHAPS. More importantly, we show by ligand binding assay that the serotonin<sub>1A</sub> receptor solubilized this way is functionally active. This constitutes the first report describing the solubilization of functional serotonin<sub>1A</sub> receptors expressed in neuronal cells.

## 2. Materials and methods

### 2.1. Materials

CHAPS, EDTA, MgCl<sub>2</sub>, MnCl<sub>2</sub>, NaCl, PMSF, Tris, PEG, polyethylenimine, fetal calf serum, penicillin, streptomycin, gentamycin sulphate, sodium bicarbonate and serotonin were obtained from Sigma Chemical Co. (St. Louis, MO). DMEM (Dulbecco's Modified Eagle Medium) and geneticin (G-418) were from Life Technologies (Grand Island, NY). [<sup>3</sup>H]8-OH-DPAT (specific activity 106.0 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, UK). BCA reagent for protein estimation was obtained from Pierce (Rockford, IL). All other chemicals used were of the highest purity available. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

### 2.2. Methods

#### 2.2.1. Cells and cell culture

The intronless human genomic clone G-21 (Fargin et al., 1988) which encodes the human serotonin<sub>1A</sub> receptor was used to generate stable transfectants in HN2 cells which are a hybrid cell line between hippocampal cells and mouse neuroblastoma (Lee et al., 1990). These cells expressing the human serotonin<sub>1A</sub> receptor, originally referred to as HN2-5 (Banerjee et al., 1993), were a generous gift from Dr. Probal Banerjee (College of Staten Island, City University of New York). These cells are referred to as HN2-5-HT<sub>1A</sub>R (Paila and Chattopadhyay, 2006). Cells were grown in DMEM supplemented with 3.7 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin sulfate, and 200 μg/ml geneticin in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C.

#### 2.2.2. Preparation of cell membranes

Cell membranes were prepared as described earlier (Paila and Chattopadhyay, 2006). Confluent cells were harvested by treatment with ice-cold buffer containing 10 mM Tris, 5 mM EDTA, 0.1 mM PMSF, pH 7.4. Cells

were then homogenized at 4 °C at maximum speed with a Polytron homogenizer. The cell lysate was centrifuged at  $500 \times g$  for 10 min at 4 °C and the resulting postnuclear supernatant was centrifuged at  $40,000 \times g$  for 30 min at 4 °C. The pellet thus obtained was suspended in 50 mM Tris buffer, pH 7.4, flash frozen in liquid nitrogen and stored at  $-70$  °C till further use. Total protein concentration in isolated membranes was determined using the BCA assay (Smith et al., 1985).

### 2.2.3. Solubilization of cell membranes

Cell membranes were treated with 5 mM CHAPS and varying concentrations of NaCl or protein, in buffer containing 50 mM Tris, 1 mM EDTA, 10 mM  $MgCl_2$ , pH 7.4 for 30 min at 4 °C with occasional shaking. Membranes were briefly sonicated (5 s) using a Branson model 250 sonifier fitted with a microtip and mildly homogenized using a hand-held Dounce homogenizer (five times) at the beginning of the incubation. The homogenization was repeated at the end of the incubation period. After incubation for 30 min, the contents were centrifuged at  $100,000 \times g$  for 1 h at 4 °C. The clear supernatant was subjected to PEG precipitation. PEG precipitation of the CHAPS solubilized cell membranes was performed to remove NaCl from the solubilized extract since the agonist binding of the serotonin<sub>1A</sub> receptor was found to be inhibited by NaCl (Harikumar and Chattopadhyay, 1998). PEG precipitation was carried out by diluting the solubilized extract with equal volume of 40% (w/w) PEG-8000 prepared in buffer containing 50 mM Tris, 1 mM EDTA, 10 mM  $MgCl_2$ , pH 7.4. Following vigorous vortexing and incubation on ice for 10 min, samples were centrifuged at  $15,000 \times g$  for 10 min at 4 °C. The pellet was carefully rinsed with 50 mM Tris, pH 7.4 buffer and resuspended in the same buffer and used for radioligand binding assays immediately.

### 2.2.4. Agonist binding assays

Agonist binding assays of cell membranes and PEG-precipitated CHAPS solubilized cell membranes were performed as described earlier (Paila and Chattopadhyay, 2006). Briefly, tubes in duplicate containing membranes were incubated in a total volume of 1 ml of buffer containing 50 mM Tris, 1 mM EDTA, 10 mM  $MgCl_2$ , 5 mM  $MnCl_2$ , pH 7.4 in the presence of 0.29 nM [<sup>3</sup>H]8-OH-DPAT for 1 h at 25 °C. Non-specific binding was determined by performing the assay in the presence of 10 μM unlabeled serotonin. The incubation was terminated by rapid filtration under vacuum in a Brandel cell harvester (Gaithersburg, MD) through Whatman GF/B 2.5 cm diameter glass microfiber filters (1.0 μm pore size), which were presoaked in 0.15% (w/v)

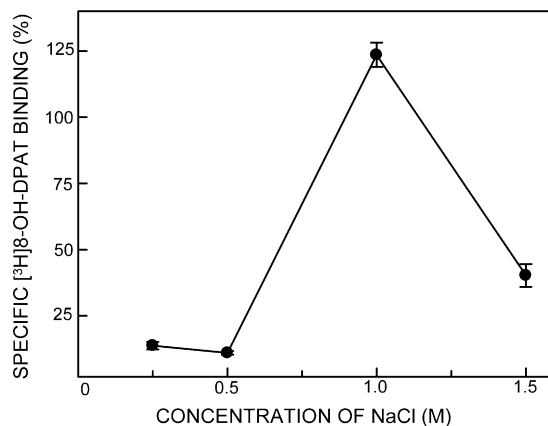


Fig. 1. Effect of NaCl concentration on the solubilization efficiency of the human serotonin<sub>1A</sub> receptor stably expressed in HN2 cells. Values are expressed as percentages of specific binding of the agonist [<sup>3</sup>H]8-OH-DPAT obtained for control membranes without solubilization. The concentration of CHAPS used was 5 mM and the total protein concentration was 2 mg/ml in all cases. Specific binding is normalized to the amount of total protein used in the assay. The data points are means  $\pm$  S.E. from at least three independent experiments. See Section 2 for other details.

polyethylenimine for 1 h (Bruns et al., 1983). The filters were then washed three times with 3 ml each of ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid. The specific [<sup>3</sup>H]8-OH-DPAT binding shown in Figs. 1 and 2 are normalized to the amount of total protein used in the assay.

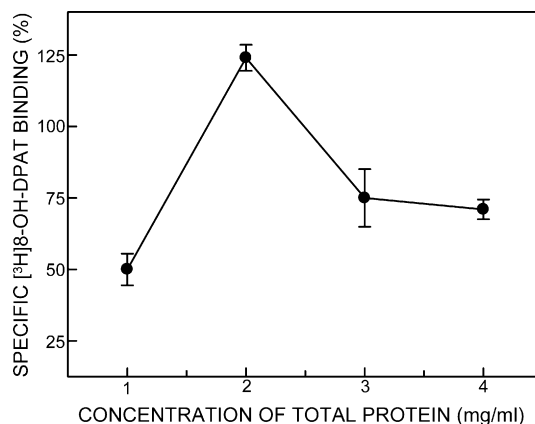


Fig. 2. Effect of total protein concentration on the solubilization efficiency of the human serotonin<sub>1A</sub> receptor stably expressed in HN2 cells. Values are expressed as percentages of specific binding of the agonist [<sup>3</sup>H]8-OH-DPAT obtained for control membranes without solubilization. The concentration of CHAPS used was 5 mM and NaCl concentration was 1 M in all cases. Specific binding is normalized to the amount of total protein used in the assay. The data points are means  $\pm$  S.E. from four independent experiments. See Section 2 for other details.

### 3. Results

The choice of a suitable detergent is an important consideration in the process of solubilization (Kalipatnapu and Chattopadhyay, 2005). The mild, non-denaturing, zwitterionic detergent CHAPS was previously found to be the most efficient detergent in functional solubilization of the serotonin<sub>1A</sub> receptor upon comparison of the solubilizing efficiencies of 14 different detergents (Banerjee et al., 1995). CHAPS is a derivative of the naturally occurring bile salts and combines useful features of both the bile salt hydrophobic group and the *N*-alkyl sulfobetaine-type polar group (Hjelmeland, 1980). CHAPS has been shown to be an efficient detergent for solubilization since it is effective in breaking protein–protein interactions. Interestingly, we have previously shown that the critical micelle concentration (CMC) of CHAPS is dependent on the salt concentration of the medium and there is significant reduction in CMC with increasing salt concentration. For example, the CMC of CHAPS decreases from 6.41 mM in absence of any salt to 4.34 mM in presence of 1 M NaCl which amounts to a reduction of ~32% (Chattopadhyay and Harikumar, 1996). PEG precipitation of the CHAPS-solubilized membrane was performed to remove NaCl from the solubilized extract since the agonist binding of the serotonin<sub>1A</sub> receptor is inhibited by NaCl (Harikumar and Chattopadhyay, 1998). This procedure constitutes a method in which the detergent along with salt is rapidly removed (Ofri et al., 1992) thereby overcoming the problem of long dialysis periods (Medrano et al., 1989). We have previously shown that 5 mM CHAPS is suitable for optimal solubilization of the hippocampal serotonin<sub>1A</sub> receptor. Higher concentration of CHAPS resulted in relatively inefficient solubilization, irrespective of NaCl concentration (Chattopadhyay et al., 2002). The solubilization efficiency of serotonin<sub>1A</sub> receptors from HN2-5-HT<sub>1A</sub>R cell membranes, as monitored by the specific binding of the agonist [<sup>3</sup>H]8-OH-DPAT as a function of NaCl concentration is shown in Fig. 1. Solubilization efficiencies were calculated as percentages of specific [<sup>3</sup>H]8-OH-DPAT binding in solubilized membranes, normalized to specific [<sup>3</sup>H]8-OH-DPAT binding in control membranes in the absence of solubilization (*i.e.*, without CHAPS treatment). Fig. 1 shows that the solubilization efficiency is marginal up to 0.5 M NaCl. The extent of solubilization shows a sharp increase when NaCl concentration is increased to 1 M. The solubilization efficiency appears to decrease considerably when NaCl concentration was increased to 1.5 M. Optimal solubilization is therefore achieved using 5 mM CHAPS in presence of 1 M NaCl. The concept of micelle formation

is relevant to solubilization and reconstitution studies of membrane proteins since it appears that there is some correlation between the ability to form micelles and the concentration of detergent required for solubilization (Rivnay and Metzger, 1982). As mentioned above, although this concentration of CHAPS is lower than literature CMC of CHAPS, we have previously shown that CHAPS exists in micellar form at this concentration (5 mM) in presence of 1 M NaCl (Chattopadhyay and Harikumar, 1996).

The process of solubilization involves multiple changes in organization of the membrane and is dependent on a host of membrane parameters (Kalipatnapu and Chattopadhyay, 2005). The ratio of detergent to protein used during solubilization represents a crucial factor for optimal solubilization. The solubilization efficiency of the serotonin<sub>1A</sub> receptor in HN2-5-HT<sub>1A</sub>R cell membranes was found to depend on the total concentration of the protein in the membrane when the concentration of CHAPS was kept constant. Fig. 2 shows that changing the protein concentration (while keeping the CHAPS and NaCl concentrations fixed at 5 mM and 1 M, respectively) alters the solubilization of HN2-5-HT<sub>1A</sub>R cell membranes. Solubilization was carried out with total protein concentration in the range of 1–4 mg/ml. Fig. 2 shows that solubilization efficiency is moderate when the protein concentration of 1 mg/ml is used with a sharp increase when the concentration is increased to 2 mg/ml. With further increase in protein concentration to 3 and 4 mg/ml, the solubilization efficiency is found to reduce. Under this condition, a total protein concentration of 2 mg/ml was therefore found to be optimal. These results show that careful control of salt and protein concentration is crucial for optimal solubilization of membrane receptors expressed in neuronal cells in culture.

### 4. Discussion

It is estimated that up to 50% of clinically prescribed drugs act as either agonists or antagonists at GPCRs which points out their immense therapeutic potential (Karnik et al., 2003). A useful approach for performing pharmacological studies on GPCRs is to use a functional receptor system that converts receptor–ligand interaction into a cellular signal which allows to monitor the relationship between concentration and response (Kenakin, 1997). With the advent of molecular biology, there have been an increasing number of genetically engineered recombinant receptor systems for the study of drug–receptor interactions. This has led to a corresponding increase in the testing of new drugs in recombinant receptor systems. However, differences in host mem-



brane lipid composition often complicate interpretation of drug testing results in such systems, and can lead to receptors with characteristics different from native receptors. For example, it has earlier been reported that although the rat cortical serotonin<sub>1A</sub> receptor exists only in the high affinity state in its native environment, it displays both high and low affinity when expressed in HEK293 cells (Watson et al., 2000). It is therefore judicious to work with cellular systems in which the membrane lipid composition closely mimics the native lipid environment. Our choice of HN2 cells for solubilizing serotonin<sub>1A</sub> receptors is based on the observation that cell lines of neural origin represent realistic models for understanding signal transduction in neuronal cells (Lee et al., 1990).

Since native tissues (of neuronal origin in particular) often have very low quantities of a specific type of receptor, solubilization and purification of neuronal receptors from native sources continue to be challenging issues in contemporary membrane biology. It is in this context that membrane receptors solubilized from a cell line with native-like membrane lipid environment gains significance. In the present study, we have standardized conditions for the optimum solubilization of active serotonin<sub>1A</sub> receptors from HN2-5-HT<sub>1A</sub>R cell membranes. We could achieve optimum solubilization using 5 mM CHAPS in presence of 1M NaCl and with a total protein concentration of 2 mg/ml. The effective solubilization of the serotonin<sub>1A</sub> receptor from neuronal cells reported here represents a crucial step toward the purification of the receptor in native-like membrane environment. Effective solubilization and purification of membrane receptors with optimum ligand binding activity and intact signal transduction components represent important steps in understanding structure–function relationship and pharmacological characterization of a specific receptor, and may constitute the first step in the detailed molecular characterization of GPCRs.

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