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Serotonin (5-HT) Receptor Subtypes Mediate Regulation of Neuromodulin Secretion in Rat Hypothalamic Neurons

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Abstract

Serotonin (5-HT), the endogenous nonselective 5-HT receptor agonist, activates the inositol -1,4,5- triphosphate / calcium ($InsP3/Ca^{2+}$) signaling pathway and exerts both stimulatory and inhibitory actions on cAMP production and neuromodulin secretion in rat hypothalamic neurons. Specific mRNA transcripts for 5-HT1A, 5-HT2C and 5-HT4 were identified in rat hypothalamic neurons. These experiments were supported by combined techniques such as cAMP and a Ca^{2+} assays in order to elucidate the associated receptors and signaling pathways.

The cAMP production and neuromodulin release were profoundly inhibited during the activation of the Gi-coupled 5-HT1A receptor. Treatment with a selective agonist to activate the Gq-coupled 5-HT2C receptor stimulated InsP3 production and caused Ca^{2+} release from the sarcoplasmic reticulum. Selective activation of the Gs-coupled 5-HT4 receptor also stimulated cAMP production, and caused an increase in neuromodulin secretion.

These findings demonstrate the ability of 5-HT receptor subtypes expressed in neurons to induce neuromodulin production. This leads to the activation of single or multiple G-proteins which regulate the $InsP3/Ca^{2+}$/PLC-γ and adenyl cyclase / cAMP signaling pathways.

Keywords: neuromodulin signaling, selective serotonin agonists, rat hypothalamus, cAMP, intracellular Ca^{2+}

Introduction

Neuromodulin (known as P-57, GAP-43, and B-50) is a neural specific, membrane associated protein which has been implicated in the regulation of neurite outgrowth, and in synaptic plasticity (Casoli *et al*, 2001). Neuromodulin is a major presynaptic substrate for the protein kinase C (PKC) phosphorylation of neuromodulin. This decreases its affinity for calmodulin, suggesting that neuromodulin may function to bind and concentrate calmodulin within neurons, releasing it locally in response to phosphorylation by PKC (Chapman *et al*, 1992). PKC is activated by the PLC-γ signaling pathway. PLC-γ binds phosphatidylinositol 4,5-bisphosphate (PIP2), which anchors the enzyme to the plasma membrane and enhances its catalytic activity for cAMP production. The calcium buffering capacity of patients suffering from neurodegenerative diseases has been reported to be diminished, probably due to either a deficit or deficiency in intracellular Ca^{2+}binding structures such as the neuromodulin-calmodulin complex (Ibaretta *et al*, 1997).

The serotoninergic system is one of several neuronal inputs that innervate hypothalamic neurons (Almero *et al*, 2000). In hypothalamic neuronal cells, it has been shown that serotonin receptors are involved with the secretion of neuromodulin. The expression of serotonin receptors increases intracellular Ca^{2+}levels that can regulate neuromodulin secretion and calmodulin signaling in the presence of PKC (Lee *et al*, 2002; Kim *et al*, 2003).

In this study, the synaptic relationship between serotoninergic terminals and neuromodulin was investigated in rat hypothalamic neurons using RT-PCR and western blotting analysis. We traced the signaling pathway for the regulation of neuromodulin secretion by serotonin receptor subtypes (Chapman *et al*, 1985). These observations led to the idea that the networks linking the neurotransmitters and the secreted peptide hormones from the hypothalamic neuron cells regulate neuronal plasticity.

Materials & Methods

Materials

5-hydroxytryptamine was purchased from Sigma Aldrich (ST Louise, MO). Selective serotonin agonists, HT1A (Ipsapirone), HT2C (MK 212) and HT4 (Cisapride) were purchased from Tocris Bioscience(Ellisville,MO).

Animal preparation and primary neuronal cell culture

Male Wistar rats weighting 200 g (12 weeks-old) were housed in air-conditioned animal quarters, with lights on,

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and given food and water *ad libitum*. The tail was grasped and cervical dislocation was performed to terminate the animals, followed by decapitation using a guillotine. The scalp was scraped the scalp from the mastoid area to expose the skull. The skull was incised along the external auditory meatus and the brain was removed. The borders of the excised hypothalamus were delineated by the anterior margin of the optic chiasm, the posterior margin of the mammillary bodies, and laterally by the hypothalamic sulci. The separated hypothalamus were split into two pieces each and placed in ice cold HBSS with 0.002% deoxyribonuclease and chopped using 2 blades. The tissues were added to 20 ml HBSS with trypsin (final concentration 0.01%) in a 37°C water bath for 15 minutes and shaken. This was followed by the addition10% FBS in NB media (1:1 vol / vol) and pipetting 10 times with a Pasteur pipette. Tissue was then centrifuged at 1000 rpm for 5 minutes and the cells were resuspended in neurobasal media containing B27, penicillin / streptomycin, and 2mM Glutamine. Cells were plated in a 6 well plate pre-coated with poly-D-lysine, at a density of 1×10^6 cells / well. The stabilized tissue was treated with neurobasal medium only and 25 μM of serotonin in neurobasal medium for 2 hours at 37°C with 5% CO_2. The hydrophobic impurities were removed using an Oasis HLB cartridge and washing the tissue with 0.5% acetonitrile and 0.1% tetrafluoroacetic acid. The stabilized tissues were finally treated with 25 μM of the selective serotonin agonists, HT1, HT2 and HT4 respectively for 2 hours at 37°C with 5% CO_2.

LC-MS/MS and Database analysis

The purified sample was dried in a speedvac for 2 hours and eluted in distilled water to 100 μl of total volume with a concentration of about 100 μg/ml of protein. The sample was stored at -200°C overnight and 90 μl of sample was analysed using chromatographic analysis. The chromatographic analysis was performed on an AKTA FPLC system (Amersham Bioscience, UK) consisting of an isocratic pump, an auto-sampler with injector, columns, tubing and an ultraviolet detector controlled by Unicorn systems. The prepared 90 μl samples were injected onto a C-4 narrowanalytical column and eluted with 0.1% acetonitrile and tetrafluoroacetic acid or distilled water at a flow rate of 1 mL/min. The absorbance of the eluant at 215 nm was monitored. Finally, the column was washed out with 20% ethanol for 1 hour to remove the remnants.

Ten micrograms of the secretome were taken and denaturated at 900°C for 20 minutes.addition, the samples were reduced by adding 10 mM of DTT, and incubated at 560°C for 20minutes. The samples underwent alkylation for another 20 minutes in a dark room with the addition of 100 mM of iodoacetamide. Finally, the samples were digested with 0.1 μg of trypsin overnight.

For these experiments, the mass spectrometer was connected on-line with an AKTA Basic liquid chromatograph (GE Healthcare, NJ) by using an RP-C18, 300 m ID 5 cm column (Dionex, CA). Peptides were eluted from the column using a linear gradient of acetonitrile with 0.2% formic acid from 5 to 60% for 50 min, at a flow rate of 3 L/min. To acquire the LC-MS/MS spectra the first quadrupole was used to select the precursor ion within a window of 4 Th. A pressure of $\sim3\times10^{-2}$Pa of collision gas (argon) was used in the hexapole collision cell to yield the fragment ions. The doubly- and triply- charged precursor ions to be fragmented were selected automatically once their intensity rose above a defined threshold (8 conts s^{-1}). The instrument reverted to MS mode once the total ion count decreased below 2 count sec^{-1} or when the MS/MS mode had been maintained for 4 sec. Data acquisition and processing were performed using a MassLynx system (version 3.5) from Waters (MA). Protein identifications were based on manual interpretation of the MS/MS spectra[4]). The program Selestact (CIGB, Havana, Cuba) coded in C for the console use was developed to scan the Swiss-Prot sequence database, and calculate the number of proteins present.

RT-PCR analysis of 5-HT receptor subtype

The total RNA was extracted from the selective serotonin agonist treated cells using RNA RT-PCR Kits (Takara, Japan) The RNA was digested with DNAse in a low-salt buffer to remove any remaining DNA. Reverse transcription was performed using AVM reverse transcriptase XL (Takara, Japan). Briefly, 5 μg of total RNA was used as a template; the first strand of cDNA was made using 1 μl of dNTP mixture in a 10 μl reaction volume with 0.25 μl of RNAse inhibitor. Heat denaturation of reverse transcriptase was performed at 99°C for 5 minutes. Reverse transcription was performed at 420°C for 30 minutes. A 0.5 μl aliquot of cDNA was used as a template. The primers used were as follows:

5-HT1A receptor forward: CATTTCTTTTTCCCTCCCTTCC
reverse: CTCCCTTCCTTCCGTATTCCC
5-HT2C receptor forward: GGCATCCTTCCACTTCTGTAGTC
reverse: GGCATCCTTCCACTTCTGTAGTC
5-HT4 receptor forward: GAGTTCCAACGAGGGTTTCAG
reverse: AATGCGATGCGTAGAGGGG

The expected sizes of 5-HT1, 5-HT2C, 5-HT4 were 321, 347 and 315 nucleotides, respectively. The PCR conditions were: denaturing at 94°C for 2 minutes, followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. The PCR products were analyzed by electrophoresis using 2% agarose gels.

Western Blot

The secretomes containing an equal amount of protein were separated by SDS-PAGE and blotted onto nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Buckinghamshire, England). The membrane was incubated with an antibody as specified, followed by a secondary antibody conjugated with horseradish peroxidase. Specific antigen- antibody complexes were detected by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL). GAP-43 (anti- neuromodulin) antibodies (Santa Cruz, USA) were used in this study.

Determination of cAMP Level

cAMP was assayed using the cAMP EIA system (Amersham) according to the method specified by the manufacturer. To determine the total cAMP level, selective serotonin agonist treated samples were heated for 5 minutes followed by centrifugation at 12000 g for 2 minutes at 4°C. The cAMP levels of supernatants were determined as the total cAMP levels which were expressed in fmol / OD unit (at 600 nm) of the sample. The pellets were suspended in 20 μl of H_2O, and heated for 5 minutes at 100°C. Samples were centrifuged at 12000 g for 2 minutes at 4°C, and supernatants were removed. The reaction was terminated using 60 μl of 80% ice cold ethanol and then centrifuged 12000 g for 10 minutes at 4°C. Thesupernatants were dried, and the pellets were suspended in 10 μl of H_2O and used for the determination of intracellular cAMP levels. The intracellular concentration of cAMP was calculated on the assumption that an OD600 of 1.4 corresponds to 10^9 cells / ml (Miller, 1972) and the volume of each cell is 2×10^{-12}ml (Joseph *et al*, 1982).

Ca^{2+} Assays

Receptor signaling activity was measured in intact cells using a fluorometric plate-based assay for detecting changes in intracellular calcium. Cells were cultured in a HEPES-buffered neurobasal medium containing B27, penicillin / streptomycin and 2mM Glutamine in 100 mm dishes (1.5-2.5×10^6 cells / dish); they were treated with selective serotonin agonists for 2 hours in black-walled, clear-base 96-well plates before the calcium assay. The assay was performed with the use of a calcium assay kit, according to the recommendations of the manufacturer (Shimadzu, Japan). Briefly, cells were washed once in Hanks balanced saline supplemented with 2.5mM probenecid (pH7.4) and incubated with 5 μM fura 2-AM at 37°C for 60 minutes. Fura 2-AM is light-sensitive, so the tube containing the cells was covered with aluminum foil during loading. After incubation, cells were immediately placed in a RF-5301 PC fluorometer equipped with a multichannel injector (Shimadzu, Japan) and set at λexnm, λem. Fluorescence measurements were taken at 0.02intervals before and after agonist addition for a total of 60per well. The raw data were analysed using the Softmax Pro V software package (Shimadzu, Japan). The [Ca^{2+}] values were calculated using Schaeffer's method (Schaeffer and Blaustein, 1989). Functional responses were measured as peak fluorescence levels after subtraction of the baseline.

Results

LC-MS analysis

From MS-MS analysis, there was no identified protein in the control (treated with neurobasal medium only). Despite this, we identifed 6 membrane proteins in the serotonin treated secretome of the rat hypothalamic neurons (Table 1). tneurons. We concluded that neuromodulin is secreted due to serotonin signaling in rat hypothalamic cells.

Table 1. 6 proteins including neuromodulin were identified from this study known as in cellular membranes.

IPI Reference	Protein	Peptide	Function
IPI 00213220.1	Neuromodulin	2	neuronal plasticity
IPI 00231651.6	Brain acid soluble protein	3	axon elongation
IPI 00476991.1	Neural cell adhesion molecule 1	1	fasciculation
IPI 00230937.4	Phosphatidyletanolamine binding protein	1	cholinergic
IPI 00231801.3	Acyl coA binding protein	2	GABAergic
IPI 00193173.4	Serum albumin precursor	1	osmoregulation

Verification of the expression of selective serotonin receptor subtypes by RT-PCR

To verify the results obtained from western blotting of neuromodulin, the receptor expression in the hypothalamic neuronal cells was subjected to RT-PCR analysis. Using gene specified primers and the total RNA extracted from the treated cells, we were able to obtain the purified bands at the expected sizes.

Inhibitory effect of the Gi-coupled 5-HT1A receptor on cAMP production and neuromodulin release

5-HT1A receptor expression in rat hypothalamic neurons is known to be lowered by the production of cAMP. We further investigated whether 5-HT1A receptor expression down-regulated neuromodulin secretion. As shown in Fig. 1, neuromodulin release was lower than observed after 5-HT4 receptor expression. There was a decrease in intracellular cAMP level following 5-HT1A receptor

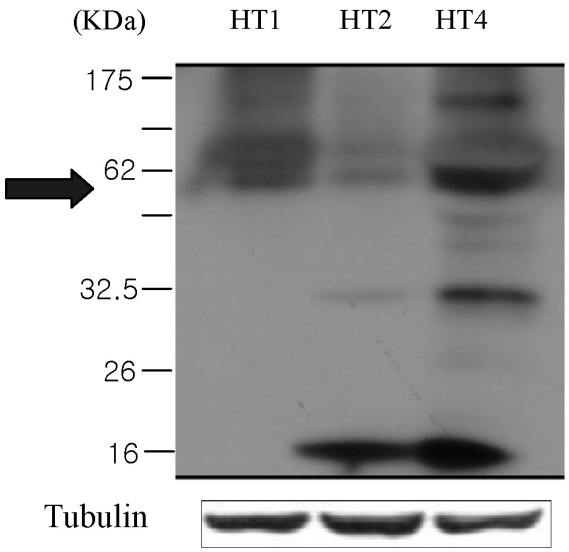

Fig. 1. Selective activation of the Gs-coupled 5-HT4 receptor caused a robust increase in neuromodulin release. An arrow Indicates neuromodulin bands. Tubulin was used as a loading control.

Table 2. Assay measuring cAMP levels in rat hypothalamic cells after selective serotonin agonist treatment. The experiments have been performed in triplicate.

Sample	Individual Value			AVE	OD-NSB	%B/B0	cAMP
Control	0.8	0.9	0.9	0.9	0.813	122.81	3.75
HT1	0.9	0.8	0.9	0.9	0.806	120.01	4.02
HT2	0.405	0.411	0.408	0.408	0.321	35.35	389.01
HT4	0.381	0.384	0.387	0.384	0.297	31.72	471.57

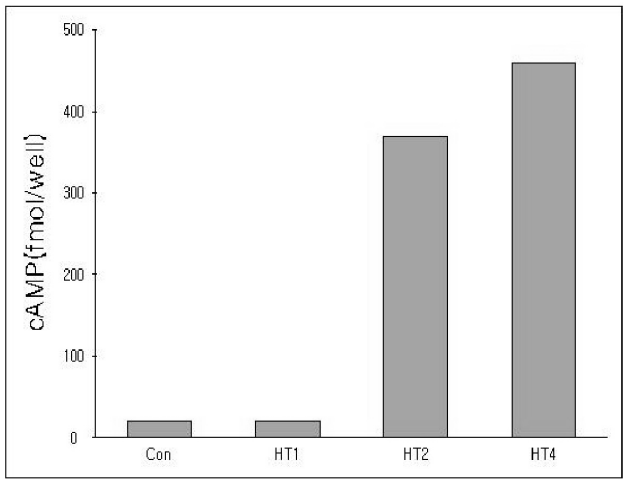

Fig. 2. The rate of cAMP production in rat hypothalamic cells was inhibited during activation of the Gi-coupled 5-HT1A receptor, but increased in Gq and Gs-coupled 5-HT2C and 4 receptor activation.

expression compared to 5HT-2 or 5-HT-4 expression (Fig. 2). It is interesting to note that selective serotonin agonists modulated the production of cAMP by GPCR receptor regulation of neuromodulin secretion. As shown in Table 2, it can be stated that selective 5-HT1A agonist decreased the intracellular cAMP level. These results indicate that the 5-HT1A agonist down-regulates the production of cAMP negatively, affecting relative neuromodulin secretion.

Effect of the Gs-coupled 5-HT4 receptor on rat hypothalamic neuronal Ca^{2+} and neuromodulin release

As shown in Fig. 1 and Fig. 3, when the rat hypothalamus was stimulated by the 5-HT4 agonist, the level of [Ca^{2+}] increased to 448 nM which provoked PKC signaling and the release of neuromodulin. This secreted neuromodulin can bind calmodulin and form Ca^{2+} binding structures in the neuronal cells. Table 3 shows that the intracellular Ca^{2+} was increased most by the selective HT2C agonist and next by the selective HT4 agonist to regulate PKC signaling and neuromodulin secretion.

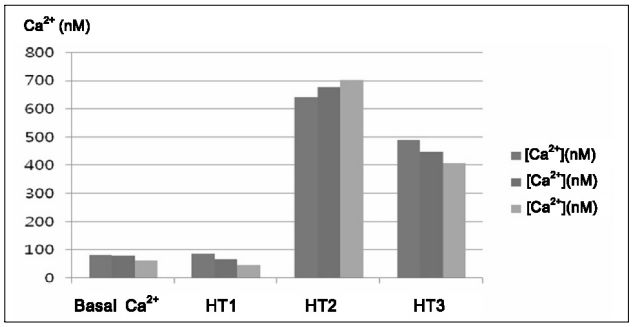

Fig. 3. The 5-HT4 receptor subtype expressed in rat hypothalamic neurons was able to activate the Ca^{2+} signaling pathway differentially from the 5-HT1A receptor.

Table 3. Intracellular Ca^{2+}measurements in rat hypothalamic cells after selective serotonin agonist treatement. Data are expressed as mean and S.D. (N=3). $p<0.05$

	[Ca^{2+}](nM)			Mean	SD
Basal Ca^{2+}	83	79	63	75	10
HT1	87	68	46	67	14
HT2	640	675	701	672	30
HT4	489	447	408	448	38

Disscussion

In this study, we used selective serotonin agonists to investigate neuromodulin signaling in the rat hypothalamus (Bonnin *et al*, 2006, Wada *et al*, 2006). 5-HT1A receptor

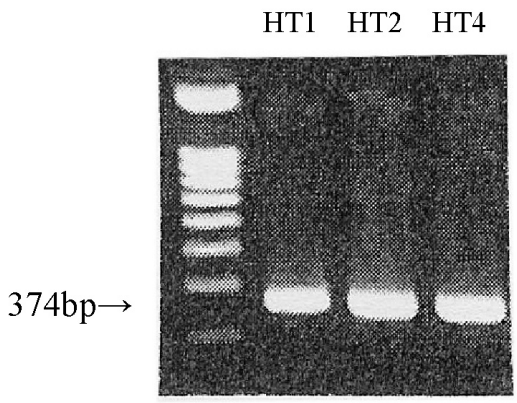

Fig. 4. Expression of selective serotonin receptors in rat hypothalamic cells were demonstrated using gene specified primers and were confirmed by the purified bands.

expression significantly inhibited cAMP production and neuromodulin release (Fig. 2) (Liu *et al*, 2007, O'conner *et al*, 2007). On the contrary, 5-HT4 receptor expression activated PKC signaling and increased the intracellular Ca^{2+}level as well as neuromodulin secretion. Consequently, we focused on the effect of the 5-HT4 receptor agonist on neuromodulin secretion in rat hypothalamic neuronal cells. Previous studies suggested that the regulation of intracellular Ca^{2+} is important for maintaining neuronal plasticity. Calmodulin is involved in maintaining the Ca^{2+} buffering capacity in neuronal cells by establishing the Ca^{2+}binding structure. Earlier studies indicated that neuromodulin has a binding site for calmodulin and forms the Ca^{2+}binding structure. As shown in Fig. 1, the incubation of rat hypothalamic cells with the 5-HT4 agonist for 2 hours activated neuromodulin secretion. These results indicate that the selective 5-HT4 receptor affects neuromodulin secretion, initiating the signaling of neuronal

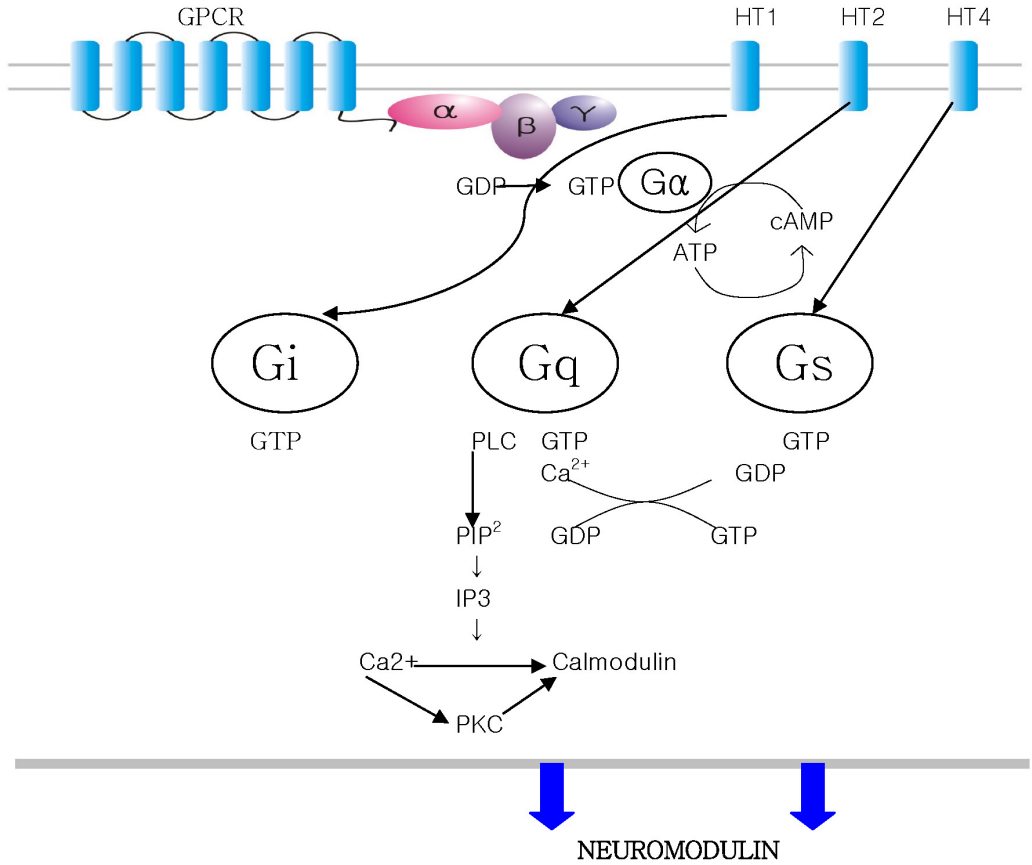

Fig. 5. A proposed model: 5-HT1A receptor expression significantly inhibited cAMP production and neuromodulin release. Conversely, 5-HT4 receptor expression activated PKC signaling and increased the intracellular Ca2+level and neuromodulin secretion. The selective 5-HT2C receptor may affect cAMP regulation: this is in accord with the concept that intracellular cAMP levels are responsible for neuromodulin releasing.

cells with PKC and Ca^{2+}. This also indicates that the activated selective 5-HT2C receptor may affect cAMP regulation, in accord with the concept that intracellular cAMP levels are responsible for neuromodulin release (Xie *et al*, 2005). Thus, we measured cAMP production using the selective 5-HT2C receptor in the treated cells. The results showed that, in the presence of GPCR receptor activation, the 5-HT2C receptor acts as a strong intracellular inducer of cAMP which is a positive regulator of endogenous neuromodulin secretion (Fig. 2 and 5) (Cai *et al*, 2007, Simler *et al*, 2007). On the other hand, 5-HT1A receptor expression alone decreased cAMP production in these cells. These results suggest that the 5-HT2C receptor might directly affect the activity of adenylate cyclase and ATP. The increased levels participate in activating Ca^{2+} signaling and consequently these ions bind to calmodulin and neuromodulin. This results in the regulation of Ca^{2+} buffering in neuronal cells.

In conclusion, the most important result of this study was that selective 5-HT4 receptor expression significantly activated neuromodulin secretion in rat hypothalamic cells (Papageorgiou *et al*, 2007). This excitatory effect may be due to the PKC and Ca^{2+} signaling in neuronal cells. Selective 5-HT2C receptor activation increased the intracellular cAMP levels, thereby linking to the intracellular Ca^{2+} mobilization to calmodulin. On the other hand, 5-HT1A receptor activation resulted in diminished cAMP production, causing decreased neuromodulin secretion in hypothalamic neuronal cells. Therefore, it is possible that selective serotonin receptor subtypes are involved in the regulation of neuromodulin secretion mediated by signaling pathways which utilize Ca^{2+} mediated binding to calmodulin in hypothalamic neuron cells.

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