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Heterologous Expression of the Serotonin 5-HT_{1A} Receptor in Neural and Non-neural Cell Lines

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Stable expression of neuronal receptors in cell lines of neural origin is important for studies of neurotransmitter mediated signal transduction. We have achieved this for the first time in three cell lines which are derived from various tissues of neural origin (hippocampus, HN2; chinese hamster brain explant, NCB-20; rat dorsal root ganglion, F-11). Following electroporation assisted transfer of a construct containing the hippocampal serotonin 5-HT_{1A} receptor (5-HT_{1A}R) DNA, one neural cell line, NG-108-15 (murine neuroblastoma x C6 glioma), failed to express the transfected activity, while three others as well as the non-neural CHO (chinese hamster ovary) cells expressed high levels of the receptor. Upon normalization to coexpressed human ß-hexosaminidase B activity, it was found that the human 5-HT_{1A}R, which is normally concentrated in the hippocampus and at a lesser density in the brain, was expressed at the highest level (15.7 x 10^4 receptors/cell) in the HN2 followed by the NCB-20 (8.3 x 10^4 receptors/cell), F-11 (4.4 x 10^4 receptors/cell) and lastly the non-neuronal CHO (4.2 x 10^4 receptors/cell) cells. Ten-twelve days after passage, a striking increase in expression of the receptor was observed only in the cell lines of neural origin. By contrast, there was no appreciable increase in expression of the transfected 5-HT_{1A}R in the non-neural CHO cells over time. This late increase in expression was eliminated in cells which had been maintained in low glucose (1 g/L) for the first two days after passage, thus establishing a vital role of glucose in expression of the transfected 5-HT_{1A}R in cell lines of neural origin. In all cases the 5-HT_{1A}R was negatively coupled to adenylate cyclase, as evidenced by an agonist mediated decrease in prostaglandin E_1 stimulated cyclic AMP levels.

Early research on receptor proteins was based on the dogma that each receptor is faithfully coupled to one class of G-proteins (G_s, G_i or G_o) which in turn led to signal transduction via one type of effector molecule (adenylate cyclase, phospholipase C or G-protein coupled ion channels). Transfection of the dopamine, ß-adrenergic or serotonin receptor cDNAs in COS-7, HeLa, pituitary GH_4C_1 and mouse L cells has resulted in cell type dependent differential signal transduction by the same receptor. Thus, the serotonin 5-HT_{1A} receptor stimulated the phospholipase C-inositol tris-phosphate cascade in mouse L cells (1) but inhibited Ca^{2+} release in pituitary GH_4 cells. In another study it was shown in membranes obtained from CHO cells transfected with the α_2-adrenergic receptor (α_2AR) cDNA that low concentrations of an agonist promoted α_2AR-G_i coupling whereas high concentrations of the agonist mediated α_2AR-G_s

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coupling (2). Various receptors are asymmetrically distributed in a mammalian body, and one way of understanding the mechanism of signal transduction as mediated by a receptor is by transfection of a DNA construct encoding the receptor in a cell line which is derived from the tissue of natural occurrence of the same receptor. Thus a neural receptor (e.g. the 5-HT_{1A}R or the opoid receptors) can be best studied by transfection of the corresponding cDNA in a cell line of neural origin (e.g. the NG 108-15). While transfection of various receptors in CHO, NIH 3T3, COS-7, HeLa, and pituitary GH_4 cells have been achieved (3-6), transfection of cell lines of neural origin has so far remained an invincible challenge. Ann and coworkers showed that transfection of NG108-15 neuroblastoma x glioma cells with antisense sequences of a portion of the rat cDNA for the cell adhesion molecule, OBCAM, which bears homologies to an opioid binding protein, greatly reduced diprenorphine binding. However such transfections with the sense DNA failed to produce increased diprenorphine binding over untransfected NG108-15 cells (7).

For the first time, we have achieved efficient transfection of neuroblastoma cell lines, such as NCB-20 (mouse neuroblastoma x chinese hamster brain explant), F-11 (mouse neuroblastoma x rat dorsal root ganglion) and HN2 (mouse neuroblastoma x hippocampal cells) by using a DNA construct encoding the hippocampal, serotonin 5-HT_{1A} receptor. Even though both HN2 (hippocampus) and NCB-20 (brain) cells are derived from 5-HT_{1A} receptor enriched tissues, these receptors were completely lost during the process of creation of these cell lines and therefore, the background level of endogeneous 5-HT_{1A} receptors was negligible in the transfected cells. In order to compare with the expression and function of this receptor in non-neural cells, we also transfected the CHO cells with the same cDNA construct.

MATERIAL AND METHODS

The G-21 clone in pBCI vector was a generous gift from Drs. John Raymond and Robert Lefkowitz (Duke University Medical Center, Durham, NC). ß-hexosaminidase ß-chain cDNA (ßpCD) was a gift from Dr. Don Mahuran (Hospital for Sick Children, University of Toronto, Toronto, Canada).

The G-21pBCI construct which is under the control of the RSV long terminal repeat was cotransfected with a ß-hexosaminidase ß-chain cDNA construct, ßpCMV6b, and a gene construct, pSVneo, which confers neomycin resistance. The ß-hexosaminidase ß-chain cDNA construct, ßpCMV6b, was prepared by digestion of ßpCD with Xho 1 followed by ligation of the 2 kb fragment generated into a Sal 1 site in the multiple cloning region of a vector pCMV6b which is driven by the cytomegalovirus promoter. G-21pBCI (10 µg) was linearized by digestion with Pvu I, purified by phenol extraction and ethanol precipitation and then mixed with 5 µg of ßpCMV6b, 3 µg of pSVneo and 500 µg of salmon sperm DNA (all sterilized by ethanol precipitation) and the total volume was adjusted to 200 µl with 100 mM phosphate buffered saline (PBS). The DNA solution was transferred to a Bio-Rad electroporation cuvette (0.8 ml with 0.4 cm path length), overlayered with a suspension of 5-10 x 10^6 cells in 0.6 ml PBS and then subjected to an electrical pulse using a Bio-Rad Cell Porator. Electroporated cells were transferred to the respective growth media within ten minutes and maintained for three days without Geneticin, following which, the cells were passaged to five-fold dilution and maintained in media containing 400 µg/ml Geneticin. After 2-4 weeks, individual colonies were harvested and grown in progressively larger plates starting from 24-well plates in order to finally obtain clones which were maintained in 10-cm plates.

Plates of cells which were initially seeded at 5 x 10^5 per 10-cm plate were harvested and washed once with a cold (4 °C) swelling buffer (10 mM Tris-HCl, pH 7.4, 5 mM EGTA) and then homogenized in the same buffer using a Potter-Elvehjem homogenizer in ice. The lysate thus obtained was centrifuged at 300, 000 x g for 5 min, the supernatant discarded and the pellet washed once with the same buffer and then resuspended in buffer A_1 (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) by Potter-Elvehjem homogenization and stored at -80 °C until assay. An aliquot

(~1/10 th of the total number of cells) of each cell pellet was transferred to a microfuge tube pelleted at setting "7" at 4 °C, washed once with PBS and then stored at -80 °C for later analysis of RNA and ß-hexosaminidase activity.

Binding assays were carried out in duplicate using 100-200 µg of membrane protein in a total volume of 1 ml in the presence of 1.2-5 nM [^{3}H] 8-OH-DPAT according to our earlier report (8). Number of receptors per cell was calculated as follows:

Number of cells per dish	= x
Total membrane protein obtained from one dish	= y mg
Therefore, number of cells per mg membrane protein	= x/y
Specific [^{3}H] 8-OH-DPAT binding activity	= z moles/mg protein
Assuming one [^{3}H] 8-OH-DPAT molecule binds to one receptor molecule,	
number of receptors per cell	= (z x Avogadro no.) / (x/y)

mRNA (poly A$^+$) was isolated from both fibroblasts and lymphoblastoid cells (typically 1-5 x 10^6 cells) with the help of the FASTTRACK kit (Invitrogen, San Diego, CA). Generally 5 µg or more mRNA (poly A$^+$) was thus prepared and stored under ethanol at -80 °C.

Northern blots were obtained by using 0.5 µg of mRNA (poly A$^+$) (9). Serotonin 5-HT_{1A}R cDNA probe was released from G-21pBCI by digestion with Hind III and Bam HI, resolved by agarose gel electrophoresis, purified using Geneclean and labeled with α[^{32}P] dCTP by random priming in the presence of the Klenow fragment (using the Oligonucleotide Labeling Kit, Pharmacia, Alameda, CA). Prehybridized blots were hybridized overnight at 42 °C with radiolabeled cDNA. Following this, blots were washed twice with 2X SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at 42 °C and then twice with 0.1X SSC plus 0.1% SDS at 65 °C. Autoradiography was carried out at -80 °C with enhancing screens.

Assays for cAMP were conducted using cells plated in six-well costar plates at 70-80% confluence in serum-free medium which was supplemented with various drugs plus 50 µM IBMX for 30 min. Following this, the medium was aspirated, 500 µl of cold (4 °C) 5% trichloroacetic acid (TCA) added to each plate and the cells harvested on ice. Each cell extract was sonicated and centrifuged at 10, 000 x g, the supernatant obtained freed from TCA by ether extractions, and the pellet saved for protein assay. The supernatant thus obtained was blown dry, the residue reconstituted in 100 µl of water and assayed for cAMP as reported earlier (10). The initial protein pellet from TCA-treatment was dissolved in 1 N NaOH (100 µl) and an aliquot of the solution assayed for protein (11).

Total ß-Hex activity was estimated by incubation at 37 °C for 15 min in the presence of 0.4 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside (4-MU-GlcNAc), 0.1 M citrate-phosphate buffer (pH 4.4), 0.5% bovine serum albumin in a total volume of 200 µl. Heat inactivation of ß-Hex A was carried out by preincubation of tubes for 90 min at 50 °C before addition of the substrate (4-MU-GlcNAc) (the tubes are placed on ice for 5 min before substrate addition) and incubation at 37 °C for 15 min. Following 37-°C incubation, 2 ml of 0.17 M glycine-carbonate buffer (pH 10.0) was added and fluorescence emission measured at 460 nm (with excitation wave length set at 360 nm).

About 2-3 µg of each double stranded circular plasmid sample was sequenced using [^{35}S] dATP and the SEQUENASE kit (Perkin Elmer, Norwalk, CT) (12).

RESULTS

Highest levels of expression of the serotonin 5-HT_{1A} receptor in cell lines derived from hippocampus and brain : All the neurotumor cell lines tested here originated from murine neuroblastoma which had been hybridized to various cell types as indicated in the text. Efficiency of transfection of the pBCI-5-HT_{1A} construct seemed to have no correlation with the levels of stable expression of the 5-HT_{1A} receptor on each cell. Thus, even though several Geneticin resistant, ß-Hex B expressing clones were obtained from NG-108-15 (C6 glioma x murine neuroblastoma), F-11 (rat dorsal root ganglion) and CHO (chinese hamster ovary) cells,

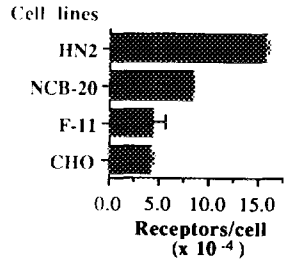

Fig. 1. Maximal expression of human serotonin 5-HT_{1A} receptor following stable transfection. While expression of the receptors was relatively constant over passage in the non-neural CHO cells, maximal expression of the receptors was observed in the neurotumor cell lines (F-11, NCB-20 and HN2) during passage 4, 5 and 6.

the NG-108-15 cells did not express the receptor, whereas the highest levels of 5-HT_{1A}R expression as observed after normalization to overexpressed ß-Hex B activity was only 4.4 x 10^4 and 4.23 x 10^4 receptors/cell, respectively, for the F-11 and CHO cells. This was in sharp contrast to the HN2 and NCB-20 cells which, though transfected at a much lower efficiency, yielded highly active clones expressing 15.7 x 10^4 (HN2, hippocampus) and 8.3 x 10^4 (NCB-20, brain explant) receptors per cell (Fig. 1). This closely mimicked the natural expression of the serotonin 5-HT_{1A} receptor in the mammalian system where the receptor is concentrated mainly in the hippocampus and the brain.

Message level in the transfected cells : While no cell line known so far expresses the serotonin 5-HT_{1A} receptor, following transfection of the G-21pBCI construct, detectable message levels were obtained after 2-3 passages (Fig. 2a). None of the untransfected cells hybridized to the G-21 probe thus confirming the absence of the receptor in the untransfected cells.

Late increase in [^{3}H] 8-OH-DPAT binding activity in cell lines of neural origin :

[^{3}H] 8-OH-DPAT binding assays on membrane preparations revealed that the expression of the serotonin 5-HT_{1A} receptor in the CHO cells remained fairly constant after confluence, i.e between six and twelve days. In sharp contrast, in the cell lines of neural origin, a dramatic increase (3-5 fold) in [^{3}H] 8-OH-DPAT binding activity was observed 10 days after passage (Fig. 2b). As reported earlier for 3T3 cells transfected with the G-21 clone (5), a passage dependence of expression of the serotonin 5-HT_{1A} receptors was also observed in this study. Thus in the experiment shown in Fig. 2b, the NCB-20T8 cells were at the peak of expression of the serotonin 5-HT_{1A} receptor but the other transfectants were not, and the [^{3}H] 8-OH-DPAT binding activity per mg membrane protein of the NCB-20T8 (at passage 5) was higher than that of the HN2-5 clone (at passage 2). However, the HN2-5 clone showed two to three fold higher expression of this activity at passage 4 and 5 as shown in Fig. 1. Also, the number of cells per mg membrane protein was widely different for the different cell lines (e.g. 10^7 NCB-20 cells produces 1 mg membrane protein while only 6 x 10^6 HN2 cells produces 1 mg of membrane protein). Therefore, the [^{3}H] 8-OH-DPAT binding activity shown in Fig. 2b is not at a passage when the highest number of receptors are expressed by the cells. Nevertheless, this late increase in [^{3}H] 8-OH-DPAT binding activity was observed in the transfected neurotumor cell lines during all passages.

Elimination of the late induction of 5-HT_{1A}R expression in neural cells by glucose deprivation : Replenishment of medium on the fifth day after passage caused a

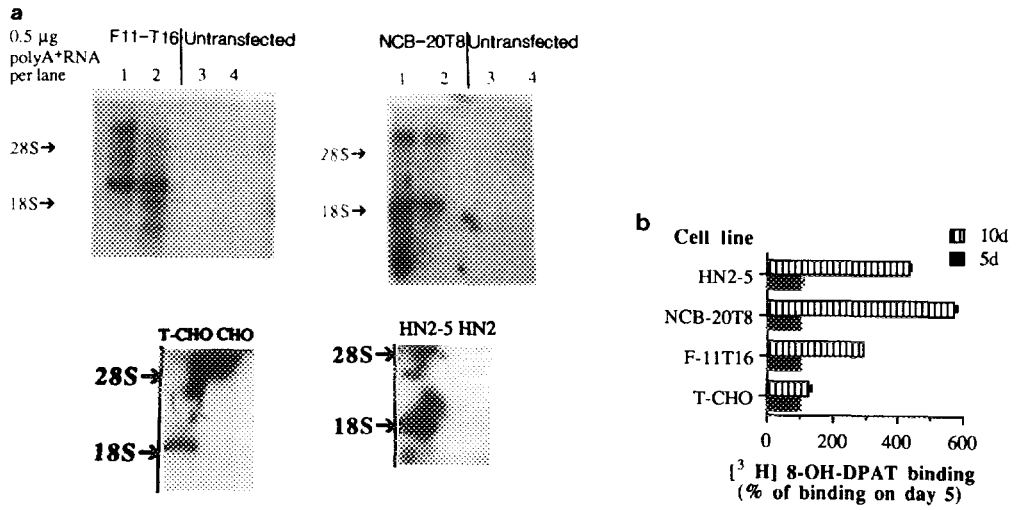

Fig. 2. **(a) Message levels observed following Northern blot analysis using the G-21 coding sequence as a probe.** Two samples of RNA per cell line (transfected, in lanes 1 and 2, and untransfected, in lanes 3 and 4) for F-11 (upper left), NCB-20 (upper right) and one sample per cell line from CHO (lower left) as well as HN2 (lower right) cells before (right lane) and after transfection (left lane) were resolved by agarose gel electrophoresis and probed using the Hind III, BamHI fragment of G-21pBCI. G-21 message was observed only in the RNA samples from transfected cells. The upper band is due to multimeric RNA molecules synthesized under the influence of the RSVLTR.

(b) Striking increase in [^{3}H] 8-OH-DPAT binding activity following confluence only in the transfected neurotumor cells : While no appreciable change in [^{3}H] 8-OH-DPAT binding activity over time was observed in the transfected CHO cells, [^{3}H] 8-OH-DPAT binding activity in the transfected neurotumor cell lines F-11, NCB-20, and HN2 increased several fold following confluence in the same medium that the cells were plated in.

decrease in 5-HT$_{1A}$R expression in NCB-20 and F-11 without any effect on the transfected CHO cells (Fig. 3a). Since this suggests that the increase in 5-HT$_{1A}$R expression is related to a stress caused by depletion of of one or more constituents of the cell medium, effect of partial glucose

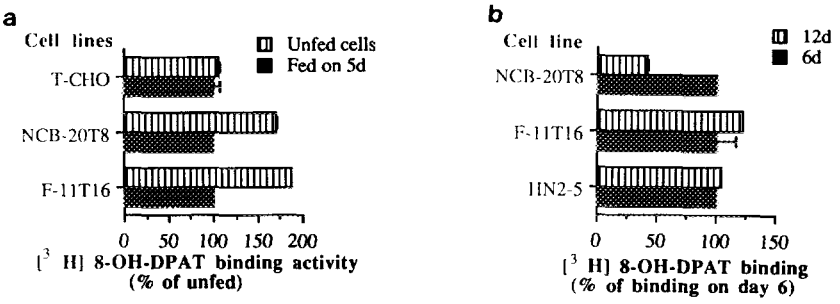

Fig. 3. ***(a) Effect of replenishment of growth medium on the expression of* [^{3}H] 8-OH-DPAT binding activity.** The cells were fed (old medium replaced with fresh) on day 5 after passage and then harvested on day 7. The time dependent increase in [^{3}H] 8-OH-DPAT binding activity in the transfected F-11 (F-11T16) and NCB-20 (NCB-20T8) clones was inhibited upon feeding while the transfected CHO (T-CHO) cells remained unaffected.

(b) *Effect of partial glucose deprivation on the expression of* [^{3}H] 8-OH-DPAT binding activity. The cells were passaged in low glucose (1 g/l)containing media and after two days, a small aliquot (less than 1 ml) of sterile glucose solution in growth medium was added to the culture in order to adjust the glucose concentration to 4.5 g/l.

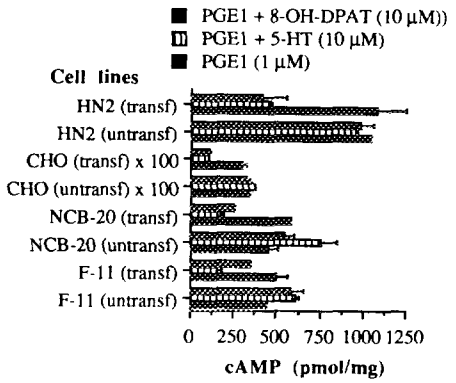

Fig. 4. **Serotonin 5-HT_{1A} receptor mediated inhibition of intracellular cAMP following transfection.** Prostaglandin E_1 stimulated adenylate cyclase activity in both transfected (transf) and untransfected (untransf) cells was further modulated in the presence of either the 5-HT_{1A} specific ligand 8-OH-DPAT or serotonin (10 μM of each).

depletion was tested on the transfected cells. When all the transfected cells were passaged in low glucose (1 g/l as opposed to 4.5 g/l in normal DMEM) and maintained in the same medium for two days before addition of 3.5 g/l of glucose to the medium, it was observed that the increase in [^{3}H] 8-OH-DPAT binding activity at 10-12 days was completely eliminated (Fig.3b). This indicates that glucose plays a vital role in the late induction of serotonin 5-HT_{1A}R expression in the neurotumor cells.

The expressed 5-HT_{1A}R was negatively coupled to adenylate cyclase in all the cell lines : While both serotonin (5-HT) and 8-OH-DPAT caused an increase in prostaglandin E_1 stimulated cAMP levels in the untransfected NCB-20 and F-11 cells, both these 5-HT_{1A} agonists were found to cause inhibition of cAMP in all the transfected cells (Fig. 4). This was in agreement to the earlier observation that forskolin-stimulated cAMP levels in pBCI-5-HT_{1A} transfected HeLa cells are inhibited in the presence of 8-OH-DPAT (3).

Cell lines of neural origin such as NCB-20, F-11, NG 108-15, PC-12, HN2 and various others (e.g. the pituitary GH_4 cells) have been considered as models for neuronal cells in studies to understand signal transduction in mammalian systems. It has been shown that both NCB-20 and F-11 cells contain bradykinin receptors which are positively coupled to the phospholipase C-inositol phosphate system (13, 14). Presence of a serotonin 5-HT_1 like receptor and prostaglandin E_1 receptor which are positively coupled to adenylate cyclase and also an opiate receptor have been demonstrated in the NCB-20 cells (15-17). Surprisingly, there was yet no report describing transfection mediated expression of any receptor or biologically active molecule in any of these cell lines. Therefore, we achieved this task for the first time before commencing our study aimed at understanding regulation of expression of a neuron-specific receptor and its signal transduction properties in a hybrid, neurotumor cell line which originally did not express the receptor.

The low levels of [^{3}H] 8-OH-DPAT activity at the initial stages following passage of the transfected neurotumor cells is an important observation without which successful expression of the G-21 clone in neural hybrid cell lines could never be demonstrated. Also, the striking increase in the number of expressed receptors at cessation of cell proliferation is the second novel

observation which strongly indicates that the brain specific 5-HT_{1A} receptor is regulated in a unique way in cell lines of neural origin. Cell-cell contact regulated cessation of cell proliferation and initiation of synaptogenesis in neural cells have been reported earlier (18). Thus the late increase in expression of the serotonin 5-HT_{1A} receptors in cell lines of neural origin seems to correlate with the process of synaptogenesis.

In summary, we have achieved stable expression of the hippocampus and brain specific, human serotonin 5-HT_{1A} receptor in cell lines of neural origin and also demonstrated that the highest expression of this transfected receptor occurs in hippocampus and brain derived neural cell lines. Our G-21pBCI transfection experiments show a striking increase in expression of the 5-HT_{1A} receptor at confluence in cell lines of neural origin but not in the CHO cells, and this increase in expression is eliminated upon partial glucose deprivation even at the early stages of cell proliferation.

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