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Research Articles

Ectopic Expression of the Serotonin 1c Receptor and the Triggering of Malignant Transformation

DAVID JULIUS, THOMAS J. LIVELLI, THOMAS M. JESSELL, RICHARD AXEL

Neurotransmitter receptors are usually restricted to neuronal cells, but the signaling pathways activated by these receptors are widely distributed in both neural and nonneural cells. The functional consequences of activating a brain-specific neurotransmitter receptor, the serotonin 5HTlc receptor, in the unnatural environment of a fibroblast were examined. Introduction of functional 5HTlc receptors into NIH 3T3 cells results, at high frequency, in the generation of transformed foci. Moreover, the generation and maintenance of transformed foci requires continued activation of the serotonin receptor. In addition, the injection of cells derived from transformed foci into nude mice results in the generation of tumors. The serotonin 5HTlc receptor therefore functions as a protooncogene when expressed in NIH 3T3 fibroblasts.

NEUROTRANSMITTERS MEDIATE RAPID INTERCELLULAR communication within the nervous system by interacting with cell surface receptors. These receptors often trigger second messenger signaling pathways that regulate the activity of ion channels. Although neurotransmitter receptors by definition have been restricted to the nervous system, their second messenger systems have been observed in both neural and nonneural cells. These observations raise the question as to the functional consequences of introducing a neurally restricted transmitter receptor into nonneural cells.

Serotonin is one example of a neurotransmitter that mediates diverse neural functions by binding to multiple receptor subtypes (*1*). Moreover, individual serotonin receptor subtypes couple to different intracellular signaling systems. The 5HTlc and 5HT2 receptors activate phospholipase C (*2*), whereas the 5HTla and 5HTlb receptors modulate adenylate cyclase activity (*3*). In neurons that express the 5HTlc and 5HT2 receptors, receptor activation by serotonin is likely to generate inositol polyphosphates that release intracellular Ca^{2+} (*2*, *4*). In other neurons that express the 5HTla receptor, changes in cyclic adenosine monophosphate levels or activation of G proteins appears to regulate K^+ channel function (*5*).

We have recently cloned and characterized the genes encoding the 5HTlc and 5HT2 receptors (*6*, *7*). These proteins are members of the family of G protein–coupled receptor molecules that traverse the membrane seven times. Introduction of the 5HTlc gene into

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fibroblasts results in the appearance of high-affinity serotonin binding sites on the cell surface (*6*). Moreover, agonist binding in fibroblasts triggers second messenger pathways that result in marked increases in intracellular Ca^{2+} (*4*, *6*).

These studies establish that fibroblasts express intracellular signaling pathways activated by the 5HTlc receptor. However, in fibroblasts, this second messenger pathway may be coupled to effector proteins that differ from those in neurons and which may mediate distinct cellular functions. We have therefore examined the functional consequences of activating the 5HTlc receptor in NIH 3T3 fibroblasts transfected with a functional cDNA encoding this neurotransmitter receptor. We demonstrate that NIH 3T3 cells that express high levels of the 5HTlc receptor form foci in cell culture. Moreover, the formation of foci is dependent on activation of the 5HTlc receptor by serotonin. In addition, the introduction of transformed foci into nude mice results in the rapid appearance of tumors. Thus, activation of the same receptor in fibroblasts and neurons elicits distinct phenotypes. In neurons the 5HTlc receptor may be involved in neurotransmission via the regulation of ion channel function, whereas in fibroblasts the same receptor alters the growth properties of cells and results in malignant transformation.

Expression of the 5HTlc receptor leads to focus formation. To ascertain whether activation of the 5HTlc receptor alters the growth properties of transfected fibroblasts, we first cloned the 5HTlc receptor cDNA into the expression vector, pMV7 (*8*). This vector contains the Moloney leukemia virus long terminal repeat (LTR), which directs transcription of the 5HTlc cDNA, as well as an independent expression cassette encoding neomycin phosphotransferase. This plasmid, pMV7-SRlc, was then introduced into NIH 3T3 fibroblasts. Cells were allowed to grow to confluence and foci were scored after 2 to 3 weeks (*9*). In parallel, the efficiency of stable transfection was determined by scoring the frequency of neomycin-resistant colonies (Table 1).

Transfection of NIH 3T3 cells with pMV7-SRlc results in focus formation at a frequency of 75 foci per microgram of plasmid DNA. Cells within foci do not exhibit gross alterations in their morphology and do not grow in soft agar (Fig. 1). The efficiency with which foci are generated is one-tenth that observed for the generation of neomycin-resistant colonies (Table 1). In separate experiments, we have established that the 5HTlc receptor is expressed in 30 percent of the Neo^r colonies. Thus, about one-third of the clones that express receptor are capable of generating foci.

Several observations indicate that the formation of foci results from the expression and activation of 5HTlc receptors on the surface of fibroblasts. First, the formation of foci is dependent on transfection with pMV7-SRlc. Transformed foci are never observed after transfection with the Neo^r expression vector alone, although both plasmids generate neomycin-resistant colonies with similar efficiency (Table 1).

Second, cells within transformed foci exhibited a high density of 5HTlc receptors, which activate second messenger systems within the fibroblast. Seven independent lines derived from transformed foci expressed between 20,000 and 100,000 binding sites for the serotonin ligand ^{125}I-labeled lysergic acid diethylamide (^{125}I-LSD) (*6*, *10*) (Table 2, also see below). In contrast, no specific binding was observed to untransformed NIH 3T3 cells. LSD binding was blocked (>90 percent) by the serotonin antagonist mesulergine ($10^{-6}M$). Thus, all foci analyzed exhibit 5HTlc receptors.

Serotonin also activates intracellular signaling pathways in cells derived from all transformed foci. In excitable cells, activation of the 5HTlc receptor results in the release of inositol polyphosphates and an elevation in cellular Ca^{2+} (*4*, *6*). Cells from individual foci were loaded with the Ca^{2+}-sensitive dye indo-1 and the Ca^{2+} change was measured by analysis in a fluorescence-activated cell sorter (*11*).

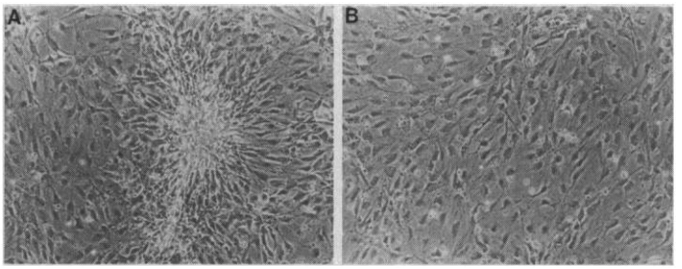

Fig. 1. NIH 3T3 cells expressing 5HT1c receptors exhibit a transformed phenotype in culture. NIH 3T3 cells transfected with the 5HT1c receptor expression plasmid pMV7-SR1c (**A**) or with the expression vector pMV7 alone (**B**) were maintained as a confluent culture for 2 to 3 weeks in medium containing 3 percent calf serum and 1 μM 5HT. A single focus is illustrated for NIH 3T3 cells transfected with pMV7-SR1c (A). Foci were not observed after transfection with the vector alone (B).

Table 1. NIH 3T3 fibroblasts were transfected with the Neo^r expression vector, pMV7, or with pMV7 containing a functional 5HTlc receptor cDNA (pMV7-SR1c). Portions of the cells, 48 hours after the addition of DNA, were placed into several petri dishes to determine the efficiency of transfection and focus formation. The efficiency of transfection was determined by scoring Neo^r colonies in medium containing 10 percent calf serum and G418 (0.5 mg/ml). The efficiency of focus formation in the presence or absence of the serotonin antagonist mesulergine (MS) (1 μM) was scored in medium containing 3 percent calf serum and 0.1 μM serotonin. The serotonin and mesulergine were replenished every 24 to 48 hours.

Experiment	DNA	Neo^r colonies (No.)	Foci (No.)	
			−MS	+MS
1	pMV7	600	0	0
	pMV7-SR1c	680	75	0
2	pMV7	835	0	0
	pMV7-SR1c	260	27	0

Indo-1 undergoes a characteristic and quantitative shift in its fluorescence emission spectrum as a function of Ca^{2+} concentration and serves as a measure of intracellular-free Ca^{2+} (*12*). When exposed to serotonin, these cells showed a marked increase in intracellular Ca^{2+}, whereas control untransfected NIH 3T3 cells did not respond to serotonin (Fig. 2).

Moreover, the appearance of transformed foci depends on activation of the 5HTlc receptor by serotonin. NIH 3T3 cells were transfected with the plasmid pMV7-SRlc, plated in the presence of the serotonin antagonist mesulergine, and scored for foci three weeks later. Mesulergine completely blocked the generation of foci by pMV7-SRlc (Table 1), but had no effect on the generation of foci by plasmids containing the activated *ras* oncogene (*13*). The ability of pMV7-SRlc to generate neomycin-resistant colonies was unaffected by mesulergine (Table 1). We also attempted to prevent focus formation simply by omitting serotonin from the growth medium. However, we found that the serum component of our growth medium contained enough serotonin to activate 5HTlc receptors on transformed cells.

We then showed that the maintenance of the transformed phenotype requires the continued presence of serotonin. Cells derived from two individual transformed foci, F12 and F15, were plated on a confluent lawn of NIH 3T3 cells, and the ability of these transformed cells to generate foci was scored in the presence or absence of mesulergine (Fig. 3). Cells plated either in serum or serum supplemented with serotonin generated transformed foci at high frequency. The addition of mesulergine to the growth medium

reduced the ability of these cells to form foci by two orders of magnitude. No foci were observed under any conditions with untransformed NIH 3T3 cells. Moreover, neither serotonin nor mesulergine had an effect on the generation of foci by cells transformed with the activated *ras* oncogene (Fig. 3). Taken together, these results indicate that the formation and maintenance of foci depend on the expression and activation of serotonin 5HTlc receptors on fibroblasts.

Transformed foci form tumors in nude mice. To determine whether cells expressing 5HTlc receptors are tumorigenic in vivo, we injected NIH 3T3 cells transfected with pMV7-SRlc into nude mice (*14*). In initial experiments, approximately 10^6 cells from a population of several hundred neomycin-resistant colonies were injected subcutaneously at two sites. Tumors appeared at both sites within 4 weeks. In further experiments, 10^6 cells from each of the seven focus-derived cell lines, as well as parental NIH 3T3 cells were injected separately into nude mice. Cells derived from two of the seven foci, F12 and F15, generated tumors at both sites of injection within 2 to 3 weeks (Table 2). No tumors were observed even after 6 weeks in mice injected with either parental NIH 3T3 cells or cells derived from the other five foci. Thus, expression of 5HTlc receptor in fibroblasts results in the formation of foci which are tumorigenic in nude mice. If we broadly define a protooncogene as a cellular gene which, when mutated or inappropriately expressed, renders cells tumorigenic, then the gene encoding the 5HTlc receptor is a protooncogene in 3T3 fibroblasts.

To determine whether receptor density correlates with tumorigenic potential, we examined the levels of 5HTlc receptors on foci and on tumor-derived cell lines (Table 2). The two focus-derived cell lines that were tumorigenic (F12 and F15) expressed more (greater density) receptors than cell lines that did not form tumors. Moreover, the cell lines established from the F15 tumor (F15T) expressed seven times the number of receptors present on the parental focus. The tumor derived from F12 focus (F12T) expressed 1.5 times the density of receptors as its parent focus, F12. The tumor cell line P_0, derived from a population of pMV7-SRlc transformants expressed the highest number of binding sites for ^{125}I-LSD (Table 2, lower). Scatchard analysis revealed that P_0 membranes bind ^{125}I-LSD with a site density (B_{max}) equal to 18 pmol per milligram of protein and an average dissociation constant (K_d) equal to 2.2 n*M* (Fig. 4). From the kinetic analysis, we calculate that P_0 expresses approximately 8×10^5 receptors per cell. The observation that the foci with the highest

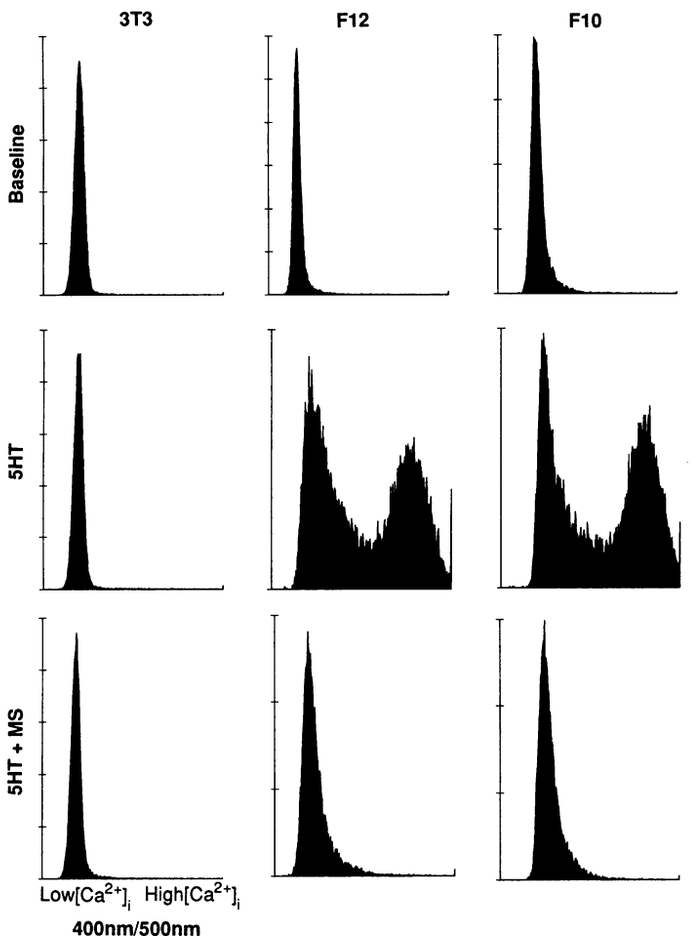

Fig. 2. Activation of 5HT1c receptors on focus-derived cells elevates intracellular Ca^{2+} in a mesulergine-sensitive manner. Focus-derived (F10 and F12) and control (NIH 3T3) cells were loaded with the Ca^{2+}-sensitive dye indo-1. Changes in intracellular Ca^{2+} were determined in a flow cytometer by monitoring the ratio of fluorescence emission at 400 and 500 nm. In each case, the resting Ca^{2+} level in the absence of agonist (serotonin, 1 μ*M*) is shown (baseline). In the presence of 1 μ*M* mesulergine (MS), the response to serotonin (5HT) is markedly attenuated. Each histogram depicts the response of 15,000 cells.

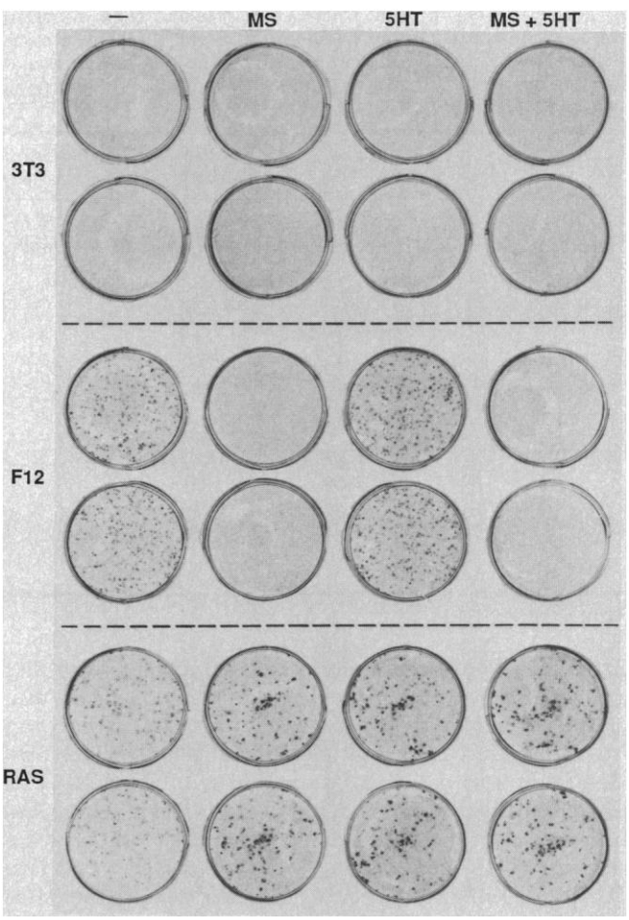

Fig. 3. Focus-derived cells do not exhibit a transformed phenotype in the presence of the serotonin receptor antagonist mesulergine. Approximately 200 cells from the focus-derived cell line, F12, or 200 cells from a focus generated by the activated *ras* oncogene (ras) were plated together with 10^5 NIH 3T3 fibroblasts (*29*). As controls, 10^5 NIH 3T3 cells were plated alone. The frequency of focus formation was determined in medium containing 3 percent calf serum. The conditions were as follows: medium alone (−); medium containing 1 μ*M* mesulergine (MS); medium supplemented with 0.1 μ*M* serotonin (5HT); and medium supplemented with both 1 μ*M* mesulergine and 0.1 μ*M* serotonin (MS + 5HT).

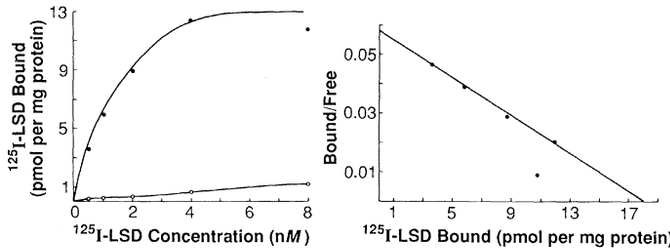

Fig. 4. Binding of ^{125}I-LSD to membranes derived from the P_0 cell line. ^{125}I-LSD was incubated with membranes prepared from the tumor-derived cell line P_0 at the concentrations shown. Each sample contained 6.8 μg of membrane protein in a total volume of 1 ml, which at 1 nM ^{125}I-LSD was within the linear range of binding. Specific binding (●) represents the difference between the total amount of labeled ligand bound and the amount bound nonspecifically (○) in the presence of mesulergine (2.5 μM). The data are presented as a saturation plot (left) and a corresponding Scatchard plot (right). In this experiment, the K_d was determined to be 2.2 nM and the B_{max} was 18 pmol per milligram of protein.

Table 2. Binding of LSD to membranes from transformed cell lines. ^{125}I-LSD (1 nM) was incubated (1-ml volume) with membranes from the focus- and tumor-derived cell lines indicated. Binding under these nonsaturating conditions provides a relative value for receptor densities on the various cell lines. Specific binding, defined as the difference between the total labeled ligand bound and the amount bound in the presence of mesulergine (1 μM), was greater than 88 percent. Membranes prepared from untransfected NIH 3T3 cells did not show specific binding of ^{125}I-LSD. Each sample contained from 5 to 130 μg of membrane protein in 1 ml (final volume). Less than 7 percent of the labeled ligand was bound. Values were determined in separate experiments.

Cell line	^{125}I-LSD bound (fmol/mg)*	Relative binding	Tumorigenicity (in vivo)
F2	154	1.0	ND
F3	242	1.6	ND
F5	316	2.1	−
F7	147	[1]	−
F10	397	2.7	−
F12	738	5.0	+
F15	1036	7.0	+
F17	178	1.2	−
F12	867	[1]	
F12 tumor	1200	1.4	
F15	324	[1]	
F15 tumor	2375	7.3	
P_0 tumor	6800		

*Per milligram of protein.

number of receptors form tumors in mice, together with the fact that tumors express even higher levels of receptor suggests that the formation of tumors may be associated with the selective expansion of those cells which express the greatest number of 5HTlc receptors.

Ligand-independent focus formation by tumor cells. Cell lines derived from tumors retain the ability to form foci in culture. To test whether the formation of these foci requires the continued activation of the 5HTlc receptor, we plated tumor cell lines on a lawn of NIH 3T3 cells in the presence or absence of mesulergine. Mesulergine had no effect on the ability of the tumor-derived cell lines to form foci (Table 3), in marked contrast with the finding that growth of parental foci is almost completely blocked by this antagonist. Thus, the generation of foci by tumor cell lines appears to be ligand-independent.

One possible explanation for these observations is that passage of cells in the mouse resulted in the selection of mutant receptors that were constitutively active in the absence of ligand. To exclude this possibility, we demonstrated that tumor-derived cell lines express

Table 3. Mesulergine resistance of tumor-derived cell lines. Between 500 and 1000 cells from focus- or tumor-derived cell lines were plated together with 2×10^5 normal NIH 3T3 cells (*29*). Cultures were maintained in the absence or presence of 1 μM mesulergine (MS). Foci were counted after 11 days.

Cell line	Foci	
	−MS	+MS
3T3	0	0
F12	819	8
F12 tumor	726	766
F15	534	4
F15 tumor	748	738

wild-type 5HTlc receptors, which activate intracellular signaling pathways only in the presence of agonist. Each of the three tumor cell lines was loaded with the Ca^{2+}-sensitive dye indo-1 and then exposed to serotonin. In all three tumor cell lines elevation of intracellular calcium was increased after the addition of serotonin (Fig. 5). Moreover, the response to serotonin could be completely blocked by mesulergine. In the absence of ligand, the resting Ca^{2+} in tumor cells was similar to that observed in untransformed NIH 3T3 cells. Thus, the serotonin receptors on the surface of these tumor cells appeared functionally normal and triggered intracellular signaling events only in response to the addition of serotonin. These experiments suggest that, whereas the 5HTlc receptor is required for the initial generation of foci, activation of the receptor is no longer required for focus formation after cells have been passaged as tumors in nude mice.

Neurotransmitters may act as growth factors. Several neurotransmitter receptors are restricted to neurons. However, the signaling pathways activated by these receptors are expressed in nonneural cells where they may regulate growth and differentiation rather than ion channel activity. Our data provide an example of the consequences of activating a neurotransmitter receptor in the unnatural environment of a fibroblast. The 5HTlc receptor is normally restricted to cells of the central nervous system (*6*, *15*). Activation of this neurotransmitter receptor in fibroblasts leads to focus formation and malignant transformation. It is likely that the transformation of fibroblasts results, at least in part, from the ligand-induced activation of phospholipase C and the subsequent liberation of inositol polyphosphates and diacylglycerol. The finding that focus formation is dependent on receptor activation by serotonin provides selectable genetic systems to identify receptor mutants and therefore permits a somatic cell genetic analysis of neurotransmitter receptor function. Finally, the observation that the 5HTlc receptor is a conditional protooncogene has allowed us to dissect the processes of tumorigenesis. Our studies suggest that the tumors we observe are the consequence of multiple, sequential, heritable events.

Several lines of evidence indicate that the generation and maintenance of transformed foci requires the activation of the serotonin receptor. (i) The introduction of a functional 5HTlc receptor cDNA into NIH 3T3 cells results in the generation of transformed foci at high frequency. Foci are not observed after transfection with expression vectors lacking the 5HTlc receptor cDNA. (ii) The generation of foci after DNA transfer is completely blocked by the serotonin antagonist mesulergine. (iii) Transformed foci expressing the 5HTlc receptor fail to reestablish foci when replated in mesulergine. (iv) The injection of cells derived from transformed foci into nude mice results in the generation of tumors. The serotonin 5HTlc receptor therefore functions as a protooncogene in NIH 3T3 fibroblasts.

Second messengers and malignant transformation. The second messengers responsible for tumorigenicity in fibroblasts transformed with the 5HTlc receptor are not known. The ability of serotonin to mobilize intracellular Ca^{2+} in these cells is likely to

result from activation of phospholipase C and the subsequent liberation of inositol trisphosphate (IP_3) (*2, 6, 16*). In fibroblasts, the activation of phospholipase C also results in the liberation of diacyl glycerol, an endogenous activator of protein kinase C (*17*). Either the generation of IP_3 or the activation of protein kinase C may contribute to transformation. Recent studies have shown that the introduction and overexpression of the protein kinase C gene in rat fibroblasts results in the generation of transformed foci (*18*). The 5HTlc receptor may therefore alter the growth properties of fibroblasts, in part, via protein kinase C activation.

In the nervous system, activation of 5HTlc receptor is likely to modulate neuronal excitability while in fibroblasts this receptor functions as a protooncogene. These distinct cellular responses may result from the activation of the same transduction machinery in different cell types. One line of evidence in favor of a common transduction pathway is the observation that activation of 5HTlc receptors elicits an elevation in intracellular Ca^{2+} independent of the cell type in which it is expressed (*6*). Thus, the distinct phenotypic consequences of receptor activation in fibroblasts and neurons may reflect different ways in which different cell types are programmed to respond to the same set of signaling events.

The oncogene *mas* is also a member of the family of G-coupled receptors that traverse the membrane seven times (*19*). Recent studies indicate that *mas* encodes a neuron-specific angiotensin receptor that appears to activate a phospholipase C pathway (*20*). Thus, two neurotransmitter receptors that couple to phospholipase C function as oncogenes in vitro in NIH 3T3 fibroblasts. In vivo, however, both the 5HTlc receptor (*6, 15*) and *mas* (*21*) are largely restricted to postmitotic cells of the central nervous system. Activation of the 5HTlc receptor in the natural environment of a nondividing neuron is therefore unlikely to lead to proliferation.

The ability of the serotonin receptor to alter the growth properties of fibroblasts in vitro raises the question as to whether neurotransmitters may function as growth factors in vivo in non-neuronal cells. Activation of other serotonin receptor subtypes triggers mitogenesis in cultures of aortic smooth muscle cells (*22*), Chinese hamster lung fibroblasts (*23*), and human B cells (*24*). Peptide neurotransmitters have also been shown to stimulate DNA synthesis or cell division in multiple cell types in culture (*25*). Moreover, the mitogenic actions of both thrombin and bombesin on cultured fibroblasts is blocked by pertussis toxin (*26, 27*) under conditions that also inhibit ligand-activated hydrolysis of inositol lipids (*26*). These observations further suggest that phospholipase C may be directly involved in regulating cell growth. Thus, the distinction between a neurotransmitter receptor, a growth factor receptor, and an oncogene may depend critically on the cellular environment.

The observation that expression of the 5HTlc receptor elicits focus formation only in the presence of ligand now permits the selection of mutants affecting the components of the signal transduction machinery. For example, foci that emerge in the presence of serotonin antagonists are candidates for receptor mutants that either do not bind antagonists or are constitutively activated in the absence of agonists. Negative selection conditions designed to kill cells proliferating within foci can also be imposed to isolate mutant receptors that no longer respond to serotonin. In addition to the isolation of mutants that directly affect receptor function, this selection system may facilitate the isolation of mutants affecting components of the signal transduction machinery that function downstream of the receptor, for example, phospholipase C or G proteins. Thus, the receptor-mediated transformation system we have developed should facilitate a somatic cell genetic analysis of neurotransmitter-mediated signaling.

When foci expressing high levels of the 5HTlc serotonin receptor are introduced into nude mice, tumors appear with a relatively short latent period. The phenotype of the tumor cells differs from that of the parental foci in two ways. (i) Tumor cells have a two to seven times greater density of receptors on their surfaces than the parental foci. (ii) Cell lines derived from tumors have acquired the capacity to form foci in the absence of serotonin, whereas in parental cells focus formation requires receptor activation. Thus, the 5HTlc receptor gene is responsible for the generation of foci, but does not seem to be required once the foci have been passaged as tumors.

One interpretation of our findings is that the tumors observed are the consequence of several sequential events. The presence of 5HTlc receptors may "initiate" tumorigenesis by facilitating the growth of fibroblasts in the mouse. Variant cells expressing high levels of receptor may be selected by virtue of a higher rate of proliferation. Individual cells within this expanded population may then acquire a second independent mutation which leads to malignant transformation and the generation of tumors. This second event may be sufficient to elicit focus formation and malignant transformation independent of the activation of the 5HTlc receptors despite their continued presence. This cascade of events in consistent with a "multistage" hypothesis of carcinogenesis (*28*).

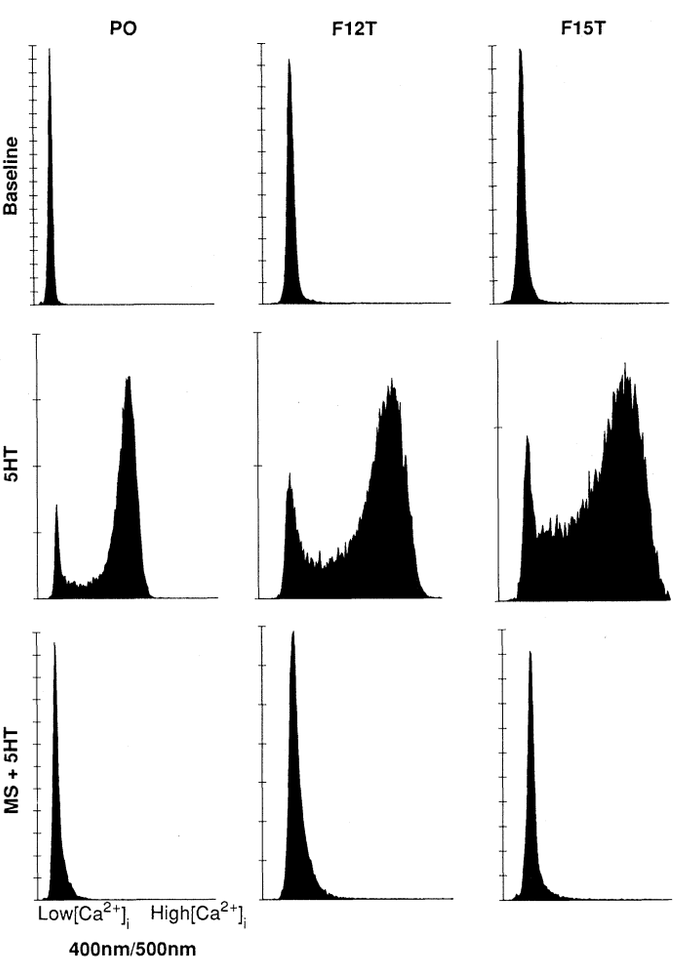

Fig. 5. Activation of 5HT1c receptors on tumor-derived cells elevates intracellular Ca^{2+} and is blocked by mesulergine. Tumor-derived cells (P_0, F12T, F15T) were loaded with the Ca^{2+}-sensitive dye indo-1. Changes in the amounts of intracellular Ca^{2+} were assayed in a flow cytometer to measure the ratio of fluorescence emission at 400 and 500 nm. In each case, the resting intracellular Ca^{2+} level in the absence of agonist (serotonin, 1 μM) is shown (baseline). In the presence of 1 μM mesulergine (MS) the response to serotonin is attenuated. Each histogram represents the response of 15,000 cells.

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29. NIH 3T3 cells (10^5) were mixed with approximately 100 focus- or tumor-derived cells and plated in DMEM (with 10 percent calf serum) in a 35-mm dish (Falcon). After 24 hours, the medium was changed to DMEM, 3 percent calf serum and 0.1 μ*M* 5HT or 1 μ*M* MS (or both). The 5HT and MS were replenished every 24 hours, and the medium was replaced every 48 hours. Control dishes with only NIH 3T3 cells were maintained under the same conditions. Foci were fully established in 6 days.

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