The Journal of Neuroscience, December 1992, 12(12): 4775-4782

# Serotonin Agonists Increase Transferrin Levels via Activation of 5-$HT_{1C}$ Receptors in Choroid Plexus Epithelium

**Teresa M. Esterle and Elaine Sanders-Bush**

Department of Pharmacology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232

**Choroid plexus epithelial cells are enriched in mRNA for proteins such as the iron carrier transferrin, which acts as a trophic factor in the brain. Choroid plexus epithelial cells also have a high density of 5-$HT_{1C}$ receptors linked to activation of the phosphoinositide (PI) hydrolysis second messenger system. The present studies show that the 5-$HT_{1C}$/5-$HT_2$ receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) potently increases PI hydrolysis and the levels of transferrin in primary cultures of rat choroid plexus epithelial cells. These effects are blocked by the 5-$HT_{1C}$/5-$HT_2$ receptor antagonists mesulergine and mianserin, but not by the 5-$HT_2$ receptor–selective antagonist spiperone. Similarly, mesulergine and mianserin, but not spiperone, block the increases in transferrin levels and PI hydrolysis elicited by 5-carboxamidotryptamine (5-CT), a 5-$HT_1$ receptor–selective agonist, and by serotonin. We conclude, therefore, that 5-$HT_{1C}$ receptor activation in the choroid plexus leads to an increase in the production of transferrin. By promoting transferrin synthesis in the choroid plexus, 5-HT may indirectly influence brain development and differentiation.**

Choroid plexus contains a monolayer of cuboidal epithelial cells with three main functions: to serve as the blood–cerebrospinal fluid (CSF) barrier, to regulate CSF production, and to modulate CSF composition (Cserr, 1975). The choroid plexus is enriched in a variety of mRNA species encoding transport proteins such as transferrin (Dickson et al., 1985; Aldred et al., 1987b), transthyretin (Dickson et al., 1985; Stauder et al., 1986), and ceruloplasmin (Aldred et al., 1987a). Since these proteins are enriched in the CSF relative to the plasma, it is thought that the choroid plexus synthesizes and releases them into the CSF. Consistent with this, we have recently shown that choroid plexus cells in primary culture synthesize transferrin and release it into the medium (Tsutsumi et al., 1989). Transferrin is an important source of iron (Laskey et al., 1988), and has also been shown to have growth factor-like actions independent of its ability to supply iron (Toran-Allerand, 1980; Beach et al., 1983; Ekblom et al., 1983; Espinosa de los Monteros et al., 1989). Transferrin mRNA levels are high in choroid plexus and moderately so in oligodendrocytes, but are negligible in other brain structures (Toran-Allerand, 1980; Bloch et al., 1985). The choroid plexus may thus be an important source of transferrin for brain cells.

Choroid plexus receives serotonergic innervation from the raphe, and the basolateral surface of the epithelial cells may also be exposed to 5-HT in the blood (Moskowitz et al., 1979; Napoleone et al., 1982). The epithelial cells have a high density of 5-$HT_{1C}$ receptors that are apparently localized to the apical surface (Giordano and Hartig, 1987). Thus, 5-HT in the CSF could play an important role in the choroid plexus. 5-HT is released into the CSF via a group of neurons, termed supraependymal fibers, that release their contents directly into the CSF (Aghajanian and Gallager, 1975; Tramu et al., 1983; Matsuura et al., 1985). The concentration of 5-HT in the CSF has been reported to be in the 10–100 nM range (Le Quan-Bui et al., 1982; Volicer et al., 1985; Linnoila et al., 1986), which would be sufficient to activate the 5-$HT_{1C}$ receptor. 5-$HT_{1C}$ receptors in choroid plexus are capable of activating the phosphoinositide (PI) hydrolysis signal transduction pathway (Conn and Sanders-Bush, 1986; Conn et al., 1986). Furthermore, these receptors become supersensitive when 5-HT neurons are destroyed (Conn et al., 1987). These findings, suggesting that 5-$HT_{1C}$ receptors in choroid plexus are innervated and functionally active, have spurred interest in a possible physiological role for these receptors. Our studies of the regulation of transferrin synthesis showed that 5-HT applied to primary cultures of choroid plexus cells increases transferrin production and increases the steady-state level of transferrin mRNA (Tsutsumi et al., 1989). Thus, 5-HT may indirectly influence brain development by increasing the production of a trophic factor by the choroid plexus. The present studies were designed to identify the receptor that mediates this effect.

## Materials and Methods

*Materials and animals.* Twenty-day-old male Sprague–Dawley rats were purchased from Sasco, Inc. (Omaha, NE). Cell media, dialyzed calf serum, fetal bovine serum, gentamicin, L-glutamine, trypsin, and vitamin and amino acid supplements were obtained from GIBCO/Bethesda Research Laboratories Life Technologies, Inc. (Grand Island, NY). $^3$H-myo-inositol, $^3$H-thymidine, $^{32}$P-αCTP, and Na$^{125}$I were obtained from New England Nuclear Corp. (Boston, MA). 1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane (DOI), 5-carboxamidotryptamine (5-CT), mianserin, and spiperone were purchased from Research Biochemicals, Inc. (Wayland, MA), and mesulergine, from Sandoz Pharmaceuticals (East Hanover, NJ). Transferrin and rabbit anti-rat transferrin primary antibody were purchased from Organon Teknika Corp. (West Chester, PA), and pronase, from Boehringer Mannheim Biochemicals (Indianapolis, IN). The Riboprobe Gemini System was purchased from Promega Corporation (Madison, WI), and Nytran membranes, from Schleicher and Schuell (Keene, NH). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

*Epithelial cell preparation.* The preparation is a modification of the method of Crook et al. (1981). Choroid plexi were removed from brains

Received Jan. 30, 1992; revised May 25, 1992; accepted June 29, 1992.

The graduate work of T.M.E. was supported by a University Graduate fellowship from Vanderbilt University. Research of both E.S.-B. and T.M.E. was supported by NIMH Grant MH 34007.

Correspondence should be addressed to Elaine Sanders-Bush, Ph.D., Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-6600.

Copyright © 1992 Society for Neuroscience 0270-6474/92/124775-08$05.00/0

and washed with Hanks' buffer. Tissue was partially digested in Hanks' buffer containing pronase (330 μg/ml) and type I DNase (260 μg/ml) at 37°C for 10 min. The tissue was allowed to settle and the supernatant removed. Cells were liberated by swirling gently in fresh Hanks' containing DNase (130 μg/ml). The procedure was repeated twice. The pooled cells were spun at 300 × *g*, and the pellet was resuspended and distributed into 11-mm wells containing minimum essential medium with D-valine substituted for L-valine, 15% dialyzed calf serum, and gentamicin (10 μg/ml). On the fourth day of culture and every 2 d thereafter, the medium was replaced with Ham's F12 without serum. The majority of the cells had typical epithelial morphology, with only a few contaminating cells that were morphologically similar to fibroblasts (see next section).

*Fibroblast preparation.* Choroid plexi were treated with pronase exactly as described above. After the pooled cells were spun down, the supernatant was harvested and spun for 10 min at 900 × *g*, and the pellet was resuspended and distributed into 35-mm plates containing Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum, L-glutamine (2 mM), gentamicin (10μg/ml), and vitamin and amino acid supplements. The cells had the characteristic morphology of fibroblasts, divided rapidly in culture, and stained intensely when incubated with antibodies to fibronectin. The first plating contained some epithelial contamination, which disappeared upon splitting. For studies of transferrin mRNA and protein, cells were transferred to F12 without supplements 48 hr before initiation of treatment. For phosphoinositide hydrolysis studies, medium was replaced with DMEM containing 2% dialyzed calf serum 24 hr before the addition of $^{3}$H-myo-inositol.

*Drug treatment.* Beginning on the fourth day of culture, cells were treated for 4 d with either drug or vehicle, with a change of medium on the seventh day. On the last day of treatment, $^{3}$H-myo-inositol was added. The following day, phosphoinositide hydrolysis was measured by incubating with 10 mM lithium chloride for 45 min. Medium was collected for analysis of transferrin content, and cells were harvested for measurement of $^{3}$H-inositol monophosphate ($^{3}$H-IP) formation.

*Radioimmunoassay.* Transferrin in the medium was measured by radioimmunoassay as described previously (Tsutsumi et al., 1989). Briefly, medium was incubated with rabbit anti-rat transferrin primary antibody and $^{125}$I-transferrin for 60 min, followed by an additional incubation with a secondary goat anti-rabbit antibody. The complex was precipitated at 4°C with 13% polyethylene glycol and spun at 2000 × *g*, and the pellet was analyzed for radioactivity using a Beckmann gamma counter. Samples were normalized for DNA content using a fluorescence method (Karsten and Wollenberger, 1977). Control experiments showed that lithium chloride, which was added for the phosphoinositide hydrolysis assay, did not interfere with the transferrin radioimmunoassay.

*Phosphoinositide hydrolysis.* The procedure was based on that of Berridge (1982) as modified by Tsutsumi and Sanders-Bush (1990). For acute studies, cells were incubated overnight with 1 μCi/ml $^{3}$H-myo-inositol in CMRL 1066 medium, which has only 0.2% of the inositol contained in F12. Labeled cells were incubated with 10 mM lithium chloride and 10 μM pargyline for 15 min, and then with agonist or vehicle for 30 min. The reaction was stopped by removal of medium and addition of 50 μl of methanol. Cells were disrupted by sonication in a buffer containing 0.15% trypsin. The inositol phosphates were extracted with chloroform/methanol, and $^{3}$H-IP was separated by anion exchange chromatography. $^{3}$H-IP was collected and counted in a Packard 1900CA liquid scintillation counter. For chronic studies, cells were labeled on the fourth day of drug treatment (see above) with $^{3}$H-myo-inositol (5 μCi/ml) overnight, and were then incubated with 10 mM lithium chloride for 45 min. No additional agonist or antagonist was added in these studies. All further procedures were the same as already described.

*$^{3}$H-thymidine assay.* Choroid plexus epithelial cells were removed from serum and kept in Ham's F12 for 2 d. Cells were then incubated 24 hr with 2 μCi/ml $^{3}$H-thymidine in F12 containing vehicle or drug. Plates were rinsed with phosphate-buffered saline, and cells were fixed with two 10 min methanol washes. The cells were washed by submerging eight times in tap water, dried, and solubilized for 2 hr at 60° in 0.2*N* NaOH/1% SDS. The samples were counted in a Packard 1900CA liquid scintillation counter using Aquasol scintillation fluid (Du Pont New England Nuclear, Boston, MA).

*Northern analysis.* Cells plated in 35-mm wells were maintained and treated with drugs as described previously. After 4 d of treatment, RNA was isolated using a modification of the method of MacDonald (1987). Medium was removed, and buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) was added. The cell lysate was sheared five times through a 22-gauge needle, layered on a cushion of 5.7 M cesium chloride, and centrifuged at 140,000 × *g* for 23 hr at 20°C. The resulting pellet was resuspended, precipitated with ethanol, and quantified by measuring absorbance at 260 nm. Aliquots representing 5 μg of total RNA were separated in a 1.2% agarose, 18% formaldehyde, 20 mM 3-[*N*-morpholino]-propane sulfonic acid gel for 4 hr at 25 V. The gel was capillary blotted overnight to a Nytran membrane, and the RNA was immobilized by baking 1 hr at 80°C.

*Hybridization.* cRNA probes were prepared by *in vitro* transcription using the Promega Gemini II system. The transferrin cRNA probe was synthesized from a plasmid (provided by Dr. Michael Skinner, Vanderbilt University) containing a 390 base pair (bp) fragment of rat transferrin cDNA (Huggenvik et al., 1987). The cyclophilin probe was made from a plasmid with a 680 bp insert (Danielson et al., 1988) (provided by Dr. Robert Coffey, Vanderbilt). Probes were labeled to a specific activity of $10^{8}$ cpm/μg using $^{32}$P-αCTP and were purified on a 9 M urea, 4% agarose, 90 mM Tris/90 mM boric acid/10 mM EDTA gel. Blots were prehybridized at 42°C for 6 hr in 10 ml of buffer [50% formamide, 10% dextran sulfate, 5× SSPE (750 mM NaCl, 500 mM $NaH_2PO_4$, 5 mM EDTA), 1% SDS], after which $10^{7}$ cpm of probe was added along with 1 mg of sheared salmon sperm DNA. Hybridization was allowed to proceed for 16 hr. Blots were washed three times at 65°C in 2 × saline–sodium citrate (SSC), 0.1% SDS for 30 min, followed by three more 30 min washes at 65°C in 0.2× SSC, 0.1% SDS. The blots were then exposed at −70°C to Kodak X-OMAT film, and bands were quantified using an LKB laser densitometer.

## Results

*Effects of 5-HT receptor agonists.* 5-HT, 5-CT, and DOI increased PI hydrolysis in cultured choroid plexus epithelial cells (Fig. 1). 5-HT and 5-CT were full agonists, producing an average ninefold increase over basal. 5-CT was less potent than was 5-HT. DOI, on the other hand, was essentially equipotent with 5-HT, but produced a submaximal response (60%), apparently acting as a partial agonist in this system. The order of potency (5-HT = DOI ≫ 5-CT) is consistent with activation of the 5-HT$_{1C}$ receptor.

In additional experiments, cells were treated with DOI, 5-CT, or 5-HT for 4 d and PI hydrolysis and transferrin production were measured. Cells were treated with drug(s) as usual on day 4, and $^{3}$H-myo-inositol was added as well. The next day, lithium chloride was added to start the accumulation of IP; no additional drugs were added. After 45 min, the medium was removed for analysis of transferrin content, and the cells were harvested for measurement of $^{3}$H-IP formation. DOI (Fig. 2) and 5-CT (Fig. 3) dose-dependently increased transferrin production and PI hydrolysis. Consistent with the acute studies, DOI was more potent than was 5-CT. The indicated concentration of drug was added daily, so the actual concentration at the time of the PI hydrolysis assay was probably larger than indicated. This presumably explains the apparent differences in potencies in the chronic versus the single-dose experiments. Although a comparison of Figures 2 and 3 suggests that DOI and 5-CT elicit different maximum transferrin responses, additional studies in which the two drugs were analyzed concurrently revealed no significant difference. This is unexpected in light of the evidence that DOI is a partial agonist for eliciting PI hydrolysis.

In some experiments, cells were harvested and assayed for transferrin content. The intracellular levels of transferrin were below the limit of detection (≈10 ng) even in the presence of DOI. When two wells of cells were combined, the amount of transferrin was still <10 ng, while aliquots representing one-third of the medium from one well contained between 30 and

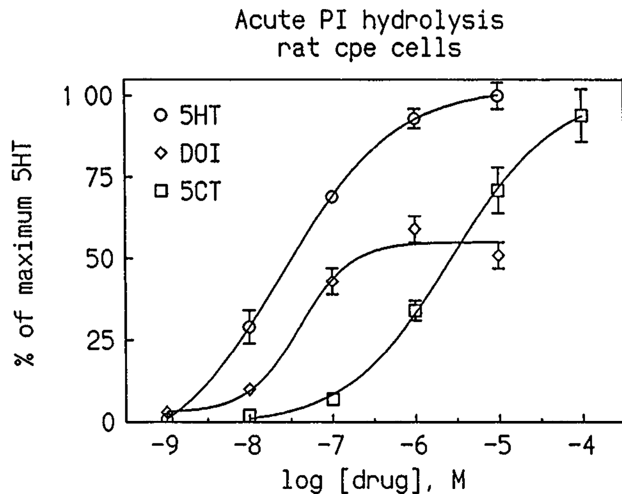



*Figure 1.* Activation of PI hydrolysis by 5-HT agonists. Cells on day 5 of culture were treated for 30 min with agonist as described in Materials and Methods. Values were expressed as percentage of the maximum 5-HT response for each cell preparation, which varied from 5- to 16-fold above basal. Experiments with 5-HT were performed on two different cell preparations ($n = 8$), except the points at −8 and −9, which represent one preparation ($n = 3$). Points for DOI and 5-CT are from three different preparations ($n = 9$) except for two points, −5 for DOI and −4 for 5-CT, which represent two preparations ($n = 6$). Error bars represent SEM. The curves were fit by a computer program (GRAPHPAD INPLOT, La Jolla, CA), using the equation for a sigmoid curve. The calculated $EC_{50}$ value for 5-HT was 30 nM; for DOI, 50 nM, and for 5-CT, 300 nM.

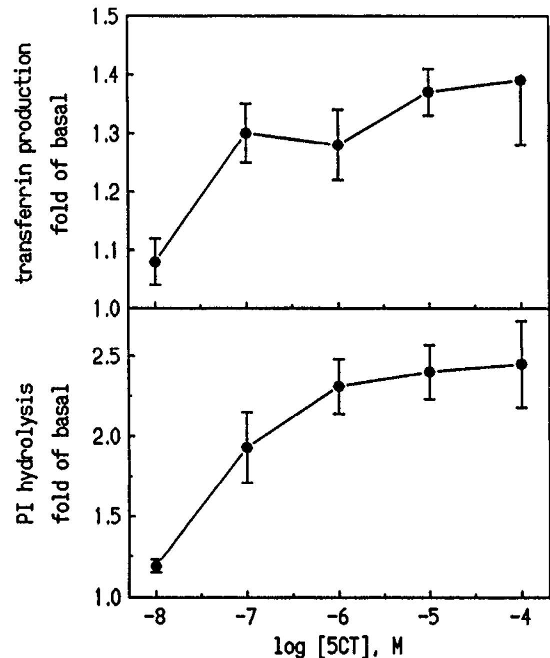



*Figure 3.* Effect of 5-CT on transferrin levels and PI hydrolysis in the same cells. Cells were treated for 4 d with 5-CT. Transferrin levels and PI hydrolysis were measured as described in Materials and Methods. Points represent four different cell preparations ($n = 16$) except for −7 and −4 (two preparations, $n = 8$). Error bars represent SEM.

60 ng of transferrin. Transferrin levels in the medium were corrected for the amount of DNA in the wells. DNA levels were not affected by drug treatment (data not shown). In addition, neither DOI nor 5-HT altered the incorporation of $^3$H-thymidine into DNA. Although serum alone increased $^3$H-thymidine incorporation, there was no additive effect when DOI was present (Table 1).

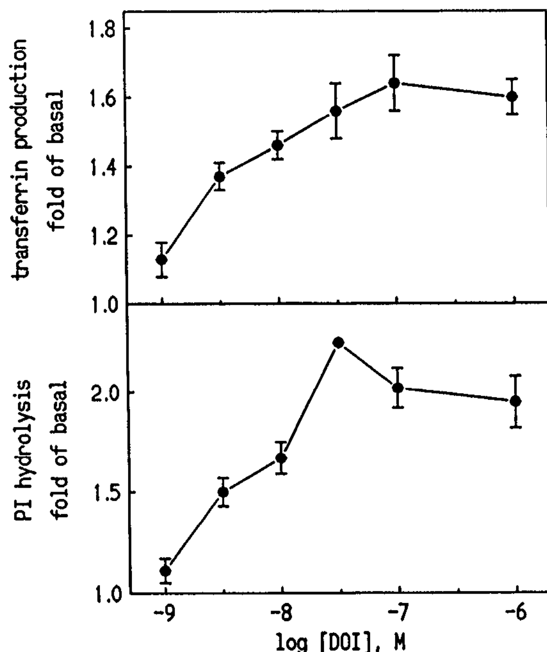



*Figure 2.* Effect of DOI on transferrin production and PI hydrolysis in the same cells. The indicated concentration of DOI was added daily for 4 d. Transferrin production and PI hydrolysis were then measured as described in Materials and Methods. Points represent data from five different cell preparations ($n = 20$) except for −7 (four preparations, $n = 16$), −7.5 (three preparations, $n = 12$), and −6 (two preparations, $n = 8$). Error bars represent SEM.

*Antagonism of the effects of 5-HT agonists.* The $5\text{-HT}_{1C}/5\text{-HT}_2$ receptor antagonists mesulergine and mianserin blocked both transferrin production and PI hydrolysis in response to DOI (Fig. 4) and 5-CT (Fig. 5), while spiperone, a $5\text{-HT}_2$ receptor–selective antagonist, had no effect. Similarly, mesulergine and mianserin, but not spiperone, blocked the effect of 5-HT on transferrin levels (Fig. 6). The antagonists had no effect on basal transferrin levels. To examine the possibility that spiperone's inability to block reflects inactivation of the drug, aliquots of the conditioned medium were removed and the ability to block

**Table 1. $^3$H-thymidine incorporation in rat choroid plexus epithelial cells**

| Treatment | cpm |
|---|---|
| Experiment 1 | |
| Vehicle | 6689 ± 422 (4) |
| 1 μM DOI | 6683 ± 480 (4) |
| 1 μM 5HT | 6702 ± 170 (4) |
| Experiment 2 | |
| Vehicle | 5539 ± 182 (4) |
| 3% serum | 7820 ± 184 (4) |
| 3% serum + 1 μM DOI | 7995 ± 148 (4) |

Cells were incubated with $^3$H-thymidine for 24 hr in the presence of vehicle, drug, and/or serum as described in Materials and Methods. Number of replications is indicated in parentheses. Values for experiment 1 are representative of three separate experiments. Values for experiment 2 are representative of two experiments. Data were subjected to one-way ANOVA using the GRAPHPAD INSTAT computer program. Serum treatment increased $^3$H-thymidine incorporation ($p < 0.001$), but the addition of drug had no effect, in either the presence or absence of serum ($p > 0.05$).

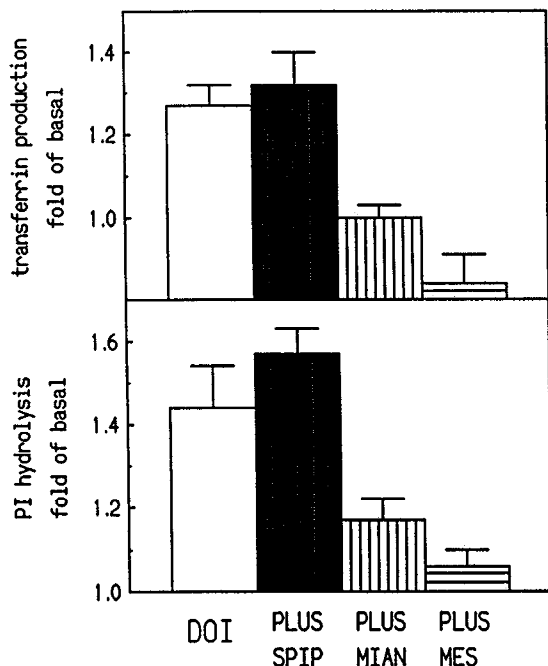
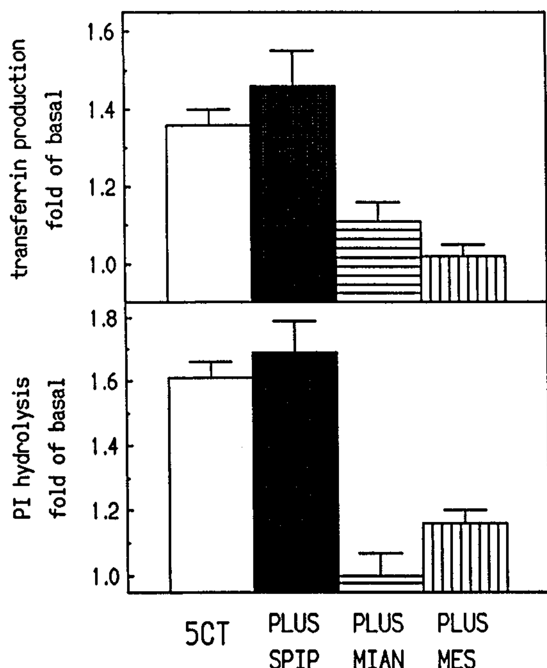



*Figure 4.* Effect of 5-HT receptor antagonists on DOI-stimulated increases in transferrin levels and PI hydrolysis. Cells were treated daily for 4 d with 3 nM DOI in the presence or absence of 1 μM concentrations of antagonists. Transferrin levels and PI hydrolysis were measured as described in Materials and Methods. Antagonist data were expressed as $n$-fold over the basal value obtained with the respective antagonist for that experiment, although overall there was no difference between basal values of vehicle- and antagonist-treated wells. Values represent four different cell preparations for mesulergine and spiperone ($n = 16$) and eight preparations for mianserin ($n = 32$). Data were subjected to one-way ANOVA using the GRAPHPAD INSTAT computer program, followed by post-tests to determine Bonferroni $p$ values. Mesulergine significantly decreased DOI-stimulated transferrin ($p < 0.001$) and PI hydrolysis ($p < 0.01$) as did mianserin ($p < 0.01$ for both), while spiperone had no significant effect on either transferrin or PI hydrolysis ($p > 0.05$). Error bars represent SEM.



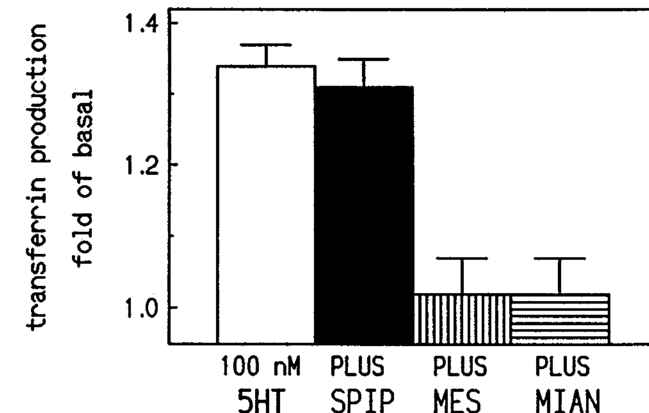




*Figure 6.* Effect of antagonists on 5-HT–stimulated increases in transferrin levels. Cells were treated for 4 d with 100 nM 5-HT in the presence or absence of mesulergine, mianserin, or spiperone (1 μM). Transferrin levels were measured as described in Materials and Methods. Values represent three (mianserin, $n = 24$; spiperone, $n = 12$) or four (mesulergine, $n = 16$) different cell preparations. Data were subjected to one-way ANOVA using the GRAPHPAD INSTAT computer program, followed by post-tests to determine Bonferroni $p$ values. Mesulergine and mianserin significantly inhibited 5-HT–stimulated increases in transferrin production ($p < 0.001$), while spiperone had no effect ($p > 0.05$). Error bars represent SEM.

5-HT–stimulated PI hydrolysis was determined in NIH3T3 cells transfected with 5-$HT_2$ receptor mRNA (Julius et al., 1990). Spiperone from the medium reduced the 5-fold PI hydrolysis response of 200 nM 5-HT to 1.5-fold of basal, indicating that the drug was indeed still active. The possibility that mesulergine actually did not block transferrin production but instead blocked secretion of the protein into the cell culture medium was tested by analyzing the cellular levels of transferrin. Transferrin in cells was below the limit of detection regardless of drug treatment (data not shown). If mesulergine were blocking transferrin release, cell levels of transferrin should have increased to readily detectable values.

The studies described so far utilized only single concentrations of antagonist. In additional experiments, increasing concentrations of mesulergine were tested with a near-maximal (33 nM) concentration of DOI. Mesulergine potently and dose dependently ($IC_{50}$ = 3 nM) blocked the ability of DOI to increase PI hydrolysis and to increase transferrin levels, with complete inhibition at 100 nM (Fig. 7).

*Time course studies.* Transferrin measurements were routinely performed after 4 d of drug treatment, because preliminary studies suggested that increases in transferrin levels were delayed in onset. Additional experiments were designed to determine whether continuous agonist activation was needed in order to

←

*Figure 5.* Effects of 5-HT receptor antagonists on 5-CT–stimulated increases in transferrin levels and PI hydrolysis. Cells were treated daily for 4 d with 1 μM 5-CT in the presence or absence of 1 μM concentrations of antagonists. Transferrin levels and PI hydrolysis were measured as described in Materials and Methods. Antagonist data were expressed as $n$-fold over respective basal. Values represent four (mesulergine, $n = 16$) or five (spiperone and mianserin, $n = 20$) different cell preparations. Data were subjected to one-way ANOVA using the GRAPHPAD INSTAT computer program, followed by post-tests to determine Bonferroni $p$ values. Mesulergine and mianserin significantly reduced 5-CT–induced PI hydrolysis and transferrin production ($p < 0.001$ in all cases), but spiperone had no effect ($p > 0.05$). Error bars represent SEM.

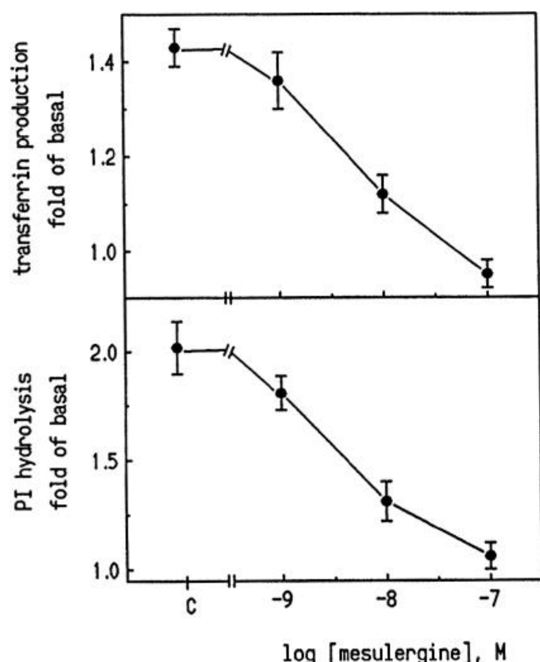



*Figure 7.* Dose-dependent effect of mesulergine on DOI-stimulated increases in transferrin levels and PI hydrolysis. Cells were treated for 4 d with 33 nM DOI in the presence or absence of increasing concentrations of mesulergine. Transferrin levels and PI hydrolysis were measured as in Materials and Methods. Points represent four different cell preparations ($n = 16$), except −9 (three preparations, $n = 12$). Error bars represent SEM.

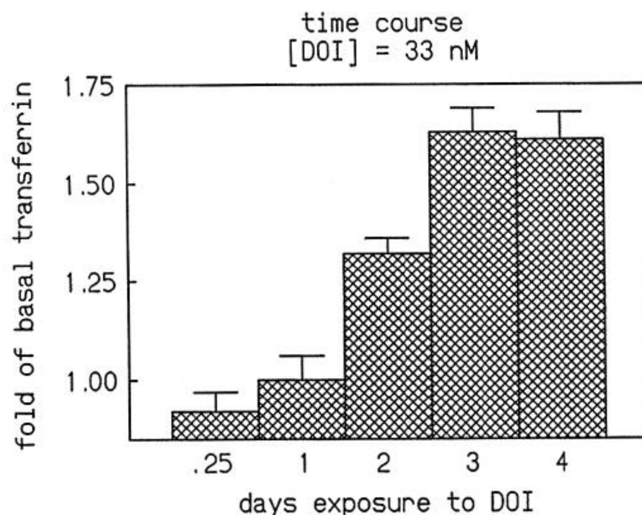



*Figure 8.* Varying time of exposure to DOI. Cells were treated with 33 nM DOI, and then 1 μM mesulergine was added at various times afterward to block the effect of the agonist. Transferrin levels in the medium were measured after 4 d as described in Materials and Methods. Values represent six different cell preparations ($n = 24$), except for 3 d (three preparations, $n = 12$) and 2 d (five preparations, $n = 20$). Error bars represent SEM.

have an effect, or if an initial stimulation with DOI was sufficient to produce a delayed increase in transferrin. Because DOI could not be washed out of the cells (data not shown), 1 μM mesulergine was added at various times after the addition of DOI to block the effect of the agonist during the subsequent incubation. As demonstrated in Figure 8, 3 d of exposure to DOI was needed to give a maximum effect, although a significant increase was seen after 2 d. These studies demonstrate that an initial brief stimulation of the cells with DOI was not sufficient to increase transferrin production. Instead, the continued presence of the drug is necessary, perhaps indicating that global, long-term changes in cell function (e.g., differentiation) must occur before transferrin production is seen.

*Characteristics of fibroblasts from rat choroid plexus.* The major contaminating cells in the primary cultures of epithelial cells had morphological features of fibroblasts. To determine whether the fibroblasts contributed to either the transferrin or the PI hydrolysis response, the cell isolation procedure was modified to establish cultures of fibroblasts. Unlike epithelial cells, the fibroblasts had negligible levels of transferrin mRNA (Fig. 9), and transferrin levels in the cell culture medium were undetectable, even after 4 d of treatment with DOI. 5-HT increased PI hydrolysis in these cells, with an $EC_{50}$ of 250 nM. In contrast to the effect in the epithelial cells, 5-HT–induced PI hydrolysis in fibroblasts was blocked by spiperone (Fig. 10).

## Discussion

Choroid plexus contains a high density of $5\text{-HT}_{1C}$ receptors (Yagaloff and Hartig, 1985) that are linked to the PI hydrolysis signaling cascade (Conn and Sanders-Bush, 1986; Conn et al., 1986). 5-HT applied to primary cultures of choroid plexus cells increases the level of transferrin, an iron carrier protein that also has growth factor-like effects (Tsutsumi et al., 1989). The present studies show that the $5\text{-HT}_1$ receptor–selective agonist 5-CT and the $5\text{-HT}_{1C}/5\text{-HT}_2$ receptor–selective agonist DOI in-

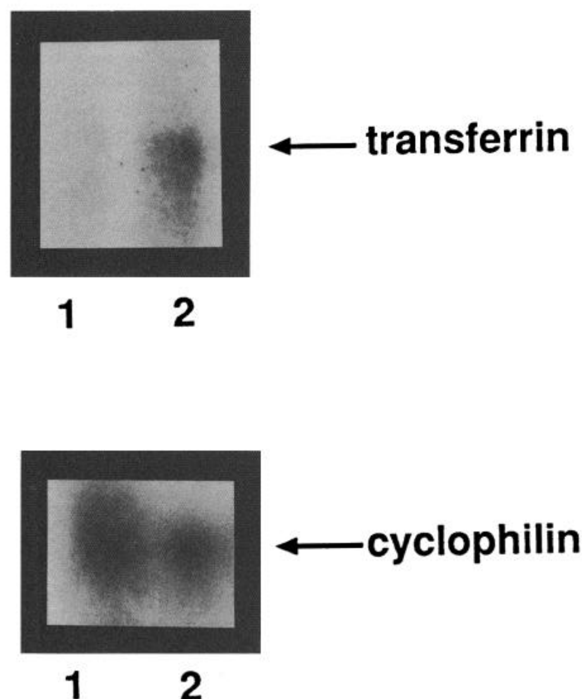



*Figure 9.* Transferrin mRNA levels in choroid plexus fibroblasts. Total RNA was harvested from fibroblasts (*lane 1*) and epithelial cells (*lane 2*) as described in Materials and Methods. Ten micrograms of fibroblast and 5 μg of epithelial cell RNA were loaded onto a formaldehyde/agarose gel and processed as described in Materials and Methods. Nytran membrane blots were hybridized with cRNA probes specific for transferrin and cyclophilin (used as an internal control). This blot is representative of two separate experiments.

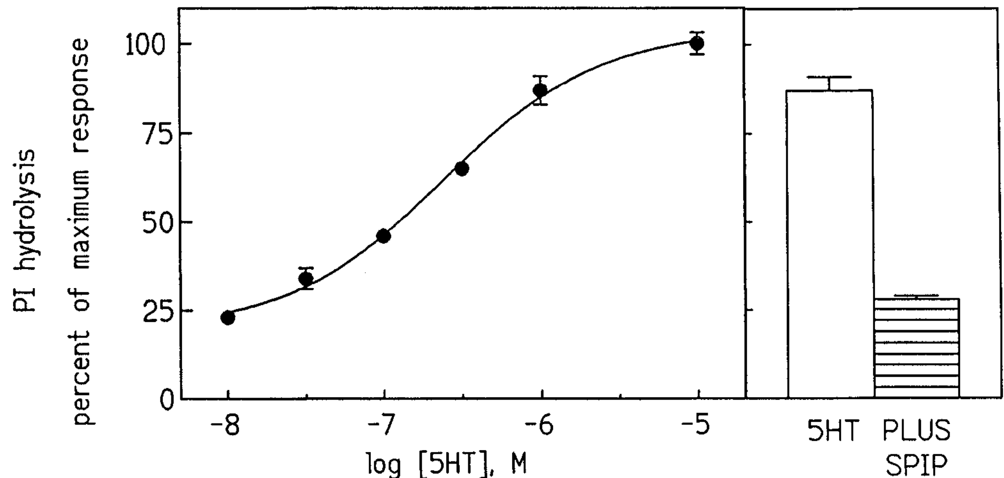



*Figure 10.* 5-HT–mediated PI hydrolysis in choroid plexus fibroblasts. In the *left panel,* cells were treated with increasing concentrations of 5-HT, and PI hydrolysis was measured as described in Materials and Methods. In some of the cells treated with 1 μM 5-HT, spiperone (1 μM) was added simultaneously. The results of these experiments are shown in the *right panel.* Values were expressed as percentage of the maximum 5-HT response for each cell preparation, which was approximately sevenfold above basal. Points represent three cell preparations ($n = 12$), except −7.5 and −6.5 (two preparations, $n = 8$). Error bars represent SEM.

creased transferrin levels when added to the incubation medium. Furthermore, these effects were potently blocked by $5\text{-HT}_{1C}$/$5\text{-HT}_2$ receptor antagonists, but not by the $5\text{-HT}_2$ receptor–selective antagonist spiperone. We conclude, therefore, that activation of the $5\text{-HT}_{1C}$ receptor increases the levels of transferrin in cultures of choroid plexus epithelial cells. This effect is apparently not secondary to an increase in cell number, since the transferrin values were corrected for DNA content. Furthermore, a direct comparison of DNA levels revealed no difference between control and DOI-treated cells, and the incorporation of $^3$H-thymidine was not altered by the addition of 5-HT agonists. Interestingly, $5\text{-HT}_{1C}$ receptor activation causes DNA replication and cell transformation in fibroblasts that have been transfected with the $5\text{-HT}_{1C}$ receptor cDNA (Julius et al., 1989). The present studies show that, rather than acting as a mitogenic stimulus, $5\text{-HT}_{1C}$ receptor activation in choroid plexus has effects analogous to the recently described action of 5-HT in astrocyte cultures. In these cells, 5-HT increases the levels of another trophic substance, S100β, apparently by interacting with $5\text{-HT}_{1A}$ receptors (Whitaker Azmitia and Azmitia, 1989; Azmitia et al., 1990).

The present studies utilized a primary cell culture system as a model of choroid plexus epithelial cells, but it is possible that a contaminating cell type may have contributed to the observed effects. Culture conditions were optimized to minimize contamination of the epithelial cultures with fibroblasts; however, it is still possible that 5-HT may have interacted with remaining fibroblasts or an unknown cell type to increase transferrin. To test this possibility directly, cultures of fibroblasts were established; after passage, no epithelial cells were evident in these cultures. Transferrin mRNA levels were undetectable in the fibroblast cultures, and in addition, transferrin protein was not present in controls or in cultures treated with DOI. Therefore, the fibroblasts do not appear to contribute directly to the increased transferrin level found after treatment with 5-HT receptor agonists.

Previous studies were unsuccessful in demonstrating blockade of the 5-HT–induced transferrin increase by $5\text{-HT}_{1C}$ receptor antagonists (Tsutsumi and Sanders-Bush, 1990). However, the present studies show a reproducible and complete blockade of DOI, 5-CT, and 5-HT. The explanation for this discrepancy is not entirely clear. In the original studies, a high concentration of 5-HT was used, which may have other effects on the cultured cells that mask the $5\text{-HT}_{1C}$ receptor–mediated effect. One interesting possibility is that 5-HT acts on another 5-HT receptor; however, no other 5-HT receptors were detected in binding studies of intact choroid plexus (Pazos and Palacios, 1985; Pazos et al., 1985; Yagaloff and Hartig, 1985). In fibroblast cultures isolated from choroid plexus, a $5\text{-HT}_2$ receptor–mediated PI hydrolysis response has been characterized (Barker et al., 1991; present results), although the density of sites is too low for radioligand binding analyses. High concentrations of 5-HT would activate this receptor; however, the antagonists used should also block $5\text{-HT}_2$ receptor–mediated effects. At high concentrations, 5-HT may have non-receptor-mediated effects. This was the case with 5-HT antagonists; Tsutsumi and Sanders-Bush (1990) found that high concentrations of mianserin and other 5-HT antagonists increased basal transferrin levels. In the present studies, where lower concentrations of antagonists were used, no change in transferrin levels was detected. Given the possibility of nonreceptor effects, the present demonstration that the effects of 5-HT agonists can be blocked by selective antagonists is especially important.

The DOI-induced increase in transferrin is delayed, suggesting that the synthesis of transferrin may reflect or be secondary to other changes in the cell. We have evidence that DOI treatment increases levels of c-*fos* mRNA in the cells (T. M. Esterle and E. Sanders-Bush, unpublished observations). Since Fos protein is known to increase the transcription of genes that have an AP-1 regulatory region (Morgan and Curran, 1988), it is possible that the transcription of specific target genes may be influenced by treatment with a $5\text{-HT}_{1C}$ receptor agonist. Whether transferrin is one of the target genes whose transcription is influenced remains to be seen. The 5′ upstream region of the transferrin gene has two regions with some homology to the AP-1 site, and Tsutsumi et al. (1989) showed that 5-HT increases transferrin mRNA in choroid plexus cultures. Even if the transferrin gene is not a direct target, Fos may increase the transcription of other genes that contribute to increased synthesis of transferrin. The finding that more than 2 d is required to elicit a full transferrin response suggests that multiple intervening events are invoked between receptor activation and protein production.

In conclusion, the present studies demonstrate that activation of the $5\text{-HT}_{1C}$ receptor in primary cultures of choroid plexus epithelial cells results in an increased level of transferrin, an iron-carrying protein that also has growth factor-like effects.

Thus, 5-HT may indirectly serve as a growth or trophic factor in the choroid plexus. 5-HT has been shown to have growth factor-like effects in a number of other systems. In embryonic rat brain, it influences the development of its target neurons (Lauder, 1983; Lauder et al., 1988). 5-HT also increases $^3$H-thymidine incorporation in fibroblasts via activation of the 5-$HT_{1B}$ receptor (Seuwen et al., 1988), and in aortic smooth muscle cells following pertussis toxin-sensitive activation of PI hydrolysis (Nemecek et al., 1986). Further, in fibroblasts transfected with 5-$HT_{1C}$ or 5-$HT_2$ receptor cDNA, 5-HT acts as a protooncogene, presumably by activating PI hydrolysis in these cells (Julius et al., 1990). By contrast, 5-$HT_{1C}$ receptor activation had no effect on $^3$H-thymidine incorporation in our primary cultures of choroid plexus epithelial cells that are presumably differentiated in culture. Our studies suggest that 5-HT's growth factor-like activity is manifested as an ability to maintain the differentiated phenotype in primary cultures of choroid plexus epithelial cells. The demonstration that the 5-$HT_{1C}$ receptor mRNA in choroid plexus is expressed early in fetal development (Roth et al., 1991), combined with the present evidence that activation of the 5-$HT_{1C}$ receptor has trophic effects, suggests the possibility that 5-HT acts as a developmental signal in the choroid plexus. Future studies should address this possibility by exploring the role of the 5-$HT_{1C}$ receptor in developing animals.

## References


Aghajanian GK, Gallager DW (1975) Raphe origin of serotonergic nerves terminating in the cerebral ventricles. Brain Res 88:221–231.

Aldred AR, Grimes A, Schreiber G, Mercer JF (1987a) Rat ceruloplasmin. Molecular cloning and gene expression in liver, choroid plexus, yolk sac, placenta, and testis. J Biol Chem 262:2875–2878.

Aldred AR, Dickson PW, Marley PD, Schreiber G (1987b) Distribution of transferrin synthesis in brain and other tissues in the rat. J Biol Chem 262:5293–5297.

Azmitia EC, Dolan K, Whitaker-Azmitia PM (1990) S-100β but not NGF, EGF, insulin or calmodulin is a CNS serotonergic growth factor. Brain Res 516:354–356.

Barker EL, Burris KD, Sanders-Bush E (1991) Phosphoinositide hydrolysis linked 5HT2 receptors in fibroblasts from choroid plexus. Brain Res 552:330–332.

Beach RL, Popiela H, Festoff BW (1983) The identification of neurotrophic factor as a transferrin. FEBS Lett 156:151–156.

Berridge MJ, Downes CP, Hanley MR (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J 206:587–595.

Bloch B, Popovici T, Levin MJ, Tuil D, Kahn A (1985) Transferrin gene expression visualized in oligodendrocytes of the rat brain by using *in situ* hybridization and immunohistochemistry. Proc Natl Acad Sci USA 82:6706–6710.

Conn PJ, Sanders-Bush E (1986) Agonist-induced phosphoinositide hydrolysis in choroid plexus. J Neurochem 47:1754–1760.

Conn PJ, Sanders-Bush E, Hoffman BJ, Hartig P (1986) A unique serotonin receptor in choroid plexus is linked to phosphatidylinositol turnover. Proc Natl Acad Sci USA 83:4086–4088.

Conn PJ, Janowsky A, Sanders-Bush E (1987) Denervation supersensitivity of 5-HT-1c receptors in rat choroid plexus. Brain Res 400: 396–398.

Crook RB, Kasagami H, Prusiner SB (1981) Culture and characterization of epithelial cells from bovine choroid plexus. J Neurochem 37:845–854.

Cserr H (1975) Physiology of the choroid plexus. In: The choroid plexus in health and disease (Netsky MG, ed), pp 175–195. Charlottesville: University of Virginia.

Danielson PE, Forss-Petter S, Brow MA, Calavetta L, Douglass J, Milner RJ, Sutcliffe JG (1988) p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 7:261–267.

Dickson PW, Aldred AR, Marley PD, Tu GF, Howlett GJ, Schreiber G (1985) High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. Biochem Biophys Res Commun 127:890–895.

Ekblom P, Thesleff I, Saxen L, Miettinen A, Timpl R (1983) Transferrin as a fetal growth factor: acquisition of responsiveness related to embryonic induction. Proc Natl Acad Sci USA 80:2651–2655.

Espinosa de los Monteros A, Pena LA, de Vellis J (1989) Does transferrin have a special role in the nervous system? J Neurosci Res 24: 125–136.

Giordano J, Hartig P (1987) Activation and regulation of the serotonin 5HT-1c receptor on the choroid plexus by CSF-borne serotonin. Neuroscience 13:1236 (abstract).

Huggenvik JI, Idzerda RL, Haywood L, Lee DC, McKnight GS, Griswold MD (1987) Transferrin messenger ribonucleic acid: molecular cloning and hormonal regulation in rat Sertoli cells. Endocrinology 120:332–340.

Julius D, Livelli TJ, Jessell TM, Axel R (1989) Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation. Science 244:1057–1062.

Julius D, Huang KN, Livelli TJ, Axel R, Jessell TM (1990) The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. Proc Natl Acad Sci USA 87:928–932.

Karsten U, Wollenberger A (1977) Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. Anal Biochem 77:464–470.

Laskey J, Webb I, Schulman HM, Ponka P (1988) Evidence that transferrin supports cell proliferation by supplying iron for DNA synthesis. Exp Cell Res 176:87–95.

Lauder JM (1983) Hormonal and humoral influences on brain development. Psychoneuroendocrinology 8:121–155.

Lauder JM, Tamir H, Sadler TW (1988) Serotonin and morphogenesis. I. Sites of serotonin uptake and -binding protein immunoreactivity in the midgestation mouse embryo. Development 102:709–720.

Le Quan-Bui KH, Elghozi J, Devynck M, Meyer P (1982) Rapid liquid chromatographic determination of 5-hydroxyindoles and dihydroxyphenylacetic acid in cerebrospinal fluid of the rat. Eur J Pharmacol 81:315–320.

Linnoila M, Jacobson KA, Marshall TH, Miller TL, Kirk KL (1986) Liquid chromatographic assay for cerebrospinal fluid serotonin. Life Sci 38:687–694.

MacDonald RJ, Swift GH, Przybyla AE, Chirgwin JM (1987) Isolation of RNA using guanidinium salts. Methods Enzymol 152:219–227.

Matsuura T, Takeuchi Y, Kojima M, Ueda S, Yamada H, Nojyo Y, Ushijima K, Sano Y (1985) Immunohistochemical studies of the serotonergic supraependymal plexus in the mammalian ventricular system, with special reference to the characteristic reticular ramification. Acta Anat 123:207–219.

Morgan JI, Curran T (1988) Calcium as a modulator of the immediate-early gene cascade in neurons. Cell Calcium 9:303–311.

Moskowitz MA, Liebmann JE, Reinhard JF, Schlosberg A (1979) Raphe origin of serotonin-containing neurons within choroid plexus of the rat. Brain Res 169:590–594.

Napoleone P, Sancesario G, Amenta F (1982) Indoleaminergic innervation of rat choroid plexus: a fluorescence histochemical study. Neurosci Lett 34:143–147.

Nemecek GM, Coughlin SR, Handley DA, Moskowitz MA (1986) Stimulation of aortic smooth muscle cell mitogenesis by serotonin. Proc Natl Acad Sci USA 83:674–678.

Pazos A, Palacios JM (1985) Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Res 346:205–230.

Pazos A, Hoyer D, Palacios JM (1985) The binding of serotonergic ligands to the porcine choroid plexus: characterization of a new type of serotonin recognition site. Eur J Pharmacol 106:539–546.

Roth BL, Hamblin MW, Ciaranello RD (1991) Developmental regulation of 5HT2 and 5HT1C mRNA and receptor levels. Dev Brain Res 58:51–58.

Seuwen K, Magnaldo I, Pouyssegur J (1988) Serotonin stimulates DNA synthesis in fibroblasts acting through 5HT-1b receptors coupled to a $G_i$-protein. Nature 335:254–256.

Stauder AJ, Dickson PW, Aldred AR, Schreiber G, Mendelsohn FA, Hudson P (1986) Synthesis of transthyretin (pre-albumin) mRNA in choroid plexus epithelial cells, localized by *in situ* hybridization in rat brain. J Histochem Cytochem 34:949–952.

Toran-Allerand CD (1980) Coexistence of α-fetoprotein, albumin and

transferrin immunoreactivity in neurones of the developing mouse brain. Nature 286:733–735.
Tramu G, Pillez A, Leonardelli J (1983) Serotonin axons of the ependyma and circumventricular organs in the forebrain of the guinea pig. An immunohistochemical study. Cell Tissue Res 228:297–311.
Tsutsumi M, Sanders-Bush E (1990) Serotonin-induced transferrin production by choroid plexus epithelial cells in culture: role of 5-HT1c receptor. J Pharmacol Exp Ther 254:253–257.
Tsutsumi M, Skinner MK, Sanders-Bush E (1989) Transferrin gene expression and synthesis by cultured choroid plexus epithelial cells: regulation by serotonin and cyclic 3′,5′-adenosine monophosphate. J Biol Chem 264:9626–9631.
Volicer L, Direnfeld LK, Freedman M, Albert ML, Langlias PJ, Bird ED (1985) Serotonin and 5-hydroxyindoleacetic acid in CSF. Difference in Parkinson's disease and dementia of the Alzheimer's type. Arch Neurol 42:127–129.
Whitaker Azmitia PM, Azmitia EC (1989) Stimulation of astroglial serotonin receptors produces culture media which regulates growth of serotonergic neurons. Brain Res 497:80–85.
Yagaloff KA, Hartig PR (1985) $^{125}$I-lysergic acid diethylamide binds to a novel serotonergic site on rat choroid plexus epithelial cells. J Neurosci 5:3178–3183.