Overexpression of 5-HT_{1B} Receptor in Dorsal Raphe Nucleus Using Herpes Simplex Virus Gene Transfer Increases Anxiety Behavior after Inescapable Stress

Michael S. Clark, Timothy J. Sexton, Molly McClain, Daniel Root, Ruth Kohen, and John F. Neumaier

Department of Psychiatry and Behavioral Sciences and Harborview Medical Center, University of Washington, Seattle, Washington 98195

5-HT_{1B} autoreceptors have been implicated in animal models of stress and are regulated selectively by serotonin-selective reuptake inhibitors such as fluoxetine. These terminal autoreceptors regulate serotonin release from dorsal raphe nucleus (DRN) projections throughout rat forebrain. However, it has not been previously possible to manipulate 5-HT_{1B} autoreceptor activity selectively without also changing 5-HT_{1B} activity in other neurons mediating different behavioral responses. Therefore, we have developed a viral-mediated gene transfer strategy to express hemagglutinin-tagged 5-HT_{1B} and manipulate these autoreceptors in DRN. Green fluorescent protein (GFP) was coexpressed from a separate transcriptional unit on the same amplicon to assist in monitoring infection and expression. We confirmed the expression and biological activity of both transgenic proteins *in vitro*. When injected directly into DRN using stereotaxic procedure, HA-5-HT_{1B} receptors were expressed in serotonergic neurons and translocated to the forebrain. The effect of DRN expression of HA-5-HT_{1B} on stress-induced behaviors was compared with control rats that received GFP-only amplicons. There was no change in immobility in the forced swim test. However, HA-5-HT_{1B} expression significantly reduced entrances into the central region of an open-field arena after water-restraint stress without altering overall locomotor activity, but not in the absence of stress exposure. HA-5-HT_{1B} expression also reduced entries into the open arms of the elevated plus maze after water restraint. Because these tests are sensitive to increases in anxiety-like behavior, our results suggest that overactivity of 5-HT_{1B} autoreceptors in DRN neurons may be an important mediator of pathological responses to stressful events.

Key words: herpes simplex virus; HSV; dorsal raphe nucleus; autoreceptor; hemagglutinin; forced swim test; open-field test; elevated plus maze

5-HT_{1B} autoreceptors are localized in the terminals of serotonergic axonal projections from midbrain raphe nuclei throughout the rat forebrain (Jacobs and Azmitia, 1992). These inhibitory autoreceptors respond to extracellular serotonin (5-HT) by reducing release of 5-HT from axonal terminals acutely and by reducing the amount of 5-HT synthesized over time (Hoyer and Middlemiss, 1989; Hjorth et al., 1995). Therefore, 5-HT_{1B} autoreceptors constitute a negative feedback system that regulates 5-HT neurotransmission on the basis of local conditions at the site of release.

5-HT_{1B} receptors are also synthesized in many other neurons widely distributed throughout the forebrain, where they inhibit the release of various other neurotransmitters such as acetylcholine, glutamate, and GABA (Barnes and Sharp, 1999). Because there are only ~20,000 serotonergic neurons in rat brain (Wiklund et al., 1981), the proportion of 5-HT_{1B} receptors in rat CNS that are autoreceptors in serotonergic neurons is very small. Indeed, chemical lesioning of the serotonergic system has small and inconsistent effects on total 5-HT_{1B} binding density (Verge et al., 1986; Offord et al., 1988; Sexton et al., 1999).

Received Dec. 21, 2001; revised March 5, 2002; accepted March 5, 2002.

This work was supported by the National Institute of Mental Health (MH 57049 and 63303) and the University of Washington Royalty Research Fund. We thank Dr. Rachael Neve for her generous gifts of the parent HSV amplicon and related packaging materials as well as her advice and assistance.

Correspondence should be addressed to Dr. John F. Neumaier, Harborview Medical Center, Psychiatry, Box 359911, 325 Ninth Avenue, Seattle, WA 98104-2499. E-mail: neumaier@u.washington.edu.

Copyright © 2002 Society for Neuroscience 0270-6474/02/224550-13$15.00/0

Pharmacological and null mutation ("knock-out") strategies have implicated 5-HT_{1B} receptors in a number of physiological processes and complex behaviors (Hen et al., 1993). However, in most cases it has not been possible to ascribe the 5-HT_{1B} effects to a particular population of neurons, because 5-HT_{1B} receptors on various neuronal terminals are intermingled in practically all forebrain areas. This has made it difficult to study the specific cellular mechanisms by which 5-HT_{1B} receptors are involved in brain functions.

5-HT_{1B} autoreceptors in forebrain are coded for by messenger RNA expressed primarily in dorsal raphe nucleus (DRN) (Hamblin et al., 1992; Neumaier et al., 1996b, 2000; Roberts et al., 1998). Although these neurons project very diffusely to forebrain cortical and subcortical structures, the cell bodies are closely packed in a small, midline nucleus (Kosofsky and Molliver, 1987). 5-HT_{1B} terminal autoreceptors appear to be involved in the adaptation of DRN neurons to serotonin-selective antidepressants, which are also effective in many anxiety disorders (Bergqvist et al., 1999; Sayer et al., 1999). 5-HT_{1B} mRNA is selectively downregulated in DRN but not in hippocampus, striatum, or frontal cortex by either fluoxetine or paroxetine in a time-dependent and reversible manner (Neumaier et al., 1996a; Anthony et al., 2000). Learned helpless rats (an animal model of depression involving inescapable stress) have a reversible deficit in 5-HT release in prefrontal cortex (Sherman and Petty, 1980; Petty et al., 1992) and increased 5-HT_{1B} mRNA in DRN (Neumaier et al., 1997). These observations suggest that increased 5-HT_{1B} autoreceptor activity induces depressive and related anx-

iety symptoms and that downregulation of DRN 5-HT$_{1B}$ autoreceptors by antidepressants may be important in normalizing serotonergic neurotransmission and relieving symptoms of depression or anxiety (Briley and Moret, 1993).

We propose to test the hypothesis that 5-HT$_{1B}$ autoreceptor overactivity alters the behavioral responses to inescapable stress. In this study we manipulated the 5-HT$_{1B}$ autoreceptors selectively while avoiding direct effects on the 5-HT$_{1B}$ heteroreceptors expressed in nonserotonergic neurons throughout the forebrain. To accomplish this we used replication-deficient herpes simplex virus type 1 (HSV) (Geller et al., 1990; Neve and Geller, 1995; Neve, 1999a) to overexpress 5-HT$_{1B}$ autoreceptors in DRN neurons. In this study we describe the development of a dual expression vector carrying both epitope-tagged 5-HT$_{1B}$ receptors and green fluorescent protein (GFP) on separate transcriptional cassettes and have characterized the effects of 5-HT$_{1B}$ gene transfer into DRN neurons on behavioral responses to inescapable stress using the forced swim test (FST), open-field test (OFT), and elevated plus maze (EPM) test.

MATERIALS AND METHODS

Plasmid construction

To introduce a hemagglutinin (HA) epitope tag into the N terminus of the rat 5-HT$_{1B}$ gene, plasmid MGIIB (Hamblin et al., 1992) was used as a template to PCR clone the rat 5-HT$_{1B}$ full-length sequence using an upstream primer (5′-TTCTAGAGCTATGTACCCATATGACGTCCCAGACTACGCCGAGGAGCAGGGTA-3′) that introduced an *Xba*I site and an in-frame HA epitope. The downstream primer (5′-GAGATGCATGATGGAAGCAGT-3′) corresponded to the single *Nsi*I site downstream of the translation start point. The resulting fragment was cloned into pCR-Script AMP SK+ (Stratagene, La Jolla, CA) as described by the manufacturer. This sequence was confirmed in its entirety by automated DNA sequencing. The *Xba*I/*Nsi*I fragment of this plasmid was then ligated into the *Xba*I/*Nsi*I fragment of an intermediate plasmid created by ligation of the *Hin*dIII fragment of the rat 5-HT$_{1B}$ cDNA, MG11B (Hamblin et al., 1992), into *Hin*dIII cut pGEM3Zf+ (Promega, Madison, WI). The resulting plasmid, pHA1B, was cut with *Hin*dIII, blunted with Klenow fragment, cut with *Eco*RI, and ligated into *Eco*RI/*Sma*I cut pCI (Promega). This plasmid, pCI-HA1B, produces hemagglutinin-tagged 5-HT$_{1B}$ under control of the CMV promoter/enhancer. To produce a version of this plasmid that coexpresses enhanced GFP (EGFP), plasmid pEGFP-C1 (Clontech) was cut with *Bam*HI/*Bgl*II and recircularized to eliminate most of the polylinker. The resulting plasmid was then cut with *Xba*I, blunted with Klenow fragment, and cut with *Nhe*I, and the GFP cDNA was isolated and ligated into *Nhe*I/*Sma*I cut pCI (Promega). This plasmid, pCMV-GFP, produces GFP under control of the CMV promoter/enhancer. pCMV-GFP was cut with *Bgl*II/*Bam*HI, and the 3.3 kb fragment was isolated. This fragment was ligated into *Bam*HI cut pCI (Promega) to generate pCI$_{GFP}$, containing two transcriptional units in tandem, with the first possessing a polylinker for introduction of a desired gene and the second expressing GFP. The pCI$_{GFP}$ was cut with *Eco*RI/*Sma*I, and the HA-5-HT$_{1B}$ fragment used to produce pCI-HA1B was ligated into it to produce pCI$_{GFP}$-HA1B. All plasmids were identified by multiple restriction cuts.

Amplicon construction and packaging

To produce an HSV amplicon producing both HA-5-HT$_{1B}$ and GFP, pCI$_{GFP}$-HA1B was cut with *Xba*I and *Bgl*II. This digest was then partially digested with *Bam*HI, and the 3.8 kb fragment was isolated. The resulting fragment was ligated into *Xba*I/*Bam*HI cut pHSV-PrPUC (generously provided by Dr. Rachael Neve, McLean Hospital, Boston, MA). This reconstructed amplicon contains two transcriptional units terminated by SV40 polyadenylation sites, the first producing HA-5-HT$_{1B}$ from an HSV promoter/enhancer and the second producing GFP from a CMV promoter/enhancer. To make an amplicon producing only GFP, the *Nhe*I/blunted *Xba*I fragment of pCMV-GFP was ligated into *Xba*I/(blunted)-*Bam*HI cut pHSV-PrPUC. Each plasmid was identified by multiple restriction cuts. HSV amplicons were then packaged either by Dr. Rachael Neve or in our laboratory as described previously (Neve, 1999b).

Cell culture infections and transfections

PC12 cells were infected with packaged HSV amplicons as described previously (Neve, 1999b). Briefly, PC12 cells were grown to ~80% confluence in DMEM containing 10% fetal bovine serum and penicillin/streptomycin/amphotericin B (100 U/ml, 100 μg/ml, and 0.25 μg/ml, respectively). Cells were harvested by a brief trypsin/EDTA treatment and passed through a 21 gauge syringe to dissociate aggregates. After counting on a hemocytometer, 3×10^5 cells were plated onto poly-D-lysine-coated 24-well cell culture dishes, grown for 24 hr, and then treated with varying concentrations of packaged HSV amplicon stocks (maximum of 1 μl of virus per milliliter of medium). After 24 hr, cells were fixed in 4% paraformaldehyde/sodium phosphate buffer for later fluoromicroscopy and immunocytochemistry. Viral titer was determined from the number of GFP or hemagglutinin/GFP-positive cells.

HeLa, COS7, and CA77 cells were maintained as described previously (Hamblin et al., 1992; Clark et al., 1995; Zhukovskaya and Neumaier, 2000). HeLa cells were transfected essentially as described previously (Tverberg and Russo, 1992). Briefly, cells were grown to ~80% confluence and harvested by a brief trypsin/EDTA treatment. Cells ($\sim 1 \times 10^6$ cells per chamber) were suspended in ice-cold Ca^{2+}/Mg^{2+}-free PBS, chilled 10 min on ice, and electroporated (0.300 kV, 1000 μF) in an Electroporator 2 (Invitrogen, Carlsbad, CA) in the presence of 20 μg of transfection DNA. After electroporation, cells were mixed very gently and placed on ice for another 10 min before plating. COS7 cells were similarly transfected, except that the electroporation conditions were 0.330 kV at 500 μF. CA77 cells were grown to 80% confluence and infected with 3 ml of viral particles per 3 ml of medium per well in six-well dishes. Twenty-four hours after transfection or infection, cells were fixed in 4% paraformaldehyde or collected by centrifugation at $500 \times g$ for 10 min for subsequent RNA extraction.

Reverse transcribed-PCR

Total RNA from fresh CA77 cells infected with either pHSV-HA1B/GFP or pHSV-GFP was purified using RNeasy columns (Qiagen, Valencia CA) and DNase I treatment. Total RNA from DRN was prepared from a 1 mm tissue punch containing DRN from a 2-mm-thick fresh brain slice that contained the anterior DRN (approximately −6.5 to −8.5 mm relative to bregma). The punched tissue was processed in RNAlater (Ambion, Austin, TX), and total RNA was isolated as described for CA77 cells, using the manufacturer's recommended procedures followed by DNase I treatment. RNA was quantified with RiboQuant (Molecular Probes, Eugene, OR), and control DNA was quantified with PicoGreen assays (Molecular Probes). Total RNA (1.5 μg for CA77 cells; 0.25 μg for DRN) was reverse transcribed into first-strand cDNA using oligo-dT primer and Moloney murine leukemia virus (Promega) in a final volume of 20 μl. HA-5-HT$_{1B}$ was selectively amplified by 35 cycles of PCR using a pair of primers that are specific for the hemagglutinin tag (5′-ACCCATATGACGTCCCA-3′) and the 5-HT$_{1B}$ sequence (5′-ACCGTGTACATGGTGCT-3′), yielding a 350 nucleotide PCR product. Total 5-HT$_{1B}$ reverse transcribed (RT)-PCR was similarly amplified using primers 5′-GGTCTTTTCACAGGTAGGTCAA-3′ (upstream) and 5′-TTGACCTACCTGTGAAAAGACC-3′ (downstream), yielding a 578 nucleotide PCR product. PCR products were resolved using 1.3% Agarose gels and stained with SYBR Gold (Molecular Probes) before photography.

Quantitative reverse transcribed-PCR

5-HT$_{1B}$ mRNA was quantified from first-strand cDNA prepared from DRN as described above using real time quantitative PCR with a LightCycler Instrument (Roche, Indianapolis, IN) with SYBR Green detection of PCR product. A 61 nucleotide PCR product was amplified using primers5′-CCAAAAGGGCGGCCA-3′(upstream)and5′-TGGCAGCGAAATCGAGATG-3′ (downstream) from 1 μl of template containing either first-strand cDNA or known amounts of MG11B control template ($1 \times 10^{-7} - 1 \times 10^{-4}$ ng per reaction). The thermal cycling procedures and quantitation procedures were based on the manufacturer's recommendations. Briefly, a standard curve constructed from the control template reactions was used to calculate the amount of first-strand cDNA present in the samples. Each duplicate determination was analyzed in three independent assays to calculate the relative amount of first-strand cDNA from each tissue sample in a blinded manner. Total 5-HT$_{1B}$ mRNA determinations from each brain sample were standardized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR quantitation from the same preparation, using the following primers: 5′-

AACGACCCCTTCATTGAC-3′ (upstream) and 5′-TCCACGACATACTCAGCAC-3′ (downstream). After the code was broken, treatment group averages were calculated and are expressed as percentage of control (pHSV-GFP). The efficiency of the RT reaction was not calculated, but all samples were prepared in parallel at each step.

cAMP determination

cAMP levels were assayed as described previously (Kohen et al., 1996). Briefly, JEG-3 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin under 10% CO_2. Cells were seeded into 24-well plates and grown to a density of ~50,000 cells per well. One to four hours before transfection, the medium was replaced with 250 μl of DMEM supplemented with 10% dialyzed fetal bovine serum and 1% penicillin–streptomycin, after which the cells were switched to 5% CO_2. Cells were transiently transfected by a calcium phosphate precipitation method as described previously (Heidmann et al., 1998). Transfected DNA consisted of 1 ng of 5-HT_{1B} (MG11B) or pHSV-HA1B/GFP plasmid (except for controls in which no receptor was transfected), 50 ng of Rous sarcoma virus (RSV)-β-galactosidase plasmid, 2.5 ng RSV-cAMP responsive element (CRE)-luciferase plasmid (Mellon et al., 1989), and plasmid Bluescript II KS(−) (Stratagene) as carrier DNA for a total of 250 ng of DNA in 25 μl per well. Twenty hours after transfection, cells were washed once with PBS, supplemented with 500 μl of serum- and serotonin-free medium (Complete Medium, Cellgro, Herndon, VA) with 1% penicillin–streptomycin, and switched back to 10% CO_2. After another 24 hr, triplicate wells were supplemented with 25 μl of forskolin (Calbiochem, San Diego, CA) for a final concentration of 1 mM, and with 25 μl of 2 mM ascorbic acid alone or ascorbic acid containing 5-HT (Sigma, St. Louis, MO) for a final concentration of 1×10^{-11} M to 1×10^{-6} M. Five hours later, cells were harvested in 100 μl of lysis buffer containing 100 nM KPO_4, 6 mM $MgSO_4$, 1 mM dithiothreitol, and 0.1% Triton X-100. To 350 μl of luciferase assay buffer (100 nM KPO_4, 4 mM ATP, 6 mM $MgSO_4$), 25 μl of cell extract was added and incubated at room temperature for 30 min. Luciferase activity was then assayed using an Autolumat LB 953 luminometer (EG and G Berthold, Bundoora, Australia) as described elsewhere (Migeon and Nathanson, 1994). Data were analyzed using the program Prism (GraphPad Software, San Diego, CA).

Stereotaxic injections and animal care

All animal procedures were approved by this institution's animal care committee and handled in accordance with National Institutes of Health guidelines. Male Sprague Dawley rats (180–250 gm) were anesthetized with pentobarbital (0.9 mg/kg, i.p.) or isoflurane (2–3% in oxygen), the scalp fur was shaved, the animal was placed in a Stoelting stereotaxic device, and the surgical site was cleaned with betadine. After scalp incision, skull landmarks were visualized by scraping of the periosteum. A small hole was bored at the site of injection. To avoid penetration of the third ventricle, the DRN (−7.7 from bregma, midline, 6.6 mm deep) was approached from an angle 20 or 25° off midline. The needle was slowly advanced over the course of 5 min, and 2 μl of viral particles (~200,000 infective units) was injected from a Hamilton syringe (#30 needle) over 10 min using a microprocessor-controlled pump (World Precision Instruments, Sarasota, FL). The needle was left in place for 10 min after the injection and then withdrawn slowly over 10 min. This injection volume and procedure correspond to previous studies with pHSV-PrPUC-based amplicons (Carlezon et al., 1997; Song et al., 1998). The skin was closed with surgical methylacrylate glue, and in later injections the closure was augmented with sterile 3-0 monofilament nylon sutures (Ethicon); the rats were monitored until they recovered spontaneous movement. Animals were allowed to recover for 48–96 hr before being killed. For immunohistochemistry, rats were injected with heparin (1000 U, i.p.), deeply anesthetized with pentobarbital, and intracardially perfused with Tyrode's solution followed by 4% paraformaldehyde. The brains were removed, post-fixed for 2 hr in 4% paraformaldehyde, and stored in PBS at 4°C 1–2 d before being processed further. For immunoblot analysis, fresh tissue was harvested, immediately frozen, and stored at −70°C. For RT-PCR, fresh tissue was processed as described below. For all elevated plus maze and pre-stress open-field testing, injection location was confirmed in a blinded manner on perfused 40 μm tissue slices prepared on a vibratome. Animals were excluded if >50% of GFP-expressing neurons were outside the DRN or if there was any evidence of trauma distorting any anatomic structures nearby.

To determine the number of infected neurons, 40 μm sequential vibratome sections through the entire DRN were cut from perfused tissue and mounted on slides. GFP-positive cell bodies within the DRN were then counted manually. Although counting of the same GFP-positive cell body in two sequential sections was theoretically possible, the thickness of the slices relative to cell body size suggests that the error introduced by counting a single cell twice is small relative to the number of total neurons infected.

Immunocytochemistry, immunohistochemistry, and microscopy

For immunocytochemistry of cell cultures, the medium was aspirated, and cultures were rinsed briefly in PBS and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were rinsed briefly in PBS blocked with 0.3% gelatin (bovine) in PBS–0.025% Triton X-100 for 1 hr to overnight. Wells were incubated with mouse monoclonal anti-hemagglutinin antibody (1:1000 in 0.3% gelatin PBS–0.025% Triton X-100) (HA.11, Babco, Richmond, CA), rinsed three times for 10 min with PBS–0.025% Triton X-100, and then incubated with a goat anti-mouse Cy3-conjugated antibody (1:500 in 0.3% gelatin PBS–0.025% Triton X-100) (Jackson ImmunoResearch, West Grove PA) for 1 hr at 37°. Wells were then rinsed three times for 10 min with PBS–0.025% Triton X-100 and rinsed briefly with deionized water. Excess water was removed, and the bottoms of the wells were coated with Gel/Mount (Biomeda, Foster City, CA). Immunofluorescence was visualized with a Nikon inverted fluorescence microscope using an FITC filter for detection of GFP fluorescence and a rhodamine filter for detection of Cy3.

For immunohistochemistry, free-floating sections (40 μm) were prepared on a Leica VT1000S and rinsed in PBS. Sections were permeabilized in PBS–0.5% Triton X-100 for 30 min and then blocked with 0.3% gelatin (bovine) in PBS–0.025% Triton X-100 for 1 hr at room temperature or overnight at 4°C. They were then incubated with one or more primary antibodies concurrently: mouse monoclonal anti-hemagglutinin antibody (HA.11, Babco,), guinea pig anti-5-HT_{1B} (Chemicon, Temecula, CA), and guinea pig anti-5-HT_{1A} (Chemicon). All antibodies were diluted 1:1000 in 0.3% gelatin in PBS–0.025% Triton X-100 and incubated overnight at room temperature with gentle agitation. Sections were then rinsed three times for 10 min with PBS–0.025% Triton X-100 and incubated with secondary antibodies [goat anti-mouse Alexa-633 conjugate and/or goat anti-guinea pig Alexa-568 conjugate (Molecular Probes)], again concurrently, in 0.3% gelatin PBS–0.025% Triton X-100 for 1 hr at room temperature. Secondary antibodies were diluted as follows: Alexa-568 conjugate diluted to 5 μg/ml for anti-5-HT_{1A} and to 10 μg/ml for anti-5-HT_{1B}, and Alexa-633 conjugate diluted to 20 μg/ml. Sections were then rinsed three times for 10 min with PBS–0.025% Triton X-100, rinsed briefly with deionized water, and mounted on glass slides with Prolong Antifade mounting medium (Molecular Probes). The sections were analyzed using a Bio-Rad Radiance 2000 confocal system (Bio-Rad, Hercules, CA) and an associated Nikon fluorescence microscope using an argon/krypton laser and red laser diode with appropriate Performance filters (Bio-Rad) for detection of GFP, Alexa-568, and Alexa-633 fluorescence.

Immunoblot analysis

Frozen tissue was crushed on dry ice, placed into boiling 5% SDS/50 mM Tris-HCl, pH 8.0, for 5 min, sonicated for 10 sec, and spun at 10,000 × *g* for 10 min. The supernatant was retained and assayed for protein concentration by the BCA Protein Assay (Pierce Biochemical, Rockford, IL). Protein samples (50–100 μg) were separated on a 10% SDS-PAGE gel. Western blotting using nitrocellulose was performed at 4°C in a Bio-Rad blotting apparatus at 15 mV overnight or 35 mV for 2 hr. Blots were blocked in 5% instant milk in 50 mM Tris–0.9% saline containing 0.025% Tween 20 (TBST) for 1–2 hr, incubated in anti-hemagglutinin primary antibody (1:1000 in 5% non-fat instant milk-TBST for 1 hr at room temperature) (HA.11, Babco), and rinsed three times for 10 min in TBST. Blots were incubated in anti-mouse-HRP-conjugated secondary antibody (1:1000 in 5% non-fat instant milk–TBST for 1 hr at room temperature) and rinsed three times for 10 min in TBST. Blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce Biochemicals) for 5 min and exposed to film. Biotin-conjugated molecular weight markers were visualized by further incubating the blot in anti-biotin-HRP-conjugated antibody (New England Biolabs, Beverly, MA) at 1:1000 for 1 hr, rinsing three times for 10 min in TBST, and redeveloping in chemiluminescent substrate.

Behavioral testing procedures

Forced swim test. The FST was performed as described previously (Porsolt et al., 1977; Detke et al., 1995). The FST container was a 40-cm-tall

Plexiglas cylinder with a 20-cm-diameter base, mounted on a Plexiglas base. It was filled with tap water (25°C) to 30 cm, a level deep enough to prevent the rat from resting on its extended tail. The forced swim stress session consisted of placing the rat in the chamber for 15 min, followed by towel drying the rat under warm lamps and returning it to the home cage. The following day (between the hours of 9 and 11 A.M.), the test session was performed by putting the rat into the water and videotaping its behavior for 5 min. Each cylinder was cleaned between animals. FST behaviors were scored in a blinded manner, by a different experimenter (J.N.), using a time-sampling method (Detke et al., 1995). Every 5 sec the animal's behavior was scored as climbing, swimming, or immobile; a total of 60 observations were made during the 5 min test session. Statistical comparison of between group differences was performed with the Mann–Whitney *U* test using GB-Stat software, with $p \leq 0.05$ considered significant. Increased immobility time was considered to represent behavioral depression or behavioral despair but could also be operationally defined as a behavioral pattern that is preventable by treating the animal with antidepressants (Porsolt, 2000).

Water-restraint stress. Some animals were stressed for 15 min by water restraint on the third day after viral particle injection, as modified from a previously described procedure (Pare, 1994). Animals were loosely restrained in an envelope constructed of plastic mesh so that they could not make gross body movements, and they were suspended to the level of their necks in 25°C water for 15 min (using an FST chamber). The animals were then wiped briefly with a towel, dried under a lamp, and returned to their home cage.

Open-field test. Animals were either tested 3 d after viral particle injection (no stress exposure) or 24 hr later after exposure to water-restraint stress. The open-field test (OFT) was performed using a 45-cm-square black Plexiglas enclosure with 30-cm-tall walls set on a non-reflective black plastic base divided into a grid of nine equal squares. The OFT arena was located in a small, quiet, light-proof room with video monitoring so that the researcher could leave the room immediately after placing the rat in the center of the arena. Animals were tested between 4 and 6 P.M. under low illumination red light, to which Sprague Dawley rats are blind, thereby simulating darkness and increasing locomotor activity. Behavioral data were collected by videotape for 10 min; the tape was scored by a different experimenter (J.N.) in a blinded manner. The number of entries into the central square over the first 3 min and the total squares entered over 10 min were counted. The procedure and analysis used were based on previous studies suggesting that centroid entering on initial placement in the maze was most sensitive to stress-induced anxiety states (Pare, 1994; Izumi et al., 1997; Durand et al., 1999). Furthermore, factor analysis suggests that center entering assesses approach/avoidance toward aversive stimuli, which is considered a reliable index of fearfulness/anxiety and responds to anxiolytic agents (Ramos et al., 1997). Statistical comparison of between group differences was performed with the Mann–Whitney *U* test using GB-Stat software, with $p \leq 0.05$ considered significant.

Elevated plus maze. The EPM apparatus was constructed in this lab from black Plexiglas with nonreflective painted surfaces. The maze consisted of four runways (10 × 40 cm) joined by a central 10 × 10 cm square, 50 cm above the floor. Opposing arms were either open (having only a 0.5 cm lip) or enclosed by 40-cm-high walls. The maze was illuminated by a dim lamp above the apparatus (12 lux). Experimental methodology was based on previously published studies of the EPM (Handley and McBlane, 1993; Hogg, 1996). Percentage open arm entries was the key parameter assessed, because factor analysis had previously shown this index to be associated with fearfulness/anxiety (Ramos et al., 1997). Animals were introduced into the center square facing an open arm, and behavior was video recorded for 5 min and analyzed using the SMART computer analysis program (San Diego Instruments, San Diego, CA). The number of entries into open or closed arms and total distance traveled were measured. Statistical comparison of between group differences was performed with the Mann–Whitney *U* test using GB-Stat software, with $p \leq 0.05$ considered significant. Open arm time, closed arm time, open entries, and closed entries were also recorded. The center square was not considered to be part of either the open or closed arms. To confirm our ability to detect anxiolytic and anxiogenic effects in our EPM apparatus, we used the protocol of Grahn et al. (1995) to assess our methodology. Compared with vehicle alone, we found that diazepam (2 mg/kg, with 4 d pretreatment to allow tolerance to motor effects of the drug) increases and methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (a benzodiazepine inverse agonist, 0.4 mg/kg,) decreases percentage open arm entries while not altering total locomotion, as seen with our viral injections (data not shown). This finding indicates that we can detect both anxiolytic and anxiogenic effects in our EPM apparatus.

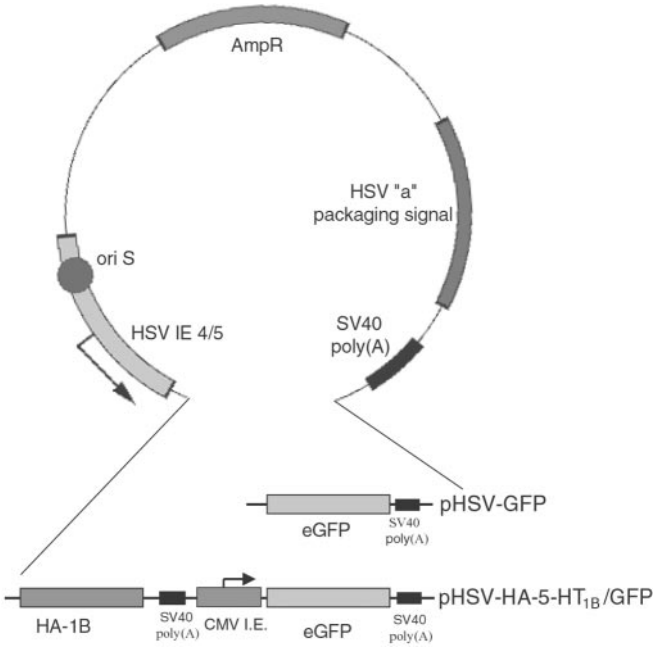

Figure 1. Amplicon maps used for HA-5-HT$_{1B}$ and GFP expression. The plasmids pHSV-HA1B/GFP and pHSV-GFP were constructed as described in Materials and Methods and were confirmed by sequence analysis. Note that either GFP alone or the HA-5-HT$_{1B}$ and GFP sequences were inserted into the pHSV-PrPUC backbone provided by Dr. Rachael Neve (Neve and Geller, 1995). In the latter case, the HA-5-HT$_{1B}$ and GFP gene sequences were interrupted by an SV40 polyadenylation signal; the two genes have different promoter/enhancers controlling expression (HSV IE 4/5 and CMV IE, respectively), to reduce competition effects. *ori S*, HSV origin of replication; *AmpR*, ampicillin resistance gene.

RESULTS

Coexpression of HA-5-HT$_{1B}$ and GFP *in vitro* using multiple promoter/enhancer elements

Because 5-HT$_{1B}$ autoreceptors in DRN neurons are translocated to axon terminals in forebrain (Hoyer and Middlemiss, 1989; Boschert et al., 1994; Ghavami et al., 1999), they may be difficult to detect in the cell body. Accordingly, it would be useful to express both the receptor and a marker protein that can be detected in the cytosol of the transfected cell. GFP, which can be detected easily both *in vivo* and *in vitro*, can provide such a cytosolic marker.

To accomplish our goal, we first introduced a hemagglutinin epitope into the N terminus of the rat 5-HT$_{1B}$ cDNA to facilitate detection of expressed transgenic receptor with commercially available antibodies. The modified cDNA was then cloned into the multiple cloning site of plasmid pCI$_{GFP}$, in which a complete expression unit containing the GFP gene driven by the CMV immediate/early (IE) promoter/enhancer, and terminated with the SV40 polyadenylation site, was inserted into the single *Bam*HI site of pCI (Fig. 1). The resulting plasmid expressed both GFP and HA-5-HT$_{1B,}$ each from a separate transcriptional unit. To determine whether this plasmid, pCI$_{GFP}$-HA1B, did indeed express both genes, it was transfected into COS7 cells by electroporation. After 72 hr, all cells that displayed GFP fluorescence also displayed HA immunoreactivity, which appeared to be concentrated at the cell membrane (Fig. 2*A*,*C*). In contrast, cells

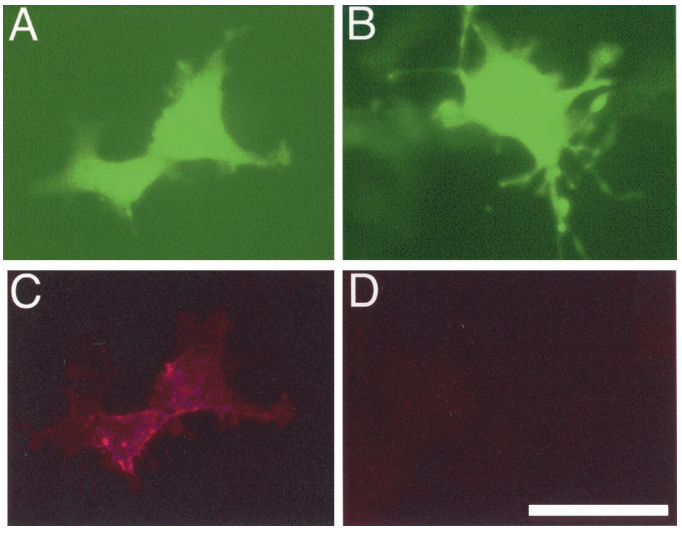

Figure 2. COS cells transfected with pCI$_{GFP}$-HA1B show dual expression. Cells were transfected with pCI$_{GFP}$-HA1B or pCI$_{GFP}$ by electroporation as described. *A* and *C* show pCI$_{GFP}$-HA1B-transfected cells; *B* and *D* show pCI$_{GFP}$-transfected cells. *A* and *B* show GFP fluorescence; *C* and *D* show hemagglutinin immunoreactivity. The HA-tagged 5-HT$_{1B}$ receptor could be detected only in the pCI$_{GFP}$-HA1B-transfected cells, and there was no apparent interaction between GFP and HA-5-HT$_{1B}$ expression in the same cells. Scale bar, 20 μm.

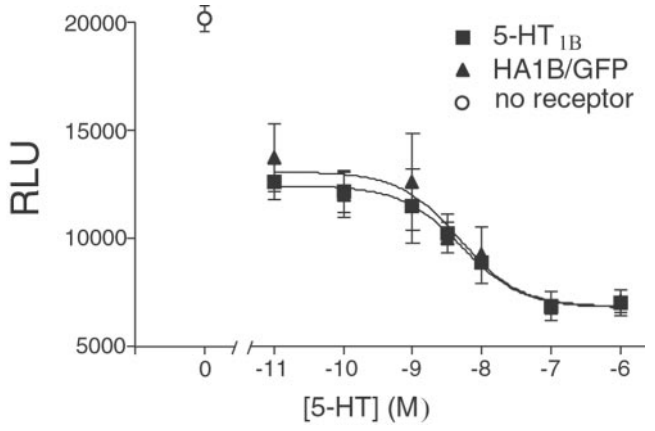

Figure 3. HA-5-HT$_{1B}$ receptors inhibit adenylate cyclase in JEG-3 cells. Adenylate cyclase activity was assayed using a luciferase reporter gene assay as described in Materials and Methods. Data points represent SD of triplicate determinations; two replicate assays were performed. The curve fits and EC$_{50}$ determinations were calculated using Prism 2.0. HA tagging of the 5-HT$_{1B}$ receptor did not appear to reduce its ability to inhibit adenylate cyclase activity, and coexpression of GFP from the same plasmid did not impair the level of HA-5-HT$_{1B}$ expression or function. *RLU*, Relative light units.

transfected with pCI$_{GFP}$ displayed only GFP fluorescence (Fig. 2*B*,*D*). Transfection of pCI$_{GFP}$-HA1B also led to expression of both gene products in HeLa cells (data not shown).

HA-5-HT$_{1B}$ receptors coexpressed with GFP decrease cAMP accumulation and forskolin-stimulated CRE activity

In mammalian cell culture, the rodent 5-HT$_{1B}$ receptor is known to inhibit adenylate cyclase via activation of G$_{i\alpha}$ (Barnes and Sharp, 1999), thereby reducing the production of cAMP. To determine whether the HA-tagged 5-HT$_{1B}$ receptor retains this activity when coexpressed with GFP, the ability of the receptor to suppress forskolin-stimulated CRE-mediated expression of a reporter gene (Mellon et al., 1989) was determined. As can be seen in Figure 3, transfection into JEG-3 cells with either pCI$_{GFP}$-HA1B or MG11B (wild-type receptor) produced 5-HT-responsive reductions in luciferase expression with an EC$_{50}$ of 5.1 and 4.8 nM, respectively, agreeing with previously published values of ~6 nM (Hamblin et al., 1992). Therefore, the HA epitope tag on the 5-HT$_{1B}$ amino terminus and coexpression of GFP do not appear to change the apparent affinity or coupling efficiency of the receptor.

HA-5-HT$_{1B}$ and GFP can be coexpressed from a single HSV amplicon *in vitro*

To produce an HSV amplicon from pCI$_{GFP}$-HA1B, the 3.8 kb restriction fragment containing 5′-HA-5-HT$_{1B}$–SV40 polyA–CMV I/E–GFP–SV40 polyA-3′ was ligated into *Xba*I/*Bam*HI cut pHSV-PrPUC. In the resulting plasmid, pHSV-HA1B/GFP, HA-5-HT$_{1B}$ cDNA is expressed from the HSV IE 4/5 promoter/enhancer, and GFP is expressed from the CMV promoter/enhancer transferred from pCI$_{GFP}$-HA1B (Fig. 1). The amplicon was then packaged using replication-deficient HSV as described previously (Neve and Geller, 1995; Neve, 1999b). The recombinant viral particles carrying pHSV-HA1B/GFP were titered using PC-12 cells by assaying for HA immunocytochemistry and GFP fluorescence, and they typically contained 1–2 $\times$ 10^8 infective units per milliliter. CA77 cells were infected with 3 μl of viral particles per well, with 50–90% infection rates. Infected CA77 cells were examined for the presence of 5-HT$_{1B}$ RNA. RT-PCR amplification of polyadenylated RNA from cells infected with pHSV-HA1B/GFP produced a robust RT-PCR product, whereas those infected with pHSV-GFP RNA had low levels of endogenous 5-HT$_{1B}$ mRNA (Clark et al., 1995), similar to that in vehicle-treated cells (Fig. 4*A*). The HA-specific RT-PCR product was evident only in pHSV-HA1B/GFP-infected cells. HA specific antibodies labeled a single band of 65 kb only in pHSV-HA1B/GFP infected cells (Fig. 4*B*). Taken together, these *in vitro* studies showed that high levels of functional HA-5-HT$_{1B}$ and GFP could be expressed from separate promoter/enhancer elements using HSV amplicons.

HSV amplicons are capable of expressing HA-5-HT$_{1B}$ and GFP gene products *in vivo*

To determine whether pHSV-HA1B/GFP is capable of infecting mammalian brain and inducing gene expression of both HA-5-HT$_{1B}$ and GFP, we injected viral particles into the rat DRN by stereotaxic surgery. After microinjection into the DRN, animals were returned to home cages for 4 d and then euthanized. The brains were either prepared for immunocytochemistry or rapidly frozen for subsequent Western blot. Tissue sections were examined for GFP fluorescence and immunostained for the presence of the HA epitope. Large numbers of GFP-positive neurons were detected at the sites of injection (Fig. 5*A*). In six consecutive brains examined 4 d after gene transfer, 930 ± 160 GFP-positive cells were counted (mean ± SEM). These counts may be an underestimate of total transgene expression because gene expression peaks on day 3 and declines gradually thereafter (Carlezon et al., 2000a; Pliakas et al., 2001), but these numbers are comparable to previous studies that used gene transfer with this vector or similar strategies (Carlezon et al., 1997; Fabre et al., 2000; Pliakas et al., 2001). There are ~11,000–15,000 serotonergic

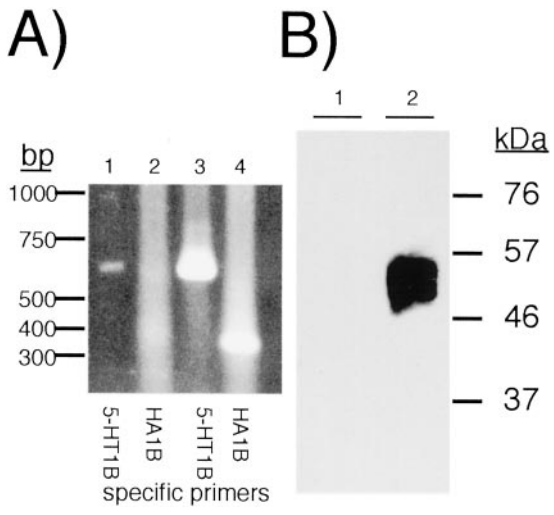

Figure 4. *In vitro* expression of pHSV-HA1B/GFP in CA77 cells. *A*, Twenty-four hours after infection with pHSV-GFP (*lanes 1*, *2*) or pHSV-HA1B/GFP (*lanes 3, 4*) (3 μl per well in six-well tissue culture plates; 1–2 × 10^8 infective units per milliliter), CA77 cells were harvested and processed for either immunoblot analysis or poly-A RNA extraction. GFP expression and HA immunoreactivity were robustly detected in ~50–90% of cells (data not shown). RT-PCR of poly-A RNA from these cells showed the presence of 5-HT$_{1B}$ mRNA using total 5-HT$_{1B}$ primers for amplification (*lanes 1*, *3*) and HA-specific primers (*lanes 2*, *4*). Vehicle and pHSV-GFP-treated CA77 cells express low levels of 5-HT$_{1B}$ RNA (*lane 1*) and no HA epitope (*lane 2*). pHSV-HA1B/GFP-treated cells showed dramatically more total 5-HT$_{1B}$ message (*lane 3*) and a strong HA-5-HT$_{1B}$-specific PCR product (*lane 4*). Vehicle-treated CA77 cells showed low levels of 5-HT$_{1B}$ mRNA, similar to pHSV-GFP (data not shown). *B*, Protein samples (5 μm) from pHSV-GFP (*lane 1*)- or pHSV-HA1B/GFP (*lane 2*)-infected CA77 cells were separated by PAGE and immobilized on membranes by Western blot. HA-specific immunoreactive protein was detected only in pHSV-HA1B/GFP-infected cells.

neurons in the full rostral–caudal extent of the nucleus (Wiklund and Bjorklund, 1980; Vertes and Crane, 1997). Therefore, we estimate that ~10% of serotonergic neurons in the anterior dorsal raphe nucleus, the area targeted in the injections, expressed HA-5-HT$_{1B}$. As shown in confocal micrographs, individual DRN neurons infected with pHSV-HA1B/GFP coexpressed GFP and HA-5-HT$_{1B}$ signals (Fig. 5*B*,*C*). Furthermore, most of the transgene-expressing DRN neurons were serotonergic, as indicated by 5-HT$_{1A}$ immunostaining (Fig. 5*D*,*E*). In some cases, either GFP or anti-HA label was apparent in a particular neuron or a specific confocal plane, but in most cases both signals colocalized in the same neurons (Fig. 5*E*). Specifically, immunolabeling was most intense near the surfaces of the tissue section, likely because of antibody penetration, whereas GFP expression was equally intense at all depths within a tissue section.

5-HT$_{1B}$ immunostaining was much more intense in GFP-expressing cells than in other serotonergic cells expressing only endogenous 5-HT$_{1B}$ receptor within the DRN (Fig. 6*A–D*). In a subset of injected brains, the DRN was removed by punch, total RNA was extracted, and total 5-HT$_{1B}$ mRNA was quantified by real-time RT-PCR. This was normalized for GAPDH mRNA. DRNs that were injected with pHSV-HA1B/GFP had approximately threefold more 5-HT$_{1B}$ mRNA than pHSV-GFP-injected brains (Fig. 6*E*). This reflects total expression in the DRN; therefore, the infected neurons probably had significantly higher levels of expression than suggested above.

Because the 5-HT$_{1B}$ receptor is translocated to axon terminals, only a small proportion of 5-HT$_{1B}$ protein would be expected in the soma, whereas the majority would be expected to be present diffusely among the termination zones of DRN axonal projections such as frontal cortex and striatum (Molliver, 1987). To determine whether midbrain DRN infection with pHSV-HA1B/GFP and pHSV-GFP induces gene expression and protein translocation to forebrain, immunocytochemistry was performed on striatal sections for HA epitope. HA immunoreactive fibers with morphology characteristic of DRN projections (Kosofsky and Molliver, 1987) are seen in the striatum of pHSV-HA1B/GFP-injected animals (Fig. 7*A*) but not pHSV-GFP-injected animals (Fig. 7*B*). To confirm this data, protein samples from frontal cortex and striatum were subjected to PAGE and immunoblot analysis. As can be seen in Figure 7*C*, rat forebrain contains an HA-immunoreactive band at 65 kDa, approximately the same size as photoaffinity-labeled CNS 5-HT$_{1B}$ receptor (Hamblin et al., 1988) and slightly larger than that seen *in vitro* (Fig. 4*B*). The HA immunoreactivity was more intense in striatum than frontal cortex, perhaps reflecting the greater density of 5-HT terminals in striatum. These findings strongly suggest that HA-5-HT$_{1B}$ receptors introduced by viral-mediated gene transfer are translocated from DRN to serotonergic axon terminals in forebrain.

HA-5-HT$_{1B}$ overexpression in rat DRN alters anxiety-related behavior after an inescapable stressor

5-HT$_{1B}$ terminal autoreceptors are downregulated by antidepressants (Artigas et al., 1996; Sayer et al., 1999) and may be upregulated in learned helpless rats (Edwards et al., 1991; Neumaier et al., 1997) but are difficult to manipulate in behavioral models without also impinging on 5-HT$_{1B}$ heteroreceptors. Therefore we sought to determine whether increasing 5-HT$_{1B}$ mRNA in DRN would induce behaviors relevant to the symptoms of depression and anxiety. We first hypothesized that because antidepressants reduce immobility in the Porsolt FST (Porsolt et al., 1977), overexpression of 5-HT$_{1B}$ autoreceptors would increase immobility in the same test. Animals received stereotaxic injections of viral particles containing either pHSV-HA1B/GFP or pHSV-GFP into DRN and were subjected to forced swim 3 d later and tested the following morning. We used the pHSV-GFP amplicon as a control treatment because it controlled for the surgical procedure, infection with viral particles, the presence of viral particle constituents, transgenic RNA expression, and expression of GFP. We believe that this represents a good control strategy for viral-mediated gene transfer studies in rat brain. The animals' behavior was coded as climbing, swimming, or immobile, as described previously (Detke et al., 1995). There were no statistically significant changes in any of these behaviors in control or experimental animals (Fig. 8).

The OFT has been used to model "emotionality" or behavioral anxiety in rodents, whose open-field behavior is altered by both stressors and antidepressants (Stockert et al., 1988; Kelly and Leonard, 1994; Pare, 1994; Meerlo et al., 1996; Izumi et al., 1997; Ramos et al., 1997; Durand et al., 1999). Factor analysis suggests that center entries are most related to approach/avoidance toward potentially aversive stimuli, which responds more strongly to anxiolytic drugs such as diazepam and is considered an index of anxiety/fearfulness (Ramos et al., 1997). Thus, we hypothesized that open entries would be altered in animals overexpressing 5-HT$_{1B}$ in the DRN. Animals were injected in DRN with viral particles carrying either pHSV-HA1B/GFP or pHSV-GFP, housed in routine conditions for 3 d without specific stress exposure, and then tested with the OFT. In the absence of specific stress exposure, pHSV-HA1B/GFP-treated animals showed

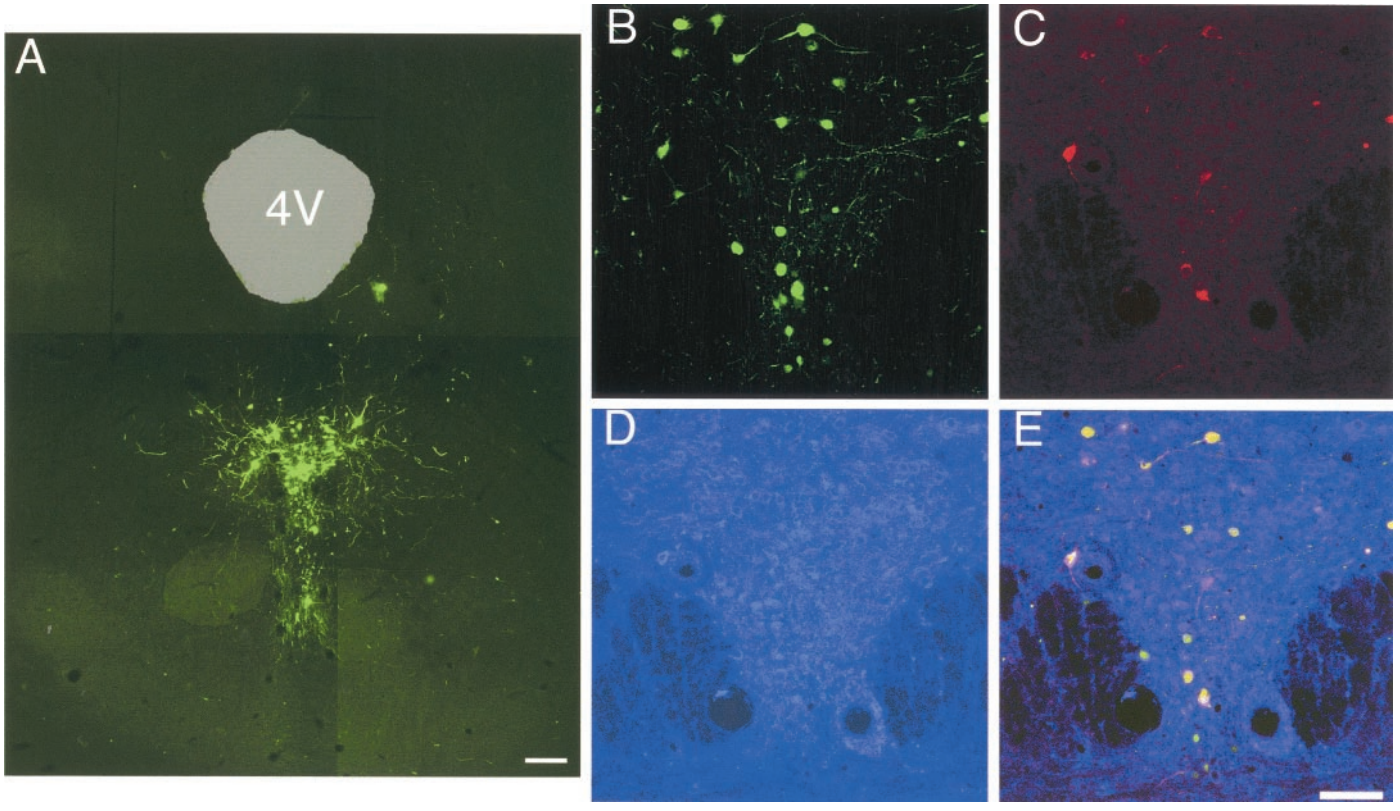

Figure 5. Coexpression of HA-5-HT$_{1B}$ and GFP *in vivo*. pHSV-HA1B/GFP viral particles were injected stereotaxically into DRN, and animals were killed 4 d later for evaluation of transgene expression by immunostaining and confocal microscopy. *A*, A composite image of several 10× fields shows clear GFP localization within the anatomic region of the DRN. The fourth ventricle (*4V*) has been colored light green for clarity. Scale bar, 100 μm. *B*, A 20× image of DRN shows GFP fluorescence in cells and beaded fibers. *C*, HA-5-HT$_{1B}$ immunostaining of the same region shown in *B*. *D*, 5-HT$_{1A}$ immunostaining of the same region shown in *B* and *C*. Because of the lower laser strength available for the secondary dye used (Alexa-633), the intensity of positive staining, typically visible as rings around darker nuclei, is relatively low. *E*, A composite image of all three signals displays cells positive for GFP, HA-5-HT$_{1B}$, and 5-HT$_{1A}$, demonstrating that serotonergic neurons have been infected and that these neurons produce both viral transgene products *in vivo*. A count of cells positive for both GFP and 5-HT$_{1A}$ in images obtained for this study showed that 80% of GFP-positive neurons were also positive for 5-HT$_{1A}$. Scale bar, 100 μm.

greater exploration of the center of the open field, with 35% more entries into the center square as compared with pHSV-GFP-treated animals (Fig. 9*A*) ($p = 0.05$). There was no difference in total locomotor activity between groups as shown by total square entries (Fig. 9*B*), indicating that overexpression of 5-HT$_{1B}$ receptors in DRN did not alter general locomotor activity. In a separate experiment, animals received viral injections followed by water-restraint stress 3 d later and were then tested in the OFT 24 hr later. This stress paradigm differs from the FST in that it prevents gross body movements (Pare, 1994). The pHSV-HA1B/GFP-injected animals entered the central region of the arena 30% less frequently than the pHSV-GFP-treated animals (Fig. 9*C*) ($p = 0.044$). Total locomotor activity, as determined by total square entries, was not affected by HA-5-HT$_{1B}$ expression (Fig. 9*D*). This suggests that the animals that received pHSV-HA1B/GFP were more sensitive to water-restraint stress than GFP controls.

To further examine the effects of 5-HT$_{1B}$ overexpression in DRN on stress-induced behaviors, we used the EPM, another commonly used test for anxiety-like behaviors (Handley and McBlane, 1993; Hogg, 1996). As in the OFT, we assessed behavior most associated with indices of anxiety. On the basis of factor analysis (Ramos et al., 1997) and response to anxiolytics (Handley and McBlane, 1993; Hogg, 1996), we chose to examine percentage open arm entries as our primary measure. Indeed, our preliminary studies with diazepam and the benzodiazepine inverse agonist methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (Grahn et al., 1995) demonstrated that this parameter was the most robust and least variable indicator of anxiety in our protocol (data not shown). Animals were exposed to water-restraint stress 3 d after viral vector injection into DRN and were tested in the EPM 24 hr later. Animals treated with pHSV-HA1B/GFP demonstrated greater anxiety-like behavior than pHSV-GFP controls, as indicated by a 20% reduction in percentage entries into the open arms of the maze (Fig. 10*A*) ($p = 0.047$). 5-HT$_{1B}$ overexpression did not significantly affect total distance traveled in the EPM (Fig. 10*B*).

DISCUSSION

Validation of HA-5-HT$_{1B}$ and GFP dual expression

Several recombinant viral vectors have been developed for use in the mammalian CNS, including gene transfer systems derived from adenovirus, adeno-associated virus, HSV, and others (Zlokovic and Apuzzo, 1997). The HSV system used here possesses many advantages for delivery of genes into postmitotic neurons, including neurotropic specificity, high infectivity, efficient extrachromosomal gene expression, and low toxicity (Neve and Geller, 1995). HSV amplicons have been used previously to introduce

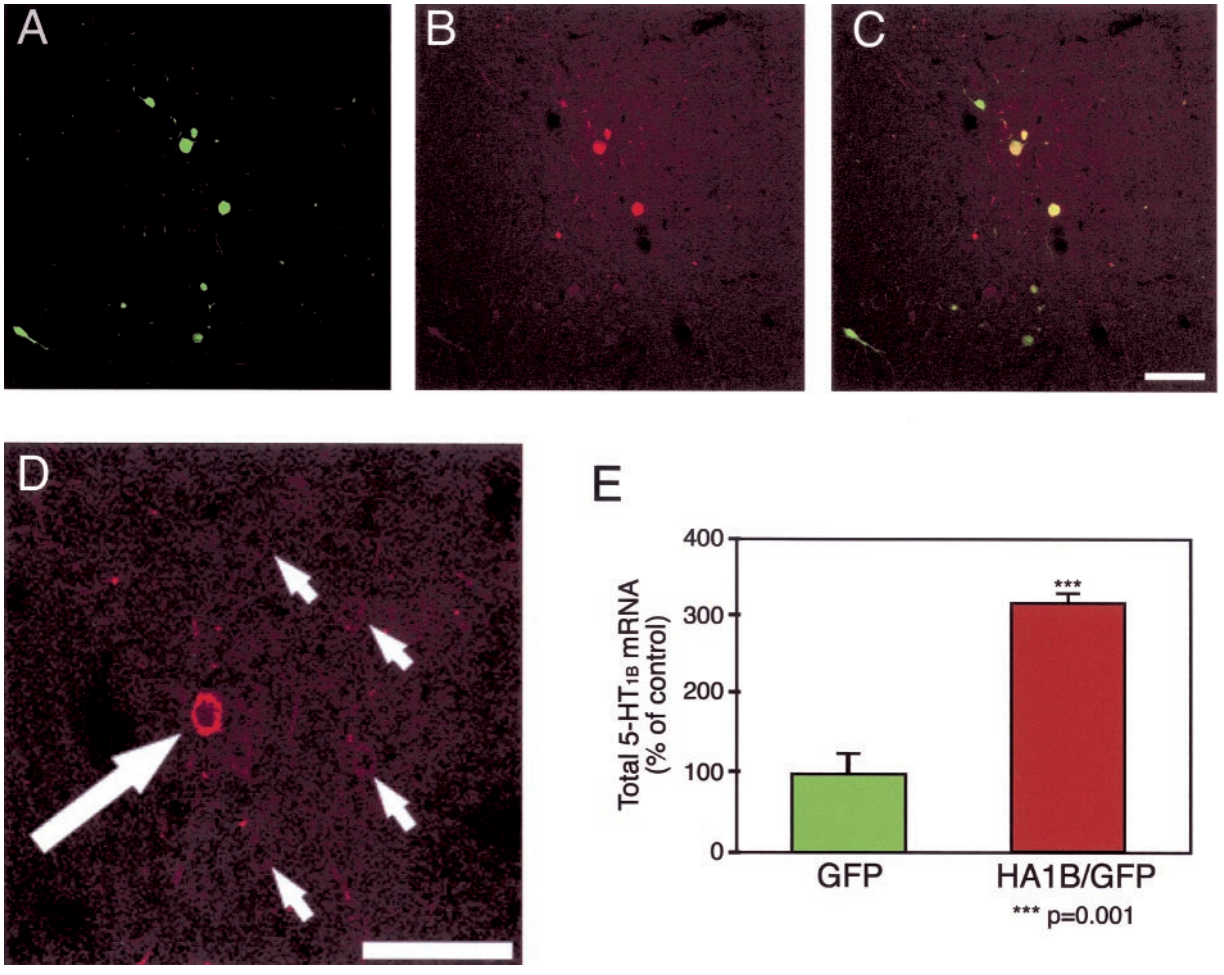

Figure 6. Injection of pHSV-HA1B/GFP increases 5-HT$_{1B}$ expression *in vivo*. Sections through the DRN were taken from animals injected at that site with pHSV-HA1B/GFP viral particles, immunostained for 5-HT$_{1B}$, and examined by confocal microscopy. *A*, GFP-containing cells and associated fibers may be clearly seen in the DRN, as shown previously. *B*, 5-HT$_{1B}$ immunoreactivity is detected within both GFP- and non-GFP-containing cells. *C*, When GFP fluorescence and 5-HT$_{1B}$ immunoreactivity are overlapped, GFP-positive cells are shown to typically display intense 5-HT$_{1B}$ immunoreactivity, as indicated by the *yellow-green coloration* of these cells. *A–C*, Magnification: 20×. Scale bar, 100 μm. *D*, In another focal plane from the same section shown in *A–C*, 5-HT$_{1B}$ immunoreactivity is much more intense in a GFP-positive cell (indicated by a *large white arrow*) compared with endogenously expressed 5-HT$_{1B}$ protein in non-GFP-positive cells (*small arrows*). The GFP-positive cell displays much greater 5-HT$_{1B}$ immunoreactivity, although both types of cells display what appears to be membrane-bound localization of 5-HT$_{1B}$ signal. Magnification: 40×. Scale bar, 100 μm. *E*, DRN punches from animals injected with either pHSV-GFP or pHSV-HA1B/GFP viral particles were analyzed by quantitative RT-PCR for 5-HT$_{1B}$ message content ($n = 9$ for each group). 5-HT$_{1B}$ mRNA levels, normalized for GAPDH mRNA expression, were 3.16-fold times higher in animals injected with pHSV-HA1B/GFP than in animals injected with pHSV-GFP (***$p = 0.001$; Student's t test).

transgenes for the purpose of overexpressing an endogenous protein of interest in discrete structures of the rat brain (Chiocca et al., 1990; Geller et al., 1991; Wood et al., 1994; Carlezon et al., 1997, 2000b; Song et al., 1998; Neve, 1999a). Double infection with two HSV vectors has been used to introduce two genes of interest into primary cortical neurons in culture (Coopersmith and Neve, 1999). Although using two viral amplicons simultaneously can lead to dual infection of some neurons, essentially all neurons that are infected with pHSV-HA1B/GFP should express both genes. This report is the first to use a single amplicon to introduce and express two genes of interest in rat brain. When viral particles were injected into DRN in rat midbrain, expression of HA-5-HT$_{1B}$ and GFP in individual neurons could be detected, and HA immunoreactivity was found in the striatum and frontal cortex only when HA-5-HT$_{1B}$/GFP was injected into DRN. We have validated that both genes are expressed *in vitro* and *in vivo*, preserving protein activity and receptor localization.

This approach made it possible to manipulate 5-HT$_{1B}$ presynaptic autoreceptors separately from 5-HT$_{1B}$ postsynaptic heteroreceptors in forebrain tissue. Such discrimination is critical in examining the role of DRN 5-HT$_{1B}$ autoreceptors in depression and anxiety, because most of the 5-HT$_{1B}$ receptors in the brain are postsynaptic heteroreceptors, located on nonserotonergic neurons, that are intermingled with 5-HT$_{1B}$ autoreceptor-containing serotonergic fibers (Verge et al., 1986; Offord et al., 1988; Jacobs and Azmitia, 1992; Sexton et al., 1999). Manipulating expression of the autoreceptor population selectively offers the opportunity to examine the behavioral role of a small but very important subpopulation of 5-HT$_{1B}$ receptors that has not been possible using other genetic or pharmacologic techniques. Fabre et al. (2000) recently used nonviral gene transfer to alter serotonin transporter expression in the rat DRN, observing alterations of circadian rhythms in animals transfected with an antisense-expressing plasmid. The nonviral approach transfects both glia

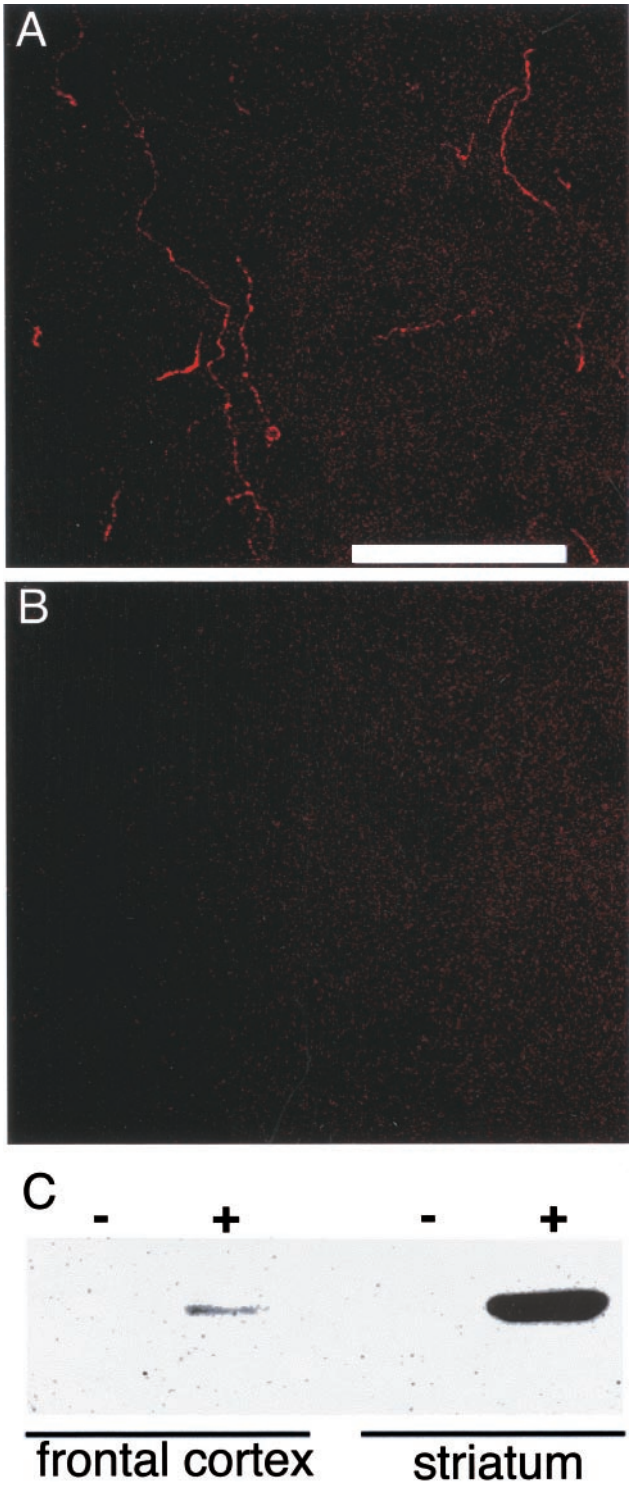

Figure 7. HA-5-HT$_{1B}$ is translocated to the forebrain. Striatal sections of animals injected in the DRN with either pHSV-HA1B/GFP or pHSV-GFP viral particles were immunostained to detect the presence of HA-5-HT$_{1B}$ immunoreactivity and examined by confocal microscopy. *A*, In animals injected with pHSV-HA1B/GFP, beaded fibers with anti-HA immunoreactivity may be clearly seen. The fibers demonstrate typical pleiomorphic varicosities, suggesting multiple sites of neurotransmitter release that are characteristic of DRN axons (Kosofsky and Molliver, 1987). *B*, In animals injected with pHSV-GFP, only background is present. In neither case was GFP detected (data not shown). Images shown are flattened, 60× confocal stacks. Scale bar, 50 μm. *C*, Western blot of HA-immunostained protein from terminals field of DRN axonal projections to forebrain. Protein samples from frontal cortex (*lanes 1*, *2*) or striatum (*lanes 3*, *4*) after DRN injection of pHSV-GFP (−) or pHSV-HA1B/GFP (+) viral particles. The single immunoreactive HA-5-HT$_{1B}$ band migrated at an apparent size of 65 kDa, perhaps reflecting glycosylation and/or other posttranslational modifications of the 49 kDa predicted protein.

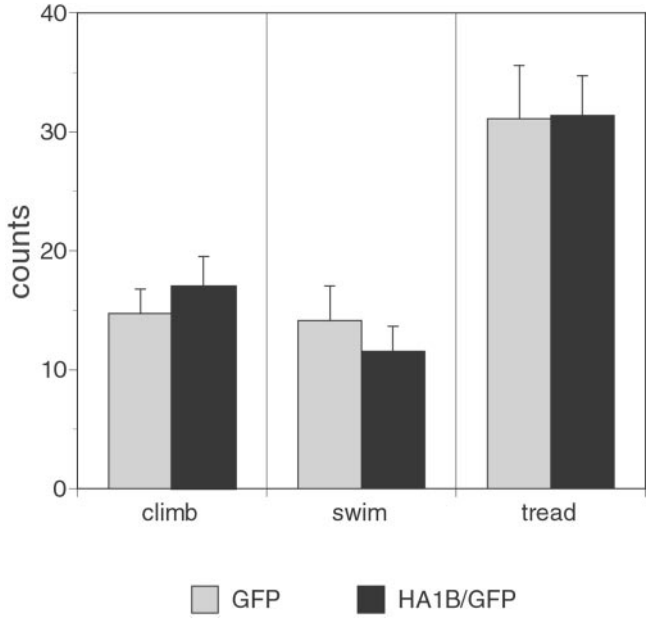

Figure 8. HA-5-HT$_{1B}$ expression in DRN neurons did not alter immobility in the forced swim test. Animals received injections of pHSV-HA1B/GFP (n = 11) or pHSV-GFP (n = 8) in DRN and were subjected to the standard FST procedure on days 3 and 4 as described in Materials and Methods. Behaviors were counted as swimming, climbing, or immobile as described in Materials and Methods. Numerical data are as follows and are presented as mean ± SEM (*climb*: GFP 14 ± 2.0, HA1B/GFP 17 ± 2.5; *swim*: GFP 14 ± 2.9, HA1B/GFP 12 ± 2.1; *tread*: GFP 31 ± 4.5, HA1B 31 ± 3.4). There were no significant differences in these behaviors between treatment groups.

and neurons. However, both viral and nonviral methods are well suited to altering gene expression in DRN because of its small size and mostly homogenous neuron type (i.e., 70% serotonergic). Although our infection rate of 10% of DRN neurons appears fairly low, previous studies using viral-mediated gene transfer demonstrating robust biological effects have often targeted larger brain regions than the DRN while infecting approximately as many neurons (Chiocca et al., 1990; Geller et al., 1991; Wood et al., 1994; Carlezon et al., 1997, 2000b; Song et al., 1998; Neve, 1999a). Because DRN has only 11,000–15,000 serotonergic neurons and we targeted the anterior section of the nucleus, we achieved an equal or higher proportion of transgene-expressing neurons within the region of interest compared with previous studies. However, our findings do not rule out the possibility of differential involvement of 5-HT$_{1B}$ autoreceptors in anxiety behaviors between subregions of the DRN or in other raphe nuclei such as the median raphe nucleus. Further investigation will likely prove useful in addressing these issues.

Analyses of behavior after HA-5-HT$_{1B}$/GFP infection of dorsal raphe nucleus

The second purpose of this study was to characterize the behavioral effects of HA-5-HT$_{1B}$/GFP expression in DRN neurons. We

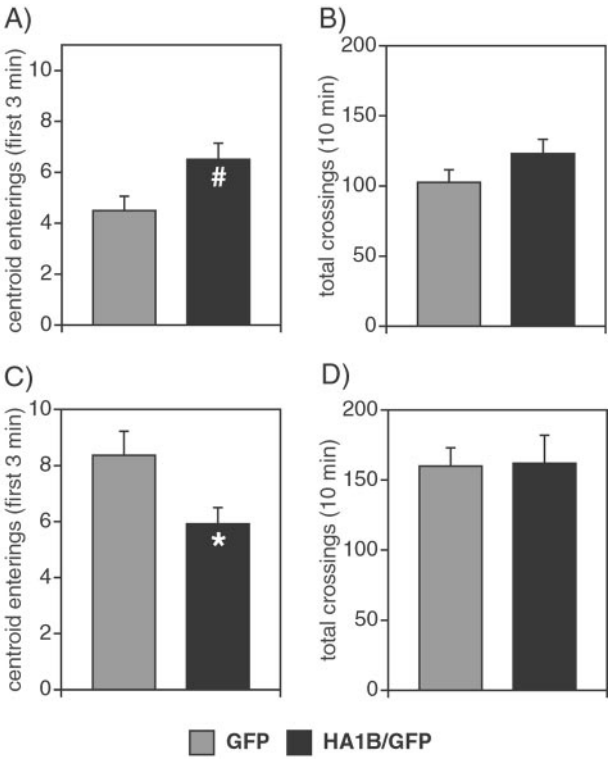

Figure 9. HA-5-HT$_{1B}$ expression in DRN neurons increased avoidance of the center of an open field only after water-restraint stress. Animals received injections of pHSV-HA1B/GFP or pHSV-GFP and either were tested in the OFT 3 d later (*A*, *B*) or subjected to water-restraint stress on day 3 and tested in the OFT 24 hr later (*C*, *D*). The numbers of entries into the central square during the first 3 min were counted and are shown as mean ± SEM (*A*, GFP 4.5 ± 0.56, HA1B/GFP 6.5 ± 0.64; *C*, GFP 8.4 ± 0.86, HA1B/GFP 5.9 ± 0.58). HA-5-HT$_{1B}$ expression increased central square entries in the absence of a specific stress exposure (#p = 0.05) but reduced entries into the central region after stress by 30% (*p = 0.044). The total number of zone crossings, shown as mean ± SEM (*B*, GFP 102 ± 9, HA1B/GFP 123 ± 10; *D*, GFP 160 ± 13, HA1B/GFP 162 ± 20), was not different between pHSV-HA1B/GFP and pHSV-GFP. n = 8–14 animals in each treatment condition.

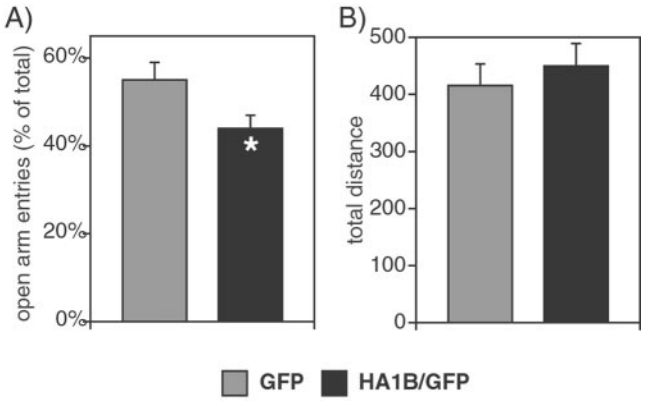

Figure 10. HA-5-HT$_{1B}$ expression in DRN reduced open arm entries in the EPM 24 hr after water-restraint stress. Animals received injections of pHSV-HA1B/GFP (n = 13) or pHSV-GFP (n = 8), were subjected to water-restraint stress 3 d later, and were tested in the EPM 24 hr later. The rat's behavior was recorded and analyzed by computer-assisted video monitoring. *A* shows the percentage of open arm/total arm entries; HA-5-HT$_{1B}$-expressing animals had significantly reduced percentage of entries into open arms (GFP 55 ± 4%; HA1B/GFP 44 ± 3%; mean ± SEM shown); *p = 0.047. *B*, There was no significant difference in total distance traveled between treatment groups (GFP 415 ± 38 cm; HA1B/GFP 450 ± 40 cm; mean ± SEM shown). Other parameters measured include percentage open time (GFP 29.6 ± 8.9; HA1B/GFP 22.3 ± 4.0), percentage closed time (GFP 51.5 ± 8.1, HA1B/GFP 54.2 ± 5.2), open entries (GFP 8.2 ± 1.0, HA1B/GFP 7.2 ± 1.1), and closed entries (GFP 7.4 ± 1.5, HA1B/GFP 9.1 ± 1.2); mean ± SEM shown. Although these parameters did not reach statistical significance ($p > 0.05$), all trends are consistent with anxiogenic effects in HA-5-HT$_{1B}$-expressing animals.

examined several behavioral paradigms to elucidate the role of 5-HT$_{1B}$ autoreceptors in depression and anxiety. The FST has been used to predict the antidepressant activity of drugs, whereas the OFT and EPM have been used to detect changes in anxiety-like behavior (Porsolt, 2000). We did not detect an effect of HA-5-HT$_{1B}$/GFP expression in DRN on immobility or struggling behaviors using the FST. This negative result could be explained by several interpretations. First, the FST may be more sensitive in detecting antidepressant activity than prodepressant activity. It is also possible that forced swim stress does not activate DRN mechanisms to the same extent as other stress procedures. Forced swim does cause region-specific changes in 5-HT release and metabolism immediately after forced swim, but no changes were detected 24 hr later (Kirby and Lucki, 1998), suggesting that forced swim either does not alter 5-HT$_{1B}$ receptor activity or does so at a different time point than we tested using the standard FST procedure. Previously we found 5-HT$_{1B}$ mRNA to be elevated in rats displaying learned helplessness in shuttle box testing after inescapable restraint and tail shock (Neumaier et al., 1997). Tail shock stress alters DRN function for at least 24 hr (Maier et al., 1995; Sutton et al., 1997; Grahn et al., 1999). Different stress paradigms have variable effects on monoamine activity in general and have different impacts on dorsal versus median raphe activity in particular (Adell et al., 1997; Durand et al., 1999). Thus, it is important to consider a range of behavioral measures in assessing the role of 5-HT$_{1B}$ autoreceptors in depression- and anxiety-related behavior.

Toward this end we also analyzed the effect of water-restraint stress on OFT and EPM behavior after HA-5-HT$_{1B}$/GFP infection of DRN. We used water restraint because it has previously been found to be useful in a rodent model of stress-induced depression, Wistar Kyoto rats (Pare, 1994), and was similar to our FST procedure. Using this assay, we found a significant reduction in central square entries after an inescapable stressor in the pHSV-HA1B/GFP group as compared with the pHSV-GFP control group. OFT procedures differ widely between different research groups, particularly in the size, shape, and lighting of the testing arena and in the behavioral outcomes measured. In this study, rats were tested in the afternoon to maximize the potential contribution of 5-HT$_{1B}$ autoreceptors (Sayer et al., 1999) and under low intensity red illumination because this leads to greater overall locomotor activity. A number of behaviors have been measured in previous OFT studies, including total locomotor activity, central entries, rearing, defecation, and others (Plaznik et al., 1988; Stockert et al., 1988; Kelly and Leonard, 1994; Pare, 1994; Meerlo et al., 1996; Izumi et al., 1997; Durand et al., 1999). We chose to measured central square entries, which reflects an approach/avoidance conflict, because this was recently shown to be particularly sensitive to stress-induced anxiety states (Ramos et al., 1997; Durand et al., 1999). There was no change in overall animal locomotor activity in the OFT, ruling out a nonspecific change in locomotor activity. Similarly, rats with 5-HT$_{1B}$ overex-

pression in DRN who were stressed by water restraint avoided the open arms of the EPM, consistent with increased anxiety-like behavior, but had no greater total locomotor activity than GFP controls. Therefore, 5-HT$_{1B}$ overexpression in DRN combined with exposure to a stressor increased anxiety-like behavior 24 hr later, suggesting that the 5-HT$_{1B}$ overexpression induced an enduring change in behavior after stress.

There are at least two possible interpretations of this data. Increasing 5-HT$_{1B}$ autoreceptor expression in serotonergic DRN neurons either makes the animals more anxious directly or increases the impact of stress on anxiety behaviors. When we examined open-field behavior in the absence of stress exposure, pHSV-HA1B/GFP did not decrease entries into the center of the open field. Indeed, 5-HT$_{1B}$ overexpression in DRN in the absence of stress increased exploration of the central square. Although it is not clear why increased 5-HT$_{1B}$ autoreceptor expression might lower anxiety behavior in an unstressed animal, we have recently observed that 5-HT$_{1B}$ mRNA is elevated in the stress-resistant group from two models of differential stress susceptibility when the animals had not been stressed (Neumaier et al., 2002). The findings suggest a complex role for the 5-HT$_{1B}$ autoreceptor in modulating anxiety. Our working hypothesis is that the impact of 5-HT$_{1B}$ overexpression in DRN is dependent on context (in this case, exposure to stress). Thus in the absence of stress, 5-HT$_{1B}$ autoreceptor overexpression may increase an approach toward potentially aversive stimuli, whereas in the presence of stress, these stimuli may provoke increased anxious behavior. Because serotonin release is increased or decreased by stress in different brain regions at different time points (Adell et al., 1997; Amat et al., 1998; Kirby and Lucki, 1998), there are likely to be discrete regulatory mechanisms that have yet to be elucidated.

Although OFT, EPM, and FST behaviors may involve different behavioral circuits and do not necessarily change in a unified manner (Plaznik et al., 1988; West and Weiss, 1998; Durand et al., 1999; Page et al., 1999), forced swim appears to have less impact on 5-HT$_{1B}$ autoreceptor mechanisms than inescapable water-restraint (this study) or inescapable restraint with tail shock (Neumaier et al., 1997). Controllability of stress is especially important in activating the DRN (Grahn et al., 1999). This may explain why we detected larger effects of water restraint than forced swim in this study: the combination of water stress and restraint may have potently impacted the dimension of controllability. The behavioral measures used in this study may be less dependent on the amygdala, which receives serotonergic innervation predominantly from DRN, than other models such as learned helplessness, fear conditioning, and social interaction indices of stress (Maier et al., 1993; Gonzalez et al., 1996; Amat et al., 1998). It will be interesting to examine the effect of HA-5-HT$_{1B}$/GFP expression using other stress paradigms such as inescapable tail shock, and in other testing paradigms, to understand the role of 5-HT$_{1B}$ autoreceptors more fully.

In summary, we have used a modification of HSV-based gene delivery to overexpress 5-HT$_{1B}$ mRNA in DRN and to express GFP as a vital marker of neuronal infection. The dual expressing amplicon led to functional expression of membrane-bound, epitope-tagged 5-HT$_{1B}$ receptors *in vitro* and *in vivo*. Overexpression of HA-5-HT$_{1B}$/GFP in DRN had marked effects on stress-sensitive behaviors in the open-field paradigm. This manipulation is particularly well suited to studying the 5-HT$_{1B}$ autoreceptor in DRN because only the region of interest is directly altered, and exogenous agonist treatment is not necessary. Because 5-HT$_{1B}$ autoreceptors are thought to be predominantly active in the axon terminals and not in the vicinity of DRN (Pineyro et al., 1995), HA-5-HT$_{1B}$/GFP effects in animal models of depression or anxiety can be attributed mainly to these axonal projections. Although the cellular specificity of acute gene transfer strategies is still incomplete, it offers some advantages over stem cell approaches (e.g., null mutant or "knock back in" mice) that have either no cellular specificity at all or incomplete regional specificity. Viral-mediated gene transfer also allows one to test hypotheses efficiently in various animal strains with different genetic backgrounds. Combining stem cell and acute gene transfer strategies will help circumvent anatomical complexity and the lack of sufficiently selective drugs to study the cellular basis of the involvement of serotonin in behavior.

REFERENCES

Adell A, Casanovas JM, Artigas F (1997) Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. Neuropharmacology 36:735–741.

Amat J, Matus-Amat P, Watkins LR, Maier SF (1998) Escapable and inescapable stress differentially alter extracellular levels of 5-HT in the basolateral amygdala of the rat. Brain Res 812:113–120.

Anthony JP, Sexton TJ, Neumaier JF (2000) Antidepressant induced regulation of 5-HT$_{1B}$ mRNA in rat dorsal raphe nucleus reverses rapidly following drug discontinuation. J Neurosci Res 61:82–87.

Artigas F, Romero L, de Montigny C, Blier P (1996) Acceleration of the effect of selected antidepressant drugs in major depression by 5-HT$_{1A}$ antagonists. Trends Neurosci 19:378–383.

Barnes NM, Sharp T (1999) A review of central 5-HT receptors and their function. Neuropharmacology 38:1083–1152.

Bergqvist PB, Bouchard C, Blier P (1999) Effect of long-term administration of antidepressant treatments on serotonin release in brain regions involved in obsessive-compulsive disorder. Biol Psychiatry 45:164–174.

Boschert M, Amara DA, Segu L, Hen R (1994) The mouse 5-hydroxytryptamine$_{1B}$ receptor is localized predominantly on axon terminals. Neuroscience 58:167–182.

Briley M, Moret C (1993) Neurobiological mechanisms involved in antidepressant therapies. Clin Neuropharmacol 16:387–400.

Carlezon Jr WA, Boundy VA, Haile CN, Lane SB, Kalb RG, Neve RL, Nestler EJ (1997) Sensitization to morphine induced by viral-mediated gene transfer. Science 277:812–814.

Carlezon Jr WA, Nestler EJ, Neve RL (2000a) Herpes simplex virus-mediated gene transfer as a tool for neuropsychiatric research. Crit Rev Neurobiol 14:47–67.

Carlezon Jr WA, Haile CN, Coppersmith R, Hayashi Y, Malinow R, Neve RL, Nestler EJ (2000b) Distinct sites of opiate reward and aversion within the midbrain identified using a herpes simplex virus vector expressing GluR1. J Neurosci 20:RC62(1–5).

Chiocca EA, Choi BB, Cai WZ, DeLuca NA, Schaffer PA, DiFiglia M, Breakefield XO, Martuza RL (1990) Transfer and expression of the lacZ gene in rat brain neurons mediated by herpes simplex virus mutants. New Biol 2:739–746.

Clark MS, Lanigan TM, Page NM, Russo AF (1995) Induction of a serotonergic and neuronal phenotype in thyroid C-cells. J Neurosci 15:6167–6178.

Coopersmith R, Neve RL (1999) Expression of multiple proteins within single primary cortical neurons using a replication deficient HSV vector. Biotechniques 27:1156–1160.

Detke MJ, Rickels M, Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. Psychopharmacology 121:66–72.

Durand M, Berton O, Aguerre S, Edno L, Combourieu I, Mormede P, Chaouloff F (1999) Effects of repeated fluoxetine on anxiety-related behaviours, central serotonergic systems, and the corticotropic axis in SHR and WKY rats. Neuropharmacology 38:893–907.

Edwards E, Harkins K, Wright G, Henn FA (1991) 5-HT1b receptors in an animal model of depression. Neuropharmacology 30:101–105.

Fabre V, Boutrel B, Hanoun N, Lanfumey L, Fattaccini CM, Demeneix B, Adrien J, Hamon M, Martres MP (2000) Homeostatic regulation of serotonergic function by the serotonin transporter as revealed by non-viral gene transfer. J Neurosci 20:5065–5075.

Geller AI, Keyomarsi K, Bryan J, Pardee AB (1990) An efficient deletion mutant packaging system for defective herpes simplex virus vectors: potential applications to human gene therapy and neuronal physiology. Proc Natl Acad Sci USA 87:8950–8954.

Geller AI, During MJ, Neve RL (1991) Molecular analysis of neuronal physiology by gene transfer into neurons with herpes simplex virus vectors. Trends Neurosci 14:428–432.

Ghavami A, Stark KL, Jareb M, Ramboz S, Segu L, Hen R (1999)

Differential addressing of 5-HT1A and 5-HT1B receptors in epithelial cells and neurons. J Cell Sci 112:967–976.
Gonzalez LE, Andrews N, File SE (1996) 5-HT1A and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain Res 732:145–153.
Grahn RE, Kalman BA, Brennan FX, Watkins LR, Maier SF (1995) The elevated plus-maze is not sensitive to the effect of stressor controllability in rats. Pharmacol Biochem Behav 52:565–570.
Grahn RE, Will MJ, Hammack SE, Maswood S, McQueen MB, Watkins LR, Maier SF (1999) Activation of serotonin-immunoreactive cells in the dorsal raphe nucleus in rats exposed to an uncontrollable stressor. Brain Res 826:35–43.
Hamblin MW, Adriaenssens PI, Ariani K, Tan GL, Ciaranello RD (1988) Photoaffinity labeling of rat brain 5-HT_{1B} receptors with [^{125}I]iodocyanopindolol diazirine. Soc Neurosci Abstr 14:313.
Hamblin MW, McGuffin RW, Metcalf MA, Dorsa DM, Merchant KA (1992) Distinct 5-HT_{1B} and 5-HT_{1D} serotonin receptors in rat: structural and pharmacological comparison of the two cloned receptors. Mol Cell Neurosci 3:578–587.
Handley SL, McBlane JW (1993) An assessment of the elevated X-maze for studying anxiety and anxiety-modulating drugs. J Pharmacol Toxicol Methods 29:129–138.
Heidmann DE, Szot P, Kohen R, Hamblin MW (1998) Function and distribution of three rat 5-hydroxytryptamine 7 (5-HT7) receptor isoforms produced by alternative splicing. Neuropharmacology 37:1621–1632.
Hen R, Boschert U, Lemeur M, Dierich A, Ait Amara D, Buhot MC, Segu L, Misslin R, Saudou F (1993) 5-HT1B receptor "knock-out": pharmacological and behavioral consequences. Soc Neurosci Abstr 19:632.
Hjorth S, Suchowski CS, Galloway MP (1995) Evidence for 5-HT autoreceptor-mediated, nerve impulse-independent, control of 5-HT synthesis in the rat brain. Synapse 19:170–176.
Hogg S (1996) A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 54:21–30.
Hoyer D, Middlemiss DN (1989) Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. Trends Pharmacol Sci 10:130–132.
Izumi J, Washizuka M, Hayashi-Kuwabara Y, Yoshinaga K, Tanaka Y, Ikeda Y, Kiuchi Y, Oguchi K (1997) Evidence for a depressive-like state induced by repeated saline injections in Fischer 344 rats. Pharmacol Biochem Behav 57:883–888.
Jacobs BL, Azmitia EC (1992) Structure and function of the brain serotonin system. Physiol Rev 72:165–229.
Kelly JP, Leonard BE (1994) The effect of tianeptine and sertraline in three animal models of depression. Neuropharmacology 33:1011–1016.
Kirby LG, Lucki I (1998) The effect of repeated exposure to forced swimming on extracellular levels of 5-hydroxytryptamine in the rat. Stress 2:251–263.
Kohen R, Metcalf MA, Khan N, Druck T, Huebner K, Lachowicz JE, Meltzer HY, Sibley DR, Roth BL, Hamblin MW (1996) Cloning, characterization, and chromosomal localization of a human 5-HT6 serotonin receptor. J Neurochem 66:47–56.
Kosofsky BE, Molliver ME (1987) The serotoninergic innervation of cerebral cortex: different classes of axon terminals arise from dorsal and median raphe nuclei. Synapse 1:153–168.
Maier SF, Grahn RE, Kalman BA, Sutton LC, Wiertelak EP, Watkins LR (1993) The role of the amygdala and dorsal raphe nucleus in mediating the behavioral consequences of inescapable shock. Behav Neurosci 107:377–388.
Maier SF, Busch CR, Maswood S, Grahn RE, Watkins LR (1995) The dorsal raphe nucleus is a site of action mediating the behavioral effects of the benzodiazepine receptor inverse agonist DMCM. Behav Neurosci 109:759–766.
Meerlo P, Overkamp GJ, Benning MA, Koolhaas JM, Van den Hoofdakker RH (1996) Long-term changes in open field behaviour following a single social defeat in rats can be reversed by sleep deprivation. Physiol Behav 60:115–119.
Mellon PL, Clegg CH, Correll LA, McKnight GS (1989) Regulation of transcription by cyclic AMP-dependent protein kinase. Proc Natl Acad Sci USA 86:4887–4891.
Migeon JC, Nathanson NM (1994) Differential regulation of cAMP-mediated gene transcription by m1 and m4 muscarinic acetylcholine receptors. Preferential coupling of m4 receptors to Gi alpha-2. J Biol Chem 269:9767–9773.
Molliver ME (1987) Serotonergic neuronal systems: what their anatomic organization tells us about function. J Clin Psychopharmacol 7:3S–23S.
Neumaier JF, Root DC, Hamblin MW (1996a) Chronic fluoxetine reduces serotonin transporter mRNA and 5-HT1B mRNA in a sequential manner in the rat dorsal raphe nucleus. Neuropsychopharmacology 15:515–522.
Neumaier JF, Szot P, Peskind E, Dorsa DM, Hamblin MW (1996b) Serotonergic lesioning differentially effects presynaptic and postsynaptic 5-HT_{1B} receptor mRNA levels in rat brain. Brain Res 722:50–58.
Neumaier JF, Petty F, Kramer GL, Szot P, Hamblin MW (1997) Learned helplessness increases 5-hydroxytryptamine 1B receptor mRNA levels in the rat dorsal raphe nucleus. Biol Psychiatry 41:668–674.
Neumaier JF, Sexton TJ, Hamblin MW, Beck SG (2000) Corticosteroids regulate 5-HT1A but not 5-HT1B receptor mRNA in rat hippocampus. Mol Brain Res 82:65–73.
Neumaier JF, Edwards E, Plotsky PM (2002) 5-HT1B mRNA regulation in two animal models of altered stress reactivity. Mol Psychiatry, in press.
Neve RL (1999a) Overview of gene delivery into cells using HSV-1-based vectors. In: Current protocols in neuroscience (Hall ZW, ed), Section 4.12. New York: Wiley.
Neve RL (1999b) Generation of high-titer defective HSV-1 vectors. In: Current protocols in neuroscience (Hall ZW, ed), Section 4.13. New York: Wiley.
Neve RL, Geller AI (1995) A defective herpes simplex virus vector system for gene delivery into the brain: comparison with alternative gene delivery systems and usefulness for gene therapy. Clin Neurosci 3:262–267.
Offord SJ, Ordway GA, Frazer A (1988) Application of [^{125}I]iodocyanopindolol to measure 5-hydroxytryptamine$_{1B}$ receptors in the brain of the rat. J Pharmacol Exp Ther 244:144–153.
Page ME, Detke MJ, Dalvi A, Kirby LG, Lucki I (1999) Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. Psychopharmacology 147:162–167.
Pare WP (1994) Open field, learned helplessness, conditioned defensive burying, and forced-swim tests in WKY rats. Physiol Behav 55:433–439.
Petty F, Kramer G, Wilson L (1992) Prevention of learned helplessness: in vivo correlation with cortical serotonin. Pharmacol Biochem Behav 43:361–367.
Pineyro G, de Montigny C, Blier P (1995) 5-HT1D receptors regulate 5-HT release in the rat raphe nuclei. In vivo voltammetry and in vitro superfusion studies. Neuropsychopharmacology 13:249–260.
Plaznik A, Tamborska E, Hauptmann M, Bidzinski A, Kostowski W (1988) Brain neurotransmitter systems mediating behavioral deficits produced by inescapable shock treatment in rats. Brain Res 447:122–132.
Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon Jr WA (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. J Neurosci 21:7397–7403.
Porsolt RD (2000) Animal models of depression: utility for transgenic research. Rev Neurosci 11:53–58.
Porsolt RD, Le Pichon M, Jalfre M (1977) Depression: a new animal model sensitive to antidepressant treatments. Nature 266:730–732.
Ramos A, Berton O, Mormede P, Chaouloff F (1997) A multiple-test study of anxiety-related behaviours in six inbred rat strains. Behav Brain Res 85:57–69.
Roberts C, Belenguer A, Middlemiss DN, Routledge C (1998) Differential effects of 5-HT1B/1D receptor antagonists in dorsal and median raphe innervated brain regions. Eur J Pharmacol 346:175–180.
Sayer TJ, Hannon SD, Redfern PH, Martin KF (1999) Diurnal variation in 5-HT1B autoreceptor function in the anterior hypothalamus in vivo: effect of chronic antidepressant drug treatment. Br J Pharmacol 126:1777–1784.
Sexton T, McEvoy C, Neumaier J (1999) (+)3,4-Methylenedioxymethamphetamine ("Ecstasy") transiently increases striatal 5-HT1B binding sites without altering 5-HT1B mRNA in rat brain. Mol Psychiatry 4:572–579.
Sherman AD, Petty F (1980) Neurochemical basis of the action of antidepressants on learned helplessness. Behav Neural Biol 30:119–134.
Song S, Wang Y, Bak SY, During MJ, Bryan J, Ashe O, Ullrey DB, Trask LE, Grant FD, O'Malley KL, Riedel H, Goldstein DS, Neve KA, LaHoste GJ, Marshall JF, Haycock JW, Neve RL, Geller AI (1998) Modulation of rat rotational behavior by direct gene transfer of constitutively active protein kinase C into nigrostriatal neurons. J Neurosci 18:4119–4132.
Stockert M, Serra J, De Robertis E (1988) Effect of olfactory bulbectomy and chronic amitryptiline treatment in rats. 3H-imipramine binding and behavioral analysis by swimming and open field tests. Pharmacol Biochem Behav 29:681–686.
Sutton LC, Grahn RE, Wiertelak EP, Watkins LR, Maier SF (1997) Inescapable shock-induced potentiation of morphine analgesia in rats: involvement of opioid, GABAergic, and serotonergic mechanisms in the dorsal raphe nucleus. Behav Neurosci 111:816–824.
Tverberg LA, Russo AF (1992) Cell-specific glucocorticoid repression of calcitonin/calcitonin gene-related peptide transcription. Localization to an 18-base pair basal enhancer element. J Biol Chem 267:17567–17573.

Verge D, Daval G, Marcinkiewicz M, Patey A, el Mestikawy S, Gozlan H, Hamon M (1986) Quantitative autoradiography of multiple 5-HT1 receptor subtypes in the brain of control or 5,7-dihydroxytryptamine-treated rats. J Neurosci 6:3474–3482.

Vertes RP, Crane AM (1997) Distribution, quantification, and morphological characteristics of serotonin-immunoreactive cells of the supralemniscal nucleus (B9) and pontomesencephalic reticular formation in the rat. J Comp Neurol 378:411–424.

West CH, Weiss JM (1998) Effects of antidepressant drugs on rats bred for low activity in the swim test. Pharmacol Biochem Behav 61:67–79.

Wiklund L, Bjorklund A (1980) Mechanisms of regrowth in the bulbospinal serotonin system following 5,6-dihydroxytryptamine induced axotomy. II. Fluorescence histochemical observations. Brain Res 191:109–127.

Wiklund L, Leger L, Persson M (1981) Monoamine cell distribution in the cat brain stem. A fluorescence histochemical study with quantification of indolaminergic and locus coeruleus cell groups. J Comp Neurol 203:613–647.

Wood MJ, Byrnes AP, Kaplitt MG, Pfaff DW, Rabkin SD, Charlton HM (1994) Specific patterns of defective HSV-1 gene transfer in the adult central nervous system: implications for gene targeting. Exp Neurol 130:127–140.

Zhukovskaya NL, Neumaier JF (2000) Clozapine downregulates 5-hydroxytryptamine($_6$) (5-HT_6) and upregulates 5-HT_7 receptors in HeLa cells. Neurosci Lett 288:236–240.

Zlokovic BV, Apuzzo ML (1997) Cellular and molecular neurosurgery: pathways from concept to reality–part II: vector systems and delivery methodologies for gene therapy of the central nervous system. Neurosurgery 40:805–812.