Increased Expression of 5-HT$_6$ Receptors in the Rat Dorsomedial Striatum Impairs Instrumental Learning

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A number of studies have shown that systemic 5-HT$_6$ receptor antagonists can improve learning and memory, but the mechanism for these observations is not known. As striatum normally expresses 5-HT$_6$ receptors abundantly and is important in consolidating stimulus-response learning, we used targeted gene delivery to further increase the expression of 5-HT$_6$ receptors in rat striatum and then examined learning. Increased 5-HT$_6$ expression had no effect on performance in the Morris water maze, a hippocampal-dependent learning paradigm, and did not alter the latency to approach or consume sucrose tablets. However, rats with increased 5-HT$_6$ expression failed to acquire a reward-based instrumental learning task, a striatum-dependent learning model, during 3 days of successive sessions as compared to sham surgery or GFP-expressing control rats. This behavioral deficit was observed in rats overexpressing 5-HT$_6$ receptors in the dorsomedial striatum, but not in rats with increased dorsocentral striatal expression. The 5-HT$_6$ receptor-associated deficit was reversed by administration of a 5-HT$_6$ antagonist, SB-258585, before each training session. When animals learned the instrumental learning task before gene transfer, increased 5-HT$_6$ receptor expression had no effect on long-term recall or performance of the task or on extinction of operant responding. Thus, 5-HT$_6$ receptor activity in rat striatum disrupts acquisition of new instrumental learning but does not impair memory or performance of reward-motivated behavior once established.
Neuropsychopharmacology (2007) **32,** 1520–1530; doi:10.1038/sj.npp.1301284; published online 27 December 2006

Keywords: memory; cognition; serotonin; operant; viral gene transfer; behavior

INTRODUCTION

Serotonin has received increasing attention as a modulator of learning and memory mechanisms, but the multiplicity of serotonin receptors and the difficulty in selectively modulating some of these receptors has impeded the understanding of how this neurotransmitter modulates critical learning behaviors. 5-HT$_6$ receptors are one of the most recently identified serotonin receptor subtypes (Monsma *et al*, 1993; Plassat *et al*, 1993; Ruat *et al*, 1993), and are coupled to a G stimulatory protein, inducing cAMP through adenylate cyclase stimulation (Monsma *et al*, 1993). Several *in situ* hybridization studies have shown robust mRNA expression in rat and human striatum, nucleus accumbens, cortex and olfactory tubercle, with moderate expression in the hippocampus and thalamus (Gerard *et al*, 1996; Ward and Dorsa, 1996), whereas expression in mouse brain is very low compared to rat or human brain (Hirst *et al*, 2003). Immunohistochemical localization of 5-HT$_6$ receptors has

been detected in the above regions as well as the cerebellum and substantia nigra (Gerard *et al*, 1997; Hamon *et al*, 1999).

Over the last 10 years, increasing evidence indicates that 5-HT$_6$ receptors play a role in memory consolidation (Meneses, 2001; Rogers and Hagan, 2001; Woolley *et al*, 2001; Foley *et al*, 2004; King *et al*, 2004; Mitchell and Neumaier, 2005). SB-271046, a 5-HT$_6$ antagonist, has been shown to improve memory consolidation of passive avoidance, novel object recognition and autoshaping (Meneses, 2001; Woolley *et al*, 2001; Foley *et al*, 2004). In general, it appears that 5-HT6 blockade is more consistently effective in alleviating memory deficits rather than increasing memory in normally functioning animals.

Another 5-HT$_6$ selective antagonist, BGC20-761, enhances memory consolidation and reverses scopolamine-induced memory deficit in social and visuospatial memory tasks (Mitchell *et al*, 2006). The mechanism of enhanced consolidation is not completely understood; however, microdialysis studies have shown that 5-HT$_6$ inhibition increases acetylcholine and glutamate release in the cortex and hippocampus (Dawson *et al*, 2001; Riemer *et al*, 2003). Although a number of different antagonists have been tested, 5-HT$_6$ receptor activation in learning has not been investigated, and the anatomical basis for 5-HT$_6$ receptor effects on memory and learning have not been examined.

The striatum has the highest density of 5-HT$_6$ expression in rat brain (Gerard *et al*, 1997; East *et al*, 2002), where

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receptors have been identified on medium spiny neurons (Ward and Dorsa, 1996), which make 95% of the neurons in this region. The dorsomedial striatum has been shown to be important for instrumental learning such as habituation to lever pressing (Hartley and Burgess, 2005), which may be influenced by serotoninergic activity (Harrison et al, 1999). Therefore, this region seemed like a logical place to investigate the effects of altered 5-HT$_6$ expression on learning behavior. To examine the contribution of 5-HT$_6$ receptors in the striatum to instrumental learning, we used viral-mediated gene transfer to increase 5-HT$_6$ receptor expression followed by behavioral testing. This approach has the advantage of regional, temporal, and pharmacological specificity that could not have been easily achieved by other means, and does not disrupt the spatial and temporal patterning of information involved in endogenous serotonin neurotransmission. The present results suggest that serotonin neurotransmission via striatal 5-HT$_6$ receptors has a potent effect on instrumental learning.

MATERIALS AND METHODS

Construction of the 5-HT$_6$ Viral Vector

We used a modified version of the herpes simplex virus amplicon that expresses a desired transgene and green fluorescent protein (GFP) under separate transcriptional control that has been previously described (Clark et al, 2002). A hemagglutinin (HA) epitope tag coding sequencing was added to the N-terminus of the rat 5-HT$_6$ receptor sequence by PCR cloning. This PCR product was subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA). From the TOPO plasmid, the inserted PCR product was cut out using BglII and AvrII sites engineered into the PCR primers. This fragment was inserted into the viral amplicon behind the HSV promoter; GFP expression was under the control of the CMV I/E promoter/enhancer. The HA-5-HT$_6$ sequence of the pHSV-HA6/GFP amplicon was confirmed in its entirety. The pHSV-HA6/GFP amplicon (Figure 2a) was packaged into HSV viral particles as previously described (Clark et al, 2002) yielding approximately 1×10^8 infective units/ml.

Confirmation of Transgene Function

Functional characteristics of the HA-modified 5-HT$_6$ receptor was evaluated using a luciferase-based cAMP assay (Kohen et al, 2001). PC12 cells were seeded onto 24-well plates, and grown to 50% confluence in Dulbecco's modified Eagle's media supplemented with 12% fetal calf serum and ampicillin. Using FuGENE (Roche, Palo Alto, CA), cells were transfected with 0.5 ng/well of rat wild-type 5-HT$_6$ or pHSV-HA6/GFP plasmid DNA. All wells were also transfected with 0.5 ng of plasmid containing the RSV-cAMP responsive element (CRE)-luciferase and 0.1 ng of plasmid containing the β-galactose gene (gifts from Mark Hamblin). At 24 h before the assay, media was aspirated and replaced with serum-free media. Triplicate wells were incubated with serial dilutions of 5-HT (Perkin-Elmer, Boston, MA) added to Optimem media (Invitrogen) for 4 h. Some wells were also treated with the 5-HT$_6$ antagonist, BGC20-761 (Tsai et al, 2000; Mitchell et al, 2006). Cells were rinsed with

phosphate-buffered saline (PBS) and lysed with a proprietory lysing buffer (Promega, Madison, WI). Luciferase activity was measured with a commercial assay system (steadylite, Promega), using a Perkin-Elmer Fusion microplate reader to detect luminescence. Luciferase luminescence was quantified as a percent increase over control (no 5-HT added). Transfection efficiency was measured by an X-gal assay. Briefly, the cells were rinsed several times with phosphate-buffered saline, fixed with 0.5% glutaraldehyde for 30 min, and incubated for 24 h in an X-gal Reaction buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl, and 2 mM X-Gal in PBS).

Animals

In all, 160 male Long-Evans rats, 300 g in weight, were ordered from Charles River Laboratories and housed according to the guidelines of the IACUC of University of Washington. Rats were pair-housed, kept in a 12:12 light/dark schedule (lights on at 0600 hours) and fed ad lib food and water unless otherwise specified.

Stereotaxic Surgery

Surgery was performed under aseptic conditions and general anesthesia was maintained using 2–3% isoflurane. The scalp of the rat was shaved and wiped with Betadine and secured into a stereotaxic device (Stoelting, Woodale, IL) with blunt ear-bars. After a vertical scalp incision, the skin covering the skull was retracted and bregma was located. Jeweler's screws were drilled into two caudal regions of the skull to provide anchorage. Two single barrel 26-gauge guide cannulas with dummy cannulas in place (Plastics One, Roanoke, VA) were inserted at the dorsomedial striatum A/P −0.8, M/D ±2.8 from the bregma (Paxinos and Wats, 1982), advanced to 5.0 mm below dura, and secured with dental cement (Supplementary Figure 1). Rats from an additional set were implanted with cannulas in the dorsal central striatum A/P −0.2, M/D ±3.3 as an anatomical control (Supplementary Figure 2). The skin around the dental cement was sutured and antibiotic ointment was applied to the scalp. The cannulas were covered by screw-on plastic caps. Each rat received buprenorphine (s.c. 0.5 mg/kg) to reduce post-surgical pain. The rats were allowed to recover over the course of 3 days.

Viral Vector Microinjections and Feeding Schedule

We used a viral vector injection method modified for the striatum that has been previously described by this and other labs (Carlezon et al, 2000a, b; Clark et al, 2002; Neumaier et al, 2002). At 4 days after surgery, the rats were started on a food-restricted diet of 20 g of rat chow pellets and ad lib water for 10 days before operant conditioning was started. During the reduced feeding period, the rats were housed singly so that their food intake could be accurately monitored. The rats were weighed daily to ascertain a consistent weight loss. The rats typically lost 10–15% of their original weight. While the rats were being tested, they were kept on the same diet, being fed at 1700 hours each day.

At 3 days before operant conditioning, rats were briefly anesthetized with 2% isoflurane and a 33-gauge infusion cannula was inserted through each implanted guide cannula and extended 1 mm below the tip of the guide cannula. Rats were infused bilaterally with 2 μl of either GFP-only (control; 200 000 infectious units in 10% sucrose) or HA6/GFP viral vector (200 000 infectious units in 10% sucrose) at a rate of 0.2 μl/min under the control of microprocessor-controlled pumps (World Precision Instruments, Sarasota, FL). The infusion cannula was left in place for at least 5 min after active infusion to ensure accurate dosing. Rats from one set, the 'sham surgery' group, were implanted with guide cannulas but did not receive viral infusions. These injection volumes and parameters produce a transgene expression zone of about 500 μm in length and 200 μm in width.

Operant Conditioning Procedure

The afternoon before testing, rats were presented with five sucrose pellets (45 mg, Bio-Serv, Frenchtown, NJ) and the intervals to approach and consumption of the first pellet were recorded. Rats from one set were briefly anesthetized 1 day before behavioral testing but did not receive surgery and acted as the 'no surgery' control group.

The animals were brought to the testing room at 0930 hours every day and left for an hour before training or testing. The rats were weighed before each conditioning session. Operant boxes (Med Associates, St Albans, VT) were equipped with a house light, a stimulus light above a retracting lever, and a food receptacle. All boxes were kept in sound-attenuating chambers (Med Associates) equipped with fans providing background noise and temperature control. Experimental events were controlled and data were recorded by MED-PC 4.0 (Med Associates) software on an IBM-compatible computer. Programs written in MedState Notation were used to train rats for reward-based operant conditioning and extinction.

The rat was placed in the operant chamber 5 min before the start of a session. Each session was initiated by the activation of the house light, which stayed on for the duration of the procedure. During the first training session (day 1), one sucrose pellet was presented in the food receptacle every 40 s. The rat received a total of 10 pellets over the course of 7 min before the first trial (lever presentation) began. The stimulus light over the retractable lever was activated at the same time the lever was presented. The stimulus light remained on and the lever was presented for 10 s. If the animal failed to press the lever during this time, the light turned off and the lever retracted. If the rat pressed the lever during the 10 s, a pellet was presented in the food receptacle and the lever was retracted. The lever was presented every 50 s for 50 trials (lever presentations). The rate of success was calculated as the number of successful lever presses as a ratio of the number of trials (lever presentations).

During the next training session of operant conditioning (day 2), three pellets were inserted into the receptacle during the first 2 min before the lever was presented for 40 trials. The last day of conditioning (day 3) follows the same procedure as day 2. Each rat was trained in a different box

for each conditioning session. The boxes were cleaned with a detergent at the end of each session.

A separate over-training plus extinction schedule was also given to some rats. In this experiment, untreated rats were given one session per day of the instrumental training procedure as described above for 7 days in total. At this point, all rats were achieving at least 90% successful lever presses. After the seventh day, the rats were infused intrastriatally with either pHSV-GFP-only or pHSV-GFP/HA6 viral vector and allowed to recover for 2 days. Rats were then tested on the operant conditioning schedule once daily for 2 days to assess the effect of increased 5-HT$_6$ expression on previously consolidated memory and performance of the lever-pressing task. Rats were then tested for extinction, where the rats were presented with the lever and cue light 50 times per session but no rewarding food pellet was delivered upon pressing of the lever. Rats were given one extinction session per day for 3 days.

Immediately after the end of the last session, rats were either narcotized with CO_2, decapitated, and the brains removed for histological confirmation of cannula placement or anesthetized with a pentobarbital/xylazine solution and perfused transcardially with 1% heparin phosphate-buffered saline, then 4% paraformaldehyde (for immunohistochemical analysis). Each rat's brain was removed, post-fixed overnight in 4% paraformaldehyde, and then sliced into 40 μm sections with a vibratome. Sections were checked for cannula placement into the medial striatum and GFP fluorescence, indicating adequate viral infection and GFP expression in neurons.

Drug Treatment

In order to reverse the physiological effects of increased 5-HT$_6$ expression, 5-HT$_6$ antagonist were given to rats by various administration paradigms. Some pHSV-GFP-only and pHSV-HA6/GFP infused groups received the 5-HT$_6$ antagonist, SB-258585 (i.p. 5 mg/kg, Sigma), or saline 10 min before the start of training on both day 1 and day 2 sessions, while some groups received the 5-HT$_6$ antagonist, BGC20-761 (10 mg/kg i.p.), or SB-258585 (5 mg/kg i.p.) *after* the training session on day 2 only. A separate group of infused rats received SB-258585 intrastriatally via the guide cannula immediately after training on day 2. Rats were habituated to the infusion procedure for 2 days beforehand, by connecting plastic tubing to the awake rat's cannula guides. The rat was then allowed to move freely in its homecage. On the day of infusion, 1 μg per side of SB-258585 (in 2 μl of aCSF) was infused over the course of 10 min via a microprocessor-controlled pump.

Morris Water Maze

The apparatus used for behavior consisted of a galvanized steel tank, painted white, with dimensions 202 cm in diameter × 60 cm in height. A platform, also painted white and 34.5 cm in height × 11.5 cm in diameter was placed within the tank in one of the quadrants. The tanks was filled with warm water (24°C), made opaque by nontoxic, white tempura paint, to a depth of 36 cm. A solid-state monochromatic video camera recorded animal movement within the tank, data were then analyzed offline by the SMART

Tracking program (San Diego Instruments). Visual cues (large posterboard geometric shapes) were placed in the south and east corners of the room.

The rats were habituated to the water maze the day before viral infusions by placing each animal into the tank (platform removed) and allowing it to swim for 90 s.

The learning trials began 3 days after viral infusion. Each rat was given four trials per day, with starting placements next to the north, south, east and west wall, always facing the inside of the tank. The rats were given 90 s to find and mount the platform. The rat was allowed to stand on the platform for 30 s before removal to a holding cage with a warming lamp. The next trial began 60 s later. If the rat failed to find the platform in 90 s, then it was guided to the platform. Trials were given for four cumulative days where starting placements for each trial were randomized. On the last day, an additional probe trial was performed (where the platform was removed) to assess spatial bias. In addition, a trial where the platform was 1 cm above the water was performed to test visual acuity. The SMART tracking program analyzed time to find the platform (latency), swim speed, swim path, and cumulative distance.

Immunohistochemistry

Floating sections (40 μm) were immersed in a 0.5% Triton-X PBS solution for 10 min, then blocked in 10% normal goat serum (NGS) Triton/PBS for 4 h. Sections were transferred in 5% NGS Triton/PBS containing 5-HT₆ receptor antibody (1:500, rabbit, Novus, Littleton, CO) and gently agitated at 4°C for 72 h. After 4–5 rinses in PBS, sections were incubated with Alexa 568-conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen). The sections were rinsed in several changes of PBS before being mounted on slides and cover-slipped with Vectashield mounting medium (Vectorlabs, Burlingame, CA). Slides were visualized with 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.

For chromogenic staining, sections were blocked in NGS Triton/PBS, then placed in a 5-HT₆ receptor antibody solution and incubated for 72 h at 4°C. The sections were rinsed, then immersed in biotinylated secondary antibody solution (1:200, goat anti-rabbit, Vector), followed by immersion in an avidin–biotin peroxidase solution (Vector Elite ABC kit) for 2 h and then reacted with diaminobenzidine (0.05%) with nickel (0.25%) and H_2O_2 (0.0015%). The sections were then mounted on glass slides, and dehydrated in increasing concentrations of ethanol. The slides were then immersed in xylene and cover-slipped with Permount.

Statistics

Data from the instrumental training sessions was analyzed for significance with a 2×2 and repeated measures ANOVA, followed by a Newman–Keuls *post hoc* test, using the Statistica suite, v.6 (Statsoft, Tulsa, OK), where significance was set at $p < 0.05$. Morris water maze (MWM) data were analyzed for statistical significance using two-way (group × day) ANOVA followed by Newman–Keuls *post hoc* tests.

RESULTS

Activation of HA-Tagged 5-HT₆ Receptors Produce Levels of cAMP Induction Similar to Wild Type

5-HT₆ receptors are normally located in dendritic and ciliary processes of neurons, with little expression adjacent to the cell body (Gerard *et al*, 1997). In order to visualize neurons expressing transgenes, the GFP protein was coexpressed along with HA-5-HT₆ receptor from the viral amplicon. GFP expression has been shown to be present throughout the cytosol in infected cells (Clark *et al*, 2002). To allow direct observation of transgenic 5-HT₆ expression, we 'tagged' the 5-HT₆ receptor with HA to identify 5-HT₆-overexpressing cells. In order to confirm that the HA tag did not interfere with the pharmacological functioning of the receptor, a cAMP accumulation assay was performed comparing wild-type to tagged receptor.

We performed a cAMP accumulation assay visualized by luciferase to compare the functionality of the HA-tagged receptor. PC12 cells transfected with pHSV-HA6 or wild-type 5-HT₆ receptor plasmid had no observable changes in health or growth. Transfection efficiency was about 30% per well based on GFP expression and HA immunostaining (data not shown). Cells transfected with luciferase and β-galactosidase plasmids only exhibited negligible increases in cAMP-induced luciferase luminescence when incubated with increasing concentrations 5-HT (data not shown). While there have been contradictory reports of endogenous 5-HT receptor expression in PC12 cells (Furukawa *et al*, 1992; Quinn *et al*, 2002), the absence of 5-HT-induced luminescence in luciferase-only treated cells indicates that PC12 cells do not exhibit endogenous serotonin receptors that stimulate adenylate cyclase in numbers significant enough to confound measurement of cAMP induction from transfected 5-HT₆ receptors.

The expression of luciferase, coupled to the CRE element, did not differ appreciably in cells transfected with the wild-type or tagged 5-HT₆ receptor, as shown by Figure 1. The
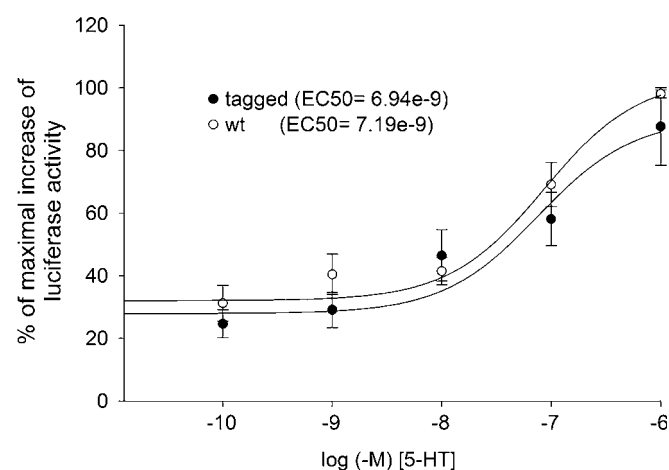
Figure I Luciferase assay measuring adenylate cyclase activity in PC12 cultures transfected with HA-tagged and or wild-type 5-HT₆ receptor. Activity is expressed as a percentage of baseline luciferase luminescence, $n = 5$ assays/dose. EC_{50} determinations were calculated with Sigma plot. Note that there was a modest amount of spare receptor activity in this overexpression system, but that the HA tag did not alter the functional characteristics of the 5-HT₆ receptor.

EC$_{50}$ of 5-HT for the wild-type receptor was 7.1 nM, while that for the tagged receptor was 6.9 nM, and are comparable to previous reports of wild-type rat 5-HT$_6$ receptor EC$_{50}$ as measured by cAMP accumulation assays (6.91 nM) (Boess *et al*, 1997). This indicates that HA-tagged 5-HT$_6$ receptor shows similar affinity for 5-HT as wild type. Furthermore, a specific 5-HT$_6$ antagonist, BGC20-761 (Mitchell *et al*, 2006), blocked 5-HT-induced luciferase expression equivalently in cells transfected with either wild-type or tagged 5-HT$_6$ receptors (data not shown). Therefore, the presence of an amino terminal epitope tag did not appear to alter the pharmacology of the 5-HT$_6$ receptor.

Striatal Neurons Infected by HSV Amplicons Express HA-5-HT$_6$ and GFP Proteins

Construction of the pHSV-HA6/GFP transgene amplicon is shown in Figure 2a. Expression of gene products from HSV viral vectors has been shown to peak about 3 days after injection and slowly decrease (Carlezon *et al*, 2000a). Furthermore, we have observed that expression levels of HA-5-HT$_{1B}$ receptor using the same vector in nucleus accumbens was comparable from days 2 to 10 after viral infection (data not shown), so transgene expression is quite stable over the duration of the behavioral experiments used in this report. At 6 days after intrastriatal infusion, brains injected with pHSV-GFP-only vector exhibited GFP-positive fluorescent cell bodies and processes in the medial striatum, as depicted in Figure 2b; no expression was apparent in cells with glial morphology. The area with greatest GFP expression was found directly below the guide cannula indentation, with moderate GFP expression observed adjacent to the guide cannula. Animals injected with the

pHSV-HA6/GFP vector exhibited GFP expression in similar numbers of cells as the pHSV-GFP-only injected brains. Figure 2b shows increased 5-HT$_6$ immunostaining within the medial striatum after injection of pHSV-HA6/GFP, both in the cell body and in the surrounding dendrites; most infected neurons show strong colocalized GFP fluorescence. Although immunostaining of 5-HT$_6$ receptors can be seen throughout the striatum and cortex, immunostaining is primarily located within the dendrites of neurons. Figure 2d demonstrates the neuroanatomical localization of 5-HT$_6$ immunohistochemical staining in control tissue, whereas Figure 2e shows increased staining in transfected tissue. Figure 2c shows the absence of specific staining in tissue processed without the 5-HT$_6$ antibody. Figure 3 demonstrates striatal localization of the increased 5-HT$_6$ expression directly beneath the infusion sites (Figures 3a and b).

5-HT$_6$ Receptor Overexpression in the Dorsomedial Striatum Decreased Learning of Operant Conditioning but had No Effect on Spatial Learning

Four groups of rats were food-restricted and trained to perform an instrumental learning procedure, pHSV-GFP-only or pHSV-HA6/GFP infused rats, as well as sham surgery rats and untreated rats, to serve as controls for viral infusion and surgery. After the viral vector infusions, rats were carefully monitored for changes in food intake and weight. No significant differences in feeding or weight were observed between groups. Additionally, there was no apparent neophobia or decreased appetite in any rat group, as tested by approach and consumption of sugar pellets 2 days after viral vector infusion (Figure 4a).
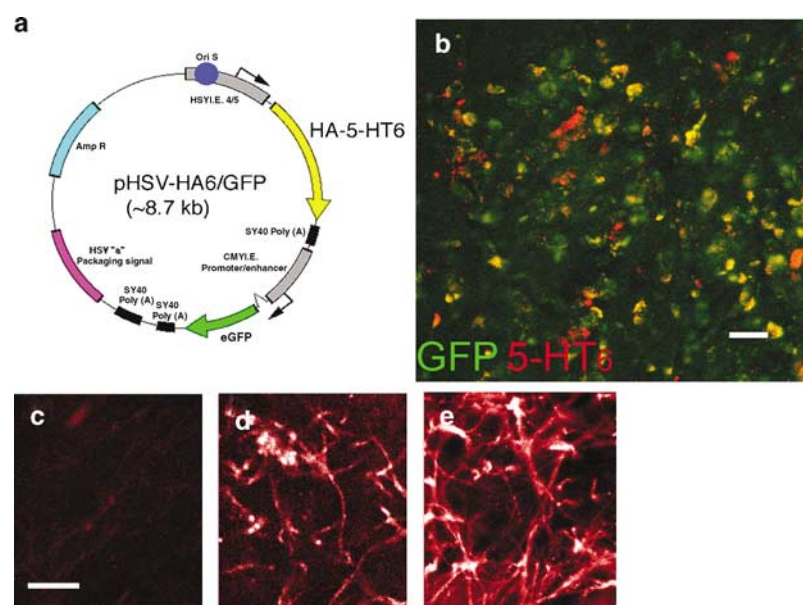

Figure 2 5-HT$_6$ immunohistochemistry transfected striatal sections. (a) Amplicon map of pHSV-HA6/GFP; GFP expression is driven by a CMV promoter and HA-5-HT$_6$ expression by an HSV promoter. (b) Photomicrograph of striatal infusion site: GFP (green) and 5-HT$_6$ immunohistochemical staining (red); generally infected neurons lie within 200–300 μm of the center of the injection site. scale bar indicates 50 μm; (c) no antibody control tissue, scale bar indicates 10 μm; (d) 5-HT$_6$ antibody in control striatum that received GFP-only vector; (e) 5-HT$_6$-like immunostaining after pHSV-HA6/GFP gene transfer into striatum. Note that punctate 5-HT$_6$ immunolabeling of endogenous receptors is apparent in control or uninfected neurons, while immunostaining is significantly increased in neurons after infection with pHSV-HA6/GFP.
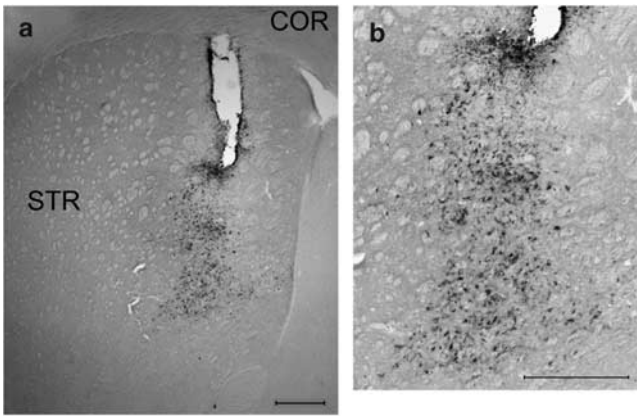
Figure 3 Distribution of 5-HT$_6$ expression following intrastriatal injection of HSV-HA6/GFP vector (a) Low magnification view of 5-HT$_6$ immunohistochemical staining localization in the striatum. COR, cortex; STR, striatum. Scale bar indicates 500 μm; (b) high magnification view of 5-HT$_6$ immunohistochemical staining localization below injection site. Scale bar indicates 500 μm. Note that increased 5-HT staining is apparent over a significant area within striatum; no GFP neurons were visible in similar section in cortex, and the tearing from the cannula occurs primarily at the time of dissection.
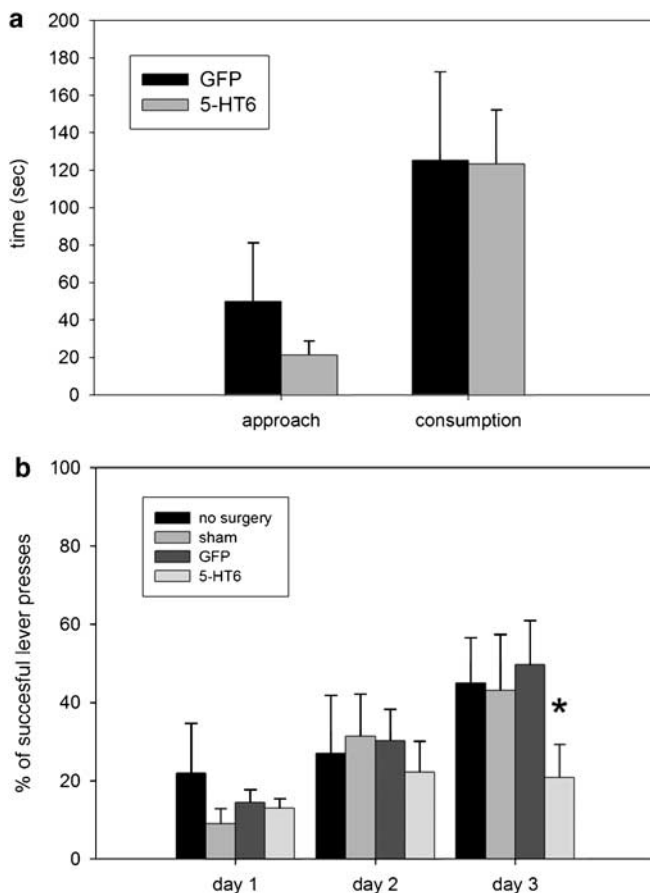

Figure 4 Increased expression 5-HT$_6$ on behavior. Data are presented as means ± SEM. (a) Effect of increased striatal 5-HT$_6$ expression on mean time to approach and consumption of one 45 mg sugar pellet. $n = 6$ for each group. (b) Effect of striatal 5-HT$_6$ overexpression in conditioned learning: no surgery rats ($n = 8$), sham: sham surgery ($n = 8$), GFP, pHSV-GFP-only vector ($n = 12$); 5-HT$_6$, pHSV-HA6/GFP vector ($n = 12$). *Significantly differently from GFP ($p < 0.05$).

All treatment groups showed comparable, low rates of successful operant responses during the first session (Figure 4b). All treatment groups showed some increase in successful responses. On day 3, pHSV-GFP-only rats demonstrated significant increases in success rates (50 ± 4%), but pHSV-HA6/GFP rats' performance did not improve (20 ± 5%). Sham and no surgery rats exhibited sequential daily improvement in acquiring the instrumental task equivalent to that of the pHSV-GFP-only rats. There was an overall effect of days of training on the rate of successful operant responses for all groups ($p < 0.05$, $F_{(2,70)} = 25.2$) except those with increased 5-HT$_6$ expression; these animals were significantly different from GFP controls ($p < 0.05$, $F_{(3,39)} = 4.48$), as well as the sham and untreated control groups.

In order to assess whether increased expression of striatal 5-HT$_6$ affected the performance of lever pressing for food rewards, two groups of cannulated rats were trained to perform the instrumental learning task to a success rate of 90% or above and then pHSV-GFP-only or pHSV-HA6/GFP viral vectors were infused after training. In 2 days of subsequent probe tests, there was no difference in the number of responses between the two groups (Figure 5). This indicates that increased 5-HT$_6$ expression in striatum did not block the performance of the food reward task once already learned. These rats were then given three additional daily sessions of extinction training during which lever presses were no longer rewarded. Both GFP-only control and increased 5-HT$_6$ expressing animals had a similar rate of extinction learning as indicated by reduced rates of lever pressing.

We also tested the effects of increased striatal expression of 5-HT$_6$ receptors in a spatial learning task, the MWM (Morris, 1984). Rats with increased striatal 5-HT$_6$ receptor expression learned to navigate the MWM at the same rate as pHSV-GFP-only control rats; more specifically, they showed no difference in memory of platform location over 4 days as
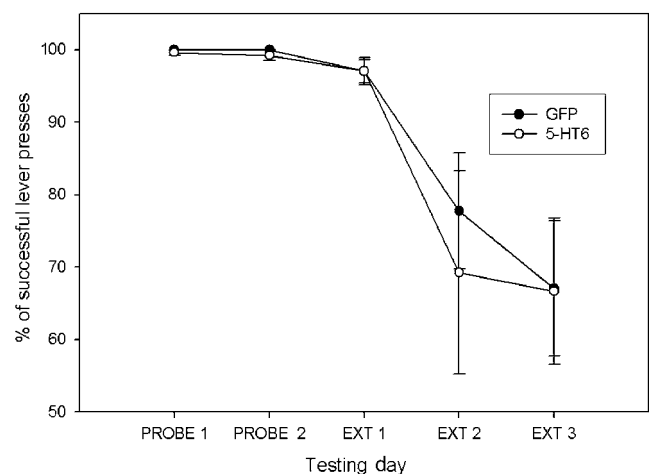

Figure 5 Effect of 5-HT$_6$ receptors on performance of previously acquired instrumental conditioning. Data are presented as means ± SEM. Groups are as follows: pHSV-GFP-only ($n = 7$): GFP or pHSV-HA6/GFP ($n = 7$): 5-HT$_6$. Data points PROBE 1 and PROBE 2 demonstrate lever-pressing rates of reward-based lever pressing on two separate days, whereas EXT 1, 2, and 3 demonstrate nonreward-based lever pressing over 3 days.
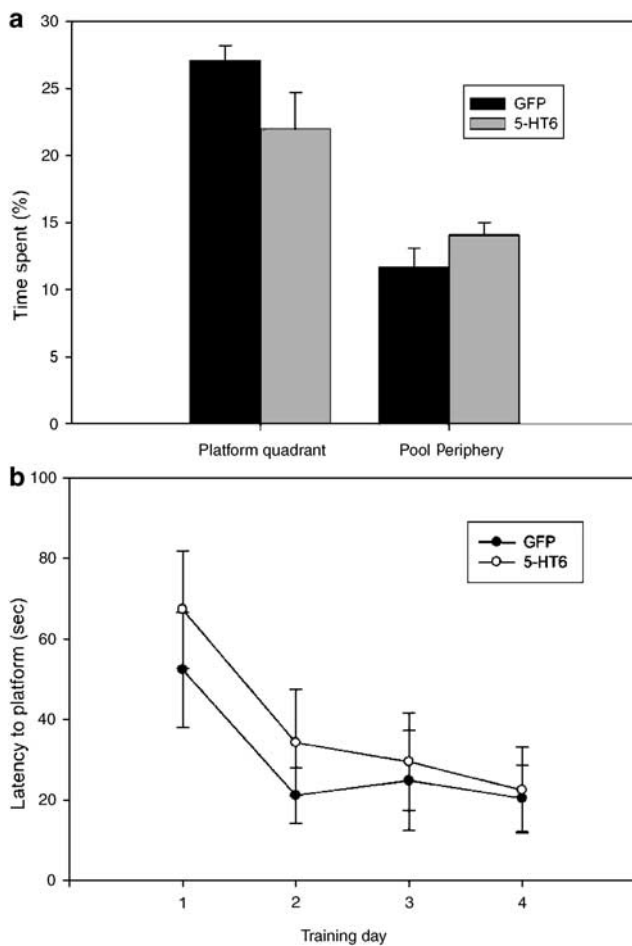
Figure 6 Morris water maze training. (a) Probe test: percent time spent in platform quadrant and time spent in pool periphery (thigmotaxis). (b) Latency to reach the submerged platform. Data are presented as means $\pm$ SEM. Groups are as follows: GFP, pHSV-GFP-only vector ($n = 7$); 5-HT$_6$, pHSV-HA6/GFP vector ($n = 7$). Shown are means $\pm$ SEM.

compared to controls (Figure 6). Additionally, there were no differences in speed or path length between groups (data not shown).

Learning Impairment from Increased 5-HT$_6$ Receptor Expression in the Dorsomedial Striatum is Reversed by Administration of 5-HT$_6$ Antagonists

Increased 5-HT$_6$ expression prevented rats from acquiring the operant conditioning task during 3 days of training. To determine whether this could be attributed to overactivity of 5-HT$_6$ receptors in the striatum *following* the training session, we administered the 5-HT$_6$-selective antagonists (BGC20-761 or SB-258585) either intrastriatally or intraperitoneally immediately after the training session on day 2, but these two paradigms did not improve lever-pressing success rates in the pHSV-HA6/GFP-expressing rats (day 3 success rate—pHSV-HA6 + SB258585 after day 2 session: $17 \pm 12\%$, pHSV-HA6 + intrastriatal BGC20-761 on day 2: $22 \pm 11\%$). We next tested the effects of SB-258585 (5 mg/kg i.p.) *vs* saline given immediately before each conditioning session on days 1 and 2. pHSV-HA6/GFP and pHSV-GFP-only rats given pre-session saline performed similarly to
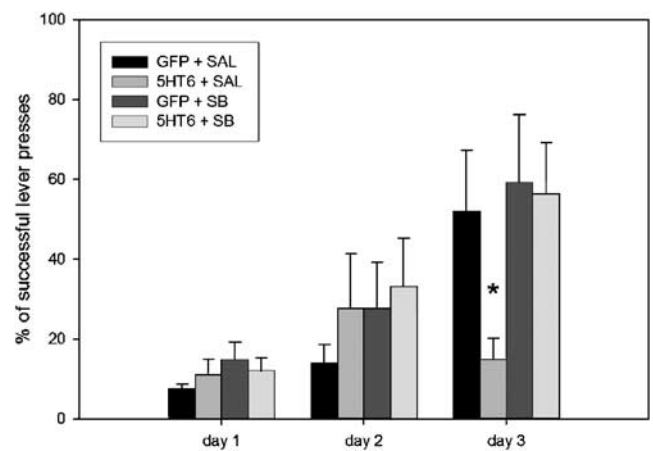

Figure 7 Effect of striatal 5-HT$_6$ overexpression and administration of a 5-HT$_6$ antagonist, SB-258585, on instrumental learning. Data are presented as means $\pm$ SEM. Groups: GFP SAL, pHSV-GFP-only vector + i.p. saline ($n = 12$); 5-HT$_6$ SAL, pHSV-HA6/GFP vector + i.p. saline ($n = 12$); GFP SB, pHSV-GFP-only vector + SB-258585 (5 mg/kg, i.p.) ($n = 9$); 5-HT$_6$ + SAL: pHSV-HA6/GFP vector + SB-258585 (5 mg/kg i.p.) ($n = 9$). *$p < 0.05$ *vs* GFP + SAL ($F_{(3,39)} = 3.66$).

each other on days 1 and 2. While saline-injected pHSV-HA6/GFP rats did not improve significantly on day 3 ($14 \pm 3\%$), the saline-injected pHSV-GFP-only control rats' success rates increased ($52 \pm 15\%$, $p < 0.05$ ($F_{(3,39)} = 3.66$)) (Figure 7). These data replicate the results shown in Figure 3b. In contrast, the pHSV-HA6/GFP rats given pre-session SB-258585 displayed success rates comparable to both the saline-treated pHSV-GFP-only rats and the SB-258585-treated pHSV-GFP-only rats ($p > 0.05$ ($F_{(3,39)} = 1.56$)) (Figure 7). As the animals were tested on day 3 in a drug-free state, the antagonist was probably influencing learning or memory consolidation during days 1 and 2 rather than performance on day 3.

In order to test whether SB-258585 modulates general motor activity, we measured noncontingent lever pressing in control rats expressing GFP that were administered SB-258585 (5 mg/kg i.p.) immediately before a session where the lever was presented but no reward was given after it was pressed. These rats did not show improvement in lever pressing over the course of 3 days (data not shown). This experiment demonstrates that SB-258585 did not affect motor activity levels as represented by spontaneous lever pressing.

Increased 5-HT$_6$ Expression in the Dorsal Central Striatum has No Effect on Instrumental Conditioning

Two separate groups of rats were implanted with cannulas in either the dorsomedial striatum, the same region targeted in the above experiments, or the dorsal central region of the striatum. These rats were infused with either pHSV-GFP or pHSV-HA6 vectors. While increased expression of 5-HT$_6$ receptors in the dorsomedial region was associated with impairment in the learning of the operant task as compared to control rats (*post hoc* Tukey test, $p < 0.043$) (Figure 8), there was no impairment associated with increased 5-HT$_6$ receptors in the dorsal central region; suggesting that 5-HT$_6$ receptors in the dorsomedial region have a more profound
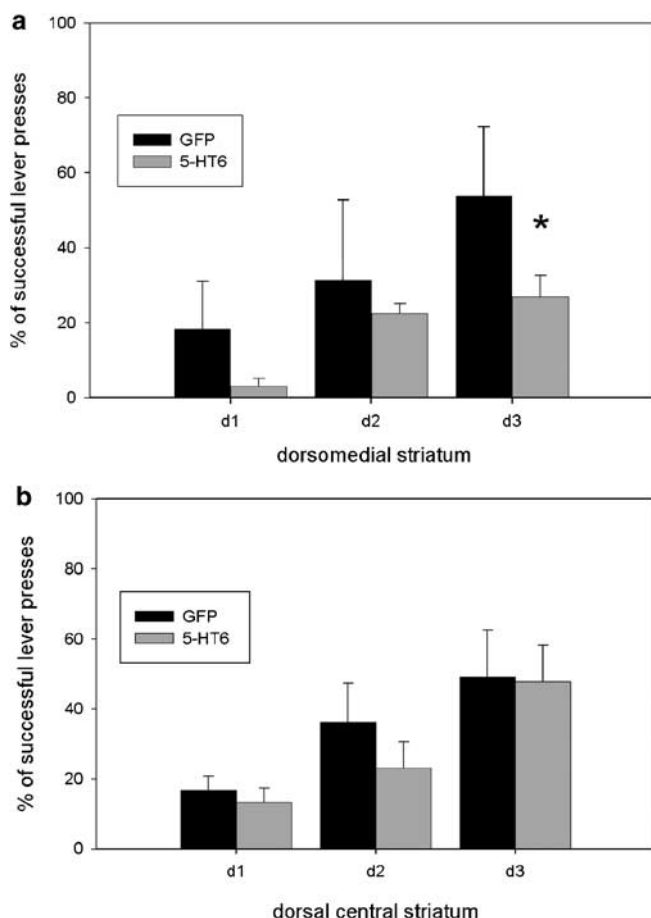
Figure 8 Increased expression of 5-HT$_6$ on behavior: comparison of distinct striatal subregions. Data are presented as means ± SEM. (a) Effect of increased 5-HT$_6$ expression in the dorsomedial striatum. $n = 8$ for each group. (b) Effect of increased 5-HT$_6$ expression in the dorsal central striatum. $n = 11$ for each group. *Significantly differently from GFP ($p < 0.05$).

effect on instrumental learning, at least under these conditions.

DISCUSSION

This is the first report of the behavioral effects of increased activation of 5-HT$_6$ receptors *in vivo* through increased expression. Previous work from this lab has demonstrated the potential uses of viral-mediated gene transfer to investigate the functions of other serotonin receptors in discrete brain regions (Clark *et al*, 2002, 2004; Neumaier *et al*, 2002; Hoplight *et al*, 2006). We confirmed that the HA-tagged 5-HT$_6$ receptor functioned identically to wild-type receptor *in vitro*, and the anatomical pattern of distribution of the transgenic 5-HT$_6$ receptors appears to closely match the normal neuronal distribution of these receptors in striatum, albeit at higher levels. As altered receptor expression level may be important in pathophysiological mechanism, viral gene transfer offers a novel method for manipulating receptors in a brain region of interest, and may eventually be relevant for therapeutic interventions in the future. The synthesis of 5-HT$_6$ selective agonists have been described (Beyer *et al*, 2005), but increased expression

using viral vectors allows the investigation of the effects of endogenous 5-HT released during behavior; this preserves the information contained in the temporal and spatial patterning of 5-HT release and action at the site of increased receptor expression.

The 5-HT$_6$ receptor has been implicated in memory function because antagonists can enhance memory consolidation (Rogers and Hagan, 2001; Woolley *et al*, 2001; Mitchell and Neumaier, 2005), however the effects of increased stimulation of 5-HT$_6$ receptors on learning has not been previously reported. As 5-HT$_6$ receptors are prominently expressed in rat and human striatum normally, we focused on this region using a stimulus-reward learning paradigm that depends on normal striatal function (Hartley and Burgess, 2005). Increased striatal expression of the 5-HT$_6$ receptor had no effect on the number of successful lever presses on day 1 and day 2, suggesting that 5-HT$_6$ activity did not impair initial exploratory behavior. However, on day 3, animals in all three control groups markedly increased their successful lever-pressing rate, whereas rats with increased 5-HT$_6$ expression did not. This suggests that increased 5-HT$_6$ expression reduced acquisition or consolidation of instrumental learning. However, the fact that the antagonist had significant effects only when it was given before a session points to an effect in acquisition.

It is unlikely that the impairment in learning in HA6/GFP-treated animals was due to nonspecific effects of increased 5-HT$_6$ expression because SB-258585, a selective antagonist, reversed the impairment in these animals. We gave the antagonist before the conditioning session on days 1 and 2, but observed robust increase in successful responses on day 3; the improved learning was not simply an acute performance enhancement by the drug. We expected that giving a 5-HT$_6$ antagonist immediately after the trial might enhance memory consolidation based on previous studies of these drugs on memory consolidation, but it was necessary to give the drug before the conditioning session to reverse the effects of increased 5-HT$_6$ receptor expression. We did not see an increase in the performance in GFP rats given 5-HT$_6$ antagonist as compared to those given saline, a result different from that of Meneses (2001), possibly due to the specific antagonist and dose used in the present study.

We also cannot rule out an effect of SB-258585 in other brain regions in reversing the effects of 5-HT$_6$ over-expression in striatum, although it seems most likely to have been an effect in the striatum. While we did not detect a significant enhancement in operant learning in pHSV-GFP-only animals treated with SB-258585, a majority of previous studies have only detected a significant effect of 5-HT$_6$ antagonists on memory consolidation using impairment experimental models such as scopolamine administration or aging (Foley *et al*, 2004; Mitchell and Neumaier, 2005; Mitchell *et al*, 2006). Thus, it is not surprising that the effects of SB-258585 were selective for the animals with increased 5-HT$_6$ receptor expression and learning deficits.

In order to ascertain that this memory deficit affected instrumental learning involving striatal circuits, we trained rats infused with pHSV-HA6/GFP or pHSV-GFP-only viral vectors in a MWM. The MWM is a hippocampal-dependent learning task where visuospatial cues allow rats to navigate towards a hidden platform with a pool of water (Morris,

1984; Brandeis et al, 1989). Increased 5-HT$_6$ expression did not impair memory retention of the platform location in a probe test, thus indicating that 5-HT$_6$ expression is affecting primarily striatal-based learning and not attention or other global factors that might also impair spatial learning or general motor function.

While enhanced memory consolidation via systemic administration of 5-HT$_6$ antagonists has received much attention (Mitchell and Neumaier, 2005), the present data focus on 5-HT$_6$ receptors in striatum only, and the contribution of these localized receptors to acquisition vs consolidation needs to be addressed with additional experimentation. Other labs have shown that long-term retention of MWM training was improved with systemic administration of a 5-HT$_6$ receptor antagonist (i.p.) or 5-HT$_6$ antisense oligonucleotides (i.c.v.), but had no effect on acquisition (Woolley et al, 2001), as rats with decreased 5-HT$_6$ receptor activity showed no difference in performance after several trials given on the same day, but did show improvement in trials given the day afterwards. However, a more recent study has demonstrated improvement in tasks measuring executive function, such as set-shifting, with administration of 5-HT$_6$ antagonists (Hatcher et al, 2005). In contrast, no studies, to our knowledge, have demonstrated a role of 5-HT$_6$ receptors in memory retrieval or extinction. Indeed, when rats over-trained on the instrumental learning task were infused with either pHSV-GFP-only or pHSV-HA6/GFP vector, we found no difference in performance of the previously learned task, demonstrating that increased levels of 5-HT$_6$ receptors in the striatum had no effect on the recall or performance of acquired, well-learned tasks. Furthermore, when rats subsequently underwent extinction of the instrumental conditioning, the rate of extinction was the same in both groups, suggesting that this type of learning was not sensitive to increased 5-HT$_6$ activity in striatum.

The striatum has been shown to be an important region for specific types of acquisition and memory consolidation, most notably learning involving habituated activity such as lever pressing (Yin et al, 2005). As stated previously, 5-HT$_6$ receptors are localized in medium spiny GABAergic neurons expressing enkephalin, substance P, and dynorphin receptor subtypes (Ward and Dorsa, 1996). Such cell types comprise 95% of neurons within the striatum (Wilson and Groves, 1980). Based on the homogenous distribution of medium spiny neurons and the pattern of transgene expression, HSV-mediated expression of 5-HT$_6$ receptors occurred predominantly in these striatal neurons. The HSV virus is neurotropic, gaining entry into neurons by binding to Nectin-1/HveC (Richart et al, 2003) and does not infect glial cells at an appreciable rate (Carlezon et al, 2000b); we have never observed any GFP in typical astrocytic cells. In a previous study involving medium spiny neurons in nucleus accumbens shell (Neumaier et al, 2002), we observed rare retrograde infection of neurons that project to the viral infusion site. Thus, we believe that increased 5-HT$_6$ receptor expression was extremely localized to medium spiny neurons at the injection site in this study as well. Immunohistochemical distribution of 5-HT$_6$ immunoreactivity within virally infected neurons in transfected brains was localized in the dendrites (as in endogenously expressing neurons) but was also distributed on the cell

body more intensely than endogenous 5-HT$_6$ receptors. Therefore, it is possible that this factor may also contribute to the changes in learning behavior. However, transgene receptors have been routinely detected in cell bodies after viral overexpression and may simply reflect the high receptor production during peak expression. Additionally, it is possible that a small number of other neuronal subtypes, such as cholinergic interneurons, may also be expressing transgene 5-HT$_6$ receptors and that such expression is contributing to the deficit in instrumental learning. The development of viral vectors, which express genes only in certain neuronal phenotypes, would allow further addressing of specific subpopulations' roles in striatal functioning.

Recent studies have attempted to dissect the roles of specific subregions of the striatum, such as the dorsomedial and dorsolateral striatum, in instrumental learning. Featherstone and McDonald (2005) have shown that lesioning of the dorsolateral regions causes deficits in acquisition of a learning task while dorsomedial lesions produce errors of commission and loss of habitation to previously learned tasks. Based on evidence that the dorsomedial striatum is strongly linked to stimulus-response goal-directed learning (Rogers et al, 2001), this region may also influence habituation. As 5-HT$_6$-infused rats did not show a deficit in lever pressing until day 3, when lever-pressing rates reach levels that indicate a habituated behavior, it appears that 5-HT$_6$ overexpression in the dorsomedial striatum may be interfering with habituation of the goal-directed behavior. In contrast, when 5-HT$_6$ receptors were overexpressed in the dorsal central region of the striatum, the rats demonstrated intact learning of the instrumental conditioning. Pharmacological blockade of the medial prefrontal cortex, a region which projects to the dorsomedial striatum (Sesack et al, 1989), produces also deficits in instrumental conditioning (Baldwin et al, 2000). The dorsal central region receives inputs from the agranular cortex and lateral orbital cortex, regions important for reversal learning but not set-shifting or habituation (Reep et al, 2003; Dalley et al, 2004), and thus it is likely that these subregional differences in corticostriatal projections may underlie the differential behavior observed (Rogers et al, 2001).

Increased stimulation of 5-HT$_6$ receptors has been shown to enhance extracellular GABA levels in the striatum (Beyer et al, 2005). The decrease in habituated learning observed in the present study may be due to changes in local signaling networks, as increased GABAergic firing inhibits cholinergic interneuron activity (DeBoer and Westerink, 1994; Kikuchi et al, 1998), which has been shown to be integral to instrumental learning (Ragozzino, 2003). Alternatively, 5-HT$_6$ overexpression may be inhibiting learning primarily through direct and indirect basal ganglia pathways (Graybiel, 1986). Both pathways are GABAergic circuits, which ultimately influence the cortex, which provides glutamatergic input to the striatum. Increased 5-HT$_6$ receptor expression may in fact be strengthening GABAergic inhibition of the basal ganglia, thus suppressing the learning feedback loop. Studies have shown that 5-HT$_6$ blockade increases glutamate and acetylcholine release in cortex and hippocampus (Riemer et al, 2003). Woolley et al (2004) has proposed that 5-HT$_6$ inhibition causes a disinhibition of striatal direct and indirect circuits, and

increased neuronal firing in the cortex. Thus in the case of increased 5-HT$_6$ activity via viral vector gene transfer, there may be decreased cortical stimulation and feedback, which would attenuate learning through dampening of excitatory signaling. Further studies will need to be conducted in order to verify such a proposed mechanism, which presumes that virally mediated expression is primarily occurring in medium spiny neurons that naturally express 5-HT$_6$ receptors. In any event, the experiments detailed in this report suggest that the 5-HT$_6$ receptors in striatum may play a more important role in cognitive function than has previously been appreciated.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)