Bidirectional Changes in Ethanol Consumption in Rats with Site-Specific Antisense Down-Regulation of 5-Hydroxytryptamine<sub>2A</sub> Receptors in Brain

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ABSTRACT

The 5-hydroxytryptamine (5-HT)<sub>2A</sub> receptor is an important component of the neural substrates underlying ethanol (EtOH) intake and behaviors related to anxiety and stress. Paradoxically, both 5-HT<sub>2A</sub> agonists and antagonists have been shown to reduce EtOH intake, however the mechanisms underlying these effects are not understood. This inconsistency could possibly be explained by their chronic down-regulation of the 5-HT<sub>2A</sub> receptor. To further address these findings, the present study sought to functionally characterize the role of localized 5-HT<sub>2A</sub> receptors in regulating EtOH ingestion by producing central nervous system site-specific receptor down-regulation through infusion of antisense oligonucleotide (ASO). Rats were infused with 5-HT<sub>2A</sub> receptor ASO into the lateral ventricle (i.c.v.), prefrontal cortex (PFC), central amygdaloid nucleus, medial and lateral division (CeA/L), dorsal raphe nucleus (DRN), or hippocampus (HIP) for a period of 26 days. Subjects were tested for EtOH intake and behaviors related to anxiety and stress. ASO administration i.c.v. and into the CeA/L significantly reduced EtOH intake. PFC 5-HT<sub>2A</sub> ASO administration increased EtOH intake. Administration of 5-HT<sub>2A</sub> ASO into the DRN and HIP had no effect on EtOH intake. Intracerebroventricular ASO administration increased activity in a novel open field and increased anxiety-like behavior in the elevated plus maze. PFC ASO administration produced an anxiogenic effect in the elevated plus maze. Intracerebroventricular, PFC, and CeA/L ASO infusions altered adrenocortical function. These differential behavioral effects specific to the anatomical locations targeted for 5-HT<sub>2A</sub> receptor down-regulation may help resolve a long-standing, apparent inconsistency in the role of 5-HT<sub>2A</sub> receptors in EtOH consumption.

The central 5-HT system is a viable target for the pharmacotherapeutic treatment of alcoholism and alcohol abuse. Moderate success in reducing EtOH intake at both the preclinical and clinical level has been achieved through the use of selective 5-HT reuptake inhibitors, which are also effective in treating anxiety and stress disorders (Naranjo et al., 1990; Knapp et al., 1993; McBride et al., 1993). Additionally, the comorbidity of psychological disorders related to stress and anxiety has long been reported to exist in many subpopulations of alcoholics, and in many cases are thought to contribute to the etiology and/or maintenance of alcoholism. Interestingly, several of the compounds used to treat alcoholism and alcohol abuse are also effective at treating anxiety and depression. One common pharmacological effect that many of these compounds produce is a down-regulation of the 5-HT<sub>2A</sub> receptor, and this receptor has been specifically implicated in mediating EtOH intake (Overstreet et al., 1997; Wilson et al., 1998; Maurel et al., 1999).

Interestingly, both 5-HT<sub>2A</sub> agonists and partial agonists such as DOI and 1-(3-chlorophenyl)piperazine (Pohorecky et al., 1998; Maurel et al., 1999), and 5-HT<sub>2A</sub> antagonists such as amperozide, FG 5974, MDL 100,907, mianserin, and ritanserin (Meert and Janssen, 1991; Meyers et al., 1993; Pohorecky et al., 1998; Roberts et al., 1998; Maurel et al., 1999) are capable of reducing EtOH consumption. Several factors, however, must be considered in the interpretation of these findings. First, it cannot be ascertained that 5-HT<sub>2A</sub> ligands that affect EtOH-related behaviors work predominately through the 5-HT<sub>2A</sub> site, because these compounds generally show at least some pharmacological activity at other 5-HT receptors (Di Giovanni et al., 1999; Gobert and Millan, 1999).

Second, reductions in EtOH intake produced by 5-HT<sub>2A</sub> drugs are not only dose-related but also seem to vary across different brain regions.
strain (including genetically preferring animal models) (Overstreet et al., 1997), drinking paradigm used (Roberts et al., 1998), and the subject's basal EtOH intake prior to testing (Meert and Janssen, 1991).

It is also not known which CNS structures are the most dominant in the 5-HT2A receptor's mediation of EtOH intake, or stress- and anxiety-related behaviors. 5-HT2A receptors are found with highest density in the PFC, the piriform cortex, and the claustrum (Pazos et al., 1985). Additionally, 5-HT2A receptors are located in several structures associated with reward and reinforcement pathways, as well as with some structures associated with stress and anxiety, such as the PFC, amygdala, DRN, and HIP.

Given that many of the ligands that have a pharmacological profile for the 5-HT2A receptor also show pharmacological activity at other 5-HT receptors, new methods of selectively attenuating activity of the 5-HT2A receptor would be valuable in clarifying its role in EtOH-related behaviors. The use of ASO has now made it possible to selectively inhibit expression of specific subpopulations of the 5-HT receptor family. ASO treatment is emerging as a viable technology, capable of providing anatomic and mechanistic data for the validation of drug targets (Bennet and Cowsert, 1999). The present studies used chronically administered phosphodiester-backbone ASO, to the 5'-untranslated region of the mRNA coding for the 5-HT2A receptor to produce brain area-specific down-regulation of 5-HT2A receptors. This allows for down-regulation of the 5-HT2A receptor in a manner analogous to that following pharmacological treatment (Sibille et al., 1997), but with much greater selectivity than 5-HT2A antagonists.

The current experiments were designed as a set of initial behavioral studies using ASO technology. The primary focus of these investigations was to more accurately identify and define the specific CNS structures in which the 5-HT2A receptor may mediate both voluntary EtOH intake and the behavioral responses to acutely administered EtOH. Given that the 5-HT2A receptor has also been linked to anxiety and stress disorders, and these may be related to alcoholism and alcohol abuse, a secondary objective of this study was to further elucidate the role of this receptor in behaviors that are thought to be valid indexes of stress and anxiety in rats. Such testing included measurements of spontaneously occurring behaviors in the open field and the elevated plus maze.

Rats were implanted with osmotic minipumps and ASO directed at the 5-HT2A receptor was infused into localized brain sites for a period of 26 days during which time behavioral testing was conducted. The results from the present study indicate that the 5-HT2A receptor both positively and negatively regulates EtOH consumption and the direction of change in EtOH consumption varies depending upon the neuroanatomical location of these receptors.

### Materials and Methods

#### Animals

A total of 80 male Long Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) were used in the subsequent experiments. Subjects were individually housed in wire mesh hanging cages with Rodent Chow (Purina, St. Louis, MO) and tap water available ad libitum. Subjects were approximately 8 weeks old and 225 g on arrival. Vivaria were maintained at a temperature of 23 ± 1°C and lights were maintained on a 12-h light/dark schedule with lights on at 8:00 PM, so all behavioral testing could be conducted during the dark phase of the light cycle to avoid disruption of the biological circadian rhythm. All animals were tested between 11:00 AM and 3:00 PM. Subjects were allowed 2 weeks to habituate to the colony rooms prior to testing. All testing procedures were in compliance with National Institutes of Health and Rutgers University guidelines for the care and use of laboratory animals.

#### Oligonucleotide Design and Preparation

The ASO sequence was a phosphodiester oligonucleotide, 22 bases in length. This sequence, 5’-AGT CAT TAT AGA GCC TCG G 3’, corresponded to the 5’-untranslated region of the 5-HT2A receptor mRNA, and did not share significant homology with any known sequence according to a gene databank search (GenBank). A control sequence with the same s-oligonucleotide base composition, 5’-TCT CAT TAT AGA GCC TGC C 3’, was also tested to ensure that the sequence was producing a specific effect in the stability and translational efficiency of the 5-HT2A receptor mRNA, and not merely a nonspecific toxic effect. Thermodynamic comparisons of hybridization rates between the ASO and MSO sequences revealed that the free energy generated by bond formation in physiological buffer was −24.88 kcal/mol for ASO and −17.95 kcal/mol for MSO (Peyret et al., 1999; HYTHER shareware, www.JSL1.chem.wayne.edu), suggesting that hybridization affinities for ASO to target were vastly greater than the MSO to target. The ASO was diluted in nuclease-free H2O (10 μg/3 μl) and then injected into an Alzet 2004 osmotic minipump (Alza Corporation, Palo Alto, CA). The pump flow rate was 0.25 μl/h allowing for the infusion of a dose of 20 μg of oligonucleotide per 24 h, and the maximum life of the pump was 28 days. However, all experiments were stopped, and animals sacrificed on, day 26 postpump implant. It was theorized that the behavioral effects of the antisense would not be seen until approximately 48 h post-ASO administration given that the half-life of the 5-HT2A receptor is estimated at 2.5 days. At the conclusion of each experiment residual oligonucleotide solution from the minipumps was analyzed against an aliquot that had been kept at −70°C for 1 month. For this, agarose gels (1.2%) were used and samples were electrophoresed at 85 V for 30 min, and then bands were visualized using ethidium bromide fluorescence. Bands from control or experimental samples were compared for concentration and integrity. Loss of oligonucleotide was never more than 5%, and smaller molecular weight bands or ladders (evidence of oligonucleotide degradation) were not detected.

#### Surgical Procedures

All surgery was performed under sterile conditions. Rats were anesthetized with a ketamine/xylazine combination and placed in a stereotaxic apparatus. A 2-cm incision anterior to the scapula was made and two subcutaneous pockets were created. A single pump was then placed into each cavity. Polyethylene tubing attached to the pumps was threaded under the skin up to the site of cannula implantation. Rats were then bilaterally implanted with delivery cannula (22 gauge; Plastics One, Roanoke, VA) targeted to the area of interest and secured with cranioplastic cement and three stainless steel screws. Implantation sites were based upon coordinates taken from Paxinos and Watson (1986). Stereotaxic coordinates for the lateral ventricle, PFC, CeA/RL, DRN, and HIP, respectively, are presented as anteroposterior, mediolateral, dorsoventral: ±1.0, ±1.5, −3.5; ±3.2, ±1.5, −0.7; −2.3, ±4.2, −7.6; −7.8, 3.25, −6.75 (15° angle); −4.8, ±5.2, −6.5. No sign of tissue damage as a result of pump implantation or oligonucleotide delivery was evident in brain histology performed at the conclusion of the experiment.

#### In Vivo Pretesting of ASO and MSO

Extensive in vivo pretesting was conducted on the ASO and MSO sequences prior to the initiation of these experiments. Both dose and
time course studies were conducted. Three important conclusions were derived from these preliminary in vivo investigations. First, data from these studies was used to select the optimal dose of ASO and MSO in the current study. Second, 5-HT<sub>2A</sub> binding studies from the time course investigations demonstrated that the same level of reduction 5-HT<sub>2A</sub> receptor expression was achieved following 5, 10, 15, or 25 days of ASO infusion. This suggested that ASO produced maximum receptor reduction after 5 days and this same level is maintained following 25 days of infusion. Third, the initial in vivo studies also allowed for the verification of MSO as a control and eliminated the necessity for an additional vehicle only control group. Extensive pretesting (personal observations, this laboratory) showed that 5-HT<sub>2A</sub> receptor expression between vehicle control and MSO control was identical. An additional verification that MSO was an appropriate control is the data in the current series of studies demonstrating that there were no differences in behavior between pre- and post-ASO infusion.

**General Procedures and Time Course for Behavioral Experiments**

An outline and chronology of the standard experiments conducted is provided in Table 1. The maximum life of the pumps used in this study is 28 days, therefore all testing was completed by, and all subjects were sacrificed on the 26th day following pump implantation. Briefly, upon arrival, subjects were given 1 week to habituate to the colony room and to handling. Subjects then received a minimum of 2 weeks of 24-h access to EtOH and water in two-bottle choice paradigm. After stable intake of a 6% EtOH was achieved, subjects were implanted with pumps. Following surgery, subjects were returned to their home cages and the 24-h EtOH/water choice paradigm was continued for 12 days (days 1–12 postsurgery). Subjects were then given 1 or 2 days of rest, followed by 2 to 3 days testing saccharin versus water preference (generally days 14–16 postsurgery) in a 1-h access paradigm. One day of rest ensued saccharin testing followed by 4 days of quantification of behaviors displayed in an open field following both saline and EtOH injection (generally tested on days 18–21). One to 2 days of rest was then given followed by 1 day of evaluation of behavior in the EPM (generally tested around day 23). All subjects received identical time courses and procedures through approximately day 23 postpump implantation (Table 1). At this point, determination of further testing in each group was based on the brain structure targeted, current theories as to the role of 5-HT and the 5-HT<sub>2A</sub> receptor in this region, time constraints of oligonucleotide delivery, and most importantly an evaluation of any changes in EtOH consumption during the drinking phase. Therefore, not all groups were subjected to the same procedures during the final days of the experiment. These additional tests included a test of nociceptin (DRN), measurements of blood plasma corticosterone levels (i.e., CeA/L), and response to 5-HT agonist-induced DOI-head shakes (i.e., c.v.). The possibility must be considered that a subject’s experience in some of the earlier testing procedures may have influenced their behavior in subsequent tests. Great care was taken to minimize these effects across groups, and it must be emphasized that the schedules and procedures were held identical for all groups.

**Oral Self-Administration of EtOH**

After approximately 1 week of habituation to the colony room and daily handling, daily consumption of water and EtOH solution were determined using a two-bottle 24-h access choice procedure. Continuous testing was then conducted for approximately 2 weeks prior to surgical pump implantation. Two 100-ml drinking tubes equipped with 8-cm stainless steel-ball-tipped sipper tubes were attached to the front of each cage. The drinking tubes were weighed and refilled with fresh solutions every day at approximately 4 h into the dark cycle, and bottle placement was rotated daily to control for position bias. Starting at 3% (v/v), the concentration of EtOH was increased by 1% every second day up to 6%. The weight of the fluid consumed was converted to (g of EtOH)/(kg of body weight) consumed, and an intake ratio, defined as (ml of EtOH/ml of EtOH + ml of water) was calculated. After reaching a criterion of 7 to 10 days of stable 6% (v/v) EtOH consumption (stable was defined as a minimum of five sequential days of consumption with a maximum of 20% variance across days) subjects were surgically implanted with the ASO delivery pumps and cannula. EtOH was immediately available following surgery and changes from presurgery levels were evaluated. The EtOH administration routine was kept identical to the presurgical procedure and lasted for first 12 days postsurgery.

**Saccharin Testing**

In paradigms where a pharmacological manipulation results in alterations of EtOH intake, the same manipulation is frequently tested on saccharin intake as well. This allows for confirmation as to whether the observed effects were specific for EtOH, or generalized to other preferred solutions as well. Control and experimental rats were tested for saccharin ingestion over 3 days in a 1-h limited access two-bottle choice paradigm. One bottle contained 0.1% saccharin solution in tap water (w/v), and the second contained tap water. The drinking tubes were refilled with fresh solutions daily, and their positions were switched daily to control for position bias.

**Open Field Test**

The quantification of spontaneously occurring behaviors that are displayed in an open field arena is an important tool in determining the effects of CNS manipulations. EtOH specifically is known to produce biphasic effects in locomotor activity, in that at low-to-moderate doses, it initially acts as a stimulant (generally in the first 5 min) and then as a depressant (after 5–10 min). This effect of EtOH is thought to be an index of its reinforcing properties, and therefore an important component of EtOH’s pharmacology. In the current experiment, testing occurred once a day for four consecutive days and behaviors were quantitated as originally described by. Days 1 and 2 consisted of a 5-min habituation session. Trials on the 3rd and 4th day were 10 min in length, and were preceded by an i.p. injection of saline or 0.75 g/kg EtOH (11%), respectively. The open field arena was 100 × 100 cm with the floor divided into 16 quadrants. Within the floor, there were eight equidistantly distributed 4-cm-diameter holes. Briefly, the onset, frequency, and total duration of the following behaviors were evaluated: crossover activity, rearing, grooming, headpoke, and center tendency. A crossover, a measure of locomotion, was counted when the rat crossed with all four legs from one square to another. A rear was counted when a rat stood on its hind limbs, with its forelimbs completely off the floor. A headpoke, believed to reflect exploratory behavior, was counted when the animal inserted its head below eye level into a hole in the floor. Center

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**TABLE 1**

<table>
<thead>
<tr>
<th>Time course of procedures and order of testing for subjects in the current experiment</th>
<th>The table summarizes daily testing schedule and overall chronology of subjects treated chronically with 5-HT&lt;sub&gt;2A&lt;/sub&gt; antisense given i.c.v. or targeted to the specific brain areas listed.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chronic</strong></td>
<td><strong>Test/Procedure</strong></td>
</tr>
<tr>
<td>Presurgery days 1 to 7</td>
<td>Adjustment to the colony room</td>
</tr>
<tr>
<td>Presurgery days 8 to 21</td>
<td>Baseline EtOH intake</td>
</tr>
<tr>
<td>Surgical Pump Implantation</td>
<td></td>
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<tr>
<td>Postsurgery days 1 to 12</td>
<td>EtOH intake</td>
</tr>
<tr>
<td>Postsurgery day 13</td>
<td>No testing</td>
</tr>
<tr>
<td>Postsurgery days 14 to 16</td>
<td>Saccharin preference</td>
</tr>
<tr>
<td>Postsurgery day 17</td>
<td>No testing</td>
</tr>
<tr>
<td>Postsurgery days 18 to 21</td>
<td>Open field behaviors</td>
</tr>
<tr>
<td>Postsurgery day 22</td>
<td>No testing</td>
</tr>
<tr>
<td>Postsurgery day 23</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>Postsurgery days 24 to 26</td>
<td>Additional group specific testing</td>
</tr>
<tr>
<td>Postsurgery day 26</td>
<td>Sacrifice and brain removal for functional receptor binding</td>
</tr>
</tbody>
</table>
behavior was counted when all four legs of the rats were in one of the four center squares (total area 50 × 50 cm). The behaviors were quantified with the aid of an IBM-XT computer equipped with a manually operated interface. The observer remained in the room approximately 2 m from the testing chamber for the entire session.

**Elevated Plus Maze Test**

As previously mentioned, alcoholism and alcohol abuse has often been linked to stress and anxiety, and the 5-HT2A receptor has been implicated in all of these conditions. The EPM is considered a valid and robust measurement of anxiety and stress in preclinical experimentation, and therefore subjects in the current experiments were tested in this procedure. The apparatus was made of Plexiglas with the arms elevated 50 cm from the floor. It had two opposite open arms, 50 × 10 cm, and two opposite enclosed arms of the same dimensions, but with 40-cm-high Plexiglas walls. The arms were interconnected by a central square forming a plus sign. Subjects were placed on the central square and remained on the maze for a single 5-min trial. To enhance aversiveness of the test, testing was conducted under bright fluorescent lighting. The latency of arm entries, the number of entries onto, and times spent on, open and enclosed arms were quantified with the aid of an IBM-XT computer equipped with a manually operated interface.

**Tail-Flick Test**

The tail-flick test is a test of nociceptin and consists of direct application of a concentrated heat source to a rat's tail and measures the latency for the subject to remove its tail from the heat source. 5-HT2A receptors specifically in the DRN have been implicated in nociceptin, therefore subjects receiving ASO in to this region were tested in this paradigm as a verification of receptor knockdown. The latency (in s) for the subject to retract its tail away from a focused beam of light was measured. Each subject received three consecutive trials with a 1-min rest period between each trial.

**DOI-Induced Head Shakes**

The first group of subjects to receive ASO to the 5-HT2A receptor was administered ASO into the lateral ventricle. As an i.v. test of 5-HT2A receptor function to confirm a reduction in 5-HT2A receptor function produced by ASO, rats were tested using a tail-flick analgesiometer (Columbus Instruments, Columbus, OH). The latency (in s) for the subject to retract its tail away from a focused beam of light was recorded. Each subject received three consecutive trials with a 1-min rest period between each trial.

**Restraint Stress**

5-HT and 5-HT2A receptors have been implicated in stress and anxiety responses, which as previously mentioned, may be related to EtOH intake. Therefore, some groups of animals were tested in a restraint stress paradigm and blood plasma corticosterone, an index of the stress response, was analyzed. Subjects were removed from their home cage and a tail blood sample was immediately collected into heparinized tubes. Following this, subjects were placed into wire mesh cylindrical restrainers for 30 min, after which a second tail blood sample was collected.

**Plasma Corticosterone Determination**

Tail blood was centrifuged at 2000g for 10 min. Plasma was collected and frozen at −70°C until analysis. Duplicate 10-μl samples of plasma were used for quantification of corticosterone concentration using ICN radioimmunoassay kits (Irvine, CA).

**5-HT2A Receptor Binding**

Two different procedures were used to determine the effectiveness of 5-HT2A ASO treatment. In subjects receiving i.c.v. 5-HT2A ASO, [3H]mesulergine binding was conducted using PFC homogenate membranes. For subjects that received ASO targeted to specific brain areas, autoradiographic receptor binding was carried out using 125I-LSD.

**Filtration Receptor Binding**

In subjects receiving 5-HT2A ASO i.c.v., rats were sacrificed at the conclusion of the experiment and the brain was removed and placed on ice. The prefrontal cortex was dissected and immediately homogenized with a Brinkman homogenizer in 40 volumes of Tris-HCl (50 mM, pH 7.9). Tissue from each rat was individually analyzed. Tissues were then centrifuged at 25,000g, and the procedure was repeated for a total of three washes and frozen in pellet form at −70°C until the binding assay was performed. Tissues were resuspended at 1 mg of protein/ml [protein concentrations were determined using the kit supplied by Bio-Rad, Richmond, CA]. The binding assay was performed in a final volume of 250 μl; 5-HT2A receptors were labeled with [3H]mesulergine (64 nM), and nonspecific binding was determined using spiroperidol (400 nM). The binding reaction was incubated for 3 h at room temperature. Membranes were washed three times using a Brandel cell harvester and collected onto filters, which were then dried and counted at 65% efficiency in OptiFluor scintillation cocktail.

**Quantitative Autoradiography.** To further delineate the regional effects of ASO treatment on 5-HT2A receptors, brains of ASO-treated rats were processed for the autoradiographic receptor binding with 125I-LSD. Following 26 days of ASO or MSO infusion, subjects were sacrificed by decapitation, brains were rapidly removed, frozen on dry ice, and then stored at −70°C until sectioning. Coronal tissue sections (20 μm) were then cut using a Reichert-Jung Frigocut 2100 cryostat microtome maintained at −21°C. Brain sections were thaw-mounted on microscope slides submerged in a gelatin (1%, w/v) and chrome alum (chromium potassium permanganate; 0.1%, w/v) solution. Consecutive, superimposable sections were then used to determine total and nonspecific binding. Following thaw-mounting, the sections were stored under vacuum with desiccant at −20°C. 125I-LSD (2000 Ci/mM; PerkinElmer Life Science Products, Boston, MA) at a concentration of 50 μM in a 50 mM Tris, pH 7.4 buffer, was used to label the 5-HT2A receptor. Tissue sections were washed twice for 15 min in ice-cold buffer and then incubated in radioligand-containing buffer at room temperature. For 5-HT2A receptors, nonspecific binding was determined in the presence of spiroperidol (200 nM), which blocks 5-HT2A. Specific 5-HT2A receptor binding was determined from calculating the difference between total and nonspecific binding.

5-HT3 receptor binding was deduced from the binding of 125I-LSD in the presence of spiroperidol (200 nM). Spiroperidol displaces 5-HT2A receptor binding allowing for 125I-LSD to bind only to 5-HT3 receptors. Nonspecific binding was defined using mianserin (1 μM). Mianserin binds to 5-HT2A, 5-HT2C, and dopamine receptors. Following incubation, sections were washed twice for 15 min/wash with ice-cold buffer and then submerged once in deionized water to remove excess buffer salts. Slides were then allowed to dry overnight in a stream of cool air.

Autoradiograms were analyzed using a Cohu video camera (Imaging Research Inc., Hamilton, Canada) and a Macintosh Quadra 800 (Macintosh, Cupertino, CA) running Image (W. Rosband, Research Services, National Institute of Mental Health). Estimates of binding activity were calculated from 125I standards (Amersham Pharmacia Biotech, Piscataway, NJ) exposed along with brain sections.

**Drugs and Reagents**

Oligonucleotides were purchased from Oligos, Etc., Inc. (Wilsonville, OR). All radioligands were purchased from PerkinElmer Life
Data Analysis

To evaluate the effect of 5-HT2A ASO on EtOH intake, statistical analysis was performed across four measures: EtOH intake (g/kg), water intake (ml), EtOH intake ratio, and total fluid intake (ml). Statistical testing was conducted using a mean calculated from 3 days of presurgery baseline, and means calculated from postsurgical days 4 to 6, days 7 to 9, and days 10 to 12. All drinking measurements from postsurgery days 1 to 3 were omitted from the statistical analysis, because subjects were still recovering from surgery. Repeated measures multivariate analysis of variance (MANOVA) was conducted comparing treatment (ASO or MSO) by days (day post-surgery) on all four primary measures. Open field behaviors, saccharin intake, endocrine measures, binding, and tail-flick latencies were analyzed using independent t tests, except where noted. All statistical analysis was carried out using Systat 7.0. All data are represented as means ± S.E.M.

Results

Given the number of groups of animals and experimental procedures conducted, results are organized in a semichronological manner. First, the effects of i.c.v. ASO on receptor binding and EtOH intake are presented, followed by binding data from a within-subjects experiment conducted to measure the extent of oligonucleotide spread. Binding data and EtOH and saccharin intakes are then presented from subjects receiving ASO into the PFC, central nucleus of the amygdala, DRN, and HIP. The additional behavioral experiments conducted, such as open field and EPM, are discussed in the section following binding and EtOH intake. A summary of the behavioral tests conducted and the results from these studies is presented as Table 2.

ASO Treatment Selectively Down-Regulates 5-HT2A Receptors and Alters EtOH and Saccharin Consumption. To determine both the extent of global CNS down-regulation produced by the ASO on the 5-HT2A receptor and the functional consequences, the initial experiment administered ASO by continuous i.c.v. infusion. Following sacrifice of these animals, down-regulation of the receptor was determined in membrane preparations obtained from prefrontal cortex. Binding data were analyzed using a one-tailed t test with the prediction that ASO administration would reduce the number of 5-HT2A receptors. Intracerebroventricular administration of 5-HT2A ASO significantly reduced (–68.9%) 5-HT2A receptors [t(10) = 3.31, p < 0.01], as determined by

<table>
<thead>
<tr>
<th>Site Targeted</th>
<th>Fluid Intake</th>
<th>Locomotor Behavior (Basal, Saline, or EtOH Conditions)</th>
<th>Elevated Plus Maze</th>
<th>Corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ETOH Preference</td>
<td>g/kg</td>
<td>EtOH Ratio</td>
<td>Water and Total Fluid</td>
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<td>Decrease</td>
<td>Increase</td>
<td>N.C.</td>
<td>N.C.</td>
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<tr>
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<td>Decrease</td>
<td>Decrease</td>
<td>N.C.</td>
<td>Decrease</td>
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<tr>
<td>DRN</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
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</tbody>
</table>

N.C., no change; —, not tested.

Table 2

Summary of measures taken and results from subjects with 5-HT2A antisense given i.c.v. or to specific brain areas

This table summarizes the outcomes of behavioral tests and endocrine measures taken on subjects treated chronically with 5-HT2A antisense given i.c.v. or targeted to the specific brain areas listed, compared with subjects treated with 5-HT2A missense.
ments and then compared with identical sites in the opposite hemisphere using a one-tailed t test, with ASO predicted to decrease receptor expression (Fig. 2B). ASO-mediated receptor down-regulation was greatest ventral to the site of implantation with effects seen at a maximum distance from the targeted site of 0.5 mm \([t(10) = 3.01, p < 0.01]\). Medial \([t(11) = 1.97, p < 0.05]\) and lateral \([t(11) = 1.95, p < 0.05]\) to the targeted site, effects were seen at a maximum distance of approximately 0.3 mm. Dorsal to the area containing the largest degree of receptor down-regulation, effects were observed up to 0.1 mm \([t(10) = 2.22, p < 0.05]\).

Following the confirmation that locally administered ASO produced region-specific down-regulation, experiments were then conducted to evaluate the effect of bilateral region-specific ASO-induced 5-HT\(_{2A}\) receptor down-regulation on EtOH consumption. The following regions were targeted in independent sets of animals: PFC, CeA/L, DRN, and HIP. Each of these selected regions has been implicated in behaviors related to EtOH intake. Autoradiographic determination of receptor binding with \(^{125}\)I-LSD was analyzed on multiple CNS structures proximal to the site of implantation to verify that ASO-induced down-regulation was limited to the targeted regions.

Bilateral infusion of 5-HT\(_{2A}\) ASO into layer 5 of the PFC produced a significant reduction of 5-HT\(_{2A}\) receptors in this region. A one-tailed t test showed that 5-HT\(_{2A}\) receptors in layer 5 of both the left and right hemispheres were significantly reduced (Table 3), and bilateral structures were combined for statistical analyses \([t(67) = 2.67, p < 0.01]\). No significant changes in 5-HT\(_{2A}\) receptor binding were seen in areas proximal to the PFC, such as the nucleus accumbens and the caudate putamen.

In a manner identical to subjects receiving i.c.v. ASO, subjects given ASO infused into the PFC were given 24-h access to EtOH and water prior to surgery, and this paradigm was resumed for the 12 days immediately following surgery. It must be noted that baseline EtOH consumption of the PFC subjects was slightly lower than that of other groups in this study. While there were no procedural differences in

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**Fig. 1.** A, Effect of i.c.v. administration of 5-HT\(_{2A}\) ASO on the binding of 5-HT\(_{2A}\) receptors on PFC neuronal membranes. Columns and error bars represent the mean femtomoles of \[^{3}\text{H}]\text{mesulergine (MS)}\text{ bound per milligram of PFC tissue} (\(\bullet\), MS; \(\square\), ASO) ± S.E.M. B, i.c.v. administration of 5-HT\(_{2A}\) receptor ASO significantly reduced ingestion of EtOH intake (g/kg) (\(\bullet\), MSO; \(\square\), ASO). Data are presented as means averaged across three consecutive days of testing ± S.E.M. Groups of 10 animals were used. * \(p < 0.05\) versus ASO group. C, Subjects receiving i.c.v. 5-HT\(_{2A}\) ASO treatment displayed significantly fewer head shakes following administration of 1.0 mg/kg DOI. Groups of 10 animals were used. * \(p < 0.05\) versus ASO group.

**Fig. 2.** Evaluation of the distance of oligonucleotide spread and the resulting range of receptor down-regulation effects of oligonucleotide on receptor binding in areas proximal to the site of implantation. The largest decrease in binding relative to control was 18%, and decreases in this effect are observed at the distance (\(\square\), dorsal; \(\circ\), ventral; \(\bigcirc\), medial; and \(\triangle\), lateral) from this area increases.
the PFC group, these subjects were tested during the summer, while all others were run in the fall or winter. Seasonal differences in rats' behavior and serotonergic function have been previously reported (Valzelli et al., 1977). ASO infusion into the PFC was found to augment EtOH intake ratio and consumption. This was unexpected, given that ASO administered i.c.v. reduced EtOH intake. An initial independent t test showed no significant difference in EtOH intake ratio baseline measurements between ASO and MSO subjects. Following ASO administration, a significant main effect was observed on EtOH intake ratio [F(1,8) = 6.32, p < 0.05] (Fig. 3A). ASO infused into the PFC also produced a significant main effect on grams per kilogram of EtOH consumption [F(1,8) = 5.89, p < 0.05] (Fig. 3B). Importantly, no significant differences in pre- or postsurgery water or total fluid intake between ASO-treated and control subjects were seen at any point during testing (Fig. 3C). Correlated two-tailed t tests comparing ASO baseline measures to their day 4 to 6 postsurgery averages confirmed that there was a significant enhancement of EtOH consumption in ASO-treated subjects. In ASO-treated animals, EtOH intake ratios were significantly different from their baseline measures by days 7 to 9 [t(4) = 4.82, p < 0.01], as was their day 7 to 9 g/kg intake [t(4) = 4.18, p < 0.01].

Following the EtOH drinking phase of the experiment, PFC subjects were then tested to see whether differences in intake of a saccharin solution existed. A number of studies have shown positive correlations between EtOH and saccharin solution intake, suggesting that similar neural substrates may underlie both behaviors (Overstreet et al., 1993). It was predicted that PFC ASO-treated subjects might have significantly higher saccharin intake, and analysis by one-tailed t test showed that 5-HT$_{2A}$ ASO administration into the PFC did result in significantly higher saccharin solution (0.1%, w/v) intake on both the first [t(10) = 1.86, p < 0.05] and second day [t(11) = 1.97, p < 0.05] of testing (Fig. 4). The higher intake of saccharin solution by ASO-treated subjects paralleled the higher levels of EtOH intake observed in these same subjects.

For subjects who received 5-HT$_{2A}$ ASO directed at the central nucleus of the amygdala, an independent t test of $^{125}$I-LSD binding confirmed ASO reduction of 5-HT$_{2A}$ receptors. While 5-HT$_{2A}$ receptors in both the left and right hemispheres were significantly reduced (Table 3), bilateral structures were combined for statistical analyses. One-tailed t tests showed that the reduction in $^{125}$I-LSD binding was significantly lower in the CeM [t(76) = 4.53, p < 0.01], the CeL [t(74) = 3.42, p < 0.01], and the bed nucleus of the stria terminalis [t(75) = 5.03, p < 0.01]. Because the down-regulation was not restricted to the central nucleus of the amygdala, but also was significant for the lateral nucleus and for the bed nucleus of the stria terminalis, we refer to the area of receptor knockdown as CeA/L. No significant reductions were observed in the other proximal structures, such as the ventral medial hypothalamus.

ASO treatment directed at the CeA/L significantly decreased the consumption of EtOH in a manner similar to that seen in the i.c.v. subjects. Testing was conducted in a manner identical to the i.c.v. and PFC groups in that subjects had 24-h access to both EtOH and water and were tested again in this paradigm for the 12 days following surgery. An independent t test of EtOH intake ratio baseline readings showed no differences between ASO and MSO animals. Following ASO administration, MANOVA performed on postsurgery EtOH intake ratio values showed a significant main effect of ASO treatment [F(1,12) = 5.48, p < 0.05] on EtOH intake ratio with ASO subjects having lower EtOH intake ratio (Fig. 5A). Similar to intake ratio, an independent t test comparing presurgery baseline grams per kilogram of EtOH intake levels showed that ASO and MSO subjects were not significantly different. MANOVA analysis of grams per kilogram of EtOH consumption in the period following ASO administration showed a significant main effect of ASO treatment [F(1,12) = 5.79, p < 0.05] (Fig. 5B), with ASO subjects consuming less EtOH than MSO subjects. Further analysis of EtOH intake ratio and gram per kilogram intakes showed no other significant effects or interactions, suggesting that the maximal ASO effect was reached by day 4 and remained constant through day 12. Again, ASO infusion did not alter water consumption because no significant differences in pre- or postsurgery water intake were found. No significant differences in pre- or postsurgery total fluid intake were found.

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**TABLE 3**

$^{125}$I-LSD binding to 5-HT$_{2A}$ receptors in CNS of ASO- and MSO-infused subjects

<table>
<thead>
<tr>
<th>Targeted Structure</th>
<th>Structure Measured</th>
<th>MSO</th>
<th>ASO</th>
<th>% Change from MSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal cortex</td>
<td>PFC-L5—left</td>
<td>40.23 ± 0.04</td>
<td>28.25 ± 0.02*</td>
<td>−29.78</td>
</tr>
<tr>
<td></td>
<td>PFC-L5—right</td>
<td>39.43 ± 0.03</td>
<td>29.12 ± 0.03*</td>
<td>−26.15</td>
</tr>
<tr>
<td></td>
<td>CPU—left</td>
<td>22.77 ± 2.81</td>
<td>18.05 ± 2.85</td>
<td>−20.73</td>
</tr>
<tr>
<td></td>
<td>CPU—right</td>
<td>23.43 ± 2.27</td>
<td>20.72 ± 2.44</td>
<td>−11.56</td>
</tr>
<tr>
<td>Amygdala</td>
<td>NuAcc—left</td>
<td>22.36 ± 2.47</td>
<td>20.32 ± 1.99</td>
<td>−9.12</td>
</tr>
<tr>
<td></td>
<td>NuAcc—right</td>
<td>22.59 ± 1.92</td>
<td>19.67 ± 2.32</td>
<td>−12.93</td>
</tr>
<tr>
<td></td>
<td>CeM—left</td>
<td>32.26 ± 0.52</td>
<td>24.88 ± 0.60*</td>
<td>−27.11</td>
</tr>
<tr>
<td></td>
<td>CeM—right</td>
<td>31.48 ± 0.54</td>
<td>26.12 ± 0.40*</td>
<td>−17.04</td>
</tr>
<tr>
<td></td>
<td>CeL—left</td>
<td>31.98 ± 0.50</td>
<td>24.40 ± 0.63*</td>
<td>−23.71</td>
</tr>
<tr>
<td></td>
<td>CeL—right</td>
<td>30.75 ± 0.50</td>
<td>23.75 ± 1.02*</td>
<td>−22.76</td>
</tr>
<tr>
<td></td>
<td>BSTIA—left</td>
<td>31.21 ± 0.46</td>
<td>25.62 ± 1.32*</td>
<td>−24.31</td>
</tr>
<tr>
<td></td>
<td>BSTIA—right</td>
<td>31.77 ± 0.53</td>
<td>26.06 ± 0.36*</td>
<td>−17.97</td>
</tr>
<tr>
<td></td>
<td>VMH—left</td>
<td>46.58 ± 1.4</td>
<td>48.27 ± 1.5</td>
<td>+3.63</td>
</tr>
<tr>
<td></td>
<td>VMH—right</td>
<td>47.55 ± 1.18</td>
<td>49.01 ± 1.48</td>
<td>+3.07</td>
</tr>
<tr>
<td>Dorsal raphe nucleus</td>
<td>DR</td>
<td>55.52 ± 1.74</td>
<td>46.85 ± 0.66*</td>
<td>−16.62</td>
</tr>
<tr>
<td></td>
<td>MnR</td>
<td>56.03 ± 2.93</td>
<td>52.75 ± 1.48</td>
<td>−5.85</td>
</tr>
<tr>
<td></td>
<td>CGLV—left</td>
<td>55.15 ± 1.81</td>
<td>51.67 ± 1.62</td>
<td>−6.31</td>
</tr>
<tr>
<td></td>
<td>CGLV—right</td>
<td>55.02 ± 1.82</td>
<td>50.63 ± 1.73</td>
<td>−7.97</td>
</tr>
</tbody>
</table>

* p < 0.01 of AS versus MS subjects.
Fig. 5C). Statistical analysis using two-tailed correlated \( t \) tests confirmed that EtOH intake had significantly changed from baseline measures in ASO-treated subjects. EtOH drinking at baseline by ASO-treated subjects significantly differed from that at days 4 to 6 for intake ratio \( t(6) = 2.87, p < 0.05 \) and grams per kilogram \( t(6) = 3.09, p < 0.05 \), suggesting that 4 days of infusion significantly reduced EtOH intake.

Given the reduction in EtOH consumption shown by these subjects, it was predicted that saccharin intake might also be lower. Analysis with a one-tailed \( t \) test showed that subjects infused with ASO into the CeA/L had significantly lower saccharin intake on the 3rd day of testing compared with MSO controls \( t(13) = 2.21, p < 0.05 \), in a manner consistent with their lower EtOH intake ratio and intake (Fig. 4).

ASO administration into the DRN resulted in a significant decrease in 5-HT\(_{2A}\) receptor binding in this region \( t(65) = 2.09, p < 0.05 \) (Table 3). No significant reduction in 5-HT\(_{2A}\) receptors was seen in the CGLV or median raphe nucleus. There were no significant differences in EtOH intake ratio or grams per kilogram intake in these subjects (Table 4) in pre- or postsurgery testing.

Bilateral infusion of ASO or MSO into the HIP produced no significant differences in any of the following behavioral tests in their pre- or postsurgery measurements: EtOH intake ratio, grams per kilogram of EtOH intake, water or total fluid intake (Table 4), or saccharin intake.

5-HT\(_{2A}\) Receptor Down-Regulation Also Alters Other Behaviors Related to 5-HT. Following the postsurgery 12-day EtOH intake testing phase, subjects were then tested in a variety of behavioral paradigms, many of which are thought to be indexes of anxiety and stress, to further iden-
tify the role of the 5-HT2A receptor in specific regions of the CNS.

After completion of EtOH intake, subjects receiving i.c.v. ASO were tested in the following paradigms: plasma corticosterone and behavioral response to a novel open field and EPM behaviors. After exposure to a novel open field, ASO subjects had significantly higher elevations in plasma corticosterone compared with ASO controls \[t(12) = 2.51, p < 0.05\] suggesting they had a hypersensitive response to the new environment (Fig. 7A). Rats receiving i.c.v. 5-HT2A ASO treatment displayed 41% greater crossing activity (an index of locomotor behavior) compared with MSO control subjects \[t(6) = 2.50, p < 0.05; MSO = 31.87 ± 6.88, ASO = 77.20 ± 24.23\] in a novel open field. Subjects were next tested on an EPM, a frequently used index of anxiety in the rat. Following i.c.v. treatment with 5-HT2A ASO, analysis with a two-tailed \(t\) test revealed that these rats had a higher percentage of entries and spent a higher percentage of time on the open arms of the EPM compared with MSO controls \(t(6) = 3.05, p < 0.05\) and \(t(6) = 2.49, p < 0.05\), respectively (Fig. 6). The higher number of entries into the open arms could be attributed to the ASO subjects higher basal levels of locomotor activity, however their increased duration of stay on the open arms suggests that this was due more to an anxiogenic effect resulting from down-regulation of 5-HT2A receptors. Taken together, i.c.v. subjects had lower EtOH intake, higher levels of locomotor activity in the open field, and a higher percentage of time spent on the open arms of the EPM, suggesting that i.c.v. ASO produced and anxiolytic-like effect.

Subjects receiving ASO into the PFC were evaluated in the following behavioral paradigms at the conclusion of the

![Fig. 5](image)

**Fig. 5.** Effects of 5-HT2A receptor ASO infusion into the CeA/L on 24-h EtOH intake ratio (6% v/v) (A), EtOH intake (g/kg) (B), and water intake for the MSO- (■) and ASO (▲)-treated subjects (C). Data are presented as means averaged across three consecutive days of testing ± S.E.M. Groups of seven animals were used. *\(p < 0.05\) and **\(p < 0.01\) versus ASO group.

![Fig. 6](image)

**Fig. 6.** Left, i.c.v. ASO oligonucleotide-infused subjects spent a significantly greater amount of time on the open arms of the EPM compared with MSO controls, but the number of entries onto the open arms was not significantly different (■, MSO; ▲, ASO). Groups of four animals were used. *\(p < 0.05\). Right, PFC ASO oligonucleotide-infused subjects made significantly less entries into the open arm of the EPM, compared with MSO controls. *\(p < 0.05\).

### Table 4

<table>
<thead>
<tr>
<th>Targeted Structure</th>
<th>Fluid Intake Measures</th>
<th>MSO</th>
<th>ASO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol intake ratio</td>
<td>0.26 ± 0.04</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Ethanol intake (g/kg)</td>
<td>2.57 ± 0.56</td>
<td>2.09 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>Total fluid (ml)</td>
<td>38.06 ± 32</td>
<td>38.76 ± 1.87</td>
</tr>
<tr>
<td>Dorsal raphe</td>
<td>Ethanol intake ratio</td>
<td>0.48 ± 0.06</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Ethanol intake (g/kg)</td>
<td>2.77 ± 0.25</td>
<td>2.39 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Total fluid (ml)</td>
<td>30.99 ± 0.6</td>
<td>32.48 ± 5.15</td>
</tr>
</tbody>
</table>
EtOH and saccharin intake phases: open field behavior following saline and EtOH injections, EPM behavior and plasma corticosterone response to a novel environment. No significant differences in open field behaviors were observed during the habituation or following saline or EtOH injections. However, a two-tailed t test showed subjects infused with 5-HT$_{2A}$ ASO into the PFC made significantly fewer entries into the open arms of the EPM compared with MSO controls (Fig. 6) $[t(10) = 3.96, p < 0.01]$. Furthermore, subjects who had ASO infused into the PFC had a significantly lower elevation in plasma corticosterone following exposure to a novel open field compared with MSO controls $[t(8) = 1.96, p = 0.05]$ (Fig. 7A). Overall, subjects with reduced 5-HT$_{2A}$ receptors had higher levels of EtOH intake, no differences in open field behavior or response to EtOH, made significantly fewer entries into the open arms of the EPM (Fig. 8), and had a lower corticosterone responses to an acute mild stressor.

Subjects who received bilateral infusions of either or MSO or ASO into the CeA/L were tested in the following paradigms following the completion of the EtOH and saccharin test phase: open field behaviors following both saline and EtOH injections, EPM behaviors and plasma corticosterone response to restraint stress. No significant differences were observed in behaviors on the EPM between MSO and ASO subjects. No significant differences were seen in any open field behaviors during the habituation phase, or following saline and EtOH injections between MSO or ASO subjects. CeA/L-targeted subjects were then tested in a restraint stress paradigm, because the various regions of the CeA/L are known to be involved in the regulation of stress response. Given the effects that ASO treatment into the PFC subjects increased EtOH intake and blood plasma corticosterone levels, it was predicted that CeA/L subjects might have reduced plasma CORT because the changes in EtOH intake were opposite that of PFC subjects. A one-tailed t test showed that a decrease of 5-HT$_{2A}$ receptors in the CeA/L significantly reduced basal corticosterone levels $[t(9) = 2.15, p < 0.05]$ (Fig. 7B). This alteration was interesting, however, restraint stress increased plasma corticosterone levels similarly in both groups (2.5–3.4-fold). In summary, subjects receiving ASO into the CeA/L had decreased EtOH intake and intake ratio, no alterations of open field behaviors under saline or EtOH conditions, and no differences in EPM performance, but they did have significantly lower plasma corticosterone levels.

Following EtOH intake testing, subjects who were infused with ASO or MSO into the DRN were tested for differences in the following paradigms: open field behaviors following saline and EtOH injection, EPM behaviors, and tail-flick response. No significant differences were seen in any open field behaviors during the habituation phase, or following saline and EtOH injections between MSO or ASO subjects. Also, no significant differences were seen in EPM behaviors. DRN ASO subjects were also tested in a tail-flick paradigm to measure levels of nociceptin, because 5-HT$_{2A}$ receptors in this region are thought to be involved in the regulation of this behavior (Kjorsvik et al., 1997). Following three trials, analysis with a two-tailed t test showed that subjects receiving ASO into the DRN demonstrated significantly longer latencies (in seconds, MSO = 4.42 ± 0.17; ASO = 3.04 ± 0.18) to remove their tails from a heat source $[t(14) = 2.64, p < 0.05]$ but only on the third trial. Subjects did not display any differences in latencies during the first two trials.

Post-EtOH testing, subjects infused with ASO or MSO into the HIP were tested in the open field and EPM. No significant behavioral differences were seen between HIP ASO and MSO control subjects in any of the following behavioral tests:

![Fig. 7. A, in icv. and PFC ASO oligonucleotide-infused subjects, plasma corticosterone levels prior to and immediately following exposure to a novel environment (■, MSO; □, ASO). Data are presented as means ± S.E.M. Groups of six animals were used. *p < 0.05 versus ASO group. B, basal plasma corticosterone concentration (ng/ml) from subjects infused with 5-HT$_{2A}$ receptor ASO into the CeA/L. Data are presented as means ± S.E.M. Groups of seven animals were used. *p < 0.05 versus ASO group.](image-url)
The current studies provide substantial evidence that down-regulation of the 5-HT$_{2A}$ receptor results in changes in EtOH and saccharin intake, as well as behaviors related to anxiety and stress, and the direction of change is dependent upon the CNS structure affected. These data combined with the moderate success seen using serotonergic agents to decrease alcohol intake and treat stress-related disorders implies that the down-regulation of the 5-HT$_{2A}$ receptor produced by chronic use of these compounds may be one mechanism through which they affect such behaviors (Pohorecky et al., 1998; Roberts et al., 1998; Maurel et al., 1999). Importantly, the changes observed in these experiments were produced by levels of 5-HT$_{2A}$ down-regulation ranging from 15 to 29% (Table 2), which are substantially less than the 50% reduction reported following treatment with compounds such as antidepressants (Blackshear et al., 1986). Additionally, the present results most likely reflect changes only in 5-HT$_{2A}$ receptor function, because our ASO sequence produced no alterations in 5-HT$_{3C}$ binding, which is common with many ligands that effect 5-HT$_{2A}$ receptors.

The initial feasibility study of i.c.v. ASO demonstrated that CNS-wide reduction of 5-HT$_{2A}$ receptors decreased EtOH intake in a manner comparable with effects in other studies, demonstrating that compounds that down-regulate 5-HT$_{2A}$ receptors also reduce EtOH intake (Meyers et al., 1993; Pohorecky et al., 1998; Maurel et al., 1999). Decreased EtOH intake following i.c.v. ASO infusion cannot be attributed to down-regulation of 5-HT$_{2A}$ receptors in any one specific CNS region. However, the decreased ethanol consumption could be mediated in part by access of the ASO to areas bordering the ventricles such as the amygdala. This is suggested by the current experiments demonstrating that ASO down-regulation of 5-HT$_{2A}$ receptors in the CeA/L decreased EtOH, as well as saccharin, intake. The resulting change in EtOH intake following CeA/L 5-HT$_{2A}$ receptor down-regulation was the largest seen of all brain sites tested, and like the effects seen in the i.c.v. subjects, was also comparable with decreases in EtOH intake following administration of compounds that reduce 5-HT$_{2A}$ receptor expression (Meert and Janssen, 1991; Meyers et al., 1993; Pohorecky et al., 1998; Roberts et al., 1998; Maurel et al., 1999).

The amygdala is considered a specialized autonomic and motor-projection region of the striatum (Cassell et al., 1999) and mediates stress-induced responses and expression of emotional behavior (LeDoux et al., 1990). Certain regions of the amygdala are moderately innervated with dopamine, γ-aminobutyric acid and glutamate neurons (Cassell et al., 1999). Amygdala γ-aminobutyric acid-ergic neurons appear to be important in EtOH pharmacology, as suggested by drug discrimination and self-administration studies (Hodge and Cox, 1998; Roberts et al., 1998). EtOH intake also increases amygdala glucose utilization and produces rapid changes in immunohistochemically measured transcription factors (c-Fos and FosB) in the posterior-ventral portion (Porrino et al., 1998; Bachtell et al., 1999). EtOH-induced locomotor activity is also associated with an increase in the number of amygdala Fos-like-immunoreactivity neurons (Chang et al., 1995), and this activation may mediate the degree of stimulatory response to EtOH (Demarest et al., 1998). Therefore, the EtOH’s locomotor activating effect, which has been associated with its rewarding effect, might be mediated by increased activation of neurons intrinsic to the CeA/L. Thus, our results of decreased EtOH consumption following ASO down-regulation of 5-HT$_{2A}$ receptors in the CeA/L might be explained by an increase in the basal neural activity within the CeA/L, which prevented further EtOH-induced activation.

Conversely, ASO induced down-regulation of 5-HT$_{2A}$ receptors in the PFC increased consumption of EtOH and saccharin, suggesting that 5-HT$_{2A}$ PFC receptors have a tonic inhibitory effect on intake of these solutions. The PFC is a compartmentalized, heterogeneous region, with dopamine-containing ascending projections originating in the ventral tegmental area; and the pars compacta and descending projections traced to both the core and shell of the nucleus accumbens, the periaqueductal gray area, the DRN, and the locus coeruleus (Berendese et al., 1992). Given the PFC’s anatomical connections with major pathways/structures associated with mediating reinforcement and reward, the PFC has also been implicated in the reward and reinforcement of EtOH (Bauer and Hesselbrock, 1993; Deckel et al., 1995) and 5-HT has been specifically implicated in EtOH/PFC interactions (Zhou et al., 1991, 1994; Murphy et al., 1992; McBride et al., 1993; Devoto et al., 1998). The present results underscore the importance of the 5-HT$_{2A}$ receptor in this system. While the specific neural mechanisms through which this occurs are unknown, one possibility is that 5-HT$_{2A}$ receptors increase excitatory postsynaptic potentials in PFC medial layer V pyramidal cells (Marek and Aghajanian, 1999; Marek et al., 2000), enhancing neurotransmitter release from glutamate terminals, leading to an inhibitory effect on reward processes.

5-HT$_{2A}$ receptor knockdown also produced changes in anxiety and stress behaviors and modulated plasma corticosterone levels in several experimental groups. A comparison of the behavioral changes across different treatment groups revealed that there was a consistent pattern between i.c.v. and PFC ASO subjects in relation to stress and anxiety behaviors and the direction of change in EtOH intake. The decrease in EtOH intake by i.c.v. ASO subjects was accom-
panned by increased locomotor activity, greater duration of time spent in the open arms of the EPM, and a hypersensitive reaction of plasma corticosterone following novel environment exposure. These behavioral effects are comparable with those seen following treatment with compounds that down-regulate 5-HT$_{2A}$ receptors. For example, mianserin increases time spent on the open arms of the EPM and is a releaser of spontaneously occurring behaviors in such paradigms as the open field (Benjamin et al., 1992a). Together, this suggests that i.c.v. ASO down-regulation of 5-HT$_{2A}$ receptors produced an anxiolytic-like profile, which was comparable with that seen following administration of compounds that down-regulate 5-HT$_{2A}$ receptors (Benjamin et al., 1992b). Similar anxiolytic-like effects following ASO-induced down-regulation of 5-HT$_{2A}$ receptors have been seen in mice (Sibille et al., 1997). Down-regulation of 5-HT$_{2A}$ receptors in the PFC resulted in an anxiogenic behavioral pattern that was almost the inverse of the effects seen in i.c.v. subjects. Specifically, PFC ASO subjects displayed significantly fewer open arm entries on the EPM, a blunted plasma corticosterone increase following exposure to a novel environment, and an increase in EtOH intake. It is difficult to ascertain what role either the anxiolytic or anxiogenic effects, seen in i.c.v. and PFC subjects, respectively, following ASO treatment had in the opposing changes in EtOH intake.

The data from subjects receiving ASO in the CeA/L are not as clear in identifying a pattern between anxiety-related behaviors and EtOH intake. ASO administration into the CeA/L decreased EtOH consumption, in a manner analogous to i.c.v. subjects. In contrast to i.c.v. and PFC subjects, CeA/L subjects showed no significant differences in behaviors related to stress and anxiety. This was surprising, because amygdaloid structures have been linked to the stress response (Sibille et al., 1997; Morelli and Pinna, 1999). While no changes in anxiety-related behaviors were seen following ASO infusion into the CeA/L, changes in basal plasma corticosterone were observed. However, unlike i.c.v. and PFC subjects, CeA/L 5-HT$_{2A}$ receptor down-regulation did not influence the plasma corticosterone response following a stressor. This finding is relevant, given that the CeA/L contains the highest density of corticotrophin-releasing factor-containing cell bodies in the amygdaloid complex (Van Bockstaele et al., 1998). Additionally, since antidepressant treatments decrease both plasma glucocorticoid levels and 5-HT$_{2A}$ receptor function (Lopez et al., 1997, Kozuru et al., 2000) these findings suggest CeA/L 5-HT$_{2A}$ receptor down-regulation might be a mechanism for the hypothalamic-pituitary-adrenal axis-attenuating action of antidepressants.

While 5-HT$_{2A}$ receptor down-regulation produced changes in open field and EPM behaviors and corticosterone function in different groups of subjects, it is possible that these systems, while overlapping in their physiology, are separate from those underlying EtOH intake. Certainly, further testing is warranted before any conclusions can be drawn.

In the case of the DRN, while no changes in EtOH ingestion occurred, change in nociceptin in ASO-treated subjects indicates the effectiveness of the local ASO infusion. In contrast, infusion of ASO into the HIP had no effect on any evaluated measure. Given the size of this structure, it is possible that ASO infusion into more anterior or posterior regions may have produced behavioral changes.

The current studies suggest that the 5-HT$_{2A}$ receptor can positively or negatively regulate reward systems for EtOH and saccharin intake, and anxiety-related behaviors, depending upon the CNS structure targeted. Such site-specific duality of action most likely reflects different local noradrenergic and neurochemical interactions with decreased 5-HT$_{2A}$ receptor expression. Brains from the present subjects were used to verify the extent of 5-HT$_{2A}$ receptor down-regulation produced by the ASO, limiting the identification of subsequent changes in specific alternate compensatory biological mechanisms as a result of 5-HT$_{2A}$ receptor down-regulation. However, it must be considered that such compensatory mechanisms occurred. Clearly, the observed changes did not reflect effects on thirst mechanisms, because total fluid consumption did not differ between any treatment group and their respective controls. Although food intake was not measured, body weights between ASO and control animals did not significantly differ, indicating that general caloric mechanisms were not affected.

In conclusion, down-regulation of 5-HT$_{2A}$ receptor down-regulation induces differential behavioral effects across multiple paradigms, which are unique to the brain structure targeted. These results may offer insight into the findings that selective serotonin reuptake inhibitors and both 5-HT$_{2A}$ agonists and antagonists reduce EtOH intake (Meert and Janssen, 1991; unpublished observations, this laboratory). All three types of compounds typically produce down-regulation of the 5-HT$_{2A}$ receptor, in a manner similar to the ASO sequence used in these studies.

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5-HT2A ASO and EtOH Ingestion 289