

LECOZOTAN (SRA-333): A SELECTIVE SEROTONIN_{1A} RECEPTOR ANTAGONIST
THAT ENHANCES THE STIMULATED RELEASE OF GLUTAMATE AND
ACETYLCHOLINE IN THE HIPPOCAMPUS AND POSSESSES
COGNITIVE-ENHANCING PROPERTIES

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Running Title: SRA-333 (lecozotan): a silent 5-HT_{1A} antagonist & cognition

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Abbreviations

4-Cyano-N-{2-[4-(2,3-dihydrobenzo[1,4]dioxin-5-yl)-piperazin-1-yl]-propyl}-N-pyridin-2-yl-benzamide HCl (lecozotan)
5-hydroxytryptamine_{1A} (5-HT_{1A})
5-methoxy-dimethyltryptamine (5-MeODMT)
8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)
Alzheimer's Disease (AD)
analysis of variance (ANOVA)
Chinese hamster ovary (CHO)
delayed matching-to-sample (DMTS)
ethylenediaminetetraacetic acid (EDTA)
fixed ratio (FR)
guanosine-5'-(γ -thio)triphosphate [³⁵S] (GTP γ S)
2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)
high-performance chromatography with electrochemical detection (HPLC-EC)
intramuscular (im)
intraperitoneal (ip)
N-methyl-D-aspartate (NMDA)
oral administration (po)
subcutaneous (sc)
dose that produced 50% of the maximal response (ED₅₀)
dose that produced 50% inhibition (ID₅₀)
vertical diagonal band (VDB)
Wisconsin General Test Apparatus (WGTA)

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ABSTRACT

Recent data has suggested that the 5-HT_{1A} receptor is involved in cognitive processing. A novel 5-HT_{1A} receptor antagonist, 4-cyano-*N*-[(2*R*)-[4-(2,3-dihydrobenzo [1,4] dioxin-5-yl) piperazin-1-yl] propyl]-*N*-pyridin-2-yl-benzamide hydrochloride (lecozotan), which has been characterized in multiple in vitro and in vivo pharmacologic assays as a drug to treat cognitive dysfunction, is reported. In vitro binding and intrinsic activity determinations demonstrated that lecozotan is a potent and selective 5-HT_{1A} receptor antagonist. Using in vivo microdialysis, lecozotan (0.3 mg/kg sc) antagonized the decrease in hippocampal extracellular 5-HT induced by a challenge dose (0.3 mg/kg sc) of 8 OH-DPAT and had no effects alone at doses 10-fold higher. Lecozotan significantly potentiated the potassium chloride-stimulated release of glutamate and acetylcholine in the dentate gyrus of the hippocampus. Chronic administration of lecozotan did not induce 5-HT_{1A} receptor tolerance or desensitization in a behavioral model indicative of 5-HT_{1A} receptor function. In drug discrimination studies, lecozotan (0.01-1 mg/kg im) did not substitute for 8-OH-DPAT and produced a dose-related blockade of the 5-HT_{1A} agonist discriminative stimulus cue. In aged rhesus monkeys, lecozotan produced a significant improvement in task performance efficiency at an optimal dose (1 mg/kg po). Learning deficits induced by the glutamatergic antagonist MK-801 (assessed by perceptually complex and visual spatial discrimination) and by specific cholinergic lesions of the hippocampus (assessed by visual spatial discrimination) were reversed by lecozotan (2 mg/kg im) in marmosets. The heterosynaptic nature of the effects of lecozotan imbues this compound with a novel mechanism of action directed at the biochemical pathologies underlying cognitive loss in AD.

INTRODUCTION

The multiplicity of biological data associated with the 5-hydroxytryptamine_{1A} (5-HT_{1A}) receptor subtype, since its discovery by radioligand binding in 1981 (Pedigo et al, 1981) and subsequent cloning in 1988 (Fargin et al, 1988), implicates this receptor in numerous behavioral and physiologic functions including cognition, psychosis, feeding/satiety, temperature regulation, anxiety, depression, sleep, pain perception, and sexual activity. The development of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) as a selective agonist for the 5-HT_{1A} receptor has been instrumental in defining the physiologic role of the 5-HT_{1A} receptor (Arvidsson et al, 1981) as well as its anatomic localization (Gozlan et al, 1983). However, it was not until the development of a “silent” 5-HT_{1A} receptor antagonist that systematic pharmacologic studies explored the consequences of blocking this receptor in the brain.

The first breakthrough in this area was reported in 1993 with the development of the arylpiperazine compound, WAY- 100135, which had moderate potency (Fletcher et al, 1993). The more potent and selective congener WAY-100635 was identified shortly thereafter (Forster et al, 1995). Since then it has been demonstrated that WAY-100635 lacks intrinsic activity in multiple assay systems that are sensitive to the agonist effects of 5-HT_{1A} compounds. Accordingly, WAY-100635 antagonizes the responses of 8-OH-DPAT at 5-HT_{1A} somatodendritic autoreceptors to inhibit the firing rate of dorsal raphe neurons and antagonizes the ability of 8-OH-DPAT to decrease the accumulation of cAMP at postsynaptic 5-HT_{1A} receptors in the hippocampus (Fletcher et al, 1996). Although WAY-100635 remains an important pharmacologic tool in defining 5-HT_{1A} receptor function, other 5-HT_{1A} antagonists have been developed over the last decade including WAY-405 (Schechter et al, 2000), which is

another aryl-piperazine analog, and NAD-299 (Johansson et al, 1997) and LY-426965 (Rasmussen et al, 2000), which are structural analogs of 8-OH-DPAT and pindolol, respectively.

One of the most intriguing areas of research surrounding 5-HT_{1A} receptor antagonists is the potential of these compounds to enhance cognitive ability. AD has been described as a neurodegenerative disorder characterized by multiple deficits in neurotransmitter function. Although many studies have focused upon the cholinergic hypothesis, it has become apparent that not all patients can be characterized by deficits in this system alone, as shown by the moderate efficacy produced by acetylcholinesterase inhibitors (Morris et al, 1986; Bliss and Collingridge, 1993). In fact, recent research suggests that glutamatergic deficits may occur before those observed in the cholinergic system (Bliss and Collingridge, 1993). Furthermore, and in contrast to glutamatergic and cholinergic neurotransmission, the serotonergic system may be hyperactive in the disease as a result of the enhanced turnover of serotonin (Kowall and Beal, 1991), which ultimately would reduce the firing of the cortical pyramidal-associated pathways through stimulation of 5-HT_{1A} autoreceptors. It was this finding of enhanced serotonergic turnover that lead Bowen et al (1994) to the hypothesis that 5-HT_{1A} receptor antagonists may be effective in treating the cognitive loss associated with AD.

A compelling rationale has been proposed for treating the dementia associated with AD based on data that antagonists of 5-HT_{1A} receptors have a facilitatory effect on glutamatergic transmission (Bliss and Collingridge, 1993; Bowen et al, 1994). N-methyl-D-aspartate (NMDA)-induced glutamate release from pyramidal neurons is potentiated by a 5-HT_{1A} receptor antagonist (Dijk et al., 1995). The 5-HT_{1A} antagonist WAY-100635 can alleviate cognitive deficits induced by both glutamatergic dysfunction (dizocilpine treatment-induced cognitive deficits), and cholinergic dysfunction (fornix transaction surgery) in primates (Harder et al, 1996; Harder and

Ridley, 2000). The blockade of 5-HT_{1A} receptors also enhances glutamate release from rat hippocampal slices (Van den Hooff and Galvan, 1991). Other data suggest that 5-HT_{1A} receptor antagonists can inhibit the tonic hyperpolarizing action of serotonin on pyramidal neurons in both the cortex and hippocampus (Araneda and Andrade, 1991; Van den Hooff and Galvan, 1992), which ultimately would result in an enhancement of glutamatergic neurotransmission and signaling. Thus, it is possible that 5-HT_{1A} receptor antagonists may improve cognition by removing the inhibitory effects of endogenous serotonin on pyramidal neurons and thus enhance glutamatergic activation and ensuing signal transduction.

The purpose of this study was to investigate the pharmacologic characteristics of 4-cyano-*N*-[(2*R*)-[4-(2,3-dihydrobenzo[1,4]dioxin-5-yl)piperazin-1-yl]propyl]-*N*-pyridin-2-yl-benzamide hydrochloride (SRA-333; lecozotan), a novel, selective, and potent antagonist of 5-HT_{1A} receptors. Lecozotan specifically antagonizes functional responses associated with stimulating the 5-HT_{1A} receptor both in vitro and in vivo. The ability of lecozotan to potentiate the release of acetylcholine and glutamate in vivo, as well as its positive role in relevant biological models for cognition, supports the utility of this compound as a novel strategy to treat the cognitive deficits associated with AD.

METHODS

Radioligand Binding

Membrane Preparations

5-HT_{1A} Receptor The polymerase chain reaction cloning of the human 5-HT_{1A} receptor subtype from a human genomic library has been described previously (Chanda et al, 1993). A stable Chinese hamster ovary cell line (CHO-K1) expressing the human 5-HT_{1A} receptor subtype (h5-HT_{1A} CHO cells) was employed throughout this study. Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, nonessential amino acids, and penicillin/streptomycin. Cells were grown to 95% to 100% confluence as a monolayer before membranes were harvested for binding studies. Cells were gently scraped from the culture plates, transferred to centrifuge tubes, and washed twice by centrifugation (2000 rpm for 10 min., 4°C) in buffer (50 mM Tris; pH 7.5). The resulting pellets were aliquoted and placed at -80°C. On the day of assay, the cells were thawed on ice and resuspended in buffer.

Dopamine Receptors The binding profile of lecozotan was assessed in stable CHO-K1 cells containing dopamine D₂ (DA₂), dopamine D₃ (DA₃), and dopamine D₄ (DA₄) receptor subtypes. The CHO-K1 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, nonessential amino acids, and penicillin/streptomycin. The cells were grown to 95 % confluence as a monolayer and were harvested by scraping. The harvested cells were centrifuged at low speed (1,000g) for 10 min to remove the culture media. The harvested cells were suspended in half volume of fresh physiologic phosphate-buffered saline solution and re-centrifuged at the same speed. This operation was repeated once more. The collected cells were then homogenized in 10 volumes of 50 mM Tris HCl, pH 7.4, and 0.5 mM EDTA (ethylenediaminetetraacetic acid). The homogenate was centrifuged at 40,000g for 30 min and the

precipitate collected. The pellet was resuspended in 10 volumes of Tris HCl buffer and recentrifuged at the same speed. The final pellet was suspended in a small volume of Tris HCl buffer and the tissue protein content was determined in aliquots of 10 to 25 microliter (μ l) volumes. Bovine serum albumin was used as the standard in the protein determination by the method of Lowry (Lowry et al, 1951). The volume of the suspended cell membranes was adjusted to give a tissue protein concentration of 1.0 mg/ml of suspension. The prepared membrane suspension (10 times concentrated) was aliquoted in 1.0 ml volumes and stored at -70°C until used in subsequent binding experiments.

Alpha₁ Adrenergic Receptor Cortical tissue from male Sprague Dawley rats was homogenized on ice in 50 mM Tris-HCl, pH 7.4 buffer with a polytron homogenizer. The homogenate was then centrifuged at 25,000 g for 10 min and the supernatant was discarded. The pellet was resuspended in fresh buffer, centrifuged, and then resuspended. Following a third wash of the membranes, the pellet was resuspended in buffer, aliquoted into separate vials, and stored at -70° C until subsequent assay.

Radioligand Binding Protocols

5-HT_{1A} Receptor Binding [³H]-WAY 100635 and [³H]-8-OH-DPAT were used to label 5-HT_{1A} receptors. Experiments were conducted in 96-well microtiter plates in a total volume of 250 μ l of buffer (50 mM Tris-HCl, pH 7.4). Non-specific binding was defined with 10 μ M methiothepin for [³H]-WAY 100635 binding. Competition studies were conducted using 1 nM of the ligand. For [³H]-8-OH-DPAT binding, non-specific binding was defined with 10 μ M serotonin. Competition studies were conducted using 1.50 nM of the ligand. The binding assays were initiated by the addition of 50 μ l of the harvested stable transfected 5-HT_{1A} cells (0.05 mg/sample) and were incubated at 25° C for 30 minutes. The reaction was terminated by

vacuum filtration through presoaked (0.5% polyethyleneimine) Whatman GF/B filter paper (Brandel, Gaithersburg, MD) using a Brandel 96-cell harvester. Filters were washed with ice-cold buffer (50 mM Tris-HCl, pH 7.4) and transferred to scintillation vials to which 5 ml of Opti-Fluor (Packard Instrument Company, Meriden, CT) was added. Radioactivity was measured by liquid scintillation counting using a Beckman[®] LS 6000TA liquid scintillation counter (Beckman Instruments, Fullerton, CA). Protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard. Measurements were taken at 595 nm with a Perkin Elmer Lambda 3B spectrophotometer (Perkin Elmer, Norwalk, CT). Binding data were analyzed by ReceptorFit (Lundon Software, Cleveland Heights, OH) a computer-assisted nonlinear regression analysis program.

Dopamine Receptor Binding The radioligand [³H]-spiperone was used at 0.5 nM to label DA₂, DA₃, and DA₄ receptors. Nonspecific binding was determined in the presence of 10 µM of d-butaclamol, 7-hydroxy-DPAT, or clozapine for DA₂, DA₃, and DA₄, respectively. Binding experiments were performed in a 96-well microtiter plate format, in a total volume of 200 µl. Competition experiments were performed using 7 to 9 concentrations of lecozotan, added in 20 µl volume. To each well was added 80 µl of incubation buffer made in 50 mM Tris HCl buffer (pH 7.4) containing 10 mM MgCl₂ and 0.5 mM EDTA, and 20 µl of radio isotope. The reaction was initiated by the final addition of 100 µl of tissue suspension. The reaction proceeded in the dark for 120 min at room temperature, at which time the bound ligand-receptor complex was filtered off on a 96-well unifilter with a Packard[®] Filtermate 196 Harvester. The radioactivity of the bound complex caught on the filter disk was measured after drying and addition of 40 µl Microscint[®]-20 scintillant to each well in a Packard TopCount[®] equipped with 6 photomultiplier

detectors. The unifilter plate was heat sealed and counted in a Packard TopCount^R with efficiencies of 31.0% and 60% for tritium and [¹²⁵I], respectively.

Alpha₁ Adrenergic Receptor Binding Membranes were thawed, placed in buffer, and incubated with [³H]-prazosin (0.2 nM) for 30 min at 25°C. All tubes contained either vehicle, test compound (1 to 8 concentrations), or a saturating concentration of phentolamine (10 μM) to define specific binding. All reactions were terminated by the addition of ice cold Tris buffer followed by rapid filtration using a Tom Tech[®] filtration device to separate bound from free [³H]-prazosin. Bound radioactivity was quantitated using a Wallac 1205 Beta Plate[®] counter.

Nova Screen Binding Profile A Nova Screen binding profile was determined for lecozotan at 61 neurotransmitter receptor, reuptake, ion channel, and enzyme sites. [³H]8-OH-DPAT and [³H]WAY-100635 were used as the 5-HT_{1A} agonist radioligand and the 5-HT_{1A} antagonist radioligand, respectively. Displacement of specific radioligand binding at these sites was determined at 3 concentrations of lecozotan (1, 100, and 10,000 nM).

Determination of Intrinsic Activity Using a cAMP Accumulation Assay

Assays were performed by incubating the cells with Dulbecco's Modified Eagle Medium containing 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 5 mM theophylline, and 10 μM pargyline for a period of 20 min at 37°C. Functional activity was assessed by treating the cells with forskolin (1 μM final concentration) followed immediately by a test compound (6 concentrations) for an additional 10 min at 37°C. In separate experiments, 6 concentrations of antagonist were preincubated for 20 min before the addition of 10 nM 8-OH-DPAT (Research Biochemicals Inc.) and forskolin. The reaction was terminated by removal of the media and addition of 0.5 ml ice-cold assay buffer. Plates were stored at -20°C before the assessment of cAMP formation by a cAMP SPA assay (Amersham).

Assessment of Intrinsic Activity Using [³⁵S]-GTP γ S Binding

The guanosine-5'-(γ -thio)triphosphate[³⁵S] (GTP γ S) binding assay was similar to that used by Lazareno and Birdsall (1993). Briefly, 5-HT_{1A} cloned receptor membrane fragments (as used for 5-HT_{1A} receptor binding assays) were stored at -70°C until needed. When needed, membranes were rapidly thawed, centrifuged at 40,000g for 10 min and resuspended at 4°C for 10 min in assay buffer (25 mM HEPES, 3 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 10 μ M GDP, 500 mM Dithiothreitol (Cleland's Reagent), pH 8.0). These membranes were then incubated for 30 min at 30°C with [³⁵S]GTP γ S (1 nM) in the presence of vehicle, test compound (1 to 8 concentrations), or excess 8-OH-DPAT to define maximum agonist response. All reactions were terminated by the addition of ice-cold Tris buffer followed by rapid filtration using a TomTech® filtration device to separate bound from free [³⁵S]GTP γ S. Agonists produced an increase in the amount of [³⁵S]GTP γ S bound whereas antagonists produced no increase in binding. Bound radioactivity was counted and analyzed as above.

In Vivo Microdialysis: 5-HT and Glutamate

Subjects and Surgery Male Sprague-Dawley rats (Charles River; 280-350 g) were used in all experiments. Animals were housed as a group with food and water available ad libitum and maintained on a 12-hour light/dark cycle (lights on at 6 AM) with all work performed during the light phase. After surgery, the animals were housed separately in plexiglass cages (45 x 45 x 30 cm) with free access to food and water.

Microdialysis procedures After induction of anesthesia with gaseous administration of halothane (2%) (Fluothane, Zeneca, Cheshire, UK), the animals were secured in a stereotaxic frame with ear and incisor bars. Anesthesia was maintained by continuous administration of halothane (1 to 2 %). A microdialysis probe guide cannula (CMA/Microdialysis, Stockholm, Sweden) was

implanted into either the ventral hippocampus or the dentate gyrus. Coordinates for the both brain regions were taken from Paxinos et al (1985): RC -4.8, L -5.0, V -4.4 and RC -3.8, L -1.4, V -3.6, respectively; reference points for microdialysis cannula implantation were taken from bregma (RC and L) and the dura (V). A cannula was also implanted subcutaneously (sc) at this time between the animal's shoulders. Both cannulae were secured to the skull using dental acrylic (Plastics One, Roanoke, VA, USA). The wound was sutured and the animals left to recover for 24 hour in their home cages with free access to food and water.

A pre-equilibrated microdialysis probe (OD 0.5 mm, membrane length 4 mm or 1 mm; CMA/Microdialysis, Sweden), perfused overnight in artificial cerebrospinal fluid, was inserted, via the guide cannula, into either the ventral hippocampus or the dentate gyrus, respectively, of the unrestrained rat 24 hours after surgery. The probe was perfused with aCSF (NaCl 125 mM, KCl 3.0 mM, MgSO₄ 0.75 mM, and CaCl₂ 1.2 mM pH 7.4) at a flow rate of 1 μ L/min. A 3-hour stabilization period was allowed after probe implantation, after which time microdialysis sampling was carried out by a modification of the method of Dawson and Routledge (1995). For 5-HT analysis, 20-min samples were taken throughout the experimental period and 4 preinjection samples were taken prior to drug administration. Alternatively, for glutamate determinations a 5-min sampling regimen was used throughout the experimental period and 6 presamples were taken before drug or vehicle injection to achieve a steady baseline. All subsequent samples were expressed as a percentage of these baseline concentrations. Lecozotan or vehicle was administered via the cannula (sc). Stimulation of neurotransmitter release was brought about by infusion of aCSF containing 100 mM KCl (NaCl 28 mM, KCl 100 mM, MgSO₄ 0.75 mM, and CaCl₂ 1.2 mM, pH 7.4) through the microdialysis probe.

Analysis of Microdialysates 5-HT Analysis: 5-HT was separated by reverse-phase high-performance liquid chromatography (HPLC) (C18 ODS2 column, 100 x 3.0 mm, Metachem, Torrance, CA, USA) and detected using an ANTEC electrochemical detector (ANTEC, Leyden, Netherlands) set at a potential of 0.65V vs a Ag/AgCl reference electrode. Mobile phase was delivered by a Jasco PU980 HPLC pump (Jasco Ltd, Essex, UK) at 0.6 ml/min and contained 0.15 M NaH₂PO₄ buffer at pH 4.3, 0.25 mM EDTA, 1.5 mM 1-octane sodium sulphonic acid, and 5 % isopropanol.

Glutamate Analysis: measurement of glutamate was performed using a Crystal 310 capillary electrophoresis system (Thermo BioAnalysis, NM, USA) with a Zeta laser-induced fluorescence detector (ZETA Technology, Toulouse, France) coupled with a helium-cadmium laser (Emission wavelength 442 nm; Omnichrome, CA, USA). All samples were pre-derivatized with naphthalene 2,3-dicarboxaldehyde by a modification of the method of Hernandez et al (1993). Microdialysate or standard samples (3 µl) were mixed with 50 mM boric acid buffer (pH 9.5) containing 20 mM sodium cyanide (5 µl) and 30 mM naphthalene 2,3-dicarboxaldehyde in methanol (1 µl). Samples were allowed to react for 3 min at room temperature before injection. Separations were performed according to Dawson et al. (1997) in fused silica capillaries (75 µm id, 375 µm od, 47 cm; Polymicro Technologies, NM, USA) with an applied voltage of 0.6 kV/cm. Samples (5 nl) were applied to the capillary via a high-pressure injection system. Separations used 30 mM boric acid pH 9.5 (pH was adjusted using 1 M NaOH). The capillary was rinsed with 0.1 M NaOH (1.5 min) and running buffer (1.5 min) between analyses. All data were acquired using the Atlas software package (Thermo Labsystems, Gulph Mills, PA) for the PC.

The fmol/ μ M perfusate values of 5-HT/glutamate for the first 4 baseline samples were averaged and this value was denoted as 100%. Results were analyzed by analysis of variance (ANOVA) with repeated measures followed by pairwise comparisons using Bonferroni adjustment for multiple comparisons using the Statview software application (Abacus Concepts Inc., Berkeley, CA 1996) for the PC.

In Vivo Microdialysis: Acetylcholine

Subjects and surgery Male Sprague-Dawley rats weighing between 350 to 400 g were maintained in individual cages. Animals were anesthetized with a combination of ketamine (50 mg/kg, ip) and xylazine (10 mg/kg, ip), and a single guide shaft aimed at the hippocampus (equal right and left side implants; region CA1) was implanted (co-ordinates: RC: -5.6; L: 5.0; V: 3.0 mm). Rats were allowed to recover at least a week before experimentation.

Microdialysis Procedure A concentric microdialysis probe with a 4-mm cellulose tip was inserted in the hippocampus of each rat 14 hours before the experiment. The probe was perfused with a modified Ringer's solution (142 mM NaCl, 3.9 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂, and 10mM bicarbonate, pH: 7.4) at a flow rate of 0.5 μ l/min overnight and 1 μ l/min starting 1 hour before the experiment. To enhance acetylcholine recovery it was necessary to add a very small concentration of neostigmine (0.3 μ M) to the Ringer's solution. Samples were collected every 20 min before and after injection of lecozotan.

Analysis of Microdialysates Acetylcholine was measured by reverse-phase, high-performance chromatography with electrochemical detection (HPLC-EC) using an ESA model 580 pump and mobile phase of 200 mM potassium phosphate (pH 8.0) at a flow rate of 0.6 mL/min. (Rada et al., 2001). Microdialysates were injected into a 20- μ L loop leading to a 10-cm C18 analytical column to separate acetylcholine, which was converted to betaine and hydrogen peroxide by an

immobilized enzyme reactor. Detection was accomplished with an amperometric detector (model 400; E,G&G Princeton Applied Research) that oxidized the hydrogen peroxide on a platinum electrode (BAS Co.) set at 0.5 V with respect to a Ag-AgCl reference electrode.

In Vivo Electrophysiology

Male Sprague-Dawley rats (240-265 g, Charles River) were housed in groups on a 12 hour light/dark cycle with *ad libitum* access to food and water with. On the day of the experiments, rats were initially anesthetized with halothane in an induction chamber. Anesthesia was subsequently maintained by continuously administering halothane (in oxygen at 1% to 3%) through a nose cone using a Fluotec halothane vaporizer. A heating pad was placed beneath the animal to maintain the body temperature at 37.5°C.

Rats were placed in a stereotaxic frame and the surface of the skull was exposed. Glass micropipettes (filled with pontamine sky blue) with an impedance of 3 to 6 megaohms were lowered through a craniotomy to a depth of 0.5 mm above the dorsal raphe nucleus (on the midline and -0.6 mm from the interaural zero). A hydraulic drive was used to further advance the recording electrode. Neuronal action potentials were passed through a high input-impedance amplifier and were monitored on an oscilloscope. Action potentials were discriminated from background noise and used to trigger an output, which was counted and recorded by an online computer system (Datawave).

Dorsal raphe neurons were identified by characteristic waveform activity and then spontaneous activity was monitored for approximately 10 minutes to establish a baseline rate of neuronal firing. A predetermined dose of the test compound was then administered subcutaneously and firing rate was monitored for an additional 3 minutes in order to observe any effect of the test compound. 8-OH-DPAT was then administered sc in cumulative doses at 3-

minute intervals. Only one neuron was studied in each rat to avoid residual effects. At the termination of the experiment, the pontamine sky blue was deposited for 20 minutes by a 10- μ A anodal current for histologic confirmation of the recording site. Each animal was then perfused intracardially with Mirsky's formula, after which the brain was removed, sectioned at 64 μ M and counterstained with neutral red. Data from recording sites not found within the histologic boundaries of the dorsal raphe were discarded.

All drugs were put into solution with 0.9% saline. The single pretreatment dose of lecozotan was in a volume concentration of 300 μ g/kg /0.1 ml. 8-OH-DPAT was injected in cumulative doses starting at 25 μ g/kg in a volume of 0.05 mL. All doses were expressed on the basis of active moiety. Firing rates for the baseline period and that following administration of lecozotan were compared using a paired *t* test ($p<0.05$).

Fixed-Ratio Operant Studies in Rats

Male Sprague-Dawley rats weighing 300 to 350g were housed individually and maintained at 85% of their free-feeding body weights by food presented during the session and by post session feeding. Water was freely available in the animal's home cage.

Experimental sessions were conducted in a standard operant conditioning chamber placed inside a ventilated sound-attenuating shell that was equipped with white noise to mask extraneous sounds (Med Associates, Georgia, VT). A response lever and a food trough were on the front panel of the chamber. Bioserv (Frenchtown, NJ) 45-mg precision dustless pellets could be delivered to food troughs to serve as reinforcers. The operant chambers were controlled and monitored by computers with software from Med Associates Inc.

Rats were trained to respond on the right lever under a fixed ratio-30 (FR30) schedule of food presentation. Experimental sessions consisted of three 10-minute components, each

preceded by a 10-minute timeout period during which drugs were administered. During the timeout period, the chamber was dark and there were no programmed consequences. During the response component, the house light was illuminated and lever pressing was associated with an audible feedback click. 8-OH-DPAT (0.03 to 0.3 mg/kg) was administered cumulatively sc at the start of the timeout periods. Lecozotan was administered as a 30-minute pretreatment before a cumulative 8-OH-DPAT dose-effect curve. The ED₅₀ was defined as the dose of 8-OH-DPAT that produced a 50% reduction in the response rate in the presence or absence of lecozotan. Data from multiple administrations were overlapped and combined.

Rates of responding were calculated separately in each of the 3 components of the session by dividing the total number of responses by the total time the component was in effect for each animal.

Fixed-Ratio Operant Studies in Squirrel Monkeys

Four adult male squirrel monkeys (*Saimiri sciureus*) were housed in individual cages except during experimental sessions. Each monkey had unlimited access to water and received a nutritionally balanced diet of Purina Monkey Chow, fresh fruits, vegetables, and vitamin supplements.

Experimental sessions were conducted in ventilated sound-attenuating chambers (Med Associates, Georgia, VT) that were provided with white noise to mask extraneous sounds. Monkeys sat in a Plexiglas chair similar to one used by Kelleher and Morse (1972) and faced a panel on which a response lever and colored stimulus lamps were mounted. Each press of the lever with a minimal downward force of 0.25 N was recorded as a response. A shaved portion of the tail of the monkey was secured in a stock beneath brass electrodes. Electrode paste insured a

low-resistance contact between the electrodes and the tail. A brief, low-intensity electric shock (200 msec, 3-5 mA) could be delivered through the electrodes to the tail.

Responding was maintained by termination of a visual stimulus associated with an electric shock (Kelleher et al, 1972). In the presence of a red light, the completion of a 10-response fixed ratio unit (FR10) turned off the red light and initiated a 30-sec timeout period. If the FR was not completed within a 10-sec period an electric shock was delivered every 10 seconds; a maximum of 10 shocks was delivered. Daily sessions consisted of 5 components. Each component consisted of a 10-min timeout period during which drugs could be administered, followed by a 5-min response period during which the FR10 schedule was in effect. Responding during the timeout period had no programmed consequences.

Incremental doses of 8-OH-DPAT were administered cumulatively intramuscularly (im) at the start of the 10-minute timeout period that preceded each of the 5 sequential components. Lecozotan was administered 30 minutes im or 60 minutes po before the start of the first response component.

Rates of responding were calculated separately in each of the 5 components of the session by dividing the total number of responses by the total time the component was in effect for each animal. ED₅₀ values were determined for the effects of 8-OH-DPAT alone and after pretreatment with lecozotan.

Discriminative Stimulus Effects in Pigeons

Six male white Carneaux pigeons, approximately 1 year old, were obtained from the Palmetto Pigeon Plant (Sunter, SC). All pigeons were experimentally naïve and were maintained individually in cages that were provided with continuously available water and grit. Lighting in the temperature- and-humidity-controlled vivarium was on a 12-hour light/dark cycle. All

pigeons were reduced to approximately 85% of their free-feeding body weights before key peck training and were maintained at this weight for the duration of the study.

Experimental sessions were conducted in a standard operant conditioning chamber placed inside a ventilated sound-attenuating shell that was equipped with white noise to mask extraneous sounds (Med Associates, Georgia, VT). The front panel of the chamber contained 3 response keys. The keys could be transilluminated with different colors. The left and right keys were lit white and used in the present study. Pecks that exceeded approximately 0.15 N on the key operated a feedback relay behind the front wall and were counted as a response. Below the center key was a rectangular opening (4.5 x 10 cm) that provided access to a solenoid-driven food magazine containing mixed grain. During food delivery, the magazine was illuminated.

Pigeons were trained to discriminate 8-OH-DPAT (0.1 mg/k im) from saline using a 2-key grain-reinforced drug discrimination procedure. Pigeons were initially trained to key peck on a FR schedule of food presentation (only one key illuminated at a time) with a gradual increase in the FR (final FR=30) over the training sessions. Pigeons were then trained to respond on 1 key after a saline (s) injection (im) and on the other key after a 8-OH-DPAT (d) injection (im) using a double-alternation daily injection schedule (s, d, d, s, s, d). When the injection schedule started, both keys were lit and the FR was dropped to 5, with a gradual increase to FR30. At the final schedule, completion of 30 consecutive responses on the injection-appropriate key resulted in 3-sec access to mixed grain. Responding on the incorrect key reset the FR for the injection-appropriate key. Training and test sessions were 30 min in duration. Criteria for establishing 8-OH-DPAT as a discriminative stimulus were that the first ratio (30 consecutive responses) was completed on the injection-appropriate key and that there was > 90% injection-appropriate response for the total session for 5 consecutive sessions.

Antagonism studies were conducted once or twice a week in individual pigeons. For antagonism studies, lecozotan (0.01 to 1.0 mg/kg im) was administered as a pretreatment (40 min prior to test session) followed by an injection (im) of the training dose of 8-OH-DPAT (0.1 mg/kg 30 min before test session). On test days, completion of 30 consecutive responses on either key resulted in access to mixed grain. Test sessions were conducted only if a pigeon met the criteria (first ratio correct and > 90% responding on the injection-appropriate key) on the previous day and on 4 of the 5 previous training days. Training sessions did not take place on days after test sessions to allow for a washout period for test compounds.

Rates of responding were calculated by dividing the total number of responses by the total time of the session. Baseline drug key rates were calculated by averaging rates of drug injection days preceding a test day. Baseline saline key rates were calculated by averaging rates of saline injection days preceding a test day. The percentage of drug lever responding was calculated by dividing the number of responses on the drug-appropriate key by the total number of responses in the session multiplied by 100. Compounds were considered to fully attenuate the effects of the training dose of 8-OH-DPAT in an individual animal if drug key responding was reduced to < 20%. Compounds were considered to partially attenuate the effects of the training dose of 8-OH-DPAT in an individual animal if the drug key responding was reduced to 20 to 60%. If an animal did not receive a reinforcer during a test session, the percentage of drug key responding was not analyzed. However, the rate of responding was included in the analysis.

Antagonism of 5-MeODMT-Induced Syndrome

Male Sprague-Dawley rats (200-350 g) were housed in groups (4/cage) under a 12 hour light/dark cycle with food and water available *ad libitum*.

Serotonin syndrome tests were conducted according to a modification of the procedure by Smith and Peroutka (1986). Scoring the serotonin syndrome consisted of rating each of the following behaviors: (1) forepaw treading, (2) headweaving, (3) tremor, (4) hindlimb abduction, (5) flattened body posture, and (6) Straub tail on a 4-point (0 to 3) ranked intensity scale (maximum score = 18). To evaluate acute effects, lecozotan or vehicle was administered (po) to rats ($n = 8/\text{dose}$) placed individually in observation cages (9.5. wide x 7.5. tall x 17.5. long) lined with corncob bedding. Agonist activity was evaluated by scoring subjects for the presence of the serotonin syndrome during the final 15 minutes of the 1-hour drug pretreatment interval. Antagonist activity was then determined by scoring for the presence of the serotonin syndrome for 15 minutes after challenging (ip) the subjects with 5-MeO-DMT (5-methoxy-N, N-dimethyltryptamine, 5.6 mg/kg), a nonselective 5-HT agonist. This agonist was used because it produced a more robust syndrome that could be completely blocked by full antagonists of the 5-HT_{1A} receptor. In a separate experiment, lecozotan (10 mg/kg) or vehicle (1 ml/kg) was administered (po) once daily for 7 consecutive days. Twenty-four (24) hours after the last dose, the potency of 5-MeO-DMT (0.3-5.6 mg/kg; $n = 8$ per dose) given ip was determined in a dose-response study in rats receiving either chronic vehicle or chronic lecozotan. In addition, the potency of lecozotan (1-17 mg/kg; $n = 8$ per group) given po to antagonize a challenge dose of 5-MeO-DMT (5.6 mg/kg ip) was evaluated in a separate group of rats receiving po chronic vehicle or lecozotan (10 mg/kg).

Statistically significant effects were determined by ANOVA followed by Dunnett's test ($p < 0.05$). ED₅₀ values were determined using nonlinear regression analysis with inverse prediction.

Aged Rhesus Monkey Task Performance

Male (2) and female (3) rhesus monkeys aged 21 to 29 years were well trained (> 100 individual sessions) in the delayed matching-to-sample (DMTS) task. The animals were maintained on tap water (unlimited) and standard laboratory monkey chow supplemented with fruits and vegetables. They were maintained on a feeding schedule such that approximately 15% of their normal daily (except weekends) food intake was derived from 300-mg reinforcement food pellets (commercial composition of standard monkey chow and banana flakes) obtained during experimental sessions. The remainder was made available after each test session. On weekends the animals were fed twice per day. The monkeys were maintained on a 12 hour light-dark cycle and were tested each weekday between 09:00 and 14:00. Room temperature and humidity was maintained at $72^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $52^{\circ}\text{C} \pm 2\%$, respectively. The aged female animals in the study were perimenopausal, ie, still cycling, but infrequently. Testing was performed between menstrual cycles.

Animals were tested simultaneously in their home cages using a computer-automated training and testing system. Daily sessions consisted of 96 trials. Each test unit included 4 press-key/pellet dispenser units (Med Associates) mounted in lightweight aluminum chassis that could be attached to the home cage. A trial began by the illumination of the sample press key located in the upper center of the panel. Monkeys were trained to press the illuminated sample (red, green, or yellow) area to initiate a trial. This action also extinguished the sample during a computer-specified delay interval. After the delay interval, the choice press keys (2), but not the sample, were illuminated. One of the 2 choices matched the sample, while the other (incorrect) choice was presented as one of the 2 remaining colors. Correct trials (matches) were rewarded by the delivery of a reinforcement food pellet (intertrial interval = 5 sec). The various

combinations of stimulus color were arranged so that each appeared an equal number of times as a sample, each color appeared an equal number of times as choices, and each color appeared an equal number of times in combination with each other color. Likewise, when 2 colors appeared in combination, each color was counterbalanced between left and right sides of the screen in a nonpredictable pattern. Finally, all stimulus-counterbalancing procedures were matched to length of delay interval. For each subject, baseline accuracy was normalized by adjusting the length of delay intervals to provide: zero delay (85%-100% correct); short delay (75%-84% correct); medium delay (65%-74% correct) and long delay (55%-64% correct). Baseline data were obtained following the administration of a drug vehicle (sterile saline).

Data for percentage correct were subdivided according to delay interval for each 24-trial delay component of the session. All statistical analyses were performed on raw data (% trials correct). Data were analyzed by a multifactorial ANOVA with repeated measures (SAS, JMP statistical software package). An orthogonal multicomparison t-test was used to compare individual means. Differences between means from experimental groups were considered significant at the $p < 0.05$ level (2-sided test).

Cognitive Enhancing Profile of Lecozotan in Marmosets

Animals Common marmosets (*Callithrix jacchus*), bred and housed in the primate colony at the University of Bradford School of Pharmacy, were used for these studies. All marmosets were housed in mixed-sex pairs, in which the male animals were vasectomized at least 3 months before any behavioral studies. Marmosets were fed and watered *ad libitum* throughout the studies. Their diet consisted of a mixture of commercially available primate ‘rings’ and primate ‘forage mix’ (Harlan, UK), plus varied fruits, multiseed bread, and occasional sweet treats in the form of gum feeders.

Treatments All drugs were administered (im) with saline as the vehicle. Injection volumes were kept at a maximum of 0.1 ml per marmoset per drug per day. MK-801 (dizocilpine maleate) was obtained from Sigma Aldrich. Both drugs were diluted in 0.9% sterile saline. Dizocilpine was administered at a dose of 15 µg/kg. Lecozotan was administered at doses of 1 and 2 mg/kg. Dizocilpine was administered as a 30 min pretreatment on each day when animals were learning tasks under either dizocilpine treatment alone, or dizocilpine + lecozotan treatment. Lecozotan was administered as a 20 min pretreatment on days when animals were learning tasks under either lecozotan treatment alone, or under dizocilpine + lecozotan treatment.

All animals received all treatments in a counter-balanced order. Dosing was semichronic, eg, when an animal took 2 weeks to learn a task, that animal would receive 2 weeks of daily injections during the period when the task was being acquired. If an animal took a month to learn a task, then drugs were administered throughout the month of testing on a daily basis.

Lesion surgery We have previously demonstrated that a significant and highly specific lesion of the marmoset vertical diagonal band (VDB), the source of the majority of cholinergic input to the hippocampus, is possible using a p75-saporin conjugate (Harder et al, 1998). An antibody to the

p75 human low-affinity nerve growth factor receptor, thought to be found mainly on cholinergic cell bodies, is conjugated to the neurotoxin saporin. This preparation is commercially available through Advanced Targeting Systems as ME 20.4-SAP. The preparation was diluted to 2 µg/µl in sterile 0.9% saline.

Marmosets were sedated with 0.05 ml Vetalar (ketamine solution), before being weighed and anesthetized with Saffan. Marmosets were placed in a pouch of bubble wrap on a warmed operating table with their heads placed in a stereotaxic frame. A small (approximately 2.5 cm) incision from the center of the forehead running posteriorly to the back of the head was made, and the skin retracted on either side. The stereotaxic frame was centered to Bregma, and burr holes were drilled using a small dental drill to allow placement of 1.5 µl immunotoxin in each hemisphere at the following coordinates: AP +1.15 cm, L ± 0.08 cm, V + 0.83 cm: AP +1.20 cm, L ± 0.05 cm, V + 0.98 cm. After suturing and recovery in a heated secure box, marmosets were returned to the home cage for behavioral testing, beginning 2 weeks postoperatively.

Lesion Verification Upon completion of the study the lesions administered to the subjects were verified to ensure bilateral lesions had been achieved in the marmosets. Lesion verification was achieved exactly as described by the method of Ridley et al. (1999). Briefly, after behavioral testing was completed the lesion procedure was validated using an antibody stain for the p75 NGF expressed on cholinergic neurones. Sections were examined under a light microscope at 25 times magnification. All animals who were tested in the behavioral procedure were validated and had a complete bilateral loss of cholinergic staining in the hippocampi which correlated with the cognitive deficits observed.

Behavioral testing Previous studies in many laboratories have made extensive use of the Wisconsin General Test Apparatus (WGTA) adapted for use with marmosets. Two WGTA task

types used in these studies are described below. Their neural substrates have been well delineated (Harder et al, 1998; Ridley et al, 1999).

Cortical task, the perceptually complex visual discrimination task. Previously, we have shown that acquisition of perceptually simple visual discrimination tasks is not robustly affected by dizocilpine, but if the simple visual discrimination task is made more perceptually difficult, a robust deficit is seen (Harder et al, 1998). Hence in this study marmosets were only tested on perceptually complex version tasks. Objects were painted matte black using car spray paint, such that only the shape could be used to distinguish between different objects (color and textural differences being eliminated through the painting).

Two different objects were presented to the marmoset in the WGTA: one situated over the right food well, and the other over the left food well. For every task, 2 different objects were used, and in each case, only 1 of the objects was associated with a reward. Thus, for example, object A might be the cap of a pen, painted black, and object B might be the small plastic toy from a cereal box, also painted black. On any single trial, the marmoset would see the 2 objects, A and B, positioned according to a pseudorandom schedule over the right and left food wells. On trial 1, A (the pen cap) might be on the right, and B (the toy) on the left. On trial 2, B might be on the right and A on the left.

Marmosets chose an object by moving the object to reveal the reward underneath, if the correct object was chosen. In the case of visual discrimination tasks, the task is ‘evaluative’, ie, it involves the formation of an association between a particular object and reward (Ridley et al, 1999). Thus, in the example above, the pen cap was always situated over the food reward, whether presented on the right or the left and the toy was never situated over the reward.

Over a number of trials, marmosets acquired the association, and eventually selected the ‘correct’ object (A, the pen cap) with sufficient accuracy (90% correct) to ‘pass’ the task and move onto the next task in the experimental design schedule.

Hippocampal task, the visuo-spatial discrimination task. Visuospatial discrimination tasks require a different type of learning from the visual discrimination task described above. Visuospatial tasks require ‘conditional’ (rather than associative) learning to take place for successful completion (Ridley and Baker, 1991), because all objects are equally associated with reward and a ‘rule’ must be learned instead of a simple association.

Pairs of identical objects are used in this instance. For every task, 2 pairs of identical objects were used, and in each trial, 2 identical objects were seen by the marmoset: one over the left food well and the other over the right food well. For example, objects Y and Y may be identical colored key rings, while objects Z and Z might be identical eppendorf tubes. On any single trial, the marmoset could see either the 2 key rings, or the 2 eppendorf tubes. The choice of which pair was seen was again determined by a pseudorandom schedule.

Marmosets choose an object by moving it to reveal the reward (or lack thereof) hidden underneath. The conditional rule that must be adhered to was that when objects Y and Y were seen, the reward would be hidden under the rightmost of the two identical objects; when objects Z and Z were seen, the reward would be hidden under the leftmost example of object Z. So in the example given above, when the key rings were seen, food was under the right key ring, and when the eppendorf tubes were seen, food was under the left tube. Marmosets had to reach a criterion of 90% correct to ‘pass’ the task and move onto the next.

Study design The first stage of the study was to ‘shape’ the marmosets, ie, to take experimentally naïve animals and train them to respond to objects presented in the WGTA. All animals were

trained with an identical series of tasks: 2 perceptually simple visual discrimination tasks; the first taken to a criterion of 90 correct out of 100 consecutive trials (90/100) and the second to a criterion of 27 correct out of 30 consecutive trials (27/30). This was followed by 2 visuospatial discrimination tasks; the first to a criterion of 90/100 and the second to a criterion of 27/30. Lastly, animals learned 2 perceptually complex (black object) visual discrimination tasks to a criterion level of 27/30 in both cases.

Once these tasks had been successfully mastered, marmosets were assigned to either the perceptually complex visual discrimination study, or the visuospatial discrimination study, such that there was an equal spread of learning ability across the 2 studies (by balancing learning scores [number of trials to achieve criterion] between groups).

Other marmosets, after completing the same training, were assigned to either the surgery or the control group; in such a way that there was an equal spread of learning ability between the 2 groups before surgery took place.

In all experiments, the marmosets learned a series of consecutive tasks using new objects for each task. All tasks were presented in the same order, such that interference between stimuli was kept identical for all monkeys. Treatments were given daily for the duration of learning task (as described above). Learning scores (the number of trials before the 27/30 correct criterion was reached) were averaged for each animal under each treatment so as to balance for practice effects across the tasks. At the end of the study, mean data for each animal under each treatment were analyzed and the control group compared with the lesion group.

Data analysis A repeated-measures ANOVA was used to analyze the data, using a StatviewII software package on an Apple Macintosh computer. The mean learning scores for each marmoset under each treatment provided the raw data. Post hoc analyses included

Dunnett's t test (for comparing various treatments to control/vehicle data) and Scheffé f test (for comparing different treatments to one another). In all cases, $p < 0.05$ indicated a significant difference.

All animal procedures were approved by the Animal Care Committees of Princeton University and Wyeth Research and conformed with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

Materials

All chemicals were of analytical grade and were purchased from Aldrich & Sigma Chemicals (Milwaukee, WI, USA). 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) was purchased from Research Biochemicals International (Natick, MA, USA). 4-Cyano-N-{2-[4-(2,3-dihydrobenzo[1,4]dioxin-5-yl)-piperazin-1-yl]-propyl}-N-pyridin-2-yl-benzamide HCl (lecozotan) was synthesized by Chemical Sciences, Wyeth Research, Princeton.

RESULTS

In Vitro Profile and Structure

The chemical name of lecozotan hydrochloride is 4-cyano-N-[(2*R*)-[4-(2,3-dihydrobenzo[1,4]dioxin-5-yl)piperazin-1-yl]propyl]-*N*-pyridin-2-yl-benzamide hydrochloride (Figure 1). Lecozotan displayed high affinity binding at the cloned human 5-HT_{1A} receptor ($K_i = 1.6 \pm 0.3$ nM and 4.5 ± 0.6 nM) using [³H]8-OH-DPAT as the 5-HT_{1A} agonist radioligand and [³H]WAY-100635 as the 5-HT_{1A} antagonist radioligand, respectively (Table 1). Lecozotan was selective for the 5-HT_{1A} receptor over other monoamine receptors that have previously been shown to be cross-reactive with the aryl-piperazine pharmacophore. A Novascreen profile revealed that greater than 50% displacement was only recorded at the highest concentration of 10,000 nM at 22 sites, indicating that lecozotan demonstrates significant selectivity for the 5-HT_{1A} receptor. Lecozotan did not exhibit significant activity (> 50% inhibition at any concentration) at any of the other 61 binding sites that were evaluated at that concentration except for dopamine D₄ (inhibition = 63%). Most notably, the compound was not active at various alpha-adrenergic (1A, 1B, 2A, 2B, 2C), beta-adrenergic (1, 2), adenosine (1, 2, 3), dopamine (1, 2, 3, 5), histamine (1, 2, 3), muscarinic (1, 2, 3, 4, 5), or serotonin (1B, 1D, 2A, 2C, 3, 4, 5, 6, 7) receptors.

In a clonal CHO cell line expressing the human 5-HT_{1A} receptor, lecozotan antagonized the ability of 8-OH-DPAT to inhibit forskolin-stimulated cAMP production. In this study the response to 10 nM 8-OH-DPAT, representing a submaximal concentration, was evaluated in the presence of increasing concentrations of antagonist. The calculated IC₅₀ for lecozotan was 25.1 ± 5.3 nM (Table 1). These results were corroborated using a guanosine-5'-(γ -thio)triphosphate[³⁵S]

(GTP γ S) assay where lecozotan also blocked the agonist effects of 8-OH-DPAT ($IC_{50} = 36.7 \pm 0.74$ nM). Lecozotan alone did not induce any agonist-like activity under any assay conditions.

Lecozotan Attenuates 8-OH-DPAT Induced Changes in Hippocampal 5-HT Levels Without Having Basal Effects: In Vivo Microdialysis

Lecozotan (3.0 mg/kg sc) had no effect on basal levels of 5-HT in the hippocampus of conscious rats when compared with vehicle-treated animals (Figure 2). Treatment with 8-OH-DPAT (0.3 mg/kg sc) induced a significant ($F_{1,24} = 7.9$, $p = 0.0089$) decrease in extracellular levels of 5-HT. Pretreatment with lecozotan (0.3 mg/kg sc) produced a significant ($F_{2,21} = 19.49$, $p < 0.0001$) and complete attenuation of the 8-OH-DPAT-induced decrease in hippocampal 5-HT.

Lecozotan Competitively Blocks the Inhibition of Dorsal Raphe Neuronal Firing Induced by 8-OH-DPAT

8-OH-DPAT (0.025 mg/kg sc) produced significant inhibition of dorsal raphe neuronal (DRN) firing in the anesthetized rat (Figure 3). At a dose of 0.3 mg/kg sc lecozotan produced no change in neuronal firing. The firing rates (mean action potentials per sec \pm SEM) at baseline and after lecozotan treatment were 1.07 ± 0.25 and 1.12 ± 0.28 , respectively (not shown). Pretreatment with lecozotan (0.3 mg/kg sc) significantly ($p < 0.001$) antagonized the inhibitory effects of 8-OH-DPAT (0.025 to 0.1 mg/kg sc). Cumulative doses up to 0.625 mg/kg of 8-OH-DPAT were needed to restore inhibition of neuronal firing to levels equal to those seen in the absence of lecozotan.

Lecozotan Antagonizes Fixed-Ratio Operant Responding in Rats

8-OH-DPAT (sc) produced dose-dependent decreases in response rate in all rats ($ED_{50} = 0.18$ mg/kg; 95% CI: 0.15 – 0.22 mg/kg). Pretreatment with 0.3 mg/kg of lecozotan sc produced an approximate 6-fold rightward shift of the 8-OH-DPAT dose-effect curve, with the ED_{50} value for

8-OH-DPAT increasing to 1.08 mg/kg (Figure 4a). Pretreatment with 1.0 and 3.0 mg/kg of lecozotan sc produced 12- and 20-fold rightward shifts of the 8-OH-DPAT dose-effect curve, respectively, with the ED₅₀ value for 8-OH-DPAT increasing to 3.68 mg/kg (95% CI: 2.35 – 5.77 mg/kg) at the higher dose. Pretreatment with 3, 10, and 30 mg/kg of lecozotan po produced approximately 3-, 6-, and 9.5-fold rightward shifts of the 8-OH-DPAT dose-effect curve, respectively (Figure 4b). At the highest dose (30 mg/kg po), the ED₅₀ value for 8-OH-DPAT increased approximately 10-fold to 2.08 mg/kg (95% CI: 1.33 – 3.20 mg/kg). These results suggest that there is a 10- to 30-fold separation between the effects of subcutaneously and orally administered lecozotan.

In an experiment to investigate the pharmacodynamic time course of antagonism, it was determined that significant ($p < 0.05$) blockade of 8-OH-DPAT by lecozotan was present as long as 4 hours after administration of lecozotan (1 mg/kg sc), data not shown. A single dose of 0.3 mg/kg 8-OH-DPAT sc decreased the response rate to 0.74 resp/sec in the first component of the session (10 to 20 minutes after administration of 8-OH-DPAT). Rates of responding gradually returned to baseline rates across the 1-hour session with a response rate of 2.33 resp/sec in the third component of the session (50 to 60 minutes after administration of 8-OH-DPAT). After a 2- or 4- hour pretreatment with lecozotan (1.0 mg/kg sc) the decreases in response rates observed after 0.3 mg/kg 8-OH-DPAT alone were completely antagonized for the entire session, with response rates greater than 2 resp/sec in all 3 response components.

Lecozotan Antagonizes Fixed-Ratio Operant Responding in Squirrel Monkeys

8-OH-DPAT im produced a dose-dependent decrease in response rates in all monkeys (ED₅₀ value of 0.07 mg/kg; 95% CI: 0.04 – 0.11 mg/kg). Lecozotan administered at doses of 0.01 and 0.1 mg/kg im produced dose-dependent rightward shifts of the dose-effect curve for 8-OH-

DPAT (Figure 5a). At the highest dose (0.1 mg/kg), the ED₅₀ value for 8-OH-DPAT was shifted approximately 17-fold to 1.21 mg/kg (95% CI: 0.45 – 3.25 mg/kg). Similar shifts in the 8-OH-DPAT dose-effect curves were produced by the oral administration of lecozotan at doses of 0.1, 0.3, and 1.0 mg/kg (Figure 5b). At the highest dose (1.0 mg/kg), the ED₅₀ value for 8-OH-DPAT was shifted approximately 10-fold to 0.68 mg/kg (95% CI: 0.27 – 1.72 mg/kg).

Lecozotan Blocks the Discriminative Stimulus Effects of 8-OH-DPAT in Pigeons

Once animals were trained to the stimulus cue, animals responded almost exclusively (> 98%) on the drug-appropriate key after an injection of 0.1 mg/kg 8-OH-DPAT and on the saline-appropriate key (< 2% drug-key responses) after an injection of saline (Figure 6). Subsequently, lecozotan was assessed for its ability to block the stimulus cue evoked by an acute injection of 8-OH-DPAT. Lecozotan was administered as a pretreatment (0.01 to 1 mg/kg im) to the training dose of 0.1 mg/kg 8-OH-DPAT im. Lecozotan dose-dependently decreased the percentage of responses on the 8-OH-DPAT appropriate key, consistent with its actions as a 5-HT_{1A} receptor antagonist (Figure 6). Response completely shifted to the saline-appropriate key after administration of 1.0 mg/kg lecozotan. Response rates were unaffected by lecozotan when given alone at doses of 0.01 to 1.0 (data not shown).

Lecozotan Antagonizes the 5-Methoxy-Dimethyltryptamine-Induced Syndrome in the Rat

The 5-HT syndrome is mediated by postsynaptic 5-HT_{1A} receptors and is considered a model of central 5-HT_{1A} receptor stimulation. The 5-HT syndrome for these studies was induced by 5-methoxy-dimethyltryptamine (5-MeODMT) at a dose of 5.6 mg/kg ip. Lecozotan (10 mg/kg) or vehicle (1 mL/kg) were given po once daily for 7 consecutive days to rats (Table 2). Twenty-four (24) hours after the last dose, the antagonist potency was determined using the 5-MeODMT-induced syndrome. ID₅₀ was 4.26 mg/kg (95% CI: 2.23 – 8.13) after chronic lecozotan

administration, which did not differ significantly from the acute potency or the potency following chronic vehicle (4.92 mg/kg; 95% CI: 2.70 – 9.00). Lecozotan exhibited no agonist activity by itself when administered up to 17 mg/kg. Thus, chronic lecozotan treatment did not alter the antagonist potency in this model.

In separate experiments, the agonist potency of 5-MeODMT was evaluated 24 hours after the last dose. An ED₅₀ value of 1.83 mg/kg (95% CI: 1.37 – 2.43) was calculated after ip administration of chronic vehicle. An ED₅₀ value of 1.68 mg/kg (95% CI: 1.00 – 2.82) mg/kg was observed after chronic ip administration of lecozotan (10 mg/kg). Thus, chronic lecozotan treatment did not alter the sensitivity of 5-HT_{1A} receptors.

Effects of Lecozotan on Basal and Stimulated Glutamate Release From the Dentate Gyrus.

Lecozotan produced a significant ($F_{2,17} = 4.52$, $p = 0.027$) augmentation of K⁺-induced increase in extracellular glutamate concentrations within the dentate gyrus of the rat. Administration sc of lecozotan (1.0 mg/kg) alone produced no change in basal levels of glutamate within the dentate gyrus (Figure 7). Infusion of aCSF containing 100 mM KCl produced a small but nonsignificant ($p = 0.053$) increase in extracellular glutamate levels, reaching a maximum value of 153.4 ± 51.6 % of preinfusion control levels. Pretreatment sc with a single dose of lecozotan (0.3 mg/kg) produced a significant potentiation of K⁺-induced increases in extracellular glutamate with a maximum value of 471.5 ± 83.1 % of preinfusion levels ($p = 0.012$).

Effects of Lecozotan on Basal and Stimulated Acetylcholine Release From the CA1 Region of the Hippocampus.

Lecozotan administered sc at a dose of 1 mg/kg had a small but significant effect on basal acetylcholine levels ($146 \pm 5\%$) in the CA1 region of the hippocampus compared with vehicle ($125 \pm 7\%$). When a dose of 1 mg/kg of lecozotan was administered sc before a 10-min infusion

of KCl (100 mM), extracellular acetylcholine levels increased ($p < 0.05$) to 275% \pm 27% over basal levels (Figure 8).

Lecozotan Enhances Cognitive Performance in the Aged Rhesus Monkey

Vehicle treatment was associated with a significant ($F_{3,40}=35.7$, $p < 0.001$) delay-dependent reduction in task accuracy that approximated the target accuracies indicated in the Methods section. Figure 9a shows the composite data set for the effects of lecozotan im on DMTS performance efficiency. Statistical analysis of the data revealed no significant effect of treatment alone ($F_{4,40}=1.03$, $p = 0.40$), and no significant effect of treatment's interaction with delay interval ($p = 0.49$). There also were no significant effects of treatment for sessions run 24 hours after drug administration (data not shown). On average, however, improvement in task performance efficiency was maintained for the 0.3 and the 1 mg/kg doses for trials associated with short delay intervals.

The optimal (Best) dose was defined as the dose that evoked the greatest task improvement independent of delay interval. Three (3) subjects received overall (all 96 trials) benefit (overall task accuracy greater than respective vehicle level) from 2 of the 5 doses. One (1) subject received benefit from 3 of the 5 doses, and 1 subject received benefit from only one dose. Therefore, none of the subjects could be considered a nonresponder under the conditions of the experiment. Most of the average task improvement was noted in the middle of the dose-response relationship: 2 animals received most improvement from the 1 mg/kg dose, and the other 3 animals' Best Doses were 0.1, 0.3 and 1 mg/kg. The average Best Dose for the study group was 1.1 mg/kg.

To reduce the likelihood of false positives, the Best Dose was repeated on 1 additional occasion. Both sessions were averaged and the data are presented in Figure 9b. There was a

significant effect of drug treatment, independent of delay interval ($F_{1,44}=7.31$, $p = 0.01$). When improvement in task efficiency was calculated as the percentage increase from baseline levels of performance, after Best Dose, accuracy increased by 16.5% of control for trials associated with short delay intervals. Although the treatment times delay effect was not statistically significant, the improvement in accuracy associated with Short delay trials accounted for 45% of the total task improvement.

In addition to task accuracies, 2 response latencies were measured: one to initiate the trial, and one to initiate a choice. Drug treatment did not significantly affect ($F_{2,40}=2.19$, $p = 0.13$) the duration of either response latency (data not shown).

Lecozotan Reverses Cognitive Deficits Associated with Glutamatergic Dysfunction or Cholinergic Lesions in Marmosets

Acquisition of visuo-spatial discrimination tasks after VDB lesion surgery

VDB-lesioned animals' mean learning scores indicate significant impairment ($p < 0.05$) compared with nonlesioned (control) animals (Fig.10). Lecozotan (1 mg/kg and 2 mg/kg im) had no significant effect in the control animals at either dose. In lesioned animals lecozotan-treated (1 mg/kg) animals had learning deficits significantly greater ($p < 0.05$) than similarly treated non-lesioned animals. Lecozotan 2 mg/kg, however, completely abolished the lesion-induced cognitive deficit. Mean learning scores after lecozotan 2 mg/kg treatment were not significantly different from learning scores of nonlesioned animals treated with 2 mg/kg lecozotan, and significantly ($p < 0.05$) lower than the scores of saline- and lecozotan-treated (1 mg/kg) lesioned animals. Overall, data suggest that lecozotan (2 mg/kg im) completely reverses a hippocampal cognitive deficit induced by cholinergic lesion.

Acquisition of perceptually complex visual discrimination tasks

Dizocilpine (0.015 mg/kg) caused a significant ($p < 0.001$) deficit in the marmosets' ability to acquire perceptually complex visual discrimination tasks compared with saline treatment (Fig.11A). Lecozotan 2 mg/kg alone had no significant effect on mean learning score compared with saline treatment. When lecozotan 2 mg/kg was given concurrently with dizocipline, the dizocipline-induced deficit in task acquisition was completely abolished ($p < 0.001$). Overall, data suggest that lecozotan im reverses cognitive deficits induced by glutamatergic blockade in a cortically mediated task.

Acquisition of visuo-spatial discrimination tasks

Dizocilpine 0.015 mg/kg caused a significant ($p < 0.05$) deficit in the marmosets' ability to acquire visuospatial discrimination tasks compared to saline treatment (Fig.11B). Lecozotan 2 mg/kg had no significant effect on mean learning score compared with saline treatment. When lecozotan 2 mg/kg was given concurrently with dizocipline, the dizocipline-induced deficit in task acquisition was completely abolished ($p < 0.05$). Overall, data suggest that lecozotan im completely reverses a dizocilpine-induced cognitive deficit in hippocampally mediated tasks.

DISCUSSION

In-depth pharmacologic studies in this report demonstrate that lecozotan is a novel 5-HT_{1A} receptor antagonist with intriguing procognitive properties. In vitro and in vivo assays designed to test the intrinsic activity of lecozotan demonstrate that it is a potent and selective 5-HT_{1A} receptor antagonist. This inclusive series of studies has determined the intrinsic activity of lecozotan in models indicative of pre- vs postsynaptic 5-HT_{1A} receptor function. Molecules that possess intrinsic activity at the presynaptic 5-HT_{1A} autoreceptor lead to decreases in serotonergic cell firing and subsequent reductions in 5-HT levels in terminal regions. Lecozotan demonstrated an antagonist profile, because administration of relatively high doses resulted in no change in either extracellular 5-HT levels in terminal projection areas of the serotonergic cell bodies or any change in serotonergic neuronal firing in the DRN. Instead, lecozotan completely antagonized 8-OH-DPAT-induced decreases in hippocampal 5-HT, indicating that lecozotan appeared to act as a 5-HT_{1A} receptor antagonist in vivo. These data contrast with those observed for the 5-HT_{1A} receptor partial agonists BMY-7378, NAN-190, and buspirone, which can antagonize postsynaptic 5-HT_{1A} mediated responses, but act as agonists at the somatodendritic 5-HT_{1A} autoreceptors (Greul and Glaser, 1992; Yocca, 1990).

Lecozotan demonstrated potent in vivo antagonist activity in rodents, nonhuman primates, and pigeons. Pharmacologic studies using fixed-ratio responding in rats and squirrel monkeys demonstrated that the compound is a competitive antagonist. Drug discrimination studies in pigeons revealed that lecozotan blocked the discriminative stimulus effects of the prototypical 5-HT_{1A} receptor agonist 8-OH-DPAT, but did not substitute for the discriminative stimulus effects of 8-OH-DPAT. Chronic administration of lecozotan did not appear to alter the sensitivity of 5-HT_{1A} receptors in that there was no significant shift in the potency of an agonist

to induce serotonin syndrome in male rats after continuous administration of lecozotan. Taken together these data show lecozotan to be a potent, selective, and orally bioavailable competitive 5-HT_{1A} receptor antagonist *in vivo* that produces no evidence of tolerance to its antagonist effects or alters the sensitivity of 5-HT_{1A} receptors.

Alzheimer's disease is a neurodegenerative disorder characterized by various deficits in neurotransmitter function. It is widely believed that loss of these functions contributes to the loss in memory that is the hallmark of this disease. The hippocampus is one of the major brain structures shown to play a critical role in both mnemonic and cognitive function (Eichenbaum et al, 1992; Jarrard, 1993). Degeneration of the pyramidal cells in the corticohippocampal and corticocortical systems is one of the earliest changes in AD (Braak and Braak, 1991). Whereas many studies have concentrated on the cholinergic deficits observed in this disease, glutamatergic dysfunction in Alzheimer's disease has received increasing interest as an alternative to the well-established cholinergic hypothesis (Maragos et al, 1987; Proctor et al, 1988; Myhrer, 1993; Francis et al, 1993).

Anatomical data indicate that 5-HT_{1A} receptors might control the excitability and propagation of information transmitted by the pyramidal cells to subcortical structures along glutamate pathways (Czyrak et al, 2003). Serotonin has been shown to exhibit inhibitory effects on the glutamatergic projections of the brain, in particular those of the cortex (Dijk et al, 1995; Grunschlag et al, 1997) and hippocampus (Matsuyama et al, 1996; Schmitz et al, 1995). The cellular mechanisms underlying these processes appear to involve activation of K⁺ conductance (Grunschlag et al, 1997) and/or inhibition of presynaptic Ca²⁺ entry (Schmitz et al, 1995; Strosznajder et al, 1996). Other data suggest that 5-HT_{1A} receptor antagonists can inhibit the tonic hyperpolarizing action of serotonin on pyramidal neurons in both the cortex and

hippocampus (Araneda and Andrade, 1991; Van den Hooft and Galvan, 1992). Thus, it is possible that 5-HT_{1A} receptor antagonists may improve cognition by removing the inhibitory effects of endogenous serotonin on pyramidal neurons and enhancing the glutamatergic activation in both the cortex and hippocampus. An important aspect of this hypothesis is supported by the suggestion that a functionally hyperresponsive serotonin system in AD may contribute to cognitive disturbances (McLoughlin et al, 1994).

To test the hypothesis that a 5-HT_{1A} receptor antagonist would enhance glutamatergic neurotransmission, we used lecozotan to examine excitatory transmission under basal and stimulated conditions within the terminal regions of the perforant pathway (ie, granular layer of the dentate gyrus) using *in vivo* microdialysis. Lecozotan alone produced no change in basal levels of glutamate within the dentate gyrus, but induced significant augmentation of dentate gyrus glutamate levels when administered in the presence of an infusion of K⁺ in a way similar to that observed previously with WAY-101405 (Schechter et al, 2000). Furthermore, these *in vivo* data confirm the *in vitro* observations of Matsuyama et al (1996), who demonstrated that NAN-190 could augment NMDA-induced increases in glutamate in the guinea pig dentate gyrus. Serotonergic modulation of glutamatergic neurotransmission via the 5-HT_{1A} receptor has also been observed in other brain regions (Dijk et al, 1995; Schmitz et al, 1995). Thus, lecozotan-enhanced stimulated release of glutamate in the hippocampus supports the contention that 5-HT_{1A} receptor antagonists can modulate stimulated glutamate release as part of the mechanism related to enhancing cognitive function.

The "cholinergic hypothesis," which claims that AD is primarily the result of substantial cholinergic abnormalities, has received widespread attention in the development of therapeutic strategies directed at this psychopathological illness (Bartus, 2000). A major feature of the

neuropathology of AD is the degeneration of the cholinergic magnocellular neurons in the basal forebrain. A high density of 5-HT_{1A} binding sites has been reported in the region of cholinergic cell bodies within the nucleus basalis magnocellularis. WAY-100635 reverses the choice accuracy deficit in rats with AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-induced lesions of the nucleus basalis magnocellularis (Balducci et al, 2003). Our data indicated that in addition to the ability of lecozotan to enhance stimulated glutamate efflux, the 5-HT_{1A} receptor antagonist is also able to enhance stimulated acetylcholine levels and reverse cognitive deficits induced by cholinergic lesions.

Serotonin-1A receptor antagonists potentiate N-methyl-D-aspartate (NMDA)-induced glutamate release from pyramidal neurons. Furthermore, a 5-HT_{1A} receptor antagonist has the ability to significantly elevate glutamate release when administered alone (Araneda and Andrade, 1991). Since cognitive deficits have previously been shown to be susceptible to cognition enhancing treatments in the marmoset monkey (Harder *et al*, 2000), the use of the NMDA receptor antagonist dizocilpine is appropriate for the evaluation of putative cognitive enhancers targeted at dementia. Degeneration of the cholinergic cells of the basal forebrain is the other major feature that has long been linked to cognitive dysfunction. In our marmosets, a specific and selective lesion of the cholinergic cell bodies of the VDB eliminated the majority of the cholinergic input to the hippocampus, and produced the expected cognitive deficit in visuo-spatial learning. Because the deficit obtained has been shown to be susceptible to cognitive enhancing agents, this ‘model’ is also appropriate for evaluating dementia treatments. The fact that the 5-HT_{1A} antagonist lecozotan was able to overcome both a VDB lesion and the effects of an NMDA receptor antagonist compound suggests that it is a potentially valuable cognitive-enhancing agent. In addition, WAY-100635 antagonizes the extinction of recognition memory

and counter acts scopolamine-induced performance deficits in the normal rat (Pitsikas et al., 2003). WAY-101405, an orally active 5-HT_{1A} antagonist, can reverse the deficits induced by MK-801 in rats in the radial arm maze (Boast et al., 1999). In contrast to the cognitive enhancing properties of 5-HT_{1A} antagonists, the prototypical 5-HT_{1A} agonist, 8-OH-DPAT can impair passive avoidance performance in the rat (Santucci et al., 2003; Winsauer et al., 1999). WAY-100635 has been shown to reverse the cognitive deficits in marmosets induced by a fornix lesion and reverses the cognitive deficits induced by the glutamatergic antagonist MK-801 and the muscarinic antagonist scopolamine as tested using visuospatial and visual discrimination tasks in marmosets (Harder et al., 1996; Harder and Ridley, 2000). These studies, which tested different aspects of cognition and cognitive impairment induced by various neurochemical and neuroanatomic insults, strongly suggest that 5-HT_{1A} receptor antagonism is a viable drug strategy for restoring the multiple deficits associated with AD and, as such, will represent a novel and unique treatment approach. This potential therapeutic utility is further supported by the data in aged monkeys. Here the effects of lecozotan administration on DMTS task accuracy did not rise to statistical significance, owing primarily to individual variations in drug responsiveness. However, the dose-response study revealed that, at least on average, the 1 mg/kg dose increased accuracy, particularly during short-delay trials. Selection and readministration of the individualized Best Dose was consistent with the dose-response analysis in that the average Best Dose was 1.1 mg/kg, and that there was an overall significant improvement in accuracy, which again was primarily associated with short-delay trials.

The degree of cognitive improvement induced by lecozotan in aged rhesus monkeys using DMTS, as indicated by the 16.5% increase in task accuracy above baseline, represents a very good level of efficacy compared with other compounds tested under similar conditions in

this setting (Buccafusco and Terry, 2000). The observation that on average the effect was largely relegated to trials associated with short-delay intervals suggests the possibility that lecozotan may have a selective action to improve attentional aspects of cognition (Paule et al, 1998). Further studies using behavioral paradigms more suited to assessing drug effects on attention should be used to confirm this contention. The lack of effect on task response latencies was consistent with no effect of the drug on psychomotor speed. No untoward effects of the drug were noted before or after testing. Taken together, these data demonstrate the efficacy of 5-HT_{1A} receptor antagonists in enhancing cognitive function in both rodents and primates.

Taken together, the heterosynaptic nature of the effects of lecozotan imbues this compound with a novel therapeutic approach to the treatment of the cognitive deficits associated with AD, and possibly other psychopathological disease states in need of improved therapies to treat cognitive- or memory-associated loss. Lecozotan has completed phase 1 clinical trials and is continuing clinical investigation in phase 2 as an agent to reverse cognitive loss in AD.

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REFERENCES

Araneda R and Andrade R (1991) 5-Hydroxytryptamine 2 and 5-hydroxytryptamine 1A receptors mediate opposing responses on membrane excitability in rat association cortex. *Neurosci* 40:399-412.

Arvidsson LE, Hacksell U, Nilsson JL, Hjorth S, Carlsson A, Lindberg P, Sanchez D, Wikstrom H (1981) 8-Hydroxy-2-(di-n-propylamino)tetralin, a new centrally acting 5-hydroxytryptamine receptor agonist. *J Med Chem* 24:921-923.

Balducci C, Nurra M, Pietropoli A, and Samanin R (2003) Reversal of visual attention dysfunction after AMPA lesions of the nucleus basalis magnocellularis (NBM) by the cholinesterase inhibitor donepezil and by a 5-HT_{1A} receptor antagonist WAY 100635. *Psychopharmacol* 167:28-36.

Bartus R (2000) On neurodegenerative diseases, models, and treatment strategies: Lessons learned and lessons forgotten a generation following the cholinergic hypothesis. *Exp Neur* 163:495-529.

Bliss TV and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31-39.

Boast C, Bartolomeo AC, Morris H and Moyer JA (1999) 5HT antagonists attenuate MK801-impaired radial arm maze performance in rats. *Neurobiol Learn Mem* 71:259-271.

Bowen DM, Francis PT, Chessell IP, and Webster MT (1994) Neurotransmission-the link integrating Alzheimer research? *Trends Neurosci.* 17:149-150.

Braak H and Braak E (1991) Alzheimer's disease affects limbic nuclei of the thalamus. *Acta Neuropathol* 82:239-259.

Buccafusco JJ and Terry AV Jr (2000) Multiple CNS targets for eliciting beneficial effects on memory and cognition. *J Pharmacol Exp Ther* 295:438-446.

Chanda PK, Minchin MC, Davis AR, Greenberg L, Reilly Y, McGregor WH, Bhat R, Lubeck MD, Mizutani S and Hung PP (1993) Identification of residues important for ligand binding to the human 5-hydroxytryptamine1A serotonin receptor. *Mol Pharmacol* 43:516-520.

Czyrak A, Czepiel K, Maćkowiak M, Chocyk A, and Wędzony K (2003) Serotonin 5-HT1A receptors might control the output of cortical glutamatergic neurons in rat cingulated cortex. *Brain Res* 989:42-51.

Dawson LA and Routledge C (1995) Differential effects of potassium channel blockers on extracellular concentrations of dopamine and 5-HT in the striatum of conscious rats. *Brit J Pharmacol* 116:3260-3264.

Dawson LA, Stow JM and Palmer AM (1997) An improved method for the measurement of glutamate and aspartate using capillary electrophoresis with laser induced fluorescence detection and its application to brain microdialysis. *J Chromatogr B* 694:455-460.

Dijk SN, Francis PT, Stratmann GC and Bowen DM (1995) NMDA-induced glutamate and aspartate release from rat cortical pyramidal neurons: evidence for modulation by a 5-HT_{1A} antagonist. *Br J Pharmacol* 115:1169-1174.

Eichenbaum H, Otto T and Cohen EJ (1992) The hippocampus – What does it do? *Behav Neural Biol* 57:2-36.

Fargin A, Raymond JR, Lohse MJ, Kobilka BK, Caron MG and Lefkowitz RJ (1988) The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. *Nature* 335:358-360.

Fletcher A, Bill DJ, Bill SJ, Cliffe IA, Dover GM, Forster EA, Haskins JT, Jones D, Mansell HL and Reilly Y (1993) WAY-100135: a novel, selective antagonist at presynaptic and postsynaptic 5-HT_{1A} receptors. *Eur J Pharmacol* 237:283-291.

Fletcher A, Forster EA, Bill DJ, Brown G, Cliffe IA, Hartley J E, Jones DE, McLenaghan A, Stanhope KJ, Critchley DJ, Childs KJ, Middlefell VC, Lanfumey L, Corradetti R, Laporte AM, Gozlan H, Hamon M and Dourish CT (1996) Electrophysiological, biochemical, neurohormonal

and behavioral studies with WAY-100635, a potent, selective and silent 5-HT1A receptor antagonist. *Behav Brain Res* 73:337-353.

Forster EA, Cliffe IA, Bill DJ, Dover GM, Jones D, Reilly Y, and Fletcher A (1995) A pharmacological profile of the selective silent 5-HT1A receptor antagonist, WAY-100635. *Eur J Pharmacol* 281:81-88.

Francis PT, Sims NR, Proctor AW and Bowen DM (1993) Cortical pyramidal neuron loss may cause glutamatergic hypoactivity and cognitive impairment in Alzheimer's disease. *J Neurochem* 60:1589-1604.

Gozlan H, El Mestikawy S, Pichat L, Glowinski J and Hamon M (1983) Identification of presynaptic serotonin autoreceptors using a new ligand: ^3H -PAT. *Nature*. 305:140-142.

Greuel JM and Glaser T (1992) The putative 5-HT1A receptor antagonists NAN-190 and BMY 7378 are partial agonists in the rat dorsal raphe nucleus in vitro. *Eur J Pharmacol* 211:211-219.

Grunschlag CR, Haas HL and Stevens DR (1997) 5-HT inhibits lateral entorhinal cortical neurons of the rat in vitro by activation of potassium channel-coupled 5-HT1A receptors. *Brain Res* 770:10-17.

Harder JA, Maclean CJ, Alder JT, Francis PT and Ridley RM (1996) The 5-HT_{1A} antagonist, WAY-100635, ameliorates the cognitive impairment induced by fornix transection in the marmoset. *Psychopharmacol* 127:245-254.

Harder JA, Baker HF and Ridley RM (1998) The role of the central cholinergic projections in cognition: implications of the effects of scopolamine on discrimination learning by monkeys. *Brain Res Bull* 45:319-326.

Harder JA and Ridley RM (2000) The 5-HT_{1A} antagonist, WAY-100635, alleviates cognitive impairments induced by dizocilpine (MK-801) in monkeys. *Neuropharmacol* 39:547-552.

Hernandez L, Tucci S, Gusman N and Paez X (1993) In vivo monitoring of glutamate in the brain by microdialysis and capillary electrophoresis with laser-induced fluorescence detection. *J Chrom A* 652:393-398.

Jarrard LE (1993) On the role of the hippocampus in learning and memory in the rat. *Behav Neural Biol* 60:9-26.

Johansson L, Sohn D, Thorberg S-O, Jackson DM, Kelder D, Larsson L-G, Rényi L, Ross SB, Wallsten C, Eriksson H, Hu P-S, Jerning E, Mohell N and Westlind-Danielsson A (1997) The pharmacological characterization of a novel selective 5-hydroxytryptamine_{1A} receptor antagonist, NAD-299. *J Pharmacol Exp Ther* 283:216-225.

Kelleher RT, Morse WH, Herd JA (1972) Effects of propranolol, phentolamine and methyl atropine on cardiovascular function in the squirrel monkey during behavioral experiments. *J Pharmacol Exp Ther* 182:204-217.

Kowall NW and Beal MF (1991) Glutamate-, glutaminase-, and taurine-immunoreactive neurons develop neurofibrillary tangles in Alzheimer's disease. *Ann Neurol* 29:162-167.

Lazareno S and Birdsall NJ (1993) Pharmacological characterization of acetylcholine-stimulated [³⁵S]-GTP gamma S binding mediated by human muscarinic m₁-m₄ receptors: antagonist studies. *Br J Pharmacol* 109:1120-1127.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurements with folin phenol reagent. *J Biol Chem* 193:265-275.

Maragos WF, Greenamyre T, Penney Jr JB and Young AB (1987) Glutamate dysfunction in Alzheimer's disease: a hypothesis. *Trends Neurosci* 10:65-67.

Matsuyama S, Nei K and Tanaka C (1996) Regulation of glutamate release via NMDA and 5-HT_{1A} receptors in guinea pig dentate gyrus. *Brain Res* 728:175-180.

McLoughlin DM, Lucey JV, and Dinan G (1994) Central serotonergic hyperresponsivity in late-onset Alzheimer's disease. *Am J Psychiatry* 151:1701-1703.

Morris RG, Anderson E, Lynch GS and Baudry M (1986) Selective impairment of learning and blockade of long term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* 319:774-776.

Myhrer T (1993) Animal models of Alzheimer's Disease: glutametergic denervations and alternative approach to cholinergic denervation. *Neurosci Behav Rev* 17:195-202.

Paule MG, Bushnell PJ, Maurissen JPJ, Wenger G.R., Buccafusco JJ, Chelonis JJ and Elliott R (1998) Symposium overview: the use of delayed matching-to-sample procedures in studies of short-term memory in animals and humans. *Neurotoxicol Teratol* 20:493-502.

Paxinos G, Watson C, Pennisi M and Topple A (1985) Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. *J Neurosci Meth* 13:139-143.

Pedigo NW, Yamamura HI and Nelson DL (1981) Discrimination of multiple [³H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. *J Neurochem* 36:220-226.

Pitsikas N, Rigamonti AE, Cella SG and Muller EE (2003) The 5-HT_{1A} receptor antagonist WAY 100635 improves rat's performance in different models of amnesia evaluated by the object recognition task. *Brain Res* 983:215-222.

Proctor AW, Palmer AM, Francis PT, Lowe SL, Neary D, Murphy E, Doshi R and Bowen DM (1988) Evidence of glutamatergic denervation and possible abnormal metabolism in Alzheimer's Disease. *J Neurochem* 50:790-802.

Rada P, Jensen K and Hoebel BG (2001) Effects of nicotine and mecamylamine-induced withdrawal on extracellular dopamine and acetylcholine in the rat nucleus accumbens. *Psychopharmacol* 157:105-110.

Rasmussen K, Calligaro DO, Czachura JF, Dreshfield-Ahmad LJ, Evans DC, Hemrick-Luecke SK, Kallman MJ, Kendrick WT, Leander JD, Nelson DL, Overshiner CD, Wainscott DB, Wolff MC, Wong DT, Branchek TA, Zgombick JM and Xu Y-C (2000) The novel 5-hydroxytryptamine_{1A} antagonist LY426965: effects on nicotine withdrawal and interactions with fluoxetine. *J Pharmacol Exp Ther* 294:688-700.

Ridley RM and Baker HF (1991) A critical evaluation of monkey models of amnesia and dementia. *Brain Res Rev* 16:15-37.

Ridley RM, Barefoot HC, Maclean CJ, Pugh P and Baker HF (1999) Severe learning impairment caused by combined immunotoxic lesion of the cholinergic projections to the cortex and hippocampus in monkeys. *Brain Res* 836:120-138.

Santucci AC, Shaw C (2003) Peripheral 8-OH-DPAT and scopolamine infused into the frontal cortex produce passive avoidance retention impairments in rats. *Neurobiol Learn Mem* 79(2):136-141.

Schechter LE, Smith DL, Rosenzweig-Lipson S, Marquis K, Jones D, Nguyen HQ, Dawson LA and Kelly MG (2000) Pharmacological characterization of an orally active 5-HT_{1A} receptor antagonist. *Soc Neurosci* 26:392.

Schmitz D, Empson RM and Heinemann U (1995) Serotonin and 8-OH-DPAT reduced excitatory transmission in rat hippocampal area CA1 via reduction in presumed presynaptic Ca²⁺ entry. *Brain Res* 701:249-254.

Smith LM and Peroutka SJ (1986) Differential effects of 5-hydroxytryptamine 1A selective drugs on the 5-HT behavioral syndrome. *Pharmacol Biochem Behav* 24:1513-1519.

Strosznajder J, Chalimoniuk M and Samochocki M (1996) Activation of serotonergic 5-HT1A receptor reduces Ca²⁺ -and glutamatergic receptor-evoked arachidonic acid and NO/cGMP release in adult hippocampus. *Neurochem* 28:439-444.

Van den Hooff P and Galvan M (1991) Electrophysiology of the 5-HT1A ligand MDL 73005EF in the rat hippocampal slice. *Eur J Pharmacol* 196:291-298.

Van den Hooff P and Galvan M (1992) Actions of 5-hydroxytryptamine and 5-HT1A receptor ligands on rat dorso-lateral septal neurons in vitro. *Br J Pharmacol* 106:893-899.

Winsauer PJ, Rodriguez FH, Cha AE, Moerschbaecher JM (1999) Full and partial 5-HT1A receptor agonists disrupt learning and performance in rats. *J Pharmacol Exp Ther* 288(1):335-347.

Yocca FD (1990) Novel anxiolytic agents: actions on specific subtypes of central 5-HT receptors. *Prog Clin Biol Res* 361:145-177.

Footnotes Page

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FIGURE LEGENDS

Figure1. Chemical structure of lecozotan.

Figure 2. Lecozotan administered subcutaneously (sc) significantly ($F_{2,21} = 19.49$, $p < 0.0001$) antagonized the decrease of hippocampal extracellular 5-HT levels induced by 8-OH-DPAT in rats. Data is expressed as mean \pm SEM of percent preinjection baseline values. Lecozotan had no effect up to 3 mg/kg when administered alone.

Figure 3. Effect of lecozotan on 8-OH-DPAT induced inhibition of dorsal raphe neuronal firing (DRN) in the anesthetized rat. After SRA-33 pretreatment (0.3 mg/kg), the dose of 8-OH-DPAT needed to demonstrate comparable inhibition increased to a cumulative dose of 0.625 mg/kg. .

Figure 4. 8-OH-DPAT (sc) produced decreases in fixed-ratio operant response rates in all rats. Pretreatment with lecozotan administered sc (A) and po (B) shifted the 8-OH-DPAT dose effect curve to the right. Values are means \pm SEM.

Figure 5. 8-OH-DPAT (im) produced decreases in fixed-ratio operant response rates in all squirrel monkeys. Pretreatment with lecozotan administered im (A) and po (B) shifted the 8-OH-DPAT dose effect curve to the right. Values are means \pm SEM.

Figure 6. In a discriminative stimulus effects study using pigeons, lecozotan (0.01 – 1.0 mg/kg) dose-dependently decreased the percentage of responses on the 8-OH-DPAT appropriate key,

consistent with its actions as a 5-HT_{1A} receptor antagonist. Values are means ± SEM % drug key responding. 8-OH-DPAT (im) training dose was 0.1 mg/kg. Saline was used as a vehicle.

Figure 7. Effects of lecozotan on basal and K⁺- stimulated glutamate levels in the dentate gyrus of rats. Data are expressed as means ± SEM of percent preinjection levels of glutamate; n = 6-8 per study group. Lecozotan significantly ($F_{2,17}$ 4.5, p = 0.027) augmented the K⁺ - stimulated increases in extracellular glutamate within the dentate gyrus reaching a maximum value of 471.5 ± 83.1 % at 120 min (p = 0.012). * denote statistical (p < 0.05) difference between K⁺- stimulated vs lecozotan+K⁺-stimulated responses.

Figure 8. Lecozotan (1.0 mg/kg, sc) produces a significant potentiation of 100mM K⁺ -induced increases in extracellular acetylcholine levels in the CA1 region of the hippocampus. Values are means ± SEM. * indicates significant difference (p<0.05) compared with vehicle.

Figure 9A. Lecozotan enhances cognitive function in the aged rhesus monkey as assessed by DMTS (delayed matching-to-sample) performance efficiency. Testing was initiated 30 min after a single dose administration. Statistical analysis of the data revealed no significant effect of treatment alone (p =0.40), and no significant effect of treatment's interaction with delay interval (p =0.49). **B.** The improvement in DMTS accuracy produced by the individualized Best Dose of lecozotan. These data represent the average of the Best Dose session determined from the dose-response series and a separate series in which only the Best Dose was administered. There was no significant difference between the data sets obtained for these two series (t=0.44, p=0.66). For the combined sessions, there was a significant effect of drug treatment relative to vehicle that

was independent of delay interval. Values are means \pm SEM. * indicates significant difference ($p = 0.01$) compared with vehicle. Avg=Average; Med=Medium.

Figure 10. Deficits in acquisition of a hippocampal task (visuo-spatial discrimination) in marmosets were induced by a vertical diagonal band (VDB) lesion. Deficits were completely abolished by lecozotan at 2mg/kg, but not at 1mg/kg. Values are mean learning scores \pm SEM. * indicates that learning scores for VDB-lesioned marmosets were significantly greater than the scores of non-lesioned animals when saline (vehicle) or 1mg/kg lecozotan were administered during task acquisition ($p<0.05$). † indicates that learning scores for VDB-lesioned marmosets were significantly lower (i.e. acquisition was faster) when these lesioned marmosets were treated with 2mg/kg lecozotan, compared to when they were treated with saline or 1mg/kg lecozotan ($p<0.05$). Additionally, VDB-lesioned marmosets acquired the task as quickly as unlesioned marmosets when they were treated with 2mg/kg lecozotan ($p>0.05$, NS).

Figure 11A. Deficits in acquisition of a hippocampal task in marmosets induced by dizocilpine (NMDA antagonist) are completely reversed by lecozotan (im) as assessed using complex visuo-spatial discrimination. Marmosets under dizocilpine treatment had significant (** $p<0.001$) deficits in learning scores compared with their scores under saline treatment. Lecozotan (2.0 mg/kg) significantly († $p<0.001$) reversed the dizocilpine-induced deficits. Values are means \pm SEM. **B.** Deficits in acquisition of a neocortical task in marmosets induced by dizocilpine (NMDA antagonist) are completely reversed by lecozotan (2 mg/kg) as assessed using perceptually complex visual discrimination. Marmosets under dizocilpine treatment had significant (* $p<0.05$) deficits in learning scores compared with their scores under saline

treatment. Lecozotan (2.0 mg/kg) significantly (${}^{\dagger}p<0.05$) reversed the dixocilpine-induced deficits. Values are means \pm SEM.

Table 1

In Vitro Binding and Functional Profile of Lecozotan

Compound	(Ki, nM).....					(IC ₅₀ , nM).....	
	5-HT _{1A}	α1	D ₂	D ₃	D ₄	cAMP	GTPγS
Lecozotan (nM)	1.6 ± 0.3 ^a	248	1,548	320	98	25.1 ± 5.3	36.7 ± 0.74
	4.5 ± 0.6 ^b						

Abbreviations: 5-HT_{1A} = serotonin -1A; cAMP = cyclic adenosine 3' 5'-monophosphate; GTPγS = guanosine-5'--(γ-thio) triphosphate [³⁵S]; α1 = subtype 1 of the α - adrenergic receptor; D₂ = subtype 2 of the dopamine receptor; D₃ = subtype 3 of the dopamine receptor;

D₄ = subtype 4 of the dopamine receptor; Ki = inhibition constant; IC₅₀ = concentration at which there was 50% inhibition.

a: [³H]8-OH-DPAT

b: [³H]WAY-100635

Lecozotan was evaluated in a broad NovaScreenR binding profile where it was determined that the compound was >100-fold selective with respect to 63 neurotransmitter, receptor, reuptake, ion channel, and enzyme sites.

Table 2

Potency of Lecozotan to Antagonize 5-MeODMT-induced Syndrome After Chronic Lecozotan

<i>Chronic Treatment</i>	ID₅₀ mg/kg (95% CI)
Vehicle (1 mL/kg, po)	4.92 (2.70 – 9.00)
Lecozotan (10 mg/kg, po)	4.26 (2.23 – 8.13)

Potency of 5-MeODMT to Induced Syndrome After Chronic Lecozotan

<i>Chronic Treatment</i>	ED₅₀ mg/kg (95% CI)
Vehicle (1 mL/kg, ip)	1.83 (1.37 – 2.43)
Lecozotan (10 mg/kg, ip)	1.68 (1.00 – 2.82)

Figure 1

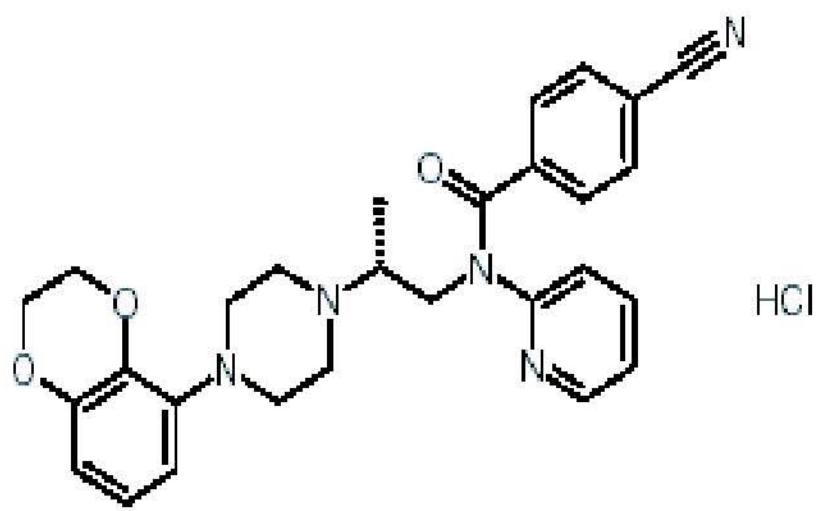


Figure 2.

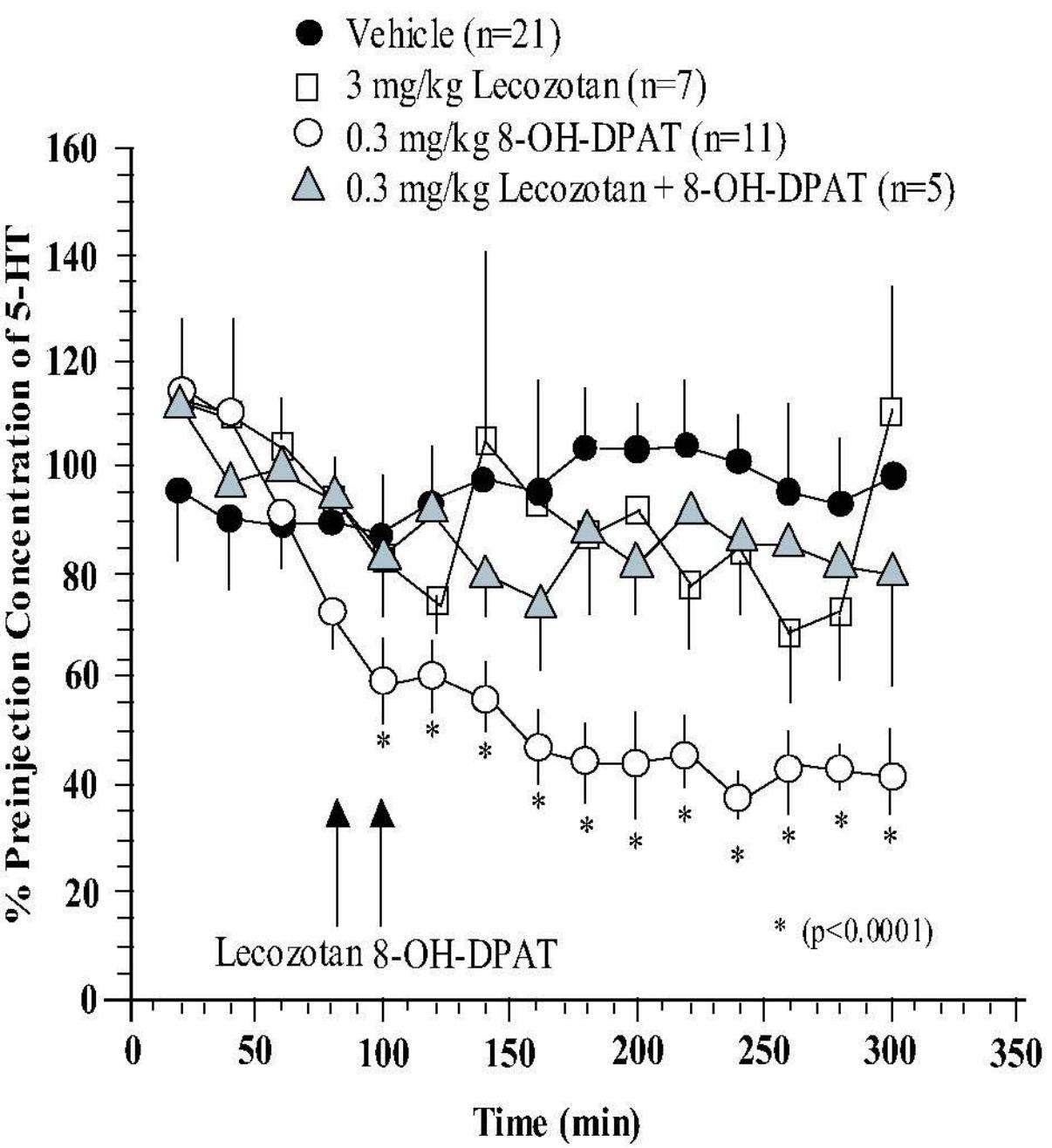


Figure 3.

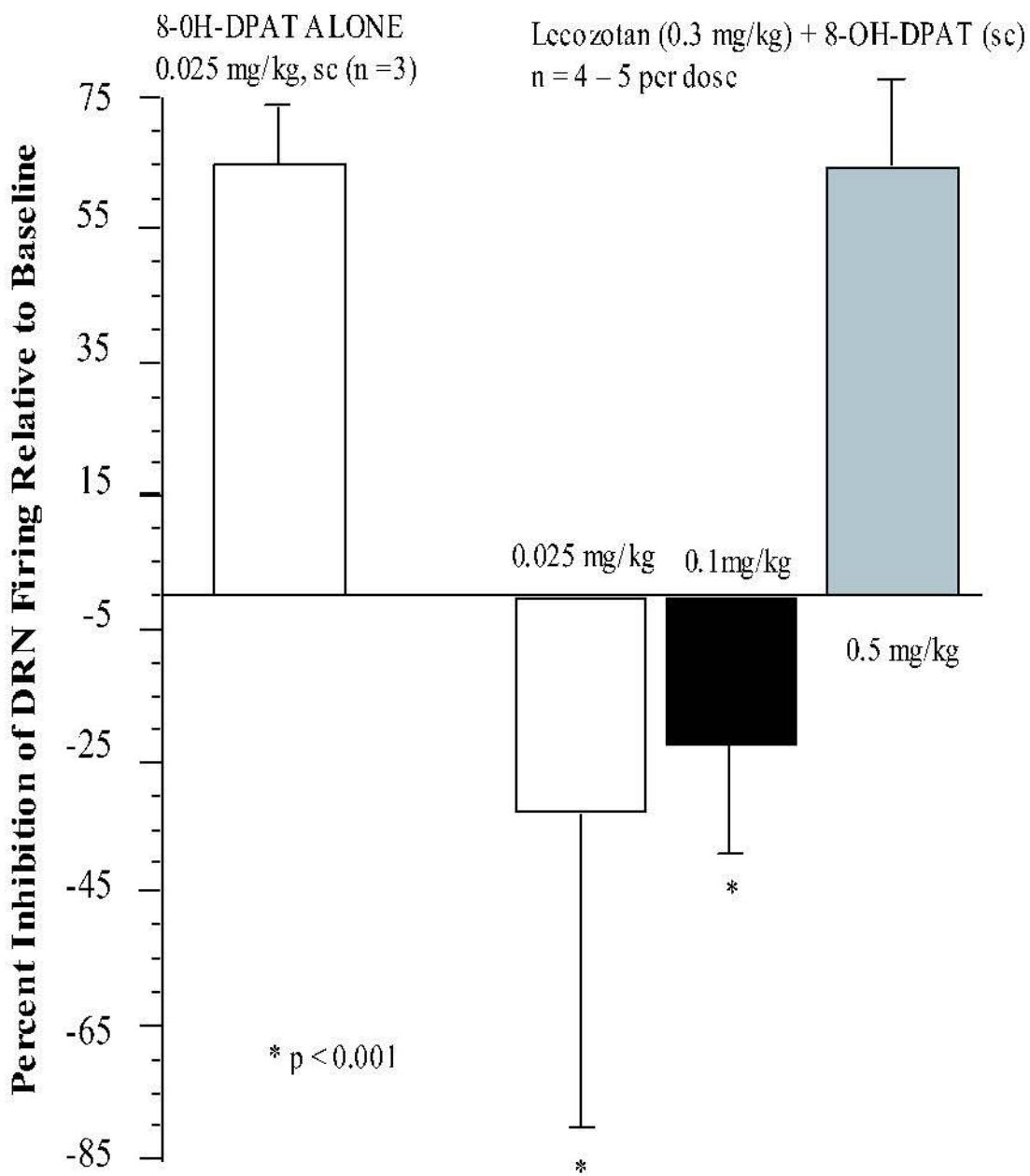


Figure 4

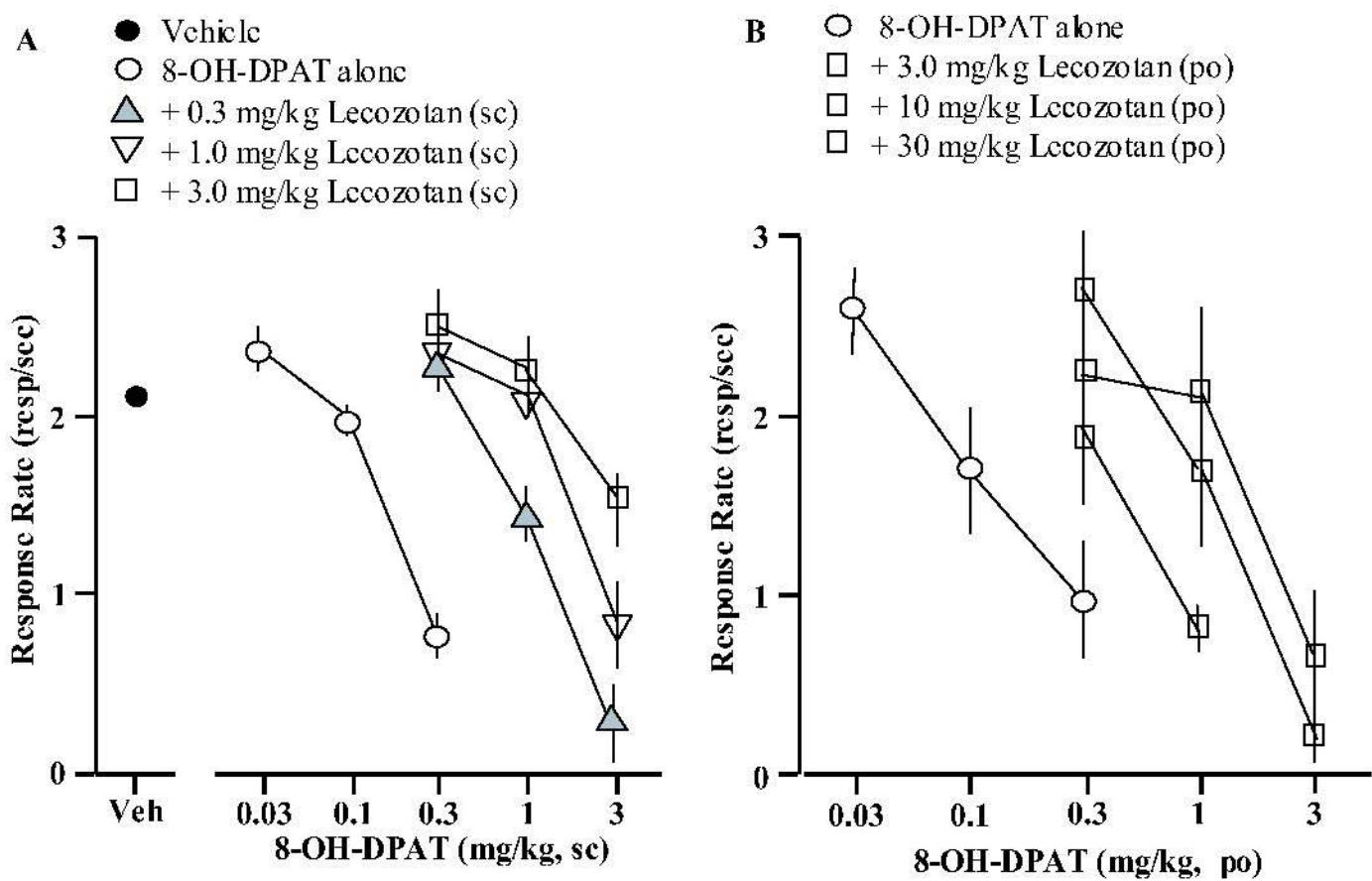


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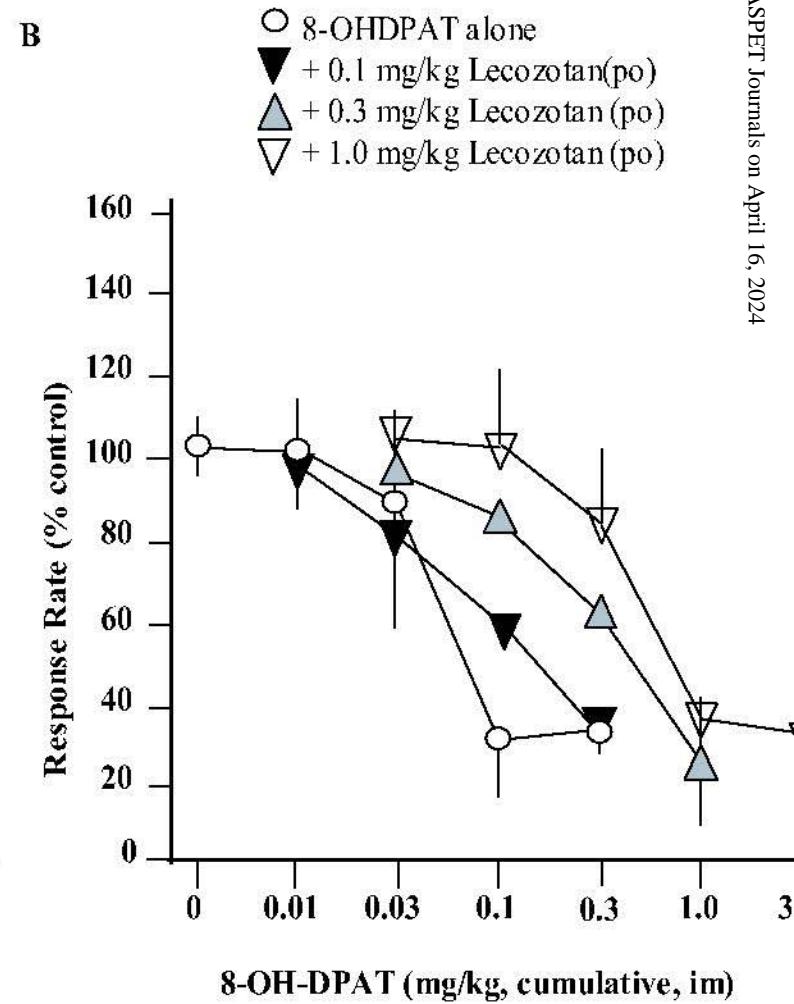
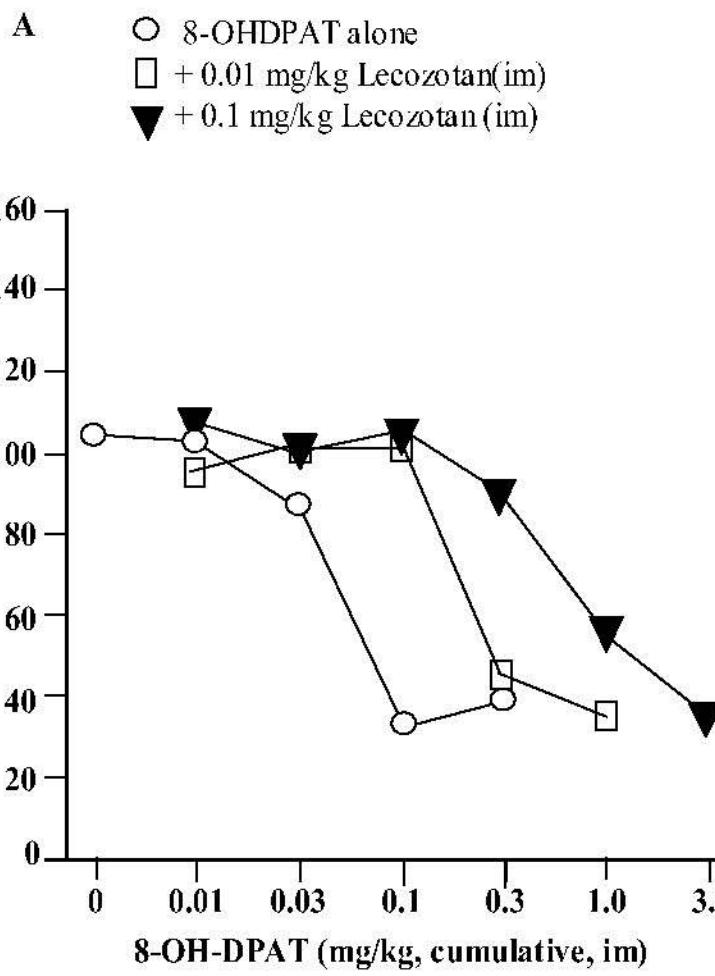


Figure 6.

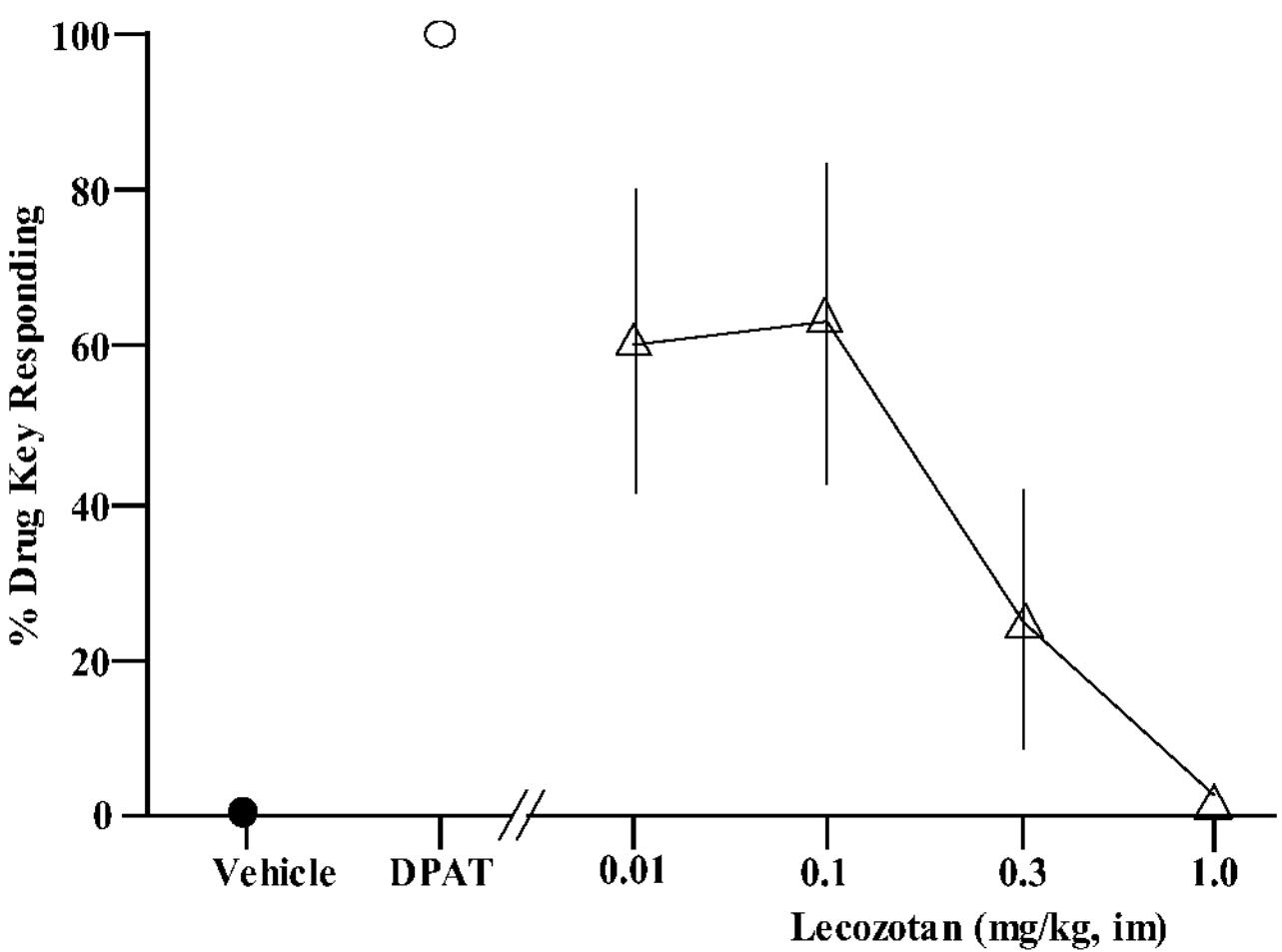


Figure 7

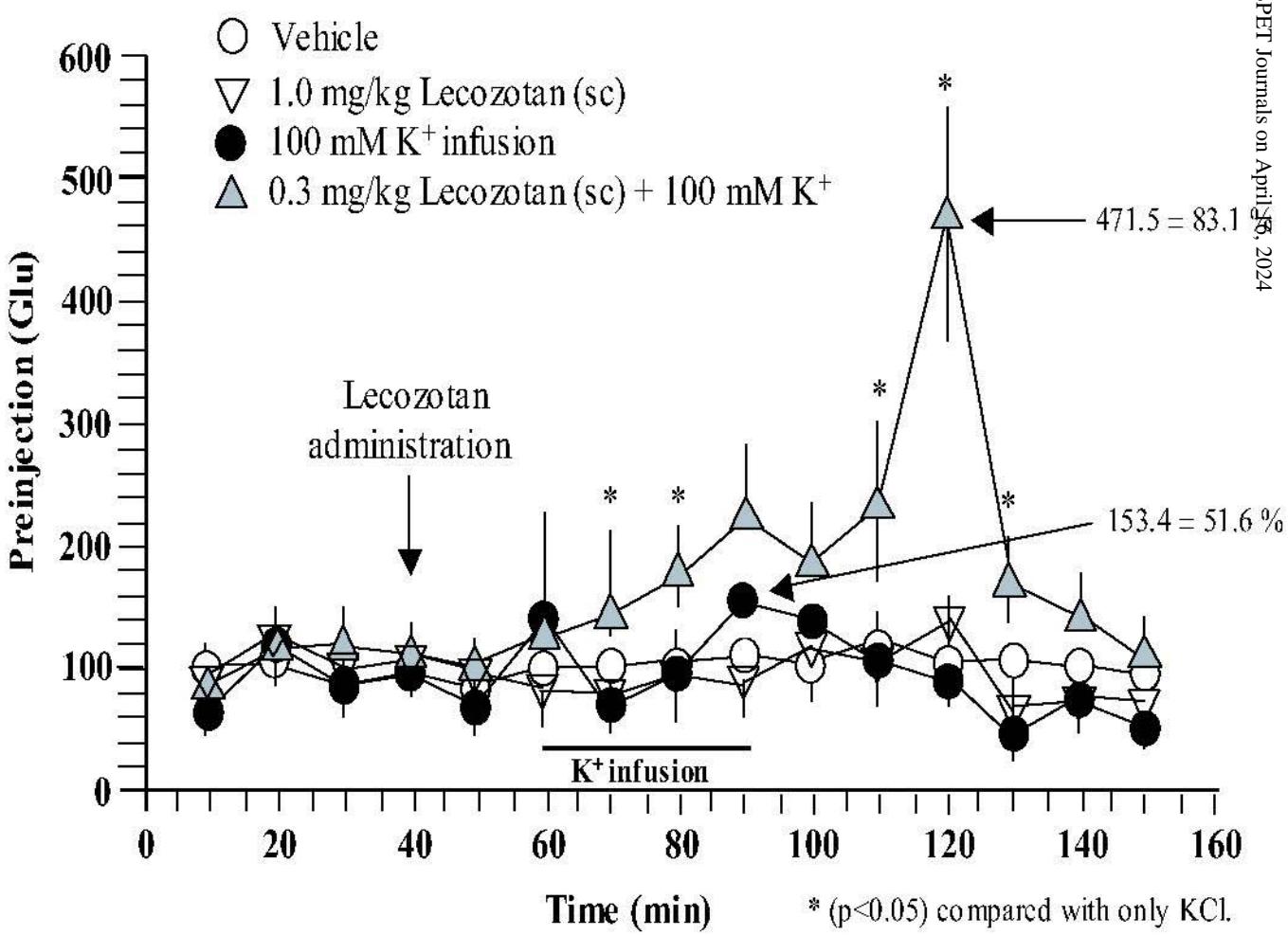


Figure 8

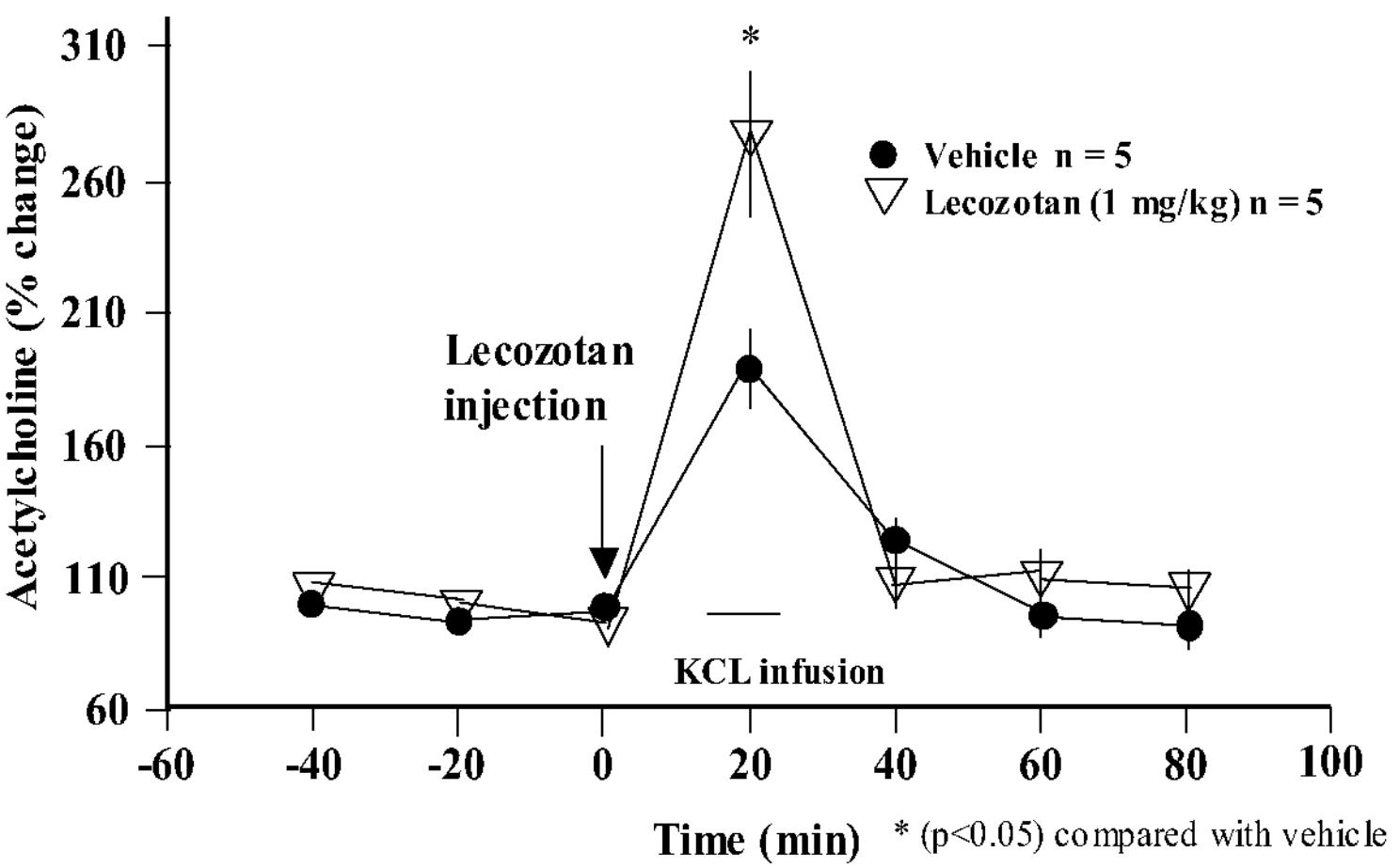


Figure 9.A

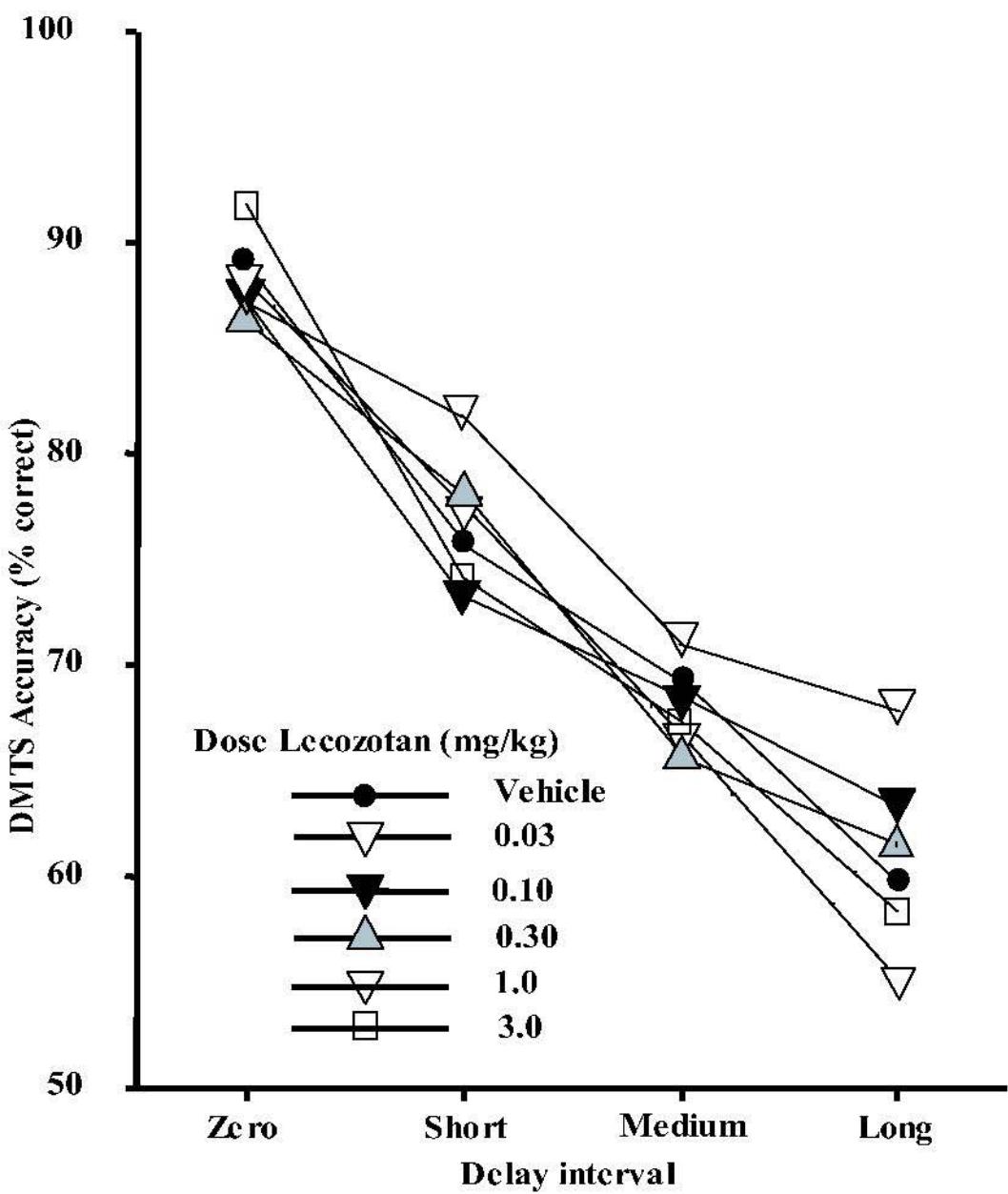
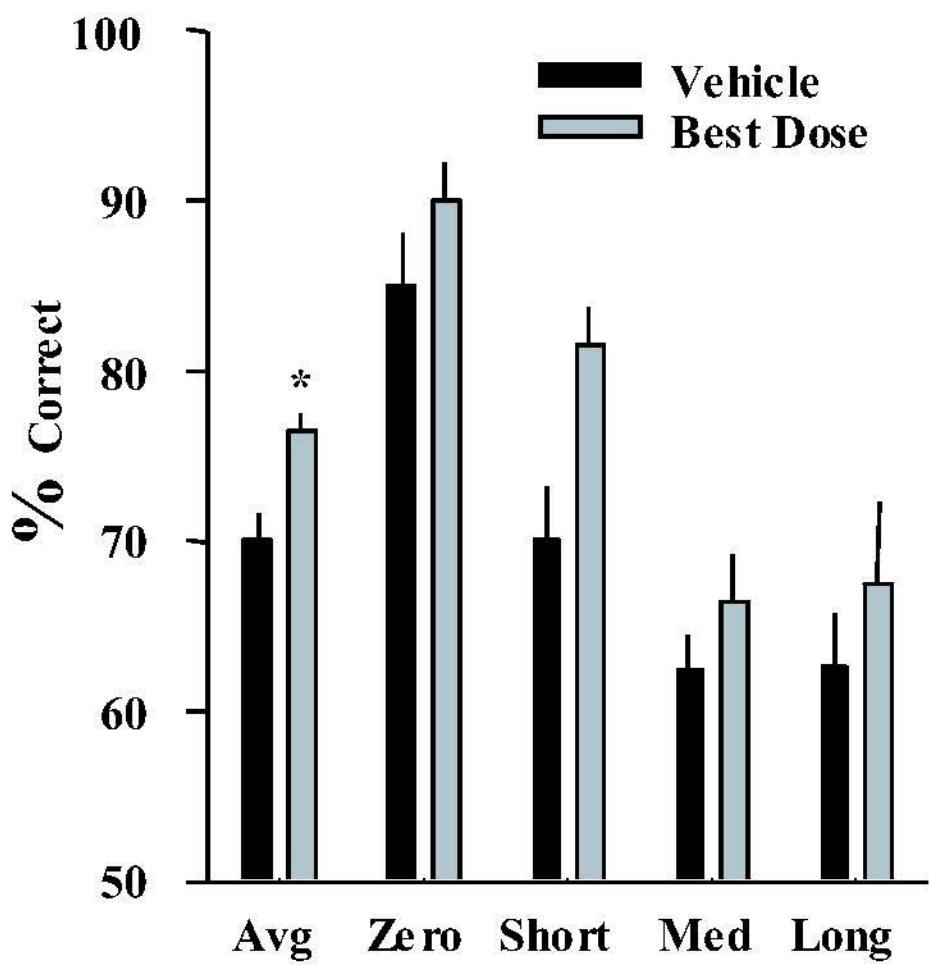


Figure 9.B



* (p<0.01) between vehicle and best dose.

Figure 10.

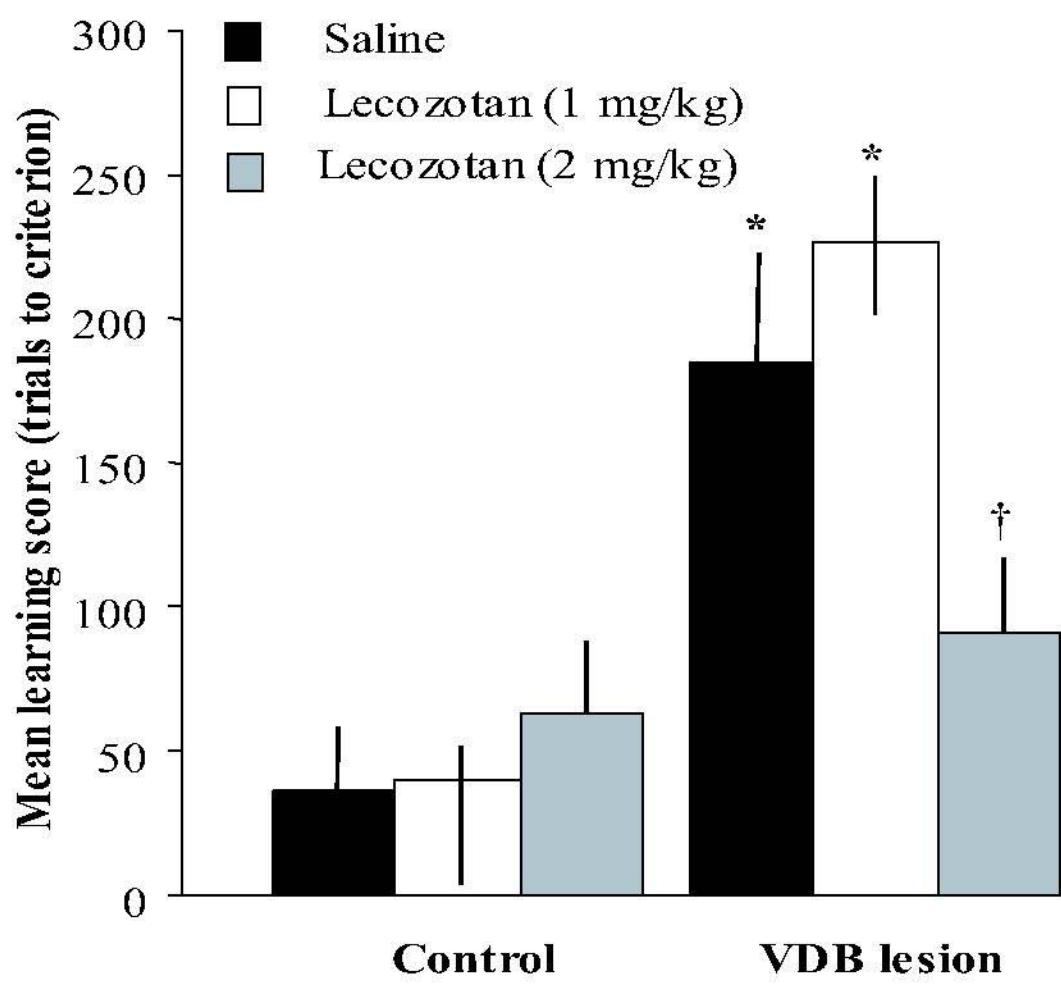


Figure 11.

