5-Hydroxytryptamine 1A Receptor Blockade Facilitates Aversive Learning in Mice: Interactions with Cholinergic and Glutamatergic Mechanisms

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ABSTRACT

The effects of 5-hydroxytryptamine 1A (5-HT1A) receptor ligands on aversive learning were examined in the passive avoidance (PA) task in mice. Anxiety and autonomic functions were investigated using the elevated plus-maze and heart rate measurements. The main findings from this study are as follows. 1) Pretreatment administration of the 5-HT1A receptor agonist 8-OH-DPAT [8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide] facilitated PA retention at low doses (0.01 and 0.03 mg/kg) but impaired PA retention at higher doses (0.1–1.0 mg/kg), consistent with previous findings in the rat. 2) Similar to the acetylcholinesterase inhibitor physostigmine, pretreatment administration of the 5-HT1A receptor antagonists [(R)-3-N,N-dicyclobutylamino-8 fluoro-3,4-dihydro-3H-1-benzopyran-5-carboxamide hydrogen(2H,3H)-trtarate monohydrate] NAD-299 (0.1–2 mg/kg) and [(N-2-4-(2-methoxyphenyl)-1-piperazinyl-ethyl-N-(2-pyrdinyl)cyclohexane carboxamide trihydrochloride] WAY-100635 (0.3–3 mg/kg) enhanced PA retention. 3) The impairment (1 mg/kg) but not the facilitation (0.03 mg/kg) induced by 8-OH-DPAT was fully blocked by NAD-299 (0.3 mg/kg). 4) 5-HT1A receptor ligands given immediate post-training failed to alter PA retention. 5) NAD-299 (0.3–1 mg/kg) blocked the impairment of PA retention caused by a) the nonselective muscarinic receptor antagonist scopolamine and b) the noncompetitive N-methyl-D-aspartate receptor antagonist MK-801 [(5R,10S)-(+-)5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate]. 6) A subthreshold dose of scopolamine completely blocked the facilitatory effect of NAD-299 on PA retention. 7) Anxiety-related behaviors and autonomic function were unchanged by NAD-299. 8) In situ hybridization showed that septal neurons expressing 5-HT1A receptor mRNA were codistributed with markers for cholinergic, GABAergic, and glutamatergic neurons. These results indicate that systemic administration of 5-HT1A receptor antagonists may be useful in the treatment of cognitive deficits such as Alzheimer’s disease.

Serotonin (5-hydroxytryptamine; 5-HT) is a biogenic amine involved in a wide range of physiological functions, including learning and memory (Ögren, 1985; Buhot et al., 2000). The ascending 5-HT neurons originating from the brainstem raphe nuclei innervate virtually all regions of the rat forebrain, allowing for widespread neuromodulatory actions (Dahlström and Fuxe, 1964) mediated by 14 identified receptor subtypes (Hoyer et al., 2002). Among the 5-HT receptor subtypes implicated in cognitive functions, the 5-HT1A receptors are of special interest because they are abundant in brain

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); PA, passive avoidance; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide; NAD-299, [(R)-3-N,N-dicyclobutylamino-8 fluoro-3,4-dihydro-3H-1-benzopyran-5-carboxamide hydrogen(2H,3H)-trtarate monohydrate] WAY-100635, N-2-4-(2-methoxyphenyl)-1-piperazinyl-ethyl-N-(2-pyrdinyl)cyclohexane carboxamide trihydrochloride; MK-801, (5R,10S)-(+-)5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate; ECG, electrocardiogram; ACh, cholinergic; VACHT, vesicular acetylcholine transporter; US, unconditioned stimulus; MSDB, medial septum/diagonal band of Broca; VGLUT2, vesicular glutamate transporter 2; ANOVA, analysis of variance; NAN-190, 1-(2-methoxyphenyl)-4-[4-(2-phthalimidomethyl)butyl]piperazine.
areas associated with cognitive functions, such as the cerebral cortex, hippocampus, and septum (Chalmers and Watson, 1991; Pompeiano et al., 1992). Moreover, the neuronal localization of 5-HT_{1A} receptors allows for modulation of both 5-HT neuronal activity as well as several other neurotransmitter systems. Stimulation of presynaptic 5-HT_{1A} receptors (autoreceptors) localized on cell bodies in the mid-brain raphe nuclei reduces raphe neuronal firing and 5-HT release (Sprouse and Aghajanian, 1987; Chalmers and Watson, 1991), resulting in reduced 5-HT neurotransmission in 5-HT terminal areas. In contrast, activation of 5-HT_{1A} receptors localized on cell bodies and dendrites of neurons postsynaptic to 5-HT nerve terminals hyperpolarizes the innervated cells (for review, see Aghajanian, 1995).

The functional significance of the 5-HT_{1A} receptor in cognition is partly based on the use of 5-HT_{1A} receptor agonists. Pretraining administration of partial agonists at the 5-HT_{1A} receptors, such as buspirone and tandospirone, disrupted acquisition of behavior in various models (Winsauer et al., 1999). Because the effects of partial 5-HT_{1A} agonists on learning seem to depend on their relative efficacy at presynaptic versus postsynaptic 5-HT_{1A} receptors (Winsauer et al., 1999), the use of full 5-HT_{1A} receptor agonist, such as 8-OH-DPAT (Foreman et al., 1993), is critical for exploring the physiological role of 5-HT_{1A} receptors. Pretraining administration of 8-OH-DPAT (Sanger and Joly, 1989; Rowan et al., 1990; Carli et al., 1993; Misane and Ögren, 2000; Lütten et al., 2005a) impaired learning and memory performance in a dose-dependent manner in the passive avoidance (PA) task in the rat. 8-OH-DPAT also retarded spatial learning in the water maze (Carli et al., 1992; Kant et al., 1998) and radial maze tasks (Winter and Petti, 1987). The impairments caused by 8-OH-DPAT in the rat seemed to be related to a stimulation of postsynaptic 5-HT_{1A} receptors (Carli et al., 1995; Misane et al., 1998; Misane and Ögren, 2003). Thus, 5-HT_{1A} receptor antagonists fully blocked the 5-HT_{1A} receptor agonist-mediated impairments, thereby supporting the involvement of the 5-HT_{1A} receptor in the action of the agonists (Carli et al., 1995; Misane et al., 1998). In contrast, facilitated learning and memory have been observed after low, presumably presynaptic, doses of 8-OH-DPAT both in the PA task (Lütten et al., 2005a) and in an operant-delayed matching to position task in the rat (Cole et al., 1994). Pretraining administration of 5-HT_{1A} receptor antagonists in rats has reported either facilitation (Sanger and Joly, 1989; Belcheva et al., 1997; Pitsikas et al., 2003), impairment (Cole et al., 1994), or no effects (Carli et al., 1997; Misane and Ögren, 2003) on learning performance in various tasks.

There are limited studies on the role of the 5-HT_{1A} receptor in cognition in mice. Similar to findings in the rat, 8-OH-DPAT, when injected before but not after training, caused an impairment in PA retention (Mendelson et al., 1993). Studies using pretraining administration of 5-HT_{1A} receptor antagonists in mice have reported variable results, including no effects (Mendelson et al., 1993; Stiedl et al., 2000b) or impairments (Galeotti et al., 2000) in the PA and fear-conditioning task. In addition, results obtained after post-training administration of 5-HT_{1A} receptor antagonists are inconsistent because both facilitation (Schneider et al., 2003) and no effects (Manuel-Apolinar and Meneses, 2004) have been reported. These inconsistent findings may be explained by differential effects on presynaptic and postsynaptic 5-HT_{1A} receptors, differences in routes of administration, and lack of receptor specificity of the compounds used as well as different designs of the studies. In addition, difference in species may contribute to the lack of clear answers regarding the physiological role of the 5-HT_{1A} receptor in cognition. The main aim of this study was to examine the role of basal 5-HT_{1A} receptor-mediated transmission by the use of two selective 5-HT_{1A} receptor antagonists, NAD-299 (Johansson et al., 1997) and WAY-100635 (Fletcher et al., 1996). The ability of the 5-HT_{1A} receptor antagonists to influence PA performance was investigated alone or in combination with 8-OH-DPAT to explore the involvement of presynaptic versus postsynaptic 5-HT_{1A} receptors. Immediate post-training administration of the 5-HT_{1A} receptor ligands investigated the possible involvement of 5-HT_{1A} receptors in memory consolidation. Because the 5-HT_{1A} receptor has been implicated in emotional functions, the ability of 5-HT_{1A} receptor blockade to affect anxiety-related behavior and autonomic responses was also studied. In situ hybridization of sections of the mouse septum was used to provide an anatomical basis for interactions between the 5-HT_{1A} receptors and cholinergic/glutamatergic neurons in the septohippocampal projection.

Materials and Methods

Materials

Animals. Adult male NMRI mice (2 months of age, weight 20–30 g; Scanbur, Sollentuna, Sweden) were used in most experiments. Adult male C57BL/6J mice (2 months of age, weight 25–30 g; Charles River, Sulzfeld, Germany) were used in the studies, which examined the potential effects of 5-HT_{1A} receptor blockade on autonomic function (heart rate) and anxiety-like behavior. The interstrain comparison was included for the reason that the C57BL/6J strain is more sensitive to anxiety-provoking events than the NMRI strain. The use of such a strain will thus increase the sensitivity of the heart rate measurements (Stiedl et al., 1999; Griebel et al., 2000). Therefore, it was also logical to compare the effects of 5-HT_{1A} antagonism on PA in the C57BL/6J versus the NMRI strain.

The animals were housed in groups of four to six in standard Macron cages (type 3: 42 × 26 × 20 cm) in a temperature- and humidity-controlled room with a constant 12-h light/dark cycle (lights on at 7:00 AM) and free access to standard lab chow and tap water up to the time of experiments. To avoid postsurgery complications, animals used for heart rate measurements were individually housed postsurgery in standard Macron cages (type 2: 22 × 16 × 13 cm).

The mice were habituated to the animal facilities and handled daily by the same experimenter for a period of five days before the experiments. Experiments were conducted in experimentally naive mice that were used only once to prevent possible carryover effects between tests. Experimental procedures involving animals and their care followed the provisions and recommendations of the Swedish animal protection legislation. The experimental procedures were approved by the local Animal Ethical Committees (Ethical Numbers N155/01, N48/02, N132/01, and 509.42502/02-01.00) and conformed to the European Council Directive (86/609/EEC).

Drugs. The following drugs were used: +/− 8-OH-DPAT (AstraZeneca R&D, Södertälje, Sweden); robalzotan (NAD-299, AstraZeneca R&D); WAY-100635 (Wyeth, Taplow, UK); (+)-scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO); physostigmine hemisulfate (Eserine; Fluka, Buchs, Switzerland); MK-801 (Sigma-Aldrich). All drugs were dissolved in sterile saline (0.9% NaCl) and injected s.c. into the scruff of the neck at a volume of 4 ml/kg. Injections occurred at specific time points prior to training (40, 30,
20, 15, 10, and 5 min) or immediately post-training as noted under Results. Saline control groups were run concurrently.

Methods

Each experiment was carried out by a single operator. The experimenter was not blind to the pharmacological treatments given. Behavioral tests were conducted during the light phase between 10:00 AM and 3:00 PM. For habituation, mice were transferred to the experimental room 30 to 60 min prior to the experiment.

Passive Avoidance. Passive avoidance is an associative learning paradigm, based on Pavlovian fear-conditioning and instrumental conditioning, and it is sensitive to changes in serotoninergic, glutamatergic, and cholinergic (ACh) neurotransmissions (Ögren, 1985; Misane and Ögren, 2000). The PA task was conducted as described earlier for rats (Ögren, 1986; Misane and Ögren, 2000), with some important modifications adapted for mice. In brief, a two-compartment standard shuttle box (adapted for mice; 10 × 16 × 18 cm; Ugo Basile, Comerio-Varese, Italy), with two communicating compartments of equal size and a stainless steel bar floor, was used. The compartments were separated by a 4 × 4-cm sliding door built into the separating wall. One compartment (the conditioning compartment) was painted black to obtain a dark chamber, whereas the other compartment (light compartment) was illuminated by a light bulb (24 V, 5 watts). The light intensity in the light versus the dark compartment was 330 and 3 lux, respectively.

The animals were handled daily for a period of five days prior to the experiments. Handling has been shown to reduce variations between animals in the PA task probably by a reduction in stress (Ögren et al., unpublished results). Fifteen minutes before the first drug injection on day 1, mice were first allowed to explore the light compartment for 60 s and, thereafter, the sliding door was opened. Once the animal crossed to the dark compartment, the sliding door was automatically closed and the animals were allowed to explore the dark compartment for an additional 60 s. After the exploration period, the animals were removed from the PA apparatus and returned to a holding cage.

To be able to study both enhancement and impairment of PA memory in the same experiment, two different shock intensities were used. To enable demonstration of facilitatory effects of drugs on PA retention, a mildly aversive electrical current (0.3 mA) served as the conditioning, and it is sensitive to changes in serotonergic, glutamatergic, and cholinergic (ACh) neurotransmissions (Ögren, 1985; Misane and Ögren, 2000). The PA task was conducted as described earlier for rats (Ögren, 1986; Misane and Ögren, 2000), with some important modifications adapted for mice. In brief, a two-compartment standard shuttle box (adapted for mice; 10 × 16 × 18 cm; Ugo Basile, Comerio-Varese, Italy), with two communicating compartments of equal size and a stainless steel bar floor, was used. The compartments were separated by a 4 × 4-cm sliding door built into the separating wall. One compartment (the conditioning compartment) was painted black to obtain a dark chamber, whereas the other compartment (light compartment) was illuminated by a light bulb (24 V, 5 watts). The light intensity in the light versus the dark compartment was 330 and 3 lux, respectively.

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PA training was conducted in a single session on day 1. The animals were treated with the test compounds as described below. After a defined time interval following injection, the animal was placed in the light compartment with the sliding door closed (i.e., no access to the dark compartment) and allowed to explore for 60 s. After 60 s, the sliding door was automatically opened and the mouse could cross over into the dark compartment. The latency to cross into the dark compartment (training latency) was recorded. Upon entering the dark compartment with all four paws, the sliding door was automatically closed and a weak electrical current was delivered via the grid floor (scrambled current: 2-s duration, 0.2-mA C57BL/6J and 0.3-mA NMRI). Some experiments used a current of 0.4 mA to explicitly study impairment of PA retention (see above). The reactions (e.g., vocalization, jumping) of the mice to the electric current were noted. After exposure to the US, the mouse remained for 30 s in the dark compartment of the PA apparatus before being removed and transferred to its holding cage. The rationale for this procedure is to avoid mice from an aversive association of the US with the handling (removal from the compartment after the US exposure).

After completion of testing, the mice were returned to their home cages. Retention latencies were determined 24 h after training (day 2). Each animal was placed into the light (safe) compartment, with access to the dark compartment within 10 s. The latency to cross into the dark compartment with all four paws was automatically measured (step-through latency; retention latency; cut-off time was 300 s).

Estimated Plus-Maze. The apparatus was elevated 1 m above the floor. It consisted of four arms (30 × 5 cm) and an entrance from the central area (5 × 5 cm) (TSE, Bad Homburg, Germany) made of dark gray polyvinyl chloride. Two opposite arms were enclosed by 10-cm high walls made from the same material, whereas two arms (open arm) remained without walls. The animal was gently released in the center of the plus-maze facing an open arm. The behavior of each mouse was monitored for 5 min by a video-tracking system (TSE). The main parameters recorded were time spent in open arms, closed arms, and central region; the total distance traveled during the experiment (locomotion index); and the number of entries to closed and opened arms (exploration index). Based on these results, the “anxiety index” was calculated as the ratio between the time spent in the closed arms and the total time of experiment. All boxes were thoroughly cleaned before each mouse was tested using 70% ethanol.

Heart Rate Measurement. Electrocardiogram (ECG) transmitters (TA10E8A-F20; Data Sciences, St. Paul, MN) were implanted i.p. with two electrodes placed s.c. as described previously (Stiedl and Spiess, 1997). The mice were allowed to recover from surgery for 14 to 21 days. Telemetry measurements were performed in the home cage 15 min after s.c. injection of NAD-299 (0.3 mg/kg). The s.c. injection was performed during brief (−60 s) isoflurane anesthesia as described previously (Stiedl et al., 2000a). ECG was continuously recorded during an 18-min period. The ECG signal (lead II) was digitized at a rate of 4 kHz and stored for later off-line analysis. Unrecognized beats in the ECG recording were edited, and artifacts were excluded from analysis. Heartbeat intervals (in milliseconds) derived from successive R-waves of the ECG signal were converted into instantaneous heart rate. For later statistical analysis, heart rate and heart rate variability were calculated in 2-min intervals.

Heart rate variability was determined by the root mean square of the sum of successive RR interval differences (Stiedl and Spiess, 1997).

In Situ Hybridization Histochemistry. Mice were decapitated, and the brains were rapidly dissected out and frozen. Coronal sections of the septal complex (bregma 1.00 to 0.60 mm) (Paxinos and Franklin, 2001) at 14-μm thickness were cut using a cryostat (Dittes, Heidelberg, Germany). The sections were thaw-mounted onto pre-treated glass slides (ProbeOn; Fisher Scientific Inc., Pittsburgh, PA) and stored at −20°C until in situ hybridization was performed. Using MacVector software (Kodak Scientific Imaging Systems, New Haven, CT), oligonucleotide probes were selected on optimum ratio of guanosine + cytosine/total nucleotide numbers (50–65%) and minimal homology (not greater than 80%) with other GenBank entered nucleotide sequences. Oligonucleotide probes complementary to nucleotides 97 to 144 of mouse 5-HT1A receptor mRNA (Gen-Bank accession number U39391), 595 to 642 of mouse vesicular acetylcholine transporter (VACHT) mRNA (GenBank accession number NM021712), 349 to 396 of mouse calcium-binding protein parvalbumin mRNA (GenBank accession number NM013645), and 760 to 807 of mouse vesicular glutamate transporter 2 (VGLUT2) mRNA (GenBank accession number AF324864) were synthesized (CyberGene, Stockholm, Sweden). The probes were labeled with [35S]dATP (BioNuclear, Stockholm, Sweden) at the 3′-end using terminal deoxynucleotidyltransferase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and purified using ProbeQuant G50 micro columns (GE Healthcare). Tissue sections were hybridized overnight at 42°C with 0.5 ng of labeled probe per slide in a cocktail as described earlier (Dagerlund et al., 1992), rinsed in 1× SSC buffer for 4 × 15 min at 56°C, and allowed to cool down to room temperature followed by rinsing in distilled water and drying in 60 and 95% ethanol. After
air-drying, the labeled sections were apposed to βmax autoradiography film (GE Healthcare). The films were exposed for 18 days (VChT and parvalbumin), 4 weeks (5-HT1A receptor), or 8 weeks (VGLUT2); developed with LX 24; and processed using the Adobe Photoshop software, version 6.0 (Adobe Systems, San José, CA). For control purposes, an excess (100×) of unlabeled probe was added to the hybridization cocktail.

Statistical Analysis. The results obtained in the PA studies were analyzed using one-way analysis of variance (ANOVA). For each significant F ratio, a post hoc testing was performed using Fisher’s protected least significant different test. The overall effects of heart rate measurements were analyzed using ANOVA for repeated measures. For each significant F ratio, Fisher’s protected least significant different test was used for post hoc analysis. In the elevated plus-maze experiment, the Student-Neumann-Keuls test was used to analyze the statistical significance among multiple groups (Kirk, 1968). A probability level of p < 0.05 was accepted as statistically significant.

Results

Passive Avoidance Experiments

Behavioral Observations. No apparent differences in the response to the electric current were observed among the treatment groups. The latencies to enter the dark compartment during training (training latencies) were in the range of 15 to 23 s in all of the experimental groups. The training latencies were not significantly affected by the different treatments (data not shown). This indicates that the drugs used under the present conditions did not interfere with motor control or sensory processing in a manner that resulted in an altered training latency.

Pretraining Administration of 5-HT1A Receptor Ligands. When tested 24 h after training, the saline-treated NMRI control groups displayed retention latencies in the range of 60 to 100 s, indicating that the mice acquired the task. The 5-HT1A receptor agonist 8-OH-DPAT (0.01 to 1 mg/kg, 15 min prior to training) exerted a biphasic effect on PA memory retention (F(5, 43) = 21.44; p < 0.01). The lowest doses of 8-OH-DPAT (0.01 and 0.03 mg/kg) improved PA retention, indicated by an increase in the retention latencies (p < 0.01 versus saline). In contrast, the higher doses (0.1–1 mg/kg) caused a significant impairment of PA retention (p < 0.05 versus saline at 0.1 and 0.3 mg/kg; p < 0.01 versus saline at 1 mg/kg), indicated by decreased retention latencies (Fig. 1).

The 5-HT1A receptor antagonist NAD-299 administered s.c. 15 min prior to training produced a dose-dependent in-
crease in PA retention in the 0.1- to 2-mg/kg dose range in NMRI mice [Fig. 1C; F(5,72) = 11.28; p < 0.001]. However, the dose-response curve of NAD-299 had an inverted U-shape (i.e., at the 3 mg/kg dose), no significant effect on PA retention was observed. The 5-HT1A receptor antagonist WAY-100635 (0.03–3 mg/kg, 30 min prior to training) also produced a significant increase in PA retention [Fig. 1B; F(5,48) = 3.92; p < 0.01]. Post hoc analysis revealed a significant effect at the 1–3 mg/kg doses in NMRI mice. However, WAY-100635 at 2 and 3 mg/kg did not show the same bell-shape effect as observed with NAD-299.

The C57BL/6J strain was included to compare PA performance with the result obtained in the anxiety related test (see below). A pilot experiment established that the shock sensitivity differed between the C57BL/6J and the NMRI strains. As shown in Fig. 1, C and D, C57BL/6J mice had approximately the same response latency at the retention test at a shock intensity that was 0.1 mA lower than the one used in the experiment with NMRI mice. NAD-299 (0.1–3 mg/kg, 15 min prior to training, 0.2 mA) produced a dose-dependent increase in PA retention in C57BL/6J mice, with a significant facilitation of PA retention at the 1 and 3 mg/kg doses [Fig. 1D; F(3,36) = 2.49; p < 0.05]. However, the shape of the dose-response curves differed between the two strains (compare Fig. 1, C and D).

**Post-Training Administration of 5-HT1A Receptor Ligands and Scopolamine.** This experiment was designed to investigate the putative effects of 5-HT1A receptor agonist and antagonists together with scopolamine on memory consolidation. When given immediately after training, 8-OH-DPAT (0.01 and 0.3 mg/kg) (shock intensity 0.4 mA) caused no statistically significant changes in PA retention [Fig. 2A; F(2,21) = 1.67; p = 0.21, N.S.]. NAD-299 (1 mg/kg), WAY-100635 (1 mg/kg), and scopolamine (0.1 mg/kg) (shock intensity, 0.4 mA) were also administered immediately post-training. However, no significant effects were found on PA retention [Fig. 2B; F(3,36) = 1.44; p = 0.25, N.S.].

**Involvement of Presynaptic and Postsynaptic 5-HT1A Receptors in the Actions of 8-OH-DPAT.** This experiment was aimed at investigating the 5-HT1A receptor-mediated mechanisms behind the biphasic effect of 8-OH-DPAT on PA retention. Based on the results from the previous experiment, a facilitatory (0.03 mg/kg) and an inhibitory (1 mg/kg) dose of 8-OH-DPAT was studied in combination with NAD-299. The facilitation in PA retention induced by the low dose of 8-OH-DPAT (0.03 mg/kg) was unaffected by pretreatment with NAD-299 (Fig. 3A; N.S.). In contrast, treatment with NAD-299 (0.3 mg/kg, 30 min prior to training) completely blocked the impairment of PA retention induced by 8-OH-DPAT (1 mg/kg, 15 min prior to training) [Fig. 3B; F(4,25) = 17.42; p < 0.01].

**Effects of Scopolamine and Physostigmine.** To investigate possible interaction between 5-HT1A receptors and muscarinic receptors in the control of PA memory retention, initial experiments compared the effects of a muscarinic receptor antagonist (scopolamine) and an acetylcholinesterase inhibitor (physostigmine). Training latencies were not affected by scopolamine [F(3,20) < 1, N.S.] or physostigmine [F(5,33) < 1, N.S.; data not shown]. Scopolamine (0.03–0.3 mg/kg; 40 min prior to training) markedly impaired PA retention [F(3,20) = 9.53; p < 0.01] with a significant effect from the 0.1 mg/kg dose (Fig. 4A; p < 0.01 versus saline). In addition, the time-dependent effects of scopolamine on PA retention were examined. Notably, scopolamine (0.1 mg/kg) injected 5, 10, or 20 min prior to training produced a similar degree of impairment in PA retention [Fig. 4B; F(3,26) = 13.93; p < 0.01]. In contrast, scopolamine, physostigmine (0.0125–0.3 mg/kg; 20 min prior to training) produced a bell-shaped increase in PA retention [Fig. 4C; F(5,33) = 8.22; p < 0.01].

**Combined Effect of 5-HT1A and Muscarinic Receptor Antagonists.** Based on the results from the previous experiments, the 0.03- and 0.1-mg/kg doses of scopolamine were selected for the subsequent interaction studies with NAD-299. The effects of NAD-299 (0.1, 0.3, and 1 mg/kg) on the scopolamine-induced impairment of PA retention were studied using different injection schedules, with NAD-299 being injected either 10 min before or 10 min after scopolamine administration. When administered 10 min before scopolamine (0.1 mg/kg; 10 min prior to training), NAD-299 (0.1–1 mg/kg) almost completely blocked the impairment of PA retention [Fig. 5A; F(4,24) = 9.45; p < 0.01]. In contrast, when administered 10 min after scopolamine (0.1 mg/kg; 40 min prior to training), the same doses of NAD-299 (0.1–1 mg/kg) partially blocked the scopolamine-induced impairment of PA retention [Fig. 5B; F(4,34) = 26.9; p < 0.01]. On the other hand, the 0.03-mg/kg dose of scopolamine, which does not
produce any significant effect on PA retention, completely blocked the facilitatory effect of NAD-299 [Fig. 5C; $F(4,25) = 15.44; p < 0.01$].

**Effect of MK-801 Alone or in Combination with NAD-299.** The NMDA receptor antagonist MK-801 (0.03–0.3 mg/kg; 30 min prior to training) impaired PA retention [$F(2,16) = 3.57; p < 0.05$] with a significant effect at the 0.3 mg/kg dose (Fig. 6A; $p < 0.05$ versus saline). NAD-299 (0.3 and 1 mg/kg) injected 10 min before MK-801 (0.3 mg/kg) blocked the MK-801-induced impairment of PA retention [Fig. 6B; $F(3,28) = 8.98; p < 0.01$]. The 0.3 mg/kg dose of MK-801, which is amnesic in the NMRI strain, did not produce any apparent ataxic effect because training latency was unaffected. Moreover, behavioral observation indicated that MK-801 at 0.3 mg/kg slightly increased motility but did not apparently affect the responsivity to the US.

**Effects of NAD-299 on Anxiety-Related Behavior and Autonomic Function.** To investigate the potential effect of NAD-299 on the emotional state of mice, experiments were performed using the elevated plus-maze to measure anxiety-related behavior. NAD-299 (0.3 and 1.0 mg/kg) had no significant effects on any parameter [e.g., number of arm crossings: $F(2,12) < 1$; N.S.; anxiety index: $F(2,12) < 1$; N.S.] as measured in the elevated plus-maze (Fig. 7, A and B). Because emotionality is known to affect autonomic function, the effect of NAD-299 (0.3 mg/kg) on heart rate and its variability was investigated in C57BL/6J mice as an indicator of sympathetic activation under baseline stress-free conditions in the home cage. ANOVA for repeated measures did not indicate any significant differences in heart rate [$F(1,15) < 1$; N.S] or heart rate variability [Fig. 7C; $F(1,15) < 1$; N.S].
In Situ Hybridization Histochemistry. In the septal complex, weak-to-medium hybridization signal was detected in the medial septum/diagonal band of Broca (MSDB) after hybridization with an oligonucleotide probe to the 5-HT₁A receptor mRNA (Fig. 8A). Labeled cells were demonstrated in all parts of the MSDB. Strong VAChT mRNA expression was observed in the MSDB with a stronger signal in the lateral than in the medial aspect of the MSDB (Fig. 8B). Many parvalbumin-labeled cells were detected in the MSDB, mainly distributed in the medial aspect of the MSDB (Fig. 8C). Medium-strong VGLUT2 mRNA expression was observed in the medial and lateral aspects of the MSDB (Fig. 8D). No hybridization signal was observed in the septal complex after the addition of an excess of unlabeled probe (data not shown).

Discussion

Because 5-HT₁ₐ receptors are localized both presynaptically and postsynaptically, the understanding of their relative role in the actions of 8-OH-DPAT is critical for the interpretation of mechanisms underlying learning. The selective 5-HT₁ₐ receptor agonist 8-OH-DPAT produced a biphasic effect on PA retention in mice probably mediated by stimulation of presynaptic and postsynaptic 5-HT₁ₐ receptors, respectively. The marked PA impairment seen at higher doses (0.1–1.0 mg/kg) of 8-OH-DPAT, which was fully blocked by NAD-299, is consistent with previous findings that stimulation of postsynaptic 5-HT₁ₐ receptors impairs...
performance in various learning and memory tasks in rodents (see Introduction). It seems likely that the impairing effects of systemically administered 8-OH-DPAT on PA are mediated mainly but not exclusively by stimulation of hippocampal 5-HT1A receptors. Thus, administration of 8-OH-DPAT into the hippocampus expressing postsynaptic 5-HT1A receptors caused deficits in aversive learning paradigms, such as PA and fear conditioning in the rat and mouse, respectively (Carli et al., 1993; Stiedl et al., 2000a). However, because both the cortex and hippocampus are involved in PA learning and retention (Santucci and Shaw, 2003), the effects of systemic 8-OH-DPAT on PA cannot be exclusively related to an action on hippocampal 5-HT1A receptors.

Low doses of 8-OH-DPAT (0.01 and 0.03 mg/kg) facilitated PA retention, most likely related to stimulation of 5-HT1A autoreceptors, located on the raphe cell level (Warburton et al., 1997). This interpretation is consistent with the observation that systemic administration of low doses of 8-OH-DPAT and intraraphe administration of 8-OH-DPAT improved learning and memory in different tasks (Cole et al., 1994; Warburton et al., 1997) and reversed the learning deficit caused by intrahippocampally administered scopolamine in rats (Carli et al., 1998). The 5-HT1A receptor stimulation results in an inhibition of raphe cell firing and a decrease of 5-HT release in forebrain areas, such as the cortex and the hippocampus. In turn, this results in a decrease in the basal activity at all 5-HT receptors innervated by the 5-HT terminal system, including the 5-HT1A receptor. The decrease in tonic activity at postsynaptic 5-HT1A receptors after 8-OH-DPAT administration will in principle be the same as the decrease in tonic activity caused by a postsynaptic 5-HT1A receptor blockade. It must be emphasized that, unlike 8-OH-
DPAT, the facilitatory action of NAD-299 on PA performance is related to its postsynaptic blocking action, either at cell body levels in the septum and/or nucleus basalis or at hippocampal/cortical neurons. Although NAD-299 certainly binds to the 5-HT_{1A} autoreceptors and blocks its action, the compound also blocks the tonic inhibitory action of 5-HT at postsynaptic 5-HT_{1A} receptors in hippocampal and/or cortical cells. This explains why NAD-299 cannot block the precognitive effect of 8-OH-DPAT. This interpretation is in line with the observation that, when NAD-299 and a low precognitive dose of 8-OH-DPAT are combined, there was no enhancement of PA retention performance compared with the effect caused by each compound separately. These results suggest that there exists an optimal dynamic range in which reduction in tonic 5-HT neurotransmission can improve learning performance.

A problem in the analysis of the role of the 5-HT_{1A} receptor in cognition is that most of the evidence is based on results obtained with 8-OH-DPAT, which has about a 10 times lower affinity for the 5-HT_{7} receptor than it has for the 5-HT_{1A} receptor (Hoyer et al., 1994). Using nonselective 5-HT receptor antagonists, the contribution of the 5-HT_{7} receptors in the impairing actions of 8-OH-DPAT in the rat seems to be negligible. Thus, methiothepin and spiperone, with much higher affinities for the 5-HT_{7} receptor than the 5-HT_{1A} receptor, failed to block the PA impairment induced by the 0.2-mg/kg dose of 8-OH-DPAT in the rat (Misane and Ögren, 2000). Moreover, the impairment of PA by 8-OH-DPAT is fully blocked by pretreatment with the selective 5-HT_{1A} antagonists NAD-299 and WAY-100635 as shown in this and previous studies (Misane and Ögren, 2000; Lütgten et al., 2005a). These findings indicate that it is not likely that 8-OH-DPAT mediates its effect on PA via stimulation of 5-HT_{7} receptors.

The physiological role of 5-HT_{1A} receptors in rodent cognition has remained unclear due to the inconsistent results reported after various 5-HT_{1A} receptor antagonists (see Introduction). However, the present results indicate that systemic administration of selective 5-HT_{1A} receptor antagonists can facilitate PA retention. This suggests that 5-HT_{1A} receptors of importance for cognitive function are tonically activated by endogenous 5-HT, contributing to a suboptimal PA performance. On the other hand, the impaired spatial learning observed in 5-HT_{1A} receptor knockout mice (Sarnyai et al., 2000) is difficult to reconcile with the present findings. This discrepancy could be explained by compensatory changes in 5-HT-related transmission in the 5-HT_{1A} receptor knockout mice (Ramboz et al., 1998).

Although approximately half as potent as a 5-HT_{1A} receptor antagonist (K_{i}: NAD-299, 0.59 nM; WAY-100635, 0.24 nM) (Johansson et al., 1997), NAD-299 was more effective in its facilitatory action than WAY-100635. The facilitatory effect of NAD-299 reached a somewhat greater magnitude than that of the acetylcholinesterase inhibitor phystostigmine. This may be due to the fact that NAD-299 is a more selective antagonist than WAY-100635, with a low affinity for both adrenergic and dopaminergic receptors (Johansson et al., 1997). Interestingly, both NAD-299 and physostigmine displayed a bell-shaped dose-response curve in the NMRI strain, which probably is related to the appearance of peripheral muscarinic side effects (tremor and salivation) and/or central cholinergic overstimulation (Yoshida and Suzuki, 1993). NAD-299 administration did not produce any muscarinic side effects, but it is possible that NAD-299 at higher doses enhances 5-HT transmission and/or stimulates 5-HT_{1A} receptors, resulting in impairment (Misane and Ögren, 2003). In this respect, NAD-299 and the "silent" 5-HT_{1A} receptor antagonist WAY-100635 may differ, because the dose-response curve of the latter did not display an inverted U shape (Fletcher et al., 1996).

The role of 5-HT_{1A} receptors in associative learning tasks has been mainly related to the acquisition or encoding phase of learning and not to the consolidation phase (Ögren, 1985; Misane et al., 1998; Stiedl et al., 2000b). Thus, immediate post-training administration of 5-HT_{1A} receptor agonists failed to significantly alter PA retention or fear conditioning (Misane et al., 1998; Stiedl et al., 2000b). The present study failed to reveal any significant changes of PA retention after immediate post-training administration of the 5-HT_{1A} receptor antagonists and 8-OH-DPAT. In contrast, the 5-HT_{1A} receptor antagonist NAN-190 (1 mg/kg) was reported to facilitate PA retention when administered immediately post-training (Schneider et al., 2003). These contradictory findings indicate that more decisive studies are needed to elucidate whether the 5-HT_{1A} receptor has a significant role in memory consolidation. Besides encoding, there is evidence that 5-HT_{1A} receptors may play a role in retrieval of aversive information, because 8-OH-DPAT injected prior to the retention test impaired PA memory retention in the rat (Misane et al., 1998).

Because the compounds were administered prior to training, the results obtained could be due to changes in noncognitive processes, such as sensorimotor function and changes in the emotional state of the animals. However, neither NAD-299 nor 8-OH-DPAT alone nor their combination influenced training latency or the responsivity to the electric current in a significant manner. Moreover, NAD-299 given at doses that facilitated PA retention did not influence anxiety-related behavior. Moreover, autonomic responses seem not to be altered after 5-HT_{1A} receptor blockade tested in the C57BL/6J strain, which is known to be a highly emotional strain (Stiedl et al., 1999; Griebel et al., 2000). An anxiety-like state is expected to enhance sympathetic activity when compared with stress-free conditions (Berntson et al., 1998). Because the heart rate and its variability were unaffected, the results did not indicate alterations in anxiety states by 5-HT_{1A} receptor blockade. The failure of NAD-299 to change the emotional state is unexpected, because studies with different strains of 5-HT_{1A} receptor knockout mice reported enhanced anxiety as measured in various anxiety tests (Ramboz et al., 1998). Thus, the modulatory effects of the 5-HT_{1A} receptor antagonist on cognitive functions in mice seem not to be directly related to changes in emotional state (i.e., increased anxiety) or autonomic functions.

There is evidence that 5-HT exerts a modulatory effect on cognition through interactions with the cholinergic system (Cassel and Jeltsch, 1995; Steckler and Sahgal, 1995; Millan et al., 2004). The present results support this hypothesis because blockade of 5-HT_{1A} receptors prevented the memory deficit caused by blockade of cholinergic muscarinic receptors by scopolamine. Scopolamine is known to induce memory deficits in humans as well as in rodents (Blokland, 1996). Furthermore, our results are in agreement with the finding that both WAY-100635 and NAD-299 attenuated the PA impairment caused by scopolamine when injected before and/or after scopolamine in rats (Misane and Ögren, 2003). Similar to the results with WAY-100635 and NAD-299 ob-
tained in rats (Misane and Ögren, 2003), NAD-299 was more effective when injected 10 min prior to scopolamine than 10 min afterward. This suggests that the temporal kinetics of 5-HT1A receptor blockade in relationship to muscarinic blockade by scopolamine is important, as noted previously in the rat (Misane and Ögren, 2003).

The ability of 5-HT1A receptor antagonists to block or attenuate the memory impairments by scopolamine or MK-801 probably involves multiple mechanisms. An immunohistochemical study in the rat provided evidence that 5-HT1A receptors are colocalized with choline acetyltransferase, a marker for cholinergic neurons, in the MSDB (Kia et al., 1996; Lütgen et al., 2005b). Because the cellular localization of the 5-HT1A receptor in the mouse is not well known, the localization of 5-HT1A receptor mRNA expressing cell bodies was investigated in the mouse MSDB, the origin of the cholinergic input to the hippocampal formation (Kiss et al., 1990; Bland and Oddie, 1998). The 5-HT1A receptor protein has been reported to have a somatodendritic localization (Kia et al., 1996); therefore, 5-HT1A receptor mRNA can be used to identify neurons possessing 5-HT1A receptors. In the MSDB, cell bodies expressing 5-HT1A receptor mRNA were found to be codistributed with cell bodies expressing VACHT (a marker for cholinergic neurons) (Roghani et al., 1994), parvalbumin (a marker for septal GABAergic neurons projecting to the hippocampus) (Freund, 1989), and VGLUT2 (a marker for glutamatergic neurons) (Lin et al., 2003).

The present anatomical data suggest that 5-HT1A receptor transmission can influence cholinergic, GABAergic, and glutamatergic transmissions in the medial septal area. The MSDB and nucleus basalis magnocellularis neurons provide the main cholinergic innervation of the hippocampus and cerebral cortex, respectively. Stimulation of 5-HT1A receptors is known to reduce the excitability of cholinergic and glutamatergic neurons (see below). This suggests that blockade of tonic 5-HT1A receptor transmission in the basal forebrain might result in a disinhibition of cholinergic septal and/or nucleus basalis magnocellularis neurons, resulting in enhancement of hippocampal/cortical cholinergic transmission. This hypothesis is supported by a recent in vivo microdialysis study showing that NAD-299 and WAY-100635 significantly increased basal ACh release in the hippocampus and cortex of freely moving rats in the dose range that displayed pro-cognitive effects and blocked cognitive deficits induced by scopolamine (Hu et al., 2003; Millan et al., 2004). Moreover, recent studies with the selective antagonist lercanidipine showed an enhancement of evoked release of ACh and glutamate in the hippocampus in the rat at doses that enhanced cognitive functions (Schechter et al., 2005). The importance of enhanced ACh transmission for the action of 5-HT1A receptor blockade is further supported by the observation that a subthreshold dose of scopolamine (0.03 mg/kg) completely antagonized the facilitatory effect of NAD-299 on PA retention.

The PA retention deficit caused by pretraining administration of MK-801 is consistent with a number of studies showing that NMDA receptors are important in the encoding phase of aversive learning (Parada-Turska and Turski, 1990; Stiedel et al., 2000a). Notably, NAD-299 attenuated the actions of MK-801 in the same dose range in which it blocked the impairments by scopolamine. Consistently, WAY-100635 attenuated the cognitive impairment induced by both MK-801 treatment and cholinergic dysfunctions (fornix transection) in the marmoset (Harder and Ridley, 2000).

In addition to enhancement of ACh and glutamate release, another site for the 5-HT1A and muscarinic/glutamatergic interactions might involve cortical/hippocampal pyramidal neurons. Both 5-HT1A and muscarinic receptors are localized on pyramidal hippocampal cells (Azmitia et al., 1996; van der Zee and Luiten, 1999). 5-HT1A receptor stimulation hyperpolarizes pyramidal neurons (Tada et al., 2004), whereas increases in ACh stimulates excitatory transmission (Dutar et al., 1995). Hippocampal CA1 pyramidal neurons are tonically inhibited by endogenous 5-HT through postsynaptic 5-HT1A receptors during conditioned fear learning (Tada et al., 2004). By blocking the hyperpolarization action of endogenous 5-HT mediated by 5-HT1A receptors, 5-HT1A receptor antagonists could compensate for the reduction of cholinergic afferent input and NMDA receptor-mediated excitatory drive on pyramidal cells in the hippocampus/cortex. A similar mechanism may explain that the PA impairment caused by the NMDA receptor antagonist MK-801 is blocked by NAD-299. Thus, 5-HT1A receptor blockade can counteract the tonic inhibition of glutamatergic pyramidal cells after activation of 5-HT1A receptors.

In summary, the present results suggest that 5-HT1A receptor antagonists can enhance cognitive functions by facilitating cholinergic and/or glutamatergic transmissions in the hippocampus/cortex, in line with earlier proposals (Benson et al., 1994). Studies in healthy human subjects using positron emission tomography with [11C]WAY-100635 also support a role for hippocampal 5-HT1A receptors in explicit memory (Yasuno et al., 2003). These findings indicate that 5-HT1A receptor antagonists may be beneficial in the treatment of cognitive loss in disorders characterized by cholinergic and glutamatergic dysfunctions, such as Alzheimer’s disease and age-related memory disorders.

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