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Linopirdine (DuP 996) Improves Performance in Several Tests of Learning and Memory by Modulation of Cholinergic Neurotransmission

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FONTANA, D. J., G. T. INOUYE AND R. M. JOHNSON. *Linopirdine (DuP 996) improves performance in several tests of learning and memory by modulation of cholinergic neurotransmission.* PHARMACOL BIOCHEM BEHAV **49**(4) 1075–1082, 1994.—The actions of linopirdine (DuP 996; 3,3-*bis*[4-pyrindinylmethyl]-1-phenylindolin-2-one) were evaluated in rats and mice in several cognitive behavioral tests, and for its effects on hippocampal acetylcholine (ACh) overflow in rats. Using mice treated with the muscarinic receptor antagonist, scopolamine, we studied the effects of linopiridine on retention of a passive avoidance task. Linopirdine (0.1 and 1 mg/kg) ameliorated the scopolamine-induced deficit, but at doses ranging from 0.01–1 mg/kg, it did not affect passive avoidance retention in normal (untreated) mice. In a scopolamine-induced hyperactivity test, linopirdine (1 mg/kg) decreased the motoric stimulation associated with the cholinergic hypofunction, without affecting locomotor activity on its own. Using rats, we studied the effects of linopirdine on performance in the Morris water maze spatial memory task. Young rats treated with atropine (30 mg/kg), a muscarinic receptor antagonist, took significantly longer to locate the submerged platform across 12 trials. Linopirdine (0.01 and 0.1, but not 1 mg/kg) ameliorated the atropine deficit. In addition, linopirdine (0.1 mg/kg) ameliorated the deficit in cognition-impaired aged rats (23–24 mo), but did not affect unimpaired aged rats. In terms of neurochemical action, linopirdine (1, 10, and 100 μM) produced a concentration-dependent increase in K^+-evoked ACh overflow from superfused rat hippocampal slices. Also, linopirdine (10 μM) similarly increased ACh release in young control rats and cognition-impaired and nonimpaired aged rats. Our results confirm and extend findings from other studies that demonstrate the cognition-enhancing action of linopirdine in rodent models. Furthermore, these results suggest that the ACh-releasing action of linopirdine may be beneficial only when cholinergic and/or cognitive function is compromised.

Spatial navigation/memory Avoidance behavior Morris water maze Aged rats Acetylcholine DuP 996 Linopirdine Rat Neurochemistry

THERE can be little doubt that acetylcholine (ACh) is necessary, although perhaps not sufficient, for cognitive processing. Many studies suggest that cholinergic dysfunction is related to cognitive impairment, and thus support the notion that cholinergic neurotransmission is necessary for cognition. For example, lesions of the major cholinergic pathways (1,9,16–18) and administration of such anticholinergic agents as scopolamine, atropine, and mecamylamine (13,21) disrupt memory. In addition, agents that increase synaptic ACh, such as the cholinesterase inhibitors, physostigmine (17), heptylphysostigmine (7) and tetrahydroaminoacridine (THA) (10) ameliorate memory deficits. Similarly, direct cholinergic agonists such as nicotine, arecoline, and oxotremorine enhance cognition in animals (1,8,15).

In humans, dysfunction of neocortical cholinergic pathways may be paramount to the etiology of such dementias as Alzheimer's disease (AD) and senile dementia of the Alzheimer's type (SDAT) (5,25). Loss of cortical cholinergic integrity is significantly correlated with the severity of dementia (24,25). Moreover, Davis and Mohs (6) and Summers et al. (22) suggested that physostigmine and THA, respectively, may ameliorate the cognitive dysfunction associated with AD. Their research supports the use of cholinergic therapy in AD.

The discovery of linopirdine represents a novel approach

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to cholinergic therapy for cognitive dysfunction. Linopirdine increases endogenous ACh, but it does not inhibit cholinesterase or increase choline, the precursor to ACh. Nickolson et al. (20) showed that linopirdine increased stimulated ACh overflow from rat hippocampal, cortical, and striatal tissue in a concentration-dependent manner. In behavioral tasks of learning and memory, linopirdine enhanced cognition in mice in a passive avoidance paradigm (3). In addition, it ameliorated a CO_2-induced retention deficit (4) and following a hypoxic challenge (10) in rats.

We conducted several studies to further characterize the behavioral and neurochemical action of linopirdine. In addition, we examined the actions of linopirdine in an aged rodent model of cognitive dysfunction.

METHODS

Mouse Behavioral Studies

Animals. Male CD-1 mice (Charles River) weighing 30–40 g were used in the passive avoidance and motor activity studies. The mice were housed in groups of eight in a climate-controlled room on a normal 12-h light–12-h dark cycle. Water and food were freely available. All animals were allowed 2–3 h to acclimate to the testing room before the experiment.

Locomotor Activity

Motor activity was monitored by means of an automated activity system (San Diego Instruments, San Diego, CA). Each cage measured 45 (width) × 24 (length) × 24 (height) in centimeters, and was equipped with three photoemittors and photodetectors. Interruptions of the same photobeam were recorded as micromovements, and the breaking of two adjacent photobeams as ambulation. Linopirdine (0.01–3.0 mg/kg), cocaine (30 mg/kg), or vehicle was administered 30 min before testing, and locomotor activity was monitored for 90 min. The psychomotor stimulant cocaine was used as a positive control for locomotor hyperactivity.

Scopolamine-induced hyperactivity. In this study motor activity was monitored by means of automated Digiscan activity system (Omni-Tech Electronics, Columbus, OH). Each cage measured 30 (width) × 30 (length) × 24 (height) in centimeters, and was equipped with 24 photoemittors and photodetectors. We produced hyperactivity by administering scopolamine (1 mg/kg, subcutaneously [SC]) 15 min before testing. Vehicle or linopirdine (0.1 or 1 mg/kg) was administered 30 min before testing. Horizontal activity was monitored for 20 min.

Statistical methods. The number of micromovements and ambulation as well as horizontal activity counts were analyzed separately using a one-way analysis of variance (ANOVA), followed by a least significant difference posthoc test.

Passive avoidance.

Apparatus. Acquisition and retention trials were carried out in an automated eight-station GEMINI™ avoidance system (San Diego Instruments). Briefly, the apparatus consisted of a two-compartment passive avoidance box. One compartment was made of clear Perspex that measured 21 (width) × 24 (length) × 28 (height) in centimeters, and was illuminated by a 12-W incandescent lightbulb. The other compartment was of identical dimensions but was made of black Perspex. The floor of both compartments was made with 6.35-mm stainless-steel rods spaced 1.25 cm apart. A Coulbourn Instruments Grid Floor Shocker (Allentown, PA) was connected to the grids to provide a scrambled foot shock. The compartments were separated by a guillotine door (Lafayette Instrument Co., Lafayette, IN). The GEMINI™ Avoidance software controlled the opening and closing of the guillotine door and recorded the latencies. These latencies were defined as the time (in seconds) it took an animal to enter all four paws into the dark compartment.

For memory disruption, the mice were treated with scopolamine (1 mg/kg, SC) 15 min before passive avoidance training. Linopirdine or vehicle (0.1 or 1 mg/kg, intraperitoneal [IP]) was administered 30 min before training. No drugs were administered before the retention test.

Training. We began the passive avoidance training session by placing the mice into the clear compartment of the two-compartment passive avoidance chamber. After a 30-s delay, the guillotine door was raised, providing access to the dark compartment. Once the mouse moved completely into the dark compartment, the guillotine door was closed and immediately a 0.1 or 0.7 mA (scopolamine study) inescapable shock was applied to the grid floor for 1 s. The mice were removed from the dark compartment and returned to their home cage. In the present study all of the mice entered the dark compartment within 90 s of being allowed access. A retention test was give 24 h later. It proceeded in a manner identical to training except that no shock was applied to the grid floor. During the retention test, the mice were allowed 300 s access to the dark compartment, and a score of 300 was given to animals that did not enter the dark compartment within this time.

Statistical methods. The retention latency (time to enter the dark compartment) was analyzed using the log rank test, a survival analysis technique. If a mouse failed to enter the dark compartment within the 300 s, the data were considered to be a censored observation. The log rank test is a powerful and sensitive statistical method for analyzing "time-to-event" data, as it accounts for censored observations in the analysis. To determine the drug effect, the overall comparison was performed to determine whether there were differences among the treatment groups. Then, pairwise comparisons were made among the scopolamine-treated groups comparing the linopirdine groups with the vehicle group.

Rat Behavioral Studies

Animals. Young (3–4-mo) male Sprague–Dawley rats (Charles River), weighing 250–300 g at testing, were used for the atropine studies in the Morris water maze (MWM) and the concentration-response ACh release study. Aged (23–24 mo) and young control (4 mo) female Wistar (Harlan, Indianapolis, IN) were used in the age study in the MWM and in a pilot study for ACh release. The aged animals were obtained as nonvirgins at 18 mo of age and were housed for an additional 5–6 mo before testing. The young animals were brought in at 3 months and housed for an additional month. All the animals were housed in pairs in a climate-controlled colony room on a normal 12-h light–12-h dark cycle (lights on 0700–1900 h). Tap water and food were made available continuously. All animals were kept in the testing room overnight before testing and throughout the experiment.

Morris water maze.

Apparatus. The MWM used was adopted from that described originally by Morris (19), and consisted of a circular pool (122 cm in diameter × 46 cm in height) constructed from black polyethylene. The platform consisted of a clear Perspex base and stand on which the square (12 cm × 12 cm) black Perspex platform rested. The water level was 35 cm, 1–2 cm above the

platform, and thus completely concealing it. The pool was divided into four quadrants corresponding to starting points arbitrarily designated north, south, east, and west. The platform was located in the center of the southwest quadrant about 24 cm from the side. Objects of high contrast (e.g., posters, figures, spotlights) were placed about the room to serve as visual extramaze cues for the animals. A ccTV camera was centered and rested (200 cm) above the pool to track the animals' swim path by means of the black pool and white rat contrast. This information was directly fed into an HSV Image Tracking System (model VP 112; San Diego Instruments) attached to a diagonal video monitor. The tracking system was controlled by a PC486 microprocessor using Hidden Platform Learning Task Software (version 2.0; San Diego Instruments). This software recorded the latency to find the hidden platform (escape latency).

Water maze procedure. Trials were started by placing the animals by hand into the water facing the wall of the pool at one of four equally spaced starting locations (north, south, east, or west) around the perimeter of the pool. Testing consisted of a block of six trials (north, south, east, west, north, east) on each of two consecutive days. During each trial the animal was allowed 90 s to find the hidden platform. When successful, the animal was given 30 s on the platform to "study" the spatial cues. If the animal failed to find the platform in this allotted time, it was given a score of 90 and placed on the platform for the 30 s. The next trial was initiated immediately after this period (intertrial interval = 30 s).

Atropine studies. In the atropine studies, atropine (30 mg/kg, IP) or vehicle was administered 15 min before the first trial on days 1 and 2. Linopirdine or vehicle was administered 30 min before testing, followed 15 min later by atropine or vehicle. The following treatment groups were used: a) vehicle + vehicle controls; b) vehicle + atropine treated controls; and c) various doses of linopirdine + atropine test groups. A single vehicle group and atropine group served as a positive and negative control, respectively. Thus, these studies were designed to determine whether the various doses of linopirdine could alleviate the cognitive deficit induced by atropine. In these studies eight animals per group were tested.

Aged animal studies. A total of 76 aged Wistar female rats (23–24 mo) were screened for performance in the MWM and compared with 15 young control rats. Screening for impairment involved the same procedure described earlier, except that no drugs were administered. An animal was denoted as "aged impaired" when it had a mean latency (trials 7–12) > 3 standard deviations (SD) from the mean performance of young animals. Aged "nonimpaired" animals were those animals having a mean latency (trials 7–12) < 1 SD from the young controls. With these operational definitions, impairment in performance was found in about 30% of the aged animals, and 30% were nonimpaired. These subjects were then assigned into either vehicle or linopirdine treatment groups. Care was taken to ensure that the treatment groups had almost identical mean latencies based on the screening data (Table 3). Drug testing took place 3–4 weeks after screening. Linopirdine or vehicle was administered 30 min before the first trial on both test days. Vehicle-treated young control animals were also included in the test for reference. The platform location was changed between screening and drug testing. In all studies the experimenter was kept blinded to the treatments.

Statistical methodology. The overall statistical significance for each experiment was computed on the mean total latency to locate the submerged platform using a one-way ANOVA. Pairwise comparisons were performed using a posthoc LSD test (26).

Rat neurochemical studies.

Superfusion protocol. Rats were decapitated and the brains rapidly removed. Hippocampi were dissected out in a Petri dish containing ice-cold Krebs' Ringer bicarbonate (KRB) gassed with 95%/5% O_2/CO_2 and then chopped into 400-μm slices. KRB was composed of (in mmol/l) NaCl 118.0, KCl 4.75, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25.0, glucose 11.0. Slices were rinsed and incubated in 5.0 ml KRB containing 0.1 μmol/l [^{3}H]-choline (2 μCi/ml, 15 Ci/mmol, Amersham, Arlington Heights, IL) at 37°C for 30 min under a stream of 95%/5% O_2/CO_2. The slices were rinsed three times and packed by removing excess KRB.

Aliquots (100 μl) of packed slices (1.5–2 mg total protein) were added to each of 20 chambers of the robotically controlled Brandel Superfusion (Gaithersburg, MD) apparatus (Model 2000) as described by Johnson et al. (14). Slices were superfused at 0.45 ml/min with KRB containing 10 μmol/l hemicholinium-3, and after a 30 min equilibrium period, 4-min fractions of perfusate were collected. At 12 (S_1) and 48 min (S_2) after the equilibrium period, slices were depolarized for 4 min by changing the perfusion medium to a KRB solution containing 20 mmol/l KCl (osmolarity maintained by reducing the NaCl concentration). Linopirdine was added 12 min before S_2. The superfusion fractions were collected for a total of 72 min. On completion of the experiment, slices were removed from the superfusion apparatus and solubilized overnight in 1 mol/l NaOH and neutralized with 1 mol/l HCl. Ready-Gel scintillation cocktail (Beckman, Fullerton, CA) was added to the fractions and the tritium content assayed by liquid scintillation spectroscopy with 34% counting efficiency.

Expression of results. The disintegration per minute (DPM), per 4-min collection period, was converted to fractional release by dividing the DPM of each 4-min fraction by the total amount of radioactivity present (sum of the fractions and tissue counts). S_1 and S_2 were calculated as the difference between potassium-evoked and basal release. The ratio of S_2/S_1 was then calculated. Results of S_2/S_1 ratios represent the mean ± SEM of four experiments done in triplicate, unless otherwise indicated. A Kruskal–Wallis test (nonparametric analysis) was used to test for an overall treatment effect. Pairwise comparisons for linopirdine-treated groups to controls were then performed.

Drugs. Atropine sulfate and scopolamine hydrobromide were purchased from Sigma Chemical Co. (St. Louis, MO). Linopirdine (DuP 996; 3,3-*bis*(4-pyrindinylmethyl)-1-phenylindolin-2-one) was synthesized by the Institute of Organic Chemistry at Syntex Discovery Research. All drugs were made fresh daily and dissolved in deionized distilled water. Injections were made at 1 ml/kg body weight. Doses refer to the free base, except for atropine and scopolamine.

RESULTS

Effects of Linopirdine on Locomotor Activity (Table 1) and Scopolamine-Induced Hyperactivity (Table 2)

Table 1 shows that linopirdine did not affect motor behavior in mice. In contrast, the psychomotor stimulant cocaine significantly increased ambulation and micromovements (both $p < 0.01$). Table 2 illustrates the antagonism of scopolamine-induced hyperactivity by linopirdine. Scopolamine (1 mg/kg) increased horizontal activity significantly ($p < 0.01$) over the

TABLE 1

EFFECTS OF LINOPIRDINE ON LOCOMOTOR ACTIVITY IN MICE

Treatment	Micromovements	Ambulations
Vehicle	114.3 ± 22.1	69.1 ± 10.7
Cocaine (30 mg/kg)	1101.7 ± 124.9*	844.6 ± 199.3*
Linopirdine (mg/kg)		
0.01	118.3 ± 22.6	64.3 ± 16.5
0.03	88.0 ± 26.6	42.2 ± 10.2
0.1	86.3 ± 20.8	46.5 ± 14.7
0.3	116.8 ± 29.8	71.0 ± 16.5
1.0	85.2 ± 20	53.3 ± 13.6
3.0	141.6 ± 36.6	81.1 ± 20.9

Data represent the mean ± SEM of activity counts over 90 min. All treatments were administered intraperitoneally 30 min before testing (n = 12/group). *$p < 0.001$ vs. vehicle-treated control subjects (posthoc LSD following ANOVA).

20-min test period. Linopirdine at 1 mg/kg significantly attenuated the actions of scopolamine, but was without effect at 0.1 mg/kg.

Effects of Linopirdine on Passive Avoidance Performance (Figs. 1 and 2)

As illustrated in Figure 1, the vehicle-treated mice receiving a 0.1-mA shock during training had a significantly longer retention latency compared with the vehicle-treated mice that did not receive shock (t = 3.7, $p < 0.001$). When we compared the various treatments groups that received the 0.1-mA shock the overall ANOVA was not significant [$F(5, 54) < 1$, NS]. Moreover, no significant differences were found between the vehicle-treated group and the linopirdine groups. Thus, linopirdine had no effect on passive avoidance retention latency when given on its own as indicated.

The scopolamine-treated animals had a significantly shorter latency than the vehicle controls ($p < 0.01$) during the retention test (Fig. 2). Overall, there was a significant difference among the scopolamine-treated groups (p = 0.0006). Pairwise comparisons revealed that both linopirdine-treated groups (0.1 and 1 mg/kg) had significantly longer latencies than scopolamine controls ($p < 0.05$ and $p < 0.01$, respectively). Thus, linopirdine reduced the passive avoidance retention deficit produced by the muscarinic receptor antagonist, scopolamine.

TABLE 2

EFFECTS OF LINOPIRDINE ON SCOPOLAMINE-INDUCED HYPERACTIVITY IN MICE

Treatment	Horizontal Activity Counts
Vehicle + vehicle	2249.6 ± 404.5*
Vehicle + scopolamine	5133.0 ± 600.3
Linopirdine (0.1 mg/kg) + scopolamine	4064.7 ± 546.3
Linopirdine (1.0 mg/kg) + scopolamine	3482.7 ± 402.7*

Data represent the mean ± SEM for activity counts over 20 min. All pretreatments were administered intraperitoneally 30 min before testing (n = 12/group). Scopolamine (1 mg/kg, subcutaneously) or saline were administered 15 min before testing. *$p < 0.01$ vs. vehicle + scopolamine-treated subjects (posthoc LSD following ANOVA).

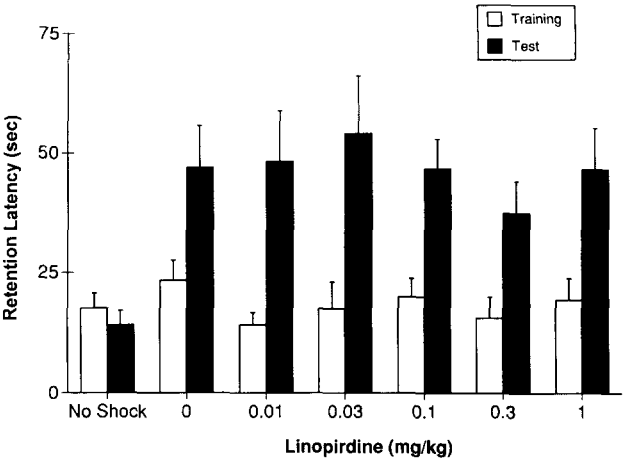

FIG. 1. Effects of linopirdine on passive avoidance behavior in mice. Linopirdine or vehicle was administered intraperitoneally 30 min before training. Passive avoidance retention was tested 24 h later. Data represent the mean ± SEM latency to step-through during training and testing (n = 10/treatment).

Effects of Linopirdine on MWM Performance in an Atropine-Induced Deficit Model (Fig. 3)

Figure 3 illustrates the effects of linopirdine on MWM performance in young rats treated with atropine. Overall, there was a significant effect of treatment on day 1 [$F(4, 45)$ = 14.3, $p < 0.01$] and day 2 [$F(4, 45)$ = 17.0, $p < 0.01$]. As shown, atropine produced a significant increase in latency to find the submerged platform relative to vehicle-treated animals on both test days (both $p < 0.01$). When we compared

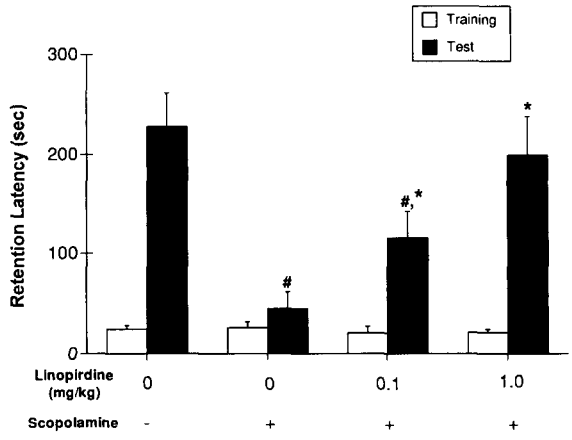

FIG. 2. Effects of linopirdine on scopolamine-induced deficit in passive avoidance behavior in mice. Linopirdine or vehicle was administered intraperitoneally 30 min before training. Scopolamine (1 mg/kg) was administered 15 min before training. Passive avoidance retention was tested 24 h later. Data represent the mean ± SEM latency to step-through during training and testing (n = 10/group). #$p < 0.05$ vs. vehicle + vehicle group; *$p < 0.05$ vs. vehicle + scopolamine group (log rank test).

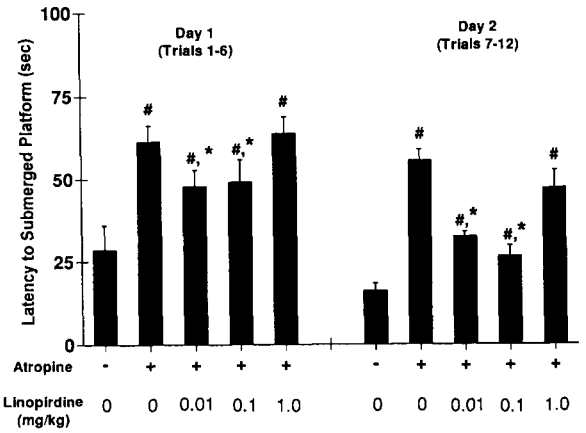

FIG. 3. Effects of linopirdine in an atropine-induced deficit model in the Morris Water Maze in rats. Linopirdine or vehicle was administered 30 min and atropine or vehicle 15 min before the first trial on each day. Eight animals were included in each group. Data represent the mean latency values (± SEM) across trials on day 1 (trials 1-6) and day 2 (trials 7-12) for each treatment group. #$p < 0.05$ vs. vehicle + vehicle group; *$p < 0.05$ vs. vehicle + atropine group (posthoc LSD following ANOVA).

the latencies of the atropine-treated groups, linopirdine at doses of 0.01 and 0.1 mg/kg significantly decreased latency relative to the atropine control group on both test days (all $p < 0.05$). Such an effect was not found at the 1.0-mg/kg dose on either test day. All of the linopirdine-treated-atropine-treated groups had significantly longer latencies than vehicle-treated controls ($p < 0.01$), indicating that the amelioration of the atropine-deficit was only partial.

Effects of Linopirdine on an Age-Related Deficit (Table 3)

We divided the aged rats into groups matched in terms of mean latency of trials 7-12 of screening. Table 3 depicts the latencies of the treatment groups before testing. By design there is no significant difference in the screening latencies between the age-impaired treatment groups (vehicle and linopirdine) and no significant differences between the age-nonimpaired groups (vehicle and linopirdine) and the young rats treatment groups. The young control animals had a significantly shorter latency than the age-impaired ($p < 0.001$), but not the age-nonimpaired rats. Likewise, the aged-nonimpaired rats had a significantly shorter latency than the age-impaired rats ($p < 0.001$).

Table 3 shows the effect of linopirdine on swim latencies in aged animals. In the overall ANOVA there was a significant group-treatment effect on latencies for day 1 [$F(5, 54) = 12.8$, $p < 0.01$] and day 2 [$F(5, 54) = 16.6$, $p < 0.01$]. As expected, pairwise comparisons revealed that the age-impaired vehicle group had a significantly longer latency than the age-nonimpaired and young vehicle groups on both test days (all $p < 0.01$). The age-impaired linopirdine-treated group was not different from the impaired vehicle-treated group on day 1, whereas it had significantly shorter latency on day 2 ($p < 0.01$). Linopirdine had no significant effects on latency in the age-nonimpaired or young animals on either test day. Thus, linopirdine improved performance in the age-impaired animals exclusively.

Effects of Linopirdine on ACh Release From Hippocampal Tissue (Fig. 4)

Linopirdine showed a concentration-dependent increase in potassium-evoked tritium overflow from young rats (Fig. 4). The increase in tritium overflow represents increased ACh release, as demonstrated in similar studies with linopirdine that showed concomitant release of both tritium and endogenous ACh as measured by high-performance liquid chromatography-electrochemical detection (ECD) (14). Maximal response was seen at 100 μmol/l (750% of control S_2/S_1), and 300 μmol/l increased tritium overflow by 160% of control (data not shown).

Effects of Linopirdine on ACh Overflow From Hippocampal Slices of Aged Rats (Table 4)

Table 4 compares the effects of linopirdine on endogenous and [^{3}H]-ACh release in hippocampal slices from young, age-impaired and -nonimpaired rats. Linopirdine, regardless of whether endogenous ACh or [^{3}H]-ACh release was measured, displayed similar augmenting effects in slices from young and age-impaired and -nonimpaired rats. No difference was observed in the basal release of ACh from all groups tested. Thus, slices isolated from either young or aged rats did not discriminate the actions of linopirdine based on K^+-evoked release of ACh.

TABLE 3

EFFECTS OF LINOPIRDINE ON SWIM LATENCIES IN YOUNG AND AGED RATS

Group (Treatment)	Pretest Screening (Trials 7-12)	Drug Test (Trials 1-6) Day 1	Drug Test (Trials 7-12) Day 2
Young (vehicle)	14.6 ± 3.5*	13.7 ± 4.8	10.3 ± 1.2
Young (linopirdine, 0.1 mg/kg)	16.7 ± 4.6	15.7 ± 3.2	13.6 ± 2.7
Aged-nonimpaired (vehicle)	21.3 ± 2.3	23.9 ± 4.7	20.0 ± 3.1
Aged-nonimpaired (linopirdine, 0.1 mg/kg)	22.9 ± 3.4	24.3 ± 7.9	16.9 ± 1.8
Aged-impaired (vehicle)	55.4 ± 4.3†,‡	48.8 ± 9.8	50.8 ± 5.9
Aged-impaired (linopirdine, 0.1 mg/kg)	56.3 ± 3.1†,‡	42.3 ± 7.4	37.9 ± 5.8§

*Values represent mean ± SEM across trials.
†$p < 0.01$ vs. young controls.
‡$p < 0.01$ vs. age-nonimpaired subjects.
§$p < 0.01$ vs. age-impaired vehicle-treated subjects.

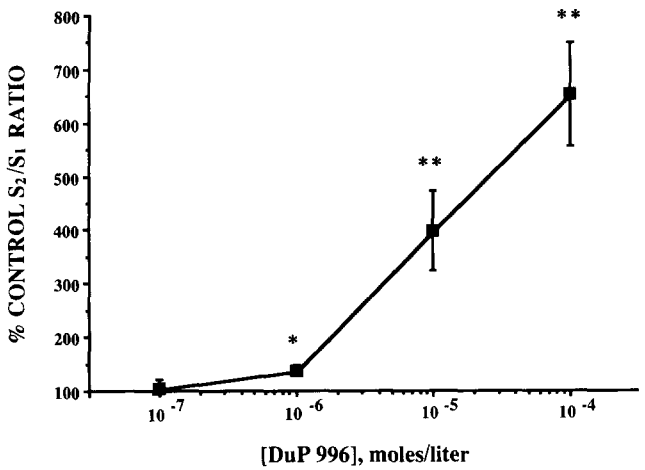

FIG. 4. Enhancement of K^+-evoked [^{3}H]-ACh release by linopirdine in rat hippocampal slices. Rat hippocampal slices were preincubated with [^{3}H]-choline and perfused with Kreb's Ringer bicarbonate (KRB). After a 30-min washout period, 4-min fractions were collected. At t = 14 min (S_1) or t = 48 min (S_2), the slices were depolarized by the addition of 20 mM KCl to the KRB for 4 min. Linopirdine was added 12 min before the second depolarization (S_2). *$p < 0.05$. n = 4, done in triplicate.

DISCUSSION

The results from our behavioral and neurochemical studies support the notion that linopirdine enhances cognition in animal models of cognitive dysfunction by means of enhancing cholinergic neurotransmission. The data show that linopirdine enhanced K^+-evoked [^{3}H]-ACh release from hippocampal slices and improved performance in behavioral tests of learning and memory when cholinergic function was compromised. Linopirdine ameliorated the retention deficit produced by scopolamine in the passive avoidance paradigm and it partially ameliorated the deficit produced by atropine in the MWM task. In addition, it improved the performance of cognition-impaired aged rats in the water maze. Thus, linopirdine displayed procognitive actions in deficit models whether pharmacologic or natural (i.e., aging).

In contrast, linopirdine did not enhance cognition in the behavioral tests of learning and memory in the absence of age-related impairment or cholinergic hypofunction. For example, in the absence of scopolamine-deficit, linopirdine did not improve retention performance in the passive avoidance paradigm. Linopirdine also failed to improve performance in the MWM in young control and age-nonimpaired rats. This finding is in agreement with a recent report from Buxton et al. (3). In the delayed-response tasks, delayed match to position and delayed nonmatch to position, linopirdine had no effect on accuracy in delayed trials on its own, but it did improve accuracy significantly in scopolamine-treated rats. Linopirdine antagonism of the actions of scopolamine was limited to the cognitive aspects of these tasks as it had no effect on the decrease in the number of trials completed resulting from scopolamine treatment. These data demonstrate that linopirdine improves cognition in the presence of cholinergic receptor blockade, but does not improve normal cognitive processing.

Several other investigators have reported procognitive actions of linopirdine in deficit models. Linopirdine (0.01–0.1 mg/kg) administered 1 min after training produced increases in passive avoidance retention after hypoxia (10) and protected against CO_2-induced retention deficit in passive avoidance. Moreover, Brioni et al. (2) reported that linopirdine facilitated acquisition of spatial discrimination in a two-platform water maze using septal-lesioned rats. In this model of cholinergic hypofunction, linopirdine reduced the number of errors in septal-lesioned rats to a level comparable to sham-operated rats (2). Other evidence is in agreement with the notion that linopirdine displayed activity mostly under situations of perturbation. Specifically, in hypoxic rats linopirdine (0.01, 0.1, and 1 mg/kg) significantly decreased glucose metabolism in the hippocampus, limbic cortex, medial septum, striatum, cortex, and other regions typically associated with cognition, but did not significantly alter cerebral glucose metabolism in these regions in naive control rats (11). Recently, linopirdine (10 mg/kg) was reported to increase c-*fos* expression in cortical areas of aged rats without affecting the expression in the same regions of young rats (12). A similar pattern

TABLE 4

COMPARISON OF LINOPIRDINE EFFECTS ON ENDOGENOUS AND [^{3}H]-ACh RELEASE FROM YOUNG AND AGED RATS

Group	Treatment	Endogenous ACh Release (S_2/S_1 ± SEM)	[^{3}H]-ACh Release (S_2/S_1 ± SEM)
Young	Control	0.9 ± 0.03	0.8 ± 0.01
	Linopirdine 10 μM	2.4 ± 0.07	1.9 ± 0.15
Age-nonimpaired	Control	0.9 ± 0.03	0.7 ± 0.09
	Linopirdine 10 μM	2.6 ± 0.03	2.4 ± 0.01
Age-impaired	Control	0.9 ± 0.01	0.8 ± 0.01
	Linopirdine 10 μM	2.4 ± 0.05	2.0 ± 0.20

Pooled hippocampi from four animals in two separate experiments were aliquoted into separate chambers with each condition done in triplicate. Endogenous ACh release was determined by high-performance liquid chromatography-ECD as described in Johnson et al. (14). Basal release of ACh in all groups ranged from 3.2–3.5 pmol/mg protein/4 min. Screening latencies from these animals were young: 15.3 ± 3.4, age-nonimpaired: 19.1 ± 2.9, age-impaired: 50.8 ± 5.3. All rats were naive to linopirdine treatment.

was demonstrated after administration of cholinesterase inhibitors. Taken together, these data suggest that the pharmacologic and procognitive actions of linopirdine are fully expressed in deficit models.

Other behavioral studies suggest that linopirdine can facilitate performance in cognition tasks in the absence of an induced deficit. In active avoidance, linopirdine enhanced acquisition in mice and rats without affecting foot shock sensitivity (4). In addition, linopirdine enhanced lever pressing acquisition for food in rats (4) and increased passive avoidance retention latency (3). These studies suggest that linopirdine can enhance cognition in the absence of impairment.

Linopirdine decreased the hyperactivity produced by scopolamine while having no effects on basal locomotor activity. Thus, a general behavioral depression cannot account for this effect. It is likely that linopirdine increased synaptic acetylcholine levels, resulting in a competitive displacement of the muscarinic receptor antagonist, thereby surmounting the cholinergic hypofunction. These data suggest in vivo facilitation of cholinergic neurotransmission. The data from our neurochemistry studies also support this, as linopirdine produced a concentration-dependent increase in K^+-evoked [^{3}H]-ACh release from hippocampal slices. Linopirdine enhanced K^+-evoked and endogenous release of ACh from hippocampal slices that was independent of age and cognitive impairment. Although it is well documented that linopirdine enhances sustained depolarization-activated release of ACh in vitro and increases extracellular levels of ACh in vivo [for review, see Zaczek and Saydoff (27)], the exact mechanism through which the drug mediates these effects remains unknown. In hippocampal synaptosomes, linopirdine facilitated stimulus-dependent release of ACh through a mechanism influenced by the neuronal membrane potential (23). We have been unable to observe an enhancement of ACh release in rat hippocampus evoked by electrical stimulation (unpublished observation; also 28). The differential effects of linopirdine on ACh release when evoked by elevated potassium concentrations or direct pulsatory current application should provide a clue as to the mechanism of action of the drug.

Thus, it appears that linopirdine can release sufficient ACh to overcome the blockade of postsynaptic muscarinic receptors that cause a cognitive impairment in young rats; and it is possible that no apparent differences exist in the presynaptic membrane potential from either young or aged rats, which may explain the similarity in basal and linopirdine-enhancement of K^+-evoked release from these animals. Consequently, the reason why the cognitive actions are limited to the age-impaired rodents remains paradoxical. Our behavioral data, however, imply that increases in ACh are effective in improving performance in learning and memory tasks only when a deficit exists.

The results from the present report confirm and extend the procognitive action of linopirdine to an aged rodent model. We also suggest that linopirdine may ameliorate cognitive dysfunction, such as memory loss, only in cases where cholinergic or cognitive function is compromised. Thus, linopirdine may be useful in treating the memory loss associated with Alzheimer's disease and age-related disorders.

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REFERENCES

1. Bartus, R. T.; Dean, R. L.; Beer, B.; Lippa, A. S. The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–416; 1982.
2. Brioni, J. D.; Curzon, P.; Buckley, M. J.; Arneric, S. P.; Decker, M. W. Linopirdine (DuP 996) facilitates the retention of avoidance training and improves performance of septal-lesioned rats in the water maze. Pharmacol. Biochem. Behav. 44:37–43, 1993.
3. Buxton, A.; Callan, O. A.; Blatt, E. J.; Wong, E. H. F.; Fontana, D. J. Cholinergic agents and delay-dependent performance in the rat. Pharmacol. Biochem. Behav. 49:1067–1073; 1994.
4. Cook, L.; Nickolson, V. J.; Stainfels, G. F.; Rohrbach, K. W.; DeNoble, V. J. Cognition enhancement by the acetylcholine releaser DuP 996. Drug Devel. Res. 19:301–314; 1990.
5. Coyle, J.; Price, D.; DeLong, M. Alzheimer's diseases: A disorder of cortical cholinergic innervation. Science 219:1184–1190; 1983.
6. Davis, K.; Mohs, R. C. Enhancement of memory processes in Alzheimer's disease with multiple dose intravenous physostigmine. Am. J. Psychiatry 41:281–289, 1982.
7. Dawson, G. R.; Bentley, G.; Draper, F.; Rycroft, W.; Iversen, S. D.; Pagella, P. G. The behavioral effects of heptyl physostigmine, a new cholinesterase inhibitor, in test of long-term and working memory in rodents. Pharmacol. Biochem. Behav. 39: 865–871; 1991.
8. Decker, M. W.; Majchrzak, M. J.; Anderson, D. J. Effects of nicotine on spatial memory deficits in rats with septal lesions. Brain Res. 572:281–285; 1992.
9. Dekker, A. J.; Connor, D. J.; Thal, L. J. The role of cholinergic projections from the nucleus basalis in memory. Neurosci. Biobehav. Rev. 15:299–317; 1991.
10. DeNoble, V. J.; Spencer, K. R.; Johnson, L. C.; Cook, L.; Myers, M. J.; Scribner, R. M. Comparison of DuP 996, with physostigmine, THA and 3,4-DAP on hypoxia-induced amnesia in rats. Pharmacol. Biochem. Behav. 36:957–961; 1990.
11. Dent, G. W.; Rule, B. L.; Tam, S. W.; DeSouza, E. B. Effects of the memory enhancer linopirdine (DuP 996) on cerebral glucose metabolism in naive and hypoxia-exposed rats. Brain Res. 620:7–15; 1993.
12. Dent, G. W.; Tam, S. W.; Grzanna, R. The memory enhancer linopirdine increases c-fos expression in cerebral cortex of aged rats. Soc. Neurosci. Abstr. 19:423.6; 1993.
13. Flood, J. F; Cherkin, A. Scopolamine effects on memory retention in mice: A model for dementia? Behav. Neural Biol. 45:169–184, 1986.
14. Johnson, R. M.; Inouye, G. T.; Eglen, R. M.; Wong, E. H. F. 5-HT_3 receptor ligands lack modulatory influence on acetylcholine release in rat entorhinal cortex. Naunyn Schmiedeberg Arch. Pharmacol. 347:241–247, 1993.
15. Levin, E. D. Nicotinic systems and cognitive function. Psychopharmacology 108:417–431, 1992.
16. Matsuoka, N.; Maeda, N.; Ohkubo, Y.; Yamaguchi, I. Differential effects ofphysostigmine and pilocarpine on the spatial memory deficits produced by twosepto-hippocampal deafferentation in rats. Brain Res. 559:233–240; 1991.
17. Matsuoka, N.; Maeda, N.; Yamazaki, M.; Ohkubo, Y.; Yamaguchi, I. Effects of FR12 1196 a novel cognition enhancer, on memory impairment of rats in passive avoidance and radial arm maze tasks. J. Pharmacol. Exp. Ther. 263:436–44; 1992.
18. Miyamoto, M.; Kato, J.; Narumi, S.; Nagaoka, A. Characteristics of memory impairment following lesioning of the basal fore-

brain and medial septal nucleus in rats. Brain Res. 419:19–31; 1987.
19. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11:47–60; 1984.
20. Nickolson, V. J.; Tamm, S. W.; Myers, M. J.; Cook, L. DuP 996 (3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one) enhances the stimulus-induced release of acetylcholine from rat brain in vitro and in vivo. Drug. Dev. Res. 19:285–300; 1990.
21. Riekkinen, M.; Sirvio, J.; Riekkinen, P. Pharmacological consequences of nicotinergic plus serotonergic manipulations. Brain Res. 622:139–146; 1993.
22. Summers, W.; Majovski, L.; Marsh, G.; Tachiki, K.; Kling, A. Oral tetrahydroaminoacridine in long-term treatment of senile-dementia Alzheimer's type. N. Engl. J. Med. 315:1241–1244; 1986.
23. Vickroy, T. W. Presynaptic cholinergic actions by the putative cognition enhancing agent DuP 996. J. Pharmacol. Exp. Ther. 264:910–917; 1993.
24. Whitehouse, P. J.; Price, D. L.; Coyle, J. I.; DeLong, M. R. Alzheimer's disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann. Neurol. 10:122–134; 1981.
25. Whitehouse, P. J.; Price, D. L.; Struble, R. G.; Coyle, J. I.; DeLong, M. R. Alzheimer's disease and senile dementia—loss of neurons in the basal forebrain. Science 215:1237–1239; 1982.
26. Winer, B. J. Statistical principles in experimental design. New York: McGraw-Hill; 1971.
27. Zaczek, R. Saydoff, J. Depolarization activated releasers of neurotransmitters as therapuetics for dementia: Preclinical characterization of linopirdine (DuP 996). Current Opin. Invest. Drugs 2: 1097–1104; 1993.
28. Zaczek, R.; Maciag, C.; Tinker, W. J. Effect of linopirdine (DuP 996) on the KCl, veratridine, NMDA and electrically induced release of [^{3}H]acetylcholine from superfused brain slices. Soc. Neurosci. Abstr. 423.7; 1993.