Facilitation of Acetylcholine Release and Cognitive Performance by an M₂-Muscarinic Receptor Antagonist in Aged Memory-Impaired Rats

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Aged memory-impaired (AI) and unimpaired (AU) 24–25-month-old Long–Evans rats were used to investigate the integrity of various cholinergic markers during normal aging and to establish if alterations can possibly relate to cognitive disabilities. AI and AU rats were classified on the basis of their performance in the Morris swim maze task. Choline acetyltransferase activity (ChAT) was not differentially altered in various cortical and hippocampal areas between these two groups. Similarly, quantitative receptor autoradiography did not reveal significant differences in ³H-pirenzepine/muscarinic M₁ and ³H-hemicholinium-3/high-affinity choline uptake binding sites in AI versus AU rats. In contrast, ³H-AF-DX 384/putative muscarinic M₂ binding was significantly increased in certain cortical and hippocampal areas of the age-impaired animals. These increments were correlated with decreased *in vivo* acetylcholine (ACh) release capacity in the AI rats. Most interestingly, the muscarinic M₂ antagonist BIBN-99 reversed, in a dose-dependent manner, the impaired ACh release as well as the cognitive deficits observed in the AI group. Similarly, BIBN-99 reversed scopolamine-induced amnesia in young animals. The efficacy of BIBN-99 likely relates to its antagonistic properties on negative muscarinic M₂ autoreceptors that are apparently increased in the AI animals, leading to altered ACh release. Taken together, these findings strengthen the role of ACh in learning and memory and may have implications for the treatment of degenerative disorders associated with impaired cholinergic functions, such as Alzheimer's disease.

[Key words: muscarinic receptor subtypes, second messengers, Alzheimer's disease, *in vivo* dialysis, Morris maze learning task, scopolamine amnesia]

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Alzheimer's disease (AD) is a common disease of the aging brain (Price, 1986; Kosik, 1992; Mullan and Crawford, 1993; Selkoe, 1993). The degeneration of forebrain cholinergic projections is certainly one of the most salient neurochemical features of AD (Davies and Maloney, 1976; Perry et al., 1977; Coyle et al., 1983), although other transmitter systems may also be altered in some, but not all, patients (Perry, 1986; Quirion et al., 1990; Francis et al., 1993). However, thus far cholinergic-based therapies of AD have generally failed (Perry, 1986; Whitehouse, 1988; Gauthier et al., 1991) or resulted in rather limited clinical improvements (Summers et al., 1986; Tariot et al., 1988; Davis et al., 1992). These therapies were mostly based on the use of precursors of acetylcholine (e.g., lecithin or choline; Perry, 1986; Whitehouse, 1988; Gauthier et al., 1991), blockers of acetylcholinesterase (e.g., tacrine; Summers et al., 1986; Tariot et al., 1988; Davis et al., 1992), or nonselective receptor subtype muscarinic agonists (e.g., bethanechol; Perry, 1986; Whitehouse, 1988; Gauthier et al., 1991).

Functional data from various laboratories (Perry, 1986; Quirion et al., 1990; McKinney and Coyle, 1991; Doods et al., 1993a) now suggest that the drugs used in these clinical trials may not have been ideal, as they can all result in the activation of presynaptically located negative M₂-muscarinic autoreceptors (Potter et al., 1984; Raiteri et al., 1984; Mash et al., 1985; Araujo et al., 1988; Pohorecki et al., 1988; Lapchak et al., 1989; Richards, 1990; Aubert et al., 1992), either by increasing acetylcholine (ACh) half-life or direct stimulation by agonists that would inhibit the subsequent release of the transmitter from remaining functional synapses (McKinney and Coyle, 1991; Doods et al., 1993a; Quirion, 1993). Moreover, it has recently been demonstrated that the normal processing of the β -amyloid precursor is under the control of cholinergic inputs (Buxbaum et al., 1992; Nitsch et al., 1992). Hence, altered cholinergic innervation could lead to an aberrant processing of β -amyloid and the possible formation of potentially neurotoxic fragments leading to neuritic plaque formation (Kosik, 1992; Mullan and Crawford, 1993; Selkoe, 1993).

Taking these recent findings into consideration, a reevaluation of the potential role of cholinergic innervation in learning and memory was deemed to be highly appropriate. Accordingly, the potential effectiveness of the selective blockade of muscarinic autoreceptors (putative M₂ subtype) on cognition was investi-

gated in a scopolamine-induced amnesia model (Levin et al., 1989) and, more critically, in a population of aged, Morris maze (Morris, 1985)-tested memory-impaired and unimpaired rats as a model more relevant to the human situation. In parallel, the capacity of the hippocampal cholinergic nerve terminals to release ACh was evaluated using *in vivo* dialysis, while the status of various cholinergic receptors and associated transduction mechanisms were investigated as an attempt to explain functional deficits. The recently characterized, potent muscarinic M₂ antagonist BIBN-99 (Doods et al., 1993a) was used in both *in vivo* dialysis and behavioral experiments. It has recently been shown that this molecule is one of the most potent and selective M₂-muscarinic blocker known thus far, having its highest affinity for the cloned m₂ (pK_i = 7.5) receptors expressed in CHO cells over the m₄ (6.8) > m₃ (6.1) > m₁ (6.0) > m₅ (5.8) subtypes (Doods et al., 1993a). Functionally, BIBN-99 was shown to antagonize vagally mediated bradycardia and to block the centrally induced pressor effects of arecoline, two well known muscarinic M₂-like effects (Doods et al., 1993b).

Materials and Methods

Animals and BIBN-99. All animals used in this study were obtained from our colony of aged Long-Evans rats purchased at young adult ages (3–6 months; males) from Charles River (St. Constant, Quebec, Canada). Animals were maintained on a 12 hr light–dark cycle (lights on at 7:00 A.M.) in temperature- and humidity-controlled rooms, and fed standard laboratory chow with access to tap water *ad libitum*. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care. Monthly sentinel evaluation insured that aged animals were generally in good health and free of infection.

BIBN-99 (>95% pure based on a battery of analytical methods; Doods et al., 1993a,b) was obtained from K. Thomae GmbH, Biberach, Germany, and stored at 4°C in its powder form until the day of the experiments.

Morris swim maze task. Young and 24–25-month-old aged rats were evaluated for their learning capacities using the well established Morris maze task (Gage et al., 1984; Morris, 1985; Pelleymounter et al., 1990), and as previously described by our group (Issa et al., 1990). Briefly, animals are required to find a submerged (2 cm) platform in a pool (1.6 m diameter) of water that has been made opaque by the addition of powdered skim milk. The animals solve the task using only distal spatial cues provided in the testing room. The animals were given 15 trials over five successive days with the platform submerged. Unless the animals were used for drug testing, all the animals were immediately run on probe trials on the fifth day with the platform elevated 2 cm above the surface of the water. This condition was used to ensure that impairments were not related to any visual defects or to the animal's inability to perform the motor demands of the task (see Gage et al., 1984; Morris, 1985; Pelleymounter et al., 1990). Animals used in the BIBN-99 studies were given probe trials immediately following termination of testing. As reported earlier by various groups (Gage et al., 1984; Morris, 1985; Issa et al., 1990; Pelleymounter et al., 1990), aged animals demonstrate great differences in learning profile, some (age unimpaired, AU) learning as easily as young 6-month-old rats while others are unable to reach criteria (age impaired, AI). See legend to Figure 1 for further details.

ChAT activity. To determine ChAT activity, dissected frontal cortex and hippocampi from AI (*n* = 8) and AU (*n* = 9) rats were homogenized and incubated for 15 min in buffer containing ¹⁴C-acetyl coenzyme A, as previously described in detail elsewhere (Araujo et al., 1988), using the method of Fonnum (1969) as modified by Tucek (1978). Data are expressed as mean ± SEM of nmol ACh/mg protein/hr. Protein content was determined as described by Lowry et al. (1951) using bovine serum albumin (Sigma Chemicals, St. Louis, MO) as standard.

Autoradiographic visualization of various cholinergic markers. Sections from AI (*n* = 6–12, depending upon markers) and AU (*n* = 6–12) rat brains were prepared as described in detail elsewhere (Quirion, 1987; Aubert et al., 1994). Briefly, adjacent 15 μm thick slide-mounted AI and AU brain sections at various cortical, hippocampal, and sub-

Figure 1. Performance of male young (6 months) and 24–25-month-old aged impaired (AI) and aged unimpaired (AU) Long-Evans rats in the Morris swim maze. Aged rats were classified as previously described (Issa et al., 1990) by comparison to the performance of the young rats. An animal was designated as impaired if the latency to find the platform on each of the test days 2–5 was >2 SD higher than the mean for the young animals. Aged unimpaired animals showed latencies over this period that were <0.5 SD higher than the mean for the young animals. A significant portion (33.2%) of the aged animals (*n* = 247) from our colony demonstrates significantly impaired performance; in contrast, 27.5% of the animals show clearly unimpaired performance. The remainder fall in between these extremes. An analysis of variance (with Scheffe post hoc tests; STATVIEW-MACINTOSH, version 5.12) shows that, as a group, the AI differ significantly from both the young and AU animals on test days 2–5; there is no difference at any time between the young and AU rats. The performance of the animals has also been measured in terms of the distance traveled before locating and climbing onto the platform. An analysis of these data shows the same pattern of differences as the latency data (data not shown). When these same animals are provided an opportunity to swim toward a platform that has been raised above the level of the water (visually cued condition) there are no differences in latency or distance measures (data not shown), indicating that the differences between the groups are not related to swimming abilities (Issa et al., 1990). **p* < 0.01 compared to Y animals.

cortical levels were incubated in the presence of 15 nM ³H-pirenzepine (83–87 Ci/mmol; Dupont Canada, Toronto, Ontario), 2.0 nM ³H-AF-DX 384 (97–105 Ci/mmol; Dupont Canada), or 10 nM ³H-hemicholinium-3 (124–137 Ci/mmol; Dupont Canada) to investigate putative muscarinic M₁, -M₂, and high-affinity choline uptake (HACU) sites, respectively. At the end of the 60 min incubation period, slides were washed in 50 mM cold Tris-HCl buffer for 3 × 4 min each, rapidly dried before being apposed, alongside high- and low-activity tritium microscopies, to Hyperfilms (Amersham Canada, Montreal, Quebec) for 2 weeks for the M₁ and M₂ sites and 6 weeks for the HACU sites. Films were then developed and quantified using an MCID System image analyzer (Aubert et al., 1994). Data were analyzed using the NUMBER CRUNCHER STATISTICAL SYSTEM (NCSS; version 4.21) and statistical significance determined using a Student's *t* test. Nonspecific binding was determined in the presence of 1.0 μM atropine (M₁ and M₂) or 10 μM HC-3 (HACU). For ³H-HC-3, nonspecific binding was subtracted from all determinations, this being only film background for M₁ and M₂ sites.

Carbachol-induced phosphoinositide production. The production of phosphoinositide (total) and metabolites [IP₃, IP₂, and diacylglycerol (DAG)] following a carbachol (1 mM; Sigma) challenge was determined as described in detail by others (Fowler et al., 1987; Dean and Beaven, 1989; Godfrey, 1989; Godfrey and Watson, 1990; Hwang et al., 1990). Briefly, AI and AU brain slices (400 μm; five or six animals per group) were prepared using a McIlwain tissue slicer and allowed to recover

for 60 min at 37°C in a 5% CO₂ chamber incubator in modified Krebs/HEPES buffer (1.18 mM NaCl, 4.65 mM KCl, 2.5 mM MgSO₄, 1.18 mM KH₂PO₄, 1.25 mM CaCl₂, 25 mM NaHCO₃, 25 mM HEPES, 10 mM dextrose, 1% bovine serum albumin, pH 7.4). Slices (approximately 4–5 slices per well) were then incubated for 60 min in 500 μ l of buffer containing 10 μ Ci/ml of ³H-cytidine (26.9 Ci/mmol; Dupont Canada) or ³H-inositol (17.9 Ci/mmol; Amersham Canada) for measurements of ³H-CDP-DAG or total ³H-IP, ³H-IP₃, and ³H-P₄, respectively. Slices were then washed twice using 10 mM LiCl and maintained in the presence of phosphatase inhibitor for 30 min before carbachol (1 mM) stimulation. The accumulation of ³H-IP and ³H-CDP-DAG was determined after 30 and 60 min of stimulation, respectively. ³H-IP (total, IP₃ and IP₄) accumulation was terminated by adding 2 ml methanol-HCl (2:0.05 by volume) containing 5 mM EDTA (Sigma), 5 mM DTPA (Sigma), and 500 μ M phytate (Sigma), while ³H-CDP-DAG production was stopped by adding 2 ml of methanol (Aldrich Chemicals, IL). For tissues labeled with ³H-inositol, the ³H-IPs are determined following extraction with ion-exchange chromatography using Dowex columns (200–400 mesh, Bio-Rad, Montreal, Quebec). ³H-IPs were eluted with increasing concentrations of ammonium formate containing 0.1 M formic acid (³H-IP₁, 0.3 M; ³H-IP₂, 0.7 M; ³H-IP₃, 1.0 M; ³H-IP₄, 1.8 M). Elution volumes and ammonium formate concentrations were determined following previous elution conditions established with respective tritiated IP standards. ³H-IP accumulation levels were expressed as the ratio of ³H-inositol incorporated in aqueous and lipidic phases (³H-IP/³H-IP + ³H-inositol + ³H-phosphatidylinositol), while ³H-CPD-DAG production was determined using the following formula: ³H-CPD-DAG/³H-CPD-DAG + ³H-cytidine. Changes in ³H-IP or ³H-CDP-DAG production following a carbachol challenge were expressed as mean $\pm$ SEM from percentage of basal values. Statistical significance ($p < 0.05$) was established by one-way ANOVA followed by the posteriori Fisher's test using an NCSS statistical package.

In vivo dialysis. As we reported earlier (Richard et al., 1991), and under sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) anesthesia, AI and AU rats were stereotactically implanted with guide cannula in the dorsal hippocampus according to the following coordinates: 3.8 mm posterior to bregma, 1.8 mm lateral to the midline, and 1.0 mm ventral to the dura (Paxinos and Watson, 1982). Four stainless steel screws were placed in the skull, and the guide cannula was protected by a plastic collar and embedded in dental acrylic anchored to the top of the head by the screws. Animals ($n = 8$ or 9 per group) were returned to the colony and allowed to recover for 4–6 d prior to the *in vivo* dialysis. Twelve to sixteen hours before these experiments, 2 mm dialysis probes (BAS-Bioanalytical Systems Inc., West Lafayette, IN) were implanted through the guide cannula. Each animal was dialyzed only once. On the day of the dialysis experiments, rats were placed individually in lidless oval cages for 1 hr before being connected to a Harvard Micro-liter syringe pump (Harvard Apparatus, South Natick, MA) and dialyzed for a 1 hr washout period with Ungerstedt-Ringer buffer (containing 75 μ M physostigmine sulfate; Sigma) at the rate of 2.30 μ l/min. Twenty minute dialysate fractions were collected into 1.5 ml glass reaction vials containing 46 μ l of 0.1 N HCl, mixed with internal standard (100 pmol of deuterated ACh; Merck Isotopes, Montreal, Quebec), frozen on dry ice, and stored at -80°C until assayed by gas chromatography/mass spectrometry (GC/MS). Eight 20 min dialysate samples were collected before BIBN-99 injections (subcutaneous in 0.9% sterile saline) to establish basal ACh release values. Animals were killed and the location of the probe verified by visual or histological (cresyl violet) examination.

ACh was assayed as described earlier using a well established GC/MS methodology (Wood and Peloquin, 1982). Briefly, frozen dialysates were lyophilized directly in glass reaction vials, reconstituted in 250 μ l of acetonitrile, capped, heated at 80°C for 40 min, cooled to room temperature, and dried under a gentle stream of nitrogen gas. Quaternary amines in the dried samples were demethylated using sodium benzenethiolate synthesized according to Jenden and Hanin (1974). The samples were then carried through a series of liquid-liquid extractions in order to concentrate the demethylated amines into ethyl acetate for injection (3–5 μ l) into the GC-MS (Hewlett-Packard 5987B). The amount of endogenous ACh in each dialysate sample was calculated from the peak area ratio of endogenous/deuterated ACh, using a regression curve derived from standards of authentic ACh prepared in the Ringer solution. Calculations were not corrected for the recovery of ACh by the probes. Significant differences between samples within experimental

Figure 2. Comparative levels of choline acetyltransferase (ChAT) activity and muscarinic M₁, M₂, and high-affinity choline uptake (HACU) binding in various brain areas of 24–25-month-old age-impaired (AI) and unimpaired (AU) rats. M₂ binding is significantly increased in various cortical areas and in the molecular layer of the dentate gyrus of the hippocampus of the AI rats, while ChAT activity, and M₁ and HACU sites are not altered. Data are mean $\pm$ SEM of 6–12 animals per group and derived from quantitative *in vitro* receptor autoradiography of M₁/³H-pirenzepine, M₂/³H-AF-DX 384, and HACU-³H-hemicholinium-3 binding, and a radioenzymatic assay for the determination of ChAT activity. Statistical analysis of binding and ChAT activity data between the AI and AU groups was performed using the Student *t* test, $p < 0.05$ being considered statistically significant. CA1–CA3, subfields of the Ammon's horn of the hippocampus; Cg, cingulate cortex; DG, granular layer of the dentate gyrus; Fr, frontal cortex; Mol, molecular layer of the dentate gyrus of the hippocampus; Par, parietal cortex; PFR, prefrontal cortex; Oc, occipital cortex. * $p < 0.05$; ** $p < 0.01$.

Figure 3. Comparative efficacies of the cholinergic agonist carbachol (1 mM) to stimulate the production of phosphoinositides (total, *IP tot*; A) and metabolites *IP₃* (B) and *IP₄* (C), and diacylglycerol (*DAG*; D) in various brain regions of 24–25-month-old AI and AU rats. The activation of this transduction pathway is associated with the stimulation of pharmacologically characterized muscarinic M₁ receptors that include the cloned m₁, m₃, and m₅ subtypes (Hulme et al., 1990). Respective changes in *IP* (total, *IP₃*, *IP₄*) or *DAG* were expressed as mean $\pm$ SEM from percentage of basal values ($n = 5$ –6 animals per group). *Fr*, frontal cortex; *Hip*, hippocampus; *Par*, parietal cortex; *Te*, temporal cortex.

groups were determined by a one-way repeated measure of variance (ANOVA) followed by a pair-wise multiple comparisons test.

Scopolamine-induced amnesia in young rats. Using 6-month-old Long–Evans rats from our colony, this well established model was used as described earlier using an eight-arm radial maze (Levin et al., 1989). Briefly, following training to criterion (less than one error for two consecutive days), male rats ($n = 7$ or 8 per group) were divided into two groups: one-third were administered sterile saline 30 min prior to the session ($n = 8$), and two-thirds were administered scopolamine (0.1 mg/kg, s.c.; RBI Inc., Natick, MA). Following a 48 hr washout period, rats in the scopolamine-treated group were further subdivided: one-half were pretreated with BIBN-99 (0.50 mg/kg, s.c.; $n = 8$) 60 min before the session, and one-half were pretreated with saline 60 min prior to the session ($n = 7$). The potential effectiveness of BIBN-99 was evaluated using repeated measures ANOVA followed by Newman–Keuls post hoc tests.

Results

Long–Evans rats 24–25 months old display considerable individual differences in spatial memory deficits (Gage et al., 1984;

Table 1. *In vivo* hippocampal release of acetylcholine (ACh) in freely moving 24–25-month-old age-impaired (AI) and unimpaired (AU) rats

	<i>N</i>	Basal ACh release (pmol/20 min)	BIBN-99 treated (0.5 mg/kg; % basal)
AU	8	4.2 $\pm$ 0.6	98 $\pm$ 7.3
AI	9	1.9 $\pm$ 0.4*	153 $\pm$ 18*

ACh release (i.e., efflux of dialyzable transmitter, mean $\pm$ SEM) was expressed either as picomoles of ACh per 20 min dialysate sample or as the percentage of the basal value. Repeated ANOVA measures revealed a significant effect for group [$F(1,11) = 10.1509$, $p = 0.00856$], time [$F(20,220) = 1.92260$, $p = 0.1219$], and group $\times$ time interaction [$F(20,220) = 1.76556$, $p = 0.02556$]. In the two BIBN-99-treated groups, post hoc multiple comparison tests contrasting the eight baseline samples (prior to drug administration) against individual time samples after drug administration revealed significant effect for samples 10 [$F(1,11) = 13.3266$, $p = 0.004$] and 11 [$F(1,11) = 13.0142$, $p = 0.004$], the drug being administered at time sample 9. ACh release returned to baseline in the AI group at time sample 12.

* $p < 0.05$.

Morris, 1985; Issa et al., 1990; Pelleymounter et al., 1990). Accordingly, over the past few years, different cohorts from our colony were evaluated in the Morris swim maze and defined as age impaired (AI) or unimpaired (AU) on the basis of their performance in this task (Fig. 1).

The status of various cholinergic markers in these two groups of rats was examined first. As shown in Figure 2, it is evident that these markers were not decreased in impaired versus unimpaired animals in various cortical and hippocampal areas. In fact, the levels of the pharmacologically defined muscarinic M₂ (³H-AF-DX 384; including cloned m₂ and m₄ subtypes) receptors were even increased in various cortical areas as well as the molecular layer of the dentate gyrus of the hippocampus (enriched in cholinergic terminals) of the AI group. Pharmacological muscarinic M₁ receptors labeled with ³H-pirenzepine, choline acetyltransferase (ChAT) activity, and high-affinity choline uptake (HACU; ³H-hemicholinium-3) binding sites were not significantly different between these two groups, at least in the brain regions studied (Fig. 2).

The pharmacologically defined M₁ receptors (coupled to the inositol cascade; Hulme et al., 1990) are apparently fully functional, as the production of inositides (*IP₃*, *IP₄*) and diacylglycerol (*DAG*) associated with their activation by carbachol is even possibly increased in certain cortical and hippocampal areas of impaired versus unimpaired animals (Fig. 3). ³H-AF-DX 384 binding data (Fig. 2) suggest possible increases in the density of M₂ sites in AI animals. Such an increment in putative M₂ binding could lead to a decrease in the capacity of releasing ACh due to an increased activation of these receptors likely acting as inhibitory autoreceptors (see introductory remarks). To investigate this possibility, we used *in vivo* dialysis to monitor hippocampal ACh release in behaving animals (Richard et al., 1991). Interestingly, basal ACh release was found to be decreased in AI versus AU male Long–Evans rats (Table 1). Next, AI and AU rats were given peripheral injections of the newly characterized muscarinic M₂ blocker BIBN-99 (Doods et al., 1993a). As shown in Table 1, this treatment produced a greater increase in hippocampal ACh release in the impaired than the unimpaired animals, suggesting a higher degree of sensitivity to M₂ receptor modulation in the AI group.

The behavioral consequences of an increased inhibitory activity of the M₂ receptor in the age-impaired group was then evaluated (Issa et al., 1990). Peripheral injections of BIBN-99 mark-

Figure 4. *A*, Effects of the muscarinic M_2 blocker BIBN-99 on the learning latency of 24–25-month-old age-impaired (AI) versus age-unimpaired (AU) and young (Y) rats as assessed using the Morris swim maze. It is evident that while AU animals performed as well as 6-month-old Long-Evans rats (and hence BIBN-99 was without effects), this drug induced a marked dose-dependent improvement of the learning ability in the AI animals, at doses of 0.25 and 0.50 mg/kg (s.c.). BIBN-99 does not appear to effect motor performance of the animals tested, as their average swim velocities remained unchanged with or without drug treatment (≈ 30 cm/sec, data not shown). The mean ($\pm$ SEM) latencies are based on the performances (three trials per day for 3 d) of AI animals treated with saline ($n = 7$) or 0.025, 0.075, 0.25, or 0.50 mg/kg, s.c., BIBN-99 ($n = 5$ animals for each dose) 1 hr before versus AU ($n = 5$) and Y ($n = 7$) animals treated with BIBN-99 (0.5 mg/kg, s.c.) 1 hr before retesting in the Morris swim maze. The data was analyzed using analysis of variance with Scheffe post hoc test. $*p < 0.05$ compared to AI (saline group). *B*, A preinjection 24 hr before testing of 0.5 mg/kg BIBN-99 to AI animals failed to modify the latency as expected on the basis of the pharmacokinetics of BIBN-99 (Doods et al., 1993b; Eberlein et al., 1993), while injections of BIBN-99 1 and 4 hr before testing were fully effective. The mean ($\pm$ SEM) latencies are based on the performances (three trials per day for 3 d) of AI

edly facilitated, in a dose- and time-dependent manner, the cognitive performance of AI animals in the Morris swim maze (Fig. 4*A,B*). In fact, at a dose of 0.25 mg/kg (s.c.) of BIBN-99, the cognitive performance of the impaired animals attained a level similar to that of unimpaired or young rats (Fig. 4*A*). Similar improvements were seen after 1 or 4 hr, but not 24 hr, post injection (Fig. 4*B*).

As an additional investigation of the behavioral effects of BIBN-99, we also administered this molecule to young rats that had been treated with scopolamine, a well established model of pharmacologically induced cholinergic hypofunction (Levin et al., 1989). As shown in Figure 4*C*, BIBN-99 potentially reversed the amnesia produced in this model, demonstrating further the potential usefulness of BIBN-99 in ameliorating cognitive deficits in a different behavioral model.

Discussion

Acetylcholine has been proposed to be involved in learning and memory processes, and in dementia of the Alzheimer's type. However, recent controversies have questioned the critical role of this neurotransmitter in cognition. Using aged, Morris maze-tested, memory-impaired and unimpaired rats as a representative model, we show that it is possible to reverse cognitive deficits in the age-impaired cohort, likely by blocking the cholinergic autoreceptors and hence increasing acetylcholine release as monitored by *in vivo* dialysis and using the selective M_2 antagonist BIBN-99. This is correlated with apparent increases in muscarinic M_2 -like 3H -AF-DX 384 binding sites in certain cortical and hippocampal areas of the impaired animals. Taken together, the results of the present study suggest that learning deficits observed in the age-impaired group may be related, at least in part, to the reduced availability of ACh in the synaptic cleft induced by increments in the density of M_2 -negative autoreceptors. They also support a critical role for ACh in learning and memory, as the model used here is not based on a drug-induced deficit (Fibiger, 1991; Hasselmo and Bowen, 1993) but rather on functional alterations associated with the aging process.

Quantitative receptor autoradiographic studies revealed that various cholinergic markers, including 3H -pirenzepine/ M_1 -muscarinic and 3H -hemicholinium-3/HACU binding, as well as ChAT activity, failed to be significantly altered in the AI and AU groups. In contrast, 3H -AF-DX384 muscarinic binding was found to be significantly increased in certain cortical areas and in the hippocampus of age-impaired versus unimpaired rats.

←

animals treated with saline 1 hr before ($n = 6$) versus BIBN-99 1, 4, and 24 hr (0.50 mg/kg, s.c.; $n = 5$ animals for each time point) before retesting. The data was analyzed using analysis of variance with Scheffe post hoc test. $*p < 0.05$ compared to saline group. *C*, For comparison the potential effectiveness of BIBN-99 in scopolamine-induced amnesia in young 6-month-old male Long-Evans rats was also evaluated. Following training to criterion (less than one error for two consecutive days), rats were divided into two groups: one-third were administered saline (Sal) 30 min prior to the session ($n = 8$) and two-thirds were administered scopolamine (Sco; 0.1 mg/kg, s.c.). Forty-eight hours later, rats in the scopolamine group were further divided into BIBN-99 (0.5 mg/kg, s.c.; $n = 8$) and saline-treated ($n = 7$) groups 60 min prior to the session. Repeated measures ANOVA revealed a significant effect for group [$F(2,30) = 9.1448$, $p = 0.0002$], trial [$F(2,60) = 11.4$, $p = 0.00001$], and group $\times$ trial interaction [$F(4,60) = 3.288$, $p = 0.016$]. Newman-Keuls post hoc tests revealed that scopolamine-treated animals made more errors during retention than saline-treated controls ($p < 0.05$). Pretreatment with BIBN-99 effectively blocked the cognitive impairment observed with scopolamine ($*p < 0.05$).

These rather unexpected findings are consistent, however, with recent reports demonstrating hyperplasia of basal forebrain cholinergic neurons in aged, memory-impaired monkeys (Rapp and Amaral, 1992; Stroessner-Johnson et al., 1992). It thus appears that cholinergic innervation may compensate for age-related learning deficits by increasing the size of the perikarya or the arborization of its terminal fields, this possibly leading to aberrant sprouting (Gage et al., 1983; Geddes et al., 1985; Masliah et al., 1991; Leanza et al., 1993). This would explain the apparent integrity of cortical and hippocampal ChAT activity in AI animals, and the observed increases in M₂ sites, a portion of which likely act as presynaptic autoreceptors regulating ACh release in cortical and hippocampal areas (Raiteri et al., 1984; Mash et al., 1985; Araujo et al., 1988; Pohorecki et al., 1988; Lapchak et al., 1989; Richards, 1990; Aubert et al., 1992; Quirion, 1993).

Pharmacologically defined M₁ receptors include the molecularly characterized m₁, m₃, and m₅ subtypes and are known to be coupled to the inositol cascade (Hulme et al., 1990). Signal transduction associated with the activation of these M₁ receptors is likely spared in the AI animals. In fact, we observed that carbachol-stimulated inositol and diacylglycerol production may even be slightly increased in cortical areas of the impaired rats. Similar results have recently been reported in regard to the coupling of M₁ receptors in Alzheimer's brains (Pearce and Potter, 1991; but see Flynn et al., 1991) as well as in aged Fischer 344 and Sprague-Dawley rats (Nalepa et al., 1989; Tandon et al., 1991). Moreover, the transduction mechanism of the pharmacological M₂ receptors (molecularly defined m₂ and m₄; coupled to the inhibition of adenylate cyclase activity) has also been reported to be spared in aging (Pradhan, 1980).

In accordance with increases in putative M₂ binding sites and normal signal transduction pathways, hippocampal *in vivo* dialysis revealed that basal ACh release was lower in the AI than the AU animals, possibly because of heightened negative M₂ autoreceptor functions. Accordingly, BIBN-99 induced a greater release of ACh in the AI than the AU rats. Most interestingly, this molecule also potently and dose dependently reversed the cognitive impairments of the AI group as assessed in the Morris maze task. In addition, BIBN-99 was found to be most effective in another well established model of learning dysfunctions, the scopolamine-induced amnesic rat. These *in vivo* dialysis and behavioral data demonstrate that a prototypic muscarinic M₂ antagonist, BIBN-99, can significantly facilitate cognitive performance in aged memory-impaired and scopolamine-induced amnesic rats. The drug possibly induces this effect by blocking the M₂ autoreceptors on cholinergic nerve terminals, thus increasing the presynaptic release of ACh to virtually eliminate the spatial memory deficit in the AI rats. In view of recent findings, however, it is likely that other systems may also be involved with the functional effects of BIBN-99. Mrzljak et al. (1993) identified M₂-receptor immunostaining on presynaptic (autoreceptors) as well as postsynaptic neuronal elements in the monkey cortex. The postsynaptically labeled cells are likely glutamatergic on the basis of their ultrastructural appearance. As cholinergic drugs have been shown to inhibit glutamate release by acting via M₂-muscarinic receptors (Marchi and Raiteri, 1989; Raiteri, 1990), BIBN-99 could relieve this inhibition and hence facilitate the release of glutamate. This is of potential significance here in view of the purported roles of excitatory amino acids such as glutamate in cognitive processes (for a recent review, see Francis et al., 1993). Thus, BIBN-99, by pos-

sibly simultaneously inducing the release of ACh and glutamate, could promote both cholinergic and glutamatergic neurotransmission leading to a facilitation of cognitive processes.

The present observations may also help to resolve some of the current controversy concerning the role of ACh in learning and memory (Fibiger, 1991; Hasselmo and Bowen, 1993). One of the key criticisms of this hypothesis was that most evidence was based on pharmacologically (scopolamine) or lesion (basal forebrain)-induced learning deficits. We have now shown that a cholinergic drug like BIBN-99 can have beneficial effects on learning parameters in both pharmacologically induced (scopolamine-induced amnesia) and "natural" models of age-related memory impairments (AI rats).

The possible relevance of these findings to diseases such as AD is certainly worthy of consideration. While significant losses in both cortical and hippocampal ChAT activities (Davies and Maloney, 1976; Perry et al., 1977; Coyle et al., 1983; Mullan and Crawford, 1993) and muscarinic M₂ receptors (Mash et al., 1985; Araujo, 1988; Aubert et al., 1992) have been observed in AD, often up to 40–45% of the normal levels of these two markers are still present, even in advanced AD cases (Mash et al., 1985; Araujo, 1988; Aubert et al., 1992). It has also been shown that post-mortem cortical and hippocampal AD tissues can synthesize and release ACh (Rylett et al., 1983; Nilsson et al., 1986, 1987), and that a proportion of muscarinic receptors is still functional in AD (Pearce and Potter, 1991). Accordingly, treatment strategies could take advantage of remaining cholinergic innervation by using appropriate pharmacological interventions. The use of peripherally injected molecules such as BIBN-99, likely by blocking the autoreceptors, could relieve the remaining feedback inhibition and hence facilitate ACh release. This effect would be in contrast to those produced by the cholinergic drugs used in most clinical trials that have resulted, in fact, in greater activities of the negative autoreceptors either by increasing the half-life of ACh in the synaptic cleft (using esterase blockers; Summers et al., 1986; Tariot et al., 1988; Davis et al., 1992) or by directly activating these autoreceptors using poorly selective agonists (Whitehouse, 1988; Gauthier et al., 1991). Accordingly, a more appropriate strategy should take into account the potential role of the muscarinic autoreceptors controlling ACh release (Potter et al., 1984; Mash et al., 1985). The neurochemical and behavioral effectiveness of BIBN-99 observed in the present study supports this contention.

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