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Choline pivaloyl ester strengthened the benefit effects of Tacrine and Galantamine on electroencephalographic and cognitive performances in nucleus basalis magnocellularis-lesioned and aged rats

V. Rispoli [a,*], R. Marra [b], N. Costa [a], L. Scipione [c], D. Rotiroti [a], D. De Vita [c], F. Liberatore [c], V. Carelli [c]

[a] *Department of Pharmacobiological Sciences, University "Magna Græcia" of Catanzaro, Complesso "Ninì Barbieri", I-88021 Roccelletta di Borgia (CZ), Italy*
[b] *CNR Institute of Neurological Sciences, I-88021 Roccelletta di Borgia, Catanzaro, Italy*
[c] *Department of "Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive", University of Rome "La Sapienza", Piazzale Aldo Moro 5, I-00185 Rome, Italy*

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Abstract

The aim of the present work was the assessment of the effects produced on the electroencephalographic (EEG) activity and the cognitive and memory performances of nucleus basalis magnocellularis (NBM)-lesioned or aged rats by the combined treatment with [2-(2,2-dimethylpropionyloxy)ethyl]trimethylammonium 2,2-dimethylpropionate (choline pivaloyl ester) (CPE) and the Cholinesterase inhibitors (ChEIs) Tacrine (THA) and Galantamine (GAL). Intraperitoneal administration of CPE combined with THA or GAL to both NBM-lesioned or aged rats, produced EEG desynchronisation, and a significant decrease in the energy of the total EEG spectrum and the lower frequency bands (delta 0.25–3 and theta 4–7 Hz) lasting many minutes. Furthermore, drug associations reversed in aged rats the scopolamine (0.2 mg/kg, i.p.)-induced increase in EEG power, slow waves and high-voltage spindle (HVS). Furthermore, the combined administration of CPE and Cholinesterase inhibitors in both NBM-lesioned or aged animals, improved performances in all behavioural tasks, enhancing object discrimination, increasing locomotory activity and alternation choice in T-maze, ameliorating retention in passive avoidance and decreasing escape latency in Morris water maze. In all test, AChEIs and CPE combinations proved to be more effective than CPE, THA or GAL given alone. In conclusion, the present work shows the ability of choline pivaloyl ester in strengthening the positive cerebral activity of THA and GAL.

Keywords: Choline; Cholinesterase inhibitors; Combined treatment; Cholinergic neurotransmission; Electroencephalography; Cognitive–behavioural functions; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder affecting major brain areas and is characterized by progressive decline in memory, impairment of cognitive functions and behavioural disturbances (Bartus et al., 1982; Katzman, 1986). The AD syndrome is associated with a severe deficit in the cholinergic neurotransmission because of a progressive degeneration in forebrain and, in particular, in the nucleus basalis magnocellularis (NBM) (Whitehouse et al., 1981, 1982). The degeneration of cell bodies in NBM causes the loss of neuronal projections to the cortex accompanied by a reduction of the brain levels of the neurotransmitter acetylcholine (ACh) and the biosynthetic enzymes Choline Acetyl transferase (ChAT) and Acetylcholinesterase (AChE) (Perry et al., 1978; Ladner and Lee, 1998).

As consequence of the loss of the cholinergic activity, impairments of attention, learning and memory functions are produced and, furthermore, many other behavioural and cognitive capacities are also affected (Coyle et al., 1983; Collerton, 1986; Bartus et al., 1982; Allen and Burns, 1995; Francis et al., 1999). Interestingly, in AD patients, cognitive and behavioural

* Corresponding author. University "*Magna Grœcia*" of Catanzaro, UNICZ, Department of Pharmacobiological Sciences, Complesso Ninì Barbieri, I-88021 Roccelletta di Borgia, CZ, Italy. Tel.: +39 961 391157; fax: +39 961 391490.
E-mail address: rispoli@unicz.it (V. Rispoli).

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impairments closely correlate with the slowing of cerebral electroencephalographic (EEG) activity, characterized by the presence of large amplitude waves associated with a decrease in high frequency components (Fenton, 1986; Brenner et al., 1988; Helkala et al., 1991; Miyauchi et al., 1994; Prichep et al., 1994; Pozzi et al., 1995; Locatelli et al., 1998).

To improve cholinergic neurotransmission, different strategies have been investigated, including the reduction of ACh synaptic hydrolysis by Cholinesterases (ChE) inhibition and the increase of ACh synthesis. To date, the only AD effective symptomatic treatment is the use of ChE inhibitors (ChEIs), whose clinical efficacy is thought to result from the enhancement of the cholinergic activity by prolonging the half-life of ACh (Krall et al., 1999; Schneider, 2001). Unfortunately, the response to AD treatment with ChEIs shows a modest average degree of benefit (Doody et al., 2001; Olin and Schneider, 2001; Birks, 2006). Nevertheless, new roles of ChE and ChEIs in brain function and AD have been recently reported (Giacobini, 2003; Racchi et al., 2004).

In this regard, advanced neurochemical knowledge of AD pathology clearly indicated that, besides cholinergic deficits, other neurotransmitter systems, such as the dopaminergic, GABAergic, glutamatergic, noradrenergic and serotoninergic ones, are recognized to be involved in learning and memory processes (Cassel and Jeltsch, 1995; Cain et al., 2000; Dringenberg, 2000). Therefore, strategies based on treatment with combinations of drugs with different therapeutic targets have been positively experimented in animal models of senile dementia. Thus, combinations of the AChE inhibitor Tacrine (THA) with drugs such as D-cycloserine, a partial agonist of the glycine B-site of the glutamate NMDA receptor (Aura et al., 1998), lithium, an indoleamines and cathecolamines release inhibitor (Arendt et al., 1999), deprenyl or pargyline, monoamine oxidase (MAO) inhibitors (Dringenberg, 2000), ondansetron, a serotonine 5-HT3 receptors agonist (Diez-Ariza et al., 2003), proved to be more effective than cholinergic enhancers alone, in reversing cognitive and behavioural impairments and/or EEG changes induced in NBM-lesioned or scopolamine manipulated rats.

Recently, we have reported that treatment with two choline esters, [2-(2,2-dimethylpropionyloxy) ethyl]trimethylammonium iodide (**1**) and [2-(2,2-dimethylpropionyloxy)ethyl]-trimethylammonium 2,2-dimethylpropionate (**2**) (Carelli et al., 2001), was found to be effective in restoring discrimination in object recognition and improving spatial memory in Morris water maze (MWM) in scopolamine-treated or NBM-lesioned rats (Rispoli et al., 2004a). Furthermore, compounds **1** and **2** were able to induce EEG desynchronisation and significant changes in the architecture of EEG tracings (Rispoli et al., 2004b). The positive effects of choline esters on cholinergic neurotransmission were attributed to their limited acetylcholinesterase (AChE) inhibitory activity. In both object recognition and spatial memory tests, ester **2** exhibited better performances than ester **1** and, furthermore, induced longer lasting EEG effects (Rispoli et al., 2004a,b). These findings, because of the importance of cholinesterases and cholinesterase inhibitors in the treatment of AD, prompted us to thoroughly investigate the interaction between **2** and AChE and Butyrylcholinesterase (BChE), (see Experimental design and procedures). Choline ester **2** proved to be a weak inhibitor of both Cholinesterases and, furthermore, showed a high affinity as substrate for AChE; indeed, AChE rapidly catalysed its hydrolysis.

Bearing in mind that AChE is present in neurons in selected areas of the CNS, it may be suggested that ester **2** could operate as a carrier able to deliver and release choline in the brain by AChE hydrolysis. Thus, the levels of the ACh precursor could be increased and the synthesis of the neurotransmitter enhanced. Numerous experimental studies were performed investigating the effects of choline administration on the synthesis and release of ACh in the brain and suggesting the treatment with choline or choline sources as a possible therapeutic approach to obviate the AD cholinergic deficiency (Wecker, 1990; Buyukuysal et al., 1995; Koppen et al., 1997). Therefore, the current state of knowledge seems to justify the study of combined treatments with ChEIs and choline (or choline sources) in order to obtain additive effects in alleviating impairment of cognitive functions in suitable animal models.

Thus, we designed the present work to evaluate the effects of the combined administration of ChEIs, such as THA or Galantamine (GAL), along with a choline source [such as ester **2**, which in the following of the paper will be named choline pivaloyl ester (CPE)], on memory and cognitive deficits and neocortical EEG activity in animal models of senile dementia.

Tacrine was used by us as a reference drug in view of its ability to reverse, in animal models, learning and memory impairments or EEG changes produced by scopolamine or NBM lesion (Riekkinen et al., 1991; Vanderwolf et al., 1993; Wang and Tany, 1998), as well as cognitive deficit associated to aging (Scali et al., 1997a; Aura et al., 1998; Stammelin et al., 1999), Furthermore, we used the AChE inhibitor Galantamine also on account of its additional property of stimulating $\alpha4\beta2$ and $\alpha7$ nicotinic ACh receptors, as well as NMDA receptors (Maelicke and Albuquerque, 2000; Maelike et al., 2001; Samochocki et al., 2003; Kihara et al., 2004; Moriguchi et al., 2004; Geerts, 2005; Texido et al., 2005), thereby potentiating the activity of both cholinergic and NMDA systems (Santos et al., 2002, Moriguchi et al., 2004) and strengthening the effects of CPE as nAChRs agonist (see Discussion).

As animal models of the AD disease we used stereotaxically NBM-lesioned or scopolamine-treated rats, as well as 27-month-old rats. AMPA-induced excitotoxic lesion of rat NBM determines loss of cholinergic neurons projecting to cortex and decrease of AChE and ChAT. Consequently, increase of EEG slowing is induced and, furthermore, cognitive and memory deficits are produced (Casamenti et al., 1986; Page et al., 1991; Riekkinen et al., 1991, 1992; Muir et al., 1993). On the other hand, central cholinergic blockade due to scopolamine produces well characterized deficits in EEG pattern, as well as in attention, information processing and memory (Sunderland et al., 1985; Beatty et al., 1986; Kopelman and Corn, 1988). Therefore, NBM lesions or scopolamine-induced amnesia provide suitable experimental models resembling AD. Regarding aging effects, different alterations in the electrophysiological activity of NBM

neurons were observed in aged rats: e.g. neuron firing is reduced, whereas neuronal response threshold to cortex stimulation is enhanced and excitatory reaction is decreased (Zhang et al., 2000). Therefore, aged rats represent no brain-manipulated models with face validity to the human equivalent.

2. Materials and methods

Tacrine hydrochloride (THA), Galantamine (Gal), Scopolamine hydrobromide and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) hydrobromide, were purchased from Tocris (Avonmouth, UK). Electric eel acetylcholinesterase (AChE), equine butyrylcholinesterase (BChE), acetylthiocholine iodide, butyrylthiocholine iodide and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (Milano, Italy). ChAT monoclonal antibodies were purchased from Boehringer (Mannheim, Germany). All other chemical and biological reagents and solvents used were of the highest analytical, commercially available grade.

A double beam UV–Vis lambda 40 Perkin Elmer spectrophotometer was used to determine the inhibition constants of CPE, whose hydrolysis rate measurements were performed by ^{1}H-NMR spectroscopy using a Bruker AVANCE 400 spectrometer.

2.1. Enzymatic tests

2.1.1. Esterase activity

Inhibition tests were carried out, according to the Ellmann method (Ellman et al., 1961), by mixing 3 mL of 0.1 M (pH 7.4) phosphate buffer containing 0.75 mmol of DTNB and 0.083 U AChE or 0.25 U of BChE, with CPE (30–300 μmol) in a polystyrene cuvette of 1 cm path length. The reaction was started by the addition of acetylthiocholine iodide or butyrylthiocholine iodide (300–900 μmol) and the changes in the absorbance at 412 nm were recorded at 25 °C between 0.5 and 1.5 min after reagent addition. Each determination was performed at least in triplicate. The recorded data were analysed with the enzyme kinetic module of SigmaPlot, version 8.02a (Systat Software , Inc.) in order to find the best fitting model of inhibition. K_i values were obtained according to Dixon's method (Dixon, 1953), reporting the reciprocal of the hydrolysis rate vs the concentration of CPE, which proved to be a competitive inhibitor of both AChE (K_i=130.8±14.5 μM, r^2=0.973) and BChE (K_i=41.2±4.4 μM, r^2=0.978).

2.1.2. CPE as substrate of AChE and BChE

CPE displayed a higher affinity for AChE than for BChE. A comparison was made of the efficiency with which the two cholinesterases catalyse the breakdown of CPE by determining the rate of hydrolysis for each reaction under identical conditions. Thus, the ^{1}H-NMR spectrum of a ca. 10^{-3} M solution of CPE (0.6 mL of 1:1 D_2O/0.1 M pH 7.4 phosphate buffer) was recorded as blank-test; afterwards, 4.4 U of BChE or AChE was added to the solution which, then, was stored at 25 °C for 30 min. Thereafter, protonic NMR spectra were recorded at 15 min intervals for 1.5 h. In both cases, the progress of CPE hydrolysis was evaluated from the progressive increase in intensity of choline and pivalate ion signals.

The NMR data were elaborated using the software WIN-NMR Bruker Daltonik GmbH, version 6.1.0.0.

Under the described conditions the AChE hydrolysis rate of pivaloylcholine (1.6×10^{-2} μmol/min/AChE Unit) was ca. 2.7 times higher than the BChE hydrolysis rate (5.9×10^{-3} μmol/min/BChE Unit).

2.2. Animals

Male Wistar 3-months young (weighing 200–250 g) and 27-month-old rats (Charles River, Italy) were used in the experiments. Animals were group-housed (12-h light/dark cycle) with *ad libitum* access to food and water. All animals were treated under standard conditions, according to the guidelines for care and use of experimental animals of the European Community Council directive (86/609/EEC). The experimental protocol and procedures were also approved by the Italian Ministry of Health.

2.3. Experimental design and procedures

A set of experiment was designed to evaluate the reversal of the EEG slowing and cognitive deficit in animal models of AD disease treated with the pivaloyl choline ester and its associations with the AChE inhibitors, Tacrine and Galantamine. Preliminarily, a dose–response study was carried out to assess behavioural and EEG effects of increasing doses of each ChEI, under our experimental conditions.

Experimental outlined in EEG study and cognitive performance were carried out on young NBM-lesioned and aged rats. The number of tested animals was seven for each drug or combination of drugs and for each dose.

For EEG study, NBM-lesioned and aged rats were divided in several groups. Five groups of NBM-lesioned animals were used as follows: three groups were treated with CPE, THA or GAL alone respectively, two groups were treated with CPE in association with THA or GAL respectively.

Finally, three groups, including intact, sham-operated and NBM-lesioned, treated with vehicle, animals served as control. Similarly, eight groups of aged rats were used as follows: one control group was vehicle-treated, three groups were treated with CPE, THA or GAL alone respectively, two groups were treated with CPE in association with THA or GAL respectively, one group was treated with scopolamine and finally one with scopolamine and CPE. Control groups of animals received the same amount of physiological saline as vehicle, which was also used for the intraperitoneal administration of drugs.

For behavioural test on attention, working/episodic and working/spatial memory, groups of seven animals each were used in every task. As stated for EEG study, NBM-lesioned and aged animals were divided in the same groups of treatment. According to the guidelines for care and use of experimental animals, and with the purpose of reducing the number of animals used, for object recognition test (ORT) and passive avoidance (PA), the same group of rats were sequentially used,

whereas for MWM and T-maze each group was performed only one of the task.

2.4. Surgical procedures

In order to perform the recording of EEG cortical activity, the animals, anesthetized with intraperitoneal chloral hydrate (400 mg/kg) were fixed in a stereotaxic frame and implanted with four surface electrodes onto the frontoparietal region of the neocortex (two electrodes for each hemisphere; coordinates: AP: 1 and 3 mm posterior to bregma; L: 2 mm from midline), which were fastened to the skull with dental cement. Other two electrodes, placed onto the bone overlying the cerebellum and onto the nasal bone, served as reference and ground respectively. After surgery, all animals were housed for 48 h to allow complete recovery. On the day of the experiment, the animals were placed in the recording cage and left for a minimum of 15 min prior to the recording session.

The bilateral and selective damage of the NBM of the anesthetized rats was stereotaxically performed (coordinates: AP=−0.9 mm from bregma; L=2.6 mm; V=7.4 mm from skull, 10 according to the atlas of Paxinos and Watson, 1980) by intracerebral infusion of excitotoxic AMPA, which was injected (1.3 μg/0.5 μl) with a Hamilton syringe through a Teflon tube connected by an injector (26 gauge, rate of microinfusion 0.1 μl/min). No AMPA was injected to the sham-operated rats. After lesion, all the animals were housed for 2 weeks; thereafter, parts of lesioned animals were stereotaxically implanted with cortical electrodes as described above. Forty-eight hours later, electrocortical activity was continuously monitored, recorded, and quantitatively analysed in both hemispheres. The remaining lesioned animals were used to perform behavioural tasks.

Brain histological preparations were performed to verify the site of injection and to quantify the neuronal loss in the NBM. Briefly, six rats, anesthetized with i.p. chloral hydrate, were transcardially perfused with 50 mL cold phosphate-buffered saline (PBS), followed by 200 mL 40% paraformaldehyde dissolved in 0.1 phosphate buffer (pH=7.4). Brains were then removed and postfixed in the latter solution overnight, followed by 20% sucrose solution in PBS for 24 h. The brains was then mounted on the freezing microtome and 30-μm-thick sections containing the nucleus basalis were cut and processed for ChAT immunohistochemistry. The free-floating sections were first rinsed in PBS and then left overnight in ChAT monoclonal antibodies containing 0.20% Triton-X-100 and incubated at 4 °C for 24 h. The primary antibody was then removed and the section exposed to the secondary antibodies, biotinylated antirat immunoglobulin (lg)G, and then to avidin–biotin–horseradish peroxidase, followed by diaminobenzidine. Stained sections were then examined.

The quantitative analysis of NBM ChAT positive cells was carried out using a computerized image analysis system (Axiophot Zeiss microscope equipped with a Vidas Kontron system). In the NBM cholinergic neurons, in both hemispheres, ChAT immunoreactivity was significantly reduced to 45% ($P<0.01$).

2.5. EEG analysis

In the EEG experiments on intact rats, the number of tested animals was seven for each drug and each dose. Control groups of animals received the same amount of the vehicle alone. Physiological saline was used for the intraperitoneal administration of drugs.

The neocortical-derived EEG activity was amplified (set at 0.3 Hz and 10 kHz), digitized and stored over the total spectral range (0.25 to 16 Hz) by a computerized apparatus (Vega 24, ESA-OTE Biomedica, Firenze, Italy). The EEG signal was continuously monitored and recorded for at least 210 min, and the EEG analysis was performed over the whole spectral range and over pre-selected frequency bands (delta 0.25–3; theta 4–7; alpha 8–12 and beta 13–16 Hz) as well. For each drug treatment five artifacts-free epochs of 4 s each (sampled during complete behavioural immobility of the animals) were selected during 30 min pre-drug (baseline) recording and eight artifacts-free epochs of 4 s each in the following 210 min post-treatment period (at 15, 30, 60, 90, 120, 150, 180 and 210 min), digitized and quantitatively analysed with a Berg-Fourier EEG recorder (ESA-OTE Biomedica) using Fast Fourier Transform (FFT). We averaged four to six EEG samples/hour and analysed averaged epochs exporting power spectral data to a Prism 3.0 graphic software for further analysis. Moreover, averages of the total spectral profile during each hour of EEG recording were processed to analyse absolute and relative power and, finally, peak-frequency into each frequency range was identified. High-voltage spindle (HVS) activity was analysed on epoch of EEG related with complete immobility of the animals. Statistical analysis of the data was performed on the EEG signal amplitude and every range of frequencies comparing each group with control by analysis of variance (ANOVA), followed, if significant, by post-hoc Tukey–Kramer test for multiple comparison. In each group, the baseline EEG activity vs EEG recording, after drug administration, was evaluated. Data are presented as mean±S.E.M.

2.6. Object recognition test

Attention and working/episodic memory were investigated by object recognition tests (ORT) carried out according to Ennaceur and Delacour (1988) and Bartolini et al. (1996). The apparatus consists of a white coloured arena (70×60×30 cm) where rats were trained to discriminate between differently shaped objects (cubes, pyramids and cylinders). The day before testing, the animals were placed in the arena and allowed to explore it for 2 min. The day after, a single session of two trials, separated by an inter-trial interval of 60 min, was carried out. In the first trial (T1), two identical objects were presented in two opposite corners of the arena and rats were left there until a criterion of 20 s of total exploration of the objects was reached; at this point the animal was removed and placed in the home cage. Exploration was defined as directing the nose toward the object at a distance <2 cm and/or touching it with the nose. During the second trial (T2), one of the objects presented in T1 was replaced by another, differently shaped, object and rats

were left in the arena for 5 min. The time spent exploring the familiar (*F*) and the novel object (*N*) were recorded separately and the difference between the two exploration times was taken as the discrimination index ($D=N-F/N+F$). To avoid object and place preference, in T2 a random changing of the carefully cleaned objects and their position in the opposite corners of the arena was done. CPE and AChEIs were administered 15 and 30 min before T1, respectively. Associations of CPE with AChEIs also were administered 30 min before T1. Memory performance evaluation in NBM-lesioned and sham-operated rats started 2 weeks after surgery.

2.7. Passive avoidance task

The experiments of Passive avoidance test (PAT) were performed in a step-through inhibitory avoidance conditioning apparatus (Ugo Basile, Comerio, Varese, Italy) consisting of a box divided into two compartments (30 × 30 × 30 cm each), one illuminated and one dark, separated by a guillotine door. The test consisted of an acquisition and a retention session which were carried out on seven rats for each session. In the acquisition trial, the animal was placed in the illuminated compartment and allowed to explore it for 10 s. Then the guillotine door was opened and the latency to enter the dark compartment recorded. As soon as the animal entered the dark compartment, the door was closed and an inescapable footshock (1.0 mA for 5 s) was given through the grid floor. Afterwards, the rats were recovered into their home cage. In the retention test, performed 24 and 72 h later, the animals were again put into the illuminated compartment and the time elapsed before entering the dark compartment was assessed as step-through latency. A cut off latency of 180 s was assessed for rats that did not pass into the dark compartment. Drugs were i.p. injected in each animal before the acquisition session as follows: CPE 15 min, THA and GAL 30 min before the trial, respectively. Step-through passive avoidance latency evaluation, in acquisition and retention sessions, on NBM-lesioned and sham-operated animals started 2 weeks after surgery, and was analysed by one-way ANOVA followed by a post-hoc Mann–Whitney *U*-test.

2.8. T-maze test

Locomotory activity and spatial working memory were assessed using a T-maze test (T-MT), which consists of a central pathway (45 cm long × 15 cm with 15 cm high walls) containing the start box and two lateral arms (30 × 15 × 15 cm) with the goal boxes; the start box opens into the central pathway of the T-maze via a sliding door. Position and orientation of the maze was not changed during the whole time of the experiments. The rat was allowed first to freely explore the maze for 5 min. After the habituation session, the animals were trained in the maze for two daily trials and then tested for the following 3 days (three trials per day). Animals were initially placed into the start box and, after the door was opened, performed the task visiting each arm. The animal was considered to have fully entered an arm when all its paws were positioned into the arm. Entry into an arm previously visited within any daily trial was scored as error. The inter-trial time was 15 min; during this time the animal was taken off and placed in the holding cage. The total number of arm entries (TTE) (locomotory activity) as well as the spontaneous

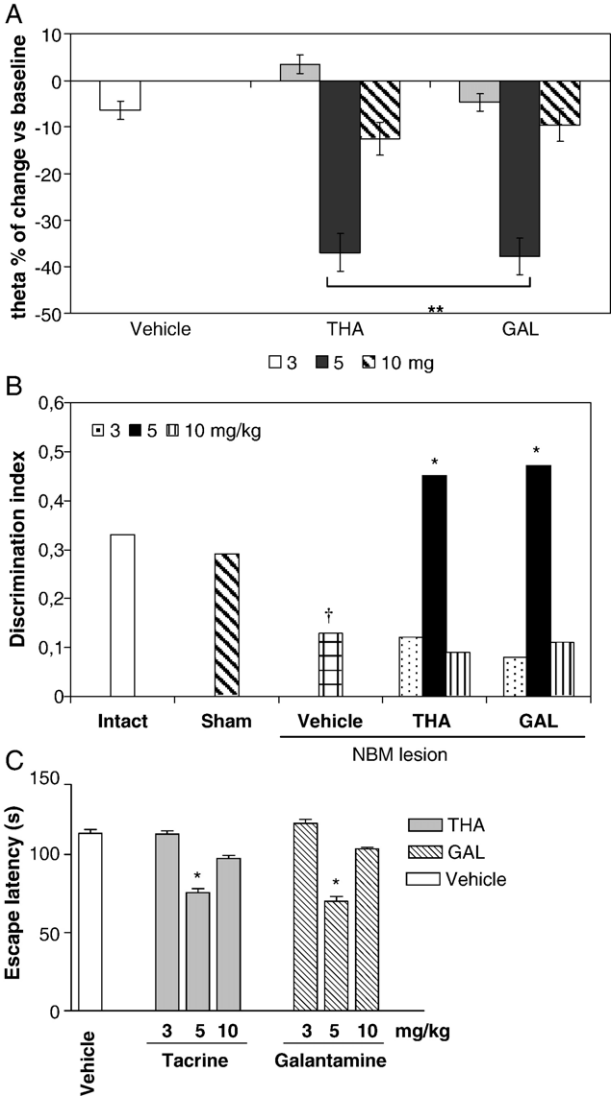

Fig. 1. Dose–response curve of THA (3, 5 and 10 mg/kg) and GAL (3, 5 and 10 mg/kg), after intraperitoneal administration, on EEG activity and performance in learning and memory tasks in NBM-lesioned rats. Groups of seven animals were used for each experiment. (A) Effects of AChEIs on EEG theta component (4–7 Hz) Data are expressed as percent change of EEG activity (mean + S.E.M.) vs baseline. $^{**}p<0.001$ vs vehicle. Statistical analysis of the data was performed on the EEG theta power (ANOVA), followed by post-hoc Tukey–Kramer test for multiple comparison (B) Effects of AChEIs on object recognition performance expressed as discrimination index ($N-F/N+F$, where *F* is the time spent exploring the familiar object and *N* the novel one). In each set of experiments seven animals were employed. Drugs were administered 30 min before trial 1. Two-tailed Student's *t*-test and Tukey–Kramer test for multiple comparison were performed. $^{\dagger}p<0.005$ vs intact vehicle treated rats and sham; $^{*}p<0.001$ vs NBM-lesioned vehicle-treated rats. (C) Effects of AChEIs on Morris water maze test expressed as escape latency (s). Two daily trials for five consecutive days were given. AChEIs were injected 30 min before starting trial 1. Values represent mean + S.E.M. of the total escape latencies for two trials for five consecutive days. $^{*}p<0.01$ vs vehicle. Two-way ANOVA test for repeated measurement was used.

alternation behaviour (defined as successive entries into the two arms, without repetitions), related to spatial/working memory, were scored. Spontaneous alternation performance was defined as the ratio of actual to possible correct alternations multiplied by 100 (SAP%). Statistical significance was performed with ANOVA for repeated measure followed by Tukey–Kramer's post hoc test.

Behavioural assessment for the NBM-lesioned animals and sham-operated control started 2 weeks after surgery.

2.9. Morris water maze test

The spatial memory ability in rats was assessed by an Morris water maze (MWM) apparatus (Morris, 1984), consisting of a swimming pool (diameter 150 cm, height 50 cm) which was filled with cloudy water at room temperature to a depth of 30 cm. The pool was ideally divided into four quadrants of equal area and a glassy, transparent platform (diameter 15 cm), hidden 1 cm below the water surface, was placed at the centre of a quadrant and its location changed every day. The rat was placed in one of four starting locations around the perimeter of the pool, in a quadrant opposite the platform, and the time taken to reach the platform (escape latency) was measured. If the animal failed to do so within 180 s, it was placed on the platform for 20 s and then placed in the home cage. The rat was subjected to two daily trials for five consecutive days with an inter-trial interval of 15 min. The point of entry of the rat in the pool and the location of the escape platform remained unchanged between trials 1 and 2, but were changed every day. The decrease in escape latency from day to day in trial 1 represents the reference long-term memory while that from trials 1 to 2 is consistent with working, or short-term memory. NBM-lesioned animals were trained to find the hidden platform 2 weeks after surgery. The escape latency was measured and recorded, whereas the swimming speed, a parameter reflecting motor activity, was only observed. The escape latency to reach the hidden platform was used as a measure of spatial navigation performance. ANOVA with repeated measurements was used to analyse latency values.

3. Results

3.1. Curve dose–response determination

Dose–response study for pivaloylcholine was previously reported (Rispoli et al., 2004a). A dose–response study was carried out to find the dose of AChEIs which produced the better effects on EEG activity and behavioural performances. In order to use the minimum number of animal as possible, the effects of several doses of drugs on EEG activity, as well as on cognitive performances, were studied only in NBM-lesioned rats. Animals, divided in groups, were treated with THA or GAL (seven animals for group and for each dose) 2 weeks later the surgery. The effects of different doses of AChEIs on learning were assessed by two tasks: attention and short-term memory by object recognition test and working/spatial memory by Morris water maze, respectively. For both THA and GAL, the best dose proved to be 5 mg/kg, i.p., which, while not producing any sign

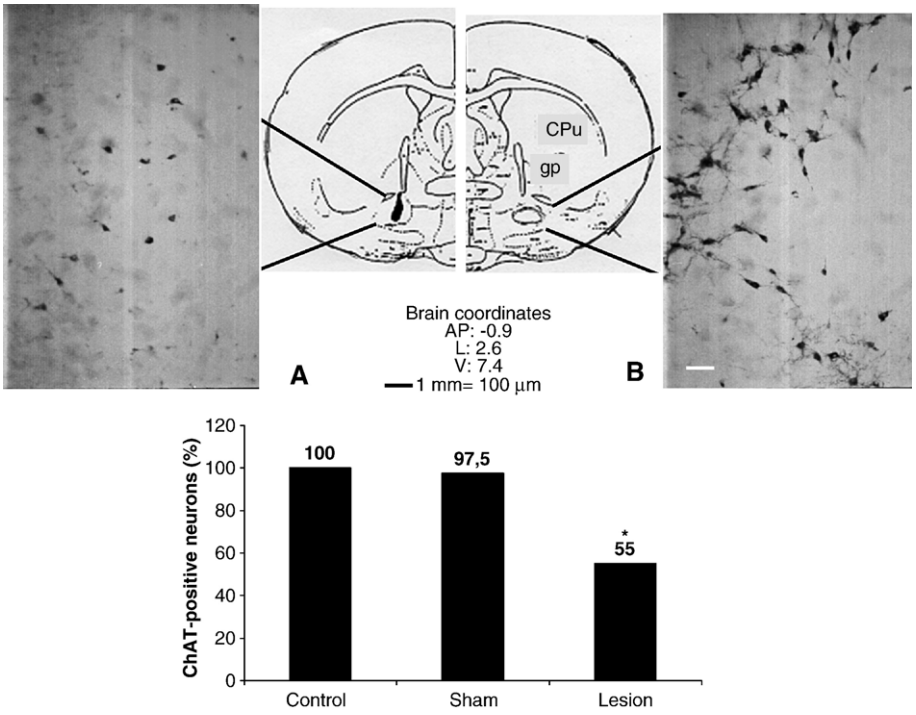

Fig. 2. Low power photomicrographs (40× magnification, 1 mm=100 µm) of a coronal section through the rat NBM. ChAT immunostaining was used to show the neuronal loss in NBM after focal infusion of AMPA (1.3 µg/0.5 µl; rate of microinfusion 0.1 µl/min). Neuronal loss of 45% ($p<0.01$ vs control intact) was observed into the area which projects to frontal cortex. Section in the left panel A depicts neuronal loss in the damaged area (black area in drawing). Section in the right panel B represents the same area of forebrain in sham-operated animals which serves as control. Cpu and gp, are caudate–putamen and pallidus, respectively.

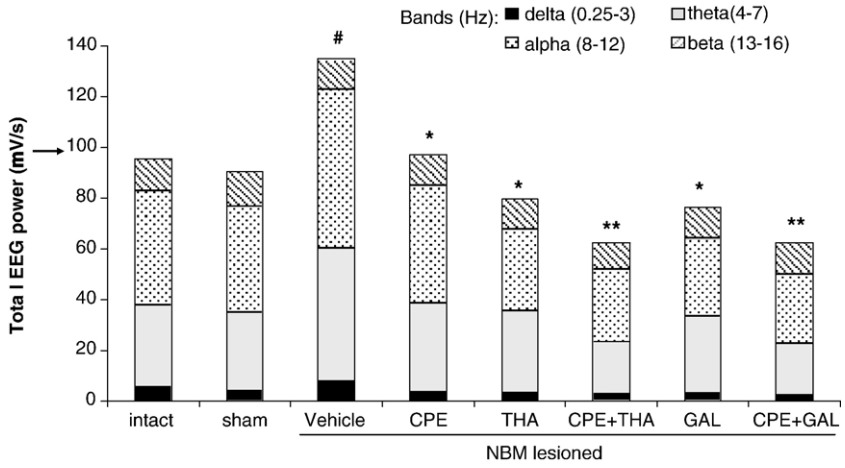

Fig. 3. Quantitative analysis of total EEG spectra (μV/s) obtained from rat frontoparietal cortex of NBM-lesioned rats treated with: CPE (60 μmol/kg), THA (5 mg/kg), GAL (5 mg/kg) alone and CPE (60 μmol/kg)+THA (5 mg/kg), CPE (60 μmol/kg)+GAL (5 mg/kg). Results are the average of the whole EEG power recorded during 210 min. The contribution of every EEG range of frequency is indicated and expressed as total power fraction. Groups of seven rats for each experiment were used. Tukey–Kramer's post-hoc after significant repeated ANOVA measures were used. $^{\#}p<0.01$ vs intact rats control; $^{*}p<0.01$ and $^{**}p<0.001$ vs NBM-lesioned animals.

of cholinergic hyperactivity, was nevertheless able to significantly modify the baseline EEG pattern and produce improvement in learning, recognition and spatial navigation performances. In fact, lower doses (<2–3 mg/kg, i.p.) of THA and GAL neither improved behavioural performance nor changed EEG activity, whereas at higher doses (10 mg/kg), rats exhibit worse behavioural performances, failing in object recognition in ORT and showing a longer escape latency in MWM (Fig. 1). Furthermore, both ChEIs produced EEG single spikes characterized by high-voltage (>200 μV) and in some animals seizures. Moreover, cholinergic overstimulation effects, such as salivation, chromodracriorrhoea, diarrhoea and tremor, were also observed. In accordance with other cognitive enhancers, THA and GAL also displayed a typical bell-shaped dose–response curve (Yoshida and Suzuki, 1993) (Fig. 1).

Table 1
Incidence of neocortical high voltage spindle (HVS) in young intact rats and in NBM-lesioned animals

Group	HVS	
	Baseline	After treatment
Young intact		
Vehicle	9.5±1.9	8±2.3
NBM lesioned		
Vehicle	32±2.3$^{\#}$	28±2.6
CPE	35.5±3.5	20±2.9
THA	34±3.2	18±1.5*
THA+CPE	37±2.9	13±1.6**
GAL	35±3.6	12±3.3*
GAL+CPE	33.5±2.3	13±1.6**

HVS activity was recorded in NBM-lesioned rats before (baseline) and after i.p. administration of CPE (60 μmol/kg), THA (5 mg/kg), GAL (5 mg/kg) and associations of CPE (60 μmol/kg) together AChEIs (5 mg/kg each). The number of animal was seven for each group. Values represent the number of HVS events expressed as mean±S.E.M. ANOVA analysis followed post hoc Tukey–Kramer test was performed. $^{\#}p<0.001$ vs young intact rats; $^{*}p<0.01$; $^{**}p<0.001$ vs baseline.

3.2. EEG effects in NBM-lesioned rats

Neocortical EEG pattern of NBM-lesioned animals showed large amplitude EEG waves and synchronised EEG activity when compared with young intact and sham-operated animals. Indeed, neuronal loss (45%; $P<0.01$) (Fig. 2), was correlated with a significant ($P<0.01$) increase in the energy (μV) of the EEG power as well as in the slow wave frequencies (Fig. 3) and HVS activity ($P<0.001$) (Table 1). Analysis of the spectrum bands showed that the amplitude increased over the whole

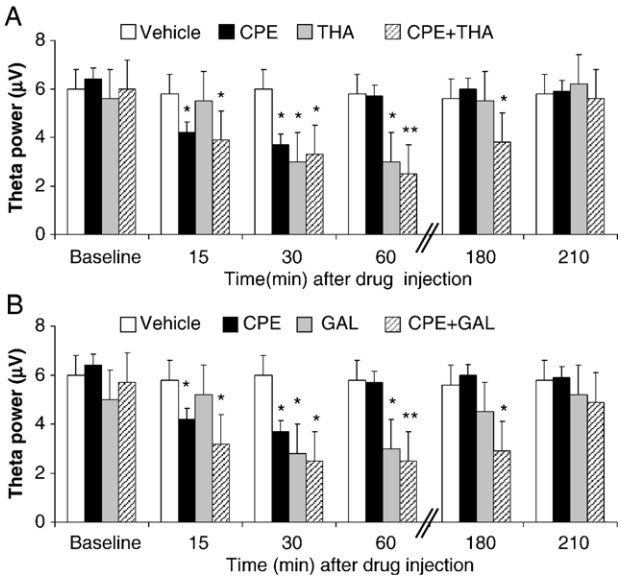

Fig. 4. Time course of the change of theta component (4–7 Hz) of total EEG spectrum in NBM-lesioned rats after i.p. administration of CPE (60 μmol/kg), and: (A) THA (5 mg/kg), CPE (60 μmol/kg)+THA (5 mg/kg); (B) GAL (5 mg/kg), CPE (60 μmol/kg)+GAL (5 mg/kg). Groups of seven rats for each experiment were used. Theta power (μV): mean+S.E.M. vs respective baseline (in all experiments baseline was recorded 30 min before drug administration); Tukey–Kramer's post-hoc after significant repeated ANOVA measures were used. $^{*}p<0.01$ and $^{**}p<0.001$ vs vehicle.

Table 2
Effects on theta component (4–7 Hz) of EEG total spectrum and HVS activity after i.p. administration in aged rats of CPE (60 μmol/kg), THA (5 mg/kg), GAL (5 mg/kg) and associations of CPE (60 μmol/kg) together AChEIs (5 mg/kg each)

	% of EEG theta changes ϑ (4–7 Hz) vs baseline	HVS	
		Baseline	After treatment
Vehicle	−10±2.5	44.5±4.7	41.5±3.9
Scopolamine	+62.5±4.3$^{\#}$	47.5±2.6	72.5±3.3***
Scopolamine+CPE	+15.5±3.6†	39.5±2.5	33.5±1.9
CPE	−30.5±2.6*	45.5±5.3	27.5±3.6*
THA	−32.0±3.9*	41.5±3.3	22.0±2.9**
THA+CPE	−37.5±3.7**	41.5±4.3	18.0±2.3***
GAL	−34.0±2.9*	39.5±3.6	17.5±2.5**
GAL+CPE	−40.0±3.3**	43.5±4.3	19.5±3.9***

Scopolamine (0.2 mg/kg, i.p.) evoked increase in theta band and HVS activity, and was antagonized by CPE (60 μmol/kg). The number of animals was seven for each group. Theta power was the mean value of the whole recording time after drugs administration and was expressed as %±S.E.M. vs baseline, HVS activity was the mean of event number±S.E.M. vs baseline. ANOVA analysis followed post hoc Tukey–Kramer test was performed. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ vs baseline; $^{\#}p<0.001$ vs vehicle; $^{\dagger}p<0.001$ vs scopolamine.

frequency range (0.25–16 Hz) but it was more pronounced at the lower frequencies (0.25–3 and 4–7 Hz) (Fig. 3). The lowest spectrum components in lesioned animals increased as follows: delta 58%, theta 55% vs control intact animals. No significant difference in distribution of EEG activity was recorded in NBM-lesioned animals treated with vehicle when compared with NBM-lesioned and not treated one.

Combined treatment of CPE (60 μmol/kg, i.p.) and THA (5 mg/kg, i.p.) or GAL (5 mg/kg, i.p.) on lesioned animals produced, 15 min after administration, long lasting EEG desynchronisation and a considerable fall ($P<0.001$) in the energy of the total EEG power (Fig. 3). Quantitative EEG analysis revealed a significant decrease ($P<0.001$) in lower frequency bands (4–7 Hz) (Fig. 4A and B) and reduced incidence of HVS ($P<0.001$) (Table 1). EEG effects were observed at least for 180 min. THA and GAL, given alone,

Table 3
Effects of CPE, THA, GAL and their associations on object recognition performance in NBM-lesioned rats

	T2 object		D
	Familiar (s±S.E.M.)	Novel (s±S.E.M.)	
Intact rats	5.2±1.6	10.0±1.3*	0.31
Sham-operated rats	6.0±1.5	12.5±2.3*	0.29$^{\#}$
NBM-lesioned rats			
Vehicle	10.5±1.7	12±1.9	0.09†
CPE	7.3±1.5	14.0±2.3**	0.42$^{\#}$
THA	6.5±1.3	15.3±2.3**	0.48$^{\#}$
THA+CPE	6.3±1.3	17.5±1.7***	0.60$^{\#\#}$
GAL	7.5±1.5	16.3±1.9**	0.50$^{\#}$
GAL+CPE	5.5±1.9	16.3±1.9***	0.65$^{\#\#}$

T2=second exploration session. F=exploration time of the familiar object. N=exploration time of the novel object. D=discrimination index ($N-F/N+F$). In each set of experiments seven animals were employed. CPE (60 μmol/kg) was administered 15 min before trial 1, THA (5 mg/kg), GAL (5 mg/kg), THA+CPE (5 mg/kg+60 μmol/kg) and GAL+CPE (5 mg/kg+60 μmol/kg) were administered 30 min before trial 1. *$p<0.05$; **$p<0.01$ and ***$p<0.005$ N vs F, (two-tailed Student's t-test); $^{\dagger}p<0.001$ vs intact rats; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs lesion and vehicle-treated animals (Tukey–Kramer test for multiple comparison).

Table 4
Effects of CPE, THA, GAL and their associations on object recognition performance in aged rats

	T2 object		D
	Familiar (s±S.E.M.)	Novel (s±S.E.M.)	
Young rats	5.2±1.6	10.0±1.3*	0.31
Aged rats			
Vehicle	15.5±2.6	13.5±2.3	0.15†
CPE	12.5±2.3	21.3±2.6**	0.29$^{\#}$
THA	10.3±1.9	21.5±2.9**	0.32$^{\#}$
THA/CPE	10.5±1.5	24.0±2.3***	0.38$^{\#\#}$
GAL	11.3±1.9	23.5±2.9**	0.34$^{\#}$
GAL/CPE	10.0±1.6	27.3±2.3***	0.39$^{\#\#}$

T2=second exploration session. D=discrimination index ($N-F/N+F$). F=exploration time of the familiar object; N=exploration time of the novel object. In each set of experiments seven animals were used. CPE (60 μmol/kg) was administered 15 min before trial 1, THA (5 mg/kg), GAL (5 mg/kg), THA+CPE (5 mg/kg+60 μmol/kg) and GAL+CPE (5 mg/kg+60 μmol/kg) were administered 30 min before trial 1. *$p<0.05$; **$p<0.01$ and ***$p<0.005$ N vs F; (two-tailed Student's t-test); $^{\dagger}p<0.001$ vs young rats $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs vehicle (Tukey–Kramer test for multiple comparison).

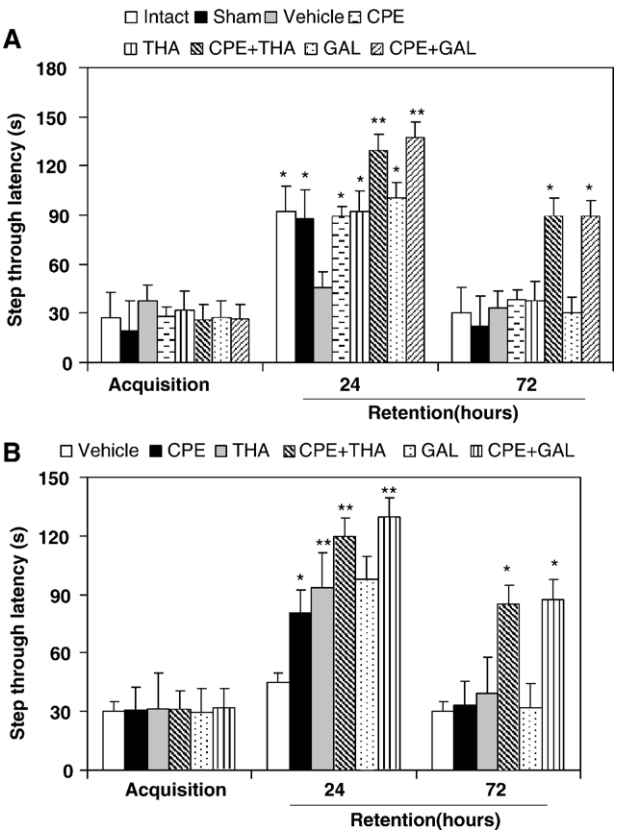

Fig. 5. Effects of CPE (60 μmol/kg), THA (5 mg/kg), GAL (5 mg/kg) and combination of CPE (60 μmol/kg) with AChEIs (5 mg/kg each) on retention of passive avoidance task in (A) NBM-lesioned rats and (B) aged rats. ANOVA was used to compare training escape in acquisition session and retention latency, followed by a post-hoc Mann–Whitney U-test. Groups of seven animals for each experiment were used. Step through latency (s) expressed as mean+S.E.M. *$p<0.01$, **$p<0.001$ vs acquisition and vehicle. All drugs were i.p. injected.

displayed less EEG effects, in terms of latency (30 min vs 15 min after injection) and lasting (about 120 min vs 180), than in combinations with CPE (Fig. 4). In fact, ANOVA analysis showed a very significant Drug × Time [$F_{4,140}=5.35$, $P<0.001$] and Drug × Drug interactions [$F_{30,140}=7.25$, $P<0.0001$].

3.3. EEG effects in 27-month-old rats

CPE alone produced, in aged rats, significant EEG theta band changes and furthermore, it was able to reduce incidence of HVS, in the same way of THA and Gal (Table 2). Administration in aged rats of CPE in association with THA or GAL, shifted EEG large-amplitude and synchronised activity to low amplitude and desynchronisation. Significant changes were found regarding the EEG theta power and HVS incidence (Table 2). The energy of the total power spectrum and delta and theta activity decreased significantly ($P<0.01$) as well as HVS activity ($P<0.001$) (Table 2). EEG changes were observed for at least 150 min. Treatment of aged rats with scopolamine (0.2 mg/kg, i.p.) increased EEG slow frequencies and the incidence of HVS (Table 2). These EEG effects were antagonized by CPE, THA and GAL alone and their association as well (Table 2).

3.4. Behavioural performance assessment

3.4.1. Object recognition test

The effects of single administration of CPE, THA or GAL as well as of their associations, on object recognition in NBM-lesioned and aged rats, are summarized in Tables 3 and 4 respectively. In T1, NBM-lesioned and aged rats, after drug treatments too, did not show significant differences, compared with intact and young animals, in the time need to reach criterion. In T2, intact and young rats were able to discriminate between the familiar and novel objects as shown by the longer exploration time devoted to the latter and the larger discrimination index. Otherwise, NBM-lesioned and aged animals were unable to discriminate between the familiar and novel objects, spending more time than intact and young rats in exploring the familiar object. Administration of CPE together with THA or GAL to lesioned animals was able to restore discrimination between familiar and novel objects, displaying larger discrimination indexes, compared with animals treated with CPE or AChEIs alone. Likewise, after drug treatment, aged rats also showed an increased locomotory activity and a better memory performance, showing enhanced attention in novel objects ($P<0.005$ vs familiars objects), as evident from larger discrimination indexes compared with saline treated animals ($P<0.001$ vs saline).

The results obtained in this task clearly showed the positive effects of combined drugs treatment in restoring episodic memory in impaired animals.

3.4.2. Passive avoidance task

Acquisition and retention scores for NBM-lesioned and aged rats are reported in Fig. 5. NBM-lesioned rats exhibited significantly shorted latency time in retention trial, in comparison with intact and sham-operated animals. The treatment with the single drugs (CPE, THA or GAL) largely prolonged the retention latency times (Fig. 5A). Significantly, a synergistic restoring effect on memory impairment was detected

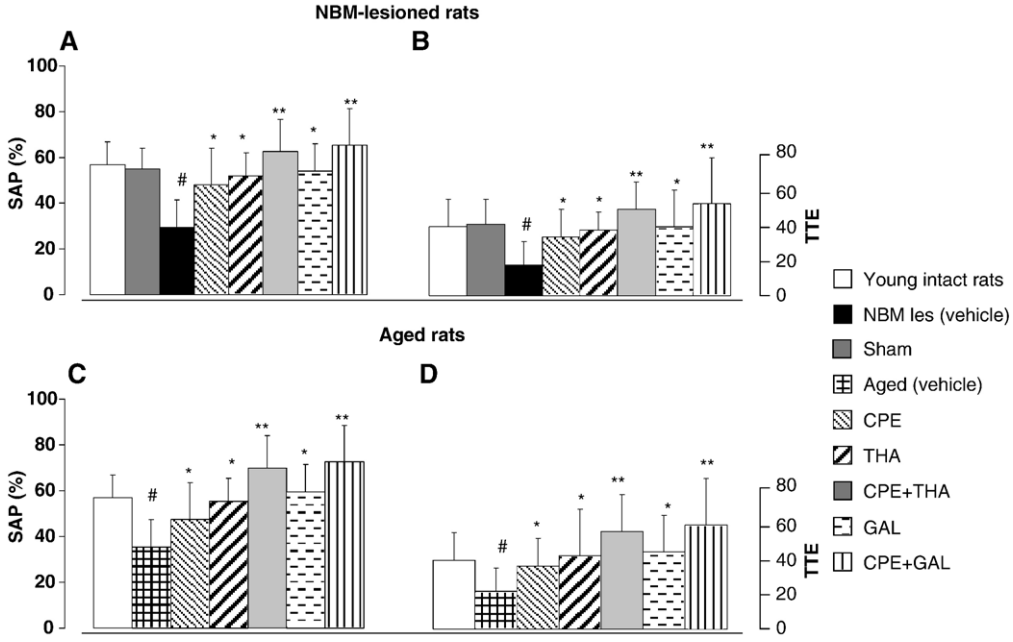

Fig. 6. Effects of CPE (60 μmol/kg), THA (5 mg/kg), GAL (5 mg/kg) and combination of CPE (60 μmol/kg) with AChEIs (5 mg/kg each) in T-maze test on NBM-lesioned rats (histograms A and B) and aged animals (histograms C and D). Short-term memory expressed as spontaneous alternation performance (SAP %±S.E.M.) (A and C); locomotor activity expressed as total number of arm entries (TTE±S.E.M.) (B and D). Statistical significance was performed with ANOVA for repeated measure followed by Tukey–Kramer's post hoc test. Histograms A and B: $^{\#}p<0.05$ vs intact and sham, $^{*}p<0.05$, $^{**}p<0.01$ vs lesioned and vehicle-treated animals. Histograms C and D: $^{\#}p<0.05$ vs young rats, $^{*}p<0.05$ and $^{**}p<0.01$ vs vehicle. Groups of seven animals for each experiment were used. All drugs were i.p. injected.

when CPE was associated with THA or GAL (Fig. 5A). In fact, ANOVA revealed a main effect of drug [$F_{3,21}=21.12$; $P<0.001$] and treatment (lesion vs control) [$F_{5,41}=26.73$; $P<0.001$] and a significant drug×treatment interaction [$F_{28,139}=15.55$; $P<0.0001$]. Interestingly, a significant ($P<0.01$) retention latency was still observed after 72 h from the acquisition session, in animals which received AChEIs in association with CPE, while a lower retention latency was observed when CPE and AChEIs were given alone (Fig. 5A).

Likewise, aged rats showed prolonged retention latency times following CPE or AChEIs single administration and, as expected, the memory performance was increased by combined drugs treatment (Fig. 5B). ANOVA showed an effect of drug [$F_{3,21}=27.56$; $P<0.001$] as well as of treatment (aged vs young) [$F_{5,47}=19.25$; $P<0.001$] and a significant drug×treatment interaction [$F_{33,132}=1.78$; $P<0.001$]. Furthermore, drug associations were able to produce significant prolongation of latency time in aged rats still 72 h after the acquisition session (Fig. 5B). The results obtained in this task demonstrate that CPE, AChEIs and their combinations, administered to NBM-lesioned or aged rats, significantly improved the acquisition of the conditioned response.

3.4.3. T-maze test

The scores obtained by NBM-lesioned and aged rats in the three daily TTE and SAP performances are reported in Fig.

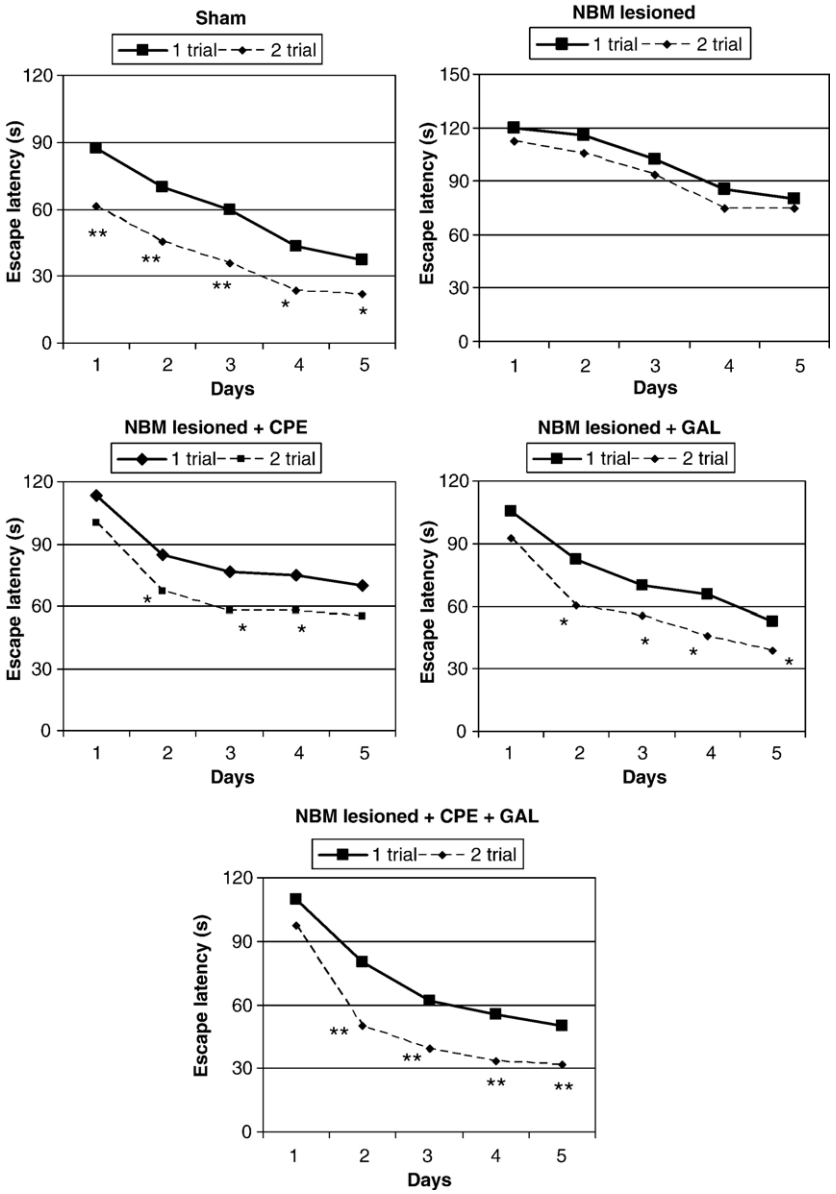

Fig. 7. Effects of CPE (60 μmol/kg), GAL (5 mg/kg) and combination of CPE (60 μmol/kg)+GAL (5 mg/kg) on spatial memory (escape latency to reach the hidden platform) in MWM task in NBM-lesioned rats. Two daily trials for five consecutive days were given. Drug administration produced shortness of escape latencies from trial 1 to trial 2 and, furthermore, reduced day by day in each trial the time to find the platform. Escape latencies were calculated from each of two daily trial average on seven animals for each group. CPE and GAL (and their association) were i.p. administered respectively 15 and 30 min before the first trial of each daily session. ANOVA analysis for repeated measurements were performed on each trial. *$p<0.01$ and **$p<0.001$ vs trial 1.

6A–D. Both lesioned and aged animals showed decrease in spontaneous alternation, in comparison with intact or young rats, respectively. Treatment with single CPE, THA or GAL was able to improve, in both lesioned and aged animals, behavioural performances as proved by the reduction of the number of choice errors in visiting maze arms ($P<0.05$) and the increase of arm entries. Administration of associations of CPE with THA or GAL still more enhanced, in both NBM-lesioned and aged rats, the spontaneous alternation performance ($P<0.01$) and significantly stimulated locomotory activity. The better performances shown in this task by both drug treated NBM-lesioned and aged rats reflect an improved spatial and short-term memory. In fact, the increased total entries in each compartment were estimated as measure of increased locomotory activity, as well as the improved behaviour in alternation opportunities was related to enhanced spatial/working memory.

3.4.4. Morris water maze test

Sham-operated rats trained in spatial navigation show a marked reduction in escape latencies from the first to the second trials and day by day. In contrast, compared with sham-operated rats, NBM-lesioned animals did not exhibit a significant progressive reduction of the escape latency day by day and from the first to second trial ($P<0.01$) (Fig. 7). Combined treatments with CPE and GAL restored, in lesioned rats, spatial navigation performance, showing shorter latency to reach the hidden platform, from days 1 to 5, in comparison with those treated with AChI only. ANOVA (trial by day) with trial and day as repeated measurements showed significant effects of group [$F_{1,4}=26.57$; $P<0.001$] and of group by day [$F_{24,136}=15.37$; $P<0.001$] (Fig. 7). Comparable results were obtained by combined treatments with CPE and THA.

The ANOVA analysis on escape latencies in spatial navigation in both trials on the 1st day revealed a highly significant group effect. Post-hoc Tukey's test showed that NBM-lesioned rats were significantly impaired in their ability to find the platform (Fig. 7).

Compared with young animals, aged rats did show significant increase in escape latency to reach the hidden platform (Fig. 8). The small reduction in escape latency, showed day by day by aged animals, is probably only due to training which improved learning. CPE administered i.p. in association with GAL significantly increased, day by day, in aged rats, spatial navigation performance and memory better than AChEI injected alone (Fig. 8). ANOVA analysis (trial by day) with trial and day as repeated measurements (Tukey–Kramer test for multiple comparison) on escape latency showed significant effects of group [$F_{1,4}=32.25$; $P<0.001$] and group by day [$F_{24,136}=19.55$; $P<0.001$].

In the MWM task, the escape latency is affected by the swimming speed. We found that aged rats tend to swim more

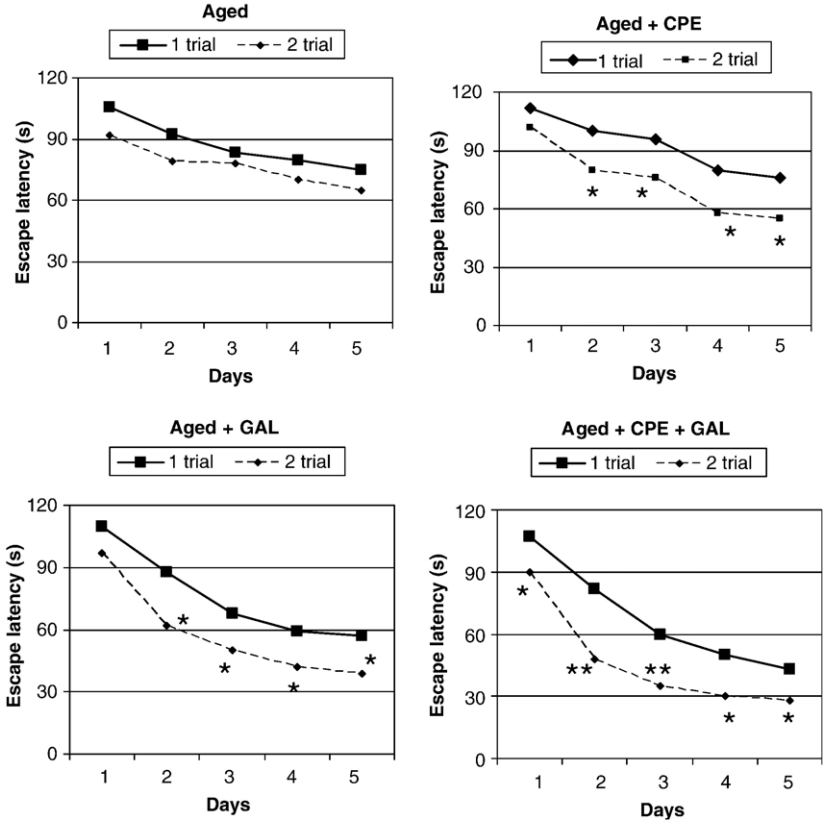

Fig. 8. Effects of CPE (60 μmol/kg), GAL (5 mg/kg) and combination of CPE (60 μmol/kg)+GAL (5 mg/kg) on spatial memory (escape latency to reach the hidden platform) in MWM task in aged rats. Two daily trials for five consecutive days were given. Escape latencies were calculated from the each daily trial average on seven animals for each group. CPE and GAL (and their association) were i.p. administered respectively 15 and 30 min before the first trial of each daily session. ANOVA analysis for repeated measurements were performed on each trial. *$p<0.01$ and **$p<0.001$ vs trial 1.

slowly than young ones, whereas NBM-lesion did not influence rat's swimming speed.

4. Discussion

The present paper reports the results of thorough EEG measurements and several behavioural tasks carried out in order to obtain as much as complete information on the pharmacological effects of the drugs used.

Treatment of aged, as well as NBM-lesioned rats, with CPE, THA or GAL alone, and more significantly with their associations, produced positive EEG changes and improved performance in cognitive tasks. Much more long lasting EEG effects were produced by drug combined treatment than by CPE or AChEIs alone. Indeed, administration of combinations of drugs resulted in more pronounced positive effects on the EEG pattern, reducing HVS activity, EEG slow wave components and, in particular, the theta band.

Furthermore, associations of CPE with AChEIs effectively enhanced attention, improved working/episodic memory performance in recognition of familiar objects test, and increased retention on escape latency in passive avoidance.

It has been demonstrated that object discrimination requires the integrity of cortical cholinergic system in the brain (Scali et al., 1994, 1997b; Bartolini et al., 1996), as well as loss of cholinergic neurons in the NBM produced passive avoidance deficits (Riekkinen et al., 1992). We can hypothesize that restored and improved recognition, showed by both NBM-lesioned and aged rats, is consistent with the relationship existing between the enhanced state of attention and the increased ACh brain levels which betters short-term memory.

Moreover, our results show that, in both aged and NBM-lesioned rats, treatment with drug associations clearly induced a better behaviour in the T-maze task. Indeed, in lesioned and aged rats locomotory activity was increased while choice errors in spontaneous alternation performance diminished, indicating an enhanced spatial working memory. In addition, animals showed also an improved short-term memory after drug treatment.

In the MWM task, the time spent by rats to escape from water was scored to evaluate spatial memory performance. The results revealed that the ability to find the hidden platform, impaired in NBM-lesioned and aged rats, was restored and significantly enhanced by treatment with CPE and AChE inhibitor combinations, which, therefore, showed effectiveness in strengthening spatial memory function.

The impairment in spatial learning shown by old rats compared to young animals supports the hypothesis that the age-related spatial memory deficit should be due to impaired functioning of the septo-hippocampal system. In fact, several studies have shown that hippocampal function may be compromised during ageing (Barnes, 1994; Foster, 1999; Barnes et al., 2000; Erickson and Barnes, 2003).

Surprisingly, less or no difference between NBM-lesioned rats and aged ones were observed in this memory function. Apparently, that could seem in contrast with the opinion that cognitive processes underlying behaviour in NBM-lesioned rats are different from the ones involved during aging. Actually, we selectively destroyed, in young rats, the cholinergic area of the NBM projecting to frontal cortex, producing changes in EEG neocortical activity and impairing arousal and attention. In fact, it is well known that the nucleus basalis controls the functioning of the frontal cortex and play an important role in attention (Jakala et al., 1992; Muir et al., 1994, 1995); consequently, lesions of the nucleus basalis could impair behaviour in young rats even if their septo-hippocampal system was functioning. In fact, since no learning process occurs without attention, spatial learning requires the integrity of both nucleus basalis-frontal cortex and septo-hippocampal cholinergic systems for a correct functioning. Therefore, it is possible that the NBM lesions produce in rats learning dysfunction like that due in aged rats to disruption of the septo-hippocampal system.

The improved performances in all behavioural tasks, such as spontaneous alternation, spatial navigation and acquisition of conditioned avoidance response, as well as attention in object recognition, were correlated with neocortical activation as revealed by EEG activity. Many evidences support that cholinergic forebrain system is strongly involved in the maintenance of desynchronised EEG activity (Semba, 1991; Detari et al., 1999). Pioneering and elegant experimental studies have already shown a substantial relationship among the increased activity of cholinergic neurons in the NBM, the release of ACh in the cortex and EEG desynchronisation. In fact, the amount of ACh, released from the cortex, is high during EEG activation and decrease during slow, synchronised EEG period (Celesia and Jasper, 1966). Accordingly, pharmacological researches have shown that EEG activation is reliably induced by cholinergic agonists and reduced by cholinergic muscarinic antagonists (Funderbuck and Case, 1951; Cuculic et al., 1968; Metherate et al., 1992).

Actually, the reduction in lesioned-rat brain of theta EEG rhythmic activity, observed after treatment with CPE/AChEIs, could be the result of increasing activity of surviving basal forebrain cholinergic neurons (Buzsaki et al., 1988; Cape and Jones, 2000). A modulating role of the basal forebrain cholinergic neurons in the cortical theta activity is indeed well known (Holsheimer, 1982; Borst et al., 1987; Mann et al., 2000). Another consideration must be made: normally, NBM through cholinergic output inhibits the thalamic spindle pacemaker (Steriade and Llinas, 1988; Buzsaki et al., 1988; Steriade et al., 1990; von Krosig et al., 1993). In brain, a reduction in spindling is strongly associated with cortical activation and arousal and attention state (Buzsaki et al., 1988; Steriade et al., 1990). In our experiments, the significant reduction in HVS and increase in arousal state, observed by EEG in NBM-lesioned rats after treatment with AChEIs/CPE, clearly appears related to ACh accumulation in the brain.

The ability shown by CPE in strengthening the effects of AChEIs in restoring EEG architecture and inducing a more pronounced activity in attention and memory tasks, could be of clinical relevance because AChEIs currently used in therapy are

often unable to produce significant benefits. A hypothesis of mechanism of action able to explain the enhancement of pharmacological effects induced by the associations of AChEIs with CPE, could be the following: it is beyond doubt that ACh levels at the synaptic cleft in the brain are increased by AChEIs and, furthermore, that enhanced choline concentration in the brain, following AChE and BChE hydrolysis of CPE, could contribute to increase ACh synthesis and improve the impaired cholinergic activity.

In this regard, a choline role in the neurotransmission has been recently recognised; experimental evidences have clearly shown that choline acts as an efficient and selective agonist of presynaptic and postsynaptic α7 nicotinic acetylcholine receptors (α7nAChRs) (Mandelzys et al., 1995; Papke et al., 1996; Alkondon et al., 1997). These subtypes of functional nAChRs are highly expressed in the basal forebrain cholinergic neurons that project to the hippocampus and the cortex (Alkondon and Albuquerque, 1994; Breese et al., 1997). More importantly, choline activation of α7nAChRs gives rise to significant effects on calcium homeostasis and acetylcholine release, two processes critically involved in cognitive and memory functions.

Furthermore, recent papers showed that choline can regulate the neurons of hypothalamic tuberomammillary (TM) nucleus, via activation of α7nAChRs expressed in such neurons (Uteshev et al., 2003; Uteshev and Knot, 2005). Significantly, recent studies provided strong evidence that hystaminergic hypothalamic TM neurons are involved in cognition, alertness and behavioural functions (Brown et al., 2001; Haas and Panula, 2003).

In this context, it is pertinent to bear in mind that the only essential feature for the α7nACh receptor activity appears to be the charged nitrogen whereas other structural elements are permissive (Papke et al., 1996); therefore, CPE itself could act as an agonist at α7nAChRs. Furthermore, it is important to point out that, recently, it have been reported that agonists for nAChRs increase the synthesis of neurotrophic factors (Belluardo et al., 2000). Lately, the α7nAChRs agonist property of choline has been associated with its ability to offer neuroprotection in many experimental models. Indeed, choline has been shown to protect differentiated PC-12 cells from cytotoxicity induced by growth factor deprivation (Jonnala et al., 2003).

These recent findings prompted us to investigate the relationship between CPE and brain expression of neurotrophic factors. Preliminary data show an increased expression of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in brain of NBM-lesioned animals, after chronic short-term administration of CPE.

Therefore, the main findings of this work demonstrate that the short-term combined treatment with pivaloylcholine ester can strengthen the positive effects on cognitive functions and EEG activity of cholinesterase inhibitors such as THA and GAL, without producing any significant cholinergic hyperreactivity. The last aspect could be of great interest in treatment of neurological disorders, such as AD, in order to have a better and safer pharmacological activity.

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