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Role of molecular isoforms of acetylcholinesterase in learning and memory functions

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Abstract

In the present study, activity of salt soluble (SS) G1 and detergent soluble (DS) G4 molecular isoforms of acetylcholinesterase (AChE) has been investigated in rat brain areas in trained (learned), scopolamine (amnesic) and Tacrine (anti-dementic) treated rats to find out their role in learning and memory functions. AChE was estimated spectrophotometrically at 412 nm in rat brain areas. Isolation and partial purification of molecular isoforms G1 and G4 of AChE was done by gel filtration chromatography.

Passive avoidance was used to test learning and memory functions. AChE activity was altered in both the fractions SS and DS of different brain areas following passive avoidance in control, scopolamine treated, tacrine treated and tacrine treatment in scopolamine pretreated rats. The peak AChE activity obtained in the DS (fraction 9) and the SS (fraction 13) fraction following gel filtration chromatography. On the basis of molecular weight fraction 9 (DS) and 13 (SS) represent the G4 and G1, respectively. The pattern of changes in the AChE activity of G1 isoform (fraction 13 of SS) and G4 isoform (fraction 9 of DS) in brain areas were similar to those of SS and DS fraction, respectively. In hippocampus, AChE activity in the fraction G1 isoform (fraction 13 of SS) was decreased only in tacrine treated rats but AChE activity in the G4 isoform (fraction 9 of DS) was decreased in both trained and tacrine treated rats. Changes in activity of G4 isoform of AChE in hippocampus could be correlated with passive avoidance learning, scopolamine induced deficit in passive avoidance and reversal of scopolamine deficit by tacrine.

Keywords: Acetylcholinesterase; Hippocampus; Learning; Memory; Dementia

1. Introduction

The vast experimental as well as clinical studies clearly indicate an undisputed major role of acetylcholine (ACh) in the regulation of cognitive functions (Blockland, 1996). The therapeutic strategies to combat miseries of cognitive disorder have been aimed to improve ACh activity. Therefore, the cholinergic receptor agonists (muscarinic and nicotinic) and enhancer of endogenous level of ACh (synthesis promoters and inhibitors of its metabolizing enzyme) have been tried to treat senile dementia of Alzheimer type. Among the various approaches attempted, the inhibition of AChE is the most successful one (Giacobini, 1996). The inhibitors of AChE increase the efficiency of cholinergic transmission by preventing the hydrolysis of released ACh, thus making more ACh available at the cholinergic synapse. AChE metabolizes acetylcholine into acetyl Co A and choline. The enzyme AChE has a catalytic triad, which is made up by one glutamic acid, one histidine and one serine residue. Reacting with the histidine residue, the glutamic carboxylic group activates the serine hydroxy group, which then hydrolyses the ACh ester function by nucleophilic attack (Taylor and Lappi, 1975; Shafferman et al., 1992).

AChE exists into different molecular forms which can be distinguished on the basis of their shapes e.g., collagen-

Abbreviations: AChE, acetylcholinesterase; SS, salt soluble; DS, detergent soluble.

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tailed asymmetric forms (An) and globular forms (Gn). The latter is present in the mammalian brain in different multiples of the monomer subunit e.g., monomer, dimer and tetramer. It has also been found that these isomeric forms are differentially localized in the neuron. The G1 form is cytosolic and the G4 form is membrane bound by hydrophobic amino acid sequences or glycophospholipids (Heller and Hanahan, 1972; Inestrosa and Perelman, 1990; Massoulie et al., 1992; Taylor and Radic, 1994). The detergent soluble (DS) and salt soluble (SS) fraction of AChE contain predominantly G4 and G1 form, respectively (Rieger and Vigny, 1976).

In spite of extensive studies on various biochemical aspects of AChE, information on its molecular isoforms of AChE and their involvement in learning and memory functions are quite inadequate. The pattern of AChE activity in rat brain areas differs on the basis of gender and age (Das et al., 2001). We have also found significant changes in AChE activity in rodent brain following ovariectomy (Das et al., 2002a,b) and various type stresses (Das et al., 2000, 2002a,b). Existing cholinesterase inhibitors have their side effects, which limit their therapeutic benefits (Giacobini, 1996). In this context, the use of inhibitors of specific molecular isoform of AChE, might provide a better cognitive enhancer drug. Therefore, the present study was designed to study the activity of molecular isoforms of AChE in the brain regions in rats subjected to passive avoidance test with an aim to determine role of the molecular isoforms of AChE in the learning and memory functions.

2. Methods

2.1. Animals

The experiments were carried out with adult Sprague–Dawley male rats of 3–4 months (wt. 175–200 g). The animals were kept in polyacrylic cage ($38 \times 23 \times 10$ cm) with 1–2 rats per cage and maintained under standard housing conditions (room temperature 24–27 °C and humidity 60–65%) with 12 h light and dark cycle. There were 6 animals in each group. The food in form of dry pellets and water were available ad libitum.

The animals were procured from the Laboratory Animal Services Division of Central Drug Research Institute and experiments were performed according to internationally followed ethical standards and approved by the research ethics committee of Central Drug Research Institute.

2.2. Learning and memory functions

Learning and memory was studied by the single trial passive avoidance test. Avoidance responses were studied in the control, scopolamine (amnesic) and tacrine (anti-dementic) treated groups.

2.3. Passive avoidance test

The rats were subjected to single trial passive avoidance test as described by Brioni et al. (1997). Briefly an animal was placed in the lighted compartment of a computerized shuttle box (Columbus Instruments, Ohio, USA) provided with a software program PACS 30. An automated guillotine door isolated the compartment lighted at intensity of 8 (scale of 0—off and 10—brightest provided in the PACS 30 software) from the dark compartment. After an acclimatization period of 30 s the guillotine door automatically opens and the animal was subjected to a trial of 270 s. An entry into the dark compartment automatically shuts the door and the subject was punished with a single low intensity foot shock (0.5 mA; 5 s). Infrared sensors monitor the transfer from one compartment to another, which was recorded as transfer latency time (TLT) in seconds. The 1st trial is for acquisition and retention is tested by a 2nd trial was given 24 h after the 1st trial. The shock is not delivered in the 2nd trial. The criterion for learning was taken as an increase in the TLT on 2nd trial (retention) as compared to 1st trial (acquisition).

2.4. Administration of amnesic and anti-dementic drugs

In passive avoidance test a reversible amnesia was induced by administering Scopolamine (3 mg/kg, i.p.) was administered 5 min prior to 1st trial (acquisition) to produce a reversible amnesia i.e. impaired learning and memory indicated by no significant increase in the transfer latency time on 2nd trial (after 24 h) as compared to 1st trial.

Tacrine, an anti-dementic drug was studied for its effect on scopolamine amnesia and AChE activity in the brain regions. Tacrine was freshly dissolved in normal saline and was administered intraperitonially (i.p.), at a dose of 1.25, 2.5 and 5.0 mg/kg, 30 min prior to 2nd trial of the passive avoidance test in scopolamine induced amnesic rats.

2.4.1. Study on AChE activity

AChE acivity was assayed in the brain regions of rats not subjected to passive avoidance (control), and all the groups of the rat subjected passive avoidance test. The groups in the later category included learned passive avoidance but received no treatment (trained), treated with scopolamine (amnesic) and tacrine (5 mg/kg, i.p.) in scopolamine treated rats. The ex-vivo effect of tacrine at doses 1.25, 2.5 and 5.0 mg/kg, i.p. on AChE was also studied in control rats for per se effect. The tacrine treated rats were sacrificed 1 h after the administration in control group and 30 min after 2nd trial in the groups subjected to passive avoidance.

2.4.2. AChE Assay in brain

The biochemical study was focussed on the kinetic profile of AChE and its molecular isoforms of AChE in the brain regions.

2.5. Perfusion

Since the enzyme acetylcholinesterase is also present in plasma and erythrocytes, rats were perfused under mild ether anesthesia through heart with pre cooled normal saline (0.9% NaCl) to reduce blood-borne cholinesterase from the brain according to the method described by Edwards and Brimijoin (1982).

2.6. Tissue preparation

The brain was exposed from its dorsal side by incising the skull. The whole brain was then carefully removed from each rat, chilled for 15 min and thereafter, dissected into different regions over ice according to Glowinski and Iversen (1966). Brain was dissected into nine different regions, viz. (1) frontal cortex, (2) cerebral cortex, (3) striatum, (4) hippocampus, (5) thalamus, (6) hypothalamus, (7) pons, (8) medulla and (9) cerebellum.

2.7. Homogenization and extraction of brain AChE

A cocktail of protease inhibitors, leupeptin (40 μg/ml), pepstatin (20 μg/ml), bacitracin (1 mg/ml), benzamidine hydrochloride (2 mM), ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (10 mM), and *N*-ethylmaleimide (5 mM) were added to the each sample preparation to prevent the protein degradation (Silman et al., 1978). A 10% (w/v) homogenate of different regions of brain samples was prepared by homogenizing on ice (30 mmol/l sodium phosphate buffer, pH 7.0) using an Ultra-Turrax T25 (USA) homogenizer at a speed of 9500 rpm thrice at an interval of a few seconds.

2.8. Differential ultracentrifugation

The brain homogenates were centrifuged at 100,000 × *g* at 4 °C in an Beckman Ultracentrifuge (LE 80, USA) using a fixed angle rotor (80 Ti) for 60 min. Supernatant was collected and stored at 4 °C, which constituted the salt soluble (SS) fractions. The pellet was re-suspended in 1% Triton X-100 (1% w/v in 30 mmol/l. Sodium phosphate buffer, pH 7.0) and incubated at 4 °C for 60 min. These samples were then centrifuged at the same speed, i.e., 100,000 × *g* at 4 °C for 60 min. Supernatant was collected and stored at 4 °C, which served as detergent soluble (DS) fractions. This double-extraction method recovered 80–90% of the total AChE activity (Moral-naranjo et al., 1996). Aliquots of the supernatants were diluted in a ratio of 1:10 at the time of activity estimation.

2.9. Enzyme assay

AChE activity was estimated in SS and DS fractions according to the method of Ellman et al. (1961). However, a minor change in final concentration (1 mmol/l) of substrate, acetylthiocholine iodide and chromophore, 5′,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (1 mM), led to a better kinetic profile and henceforth, studies were performed with the modified concentrations, (Das et al., 2000). A kinetic profile of the enzyme activity was studied spectrophotometrically (Shimadzu, USA) at 412 nm at an interval of 15 s. The assay for each sample was run in duplicate and each experiment was performed thrice. One unit of AChE activity was defined as the number of micromoles (μmol) of acetylthiocholine iodide hydrolyzed per minute (min) per milligram (mg) of protein. The specific activity of AChE is expressed in μmoles/min/mg of protein.

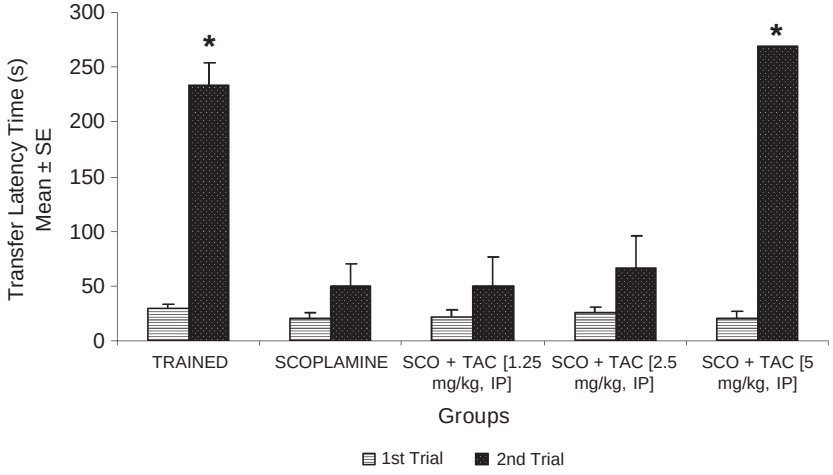

Fig. 1. Transfer latency time in the passive avoidance in trained, amnesia (scopolamine, 3.0 mg/kg, i.p.), scopolamine (SCO)+tacrine (TAC) treated groups. The trained and tacrine (5.0 mg/kg, i.p) group showed significant memory as indicated by increase (*$p<0.01$) in transfer latency time in 2nd trial as compared to from 1st trial.

Table 1
Effect of tacrine in on AChE activity (μmol/min/mg) in DS fraction of different brain regions—frontal cortex (FC), cerebral cortex (CC), striatum (ST), hippocampus (HP), hypothalamus (HY), thalamus (TH), pons (PN), medulla (MD) and cerebellum (CB) in scopolamine (3 mg/kg, i.p.) pretreated (24 h) rats

Region	Control	Tacrine 1.25 mg/kg	Tacrine 2.5 mg/kg	Tacrine 5.0 mg/kg
FC	0.280 ± 0.012	0.314 ± 0.019	0.273 ± 0.011	0.260 ± 0.005
CC	0.195 ± 0.007	0.253 ± 0.020**	0.207 ± 0.006	0.190 ± 0.012
ST	0.706 ± 0.022	0.735 ± 0.042	0.665 ± 0.048	0.659 ± 0.042
HP	0.283 ± 0.006	0.266 ± 0.039	0.257 ± 0.011	0.203 ± 0.013*
HY	0.215 ± 0.016	0.204 ± 0.009	0.201 ± 0.013	0.181 ± 0.010
TH	0.293 ± 0.007	0.367 ± 0.032*	0.317 ± 0.034	0.289 ± 0.013
PN	0.267 ± 0.014	0.259 ± 0.015	0.257 ± 0.008	0.230 ± 0.007*
MD	0.250 ± 0.016	0.253 ± 0.011	0.246 ± 0.005	0.240 ± 0.014
CB	0.138 ± 0.005	0.150 ± 0.006	0.147 ± 0.012	0.134 ± 0.010

Significantly different (*$p<0.05$, **$p<0.01$) from the control group.

2.10. Protein estimation

Protein was measured in all the brain samples, after overnight precipitation with trichloroaceteic acid (TCA), by the method of Lowry et al. (1951) and Wang and Smith (1975) in the salt soluble and detergent soluble fractions, respectively. Bovine serum albumin (BSA) (1 mg/ml) was used as standard and measured in the range of 0.01–0.1 mg/ml.

2.11. Isolation and partial purification of molecular isoforms of AChE

The DS and SS fractions were frozen at −80 °C prior to lyophilize/concentrate in the Freeze dryer (Flexi-Dry™ μP, USA) to obtain a final concentration of approx. 8 mg of protein/ml for gel filtration chromatography.

2.12. Gel filtration chromatography

Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech AB, Sweden) was equilibrated with phosphate saline buffer (PBS, pH 7.0, 50 mmol/l) in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech AB, Sweden) to partially purify AChE from brain homogenate fractions. The void volume (V0) of the column was determined by using Blue dextran, Mr 2,000,000. The concentrated DS and SS fractions were filtered through 0.2 μm syringe filter. An aliquot (200 μL) of filtrate was loaded on the column. The proteins were eluted with PBS at the flow rate of 0.4 ml/min. Fractions of 1.0 ml were collected and AChE activity and protein concentration was estimated in each fraction as described earlier. Approximate molecular size of AChE was determined by using molecular weight protein markers such as: Thyroglobulin, Mr 669,000; Apoferritin, Mr 443,000; Alcohol dehydrogenase, Mr 150,000; Albumin Bovine, Mr 66,000; Carbonic anhydrase, Mr 29,000, which were passed through the same column and eluted under similar conditions. Fractions with high AChE activity were pooled, frozen and concentrated using Freeze dryer (Flexi-Dry™ μP, USA).

2.12.1. Statistical analysis

The data was analyzed by One way analysis of variance (ANOVA) followed by Dunnett's test to determine the significance of difference.

3. Results

3.1. Passive avoidance test

3.1.1. Effect of scopolamine and tacrine in scopolamine pretreated rats on passive avoidance

The transfer latency time was significantly increased on the 2nd trial (233.08 ± 20.34) as compared to 1st trial (28.85 ± 4.73) in control-trained group. But in scopol-

Table 2
Effect of tacrine on AChE activity (μmol/min/mg) in SS fraction of different brain regions—frontal cortex (FC), cerebral cortex (CC), striatum (ST), hippocampus (HP), hypothalamus (HY), thalamus (TH), pons (PN), medulla (MD) and cerebellum (CB) in scopolamine scopolamine (3 mg/kg, i.p.) pretreated (24 h) rats

Region	Control	Tacrine 1.25 mg/kg	Tacrine 2.5 mg/kg	Tacrine 5.0 mg/kg
FC	0.063 ± 0.003	0.059 ± 0.004	0.056 ± 0.008	0.043 ± 0.006*
CC	0.049 ± 0.003	0.054 ± 0.009	0.051 ± 0.004	0.0390 ± 0.005
ST	0.112 ± 0.009	0.113 ± 0.012	0.102 ± 0.011	0.083 ± 0.008*
HP	0.070 ± 0.005	0.081 ± 0.011	0.063 ± 0.003	0.037 ± 0.002**
HY	0.085 ± 0.006	0.075 ± 0.002	0.070 ± 0.006*	0.065 ± 0.004**
TH	0.094 ± 0.005	0.095 ± 0.008	0.089 ± 0.012	0.064 ± 0.004**
PN	0.094 ± 0.003	0.102 ± 0.012	0.087 ± 0.004	0.085 ± 0.013
MD	0.105 ± 0.003	0.103 ± 0.009	0.083 ± 0.012	0.081 ± 0.006*
CB	0.056 ± 0.005	0.078 ± 0.014	0.064 ± 0.006	0.053 ± 0.007

Significantly different (*$p<0.05$, **$p<0.01$) from the control group.

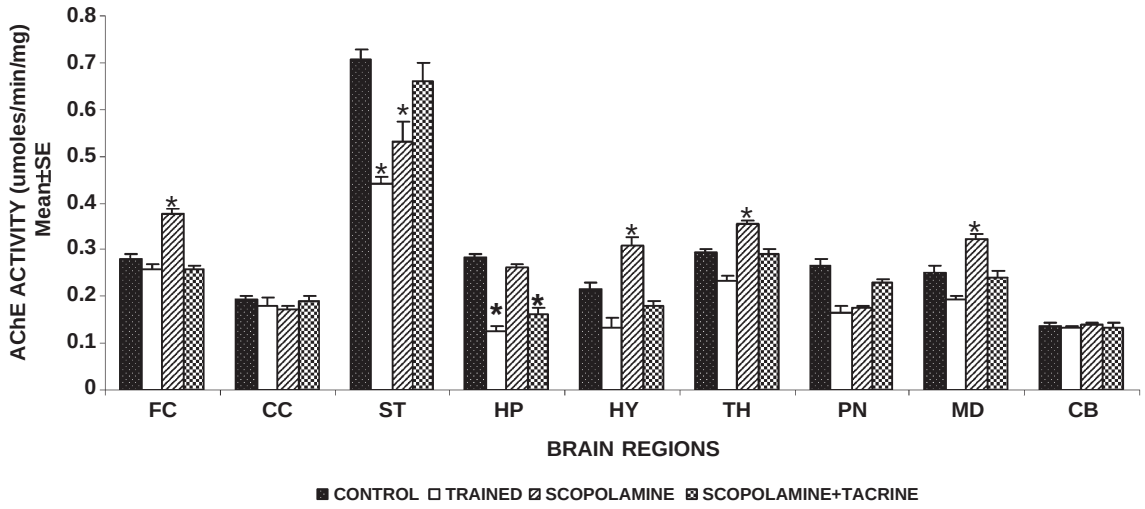

Fig. 2. AChE activity in DS fraction of different brain regions—frontal cortex (FC), cerebral cortex (CC), striatum (ST), hippocampus (HP), hypothalamus (HY), thalamus (TH), pons (PN), medulla (MD) and cerebellum (CB) in control, control-trained scopolamine and scopolamine+tacrine (5.0 mg/kg) groups. *AChE activity is significantly different, $p<0.01$, from control.

amine treated group there was no significant increase on the 2nd trial (39.43 ± 12.11) as compared to 1st trial (20.21 ± 5.19). Tacrine at dose of 5.0 mg/kg, i.p in scopolamine pretreated rats significantly increased the transfer latency time on 2nd trial (270 ± 0.0) as compared to 1st trial (20.6 ± 6.8). However, tacrine at doses 1.25 and 2.5 mg/kg, i.p did not revert back the decreased TLT in rats pretreated with scopolamine (Fig. 1). There was no significant difference in transfer latency time among the 1st trial of different groups. However, transfer latency time of 2nd trial in control-trained and tacrine (5.0 mg/kg) groups was significantly higher than scoplolamine, tacrine (1.25 mg/kg) and tacrine (2.5 mg/kg) groups.

3.1.2. Effect of tacrine on brain AChE activity in scopolamine pretreated rats

Tacrine was administered in doses 1.25, 2.5 and 5.0 mg/kg, i.p. in rats which received scopolamine 24 h earlier and were not subjected to passive avoidance. AChE acivity was assayed after 1 h of tacrine administration. Tacrine (5.0 mg/kg, i.p) group showed significant increase in AChE activity in DS fractions of striatum, thalamus, pons and medulla and decrease in cerebral cortex and hippocampus (Table 1) and decrease in SS fractions in almost all the regions (Table 2) as compared to control group. The lower doses (1.25 and 2.5 mg/kg, ip) of tacrine could not show any significant decrease in AChE activity except in SS fraction of hypothalamus by 2.5 mg/kg dose.

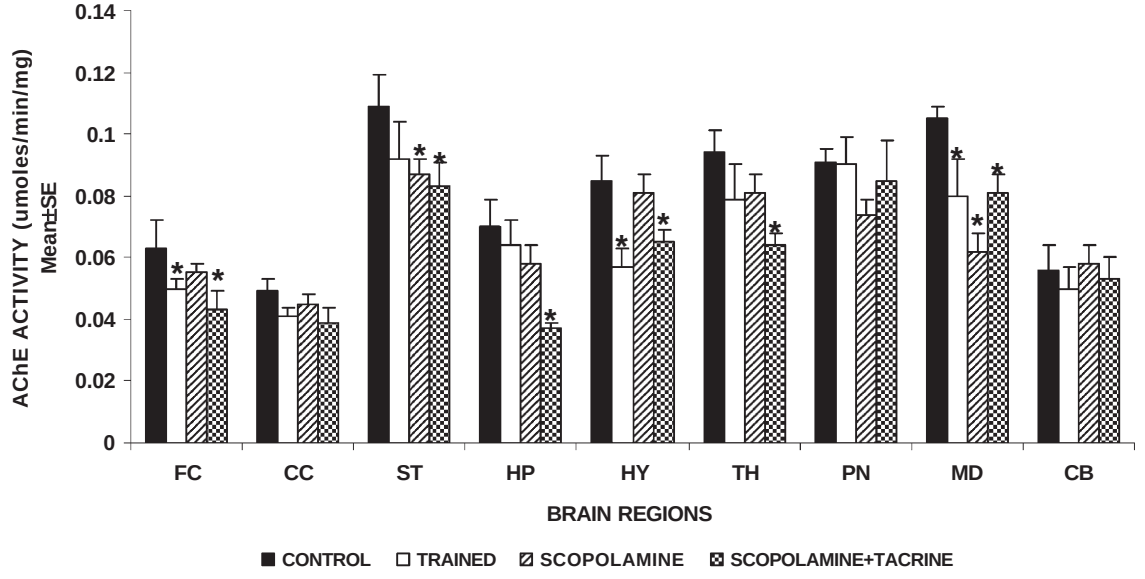

Fig. 3. AChE activity in SS fraction of different brain regions—frontal cortex (FC), cerebral cortex (CC), striatum (ST), hippocampus (HP), hypothalamus (HY), thalamus (TH), pons (PN), medulla (MD) and cerebellum (CB) in control, control-trained scopolamine and scopolamine+tacrine (5.0 mg/kg) groups. *Significant ($p<0.01$) difference in AChE activity from control group.

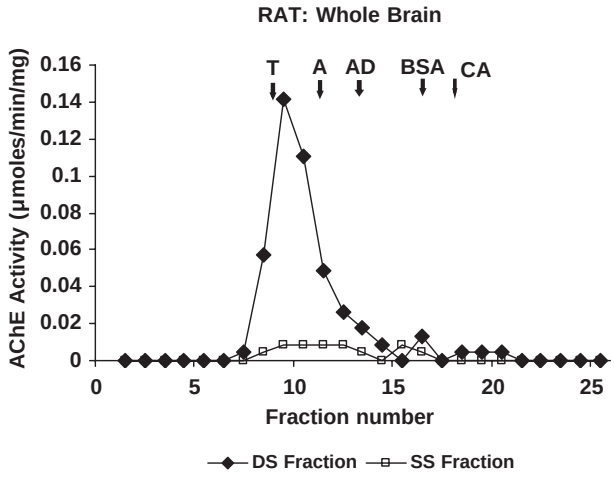

Fig. 4. Elution profile of AChE activity in the DS and SS fraction of rat whole brain is depicted. The arrows denote the position of peak fractions of molecular weight (Mr in kDa) markers: T, thyroglobulin (669); A, apoferritin (400); D, alcohol dehydrogenase (150); B, Bovine Serum Albumin (66) and C, carbonic anhydrase (29).

3.1.3. AChE activity in the brain regions of control, control-trained, scopolamine-amnesia and tacrine treaed amnesic rats in passive avoidance test

Trained group showed significant decrease in AChE activity in DS fractions of striatum and hippocampus and in SS fractions of frontal cortex, cerebral cortex, hypothalamus, thalamus and medulla as compared to control group (Figs. 2 and 3).

Scopolamine amnesia group showed significant increase in AChE activity in DS fractions of frontal cortex, hypothalamus, thalamus and medulla, and significant decrease in AChE activity in detergent soluble fraction of striatum. AChE acivity in SS fraction of striatum, pons and medulla was decreased in amnesic rats as compared to control group.

Tacrine (5.0 mg/kg, i.p.) treated amnesia group showed significant increase in AChE activity in DS fractions of striatum, thalamus, pons and medulla and decrease in cerebral cortex and hippocampus and decrease in SS fractions in almost all the regions as compared to control group.

3.2. Molecular isoforms of AChE

3.2.1. Gel filtration chromatography of DS and SS fractions of AChE from various brain regions

The molecular isoforms of AChE was isolated and identified in the DS and SS fractions of AChE in the various brain regions. AChE activity assay from all the fraction fractions revealed two major peaks, corresponding to fractions 8–10 and 12–14. The highest AChE activity were eluted in fraction number 9 and 13 of DS and SS fractions of whole brain, respectively (Fig. 4). On the basis of Ve/Vo ratio a putative molecular mass of Mr 600,000 and Mr 150,000 was calculated from the protein markers graph for the DS-fraction fraction 9 and SS-fraction fraction 13 of AChE in various brain regions.

This study was performed in the DS and SS fractions of AChE from various regions of control, trained on passive avoidance test, scopolamine (3.0 mg/kg, i.p.) treated (amnesic) and tacrine (5.0 mg/kg, i.p.) treated amnesic adult male rat groups. Specific activities of AChE (µmoles/min/mg) of fraction fraction 9-DS Fraction (Table 3) and 13-SS Fraction (Table 4) showed a significant decrease in almost all the regions except cerebellum in the fraction fraction 13-SS fractions upon tacrine treatment in amnesic rats and also in the hippocampus, cerebral cortex and medulla of fraction fraction 9-DS fraction as compared to control group. In the control-trained group, the AChE specific activity of fraction fraction 9-DS Fraction was significantly decreased in the striatum and hippocampus and fraction fraction 13-SS fractions in frontal cortex, cerebral cortex, hypothalamus, thalamus and medulla as compared to control group. AChE specific activity was significantly increased in the frontal cortex, hypothalamus and

Table 3
AChE activity profile of the fraction number 9 (G4 isoform) of DS Fraction in rat brain regions of control, control-trained, scopolamine and scopolamine+tacrine groups ($n=6$)

Brain regions	AChE activity (µmol/min/mg) Mean ± SE			
	Control	Control-trained	Scopolamine	Scopolamine+ tacrine
Frontal cortex	0.257 ± 0.015	0.314 ± 0.020	0.323 ± 0.011*	0.276 ± 0.013
Cerebral cortex	0.245 ± 0.011	0.361 ± 0.026	0.206 ± 0.020	0.194 ± 0.009*
Striatum	1.804 ± 0.022	1.266 ± 0.023*	2.110 ± 0.096	2.417 ± .063*
Hippocampus	0.385 ± 0.021	0.231 ± 0.016*	0.366 ± 0.024	0.097 ± 0.009*
Hypothalamus	0.321 ± 0.025	0.241 ± 0.032*	0.441 ± 0.020*	0.281 ± 0.029
Thalamus	0.231 ± 0.034	0.296 ± 0.036*	0.658 ± 0.045*	0.631 ± 0.054*
Pons	0.165 ± 0.011	0.156 ± 0.018	0.123 ± 0.009*	0.289 ± 0.018*
Medulla	0.197 ± 0.011	0.189 ± 0.023	0.159 ± 0.011*	0.156 ± 0.010*
Cerebellum	0.170 ± 0.021	0.142 ± 0.023	0.185 ± 0.019	0.156 ± 0.020

Significantly different (*$p<0.05$) from the control group.

Table 4
AChE activity profile of the fraction number 13 (G1 isoform) of SS Fraction in rat brain regions of control, control-trained, scopolamine and scopolamine+tacrine groups ($n=6$)

Brain regions	AChE activity (μmol/min/mg) Mean ± SE			
	Control	Control-trained	Scopolamine	Scopolamine+tacrine
Frontal cortex	0.085 ± 0.005	0.034 ± 0.006*	0.085 ± 0.009	0.017 ± 0.004*
Cerebral cortex	0.084 ± 0.003	0.042 ± 0.006*	0.063 ± 0.010	0.021 ± 0.006*
Striatum	0.307 ± 0.021	0.192 ± 0.032	0.115 ± 0.016*	0.038 ± 0.009*
Hippocampus	0.044 ± 0.004	0.033 ± 0.003	0.033 ± 0.006	0.011 ± 0.002*
Hypothalamus	0.230 ± 0.018	0.038 ± 0.009*	0.192 ± 0.019	0.077 ± 0.011*
Thalamus	0.142 ± 0.016	0.085 ± 0.010*	0.113 ± 0.021	0.056 ± 0.009*
Pons	0.230 ± 0.015	0.192 ± 0.018	0.115 ± 0.011*	0.113 ± 0.010*
Medulla	0.186 ± 0.021	0.111 ± 0.010*	0.037 ± 0.009*	0.074 ± 0.011*
Cerebellum	0.152 ± 0.023	0.191 ± 0.032	0.201 ± 0.033	0.150 ± 0.022

Significantly different (*$p<0.05$) from the control group.

thalamus of fraction fraction 9-DS fraction and decreased in striatum, pons and medulla of fraction fraction 13-SS fractions in amnesia as compared to control groups.

4. Discussion

The central cholinergic neurons are important in the acquisition and post-acquisition (consolidation) performance of a variety of learned behaviors. There is substantial clinical evidence that muscarinic receptor blockade by drugs like scopolamine results into disruptions of behavioral inhibition, working (short term) memory, retrieval from reference (long term) memory, attention, decisional processes, movement and strategy selection, and altered sensory processing (Fibiger, 1990). These alterations are the major characteristic of dementia (Bartus et al., 1982). Cholinergic enhancers particularly anti-cholinesterase drugs are mainstay of anti-dementia drug therapy at present (Enz et al., 1993; Giacobini, 1996; Siddiqui and Levey, 1999).

In passive avoidance test, animals on exposure to first trial acquire the information that entry into dark chamber results into noxious experience of electric shock and cognitive ability of the animal is reflected in avoiding the entry into dark chamber (a judgment based on successful retention and recalling of the acquired information). Scopolamine administration prior to first trial (acquisition) resulted into impaired learning (amnesia) i.e., no significant increase in the transfer latency time on second trial (retention). In active avoidance test, it was observed that the consolidation process of learned task rather than the recalling was more vulnerable to the effect of scopolamine (Das et al., 2003).

In scopolamine treated rats, administration of tacrine, an anticholinesterase anti-dementic drug used clinically in the patients of Alzheimer's disease, prior to retention trial (2nd trial) reversed the effect of scopolamine. These animals showed successful retention and recalling of the acquired information (learning and memory) as indicated by an increase in the transfer latency time in a dose dependent manner.

To establish co-relationship between different molecular isoforms of AChE and learning and memory function, AChE activity was determined in the DS and SS fractions of various brain regions in scopolamine and tacrine treated rats. In the control group AChE activity profile in the various brain regions was similar to that obtained in the adult male rats in age related study described earlier (Das et al., 2001).

It is pertinent to mention that lower doses of tacrine (1.25 and 2.5 mg/kg, i.p) that were ineffective against scopolamine induced impairment in passive avoidance did not affect AChE activity en large in the brain areas of rats pretreated with scopolamine 24 h earlier but not subjected to passive avoidance test. Effective dose of tacrine (5 mg/kg) caused significant inhibition on AChE activity in DS and SS fraction of brain areas which correlates inhibition of AChE activity with anti-scopolamine effect of tacrine. Therefore, subsequent studies in rats subjected to passive avoidance were conducted with the effective dose of tacrine, i.e. 5 mg/kg.

It appeared that scopolamine treatment resulted into a significant influence on AChE activity during passive avoidance learning. It was interesting to note that scopolamine reversed the activity of DS fraction of hippocampus which was observed in the trained control rats but the AChE in SS fraction remained unchanged in trained as well as scopolamine amnesic rats. In tacrine treated group AChE activity profile for DS as well as for SS fraction was quite different from the scopolamine amnesia. Tacrine was effective in countering the scopolamine-induced changes in DS fraction. After tacrine treatment AChE activity in DS fraction of hippocampus was similar to that of trained group. This observation suggests that the effectiveness of tacrine against scopolamine amnesia may depend upon its inhibitory influence on AChE activity in particularly in hippocampal DS fraction.

To further confirm the role of different molecular isoforms of AChE, the isoforms were partially purified, isolated and identified by gel filtration chromatography. The peak AChE activity obtained in the fraction fraction 9 of the DS Fraction, which corresponds to the molecular weight of

600 kDa on the basis of elution profile obtained with known molecular weight marker proteins (thyroglobulin, 669 kDa; apoferritin, 400 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa and carbonic anhydrase, 29 kDa) in our experimental set up. In SS Fraction, peak activity of AChE was obtained in the fraction fraction 13, which corresponds to molecular weight of 150 kDa. As mentioned earlier the DS and SS Fraction predominantly contain G4 and G1 molecular isoforms of AChE (Rieger and Vigny, 1976). The reported molecular weight of G4 and G1 are 600 and 150 kDa, respectively (Adamsom, 1977). Therefore, on the basis of molecular weight and activity profile of fraction fraction 9 of DS Fraction and 13 of SS Fraction represents the G4 and G1, respectively.

To correlate the activity of G4 and G1 molecular isoforms of AChE with learning and memory functions, the activity profile of fractions of DS and SS Fraction obtained in trained, demented and demented treated with tacrine groups of adult rats. The pattern of significant changes observed in G4 and G1 isoforms in the brain regions in the different groups were of similar pattern to that found in the studies performed with DS and SS fractions in similar groups as described earlier. These results clearly indicates that the changes in the activities of DS and SS fractions observed in different setups described earlier represent the alterations in the activity of G4 and G1 molecular isoforms, respectively.

In these experiments the extraction was performed in the anti-protease medium to preserve the intrinsic pattern of molecular isoforms of AChE present in the intact brain tissue. EGTA serve to inhibit the Ca^{2+} activated protease (Dayton et al., 1976), while *N*-ethylmaleimide acts by alkylating free sulfhydryls on extraneous proteins, which otherwise produce background by reduction of DTNB. Bacitracin has a potent effect in preventing degradation of glucagon by microsomal extracts (Desbuquois et al., 1974). The other two polypeptide inhibitors, leupeptin and pepstatin, in combination, have been shown to be very effective in blocking the action of lysosomal proteases from rat liver, the additional presence of an alkylating agent suppressing proteolysis completely (Huisman et al., 1974; Dean, 1976).

In a recent study conducted by Zhao and Tang (2000) demonstrated the isolation of G4 and G1 molecular isoforms from rat cortex involving size exclusion (gel filtration) chromatography. These workers reported the molecular weights (kDa) of G4 and G1 molecular isoforms as 239 and 68, respectively. The greatest discrepancy which lies in this piece of work is the lack of use of protease inhibitors apart from the use of detergent in the extraction procedure, it may be due to this the molecular weight assigned by them for G4 and G1 are not in correlation to the other reports published earlier (Adamsom, 1977). The lack of use of detergent in the extraction procedure do not extract the G4 isoform is already well known phenomenon.

There are studies which attribute the possibility of changes in the isoform distribution of AChE as a biochemical marker for AD (Atack et al., 1983; Fishman et al., 1986; Younkin et al., 1986; Siek et al., 1990; Arendt et al., 1992; Scheff et al., 1992). But till this date no studies have demonstrated changes of sufficient magnitude or specificity to warrant their use as diagnostic marker. An anomalous AChE isoform has been detected on the CSF of some patients with AD (Navaratnam et al., 1991; Smith et al., 1982), but this isoform was found to be also present in the CSF of other dementias and in many controls (Shen and Zhang, 1993; Shen, 1997).

The change in AChE isoform distribution may be a consequence of several events occuring in the AD brain. The loss of the major G4 isoform correlates with the overall loss of AChE activity observed by histo-chemical staining and is mostly associated with axonal fibers in the cortex (Mesulam, 1995). Thus, the loss of G4 may be a consequence of the cellular and axonal degeneration known to occur in the cortex (Davis and Yamamura, 1978). However, the activity of AChE is increased around the amyloid plaques early in the process of amyloid deposition (Friede, 1965; Ulrich et al., 1990; Moran et al., 1993). The significance of the increase in AChE levels around amyloid plaques is unclear. It has been suggested that AChE associated with Aβ, may accelerate amyloid deposition (Inestrosa et al., 1996), although direct evidence for this hypothesis is still lacking. But in our present study we did not find any effect of amyloid β fragments on the AChE activity in-vitro, however, the regulation at the level of AChE gene expression cannot be ruled out. Luo et al. (1994) had demonstrated that AChE expression can be regulated by intracellular calcium in C2–C12 muscle cells. Also, Sberna et al. (1997) have shown that amyloid peptides can increase AChE levels by stimulating L-type channels, thereby disrupting calcium homeostasis, using P19 embryonal carcinoma cells. There are also reports of calcium-dependent increases in AChE gene expression in brain of stressed mice and in brain slices after exposure to cholinesterase inhibitors.

The profile of activity of AChE isoforms in various brain regions obtained in different setup in this study showed a varied pattern and that is expected because of extensive cholinergic innervation in the brain regions involved in regulation of different type of functions. It seems that changes occurring in AChE activity of both the isoforms in brain areas other than hippocampus are not directly related with learning and memory functions. Inhibited AChE activity could contribute to learning and memory by increasing ACh level. In striatum AChE activity of G4 (fraction 9) was decreased in learned rats but tacrine treated rats it was higher. A higher AChE activity can not be correlated with anti-dementic activity of tacrine. In pons G4 activity was low in tacrine treated as well as in scopolamine amnesic rats while the effect of tacrine and scopolamine are reverse on learning and memory. However, activity of G1 (fraction 13) in frontal and cerebral cortex was decreased in trained and tacrine treated rats but in hippocampus G1 activity in trained rats was unaffected. Since hippocampus

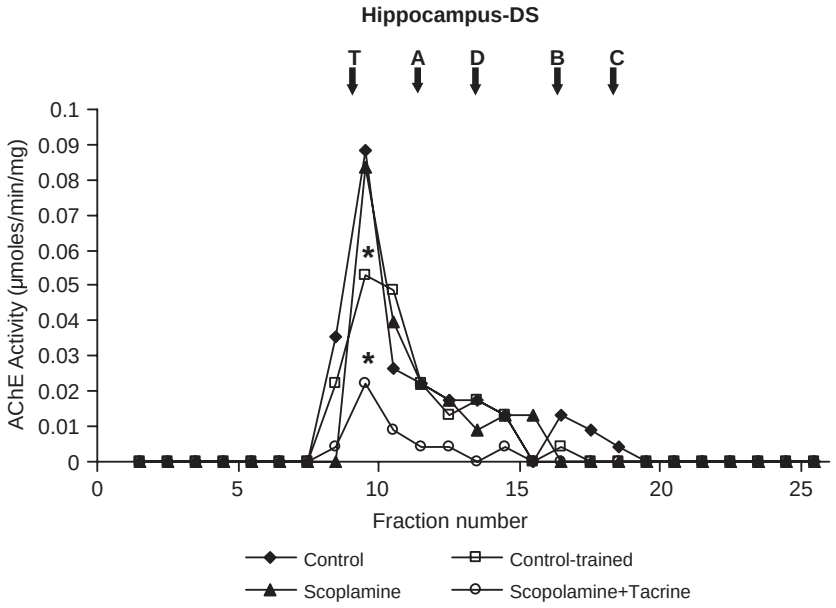

Fig. 5. AChE activity of fraction fraction 9 of DS of hippocampus in control, control-trained, scopolamine and scopolamine+tacrine (5.0 mg/kg) groups. *Significantly ($p<0.01$) decreased from control.

play the key role in learning and memory function, low G1 activity in frontal and cerebral cortex might not be involved directly as a major factor in learning.

As far as learning and memory is concerned the most important area is hippocampus. The lesions in hippocampus in clinical situations e.g. Alzheimer's disease or experimental animal results in cognitive deficits (Fibiger, 1990). The changes observed in AChE activity in hippocampal fractions in this study can also be correlated with learning and memory functions. The activity in DS fraction was decreased following the training that might be contributory factor for learning. Scopolamine, a cholinergic muscarinic receptor antagonist, produces cognitive deficits in human beings very similar to senile dementia and in experimental animals (Fibiger, 1990). Scopolamine in this study also caused impairment in passive avoidance. These scopolamine treated rats did not show decrease in the AChE activity in DS fraction of hippocampus as observed in the trained rats. It seems that during training a decrease in AChE activity (DS fraction) might lead to increase the cholinergic activity to facilitate learning. Tacrine by inhibiting the AChE activity in hippocampus restored cholinergic enhancement and reversed the scopolamine induced amnesia. Among the two fractions, DS fraction seems to play a major role in learning because SS fraction was not affected by training and amnesia in the hippocampus. It was further confirmed in the study following isolation and identification of molecular isoforms of AChE. The activity of hippocampal G_4 (Fig. 5) and G_1 (Fig. 6) isoforms was affected in training, amnesia and tacrine

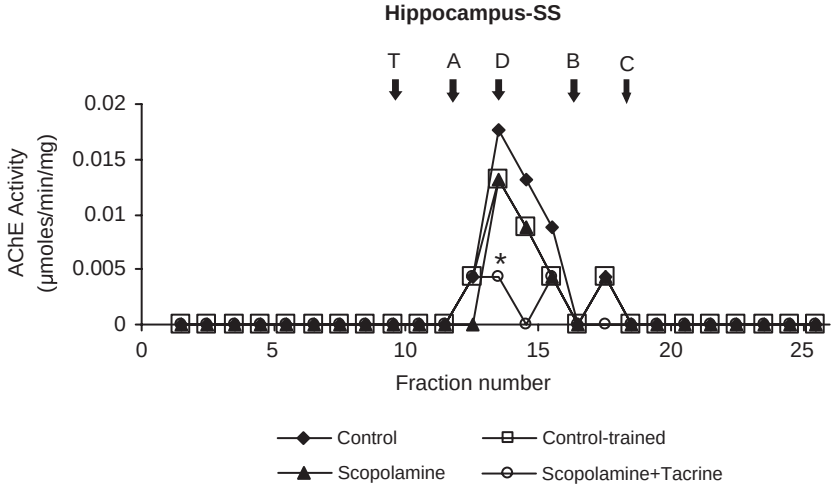

Fig. 6. AChE activity of fraction fraction 13 of SS of hippocampus in control, control-trained, scopolamine and scopolamine+ tacrine (5.0 mg/kg) groups. *Significantly different, $p<0.01$, from control.

treatment in dementic rats in a manner similar to that observed with DS and SS fraction, respectively. Therefore it may be suggested that Hippocampal G_4 isoform seems to play a predominant role in learning and memory, as the changes in activity correlate with events of training, amnesia and anti-dementic treatment.

Thus G_4 isoform of AChE emerges as a potent molecular target for development of effective and safe anti-dementic drug acting as anticholinesterase agents.

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