

Pharmacokinetic and Pharmacodynamic Properties of Cholinesterase Inhibitors Donepezil, Tacrine, and Galantamine in Aged and Young Lister Hooded Rats

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ABSTRACT:

Physiological alterations that may change pharmacological response accompany aging. Pharmacokinetic/pharmacodynamic properties of cholinesterase inhibitors (ChEIs) used in the treatment of Alzheimer's disease, donepezil, tacrine, and galantamine, were investigated in an aged Lister hooded rat model. Intravenous and oral 6-h blood sampling profiles in old (30 months old) and young (7 months old) rats revealed pharmacokinetic changes similar to those in humans with an approximately 40% increase in C_{max} of galantamine and prolonged $t_{1/2}$ (1.4-fold) and mean residence time (1.5-fold) of donepezil. Tacrine disposition was maintained with age, and area under the concentration-time curve and clearance in old rats were similar to those in young rats for all drugs tested as was bioavailability. Old rats showed a trend of increased pharmacodynamic sensi-

tivity (<20%) to ChEIs in cholinesterase activity assays, which was attributed to pharmacokinetic effects because a trend of higher blood and brain concentrations was seen in the old rats although brain/blood ratios remained unaffected. Enhanced cholinergic-mediated behaviors such as tremor, hypothermia, salivation, and lacrimation were also observed in the old rats, which could not be accounted for by a similar magnitude of change in pharmacokinetics. A decrease in expression of muscarinic acetylcholine receptor subtype 2 detected in old rat brains was postulated to play a role. Greater age effects in both pharmacokinetics and pharmacodynamics of donepezil and tacrine were seen in previous studies with Fischer 344 rats, indicating a potential risk in overreliance on this rat strain for aging studies.

Introduction

The world population is aging at an alarming rate with the number of people aged 60 years and older projected to triple from 2000 to 2050, reaching nearly 2 billion (United Nations, World Population Ageing: 1950–2050, <http://www.un.org/esa/population/publications/worldageing19502050>). Elderly individuals, having an increased vulnerability to disease, consume a disproportionately larger share of medicines compared with any other age group. Changes in pharmacological response are known to occur with aging (McLean and Le Couteur, 2004; Hilmer, 2008), and it is vital to understand and

preferably predict and manage them to reduce incidents of adverse drug events or states of poor efficacious response.

The effect of age on pharmacokinetics has been extensively covered by various reviews (Cusack 2004; McLean and Le Couteur, 2004; Turnheim, 2004; Klotz 2009). The decline in glomerular filtration rate with aging is probably the most influential change, affecting excretion of most drugs. Also accompanying aging are decreases in liver size and blood flow, which may reduce hepatic clearance of drugs, but factors such as binding affinity with liver enzymes and plasma/tissue proteins also define drug uptake into liver, resulting in drug-specific alterations in metabolism and bioavailability. Cytochrome P450 enzymes involved in phase I metabolism have shown some dysregulation with age with varying trends in activity reported. Phase II enzymes involved in conjugation reactions, in contrast, are reportedly maintained with aging. Drug distribution is known to change in elderly individuals because of body fat/water composition alterations, and some reports on decreased transporter activity (e.g., P-glycoprotein) in the blood-brain barrier with aging could alter the brain permeability of drugs (Toornvliet et al., 2006; Bartels et al., 2009).

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ABBREVIATIONS: PK, pharmacokinetic; PD, pharmacodynamic; LH, Lister hooded; ChEI, cholinesterase inhibitor; AD, Alzheimer's disease; ACh, acetylcholine; ChE, cholinesterase; F344, Fischer 344; IS, internal standard; LC-MS/MS, liquid chromatography with tandem mass spectrometric detection; MRT, mean residence time; AUC, area under concentration-time curve; TIS, TurbolonSpray; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; mAChR, muscarinic acetylcholine receptor; ANOVA, analysis of variance.

Pharmacodynamic changes with age are less clearly elucidated compared with pharmacokinetic changes. Investigations on pharmacodynamic changes should measure drug concentrations in blood or plasma to ensure that sensitivity alterations are not due to pharmacokinetics. Elderly individuals are generally more sensitive to pharmacotherapy although bidirectional pharmacodynamic alterations have been observed (Mangoni and Jackson, 2003). The central nervous system is especially vulnerable with aging and centrally acting drugs consistently shown to produce sensitized reactions in the elderly independent of pharmacokinetic changes include benzodiazepines (midazolam), opioids (morphine), and anticholinergic tricyclic antidepressants (imipramine and amitriptyline) (Albrecht et al., 1999; Lotrich and Pollock, 2005; Villesen et al., 2007). Mechanisms by which these pharmacodynamic alterations occur are still relatively unclear but are usually attributed to changes in drug-receptor interactions, altered postreceptor signaling, and impaired homeostatic mechanisms (Shi et al., 2008).

Studying and establishing trends in the aged pharmacological response is fraught with roadblocks. A key challenge lies with the heterogeneity in health status of the elderly population and confounding factors such as gender (because a higher proportion of women reach old age than men), pharmacogenetics, and environmental influences (e.g., smoking, diet, and coadministered drugs). The use of aged animal models provides an attractive alternative for studying aged pharmacological responses because of their short life-span and close physiological resemblance to humans. Their functional decline with aging mimics that of humans with some exceptions. Rats, for example, experience a decline in cytochrome P450 enzyme metabolism with age, which does not occur with the same consistency in humans (Schwartz, 2007). In contrast, the decreased glomerular filtration rate seen in elderly humans is not apparent in rats, with the exclusion of some albino strains that undergo chronic progressive nephropathy, the manifestation of which is more dependent on strain and sex than on age (Goldstein et al., 1988; Baylis and Corman, 1998). Nevertheless, prior pharmacokinetic (PK)/pharmacodynamic (PD) studies performed with anesthetics and analgesics in aged animal models have shown good correlation to human responses (Hovinga et al., 1992; Jourdan et al., 2002).

An aged Lister hooded (LH) rat model was used to evaluate the PK/PD properties of cholinesterase inhibitors (ChEIs), donepezil (Ari-cept), tacrine (Cognex), and galantamine (Razadyne) used in the treatment of Alzheimer's disease (AD). AD is a debilitating neurodegenerative disease characterized by cholinergic deficiency, and ChEIs function to alleviate symptoms of AD by preventing the breakdown of the neurotransmitter acetylcholine (ACh). Because of their prevalent use among the elderly, pharmacokinetic information comparing young and aged patients with AD is readily available (Jann et al., 2002; Farlow 2003, Piotrovsky et al., 2003). Similar information from rats has not been obtained. Oral and intravenous PK profiles and PD measurements in terms of cholinesterase (ChE) inhibition and induced cholinergic behaviors were therefore performed in LH aged and young rats to evaluate the potential of this model for prediction of PK/PD changes with age of ChEIs in humans. Results were compared with similar studies done with aged Fischer 344 (F344) rats (Kosasa et al., 1999) to identify any issues with regard to strain selection in aging studies.

Materials and Methods

Drugs and Chemicals. Donepezil hydrochloride and galantamine hydrobromide salts were obtained from Manus Aktveva (Gujarat, India). Tacrine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). A proprietary GlaxoSmithKline compound with characteristics similar to those of

compounds analyzed was used as an internal standard (IS) in LC-MS/MS analysis (GlaxoSmithKline, Harlow, UK). Solvents acetonitrile, methanol, ethanol, and ammonium acetate were obtained from Merck (Darmstadt, Germany).

Animal Maintenance. Young (7 months old, 350–450 g) and old (30 months old, 350–500 g) male LH rats obtained from Harlan (Loughborough, Leicestershire, UK) were approximately 3 months old on arrival. They were housed in groups of three to five in individual ventilated cages at controlled temperature ($20 \pm 1^\circ\text{C}$) and humidity ($40 \pm 2\%$) in a specified pathogen-free facility until time of use. Rooms were on a 12-h light/dark cycle, and animals were allowed ad libitum access to water and food (Teklad Irradiated Global 14% Protein Maintenance Diet, 2914; Harlan). Old animals that developed tumors or signs of disease were not used in the study.

Animals that underwent cannulation surgery for pharmacokinetic studies were housed individually in clear cages and fitted with elastic harnesses attached to a counter-weighted swivel system allowing free movement (Instech Laboratories, Plymouth Meeting, PA). They were monitored daily for weight changes, and their cannulas were flushed with 100 U/l heparinized saline (made with heparin sodium salt from Sigma-Aldrich) to prevent clotting. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (protocol 080342) and Singapore National Advisory Committee for Laboratory Animal Research guidelines for the use and care of animals for scientific purposes and GlaxoSmithKline animal research ethical standards.

Pharmacokinetic Studies of Donepezil, Tacrine, and Galantamine in Old and Young Rats. Animals underwent surgery during which they were dosed subcutaneously with analgesic (Torbugesic, 10 mg/kg, 0.05 ml/100 g) and antibiotic (Baytril, 1 mg/kg, 0.1 ml/100 g) and handmade cannulas (PE50 and PE10 polyethylene tubing; BD, Franklin Lakes, NJ) were implanted into the right jugular and left femoral vein, respectively, under inhaled isoflurane anesthesia. After a day of recovery, rats were given a 1-h intravenous infusion of postfiltered drug solution dissolved in 2% dimethyl sulfoxide (Sigma-Aldrich) and hydroxy- β -cyclodextrin (Acros Organics, Geel, Belgium) (10% w/v in 0.9% saline) at 1 mg/kg and 10 ml/kg/h via the femoral cannula with the aid of a syringe pump. A predose blood sample was taken from the jugular cannula followed by sampling at 15, 30, 45, 60, 65, 75, 90, 120, 150, 180, 240, 300, and 360 min time points. To assess bioavailability, rats went through a 1-day washout period before being orally gavaged (3 mg/kg, 5 ml/kg; 1% methyl cellulose from Sigma-Aldrich as vehicle) with the same drug they were dosed intravenously with before. Blood samples were collected at predose and 30, 60, 90, 120, 180, 240, 300, and 360 min postdose. A blood volume of 120 μl was collected per time point and diluted with an equal volume of water before being kept at -80°C until LC-MS/MS analysis.

Pharmacokinetic Analysis. Pharmacokinetic analysis of the intravenous profiles was performed with WinNonlin software (Pharsight, Mountain View, CA) using a noncompartmental model. Pharmacokinetic parameters obtained included peak concentration in blood (C_{max}), blood clearance (CL_b), volume of distribution (V_d), terminal half-life ($t_{1/2}$), mean residence time (MRT), area under the blood concentration-time curve per unit dose ($\text{AUC}_{(0-t)}/\text{dose}$) with t being 6 h or time of the last quantifiable concentration and $\text{AUC}_{0-\infty}/\text{dose}$ representing AUC per unit dose extrapolated to infinity. AUCs were calculated using linear/log trapezoidal interpolation. Pharmacokinetic analysis for the oral profiles was performed in Microsoft Excel with AUC values calculated by the linear trapezoidal rule. Additional pharmacokinetic parameters include time to reach C_{max} (T_{max}) and bioavailability (F_{po}), which was calculated by the equation

$$F_{\text{p.o.}}(\%) = \frac{\text{AUC}_{\text{p.o.}} \times \text{dose}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}} \times \text{dose}_{\text{p.o.}}} \times 100$$

where $\text{AUC}_{\text{i.v.}}$ was obtained from the prior intravenous profile in the same rat unless stated otherwise.

Terminal Sampling of Brain, Blood, and Plasma for Measurement of Cholinesterase Activity and Drug Concentration. Young and old rats were dosed orally with donepezil, tacrine, galantamine, or vehicle alone (1% methylcellulose) at 3 mg/kg (5 ml/kg; $n = 3$). The rats were decapitated after 1 h, and trunk blood was collected into EDTA-coated tubes (BD). A portion of blood was aliquoted into Eppendorf tubes and centrifuged to isolate plasma.

TABLE 1
 LC-MS/MS conditions for analysis of donepezil, tacrine, galantamine, and IS

	Donepezil	Tacrine	Galantamine	IS
MRM transition	380.10→91.00	198.92→170.31	288.04→213.30	339.18→257.20
Declustering potential (V)	86	81	56	86
Collision energy (V)	63	143	33	39
Collision exit potential (V)	8	16	14	20
HPLC conditions	Cassette analysis (donepezil + tacrine + IS)		Cassette analysis (galantamine + IS)	
Column	Discovery Cyano HPLC column (5 cm × 4.6 mm, particle size 5 μm, Supelco; Sigma-Aldrich)		Hypurity C18 column (5 cm × 4.6 mm, particle size 5 μm; Thermo Fisher Scientific, Waltham, MA)	
Gradient elution conditions ^a	1 → 90% B (0–1.2 min), 90% B (1.2–1.6 min), 1% B (1.61–2 min)		1 → 90% B (0–1.2 min), 90% B (1.2–1.6 min), 1% B (1.61–2 min)	
Injection volume (μl)	3		6	

MRM, multiple reaction monitoring; HPLC, high-performance liquid chromatography.

^a B, organic mobile phase, acetonitrile.

Whole brains were extracted and briefly rinsed with water before being dissected in halves and stored in tubes. All samples were kept at -80°C until LC-MS/MS analysis.

Sample Preparation and LC-MS/MS Analysis. All brain tissues were weighed and homogenized in 1 volume of water with an ultrasonicated homogenizer (TOMTEC Autogiser; Receptor Technologies, Adderbury, Oxon, UK). Calibration standards of each compound were prepared from a 100 μg/ml stock in ethanol at concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml to a final volume of 50 μl. These were spiked with 100 μl of blood (diluted 1:1 with water) or 50 μl of brain homogenate from an undosed rat to account for matrix effects. Likewise, sample volumes of 100 and 50 μl of diluted blood and brain homogenate, respectively, were combined with 50 μl of ethanol in 1.4-ml micronic tubes (Micronic, Lelystad, The Netherlands). Protein precipitation for which 350 μl of IS solution (100 ng/ml IS compound in 80% acetonitrile with 20% 10 mM ammonium acetate) was added to samples and standards was performed. The tubes underwent vigorous shaking for 20 min and centrifugation for 15 min at 3220g and room temperature before LC-MS/MS analysis was performed directly from the supernatant solution. A total blank (without analyte and IS) and blank (without analyte) were injected at the start of the standard line and again at the end to ensure that there was no carryover of analytes. Washes were included between standards and samples and between different sample groups.

For LC-MS/MS analysis, samples were introduced with a CTC Analytics HTS Pal autosampler (Presearch, Basingstoke, Hampshire, UK) to an 1100 series binary pump high-performance liquid chromatography system (Agilent Technologies, Waldbronn, Germany) interfaced with an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray (TIS) interface (Applied Biosystems, Streetsville, ON, Canada). All runs were performed with the column thermostat set to 40°C , an eluent flow rate of 1 ml/min and a 2-min run time, using mobile phases 10 mM ammonium acetate with 0.1% formic acid (solvent A) and acetonitrile (solvent B). The positive ionization mode was used with the following: TIS source temperature, 690°C ; TIS voltage, 5500 V; curtain gas, 20 psi; nebulizing gas (GS1), 50 psi; TIS (GS2) gas, 70 psi; and collision-activated dissociation gas, 4 psi. LC-MS/MS conditions and cassette groupings for the compounds analyzed are listed in Table 1.

Peak quantification was performed in Analyst software (Applied Biosystems, Foster City, CA), and all standards had to pass $\pm 20\%$ acceptance criteria for runs to be accepted. Peak area ratios (analyte/internal standard) were quantified against the calibrated standard concentration line obtained using $1/x^2$ weighted linear regression enabling calculations of the compound levels present in the samples.

ChE Enzyme Activity Assay. ChE activity was measured using the colorimetric assay of Ellman et al. (1961) modified into a high-throughput 96-well plate format by Padilla et al. (1999). Half-brains collected previously from animals dosed with donepezil, tacrine, galantamine, and vehicle were homogenized in 10 volumes of 0.1 M sodium phosphate buffer (pH 8.0) and 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA) (homogenizing buffer). The homogenate was centrifuged for 10 min at 1000g at 4°C , and protein from supernatant was quantified with a BCA protein assay kit (Pierce Chemical, Rockford, IL) to ensure that all samples contained approximately similar protein levels. Supernatant was kept at -80°C in aliquots.

On the day of assay, undiluted plasma (5 μl) or brain preparations diluted $5\times$ with homogenizing buffer (10 μl) were preincubated at 37°C for 10 min with 0.33 M 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich) in 0.1 M sodium phosphate buffer (pH 8.0) (working buffer). In wells in which only AChE activity was to be measured, 10 μl of tetra(monoisopropyl)pyrophosphotetramide (selective BChE inhibitor; Sigma-Aldrich) was included (final concentration in well: 0.1 mM). After preincubation, 10 μl of substrate, acetylthiocholine iodide (Sigma-Aldrich), was added to the wells (final concentration in well: 1 mM). The plate was immediately put in a microplate reader (Infinite F500; Tecan, Männedorf, Switzerland) heated to 37°C and read in a kinetic format with the plate set to shake for 5 s followed by a read at 412 nm, this process repeating every 2 min up to 12 min (six reads). Tissue and substrate blanks were included for every plate that excluded brain homogenate/plasma and acetylthiocholine iodide, respectively. All samples were done in triplicate wells. Final volume in each well was 200 μl.

For calculation of activity, the mean of the slopes (change in absorbance per minute, $\Delta A/\text{min}$) for each triplicate set of samples was taken and converted to micromoles per minute per milliliter (plasma) or micromoles per minute per gram (brain) with the following equation:

$$\text{Activity} = [(\text{average } \Delta A - \Delta A_{\text{blank}}) / \text{min}] / \text{factor} (\Delta A / -\text{SHnmol}) / \text{sample vol (ml)} / \text{sample dilution (1 for plasma, 55 for brain)} / 1000 \text{ (nmol to } \mu\text{mol)}$$

where factor is an instrument-specific calculation factor determined by establishing a free sulfhydryl standard curve that measures ΔA per unit of $-\text{SH}$. BChE activity was calculated by taking total ChE activity minus residual activity in the presence of the specific BChE inhibitor (i.e., AChE activity).

Observation of Cholinergic-Mediated Behavior in Tacrine-Dosed Animals. Young and old animals ($n = 6$) were dosed orally with tacrine at 10 and 30 mg/kg at 5 ml/kg dose volume with 1% methyl cellulose as vehicle that was given alone to a control group of animals. Doses were given in a random fashion and behavior was graded by a person unaware of the treatment administered. Behavioral grading of tremor and lacrimation was performed as shown in Table 2 on a 0 to 3 scale, which was devised previously by Dronfield et al. (2000). Temperature was taken by means of a rectal probe (Bioseb, Chaville, France) inserted by 5 cm into the rectum, which gave a steady reading within 10 s that was promptly recorded. Salivation was measured by collecting saliva on a preweighed cotton bud by wiping the bud in and around the mouth for 6 s and reweighing it. Responses were recorded before dosing and at 0.5, 1, 2, 4, and 6 h after dosing.

Immunoblotting Experiments to Quantify Expression of Muscarinic Acetylcholine Receptor Subtypes in Rat Brains. Six young and six old rat half-brains were dissected free of meninges and white matter before gentle homogenization with a glass and mortar (10 plunges) in $1\times$ CST lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) with protease inhibitors, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma-Aldrich), and one complete mini Roche tablet (Roche Applied Science, Indianapolis, IN). Sample buffer (Laemmli sample buffer; Bio-Rad Laboratories) with 2-mercaptoethanol was then added to the samples, followed by boiling at 100°C for

TABLE 2

Grading scale for cholinergic-mediated behaviors of tremor and lacrimation

	0	1	2	3
Tremor	No tremor	Obvious tremor in two limbs or slight tremor in four limbs	Obvious tremor in four limbs (shaking movements)	Severe tremor in four limbs (vigorous jerking movements)
Lacrimation	No lacrimation	Lacrimation in one eye	Lacrimation in both eyes (watery but not flowing)	Severe lacrimation in both eyes

3 to 5 min to denature proteins. The samples and a Precision Plus Protein Dual Color Standard (0–250 kDa range; Bio-Rad Laboratories) were loaded in 10% polyacrylamide gels, and electrophoresis was performed at 120 V for 1 h. Samples were transferred onto nitrocellulose membranes, and these were blocked in 10 mM phosphate-buffered saline (pH 7.4), 0.1% Tween 20, and 5% skim milk (PBSTM) before immunoblotting with primary antibody in PBSTM with 5% bovine serum albumin at 1:1000 overnight at 4°C. Primary antibodies used for M1 muscarinic acetylcholine receptors (mAChRs) (rabbit polyclonal) were from Alomone Labs (Jerusalem, Israel), and M2–M4 mAChR antibodies (rabbit polyclonal) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After washings in PBSTM and incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), immunoreactive bands on the membranes were detected by enhanced chemiluminescence and quantified by an image analyzer (UVItec, Cambridge, UK). Membranes were then stripped and reblotted with anti- β -actin (1:5000, mouse monoclonal; Sigma-Aldrich) to control for sample loading across lanes. Normalized immunoblot optical densities are expressed in arbitrary units.

Statistical Analysis. Data are presented as means \pm S.D. All statistical analysis was performed using Statistica software (StatSoft Ltd., Bedford, UK). Comparisons between young and aged groups were made using unpaired/

independent *t* tests (two-tailed). A one-way ANOVA followed by a Dunnett's post hoc test was used to compare variance of treatment groups from the control group (cholinesterase activity). For repeated measurements (e.g., cholinergic grading or intravenous/oral pharmacokinetic profiles), a repeated-measures ANOVA was used with planned comparisons made between groups to demonstrate treatment or age effects. $p < 0.05$ was considered significant.

Results

Pharmacokinetic Profiles of ChEIs Dosed Intravenously. Of the three drugs tested, only galantamine showed a notably different concentration-time profile with 1-h intravenous infusion at 1 mg/kg and 10 ml/kg (Fig. 1C). Significantly higher blood concentrations of drug were reached in the old rats from 0.25 to 2 h with a C_{max} 1.4-fold greater than that reached in the young group after which drug concentrations declined to levels similar to those seen in the young rats. For the other drugs, concentration-time profiles were similar between young and old rats, with slight but significant differences in concentrations seen at certain time points (Fig. 1, A and B).

Pharmacokinetic parameters obtained from noncompartmental analysis of the intravenous concentration-time profiles are presented in Table 3.

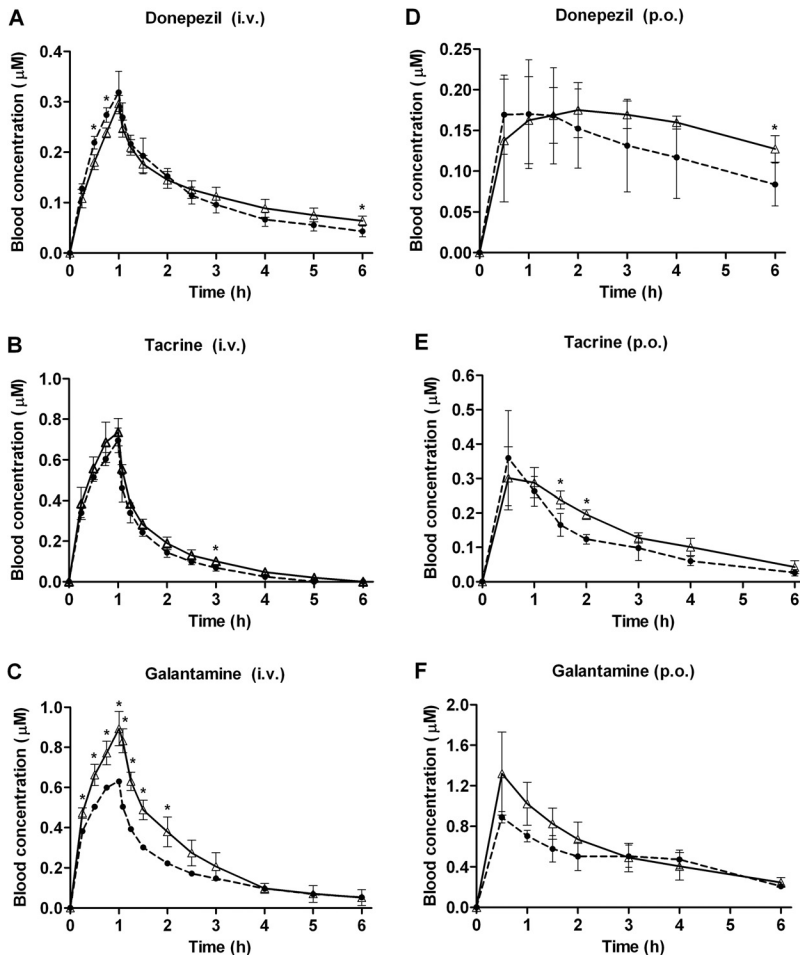


FIG. 1. Blood concentration-time profiles of donepezil, tacrine, and galantamine dosed intravenously in a 1-h infusion (A, B, and C) and orally (D, E, and F) in young (●) and old (△) LH rats. Points represent the mean \pm S.D. ($n = 3$) except for the donepezil old group ($n = 5$) and the galantamine intravenously dosed young group ($n = 2$). A repeated-measures ANOVA was performed, and planned comparisons between young and old groups showed significance ($p < 0.05$) at certain time points (*).

TABLE 3

Pharmacokinetic parameters in blood after intravenous and oral administration of donepezil, tacrine, and galantamine

Values represent means \pm S.D. $n = 3$ except for donepezil old group ($n = 5$). For the galantamine intravenously dosed young group ($n = 2$), both values are shown. T_{max} is expressed as median and range. $F_{p.o.}$ was calculated for galantamine-dosed young rat 3 using average $AUC_{i.v.}$ from young rat 1 and 2.

Parameters	Donepezil		Tacrine		Galantamine	
	Young	Old	Young	Old	Young	Old
Intravenous						
C_{max} (μM)	0.32 \pm 0.04	0.30 \pm 0.02	0.70 \pm 0.06	0.74 \pm 0.07	0.59, 0.69	0.91 \pm 0.07*
CL_b (ml per min/kg)	52 \pm 7	43 \pm 5	93 \pm 9	77 \pm 8	44, 41	34 \pm 6
V_d (l/kg)	9.2 \pm 0.6	11.8 \pm 0.8**	5.1 \pm 0.5	5.2 \pm 0.7	5.5, 5.0	2.9 \pm 0.1**
$t_{1/2}$ (h)	2.5 \pm 0.3	3.6 \pm 0.3**	0.9 \pm 0.2	1.1 \pm 0.1	1.9, 1.9	1.2 \pm 0.4
MRT (h)	3.0 \pm 0.4	4.6 \pm 0.5**	0.9 \pm 0.2	1.1 \pm 0.1	2.1, 2.0	1.5 \pm 0.3
$AUC_{(0-t)}$ /dose (min \cdot kg/l)	15.9 \pm 1.7	16.4 \pm 1.6	10.2 \pm 1.0	12.4 \pm 1.4	20.4, 21.8	28.9 \pm 4.6
$AUC_{(0-\infty)}$ /dose (min \cdot kg/l)	19.5 \pm 2.9	23.7 \pm 3.0	10.8 \pm 1.0	13.0 \pm 1.2	22.7, 24.1	29.9 \pm 5.3
% extrapolation	18 \pm 3	30 \pm 3	5 \pm 2	5 \pm 2	10, 10	7 \pm 3
Oral						
C_{max} (μM)	0.18 \pm 0.06	0.20 \pm 0.02	0.36 \pm 0.13	0.31 \pm 0.08	0.89 \pm 0.06	1.33 \pm 0.40
T_{max} (h)	1.0 (0.5–1.5)	1.0 (0.5–4.1)	0.5 (0.5–1.0)	0.5 (0.5–1.0)	0.5 (0.5–0.5)	0.5 (0.5–1.3)
$AUC_{(0-t)}$ ($\mu M \cdot h$)	0.76 \pm 0.27	0.91 \pm 0.09	0.70 \pm 0.15	0.89 \pm 0.08	2.89 \pm 0.38	3.45 \pm 0.53
AUC_t /dose (min \cdot kg/l)	5.8 \pm 2.1	6.9 \pm 0.8	2.8 \pm 0.6	3.5 \pm 0.3	17.4 \pm 2.3	19.3 \pm 2.6
$F_{p.o.}$ (%)	36 \pm 10	43 \pm 8	27 \pm 7	29 \pm 2	83 \pm 13	67 \pm 10

* $p < 0.05$.

** $p < 0.01$.

Despite the higher C_{max} observed in galantamine-dosed old rats, changes in AUC , $t_{1/2}$, and CL_b were not significant because of the greater variability among the old rats. However, a decrease in V_d in old rats reached significance ($p < 0.01$). In contrast with galantamine, a significantly higher V_d of donepezil was found in the old rats ($p < 0.01$). This was accompanied by a significant prolongation of $t_{1/2}$ and MRT by 1.4- and 1.5-fold, respectively ($p < 0.01$). No significant change in AUC or CL_b was observed. Pharmacokinetic parameters of tacrine showed no significant differences between young and old rats.

It should be noted that larger extrapolations were made for the concentration-time profile of donepezil because it did not reach baseline levels at 6 h. Pharmacokinetic parameters derived should therefore be subject to caution on interpretation.

Pharmacokinetic Profiles of ChEIs Dosed Orally. Oral dosing commonly results in a more variable concentration-time profile because of the differing extents of first-pass effect between animals. This was observed in the oral pharmacokinetic profiles for which higher S.D.s were obtained compared with intravenous pharmacokinetic profiles. Old rats dosed with donepezil and tacrine showed significant but slight increases in blood drug concentrations at certain

time points with a delayed return back to basal levels more so for donepezil than for tacrine (Fig. 1, D and E). A shift in T_{max} can be seen in the average blood concentration-time curves of donepezil but as a prolonged T_{max} (up to 4-fold of the median) was only seen in one old rat of five; the median T_{max} values in both age groups were the same (Table 3). There was a trend of slightly elevated blood concentrations of galantamine in the old rats during the first 2 h after dosing with similar levels attained after 3 h (Fig. 1F). No significant changes were detected with age for all pharmacokinetic parameters obtained, including bioavailability ($F_{p.o.}$) (Table 3).

Cholinesterase Activity in Plasma and Brain of Young and Old Rats. ChE activity levels were 20-fold higher in the brain (12–15 μmol per min/g) compared with those in the plasma (0.4–0.8 μmol per min/ml) (Fig. 2). A major proportion of total ChE activity in brain was derived from AChE (AChE/BChE ratio ~ 12.2), whereas equivalent levels of AChE and BChE were found in plasma (AChE/BChE ratio ~ 1.2). In the aged animals, total plasma ChE activity levels appeared to be significantly enhanced (by $\sim 40\%$), with BChE activity showing a greater elevation (2.1-fold) than AChE activity (1.2-fold) compared with the respective ChE activity in young rat plasma. In

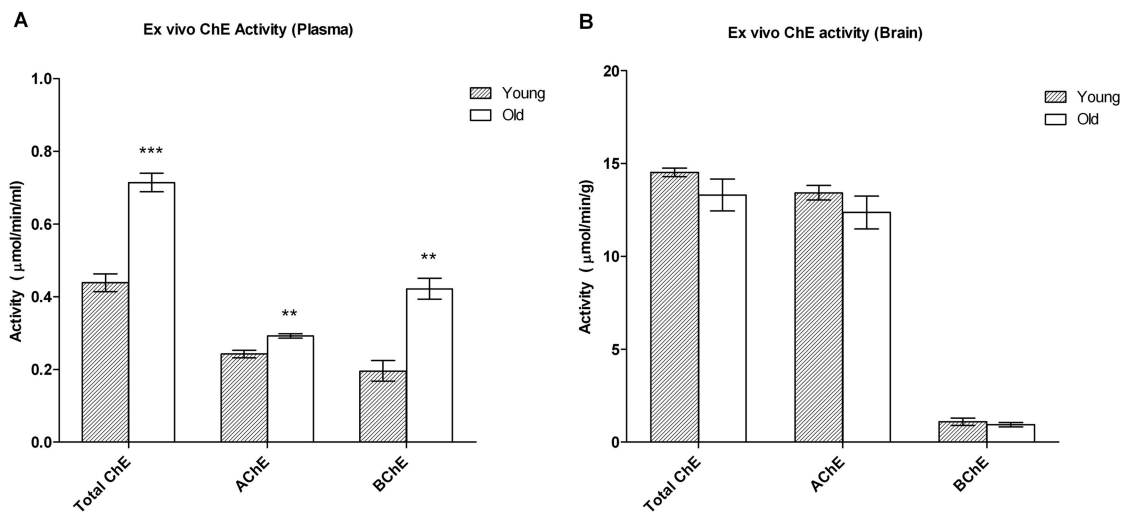


FIG. 2. Total ChE, AChE, and BChE activity in plasma (A) and brain (B) of young and old LH rats. Data are expressed as the mean \pm S.D.; $n = 3$. **, $p < 0.01$; ***, $p < 0.001$.

contrast, brain ChE activity levels were not significantly different between young and aged animals.

Effects of Donepezil, Tacrine, and Galantamine on Cholinesterase Activity in Plasma and Brain of Young and Old Rats. Plasma and brain isolated from young rats 1 h after treatment with the various ChE inhibitors (3 mg/kg) showed significant inhibition of total ChE activity only after donepezil treatment (Fig. 3). This inhibition was greater in the plasma than in the brain (81.6 versus 92.6%). Most of the inhibition of donepezil was derived from AChE inhibition rather than from BChE inhibition. Tacrine was observed to have the greatest BChE-inhibiting effect among the drugs, which is significantly discernible in the plasma rather than the brain because BChE is found at higher levels there.

ChE inhibition showed a trend of enhancement in the old rats for both plasma and brain tissues (Fig. 3). However, absolute ChE activity levels in the plasma remained higher in the old rats than in the young rats (data not shown). The percent enhancement ranged from a 3.2% increase in tacrine-induced brain AChE inhibition to a 10.5% increase in galantamine-induced brain AChE and total ChE inhibition to an 18.0% increase in donepezil-induced plasma total ChE inhibition. Although these enhancements were slight, they led to significant inhibition of total ChE in the old rats, which was previously undetected.

Brain and Blood Concentrations of Donepezil, Tacrine, and Galantamine. Brain and blood concentrations together with brain/blood ratios of donepezil, tacrine, and galantamine at 1 h postdose can be seen in Table 4. Corrected brain concentration refers to measured brain concentration minus residual blood volume present in brain, which is 15 μg as derived from the literature (Brown et al., 1986). Apart from a significantly higher brain concentration of donepezil

detected in the old rats ($p < 0.05$) at a magnitude of 1.8-fold greater than that in the young rats, other changes in concentrations were nonsignificant. However, there was a trend of higher blood and brain drug concentrations observed in the old rats compared with those in the young rats across treatments. Brain/blood ratios were found to be similar in young and old rats for all drugs.

Cholinergic-Mediated Behavior in Tacrine-Dosed Young and Old Rats. To further investigate aging changes in the pharmacodynamic response to ChEIs, a simple assessment of cholinergic-mediated behavior (tremor, hypothermia, lacrimation, and salivation) in response to tacrine administration (oral, 10 and 30 mg/kg) was performed in young and old rats. The results are shown in Fig. 4.

Higher baseline cholinergic activities were observed in the old rats, indicated by the presence of subtle tremor in limbs and consistent hypothermia in vehicle-dosed old rats. For all effects, the response to tacrine was significantly prolonged in the old rats compared with that in the young rats. This was more striking at the 10 mg/kg dose level at which responses in the young rats tended to recover to baseline levels by 6 h, whereas old rats continued exhibiting cholinergic-mediated behavior. With regard to magnitude of response, salivation underwent the most observable enhancement at both dose levels with old rats producing almost 3 times the amount of saliva as tacrine-dosed young rats, the latter of which showed no significant differences in salivation from that of the vehicle-dosed group (Fig. 4D).

At the 30 mg/kg dose level, lacrimation was not seen in the young rats until 2 h postdose, whereas old rats exhibited signs of lacrimation at 0.5 h (Fig. 4C). In contrast, tremor and hypothermia manifested more slowly in the old rats than in the young rats. This was only discernible at the 10 mg/kg dose level at which a significant treatment effect in terms of tremorigenic response was seen at 2 h in the old rats

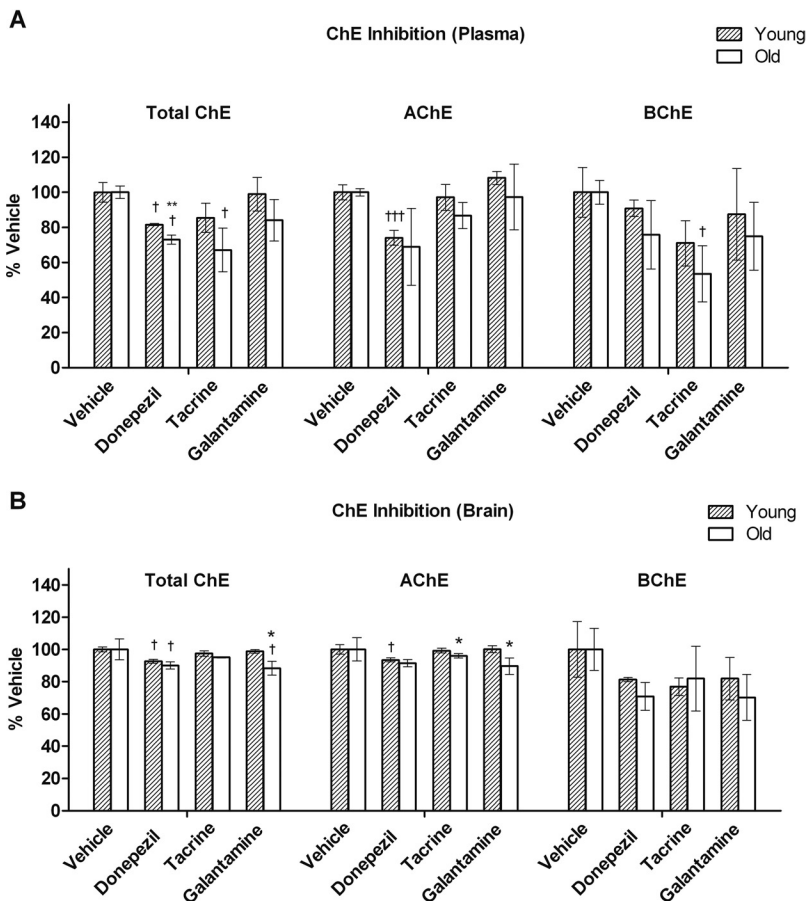


FIG. 3. ChE, AChE, and BChE inhibition in plasma (A) and brain (B) 1 h after oral dosing with donepezil, tacrine, and galantamine represented as percent activity of vehicle-dosed rats. Values are means \pm S.D.; $n = 3$. †, $p < 0.05$ versus the respective control (Dunnett's post hoc test); *, $p < 0.05$ versus the corresponding young group (independent t test).

TABLE 4

Blood and brain concentrations and brain/blood ratios of donepezil, tacrine, and galantamine at 1 h after oral administration

Values represent means \pm S.D.

Drug	Blood Conc.		Corrected Brain Conc.		Brain/Blood Ratio	
	Young	Old	Young	Old	Young	Old
	μM					
Donepezil	0.13 \pm 0.01	0.21 \pm 0.11	0.57 \pm 0.09	1.02 \pm 0.23*	4.4:1 \pm 0.3	5.3:1 \pm 1.8
Tacrine	0.11 \pm 0.03	0.19 \pm 0.12	1.24 \pm 0.30	1.72 \pm 0.93	11.0:1 \pm 1.2	9.5:1 \pm 0.7
Galantamine	0.42 \pm 0.02	0.58 \pm 0.21	0.73 \pm 0.04	1.14 \pm 0.39	1.7:1 \pm 0.2	2.0:1 \pm 0.0

* $p < 0.05$.

($p < 0.017$) compared with 1 h in the young rats ($p < 0.004$). Likewise, a significant decrease in temperature in response to 10 mg/kg tacrine was detected at 1 h in the old rats and at 0.5 h in the young rats.

Expression of mAChR Subtypes in Old and Young LH Rat Brain. Enhanced cholinergic responses may arise from an increase in mAChR-mediated response or decline in mAChR autoregulation of ACh release. Expression of mAChR subtypes in brain was determined for young and old rats by immunoblotting, and results can be found in Fig. 5. It was found that only M2 mAChR expression decreased significantly, whereas M1, M3, and M4 receptor subtypes showed no significant change in expression with age. The expression of muscarinic M2 receptors in the old rats was decreased to a level approximately two-thirds of that in the young.

Discussion

Human studies investigating the effect of age on the disposition of the ChEIs used in this article showed that only donepezil and galantamine underwent significantly different pharmacokinetics in the elderly (Table 5). Prolonged $t_{1/2}$, T_{max} , and MRT of donepezil, attributed to a significantly larger V_d because oral clearance was maintained, was observed in 6 elderly patients (68–82 years) versus 12 young healthy adults (20–27 years) after a single 2-mg oral dose (Ohnishi et al., 1993). For galantamine, C_{max} was 30 to 40% higher in older patients with AD than in healthy younger adults although the AUC and $t_{1/2}$ remained unchanged (Jann et al., 2002). A population

pharmacokinetic modeling study using data from 15 clinical trials revealed a 30% reduction in galantamine clearance with age when healthy subjects (median age 25 years) were compared with patients with AD (median age 75 years), with hepatic impairment having a greater impact on clearance over renal insufficiency (Piotrovsky et al., 2003).

Pharmacokinetic studies of ChEIs performed in old and young LH rats showed no change in tacrine disposition, a significant prolonging of $t_{1/2}$ and MRT of donepezil, and a significant 1.4-fold (~40%) increase in C_{max} of galantamine in the old rats after intravenous infusion of the respective drugs at 1 mg/kg. No differences in clearance or AUC were observed for all drugs, whereas alterations in V_d of galantamine and donepezil were detected in contrasting directions. The smaller V_d of galantamine observed in old rats probably contributed to the greater C_{max} achieved during the initial phase of drug absorption (0.25–2 h) because the decreased distribution into tissue compartments would result in higher blood concentrations. Donepezil, on the other hand, having a larger V_d in the old rats, takes a longer time to penetrate and dissociate from tissue compartments to be subsequently cleared, resulting in a longer $t_{1/2}$ and a prolonged T_{max} seen in one old rat after oral administration. The different directional changes in V_d may be attributed to galantamine having a lower lipophilicity (clogP: galantamine 1.02 versus donepezil 4.60) and slightly higher polar surface area than donepezil (galantamine 41.9 versus donepezil 38.8). Galantamine would therefore associate less extensively to fatty tissue compartments than donepezil and bind to

TABLE 5

Pharmacokinetic properties of donepezil, tacrine, and galantamine in human and changes in PK parameters of the ChEIs with age in humans, F344 rats, and LH rats

Pharmacokinetic Parameters (Human)		Mode of Metabolism/Excretion	Change in PK Parameters with Age?		
			Humans	F344 Rats	LH Rats
Donepezil pMW: 379.504 clogP: 4.60	F : 100%	Hepatic (CYP2D6/3A4);	AUC \leftrightarrow	Brain/plasma concentration \uparrow (at single time point)	AUC \leftrightarrow
	Protein binding: 96% T_{max} : 3–5 h $t_{1/2}$: 70 h V_d : 12 l/kg CL: 0.13 l per h/kg	57% (17% unchanged) in urine, 15% in feces	$t_{1/2}$ \uparrow T_{max} \uparrow MRT \uparrow V_d \uparrow		$t_{1/2}$ \uparrow T_{max} \leftrightarrow MRT \uparrow V_d \uparrow
Tacrine pMW: 198.270 clogP: 3.27	F : 17–37%	Hepatic (CYP1A2); 57% excreted in urine, 23% in feces	\leftrightarrow	Brain/plasma concentration \uparrow (at single time point)	\leftrightarrow
	Protein binding: 55% T_{max} : 1–2 h $t_{1/2}$: 2–4 h V_d : 5.02 l/kg CL: 2.42 l per h/kg				
Galantamine pMW: 287.362 clogP: 1.02	F : 85–100%	Hepatic (CYP2D6/3A4); 95% (32% unchanged) in urine, 5% in feces	AUC \leftrightarrow ($t_{1/2}$ \leftrightarrow) C_{max} \uparrow CL \downarrow	No data available	AUC \leftrightarrow ($t_{1/2}$ \leftrightarrow) C_{max} \uparrow V_d \downarrow CL \leftrightarrow
	Protein binding: 18% T_{max} : 1–2 h $t_{1/2}$: 5–7 h V_d : 2.9 l/kg CL: 0.34 l per h/kg				

pMW, parent molecular weight.

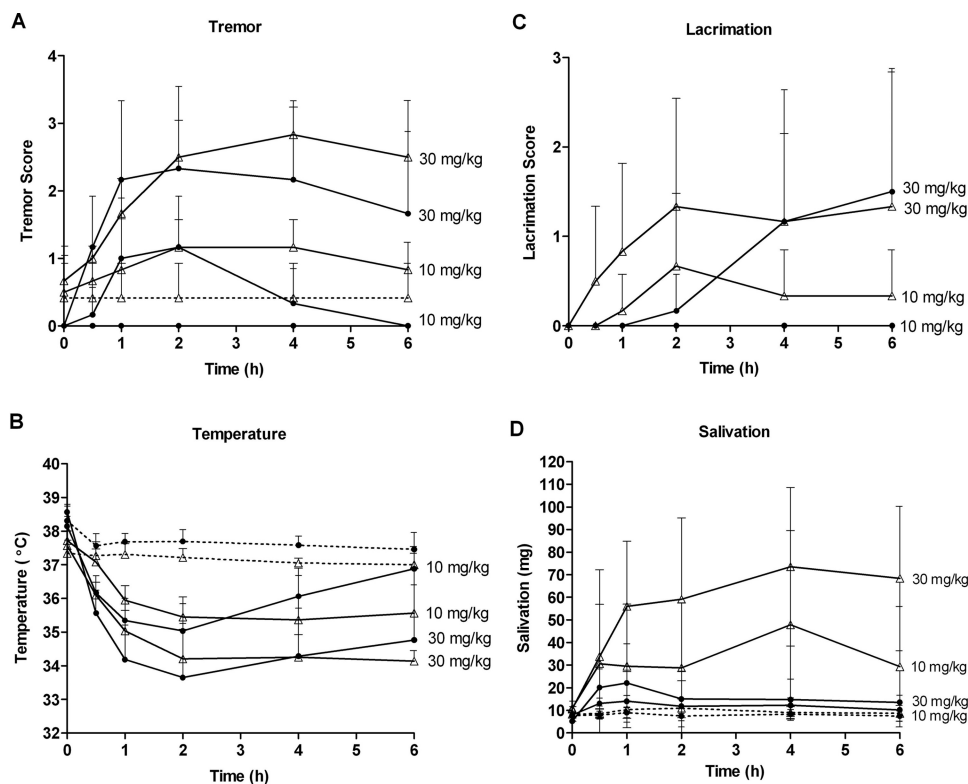


FIG. 4. Cholinergic-mediated behaviors induced by tacrine 6 h after oral dosing at 10 and 30 mg/kg in young (●) and old (△) LH rats. Dashed lines show responses of vehicle-dosed rats. A, tremor. B, rectal temperature. C, lacrimation. D, salivation. Lacrimation scores of vehicle-dosed rats were zero and therefore are not shown. Data represent the mean and above S.D. bars ($n = 6$ and $n = 12$ for vehicle groups).

polar regions with greater affinity. Because body fat content is known to increase with age, whereas body water declines (Beaufrère and Morio, 2000), contrasting directional movements of these drug levels in the blood and tissues may result. Pharmacokinetic parameters obtained from oral profiles of the ChEIs were similar between age groups although old rats exhibited slightly elevated blood concentrations of donepezil and tacrine at certain time points. Calculated bioavailability was maintained with age for all drugs, indicating a sustained metabolic clearance with age of the ChEIs in the LH rat.

The lower clearance of galantamine reported in the elderly group of patients with AD from the study of Piotrovsky et al. (2003), which was not observed in the aged LH rat, may have stemmed from the greater proportion of females used in this group (57.5% as opposed to 25.3% in the young healthy subject group). Females experience a reduced rate of renal and metabolic clearance because these functions are components of body weight (Piotrovsky et al., 2003). The LH aged rat therefore provides a fairly robust model for estimating alterations in pharmacokinetics of donepezil, tacrine, and galantamine in older humans.

To measure pharmacodynamic responses to the ChEIs, ChE activity assays were performed in plasma and brain tissue of LH rats. Without drug interference, ChE activity in plasma was enhanced in old rats by nearly 40%, whereas brain ChE activity remained unchanged. This result was in line with previous findings in F344 rats (Kosasa et al., 1999). In elderly subjects, the plasma BChE activity was enhanced although AChE activity was maintained (Hubbard et al., 2008). Upon dosing with the ChEIs, donepezil exerted the greatest inhibitory effect on total ChE, derived mostly from AChE inhibition. This finding correlates with its higher potency and greater selectivity for AChE over BChE in contrast with tacrine, which has similar affinities for both ChEs, resulting in a greater incidence of peripheral side effects associated with its use (Ogura et al., 2000). Galantamine had the least effect on ChE inhibition because of its lower potency (Geerts et al., 2005). In the old rats, ChE inhibition showed a trend toward enhance-

ment in plasma and brain tissues with some significant differences reported in magnitudes of $<20\%$. Concentrations of the drugs in blood and brain from the same rats were mostly maintained with age aside from donepezil, which was present at 1.8-fold higher concentrations in brain of the old rats. In addition, blood/brain ratios remained unchanged between age groups, indicating a maintenance of blood-brain barrier integrity with age. However, a trend for increased blood and brain concentrations of the ChEIs observed in the old rats makes it difficult to rule out pharmacokinetic changes as a factor for the slightly enhanced pharmacodynamic response seen.

Of interest, prior investigations in F344 rats revealed equivalent plasma but lower brain ChE activity levels compared with those in LH rats (F344 5–8 $\mu\text{mol per min/g}$ versus LH 12–15 $\mu\text{mol per min/g}$). Furthermore, donepezil (2.5 mg/kg) inhibited total ChE activity in brain by 40% at 1 h postdose in F344 rats and enhancement of ChE inhibition by $\sim 30\%$ was seen in old versus young F344 rats (Kosasa et al., 1999). Donepezil concentrations in brain and plasma were 3- to 4-fold higher in the old F344 rats accompanied by increased brain/plasma ratios. These findings demonstrate that differences in aging physiology can occur between strains; F344 rats, for example, undergo chronic progressive nephropathy, which could lead to lower levels of drug clearance. This observation exacerbates concerns regarding the overuse of the F344 strain for aging studies (Weindruch and Masoro, 1991).

Behaviors such as tremor, hypothermia, lacrimation, and salivation induced by tacrine administration result from an overstimulation of mAChRs by ACh accumulation. Prior pharmacological studies using centrally and peripherally acting mAChR agonists and antagonists have established that tremor and hypothermia are centrally mediated effects, whereas salivation and lacrimation are mediated in the peripheral nervous system (Dronfield et al., 2000). The old LH rats exhibited 1) a trend of increased baseline cholinergic responses with consistent hypothermia and the presence of subtle limb tremor, 2) enhanced peripherally mediated cholinergic behavior (sal-

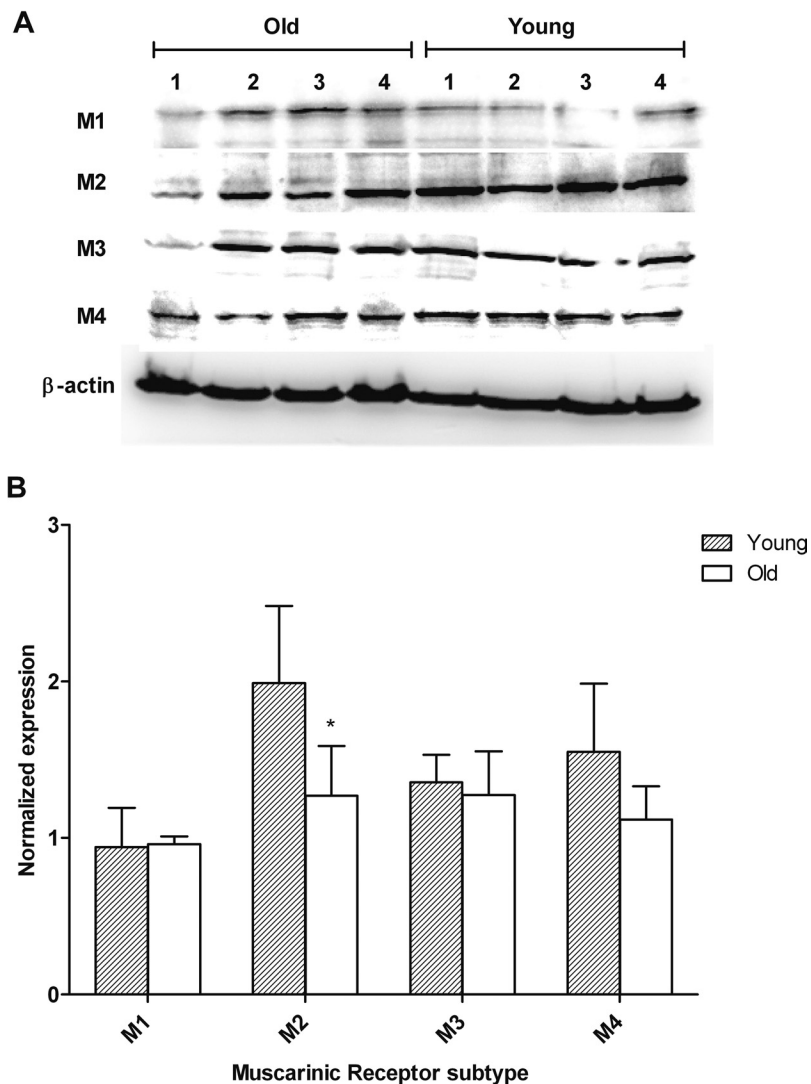


FIG. 5. Muscarinic acetylcholine receptor subtype expression in young and old LH rat brain. A, representative Western blots of M1–M4 receptor subtypes from four old and four young rats. B, graphical representation of normalized expression. Data presented the mean \pm S.D.; $n = 6$. *, $p < 0.05$ versus young group (independent t test).

ivation and lacrimation) and less sensitive centrally mediated cholinergic behavior (tremor and hypothermia) in terms of response time and magnitude of effect during the initial 2 h after dosing with tacrine, and 3) a prolonged cholinergic response with protracted return back to baseline in the older animals after dosing.

Observations 2 and 3 may be ascribed to pharmacokinetics, but prior pharmacokinetic oral profiles of tacrine revealed similar T_{max} (0.5–1 h) in both age groups with blood concentrations in old rats returning to levels similar to those in the young rats after 6 h. A disparity in dose levels in cholinergic behavior studies compared with oral profiles (10 and 30 mg/kg versus 3 mg/kg) could be a possible source of contention because a higher dose can overwhelm clearance mechanisms, leading to greater tacrine accumulation in the bloodstream. Nevertheless, Pedigo et al. (1984) observed that intracerebroventricular injection of oxotremorine (muscarinic agonist) also produced age-related hypothermic sensitivity, suggesting that other mechanisms may be involved.

The decrease in M2 mAChR expression detected in the aged LH rat brain may play a role in the altered pharmacodynamics of tacrine observed in the old rats. From previous knockout studies in mice, the M2 mAChR subtype was identified to be critical for mediating fine whole body tremors and hypothermia (Bymaster et al., 2003). Its age-related decline in expression may therefore account for the slower manifestation and slightly smaller magnitude of central responses in

old rats compared with those in young rats (observation 2). In contrast, M2 mAChRs also act as autoreceptors inhibiting ACh release in the hippocampus and cortex, and its down-regulation could therefore contribute to observations 1 and 3, which manifest at high ACh levels. Of interest, lower baseline body temperatures have similarly been reported in the elderly population, attributed mostly to impaired homeostatic mechanisms (Sund-Levander and Grodzinsky, 2009).

At present, determination of the aged pharmacological response of a drug is achieved through controlled clinical trials before it enters the market. The underlying problem with this approach is that a general underrepresentation of the elderly population often occurs. For cancer drugs in particular, it was reported that only 22 to 36% of patients 65 years and older took part in clinical trials, although they represent nearly 60% of the population with cancer (Lewis et al., 2003). Aside from exclusion criteria based on the presence of comorbidities and concurrent drug intake, other factors involving logistic and financial impositions, poor compliance, lack of social support, and widespread ageist behaviors of physicians who associate older age with poorer outcomes act as deterrents for the recruitment of elderly individuals into clinical trials (Siu, 2007). The lack of appropriate evidence-based profiling of drug responses in elderly individuals consequently contributes to an increased incidence of adverse drug events or a dampened efficacious response among the older population once the drug

enters the market, especially for drugs with a narrow therapeutic range.

The aged LH rat model therefore provides a useful alternative for studying age-related changes in pharmacokinetics and pharmacodynamics. However, further testing with drugs of varying pharmacological properties is required to ascertain its translational potential.

Authorship Contributions

Participated in research design: Goh, Browne, and Chen.

Conducted experiments: Goh, Aw, and Lee.

Performed data analysis: Goh,

Wrote or contributed to the writing of the manuscript: Goh, Browne, and Chen.

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