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Brain cholinergic impairment in liver failure

María-Salud García-Ayllón,[1,2,*] Omar Cauli,[3,*] María-Ximena Silveyra,[1,2] Regina Rodrigo,[3] Asunción Candela,[4] Antonio Compañ,[4] Rodrigo Jover,[5] Miguel Pérez-Mateo,[5] Salvador Martínez,[1] Vicente Felipo[3] and Javier Sáez-Valero[1,2]

[1]Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC, San Juan de Alicante, [2]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), [3]Laboratory of Neurobiology, Fundación Centro de Investigación Príncipe Felipe, Valencia, [4]Departamento de Patología y Cirugía, Universidad Miguel Hernández, San Juan de Alicante and [5]Department of Gastroenterology, Hospital General Universitario de Alicante, Alicante, Spain

*These authors contributed equally to this study.

Correspondence to: Javier Sáez-Valero, Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, E-03550 San Juan de Alicante, Spain
E-mail: j.saez@umh.es

The cholinergic system is involved in specific behavioural responses and cognitive processes. Here, we examined potential alterations in the brain levels of key cholinergic enzymes in cirrhotic patients and animal models with liver failure. An increase (~30%) in the activity of the acetylcholine-hydrolyzing enzyme, acetylcholinesterase (AChE) is observed in the brain cortex from patients deceased from hepatic coma, while the activity of the acetylcholine-synthesizing enzyme, choline acetyltransferase, remains unaffected. In agreement with the human data, AChE activity in brain cortical extracts of bile duct ligated (BDL) rats was increased (~20%) compared to controls. A hyperammonemic diet did not result in any further increase of AChE levels in the BDL model, and no change was observed in hyperammonemic diet rats without liver disease. Portacaval shunted rats which display increased levels of cerebral ammonia did not show any brain cholinergic abnormalities, confirming that high ammonia levels do not play a role in brain AChE changes. A selective increase of tetrameric AChE, the major AChE species involved in hydrolysis of acetylcholine in the brain, was detected in both cirrhotic humans and BDL rats. Histological examination of BDL and non-ligated rat brains shows that the subcellular localization of both AChE and choline acetyltransferase, and thus the accessibility to their substrates, appears unaltered by the pathological condition. The BDL-induced increase in AChE activity was not parallelled by an increase in mRNA levels. Increased AChE in BDL cirrhotic rats leads to a pronounced decrease (~50–60%) in the levels of acetylcholine. Finally, we demonstrate that the AChE inhibitor rivastigmine is able to improve memory deficits in BDL rats. One week treatment with rivastigmine (0.6 mg/kg; once a day, orally, for a week) resulted in a 25% of inhibition in the enzymatic activity of AChE with no change in protein composition, as assessed by sucrose density gradient fractionation and western blotting analysis. In conclusion, this study is the first direct evidence of a cholinergic imbalance in the brain as a consequence of liver failure and points to the possible role of the cholinergic system in the pathogenesis of hepatic encephalopathy.

Keywords: cirrhosis; hepatic encephalopathy; cerebral cortex; acetylcholinesterase; acetylcholine

Abbreviations: ACh = acetylcholine; AChE = acetylcholinesterase; BDL = bile duct ligated; BDL + HD = BDL plus a diet containing ammonium; BuChE = butyrylcholinesterase; ChAT = choline acetyltransferase; G_1 = AChE monomeric form; G_2 = AChE dimeric form; G_4 = AChE tetrameric form; HD = hyperammonemic diet; HE = hepatic encephalopathy; NC = non-cirrhotic; NL = non-ligated; PCS = portacaval shunt; PF = pair-fed.; QRT-PCR = quantitative reverse transcription-polymerase chain reaction

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Alteration of normal brain function is a characteristic complication of both acute and chronic liver failure and is known as hepatic encephalopathy (HE). HE is characterized by deficits in several neurotransmitter systems in the brain. In particular, significant alterations in glutamatergic and monoaminergic mechanisms, as well as endogenous opioid

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neurotransmitter, γ-amino butyric acid and serotoninergic systems have been reported in both animal models of HE and post-mortem brain tissue from patients with hepatic liver disease (Butterworth, 1996; Felipo and Butterworth, 2002; Lozeva *et al.*, 2004). However, thus far, there have been few studies on alterations in the cholinergic system during liver failure, with no significant conclusions (Rao *et al.*, 1994; Kabatnik *et al.*, 1999; Jamal *et al.*, 2007; Swapna *et al.*, 2007). In fact Butterworth and co-workers (Rao *et al.*, 1994) fail to demonstrate changes in the levels of cholinergic enzymes in human and experimental portal-systemic encephalopathy.

The cholinergic system, and specifically the neurotransmitter acetylcholine (ACh), is involved in specific behavioural responses and cognitive processes in both healthy subjects and those with neurological dysfunction (Bartus *et al.*, 1982). Therapies designed to restore cholinergic balance are based on the importance of cholinergic function in cognition. The levels of ACh are continuously regulated by choline acetyltransferase (ChAT), the enzyme which synthesizes ACh and by acetylcholinesterase (AChE), which rapidly degrades the neurotransmitter at cholinergic synapses terminating synaptic transmission.

The present study investigates alterations in the levels of both cholinergic enzymes, AChE and ChAT, in post-mortem human brain tissue from patients deceased from hepatic coma, as well as the levels of both enzymes and the neurotransmitter ACh in cerebral cortex from several animal models with and without liver failure. The ability of rivastigmine, a widely prescribed reversible AChE inhibitor, to revert cognitive deficits in bile duct ligated (BDL) rats was also investigated.

Patients and Methods

Human brain samples

Small pieces (~0.2 g) of prefrontal cortex tissue, corresponding to Brodmann area 9, were obtained post-mortem from four alcoholic cirrhotic patients deceased from hepatic coma [3M/1F; average age 68 (60–86) years] and four control subjects free from any known hepatic, neurologic and psychiatric disorders [2M/2F; average age 66 (60–73) years]. Studies in our laboratory have shown that for a *post-mortem* interval >72 h, storage at −20°C or repeated cycles of freeze-thawing caused degradation of AChE, which confounded analysis. Thus, only samples which *post-mortem* delay <12 h (for all samples, with no differences between groups) were included. Samples were stored at −80°C until analysis. These studies were approved by the local ethics committee.

Animals and tissue preparation

All animal procedures were approved by the Universidad Miguel Hernández's Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250–300 g at the times of surgery were used. Several rat groups were used in this study: non-ligated (NL) controls, BDL, BDL + hyperammonemic diet (BDL+HD), hyperammonemic diet (HD) without liver failure and portacaval shunted (PCS) rats.

Liver injury in BDL was induced by common bile duct-ligation as previously described (García-Ayllón *et al.*, 2006; Jover *et al.*, 2006). In NL controls, a midline incision was performed without BDL. Hyperammonemia was induced in HD and BDL + HD rats by feeding with a diet containing ammonium for 1 week until sacrifice (Azorín *et al.*, 1989; Jover *et al.*, 2006). BDL + HD rats were given the ammonium diet the third week after the BDL. An additional control group was included, sham pair-fed (PF) rats with equal control diet consumption as that of the ammonium-containing diet eaten by HD rats.

For behavioural tests, sham and BDL subgroups were treated with the cholinesterase inhibitor rivastigmine (0.6 mg/kg; Exelon®, Novartis Farmacéutica S.A., Spain) or vehicle (saline), both administered 2 weeks after BDL, once a day, orally, for a week. Thus, all BDL rats were sacrificed 3 weeks after surgery, brains removed and cerebral cortices dissected and stored at −80°C. In PCS rats, portacaval anastomosis was performed according to Lee and Fisher (1961). Rats were sacrificed 4 weeks after the surgery.

Brain homogenization and extraction

Approximately 0.2 g of human prefrontal cortex and rat cortices were homogenized (10% w/v) in ice-cold Tris–saline buffer (50 mM Tris–HCl, 1 M NaCl and 50 mM $MgCl_2$, pH 7.4) supplemented with a cocktail of proteinase inhibitors (García-Ayllón *et al.*, 2006). The homogenates were centrifuged at 100 000×g at 4°C for 1 h to recover a salt soluble fraction. The pellets were re-extracted with an equal volume of Tris–saline buffer containing 1% (w/v) Triton X-100, and the suspension was centrifuged at 100 000×g at 4°C for 1 h to recover a detergent soluble fraction, rich in membrane-bound enzyme. This method of extraction allows greater extraction of total AChE activity (Sberna *et al.*, 1998). For the assay of enzyme activities equal volumes of salt and detergent supernatants were combined.

Enzyme assays and protein determination

A modified microassay version of the Ellman method was used to measure AChE and the structurally related butyrylcholinesterase (BuChE) (Sberna *et al.*, 1998; García-Ayllón *et al.*, 2006). ChAT was assayed by the procedure established by Fonnum (Sberna *et al.*, 1998). Protein concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA).

Determination of ammonia

Ammonia was measured in plasma and cerebral cortex. Briefly, blood (150 µl) was taken from the tail vein in the morning, 21 days after surgery (or 28 days for PCS rats). The cerebral cortex was homogenized as previously described (Jover *et al.*, 2006) and both blood and cortical extracts deproteinized (Jover *et al.*, 2006). Ammonia was measured in the neutralized supernatants by fluorimetry (Fluoroskan Ascent Labsystems, Helsinki, Finland).

Sedimentation analysis of AChE molecular forms

AChE species were analysed by ultracentrifugation on a continuous sucrose gradient (5–20% w/v) containing 0.5% (w/v) Triton X-100, as previously described (Sberna *et al.*, 1998). The AChE present in brain is mainly distributed as tetramers (G_4) and light globular species (dimers, G_2; monomers, G_1). Peaks associated with the major forms, were identified and the relative contribution of each molecular form of AChE was also calculated.

Detection of AChE subunits by western blotting

AChE variants were detected by immunoblotting. Briefly, rat brain extracts (30 µg) were resolved under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide slab gels. After electrophoresis, proteins were blotted onto nitrocellulose membranes, blocked with 5% non-fat milk and incubated overnight with a goat anti-AChE polyclonal antibody (E-19, Santa Cruz Biotechnology, Santa Cruz, CA). The nitrocellulose membrane strips were then incubated with a horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG secondary antibody (Santa Cruz Biotechnology) and immunoreactive AChE was detected using the ECL-Plus kit (Amersham Life Science, Arlington Heights, IL, USA) in a Luminescent Image Analyzer LAS-1000 Plus (Fujifilm, Stamford, CT, USA). Molecular weight markers were used to determine protein size (Sigma-Aldrich Co., St Louis, MO, USA). For semi-quantitative analysis, the intensity of AChE bands was measured with Science Lab Image Gauge v4.0 software provided by Fujifilm.

AChE histochemistry and immunocytochemistry

Animals were perfused through the heart with 0.1 M phosphate buffer (PB), pH 7.4, followed by 4% paraformaldehyde in PB containing 1% $CaCl_2$. The brain was then removed and immersed in the same fixative overnight. The brains were serially sectioned at 50 µm in a sliding microtome (Microm, Heidelberg, Germany) coupled to a freezing unit. AChE staining was examined by immunohistochemical analysis with the anti-AChE antibody E-19 (1:100 dilution). Similar sections were also incubated with the polyclonal anti-ChAT antibody (1:200 dilution; Chemicon International, Temecula, CA, USA).

RNA isolation and analysis of AChE and ChAT transcripts by QRT-PCR

Total RNA from NL and BDL rat brain was isolated using TRIzol® Reagent in the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen™ Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions. cDNAs were synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen™ Life Technologies) according to the manufacturer's instructions using 5 µg of total RNA and oligo (dT)12-18. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) amplification was performed using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Power SYBR® Green PCR Master Mix according to the manufacturer's instructions (see primer sequence in Table 1). Transcript levels for AChE (R and T transcripts) and two splice forms of ChAT, the cholinergic (cChAT) and the peripheral (pChAT) were calculated using the relative standard curve method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Microdialysis and perfusate analysis of ACh

On day 18 after the BDL surgery, a microdialysis guide (CMA/12 guide cannula; CMA/Microdialysis AB, Solna, Sweden) was implanted 3 mm above the coordinates in the prefrontal cortex (AP 3.7, ML −0.8, DV −1.0, from brain surface). Three days later a 3 mm long microdialysis probe (CMA/12, Solna, Sweden) was lowered and perfused (3 µl/min) with artificial cerebrospinal fluid (145 mM NaCl, 3.0 mM KCl, 2.26 mM $CaCl_2$, 2 mM phosphate, pH 7.4) containing 2 µM of the cholinesterase inhibitor neostigmine chloride (Damsma *et al.*, 1985; Chang *et al.*, 2006). As AChE is very efficient at removing extracellular ACh, obscuring differences in ACh release, inclusion of the AChE inhibitor in the microdialysis perfusate was necessary to observe functional changes in ACh release (Rao *et al.*, 1994). As extracellular ACh levels in the prefrontal cortex are influenced by circadian rhythm (Mitsushima *et al.*, 1996), time of sampling was controlled and limited to a short period during the light phase (between 11:00 AM and 2:00 PM). Following a stabilization period of at least 2 h, samples were collected every 20 min. ACh in the perfusate was measured using high-performance liquid chromatography with electrochemical detection, as previously described (Damsma *et al.*, 1985). ACh values presented are uncorrected for probe recovery (32% of recovery) and expressed in nanomolars.

Behaviour studies

Behavioural tests were performed to analyse memory and learning functions (active avoidance and object recognition tests) and motor coordination (rotarod). The tests were performed 3 h after the final administration of rivastigmine.

Active avoidance

The active avoidance task is designed to test the ability of the rats to avoid an aversive event by first learning to perform a specific behaviour in response to a stimulus cue. The test was performed in one single day and consisted of 50 trials per animal, as previously described (Aguilar *et al.*, 2000).

Object recognition test

This test exploits the tendency of rats to preferentially explore novel elements of their environment. Thus, when a rat is presented with both a novel and recently presented familiar object, it will spend significantly more time exploring the novel object. The familiar object was presented in a previous training session 4 h before the test. The percentage of time exploring the non-familiar object in the training session over total exploration time (exploration time of the familiar plus the non-familiar objects) was represented.

Table 1 Primer design and optimization for QRT-PCR

Primer	Sequence	Optimun concentration (nM)	Amplicon lengh (nt)
GAPDH			
forward	TGGGAAGCTGGTCATCAAC	100	78
reverse	GCATCACCCCATTTGATGT	100	
AChE-T			
forward	CAGCAATACGTGAGCCTGAA	100	123
reverse	CTCGTCCAGCGTGTCTGT	100	
AChE-R			
forward	CTCAGCGCCACAGGTAGG	200	76
reverse	TCTCTCCCGTCCTTCCAAC	200	
cChAT			
forward	GGTGTGGTGTGTGAGCATTC	200	167
reverse	TGGGTTTCTGGGGAACATT	200	
pChAT			
forward	CTGCTGGACAGGATGACAAG	200	104
reverse	TGGGTTTCTGGGGAACATT	200	

Rotarod

The rotarod test assesses the ability of rats to stay on a rotating drum to evaluate motor coordination functions. An accelerating rotarod was used (Ugo Basile, Comerio, Italy). Two consecutive days before testing, each rat was placed on the rotarod which was switched off for 3 min. The rotarod speed was then increased from 4 to 40 r.p.m. over 300 s. The time at which each animal fell off the rungs was recorded, with a maximum cut-off of 600 s.

Statistical analysis

Measurements are expressed as means ± SEM. Data were analysed by a Student's *t*-test or by an one-way analysis of variance followed by a post hoc Tukey–Kramer test for multiple comparisons between groups. Statistical significance was designated as $P < 0.05$.

Results

Assay of AChE activity revealed that frontal cortex extracts from HE patients had higher enzyme activity (33% increment; $P = 0.02$) compared to non-cirrhotic (NC) controls, while ChAT activity remains unaltered (Fig. 1A and B). The specificity of the changes in AChE activity was supported by the observation that the activity of the related enzyme BuChE, in extracts from cirrhotic individuals, was not different from that in control subjects (Fig. 1C). Additionally, as AChE is expressed as several molecular forms (Massoulié *et al.*, 1993; Taylor and Radic, 1994), we determined whether the observed increase in AChE activity in cirrhotic subjects was due to an increase of a particular molecular form. Brain frontal cortex supernatants were fractionated on sucrose density gradients to separate AChE species. In HE samples, there was a small increase in the major AChE tetrameric form peak ($P = 0.036$) with respect to control samples (Fig. 1D).

Next, we examined the levels of these enzymes in rat brain from several pathological models (Fig. 2). In agreement with the human data, AChE activity in cortical extracts of cirrhotic BDL rats was increased (~20% increase; $P = 0.004$) compared to NL controls, while the levels of ChAT and BuChE remain unaltered (Fig. 2A–C). Activity levels were similar in BDL and BDL + HD rats, while neither change was found in HD rats without liver disease fed with an ammonium-containing diet for 1 week (Fig. 2A–C), nor in sham PF rats (not shown). In good agreement with previous observations (Huang *et al.*, 2004; Jover *et al.*, 2006), the trend was for plasma ammonia levels to increase in BDL rats, but only BDL + HD and HD rats were hyperammonemic (Fig. 2D). More interestingly, only BDL + HD rats showed increased ammonia in the cerebral cortex (Fig. 2E). The lack of correlation between AChE alterations and ammonia levels was corroborated in the PCS group. After 4 weeks of portacaval anastomosis, these rats' large increases in ammonia levels were observed in both plasma and cortex (Fig. 2D and E), while cholinergic markers were unaffected (Fig. 2A–D).

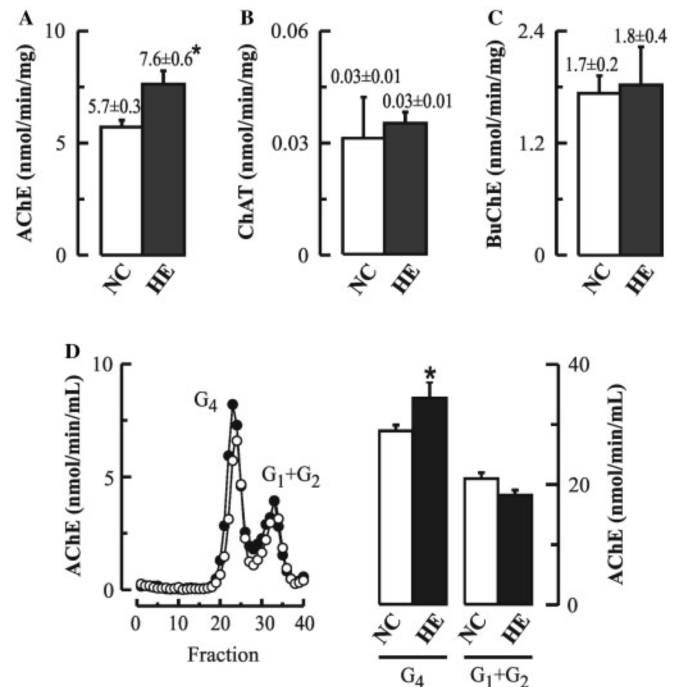

Fig. 1 (**A**) AChE, (**B**) ChAT and (**C**) BuChE activity levels in frontal cortex extracts from NC controls ($n = 4$) and patients with HE ($n = 4$). (**D**) Representative profiles of AChE molecular forms (G_4 = tetramers; $G_1 + G_2$ = monomers + dimers) and the activity of each AChE molecular form are also shown. Values are means ± SEM. $^*P < 0.05$.

The major AChE tetrameric form was significantly increased in the cirrhotic rat cortices from BDL rats compared to controls ($P = 0.039$; Fig. 3). A similar trend was observed for BDL + HD rats (Fig. 3).

We also assessed whether the subcellular location of both, AChE and ChAT were modified by the BDL condition (Fig. 4). Immunoreactivity to AChE and ChAT showed consistent and similar expression in both experimental and control animals. While strong immunoreactive signal was observed in the perinuclear area of pyramidal cells in frontal cortex layer V, a weak reaction was localized in the cytoplasm of these cells, staining within basal and apical dendrites. Some small cells around the pyramidal neurons also show perinuclear labelling with very weak cellular signal. No difference observed in the localization and intensity of the AChE and ChAT immunoreactivity in all the analysed coronal sections of control NL ($n = 3$), BDL ($n = 3$) and BDL + HD ($n = 3$) rats.

Some of the molecular heterogeneity of AChE derives from alternative RNA splicing, generating different polypeptide encoding transcripts with the same catalytic domain, but distinct C-terminal peptides that determine the ability of the molecule to form oligomers (Massoulié *et al.*, 1993; Taylor and Radic, 1994; Grisaru *et al.*, 1999). To determine if AChE expression is altered in BDL rats, we performed QRT-PCR analysis of the AChE mRNA.

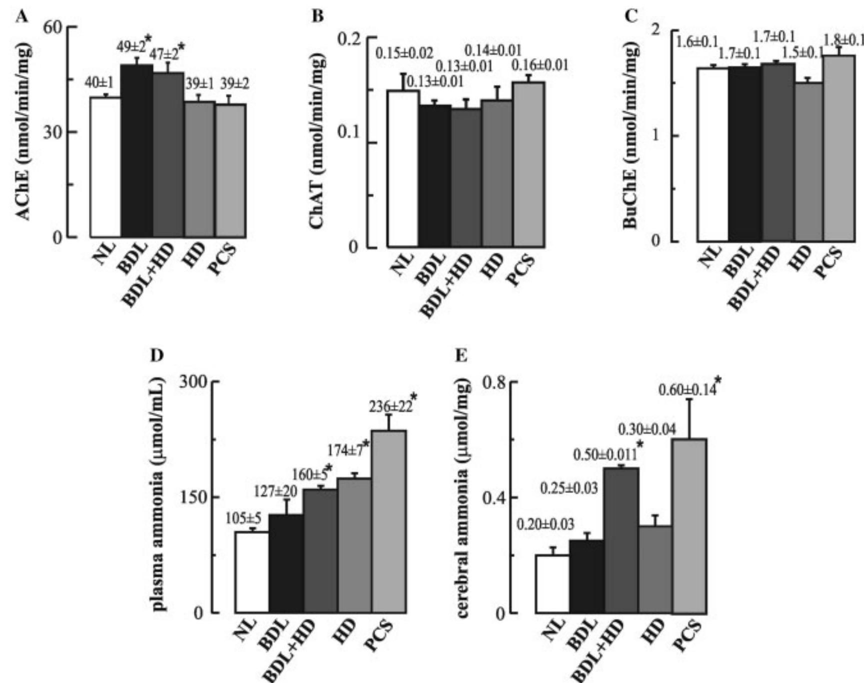

Fig. 2 Levels of (**A**) AChE, (**B**) ChAT and (**C**) BuChE in cortical extracts from sham NL controls, BDL rats without and with HD (BDL + HD), HD without liver disease fed with an ammonium-containing diet for 1 week and PCS rats. (**D**) Plasma and (**E**) cerebral ammonia levels were also determined for each rat group. Values are means ± SEM. $^*P < 0.05$, significantly different from NL group; there were no statistically significant differences between BDL and BDL + HD groups (at least $n = 6$ for each group).

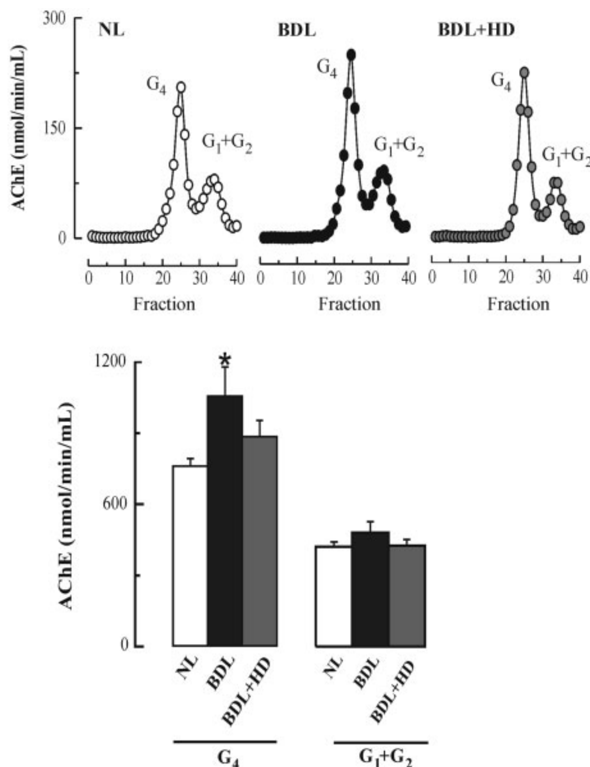

Fig. 3 Representative profiles of AChE molecular forms (G_4 = tetramers; $G_1 + G_2$ = monomers and dimers) and the activity of each molecular AChE form, for sham NL controls, BDL rats and BDL rats with HD (BDL + HD) ($n = 6$ for each group). Values are means ± SEM. $^*P < 0.05$, significantly different from NL group.

The levels of the T-transcript, the major transcript in mammalian brain that encodes subunits which produce monomeric and tetrameric forms, was unaltered in cortices from BDL rats compared to controls (Fig. 5A). Similarly, levels of the R-transcript which encodes monomeric soluble subunits and is normally present at low levels in the mammalian brain (Kaufer *et al.*, 1998) did not vary in BDL animals compared to controls (Fig. 5A). QRT-PCR analysis was used to determine the mRNA levels for both the conventional cholinergic ChAT transcript, the major variant found in both central and peripheral neurons [protein product is called ChAT of the common type (cChAT)], and also transcriptional levels for the minor splice variant [protein product designated ChAT of a peripheral type (pChAT)], which is predominantly localized in peripheral neurons (Tooyama and Kimura, 2000). As expected for the unmodified ChAT enzyme activity, the levels of both cChAT and pChAT were unaltered in cortices from BDL rats compared to NL controls (Fig. 5A).

As AChE is present as both active and inactive subunits (Rotundo, 1988; Chatel *et al.*, 1993; García-Ayllón *et al.*, 2006, 2007), we performed immunoblotting to assess whether altered activity levels correlated with increases in the total pool of AChE protein. Rat cortical extracts were analysed by SDS–PAGE under fully reducing conditions, followed by western blotting using the anti-AChE antibody E-19. This polyclonal antibody was raised against a peptide mapping to the amino terminus of AChE, common to all AChE forms and thus presumably detects all species, including inactive

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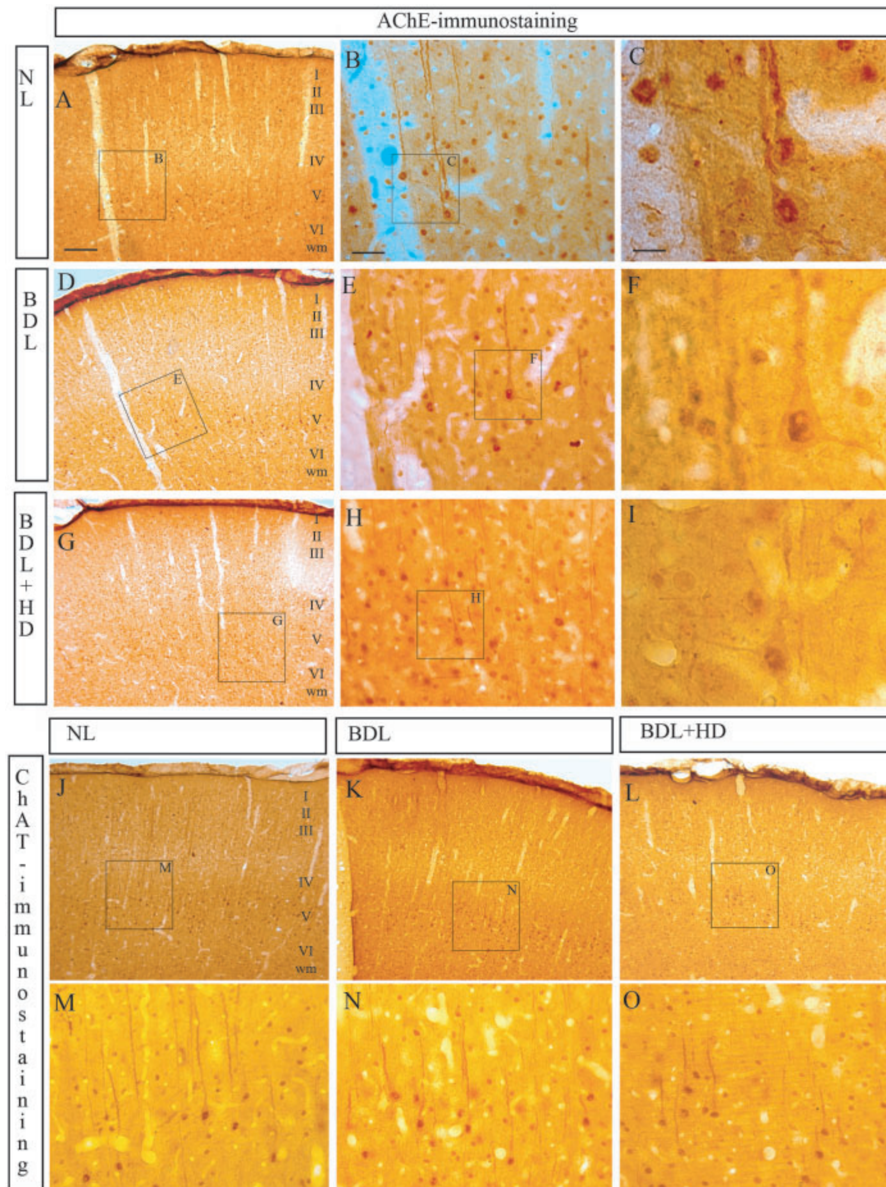

Fig. 4 AChE and ChAT detection in the cerebral cortex of control and experimental rats. (**A**–**I**) AChE immunoreactivity in brain frontal cortex in normal (NL) rats (**A**–**C**), BDL rats, (**D**–**F**) and BDL + HD rats (**G**–**I**). The immunopositive staining is mainly localized in the perinuclear area of layer V pyramidal neurons in both control and experimental samples. Inserts show the progressive higher power pictures in the localized areas for each rat model. Scale bar: 250 microns in (**A**), (**D**) and (**G**); 100 microns in (**B**), (**E**) and (**H**); and 30 microns in (**C**), (**F**) and (**I**). (**J**–**O**) ChAT immunoreactivity of frontal cortex in normal (NL) rats (**J** and **M**), BDL rats (**K** and **N**) and BDL + HD rats (**L** and **O**). Immunopositive staining is mainly localized in the perinuclear area of layer V pyramidal neurons in control and experimental samples. Inserts show progressive higher power pictures in localized areas for each rat model. Scale bar: 250 microns in (**J**), (**K**) and (**L**); 100 microns in (**M**), (**N**) and (**O**). No significant staining was found in cortical superficial layers in pyramidal cells or interneurons, only apical processes of pyramidal neurons could be followed from layer V. Not staining was observed in the white matter.

subunits (García-Ayllón *et al.*, 2006). E-19 detected three major bands of ~77, 70 and 60 kDa (Fig. 5B). Neither the intensity of the individual bands nor their relative banding pattern was altered in BDL rats compared to controls.

In an attempt to assign immunopositive AChE bands to specific AChE species, immunoblots were performed for the G_4 and $G_1 + G_2$ peaks from sucrose density gradients (Fig. 5C). In both NL and BDL rats, the majority of the G_4 peak was 70 kDa, while blots of material from the $G_1 + G_2$ peak showed all three AChE bands, similar to total extracts. There was no correlation between increased G_4 cortical AChE activity in BDL with immunoreactivity as much of the AChE immunoreactivity was associated with unaltered protein that was present in the $G_1 + G_2$ peak and may correspond to both active and inactive subunits (García-Ayllón *et al.*, 2007).

Complementary to the imbalance in cholinergic enzymes and increased catalytic AChE activity in cerebral cortex extracts, extracellular ACh levels obtained during *in vivo*

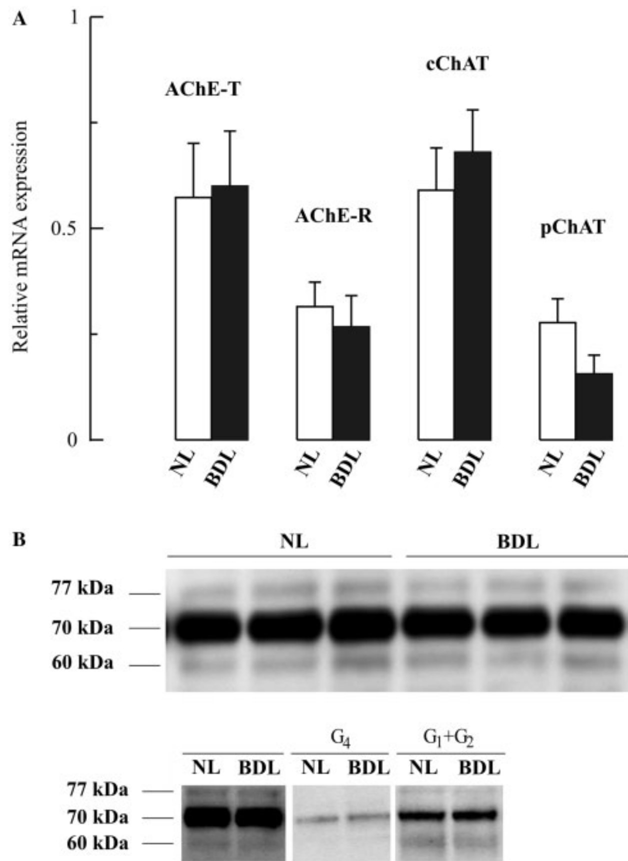

Fig. 5 Immunodetection of AChE subunits and detection of AChE and ChAT transcripts in brain cortex from NL and BDL rats. (**A**) Relative mRNA levels of the transcripts for AChE (Tand R; labelled AChE-Tand AChE-R in the figure) and ChAT (cholinergic and peripheral; cChATand pChAT) were analysed by QRT-PCR. Values were calculated using relative standard curves and normalized to GAPDH control from the same cDNA preparations. Specifity of the PCR products was confirmed by dissociation curve analysis. (**B**) Three major AChE bands of ~77, 70 and 60 kDa were identified with the antibody E-19 in NL and BDL brain cortical extracts (equivalent amounts of protein were loaded in each lane). None of the major AChE immunopositive bands were altered when comparing NL and BDL animals. (**C**) Representative immunoblot of individual G_4 and $G_1 + G_2$ AChE peak-fractions separated by sucrose gradient centrifugation from NL and BDL brain cortical extracts (equivalent volume of G_4 and $G_1 + G_2$ AChE peak-fractions were loaded in each lane).

microdialysis from cerebral cortex, showed a 52% decrease in BDL and a 66% decrease in BDL + HD rats compared to NL rats ($P < 0.001$; Fig. 6). All microdialysis fractions were performed with the addition of 2 μM of the cholinesterase inhibitor neostigmine in the perfused solution, which is necessary to avoid degradation of ACh in the extracellular space.

To assess potential behavioural changes, we performed an active avoidance test in the animal models to analyse memory and learning functions. Cirrhotic BDL and BDL + HD rats were the only groups that demonstrated a decrease in the number of attempts made to avoid foot shock compared to control rats ($P < 0.02$; Fig. 7).

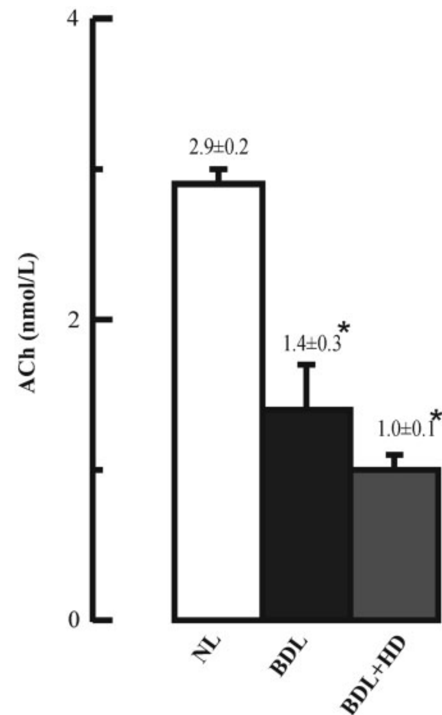

Fig. 6 Levels of ACh in cortical extracts from sham NL controls, and BDL rats without and with HD (BDL + HD). ACh values are uncorrected for probe recovery (32% of recovery) and represent means ± SEM. *$P < 0.05$, significantly different from NL group; there were no statistically significant differences between BDL and BDL + HD groups ($n = 6$ for each group).

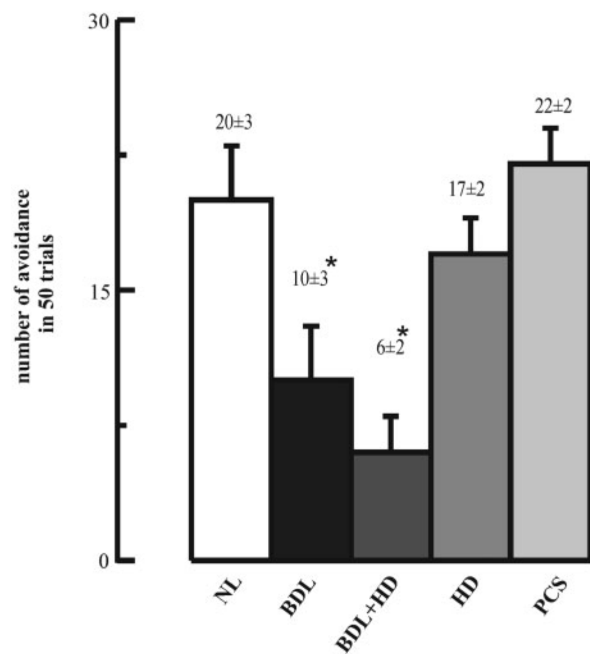

Fig. 7 Result of the active avoidance test in sham NL controls, BDL rats, BDL rats + HD (BDL + HD), rats fed with a HD without liver disease, and PCS rats. Only cirrhotic BDL and BDL + HD rats showed a decrease in the number of attempts made to avoid the foot shock as compared with NL controls (*$P < 0.02$). Values are means ± SEM (at least $n = 6$ for each group).

We proposed that the impairment in the ability of rats to learn the active avoidance task was dependent on cholinergic imbalance, which in turn leads to memory deficits. Thus, we tested whether rivastigmine, a widely prescribed reversible AChE inhibitor, was able to improve this deficit, and extended our analysis to include another memory test, the object recognition test. In addition, motor coordination of the rats was assessed using the rotarod. We concentrated our analysis on the BDL group administered rivastigmine 2 weeks after bile duct-ligation, once a day (0.6 mg/Kg daily), orally, for a week. AChE measurements in frontal cortex extracts from rats sacrificed after performing behaviour tests, demonstrated a 25% of inhibition of AChE enzymatic activity, similar to previous observations (Amenta *et al.*, 2006). Up-regulation of AChE in response to chronic inhibition is well recognized (Chiappa *et al.*, 1995; Friedman *et al.*, 1996; Keller *et al.*, 2001). As this may interfere with the therapeutic response, we determined whether rivastigmine treatment modified protein composition by fractionating the different AChE molecular forms on sucrose density gradients. These analyses revealed that peaks corresponding to the major AChE tetramers and to the minor light forms were decreased similarly in both rivastigmine-treated BDL and control animals (Fig. 8A). Rivastigmine can also affect AChE activity, thus we extended our examination to include western blotting analysis. Neither the intensity of the individual bands nor their relative banding pattern was altered in rivastigmine-treated rats compared to vehicle-treated controls (Fig. 8B).

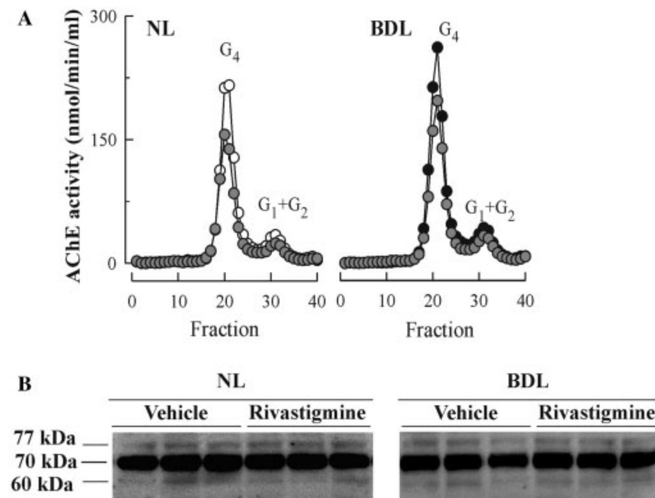

Fig. 8 Change in levels of brain AChE species in response to rivastigmine or vehicle treatment in NL and BDL rats (**A**) representative profiles of AChE molecular forms. (**B**) Immunodetection of AChE variants analysed by western blot with the anti-N-terminal AChE antibody N-19 (equivalent amounts of protein were loaded in each lane). None of the major AChE immunopositive bands were altered when comparing rivastigmine and vehicle treated animals.

Interestingly, rivastigmine restored learning abilities in BDL rats tested for active avoidance ($P = 0.003$; Fig. 9A). However, treatment with rivastigmine demonstrated trends to negatively affect memory performance in control rats tested for object recognition, as compared with sham-vehicle treated rats ($P = 0.16$; Fig. 9B). Despite this tendency, BDL-rivastigmine treated rats displayed restored learning abilities compared with BDL-vehicle treated rats ($P = 0.04$; Fig. 6B). Rats with BDL have previously been shown to have decreased motor activities (Chan *et al.*, 2004; Jover *et al.*, 2006), correlating with some of the typical signs found in patients with HE developed during chronic liver disease. In the current study, the BDL rats demonstrated mild impairment

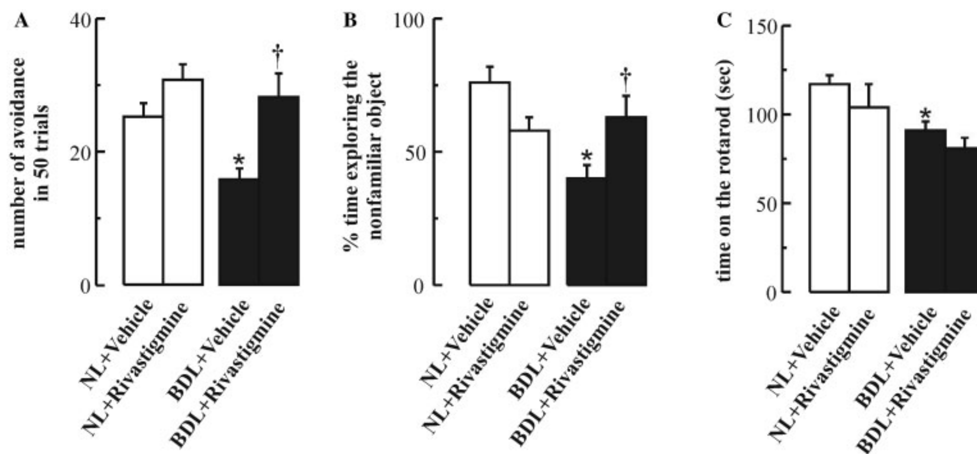

Fig. 9 Effect of the AChE inhibitor rivastigmine on conditioned stimuli response (avoidances) in the active avoidance test (**A**), the object recognition test (**B**) and on the rotarod motor coordination test (**C**), for NL and BDL rats ($n = 10$ for each subgroup). $^*P < 0.05$ for a difference between NL-vehicle and BDL-vehicle rats; $^\dagger P < 0.05$ for a difference between BDL-vehicle and BDL-rivastigmine treated rats. Note that BDL-rivastigmine treated rats show differences in the number of avoidances and in the percentage of time exploring the non-familiar object, as compared with BDL-vehicle rats. No differences in the time on the rotarod were observed between BDL-rivastigmine treated rats and BDL-vehicle rats.

of motor coordination, determined by failure to stay on the rotarod ($P = 0.02$; Fig. 9C). This was not reversed by rivastigmine treatment signifying the specificity of its effects in learning abilities.

Discussion

This is the first study that describes an alteration in AChE activity in human and rat brain cortex as a consequence of liver failure. In the animal model, increased AChE leads to a pronounced decrease in the levels of the neurotransmitter ACh which plays a critical role in cognitive function. Thus, our data suggests that impairment of the brain cholinergic system induced by liver disease may be associated with failure in learning and memory functions. Interestingly, it has been demonstrated that disrupting the cholinergic balance itself by transgenic expression of AChE in mice causes progressive decline (Beeri *et al.*, 1995).

Hyperammonemia is considered one of the main factors responsible for HE-neurological alterations (Felipo and Butterworth, 2002). While the contribution of hyperammonemia to brain cholinergic disturbances cannot be completely ruled out, our data suggests that high ammonia levels are not a precipitant factor in brain AChE changes. There is no correlation between increased ammonia levels and increased brain AChE activity level changes in the animal models examined. BDL rats, an animal model of liver failure, showed the same degree of alteration in both, AChE and ACh levels, as the BDL + HD animals, a model of liver failure and HE. Nonetheless, only BDL + HD rats showed increased ammonia in the cerebral cortex. Neither HD fed nor PCS rats (which display a large increase in brain ammonia) demonstrated changes in the levels of cholinergic markers. Therefore, only cirrhotic animals, independent of the degree of hyperammonemia, mimic the increase in brain AChE levels in human alcoholic cirrhotic subjects.

It has recently been reported that ethanol can cause cholinergic imbalance (Jamal *et al.*, 2007). Moreover alterations in AChE kinetic properties have been described in thioacetamide-induced HE (Swapna *et al.*, 2007). Interestingly, significant AChE increases after acute ethanol exposure have been demonstrated in the zebrafish brain, whereas ethanol *in vitro* did not alter enzymatic activity (Rico *et al.*, 2007). Further studies addressing the possibility that other precipitant factors, such as manganese (Krieger *et al.*, 1995), may contribute to cholinergic imbalance symptoms are warranted as it has been suggested that manganese may modulate cholinergic systems, including affecting AChE activity (Finkelstein *et al.*, 2007). Moreover, we have recently showed in BDL rats a pronounced decrease in liver and plasma AChE levels, with selective loss of the G_4 specie (García-Ayllón *et al.*, 2006). The decrease of serum G_4 levels in BDL rats correlates with a decrease of the tetrameric species in the cerebrospinal fluid (unpublished observation). Thus, the possibility that overall decreases in peripheral AChE activity in BDL rats may influence cerebrospinal fluid contents and trigger a central response, resulting in the accumulation of AChE in the brain, also deserves consideration.

Alterations in AChE activity have not been previously observed in brains of deceased cirrhotic patients, nor in portacaval-shunted rats (Rao *et al.*, 1994). Discrepancy within human studies may be due to differences in protein extraction protocols or handling/measurement of samples. In the study by Rao *et al.* (1994) both extraction and assay buffers were detergent-free; while homogenization of brain without detergent releases AChE activity, most of the brain AChE corresponds to amphiphilic species that requires incubation with detergents, such as Triton X-100, for optimized extraction and expression of full catalytic activity (Massoulié *et al.*, 1993; Sáez-Valero *et al.*, 1993). Thus, we believe that the use of detergent-free buffers by our colleagues may have contributed to the divergent results. We present evidence that in both, cirrhotic humans and rats, increased AChE activity is associated with a parallel increased in the major amphiphilic tetrameric molecular form.

AChE is widely distributed within different brain regions and is expressed as several molecular forms with a specific pattern that is altered in many pathological processes (Massoulié *et al.*, 1993). The different molecular forms of AChE may thus reflect specific physiological functions of the enzyme with different regulatory requirements. The BDL-induced increase in G_4, which is the major AChE form in the brain, was not accompanied by a change in its T-transcript. AChE levels are regulated at transcriptional, post-transcriptional and post-translational levels, leading to complex expression patterns which are modulated by physiological and pathological conditions through mechanisms that are not fully understood. In previous studies, we have demonstrated by immunoblotting that both active and inactive AChE species can be detected (García-Ayllón *et al.*, 2006, 2007). In this study, neither the intensity of the individual bands nor their relative banding patterns were altered by the BDL conditions. The immunoblotting assays revealed a complex AChE banding pattern with no direct relationship between specific molecular forms and enzymatic activity. It is thus not possible to correlate increased G_4 activity with altered immunoreactivity.

While the different molecular forms of AChE may have specific physiological functions (Massoulié *et al.*, 1993; Taylor and Radic, 1994), within the brain, hydrolysis of ACh is probably mediated through the G_4 plasma membrane-bound form (Lazar and Vigny, 1980; Taylor *et al.*, 1981). Immunohistochemical examination of BDL and NL rat brains show that the subcellular localization of both, AChE and ChAT, and thus the accessibility to their substrates, appears unaltered by the pathological condition. Therefore, the excessive levels of the cholinergic G_4 AChE in the brain of cirrhotic subjects may have direct physiological consequences causing a decrease of

extracellular ACh levels in the brain. Finally, other cholinesterase activities present in the brain such as BuChE may also contribute to ACh hydrolysis. However, the regulation of each AChE form and of the BuChE is probably controlled by different mechanisms (García-Ayllón *et al.*, 2007). Our data indicates that only the tetrameric AChE form, the 'true' cholinergic species, is affected during liver failure.

Cholinesterase inhibitors are widely used for treating cholinergic dysfunction associated with dementia (Giacobini, 2002). Rivastigmine, has an advantage over other cholinesterase inhibitors as it is metabolized to an inactive metabolite at the site of action, bypassing hepatic metabolic pathways and as a result has unaltered bioavailability in subjects with renal or hepatic impairment. We found sustained inhibition of AChE enzymatic activity in the rat brain after 1 week of rivastigmine treatment. Despite the conserved AChE catalytic domain, *in vitro* studies have shown that some AChE inhibitors display differential selectivity for different AChE species (Ogane *et al.*, 1992; Zhao and Tang, 2002; Rakonczay, 2003). Rats treated with rivastigmine for 1 week demonstrate similar inhibition of both tetrameric and light AChE species. This was not associated with significant changes in AChE protein composition. In addition, AChE up-regulation has been demonstrated in reaction to chronic inhibition; rats administered with the organophosphate insecticide chlorpyrifos show a dose-dependent decrease in the activity of brain AChE, while immunoreactive AChE protein increased after 3 weeks of treatmen with the higher doses (Chiappa *et al.*, 1995). In our treatment conditions, and using SDS–PAGE/western blotting analysis, stable levels of AChE protein were observed, discarding early up-regulation responses of the AChE protein within 1 week of rivastigmine treatment. More interestingly, we demonstrate the ability of this cholinesterase inhibitor to improve the memory deficit observed in cirrhotic rats.

The majority of HE patients are usually treated for their hepatic manifestations rather than their neuropsychiatric symptoms and therefore represent an untreated 'psychiatric' population. It has been suggested in a case report that HE may trigger a central anticholinergic syndrome that can be treated with the AChE inhibitor physostigmine (Kabatnik *et al.*, 1999). Therapies based on the use of cholinesterase inhibitors for treating neurological manifestations associated with liver diseases thus deserve further consideration.

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