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Behavioural effects and regulation of PKCα and MAPK by huprine X in middle aged mice

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ABSTRACT

The behavioural effects of huprine X, a new anticholinesterasic inhibitor, as well as its effects on the regulation of protein kinase C (PKC), mitogen activated protein kinase (MAPK) and α-secretase (ADAM10 and TACE/ADAM17) related to amyloid precursor protein (APP) processing remain to be established. In the present work, 12 month old 126/Sv×C57b/6 male mice which received chronic i.p. treatment with either saline, huprine X (0.04 μmol kg^{-1}) or huprine X (0.12 μmol kg^{-1}), were submitted to a battery of behavioural tests and thereafter the brains were dissected to study the neurochemical effects induced by huprine X. The results show that, in a dose dependent manner, huprine X facilitates learning and memory in the Morris water maze and improves some indicators of emotionality without inducing adverse effects, affecting motor activity nor anxiety-like behaviours, as measured in the open-field and corner tests. Moreover activation of downstream PKC/MAPK signaling pathways may underly these behavioural effects as well as the stimulation of the non-amyloidogenic processing of APP. Results obtained herein using a sample of aged animals strongly suggest that huprine X constitutes a promising therapeutic agent for the treatment of cholinergic dysfunction underlying aging and/or dementias.

1. Introduction

Experimental and neuropathological evidence support the cholinergic hypothesis of geriatric memory dysfunction postulated already 30 years ago (Bartus et al., 1982) which establishes that a serious loss of neurotransmission in the basal forebrain ascending cholinergic system contributes significantly to both age-related and Alzheimer disease induced impairments in cognitive abilities (Bartus, 2000). Accordingly, enhancement of the central cholinergic neurotransmission has been regarded as one of the most promising strategies for treatment of Alzheimer disease, mainly by means of reversible AChE inhibitors (AChEI). However, some of these drugs seem not to be exempt of CNS adverse effects, related to cholinergic stimulation in the brain, such as indirect gastrointestinal adverse effects (nausea, vomits, diarrhoea) and weight loss (Raskind et al., 2000) and extrapyramidal side effects (Carriero et al., 1997; Mayorga et al., 1997) which could be critical disadvantages when considering their therapeutic potential in some dementias (i.e. dementia with Parkinson's disease). In addition, anxiolytic-like effects and reduction of neophobia have been also reported for some AChEI such as physostigmine (Sienkiewicz-Jarosz et al., 2003). At the molecular level, it has been demonstrated that protein kinase C (PKC) plays an important role in the transduction mechanisms related to the regulation of the amyloid precursor protein (APP) metabolism (Racchi et al., 2003). Thus, in vitro studies have established the involvement of PKC and PKC-coupled receptors in the non-amyloidogenic α-secretase pathway of the APP cleavage (Buxbaum et al., 1993). Furthermore MAPK has been implicated in both PKC and tyrosine kinase receptor regulation of APP catabolism (Mills et al., 1997; Desdouits-Magnen et al., 1998). In addition, several reports seem to indicate that AChEIs may affect the secretory processes of APP via activation of both PKC, especially PKCα, and mitogen-activated protein kinase (MAPK) (Peng et al., 2006; Zhang et al., 2004; Bar-Am et al., 2004; Yogev-Falach et al., 2002). Moreover, the levels in membrane compartment of ADAM10, one of the more prominent candidates for α-secretase activity, are also increased after AChEI treatment (Zimmermann et al., 2004).

Among the different chemical species of AChEIs currently available, (±)-12-amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride, the so-called huprine X, a huperzine A-tacrine hybrid, has shown a highly potent and selective inhibitory action on acetylcholinesterase both "in vitro" and "ex vivo" (Camps et al., 2000b). Its affinity for AChE is one of the

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highest yet reported with an inhibition constant (K_I) of 26 pM, that is, 180 times that of huperzine A, 1200 times that of tacrine, and 40 times that of E2020 (donepezil, Aricept) the most selective AChEI currently approved for therapeutical use (Camps et al., 2000a). Recently some new huprines have been synthetized with similar activities against human recombinant AChE (Ronco et al., 2009). In addition huprine X has an agonistic action on muscarinic M_1 and nicotinic receptors (Román et al., 2002, 2004) and exhibited a "tight binding" character in experiments of reversibility of bovine AChE inhibitory activity (Camps et al., 2000b). However, the 'in vivo' effects of huprine X remain to be established.

Therefore, the behavioural effects of huprine X on both aging and/or animal models for Alzheimer's disease are of special interest as well as those studies investigating its effects on the regulation of PKC, MAPK and secretase levels related to APP processing. Thus, the first aim of the present work was to describe the 'in vivo' effects of chronic administration of two range of doses on normal aged animals by means of a battery of tests assessing spatial reference learning and memory, locomotor activity, anxiety-like behaviour, neophobia and emotionality. Thereafter, in the same animals, we determined the effects of huprine X on the above mentioned molecular substrates. Thus, we studied both cytosolic and membrane fractions obtained from the hippocampus and the cortex of control and huprine X treated mice and investigated the effects of huprine X on the expression and distribution of the PKC, especially PKCα, the MAPK levels as well as its effects on APP processing and the trafficking of both α-secretases (ADAM 10 and TACE/ADAM 17).

2. Methods

2.1. Animals

Twenty-nine 12-month-old 129/Sv×C57b/6 male mice were maintained and behaviourally assessed in the facilities of the Medical Psychology Unit. Four to five animals were housed per cage in standard plastic type Macrolon cages (35×35×19 cm, with 2 l of wood cuttings as bedding) up to the time of the experiment. They were maintained at room temperature (22±2 °C) with 60±10% relative humidity and a 12 h light/dark (LD) schedule with lights on at 08:00 h. The animals had lab chow and tap water "ad libitum" until the moment of the test. All the research was conducted in compliance with the Spanish legislation on 'Protection of Animals Used for Experimental and Other Scientific Purposes' and in accordance with the EU Directive 08-88 on this subject.

2.2. Drug treatment

(±)-12-Amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride (huprine X) was obtained from the Laboratori de Química Farmacèutica (Facultat de Farmàcia, Universitat de Barcelona). The drug was dissolved in a vehicle of 0.9% saline solution for chronic intraperitoneal injection in a volume of 1 ml kg^{-1}. At the age of 12 months, the animals received chronic treatment with either huprine X (0.04 and 0.12 μmol kg^{-1}) or saline at 15.00 h daily for 14 days and then the drug treatment continued throughout an additional 7-day period for behavioural testing. In each cage, animals were randomly selected to receive one of the doses of huprine X or saline.

2.3. Assessment of behavioural effects induced by huprine X

During the daily sessions of drug treatment presence or absence of adverse effects such as diarrhoea, tremulous jaw movements was recorded by visual observation of each single animal. Weight was monitored daily to control putative weight loss. Fourteen days after the start of the chronic treatment animals were successively confronted with the following battery of behavioural tests (modified from Giménez-Llort et al., 2002): 1) several spatial learning and memory tests in the Morris water maze: place learning for reference memory, removal and cue learning; 2) open-field test; 3) corner test. Behaviour was evaluated by both direct observation and analysis of video-tape recorded images by an observer unaware of the animal's treatment. The experiments were performed under dim white light (20 lx) during their light phase of the LD cycle (from 10:00 h to 13:00 h).

2.3.1. Morris water maze tests

Two paradigms in the Morris water maze were carried out (Giménez-Llort et al., 2002). The mice were trained to locate a platform (7 cm diameter) in a circular pool (Intex Recreation Corp. CA, USA; 91 cm diameter; 20 cm height, 25 °C opaque water) located in a black test room with distal cues.

2.3.1.1. Days 1–5, place learning. This place task consisted of progressive training of animals to find the location of the platform until all the three experimental groups performed equally. The procedure involved four trial sessions per day, with trials spaced 15 min apart. The mouse was gently released (facing the wall) from one starting point randomly selected (N, S, E or W) and allowed to swim until they located the platform submerged 1.5 cm in a fixed position (SW quadrant and 10 cm away from the wall). The escape latency was recorded. Mice that failed to find the platform within 60 s were placed on it for 10 s, the same period that was allowed for the successful animals.

2.3.1.2. Day 5, removal. The retention and level of accuracy of the precise location of the platform position achieved were measured in a probe trial or 'removal', one and a half hour after the end of the last session of place task. The procedure consisted of removing the platform from the maze and release the mouse from the north starting point and let the animal navigate for 60 s. The time spent in each quadrant and the navigation trajectories (Lang et al., 2003) were measured by analysis of the video-tapes.

2.3.1.3. Day 6, cue learning or visual platform. In this task, the platform was elevated 1 cm above the water level, with its position in the NW and indicated by a visible stripped flag (5×8×15 cm), whereas external maze cues were removed from the walls. Four trials spaced 15 min apart were administered in one single day. The escape latency was recorded.

2.3.2. Open field test

Animals were assessed for locomotor activity and anxiety/emotionality in an open field (woodwork, white, 50×50×35 cm height). The animals were placed in the centre of the apparatus and were observed for 5 min. The latencies to leave the centre (5×5 cm central square), to reach the peripheral zone (a 5 cm wide square ring next to the walls) and to perform the first rearing were noted. Horizontal (number of crossings) and vertical (number of rearings) locomotor activity, the number and duration of groomings, the number of defecation boli and the presence of urination were also recorded. The apparatus was cleaned thoroughly before testing the following animal.

2.3.3. Corner test

Animals were weighted and placed in the centre of a cage filled with 2 l of clean wood cuttings. One cage was used per animal. Number of visited corners and number of rearings were recorded for 30 s. The number of defecation boli was also recorded, while urination was undetectable because of the beddings.

2.4. Assessment of neurochemical effects induced by huprine X

2.4.1. Antibodies

The immunostaining reactions were performed with the following antibodies: 22C11 (Chemicon, CA, USA; dilution 1:1000) raised against an N-terminal epitope of APP, anti-ADAM10 (Affinity BioReagents USA; dilution 1:1000) for ADAM 10, anti-TACE/ADAM17 (Affinity BioReagents, USA; dilution 1:1000) for TACE/ADAM17, anti-PKCα (Biosciences Transduction Laboratories, UK; dilution 1:1000) for PKCα, anti-phospho PKCα (Biosciences Transduction Laboratories, UK; dilution 1:2000) for phospho PKCα, anti-p-44/42 MAPK (Cell Signalling, USA; dilution 1:1000) for p-44/42 MAPK, anti-phospho-p-44/42 MAPK (Cell Signalling, USA; dilution 1:1000) for phospho-p-44/42 MAPK, anti-β-Actin (Sigma-Aldrich, USA; dilution 1:3000) for β-Actin and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Ambion, USA; dilution 1:40,000) for GAPDH .

2.4.2. Western blotting

After completion of drug treatment and behavioural studies, animals were decapitated; the hippocampus and cortex were dissected out on ice and frozen at −80 °C until subsequent analysis.

Tissue was homogenized using a Polytron apparatus in ice-cold, freshly prepared homogenization buffer 1:20 dilution [1 M Tris–HCl (pH 7.5), 0.5 M EDTA (pH 8); 1% Triton X-100, protease inhibitor cocktail and phosphatase inhibitor cocktail]. Homogenates were centrifuged at 1000 g for 15 min at 4 °C to remove crude nuclear material, then cytosolic and particulate fractions from the supernatant were separated by ultracentrifugation at 100,000 g for 60 min at 4 °C. The particulate fractions were resuspended in the above homogenization buffer. Proteins determined by Bradford assay (30 μg/lane) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes by transference (100 V 2 h at 4 °C). The membranes were blocked overnight at 4 °C and rinsed 3 times during 5 min with TBST (2.5 mM Tris–HCl pH 7.5, 0.09% NaCl, 0.2% Tween-20). They were then incubated with either primary antibodies (APP22C11, anti-PKC-α, anti-phospho-PKC-α, anti-p-MAPK, anti-p-phospho-MAPK, anti-ADAM10, anti-TACE or anti-GAPDH, which was used as a control from the correct separations from both fractions: the cytosolic and the particulate), for 2 h at 25 °C, rinsed twice with TBST and incubated with appropriate secondary antibody (horseradish peroxidase) for 1 h at 25 °C. β-Actin was assayed simultaneously using the β-actin monoclonal antibody as the primary antibody. The immunoblots were developed with western blotting detection ECL reagents. Quantification of results was accomplished using the computerized imaging program Quantity One 4.6.2 (Bio-Rad, Hercules, CA, USA), and the optical density of each sample was corrected using the optical density of the corresponding β-actin band.

2.5. Statistics

Results in behavioural experiments are expressed as mean ± standard error of the mean (S.E.M.) or as incidence of certain behaviours (defecation and urination). Differences between 'doses' and 'dose × interval' interactions in the different Morris water maze tests were analysed by ANOVA for repeated measures. Difference between 'mean latency on day 3' minus 'mean latency on day 1' was calculated and analysed with ANOVA followed by post-hoc Duncan ' s. Paired *t*-test was used to compare differences between trials of the place learning *versus* the last trial of this paradigm. Differences in the incidence of defecation boli in the corner test were measured by Chi-Square test. All the analysis were performed according to the SPSS (version 15.0) software. In all cases, statistical significance was considered at $P<0.05$. All neurochemical experiments were performed by triplicate and expressed as mean ± standard error of the mean (S.E.M.). Graph Prism software version 4 (Graph-Pad Software Inc., San Diego California) was used for statistical analysis. Differences between 'doses' were analysed by One-way ANOVA followed by post-hoc Bonferroni or Student´s *t*-test comparisons. Statistical significance was considered at $P<0.05$.

3. Results

3.1. Assessment of behavioural effects induced by huprine X

Table 1 summarises the behavioural effects induced by huprine X.

3.1.1. Side effects of huprine X

Absence of diarrhoea and tremulous jaw movements was observed all along the experimental procedures. No statistical differences on weight were found between the different groups. All animals showed a similar reduction of percentage of weight at the end of the treatment as compared to the initial one [$F_{(2,28)}=2.11$, *n.s.*] probably due to the i.p. schedule itself.

3.1.2. Effect of huprine X on the Morris water maze

Repeated Measure ANOVA showed a statistically significant 'day' effect, [$F_{(4,104)}=79.838$, $^{*}P<0.01$] and 'day × dose' interaction effect [$F_{(8,104)}=2.560$, $^{\blacktriangle}P<0.05$] which indicates dose-dependent differences in the acquisition of the task (see Fig. 1A). ANOVA showed a 'dose effect' in the group of animals treated with the highest dose of huprine X as they showed a higher acquisition efficiency (day 3 *versus* day 1, $F_{(2,28)}=4.112$, $\#P<0.05$) already in the third daily training session as compared to the other two treatment groups. The animals treated with the lowest dose achieved this level of performance on day 4 and the control group did it on the last day of the place task (day 5).

The better 'day by day' performance of the animals treated with the highest dose of huprine X results from a better 'trial by trial' performance on these days (see Fig. 1B, ANOVA, Day 3, trial 2, $F_{(2,28)}=5.194$, $P<0.05$ and Day 4, trial 2, $F_{(2,28)}=3.297$, $P<0.05$, with both Duncan's test $^{*}P<0.05$, highest dose *versus* the other two groups).

Besides, at the end of the place learning task (day 5, trial 4) all the animals were able to solve the maze with exactly the same accuracy (latency of time). However, the animals treated with the high dose of huprine X exhibited a performance similar to that of this last trial from the first trial of the third day (any previous trial *versus* last trial, all *t*'s > 1.882, *df* 8, $^{a}P<0.05$), those with the lowest dose of huprine X did it from the 4 trial of day 3 (previous trials, all *t*'s > 2.804, *df* 9) while the control group did not achieve that level of efficiency until trial 3 of day 4 (previous trials, all *t*'s > 2.574, *df* 9, $^{c}P<0.05$).

On the probe trial (Fig. 1C), preference for the trained quadrant was measured. Control animals showed higher preference for both the trained and the adjacent right quadrants as compared to the other quadrants, resulting of a search strategy less focused on the platform area (Fig. 1D). In contrast, in the groups treated with huprine X the higher preference for the trained quadrant was clear (All, $F_{(3,39)}<49.57$, $P<0.001$; post-hoc Duncan's comparison, $P<0.05$).

In the cue learning task (Fig. 1A and B), all the groups showed a similar efficiency to reach the visible platform through trials (Repeated measures ANOVA, 'trial' effect: $F_{(3,78)}=2.175$ $P<0.001$, 'trial × dose' effect: $F_{(6,78)}=4.350$ n.s.; 'dose' effect, $F_{(1,26)}=1.242$ n.s.) indicating lack of differences on motivation.

3.1.3. Effect of huprine X on the open-field and corner tests

As summarized in Table 1, no statistically significant differences were observed in any of the variables studied either in the open-field nor the corner test [One-way ANOVA, all *F*'s$_{(2,26)}<2.816$, n.s.]. However, a slight trend to reduce horizontal locomotor activity in the groups treated with huprine X was observed. No differences could be found in the grooming behaviour or in other anxiety-like measures such as low preference for the central open-field area (latency to leave the centre or to reach the periphery). In the corner test for neophobia, a similar number of corners visited and rearings were recorded.

Table 1
Behavioural effects of huprine X in middle aged mice.

	Saline ($n=10$)		Huprine X ($n=10$) 0.04 μmol kg^{-1}		Huprine X ($n=9$) 0.12 μmol kg^{-1}		Effect
A. Weight							
% versus initial weight	92.2 ± 2.2		94.0 ± 1.8		90.9 ± 1.7		
B. Morris water maze							
Place task	See Fig. 1A and B						
Removal	See Fig. 1C and D						
Cue learning	See Fig. 1A and B						
	Mean ± SEM		Mean ± SEM		Mean ± SEM		
C. Open-field							
Latency to leave the center (s)	24.0 ± 7.7		44.2 ± 20.7		21.4 ± 3.4		n.s.
Latency to the peripheria (s)	50.3 ± 28.2		65.8 ± 22.7		56.3 ± 10.5		n.s.
Total number of crossings	102.1 ± 17.1		77.6 ± 13.6		55.8 ± 16.1		n.s.
Total number of rearings	11.8 ± 3.7		9.11 ± 1.9		9.13 ± 3.1		n.s.
Number of groomings	3.7 ± 1.0		2.4 ± 0.5		2.4 ± 0.6		n.s.
D. Corner							
Number of visited corners	7.3 ± 0.9		5.3 ± 0.47		6.2 ± 0.4		n.s.
Number of rearings	1.7 ± 0.4		2.2 ± 0.51		1.0 ± 0.5		n.s.
	Incid	Mean ± SEM	Incid	Mean ± SEM	Incid	Mean ± SEM	
E. Defecation							
A. in the open-field	8/10	1.5 ± 0.4	5/10	1.0 ± 0.4	6/8	1.8 ± 0.5	n.s.
B. in the corner test	6/10	0.7 ± 0.2	1/10	0.1 ± 0.1	1/8	0.1 ± 0.1	1.2
F. Urination							
A. in the open-field	2/10		1/10		1/8		n.s.

Statistical analysis:
Chi-square, incidence of defecation boli in the corner test, 2 degrees of freedom, 7.543, $P=0.023$.
Oneway ANOVA, mean of defecation boli in the corner test, $F_{(2,25)}=4.76$ $P=0.018$, Duncan's post-hoc comparisons, animals treated with any of both doses of huprine X are different from saline with $P<0.05$.

In these behavioural tests, both doses of huprine X induced a trend to reduce the incidence and number of defecator and urination behaviours, in the open-field, while reaching statistical significance in the corner test (see statistical significance in the legend).

3.2. Assessment of neurochemical effects induced by huprine X

3.2.1. Separation of membrane and cytosolic fractions

Firstly, we determined if the procedures of homogenization and centrifugation used allowed us a good separation between cytosolic and membrane fractions. For that purpose, we used the anti-GAPDH since it is well known that its presence, a key cytosolic enzyme involved in glycolysis, clearly allows distinguishing these two cellular fractions. In all cases the results revealed its presence in cytosol but not in the membrane fractions (Fig. 2).

3.2.2. Effects of huprine X on both PKCα and phospho-PKCα in mice cortex and hippocampus

The levels of PKCα and phospho-PKCα were determined in the cytosolic and the membrane fractions from both cortex and hippocampus of huprine X and vehicle treated mice. Fig. 3 shows that the chronic treatment with huprine X (0.04 μmol kg^{-1}) increased the phospho-PKCα/PKCα ratio both in the cytosolic and membrane fractions of hippocampus, but only 0.12 μmol kg^{-1} did it in the cytosol. However, in the cortex none of the two treatments with huprine X was able to modify the phospho-PKCα/PKCα ratio as compared with vehicle treated animals.

3.2.3. Effects of huprine X on both MAPK and phospho-MAPK in mice cortex and hippocampus

In order to determine the possible role of MAPK signaling pathway on the regulation of APP processing by huprine X, the stimulation of MAPK cascade by means of anti-44/42 MAPK and anti-phospho-44/42 MAPK determinations was also investigated. The results show that the highest dose of huprine X (0.12 μmol kg^{-1}) increased the phospho-MAPK/MAPK ratio in the membrane fraction of cortex and hippocampus. In contrast, in the cytosol the two doses of huprine X increased this ratio in cortex but not in the hippocampus (Fig. 4).

3.2.4. Effects of huprine X on APP processing in mice cortex and hippocampus

The levels of membrane-bound APP (holo-APP) in the cortex and hippocampus of vehicle and drug-treated mice were also determined. Fig. 5 illustrates the effects of huprine X on the APP processing. Thus, it can be seen that APP levels were significantly reduced in membrane fraction of both the cortex and hippocampus of mice treated with the highest dose (0.12 μmol·kg^{-1}) of huprine X compared with the vehicle treated animals. Representative immunoreactivity from huprine X and vehicle treated mice is also presented in Fig. 5.

3.2.5. Effects of huprine X on the expression of ADAM 10 and TACE/ADAM 17 secretases in mice cortex and hippocampus

The levels of the two metalloproteases ADAM 10 and TACE/ADAM 17, considered as two putative candidates of α-secretase enzymes (Allinson et al., 2003) were also measured in membranes by western blot analysis in the huprine X treated animals and compared with levels in vehicle treated mice. No changes in ADAM 10 were found in the steady-state levels in any of the groups treated with huprine X, neither on cortex nor on hippocampus. However, a selective increase in the steady-state levels of TACE/ADAM17 was observed in both cortex and hippocampus in the huprine X treated mice albeit it only reached statistical significance with the highest dose (Fig. 6).

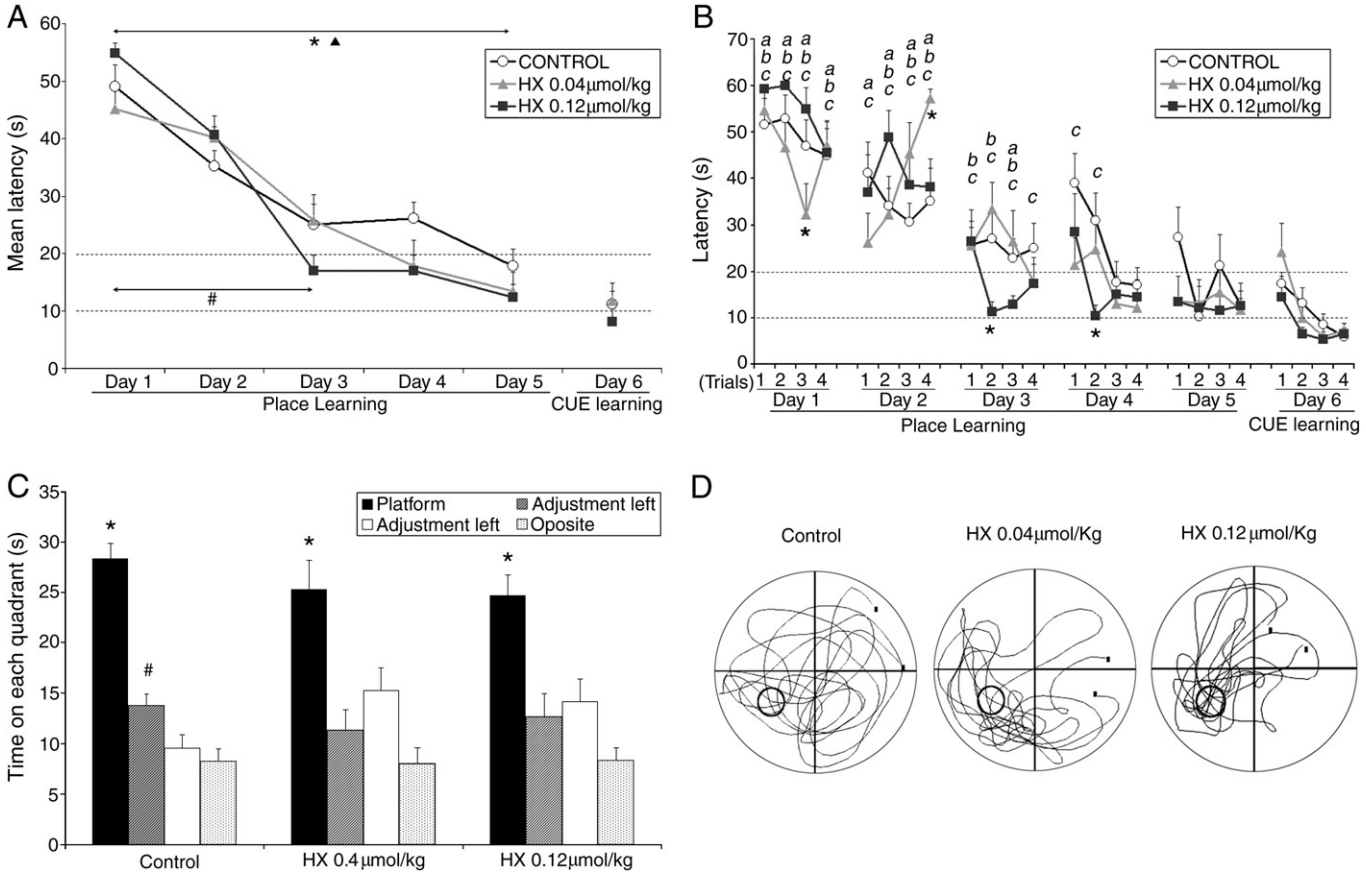

Fig. 1. Effects of huprine X on learning and memory in several tasks in the Morris water maze. Circle symbol: saline, $n=10$; triangle symbol: huprine X (0.04 µmol kg^{-1}), $n=10$; square symbol: huprine X (0.12 µmol kg^{-1}), $n=8$. Results are mean ± SEM. (**A**) Day-by-day representation of the place and cue tasks: Mean total latency to reach the platform in the four trials per session during the place learning and cue tasks. * ANOVA, 'day' effect, $P<0.01$; ▲ ANOVA, 'day × dose' effect, $P<0.05$; # ANOVA, difference day 3 minus day 1, $P<0.05$ and post-hoc Duncan's test: huprine X (0.12 µmol kg^{-1}) *versus* the other two treatment groups, $P<0.05$. (B) Trial-by-trial representation of the place task and cue tasks: Latency to reach the platform in the four trials per session during the place learning. In the place task, *$P<0.05$ *versus* the other two groups; ^{a,b,c} $P<0.05$ *versus* the last trial (day 5, trial 4) for huprine X (0.12 µmol kg^{-1}), huprine X (0.04 µmol kg^{-1}) and saline, respectively. (C) Removal: Time spent in platform, the trained quadrant where the platform was previously located; the adjacent right; opposite and adjacent left quadrants during the free swim trial (session 5 day 19). *$P<0.05$ *versus* all the other quadrants, #$P<0.05$ *versus* the adjacent right quadrant (Duncan's test). (D) Representative searching strategies shown in that group of treatment.

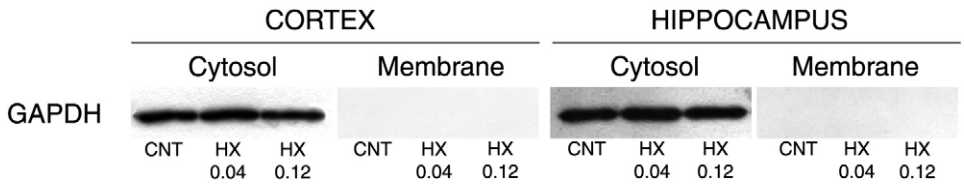

Fig. 2. Representative western blot of levels of GAPDH in cytosol and membrane fractions of cortex and hippocampus of mice. Control (CNT), huprine X 0.04 μmol kg^{-1} (HX 0.04) and huprine X 0.12 μmol kg^{-1} (HX 0.12).

4. Discussion

The present study is the first one to provide a characterization of the behavioural and neurochemical effects of huprine X, a huperzine A-tacrine hybrid that exhibits high selectivity and inhibitory potency on acetylcholinesterase (Camps et al., 2000a) as well as an agonistic action on muscarinic M_1 and nicotinic receptors (Román et al., 2002, 2004). Our previous results demonstrated that huprine X has 180 times higher affinity to acetylcholinesterase than huperzine A (Camps et al., 2000b). Taking that into consideration, the low dose of huprine X (0.04 μmol kg^{-1}) was chosen as that equivalent to that of huperzine A used previously in "in vivo" studies (Zhang et al., 2004). In addition, a higher dose (12 μmol kg^{-1}) of huprine X was also used in the present work.

The behavioural assessment first considered the effects of huprine X in learning and memory because of the known cognitive enhancing properties of cholinomimetics. Morris water maze was chosen to assess the cognitive effects of huprine X because some studies in rodents and humans have also implicated acetylcholine not only as a modulator of memory but also of visuospatial abilities (Schildein et al., 2000).

Since some AChEI drugs have been reported to cause motor dysfunction and exert some anxiolytic actions, the effects of huprine X on locomotor activity and emotionality/anxiety-like behaviours were also studied by means of the open-field and corner tests (Giménez-Llort et al., 2007). The results showed that daily injections of 0.04 and 0.12 μmol kg^{-1} of huprine X for 2 weeks exert cognitive and emotional effects in middle aged mice without the presence of secondary motor deficits.

Animals receiving chronic treatment of huprine X achieved an optimised performance in the place acquisition in the maze earlier than saline treated animals, and in a dose dependent manner. The group treated with 0.04 μmol kg^{-1} huprine X reached performance levels similar to those of the last trial on day 4 and those treated with 0.12 μmol kg^{-1} huprine X did it already on day 3. The evaluation of the performance of each group on a trial-by-trial basis evidenced the better performance shown by huprine X treated animals was mainly related to the inter-trial acquisition (short-term memory). The quadrant preferences and search strategies during the removal trial also indicated that animals treated with huprine X were more selective for the platform quadrant (focal searching and scanning, Lang et al., 2003) while the control group performed a random swimming. Finally, all animals were equal in the cue learning which is aimed to reveal putative effects on motor abilities, attention and motivational aspects.

Altogether, these paradigms in the Morris water maze showed that huprine X was able to facilitate cognitive performance of middle aged mice at very low range of doses, which is much lower than those reported for tacrine (5 μmol kg^{-1}, Cheng and Tang, 1998), huperzine A

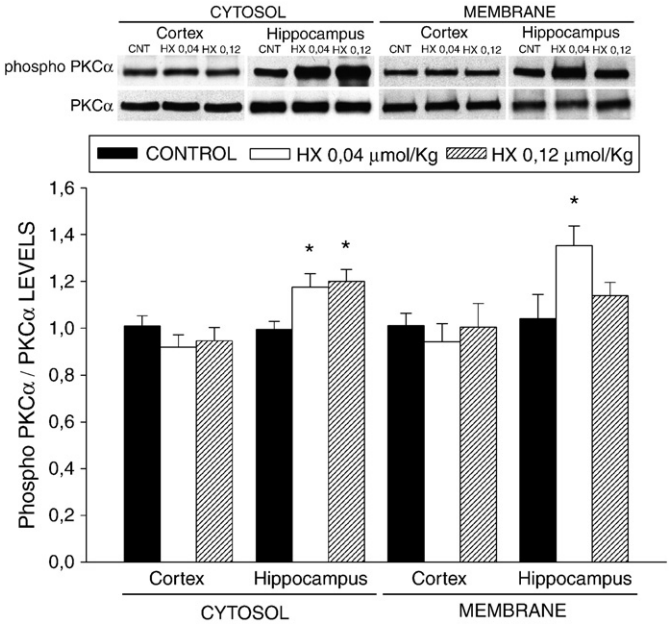

Fig. 3. Effects of huprine X on phospho-PKCα/PKCα ratio. Representative western blot levels of phospho-PKCα and PKCα on cytosol and membrane fractions of both cortex and hippocampus of vehicle and huprine X (0.04 or 0.12 μmol kg^{-1}) treated mice. Densitometric analysis is expressed as phospho-PKCα/PKCα ratio after normalizing to levels of β-actin. Data are presented as mean ± SEM; *$P<0.05$ *versus* vehicle treated animals; $n=6$ mice in each group; each experiment was performed by triplicate.

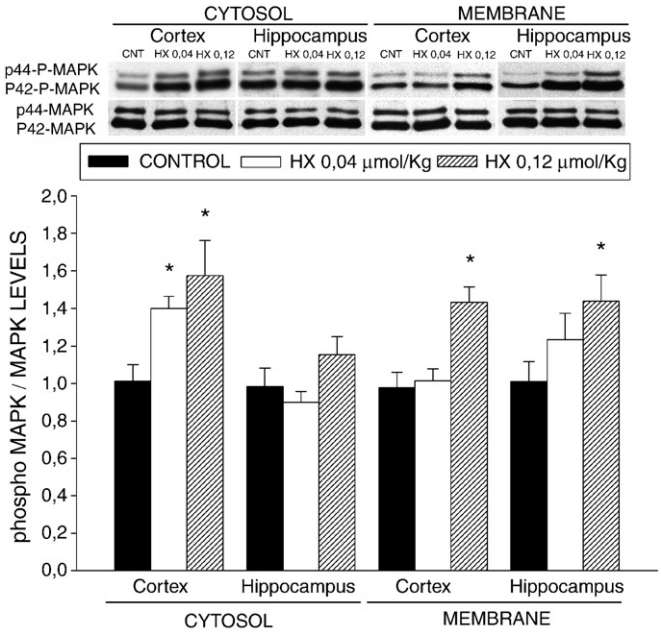

Fig. 4. Effects of huprine X on phospho-MAPK/MAPK ratio. Representative western blot levels of phospho-MAPK and MAPK on cytosol and membrane fractions of both cortex and hippocampus of vehicle and huprine X (0.04 or 0.12 μmol kg^{-1}) treated mice. Densitometric analysis is expressed as percent of the respective control untreated mice. Previously the blots were normalized with levels of β-actin. Data are presented as mean ± SEM; *$P<0.05$ *versus* vehicle treated animals; $n=6$ mice in each group; each experiment was performed by triplicate.

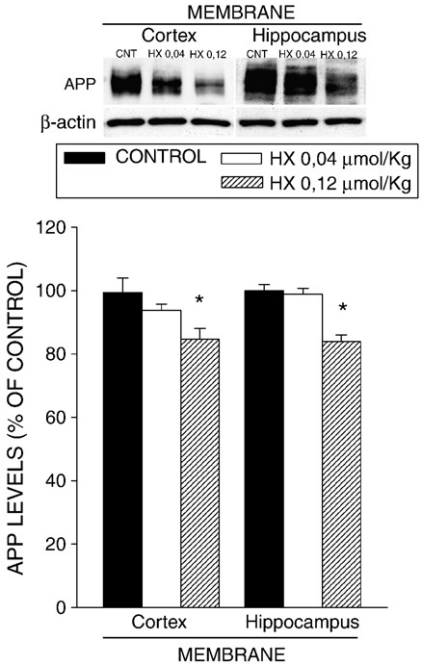

Fig. 5. Effects of huprine X on holo-APP. Representative western blot levels of holo-APP on membrane fractions of both cortex and hippocampus of vehicle and huprine X (0.04 or 0.12 µmol kg^{-1}) treated mice. Densitometric analysis is expressed as percent of the respective control untreated mice after normalizing to levels of β-actin. Data are presented as mean ± SEM; *$P<0.05$ *versus* vehicle treated animals; $n=6$ mice in each group; each experiment was performed by triplicate.

(0.8 µmol kg^{-1}, Cheng and Tang, 1998; Zhang et al., 2004) and even for structurally non related AChEI such as galantamine (5–10 µmol kg^{-1}, Van Dam and De Deyn, 2006) and donepezil (0.26–2.6 µmol kg^{-1}, Dong et al., 2005). The agonistic activity of huprine X on both M_1 and nicotine receptors may also partly account for these cognitive enhancing effects

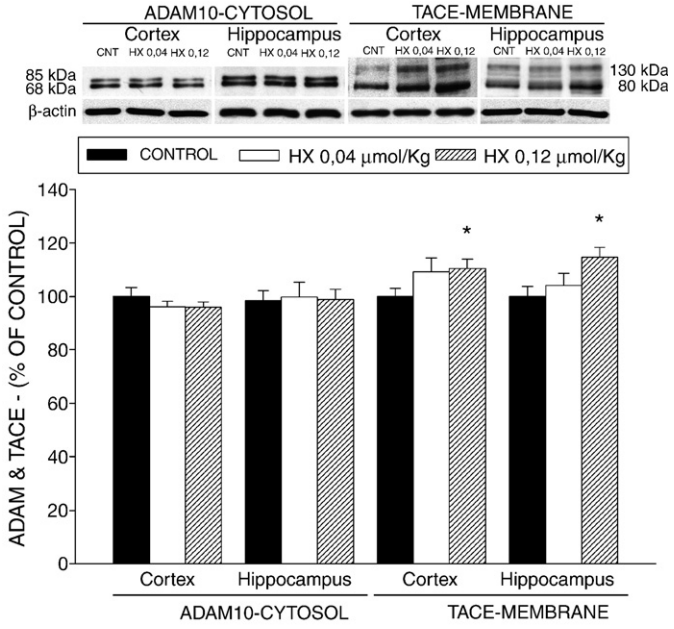

Fig. 6. Effects of huprine X on ADAM10 and TACE/ADAM17. Representative western blot levels of ADAM10 and TACE/ADAM17 on membrane fraction of both cortex and hippocampus of vehicle and huprine X (0.04 or 0.12 µmol kg^{-1}) treated mice. Densitometric analysis of ADAM10 and TACE/ADAM17 is expressed as percent of the respective control untreated mice after normalizing to levels of β-actin. Data are presented as mean ± SEM; *$P<0.05$ *versus* vehicle treated animals; $n=6$ mice in each group; each experiment was performed by triplicate.

(Román et al., 2002, 2004) as it has been demonstrated with both muscarinic and nicotinic agonists (Buccafusco and Terry, 2000). It is important to note, that this facilitatory effect of huprine X on cognition was assessed in middle aged animals without experimentally induced cognitive impairments. Whether these nootropic effects are able to ameliorate cognitive dysfunction caused by advanced aging and by β-amyloid peptide remains to be studied. However, huperzine A has already demonstrated to be able to relieve memory deficits in aged subjects and patients with Alzheimer's disease without any remarkable side effects (Tang and Han, 1999) and to attenuate cognitive dysfunction and neuronal degeneration caused by β-amyloid 1-40 in rat (Wang et al., 2001).

Evaluation of adverse effects is a relevant aspect to be explored in drugs with psychotropic properties which are likely to be used as cognitive enhancers in the elderly or therapeutical agents in several kinds of dementia, to the extent that they are already important items considered in the Physicians' desk Reference. Unfortunately, most AChEI drugs are not exempt from adverse effects such as nausea, vomits, anorexia and diarrhoea mainly due to fast cholinergic central administration (Raskind et al., 2000) but can be attenuated with a slower increase of the drug treatment. Some can also exert undesirable extrapiramidal effects. For instance, tacrine produces a dose-related suppression of open-field motor activity (Carriero et al., 1997) while, as mentioned above, the cognitive improvement induced by huperzine A seems to be free of these side effects. In our study, huprine X has effects on learning and memory performance of middle aged mice without inducing diarrhoea, affecting weight, nor their motor activity patterns. The slight trend to reduce the horizontal activity observed in the open-field is far from the drastic motor effects induced by tacrine (Carriero et al., 1997), although the possibility that higher doses of huprine X may have this effect cannot be ruled out. In addition, tremulous jaw movements are motor deficits usually induced by administration of tacrine (Carriero et al., 1997) and were not observed during none of the daily sessions of huprine X administration nor during any of the behavioural tests, which supports the lack of motor disturbances in animals treated with this compound. Thus, the corner test and also the open-field test were aimed to assessing the effects of huprine X in anxiety-like and neophobic behaviours as well as to record putative side effects on motor function as demonstrated by other AChEIs (i.e. tacrine). It is suggested that the calming and sedative effects of AChEIs observed in patients with Alzheimer's disease may be directly related to their anxiolytic action, independent of an improvement in cognitive functions, which in turn may decrease disorientation-induced distress and anxiety (Sienkiewicz-Jarosz et al., 2003). In fact, some anxiolytic-like effects have been described after treatment with AChEIs. For instance, it has been suggested that these emotional effects induced by tacrine are due to its MAO-A inhibitory activity or its established activity on the serotonergic system (Hallam et al., 2004; Adem et al., 1989). The effects of huperzine A in the serotonergic system have not been studied yet. In the present work, no group differences were found in the anxiety-like behaviours measured in the open-field test. Grooming, considered a strategy to cope with stress or the animal's fear to novelty and to be anticipated (latency) and increased (number) in anxious animals (reviewed by Steimer and Driscoll, 2005) was not modified by treatment with huprine X although a trend to reduce this behaviour in the open-field test was observed in the animals treated with the lowest dose of huprine X. On the other hand, in the corner test to measure neophobia, the number of defecation boli was drastically reduced in the groups treated with huprine X. The number of defecations in novel or stressful situations is a measure that depends on the autonomous nervous system and it is related to the animal's response to stress (Gray and McNaughton, 2000; Steimer and Driscoll, 2005). Therefore, these results suggest that huprine X exerts some emotional effects as it reduces emotional reactivity of animals in an unknown environment. It is important to note that the habituation

to handling during the 21 days of chronic treatment, which has been reported to decrease anxiety-like behaviours and to prevent the effects of benzodiazepines at the behavioural and neurochemical levels (Boix et al., 1990) may have reduced the anxiety of all the groups of animals so that it is difficult to find the differences when assessed in the tests.

At the end of this behavioural battery of tests, brains were dissected in order to perform the subsequent molecular studies. Many recent findings have demonstrated that there is a strong relationship between the PKC isozyme signaling deficits and the associative memory storage for several animal models (Alkon and Rasmussen, 1988; Alkon et al., 1998) that could participate as a molecular locus for the short-term memory loss of AD. Among the different PKC isoforms (Nishizuka, 1989) we decided to focus on PKCα isozyme because it has been reported to be involved in the constitutive and phorbol ester secretion of soluble APP (Benussi et al., 1998). In addition, it seemed of interest to investigate not only the effects of the huprine X on the regulation of PKCα but also on MAPK activation, the APP processing, as well as the trafficking of both ADAM 10 and TACE/ADAM 17. Their expression and subcellular distribution were studied, always in brain samples from the same animals used in the behavioural studies.

First, neurochemical studies were devoted to verify the accuracy of the procedure of separation of cytosolic and membrane fractions. It is well-known that GAPDH is one of the key enzymes involved in glycolysis and it catalyses the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate. Thus, the presence of GAPDH in the cytosol, but not in the membrane, can be used as an indication of the separation of the two fractions. In all our experiments, it was seen almost a complete separation of both fractions in both the huprine X and vehicle treated mice.

The present study demonstrates that the chronic treatment by huprine X, in addition to improve learning and memory in a dose dependent manner, also increased the expression of phospho-PKCα on both fractions of the mice hippocampus. This was shown as an increase of phospho-PKCα/PKCα ratio. However, these effects were only observed in the membrane fraction of hippocampus of the mice treated with the lowest dose of the anticholinesterasic. This observation agrees with previous results obtained with rasagiline, another potent inhibitor of acetylcholinesterase (Bar-Am et al., 2004). Direct and indirect receptor-mediated activation of PKC has been shown to increase the release of soluble APP (sAPPalpha) and reduce the secretion of β-amyloid peptides. Experimental evidence suggests that specific isoforms of PKC, such PKCα and PKCε are involved in the regulation of APP metabolism (Racchi et al., 2003). In addition many recent findings point out that PKC signaling deficits as a common mechanistic basis for all the major elements in the neurodegenerative pathophysiology of Alzheimer's disease (Alkon et al., 2007). Thus, compounds, as huprine X, that are able to modulate this pathway could be of beneficial interest for treating both neurodegeneration and its symptomatic memory loss in aging and/or Alzheimer's disease.

It has been demonstrated that huperzine A, one of the parent compounds from which huprine X was designed (Camps et al., 2000b), also stimulates MAPK phosphorylation in neuroblastoma SK-N-SH-APPwt cells (Peng et al., 2007). Several reports have shown that MAPK could act as an important mediator in the regulation of shedding of membrane proteins with the inclusion of APP (Desdouits-Magnen et al., 1998; Mills et al., 1997). Thus, it was of interest to determine the influence of huprine X on p42/44 MAPK pathway. Our results clearly demonstrate that the animals chronically treated with the new anticholinesterasic compound showed an increase in the immunoreactivity of the phosphorylated MAPK/MAPK ratio. The huprine X induced-increase of the MAPK activity was more evident in the membrane than in the cytosolic fraction, at least in the hippocampus indicating a probable translation of the active conformation of these enzymes from cytosol to the membrane. It has been demonstrated that the stimulation of sAPPalpha release is highly regulated through the activation of various signaling pathways, including PKC, MAPK and others (Mills and Reiner, 1999). Thus the activation of MAPK induced by huprine X could also participate in the stimulation of sAPPalpha release. These findings are in line with previous data obtained in the PC12 cells incubated with the propargylamine derivatives, TV 3326 and TV 3279, that are two new potent anticholinesterasic compounds (Yogev-Falach et al., 2002).

The altered processing and/or increased expression of full length APP and therefore the increase in generation of β-amyloid peptide may play a central role in the process of amyloidogenesis (Mills and Reiner, 1999). Thus, it seemed interesting to determine the holoAPP levels in the membrane of control and treated animals. Our results showed that huprine X induced a small but significant decrease of full length APP in the membrane of both cortex and hippocampus in the treated mice compared with saline treated animals. The observation that huprine X is able to decrease the levels of membrane bound APP (holo APP) indicated that it could be of value in accelerating non-amyloidogenic APP processing, thereby reducing β-amyloid levels. These results are consistent with the finding that the mice treated with other AChEI such as rasagiline and/or TV 3326 and TV 3279 also reduced full-length APP levels in the hippocampal membrane fractions (Bar-Am et al., 2004) and are suggestive of potential beneficial therapeutic effects on amyloidogenic processes related to aging or Alzheimer's disease.

Related to this, several studies have demonstrated that ADAM10 and TACE/ADAM17 can be considered likely candidates for α-secretase APP cleavage (Lammich et al., 1999; Nunan and Small, 2000). In our experiments we found no changes in membrane ADAM10 steady-state levels in both cortex and hippocampus following the treatment of the mice with huprine X. However, a slight but significant selective increase in membrane TACE/ADAM17 was observed after the treatment with the anticholinesterasic drug. Similar results have been recently obtained with the selective M_1 receptor agonist AF267B in the 3xTg-AD mice (Caccamo et al., 2006). All these results are consistent with previous reports indicating that TACE/ADAM17 is inducible by activation of muscarinic receptors, whereas ADAM10 is the major constitutive enzyme (Allinson et al., 2003). Therefore, both the AChE inhibition and the known agonistic action of huprine X on muscarinic M_1 receptors (Román et al., 2002) may account for this effect.

In summary, huprine X, in a dose dependent manner, showed a facilitatory effect on learning and memory, improved some indicators of emotionality without affecting motor activity and anxiety-like behaviours. Moreover, since the major way to stimulate the non-amyloidogenic cleavage of APP is either directly by activation of PKC isoenzymes and/or indirectly through PKC activation of MAPK pathway (Alkon et al., 2007), the increases of both phospho-PKCα and phospho-42/44 MAPK observed at least in the animals treated with the highest dose of huprine X, could participate in the rise of membrane holoAPP processing through the induction of α-secretase activity.

In conclusion, it can be speculated that huprine X directly or indirectly could activate muscarinic and nicotinic receptors, thereby initiating downstream PKC/MAPK signaling pathways which mediate non-amyloidogenic α-secretase cleavage of APP to form αAPPs in the treated mice and contribute to the improvement of memory and learning activities observed in the middle aged mice treated with this new anticholinesterasic drug. Consequently, present results infer us to develop further studies to analyze the effects of huprine X in a more specific cognitively impaired animal such as a transgenic mouse model of Alzheimer's disease.

5. Conflict of interest

The authors have no conflicts of interest to report. I certify hereby that this work has never been published and it is not in current process publication.

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