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ORIGINAL PAPER

Protein τ-Mediated Effects on Rat Hippocampal Choline Transporters CHT1 and τ-Amyloid β Interactions

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Abstract It is suggested that intracellular tau protein (τ), when released extracellularly upon neuron degeneration, could evoke direct toxic effects on the cholinergic neurotransmitter system through muscarinic receptors and thus contribute to the pathogenesis of Alzheimer's disease. In this study, we evaluated the in vitro effects of six naturally occurring monomeric τ isoforms on rat hippocampal synaptosomal choline transporters CHT1 (large transmembrane proteins associated with high-affinity choline transport and vulnerable to actions of amyloid β peptides (Aβ) applied in vitro or in vivo). Some τ isoforms at nM concentrations inhibited choline transport in a dose- and time-dependent saturable manner ($352 = 441 > 410 = 383 > 381 = 412$) and effects were associated with changes in the Michaelis constant rather than in maximal velocity. Moreover, the actions of τ 352/441 were not influenced by previous depolarisation of synaptosomes or by previous depletion of membrane cholesterol. Specific binding of [3H]hemicholinium-3 was not significantly altered by τ 352/441 at higher nM concentrations. Results of in vitro tests on CHT1 transporters from cholesterol-depleted synaptosomes supported interactions between Aβ 1-40 and τ 352. In addition, we developed surface plasmon resonance biosensors to monitor complexes of Aβ 1-42 and τ 352 using a sandwich detection format. It seems, therefore, that protein τ, similar to Aβ peptides, can contribute to the pathogenesis of Alzheimer's disease through its actions on CHT1 transporters. However, the interaction mechanisms are quite different (τ probably exerts its effects through direct interactions of microtubule binding repeats with extracellular portions of the CHT1 protein without influencing the choline recognition site, Aβ rather through lipid rafts in the surrounding membranes). An N-terminal insert of τ is not necessary but the N-terminal projection domain plays a role. The developed biosensor will be used to detect Aβ–τ complexes in cerebrospinal fluid in order to evaluate them as prospective biomarkers of Alzheimer's disease.

Keywords Tau protein · Amyloid β peptide · Choline transporter · Interactions

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Introduction

Amyloid β peptide (Aβ) and tau protein (τ) both have key roles in Alzheimer's disease (AD), forming the two hallmark pathologies visible in the brains of demented people. More specifically, Aβ is a basic component of extracellular amyloid plaques and τ of intracellular neurofibrillary tangles. However, recent research indicates that Aβ can accumulate initially in AD, especially intracellulary [1], and that intracellular τ, when released extracellularly upon neuronal death, can be neurotoxic, e.g. through direct effects on acetylcholine muscarinic receptors [2–8]. In particular, τ can bind to muscarinic M1/M3 receptors and it appears that monomeric τ interacts with the receptors with a higher affinity than the aggregated form [5], and only dephosphorylated τ can stimulate them [8]. Toxic effects of τ are probably associated with its direct binding to receptors at a site different from that for acetylcholine (τ has a higher affinity for M1/M3 receptors than acetylcholine

itself and neurotransmitter cannot displace bounded τ) and with an ability of τ to repeatedly stimulate the receptors, in contrast to the desensitisation evoked by acetylcholine [7]. Through activation of the receptors, τ mediates an increase in intracellular calcium [2–6]. While antagonists of M1/M3 receptors inhibit τ-mediated increases in intracellular calcium [5, 6], agonists decrease the phosphorylation of τ [2–4]. In addition, exogenous τ promotes the phosphorylation of endogenous τ and its release into the extracellular medium where it can be dephosphorylated [8]. The presence of extracellular τ has recently been revealed in microdialysis samples of patients with brain injury [9]. However, the presence of τ in the cerebrospinal fluid of healthy controls or in experiments on cultured cells demonstrating an active release of intracellular τ into the extracellular space suggest that extracellularly localized full-length monomeric τ should not be observed only under pathological conditions in vivo or *post mortem* [10, 11].

Although the mechanisms of interaction between τ and M1/M3 receptors are not yet known in detail, it seems very probable that the microtubule binding domains of τ could play a role here. It is well known that τ, with an overall basic character, consists of two major parts: the N-terminal projection domain (up to residue 120 it is acidic and projects away from the acidic microtubule surface; it is suggested that it may interact with other cytoskeletal elements and the plasma membrane) and the C-terminal part containing positively charged microtubule-binding domains facilitating electrostatic interactions [12–17]. There are 6 natural isoforms of human τ encoded by a single gene located on chromosome 17q21–22 and generated by alternative mRNA splicing. These isoforms differ in the absence (0 N) or presence of 1 (1 N) or 2 (2 N) N-terminal amino acid inserts and either 3 (3R) or 4 (4R) 18 amino acid microtubule-binding repeats located at the C-terminal. A function for the N-terminal inserts containing negatively charged amino acids is not yet known; nevertheless, it seems that the N-terminal projection domain could either act as a regulatory domain to modulate the affinity of the C-terminal binding domain (e.g., weaken the affinity of τ for microtubules [12]) or regulate the solubility of τ. The soluble conformation can be stabilised via an association of the extreme C-terminus to microtubule-binding repeats as well as an association of the N-terminal to the C-terminal region [13, 14]; the insoluble conformation based on polymerisation of τ could be associated with an interaction of the extreme N-terminus with microtubule-binding repeats and when the C-terminus is free [14]. On the other hand, the function of the C-terminal repeats is more clearly established. The binding of τ to microtubules seems to be specific and saturable; moreover, the increased number of repeats enhances its affinity (e.g., each repeat binds weakly, the first repeat binds more tightly than the other repeats and each repeat binds to a separate tubulin monomer [14]).

While the specific binding to microtubules probably reflects the main physiological role of τ in stabilising microtubules and regulating axonal transport, mutual interactions of τ and Aβ appear to be markedly involved in the pathogenesis of AD, in addition to their separate toxic effects. Specifically, it is suggested that the postsynaptic toxicity of Aβ may be dependent on τ localised in dendrites and that this mechanism involves several postsynaptic receptors. On the other hand, the presynaptic toxicity of Aβ could be associated with Aβ-mediated hyperphosphorylation of τ, leading to elimination of its binding to microtubules and its increasing localisation in soma or dendrites [18]. It also seems that the Aβ-induced impairment of long-term hippocampal potentiation requires τ and that hyperphosphorylated τ probably plays a role here [19]. Moreover, in vitro experiments support a direct interaction of Aβ (through residues 25–35) and the τ region involved in microtubule binding (residues 307–325). This interaction facilitates the aggregation of τ but decreases that of Aβ [20]. The results of surface plasmon resonance (SPR) assays suggest 1,000-fold higher affinities of τ for Aβ 1-40/1-42 than of τ for itself [21].

Data in the literature report a high vulnerability of basal forebrain cholinergic neurons to actions of Aβ through τ and suggest a possible involvement of other components of the cholinergic system [22–24], in addition to muscarinic M1/M3 receptors [2–8]. Our previous research has been focused on hippocampal CHT1 transporters localised specifically on presynaptic cholinergic nerve terminals and involved in high-affinity choline uptake (HACU) transport. It is well known that CHT1 transporters play a key regulatory role in the synthesis of the neurotransmitter acetylcholine and that they are very vulnerable to Aβ effects. Namely, in a dose- and time-dependent manner, peptides inhibit HACU transport, estimated by means of the substrate [3H]choline, and the specific binding of competitive inhibitor [3H]hemicholinium-3 ([3H]HC-3). Moreover, previous depolarisation or depletion of membrane cholesterol enhances the effects mediated by Aβ on CHT1 [25–27].

The aim of this study is to evaluate acute effects of six naturally occurring monomeric τ isoforms on CHT1 and compare them with those mediated by Aβ. Since we will evaluate naturally created Aβ–τ complexes in human cerebrospinal fluid as prospective biomarkers of AD in our future research, the second aim of the study is to estimate interactions of Aβ and τ in in vitro experiments. Interactions of Aβ 1-40 and τ 352 are studied on CHT1 from cholesterol-depleted synaptosomes. The formation of complexes between Aβ 1-42 in various concentrations and τ 352 is studied by SPR biosensor technology using a sandwich assay.

Materials and Methods

Animals and Brain Tissue Preparation

Experiments were carried out on male Wistar rats aged 3–4 months (51 animals in total weighing 350–400 g). Rats were housed in groups of two under a 12-h light/dark cycle. Food (standard pellet diet) and water were available ad libitum. All experiments were carried out in accordance with Animal Protection Law 246/1992 and Regulation 204/2004 (Czech Republic) concerning the use of animals in experimental procedures. Animals were euthanized by cervical dislocation and decapitation; the brains were quickly removed from the skulls and washed in redistilled water for 10 s. Both hippocampi (i.e., hippocampus proper, dentate gyrus and subiculum) were dissected on an ice-cold plate, weighed, transferred to ice-cold 0.32 M sucrose and immediately used for the preparation of synaptosomes.

Preparation of Synaptosomes

The hippocampi were homogenised in ice-cold 0.32 M sucrose (10 ml/g tissue) with a glass-Teflon Potter′s Braun homogeniser (10 up- and down-strokes at 800 rpm). The homogenate was centrifuged (Universal 32R centrifuge, 1,000*g* for 10 min, 4 °C) and the first supernatant was removed and stored in a refrigerator. The pellet was resuspended in 0.32 M sucrose (5 ml/g original tissue), centrifuged under the above-mentioned conditions and the second supernatant was removed again. The combined supernatants were centrifuged at 12,000*g* for 20 min at 4 °C (Beckman J2-HS centrifuge), and the pellet was resuspended in 0.32 M sucrose (5 ml/g original tissue) and re-centrifuged. The final pellet was resuspended in the original volume of sucrose (10 ml/g tissue) by means of ultraturrax (IKA-Labortechnik) and 20 µl of the resulting synaptosomal suspension was used to estimate protein concentrations (Bradford method). The synaptosomes (final concentrations equalled 2 mg/ml of total proteins in all cases) were treated as follows: (1) immediately used in current experiments; (2) pre-incubated either with 55 mM NaCl (basal conditions) or 55 mM KCl (stimulated conditions) for 10 min at 37 °C, centrifuged at 12,000*g* for 20 min at 4 °C, resuspended in the original volume of 0.32 M sucrose and used immediately; or iii) pre-incubated either with water (undepleted synaptosomes) or 10 mM methyl-β-cyclodextrin (MβCD; cholesterol-depleted synaptosomes) for 15 min at 37 °C, centrifuged at 12,000*g* for 20 min at 4 °C, and the pellets resuspended again in the original volume of 0.32 M sucrose and used immediately.

Stock Solutions

Six isoforms of τ (352, 381, 383, 410, 412 and 441, all of Sigma) were freshly diluted in redistilled water (5 µM stock solutions) and used immediately. The amyloid β fragment 1-40 (Aβ 1-40, Sigma) was freshly diluted in 10 % dimethylsulphoxide (DMSO) and used immediately. Our previous experiments suggest that especially monomeric amyloid and small oligomers (e.g. dimers), but not fibrils, are presented under these conditions [25].

HACU Experiments

Aβ 1-40 and/or isoforms of τ were pre-incubated with synaptosomes (final concentrations of synaptosomal proteins equalled 200 µg/ml), resuspended in Krebs–Ringer-HEPES-glucose buffer (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM glucose and 10 mM HEPES, pH = 7.4) for 10–60 min and subsequently with 10 nM [3H]choline ([methyl-3H]choline chloride, Perkin Elmer Life Science, specific activity 80.6 Ci/mmol) for 4 min, both at 37 °C. The incubation was terminated by rapid cooling and filtration under vacuum (Whatman BF/B filters). HACU was defined by its sensitivity to unlabelled HC-3 (Sigma) and was calculated as the difference between uptake in samples incubated with and without 1 µM HC-3. The Lineweaver–Burk plot analysis was performed with 0.01–1 µM [3H]choline.

Experiments with [3H]HC-3

Two isoforms of τ (352 and 441) were pre-incubated with synaptosomes (final concentrations of synaptosomal proteins equalled 200 µg/ml) resuspended in glycylglycine buffer (50 mM glycylglycine, 200 mM NaCl, pH = 7.8) for 60 min and subsequently with 20 nM [3H]HC-3 ([methyl-3H]hemicholinium-3 diacetate salt, American Radiolabeled Chemicals, specific activity 169.9 Ci/mmol) for 30 min, both at room temperature. Filtration was performed analogously to the HACU experiments. Parallel incubation in the presence of 10 µM HC-3 was used to define the nonspecific binding.

Statistical Analysis

Two-way and one-way ANOVA for global and Student′s *t* test (pooled variance) for pairwise comparisons were used. The least-squares linear regression analysis was applied for estimation of Lineweaver–Burk plots. Data in the Tables and Figures are presented as mean ± S.D

Reagents for SPR Biosensor Experiments

11-mercapto-tetra(ethyleneglycol)-1-undecanol: HS–$(CH_2)_{11}$–$(O(CH_2)_2)_4$–OH (OH–EG_4–AT), and 11-mercapto-undecyl-hexa(ethyleneglycol) acetic acid: HS–$(CH_2)_{11}$–$(O(CH_2)_2)_6$–OCH_2–COOH (COOH–EG_6–AT), were purchased from ProChimia, Poland. Bovine serum albumin (BSA) and ethanolamine hydrochloride (EA) were purchased from Sigma-Aldrich, Czech Republic. 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were both included in the Amine Coupling Kit from Biacore, Sweden. Beta amyloid 1-42 (Aβ 1-42) was purchased from American Peptide Company, USA. A monoclonal antibody against Aβ 1-16 (clone 6E10) [anti-Aβ (1-16)] was obtained from Covance, USA. Tau 352 (τ 352) was obtained from rPeptide, USA. A monoclonal antibody against τ (clone RB-41) (anti-τ) was purchased from Abnova, Taiwan. Buffers: 10 mM sodium acetate pH 5.0 (SA), 10 mM phosphate, 2.9 mM KCl, 137 mM NaCl, pH 7.4 (PBS) and 10 mM phosphate, 2.9 mM KCl, 0.75 M NaCl, pH 7.4 (PBNa) were purchased from Sigma-Aldrich, Czech Republic.

SPR Biosensor

Surface plasmon resonance (SPR) is a direct, label-free and real-time method that measures refractive index changes produced by the binding of an analyte to its biospecific partner (receptor) immobilised on the sensor's surface. In the reported work, we used a four-channel SPR sensor developed at the Institute of Photonics and Electronics, Prague [28, 29].

SPR Chip-Surface Functionalisation

Initially, the gold surface of the SPR chip was cleaned by washing with absolute ethanol and deionised water, dried with a stream of pure nitrogen and placed in ozone cleaner in order to remove organic contaminants. After the cleaning process, the chip was washed with deionised water and absolute ethanol and dried with a stream of nitrogen. In order to create a mixed self-assembled monolayer (SAM) of alkanethiols on the chip's surface, the cleaned chip was immersed in an ethanolic solution of OH–EG_4–AT and COOH–EG_6–AT alkanethiols mixed in a ratio of 7:3 (with a final concentration of 200 μM) and stored in a dark place at room temperature for up to 1 day. The COOH–EG_6–AT alkanethiols terminating with a carboxylic head group were used to covalently attach antibody (receptor) by amino coupling, while OH–EG_4–AT alkanethiols terminated with a hydroxyl group were used to form a stable low-fouling background. After the formation of a mixed SAM, the chip was removed from the solution, rinsed with absolute ethanol and deionised water, dried with a stream of nitrogen and immediately mounted to the SPR instrument. In the following step, the terminal carboxylic groups on the mixed SAM-coated surface were activated in situ by injecting deionised water followed by a (1:1) mixture of NHS (11.51 mg/ml) and EDC (76.68 mg/ml) for 5 min and then deionised water again. Covalent attachment of the first antibody against protein τ (anti-τ) was performed as follows: SA buffer was passed across the activated surface followed by anti-τ at a concentration of 2.5 μg/ml for 10 min and then SA buffer again. Subsequently, the high ionic strength PBNa buffer was passed along the sensor surface to remove any non-covalently bound anti-τ. In the next step, 5 μg/ml BSA was injected to saturate the surface coated with anti-τ and to minimise nonspecific adsorption. After running with SA buffer, non-covalently bound BSA was washed away from the surface with PBNa buffer. Finally, the sensor surface was treated with 1 M ethanolamine hydrochloride to deactivate residual carboxylic groups.

SPR Detection Format-Sandwich Assay

The detection of τ 352 and Aβ 1-42 complexes was carried out using a sandwich assay. Monoclonal anti-τ antibody was used as a first primary antibody and monoclonal anti-Aβ (1-16) antibody was applied as the second primary antibody. Freshly prepared complexes with a fixed concentration of τ 352 [5 nM (∼184 ng/ml)] and Aβ 1-42 (in concentrations varying from 5 nM [∼22.5 ng/ml) to 1,000 nM (∼4514.1 ng/ml)] diluted in PBS containing BSA (250 μg/ml) (PBS + BSA) were incubated on a shaker at room temperature. After 15 min of incubation, mixtures of τ 352 and various concentrations of Aβ 1-42 were passed across individual channels for 20 min. Then, PBS + BSA buffer was injected to reach the baseline, followed by PBNa buffer to wash away any non-specifically bound molecules. The PBS + BSA solution was then injected again. In each experiment, three sensing channels were used to attach the complex of τ 352 (5 nM) and Aβ 1-42 in different concentrations (5, 10, 50, 100, 500 and 1,000 nM) while the fourth was used as a reference channel through which pure τ 352 (5 nM) or pure Aβ 1-42 (1,000 nM) or buffer was run. Finally, the second primary antibody anti-Aβ (1-16) (5 μg/ml) was run over the coated surface for 15 min followed by washing with PBS + BSA buffer. The sensor response to the anti-Aβ (1-16) antibody corresponded to the amount of Aβ 1-42 (in concentrations ranging from 5 to 1,000 nM) that formed complexes with τ 352 (5 nM). A calibration curve for detection of the second primary anti-Aβ (1-16) antibody was established.

Results

Table 1 demonstrates the in vitro effects of six monomeric naturally occurring τ isoforms (352, 381, 383, 410, 412 and 441, all at 25 and 100 nM concentrations) on HACU during 30 min incubations at 37 °C. Some isoforms were able to significantly inhibit HACU values (352/441 > 383/410 > 381/412) under the above-mentioned conditions. However, the actions of the most effective isoforms 352/441 were not markedly enhanced at higher nM concentrations (Fig. 1) or during pre-incubations longer than 40–45 min (Figs. 2, 3). A detailed analysis of all the above-mentioned results revealed maximal drops in HACU levels to about 70 % of the original. Lineweaver–Burk plot analysis of HACU levels generated by isoforms 352/441 at 100 nM concentrations pre-incubated for 40 min at 37 °C revealed changes in the Michaelis constant (the K_M increased to approximately 140 %) rather than in the maximal velocity of transport (the V_{max} increased to approximately 108.3 %, data not shown).

Table 2 shows the effects of isoforms τ 352/441 on HACU levels estimated on non-depolarised or depolarised synaptosomes. Depolarisation by 55 mM KCl increased HACU levels from 113 to 129 %. However, the effects of both isoforms (100 nM concentrations pre-incubated for 40 min) were very small in these experiments (drops to 94 and 93 % with τ 352 or to 92 and 91 % with τ 441) when compared to those mentioned above. On the other hand, the results of two-way ANOVA supported the significant effects of depolarisation and of τ in both cases. Nevertheless, they did not indicate any marked differences in the effects of τ under basal and stimulated conditions or between the two isoforms.

Figure 4 demonstrates the effects of τ 352/441 on the specific binding of (3H)HC-3. Although moderate decreases were observed in both cases (to 85 % maximum with τ 352 and to 88 % with τ 441), the result of ANOVA did not support any significant differences.

Tables 3 and 4 show the HACU experiments performed on cholesterol-depleted synaptosomes. Previous depletion of membrane cholesterol (by 10 mM MβCD pre-incubated with synaptosomes for 15 min at 37 °C) decreased HACU values to approximately 70 %. Isoforms of τ inhibited HACU values at 50 nM concentrations after pre-incubation for 40 min; however, no differences between undepleted

Table 1 The effects of six τ isoforms on HACU

τ Isoforms	Concentrations (nM)	n	HACU (%)
H_2O	0	20	100.0 ± 11.9
352 (0N3R)	25	6	89.1 ± 8.6*
	100	4	77.4 ± 11.1***
381 (1N3R)	25	6	98.2 ± 12.1
	100	4	89.5 ± 4.8
383 (0N4R)	25	6	91.9 ± 7.9
	100	4	81.8 ± 4.1**
410 (2N3R)	25	6	93.6 ± 6.7
	100	4	83.7 ± 6.4**
412 (1N4R)	25	6	98.7 ± 12.8
	100	4	106.3 ± 11.2
441 (2N4R)	25	6	83.4 ± 11.6**
	100	4	84.7 ± 11.6**
			ANOVA: F(12, 67) = 3.76, $p = 0.0002$

Six naturally occurring isoforms of human τ protein (N—amino terminal inserts, R—microtubule binding repeats) freshly diluted in redistilled water were pre-incubated with rat hippocampal synaptosomes (isolated from a total of 8 rats), resuspended in buffer for 30 min and then incubated with [3H]choline for 4 min, both at 37 °C. The HACU values were related to control samples containing redistilled water and expressed in % (levels of control samples equalled 219.7 ± 26.1 fmoles/4 min/mg of proteins). Statistical significance was calculated with respect to the control samples (* $p < 0.05$, ** $p < 0.010$, *** $p < 0.001$)

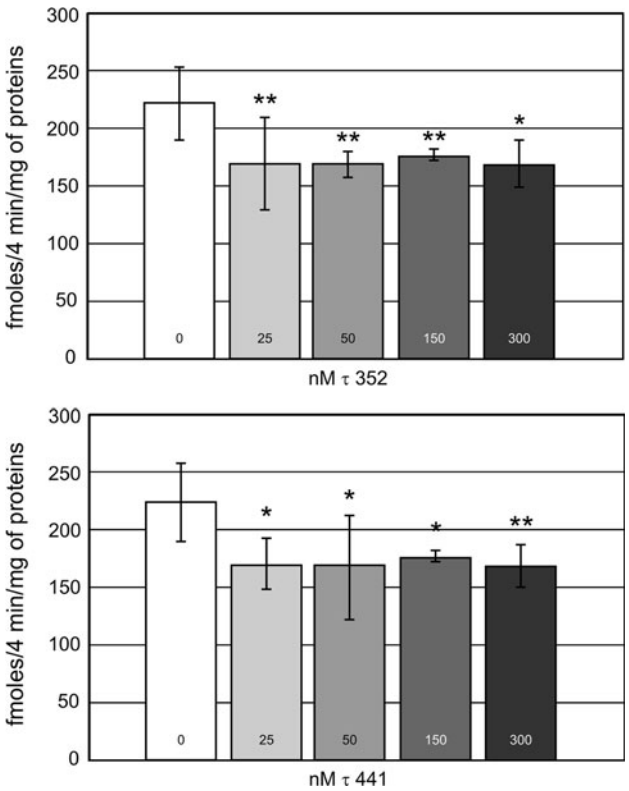

Fig. 1 Saturable effects of τ 352/441 on HACU Synaptosomes (isolated from a total of 4 rats) were pre-incubated with redistilled water or with 25–300 nM τ 352/441 in buffer for 40 min and subsequently incubated with [3H]choline for 4 min, both at 37 °C. All measurements were performed in quadruplicate. The HACU values are expressed as fmoles/4 min/mg of proteins. The results of ANOVA for τ 352: $F(4,15) = 3.36$, $p = 0.0375$, those for τ 441: $F(4,15) = 3.07$, $p = 0.0493$ (t test was calculated with respect to samples with redistilled water, * $p < 0.05$, ** $p < 0.010$)

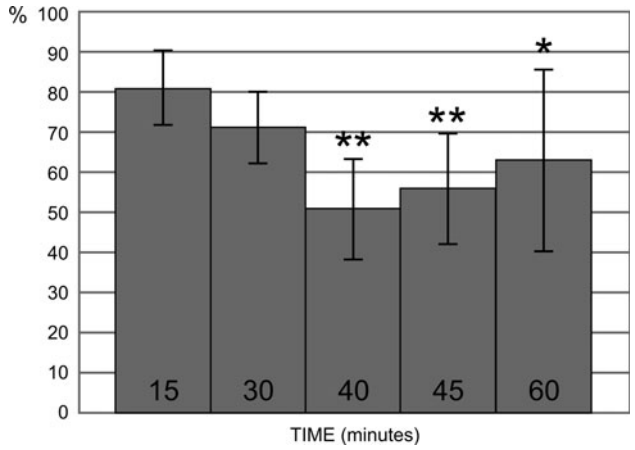

Fig. 2 Time-dependent effects of τ 352 on HACU Synaptosomes (isolated from a total of 4 rats) were pre-incubated with redistilled water or with 50 nM τ 352 in buffer for 15–60 min and subsequently incubated with [3H]choline for 4 min, both at 37 °C. The HACU values of the samples containing τ were related to the control samples pre-incubated with water for the same time and expressed in % (the levels of control samples incubated for 15 min equalled 224.2 ± 23.1 fmoles/4 min/mg of proteins). The results of ANOVA (only for τ samples) for time: $F(4,16) = 3.14$, $p = 0.0440$ (*t* test was calculated with respect to τ samples pre-incubated for 15 min, * $p < 0.050$). The results of two-way ANOVA for control and τ samples-time: $F(4,32) = 1.03$, $p = 0.4064$, τ: $F(1,32) = 15.98$, $p < 0.001$, interaction: $F(4,32) = 1.04$, $p = 0.4011$

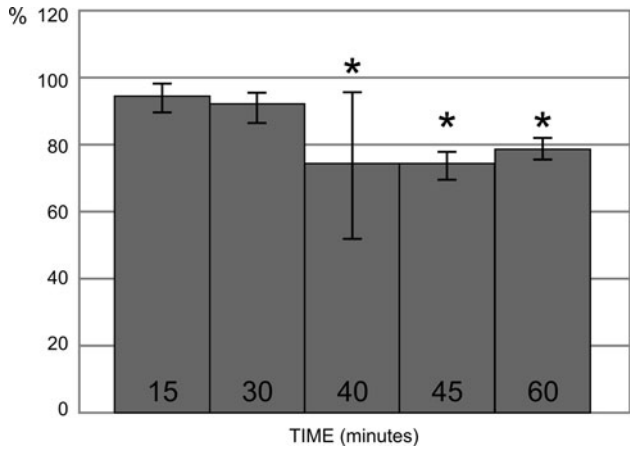

Fig. 3 Time-dependent effects of τ 441 on HACU Synaptosomes (isolated from a total of 5 rats) were pre-incubated with redistilled water or with 50 nM τ 441 in the buffer for 15–60 min and subsequently incubated with [3H]choline for 4 min, both at 37 °C. The HACU values of the samples containing τ were related to the control samples pre-incubated with water for the same time and expressed in % (the levels of control samples incubated for 15 min equalled 223.7 ± 48.1 fmoles/4 min/mg of proteins). The results of ANOVA (only for τ samples) for time: $F(4,31) = 4.47$, $p = 0.0057$ (*t* test was calculated with respect to τ samples pre-incubated for 15 min, * $p < 0.05$, ** $p < 0.010$). The results of two-way ANOVA for control and τ samples-time: $F(4,62) = 1.43$, $p = 0.2337$, τ: $F(1,62) = 63.12$, $p < 0.001$, interaction: $F(4,62) = 1.44$, $p = 0.2301$

and cholesterol-depleted synaptosomes or between τ 352 and 441 were found (Table 3). Table 4 demonstrates the marked inhibition of HACU mediated by 50 nM Aβ 1-40 pre-incubated for 40 min at 37 °C and the ability of τ 352 to eliminate it.

A sandwich assay was designed to detect the complexes between τ and Aβ previously formed in solution. This was based on binding of pre-incubated complexes of τ 352 and Aβ 1-42 to monoclonal antibody against τ immobilised on the sensor's surface and detection of a monoclonal antibody against Aβ that specifically binds to Aβ in the attached complex, as shown in Fig. 5. To demonstrate the applicability of the sandwich assay for detection of the τ and Aβ complex, the non-specific adsorption of a second anti-Aβ antibody (1-16) was investigated. In each experiment one channel was used as a reference channel; therefore, pure τ 352 (5 nM) or pure Aβ 1-42 (1,000 nM) or buffer was run along the surface with immobilised anti-τ. A specific sensor response was observed only when pure τ 352 (5 nM) was used, while for pure Aβ 1-42 (1,000 nM) or buffer no response was monitored (data not shown). However, injection of the second primary antibody anti-Aβ (1-16) resulted in negligible change in the sensor response. Figure 6 shows a representative real-time SPR sensorgram obtained for detection of anti-Aβ (1-16) in reference channels coated with pure τ (5 nM) or pure Aβ 1-42 (1,000 nM) or buffer.

The sensor response to specific binding of anti-Aβ (1-16) (5 μg/ml) to the complexes of τ 352 (5 nM) and Aβ 1-42 at various concentrations (5, 10, 50, 100, 500 and 1,000 nM) is shown in Fig. 7 (left). The calibration curve for the binding of anti-Aβ (1-16) (5 μg/ml) to captured complexes of τ 352 (5 nM) for six different concentrations of Aβ 1-42 (5, 10, 50, 100, 500 and 1,000 nM) is shown in Fig. 7 (right). Each value represents a mean ± standard deviation calculated from four different experiments. The limit of detection (LOD) for Aβ 1-42 in complex with τ 352 (5 nM) was determined as two standard deviations of the sensor response to anti-Aβ (1-16) that was bound to a blank sample (only τ 352 (5 nM) without Aβ 1-42). The LOD for Aβ 1-42 in complex with τ 352 (5 nM) was estimated to be 3 nM (~13.5 ng/ml). The chip-to-chip reproducibility of anti-Aβ (1-16) detection in the sandwich assay was determined to be better than 91 % over a concentration range from 50 to 1,000 nM.

Discussion

Interaction of τ and CHT1

Our study demonstrates the significant in vitro effects of some τ isoforms at low nM concentrations on CHT1 transporters and thus suggests that monomeric intracellular

Table 2 The effects of τ 352 or 441 on HACU under basal or stimulated conditions

τ Isoforms	Na^+HACU(fmoles/4 min/mg)	K^+HACU (fmoles/4 min/mg)	ANOVA
352 (0N3R)	H_2O: 219.2 ± 24.9 (12x)	H_2O: 248.5 ± 21.7** (12x)	$F(3,44) = 7.75$ $p < 0.001$
	τ: 205.5 ± 18.6 (12x)	τ: 230.3 ± 24.5 (12x)	
441 (2N4R)	H_2O: 220.1 ± 30.6 (12x)	H_2O: 284.9 ± 41.2*** (12x)	$F(3,40) = 14.17$ $p < 0.001$
	τ: 201.5 ± 18.5 (10x)	τ: 258.3 ± 36.6* (10x)	

Synaptosomes (isolated from a total of 8 rats) were previously incubated with 55 mM NaCl (basal conditions) or 55 mM KCl (stimulated conditions) for 10 min at 37 °C and subsequently centrifuged (2,450 g for 10 min at 4 °C). The supernatants were removed and the pellets resuspended in the original volume of 0.32 M sucrose. Two naturally occurring isoforms of τ (352 and 441) diluted in redistilled water were pre-incubated with the synaptosomes and buffer for 40 min and then incubated with [3H]choline for 4 min, both at 37 °C (final concentrations of τ were 100 nM). Statistical significance (Student's *t* test) was calculated with respect to the control samples under basal conditions (** $p < 0.010$, *** $p < 0.001$)

Results of two-way ANOVA: τ 352—depolarisation: $F(1,44) = 17.18$, $p < 0.001$, τ: $F(1,44) = 5.95$, $p = 0.0188$, interaction: $F(1,44) = 0.12$, $p = 0.7296$

τ 441—depolarisation: $F(1,40) = 36.55$, $p < 0.001$, τ: $F(1,40) = 5.07$, $p = 0.0299$, interaction: $F(1,40) = 0.16$, $p = 0.6932$

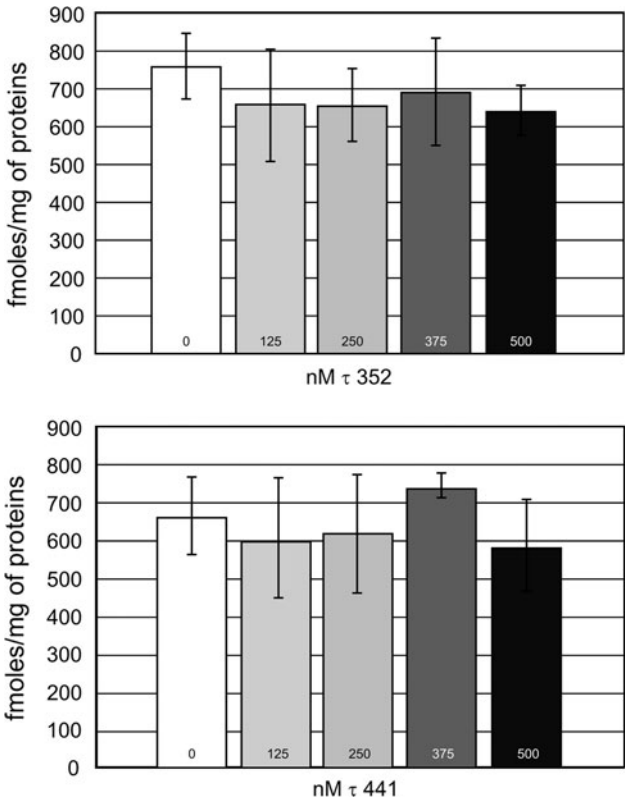

Fig. 4 The effects of τ 352/441 on specific binding of (3H)HC-3 Synaptosomes (isolated from a total of 2 rats) were pre-incubated with redistilled water or with 125–500 nM τ 352/441 for 60 min and subsequently with [3H]HC-3 for 30 min, both at room temperature. All measurements were performed in quadruplicate. The values of specific binding are expressed in fmoles/mg of proteins. The results of ANOVA for τ 352: $F(4,15) = 0.69$, $p = 0.6080$, those for τ 441: $F(4,15) = 0.92$, $p = 0.4767$

τ, when released extracellularly upon neuronal death, e.g. in AD, could contribute to impairment of the cholinergic neurotransmitter system in vivo through its actions on

Table 3 The effects of τ 352 or 441 on estimated HACU on undepleted or cholesterol-depleted synaptosomes

τ Isoforms	n	H_2O (%)	MβCD (%)	ANOVA
352 (0N3R)	27	89.2 ± 9.8	83.8. ± 15.1	$F(1,52) = 2.45$ $p = 0.1233$
441 (2N4R)	16	86.7 ± 8.6	88.9 ± 12.4	$F(1,30) = 0.33$ $p = 0.5675$

Two naturally occurring isoforms of human τ protein (352 and 441) diluted in redistilled water were pre-incubated with undepleted or cholesterol-depleted rat synaptosomes (isolated from a total of 14 rats), resuspended in buffer for 40 min and then incubated with [3H]choline for 4 min, both at 37 °C (final concentrations of τ were 50 nM). The HACU values were related to those of samples incubated with water (depletion decreased the HACU values to approximately 70 %) and expressed in %

Results of two-way ANOVA: depletion: $F(1,82) = 0.37$, $p = 0.5456$, differences between both isoforms: $F(1,82) = 0.24$, $p = 0.6279$, interaction: $F(1,82) = 2.01$, $p = 0.1597$

CHT1, in addition to those on M1/M receptors [2–8]. It is well known that CHT1 transporters play a key regulatory role in the synthesis of acetylcholine and that inhibition of CHT1 leads to the reduced neurotransmitter synthesis observed in AD, among other conditions [25]. Although the effects of monomeric τ on CHT1 are not very robust, they should not be underestimated with regard to the pronounced vulnerability of the transporters to Aβ [25–27] and to the different mechanisms of interaction between Aβ and τ (see below) suggesting additive actions on CHT1. Moreover, the effects of pathological forms of τ (e.g. hyperphosphorylated, truncated or aggregated) on CHT1 remain to be elucidated in the future.

Although the mechanism of HACU inhibition is not clear in detail, we think that a direct interaction of τ with extracellular domains of the CHT1 transporter could occur here (see the dose- and time-saturable manner of the interaction (Figs. 1, 2, 3) and the lack of sensitivity to

Table 4 Interactions of τ 352 and Aβ 1-40 estimated by means of in vitro test on CHT1 transporters of cholesterol-depleted synaptosomes

Composition	n	HACU (%)
H_2O	12	100.0 ± 12.4
500 nM Aβ 1-40	10	82.4 ± 15.4**
+ 100 nM τ 352	12	86.9 ± 16.0*
+ 500 nM τ 352	3	98.3 ± 13.1
ANOVA		$F(3, 33) = 3.28$, $p = 0.0331$

Cholesterol-depleted synaptosomes (isolated from a total of 6 rats) resuspended in buffer were pre-incubated with τ 352/441 and/or with Aβ 1-40 for 40 min and subsequently incubated with [3H]choline for 4 min, both at 37 °C. The HACU values were related to those of samples incubated with buffer (depletion decreased HACU values to approximately 70 %, i.e. the levels of control samples equalled 154. 5 ± 19.2 fmoles/4 min/mg of proteins) and expressed in %. Statistical significance (Student's *t* test) was calculated with respect to the control samples (* $p < 0.050$, ** $p < 0.010$)

depletion of membrane cholesterol (Table 3). However, τ probably does not interact with the choline recognition site (note no significant effects of τ on [3H]HC-3 binding (Fig. 4) and also that τ-mediated changes in HACU levels are associated rather with alterations in K_M). On the other hand, τ is not a very potent inhibitor (the maximum drops by approximately 30 %) and it is not clear if it partly influences all membrane-bound CHT1 transporters or effectively inhibits only 30 % of transporters displaying, e.g. a convenient conformational state [30]. Because depolarisation increases the activity of membrane-bound CHT1 transporters, including little recruitment of their occult pool [25, 30], and τ-mediated actions are not influenced by depolarisation (Table 2), the first possibility seems to be more probable. On the other hand, the effects of τ were strikingly smaller (although significant) on synaptosomes that were pre-incubated with 55 mM NaCl or KCl (Table 2), which suggests that conformational changes in extracellular CHT1 domains could also play a role in the interaction with τ.

Our results show that only some τ isoforms effectively influence CHT1 transporters. It seems that greater differences are associated with the number of N—terminal inserts rather than of R—microtubule binding repeats (Table 1). While an N-terminal insert is not necessary (τ 352 and 383 are effective 0 N isoforms), one N-terminal insert restrains the interaction (τ 381 and 412 are not effective 1 N isoforms) and two N-terminal inserts support it (τ 410 and 441 are effective 2 N isoforms). On the contrary, 3R isoforms are no better inhibitors of HACU than 4R isoforms (compare the effects of 0 N isoforms 352 and 338 or of 2 N isoforms 410 and 441), as reported in the interactions between τ and microtubules through microtubule binding domains [12]. The interaction mechanisms between some τ isoforms and CHT1 are not clear in detail; nevertheless, it seems that extracellular CHT1 domains and the C-terminus of τ with the microtubule-binding repeats could be involved. Moreover, the N-terminal projection domain could also play a role, either via modulating the affinity of the C-terminal binding domain [12] or via regulating τ solubility (e.g., the soluble conformation can be stabilised through an association of the extreme C-terminus to the microtubule-binding repeats and of the N-terminal to the C-terminal region [13, 14]; the insoluble conformation based on polymerisation of τ could be associated with an interaction of the extreme N-terminus with microtubule-binding repeats when the C-terminus is free [14]). It is important to note that an exposure to paraformaldehyde vapour leading to τ polymerisation and globular aggregates [31] totally eliminated the τ-mediated inhibition of HACU (data not shown), which suggests that the conformation of τ, in addition to that of CHT1, is very important here.

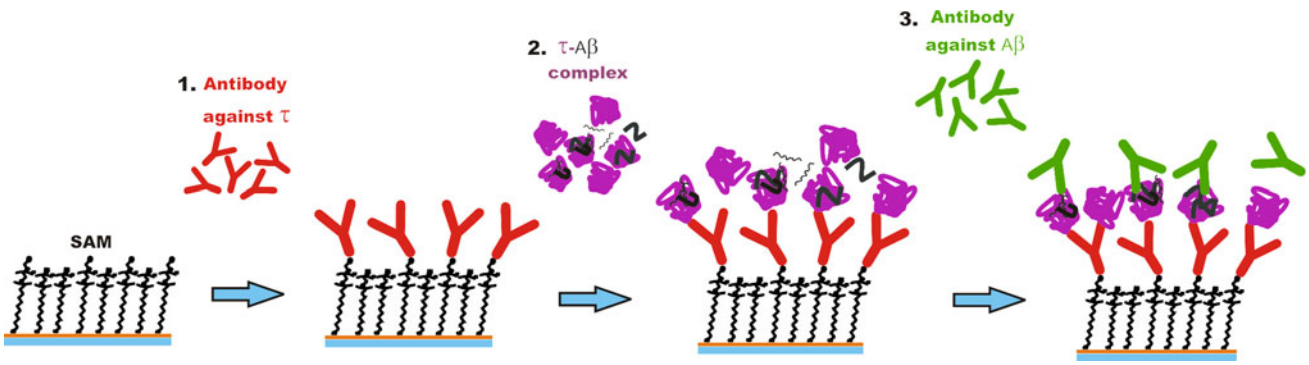

Fig. 5 Scheme of sandwich assay for SPR detection of complex between τ 352 and Aβ 1-42. First, an antibody against τ was passed over the sensor's surface and covalently attached to an activated mixed self-assembled monolayer (SAM). Second, the pre-incubated complex of τ 352 and Aβ 1-42 was passed across immobilised antibody against τ. Third, antibody against Aβ was passed over the attached complex and specifically bound to Aβ present in the captured complex

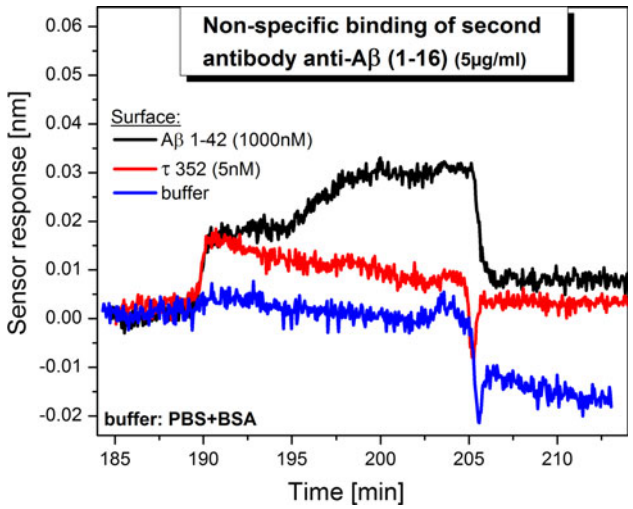

Fig. 6 Sensor response to non-specific binding of anti-Aβ (1-16). Typical sensor response to non-specific binding of anti-Aβ (1-16) (5 μg/ml) that was run through reference channels coated with pure Aβ 1-42 (1,000 nM) or pure τ 352 (5 nM) or buffer

A Comparison of the Effects of Aβ and τ on CHT1

Although both Aβ [25–27, 32] and τ (the present study) acutely inhibit HACU at nM concentrations, a more detailed comparison of the results suggests that the interaction mechanisms are probably quite different. In particular, (1) increasing concentrations and/or longer preincubations of Aβ, but not of τ, enhance HACU inhibition, (2) Aβ at nM concentrations, but not τ, can inhibit the specific binding site of HC-3, (3) Aβ-mediated inhibition of HACU is associated with alterations in V_{max}, τ-mediated inhibition rather with changes in K_M, (4) depolarisation enhances the effects of Aβ, but not those of τ, on HACU and HC-3 binding, and finally (5) disruption of lipid rafts by MβCD enhances the effects of Aβ, but not those of τ. Moreover, the Aβ conformation does not seem to play a key role in HACU inhibition, in contrast to inhibition by τ (i.e., monomeric as well as aggregated fragments of Aβ were able to markedly inhibit HACU; nevertheless, aggregated Aβ was more efficient when compared to monomeric peptides [32]). Although the interaction mechanisms of Aβ on membrane-bound proteins including CHT1 are not yet fully established, it is suggested that lipid rafts play an important role [33]. On the contrary, the results of this study demonstrate that the effects of τ on CHT1 are probably not associated with lipid rafts (Table 3).

Interactions of τ and Aβ

Experimental results have demonstrated a direct interaction of Aβ through residues 25–35 with the τ region involved in microtubule binding (residues 307–325). This interaction appears to facilitate the aggregation of τ but decrease that of Aβ [20]. Moreover, data using the SPR technique have reported higher affinities (in the nM range) of τ 441 for Aβ 1-40/1-42 than of τ 441 for itself [21]. Our experiments performed on rat hippocampal cholesterol-depleted synaptosomes suggest a loss of particular effects of Aβ 1-40 or τ 352 on CHT1 when both substances occur in a solution (Table 4). That can be interpreted as due to creation of a complex, which is in agreement with the above-mentioned studies [20, 21]. Moreover, if the microtubule-binding repeats of τ interact with Aβ and the effects of τ 352 on

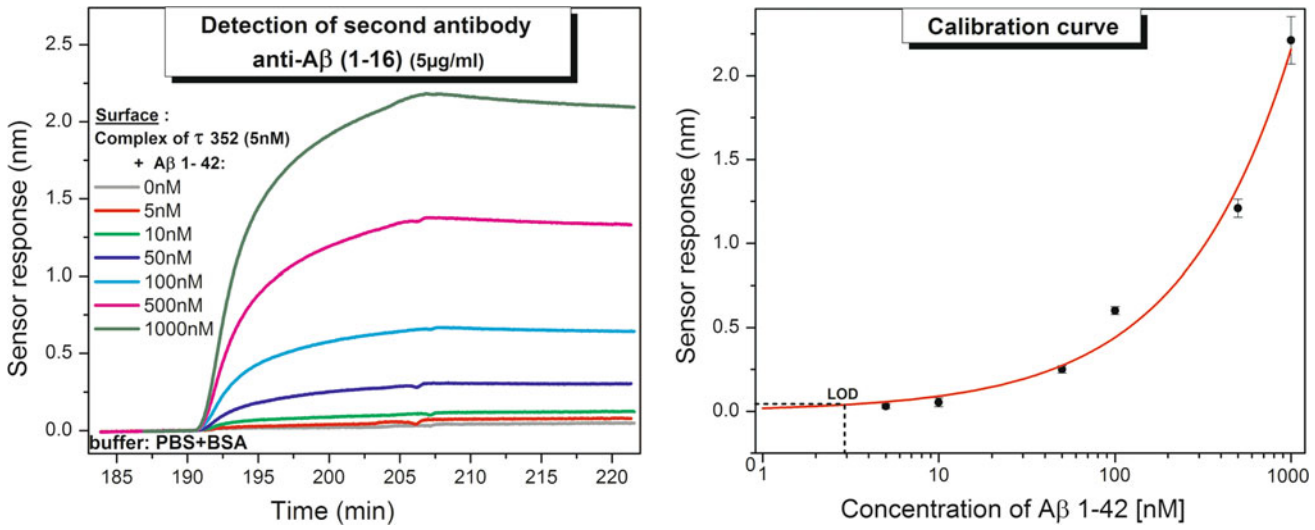

Fig. 7 Sensor response to specific binding of anti-Aβ (1-16) and calibration curve. Sensor response to binding of anti-Aβ (1-16) (5 μg/ml) to increasing concentrations of Aβ 1-42 (5, 10, 50, 100, 500 and 1,000 nM) in complex with τ 352 (5 nM) (*left*) and corresponding calibration curve (*right*)

CHT1 are eliminated in the presence of Aβ, the result supports the direct involvement of the repeats in the interaction with CHT1.

Surface plasmon resonance (SPR) experiments support the formation of a complex between τ 352 and Aβ 1-42 (Figs. 5, 6, 7). As shown in Fig. 7, the increasing sensor response to the binding of anti-Aβ (1-16) is associated with the increasing concentration of Aβ 1-42 in complex with 5 nM τ 352. The biosensor seems to be able to detect Aβ–τ complexes occurring naturally in cerebrospinal fluid.

Conclusion

Our study demonstrates significant in vitro effects of monomeric τ at low nM concentrations on CHT1 transporters, in addition to those on M1/M3 receptors reported previously in the literature. We suppose that an inhibition of high-affinity choline uptake could occur via a direct interaction of the extracellular CHT1 domains (but not near the choline recognition site) and of the C-terminus localised microtubule binding repeats of τ. An N-terminal insert is not necessary but the N-terminal projection domain of τ could play a role too. A comparison of the effects of Aβ or τ on CHT1 indicates quite different interaction mechanisms. In order to support experiments performed on CHT1 from cholesterol-depleted synaptosomes and evaluate interactions of Aβ 1-40 and τ 352, a method to detect complexes between τ and Aβ (namely τ 352 and Aβ 1-42) based on an SPR biosensor and sandwich assay was developed. The biosensor seems to be able to detect Aβ–τ complexes occurring naturally in cerebrospinal fluid, enabling their evaluation as prospective biomarkers of Alzheimer′s disease.

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