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Aβ peptides as one of the crucial volume transmission signals in the trophic units and their interactions with homocysteine. Physiological implications and relevance for Alzheimer's disease*

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Summary Amyloid peptides (Aβ) can operate as volume transmission (VT) signals since they are continuously released from cells of the central nervous system and diffuse in the extra-cellular space of the brain. They have both regulatory and trophic functions on cellular networks. In agreement with Aβ regulatory actions on glial-neuronal networks, the present paper reports new findings demonstrating that intrastriatal injections of Aβ peptides reduce striatal tyrosine hydroxylase, increase striatal GFAP immunoreactivities and lower pain threshold in experimental rats. Furthermore, it has been demonstrated that exogenous homocysteine (Hcy) binds Aβ(1-40) favouring its β-sheet conformation both in vitro and in vivo and hence the formation of β-fibrils and development of neurotoxicity.

Thus, the hypothesis is discussed that Aβ peptides represent crucial VT-signals in the brain and their action is altered by dysmetabolic signals such as high Hcy extra-cellular levels, known to be an important risk factor for Alzheimer's disease.

Keywords: β-amyloid peptides, homocysteine, volume transmission, pain threshold, Alzheimer's disease

Introduction

Protein misfolding and aggregation have been demonstrated as the molecular basis of a number of pathological conditions such as Alzheimer's and Parkinson's disease. So far a group of about 20 protein deposition diseases (amyloidoses) has been characterised, which have as common hallmark the presence of deposits of fibrillar aggregates found as intracellular inclusions (cytoplasmic and/or nuclear) or as extra-cellular plaques (Stefani, 2004). The characteristic lesions of Alzheimer's disease (AD) are the extra-cellular amyloid plaques, composed of aggregates of amyloid-β peptides and the intracellular neurofibrillary tangles, which contain accumulations of hyperphosphorylated forms of the neurofilament-associated protein tau (Ross and Poirier, 2005).

Amyloid peptides (Aβ) are fragments of the amyloid precursor protein (APP), an integral membrane protein. Aβ peptides are continuously generated by neurons and non-neuronal cells via sequential cleavage of APP by proteases (Selkoe, 2003). Several APP mRNAs arise from alternative splicing and encode forms that differ mostly by the absence (APP-695) or presence (APP-751 and APP-770) of a Kunitz protease inhibitor (KPI) domain located toward the NH_2 terminus of the protein. The APP is processed by three proteases, α-, β-, and γ-secretase, which have been implicated in the aetiology of AD. It has been shown that β-secretase and α-secretase generate and cause the release of soluble fragments of the NH_2-teminus of APP in the extra-cellular space (ECS), namely the β-APPs and α-APPs, respectively. Thus, after β-secretase proteolysis a 99-residue CTF (CT99) remains membrane bound while

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after α-secretase proteolysis an 83-residue CTF (CT83) remains. Both CT99 and CT83 are substrates for γ-secretase, which performs proteolysis in the middle of the transmembrane domain generating a 40-residue (Aβ40) and a 42-residue (Aβ42) carboxyl terminal peptides which are released into the ECS (Chang and Suh, 2005).

Thus, different fragments are produced from APP and while the C-terminal fragments are released into the cytoplasm and probably transferred to the nucleus to act as transcription factors, N-fragments made of 39–43 residues are released into ECS. It should be noted that the longer and more hydrophobic Aβ42 is much more prone to fibril formation than Aβ40, and even though Aβ42 is a minor form of Aβ, it is the major Aβ species found in cerebral plaques. Damage to neurons may be caused by intracellular and, to a lesser extent, extra-cellular highly reactive and toxic Aβ dimers and oligomers that can easily diffuse inside the cells and throughout the narrow and convoluted ECS of the central nervous system (CNS). Glial cells participate in the efficient uptake of soluble extra-cellular Aβ and in the clearance of this material at localized sites where the Aβ is concentrated (Roher et al., 2000).

Thus, the relationship between insoluble aggregates and soluble (diffusible) forms of the Aβ peptides seems of paramount importance. Recent evidence demonstrates that soluble high-molecular mass assemblies can cause impairments in the integrative functions of neuronal networks; in particular they can disrupt memory (Lesnè et al., 2006).

Against this background we have investigated from a new perspective Aβ peptides namely in the context that they are released by neurons and other cells (Selkoe, 2003) and by diffusing in the ECS work as volume transmission (VT)-signals (Agnati and Fuxe, 2000). These VT signals exert a functional role on neuronal trophism, neuroprotection, autoregulation of brain circulation and synaptic transmission (i.e., intercellular wiring transmission (WT)) (Atwood et al., 2003; Esteban, 2004). Furthermore, since Aβ peptides have also toxic actions, which appear to be a consequence of misfolding and misaggregation of monomers (Stefani, 2004a), a possible endogenous factor favouring such pathogenetic events has been investigated. Our research has been focused on homocysteine (Hcy) since experimental studies in animals and epidemiological studies in humans have clearly demonstrated a role of this sulphur-containing amino acid in several neurodegenerative diseases, in particular, in AD (Mattson and Shea, 2003; Ho et al., 2001; Seshadri et al., 2002). Thus, Hcy actions on Aβ conformation and aggregation have been evaluated.

The present paper supports the view that Aβ peptides not only operate as VT-signals, but also as modulators of specific VT-signals, such as dopamine (DA)-mediated VT-signalling in the basal ganglia.

As far as the trophic actions of Aβ are concerned the hypothesis will be advanced that the neuroprotective actions of Aβ peptides manifest themselves also as a buffering capacity of potentially toxic catabolites (such as Hcy and ROS) formed by the high rate cellular metabolism in the CNS. Above a certain threshold of toxic compound concentration the Aβ peptide buffering action becomes exhausted and the complex between Aβ peptides and toxic compounds instead becomes dangerous for CNS trophism and function. Finally, Aβ multiple functions will be discussed in the frame of the ''trophic unit'' concept (Agnati et al., 1995).

Materials and methods: in vitro experiments

Mass-spectrometric experiments

Materials. The β-amyloid 1-40 was synthesized at the Johns Hopkins School of Medicine Synthesis and Sequencing Facility. The homocysteine and alpha-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis MO, USA) and the chymotrypsin from Roche Bioscience (Indianapolis, IN, USA).

Sample. A mixture containing 10 pmol/μl β-amyloid and 100 pmol/μl homocysteine in water was prepared.

Digest. To 3 μl of the mixture were added 6 μl 25 mM ammonium bicarbonate buffer pH 8.0 and 3 μl of a 0.1 μg/μl chymotrypsin (Roche Scientific, Palo Alto, CA, USA) (Woods and Huestis, 2001).The tube was vortexed, and left to sit for 5 min at room temperature.

Mass spectrometric analysis. 0.3 μl digest was deposited on the sample target and the reaction was stopped by adding 0.3 μl saturated matrix (alpha-cyano-4-hydroxycinnamic acid), left to dry at room temperature, inserted into a MALDI-4700 TOF–TOF from applied Biosystem and the spectra acquired in positive reflection mode, as well as collision induced dissociation (Woods and Ferre, 2005). Sample and enzyme blanks were also analyzed.

Circular dichroism (CD)

Samples of Aβ1-40 (FW 4329) were prepared at final concentration of 50 μM in 10% water solution of 2,2,2-trifluoroethanol (Aldrich Chemical Co., Milwaukee, WI, USA) pH 5.5, which is a sub-optimal concentration for Aβ fibril formation (Fezoui and Teplow, 2004), in the presence or absence of L-Hcy 100 μM (Sigma-Aldrich, Milano, Italy). CD spectra, reported as molar ellipticy (degrees $cm^2\,dmol^{-1}$), were acquired on Jasco J-710 spectropolarimeter at room temperature (25°C) immediately after diluting the stocked protein sample into the solution and after 4, 6 and 24 h, respectively. Quartz cuvettes with path length of 1 mm were used. Each spectrum of the protein was achieved in the range of 185–270 nm, at 0.1 nm resolution with a scan rate of 20 nm/min. Three scans were acquired and averaged for each sample.

Materials and methods: in vivo experiments

Animals and Aβ injections. Adult male pathogen-free Sprague Dawley rats of 185–200 g (Harlan-Nossan, Milan, Italy) were used. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Aβ1-40 and Aβ1-43 (Sigma-Aldrich, Milano, Italy) were dissolved in 35% acetonitrile, 0.1% trifluoroacetic acid (TFA) solution (Stephan et al., 2001). Solutions of peptides were made up in aliquots of 6 µl and stored at −20°C.

The animals were deeply anaesthetized with halothane and the solution of Aβ was injected into striatum by means of stereotaxic instrument (coordinates: anterior, 1.2 mm; lateral, −2.5 mm; dorsal, −5.5 mm with respect to bregma, according to the stereotaxic atlas of Paxinos and Watson) with 1 µl Hamilton syringe (26G) during 3 min and waiting 3 min before withdrawal of the needle.

Protocol of treatment. Animals were randomly subdivided in four groups and assigned to one of the following treatments:

- group 1 ($n = 20$): saline intraperitoneally (i.p.), twice a day for 10 days plus, on the 5th day, intrastriatal injection of acetonitrile (35%) plus trifluoroacetate (0.1%) solution (500 nl per site);
- group 2 ($n = 20$): d/l-homocysteine (Fluka, Sigma-Aldrich, Milano, Italy) (30 mg/kg i.p.), twice a day for 10 days plus, on the 5th day, intrastriatal injection of a combination of Aβ40 (10 µg in 1 µl) and Aβ43 (5 µg in 1 µl); the total quantity per site was 7.5 µg of Aβ40 plus Aβ43 in 500 nl;
- group 3 ($n = 20$): saline i.p. twice a day for 10 days plus, on the 5th day, intrastriatal injection of a combination of Aβ40 (10 µg in 1 µl) and Aβ43 (5 µg in 1 µl); the total quantity per site was 7.5 µg of Aβ40 plus Aβ43 in 500 nl;
- group 4 ($n = 20$): d/l-homocysteine (Fluka, Sigma-Aldrich, Milano, Italy) (30 mg/kg i.p.), twice a day for 10 days plus, on the 5th day, intrastriatal injection of acetonitrile (35%) plus trifluoroacetate (0.1%) solution (500 nl per site).

Animal sacrifice. For immuno-staining and thioflavin staining rats were killed by intra-cardiac perfusion, under anaesthesia (chloral hydrate, 200 mg/kg, i.p.), with 120 ml of room temperature saline followed by 110 ml of ice-cold 4% paraformaldehyde +0.3% picric acid in 0.1 M phosphate buffer (pH 7.0). The brains were rapidly dissected out and post fixed overnight in the same fixative, rinsed in 20% sucrose in phosphate buffer at 4°C over 1 day and frozen on dry ice powder. The brains were cut, using a cryostat, throughout the entire striatum and for immunostaining and thioflavine S staininig one (thirty-micrometer-thick) out of every third section every 90 µm was used.

For determination of Hcy in plasma and brain tissue, animals were anaesthetized by aether to collect blood samples, then, always under anaesthesia, they were decapitated, the brains were dissected out and the striata were collected and frozen on dry ice powder.

Thioflavine staining. Thirty-micrometer-thick sections were processed according to thioflavin S protocol for fibrillar amyloid staining as follows: sections were floated in 0.25% potassium permanganate solution for 20 min rinsed in distilled water, floated in bleaching solution (potassium metabisulfate 1% and oxalic acid 1% solution) for 2 min, rinsed in distilled water, floated in acetic acid 0.25% for 5 s, rinsed in distilled water and floated in thioflavine S (Sigma-Aldrich, Milano, Italy) staining solution (0.025% thioflavine S in 50% ethanol) for 3–5 min and finally rinsed in two changes of 50% ethanol and two changes of distilled water. Slides were mounted with a medium suitable for immunofluorescence (30% Mowiol, Calbiochem, Darmstadt, Germany).

Immunohistochemistry

Tyrosine Hydroxylase (TH) and Glial Fibrillary Acidic Protein (GFAP) double immunofluorescence. Thirty-micrometer-thick section were washed 3 times in PBS and were incubated over night with primary antibody solution of mouse (TH) antibody (1/100, MAB318, Chemicon International, Harrow, UK) and rabbit anti Glial Fibrillary Acidic Protein (GFAP) (1/500 Dako Cytomation S.p.A., Italy) in 0.1% normal serum plus 0.3% Triton in PBS solution. Sections were then rinsed consecutively in PBS, Triton 0.2% in PBS, Triton 0.1% in PBS, BSA 0.1% in PBS, Normal Serum 0.1% in PBS, PBS and incubated with an anti-rabbit biotinylated antibody (1:200) (Amersham Pharmacia Biotech, NJ, USA) for 1 h at room temperature. After two rinses in PBS slices were incubated with red coloured fluorolink Cy3^{TM} labelled streptavidin (1:100) (Amersham Pharmacia Biotech, NJ, USA) and green coloured fluorolink Alexa Fluor 488 labelled anti-mouse (1:100) (Molecular Probes, Burlingame, CA, USA) for 1 h at room temperature. Sections were then rinsed several times in PBS and mounted with a medium suitable for immunofluorescence (30% Mowiol, Calbiochem, Darmstadt, Germany).

Microscopic observations were made by means of a Leica TCS SP2 (Leica Laser technik, Heidelberg, Germany) confocal multiband scanning laser equipment with AOBS system adapted to an inverted Leica DM IRE2 microscope interfaced with an argon-krypton laser set at a power of 8 mW in each line (488, 568, 647 nm) and operating in the single scan acquisition mode. To minimize the noise and to keep a low photobleaching rate, we selected an acquisition time of 1 s per scan and averaged 8 scans to produce each 512 × 512 pixel image. All images were recorded with a HCX PL APO 40× objective.

β-Amyloid immunohistochemistry. Thirty-micrometr-thick sections were processed according to the indirect immunoperoxidase procedure using the avidin-biotin complex technique (Agnati and Fuxe, 1985; Agnati et al., 1986).

Mouse anti Aβ (DBA srl, Segrate, Milano, Italy) were used at a dilution of 1/50. In order to minimize experimental variability, all sections to be compared were run in parallel, using the same solutions. In order to maximize the linearity of the immunocytochemical detection system, diaminobenzidine and H_2O_2 were used in optimal concentrations, and the antibody dilution was far from saturation (Sternberger and Sternberger, 1986).

Computer assisted image analysis. The semiquantitative evaluation of the intensity of immunostaining and of the size of the specific field area was performed by means of an automatic image analyser (KS300, Zeiss Kontron, Munich, Germany) according to previously published methods (Agnati et al., 2004). For each staining, the analysis was carried out on two sections per animal for a total of four slides per animal. The average value per animal was used for statistical purposes.

Data are presented as mean ± sem values. Statistical comparisons between rat groups were performed by means of the Mann–Whitney *U*-test (statistical package SPSS version 10, SPSS, Chicago, IL, USA) considering $P < 0.05$ as a threshold for significant difference.

Determination of homocysteine in the brain tissue

The frozen tissues were weighed and homogenized (1:8 w/v) in ice-cold PBS using a Labsonic U (B. Braun Biotech International, Melsurgen, Germany) homogenizer. Each homogenized tissue suspension was supplemented with N-acetyl-cysteine (2.5 M; 1:10 v/v) as an internal standard, treated with tris (2-carboxyethyl) phosphine (1:10 v/v) mixed thoroughly and incubated for 60 min at 0°C. After adding perchloric acid (0.33 M; 1:1 v/v) and mixing, the precipitated proteins were removed by centrifugation at 1200 *g* for 10 min. The supernatant (50 µl for each sample) was derivatized with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), and fluorescent detection was performed by HPLC as previously described (Fu et al., 2000; Pfeiffer et al., 1999). Data are expressed as nmol/g wet tissue.

Determination of homocysteine in plasma

Blood samples were collected in Vacutainers containing EDTA-NaF (17.5 mg; EDTA + NaF; final NaF concentration, 60 mmol/L; Becton Dickinson UK Limited) (Clark et al., 2003). Total plasma homocysteine (tHcy) was analyzed by HPLC after the reduction of plasma disulfides with tris (2-carboxyethyl)phosphine, precipitation of proteins with perchloric acid,

derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), and fluorescent detection as described previously (Pfeiffer et al., 1999) using N-acetyl-cysteine as an internal standard. Performance characteristics of the assay have been described previously (Pfeiffer et al., 1999).

Plantar test

Changes in nociception were evaluated using a Plantar test apparatus (Ugo Basile, Comerio, Italy), which measures the paw withdrawal latency from a radiant heat source directed at the proximal half of the plantar surface of the

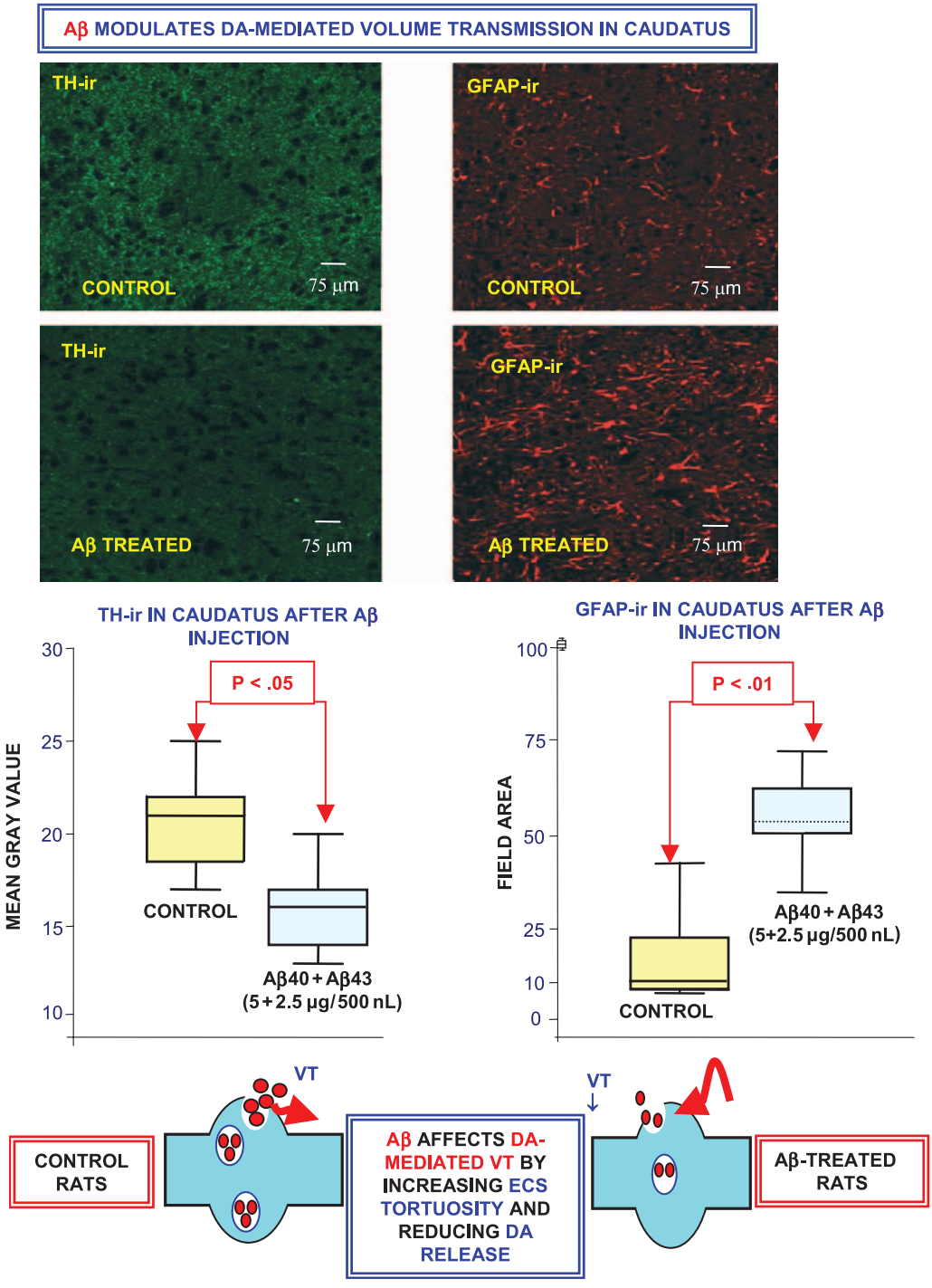


Fig. 1. In all groups of animals ($n = 12$) either saline or Homocysteine (Hcy, 30 mg/kg i.p.) was injected twice a day for 10 days. After 5 days of saline or Hcy treatment, Aβ (7.5 μg of Aβ40 plus Aβ43 in 500 nl) was microinjected into the caudate (for further details see Materials and methods). After sacrifice the immunostaining for TH and GFAP was carried out and computer-assisted image analysis of the TH and GFAP immunoreactivity was performed (for further details see Materials and methods). The plots report means ± sem only for two groups, since the mean value of the group treated with Hcy and microinjected with Aβ coincided with the mean value of the group treated with Aβ alone. Statistical analysis according to the Mann–Whitney *U*-test

hind paw. The apparatus consists of three Perspex boxes ($22 \times 17 \times 14$ cm, length × width × height) with a transparent floor through which it is possible to check the correct position of the mobile infra-red source under the plantar surface of the rat paw. Voltage to the heat source was adjusted to yield paw withdrawal latencies ranging 8–15 sec and a cut off time of 20 sec was imposed to avoid tissue damage (used intensity of 50 mW/cm^2) (Hargreaves et al., 1998). When the rats responded and withdrew their paw, the power shut off and the reaction time (the withdrawal latency of the paw) was recorded automatically. Shortening of the withdrawal latency indicated thermal hyperalgesia. On the day of the experiment, each animal was placed in plastic cages and left undisturbed for 5–10 min, after which they were subjected to three consecutive stimulations of the left hind paw at 5-min intervals. The three measurements were averaged and the mean value was used to determine group differences in paw withdrawal latency. Statistical analysis of the data was performed by treatments vs. control, non-parametric procedures (Conover, 1971).

Results

Intrastriatal injections of Aβ peptides

Diffusion of Aβ peptides. Immunoreactivity for Aβ peptides and thioflavine S staining were observed thoughout a distance of about 400 μm from the injection site in caudatus.

Tyrosine hydroxylase IR and Glial Fibrillary Acidic Protein IR (Fig. 1). By means of computer-assisted image analysis the TH and GFAP immunoreactivities were evaluated, both as intensity of the staining and specific immunoreactive field area, in fields sampled around the region where Aβ aggregates were detectable. As shown in the upper panels and plotted in the middle panels, exogenous Aβ peptides reduce TH IR in the DA terminal system of the striatum and increase the astrocyte expression of striatal GFAP IR (Fig. 1). As indicated in a schematic way this combined action likely affects the DA-mediated VT since it may cause a reduction of DA release and an increase of tortuosity of migration pathways in the ECS.

Pain threshold in the plantar test (Fig. 2). Classically basal ganglia are not a main integrative relay-station for pain sensing. However, basal ganglia can affect pain and DA neurotransmission is involved (Chudler and Dong, 1995).

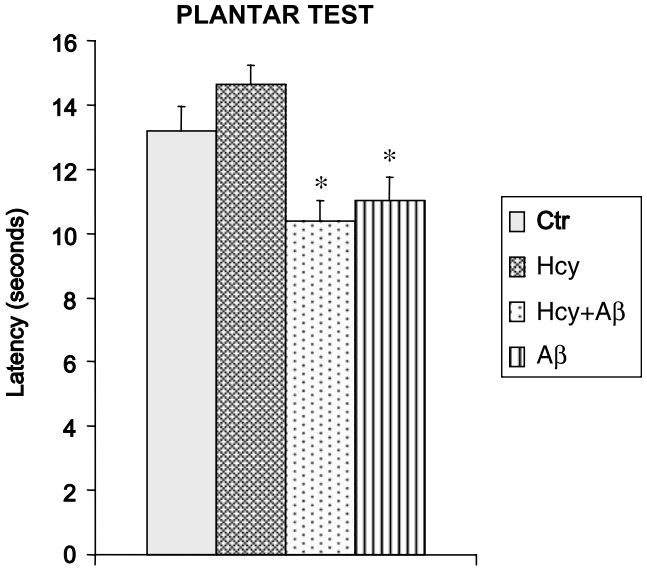


Fig. 2. Evaluation of the pain threshold by means of the "plantar test" (see Materials and methods). In all groups of animals ($n = 12$) either saline or Homocysteine (Hcy, 30 mg/kg i.p.) was injected twice a day for 10 days. After 5 days of saline or Hcy treatment, Aβ (7.5 μg of Aβ40 plus Aβ43 in 500 nl) or acetonitrile (35%) plus trifluoroacetate (0.1%) solution (500 nl) was microinjected into the caudate (for further details see Materials and methods). Bars represent means ± sem of the latencies observed in the four experimental groups. Statistical analysis according to the treatments vs. control non-parametric procedure. Asterisks mark significances ($P < 0.025$)

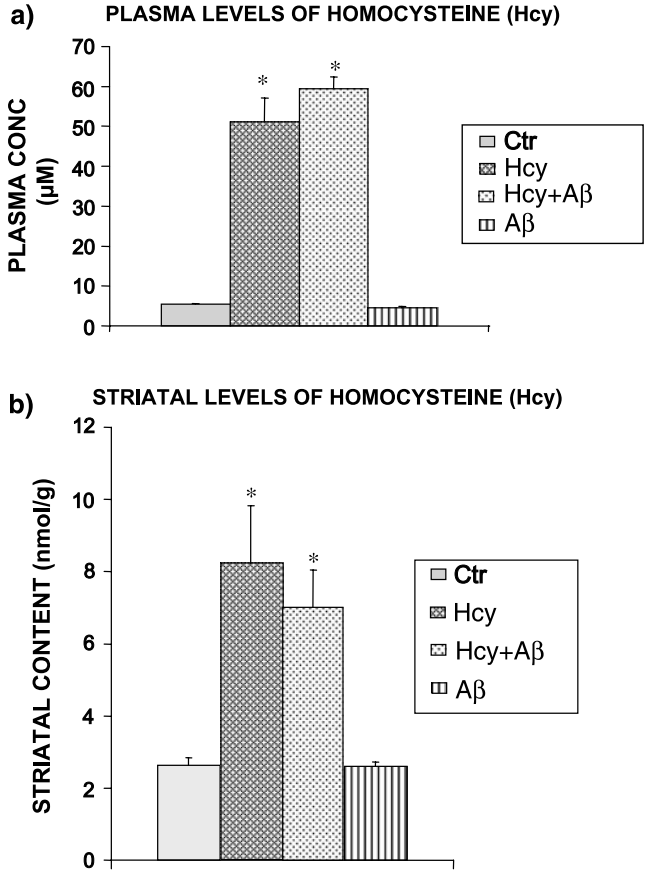


Fig. 3. In all groups of animals ($n = 6$–8) either saline or Homocysteine (Hcy, 30 mg/kg i.p.) was injected twice a day for 10 days. After 5 days of saline or Hcy treatment, Aβ (7.5 μg of Aβ40 plus Aβ43 in 500 nl) or acetonitrile (35%) plus trifluoroacetate (0.1%) solution (500 nl) was microinjected into the caudate (for further details see Materials and methods). Panel "**a**" reports the evaluations (means ± sem) of the Hcy plasma concentrations (μM) in the four groups of animals. Panel "**b**" reports the evaluations (means ± sem) of the Hcy brain concentrations (nmol/g) in the four groups of animals. Statistical analysis according to the treatments vs. control non-parametric procedures. Asterisks mark significances ($P < 0.025$)

As shown in the plot exogenous Aβ peptides in the striatum reduce the pain-threshold. This finding is in agreement with the reduction in DA neurotransmission reported in Fig. 1, which is known to affect pain threshold (Takeda et al., 2005).

Immunohistochemistry and thioflavine S staining (Fig. 4). Intrastriatally administered Aβ peptides form huge aggregates in the β-sheet conformation after Hcy sub-chronic treatment as demonstrated by immunocytochemistry (Fig. 4a) and thioflavine S staining (Fig. 4b).

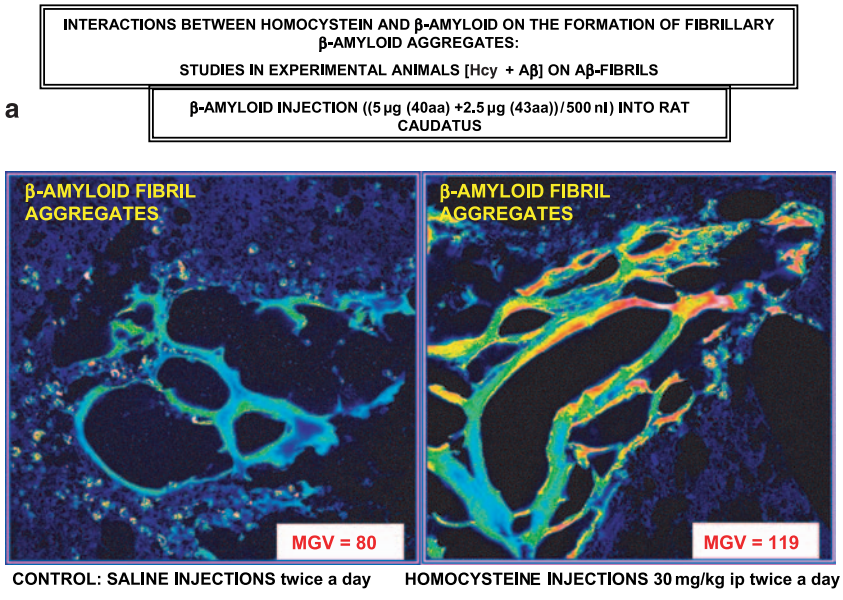
Interactions of exogenous striatal Aβ peptides with systemic homocysteine

Determination of Hcy in brain and plasma (Fig. 3). The exogenous administration of Hcy caused a significant increase in Hcy plasma level (from 5.52 ± 0.13 to $51.07 \pm 5.51\,\mu M$; Fig. 3a) and brain Hcy content (from 2.03 ± 0.22 to 6.64 ± 1.60 nmole/g; Fig. 3b). The intrastriatal injection of Aβ peptides did not significantly affect either the Hcy plasma levels or its brain content (see Fig. 3a, b), respectively.

In vitro studies on interactions of Aβ peptides with homocysteine

Mass-spectrometry (Fig. 5). Aβ peptides are shown to directly interact with Hcy. Hcy likely binds Aβ peptides due to electrostatic interactions between the Arg guanidinium group and the sulphur of Hcy.

Circular dichroism (Fig. 6). Following the binding to Hcy, Aβ peptides assume more rapidly a β-sheet conformation as demonstrated by the comparison between the left and the right panel of Fig. 6.



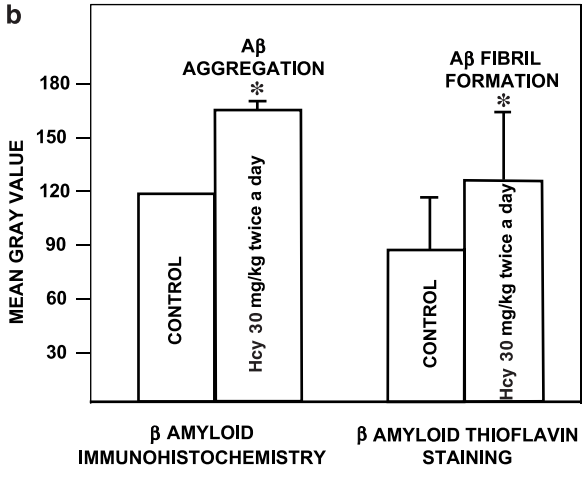


Fig. 4. In all groups of animals ($n = 12$) either saline or Homocysteine (Hcy, 30 mg/kg i.p.) was injected twice a day for 10 days. After 5 days of saline or Hcy treatment, Aβ (7.5 µg of Aβ40 plus Aβ43 in 500 nl) was microinjected into the caudate (for further details see Materials and methods). After sacrifice either Aβ-visualization by means of immunocytochemistry or the thioflavin S staining to visualize Aβ fibrils was carried out (for further details see Materials and methods). Panel "**a**" Microphoto of the thioflavin S staining in caudate after saline or Hcy treatments. Panel "**b**" Computer-assisted microdensitometric evaluation of Aβ-immunostaining and of thioflavin S staining (for further details see Materials and methods)

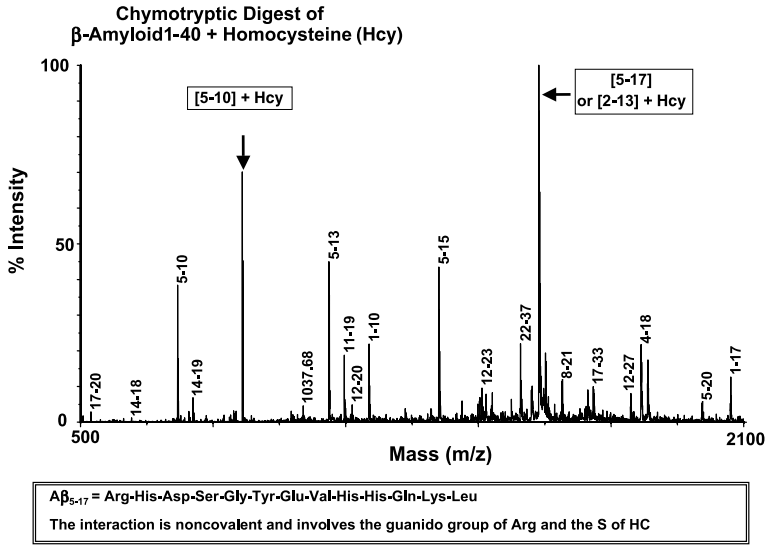


Fig. 5. Spectrum of the fragments obtained from the chymotrypsin digest of a mixture of Aβ1-40 and Hcy. Two fragments Aβ5-10 and Aβ2-13 form a noncovalent complex with Hcy through electrostatic interactions between the Arg guanidinium group and the Sulphur of Hcy (for further details see Materials and methods)

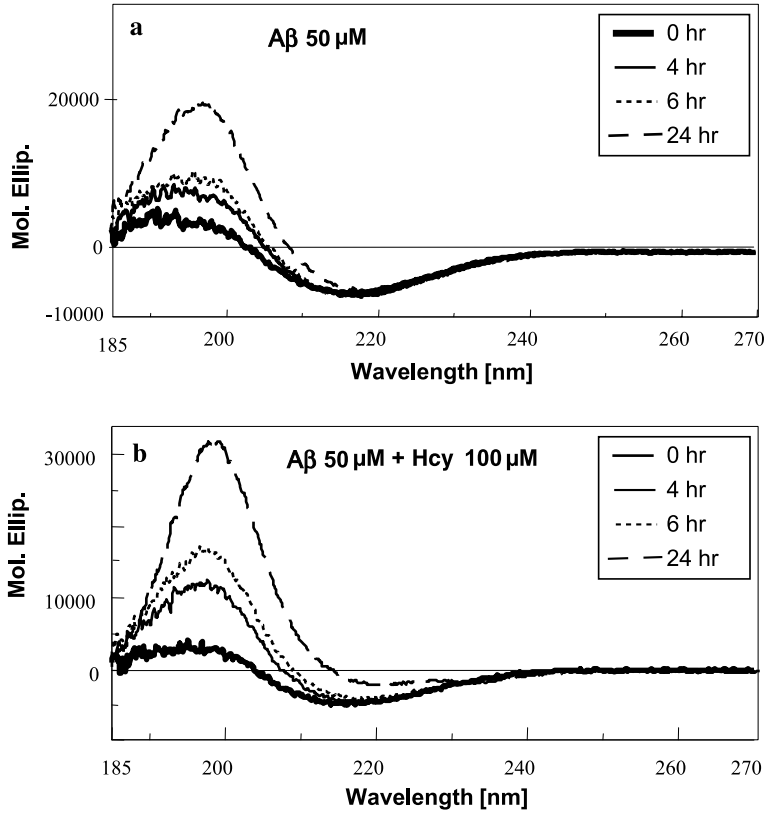


Fig. 6. **a**) CD spectra of (β-amyloid) Aβ and **b**) of Aβ in the presence of L-Hcy (100 μM) in 10% aqueous 2,2,2-trifluoroethanol. The recordings have been carried out at different time-intervals ($t = 0$, 4, 6, 24 h). For further details see Materials and methods

Discussion

It has been shown that Aβ peptides reduce synaptic efficacy of acetylcholine (ACh) and noradrenaline (NA) (Schliebs, 2005; Klingner et al., 2003). The present findings indicate an Aβ effect on DA-mediated VT in the striatum. Thus, the intra-striatal injection of Aβ peptides reduces the TH IR in the striatal DA terminal systems and at the same time enhances the astrocyte expression of GFAP IR. This combined action of Aβ probably reduces DA-mediated VT transmission (see Fig. 1) by reducing the amount of DA released from the DA terminals and by increasing tortuosity since the demonstrated reactive astrocytes are hypertrophic cells and

reduce the ECS and hence the shape/size of the pathways for the migration of VT signals in the ECS (Nicholson and Sykova, 1998; Roitbak and Sykova, 1999).

The lowering of the pain threshold (see Fig. 2) gives, at least in part, a functional correlate to the Aβ-induced alterations in the striatum. Several findings in animals and humans support a role of basal ganglia in central pain sensitivity. In fact, microinjections of morphine into the globus pallidus cause a dose-dependent, naloxone-reversible analgesia not related to a motor function suppression and increased pain sensitivity has been reported after 6-OHDA in the substantia nigra or MPTP treatment in animals (Takeda et al., 2005; Rosland et al., 1992). Furthermore, dopaminergic agonists have been used successfully to treat chronic pain in humans and electrical stimulation of the caudatus nucleus in one human patient was also effective in alleviating chronic pain (Altier and Stewart, 1999). Finally, about 10% of the parkinsonian patients have chronic pain, which is described as intermittent and difficult to localize (Chudler and Dong, 1995). A neuroanatomical correlate has recently been obtained by the demonstration that a pain pathway from primary afferent nonpeptide nociceptors reach into the globus pallidus (Braz et al., 2005).

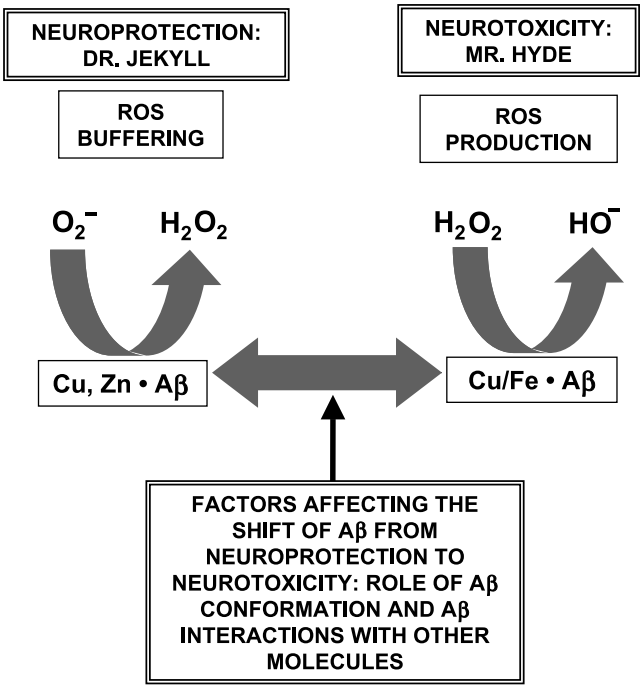


Fig. 7. Schematic representation of the possible shift of Aβ peptides from a neuroprotective and physiological role (Dr. Jekyll) to a neurotoxic pathological role (Mr. Hyde). For further details see text and Atwood et al. (2003)

It should be underlined that the Aβ-induced changes in tortuosity of the ECS pathways may cause a reduction of all types of VT, for example of the enkephalin-mediated VT in the basal ganglia with altered release of enkephalins and GABA from the D2 regulated striato-pallidal GABA terminals in the globus pallidus onto cell bodies receiving pain input (see above). Thus, it is possible that the Aβ-effects on pain threshold is in part mediated by alterations in other VT signals like enkephalins.

A major topic of this paper was the investigation of the dual role of the Aβ actions on neuronal survival, since it is well known that it can show neuroprotective as well as neurotoxic actions (Atwood et al., 2003). It has been found that Aβ can buffer potentially toxic compounds (see scheme of Fig. 7) such as ROS and heavy metals (Atwood et al., 2003). In fact, Aβ has an anti-oxidant action and under conditions that induce oxidative stress such as ischemia, hypoglycemia, traumatic brain injury APP synthesis is up regulated in vivo and in vitro. Hence, neurons respond to oxidative stress by increasing Aβ production. However, Aβ aggregates bind Aβ and prevent its anti-oxidant action. Hence, the formation of Aβ aggregates is potentially dangerous (Pike et al., 1991) and these aspects have been considered in the so called "Aβ hypothesis of AD" (Selkoe, 2003) that maintains that AD is caused by an imbalance between Aβ production and Aβ clearance that leads to a gradual cortical accumulation of the Aβ42 peptide aggregates. However, in the present paper it is suggested that a pathogenetic role in AD is played by triggering molecules that can favor and accelerate the β-sheet conformation for Aβ and the formation of aggregates that can maintain higher local concentrations of the oligomeric forms that are the potentially toxic agents. It is important to underline that the plaques themselves may not be as important neurotoxic agents as are the oligomers of Aβ that are in equilibrium with plaques. Beside their potential toxic actions oligomers have also important functional effects related to aging and senile dementia since they have been shown capable of inhibiting LTP, a basic mechanism for at least some forms of learning (Selkoe, 2003; Lee et al., 2005; Roher et al., 2000).

According to the present findings an important triggering role for these pathogenetic mechanisms is played by high concentrations of Hcy that, steadily increase with age (Agnati et al., 2005; Genedani et al., 2004).

The present paper suggests that in addition to buffering ROS and heavy metals, Aβ also buffers Hcy. In agreement with Lee's proposal it is suggested that the shift from Aβ-mediated neuroprotection (dr. Jekyll) to Aβ-mediated neurotoxicity (mr. Hyde) occurs when its buffering actions

are overwhelmed and/or alterations in the Aβ or in its proteolysis take place or, finally, when the Aβ aggregates become large enough to activate inflammatory responses and/or to manifest toxic properties possibly by releasing Aβ oligomers that cannot be completely cleared by the transport mechanisms towards the blood or towards the CSF (Zlokovic, 2005).

As previously mentioned, our attention was focused onto Hcy by the work of other groups and by our own data showing elevated Hcy plasma levels in AD and PD patients (Agnati et al., 2005; Genedani et al., 2004) as well as in view of recent epidemiological studies showing that elevated Hcy plasma levels are a risk factor for dementia and AD (Seshadri et al., 2002). In vitro and in vivo studies have been carried out to demonstrate whether Hcy could chemically interact with Aβ and whether the interaction favors the β-sheet conformation since insoluble Aβ aggregates with high β-sheet content is a key event in the pathogenesis of Alzheimer's disease (Sisodia, 1999; Pike et al., 1991). Clinical findings also support such a view since autosomal dominant forms of early onset familial AD appear to result from over-production of a more amyloidogenic form of Aβ (Aβ42), that over many years appears to drive a change in its conformation resulting in accumulation of aggregates at an early age (DeMattos et al., 2004). Thus, the role of Hcy in Aβ conformation and aggregation has been investigated in the present study both by means of in vitro and in vivo experiments. The in vivo experiments have been carried out in an animal model in which Hcy plasma and brain levels were pharmacologically enhanced to reach very high values (see Fig. 3). The in vitro experiments were carried out using enzymatic digests to generate peptide fragments which were analyzed by mass spectrometry. The peptide fragments were then further characterized with collision induced dissociation. Our fragmentation data (Fig. 5) clearly demonstrate that the only peptide fragments that bind Hcy are 5–10 and 2–13 fragments, most likely through a non-covalent interaction involving the guanido-group of Arg and the S group of Hcy. Upon this binding, Aβ acquires a β-sheet conformation as demonstrated by the circular dichroism experiments (Fig. 6). Thus, Hcy favours the potentially dangerous formation of fibrils (Makin et al., 2005; Kirkitadze et al., 2001; Fezoui and Teplow, 2002). The profibril action of Hcy has also been confirmed by in vivo studies since Aβ microinjections into the caudatus greatly increase the formation of Aβ-fibrils as demonstrated by thioflavin S staining (see Fig. 4a, b). This can be interpreted as the result of a buffering action of Aβ of a potentially toxic compound (Hcy) that has reached very high levels (pharmacological concentrations, see Fig. 3) or as a further aspect of the well known Hcy toxicity (see Mattson and Shea, 2003). In both cases, the present results are in agreement with a possible Hcy-induced shift from Aβ-neuroprotective to Aβ-neurotoxic actions according to the scheme of Fig. 7.

However, Aβ has not only neuroprotective actions but several physiological actions (Esteban, 2004; Lee et al., 2005). In fact, the proposal of a physiological role for Aβ is supported by reports showing that not only the production of Aβ is important for neuronal viability in primary cultures but also that soluble APP (sAPP) present in the brain tissue and in the CSF acts as a growth factor and regulates proliferation of progenitor cells in the adult subventricular zone (Caille et al., 2004). Furthermore, in support of a physiological role for Aβ in neurons, Kamenetz and collaborators (Kamenetz et al., 2003) showed an enhanced processing of APP upon opening of NMDA receptors and pharmacological blockade of endogenous Aβ production leads to enhanced spontaneous neuronal activity and synaptic plasticity. Thus, Aβ is released from neurons normally in response to activity and Aβ, in turn, down-regulates excitatory synaptic activity. In agreement with the Aβ role as a VT-signal it affects both the neuron which is the source of Aβ and its neighboring cells (Kamenetz et al., 2003). Thus, a question relevant to the AD pathogenesis is why the regulatory feedback loop between neuronal activity and Aβ production is broken in AD patients, resulting in unchecked accumulation of Aβ and neurotoxicity. Possibly, as Kamenetz and collaborators propose (Kametz et al., 2003), neurons may fail to be depressed by Aβ, leading to a gradual build-up of neuronal activity and further Aβ secretion or alternatively the activation of NMDA receptors possibly also via Hcy is taking over. In fact, Hcy is a modulator of NMDA receptors by acting on the glycine binding site (Lipton et al., 1997). Hence this could be a further interesting mechanism for the Aβ-Hcy functional interaction. It has been underlined that supplementation with folic acid is a feasible preventive treatment by reducing the increasing levels of Hcy found in ageing and in patients with neurodegenerative disorders. This treatment has also the great advantage not to have collateral effects (Mattson and Shea, 2003; Seshadri et al., 2002).

The trophic unit concept (see Fig. 8) could be a conceptual frame to put together in a logical way all these different findings. It should be considered that, for instance, the knockout of the Aβ precursor, APP, causes only minor neurological defects, although it presents enhanced sensitivity to kainate-induced seizures. However, we can hypothesize that Aβ has an important role for the homeostasis of the so called ''trophic unit''. It has been proposed that

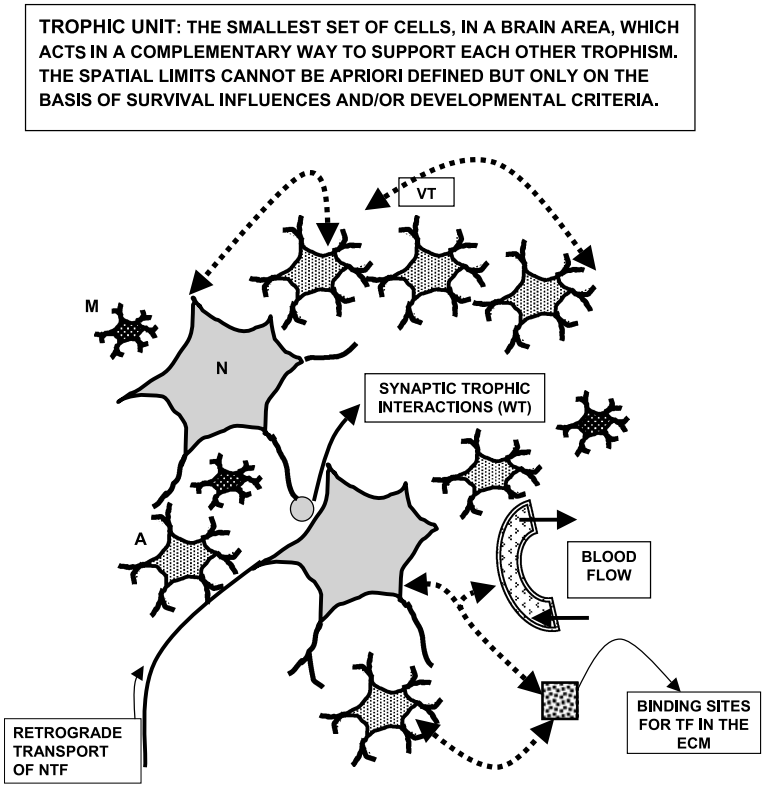


Fig. 8. Schematic representation of a "Trophic Unit". The trophic unit consists of: neurons (N, gray); astrocytes (A, weakly hatched); microglia (M, strongly hatched); blood vessels that not only supply nutrients and wash out waste products, but also give rise to release VT-signals from endothelial cells and pericytes; extra-cellular matrix (ECM) net (see above the global molecular network hypothesis) that has trophic and signalling functions for other trophic unit components, e.g., by binding and releasing trophic factors (TF). It is suggested that not only do these trophic unit elements interact (via both WT and VT) to support each other's trophism and to give out functionally integrated responses, but also is it postulated that the trophic units interact with each other. Alterations in one can affect interconnected trophic units leading to a patchy progression of trophic and functional disturbances in the brain. Some signals, such as Aβ peptides, by acting on all the components of the trophic unit can represent crucial (hub) signals for their trophism, plastic adjustments and act as controllers of their integrated outputs

the trophic unit is composed by neurons, astrocytes, microglia, endothelial cells and ECM components that functionally interact and support the physiological functions and survival of each other and hence give a reciprocal trophic support that allows the production of integrated and appropriate outputs (Agnati et al., 1995). As a matter of fact, the Aβ peptides by affecting neuronal communication and trophism, astrocyte and microglia responses, ECM composition, and microvasculature function could be one of the crucial signals for trophic unit homeostasis and integrated functions.

In agreement with the early Agnati and Fuxe proposal the trophic units tend to survive or to die according to their more or less strict interconnections and hence in some cases patchy degeneration can be observed (Agnati et al., 1995). In the AD patients a gradual evolution of the disease has been described possibly following trophic unit interconnections since Aβ plaques begin first in the basal temporal neocortex. From there, the alterations spread to adjoining neocortical areas, initially sparing the cortical regions running along the corpus callosum and primary motor and sensory cortices. The perforant pathway then becomes studded with Aβ deposits as it extends through the hippocampal formation (Lee et al., 2005). A key factor determining the vulnerability of the trophic units may be their resistance to oxidative stress leading to reduced formation of amyloid fibrils.

In conclusion, the present paper proposes a crucial role of Aβ peptides in the communication processes within and possibly among trophic units that allow not only the homeostasis of such units and the fulfillment of their integrative tasks, but also, their continuous remodeling according to the needs. Therefore, this view underlines the potential physiological and pathological roles of any alteration in the synthesis, conformation and decoding of the Aβ peptides.

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