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Amino-truncated amyloid β-peptide (Aβ5-40/42) produced from caspase-cleaved amyloid precursor protein is deposited in Alzheimer's disease brain

KAZUYA TAKEDA,*,† WATARU ARAKI,†,[1] HARUHIKO AKIYAMA,‡ AND TAKESHI TABIRA*,[1]

*Department of Vascular Dementia Research, National Institute for Longevity Sciences, NCGG, Obu, Japan; †Department of Demyelinating Disease and Aging, National Institute of Neuroscience, NCNP, Kodaira, Japan; and ‡Tokyo Institute of Psychiatry, Tokyo, Japan

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SPECIFIC AIMS

Caspase activation and apoptosis are implicated in neuronal death in Alzheimer's disease (AD). We analyzed the effects of the caspase-mediated cleavage of amyloid precursor protein (APP) on amyloid β-peptide (Aβ) production with special consideration of the generation of amino-terminally truncated Aβ.

PRINCIPAL FINDINGS

1. Evidence for altered Aβ generation from cells expressing caspase-cleaved form of APP

APP is cleaved by caspases in its cytoplasmic domain, subsequently generating APP lacking C-terminal 31 amino acids (APPΔC). Human neuroblastoma SH-SY5Y cells stably transfected with wild-type APP or APPΔC were established and designated SH-APP and SH-APPΔC cells, respectively. We selected two pairs of SH-APP and SH-APPΔC cells expressing similar APP levels (designated SH-APP-1, -2, and SH-APPΔC-1, -2 cells). SH-APP-2 and SH-APPΔC-2 expressed ~ twice as much APP as did SH-APP-1 and SH-APPΔC-1. Two types of sandwich ELISA were used to measure Aβ in conditioned media from these cells. BNT77-based ELISA detected N-terminal-intact and truncated Aβ (Aβ40total and Aβ42total), but not Aβ17-40 (p3 fragment); BAN50-based ELISA detected only N-terminal-intact Aβ (mainly Aβ1-40 and Aβ1-42). BNT77-based ELISA showed that SH-APP and SH-APPΔC cells secreted comparable levels of Aβ40total and Aβ42total. BAN50-based ELISA revealed that the amounts of Aβ1-40 and Aβ1-42 in SH-APPΔC cells were decreased to ~30% of those found in SH-APP cells. These data suggest that N-terminally truncated Aβ is increased relative to total Aβ in SH-APPΔC cells.

We next analyzed C-terminal fragments (CTF) of APP by immunoprecipitation Western blot with 4G8 antibody. Two CTF bands (~10 kDa α-CTF and ~12 kDa β-CTF) and a faint band (β′-CTF, ~11 kDa) were detected in cell lysates of SH-APP. Similarly, two bands (~6 kDa α-CTFΔC and ~8 kDa β-CTFΔC) and a faint band (β′-CTFΔC, ~7 kDa) were observed in SH-APPΔC cell samples. The relative level of β′-CTFΔC was increased in SH-APPΔC cells. Steady-state levels of β-CTFΔC and α-CTFΔC in SH-APPΔC cells were lower than those of β-CTF and α-CTF in SH-APP cells.

We compared the generation of secreted APP (sAPP) in SH-APP and SH-APPΔC cells. Immunoprecipitation Western blot showed that levels of total sAPP and sAPP-α (sAPP derived from α-secretase cleavage) were ~4-fold higher in SH-APPΔC cells, compared with SH-APP cells.

2. Increased production of amino-truncated Aβ from caspase-cleaved APP

We then analyzed altered Aβ secretion in SH-APPΔC cells using immunoprecipitation Western blot. BAN50 antibody immunoprecipitated Aβ1-40 and Aβ1-42 (band 1, comigrating with synthetic Aβ1-40/42). These immunoreactivities were decreased in media from SH-APPΔC cells, compared with SH-APP cell media. N-terminal-intact Aβ (Aβ1-40 and Aβ1-42) and the smaller fragment (band 3, most likely Aβ11-40 and Aβ11-42) were detected in media from SH-APP cells by BNT77 immunoprecipitation. In contrast, the intensities of bands 1 and 3 were reduced, and the intensity of band 2 was increased in SH-APPΔC media (**Fig. 1A**). We did not observe any band comigrating with Aβ17-40 (p3 fragment) in the BNT77 immunoprecipitates.

To identify secreted Aβ species, BNT77 immunoprecipitates were analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS). Two major peaks of Aβ1-40 and Aβ11-40 were detected in conditioned media from

[1] Correspondence: E-mail: araki@ncnp.go.jp; tabira@nils.go.jp

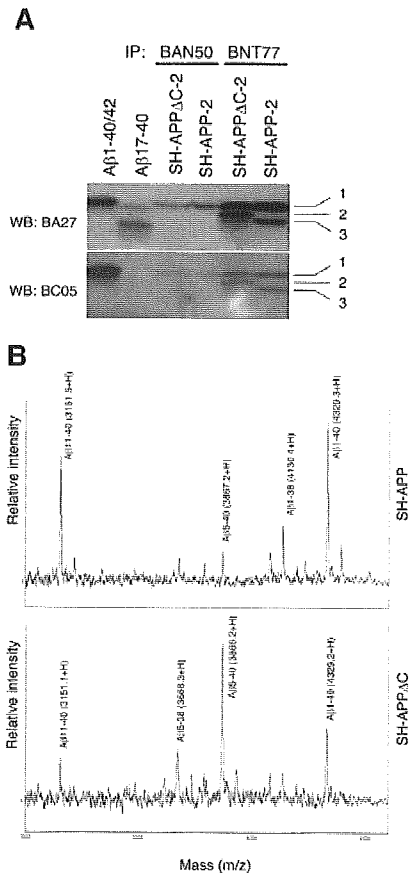

Figure 1. Immunoprecipitation Western blot and mass spectrometric analyses of secreted Aβ. *A)* Secreted Aβ peptides were immunoprecipitated with BAN50 or BNT77 antibodies and subjected to Tris-Tricine SDS-PAGE and Western blot analyses with BA27 or BC05. Synthetic Aβ1-40/42 and Aβ17-40 were simultaneously electrophoresed as markers. In BNT77 immunoprecipitates, 3 bands were detected. Band 1 corresponded to N-terminally intact Aβ1-40/42 and band 3 possibly represented Aβ11-40/42. Bands 1 and 3 were the major Aβ species in SH-APP cells. Conversely, in SH-APPΔC cells, the intensity of band 2 was markedly increased whereas that of bands 1 and 3 was reduced. In BAN50 immunoprecipitates, only band 1 was detected. *B)* Secreted Aβ was immunoprecipitated with BNT77 from conditioned media of SH-APP or SH-APPΔC cells and analyzed by MALDI-TOF-MS. Peaks were identified according to observed molecular and theoretical masses of Aβ and its variants. Aβ1-40, Aβ11-40, and some C-terminally truncated Aβ forms such as Aβ1-38 were identified in the sample from SH-APP (upper). Peak intensities of Aβ1-40 and Aβ11-40 were reduced whereas that of Aβ5-40 was markedly increased in the SH-APPΔC sample (lower). Aβ5–38 was also seen in this sample.

SH-APP cells. In contrast, the relative peak intensity of Aβ5-40 was markedly increased, whereas peak intensities of Aβ1-40 and Aβ11-40 were decreased in SH-APPΔC media (Fig. 1*B*). These data show that N-terminally truncated Aβ (starting at Arg5) is markedly increased in media from SH-APPΔC cells. Aβ11-40/42 appears to be generated through processing of APP by BACE1 and γ-secretase, as BACE1 alternatively cleaves between Tyr10-Glu11 in the Aβ sequence. Increased levels of Aβ5-40 and decreased levels of Aβ1-40 and Aβ11-40 were observed in media from HEK293 cells transiently transfected with APPΔC, compared with those transfected with APP.

We further analyzed the levels and species of intracellular Aβ in SH-APP and SH-APPΔC cells by sensitive Western blot. Aβ1-40 and Aβ1-42 levels were comparable between samples from SH-APP and SH-APPΔC cells. The intracellular Aβ1-42/Aβ1-40 ratio was ~0.4 in both cell lines. These results suggest that the generation of intracellular Aβ is unaffected by the C-terminal truncation of APP.

3. Aβ5-40/42 generation involves altered β cleavage of APP

To determine the processing mechanism by which Aβ5-40/42 is generated from APPΔC, we treated SH-APPΔC and SH-APP cells with a specific inhibitor of BACE, OM99-2. Secreted Aβ was analyzed by immunoprecipitation Western blot and mass spectrometry. In OM99-2-treated SH-APPΔC cells, Aβ1-40 secretion was significantly decreased, but the Aβ5-40 level was not altered compared with that in untreated cells. SH-APP cells treated with the inhibitor secreted significantly reduced Aβ1-40 and increased Aβ5-40 levels. These data suggest that cleavage between Phe4 and Arg5 is not mediated by BACE1. BACE1 inhibition promotes the secretion of Aβ5-40. To establish whether α-secretase-like proteases are responsible for Aβ5-40/42 generation, we incubated SH-APPΔC cells with a TACE (tumor necrosis factor-α converting enzyme) inhibitor, TAPI-1, which inhibits α-secretase. Incubation with 20 μM TAPI-1 resulted in increased Aβ1-40 and decreased Aβ5-40 secretion, suggesting that α-secretase-like proteases are involved in Aβ5-40/42 production.

4. Immunohistochemical analysis of Aβ5-40/42 in AD brain

To determine whether Aβ5-40/42 is present in human brain tissues, we generated a specific antibody to the N-terminal end region of this Aβ species (designated the Aβ5 antibody). In Western blot analyses, the Aβ5 antibody reacted with Aβ5-40, but not with Aβ1-40, whereas the BAN50 antibody recognized only Aβ1-40. The Aβ5 antibody immunostained vessels in the AD brain, indicating the deposition of Aβ5-40/42, particularly in vascular lesions with amyloid angiopathy. In nearby sections, another Aβ antibody (6E10) labeled more vessels than did the Aβ5 antibody. Amyloid angiopathy in the smaller sized vessels tended to be negative or weakly positive for Aβ5. In addition, Aβ5 antibody stained numerous neurofibrillary tangles (NFT), suggesting that Aβ5-40/42 may be deposited in the NFT. Although a small number of senile plaques were positive for Aβ5 in some cases, the staining was not as consistent as that of vessels and NFT.

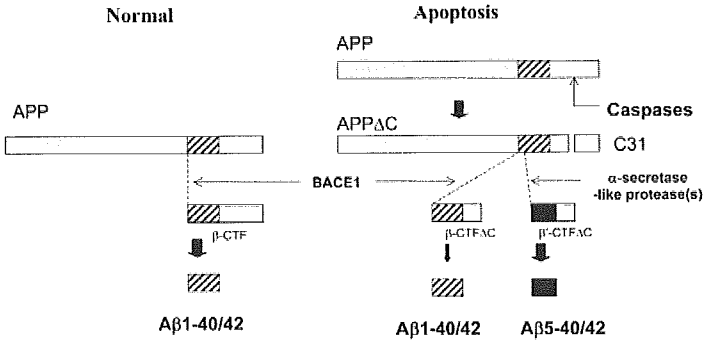

Figure 2. The possible mechanism of the generation of Aβ5-40/42. During apoptosis, APP is cleaved by caspases to form APPΔC and a C-terminal fragment consisting of 31 amino acids (C31). APPΔC is preferentially processed between Phe4 and Arg5 to generate β′-CTFΔC possibly through alternative processing by α-secretase-like protease(s). Subsequent γ-secretase cleavage results in the formation of Aβ5-40/42.

5. Cleavage at the Aβ5 site occurs when wild-type APP-expressing cells undergo apoptosis

Finally, we investigated whether APP processing to generate Aβ5-40/42 occurs during apoptosis of wild-type APP-expressing cells. SH-APP cells were exposed to MG132 or MG132 plus staurosporine. Western blots using an AB5942 antibody specific for the caspase-generated neo epitope of APP reveal that exposure to these agents leads to caspase-mediated cleavage at the cytoplasmic region of APP. We examined CTF production from caspase-processed APP by immunoprecipitation Western blot analysis with 4G8 and AB5942 antibodies. Cells treated with MG132 plus staurosporine contained significant amounts of β-CTFΔC, β′-CTFΔC, and α-CTFΔC, consistent with data from SH-APPΔC cells. The results suggest that cleavage at the Aβ5 site occurs during apoptosis in SH-APP cells.

CONCLUSIONS AND SIGNIFICANCE

Recent evidence suggests that apoptosis underlies the neuronal death seen in AD. Active forms of caspases and the caspase-cleaved APP have been detected in AD brain tissues, but it is not clear whether the caspase cleavage of APP affects Aβ formation. In this study, we have clearly demonstrated that such APP cleavage promotes the secretion of a distinct amino-truncated Aβ species (Aβ5-40/42). Our data provide the first evidentiary connection between caspase activation and the formation of amino-truncated Aβ. Our results are consistent with and expand upon data from previous studies that measured N-terminally intact Aβ, but not amino-truncated Aβ.

We used inhibitors of BACE and α-secretase to investigate the mechanism of Aβ5-40/42 generation. After treatment of SH-APP cells with a BACE inhibitor, OM99-2, Aβ1-40 levels were decreased, whereas Aβ5-40 levels were increased. Treatment of SH-APPΔC cells with TAPI-1 led to decreased Aβ5-40 and increased Aβ1-40 levels. The data strongly suggest that cleavage at the Aβ5 site is not ascribed to BACE1 activity, but mediated by α-secretase-like proteases (e.g., ADAM family proteases, including TACE and ADAM10). This is consistent with the finding that secretion of p3 (Aβ17-40), a product derived from α-secretase cleavage, is significantly increased in cells expressing APPΔC. BACE2 functions as an alternative α-secretase, but may not be involved in Aβ5-40/42 generation, since OM99-2 inhibits BACE1 and BACE2.

It has been established that wild-type APP undergoes caspase cleavage during apoptosis, so it is reasonable to assume that subsequent cleavage at the Aβ5 site occurs in apoptotic cells. We show evidence that APPΔC is generated in SH-APP cells exposed to MG-132 and staurosporine. β′-CTFΔC, which corresponds to a precursor of Aβ5-40/42, is formed in these apoptotic cells. Accordingly, we conclude that Aβ5-40/42 is generated after caspase activation (**Fig. 2**).

Our sensitive Western blot analyses indicate that the majority of intracellular Aβ consist of Aβ1-40/42 in wild-type APP- and APPΔC-expressing cells. Since two distinct pathways appear to exist for extracellular and intracellular pools of Aβ, amino-truncated Aβ peptides, including Aβ5-40/42, are likely to be produced mainly in the extracellular Aβ pathway.

Our immunohistochemical staining with the Aβ5 antibody revealed that Aβ5-40/42 species are deposited in some vessels with amyloid angiopathy in AD brain tissue. This may reflect the in vivo occurrence of caspase cleavage of APP. The observation that Aβ5 antibody labels NFT is intriguing, considering that activation of caspases is suggested to occur in neurons bearing NFT. Our data are consistent with previous reports that considerable N-terminal modifications of Aβ are seen in AD cortices and leptomeninges. Such amino-truncated Aβ species may be instrumental in the amyloidosis process.

We suggest that caspase activation in the AD brain results in the formation of APPΔC, leading to the increased production and deposition of N-terminally truncated Aβ5-40/42. Further research on the in vivo generation of this Aβ species is needed to clarify its pathological role in Aβ deposition and neuronal death in AD. FJ

Glypican-1 as an Aβ binding HSPG in the human brain: its localization in DIG domains and possible roles in the pathogenesis of Alzheimer's disease[1]

NORIFUMI WATANABE,[2] WATARU ARAKI,* DE-HUA CHUI,† TAKAO MAKIFUCHI,‡ YASUO IHARA,§ AND TAKESHI TABIRA

National Institute for Longevity Sciences, Morioka, Obu, Aichi; *Department of Demyelinating Disease and Aging, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo; †Laboratory for Alzheimer's Disease, RIKEN Brain Science Institute, Wako-shi, Saitama; ‡Department of Clinical Research, National Saigata Hospital, Ogata, Nakakubiki, Niigata; and §Department of Neuropathology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan

SPECIFIC AIMS

The amyloid deposition whose major component is the 39-43 amino acid peptide, termed β-amyloid protein (Aβ), is considered to be important in Alzheimer's disease (AD) pathology, but the precise mechanism of its accumulation in AD brain is unclear. Although there are studies of the association between Aβ and heparan sulfate proteoglycans (HSPGs), they demonstrated co-localization in plaques or binding ability of HSPGs derived from tissues other than human brain. It is not fully understood whether HSPGs are involved in neuronal cell death in AD brain. Our aims are to identify Aβ binding HSPG(s) from human brain and to investigate their roles in Aβ accumulation and neuronal cell death.

PRINCIPAL FINDINGS

1. Identification of glypican-1 as an Aβ binding HSPG from human brain

To identify HSPGs derived from the human brain with the capacity to bind Aβ, control human brain lysates were separated using anion exchange DEAE-Sepharose chromatography and fractions containing possible Aβ binding protein(s) were determined by Aβ binding assay. We found that fractions containing HSPGs exclusively showed the binding activity to fibrillar Aβ in an HS chain-dependent manner. Earlier studies reported that Aβ binding to HS chains prevents heparanase-catalyzed degradation of HS chains. Thus, we examined whether preincubation of the HSPG containing DEAE fractions with Aβ alters the sensitivity of HSPGs to heparitinase treatment. Without preincubation with Aβ, several bands (~200, ~100, ~60, ~40 kDa) were detected with 3G10 mAb in the DEAE fractions, indicating they contained plural HSPGs. Preincubation with Aβ resulted in the disappearance of the ~60 kDa band; the intensity of the other bands was relatively unchanged, suggesting that an HSPG with the ~60 kDa core protein bound to Aβ preferentially and prevented heparitinase-catalyzed degradation of HS chains. Glypicans are known as HSPGs with a ~60 kDa core protein; six glypicans (glypican-1 to -6) have been cloned. Glypican-1 is the major HSPG expressed in the adult brain. We performed the same incubation experiments to clarify the identity of the ~60 kDa band using anti-glypican-1 mAb. A 60 kDa band of glypican-1 core protein was detected in the lysates; preincubation of the lysates with Aβ resulted in a marked decrease of this 60 kDa band and the appearance of a smear band (>100 kDa) probably representing intact glypican-1. Pretreatment of lysates with heparitinase before incubation with Aβ recovered the 60 kDa band, suggesting that Aβ was unable to bind to heparitinase-treated glypican-1. These results suggest that glypican-1 derived from the human brain can bind to Aβ in an HS chain-dependent manner.

2. Aβ binding to glypican-1 depends on its aggregation state

It was reported that heparin or mouse EHS HSPG binds fibrillar Aβ (fAβ) but not non-fibrillar Aβ (non-fAβ) with high affinity. In the present in vitro analysis of Aβ binding to glypican-1, the core protein of glypican-1 could be detected after incubation with non-fibrillar Aβ but not after with fibrillar Aβ at levels similar to those observed in the untreated sample, suggesting that non-fibrillar Aβ had little or no binding ability to glypican-1. Binding of non-fibrillar Aβ to HSPGs was not observed on dot blot membranes.

3. Glypican-1 is a major HSPG in DIG domains from human brains

Glypican-1 is a GPI-anchored HSPG; most, if not all, GPI-anchored proteins are localized in special mem-

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-1040fje; doi: 10.1096/fj.03-1040fje

[2] Correspondence: National Institute for Longevity Sciences, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. E-mail: watanabn@nils.go.jp

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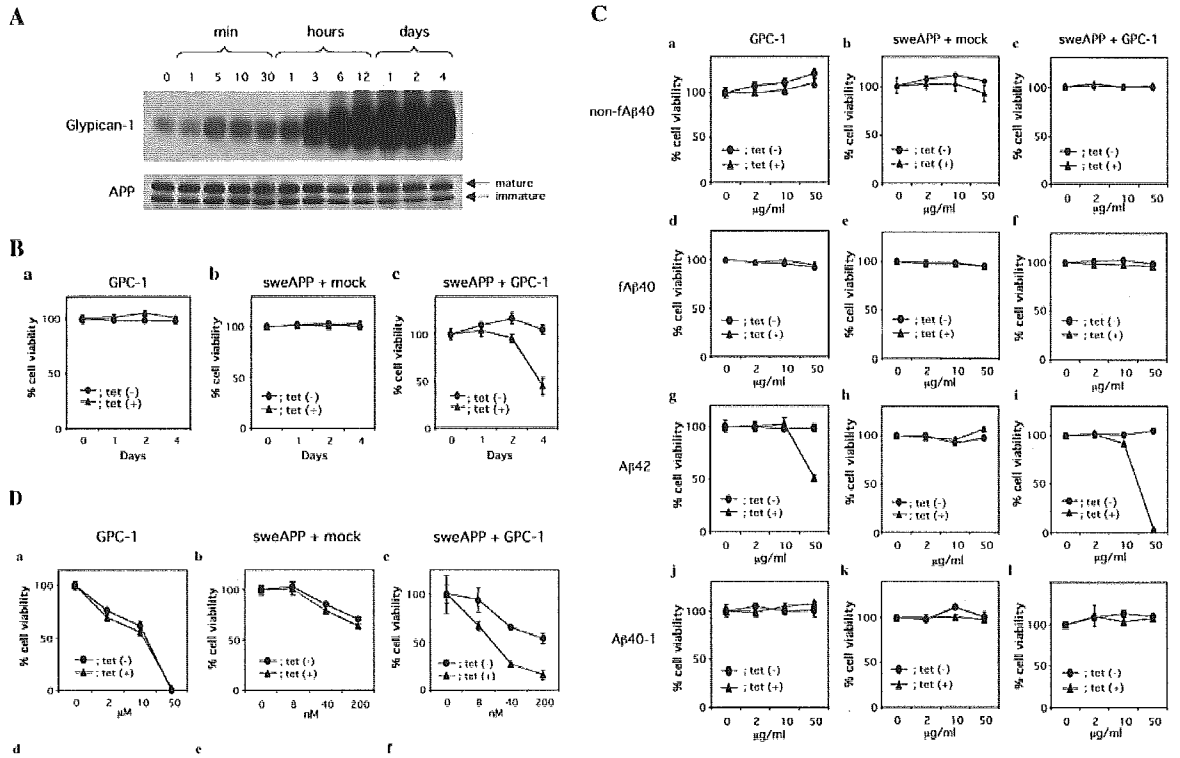

Figure 1. Time course of induction of glypican-1 expression in sweAPP-SH cells and effect of overexpression of glypican-1 on cell viability. *A)* SweAPP-GPC1-SH cells were cultured with tetracyclin and subjected to Western blot. The same membrane was reprobed by anti-APP Aβ as an internal protein control. *B)* a–c: Transfectans were cultured with (▲) or without (●) tetracyclin. *C)* After incubation in medium with (▲) or without (●) tetracyclin overnight, transfectants were cultured with non-fAβ40 (a–c), fAβ40 (d–f), Aβ42 (g–i), or Aβ40-1 (j–l) in the presence or absence of tetracyclin for 4 days. *D)* After overnight incubation in medium with (▲) or without (●) tetracycline, transfectants were treated with thapsigargin (a–c), tunicamycin (d–f), or brefeldin A (g–i). Cell viability was monitored by WST assay. Assays were performed in triplicate and mean values ± SE at 450 nm were measured. Quantitative values are expressed as % of cell viability in untreated cells.

brane domains called detergent-insoluble, glycosphingolipid-enriched (DIG) domains. We next examined whether glypican-1 is present in the DIG fraction from brain tissues. DIG was recovered in fractions 4–6, into which flotillin (a marker protein of DIG) was exclusively fractionated. Western blot with anti-glypican-1 showed that glypican-1 was mainly present in fraction 5, indicating localization of glypican-1 to the DIG domains. Western blot with 3G10 mAb indicated that HSPGs other than glypican-1 were almost undetectable in the DIG fraction. An insoluble pellet from the DIG fraction was solubilized with 6 M guanidine-HCl and the solubilized sample was used for dot blot Aβ binding assay. Aβ bound to guanidine soluble samples from the DIG fraction; this binding was significantly inhibited by heparitinase pretreatment, indicating that Aβ could bind to DIG-resided glypican-1 in an HS chain-dependent manner.

4. Co-localization of glypican-1 and Aβ in DIG fractions from AD brain

Recently, DIG domains or "rafts" have received attention with regard to the pathogenesis of AD, because accumulation of Aβ in these domains was demonstrated and appeared to correlate with the extent of Aβ deposition in the brain. Thus, it is possible that glypican-1 participates in the process of Aβ accumulation by interaction with Aβ in such specific microdomains. To clarify this, we examined whether glypican-1 and Aβ are co-fractionated in DIG fractions from AD brains. The fractions were analyzed by Western blots with antibodies to human glypican-1, Aβ, Aβ40, Aβ42, or APP. Glypican-1 was recovered mainly in DIG fractions. Full-length APP was fractionated predominantly in the high density fractions, and to a smaller extent in DIG fractions. We observed that signifi-

cant amounts of Aβ40 and Aβ42 were present in the DIG fractions as monomers and SDS stable dimers. BAN50, whose epitope is located in Aβ1-10, labeled Aβ monomers more strongly than Aβ dimers, suggesting these SDS stable dimers were formed in a way that the amino-terminal portion of Aβ was masked, modified, or deleted.

5. Preferential role of glypican-1 in Aβ42 accumulation in DIG domains

Given that Aβ binds to glypican-1 and these two proteins were accumulated in DIG domains, there may be a correlation between them. To examine this, we quantified levels of Aβ and glypican-1 in the DIG fraction using ELISA. There was a strong correlation between Aβ42 and glypican-1 (ctrl; r=0.9517, AD; r=0.8756) whereas no correlation between Aβ40 and glypican-1 (ctrl; r=0.3559, AD; r=0.0854) was observed. These results suggest that glypican-1 plays a preferential role in the accumulation of Aβ42 in DIG domains.

6. Effect of glypican-1 overexpression on cell viability

To explore the function of glypican-1 other than plaque formation, we generated transfectants that overexpressed glypican-1 in a tetracycline-inducible manner. Western blot analysis showed that the trasnsfectants were induced to express a large amount of glypican-1 protein when cultured with tetracyclin (**Fig. 1*A***). Time course experiments demonstrated that induction of glypican-1 expression was begun 5 min after addition of tetracyclin and reached maximal levels after 12 h. Cell viability of these cells with or without the induction of glypican-1 was analyzed using WST assay. As shown in Fig. 1, overexpression of glypican-1 decreased viability of cells coexpressing APP-carrying Swedish mutation of familial Alzheimer's disease (Fig. 1*Bc*). Neither glypican-1 nor Swedish APP overexpression alone affected cell viability (Fig. 1*Ba*, b). Since the production of Aβ in Swedish APP-expressing cells was increased, the observed effect of glypican-1 on cell viability may be due to enhanced Aβ toxicity by binding to glypican-1. To examine this, transfectants were cultured with exogenously added Aβ for 4 days, then cell viability was measured. The viability of all cells examined was not affected by adding non-fAβ40, fAβ40, or Aβ40-1 even though glypican-1 expression was induced (Fig. 1*Ca–f*, j–l). In contrast, Aβ42 significantly reduced cell viability only when glypican-1 was overexpressed (Fig. 1*Cg*, i). These results indicate that cells that overexpress glypican-1 become more susceptible to Aβ42 toxicity, resulting in enhanced cell death.

7. Effect of glypican-1 overexpression on ER stress

It has been reported that ER stress is an important factor in the neuropathology of a wide variety of neurological disorders, including AD. Studies have shown that neurotoxicity elicited by Aβ is at least partially mediated by ER,

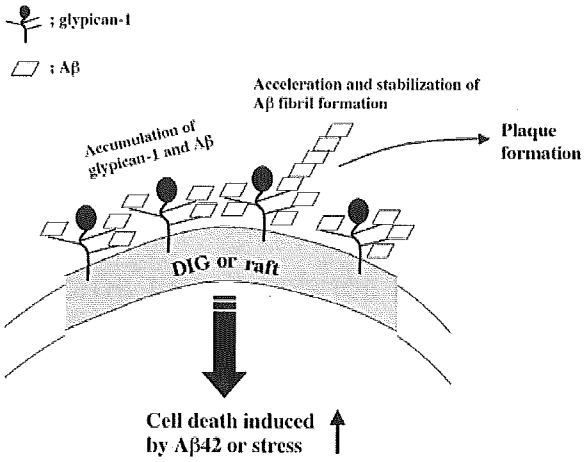

Figure 2. Proposed roles of glypican-1 in AD. Glypican-1 is involved in Aβ accumulation in DIG or raft domains; hence *1)* fibril and plaque formations are accelerated by binding to HS chains on glypican-1, and *2)* neuronal cell death induced by Aβ42 or certain stresses is enhanced when glypican-1 levels are increased.

raising the possibility that the ER stress response is influenced by overexpression of glypican-1, which may result in enhanced susceptibility of cells to Aβ42 toxicity. We examined the effect of glypican-1 expression on the stress response. Cell death induced by thapsigargin was accelerated when glypican-1 was overexpressed in cells coexpressing Swedish APP (Fig. 1*Dc*). Such an acceleration was not observed in cells that expressed glypican-1 or Swedish APP alone (Fig. 1*Da*, b). The stress response by tunicamycin and brefeldin A did not alter the cell survival even though cells were coexpressing Swedish APP and glypican-1 (Fig. 1*Dd–i*). These results suggest that glypican-1, together with Aβ, makes cells more vulnerable to some but not all stresses.

CONCLUSIONS AND SIGNIFICANCE

Although HSPGs are co-localized in senile plaques and may promote amyloid formation, deposition, and/or persistence by binding to Aβ, it remains uncertain how HSPGs are involved in AD pathogenesis and which HSPG has a pathogenic role in AD. The findings here suggest that glypican-1 binds to Aβ through HS chains and may be involved in accumulation of Aβ in DIG domains and/or the formation of plaques at an initial stage. Glypican-1 may act as a negative factor to neuronal cell survival, probably by binding with Aβ. Individuals whose expression levels of glypican-1 are relatively high might have a higher risk of AD. It is necessary to define more precisely the exact role of glypican-1 in these pathological events. A better understanding of normal and pathological functions of glypican-1 may lead to the development of new therapeutic approaches for AD. FJ

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Enhanced antidepressant efficacy of σ_1 receptor agonists in rats after chronic intracerebroventricular infusion of β-amyloid-(1–40) protein

Alexandre Urani[a,b,1], Pascal Romieu[a,2], François J. Roman[b], Kiyofumi Yamada[c,3], Yukihiro Noda[c], Hiroyuki Kamei[c,3], Hung Manh Tran[c,4], Taku Nagai[c], Toshitaka Nabeshima[c], Tangui Maurice[a,*,2]

[a] *INSERM U.336, Behavioural Neuropharmacology Group, Institut de Biologie, 4, bvd Henri IV, 34060 Montpellier, France*
[b] *PfizerGRD-Fresnes, 3/9, rue de la Loge, B.P. 100, 94265 Fresnes Cedex, France*
[c] *Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Tsuruma-cho, Nagoya 466-8560, Japan*

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Abstract

Treatment of depressive symptoms in patients suffering from neurodegenerative disorders remains a challenging issue, since few available antidepressants present an adequate efficacy during pathological aging. Previous reports suggested that selective σ_1 receptor agonists might constitute putative candidates. We here examined the pharmacological efficacy of igmesine and (+)-SKF-10,047 and the σ_1 receptor-related neuroactive steroid dehydroepiandrosterone sulfate, in rats infused intracerebroventricularly during 14 days with the β-amyloid-(1–40) protein and then submitted to the conditioned fear stress test. Igmesine and (+)-SKF-10,047 significantly reduced the stress-induced motor suppression at 30 and 6 mg/kg, respectively, in β-amyloid-(40–1)-treated control rats. Active doses were decreased, to 10 and 3 mg/kg, respectively, in β-amyloid-(1–40)-treated animals. The dehydroepiandrosterone sulfate effect was also facilitated, both in dose (10 vs. 30 mg/kg) and intensity, in β-amyloid-(1–40)-treated rats. Neurosteroid levels were measured in several brain structures after β-amyloid infusion, in basal and stress conditions. Progesterone levels, both under basal and stress-induced conditions, were decreased in the hippocampus and cortex of β-amyloid-(1–40)-treated rats. The levels in pregnenolone, dehydroepiandrosterone and their sulfate esters appeared less affected by the β-amyloid infusion. The σ_1 receptor agonist efficacy is known to be inversely correlated to brain progesterone levels, synthesized mainly by neurons that are mainly affected by the β-amyloid toxicity. The present study suggests that σ_1 receptor agonists, due to their enhanced efficacy in a nontransgenic animal model, may alleviate Alzheimer's disease-associated depressive symptoms.
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Keywords: σ_1 Receptor; Neuro(active)steroid; β-Amyloid-(1–40) protein; Alzheimer's disease; Depression; Conditioned fear stress; (Rat)

1. Introduction

Alzheimer's disease is the most common form of dementia among the elderly (Evans et al., 1989). Physiopathological features characteristic of Alzheimer's disease include abnormal extracellular accumulation of β-amyloid proteins into sensitive structures of the brain. Gradual deposition of β-amyloid protein in the form of neurotic plaques, apparition of neurofibrillary tangles as well as progressive cognitive deficits accompany the emergence of Alzheimer's disease (Selkoe, 1991). Among the most common and important complications of Alzheimer's disease is clinically relevant depression, which worsens patient disability and suffering. Prevalence of depression among Alzheimer's disease patients ranges from 0% to 86% depending on reports, but

* Corresponding author. CNRS FRE 2693, Université de Montpellier II, c.c. 090, place Eugène Bataillon, 34095 Montpellier Cedex 5, France. Tel.: +33-4-67-4-36-23; fax: +33-4-67-14-42-51.
E-mail address: maurice@univ-montp2.fr (T. Maurice).
[1] Present address: Laboratory of Biochemistry, Zentralinstitut für Seelische Gesundheit, J5 (Innenstadt), 68159 Mannheim, Germany.
[2] Present address: CNRS FRE 2693, Université de Montpellier II, c.c. 090, place Eugène Bataillon, 34095 Montpellier Cedex 5, France.
[3] Present address: Laboratory of Neuropsychopharmacology, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan.
[4] Present address: Faculty of Pharmacy, University of Medicine and Pharmacy, 41-43 Dinh Tien Hang Str., Dist. 01, Ho Chi Minh City, Viet-Nam.

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is usually considered to average about 30–50% (Zubenko and Moossy, 1988; Wragg and Jeste, 1989; Zubenko, 2000; Purandare et al., 2001; Olin et al., 2002). Depression could also be observed in association with other types of degenerative dementia, such as Parkinson's disease, Huntington's chorea, Pick's disease or vascular dementia (Zubenko and Moossy, 1988). The cause of depression observed in Alzheimer's disease patients seems to be less genetic and more structural, related to functional declines, than classic depression seen in adults (Boland, 2000; Espiritu et al., 2001). In particular, the neurodegenerative pathologies provoke anatomic damages, reduction of cerebral blood flow or receptor dysfunctions in particular brain structures, including frontotemporal areas, hippocampus or the locus cœruleus (Zubenko and Moossy, 1988; Forstl et al., 1992; Sheline et al., 1996). Alzheimer's disease patients developing major depression thus show a particular neuropathological and neurochemical context, mainly characterized by a severe central cholinergic deficit, occurring in basal forebrain structures innervating the hippocampus and neocortex (Zubenko and Moossy, 1988; Forstl et al., 1992; Sheline et al., 1996). Present therapeutic strategies using classical antidepressant treatments have produced contradictory findings and did not satisfactorily lead to clear depression reduction (Boland, 2000; Lyketsos et al., 2003; Zubenko et al., 2003). Novel therapeutic approaches with preserved antidepressant efficacy are thus needed to treat depression in patients with neurodegenerative dementia.

We have demonstrated that chronic administration of β-amyloid protein into the cerebral ventricle, using a long-term mini-pump implantation, produced memory impairments without apparent neurodegeneration (Nitta et al., 1994; Yamada and Nabeshima, 2000; Tran et al., 2002). However, numerous neurochemical and neurophysiological alterations were observed after infusion of β-amyloid protein, such as impairment of long-term potentiation (Itoh et al., 1999); functional reduction of cholinergic and dopaminergic systems (Itoh et al., 1996); changes in the ciliary neurotrophic factors levels (Yamada et al., 1995); changes in the mRNA expression of brain-derived neurotrophic factor (BDNF) (Tang et al., 2000); induction of inducible nitric oxide (NO) synthase (iNOS) and overproduction of NO in the hippocampus (Tran et al., 2001); tyrosine nitration of synaptophysin (Tran et al., 2003); impairment of endogenous antioxidant system (Kim et al., 2003); and reduced activation of protein kinase C (PKC) (Olariu et al., 2002). These observations suggested in a convergent manner that β-amyloid toxicity resulted in functional deficits affecting neuronal responses and signalling pathways within the hippocampus, sustaining the marked memory impairments.

The σ_1 receptor agonists are potent antidepressant drugs acting through a unique mechanism that suggests their potential efficacy in Alzheimer's disease-related depression (Maurice, 2002). The σ_1 receptor is a 223-amino acid protein, cloned in several animal species (Hanner et al., 1996; Kekuda et al., 1996) that appeared devoid of analogy with any other known mammalian protein. Selective σ_1 receptor ligands exert a potent neuromodulation on intracellular Ca^{2+} mobilisations and excitatory neurotransmitter systems, including noradrenergic, glutamatergic and cholinergic responses (for review, see Maurice et al., 1999). Its endogenous effector remains unidentified, but the biological relevance of this receptor is supported by the correlation observed between the σ_1 binding affinity and functional and behavioural effects of drugs, and the interaction of several endogenous systems with this receptor, including peptides of the neuropeptide Y family, or neuro(active)steroids. In particular, pregnenolone or dehydroepiandrosterone behaved as σ_1 receptor agonists, while progesterone is a potent antagonist (Maurice et al., 1999). A similar crossed pharmacology between neuro(active)steroids and σ_1 receptor ligands has been observed in animal models of depression, forced swimming or conditioned fear stress, or in the σ_1 receptor involvement in cocaine-induced conditioned place preference (Noda et al., 2000; Urani et al., 2001; Romieu et al., 2003).

In a previous study, we have reported that the antidepressant-like efficacy of igmesine or PRE-084, two σ_1 receptor agonists, measured using the forced swim test, were potentiated in mice injected intracerebroventricularly (i.c.v.) with β_{25-35}-amyloid peptide (Urani et al., 2002). This enhanced efficacy was attributed to decreased progesterone levels in the hippocampus of β_{25-35} animals and suggested that σ_1 agonists, due to their enhanced efficacy, may allow to alleviate the depressive symptoms associated with Alzheimer's disease (Urani et al., 2002). In the present study, these observations were confirmed and extended using the Alzheimer's disease model of rats chronically infused with β-amyloid-(1–40) protein (Nitta et al., 1994, 1997; Yamada et al., 1995, 1999). We examined the antidepressant-like efficacy of σ_1 receptor agonists, and particularly igmesine and the σ_1 receptor-related neuroactive steroid dehydroepiandrosterone sulfate, on the conditioned fear stress response of rats, as previously reported (Nabeshima et al., 1985; Kamei et al., 1997). The effect of the β-amyloid infusion on brain neurosteroid levels was measured in basal and stressful conditions.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats (Charles River Japan, Yokohama, Japan or breeding centre of the Faculty of Pharmacy, Montpellier, France) weighing 200–230 g at the beginning of the experiments were used. They were housed two or three per cage under standard light–dark conditions (12-h light cycle starting at 08:00 h) at a constant temperature of 23 ± 1 °C. The animals had free access to food and water and they have been handled in accordance with the guidelines established

by the Institute for Laboratory Animal Research of Nagoya University and to the European Communities Council Directive of 24 November 1986 (86-609/EEC).

On the day of surgery, a cannula attached to a mini-osmotic pump was implanted in the rat right cerebral ventricle (A 20.3 mm, L 1.2 mm, V 4.5 mm) as previously described (Nitta et al., 1994) and β-amyloid-(1–40) (Feinchemikalien, Switzerland) was continuously infused at a dose of 0.3 nmol/12 μl/day for 14 days. Control animals received 0.3 nmol/12 μl/day of β-amyloid-(40–1), the reverse sequence of (1–40). Both peptides (1–40) and (40–1) were dissolved in 35% acetonitrile–0.1% trifluoroacetic acid (vehicle). We have previously confirmed that β-amyloid-(40–1) or vehicle itself has no effect on learning behaviour at this flow rate (Yamada et al., 1999). On day 14 after the start of β-amyloid infusion, rats were submitted to the conditioned fear stress procedure. After the first behavioural session, some animals were anaesthetized with pentobarbital 6%, transcardiacally perfused with 200 ml of saline solution and their brains were quickly removed from the skull. The cerebral cortex, hippocampus and cerebellum were immediately dissected out, and subsequently stored at −80 °C until assayed.

2.2. Drugs

(+)-*N*-cyclopropylmethyl-*N*-methyl-1,4-diphenyl-1-ethyl-but-3-en-1-ylamine hydrochloride (igmesine, JO-1784) was synthesized at Institut de Recherche Jouveinal/Parke-Davis. Progesterone (4-pregnene-3,20-dione) was from Sigma/Aldrich (St. Louis, MO, USA). Dehydroepiandrosterone sulfate (5-androsten-3β-ol-17-one sulfate) and (+)-SKF-10,047 were from Research Biochemicals (Natick, MA, USA). *N,N*-Dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)ethylamine (NE-100) was provided by Taisho Pharmaceuticals (Tokyo, Japan). [1,2,6,7-^{3}H(*N*)]Progesterone (3589 GBq/mmol, 37 MBq/ml), [7-^{3}H(*N*)]pregnenolone ([^{3}H]pregnenolone, 777 GBq/mmol, 37 MBq/ml), [1,2,6,7-^{3}H(*N*)]dehydroepiandrosterone ([^{3}H]dehydroepiandrosterone, 2220 GBq/mmol, 37 MBq/ml) and [7-^{3}H(*N*)]dehydroepiandrosterone sulfate ([^{3}H]dehydroepiandrosterone sulfate, 592 GBq/mmol, 37 MBq/ml) were from New England Nuclear (Boston, MA, USA). The pregnenolone antibody was from AbCys (Paris, France); progesterone and dehydroepiandrosterone antibodies were from Biovalley (Marne-la-Vallée, France). Progesterone was suspended in sesame oil; other drugs were solubilised in distilled water or saline solution. Drugs were injected subcutaneously (s.c.) or intraperitoneally (i.p.), in a volume of 100 μl/20 g of body weight.

2.3. Conditioned fear stress procedure

The apparatus was a transparent acrylic rectangular cage (25 × 30 × 47 high cm) equipped with a metal wire floor. The cage was inserted in a sound-attenuated box and was illuminated with a 20-W bulb. Each rat was placed into the test cage and received intermittent electric shocks (0.1 Hz, 200 ms, 100 V DC) for 10 min through an isolated pulse stimulator (Nihon Koden, Tokyo, Japan). Each animal received electric footshocks in the range of 0.2–0.5 mA, because the current resistance of the animal varied between 200 and 500 kΩ (Kamei et al., 1997). The test session was performed 24 h after the first session. Animals were placed again into the test cage, but no footshock was delivered. The spontaneous motility of rats was measured using an infrared beams activity device (Scanet SV-10, Neuroscience, Osaka, Japan or Opto-varimex, Columbus Instruments, Columbus, OH, USA), in which the cage was inserted. The non-shocked control group was operated similarly, except for the absence of shock treatment. The σ_1 receptor ligands, or appropriate vehicle solutions, were administered 30 min before the test session.

2.4. Extraction and purification of neurosteroids

Brain samples were thawed, weighed and homogenized in ice-cold 10 mM phosphate buffer saline, pH 7.4. Recovery tracers ([^{3}H]progesterone, [^{3}H]pregnenolone, [^{3}H]dehydroepiandrosterone, [^{3}H]dehydroepiandrosterone sulfate, 50 Bq each) were added. Then, 10 ml of ethylacetate/isooctane, 1/1 vol/vol, was added and the tubes were vigorously stirred for 8 min. After centrifugation at 4000 × *g* for 5 min, the organic phase was removed and the extraction step was repeated twice. This organic phase was then defatted with a MeOH 90%/isooctane separation. The aqueous extracts containing unconjugated steroids were further purified by reverse-phase chromatography on C_{18} cartridges (Amersham, Les Ulis, France). The isooctane phases containing lipoidal derivatives were thrown away. Sulfate esters were hydrolysed. The aqueous phase from the first separation was

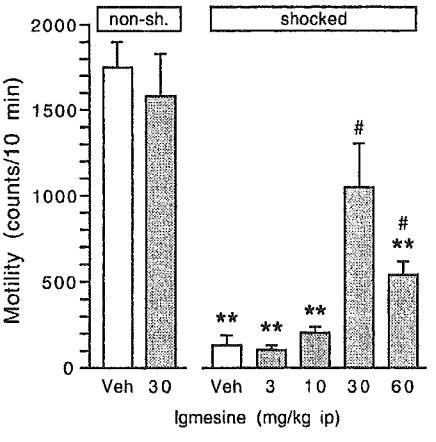

Fig. 1. Effect of the σ_1 receptor agonist igmesine in naive rats submitted to the conditioned fear stress. Igmesine was administered i.p. 30 min before the motility measurement. The number of animals per experimental group was $n=4–6$. $^*P<0.05$, $^{**}P<0.01$ compared to the vehicle (Veh)-treated non-shocked (non-sh.) group; $^{\#}P<0.05$ compared to the Veh-treated shocked group (Dunn's test).

brought to pH = 1.0 with a few drops of sulfuric acid and to a NaCl concentration of 20% by adding 2/1 vol/vol of a 30% NaCl solution. Extraction with ethylacetate was again performed as described above, and this extract, which contained steroid sulfates, was hydrolysed at 37 °C for 16 h. Ethylacetate extracts were washed once with 1 N NaOH (0.25 vol.) and twice with water (0.25 vol.). The extracts were finally dried.

The different steroids were separated using partition chromatography on Celite[545] (Prolabo, Fontenay-sous-Bois, France) microcolumns, with propanediol, 1 g, as the stationary phase. Impregnated celite was settled in 5-ml disposable glass pipettes. Extracts were taken up in 1 ml of isooctane saturated with propanediol, and deposited onto the columns. Progesterone was eluted with 19-ml isooctane, pregnenolone with 15 ml of isooctane/benzene (7/3 vol/vol) and dehydroepiandrosterone with 20 ml of isooctane/benzene (1/1 vol/vol). The recovery of the different steroids added as tracers was routinely 60–80%.

After separation, each steroid was quantified by radioimmunoassay using specific antibodies presenting minimal cross-reactions. Measurements were performed in triplicate of four dilutions of each purified sample. Results are expressed as ng/g of tissue.

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. Behavioural data were analyzed using the Dunn's multiple comparisons test after a non-parametric Kruskal–Wallis analysis of variance (ANOVA, KW values). Neurosteroid measurements were analyzed using a two-way ANOVA (F-values),

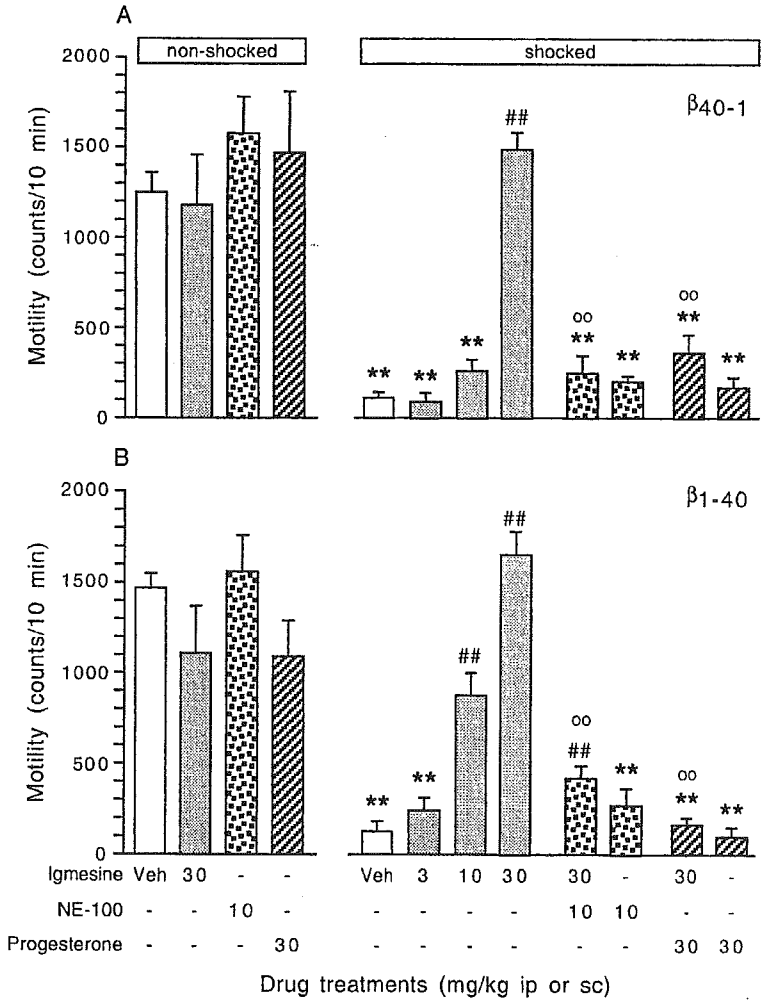

Fig. 2. Effect of igmesine on the conditioned fear stress response in rats infused during 14 days with: (A) β-amyloid-(40–1) or (B) β-amyloid-(1–40) protein. The σ_1 receptor antagonist NE-100 or the neuroactive steroid progesterone was injected i.p. or s.c., respectively, 15 min before igmesine, administered i.p. 30 min before the motility measurement. The number of animals per experimental group was $n = 4–6$. $^{**}P < 0.01$ compared to the vehicle (Veh)-treated non-shocked group; $^{\#\#}P < 0.01$ compared to the Veh-treated shocked group; $^{oo}P < 0.01$ compared to the igmesine (30)-treated shocked group (Dunn's test).

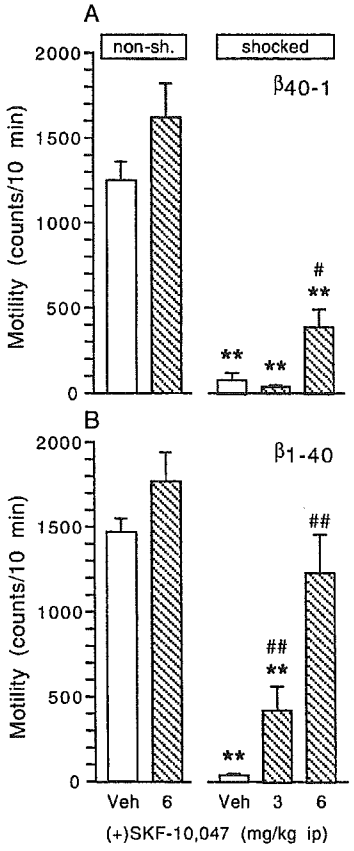

Fig. 3. Effect of the σ_1 receptor agonist (+)-SKF-10,047 on the conditioned fear stress response in rats infused during 14 days with: (A) β-amyloid-(40–1) or (B) β-amyloid-(1–40) protein. (+)-SKF-10,047 was administered i.p. 30 min before the motility measurement. The number of animals per experimental group was $n=5$. $^{**}P<0.01$ compared to the vehicle (Veh)-treated non-shocked (non-sh.) group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to the Veh-treated shocked group (Dunn's test).

with the infusion treatment and exposure to shock as independent parameters, post-hoc comparisons being made using the Welch's test. The criteria for statistical significance was $P<0.05$.

3. Results

3.1. Effects of the σ_1 receptor agonists on conditioned fear stress in β-amyloid-infused rats

Treatment with the σ_1 receptor agonist igmesine, tested in the 3–60 mg/kg i.p. dose range, resulted in an attenuation of the highly significant decrease in motility observed in rats that experienced the unavoidable electric footshock (KW = 26.84, $P<0.001$; Fig. 1). At 30 and 60 mg/kg, igmesine induced a significant ($P<0.05$), but bell-shaped effect.

Rats infused chronically with either β-amyloid-(40–1) or β-amyloid-(1–40) protein exhibited, similarly as intact animals, a highly significant decrease of motility after shock (Fig. 2A and B). In control, β-amyloid-(40–1)-treated animals, the igmesine treatment attenuated the decrease of motility, in a similar dose–response effect as compared to non-infused animals (KW = 51.07, $P<0.0001$; Fig. 2A). The σ_1 receptor agonist induced a highly significant effect at 30 mg/kg. This effect was blocked by the selective σ_1 receptor antagonist NE-100 (10 mg/kg i.p.) or the neuroactive steroid progesterone (30 mg/kg s.c.) (Fig. 2A). In β-amyloid-(1–40)-treated animals, igmesine also attenuated the decrease of motility (KW = 54.64, $P<0.0001$; Fig. 2B). The effect was even potentiated, since the compound induced a highly significant effect at a lower dose, 10 mg/kg, as well as at 30 mg/kg (Fig. 2B). The maximal effect was blocked by NE-100 or progester-

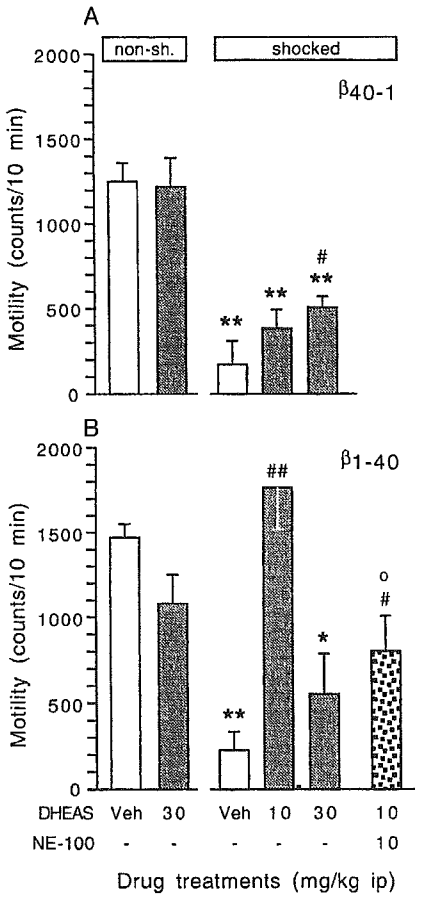

Fig. 4. Effect of the neuroactive steroid dehydroepiandrosterone sulfate on the conditioned fear stress response in rats infused during 14 days with: (A) β-amyloid-(40–1) or (B) β-amyloid-(1–40) protein. NE-100 was injected i.p. 15 min before dehydroepiandrosterone sulfate (DHEAS), administered s.c. 30 min before the motility measurement. The number of animals per experimental group was $n=5$–6. $^{*}P<0.05$, $^{**}P<0.01$ compared to the vehicle (Veh)-treated non-shocked group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to the Veh-treated shocked group; $^{o}P<0.05$ compared to the dehydroepiandrosterone sulfate (30)-treated shocked group (Dunn's test).

one (Fig. 2B). None of the compounds, tested at their highest active dose, affected the motility in non-shocked groups (Fig. 2A and B).

The anti-stress effect of the prototypic σ_1 receptor agonist (+)-SKF-10,047 was also tested in rats infused with either β-amyloid-(40–1) or β-amyloid-(1–40) protein. In β-amyloid-(40–1)-treated animals, (+)-SKF-10,047 attenuated the decrease of motility ($KW = 20.55$, $P < 0.001$; Fig. 3A). The σ_1 receptor agonist induced a significant but limited attenuation at 6 mg/kg. In β-amyloid-(1–40)-treated animals, the efficacy of (+)-SKF-10,047 to increase the shocked rats motility was potentiated, since highly significant effects were measured at 3 and 6 mg/kg ($KW = 19.15$, $P < 0.001$). At the latter dose, a complete reversion was measured (Fig. 3B).

3.2. Effect of the neuroactive steroid dehydroepiandrosterone sulfate on conditioned fear stress in β-amyloid-infused rats

The anti-stress effect of the σ_1 receptor-related neuroactive steroid dehydroepiandrosterone sulfate was examined in rats infused with either β-amyloid-(40–1) or β-amyloid-(1–40) protein. In β-amyloid-(40–1)-treated animals, dehydroepiandrosterone sulfate attenuated the decrease of motility ($KW = 19.79$, $P < 0.001$; Fig. 4A). The steroid induced a significant but limited attenuation at 30 mg/kg. In β-amyloid-(1–40)-treated animals, the dehydroepiandrosterone sulfate efficacy to increase the shocked rats motility was potentiated, since a highly significant reversion was observed at the lower dose of 10 mg/kg ($KW = 17.70$, $P < 0.001$; Fig. 4B). This motility increase was significantly, but not fully, blocked by NE-100, confirming the involvement of the σ_1 receptor in this effect (Fig. 4B).

3.3. Neurosteroid levels in β-amyloid-infused rats

Progesterone levels were measured in several brain regions, the hippocampus, cortex and cerebellum, of rats infused with β-amyloid-(40–1) or (1–40) protein (Fig. 5). In the hippocampus, the two-way ANOVA resulted in a highly significant effect of the β-amyloid treatment [$F(1,12) = 67.63$, $P < 0.001$], but not of stress [$F(1,12) = 3.28$, $P = 0.10$]. However, the treatment × stress interaction was significant [$F(1,12) = 6.58$, $P < 0.05$]. Indeed, in control β-amyloid-(40–1)-treated rats, the stress significantly increased hippocampal progesterone level (Fig. 5A). In β-amyloid-(1–40)-treated animals, the non-shocked group showed a decreased basal progesterone level, that remained unchanged in the shocked group (Fig. 5A). In turn, shocked β-amyloid-(1–40)-treated animals presented almost one third the progesterone contents of shocked β-amyloid-(40–1)-treated ones. In the cortex, highly significant effects were measured for the β-amyloid treatment [$F(1,11) = 55.80$, $P < 0.001$] and stress [$F(1,11) = 10.13$, $P < 0.01$], but not for the treatment × stress interaction. β-Amyloid-(1–40)-treated animals exhibited significantly less progesterone levels (Fig. 5B), in basal as well as stress conditions. For both β-amyloid-(40–1)- and (1–40)-treated groups, the stress only moderately increased progesterone levels. Progesterone levels in the cerebellum appeared unchanged among experimental groups (Fig. 5C).

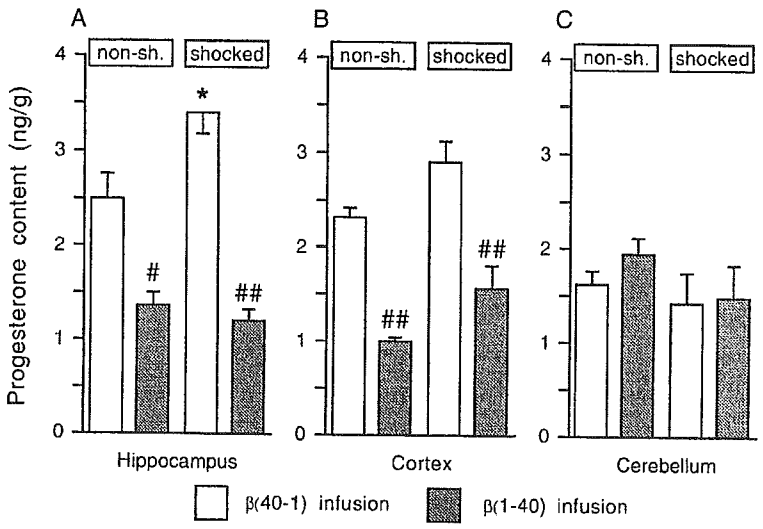

Fig. 5. Brain contents in progesterone in the hippocampus (A), cortex (B) or cerebellum (C) of rats infused during 14 days with β-amyloid-(40–1) or β-amyloid-(1–40) protein. Progesterone levels were measured in non-shocked or shocked animals, sacrificed 30 min after the test session in the conditioned fear procedure. The number of samples was $n = 3$–4 per condition. $^{*}P < 0.05$ compared to the respective non-shocked group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the respective β-amyloid-(40–1)-treated group (Welch's test).

Pregnenolone and dehydroepiandrosterone levels were measured in the different conditions in the hippocampus, cortex and cerebellum, while the levels in sulfate ester of each steroid was measured in the hippocampus (Figs. 6 and 7). In the hippocampus, significant effects for pregnenolone levels were measured for stress [$F(1,12)=11.57$, $P<0.01$] and the β-amyloid treatment × stress interaction [$F(1,12)=10.46$, $P<0.01$]. Indeed, in control β-amyloid-(40–1)-treated rats but not in β-amyloid-(1–40)-treated animals, stress significantly increased hippocampal pregnenolone level (Fig. 6A). In the cortex, a highly significant effect was measured only for stress [$F(1,11)=19.98$, $P<0.001$], indicating no change induced by the β-amyloid-(1–40)-treatment (Fig. 6B). Pregnenolone levels in the cerebellum appeared unchanged among experimental groups (Fig. 6C). In the hippocampus,

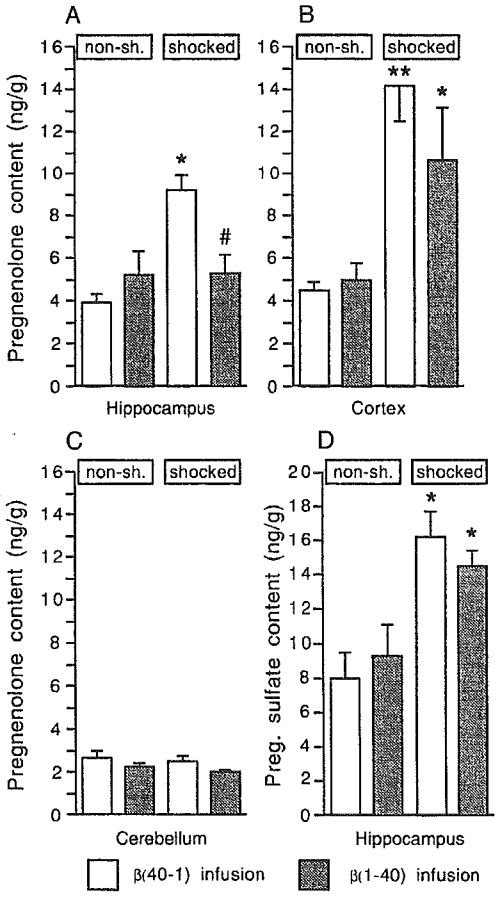

Fig. 6. Brain contents in pregnenolone in the hippocampus (A), cortex (B) or cerebellum (C) of rats infused with β-amyloid-(40–1) or β-amyloid-(1–40) protein. (D) Hippocampal content in pregnenolone sulfate in rats with β-amyloid-(40–1) or β-amyloid-(1–40) protein. Neurosteroid levels were measured in non-shocked or shocked animals, sacrificed 30 min after the test session in the conditioned fear procedure. The number of samples was $n=3$–4 per condition. $^{*}P<0.05$, $^{**}P<0.01$ compared to the respective non-shocked group; $^{\#}P<0.05$ compared to the respective β-amyloid-(40–1)-treated group (Welch's test).

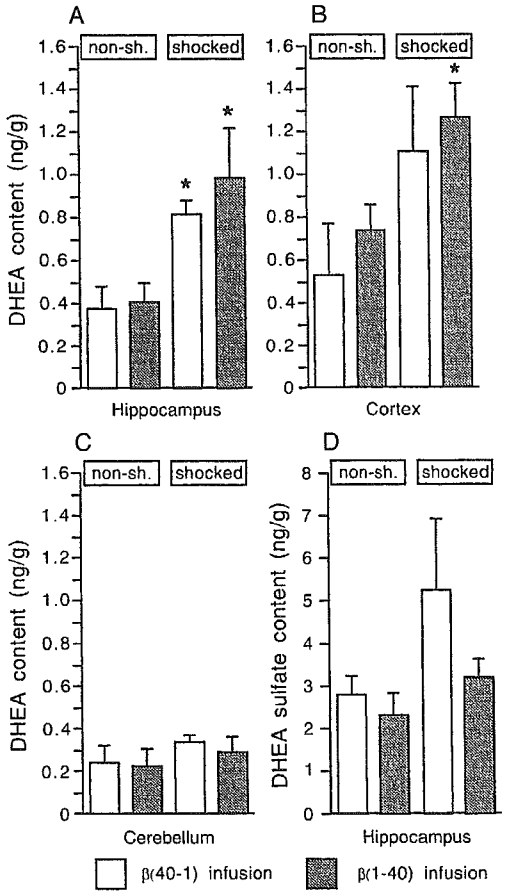

Fig. 7. Brain contents in dehydroepiandrosterone (DHEA) in the hippocampus (A), cortex (B) or cerebellum (C) of rats infused with β-amyloid-(40–1) or β-amyloid-(1–40) protein. (D) Hippocampal content in dehydroepiandrosterone sulfate (DHEAS) in rats with β-amyloid-(40–1) or β-amyloid-(1–40) protein. Neurosteroid levels were measured in non-shocked or shocked animals, sacrificed 30 min after the test session in the conditioned fear procedure. The number of samples was $n=3$–4 per condition. $^{*}P<0.05$ compared to the respective non-shocked group (Welch's test).

pregnenolone sulfate levels varied significant after stress [$F(1,11)=18.19$, $P<0.01$], but no difference was measured between β-amyloid-(40–1)- and (1–40)-treated rats (Fig. 6D).

Dehydroepiandrosterone levels were much lower in all brain structures, and significant variations were only observed after stress in the hippocampus [$F(1,12)=13.48$, $P<0.01$; Fig. 7A] and cortex [$F(1,11)=6.57$, $P<0.05$; Fig. 7B]. No difference was measured between β-amyloid-(40–1)- and (1–40)-treated rats and in the cerebellum among all experimental groups (Fig. 7C). In the hippocampus, dehydroepiandrosterone sulfate levels failed to change significantly after stress [$F(1,11)=1.78$, $P=0.21$], or according to the β-amyloid-treatment [$F(1,11)=0.27$, $P=0.61$; Fig. 7D].

4. Discussion

Major depression affects between 30% and 50% of the patients who develop Alzheimer's disease and has serious consequences not only for the evolution of the patient, in terms of increased disabilities, but also for caregivers. Results of current antidepressant therapies have produced unsatisfactory findings without fully addressing the benefits of depression reduction (Boland, 2000; Espiritu et al., 2001; Lyketsos et al., 2003). Preclinical studies are thus requested to propose alternative strategies based on novel antidepressants that may present a preserved efficacy in the demented subject. We report here, using a non-transgenic rat model of Alzheimer's disease, induced by the chronic intracerebroventricular infusion of β-amyloid-(1–40) peptide, that the effect of the selective σ_1 receptor agonists, igmesine, (+)-SKF-10,047 or dehydroepiandrosterone sulfate, was enhanced in this model compared to control animals, in the conditioned fear stress test. As compared to β-amyloid-(40–1) peptide-infused control animals, the active dose of each compound was lower by two-to three-fold in β-amyloid-(1–40)-treated rats, with an increase of the intensity of the effect observed for (+)-SKF-10,047 or dehydroepidrosterone sulfate.

Selective σ_1 receptor agonists showed antidepressant efficacy in several animal models of behavioural despair, particularly the tail suspension test or the forced swim test (Matsuno et al., 1996; Ukai et al., 1998; Urani et al., 2001), suggesting that depressive symptoms may be alleviated by the drugs acting through this receptor. Indeed, a preliminary report outlined the potential clinical efficacy of igmesine in depression (Pande et al., 1998). This antidepressant efficacy may involve the wide-range neuromodulatory action, affecting both intracellular Ca^{2+} mobilisations and responses to neurotransmitters, including glutamatergic, cholinergic and monoaminergic systems, known to be involved in the physiopathological changes sustaining depressive states (Maurice et al., 1999). The conditioned fear stress paradigm has been originally reported by Fanselow (1980). Rats exhibit a marked suppression of motility when they are replaced in the same environment in which they previously experienced an aversive electric footshock. This motor suppression is regarded as a conditioned emotional response to the environment associated with the previous footshock. Indeed, when animals returned into the same environment in which they received the aversive shock, they exhibited a marked suppression of motility. However, when they were placed in a different environment, the difference in motility between shocked and non-shocked mice was not observed (Kameyama et al., 1985).

The conditioned fear stress-induced motor suppression could be attenuated by treatments with antidepressants acting as selective serotonin reuptake inhibitors, citalopram or fluvoxamine (Hashimoto et al., 1996; Inoue et al., 1996; Li et al., 2001), suggesting that the freezing behavior is mediated by serotonergic receptor inactivation (Inoue et al., 1996). However, the conditioned fear stress-induced motor suppression was poorly sensitive to anxiolytics, such as diazepam and chlordiazepoxide (Kameyama and Nagasaka, 1982; Nagasaka and Kameyama, 1983) but attenuated by the benzodiazepine antagonist flumazenil (Izumi et al., 1999). As a result, the conditioned fear stress model may be useful for investigating the pathogenesis of mood disorders, particularly those considered to be treatment resistant, and for developing novel therapeutic drugs. It has been shown that σ_1 receptors play an important role in conditioned fear stress response (for reviews, see Kamei et al., 1998; Maurice et al., 1999). Several σ_1 receptor agonists such as (+)-SKF-10,047 and dextromethorphan attenuated the conditioned fear stress-induced motor suppression in rodents, the effects being antagonized by the σ_1 receptor antagonist NE-100 (Kamei et al., 1996). In addition, dehydroepiandrosterone sulfate and pregnenolone sulfate attenuated the conditioned fear stress-induced motor suppression in mice, via their interaction with the σ_1 receptor (Noda et al., 2000). Progesterone behaved as a potent σ_1 receptor antagonist, since it antagonized the attenuating effects of (+)-SKF-10,047, dehydroepiandrosterone sulfate and pregnenolone sulfate, similarly to what was observed with NE-100 (Noda et al., 2000). In the present study, we confirmed that (+)-SKF-10,047 or dehydroepiandrosterone sulfate are active in the conditioned fear stress in control rats. In addition, the efficacy of igmesine was demonstrated.

The chronic infusion of β-amyloid-(1–40) at a dose of 300 pmol/day provoked numerous physiopathological changes and behavioural impairments reminiscent of Alzheimer's disease (Yamada and Nabeshima, 2000). Accumulation of β-amyloid-(1–40) in the hippocampus and cerebral cortex was evident immunohistochemically following a 14-day period of infusion (Nitta et al., 1997). An interesting observation in our study is that fear conditioning is not affected by β-amyloid peptide treatment-since the stress-induced motor suppression is the same in treated animals as in intact rats-unlike other kinds of more complex memory tests. Indeed, a significant impairment of spatial reference memory formation in a water maze and a deficit of passive avoidance performance was observed in β-amyloid-(1–40)-infused animals, which was accompanied by a mild, but significant, reduction of choline acetyltransferase activity in the hippocampus (Nitta et al., 1997). Impairment of long-term potentiation (Itoh et al., 1999) and reduced activation of protein kinase C (Olariu et al., 2002) were also observed. The chronic infusion of β-amyloid-(1–40), even at very low concentrations, directly inhibited various cholinergic neuronal functions independently of apparent neurotoxicity, suggesting a possible link between chronic infusion of β-amyloid-(1–40) burden and cholinergic dysfunction in Alzheimer's disease. Using an in vivo brain microdialysis technique, we observed that KCl-and nicotine-induced increase in acetylcholine and dopamine release in the hippocampus/cerebral cortex and the striatum, respectively, is markedly impaired by the chronic infusion of β-amyloid-(1–40), although the basal levels of these neurotransmitters

in the β-amyloid-(1–40)-infused rats did not differ from those in vehicle-infused control animals (Itoh et al., 1996). The reduction of nicotine-induced ACh release may be partly due to a decrease in affinity of nicotinic ACh receptors (Olariu et al., 2001, 2002). In addition, the chronic infusion of β-amyloid-(1–40) resulted in changes in the ciliary neurotrophic factor protein levels (Yamada et al., 1995) and in the mRNA expression of BDNF in the hippocampus (Tang et al., 2000). The latter playing a major role in both the etiology of major depression and Alzheimer's disease (Tsai, 2003). All these physiological disturbances may contribute to the onset of learning and memory deficits and support the idea that infusion of β-amyloid-(1–40) in rats provoked neurotoxic changes relevant to the physiopathology of Alzheimer's disease (Yamada and Nabeshima, 2000).

The importance of neurosteroids in mood disorders and depression has been demonstrated (for review, see Van Broekhoven and Verkes, 2003). Recent reports focused on the physiopathological significance of neurosteroids in Alzheimer's disease and related dementia (Wolkowitz et al., 1997, 2003; De Bruin et al., 2002; Brown et al., 2003; Weill-Engerer et al., 2002). On one hand, neurosteroids, and particularly dehydroepiandrosterone sulfate, are expected to elicit a marked neuroprotection in the brain. On the second hand, brain structural abnormalities related to Alzheimer's disease, both β-amyloid deposits and neurofibrillary tangles, which result from the aggregation of pathologic tau proteins, affect brain neurosteroid expression. Brown et al. (2000) reported that β-amyloid-(1–42) protein increased dehydroepiandrosterone levels in human glia-derived cell lines, after a 24-h application. The authors suggested that glial cells might be able to resist to the β-amyloid-induced toxicity because of their ability to produce dehydroepiandrosterone. Direct measurements of pregnenolone, pregnenolone sulfate, dehydroepiandrosterone, dehydroepiandrosterone sulfate, progesterone and allopregnanolone were performed in individual brain regions of Alzheimer's disease patients and aged non-demented controls, including hippocampus, amygdala, frontal cortex, striatum, hypothalamus and cerebellum (Weill-Engerer et al., 2002). A general trend towards decreased levels of all steroids was observed in brain regions of Alzheimer's disease patients compared to controls. Pregnenolone sulfate levels were significantly lower in the striatum and cerebellum; dehydroepiandrosterone sulfate levels were significantly reduced in the hypothalamus, striatum and cerebellum; and progesterone and allopregnanolone levels were markedly but non-significantly reduced in several brain structures, including the hypothalamus, striatum, frontal cortex, or amygdala. A significant negative correlation was found between the levels of cortical β-amyloid peptides and those of pregnenolone sulfate in the striatum and cerebellum and between the levels of phosphorylated tau proteins and dehydroepiandrosterone sulfate in the hypothalamus (Weill-Engerer et al., 2002). Since high levels of key proteins implicated in the formation of plaques and neurofibrillary tangles were correlated with decreased brain levels of pregnenolone sulfate and dehydroepiandrosterone sulfate, the authors supported the concept of a possible neuroprotective role of these neurosteroids in Alzheimer's disease.

In the present study, we chose to measure neurosteroid levels in the hippocampus and cortex, because of: (i) the demonstrated importance of these forebrain structures in depression (Reid and Stewart, 2001); (ii) the importance of the neurosteroid/σ_1 receptor interaction within these structures (Maurice et al., 1999); and (ii) their particular vulnerability to the β-amyloid-(1–40) infusion (Nitta et al., 1997). It must be however outlined that σ_1 receptor agonists ameliorated the conditioned fear stress response through the involvement of mesolimbic dopaminergic systems (Kamei et al., 1997), and thus basal ganglia structures may also be of interest.

We observed significant decreases in brain neurosteroid levels in β-amyloid-(1–40)-infused rats, either in basal conditions or after the fear stress. Pregnenolone and dehydroepiandrosterone sulfate levels failed to increase after stress in β-amyloid-(1–40)-infused rats. Most significantly, continuous β-amyloid-(1–40) infusion caused a marked decrease of progesterone levels in the hippocampus and cortex of rats, and these alterations were not affected by the conditioned fear stress. Interestingly, the β-amyloid-mediated neurotoxic process seems to differentially affect the activity of the different enzymes involved in the steroid biosyntheses. In particular, the 3β-hydroxysteroid dehydrogenase enzyme activity is likely to be mainly affected, consistently with the important decrease in progesterone measured in β-amyloid-(1–40) rats. Since progesterone, released in stressful situations, acts as an endogenous antagonist at the σ_1 receptor, an enhanced behavioural efficacy of the σ_1 receptor agonists was observed. This mechanism has recently been demonstrated through pharmacological manipulations of the progesterone levels (adrenalectomy/castration and administration of inhibitors of the enzymes involved in progesterone synthesis and metabolism) for both the memory and behavioural despair responses (Phan et al., 1999; Urani et al., 2002). However, although this proposed mechanism might serve as an interesting basis to design new, efficient antidepressants for indications such as the Alzheimer's disease-related depression, several points must be further examined. In particular, dehydroepiandrosterone sulfate efficacy was increased in β-amyloid-(1–40)-infused rats. This increase could be due to an interaction with σ_1 receptor since it was partially blocked by NE-100, but may also originate from parallel pathways to be elucidated. At the clinical level, Wolkowitz et al. (1997, 2003) reported that dehydroepiandrosterone, administered to the patients with treatment-resistant depression for 6 months, provoked a marked improvement in depression ratings (Wolkowitz et al., 1997), but more recently, in a randomized, double-bind, placebo-

controlled study, that it allowed only a transient effect on cognitive performances, narrowly missing significance (Wolkowitz et al., 2003). The lack of effect of dehydroepiandrosterone itself in Alzheimer's disease patients encourages the use of more selective synthetic compounds, such as σ_1 receptor agonists.

In summary, the present study showed an increased antidepressant efficacy of σ_1 receptor agonists in a non-transgenic model of Alzheimer's disease, induced by the chronic infusion of β-amyloid-(1–40) in rats. This effect was coherent with decreased brain level in neurosteroids, and particularly progesterone, as previously demonstrated in mice (Phan et al., 1999, 2002; Urani et al., 2001) and in humans (Wolkowitz et al., 2003). The present results confirmed previous observations in mice injected acutely into the lateral ventricle with aggregated β-amyloid-(25–35) peptide (Urani et al., 2002). Targeting the σ_1 receptor thus appears as a promising alternative for alleviating the depressive symptoms in Alzheimer's disease patients.

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Research report

β-Amyloid (1–42)-induced learning and memory deficits in mice: involvement of oxidative burdens in the hippocampus and cerebral cortex

Jin Hyeong Jhoo[a,f], Hyoung-Chun Kim[b,*], Toshitaka Nabeshima[c], Kiyofumi Yamada[d], Eun-Joo Shin[b], Wang-Kee Jhoo[b], Wookyung Kim[b], Kee-Seok Kang[e], Sangmee Ahn Jo[e], Jong Inn Woo[f,g,1]

[a] *Department of Psychiatry, Pundang Jesaeng Hospital, Daejin Medical Center, Seongnam, South Korea*
[b] *Neurotoxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200-701, South Korea*
[c] *Department of Neuropsychopharmacology, Graduate School of Medicine, Hospital Pharmacy, Nagoya University, Nagoya, Japan*
[d] *Department of Clinical Pharmacy, Laboratory of Experimental Therapeutics, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan*
[e] *Department of Biomedical Sciences, Biomedical Brain Research Center, National Institute of Health, Seoul, South Korea*
[f] *Neuroscience Research Institute of the Medical Research Center, Clinical Research Institute of Seoul National University Hospital, Seoul National University, Seoul, South Korea*
[g] *Department of Neuropsychiatry, College of Medicine and Seoul National University Hospital, Seoul National University, Seoul 110-744, South Korea*

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Abstract

We have demonstrated that oxidative stress is involved, at least in part, in β-amyloid protein (Aβ)-induced neurotoxicity in vivo [Eur. J. Neurosci. 1999;11:83–90; Neuroscience 2003;119:399–419]. However, mechanistic links between oxidative stress and memory loss in response to Aβ remain elusive. In the present study, we examined whether oxidative stress contributes to the memory deficits induced by intracerebroventricular injection of Aβ (1–42) in mice. Aβ (1–42)-induced memory impairments were observed, as measured by the water maze and passive avoidance tests, although these impairments were not found in Aβ (40–1)-treated mice. Treatment with antioxidant α-tocopherol significantly prevented memory impairment induced by Aβ (1–42). Increased activities of the cytosolic Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and mitochondrial Mn-superoxide dismutase (Mn-SOD) were observed in the hippocampus and cerebral cortex of Aβ (1–42)-treated animals, as compared with Aβ (40–1)-treated mice. The induction of Cu,Zn-SOD was more pronounced than that of Mn-SOD after Aβ (1–42) insult. However, the concomitant induction of glutathione peroxidase (GPX) in response to significant increases in SOD activity was not seen in animals treated with Aβ (1–42). Furthermore, glutathione reductase (GRX) activity was only increased at 2 h after Aβ (1–42) injection. Production of malondialdehyde (lipid peroxidation) and protein carbonyl (protein oxidation) remained elevated at 10 days post-Aβ (1–42), but the antioxidant α-tocopherol significantly prevented these oxidative stresses. Therefore, our results suggest that the oxidative stress contributes to the Aβ (1–42)-induced learning and memory deficits in mice.
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1. Introduction

Alzheimer's disease (AD) is the most common cause of progressive cognitive impairment in the elderly [16,29,48]. The characteristic neuropathology of AD is the accumulation of senile plaques and neurofibrillary tangles in vulnerable brain regions. The senile plaques are primarily composed of amyloid beta peptide (Aβ), which is a 40–42 amino acid peptide fragment of the amyloid protein precursor that plays an important role in the development of AD. However, the mechanism by which Aβ causes neuronal injury and cognitive impairment is not yet clearly understood.

Nabeshima and his colleagues demonstrated that a continuous infusion of Aβ (1–40) into the cerebral ventricle in rats results in learning and memory deficits that were accompanied by a reduction of choline acetyltransferase activity, suggesting that accumulation of Aβ is related to cognitive

* Corresponding author. Tel: +82 33 250 6917; fax: +82 33 255 7865.
E-mail addresses: kimhc@kangwon.ac.kr (H.-C. Kim), jiwoomd@plaza.snu.ac.kr (J.I. Woo).
[1] Tel.: +82-2-760-2456; fax: +82-2-762-3176

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impairments in AD [39,40]. Memory impairment induced by Aβ (1–42) is potentiated by the long-term deprivation of estrogens in female rats [50]. In rats treated with Aβ (1–40), dysfunction of cholinergic and dopaminergic neuronal systems are observed, as evidenced by the decrease in the nicotine- and KCl-induced stimulation of acetylcholine and dopamine release in vivo [18]. Furthermore, long-term potentiation is impaired in the CA1 field of the hippocampal slices prepared from the rat brain after continuous i.c.v. infusion of Aβ (1–40) [19].

In addition, they demonstrated that the continuous infusion of Aβ (1–40) into the cerebral ventricle induced a time-dependent expression of inducible nitric oxide synthase and an overproduction of nitric oxide in the hippocampus although Aβ (40–1) had no effect [46]. The Aβ-induced overproduction of nitric oxide which reacts rapidly with superoxide radical to yield highly reactive peroxynitrite caused an increase in tyrosine nitration of a synaptic protein synaptophysin in the hippocampus [47]. We have also demonstrated that the prolonged infusion of Aβ (1–42) results in a significant reduction of the immunoreactivity of antioxidant enzymes in the rat brain areas, although the same treatment with Aβ (40–1) had little effect [25].

Evidence suggests that oxidative stresses are involved in the mechanism of Aβ-induced neurotoxicity [4–6], and AD pathogenesis [34,53]. For example, exposure to Aβ increases lipid peroxidation, protein oxidation, and the formation of hydrogen peroxide in cultured cells [2]. Similarly, increases in lipid peroxidation, protein carbonyl and oxidation of mitochondrial DNA have been observed in the brains of AD patients [31]. Yamada et al. [51] demonstrated that treatment with antioxidants, such as idebenone and α-tocopherol prevents the learning and memory deficits induced by Aβ (1–42). However, they did not find increased lipid peroxidation in the brains of the Aβ (1–42)-infused rats [51].

To examine the hypothesis that oxidative stress is involved in the learning and memory deficits evoked by Aβ, we used different experimental conditions to those of Yamada et al. [51]. We investigated the time course (2 h, 2, 4, 10, and 20 days after Aβ injection), and changed the administration method [a single i.c.v. injection of Aβ (1–42)], and used a different animal model (mice) in the present study. It has not yet been demonstrated whether Aβ (1–42)-induced changes in the activities of endogenous antioxidant enzymes contribute to oxidative stress and memory function changes in the animal models. Thus, in this study, we examined the effects of the potent antioxidant α-tocopherol on memory function, antioxidant enzyme activity, lipid peroxidation, and protein oxidation, after the administration of Aβ (1–42) or Aβ (40–1) in mice. In young mice, Aβ is initially deposited in the cingulate cortex carrying the mutant amyloid precursor protein (APP), but it rapidly encroaches on the entire cerebral cortex and hippocampus [35]. In this study, we focused on these brain regions using old mice.

2. Materials and methods

2.1. Animals and amyloid β-peptide (Aβ) administration

All animals were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals. Fifteen months old male C57BL/6 mice (Bio Genomics, Inc., Charles River Technology, Gapyung-Gun, Gyeonggi-Do, Korea) weighing about 45 ± 2 g were maintained on a 12:12 h light:dark cycle and fed ad libitum. They were adapted for 2 weeks to the above conditions before experiment.

Aβ (1–42; US Peptide, CA, USA) and Aβ (40–1; Bachem, Torrance, CA, USA) were dissolved in 35% acetonitrile containing 0.1% trifluoroacetic acid. The Aβ (1–42) or Aβ (40–1) administration [400 pmol, intracerebroventricular injection (i.c.v.)] was performed according to the procedure established by Laursen and Belknap [26]. The dose of Aβ is comparable to that of Yan et al. [52]. Briefly, each mouse was injected (without anesthesia) at bregma with a 50 μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. The injection volume was 5 μl. The injection placement or needle track was visible and was verified at the time of dissection.

2.2. Experimental design

α-Tocopherol (150 mg/kg, p.o.) was dissolved in soybean oil, and administered orally in a volume of 1 ml/kg, to Aβ (1–42)-treated mice for 27 consecutive days. The experimental schedule is shown in Fig. 1. α-Tocopherol administration began 7 days before Aβ (1–42) i.c.v. injection, and continued throughout the experimental period. The behavioural study began on day 3 after Aβ (1–42) i.c.v. injection, and carried out sequentially. In the behavioural study, α-tocopherol administration was carried after behavioural test to avoid a direct effect on performance. Mice were sacrificed at 2 h, 2, 4, 10 and 20 days after Aβ (1–42) i.c.v. injection to examine enzyme activities (Cu,Zn-superoxide dismutase, Mn-superoxide dismutase, glutathione peroxidase, and glutathione reductase) and oxidative stresses (malondialdehyde and protein carbonyl) in the brain.

2.3. Water maze test

The apparatus was a circular water, 97 cm in diameter and 60 cm height. During testing, the tank was filled with water (23 ± 2 °C) that was clouded with powered milk. A transparent platform was set inside the tank and its top was submerged 2 cm below the water surface in the center of one among the four quadrants of the maze. The tank was located in a large room with many extramaze cues that were constant throughout the study [37,39]. The movements of the animal in the tank were monitored with a video tracking system (EthoVision, Noldus, The Netherlands).