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40

# SIGNALLING OF GALANIN AND AMYLOID PRECURSOR PROTEIN THROUGH ADENYLATE CYCLASE

**RIINA MAHLAPUU**

TARTU UNIVERSITY
PRESS

Department of Chemistry, University of Tartu, Estonia
Department of Biochemistry, University of Tartu, Estonia

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Opponents: Research Professor Asko Uri (University of Tartu)
Associate Professor Tiiu Vihalemm (University of Tartu)

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# LIST OF ORIGINAL PUBLICATIONS

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II. **Mahlapuu R.**, Viht K., Balaspiri L., Bogdanovic N., Saar K., Soomets U., Land T., Zilmer M., Karelson E. and Langel Ü (2003). Amyloid precursor protein carboxy-terminal fragments modulate G-proteins and adenylate cyclase activity in Alzheimer's disease brain. Mol. Brain Res. 117, 73–82.

III. Saar K., **Mahlapuu R.**, Laidmäe E., Valkna A., Kahl U., Karelson E. and Langel Ü (2001). Characterisation of a new chimeric ligand for galanin receptors: galanin(1–13)-[D-Trp$^{32}$]-neuropeptideY(25–36)amide. Regulatory Peptides, 102(1), 15–19.

IV. Saar K., Mazarati A., **Mahlapuu R.**, Halnemo G., Soomets U., Kilk K., Hellberg S., Pooga M., Tolf B.-R., Shi T.S., Hökfelt T., Wasterlain C., Bartfai T. and Langel Ü (2002). Anticonvulsant activity of a nonpeptide galanin receptor agonist. Proc. Natl. Acad. Sci. USA, 99(10), 7136–7141.

Other publications

V. Karelson E., **Mahlapuu R.**, Zilmer M., Soomets U., Bogdanovic N. and Langel Ü. Possible signalling by glutathione and its novel analogue through potent stimulation of frontocortical G-proteins in normal aging and in Alzheimer's disease. Ann. N.-Y. Ac. Sci. (2002).

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Aβ | amyloid β |
| AC | adenylate cyclase |
| AD | Alzheimer's disease |
| AMC | 7-amino-4-methylcoumarin |
| APP | amyloid precursor protein |
| ATP | adenosine triphosphate |
| *t*-Boc | *tert*-butyloxycarbonyl |
| cAMP | cyclic adenosine monophosphate |
| Cha | cyclohexylalanine |
| CHO cells | Chinese hamster ovary cells |
| CNS | central nervous system |
| CT | carboxy-terminus |
| CTF | C-terminal fragment |
| DAG | diacylglycerol |
| DCC | dicyclohexylcarbodiimide |
| DCM | dichloromethane |
| DIEA | diisopropylethylamine |
| DMF | *N,N*-dimethylformamide |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| GAL | galanin |
| Galnon | Fmoc-β-Cha-Lys-AMC |
| GALP | galanin-like peptide |
| GALR | galanin receptor |
| GDP | guanosine diphosphate |
| G-protein | GTP hydrolase |
| GPCR | G-protein-coupled receptor |
| GSH | glutathione (reduced) |
| GTP | guanosine 5′-triphosphate |
| GTPγS | guanosine-5′-O-(3-thio)triphosphate |
| hGAL | human galanin |
| HOBT | 1-hydroxybenzotriazole |
| HPLC | high performance liquid chromatography |
| $IP_3$ | inositol triphosphate |
| NAC | N-acetyl-L-cysteine |
| NFT | neurofibrillary tangles |
| NPY | neuropeptide Y |
| $PIP_2$ | phosphatidylinositol biphosphate |
| PLC | phospholipase C |
| PS | presenilin |
| PTZ | pentylenetetrazole |

| | |
|---|---|
| PTX | pertussis toxin |
| RGS | regulators of G-protein signalling |
| ROS | reactive oxygen species |
| Sf9 cells | *Spodoptera frugiperda* cells |
| SPPS | solid phase peptide synthesis |
| TBTU | 2-(1H-bensotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate |
| TFA | trifluoroacetic acid |
| TM | transmembrane domain |

# INTRODUCTION TO THE THESIS

For many transmembrane signalling events adenylyl cyclases (ACs) are the final effector enzymes, which integrate and interpret divergent signals from different pathways. The enzymatic activity of ACs is stimulated or inhibited in response to the activation of a large number of receptors. ACs synthesize one of the major second messengers, cyclic AMP (cAMP) upon extracellular stimulation. The majority of the ACs can be modulated by the G-protein-coupled receptors of neurotransmitters and neuromodulators. AC is a crucial molecule in mediating the physiological responses of these broadly expressed neurotransmission and neuromodulation systems. The importance of ACs in signal transduction of the central nervous system (CNS) is highlighted because of the number neurotransmitters and neuromodulators in CNS are G-protein-coupled receptors.

Amyloid precursor protein (APP) is a membrane-spanning protein with a large extracellular domain and short intracellular domain. APP is the source of the amyloid-β (Aβ) peptide found in neuritic plaques of Alzheimer's disease (AD) patients and C-terminal (CT) peptides. AD, a neurodegenerative disorder is the most common form of amyloidosis and dementia in humans. APP fragments play a critical role in the cognitive dysfunction associated with AD. Alteration of G-protein associated signalling pathways in the AD post-mortem brains has been shown. Studies have demonstrated that the Aβ peptide and CT peptides of APP might be involved in the amyloidogenesis and neurodegeneration through free-radical generated profound oxidative stress.

Galanin (GAL), a 29 (30 in human) amino acid peptide is widely distributed in the peripheral and central nervous systems. GAL modulates a variety of biological actions, including cognition, and has been suggested to be aberrantly regulated in Alzheimer´s disease. In contrast to other neurotransmitters/ neuromodulators, which display a severe reduction in ligand and receptor quantity in end stage of AD, GAL and galanin receptors (GALRs) are overexpressed in this disease state, particularly in structures of the limbic system. The over-expression of GALRs in AD suggests that galaninergic systems may play a key role in limbic related behavioural dysfunction at early stages of disease. The cAMP signalling system is one of the most important mechanisms by which galanin receptor agonists or antagonists exert their diverse physiological or pharmacological effects.

This thesis focuses on adenylate cyclase directed signalling of the peptides derived from APP and of neuropeptide galanin.

# 1. NEUROPEPTIDES AND NEURODEGENERATIVE DISEASES

## 1.1. Introduction

### 1.1.1. G-protein-coupled receptors and signal transduction

The signal transduction in mammalian cells is carried out by using a variety of receptors and intracellular signals, where number is still increasing. Many of these receptors and pathways can be divided into superfamilies based on high levels of identity at the protein level and similarities in the mechanism by which the signal is transmitted into the cells. Receptors are classified into four such superfamilies: the intracellular receptor superfamily, which binds their ligands in the cytosol; and three classes of cell-surface receptor proteins, namely, G-protein-linked, ion channel-linked and enzyme-linked receptors. The most common one is the G-protein-coupled receptor (GPCR) family (Nishizuka, 1992, Helleday, 1998,).

GPCRs are involved in the recognition and transduction of messages as diverse as light, $Ca^{2+}$, odorants, small molecules including amino acid residues, nucleotides and peptides, as well as proteins. They control the activity of enzymes, ion-channels and transport of vesicles by the catalysing the GDP-GTP exchange on heterotrimeric G proteins ($G_{\alpha\beta\gamma}$) (Bockaert and Pin, 1999). The GPCRs are characterized by the same basic molecular architecture with seven hydrophobic regions of 25–35 consecutive residues connected by three inter-mediating extracellular, and three intracellular loops (Figure 1). The defining concept is that GPCRs share a common signalling mechanism, interacting with ubiquitous guanine nucleotide binding regulatory proteins (G-proteins) to regulate the synthesis of intracellular second messengers. Remarkable diversity of the primary protein sequences of GPCRs reflects their variety in physiological functions. The variety and importance of the physiological roles executed by the GPCR family has resulted in many of their members becoming important targets for drug development. A large number of modern drugs act via GPCRs (Fredriksson *et al.*, 2002).

The main role of 7-transmembrane domain (7TM) receptors is to bind ligands such as neurotransmitters and hormones and to transduce their signal intracellularly. There is a large diversity within the each family and frequently several 7TM receptors recognize the same endogenous ligand. This complexity has made the assignment of clear physiological role to each 7TM receptor difficult, especially as highly selective agonists and antagonists for most 7TM receptors are unavailable (Kilpatrick *et al.*, 1999).

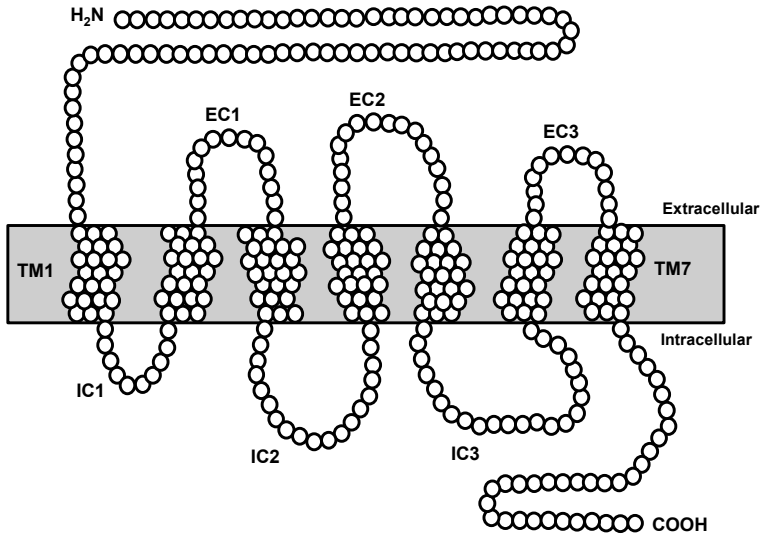



**Figure 1.** Schematic drawing of a G-protein-coupled 7-transmembrane domain receptor.

When a ligand binds to the 7TM GPCR, the guanosine diphosphate (GDP) bound to the α-subunit of the trimeric G-protein is replaced by a guanosine triphosphate (GTP) and this subunit becomes active. The α-subunit is then released and, still being anchored to the plasma membrane, migrates to adenylate cyclase (AC), which is activated, in turn, and catalyses the formation of cyclic AMP (cAMP) from ATP (Figure 2). After that the GTP on the α-subunit is hydrolysed to GDP and the AC is inactivated. cAMP activates the cAMP-dependent protein kinase (kinase A) by binding to its regulatory subunit. Protein kinase A then phosphorylates specific serine or threonine residues in selected proteins, depending on the cell type (Linder and Gilman, 1992). GPCRs also trigger another intracellular signal pathway, the inositol phospholipid pathway. An active GPCR stimulates a trimeric G-protein, which in turn activates phospholipase C-β (PLC-β). PLC-β cleaves phosphatidyl-inositol biphosphate ($PIP_2$), thereby generating inositol triphosphate ($IP_3$) and diacylglycerol (DAG) (Helleday, 1998). Both these compounds are important second messengers and stand at key points in signal transduction pathways.

Age-related changes of receptor-mediated signal transduction occur at many levels, and are known to include quantitative and qualitative changes in growth factor receptors, G-protein coupled receptors, and many other downstream signaling molecules. As major means of cellular signal transduction, the receptor tyrosine kinase system and the G-protein-coupled receptor system of senescent cells were investigated (Marshall, 2001, Yeo and Park, 2002).

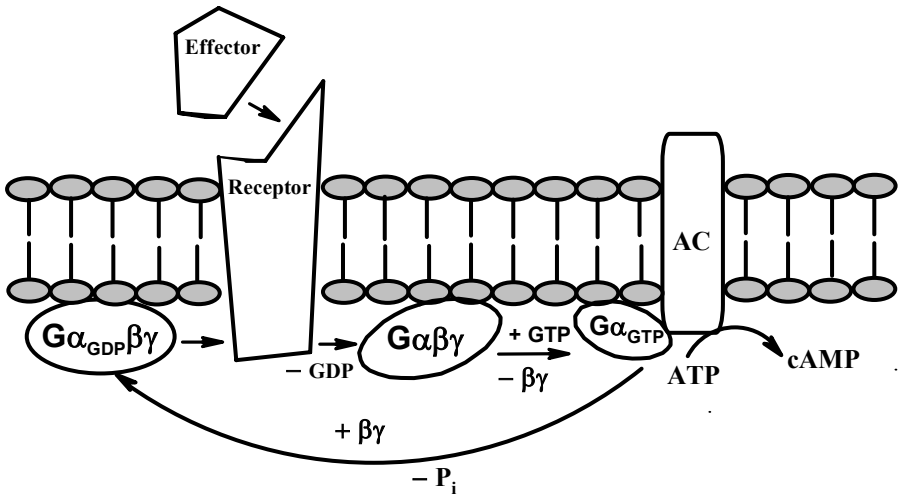



**Figure 2.** Schematic illustration of cAMP mediated signalling pathway. The GPCR are situated in the plasma membrane and, upon binding their ligands, cause replacement of GDP on the coupled G-protein by GTP. This G-protein then migrates to AC, which catalyses the transformation of ATP to cAMP.

### 1.1.2. G-protein regulation of adenylate cyclase

G-proteins are divided into two classes: heterotrimeric and monomeric (minor class). A family of heterotrimeric GTP-binding and hydrolyzing proteins plays an essential transducing role in linking many cell-surface receptors to effector proteins at the plasma membrane. G-proteins are composed of three distinct subunits: α, β and γ. The β- and γ-subunits exist as a tightly associated complexes that function as one unit. The α-subunits have a single, high-affinity binding site for guanine nucleotides (GDP or GTP). The GDP bound form of α-subunit binds tightly to βγ and is inactive, whereas the GTP-bound form of α-subunit dissociates from βγ-subunit complex and serves as a regulator of effector proteins. All α-subunits themselves carry enzymatic function. That is, these proteins possess intrinsic GTPase activity and will, at varying rates, hydrolyse the terminal phosphate of bound GTP to yield bound GDP and free inorganic phosphate ($P_i$) (Hepler and Gilman, 1992). Heterotrimeric G-protein-derived $G_{\beta\gamma}$ subunits have very diverse and complex roles in signal transduction, arising, in part, from the diversity of effectors that are regulated by these subunits. These effectors include ion channels and plethora of enzymes central to signal transduction pathways. Signal transduction enzymes, which are modulated by $G_{\beta\gamma}$ include phospholipase $A_2$, phospholipase C, mitogen-activated protein kinase, and several isoforms of adenylyl cyclase. Thus, altered

signalling via these G-protein subunits could have diverse and widespread physiological consequences. There are several parameters that can influence $G_{\beta\gamma}$ signalling. ACII, IV and VII are conditionally activated by $G_{\beta\gamma}$ derived from $G_i$ proteins, whereas ACI is inhibited (Chakrabarti *et al.*, 2001).

To date, 20 mammalian α-, 6 β-and 12 γ- subunits of G-proteins have been cloned (Hamm, 1998). The α-subunits are divided into 4 families: $\alpha_s$, $\alpha_i$, $\alpha_q$ and $\alpha_{11}$. $G_\alpha$ and βγ can activate several effector molecules. Most frequent combinations are AC activation by $\alpha_s$ and by βγ, AC inhibition by $\alpha_i$, PLC activation by $\alpha_q$ and by βγ, cGMP-specific-phosphodiesterase activation by $\alpha_q$.

Adenylate cyclase integrates positive and negative signals that act through GPCRs with other extracellular stimuli to finely regulate levels of cAMP within the cell (Simonds, 1999).

Adenylyl cyclases are a family of enzymes that upon stimulation synthesize one of the major second messengers, cyclic AMP (cAMP). Since the report of the first AC gene in 1989, tremendous efforts have been devoted to identify and characterize more AC isozymes. In the past decade, significant knowledge regarding the basic structure, tissue distribution, and regulation of AC isozymes has been accumulated. Because members of the AC superfamily are tightly controlled by various signals, one of the most important impacts of these AC isozymes is their contribution to the complexity of cellular signalling, especially in the central nervous system (CNS) where multiple signals are constantly received.

Ten mammalian ACs have been isolated and characterized. Each isoform has its own distinct tissue distribution and regulatory properties, providing possibilities for different cells to respond diversely to similar stimuli. The product of the enzymatic reaction catalyzed by ACs, cAMP, has been shown to play a crucial role for a variety of fundamental physiological cell functions ranging from cell growth and differentiation to transcriptional regulation and apoptosis. Almost every cell expresses several AC isoforms. It has been difficult to perform biochemical characterization of the different AC isoforms and nearly impossible to assess the physiological roles of the individual isoforms for intact cells, tissues or organisms (Patel *et al.*, 2001). All the AC isoforms are expressed in neural tissue, while types I and VIII are expressed exclusively in brain (Xia *et al.*, 1993, Sunahara *et al.*, 1996).

Except for the newly identified testis-specific AC (Buck *et al.*, 1999), all other AC isozymes contain 12 stretches of hydrophobic residues in conserved positions which are arranged in two sets of six, separated by a large hydrophilic domain (Figure 3). Each of these hydrophobic stretches is presumed to be a transmembrane region. The proposed structure includes a short variable amino terminus, followed by six transmembrane spans (M1), a large cytoplasmic domain (C1), a second set of six transmembrane regions (M2), and another large cytoplasmic domain (C2). The overall similarity among the different ACs

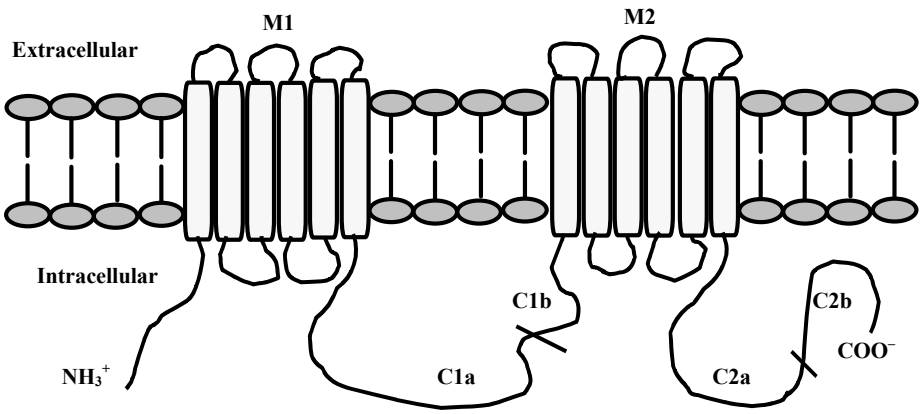



**Figure 3.** Schematic structure of membrane-bound adenylyl cyclases.

is roughly 60%: the most conserved sequences are located in the cytoplasmic domains (C1 and C2) and range from 50–90% (Patel *et al.*, 2001). Considerable homology between the ACs and guanylyl cyclases has been shown (Sunahara *et al.*, 1998, Beuve, 1999). The basic catalytic unit of a membrane-bound AC molecule consists of the C1a and C2 domains (Scholich *et al.*, 1997). The N-terminal domains of ACs are highly variable and may have a regulatory function.

The importance of ACs in signal transduction of the CNS is highlighted by three specific properties of the enzyme. Firstly, a number of indispensable neurotransmitters and neuromodulators in the CNS are ligands of GPCRs, including dopamine, serotonin and adrenaline. The fact that the majority of the AC family can be modulated by the G-protein coupled neurotransmitter and neuromodulator receptors indicates that AC is a central molecule in mediating the physiological responses of these broadly expressed neurotransmission and neuromodulation systems. The second essential property of ACs is the diversity of the AC superfamily, which allows its members to function in different signal transduction pathways of neurotransmitters, neuromodulators, and even neurotrophic factors. Date of numerous biochemical experiments suggests that different receptors are coupled to different AC isozymes, which endowe with various biochemical properties. The interaction between neurotransmitter receptors and different AC isozymes provides an important basis for AC-mediated multiple neuronal signal transductions. Thirdly, the regulation of AC activity by GPCRs represents an early stage in which extracellular signals can be transduced and integrated for neuronal information processing (Chern, 2000).

In the CNS, the transmembrane signalling system of AC modulates many cellular processes in response to extracellular signals of hormones and neurotransmitters. The pathway of regulation of AC activity involves the stimulatory

($G_s$) and inhibitory ($G_i$) G-proteins as central molecules transducing signals from activated receptors (Taussig *et al.*, 1994, Dessauer *et al.*, 1996, Harry *et al.*, 1997, Chern, 2000). While coupling of the $G_s$-protein to neurotransmitter receptor activates the AC, $G_i$ proteins mediate inhibition of this enzyme. It has also been shown that $G_i$ signalling can potentiate $G_s$ output under certain conditions (Olianas and Onali, 1999). This is mainly due to the fact that AC activity is not regulated solely by the subunits of $G_s$/$G_i$ proteins, but may be modulated by free subunits released from $G_i$/$G_o$ or $G_q$/$G_{11}$ (Clapham and Neer, 1993, Milligan *et al.*, 1998). In addition, some AC isozymes can be regulated by $Ca^{2+}$/calmodulin (Cooper *et al.*, 1995). As ACs are susceptible to more than one regulatory influence, they may serve to discriminate between convergent signals delivered by simultaneous activation of different inputs.

Recently, a new group of modulatory proteins, known as regulators of G-protein signalling, RGS, was identified. Since RGS act as potent GTPase-activating proteins, they might be engaged in switching-off the activation of any G-protein-mediated effector, adenylyl cyclase included. Hence, RGS might be considered among likely candidates to explain a specific pattern of G-protein mediated AC activity in the developing rat brain and myocardium (Ihnatovych *et al.*, 2002).

Disruptions in the AC complex are well recognized in Alzheimer's disease (AD) (Cowburn *et al.*, 1996b). It has been reported that $G_s$ protein-mediated activation of AC is decreased in the neocortex and cerebellum in AD subjects (Cowburn *et al.*, 1992). Reduced basal and stimulated AC activities have also been observed in the AD hippocampus and cerebellum (Schnecko *et al.*, 1994).

## 1.2. Galanin

Galanin (GAL) is a 29-amino acid (30 in human) neuropeptide that was originally isolated from the porcine small intestine in 1983 by Tatemoto and Mutt (Tatemoto *et al.*, 1983). This peptide is cleaved from preprogalanin, a 123-amino acid precursor molecule, to form a biologically active molecule. GAL is a widely distributed neuropeptide with a variety of physiological functions.

At present, galanin sequences from 14 species are known (Table 1). The N-terminal 14 amino acid residues of GAL are homologous throughout the species, with residue differences occurring in the C-terminal portion of the sequence. The primary sequence of human GAL (hGAL) peptide differs from the known sequences of other species by having an additional serine residue and a non-amidated carboxyl terminus (Deecher *et al.*, 1998).

**Table 1.** Amino acid sequences of galanins from different species. Bold lettering denotes amino acid differences between the hGAL sequences.

| Native peptides | Amino acid sequence |
|---|---|
| Human | GWTLN SAGYL LGPHA VGNHR SFSDK NGLTS |
| Pig | GWTLN SAGYL LGPHA **ID**NHR SF**H**DK **Y**GL**A** amide |
| Bovine | GWTLN SAGYL LGPHA **LDS**HR SF**Q**DK **H**GL**A** amide |
| Rat | GWTLN SAGYL LGPHA **ID**NHR SFSDK **H**GLT amide |
| Mouse | GWTLN SAGYL LGPHA **ID**NHR SFSDK **H**GLT amide |
| Dog | GWTLN SAGYL LGPHA **ID**NHR SF**HE**K **P**GLT amide |
| Sheep | GWTLN SAGYL LGPHA **ID**NHR SF**H**DK **H**GL**A** amide |
| Frog | GWTLN SAGYL LGPHA **ID**NHR SF**N**DK **H**GL**A** amide |
| Alligator | GWTLN SAGYL LGPHA **ID**NHR SF**NE**K **HGIA** amide |
| Quail | GWTLN SAGYL LGPHA V**D**NHR SF**N**DK **HGF**T amide |
| Chicken | GWTLN SAGYL LGPHA V**D**NHR SF**N**DK **HGF**T amide |
| Bowfin | GWTLN SAGYL LGPHA V**D**NHR S**LN**DK **H**GL**A** amide |
| Trout | GWTLN SAGYL LGPH**G ID**GHR **TL**SDK **H**GL**T** amide |
| Tuna | GWTLN **A**AGYL LGPH**G ID**GHR **TLG**DK **P**GL**A** amide |

GAL in solution may adopt a horseshoe-like shape, with two α-helices separated by a β-bend around amino acids $Gly^{12}$ and $Pro^{13}$ (Figure 4) (Rigler *et al.*, 1991).

At present, there are two known members in the galanin family of neuropeptides: GAL itself and galanin-like peptide (GALP), which was isolated from porcine hypothalamus by Ohtaki and coworkers. The peptide has 60 amino acid residues and a non-amidated C terminus. The amino acid sequence of GALP(9–21) is identical to that of GAL(1–13). A cloned porcine GALP cDNA indicated that GALP is processed from a 120-amino acid GALP precursor protein. The amino acid sequences 1–24 and 41–53 are highly conserved between human, rat, and pig. Receptor binding studies revealed that porcine GALP(1–60) had a high affinity for the GALR2 receptor ($IC_{50}$ = 0.24 nM) and a lower affinity for the GALR1 receptor ($IC_{50}$ = 4.3 nM). In contrast, GAL showed high affinity for the GALR1 ($IC_{50}$ = 0.097 nM) and GALR2 receptors ($IC_{50}$ = 0.48 nM). GALP is therefore an endogenous ligand that preferentially binds the GALR2 receptor, whereas GAL is less-selective (Ohtaki *et al.*, 1999).

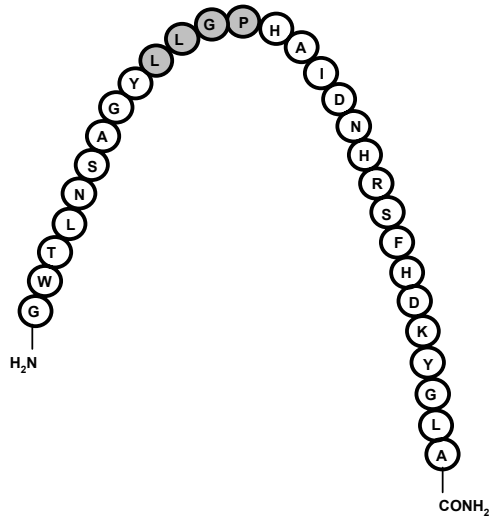



**Figure 4.** Galanin structure in solution (Rigler *et al.*, 1991). The residues participating in the bend are shown in grey.

## 1.2.1. Galanin receptors

Galanin receptors belong to the superfamily of G-protein-coupled 7TM receptors (Figure 1). High affinity galanin binding to its receptors is sensitive to GTP and to pertussis toxin-catalysed ADP-ribosylation, indicating that galanin receptors couple to effectors via the $G_i/G_o$ subfamily of G-proteins (Amiranoff *et al.*, 1989, Fisone *et al.*, 1989a, Fisone *et al.*, 1989b, Land *et al.*, 1991). Three galanin receptor (GALR) subtypes have been cloned to date.

The first known galanin receptor GALR1 has been isolated from the human Bowes melanoma cell line and other sources. Human GALR1 contains 349 amino acids with the structure of a GPCR. The highest amino acid similarities are found with human GALR2 (42%) and human GALR3 (38%) receptors. The GALR1 is reported to be coupled to an inhibitory guanine nucleotide ($G_i$) binding regulatory protein (Habert-Ortoli *et al.*, 1994, Lorimer *et al.*, 1997).

The second galanin receptor subtype GALR2 was isolated originally from the rat brain. GALR2 contains 372 amino acids, including three consensus sites for extracellular N-linked glycosylation and several intracellular phosphorylation sites distinct from GALR1. Rat GALR2 shares highest amino acid similarity with rat GALR3 (55%) and human GALR3 (58%), and less similarity with rat GALR1 (40%) and human GALR1 (40%) (Habert-Ortoli *et al.*, 1994). The cloned human GALR2 contains 387 amino acids, 15 more than rat GALR2 in the C-terminal, with only 85% similarity to this receptor. The GALR2 is mainly coupled to $G_{q/11,}$ which stimulates phospholipase C and increases intracellular calcium levels, but may be also coupled to $G_{i/o}$ (Smith *et al.*, 1997a).

A third cloned galanin receptor subtype GALR3 was first cloned from rat and described in two separate reports; the sequences described in these papers diverge in four positions for reasons that are at present unclear. Rat GALR3 contains 370 amino acids and has 36% of similarity to rat GALR1 and 55% of similarity to rat GALR2 of (Branchek *et al.*, 2000). Subtype 3 is similar to subtype 1 with respect to G-protein coupling (Deecher and Lopez, 2002).

GAL actions are mediated via high affinity $G_i/G_o$-protein-coupled receptors and involve the effector systems such as $K^+$-, $Ca^{2+}$-channels and adenylate cyclase. GALR agonists are thought to have therapeutic applications in treatment of chronic pain and prevention of ischemic damage; GALR antagonists have therapeutic perspective in the treatment of Alzheimer's disease, depression, and eating disorders (Bartfai *et al.*, 1993).

### 1.2.2. Peptidic galanin receptor ligands

Several chimeric peptides have been designed in order to modulate the biological activity of the neuropeptide GAL. Design of chimeraes was based on the knowledge that only the N-terminal part of GAL was required for recognition by the GALRs and for agonist activity. The N-terminal part of GAL was covalently connected via a hinge region (the proline kink in its structure Figure 4), to the C-terminal active parts of the other neuropeptides.

M15 was the first synthesized chimera, where GAL(1–13) and substance P(5–11) were linked to each other (Bartfai *et al.*, 1991, Langel *et al.*, 1992). Later on, a series of chimeric molecules were synthesized (Table 2). The exchange of the C-terminal portion of GAL(14–29) with the fragments of other biologically active peptide motifs (bradykinin(2–9) and neuropeptideY(25–36), respectively M35 (Kask *et al.*, 1995) and M32) has yielded several chimeric peptides, which bind to GAL receptors with higher affinity ($K_D$ = 0.01–0.04 nM) than GAL(1–13) (150 nM) and whole GAL (~1 nM) (Langel *et al.*, 1992).

**Table 2.** Selected chimeric galanin receptor ligands (X is N-terminal sequence of galanin GWTLNSAGYLGP).

| Symbol | Chimeric peptides | Amino acid sequence |
|---|---|---|
| M15 | Galanin(1–13)-substance P(5–11) | X–QQFFGLM amide |
| C7 | Galanin(1–13)-spantide | X–[D-R]PKPQQ[D-W]F[D-W]LL amide |
| M40 | Galanin(1–13)-Pro-Pro-$(Ala\text{-}Leu)_2$-Ala-amide | X–PPALALA amide |
| M35 | Galanin(1–13)-bradykinin(2–9) | X–PPGFSPFR amide |
| M32 | Galanin(1–13)-NPY(25–36)amide | X–RHYINLITRQRY amide |

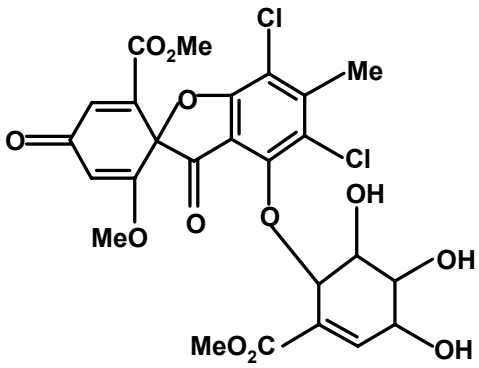
Chimeric GAL analogs distinguish between GALR subtypes providing the subtype-selective agonists. M15 peptide binds to GALR2 with high affinity ($K_d$=1 nM), GALR1 and GALR3 recognize M15 with lower affinity ($K_d$=10 and 85 nM, respectively) (Smith *et al.*, 1997a).

### 1.2.3. Non-peptidic galanin receptor ligands

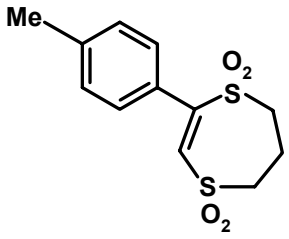
Two reports on non-peptidic ligands for galanin receptors were previously published. Because of the high level of hGALR1 in human brain, this receptor is an appropriate target for the discovery of CNS drugs for treatment of various disorders. Peptide hGALR1 ligands such as galantide (M15) (Bartfai *et al.*, 1991), M35 (Wiesenfeld-Hallin *et al.*, 1992), M40 (Langel *et al.*, 1992), and GAL peptides attached to non-peptidic units (Pooga *et al.*, 1998a) have been evaluated.

A novel fungal metabolite, Sch202596 was discovered from the fermentation of a fungal culture *Aspergillus* sp. By spectroscopy the compound was shown to be a new spirocoumaronone, related to the griseofulvin family of compounds (Figure 5). Chu *et al*. reported it to be a non-peptidic hGALR1 antagonist with $IC_{50}$ of 1.7 μM (Chu *et al.*, 1997).

The other non-peptidic ligand, 2,3-Dihydro-2-(4-methylphenyl)-1,4-dithiepine-1,1,4,4-tetroxide (Figure 5) was found in a corporate compound collection. It was the first non-peptidic hGALR1 antagonist with IC in sub-micromolar range (190 nM of $IC_{50}$) (Scott *et al.*, 2000).




Sch202596




2,3-Dihydro-2-(4-methylphenyl)-
1,4-dithiepine-1,1,4,4-tetroxide

**Figure 5.** Previously reported non-peptidic ligands for galanin receptors.

### 1.2.4. Bioeffects of galanin

Since GAL was first isolated from porcine small intestine, it was reported that this neuropeptide affects smooth muscle mobility and has strong hyperglycaemic effect (Tatemoto *et al.*, 1983). GAL exerts a number of biological effects in mammalians, some of these effects are listed in Table 3. GAL modulates feeding (Leibowitz and Kim, 1992) and sexual behaviour (Benelli *et al.*, 1994), insulin and growth hormone release (Ahrén and Lindskog, 1992), and is suggested to be involved in the pathogenesis of Alzheimer's disease (Crawley and Wenk, 1989, Kask *et al.*, 1997).

**Table 3.** Biological effects of galanin in the hippocampus (Bartfai *et al.*, 1993, Kask *et al.*, 1995, Chu *et al.*, 1997, Kask *et al.*, 1997, Mazarati *et al.*, 2000).

| Tissue/Region | Effect |
|---|---|
| Hippocampus | PTX-sensitive inhibition of ACh release<br>PTX-sensitive inhibition of mACh-R mediated PI turnover<br>Reduction of phorbol ester-stimulated protein phosphorylation<br>Inhibition of the slow cholinergic EPSP induced by the release of exogenous ACh<br>Inhibition of anoxic release of glutamate<br>Closure of N-type voltage-sensitive $Ca^{2+}$ channels<br>Decrease of $K_D$ for 5-$HT_{1A}$ receptor, reduction of 5-HT metabolism<br>Anti-seizure activity |

Recently, it has been reported that galanin is a key regulator of epileptic experimental models. Epilepsy is a neurological disorder of chronic condition of repetitive seizures. Misbalance between excitatory and inhibitory neurotransmission is regarded as a basic mechanism of epilepsy. However, classical excitatory and inhibitory transmitters are influenced by neuromodulators, including neuropeptides (Mazarati *et al.*, 2001). Neuropeptides are widely implicated in the mechanisms of epilepsy. Somatostatin, neuropeptide Y and endogenous opioid peptides have been a subject of special attention due to their abundance in the hippocampus, a key structure in limbic epilepsy, and their physiological effects. The importance of these peptides in various types of seizures is not clear. Several lines of research suggest a role for galanin in seizures. Studies have reported that galanin has anti-seizure activity. The seizure-induced depletion of galanin from the rat hippocampus is associated with the development of self-sustaining status epilepticus. The injection of galanin into the hippocampus attenuates seizure activity, whereas galanin antagonists facilitate it (Mazarati *et al.*, 1998a). Galanin-overexpressing mice have increased resistance to status epilepticus, while galanin knockout mice have lowered seizure threshold

(Mazarati *et al.*, 1998b). Galanin was recently shown to possess strong seizure-protecting activity in several animal models of epilepsy. Mazarati *et al* have demonstrated that hippocampal galanin acts as an endogenous anticonvulsant via galanin receptors (Mazarati *et al.*, 2000).

## 1.3. Alzheimer's disease (AD)

Alzheimer's disease was described for the first time by Alois Alzheimer (Alzheimer, 1907). AD is a progressive neurodegenerative disorder that affects one in four individuals aged over 85 (Evin and Weidemann, 2002). Clinically, AD is characterized by a gradual onset of memory loss followed by progressive cognitive and physical deterioration (Racchi and Govoni, 2003). Pathological changes in AD are characterized by the formation of amyloid plaques and neurofibrillary tangles leading to the extensive neuronal loss. Abnormal proteolytic processing of β-amyloid precursor protein (APP) is the important step in the progress of AD that contributes to formation of amyloid plaque, neurofibrillary tangles, leading to neuronal loss (Kourie and Shorthouse, 2000, Kourie, 2001).

A central issue in AD has been to find a link between the pathological hallmarks of AD and the degeneration of selected populations of neurons, leading to dementia. The amyloid cascade hypothesis is based on the assumption that amyloid plaque development in the brain is an early and necessary step in the neurodegenerative process that leads to dementia (Selkoe, 1991, 2001) (Figure 6).

The severe dementia and death characteristic of AD is caused by a loss of neurons in the cortex, hippocampus and basal forebrain. Neuropathological changes associated with AD include the appearance of senile plaques and neurofibillary tangles. A major component of plaques is a small aggregated peptide (Aβ) derived from APP. Mutations in three genes, APP gene on chromosome 21, the presenilin 1 (PS1) gene on chromosome 14, and the presenilin 2 (PS2) gene on chromosome 1 result in an autosomal dominant form of AD with a very early age of onset. The discovery that pathogenic mutations in these genes cause changes in the production of the Aβ peptide provides strong support for the hypothesis that APP metabolism leading to altered Aβ production or deposition is an early event in the etiology of AD (Tanzi and Bertram, 2001). It is widely accepted that Aβ lowering therapies may alter the progression of AD; therefore, the development of the specific Aβ lowering drug that can be used for treatment of AD patients is desperately needed to test the amyloid hypothesis (Roberts, 2002).

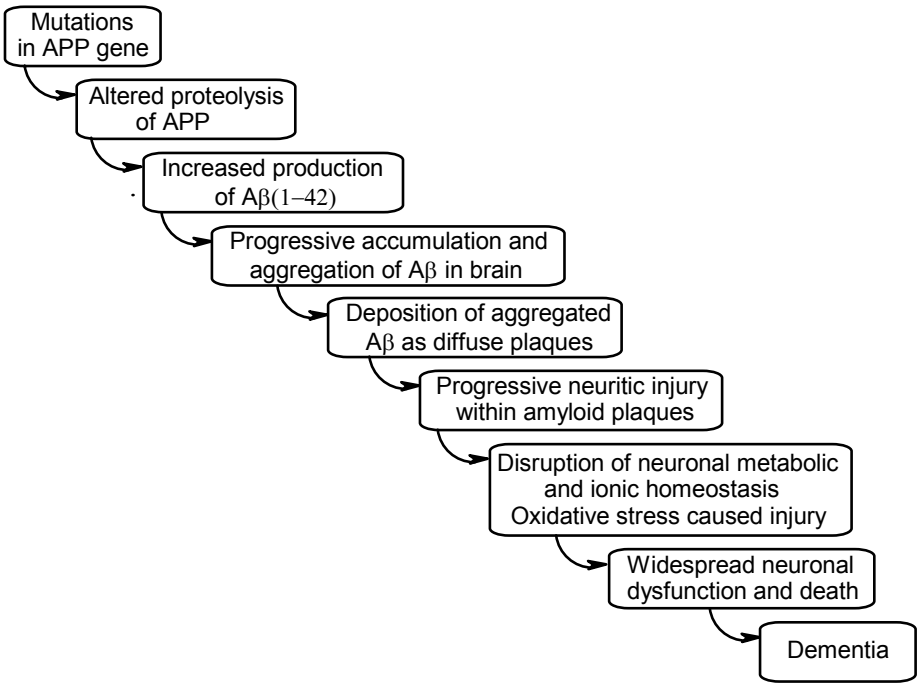



**Figure 6.** A hypotetical sequence of the pathogenetic steps of familial forms of Alzheimer's disease. Modified from Selkoe, 2001.

The progressive cognitive and behavioural symptoms, which characterize AD derive from profound functional and structural changes observed in neurons, neuronal processes and synapses, as well as astrocytosis, which accompanies these changes.

During the last 25 years the major goal on AD research has been to unravel the etiology and the shared biochemical mechanism of this syndrome to be able to identify or design small, brain-permeable molecules, which could completely inhibit at relatively early stage the molecular events that occur in most, if not all, AD patients (Selkoe, 2001).

The second goal is to find strategies and applications, which, if used early in the course of the disease, may prevent the development of further neuro-degeneration (Evin and Weidemann, 2002, Racchi and Govoni, 2003).

### 1.3.1. Pathological markers in AD

An abnormal accumulation of amyloid beta peptides (Aβ), or of the Aβ(1–42) peptide in particular, can be considered as the initial trigger of a disease process that further develops by formation of neurofibrillary tangles (NFT), leading to neuronal dysfunction, and finally the inexorable dementia and decline of the patient (Dominguez *et al.*, 2001).

The major histopathologic features of AD are senile plaques (SP), consisting primarily of Aβ peptide and NFT, which is composed of paired helical filaments containing hyperphosphorylated tau-protein. Post-mortem diagnosis reveals major degeneration of the brain cortex with amyloids in the form of large extracellular plaques, perivascular deposits, and intra-neuronal fibrillary tangles (Evin and Weidemann, 2002). Both of these filamentous proteins are essentially insoluble (Lovell *et al.*, 2002).

The types of amyloid plaques found in AD are classified as diffuse, neuritic and compact plaques. The diffuse plaques represent an early stage of plaque formation with no amyloid fibrillization, and the main component of plaques is the Aβ, a proteolytic cleavage product of membrane bound APP (Figure 7). The neuritic plaques contain fibrillar Aβ deposits, dystrophic neurites and activated glia. The compact plaques represent an end-stage in plaque formation. They lack dystrophic neurites and consist entirely of an amyloid core. The amyloid hypothesis for AD considers the Aβ peptide to be the initiator of a pathological cascade that leads to formation of amyloid plaques and neurofibrillary tangles to neuronal dysfunction, possibly to inflammatory responses, and finally to dementia of the patient (Annaert and De Strooper, 2002). The plaques, which accumulate extracellularly in the brain cause direct neurotoxic effects and/or increase neuronal vulnerability to excitotoxic insults (Kourie, 2001).

Neurofibrillary tangles consist of paired helical filaments as well as straight filaments. These filaments consist of tau protein, a microtubule-associated protein in the neuronal axons. There are six different isoforms of tau in the human brain, each of them containing numerous phosphorylation sites. Hyperphosphorylation of tau, which is typical in AD, leads to aggregation of tau-protein with subsequent formation of NFT (Goedert, 1993). The level of phospho-tau in cerebrospinal fluid may be used as a biochemical marker for AD (Blennov, et al., 2003).

Sandberg *et al*. found that SP and NFT are strongly associated with age. These lesions begin to appear in the early to late 40s, depending on the anatomic location, and become common in the 6th decade, preceding by one to two decades the age at which AD becomes clinically prevalent (Sandberg *et al.*, 2001).

Elucidating the molecular pathway involved in the generation of Aβ, particularly Aβ(1–42), is a key issue for rational therapeutic approaches to lower Aβ concentrations in AD (Vassar, 2002). Inhibition of Aβ aggregation is

the most important field on the design of therapeutic agents for AD. The first inhibitor of the Aβ(1–42) aggregation has been designed (Parker *et al.*, 2002).

### 1.3.2. β-amyloid precursor protein (APP)

APP is a integral membrane protein comprising a large extracellular domain, a membrane anchoring domain and a short intracellular C-terminal tail (Figure 7). APP has three isoforms: $APP_{770}$, $APP_{751}$ and $APP_{695}$. The brain seems to produce predominantly the 695 amino acid isoform and this isoform has received the most attention in research on AD (Turner *et al.*, 2003). The APP protein undergoes several post-translational modifications including N-glycosylation, O-glycosylation, and Tyr sulfation to give the mature form of APP (Nunan and Small, 2000, Selkoe, 2001). Following these steps, the routes of APP metabolism become more complex and result in different pathways leading to proteolytic processing of the precursor by at least three proteolytic enzymes (Nunan and Small, 2000). Near the cell surface or in a secretory vesicle a protease, α-secretase, cleaves APP in the extracellular domain and releases the ectodomain ($APP_s$-α or soluble APPα) into the extracellular space. This proteolytic cleavage occurs within the Aβ sequence, therefore preventing the formation of amyloidogenic fragments and leading to the non-amyloidogenic pathway. The Aβ peptide is formed following the cleavage by β- and γ-secretases that cleave at the N and C terminus of Aβ, respectively (Racchi and Govoni, 2003).

A major route of APP processing is via the α-secretase pathway, which cleaves on the C-terminal side of residue 16 of the Aβ sequence, generating an 83-residue C-terminal fragment (C83) (Esch *et al.*, 1990) (Figure 7). Cleavage of APP by α-secretase destroys the Aβ sequence, and this pathway mitigates amyloid formation. The C-terminally truncated form of APP released by α-secretase may have trophic actions (Small, 1998), which could antagonise the neurotoxic effects of aggregated Aβ. As it is likely that several proteases contribute to α-secretase activity, it may be difficult to regulate APP processing pharmacologically through this pathway. Therefore, most studies, which were aimed at developing of the inhibitors of Aβ production have focussed on the two other enzymes, which are directly responsible for cleavage of Aβ from APP, β- and γ-secretase (Nunan and Small, 2000).

The β-secretase cleavage generates the N-terminus of Aβ and precedes cleavage by γ-secretase. Two β-secretase cleavage products are produced: a secreted ectodomain of APP named $APP_s$-β and the C99 fragment, the membrane bound C-terminal 99 amino acids of APP (Vassar, 2002). β-Site APP cleaving enzyme (BACE) was identified through biochemical and genetic

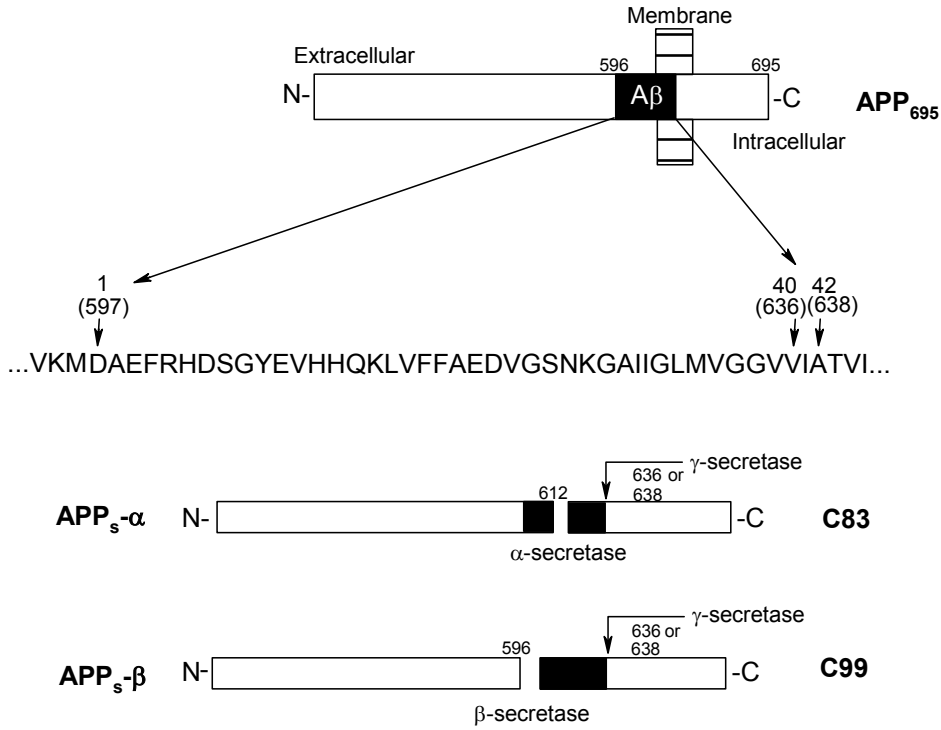



**Figure 7.** The amyloid precursor protein. Location of Aβ peptide in $APP_{695}$. The amino terminus of APP is secreted from cells upon cleavage at either the α- or β-cleavage site. Modified from Roberts, 2002.

methods as an aspartyl protease. BACE is an unusual member of the pepsin family of aspartyl proteases, which has an N-terminal catalytic domain, that contains two important aspartate residues, and is linked to a 17-residue trans-membrane domain and a short C-terminal cytoplasmic tail (Hussain *et al.*, 1999, Vassar *et al.*, 1999). β-Secretase is widely expressed in many tissues and cell lines, whereas at high levels in neurons of the brain. The β-secretase has maximal activity at acidic pH. The active site of β-secretase is located within the lumen of acidic intracellular compartments. The β-secretase is highly sequence-specific (Vassar, 2002). Site-directed mutagenesis of the amino acids surrounding this cleavage site in APP shows the sequence preferences of the β-secretase (Citron *et al.*, 1995).

An important question is whether inhibition of BACE is an appropriate strategy for therapeutic intervention in AD. It is likely that BACE has several other substrates (besides APP) and that it also has an important physiological function. Inhibition of this function could have toxic consequences (Nunan and Small, 2000).

Cleavage of the APP C99 fragment (Figure 7) by γ-secretase is the final step in the production of Aβ. The exact position of cleavage by γ-secretase is critical for the development of AD. Production of the more amyloidogenic long Aβ species via cleavage by γ-secretase, adjacent to residues 42 or 43, is closely associated with disease pathogenesis (Small, 1998, Nunan and Small, 2000).

The γ-secretase that generates the C-terminus *in vivo* is a complex of proteins containing presenilin (PS) as an integral component (Pitsi *et al.*, 2002, Cai *et al.*, 2003, Chen *et al.*, 2002, Takasugi *et al.*, 2003). Mutations in PS increase the proportion of Aβ molecules ending at amino acid 42, a fact that provided the first evidence for a connection between presenilins and γ-secretase. PS has been characterized as a complex protein with 8 transmembrane domains and multiple functions (Yu *et al.*, 1998, Soriano *et al.*, 2001). The N- and C-terminus and a large loop domain are oriented in the cytoplasm and interact with multiple cellular proteins (Roberts, 2002).

Mutations in the presenilin 1 and 2 genes that increase production of the highly amyloidogenic Aβ(1–42) are the most common cause of familial AD. Deletion of PS1 in mice reduces Aβ generation, indicating that PS1 mediates the last step in the generation of Aβ from APP by the unidentified γ-secretase. Mutating either of two conserved transmembrane aspartates in PS1 significantly reduced Aβ production and increased the APP C-terminal fragments that are γ-secretase products. These results indicate that PS1 is either an unique diaspartyl cofactor for γ-secretase or is itself γ-secretase. Furthermore, studies on the γ-secretase-like proteolytic processing of Notch and Ire1 suggest a common mechanism for the involvement of PS1 in the intramembrane proteolysis of membrane proteins (Xia, 2000).

Drugs that modulate the production of Aβ by inhibiting γ-secretase could provide an effective therapy for AD, but like most disease targets, the γ-secretase appears to have more than a single function. The use of potent inhibitors has aided the discovery and characterization of γ-secretase functions and reinforced the concept that a successful drug must demonstrate selectivity for lowering Aβ without disrupting the function of other γ-secretase targets. The discovery of drugs that can selectively inhibit β-APP cleavage is an important objective (Roberts, 2002, Tian *et al.*, 2003).

Recently, Sato *et al*. found that APP is cleaved by γ-secretase not only in the middle of the transmembrane domain (γ-cleavage) but also near the cytoplasmic membrane boundary (ε-cleavage). The major product of that process is a CTFγ of APP that begins at Val-50, according to Aβ numbering (Sato *et al.*, 2003).

The mutations in APP that cause FAD are all located near the secretase cleavage sites and affect directly the efficiency or position of the cleavages. For example, the Swedish mutation (so named because it was discovered in a Swedish family), is the amino acid substitution LysMet→AsnLeu at the positions immediately N-terminal to the β-secretase cleavage site in APP

(Mullan *et al.*, 1992). This mutation causes APP to be a much better substrate for β-secretase and dramatically increases the efficiency of cleavage at the β-secretase site, leading to greater production of Aβ. Several FAD mutations have been identified near the γ-secretase site, and they shift the position of γ-secretase cleavage causing a greater proportion of Aβ(1–42). In addition, FAD mutations near the α-secretase site appear to reduce the efficiency of α-secretase cleavage, thus providing more APP substrate for β-secretase cleavage and leading to a greater production of Aβ (Vassar, 2002).

The best characterized of these Aβ peptides is the peptide derived from C99 (Figure 7), which accumulates to high abundance in senile plaques and appears to play a central role in the etiology of AD. Whereas the several Aβ species have been studied in great detail, the other products generated by γ-secretase have received scant attention. One fragment of particular interest is the APP intracellular domain, that results from the γ-secretase cleavage of the C83, C89, or C99 fragments (Kimberly *et al.*, 2001). Multiple lines of evidence suggest that increased production and/or deposition of the Aβ peptide, derived from the APP, contributes to AD. A growing list of neurotransmitters, growth factors, cytokines and hormones has been shown to regulate APP processing. Although traditionally thought to be mediated by activation of protein kinase C, novel mechanisms of regulation, involving cholesterol-, apolipoprotein E-, and oxidative stress-activated pathways, have been identified (Mills and Reiner, 1999). In principle the full-length APP could function as a G-protein-coupled receptor, and the activation of APP may contribute to one or more of signalling cascades.

### 1.3.3. Neuropeptides in AD, possible role of galanin in AD

The family of neuropeptides includes nearly 50 known members showing a tendency of growth. The neuropeptide systems are differentially affected by neurodegeneration (Heilig *et al.*, 1995).

In AD, most neurotransmitters decline in association with neurodegeneration; however, GAL is a notable exception. GAL has been associated with cholinergic basal forebrain neurons, which degenerate in AD. The expression of GAL progressively increases in the basal forebrain in AD (Mufson *et al.*, 1993, Bowser *et al.*, 1997, Chan-Palay, 1988), and galanin-containing fibers and terminals form a dense plexus surrounding the remaining cholinergic cell bodies within the nucleus basalis of Meynert, reaching concentrations of twice that of age-matched controls (Beal *et al.*, 1990, Gabriel *et al.*, 1994). In addition, high levels of GAL continue to be expressed in the surviving neurons of the locus coeruleus in AD (Chan-Palay, 1991, Miller *et al.*, 1999). The overexpression of GAL in AD may contribute to the cognitive deficits characteristic of this disease (Steiner *et al.*, 2001). Moreover, GAL binding sites were reported to be

increased in the hippocampus (Rodriguez-Puertas *et al.*, 1997). Altogether, human studies have suggested that GAL neurotransmission is modified in several brain regions of AD brains, including cortex and hippocampus.

The results of animal studies have shown that cortical lesions up-regulate GAL synthesis in cholinergic forebrain neurons (Cortés *et al.*, 1990), GAL inhibits acetylcholine release in hippocampus (Fisone *et al.*, 1987), and central administration of GAL mostly impairs acquisition and memory retention (Ögren *et al.*, 1992, Ögren *et al.*, 1996, Ögren *et al.*, 1998, Schott *et al.*, 1998). Dysfunction of galaninergic neurotransmission may dispose PDAPP mice to be prone to cognitive defects associated with AD (Diez *et al.*, 2000).

It has been stated that somatostatin and NPY levels in cerebrospinal fluid are consistently decreased in AD. Fewer NPY cells were found in cortex, and they were distorted (Chan-Palay *et al.*, 1985). GAL levels increase with the duration of illness in AD patients (Nilsson *et al.*, 2001, Hartonian *et al.*, 2002).

Expression of neuropeptides on the brains of 26-month-old PDAPP mice was significantly changed as compared to control mice. The most common features are increases in stratum oriens (GAL, NPY, enkephalin, CCK and SP) and the supragranular layer (NPY, enkephalin, dynorphin and SP). Less common are decreases, which occur for dynorphin in the molecular layer, for CCK in mossy fibers and, most clearly, in the supragranular layer, and for SP in fibers around the granule cells. Interestingly, the latter two peptides have been shown to be mainly excitatory in the hippocampal formation. The remaining peptides, which almost always are increased, are mainly of inhibitory nature. This should lead to changes in excitability in the hippocampal formation, shifting the balance towards inhibition. An important question is still unanswered: if, and how, these global peptide changes are related to the overexpression of APP. They could represent compensatory (trophic or other) mechanisms attempting to counteract degenerative changes induced by the disease process (Diez *et al.*, 2000).

### 1.3.4. Alteration of G-protein-coupled signal transduction in AD

Extracellular signalling molecules utilize G-protein-coupled pathways for transmembrane signalling. Mutations in G-protein-coupled receptors and in G-protein $\alpha$-subunits have been identified as the cause of a variety of human disease.

The breakdown of interneuronal communication in AD is the central mechanism to the symptomatology of the disorder. This is shown by a variety of neurochemical changes in the brain of the sufferer, not least of which are alterations in aspects of cellular signal transduction. A deficit in cholinergic neurotransmission which occurs in AD is characterized by reductions in the

activity of choline acetyl transferase in certain brain areas (Procter *et al.*, 1988). The loss of nicotinic and muscarinic receptors is also demonstreated (Flynn and Mash, 1986, Flynn *et al.*, 1991). The integrity of muscarinic receptor G-protein coupling has been shown to be compromised in AD hippocampus, a region that shows typically severe senile plaque and neurofibrillary tangle pathology (Cowburn *et al.*, 1996a). Changes have been observed in the levels of neurotransmitters and their receptors in the adrenergic, glutaminergic, serotoninergic systems (Ross *et al.*, 1993). Impaired signal transduction could occur as a result of alterations in neurotransmitter receptor levels, receptor/G-protein couplings, G-protein levels, G-protein/effector enzyme coupling, effector enzyme levels, or due to actions of intracellular second messengers. AC signal transduction pathway is disrupted at a number of these components in AD brain (Yamamoto *et al.*, 2000).

The binding of a transmitter to the receptor is the primary event in the process of signal transduction. The key component in many of such processes is a family of G-proteins, which can couple to many different neurotransmitter receptors and to a variety of effector systems such as ion channels, AC and phospholipases (Birnbaumer, 1990).

The $G_s$-protein-AC dysregulation, seen in AD brain, does not appear to result from gross changes in total $G_s$ protein $\alpha$-subunit levels (McLaughlin *et al.*, 1991, Ross *et al.*, 1993, O'Neill *et al.*, 1994, Li *et al.*, 1996). In some brain regions, such as the hippocampus and angular gyrus, subtle changes in the number of large and small molecular weight $G_s\alpha$ isoforms may be important (O'Neill *et al.*, 1994, Cowburn *et al.*, 1996a).

It was reported that $G_s$-protein-stimulated AC activity is decreased in AD frontal, temporal, and occipital cortices, as well as angular gyrus and cerebellum, while basal and forskolin-stimulated activities showed no alteration (Cowburn *et al.*, 1992). Another study from the same group also showed a specific impairment of $G_s$-protein-stimulated AC activity in AD hippocampus (O'Neill *et al.*, 1994). These findings suggest that there is a specific lesion in AD brain at the level of $G_s$-protein-AC interactions. On the other hand, it was shown that basal, forskolin-stimulated AC activities, as well as $G_s$-protein-stimulated AC activity, are decreased in AD hippocampus and cerebellum (Schnecko *et al.*, 1994), indicating that both the $G_s$ protein and catalytic subunit of AC are impaired in AD brain (Yamamoto *et al.*, 2000). APP is a receptor coupled to $G_o$, and abnormal APP-$G_o$ signalling was shown to be involved in the AD disease process (Nishimoto *et al.*, 1993).

### 1.3.5. Oxidative stress in AD

Since the discovery of the neurotoxicity of Aβ peptides *in vitro*, the mechanism of its action has become the focus of attention. Theory of oxidative stress in redox changes within neurons are increasingly being implicated as an important causative agent in brain aging and neurodegenerative diseases such as amylotrophic lateral sclerosis, Parkinson's disease and AD (Olivieri *et al.*, 2001, Martindale and Holbrook, 2002).

Profound oxidative stress has been implicated in the pathogenesis of AD (Markesbery, 1997) by finding of several characteristics, such as enhanced lipid peroxidation, in specific areas of the brain in postmortem studies (Lovell *et al.*, 1995). The suggestion that high-grade oxidative stress causes the formation of oxygen radicals which results in neurodegeneration and possibly plaque formation in the central nervous system, was supported by the many studys (Frautschy *et al.*, 1991, Karelson *et al.*, 2001). Pappolla *et al*. provided evidence for the hypothesis that Aβ peptide, the major constituent of the senile plaque, is neurotoxic and that such toxicity is mediated by free radicals *in vitro* and in a transgenic mouse model of AD (Pappolla *et al.*, 1998).

There are some evidences indicating that the Aβ peptide cytotoxicity is mediated by free radical damage. Micromolar concentrations of Aβ peptide increases $H_2O_2$ concentration in cell cultures. Catalase, an enzyme that converts $H_2O_2$ to $O_2$ and $H_2O$, blocks Aβ toxicity and the cells selected for the resistance to Aβ toxicity are also highly resistant to $H_2O_2$ toxicity (Behl *et al.*, 1994, Bains and Shaw, 1997,). Evidence from a variety of studies indicates that β-amyloid enhances oxidative stress: increases in $H_2O_2$ have been detected in cells following exposure to Aβ, and both vitamin E and catalase prevent $H_2O_2$-mediated cell death. Individuals with Down's syndrome overexpress APP gene, located in chromosome 21, and develop an AD-like neurodegeneration, including the presence of senile plaques and a dramatic increase in intracellular reactive oxygen species (ROS) (Yankner, 1996). High micromolar concentration of Aβ, without the detectable pre-aggregation, produces endothelial damage, which is prevented by the enzyme superoxide dismutase (Thomas *et al.*, 1996) suggesting that $O_2^{\cdot-}$ may also play a role in Aβ toxicity. An initial report suggested that the Aβ peptide by itself generates free radicals that can damage cells (Hensley *et al.*, 1994). Amyloid fibrils reduce copper suggesting that ROS can be generated during both the initial and late step of amyloid formation. Opazo *et al*. observed that Aβ peptide (Aβ(1–40)) has copper reducing ability (Opazo *et al.*, 2003). In normal cells, the copper-reducing activity of APP and Aβ peptide should serve a favorable physiological function, possibly presenting Cu(I) to the Cu(I) transporter. In unfavorable conditions, an abnormal increase of APP or an accumulation of Aβ peptide into amyloid fibrils, may increase the reduction of copper, generating a concomitant increase in Cu(I) levels, free radicals and consequently oxidative damage (Huang *et al.*, 1999, Miranda *et al.*,

1999, Opazo *et al.*, 2003). *In vitro* activated microglia cells produce $O_2^{\cdot-}$ (Colton and Gilbert, 1987) and mediate neuronal cell death by production of NO and ROS (Boje and Arora, 1992). β-amyloid is indirectly neurotoxic by activating microglia to produce oxygen free radicals (Miranda *et al.*, 2000).

The oxidative damage described in AD brain is in correlation with extent of oxidative stress induced by the Aβ peptide. This damage is induced by free radicals that are probably generated by Aβ through the metal ion-catalyzed oxidation at the early steps of Aβ folding and later continued through different mechanisms including membrane lipid peroxidation, receptor-mediated mechanisms and activation of microglial cells. Based on the previous findings, future directions in AD treatments will focus on the use of antioxidants to contribute to the neuroprotection and potential enhancement of the intracellular antioxidant mechanisms (Miranda *et al.*, 2000). Glutathione (GSH) is a major intracellular antioxidant and its antioxidant activity depends upon the thiol group within the molecule. GSH plays a critical role in detoxification of peroxides and electrophilic toxins as substrate for GSH peroxidase and glutathione-S-transferase (Gilgun-Sherki *et al.*, 2001). Excess cerebral oxidative stress in AD might progressively deplete nervous tissue glutathione stores and this perhaps explains the observed association between plasma levels of GSH and disease severity (Fawcett *et al.*, 2002, McCaddon *et al.*, 2003, Vina *et al.*, 2004).

$H_2O_2$, UV light, Aβ(1–42) and toxic Aβ(25–35) induce a profound oxidative stress and cytotoxicity in cells. The effects are reversed when the cells are pre-treated with N-acetyl-L-cysteine (NAC). NAC strongly lowered phospho-tau levels in the presence or absence of stress treatment (Olivieri *et al.*, 2001).

# 2. AIMS OF THE STUDY

- To study the effects of the synthetic peptide Aβ(1–42), and its shorter fragments, Aβ(25–35) and Aβ(12–28), on the GTPase and adenylate cyclase activity in membrane preparations of ventral hippocampus and cerebral cortex from rat brain.
- To study the effects of APP C-terminal peptides on G-proteins and adenylate cyclase activity in the postmortem Alzheimer's disease and age-matched control brain.
- To study a new chimeric galanin-NPY peptide, galanin(1–13)-[D-Trp$^{32}$]-NPY(25–36)amide, binding to galanin receptors and its effect on adenylate cyclase activity.
- To synthesize a new low molecular weight nonpeptide ligand for galanin receptor, to examine its effects on adenylate cyclase activity and on antiepileptic activity.

# 3. METHODOLOGICAL CONSIDERATION

## 3.1. Solid Phase Peptide Synthesis (SPPS)

Solid phase peptide synthesis (SPPS) is based on the sequential coupling of α-amino and side-chain protected amino acid residues to an insoluble polymeric support. The acid-labile *t*-Boc-group or base-labile Fmoc-group is used for N-α-protection. After removal of this protecting group, the next protected amino acid is added using either a coupling reagent or pre-activated protected amino acid derivative. C-terminus of the synthesized peptide is attached to the resin via a linker and may be cleaved off to yield a peptide either in acid or amide form, depending on the used linker. Side-chain protecting groups are usually chosen to enable a simultaneous cleavage with detachment of the peptide from the resin.

### 3.1.1. Design of peptides

A 39–43 amino acids long proteolytic fragment of APP (Figure 7), Aβ, is a major component of the senile plaques associated with AD. The sequences of Aβ(1–42) and the peptides derived from it used in Paper I are shown in Figure 8. The most toxic fragments of Aβ, Aβ(25–35) and Aβ(12–28), modulate neuronal function, immune and inflammatory responses in several cell types (Ross *et al.*, 1993, Schnecko *et al.*, 1994). Aβ(25–35) is the shortest fragment that exhibits large β-sheet fibrils and retains the toxicity of the full-length peptide. Although these peptides are not naturally occurring degradation products of APP, they are widely used model substances in studies of the mechanisms of action of Aβ *in vitro* studies. Aβ(1–40) and Aβ(25–35) peptides both disrupt carbachol-induced M1 muscarinic cholinergic signal transduction in cortical neurons (Kelly *et al.*, 1996), suggesting that Aβ peptides interfere with muscarinic receptor coupling to G-proteins. These results indicate that Aβ plays an important role in the impairment of cholinergic transmission that occurs in AD, probably with the involvement of free radicals in the mechanism (Schubert *et al.*, 1995, Kelly *et al.*, 1996).

The completely conserved cytoplasmatic APP sequence (Figure 9), His657-Lys676, is reported to form a complex and to activate $G_o$, a major GTP-binding protein in the brain (Brouillet *et al.*, 1999). Connection of the APP trans-membrane sequence Thr639-Leu648 the peptide His657-Lys676 increased its potency of stimulating $G_o$ 20-fold as compared to the transmembrane or the cytoplasmic sequence alone (Nishimoto *et al.*, 1993) (Table 4).

| | 1 ↓ | 10 ↓ | 20 ↓ | 30 ↓ | 40 ↓ |
|---|---|---|---|---|---|
| Aβ(1-42) | **DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA** | | | | |
| Aβ(12-28) | | **VHHQKLVFFAEDVGSNK** | | | |
| Lys-Aβ(16-20) | | **KKLVFF** | | | |
| Aβ(25-35) | | | | **GSNKGAIIGLM** | |
| Scrambled Aβ(25-35) | | | | **IMLGSGNKGAI** | |

**Figure 8.** Amino acid sequences of Aβ(1–42), Aβ(12–28), Lys-Aβ(16–20), Aβ(25–35), and Aβ(25–35)-scrambled peptides (Paper I).

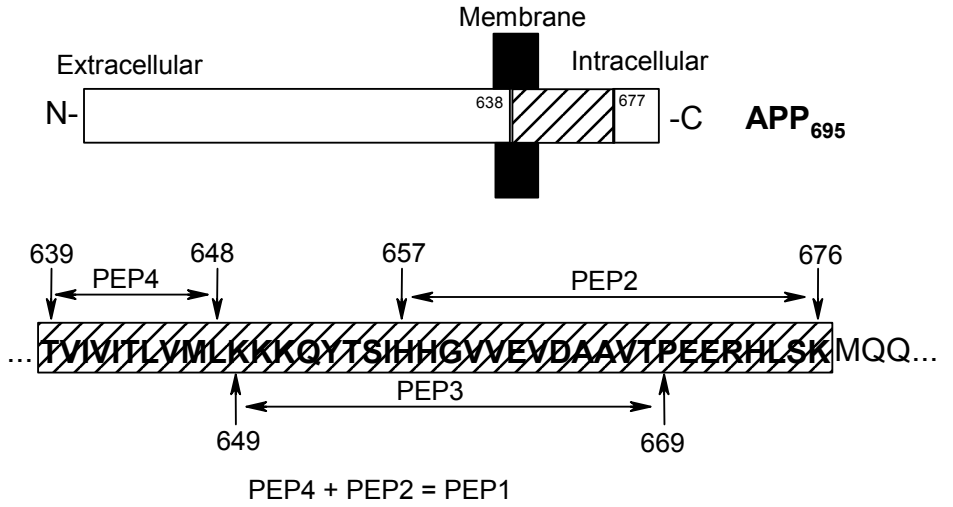



**Figure 9.** Location of the peptides PEP1, PEP2, PEP3 and PEP4 in amyloid precursor protein $APP_{695}$.

**Table 4**. The used peptide sequences from the C-terminus of amyloid precursor protein (APP) (Paper II).

| Name | Peptide | Sequence |
|---|---|---|
| PEP1 | APP(639-648)-APP(657-676) amide | TVIVITLVMLHHGVVEVDAAVTPEEHLSK amide |
| PEP2 | APP(657-676) amide | HHGVVEVDAAVTPEERHLSK amide |
| PEP3 | APP(649-669) amide | KKKQYTSIHHGVVEVDAAVTP amide |

The design of M242 was based on the findings that the substitution of one amino acid in a peptide sequence for D-Trp can stabilise/induce β-turns and thereby enhance the agonist/antagonist properties of the peptide (Balasubramaniam *et al.*, 1994, Balasubramaniam *et al.*, 1996). Such modifications in NPY have produced two specific agonists for NPY receptor subtype 5 ($Y_5$): [$^{32}$D-Trp]NPY (Balasubramaniam *et al.*, 1994, Gerald *et al.*, 1996, Hwa *et al.*, 1999) and [$^{34}$D-Trp]NPY (Parker *et al.*, 2000).

**Table 5.** Amino acid sequences of peptides used in Paper III

| Peptide | Sequence |
|---|---|
| Galanin(1–29), rat | **GWTLNSAGYLLGP**HAIDNHRSFSDKHGLT amide |
| Galanin(1–30), human | **GWTLNSAGYLLGP**HAVGNHRSFSDKNGLTS |
| Neuropeptide Y, porcine | YPSKPDNPGEDAPAEDLARYYSAL**RHYINLITRQ RY** amide |
| M32: Galanin(1–13)-neuropeptide Y(25–36)amide | GWTLNSAGYLLGPRHYINLITRQRY amide |
| M242: Galanin(1–13)-[D-Trp$^{32}$]-neuropeptide Y(25–36)amide | GWTLNSAGYLLGPRHYINLI[D-W]RQRY amide |

### 3.1.2. Synthesis of peptides

The peptides used in these studies were synthesized using *t*-Boc or Fmoc SPPS chemistry.

Peptides were synthesized in a stepwise manner in a 0.1 mmol scale manually or on the Applied Biosystem Model 431 A peptide synthesizer on solid support using *N*,*N*'-dicyclohexylcarbodiimide-hydroxybenzotriazole activation strategy. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a phenylacetamidomethyl resin to achieve the C-terminally free carboxylic acids for Aβ peptides or to a *p*-methyl-benzhydrylamine resin to obtain C-terminally amidated peptides for sequences of APP. The peptides were finally cleaved from the resin with liquid HF at 0°C for 30 min. Deprotection of the side chains, cleavage of the peptides and purification on HPLC have been described in detail earlier (Langel *et al.*, 1992).

The shorter synthetic fragments of Aβ peptides were synthesized in a stepwise manner using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) amino acid protection chemistry and Wang resin. Coupling was carried out using the standard chemistry of 2-(1*H*-bensotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and *N*-hydroxybenzotriazole (HOBt) in dimethyl-

formamide. The peptides were finally cleaved from the resin with TFA for 90 min. The peptides was purified using high-performance liquid chromatography (HPLC). The purity of the peptides was >99% as demonstrated by HPLC on an analytical Nucleosil 120–3 $C_{18}$ reversed-phase column. The molecular masses of the peptides were determined by a plasma desorption mass spectrometry and the calculated values were obtained in each case.

*Synthesis of Combinatorial Library.*
Synthesis of the combinatorial library was started from the initial lead compound, Cbz-Phe-Arg-aromatic amine (A-B-C-D), that was found by screening a library of the analogues of a tripeptide Trp-Asn-Tyr combining major pharmacophores in GAL. Modifications into all positions were included in the initial lead compound, resulting in the following lead structure: A-B-C-D, where A denoted a bulky hydrophobic group, B a hydrophobic amino acid, C an amino acid with protonated side chain (in physiological conditions), and D an aromatic amine (Table 6). All coupling steps were carried out separately for each compound. For deprotection the separate resins were pooled again. Fragment A-B-C was synthesized on 4-methylbenzhydrylamine-polystyrene resin and after cleavage from the resin, the component D was coupled to it in the solution. Synthesized compounds were purified on Sep-Pak cartridges by eluting with a stepwise gradient of acetonitrile in water. 20 fractions were obtained, freeze-dried, and screened for activity in $^{125}$I-galanin displacement assay. The structure of galnon was deduced reiteratively by using the resin samples saved in each step.

**Table 6.** The building blocks for the combinatorial library A-B-C-D, where A is a bulky hydrophobic group, B a hydrophobic amino acid, C an amino acid with protonated side chain (in physiological conditions) and D an aromatic amine coupled to the C terminus of amino acid C through an amide bond.

| A | B | C | D |
|---|---|---|---|
| Fmoc- | Phe | Lys | 7-Amino-4-(methoxymethyl)-coumarin |
| Acetyl- | Trp | His | 7-amino-4-methylcoumarin (AMC) |
| Benzyloxycarbonyl- | hPhe-* | Orn# | *m*-Anisidin |
| Adamantane-1-carbonyl- | Cha† | Dab‡ | Cyclohexylamine |

* Homophenylalanine
† Cyclohexylalanine
‡ Diaminobutyric acid
# Ornithine

### 3.1.3. Synthesis of galnon

After identification of the most active GALR ligand by combinatorial approach, galnon was synthesized following the scheme outlined in Figure 10. The first step of the synthesis was the coupling of Fmoc-Lys(*tert*-Boc)-OH to AMC. One millimole of Fmoc-Lys(Boc)-OH and 0.5 equivalents of dicyclohexylcarbodiimide were separately dissolved in dioxane, cooled on ice, and then pooled. The reaction mixture was stirred for 30 min at room temperature and then 0.5 equivalents of AMC dissolved in DMF was added to the symmetric anhydride solution. The mixture was stirred overnight. The solvents were evaporated. Fmoc-Lys(Boc)-AMC was precipitated with petrol ether/ethyl acetate mixture and dried under vacuum. The Boc group was removed with $H_2O$/trifluoroacetic acid mixture in ice bath for 5 min, followed by the evaporation of the solvents. The obtained Fmoc-Lys-AMC was coupled to chlorotrityl resin by incubation of 2–3 equivalents of Fmoc-Lys-AMC, 4–9 equivalents of diisopropylethylamine, and 1 equivalent of chlorotrityl resin for 2 h. The resin was washed and the Fmoc group was removed with piperidine/DMF. Coupling of Fmoc-Cha-OH was performed by using 2 equivalents of amino acid, TBTU, HOBt, and 4 equivalents of DIEA. Galnon was cleaved from the resin by applying trifluoroacetic acid/dichloromethane mixture in four aliquots. The filtrate was evaporated, and the obtained product was purified on Sep-Pak cartridges and analyzed by using a plasma desorption mass spectrometer.

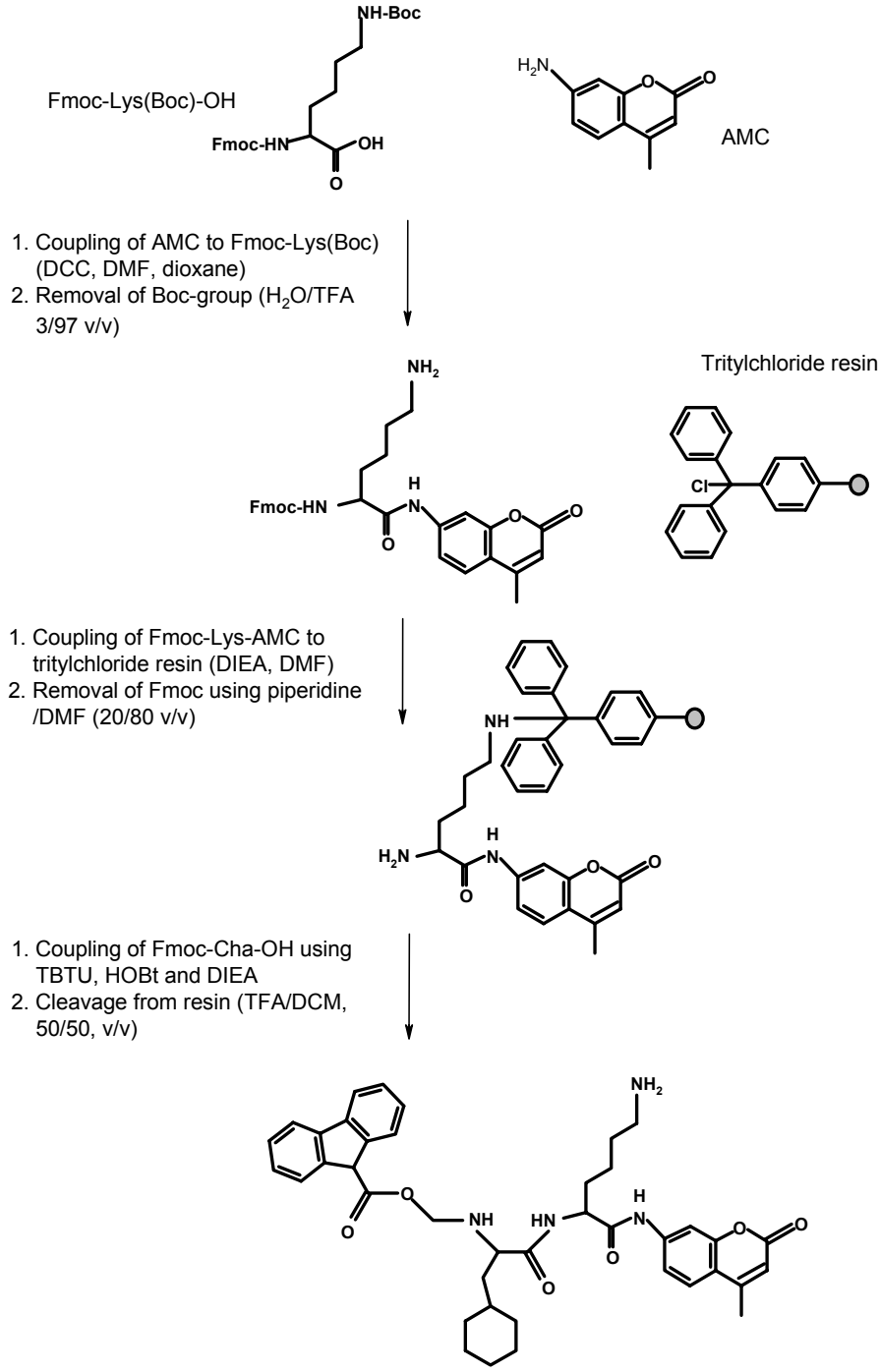



**Figure 10.** Synthesis route of galnon. Paper IV.

## 3.2. Effects of peptides on G-protein coupled cellular signalling

Various fragments of APP affect important functions in the brain. The part of the intracellular C-terminus of APP (sequence 657–676) has been shown to interact with $G_o$-proteins in brain (Simic *et al.*, 1997). It has been suggested that abnormal APP to $G_o$ signalling is involved in the development process of AD and that APP has a potential receptor function (Okamoto *et al.*, 1995). Brouillet *et al*. showed the regulation of $G_o$ GTPase activity by APP in caveolae-like compartments that are specialised in signal transduction (Brouillet *et al.*, 1999). Also, Smine *et al*. have demonstrated that presenilin-1, a product of the familial AD gene, interacts with brain $G_o$ proteins (Smine *et al.*, 1998). The described specific interactions of APP with presenilins (Xia *et al.*, 1997) may play a significant role in intramembraneous proteolysis of APP as well as in abnormal signal transduction in early onset AD (FAD).

Oxidative stress appears to contribute to neuronal dysfunction associated with AD and other CNS neurodegenerative disorders. GSH is the most abundant cellular antioxidant and free radical scavenger and the first line of defense against oxidative stress generated by reactive oxygen species (Olivieri *et al.*, 2001). In the neurons, the mechanism of neurotoxicity of Aβ fragments appears also to involve the generation of free radicals, the induction of ROS and to cause lipid peroxidation (Butterfield *et al.*, 1994, Yatin *et al.*, 1999a, Yatin *et al.*, 1999b). Different reports have shown cellular release of peroxides, superoxide and nitric oxide in response to the treatment with Aβ and its fragments (Behl *et al.*, 1994, Huang *et al.*, 1999). Spontaneous generation of free radicals by Aβ(1–40) and Aβ(25–35) themselves in free-cell system has also been reported (Hensley *et al.*, 1995).

Interaction of stimulated 7TM receptors with the α-subunits of G-proteins modulate the activity of GTPases inducing different cellular events, the synthesis of second messengers, e.g. cAMP that is generated by a membranous enzyme adenylate cyclase. It has been shown that several short amphiphilic peptides are able to directly affect the functions of G-proteins (Mousli *et al.*, 1990, Shin *et al.*, 1994, Zorko *et al.*, 1998) thereby mimicking the action of native membrane proteins.

### 3.2.1. Membrane preparation from brain tissues and Bowes cells

Membranes of ventral hippocampus and frontal cortex were prepared from Wistar rats (200–300 g), according to the previously published procedures (Valkna *et al.*, 1995). Rat hippocampus has been chosen to characterize M242

and M32, due to the regional occurrence of high-affinity receptors for both GAL (Fisone *et al.*, 1989b) and NPY (Redrobe *et al.*, 1999).

The effects of APP CT peptides on the activities of G-proteins and adenylate cyclase were studied in the membranes from the AD and age-matched control hippocampus, a region that showed high level of amyloidogenesis and profound neuronal degeneration in AD (Ball *et al.*, 1985, Shimohama *et al.*, 1999) (Paper II). Hippocampal and frontal cortical tissues of postmortal human brain were obtained from Huddinge Brain Bank, Sweden. The hippocampal and frontocortical membranes for the [$^{35}$S]GTPγS binding measurement and for the GTPase and adenylate cyclase assay were mainly prepared according to the protocol (Karelson *et al.*, 1995)**.** The protein content of the membrane preparations was determined according to Lowry *et al*. (Lowry, 1951).

Binding studies and adenylate cyclase measurements in rat hippocampus were accompanied with binding experiments in Bowes cells, which express hGALR1 (Heuillet *et al.*, 1994) and in Chinese hamster ovary cells (CHO-K1) transfected to overexpress hGALR2.

Bowes cells were propagated in Eagle's minimal essential medium with Glutamax®, supplemented with 10% foetal bovine serum, nonessential amino acids and sodium pyruvate. Cells were grown until confluent, scraped into phosphate-buffered saline and centrifuged at 1000×*g* for 10 min. The pelleted cells were lysed in lysis buffer and subsequently incubated for 10 min on ice. Centrifugation at 10 000×*g* for 10 min gave a pellet of microsomal membrane fraction, which was washed once with lysis buffer. The washed pellet was resuspended in assay buffer (Paper III).

### 3.2.2. Binding studies

Porcine [$^{125}$I]-galanin displacement experiments on membranes from rat hippocampus and Bowes cells were performed as described previously by Land *et al*. (Land *et al.*, 1991) and *N*-(propionyl-[$^{3}$H])-neuropeptide Y displacement experiments as described by Kahl *et al*. (Kahl *et al.*, 1994).

### 3.2.3. [$^{35}$S]GTPγS-binding studies

GPCRs are the most tractable and effective set of targets for therapeutic drug design (Milligan, 2003). Screening for ligands that interact with GPCRs is necessary. Measurement of the binding of GTPγS (guanosine-5´-O-(3-thio)triphosphate), a nonhydrolyzable GTP analog, to G-proteins plays a key role in the assessment of receptor-induced G-protein stimulation (Okamoto *et al.*, 1992). The binding of the [$^{35}$S]GTPγS directly reflects the receptor activation, since it measures the exhange of GDP for GTP on the G-protein (Rodriguez-

Puertas *et al.*, 2000). The [$^{35}$S]GTPγS-binding assay is based on the current model of G-protein activation cycle (Figure 2) and has been used in re-constituted systems of purified proteins, in membrane homogenates, in receptor-transfected cells, immunoprecipitation assays and *in vitro* autoradiography (Rodriguez-Puertas *et al.*, 2000).

In our studies, the brain membranes with the final protein concentration of 0.04 mg/ml were incubated in a reaction coctail containing [$^{35}$S]GTPγS. After incubation bound and free [$^{35}$S]GTPγS were separated by vacuum filtration through GF/B filters. Radioactivity was quantified by liquid scintillation counting.

### 3.2.4. GTPase activity measurements

The GTPase assay measures the inactivation reaction of the G-protein, the GTP hydrolysis, which constitutes an indirect activity that could be altered by other factors independently of the G-protein activation (Rodriguez-Puertas *et al.*, 2000). Measurement of GTPase activity was performed radiometrically according to Cassel and Selinger (Cassel and Selinger, 1976), with the modifications suggested by McKenzie (McKenzie, 1992). Membranes from the rat brain frontal cortex and hippocampus were prepared according to the protocol of McKenzie (McKenzie, 1992) with minor modifications as described previously (Zorko *et al.*, 1998).

The ice-cold reaction cocktail containing ATP (to prevent reassociation of ADP with the free $^{32}P_i$) and trace amounts of [γ-$^{32}$P]GTP to give 50 000–100 000 c.p.m. in an aliquot of the reaction cocktail (with the addition of cold GTP to give the required total concentration of GTP of 0.5 μM) was added to the diluted membranes. Incubation medium was standard TE-buffer (10 mM Tris–HCl+0.1 mM EDTA), pH 7.5. Background low-affinity hydrolysis of [γ-$^{32}$P]GTP was assessed by incubating parallel tubes in the presence of 100 μM GTP. Blank values were determined by replacing of the membrane solution with assay buffer. GTPase reaction was started by transferral of the reaction mixtures to 30°C in a water bath for 12 min. After incubation free $^{32}P_i$ was separated from $^{32}P_i$-GTP by adding activated charcoal followed by centifugation of the samples. The radioactivity of the released radioactive phosphate was determined in Packard 3255 liquid scintillation counter.

### 3.2.5. Adenylate cyclase activity measurements

Membranes of ventral hippocampus and frontal cortex were prepared from Wistar rats (200–300 g), according to previously published procedures (Valkna *et al.*, 1995). Homogenates (in 8 mM HEPES-Na, pH 7.4) of precooled ventral

hippocampus were diluted, stirred on ice for 30 min and centrifuged for 6 min at 1600×*g*. The pellets were resuspended in ice-cold protein-buffer (4 mM HEPES-Na, 1.5 mM theophylline, 8.25 mM $MgCl_2$, 0.75 mM EGTA, 7.5 mM KCl, 100 mM NaCl, pH 7.4) to a final protein concentration of 0.6–0.8 mg/ml. The basal adenylate cyclase activity was assayed at 0.04 mg/ml of membrane protein in reaction-buffer, additionally containing (in protein buffer) 100 μg/ml bacitracin, 0.03% bovine serum albumin, 10 mM phosphoenol-pyruvate and 30 μg/ml pyruvate kinase (Valkna *et al.*, 1995). In all experiments the peptides were dissolved in the reaction buffer and added to the assay mixture 2 min before the reaction was initiated by adding 10 mM ATP/10 μM GTP. The reaction was carried out at 30°C and terminated after 15 min by the addition of 100 mM EDTA, followed by boiling the samples for 3 min. Cyclic AMP content in the tubes was measured by a competitive protein saturation assay using cyclic AMP-binding protein from bovine adrenal cortex (Brown *et al.*, 1972).

### 3.2.6. PTX catalysed ADP-ribosylation

The inhibitory action of G-protein-coupled receptors on AC activity can be blocked by treatment of cells with pertussis toxin (PTX), an exotoxin from *Bordetella pertussis* with ADP-ribosyltransferase activity (Simonds, 1999).

PTX was activated by treatment with 50 mM dithiotreitol (DTT) for 30 min at 37°C. For the ADP-ribosylation reaction, the membranes from brain cortex and ventral hippocampus were treated with 10 μg/ml of PTX in the 10 mM HEPES-Na or TE-buffer (10 mM Tris–HCl+0.1 mM EDTA, pH 7.4), containing 3 μM $NAD^+$, 20 mM thymidine, 1 mM ATP and 100 mM GTP. The treatment was initiated by transferring the tissue membranes to a 37°C water bath for 30 min. After the incubation, the membranes were diluted two-fold and centrifuged at 10 000×*g* for 45 min. The obtained pellet was resuspended in ice-cold buffer and used for the GTPase or adenylate cyclase assay experiments.

### 3.2.7. Effects of antioxidants on adenylate cyclase activity

We have examined the effects of the antioxidants, glutathione and *N*-acetyl-L-cysteine, on the basal activity of adenylate cyclase as well as antioxidant induced alterations in the modulation of adenylate cyclase activity by Aβ(25–35). The effect was measured as a difference in the amount of cAMP produced by membranous adenylate cyclase in the presence or absence of $10^{-7}$ M Aβ(25–35) and in the conditions where glutathione (final concentration 1.5 mM) or *N*-acetyl-L-cysteine (0.5 mM) were added to the medium before the

peptide, and shown as percentage of changes in cAMP production against unaffected (basal) activity (=100%).

To elucidate the effects of antioxidants on the stimulation by PEP1 (Table 4) of [$^{35}$S]GTPγS-binding in control or AD hippocampal membranes, 0.01 mM of ferrous ion chelator desferrioxamine as well as 1.5 mM of GSH or 0.5 mM of *N*-acetyl-L-cysteine (NAC) were added to the medium before the peptide. The effects of the antioxidants were estimated as a difference in the stimulation of binding in the absence or presence of antioxidants. In parallel, the effect of antioxidants onto the basal [$^{35}$S]GTPγS-binding was studied.

## 3.3 *In vivo* seizure model

C57BL/6J male mice weighing 20–30 g were anaesthetized with ketamine and xylazine i.p. and stereotaxically chronically implanted into a lateral ventricle (ICV) with guide cannulae. GAL or M35 were injected ICV in freely moving mice (0.5 nmol, in 0.5 µl, over 5 min). Control animals were treated with saline.

Galnon was freshly dissolved in 50% DMSO in saline and administered i.p. in a dose of 2 mg/kg 15 min before pentylenetetrazole (PTZ), when the effect of galnon alone was studied, or 5 min after M35 injection, in the coadministration studies. Control animals were treated with 50% DMSO in saline.

Seizures were induced by i.p. injection of PTZ in a dose of 40 mg/kg when studying the effects of GAL only or galnon only or 30 mg/kg when studying the effect of M35 and galnon + M35. Seizure latency and the highest behavioral seizure score were recorded. For statistical purposes, if the animal failed to show seizure of any particular score, a latency of 900 s was assigned to this score. No behavioral side effects of galnon were observed.

# 4. RESULTS AND DISCUSSION

## 4.1. Modulation of the activity of G-proteins and adenylate cyclase by Aβ peptides in rat hippocampal membranes and by C-terminal sequences of APP in the normalaging and Alzheimer's disease hippocampus (Papers I–II)

Aβ peptide, a 39–43-amino acid long peptide, aggregates in the brain to form the amyloid depositions and seems to be a central event in AD. In Paper I, based on previous findings on alterations in signal transduction mechanisms in AD (Schnecko *et al.*, 1994, Garcia-Jimenez *et al.*, 1999), we have studied the effects of the synthetic peptide Aβ (1–42), and its shorter fragments, Aβ (25–35) and Aβ(12–28), on the GTPase and adenylate cyclase activity in membrane preparations of ventral hippocampus and cerebral cortex from rat brain. The effects of the Aβ(1–42) and the derived peptides on GTPase and AC activities are summarized in Table 5.

The dose-response curves describing the effect of Aβ(1–42) on the GTPase activity in membrane preparations from rat cerebral cortex and ventral hippocampus were bell-shaped and declined at higher concentrations of the peptide (Figure 2A and B, Paper I). The maximal effect of Aβ(1–42) on GTPase activity was about 1.5-fold above basal activity. Activation of adenylate cyclase by Aβ(1–42)in hippocampal membranes was similar to that of Aβ(25–35) (Figure 3A and B, Paper I). In nanomolar concentration range Aβ(1–42) stimulated adenylate cyclase to 30%–40% above basal activity. At the peptide concentrations above 100 nM the effect was attenuated. In membranes from the cerebral cortex, the Aβ(1–42) peptide had no significant effect on the adenylate cyclase activity.

The stimulation of GTPase by Aβ(25–35) was similar in both tissues. The maximal effect of Aβ(25–35), observed at 100 μM concentration, was two-fold above the basal activity of the enzyme. The value of Hill coefficient ($n_H$) for this activatory peptide was in some extent higher in the frontal cortex than in the ventral hippocampus, 2.4 and 1.7, respectively. The different heterogeneity in G-protein subunits in these tissues, the multiple binding to G-proteins or interaction of the peptide with membranes in region-specific manner could provide an explanation for these different $n_H$ values. The conformation, orientation and accumulation of Aβ peptides in the cell membranes can be affected by a heterogeneous composition of membrane lipids, proteins and carbohydrates (Terzi *et al.*, 1995). Ropero *et al*. have shown that cholesterol modulates GTPase activity in both $G_s$ ana $G_i$ protein families (Ropero *et al.*, 2003).

A dual response of adenylate cyclase to the neurotoxic Aβ(25–35) in membranes of rat ventral hippocampus and frontal cortex was found (Figure 3A and B, Paper I). This response includes a consistent, 20%–30% enhancement of adenylate cyclase activity at low concentrations and less or no effects at higher concentrations of the peptide.

The Aβ(25–35) and the full-length peptide Aβ(1–42) may activate different signalling systems and therefore have different effects on the signalling systems studied in our experiments. Another explanation for the different effects of two peptides might be due to the different heterogeneity of G-proteins in these brain regions. Curtain *et al*. have found that in the presence of $Cu^{2+}$ or $Zn^{2+}$, pH, cholesterol, and the length of the peptide chain influenced the interaction of these peptides with lipid bilayers (Curtain *et al.*, 2003). Aβ(1–42) and Aβ(1–40) behaved differently within the membrane (Mason *et al.*, 1999). The third explanation might be provided by the fact that these two peptides may interact differently with the membrane components.

Both of the $G_i/G_o$-proteins are present in significant quantities in the mammalian brain and serve as the major contributors to the high affinity GTPase activity (Ross *et al.*, 1993). Since we detected the significant increase in GTPase activity and the reduced stimulation of adenylate cyclase activity at relatively high concentrations (10–100 μM) of Aβ(25–35), one could assume that the peptide inhibits adenylate cyclase at higher concentrations via activation of inhibitory $G_i/G_o$-proteins. In the PTX treated membranes, an induction of GTPase activity by Aβ(25–35) was totally abolished (Fig 2A and B, Paper I) and effect at higher consentrations of Aβ(25–35) on AC was no longer attenuated (Figure 4, Paper I). This suggests that the $G_i/G_o$-proteins are activated by this peptide. The inhibitory phase in the pronounced bell-shaped effect of Aβ(25–35) on the brain adenylate cyclase activity (Figure 3A and B, Paper I) may be directly mediated by low-affinity isoform(s) of $G_i$-proteins. Apparently, potent stimulation of GTPase activity by high concentrations of Aβ(25–35) is specifically associated with those $G_i$-isoforms that inhibit the adenylate cyclase activity in the ventral hippocampus and frontal cortex. Our results suggest that the activation of adenylate cyclase by Aβ(25–35) is not mediated via $G_s$-proteins. The suppression of cyclic AMP-signalling pathway at higher concentrations of the peptide by the stimulation of inhibitory $G_i/G_o$-proteins seems to be well-supported by results of our study.

Different groups have demonstrated that the amyloid peptide causes degeneration and death of neurons by mechanism that involve free radicals and that oxidative stress becomes profocused in regions with amyloid deposition (Pappolla *et al.*, 2002). We examined whether the Aβ(25–35)-induced stimulation of AC could be influenced by the antioxidants, gluthatione or N-acetyl-L-cysteine. It is known that reduced glutathione (GSH) protects SK-N-SH human neuroblastoma cells from Aβ(25–35) toxicity (Gridley *et al.*, 1998). Incubation of membranes with antioxidants and 0.1 μM Aβ(25–35) abolished or decreased

the stimulation of adenylate cyclase activity by this fragment of Aβ (Figure 11). The significant cutback of this activation by the free radical scavengers may show that redox mechanisms is involved in the mechanism of stimulation of adenylate cyclase activity by Aβ(25–35).

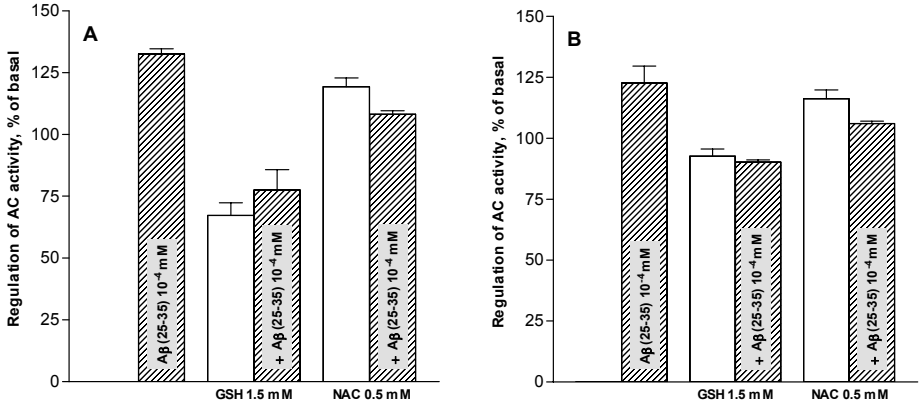



**Figure 11.** Effect of the 0.1 μM Aβ(25–35) on the adenylate cyclase activity in rat ventral hippocampal (A) and frontocortical (B) membranes in the presence of 1.5 mM GSH or 0.5 mM NAC. 100% corresponds to the basal adenylate cyclase activity.

The peptide Aβ(12–28) and the hexapeptide Lys-Aβ(16–20) had no detectable effect on the GTPase activity and generated only a slight modification of adenylate cyclase activity. The hexapeptide was capable of slight attenuation of the maximal activatory effect of Aβ(25–35) on the GTPase activity in the membranes from both tissues. These results suggest that the hexapeptide could directly or indirectly interfere with the interaction of Aβ(25–35) with G-proteins in the membranes. AC activity measurements with different concentrations of the Lys-Aβ(16–20) showed that the stimulatory effect of Aβ(25–35) could be inhibited by higher concentrations of hexapeptide (Figure 5A and B, Paper I). The inhibitory phase in the adenylate cyclase modulation by higher concentrations of Aβ(25–35) ($10^{-5}$ M) was less affected by the hexapeptide ligand. This is in accordance with a very small effect of Lys-Aβ(16–20) on the activation of GTPase by Aβ(25–35) and also corroborates the suggestion that the inhibitory effect of higher concentration of Aβ(25–35) on adenylate cyclase activation is mediated by $G_i/G_o$-proteins. The NMR-studies demonstrate the absence of direct interaction between Lys-Aβ(16–20) and Aβ(25–35) in a water environment, supporting the idea of indirect and, probably non-specific interference of the hexapeptide in the stimulatory effect by Aβ(25–35) on AC.

**Table 5.** Influence of Aβ peptides on the activity of GTPase and AC in membranes of ventral hippocampus and cerebral cortex from rat brain (Paper I).

| Aβ peptides | GTPase activity $EC_{50}$ | | Adenylate cyclase activity $EC_{50}$ | |
|---|---|---|---|---|
| | Ventral hippocampus | Cortex | Ventral hippocampus | Cortex |
| Aβ(25–35) | 26±3 μM | 29±4 μM | 1.7 nM | 2.7 nM |
| Aβ(1–42) | 1.4±0.3 μM | 1.3±0.2 μM | 2 nM | No effect |
| Aβ(12–28) | No effect | No effect | No effect | Slight increase |
| Scrambled Aβ(25–35) | No effect | No effect | No effect | No effect |

Our data show a response of G-proteins and, probably $G_i/G_o$, coupled cyclic AMP-signalling system to the neurotoxic Aβ(25–35) in the membranes from the studied brain regions. The mechanism of action of Aβ(25–35) may involve different subtypes of regulatory G-proteins, but also other transmembrane signal transduction mechanisms. The ability of Aβ and its neurotoxic fragments to initiate membrane lipid peroxidation and to enhance oxidative stress in primary neuronal cultures has been reported (Mark *et al.*, 1997). Present data suggest the influence of amyloid peptide on signal transduction in AD.

Evidence for correlating C-terminal sequences (CT) of APP (Figure 9) (Figure 1, Paper II) with neurodegeneration has come from cell transplantation models, transgenic mice, and the investigation of postmortem brains (Selkoe, 1998). The cytoplasmatic carboxy-terminus of APP (Figure 9) is suggested to regulate APP metabolism and functions in normal and AD brain. Stimulation of $G_o$-proteins and other membrane processes induced by CT fragments appear to be involved in the neurodegeneration and AD development (Selkoe, 1994, Suh, 1997, Suh *et al.*, 2000). In Paper II our aim was to study the effect of three CT sequences and transmembrane domain of APP on the [$^{35}$S]GTPγS binding to G-proteins in the hippocampal and frontocortical membranes obtained from age-matched control and AD postmortem brains. The effect of the most active CT sequence, truncated peptide PEP1, on the high-affinity GTPase and adenylate cyclase activity was examined and the involvement of plasma-membrane oxidative processes in the stimulation of [$^{35}$S]GTPγS binding by the PEP1 was elucidated.

In this study we have shown a structure-activity relationship of the tested APP sequences to the stimulation of [$^{35}$S]GTPγS binding to the membranes from AD and age-matched control hippocampus (Figure 2, Paper II). In the control membranes, the PEP1, elicited fivefold, PEP2, two-fold and the trans-membrane PEP4 2.5-fold stimulation at 10 μM concentration of the radio-

nucleotide binding. There is more than two-fold augmentative role of the same transmembrane domain for the PEP2-inducible stimulation of G-proteins in the human control hippocampus.

Decrease at the level of $G_o$- and minor types of G-proteins (Kolasa *et al.*, 2000, Cowburn *et al.*, 2001), decline in the G-protein GTP hydrolysis (Ross *et al.*, 1993, Garcia-Jimenez *et al.*, 2002) and impairment of the coupled signal transduction pathways for the more injured AD brain regions has been reported. In our experiments we have shown that PEP1 stimulation of [$^{35}$S]GTPγS binding to AD hippocampal and frontocortical membranes is significantly lower than in the corresponding control region (Figure 2 and 3, Paper II). PEP1 stimulation of high-affinity GTPase in AD hippocampus and frontal cortex revealed significant decrease compared to the age-matched control regions (Figure 4, Paper II). These data suggest that AD leads to considerable dysfunction/down-regulation of the G-proteins preferentially stimulated by the PEP1 sequence in the control regions. Certain differences in the decline of PEP1 stimulatory effect on G-proteins in AD hippocampus and frontal cortex (Figure 2 and 3, Paper II) could be explained by region-specific alterations of the membrane composition and of the G-protein garniture, induced by AD (Kolasa *et al.*, 2000, Cowburn *et al.*, 2001).

The effects of the studied peptide PEP1 on [$^{35}$S]GTPγS-binding and on GTPase and AC activities are summarized in Table 6.

**Table 6**. Influence of PEP1 on [$^{35}$S]GTPγS-binding and on the activity of GTPase and AC in hippocampal and frontocortical membranes from normalaging and Alzheimer's disease brain (Paper II).

| | Hippocampal membranes | | Frontocortical membranes | |
|---|---|---|---|---|
| | Age-matched control | AD | Age-matched control | AD |
| [$^{35}$S]GTPγS-binding $EC_{50}$ | 4.6 μM | 1.3 μM | 3.7 μM | 2.1 μM |
| GTPase activity $EC_{50}$ | 5.1 μM | 3.0 μM | 4.8 μM | 3.9 μM |
| Adenylate cyclase activity $EC_{50}$ | 7.6 μM | 11.0 μM | ND | ND |

ND: not determined

The effect of the PEP1 on the AC activity in the control and hippocampal membranes was studied. While the functional activity of $G_o$-protein, the major contributor to high-affinity GTPase, appears to be reduced, the ability of $G_i$ to inhibit cAMP signalling system is reported to be unaltered in the affected regions (Ross *et al.*, 1993, O'Neill *et al.*, 1994). O'Neill *et al.* have demonstrated a decrease in the activity of $G_s$-proteins and of the coupled cAMP system in

the more injured AD brain regions compared to controls (O'Neill *et al.*, 1994). Our studies have revealed a significant, 40%, stimulation of adenylate cyclase by the PEP1 sequence in the control hippocampal membranes. In AD hippocampal membranes this effect was significantly lower (Figure 5, Paper II). This might be explained by dysfunction of the $G_s$-proteins, which at normal conditions mediate a marked stimulatory signal from the truncated peptide to the enzyme. The 1.5-fold stimulation of [$^{35}$S]GTPγS binding by PEP1 in the membranes from Sf9 $G_s$ overexpressing cells corroborates the assumption that $G_s$ proteins are involved in the stimulation of [$^{35}$S]GTPγS binding by this peptide.

To elucidate the mechanism of stimulatory effect of the PEP1 on [$^{35}$S]GTPγS-binding in the hippocampal membranes, we investigated whether this effect could be modified by $H_2O_2$ as one of te reactive species. Reactive oxygen species have been shown to act as stimulators of signal transduction pathways (Suzuki *et al.*, 1997). It has been demonstrated that $H_2O_2$ behaves as a stimulator of [$^{35}$S]GTPγS binding to cardiac plasma membranes via the direct activation of the $G_i$ and $G_o$ protein α-subunits (Nishida *et al.*, 2000). Our results are consistent with these findings and show $H_2O_2$-stimulation of the [$^{35}$S]GTPγS binding to the control hippocampal membranes, the effect being remarkably higher than in the corresponding AD brain region (Figure 6A and B, Paper II).

We studied the effect of antioxidants on G-proteins stimulation by PEP1 in the control and hippocampal membranes. It is known that Aβ(25–35) and the other functionally active APP sequences increase the cellular level of reactive species (Yatin *et al.*, 1998, Smith *et al.*, 2000). PEP1-induced stimulation of G-proteins in the hippocampal membranes may have a complex oxidative stress mediated mechanism with the specific differences in generation of ROS between the control and AD group. In the Paper I we showed that classical free radical scavangers, GSH and NAC, protect neuronal cells and rat brain adenylate cyclase from Aβ(25–35) toxic effects (Paper I). In this study, GSH, NAC, and ferrous iron chelator desferrioxamine, caused significant decrease in the potent stimulatory effect of the truncated APP sequence on G-proteins in the hippocampal membranes (Figure 7, Paper II). Decreased protective ability of antioxidants against PEP1 stimulation in AD hippocampal membranes compared to the same control area suggests that G-proteins and coupled effectors of AD hippocampus are less protected against the PEP1-induced oxidative stress. A decline in the antioxidant capacity in the more injured regions of AD brain as hippocampus and associative cortex has been demonstrated previously (Lovell *et al.*, 1995, Karelson *et al.*, 2001). The accumulation of redox active iron in AD hippocampus, an important source of highly reactive free radicals and contributor of oxidative damage (Smith *et al.*, 1997b), might be an additional causative factor, lowering the protective effect of antioxidants against PEP1-stimulation of G-proteins.

In conclusion, the studied APP CT-fragments sequence-dependently stimulated the activity of G-proteins in the human control and AD hippocampal membranes. The PEP1 functions as a receptor in the control membranes, stimulating [$^{35}$S]GTPγS binding, activating high-affinity GTPase and transducing the stimulatory signals to adenylate cyclase. The PEP2 and transmembrane PEP4 reveal a weak stimulation of G-proteins and PEP3 is not capable to function as a signalling structure. In the membranes from the AD brain regions the stimulatory activity of the PEP1 was declined. The stimulatori effect of PEP1 on the control hippocampal G-proteins can be mediated by free radical induced mechanism prevented by antioxidants.

## 4.2. Characterisation of a new chimeric ligand for galanin receptors (Paper III)

To more caracterize AC-mediated pathways of signalling of GAL we studied chimeric peptides. In these experiments we showed the effect of a new chimeric galanin-NPY peptide, GAL(1–13)-[D-Trp$^{32}$]-NPY(25–36) amide, named M242, on AC activity and binding properties. Characterisation is given as a comparison of M242 with its parent peptide M32 (GAL(1–13)-NPY(25–36) amide) and with GAL itself (Table 5). We compared the binding properties of these peptides at two galanin receptor subtypes (hGALR1, expressed by Bowes cells, and hGALR2, overexpressed in CHO cells), and in rat hippocampal membranes. We did not address the affinities of M32 and M242 at GALR3, because it has been suggested that this receptor subtype is not functionally relevant for galaninergic signalling (Waters and Krause, 2000). We chose rat hippocampal membranes to study the effects of these peptides on cAMP formation and to get information about signal transduction.

[$^{125}$I]-Galanin displacement experiments demonstrate that all three ligands (GAL, M32 and M242) had comparable affinities at hGALR1 (<1 nM) and at hGALR2 (<10 nM) (Table 2, Paper III). When compared to M32 the affinities of M242 were slightly lower for both tested galanin receptor subtypes. At hGALR1 the difference was 1.4-fold ($K_D$ of 0.25 vs. 0.18 nM), whereas at hGALR2 it was 2.7-fold ($K_D$ of 5.84 vs. 2.18 nM). [$^{3}$H]-NPY-displacement studies indicated that M242 did not recognise hippocampal Y-receptors, while the affinity of M32 to the receptors was eightfold lower as compared to NPY itself (Table 2, Paper III).

Adenylate cyclase activity measurements in the ventral hippocampus revealed that M32 and M242 modulated cAMP production in a completely different manner. As seen from Figure 1a (Paper III) M242 modulated basal cAMP production biphasically. M32 inhibited the basal cAMP production

concentration-dependently with $IC_{50}$ value of 980 nM (maximal inhibition was 23.2%). Lower concentrations (0.1 nM–0.1 μM) of M242 caused a significant activation up to 27% of adenylate cyclase ($EC_{50}$ 1.9 nM), which declined to a 24% of inhibition at higher concentrations (0.1–10 μM) of the peptide ($IC_{50}$ 790 nM). The GAL itself inhibited cAMP production (Figure 1a, Paper III) with a maximal effect of 34.0% and an $IC_{50}$ of 0.7 nM. M32 and M242 had high affinities for galanin receptors in the ventral hippocampus. We studied the influence on these peptides on cAMP production. The stimulatory effect of M242 on cAMP production was observed in the 10–100 nM range and this was additive to the inhibitory effect of 1 nM GAL. The stimulatory effect disappeared when PLC inhibitor U-73122 (Thompson *et al.*, 1991) was included in the incubation mixture. The most likely explanation is that the stimulatory effect of M242 was a result of PLC activation leading to the activation of PKC sensitive adenylate cyclase (via inositol 1,4,5-trisphosphate as a second messenger). Studies in recombinant systems and cell-lines reveal that GALR1 and GALR3 couple to $G_i$- (Habert-Ortoli *et al.*, 1994, Smith *et al.*, 1998) and GALR2 to $G_q$-, $G_i$- and $G_{11}$- proteins (Wittau *et al.*, 2000). It seems possible that M242 activates both receptor species, GALR1 as a receptor coupled to adenylate cyclase inhibition and GALR2 as a receptor coupled to PLC activation. In ventral hippocampus GAL itself seems to be more efficient in activating GALR1 than GALR2.

In conclusion, the synthesized new peptide, GAL(1–13)-[D-$Trp^{32}$]-NPY(25–36) amide, named M242, differs remarkably from its parent peptide M32 (GAL(1–13)-NPY(25–36)). This peptide could be useful in the studies of signalling via different subtypes of galanin receptors.

## 4.3. Antiepileptic activity of a nonpeptide galanin receptor agonist (Paper IV)

Studies have reported that the first nonpeptide galanin receptor ligand Sch202596 displaces $^{125}$I-galanin with $IC_{50}$ value of 1.7 μM and the second low molecular weight galanin receptor ligand, dithiepin-1,1,4,4-tetroxide, with $IC_{50}$ value of 0.17 μM in Bowes cell membranes (Scott *et al.*, 2000). These compounds do not appear ideal for optimization because of their complex chemical structure and chemically reactive nature, respectively.

In this study we designed and synthesized Fmoc-cyclohexylalanine-Lys-amidomethylcoumarin, a low molecular weight, blood-brain barrier-penetrating galanin receptor ligand with agonist properties, named galnon. A combinatorial library of 256 compounds were synthesized and tested for the ability to displace $^{125}$I-galanin from galanin receptors in rat hypothalamic membranes. The

structure of galnon was deduced reiteratively in the synthesis. AMC-containing compounds were most active in $^{125}$I-galanin displacement assay and in the same way were identified the most active components of A and B. The synthesis of galnon is shown in Figure 9**.**

$^{125}$I-galanin was displaced by galnon in membranes from Bowes cells and rat ventral hippocampus with a $K_i$ value of 2.9 μM and 4.8 μM, respectively (Table 2, Paper IV). Decrease of $^{125}$I-galanin binding was detected at 1, 3, and 5 μM concentration of galnon by 23 ± 6%, 21 ± 10%, and 42 ± 11%, respectively.

The activity of galnon on adenylate cyclase activity was studied to elucidate whether it has agonist- or antagonist-like properties at galanin receptors. AC measurements in the rat ventral hippocampus showed that galnon inhibited both basal and forskolin-stimulated adenylate cyclase activity, like GAL (Table 2, Paper IV). Galnon (10 μM) inhibited basal cAMP production by 25%, whereas the inhibitory effect of 10 μM galanin was 36%. Inhibition of adenylate cyclase activity suggested that galnon exhibited agonist-like properties at galanin receptors.

Galanin agonists have the potential of making excellent anticonvulsants, because they may be able to inhibit a broad variety of seizures in the pathologically activated hippocampus (Mazarati *et al.*, 2000). The pentylenetetrazole model of epileptic seizures in mice was used to test the effects of systemic galnon. Galnon treatment lowered the maximal seizure score from 4.5 in control mice to 1.45, and increased the latency 3-fold of convulsions. The protection by systemic galnon from PTZ-induced seizures was comparable to galanin. Galnon abolished the proconvulsant effect of galanin receptor antagonist M35 (Figure 2, Paper IV).

Experiments in rats showed that galnon also possessed strong anticonvulsant effect against self-sustaining status epilepticus (SSSE), a particularly severe form of epileptic seizures that is resistant to conventional antiepileptic drugs (Mazarati *et al.*, 1998b)**.** The potency of galnon in our present study provides evidence that a systemically active galanin agonist can be an effective anticonvulsant in rodents and this anticonvulsant seems to act through peptide receptors.

We have shown that intrahippocampal administration of galnon shortened the duration of SSSE and decreased the time spent in seizures in a dose-dependent manner (Figure 3a, Paper IV). In the maximal dose used (5 nmol), galnon shortened SSSE duration to 28 min, from 760 min in controls. The anticonvulsant effects of galnon were abolished by pretreatment with the M35, when the latter was administered in a dose that alone did not alter the course of SSSE (0.5 nmol), as it has been previously reported (Mazarati *et al.*, 1998a), (Figure 3b, Paper IV). When galnon was injected immediately after M35, both time spent in seizures and the duration of SSSE were significantly higher than in galnon-treated rats without M35, and these parameters did not differ from those in control animals (Figure 3b, Paper IV).

We examined the anticonvulsant effects of galnon in SSSE. As shown previously, down-regulation of GALR1 by PNA1 did not affect the parameters of SSSE (Mazarati *et al.*, 2001). In PNA1-pretreated animals, galnon (1 nmol) had no effect on self-sustaining seizures, whereas in control rats injected with scrambled PNA, galnon reduced total seizure time 9-fold and duration of SSSE 7-fold (Figure 4, Paper IV).

Previously, intrathecal administration of 21-mer PNA decreased $^{125}$I-galanin binding in spinal cord (Rezaei *et al.*, 2001), suggesting that PNA1 may have caused down-regulation of GALR1 expression. It was also demonstrated by immunoprecipitation analysis that the same PNA oligomer down-regulates the expression of GALR1 *in vitro* (Pooga *et al.*, 1998b). We studied the effect of galnon on SSSE in PNA1-pretreated rats. Galnon completely failed to alter SSSE in the rats with GALR1 down-regulation and blocked SSSE in rats treated with PNAscr. This finding suggests that anticonvulsant effects of galnon may be mediated by means of GALR1.

It has been suggested that anticonvulsant activity of galanin is due to the modulation of glutamate release (Mazarati *et al.*, 2001). Differential profile of anticonvulsant effects of neuropeptides on SSSE suggests that along with common mechanisms of action, such as $G_i$-protein coupled effects on the second messenger signalling cascades and presynaptic inhibition of glutamate release, these peptides have different targets for counteracting seizure activity (Mazaraty and Wasterlain, 2002). Zachariou *et al*. (Zachariou *et al.*, 2003) have shown, that galanin agonists would be ideal analgesics because they would potentiate morphine analgesia and decrease morphine abuse potential.

We report that galnon, a nonpeptide ligand for galanin receptors, possesses agonist properties *in vitro* and *in vivo* and strong anticonvulsant properties *in vivo.*

# 5. CONCLUSIONS

1. Modulation of adenylate cyclase and GTPase activity by amyloid-β peptide Aβ(1–42), and its shorter fragments, Aβ(25–35), Aβ(12–28), were studied in isolated membranes from rat ventral hippocampus and frontal cortex. Aβ(25–35) had stimulatory effects on GTPase and AC activity in studied brain membranes, suggesting that the mechanism of action of Aβ peptides may also involve effects on G-protein mediated signal transduction. Aβ(12–28) did not affect the GTPase activity and weakly influenced AC. Activation of AC by Aβ(1–42) in hippocampal membranes was similar to that of Aβ(25–35) and no significant effect on AC in the cortical membranes was determined.

The results from this study show a response of G-protein, (probably $G_i/G_o$), coupled cAMP-signalling system to the neurotoxic Aβ peptide in the rat cortical and hippocampal membranes. The existing data suggest, besides amyloid aggregation mechanism, additional mechanisms of Aβ peptide effects in Alzheimer's disease.

2. The studied APP C-terminal fragments sequence-dependently stimulate the activity of G-proteins in the human control and AD hippocampal membranes. PEP1, consisting of transmembrane and short cytosolic sequence of APP, stimulates [$^{35}$S]GTPγS binding, activates GTPase and transduces the stimulatory signals to adenylate cyclase. PEP2 and PEP3 have a relatively weak stimulation of G-proteins. In the membranes from the AD brain regions the stimulatory activity of the PEP1 was declined. The stimulatory effect of PEP1 on the control hippocampal G-protein appears to be mediated by free radical induced mechanism and the effect is prevented by GSH, NAC and desferrioxamine.

3. The new chimeric galanin-NPY peptide, galanin(1–13)-[D-Trp$^{32}$]-NPY(25–36)amide, named M242, differs from its parent peptide M32 (galanin(1–13)-NPY(25–36)). M242 and M32 had comparable affinities at hGALR1 in Bowes cells and at hGALR2 overexpressed in CHO cells. In rat hippocampal membranes M242 has lower affinity than galanin and M32. M242 activates GALR1, as a receptor coupled to adenylate cyclase inhibition, and GALR2, as a receptor coupled to PLC activation in the hippocampal adenylate cyclase measurement assays.

4. A novel low molecular weight galanin receptor agonist, galnon, was designed, and synthesized. This compound displaces $^{125}$I-galanin with micromolar affinity at Bowes cellular and rat hippocampal membranes. Galnon inhibited basal and forskolin-stimulated adenylate cyclase activity similarly to galanin, it acts as an agonist at galanin receptors. Galnon has anticonvulsant activity in PTZ-treated mice. Galnon can be an effective anticonvulsant in rodents and deserves evaluation in a broad spectrum of seizure and epilepsy models.

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# SUMMARY IN ESTONIAN

## Galaniini ja amüloid-eellasvalgu signaaliülekanne läbi adenülaadi tsüklaasi

Adenülaadi tsüklaas on ensüüm, mille aktiivsust reguleeritakse G-valk-vahendatult erinevate retseptorite poolt ja mis omab tähtsat kohta raku signaaliülekande süsteemis.

Alzheimeri tõbi on neurodegeneratiivne kahjustus, mille patomorfoloogilisteks tunnusteks on seniilsed naastud ja neurofibrillaarsed kämbud. Need amüloidsed kogumid koosnevad peamiselt agregeerunud amüloid β-peptiididest (Aβ), mis tekivad amüloid-eellasvalgu (APP) lõhustumisel erinevate sekretaaside toimel. Lisaks Aβ peptiididele osalevad Alzheimeri tõve geneesis ka APP C-terminaalsed peptiidid. Paljud uurimused on näidanud, et Alzheimeri tõvest kahjustatud ajus on toimunud muutused raku signaaliülekande süsteemides ning on muutunud G-valkude aktiivsus. Aβ, tema toksilised fragmendid ja APP C-terminaalsed peptiidid avaldavad mõju signaali ülekandele rakus. Oksüdatiivse stressi mehhanismide vahendusel etendavad nad olulist osa Alzheimeri tõve neurodegeneratiivses protsessis.

Käesolevas töös on uuritud adenülaadi tsüklaasi aktiivsuse modulatsiooni APP-st lähtuvate peptiidide ja galaniiniga.

Antud töö raames sünteesiti Aβ lühemad fragmendid Aβ(25-35), Aβ(12–28) ja Lys-Aβ(16–20) ning uuriti nii täispika Aβ(1–42) kui ka sünteesitud fragmentide efekte adenülaadi tsüklaasi ning GTP-aasi aktiivsusele roti aju ventraalse hipokampuse ja frontaalkorteksi membraanpreparaatides. Saadud andmete põhjal võib järeldada, et Aβ ja tema fragmendid mõjutavad G-valkude poolt vahendatud signaaliülekande süsteeme. Antioksüdandid, glutatioon ja N-atsetüültsüsteiin vähendasid Aβ(25–35) mõju adenülaadi tsüklaasile nii roti aju ventraalse hipokampuse kui frontaalkorteksi membraanpreparaatides.

Uuritud APP C-terminaalsed fragmendid moduleerisid struktuursõltuvalt G-valkude aktiivsust kontroll ja Alzheimeri tõvega inimese postmortaalse aju erinevate regioonide membraanpreparaatides. Sünteesitud kimäärne peptiid (PEP1), mis sisaldas APP transmembraanset ja tsütosoolset aminohappelist järjestust, stimuleeris [$^{35}$S]GTPγS sidumist, aktiveeris GTP-aasi ja omas efekti adenülaadi tsüklaasile. Alzheimeri tõvega aju regioonides oli selle peptiidi aktiivsus vähenenud. PEP1 stimuleeriv efekt terve inimese hipokampuse G-valkudele vähenes seoses vabaradikaaliliste protsesside aeglustamisega glutatiooni, N-atsetüültsüsteiini ja desferrioksamiiniga. Teiste uuritud C-terminaalsete peptiidide mõju G-valkudele oli oluliselt nõrgem.

Neuropeptiid galaniin (GAL), isoleeritud ja iseloomustatud 1983. a. Stockholmis Tartu Ülikooli audoktori Viktor Muti laboratooriumis, omab rida bioloogilisi efekte, mis realiseeruvad G-valk-seotud galaniiniretseptorite vahen-

dusel. Paljud uurimused on näidanud, et galaniin ja galaniiniretseptorid (GALR), vastupidiselt paljudele teistele neurotransmitteritele, on Alzheimeri tõve korral üleekspresseritud. cAMP-signaalrada, mille põhikomponendiks on adenülaadi tsüklaas, on olulisim rada, mille kaudu galaniini agonistid või antagonistid avaldavad oma füsioloogilist või farmakoloogilist mõju.

Kimäärsed peptiidid on leidnud laialt kasutamist G-valk-seotud retseptorite uurimisel. Uus sünteesitud kimäärne galaniin-NPY peptiid, galaniin(1-13)-[D-Trp$^{32}$]-NPY(25-36)amiid, M242, erineb kimäärsest peptiidist galaniin(1-13)-NPY(25-36)amiid, M32, ja galaniinist endast. Sidumiskatsed näitasid, et võrreldud kolm peptiidi on sarnase afiinsusega inimese hGALR1 ja hGALR2 korral, roti hipokampaalmembraanide retseptorites on aga M242 afiinsus oluliselt madalam. M242 võib aktiveerida nii GALR1 kui GALR2. Adenülaadi tsüklaasi mõõtmise tulemused roti hipokampuse membraanides näitasid, et M242 moduleerib cAMP-i teket bifaasiliselt.

Galaniini bioloogiliste funktsioonide hulka kuulub ka antikonvulsiivne toime. Galaniini antikonvulsiivsed omadused realiseeruvad galaniini retseptorite kaudu. Tuginedes teadaolevatele galaniini farmakofooridele, disainiti selle töö raames kombinatoorne raamatukogu ning saadud tulemuste alusel sünteesiti madalmolekulaarne galaniini retseptori ligand galnon. Roti hipokampuse membraanides galnon inhibeeris adenülaadi tsüklaasi. Edasised uuringute tulemused näitasid, et galnon evib agonisti omadusi *in vitro* ja *in vivo* ning antikonvulsiivseid omadusi *in vivo*.

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Soomets U., **Mahlapuu R.**, Tehranian R., Jarvet J., Karelson E., Zilmer M., Iverfeldt K., Zorko M., Gräslund A., Langel, Ü (1999). Regulation of GTPase and adenylate cyclase activity by amyloid β-peptide and its fragments in rat brain tissue. Brain Res. 850 (1–2), 179–188.

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# CURRICULUM VITAE

## RIINA MAHLAPUU


| | |
|---|---|
| Born: | July 09, 1951 in Märjamaa, Estonia |
| Citizenship: | Estonian |
| Marital status: | Single, one child |
| Address: | Institute of Biochemistry University of Tartu<br>19 Ravila Str.<br>51014 Tartu, Estonia |
| Tel.: | 372 7 374 313 |
| e-mail: | mahla@ut.ee |


### Education

| | |
|---|---|
| 1969–1974 | Student, Department of Chemistry, Faculty of Physics and Chemistry, University of Tartu |
| 2000 | Master of Sciences (chemistry), University of Tartu, Estonia |

### Employment and professional experience

| | |
|---|---|
| 1974–1976 | teacher, Kadrina Secondary School |
| 1976–1979 | senior engineer, Department of Organic Chemistry, University of Tartu, Estonia |
| 1979–1983 | researcher, Department of Organic Chemistry, University of Tartu, Estonia |
| 1983–1988 | senior researcher, Department of Organic Chemistry, University of Tartu, Estonia |
| 1988–1996 | researcher, Department of Organic Chemistry, University of Tartu, Estonia |
| 1996–2000 | senior laboratory assistant, Institute of Biochemistry, University of Tartu, Estonia |
| 2000–present | researcher, Institute of Biochemistry, University of Tartu, Estonia |

## Publications


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# *CURRICULUM VITAE*

## RIINA MAHLAPUU


| | |
|---|---|
| Sündinud: | 9. juuli 1951 Märjamaa |
| Kodakondsus: | Eesti |
| Perekonnaseis: | Vallaline, üks tütar |
| Address: | Biokeemia Instituut |
| | Tartu Ülikool, Ravila 19 |
| | 51014 Tartu, Eesti |
| Tel.: | 372 7 374 313 |
| e-mail: | mahla@ut.ee |


### Haridus

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|---|---|
| 1969–1974 | Tartu Ülikooli keemiaosakonna üliõpilane |
| 1974 | B.Sc. keemia |
| 2000 | M.Sc. keemia |

### Erialane teenistuskäik

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|---|---|
| 1974–1976 | õpetaja Kadrina Keskkoolis |
| 1976–1979 | Tartu Ülikooli Orgaanilise keemia kateedri vaneminsener, |
| 1979–1983 | Tartu Ülikooli Orgaanilise keemia kateedri teadur, |
| 1983–1988 | Tartu Ülikooli Orgaanilise keemia kateedri vanemteadur, |
| 1988–1996 | Tartu Ülikooli Orgaanilise keemia kateedri teadur, |
| 1996–2000 | Tartu Ülikooli Biokeemia Instituudi vanemlaborant, |
| 2000–praegu | Tartu Ülikooli Biokeemia Instituudi teadur |

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