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# Model Systems and Approaches to Study the Metabolism of Alzheimer's Amyloid Precursor Protein

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It is believed that amyloid precursor protein has a central role in the etiology of Alzheimer's disease, because of the β amyloid peptide contained in its structure. Here we present different methods employed to study the metabolism of APP, such as the use of native brain slices, isolated synaptosomal fractions, specific cholinergic immunotoxins, transgenic animals and monitoring the expression of amyloid precursor protein during ontogenesis.

*Key words:* Alzheimer's disease, APP, Aβ, animal models, ontogenesis.

## Introduction

Alzheimer's disease is the most common degenerative disease of the human brain. The major risk factor in this disease is age and the incidence of the disease increases and reaches 50% in people older than 85 years. By the constant change of the demographic structure of the society toward the older group of people the number and percentage of sufferers is increasing steadily and Alzheimer's disease (AD) is becoming a serious social and economic problem.

Alois Alzheimer first described in 1906 the morphological changes in the brain characteristics for this type of dementia – degeneration of cortical neurons, extracellular neuritic plaques and intracellular neurofibrillary tangles. Neurofibrillary tangles are constituted of the abnormally phosphorylated cytoskeletal τ-protein that participates in the formation of the cytoskeleton and the core of senile plaques is composed of aggregated amyloid β peptide (Aβ).

Some 80 years later, it was clarified that Aβ is part of a larger protein – the amyloid precursor protein (APP). APP is an integral membrane glycoprotein and consists of a large extracellular portion, a transmembrane portion and a short intracellular carboxy-terminal portion. It contains the amino acid sequence of Aβ, which has 39 to 43 amino acids.

The main route of degradation of APP in a healthy brain is via an enzyme called α-secretase, which cuts within the sequence of Aβ and thus makes impossible its aggregation to form the core of senile plaques. There are alternative ways of degradation, which are much less active. There are the β- and γ-secretases that cut at the amino, respectively at the carboxy terminus of Aβ. In sufferers of AD the balance between these enzymes is impaired and β- and γ-secretase activities are increased. As a result Aβ remains intact and can be aggregated as the core of senile plaques.

Therefore, the mechanisms which regulate the processing of APP are of particular interest, since an error in the cascades that regulate, respectively modulate its secretion could lead to a change in the balance between these enzymes.

## Materials and Methods

Studies on the processing of APP are preferably carried out on cell cultures, which are often transfected. This method has its drawbacks. Cell cultures do not always reproduce exactly the processes in the brain tissue. Various cell types and various culturing conditions may affect the manner of processing of APP.

We established a relatively easy model to study the secretion of APP in native brain slices, i.e. under conditions much closer to those in the intact brain tissue [3].

For this purpose cortex of rat brain was dissected and cut on a McIlwain tissue chopper in 300 μm thick slices. They were washed in Krebs-Ringer solution at 37ºC, pH 7.4, in a cell culture incubator ($O_2/CO_2$ = 95%/5%) with repeated change of buffer. The dynamics of release of APP in the incubation medium showed that during the first 15-20 min there is a rapid increase, then it decreases and reaches a constant value after 45-60 min. For this reason we chose an equilibration time of 1h. Then, 3 slices were transferred to 250 μl Krebs-Ringer solution containing an appropriate agent, resp. Krebs-Ringer solution without additives as a control, and were incubated for 30 min.

Incubation was terminated by centrifugation whereby the slices were pelleted and the incubation medium was collected and lyophilized to concentrate the contained protein and the lyophilisates were prepared for immunoblotting.

Our first task was to prove that in the incubation medium there are only secreted forms of APP, i.e. cut from the C-terminal end. The group of Prof. Konrad Beyreuther from Heidelberg University provided us with polyclonal antibodies recognizing the C- and N-ends of APP and with them we performed immunoprecipitation once in the incubation medium and once in the slices and investigated the precipitants. We were able to demonstrate that in the incubation medium there are only secreted forms of APP truncated at the C-terminus.

## Results and Discussion

It is reported that many transmitter systems are affected in AD. The most studied one is the cholinergic system and the different types of cholinergic receptors. In patients with AD a hypofunction of the glutamate neurotransmitter system was also observed. We performed a series of experiments to establish whether glutamate is modulating the secretion of APP, using the model of native brain slices.

The stimulation with different concentrations of glutamate showed glutamate influences the secretion of APP in a concentration dependent manner (**Fig. 1**). It could be expected that, upon reaching the most effective glutamate concentration (50 μM) a plateau would be reached. The reduction of the secretion at higher concentrations

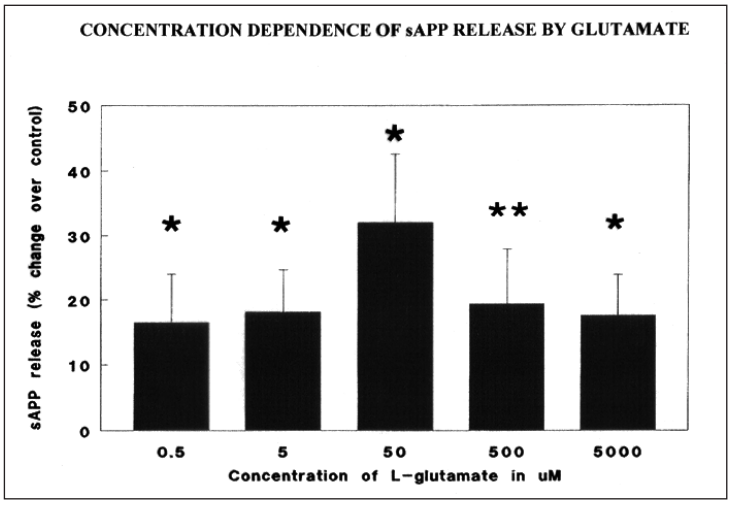


**Fig. 1.** Quantification of the effect of various concentrations of glutamate on the release of secretory forms of APP

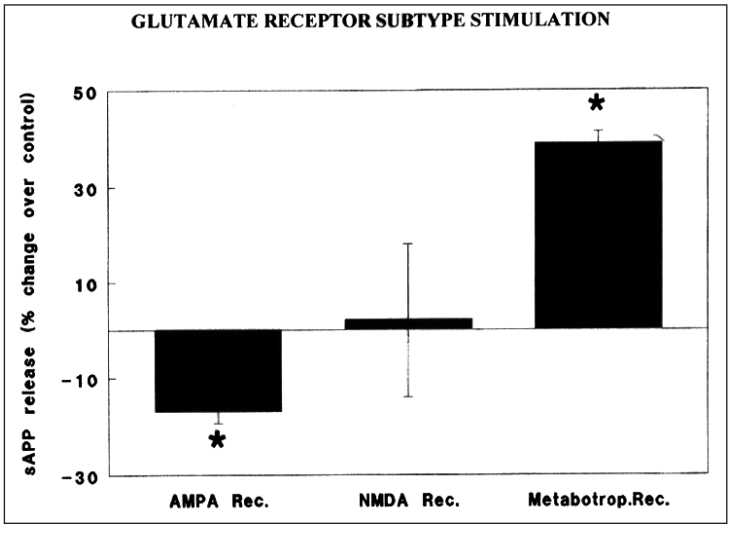


**Fig. 2.** Effect of the stimulation of different glutamate receptors on the APP secretion

could mean that at different concentrations, different subtypes of glutamate receptors are affected. To clarify this, in the next series of experiments we stimulated different glutamate receptors with their agonists.

Stimulating the AMPA-receptor results in a decrease of the secretion of APP, the stimulation of the NMDA-receptor had no effect, and the stimulation of the metabotropic Glu-receptor lead to increased secretion (**Fig. 2**). These findings can explain the biphasic character of the glutamate concentration dependence of APP release.

The data for neurotransmitter control of APP secretion directed us to choose isolated synaptosomes as the next model system for its study [5]. Synaptosomes were isolated from rat forebrain by homogenizing the tissue and separation of the homogenate

in subcellular fractions by density gradient centrifugation. Aliquots of the synaptosomal fraction were stimulated with different glutamate concentrations (**Fig. 3**).

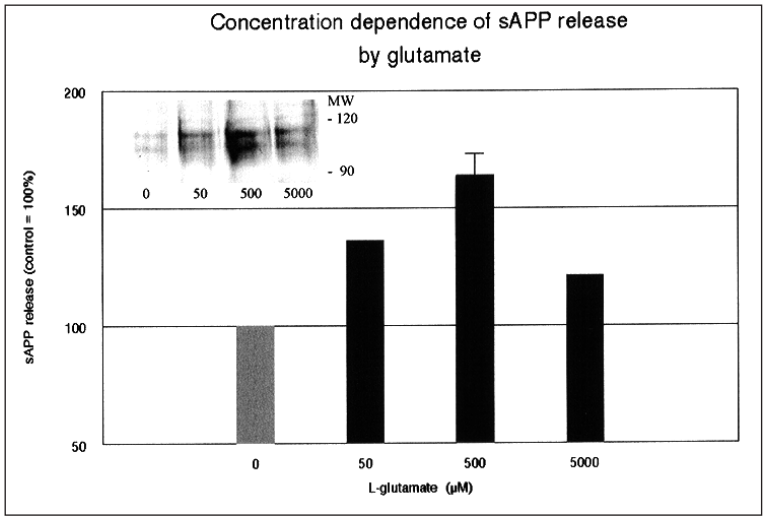


**Fig. 3.** Stimulation of sAPP release in synaptosomal fractions by different glutamate concentrations

Compared to the stimulation of native brain slices we observed a two-fold increase of the secretion. The weaker effect of glutamate on the slices may be due to the complex relationships between various cell populations; to the more difficult penetration of glutamate in the tissue; to difficulties of detecting of the APP metabolites because of their internalization into cell populations or to binding to the extracellular matrix.

Protein kinase C (PKC) participates in the metabotropic glutamate receptor cascade. In cell culture studies it has been shown that stimulation of other neurotransmitter receptors, which interact with PKC, leads to an increase in the secretion of APP. Direct stimulation of PKC brings about the same result. Therefore, we performed a direct stimulation of PKC with phorbol ester in the synaptosomal fraction.

It can be seen that direct stimulation of PKC has a stronger stimulating effect on the secretion than glutamate (**Fig. 4**). This is probably due to the fact that only part of the synaptosomal population possesses receptors responsive to stimulation by glutamate, and a larger portion are more responsive to stimulation with PKC.

These studies show that native synaptosomes and brain slices are suitable models for studying the secretion of APP, as well as the role of the different types of receptors in this process.

The model with brain slices was also used to study the effects of the vascular endothelial growth factor (VEGF) on the metabolism of APP [1]. For this purpose we used transgenic mice (Tg2576) containing human APP, carrying the double mutation of the so-called Swedish type. The mutation was named so because it was found in a large family in Sweden, where an early development of AD was observed. The children have a 50% chance to develop AD and to lose their memory about their 50th anniversary. The discovery of this family type mutation is of great importance to the study of AD, because mice transfected with this mutation develop with age morphological and behavioral changes corresponding to those of AD (plaques, memory loss) and therefore are a good animal model of the disease.

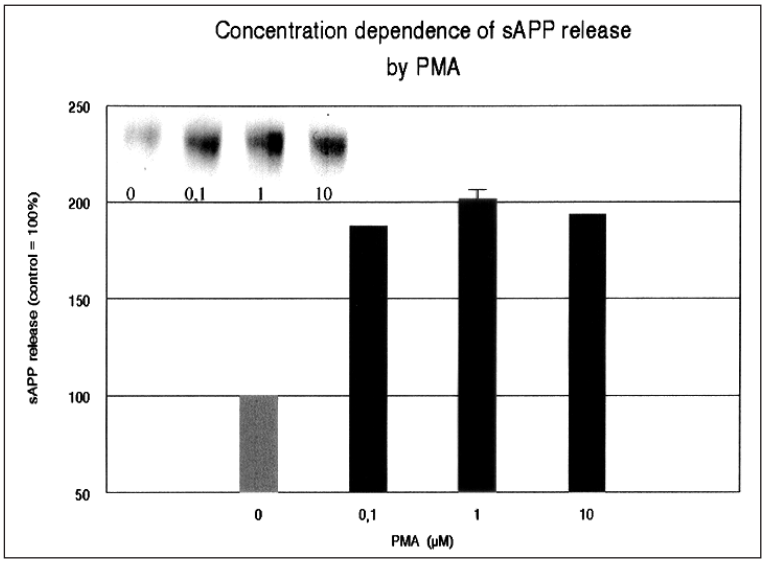


**Fig. 4.** Stimulation of sAPP release in synaptosomal fractions by different phorbol-12-miristrate-13-acetate (PMA) concentrations

Brain slices were prepared from 17-month-old animals, incubated with VEGF and the secretion of APP, Aβ formation and activity of α- and β-secretases were examined.

The results give reason to believe that VEGF is involved in the processing of Aβ – at least *in vitro*.

We established a further model system in which we used the properties of the immunotoxin 192IgG-Saporin, which consists of a monoclonal antibody against the low-affinity nerve growth factor receptor (LNGF-R), and is conjugated with the ribosome inactivating protein Saporin. The specific location of the LNGF-R on cell bodies and synaptic terminals of cholinergic neurons in the basal forebrain allows to deliver 192IgG-Saporin to that cholinergic population by an intracerebroventricular injection, without affecting cholinergic populations in other areas of the brain and non-cholinergic neurons in the basal forebrain.

The modulation of APP processing by cholinergic activity is shown in several cell cultures, transfected cell lines as well as in native brain slices, but a demonstration of receptor mediated control of APP metabolism under *in vivo* conditions is lacking. To achieve this we specifically reduced cortical cholinergic innervation in rats by partial immunolesion with 192IgG-Saporin. Subsequently the cholinergic function in lesioned rats was restored by transplantation of NGF-producing fibroblasts [6].

The total APP fraction in the cortex was not affected by this deinervation, but there was a marked reduction in the secreted APP-form accompanied by an increase in membrane-bound APP. These changes were reversible with transplantation of NGF-producing fibroblasts.

This in vivo model shows that the processing of APP in the cortex is under cholinergic control from the basal forebrain.

The same type of transgenic mice (Tg2575) were used for the creation of another animal model of AD. The cholinergic immunotoxin mu p-75-Saporin was injected in the 3rd ventricle of 6- to 8-month-old Tg2575 mice to cause cholinergic denervation. Four weeks later there was a 14-fold increase in soluble Aβ1-42 (the most active form in AD) [2].

The lesion leads to atrophy of the hippocampus and to reduction of the expression of synaptophysin, which indicates the degeneration of synaptic contacts – a feature of AD.

The cholinergic agonist carbachol counteracts these effects, indicating that the use of drugs supporting cholinergic transmission (cholinomimetics) must be taken into account in the treatment of AD.

The hippocampus (Hp) is innervated by cholinergic neurons in the basal forebrain and the disruption of their function, respectively of the cholinergic innervation of the Hp and the cerebral cortex is considered to be one of the causes of AD.

This animal model encompasses both the amyloid pathology, characteristic of Tg2575 mice, and the atrophy of the Hp, combined with synaptic dysfunction, which are also characteristic for AD. This makes the model an exceptionally suitable system for studying the etiology of AD.

Recently in a joint work with colleagues from the Paul Flechsig Institute for Brain Research, Leipzig, we conducted research on the role of the Smad family of proteins in AD (results not published). Smad proteins are transcription factors which also control the expression of APP. We used three types of transgenic animals – with increased expression of wild-type APP; with Swedish mutation; and triple transgenic: with Swedish mutation, mutation of τ-protein leading to another prominent morphological change in the brain – neurofibrillary tangles, and mutation of presenilin, which is part of the γ-secretase. A mutation in the presenilin-genes in familial forms of AD is found and it is believed that it results in increased formation of Aβ.

To get more information on the physiological role of APP, we studied the changes in the expression of APP in ontogeny [4]. The data of APP content in brain homogenate from embryonic day 20 to day 400 after birth are presented on the next figure. There is an increase during the period around the peak of synaptogenesis (**Fig. 5**).

We also examined the content of APP in isolated growth cones and synaptosomes (**Fig. 6**).

The pronounced increase of the concentration of APP in homogenate, growth cones and synaptosomes during the most intense establishment of cell contacts un-

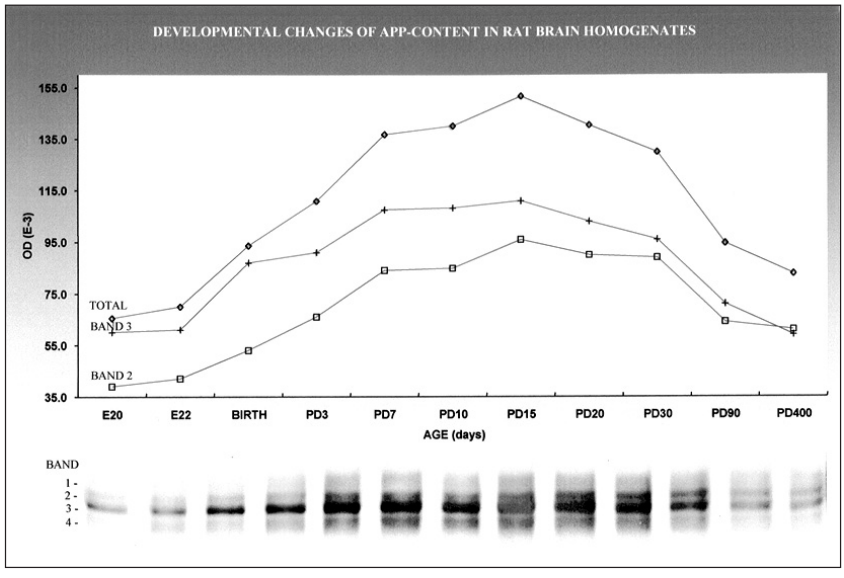


**Fig. 5.** Changes in APP content in rat forebrain during pre- and postnatal development

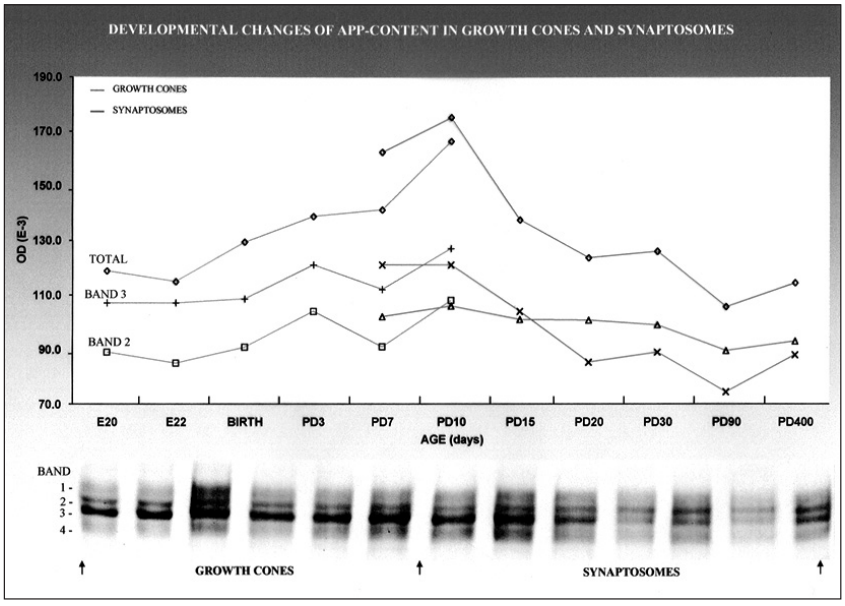


**Fig. 6.** Changes in APP content in growth cones and synaptosomes isolated from rat forebrain

derlines the importance of APP for the processes of synaptogenesis. APP is probably actively involved in finding the path from growth cones, the establishment of synaptic contacts and the maintenance of normal synaptic functions.

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