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(vypracováno na Welsh School of Pharmacy, Cardiff University, UK)

Sledování pohybu protilátky proti amyloidnímu prekurzorovému proteinu v buňce

(diplomová práce)

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Žatec, 2008 Jan Zitko

CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY

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Department of Pharmaceutical Chemistry and Drug Control

(Project of Welsh School of Pharmacy, Cardiff University, UK)

Intracellular trafficking of an anti-Amyloid Protein Precursor antibody

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Žatec, 2008

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DECLARATION

This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

PROHLÁŠENÍ

Tato práce je výsledkem mého samostatného výzkumu, pokud není vyznačeno jinak. Všechny ostatní zdroje jsou řádně citovány v seznamu použité literatury.

Signed ……. (Candidate)

Date …….

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LIST OF ABBREVIATIONS

2B12	monoclonal mouse antibody raised against amino acid sequence spanning the beta-cleavage site of APP
AD	Alzheimer's Disease
APP	β-amyloid precursor protein – Integral membrane protein expressed by many tissues. Its proteolytic cleavage by beta-secretase produces Aβ.
Avidin-FITC	avidin conjugated to fluorescein
Aβ	β-amyloid peptide – 39-43 amino acid peptide derived from APP. Primary component of amyloid plaques in AD.
BACE	beta-site APP-cleaving enzyme, also known as beta-secretase – Aspartyl protease involved in amyloidogenic cleavage of APP
Bcl-2	well known oncoprotein involved in regulation of apoptosis, it was used as a marker of endoplasmic reticulum in this project
BSA	bovine serum albumin
CNS	central nervous system
EEA1	Early Endosomal Antigen 1 – early endosomes marker
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate – fluorescent dye widely used in biological experiments
GA	Golgi apparatus
Hsp 60	Heat Shock Protein 60 **–** mitochondrial membrane marker
ICC	immunocytochemistry or immunocytochemistry experiment(s)
LAMP1	Lysosome-Associated Membrane Protein 1 – lysosomes marker
M6PR	Mannose-6-Phosphate Receptor (Cation Independent) – late endosomes marker
MAb	monoclonal antibody
MOG	astrocytoma MOG-G-UVW, cells constitutively producing APP, used as a cellular model of AD
PBS	phosphate buffer saline – see Methodological Framework for formula
TBST	Tris-buffered saline with Tween 20 – see Methodological Framework for formula
TGN	trans-Golgi network

CONTENTS

1. INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a neuropathology, clinically characterized by progressive and gradual impairment of CNS functions, especially progressive memory decline, disordered cognitive function, altered behaviour including paranoia, delusions, and loss of social appropriateness, and a progressive decline in language function. AD is the most common cause of dementia affecting 24 million people worldwide. This number is predicted to increase to 81 million by 2040 (Ferri et al., 2005). As a clinicopathological syndrome it was first described by German neurologist and psychiatrist Alois Alzheimer in 1906 (Alzheimer, 1907). Besides reporting a general neuronal dystrophy Alzheimer even described the basic pathological structures he observed in cerebral cortex – foci of extracellular accumulation of a fatty substance (today known as amyloid plaques) and intraneuronal pathological tangles (termed neurofibrillary tangles today).

However, dementia was considered to be a normal part of ageing process for following 50 years. In 1960s, Kidd and Terry were the first to investigate the neuronal tissues of dementia sufferers using electron microscopy, and both of them described the ultrastructure of amyloid plaques and neurofibrillary tangles (Kidd 1963; Terry 1963a; Kidd 1964; Terry 1963b; Terry et al. 1964). The connection between these pathological lesions and dementia became obvious and AD was recognized as a serious process leading to presenile dementia.

The hunt for the chemical basis of compounds forming the lesions took more than 20 years. Masters et al. (1985) found that the core substance of amyloid plaque was identical with the peptide named beta-amyloid (Aβ) isolated and sequenced from meningeal blood vessels of AD and Down syndrome sufferers a year before (Glenner et al., 1984). Neurofibrillary tangles were later reported to be made of microtubule-associated tau protein (among others Grundke-Iqbal et al, 1986a) and that tau protein molecules are pathologically overphosphorylated (Grundke-Iqbal et al, 1986b).

Consecutive discoveries in following years led to the formulation of so called amyloid cascade hypothesis of AD (Hardy and Higgins, 1992), which elucidates the biochemical processes leading to accumulation of Aβ in the form of senile plaques, claiming that Aβ is the main cause of AD. This hypothesis is recognized by most of the researches today, although some believe tau protein plays the major role in the pathogenesis rather than Aβ.

Genetics helped a lot to understand the ethiology of AD, although our knowledge is not thorough and complete yet. The gene encoding the beta-amyloid precursor protein (APP), the substance which Aβ is generated from, was found and located to chromosome 21 (Kang et al., 1987). This corresponded with the previously observed fact that Down syndrome (trisomy of chromosome 21) leads invariantly to presenile dementia with Aβ deposits. Mutations in APP gene are connected to the familial early-onset form of AD, however they are the less common cause in comparison with mutations in presenilin genes (PS1 and PS2) – presenilins are part of γ-secretase complex, which contributes to Aβ generation from APP. Both types of mutations are passed over in autosomal dominant way (Selkoe, 2001). Even sporadic non-familial forms of AD are supposed to be strongly influenced by genetics. There are many candidate genes, but Apolipoprotein E mutations (ApoE4 polymorphism) represent the only confirmed risk factor today (Selkoe, 2001). Research into genetic basis of AD continues and many candidate genes are tested nowadays. Marx (2007) reports a SORL1 gene, whose product regulates the trafficking of APP.

1.2 The role of amyloid precursor protein (APP) in AD

Beta-amyloid precursor protein (APP) is a 100-140 kDa integral type I glycoprotein. It's constitutively expressed and trafficked to the cell surface. APP is highly conservative in evolution and was found in all mammals tested so far (Selkoe, 2001). Alternative splicing provides 3 main isoforms of APP, with the length of 770, 751 and 695 amino acids, respectively. APP695 dominates in neuronal cells, while longer forms APP770 and APP751 in other cells including glial cells. The longer forms contain a Kunitz-type protease inhibitor domain. APP consists of a large extracellular domain (ectodomain)–

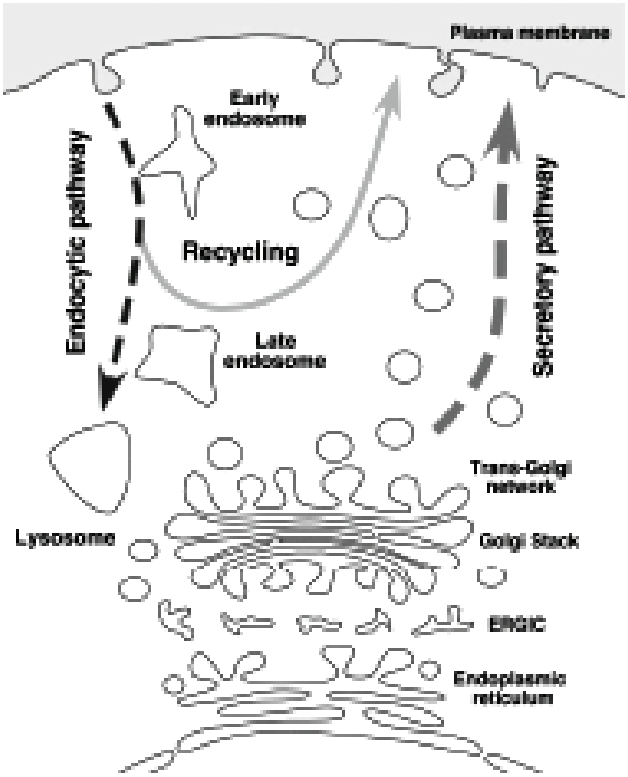


Fig. 1.1 Schematic diagram of APP trafficking (Vetrievel, 2006)

N-terminus and a small cytoplasmic tail – C-terminus. Aβ domain is situated within the transmembrane domain.Synthesised APP is trafficked to endoplasmic reticulum (ER) and continues through constitutive secretory pathway to the cell surface (it's estimated that only about 10% of nascent APP molecules are successfully delivered to the plasma membrane (Vetrievel, 2006)). APP is a subject to N-glycosylation in the ER, O-glycosylation in the Golgi apparatus (GA), phosphorylation and tyrosine sulfatation. Unlike other cell surface receptors, APP doesn't stay on the surface for long. On the plasma membrane, APP can be either proteolytically processed primarily by α-secretases (non-amyloidogenic processing), resulting in the secretion of APPsα ectodomain (Sisodia, 1992). The amount of APP molecules processed by α-secratase on the cell surface is about 30 percent (Koo et al., 1996). The rest of APP is rapidly internalized via clathrin-mediated endocytosis. The bulk of internalized APP appears to be destined for lysosomal degradation after transiting through the endosomal compartment, although a population is rapidly recycled to the cell surface for subsequent internalization and presumably secretion as well (Koo et al., 1996). It's unclear how many endocytic/recycling steps can follow, but this circulation is the place where significant portion of beta-amyloid (Aβ) is produced by amyloidogenic processing - see further (Koo et al., 1994).

1.3 Production of beta-amyloid (Aβ) and its role in AD pathology

As mentioned above in section 1.1, Aβ plays a central role in the pathology of AD according to amyloid cascade hypothesis. Amyloid plaques, one of dominant pathological changes in AD sufferers' brains, are composed mainly of highly insoluble Aβ. It might be pronounced that Aβ has toxic effect on neuronal tissues. Therefore, evidence that Aβ is produced and secreted by cultured cells in normal conditions (Haass et al., 1992) and can be isolated from biological fluids of healthy subjects (Seubert et al., 1992) was quite surprising. Considering the fact Aβ is a usual metabolite of living cells, we have to match its pathological properties rather to its over-accumulation than to it alone.

Aβ is produced by proteolytic cleavage of APP. Generally, there are two ways of proteolytic processing of APP. Amyloidogenic processing (dominant in neurons) leads to neurotoxic Aβ, while nonamyloidogenic pathway (favoured in all other cell types) produces

rather soluble substances, some of them even believed to have neurotrophic properties (see further).

Processing of APP occurs in sequential events that involve shedding of the ectodomain by either α-secretase (nonamyloidogenic pathway) or β-secretase (amyloidogenic pathway) to generate membrane-tethered α- and β-C-terminal fragments (CTFs), respectively. α-secretase cleaves APP within the Aβ domain (between residues Lys16 and Leu17 of Aβ), essentially precluding the generation of intact Aβ. A multimeric protein complex, termed γ- secretase, subsequently cleaves these α- and β-CTFs within the transmembrane domain, to generate p3 (3 kDa) and Aβ (4 kDa) peptides, respectively. Both Aβ and p3 peptides are readily secreted by cultured cells (Citron, 2002).

α-APPs, originating in the nonamyloidogenic pathway was reported to have neuroprotective and neurotrophic properties (Mattson et al., 1993).

Recently it has been discussed which form of Aβ represents the neuronal toxicity. There were cases of people having Aβ deposits but no Alzheimer's cognitive symptoms. It seems that not insoluble Aβ polymeric aggregates, but smaller and soluble oligomeric Aβ species posses the impairing ability. Amyloid plaques therefore represent inactive reservoirs, which are in equilibrium with soluble forms of Aβ (Selkoe, 2001).

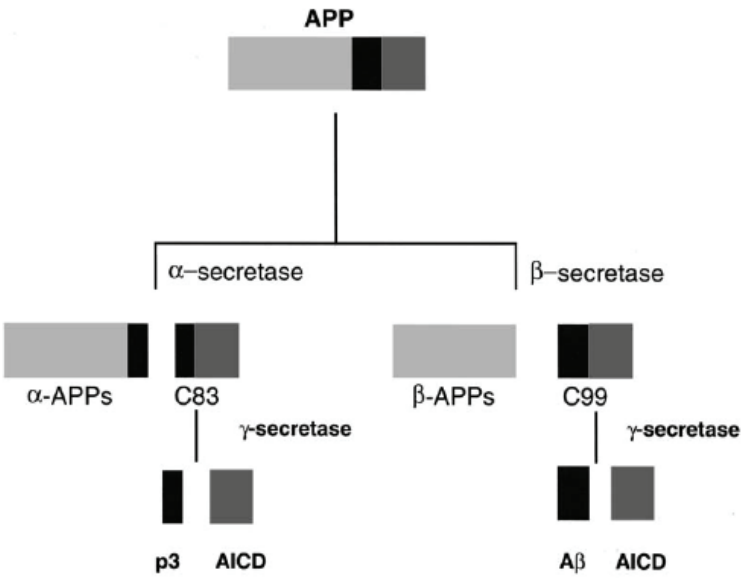


Fig. 1.2 Schematic of the APP and its metabolites relevant to Alzheimer's disease (not drawn to scale). APP can be processed along two major pathways, the α-secretase pathway and the amyloid forming β-secretase pathway. In the α-secretase pathway, α-secretase cleaves in the middle of the Aβ region to release a large soluble APP fragment, α-APPs. The C-terminal C83 peptide is metabolized to p3 and AICD (APP intracellular domain) by γ-secretase. In the amyloid forming β-secretase pathway, β-secretase releases a large soluble fragment, β-APPs. The C-terminal C99 peptide is then metabolized to Aβ and AICD by γ-secretase. β-secretase inhibitors block the formation of β-APPs and C99; γ-secretase inhibitors block the formation of p3, Aβ and AICD. (Citron, 2002)

1.4 Alpha-, Beta- and Gamma- Secretases

To date, several proteins have been identified that have **α-secretase**-like activity. They are membrane bound disintegrin and metalloproteinases including ADAM17 (also called TACE), ADAM10 and ADAM9 belonging to the adamalysin family of proteins. The constitutive α-secretase activity is primarily at the cell surface, while the regulated activity is predominantly located within the Golgi (Ling et al., 2003).

The protein with **β-secretase** activity was isolated in 1999 (Vassar et al., 1999; Hussain et al., 1999; Sinha et al., 1999; Yan et al., 1999). So called BACE1 (beta-site APP-cleaving enzyme) is a novel transmembrane aspartyl protease and is predominantly active in brain. BACE2, with 64% amino acid similarity to BACE1, is localized rather in peripheral tissues and probably does not participate in Aβ production. The physiological function of BACE2 remains unknown (Citron, 2004). BACE1 is the key rate-limiting enzyme that initiates the formation of Aβ (Vassar, 2001).

γ-secretase is a membrane bound multimeric complex. The minimal components of γ-secretase include PS1 or PS2, nicastrin, APH-1, and PEN-2 (Vetrievel et al., 2006). The role of presenilins seems to be extremely important, note the connection between AD and PS mutations. Available data indicate the presence of γ-secretase complex and enzyme activity in multiple compartments including the ER, late-Golgi/TGN, endosomes and plasma membrane. Recent studies estimated that only 6% of γ-secretase activity is at the cell-surface (Vetrievel et al., 2006).

1.5 Therapy of Alzheimer's disease (AD)

Currently, there are no disease-modifying therapies available for Alzheimer's disease (AD) (Hüll et al,. 2006). Nowadays used and recommended therapies are represented by attempts to reduce negative symptoms, especially cognitive impairment. Available treatments for AD target the cholinergic system with **acetylcholinesterase inhibitors** (AChEIs; Donepezil, Rivastigmine and Galantamine) or the glutamatergic system with competitive blockade of the NMDA receptor with memantine (Hüll et al., 2006). These two strategies are the only recommended treatments by the National Institute of Health and Clinical Excellence (NICE) guidelines for AD (NICE, 2007).

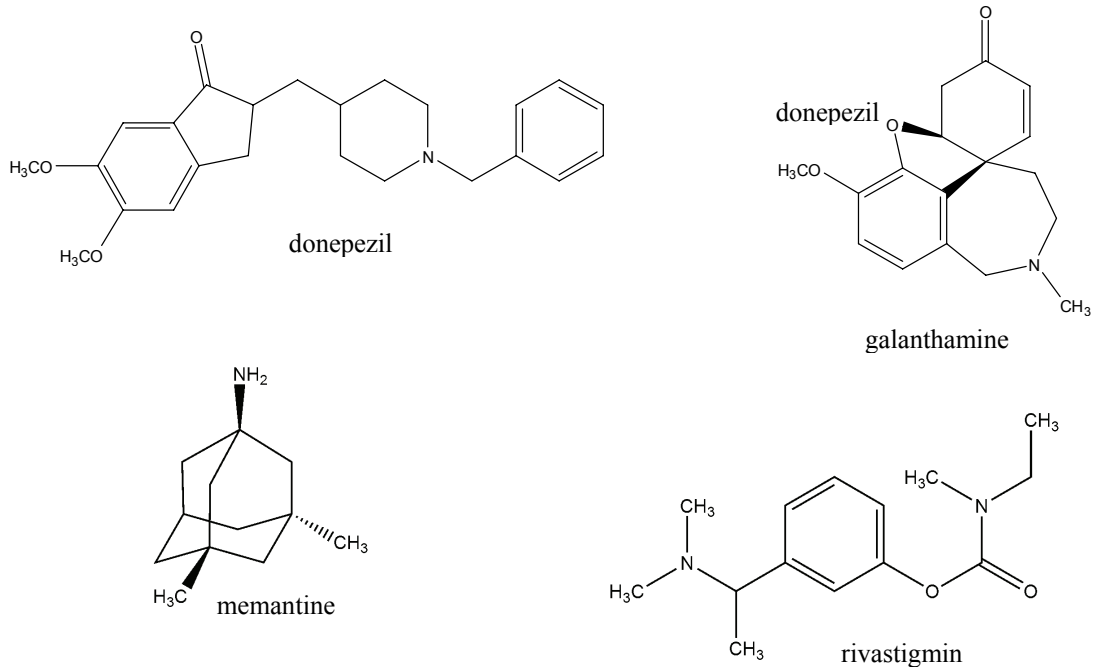


Fig. 1.3 Structural formulas of drugs approved and recommended by NICE for AD treatment

Disease – modifying therapeutic approaches are subject to intense investigation. Most of these approaches are based on amyloid cascade hypothesis. The goal is to decrease the production, accumulation or aggregation of Aβ or increase its clearance. The strategy of using β- and γ secretase inhibitors to inhibit the Aβ production is the most significant and promising approach. Potential immunotherapeutic therapies are examined today and they are considered very hopeful. These strategies are described further.

Other drugs are considered potentially valuable in AD therapy, but clinical studies performed so far haven't proved positive effects of any of them. As these potential drugs have no direct connection with my project, only a short notice, modified from Hüll et al. (2006) follows. **NSAIDs** could have positive effects on neuro-inflammatory processes accompanying AD. **HMG-CoA reductase inhibitors (statins)** seem to have a prophylactic ability, their effect being explained by various hypotheses. A recent randomised trial with patients receiving atorvastatin 80 mg/day for 12 months (Sparks et al., 2006) suggested a benefit for patients with higher baseline cholesterol or an apolipoprotein-E4 genotype. **Monoaminooxidase inhibitors (MAO-I)**, **valproate** and **arundic acid** could have protective effects on neurons. **Calcium channel antagonists** could prevent neuronal death by limiting Ca^{2+} related excitotoxicity. **Neurotrophic factors**, such as nerve growth factor (NGF), brain-derived neurotrophic factor and compounds that increase the availability of NGF (leteprinim potassium, xaliproden, paliroden) are tested.

Other strategies target tau protein phosphorylation and aggregation.

1.5.1 Beta- and Gamma-secretase inhibitors

The final step in Aβ production is the cleavage by γ-secretase, a complex with aspartyl protease activity. Inhibition of γ-secretase therefore leads to decline in Aβ production. However, γ-secretase has been reported to have a broad substrate specifity, taking part in processing of many protein substrates. Most importantly, it effects Notch signalling pathway, which is crucial in embryogenesis and regulates certain cell divisions in an adult (Guo and Hobbs, 2006). On the other hand, γ-secretase inhibitors could be designed to decrease Aβ production by some 30–40% or so, hopefully without interfering in a quantitatively meaningful way with Notch processing (Selkoe, 2001). A wide variety of γ-secretase inhibitors have been reported and some (those with small molecules, sufficient per oral availability and CNS penetration) entered clinical studies, for review see Hüll et al. (2006).

β-secretase (BACE) inhibitors present a convenient and well investigated therapeutic approach. Unlike γ-secretase, BACE inhibition seems lo lack serious adverse effects, which was demonstrated with knock out studies on mice (Roberds et al., 2001). However, even BACE has more known substrates than APP. BACE-1 inhibitors are not easy to discover because BACE-1 has a large catalytic side that may not avidly bind small molecules (suitable for CNS penetration) (Dewachter et al., 2002). For review of BACE-1 inhibitors development see Guo and Hobbs (2006). Recently a new selective non-peptidic BACE-1 inhibitor (GSK188909) with oral administration was reported by Hussain et al. (2007) and its efficacy was confirmed in vivo with orally dosed transgenic mice expressing APP.

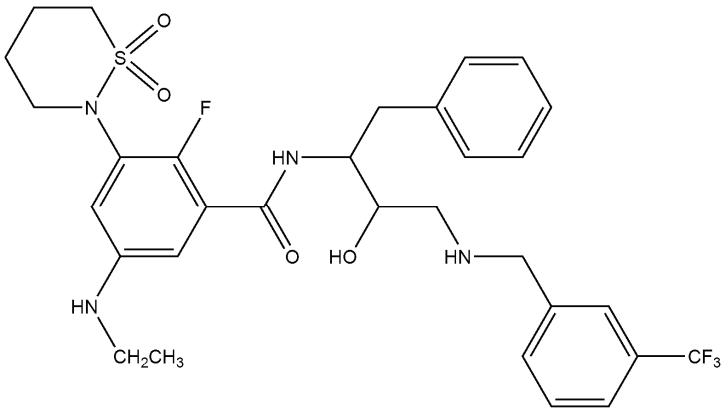


Fig. 1.4 Structural formula of GSK188909, a new non-peptidic orally available BACE-1 inhibitor (Hussain et al., 2007)

1.5.2 Immunotherapeutic approaches

It was generally considered that the brain was immune- privileged and unlikely to be affected by peripheral immune system. However, recent evidence has indicated that the CNS is not isolated or passive in its interactions with the immune system (Carson et al., 2006).

In 1999, Shenk et al. demonstrated that active immunisation of APP-transgenic mice with Aβ lead to high titres of anti-Aβ antibodies and subsequently to reduction of Aβ deposits. Similarly, other studies (Bard et al., 2000; Buttini et al., 2005) showed the benefits of passive immunisation of APP-transgenic mice with anti-Aβ antibodies. These and following investigations provided strong evidence that Aβ deposits can be reduced and cognitive functions can be improved by vaccination-based intervention. Most importantly for my thesis work, the results indicate than antibodies cross the blood-brain barrier in physiologically relevant levels.

There are three main proposed mechanisms by which anti-Aβ antibodies reduce the loads of this peptide. Opsonization of the deposits followed by phagocytosis by microglia, the catalytic transformation of the Aβ peptide into a secondary structure less compatible with amyloid fibril formation, and so called peripheral sink hypothesis, which suggests that sequestration of peripheral blood Aβ creates a concentration gradient and evokes Aβ efflux from CNS. All these mechanisms probably contribute to the final effect (Morgan, 2006).

In 2001, Phase I and II clinical trials we started with the Aβ42 immunisation. Despite encouraging preliminary results, the trial had to be halted, because minority of patients developed T-lymphocyte meningoencephalitis (Morgan, 2006). This autoimmune reaction represents the most significant drawback of possible immunotherapies of AD based on active immunisation. However, researchers continue to work and the development of safer active vaccine is in progress, e.g. by using different (shorter) Aβ immunogens and different adjuvants, trying to enhance the Th2 immune response over Th1 pro-inflammatory response. (Morgan, 2006).

Passive immunisation is much less probable to cause the meningoencephalitis. However, the risk of microhaemorrhages seems to be elevated (Morgan, 2006; Pfeifer, 2002). A humanized anti-Aβ antibody ‘Bapineuzumab’ is in Phase II trials (Hüll et al., 2006).

1.5.3 Novel therapeutic approach

This novel approach combines the positive features of BACE-1 blocking strategy and immunotherapeutic interventions, reducing the risk of adverse effects of immunotherapy such as T-lymphocyte mediated meningoencephalitis and microhaemorrhages at the same time. Briefly, the novel idea is to use a monoclonal antibody raised against immunogenic peptide representing amino-acid sequence spanning the β-secretase cleavage site of human APP. This antibody would bind to APP, preventing the β-secretase performed cleavage by steric hindrance. That means the antibody would conveniently work as a BACE-1 inhibitor without interfering with processing of other BACE-1 substrates. Moreover, optimally it wouldn't recognize Aβ itself and its deposits, thus lowering the risk of meningoencephalitis.

This approach was developed and investigated by three groups. Paganetti et al. (2005) and Arbel et al. (2005) worked with transfected cells overexpressing APP, which might not be the optimal model as most AD cases occur in people with much lower levels of APP than those associated with transfected cells (Thomas et al., 2006). Thomas et al., on the contrary, worked with cell lines constitutively producing APP on normal levels.

The work of Thomas et al. (2006) led directly to my project. They developed a monoclonal antibody (MAb) 2B12, raised against immunising peptide Ka, which represented the 15 amino acid sequence spanning the β-secretase cleavage site of human APP (Fig. 1.5).

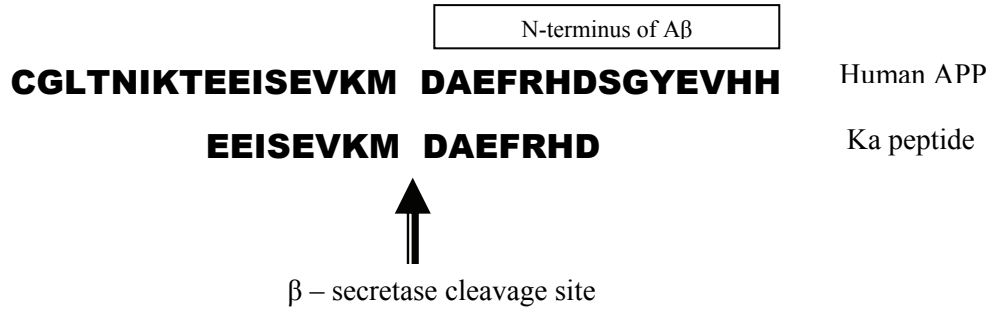


Fig. 1.5 The 30 amino acid sequence spanning the β-secretase cleavage site on human APP and the 15 amino acid sequence of the synthesised peptide (Ka) spanning the cleavage site. The Ka peptide was used as an immunising peptide to raise the 2B12 MAb.

Experiments showed that 2B12 recognized APP, but did not recognize (or recognized very weakly) Aβ40. They subsequently demonstrated that, after addition of 2B12 to standard growth media, this antibody was indeed capable of inhibiting Aβ production in neuroblastoma

(SH-SY5Y) and astrocytoma (MOG-G-UVW) cells expressing native APP, as measured by an ELISA. It was hypothesised 2B12 binds to APP exposed on the cell surface and is then internalised together with the protein (similarly as shown by Koo et al. (1996) and Yamazaki et al. (2006)).The mode of action of 2B12 is suggested as described above, i.e. binding to β-secretase cleavage site of APP and inhibiting it's cleavage by steric hindrance (see Fig. 1.6).

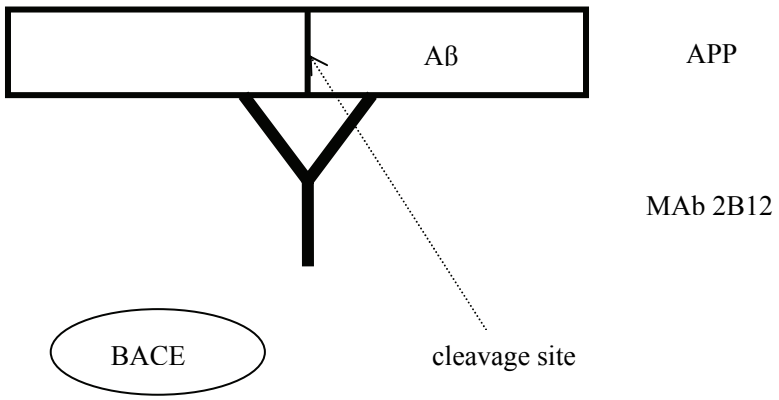


Fig. 1.6 Action of 2B12 binding to APP and preventing the BACE mediated cleavage (according to Thomas et al., 2006)

1.6 Project Aim

In 2006 Thomas et al. developed a new monoclonal antibody called 2B12 and proved that it caused a significant decrease in production of Aβ in MOG cells (Thomas et al., 2006). They hypothesized that 2B12 binds to APP exposed on the cell surface and enters the cell via natural trafficking pathways of APP. This hypothesis was confirmed by immunocytochemistry (ICC) and western-blotting experiments by Pei San Ho (2007).

The aim of this project is to make a further investigation into the fate of 2B12 inside MOG cells, especially obtain new data about the localization after the edocytosis. Live MOG cells will be exposed to 2B12 for various periods of time. Subsequently, they will be fixed and the localization of internalized 2B12 will be tested. If the 2B12 signal is located in the same compartments where APP is known to be present, it will confirm the hypothesis of 2B12 being trafficked in the form of complex with APP. The project itself consists of several partial goals as follows.

1. Repeat Pei San Ho's ICC experiments with 2B12 and get same or at least very similar results (staining) both with fixed and live MOG cells.
2. Perform ICC experiments on MOG cells with 6 different commercially available antibodies (subcellular markers for individual cell compartments) and optimize the method, e.g. find the optimal concentration for each Ab to obtain the best and the most distinctive staining.
3. Perform western-blotting analysis with MOG lysate for tested antibodies.
4. Perform colocalization experiments with 2B12 together with subcellular markers to investigate the localization of 2B12 (APP) in cells, both with fixed and living cells.

2B12 will be investigated and tested as a potential new therapeutic agent to Alzheimer's disease. Therefore this project, as well as few previous ones, was funded by Alzheimer's Society (UK).

2. METHODOLOGICAL FRAMEWORK

2.1 Chemicals and Reagents

All chemicals and reagents used in this project were purchased from Sigma-Aldrich, Poole, UK or Fisher Scientific, Leicester, UK, unless otherwise stated.

List of used purchased antibodies (subcellular markers)

- Bcl -2 (N-19): sc- 492; Santa Cruz Biotechnology, Inc. (USA); rabbit polyclonal affinity purified antibody raised against a peptide mapping at the N-terminus of Bcl-2 of human origin; concentration 200 μg/ml
- Hsp 60 (N-20): sc – 1052; Santa Cruz Biotechnology, Inc. (USA); goat polyclonal affinity purified antibody raised against a peptide mapping at the N-terminus of Hsp-60 of human origin; concentration 200 μg/ml
- LAMP-1 (H-228): sc – 5570; Santa Cruz Biotechnology, Inc. (USA); rabbit polyclonal antibody raised against amino acids 1-228 of LAMP-1 of human origin; concentration 200 μg/ml
- TGN46 (ab50595); Abcam, Inc., Cambridge, Massachusetts, USA; rabbit polyclonal affinity purified antibody raised against peptide corresponding to amino acids 426-437 of human TGN46; concentration 1000 μg/ml
- M6PR (ab12894); Abcam, Inc., Cambridge, Massachusetts, USA; rabbit polyclonal affinity purified antibody raised against peptide corresponding to amino acids 700-800 of human Mannose 6 Phosphate Receptor (Cation independent); concentration 300 μg/ml
- Anti – EEA-1 (C - terminal), Sigma-Aldrich, Saint Louis, Missouri, USA; rabbit polyclonal affinity purified antibody raised against peptide corresponding to amino acids 1391-1410 of human Early Endosomal Antigen 1; concentration 100 μg/ml

Note: Anti-Bcl-2 antibody was used as a subcellular marker for endoplasmic reticulum, where Bcl-2 protein is present. See Akao et al. (1994).

2.2 Cell Culture

As well as in previous studies (Thomas et al. (2006), Pei San Ho (2007)), astrocytoma MOG-G-UVW (MOG) was used. This is a human cell line constitutively expressing Aβ. The cell line was purchased from ECACC, Porton Down, UK. The MOG cells were grown according to Thomas et al. (2006). The growth media was a 1:1 solution of Ham's F10 and Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal Bovine Serum (FBS) (Perbio Science U.K. Ltd., Cramlington, Northumberland) and 2 mM glutamine. Cells were incubated at 37 ˚ C in 5% CO_2 in air.

2.3 Immunocytochemistry experiments (ICC)

2.3.1 Immunocytochemistry (ICC) – general procedure

Immunocytochemistry experiments were performed following the procedures developed and optimized by E.J. Kidd et al. (1998). I also followed some minor adjustments made and described by Pei San Ho (2007).

ICC experiments were designed as **indirect immunocytochemistry using biotin-conjugated secondary antibodies**. To visualize the results I used various photostable fluorophores (labelling marks) conjugated with avidin. In principle, primary antibodies, bound to their epitopes inside cells, were recognized by suitable biotinylated secondary antibodies. Thus the interaction between biotin and avidin is very strong and stable, avidin labelled by a fluorophore binds easily to the complex of the primary and secondary antibody (Fig. 2.1).

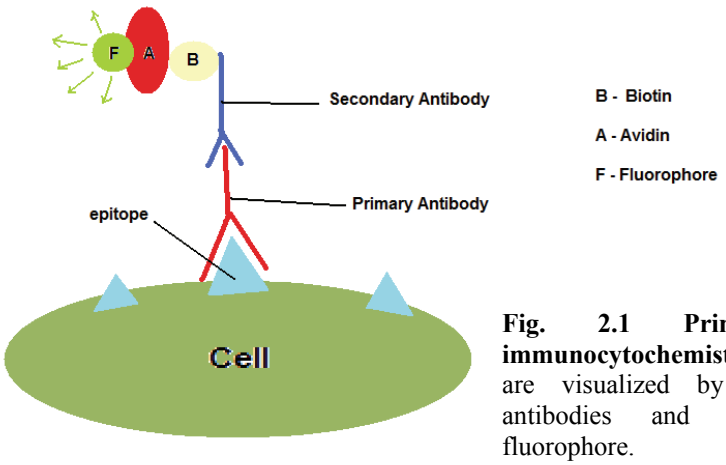


Fig. 2.1 Principles of indirect immunocytochemistry. Primary antibodies are visualized by biotinylated secondary antibodies and avidin conjugated to fluorophore.

Triton X – 100 as a detergent was used in all my ICC experiments with fixed cells, because of the fact that the permeabilization of cells leads to better and more distinctive staining with 2B12, as Pei San Ho (2007) showed.

Procedure:

MOG cells were grown on collagen (15 μg/ml) coated glass cover-slips in 24-well plates until 50% confluent and then fixed with fresh 2% formaldehyde in 0.1 M phosphate-buffered saline (PBS; 145mM NaCl, 96.4 mM NaHPO4, 21.5 mM Na2PO4, pH 7.4). The fixing procedure involved 3 washes with 0.1M PBS (pH 7.4) prior to the fixation (this is to wash off the proteins from growth media which would precipitate as artefacts), 15 min of fixation and 3 washes with O.1M PBS again to remove the formaldehyde. The cells were stored in 0.1M PBS in sealed plates (to prevent evaporation) at 4 ˚ C until the experiment.

After storage they were washed 3 times for 5 minutes with 0.1M PBS. To reduce the non-specific binding, coverslips were blocked for 30 minutes with blocking solution, which was 0.1M PBS containing 3% of serum of the animal used to raise the secondary antibody, 1% of bovine serum albumin (BSA) and 0.1% Triton X – 100. After blocking phase, primary antibodies in blocking solution (still containing Triton X – 100, see above) were added to wells, while for wells meant as controls blocking solution alone was used. Plates were incubated usually overnight, e.g. 24 – 72 hours at 4 °C in a humidified container. Wells were washed 3 times for 5 minutes with PBS after the incubation. Secondary antibodies in blocking solution were added to both controls and wells previously exposed to primary antibodies, all incubated for 2 hours at room temperature. After another washing (3 times 5 mins with PBS), all cover-slips were incubated with avidin conjugated to fluorescein (Avidin-FITC; Vector Laboratories, Burlingame, California, USA) in blocking solution for 1 hour in the dark to prevent bleaching. Washed as above, cover-slips were finally removed from the wells, dipped into distilled water to remove any remaining buffer salts, and left to dry in the dark (for about 30 min). In the end, cover-slips were mounted on glass slides previously washed with 100% ethanol using an antifade agent (Dako UK Ltd., Cambridgeshire, UK). Glass – slides were stored in the dark at 4 °C. Results were obtained using Leica DMRA2 fluorescence microscope, pictures taken by attached Leica DC500 digital camera and Leica FW 4000 software. For some pictures Leica laser confocal microscope with digital camera was used to get higher resolution. Pictures were processed by Adobe Photoshop.

2.3.2 ICC for 2B12

Immunocytochemistry for 2B12 was performed according to the general procedure described above. 2B12 was used at concentration of 5 μm/ml, blocking solution contained horse serum, secondary antibody was biotinylated anti-mouse IgG (1:270, Vector Laboratories), and Avidin-FITC (1:600, Vector Laboratories) was used for visualization.

2.3.3 Determining the optimal dilutions of purchased subcellular markers for ICC

ICC experiments for purchased antibodies were performed according to the general procedure (see 2.3.1). I worked with six different antibodies (see Fig. 2.2, or 2.1 Chemicals and Reagents for details). The type of serum used for blocking solution, and secondary antibodies are obvious from the table below. Biotin conjugated anti-rabbit IgG (Sigma-Aldrich) was used at 1:1000, biotin conjugated anti-goat IgG (Vector Lab.) at 1:270. Visualized by Avidin-FITC (1:600, Vector Laboratories). Two different negative controls (with adequate serum and secondary antibody) were used, one for antibodies used in goat serum, the other for those in horse serum.

Antibody	Raised in	Starting dilution for ICC	Block contains	2° ary Ab (biotinylated)
anti - Bcl -2	R	1:50 (4 μg/ml)	GS	anti – R
anti - Hsp 60	G	1:50 (4 μg/ml)	HS	anti – G
anti - LAMP-1	R	1:50 (4 μg/ml)	GS	anti – R
anti - TGN 46	R	1:200 (5 μg/ml)	GS	anti – R
anti - M6PR	R	1:75 (4 μg/ml)	GS	anti – R
anti – EEA1	R	1:100 (10 μg/ml)	GS	anti – R

Fig. 2.2: List of antibodies – basic data (R – rabbit, G – goat, GS – goat serum, HS – horse serum)

According to the resulting pictures the experiment was repeated for individual antibodies with different concentrations (dilutions). The aim was to determine the concentrations which produce the best and most distinctive labelling, similar to the pattern described in literature.

2.3.4 Colocalization ICC experiments

Collocalizition ICC experiments were used to investigate intracellular localization of APP and the way it's trafficked in a MOG cell. This was to be achieved by exposing MOG cells sequentially to 2B12, which detects APP, and another primary antibody labelling one of important subcellular markers associated with certain cell compartments. Mitochondria (Hsp-60), early (EEA1) and late (M6PR) endosomes, lysosomes (LAMP1), Golgi apparatus (Bcl-2) and trans-Golgi network (TGN-46).

The procedure itself followed the general procedure described in 2.3.1. First, MOG cells were exposed to 2B12 (5 μg/ml), which was detected as above by biotinylated anti-mouse IgG (1:270, Vector Laboratories) and avidin – FITC (1:600, Vector Laboratories). After washing off avidin-FITC (three times 5 minutes with PBS 0.1M), the whole process was repeated with one of the purchased antibodies (markers) using correct blocking solution and secondary antibody. The purchased antibodies were used at optimum dilution as determined in previous experiment. In the end the presence of markers was detected and visualized, unlike for 2B12, by avidin conjugated Texas Red (1:600, Vector Laboratories). As avidin - FITC emits green light and avidin – Texas Red red light, any colocalization of 2B12 and markers is represented by yellow colours, composed of green and red. Leica microscopes were set up to scan sequentially the green and red channel, and the final pictures were combined from the channels using Leica software. Leica fluorescence microscope was used for previews, final pictures with high resolution were obtained by Leica confocal laser scanning microscope.

2B12 was tested with all six purchased antibodies, each combination at least three times (n=3). Adequate controls were performed to determine the effect of non-specific staining. The system of controls is showed in Fig. 2.3.

	coverslip 1	**cover slip 2**	**cover-slip 3**	**cover-slip 4**
step 1	Ab 1	Ab 1	none	none
step 2	Ab 2	none	Ab 2	none

Fig. 2.3 System of controls performed for each pair of antibodies

2.3.5 Live Cell ICC Experiments

Live cell experiments with 2B12 were performed according to Pei San Ho (2007). Pei San Ho's experiment followed the internalization of 2B12 by MOG cells in time.

Live MOG cells on coverslips were incubated with 2B12 (or irrelevant mouse - IgG or media alone as negative controls) for various periods of time. Both 2B12 and irrelevant mouse IgG were used at 10 μg/ml and were presented in media (no PBS, no blocking solution, no Triton X - 100). Every effort was made to maintain the optimal living conditions for the cells as long as possible, e.g. added media preheated at 37˚ C, or minimizing the time cells spend outside the incubator. In this 4-hour experiment the cells were manipulated even outside the laminar flow hood, assuming that bacterial contamination won't get significant in 4-hour time. After incubation cells were washed quickly with PBS three times and fixed in 2% formaldehyde in 0.1M PBS for 15 minutes. Washed three times 5 minutes with PBS and stored in it in sealed plates at 4 ˚C until next day. On the next day the experiment was finished exactly as described in the general procedure (see 2.3.1), including the use of blocking buffer, and Triton X – 100. Visualized by biotinylated anti-mouse IgG (1:270, Vector Laboratories) and avidin-FITC (1:600, Vector Laboratories).

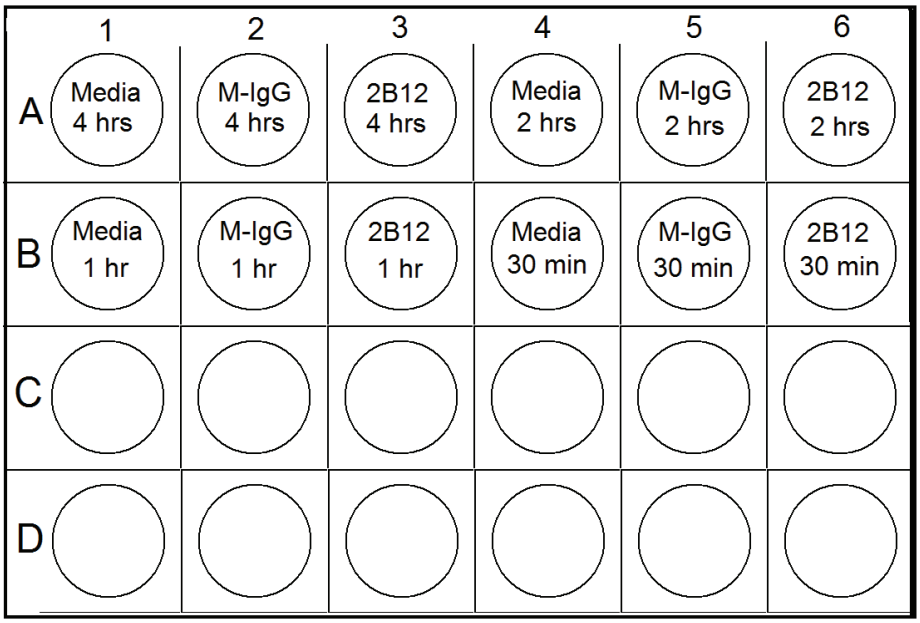


Fig. 2.4: Representation of Live Cell ICC experiment (accord. Pei San Ho, 2007)

2.4 Western Blotting

Western blotting experiments were performed according to standard protocols and as described by Kidd E.J. et al. (1998) and Thomas et al. (2006). SDS – PAGE was used to separate the proteins of MOG cell lysate.

2.4.1 MOG lysate and sample preparation

Lysates were prepared from MOG cells using lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Triton, 0.4 mM NaVO4, 50 mM NaF, 1 mM PMSF - phenylmethylsulphonyl fluoride, 20 μM phenylarsine oxide, 10mM sodium molybdate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and then concentrated through Amicon Centriplus YM-100 centrifugal filters (Thomas et al. (2006)). To prepare samples, MOG lysate was suspended in Tris (50 mM, pH 7.2) and 2x sample buffer was added with a 1:2 sample buffer : Tris ratio. Samples were homogenized, frozen and kept at minus 20 ˚C.

2.4.2 SDS – PAGE, Western Blotting and Immunoassay

Samples were thawed quickly and heated at 95 ˚C for 5 minutes before loading. The samples and molecular weight protein standard (Precision Plus Protein Standards marker, Bio-Rad Laboratories, Hercules, California, USA) were loaded on a polyacrylamide gel (7.5%, 10%, or gradient) and run in running buffer (25mM Tris base, 190mM glycine, 0.05% SDS, pH 8.3) until well separated. The proteins were then blotted to 0.2 μm nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK) using semi-dry blotting buffer (42.9mM Tris base, 38.9mM glycine, 0.038% SDS, 20% v/v methanol), washed in Tris-buffered saline with Tween 20 (TBST, 2 mM Tris, 15 mM NaCl, 0.1% Tween-20, pH 7.5) and blocked for 1 hour, at RT, in TBST supplemented with 5% w/v fat-free dried milk (5% Blotto). Blots were then incubated with tested primary antibodies in 1% Blotto overnight at 4 ˚C, then washed in TBST (2 quick washes, 2 times 2 minutes and once for 15 min, i.e. 5 washes in total), exposed to corresponding secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature, washed in TBST as above and visualized using advanced chemiluminescent detection (Super Signal®, West Dura, Perbio Science, UK). Membranes were exposed to high-performance chemiluminescent X-ray film (Amersham Biosciences) and relevant bands on the X-ray film were scanned using HP scanner. Images

were processesed using Adobe Photoshop. Data for molecular weight protein standard bands were examined using linear regression determining the logarithm of molecular weight as a linear function of logarithm of distance of the band. Using these linear functions (determined for each membrane), relevant and important bands for individual antibodies were sized.

The western blotts for individual primary antibodies (number of bands and their positions) were compared to the information from manufacturer's datasheet or other literature.

3. RESULTS

3.1 Immunocytochemisty for 2B12

ICC experiments with 2B12 (5 μg/ml) demonstrated that it had a discrete labelling pattern in MOG cells. The labelling was bright and punctuate, placed perinuclearly and in cytoplasm (Fig. 3.15). These results are very similar to the ones seen previously (Thomas et al., 2006; Pei San Ho, 2007).

3.2 Determining the optimal dilutions of purchased subcellular markers for ICC

Anti – TGN-46 produced strong, clear and very distinctive signal, marking trans-Golgi network, situated in the form of few sections in the vicinity of the nucleus. Recommended starting dilution 1:200 seemed to be too concentrated, so lower concentrations (1:400, 1:300) were engaged. These latter two produced very similar staining. 1:300 and 1:350 dilutions were used for colocalization experiments.

Anti - LAMP-1 antibody didn't give especially strong signal. At recommended starting dilution of 1:50 it produced indistinct staining (data not shown). Lower concentrations (1:75, 1:100) lead to pictures quite similar to what had been expected – perinuclear staining (areas) in cytoplasm. This antibody is supposed to visualize especially lysosomes, which is in correspondence with the fact that pictures with higher resolution taken by laser scanning confocal microscope had more punctuate staining. 1:75 and 1: 62.5 dilutions were determined for usage in colocalization experiments.

Anti – EEA-1 antibody gave relatively strong signal and produced less or more punctuate staining in cytoplasm, quite adequate to what is described in literature. The starting dilution 1:100 seemed to be too concentrated (lead to indistinct and blur staining), therefore lower concentrations (1:200, 1:250) were used for colocalization experiments.

Anti - Bcl-2 staining with the starting dilution suggested by the manufacturer (1:50) was very indistinct; lower concentrations (1:100, 1:75) produced more specific staining, but the signal was weak. Concentrations higher than suggested didn't help much either. 1:50 was used for colocalization experiments.

Anti - M6PR antibody, produced very faint signal even at concentrations higher than the starting concentration recommended by manufacturer (1:75). 1: 50 and 1:40 dilutions were tested but lead not to significantly stronger signal. However, the labelling was similar to manufacture's datasheet (although much weaker) -punctuate, accumulated around nucleus. 1:40 and 1:30 dilutions were tried for colocalization.

Anti – Hsp-60 turned out to be a great antibody. The first concentration tested (1:50) worked well and lower concentrations (1:75, 1:100) even better, labelling mitochondria around cell nucleus very clearly and distinctively. 1:100 was used in consecutive colocalization experiments.

Antibody	Starting dilution	Optimized dilution
anti – TGN-46	1:200	1:300, 1:350
anti – EEA-1	1:100	1:200, 1:250
anti – M6PR	1:75	1:40, 1:30
anti – Hsp-60	1:50	1:100
anti – LAMP-1	1:50	1:75, 1:62.5
anti – Bcl-2	1:50	1:50

Fig. 3.1 Optimized dilutions of individual antibodies for use in colocalization experiments

3.3 Western Blotting

The aim of western blotting experiments was to demonstrate that chosen primary antibodies (subcellular markers) were able do recognize specific proteins in MOG lysate. Following figures show representative results for individual antibodies, where at least 2 analyzes (n=2) – except for anti-M6PR, see below - were performed for each antibody.

Anti – TGN-46

Major band was detected at approx. 114 kDa (average of n=2). Antibody manufacturer's datasheet suggest that the antibody should detect a band of approximately 80 – 100 kDa. Few minor bands of lower molecular weights were seen.

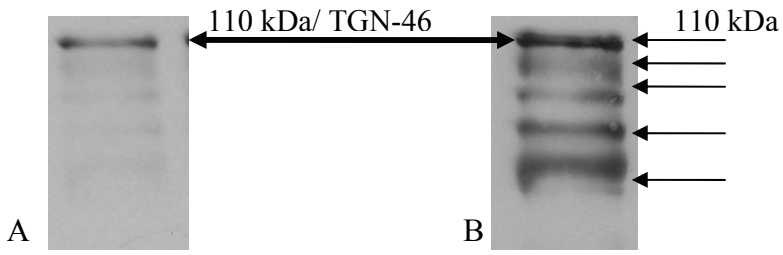


Fig. 3.2 Representative labelling of MOG cell lysate with anti – TGN-46 antibody – dilution 1: 4000 (0.25 μg/ml), secondary anti-rabbit IgG – HRP 1:10 000, two different exposures. Bands in (B) have following values (from up to down): 110 kDa, 94 kDa, 80 kDa, 70 kDa, 60 kDa).

Anti - LAMP-1

Anti-LAMP-1 antibody didn't work well in Western blot analysis. It tended to bind rather non-specifically and produced blur labelling. Attempt to reduce the non-specific bindings in order to get clearer a picture by blocking overnight was not successful. Although main band was expected at app. 125 kDa, two smeared band were detected at 102 kDa, respectively 59 kDa.

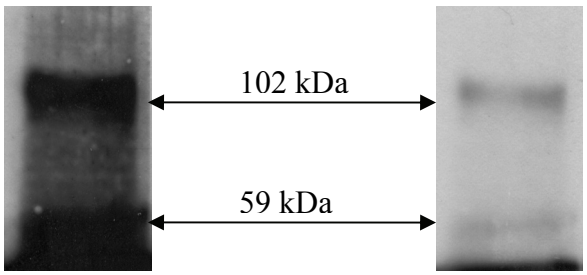


Fig. 3.3 Representative labelling of MOG cell lysate with anti - LAMP-1 antibody – dilution 1:500 (0.4 μg/ml), secondary anti-rabbit IgG-HRP 1:20 000, two different exposures

Anti – EEA-1

According to manufacturer's datasheet main band was expected at approx. 160 kDa. The antibody worked very well and labelled nice and clear bands. The molecular weight of the main band was determined as 157 kDa (average of n=2). Other bands representing possible oligomers respectively degradation products were detected at longer exposures.

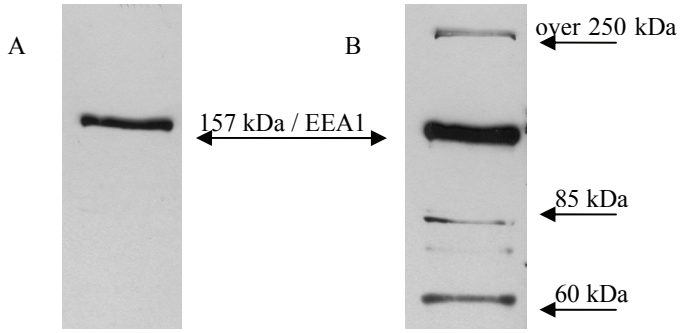


Fig. 3.4 Representative labelling of MOG cell lysate with Anti – EEA-1 antibody – dilution 1:10 000 (0.1 µg/ml), secondary anti-rabbit IgG – HRP 1:10 000, two different exposures

Anti - Bcl -2

Western blots showed a nice and clear band, having the size of 61 kDa (average of n=2). The datasheet for this antibody suggests that the strongest band should be of only 29 kDa. This band could represent a dimer of Bcl-2 protein, which could be a dominant form of Bcl-2 in MOG lysate. Another possibility is that my experiment detected only a minor band and missed the main one, which could have been lost for example because of too long blotting phase (60 mins at 0.8 mA per square centimetre).

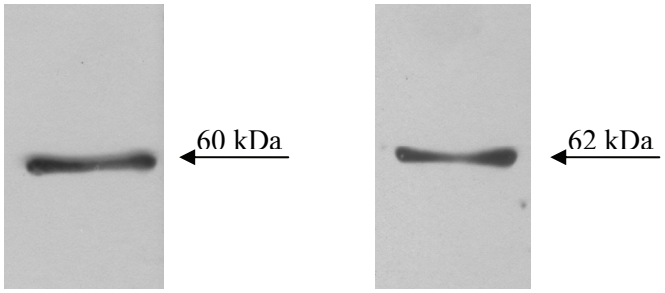


Fig. 3.5 Representative labelling of MOG cell lysate with Anti – Bcl-2 antibody – dilution 1:10 000 (0.1 µg/ml), secondary anti-rabbit IgG – HRP 1:10 000, two different exposures

Anti - M6PR

According to manufacturer's datasheet this antibody should label a band of 215 kDa (under reducing conditions). Because of the fact I was looking for such a large protein, pre-made gradient polyacrylamide gel (Bio-Rad Laboratories, Hercules, California, USA) was used for separation. However, the strongest band was detected at approx. 70 kDa, all other bands were very faint even at very long exposures (see Fig. 3..B).

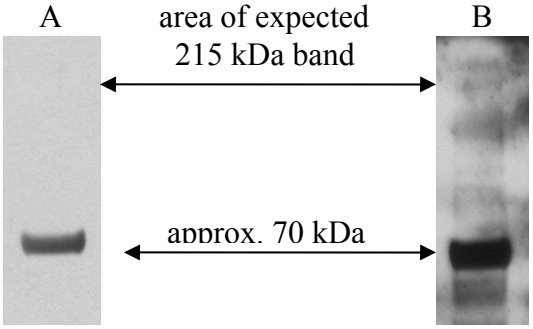


Fig. 3.6 Representative labelling of MOG cell lysate with anti - M6PR antibody – dilution 1:75 (4 μg/ml), secondary anti-rabbit IgG – HRP 1:10 000, separated on gradient polyacrylamide gel, two different exposures

Anti – Hsp-60

This antibody recognized Hsp-60 proteins of MOG lysate very strongly. The signal was so strong it was possible to lower the concentration from starting 1: 200 (1 μg/ml) to 1:10 000 (0.02 μg/ml). The main band represented proteins of molecular weight of 55 kDa (average of n=5), which is close to expected values. With slightly higher exposure times, many minor bands could be detected as well (**Fig. 3.**.B). They probably match partially phosphorylated Hsp – 60 proteins, oligomers, or degradation products.

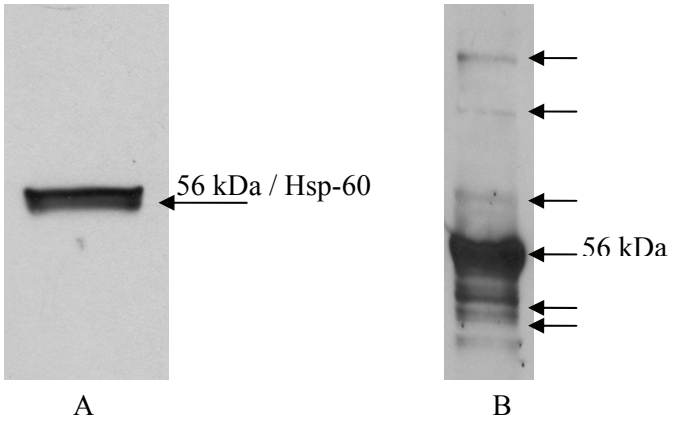


Fig. 3.7 Representative labelling of MOG cell lysate with anti – Hsp-60 antibody (A) dilution 1:10 000 (0.02 μg/ml), secondary anti-goat IgG-HRP 1: 10 000; (B) dilution 1:12 500 (0.016 μg/ml), secondary anti-goat IgG-HRP 1: 10 000, longer exposure

SUMMARY:

Tested primary antibodies against EEA-1, TGN-46 and Hsp-60 worked well in Western blot analysis. They produced clear bands with molecular weights corresponding to expected values, which suggest they are suitable for usage with MOG cells model as performed.

Anti - Bcl-2 antibody labelled a band of approx. double the molecular weight expected (dimer). Results should be revised considering the fact the little protein could have been lost in blotting phase (see above). Anti – M6PR antibody produced a misleading band - SDS-

PAGE might have not been efficient enough to separate the targeted protein of 215 kDa. Anti – LAMP-1 produced a lot of non-specific binding.

3.4 Labelling of MOG cells by individual primary antibodies (pictures)

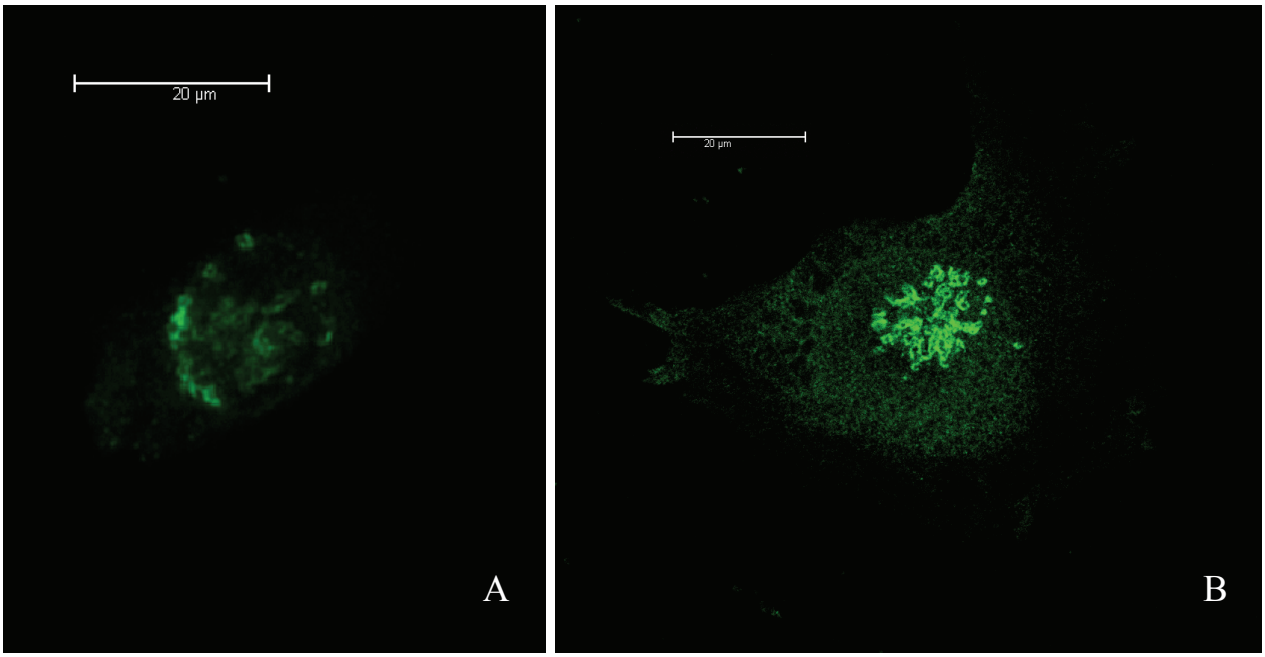


Fig. 3.8 Representative examples of the labelling by anti-TGN-46 antibody. Bright green sections represent the trans-Golgi network. With picture (A) anti-TGN-46 was used at 1:400, with picture (B) 1: 300. Visualized by biotinylated anti-rabbit antibody (1:1000) and Avidin-FITC (1:600). Picture (A) taken with fluorescent microscope, picture (B) by confocal microscope (maximum projection of z series).

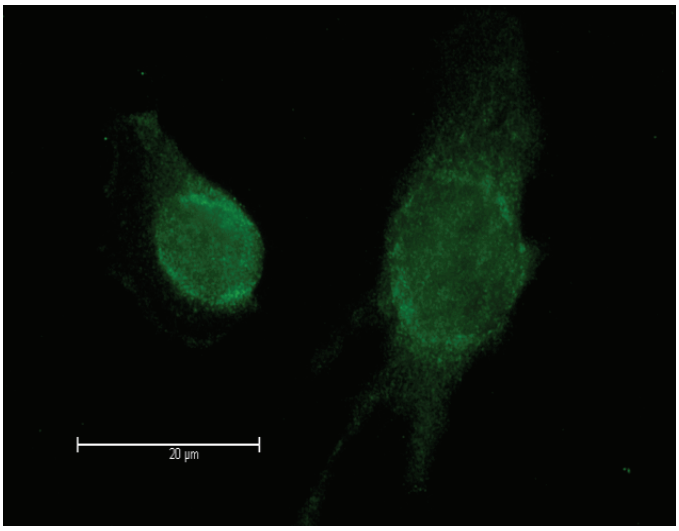


Fig. 3.9 Representative example of the labelling by anti-LAMP-1 antibody (1:100). Dotted staining, somewhat denser in the perinuclear area, represents the lysosomes.. Visualized by biotinylated anti-rabbit antibody (1:1000) and Avidin-FITC (1:600). Picture taken with fluorescent microscope.

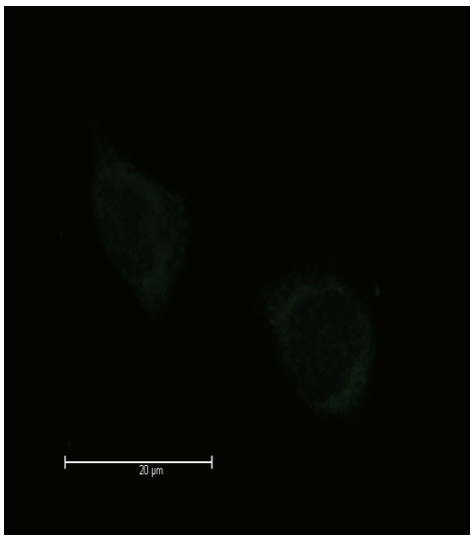


Fig. 3.10 Negative control. Visualized by biotinylated anti-rabbit antibody (1:1000) and Avidin-FITC (1:600). Therefore relevant for antibodies detected by biotinylated anti-rabbit Ab (i.e. anti TGN-46, LAMP-1, EEA-1, Bcl-2 and M6PR). Picture taken with fluorescent microscope. Controls were performed for every single experiment run, and all resulted in a faint staining similar to this one, so they won't be showed .

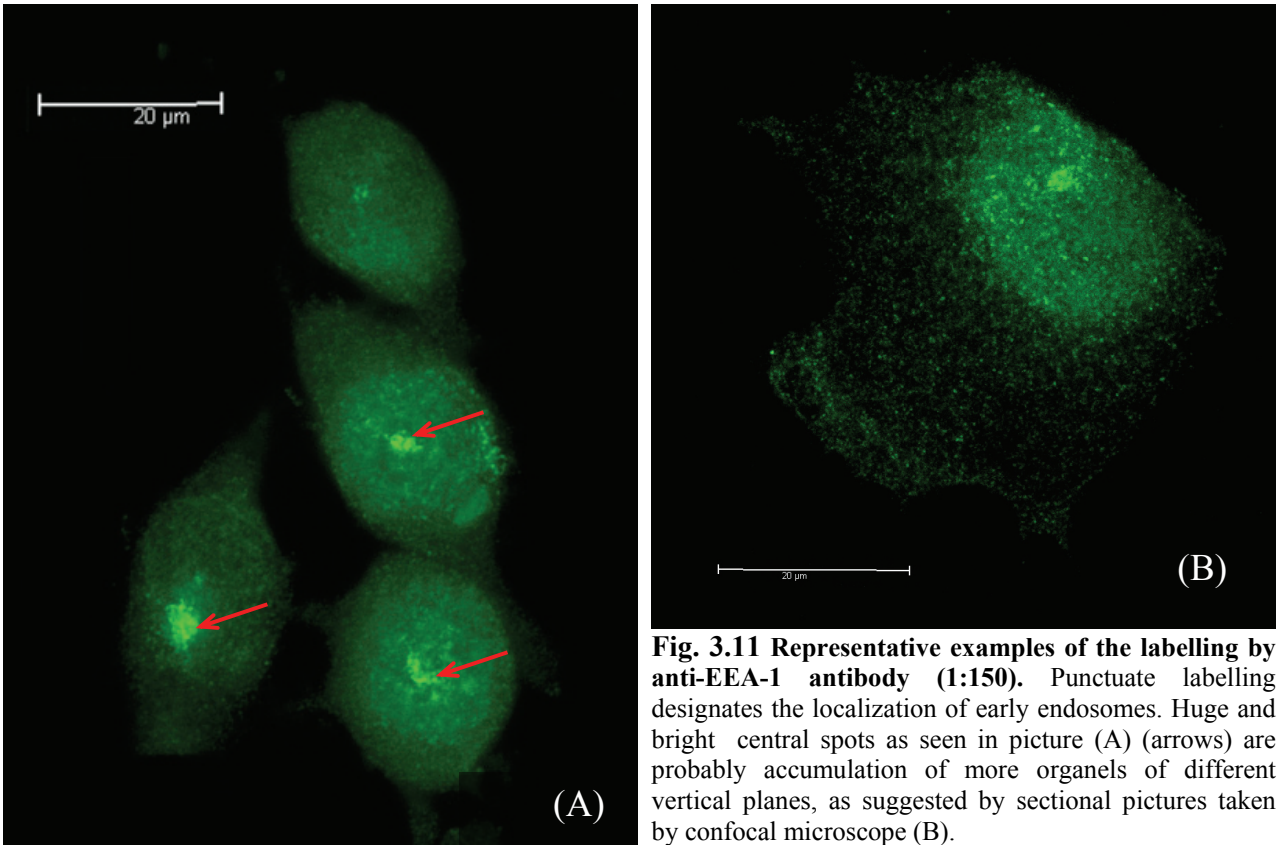


Fig. 3.11 Representative examples of the labelling by anti-EEA-1 antibody (1:150). Punctuate labelling designates the localization of early endosomes. Huge and bright central spots as seen in picture (A) (arrows) are probably accumulation of more organels of different vertical planes, as suggested by sectional pictures taken by confocal microscope (B).

Visualized by biotinylated anti-rabbit antibody (1:1000) and Avidin-FITC (1:600). Picture (A) taken with fluorescent microscope, picture (B) is one of the horizontal sections taken by confocal microscope.

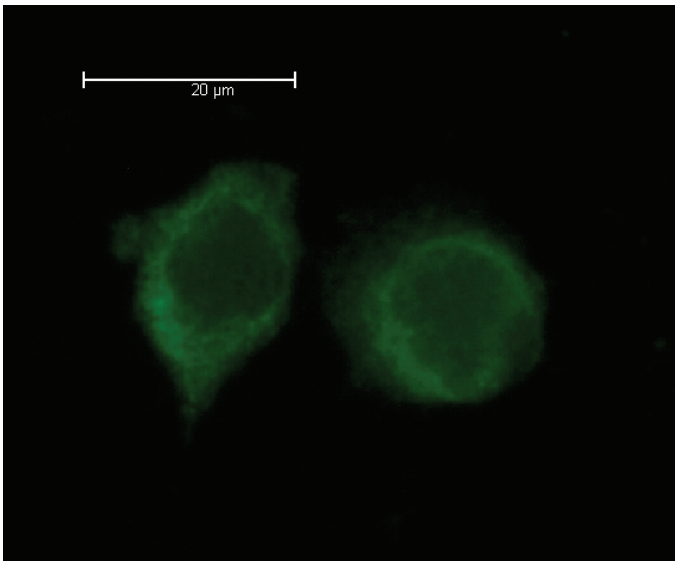


Fig. 3.12 Representative example of the labelling by anti-Bcl-2 antibody (1:75). Not very distinctive cytoplasmic staining becomes more punctuate with higher resolution under confocal microscope (see Fig. 3. in colocalization section). Visualized by biotinylated anti-rabbit antibody (1:1000) and Avidin-FITC (1:600). Picture taken with fluorescent microscope.

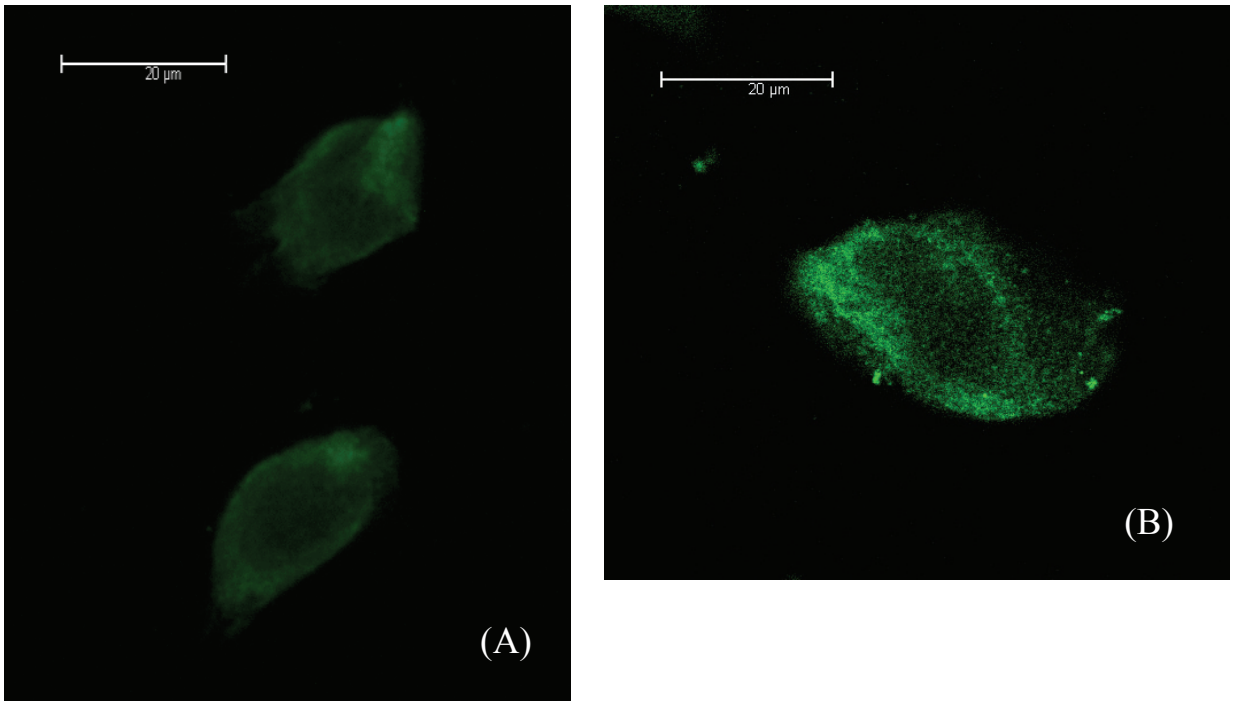


Fig. 3.13 Representative examples of the labelling by anti-M6PR antibody (1:40). Not very distinctive cytoplasmic staining (A) becomes more punctuate with higher resolution under confocal microscope (B). Labelling indicates a type of lysosomes. Visualized by biotinylated anti-rabbit antibody (1:1000) and Avidin-FITC (1:600). Picture (A) taken with fluorescent microscope, picture (B) is one of horizontal sections taken by confocal microscope.

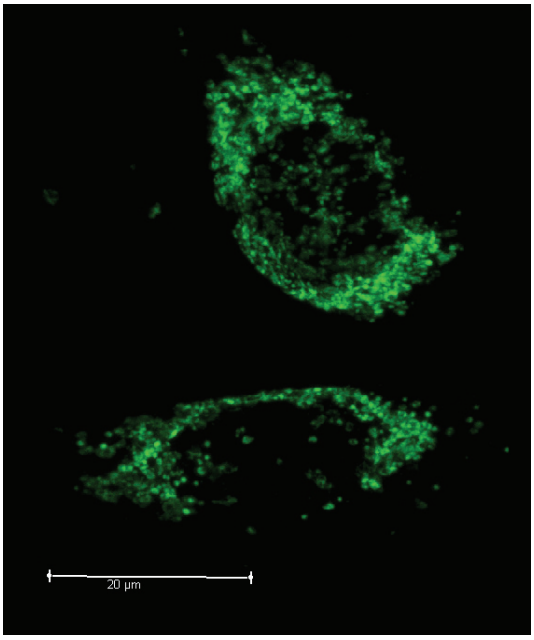


Fig. 3.14 Representative example of the labelling by anti-Hsp-60 antibody (1:100). Bright and distinct sections in the perinuclear area are mitochondria. Visualized by biotinylated anti-goat antibody (1:270) and Avidin-FITC (1:600). Picture is a maximum projection of horizontal sections taken by confocal microscope.

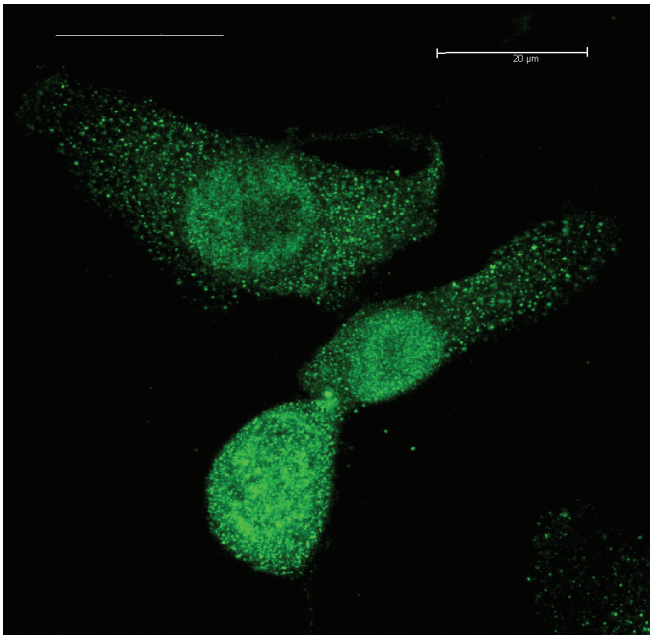


Fig. 3.15 Representative example of the labelling by 2B12 antibody (5 µg/ml). Bright and punctuate labelling pattern, placed perinuclearly and in cytoplasm, refers to the localization of APP. Visualized by biotinylated anti-mouse antibody (1:270) and Avidin-FITC (1:600). Picture is a maximum projection of horizontal sections taken by confocal microscope.

3.5 Colocalization pictures

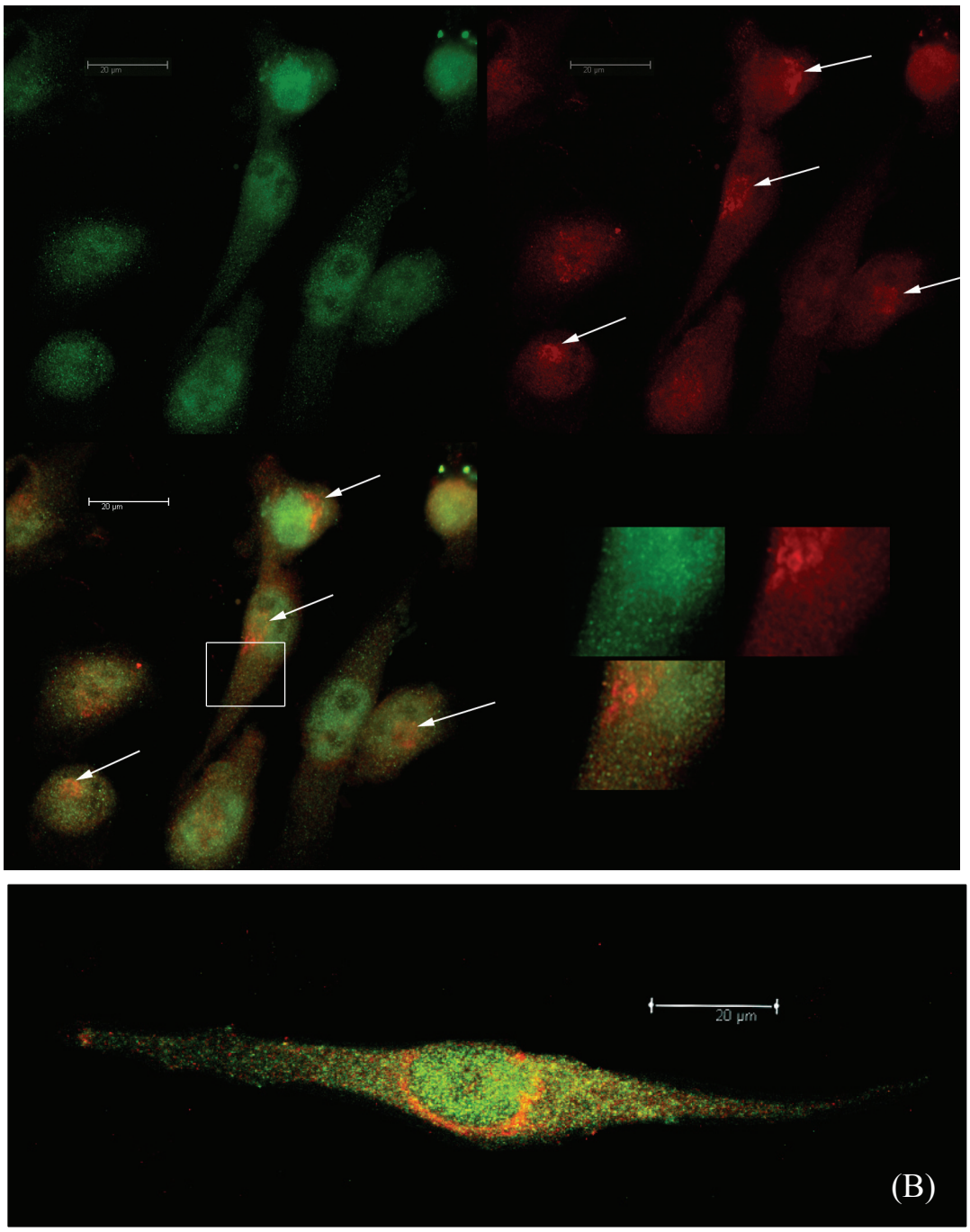
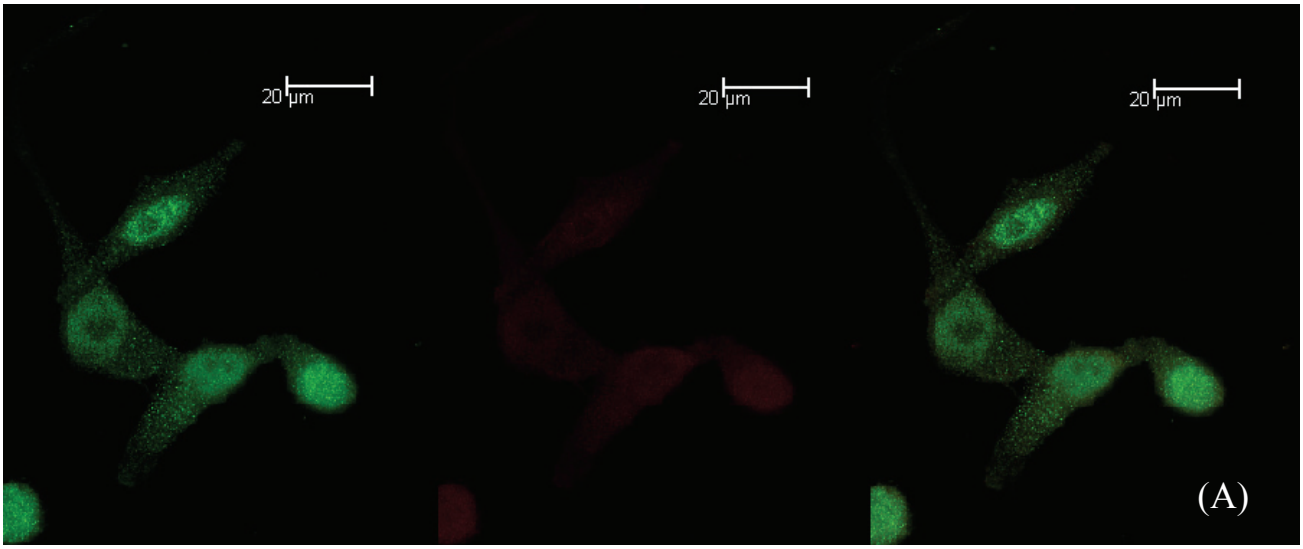
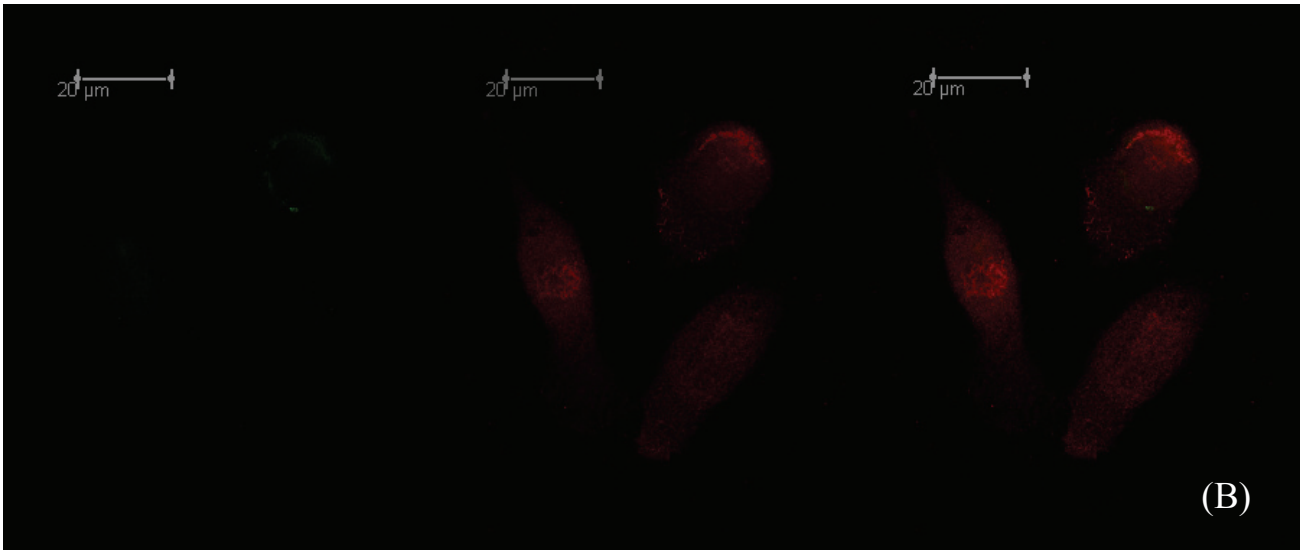


Fig. 3.16 Representative examples of labelling after treatment with 2B12 (5 mg/ml, green channel) and TGN-46 (1:100, red channel). Visualized by proper biotin-conjugated secondary antibodies and Avidin-FITC (1:600), Avidin-Texas Red (1:600), respectively. Green punctuate staining by 2B12 represents the localization of APP, solid red staining around nucleus is trans - Golgi network (see arrows). The overlay of the channels expresses the colocalization. Pictures (A) was taken with fluorescent microscope. Picture (B) is a maximum projection of Z series taken with confocal microscope (n = 3) and represents the overlay. See **Fig. 3.17** for negative controls.





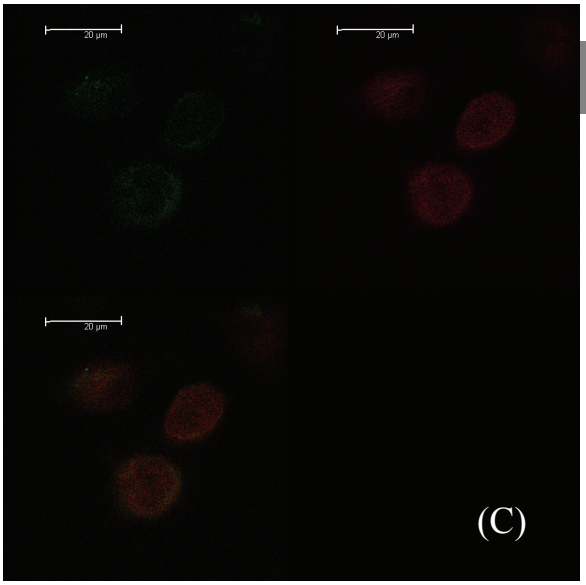


Fig. 3.17 Sample pictures of performed negative controls. Cells in figure (A) were treated with 2B12 only concerning primary antibodies. Similarly, cells in figure (B) were treated only with TGN-46. Finally, no primary antibodies were used for cells in figure (C). These controls are relevant for 2B12 – TGN-46 colocalization experiments. Analogous controls were performed for all other experiments (antibodies) as well – data not shown.

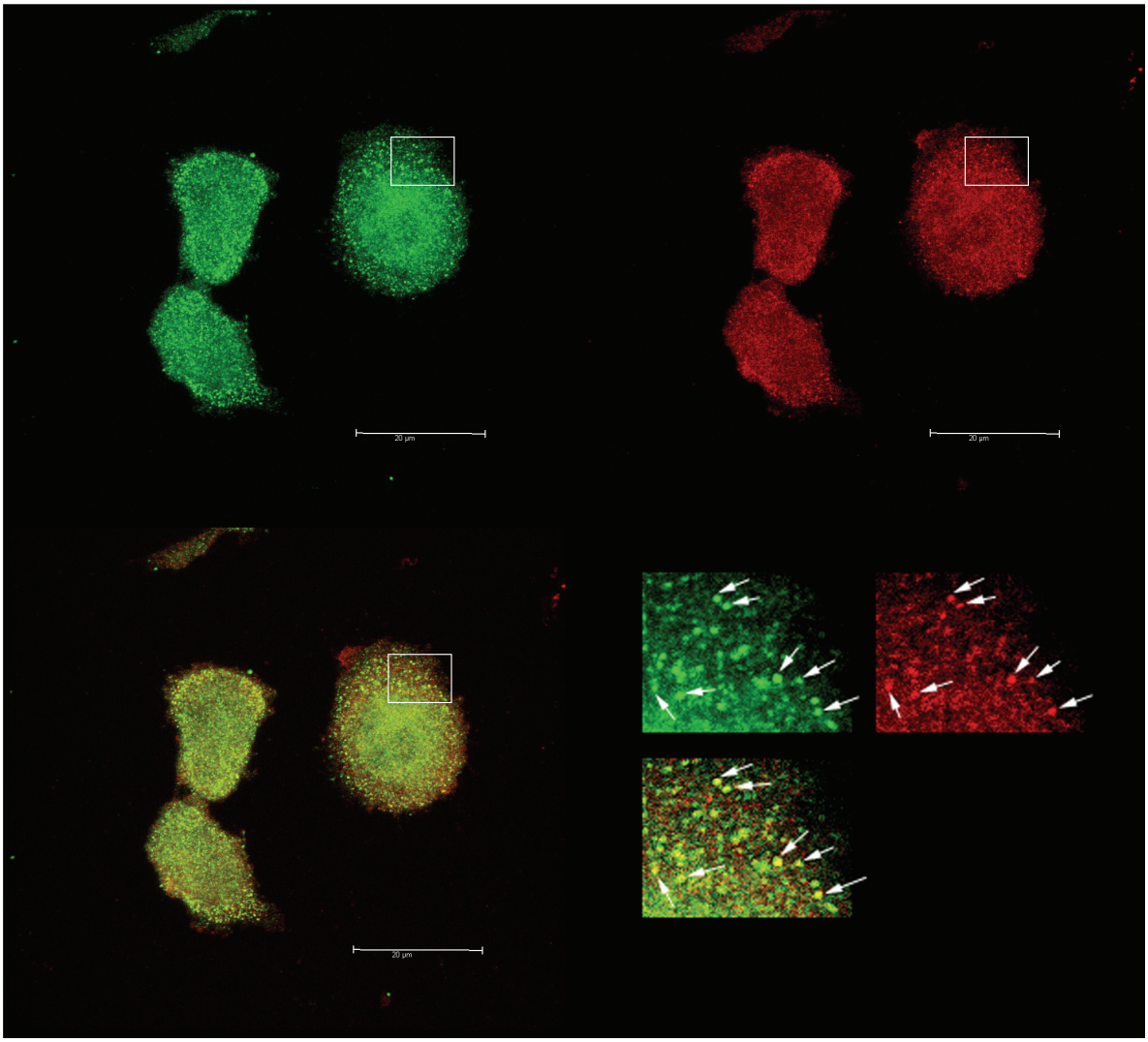


Fig. 3.18 Representative example of labelling after treatment with 2B12 (5 mg/ml, green channel) and LAMP-1 (1:100, red channel). Visualized by proper biotin-conjugated secondary antibodies and Avidin-FITC (1:600), Avidin-Texas Red (1:600), respectively. Green punctuate staining by 2B12 represents the localization of APP, red punctuate staining refers to lysosomes. The overlay of channels expresses the colocalization. Picture is a maximum projection of Z series taken with confocal microscope (n = 3). Arrows indicate the colocalization signal (yellow) located to vesicular compartments (lysosomes). Adequate controls were performed (data not shown).

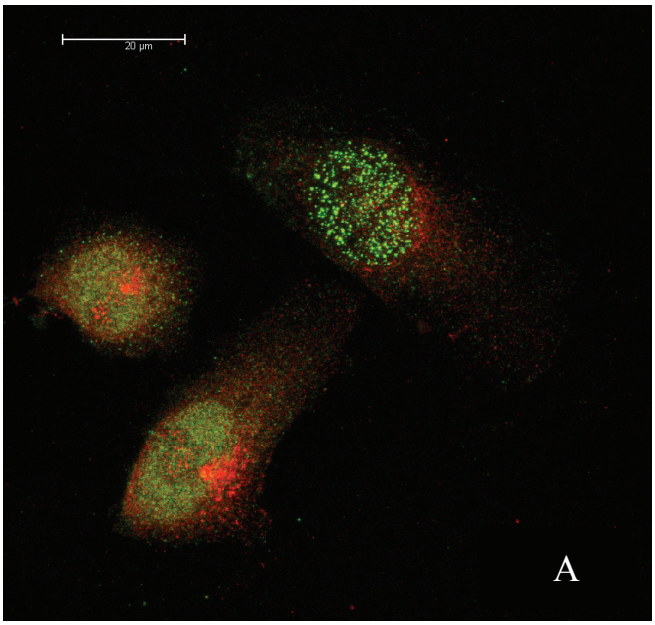
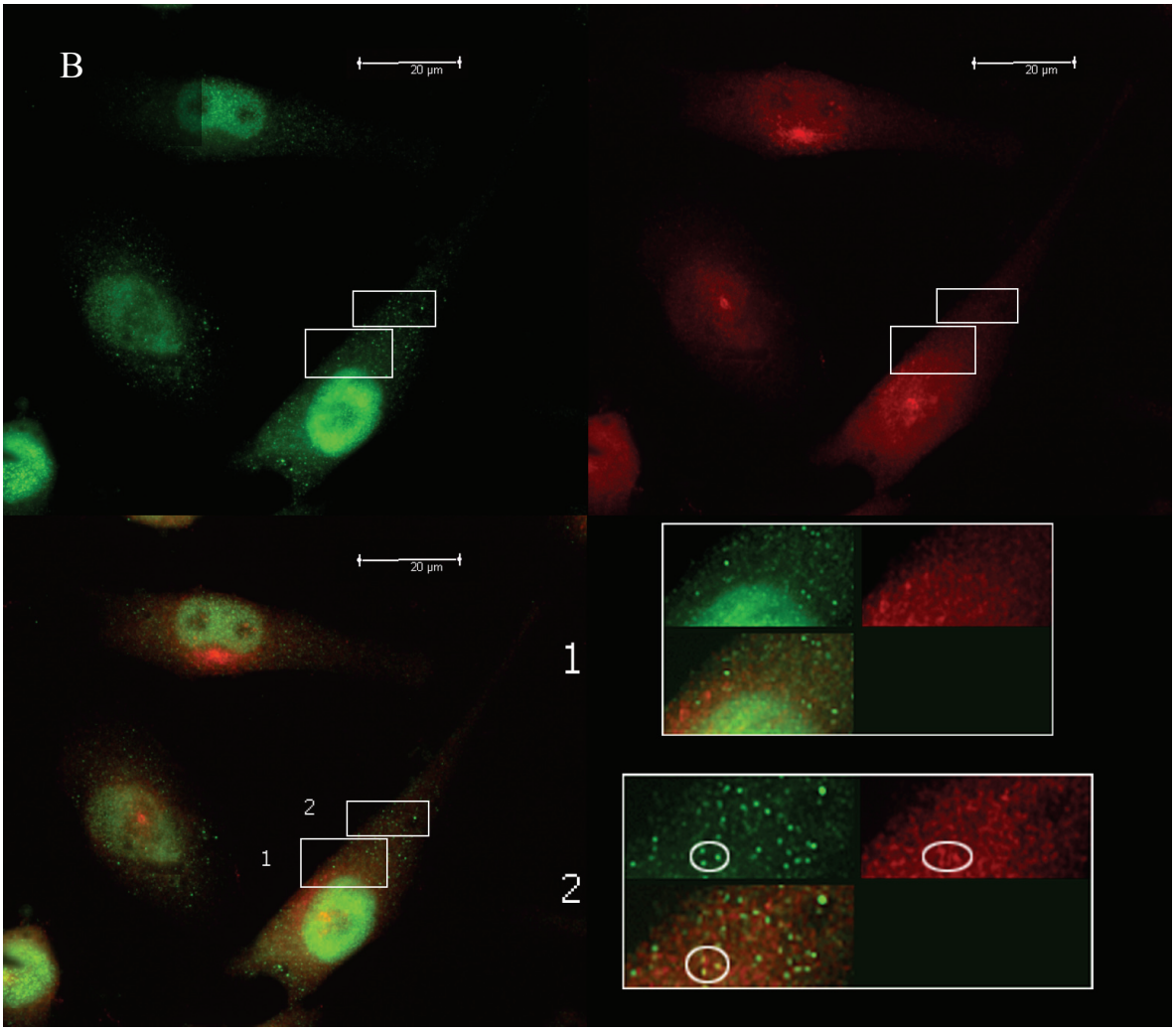


Fig. 3.19 Examples of labelling after treatment with 2B12 (5 mg/ml, green channel) and EEA-1 (1:100, red channel). Visualized by proper biotin-conjugated secondary antibodies and Avidin-FITC (1:600), Avidin-Texas Red (1:600), respectively. Green punctuate staining by 2B12 represents the localization of APP, red punctuate staining refers to early endosomes. The overlay of channels expresses the colocalization. Picture (A) is the overlay and maximum projection of Z series taken with confocal microscope (n = 3), picture (B) was taken with fluorescent microscope. In picture (B), boxes (1) and (2) are magnified. Colocalization is absent in box (1). There is a little amount of colocalization signal in box (2) – marked by the ellipses. Adequate controls were performed (data not shown).



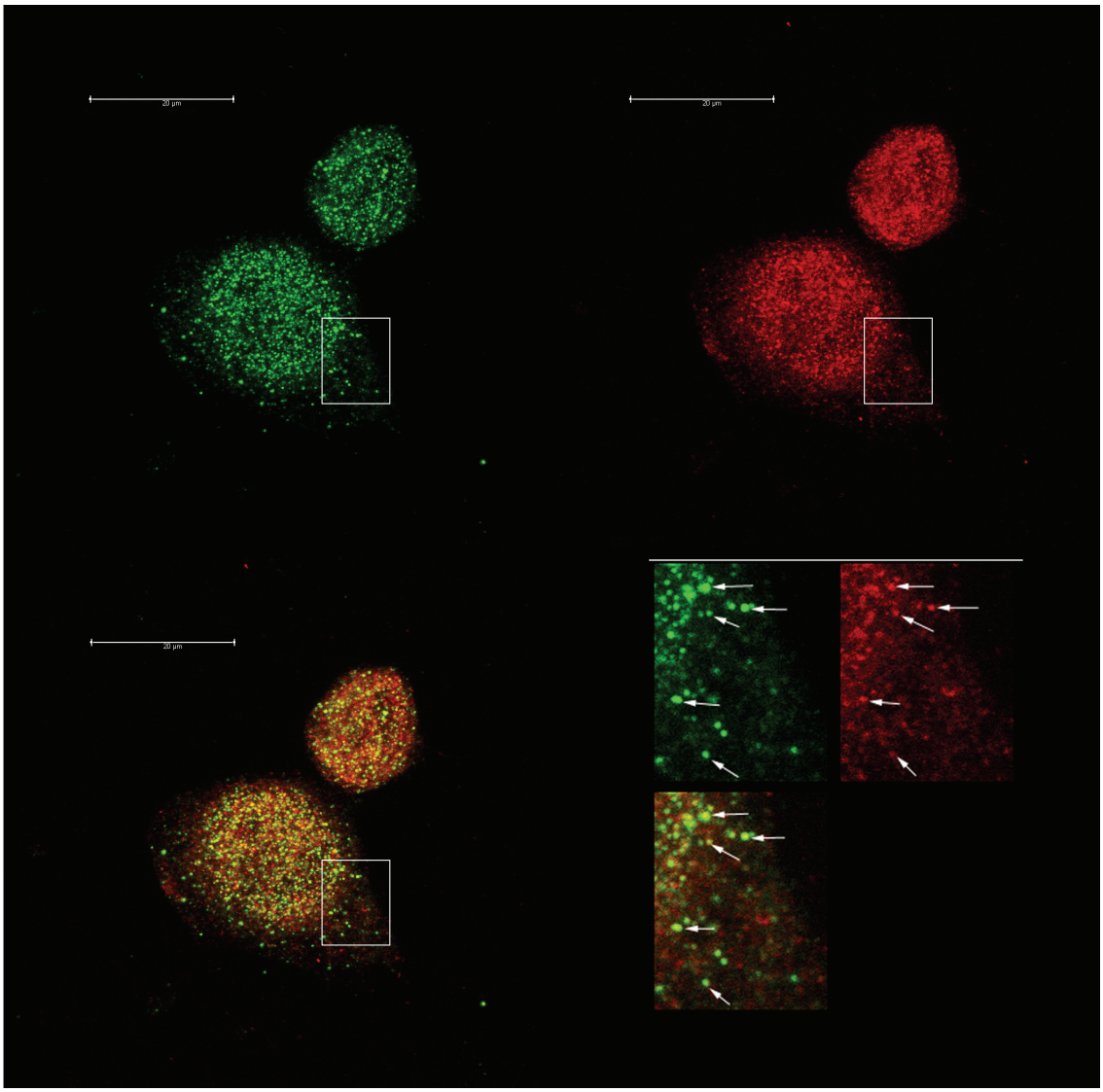


Fig. 3.20 Representative examples of labelling after treatment with 2B12 (5 mg/ml, green channel) and Bcl - 2 (1:100, red channel). Visualized by proper biotin-conjugated secondary antibodies and Avidin-FITC (1:600), Avidin-Texas Red (1:600), respectively. Green punctuate staining by 2B12 represents the localization of APP, red punctuate staining refers to early endosomes. The overlay of channels expresses the colocalization. Picture is a maximum projection of Z series taken with confocal microscope (n = 3). Adequate controls were performed (data not shown).

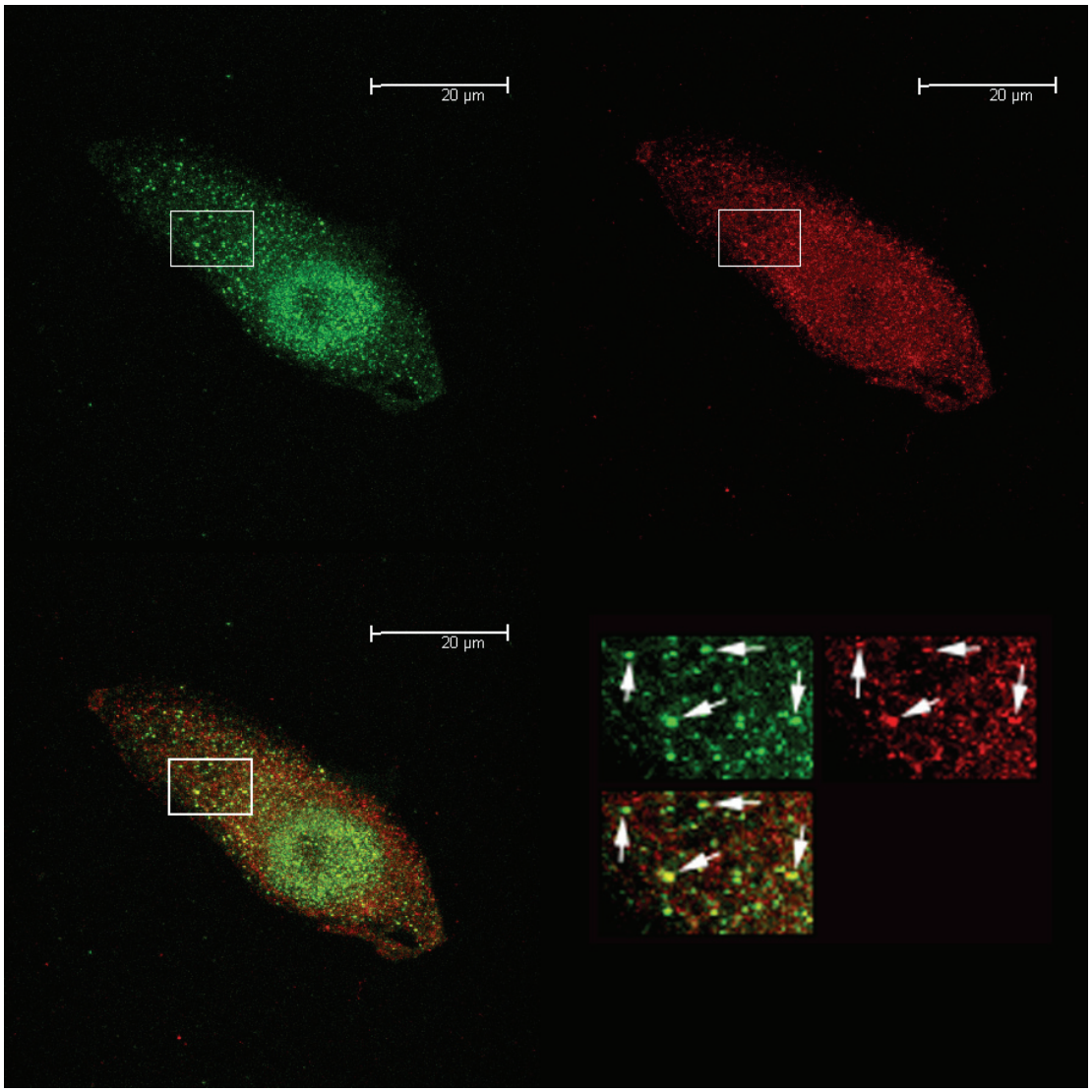


Fig. 3.21 Representative example of labelling after treatment with 2B12 (5 mg/ml, green channel) and M6PR (1:100, red channel). Visualized by proper biotin-conjugated secondary antibodies and Avidin-FITC (1:600), Avidin-Texas Red (1:600), respectively. Green punctuate staining by 2B12 represents the localization of APP, red punctuate staining refers to late endosomes. The overlay of channels expresses the colocalization. Picture is a maximum projection of Z series taken with confocal microscope (n = 3). Arrows indicate the colocalization signal (yellow) located to vesicular compartments (late endosomes). Adequate controls were performed (data not shown).

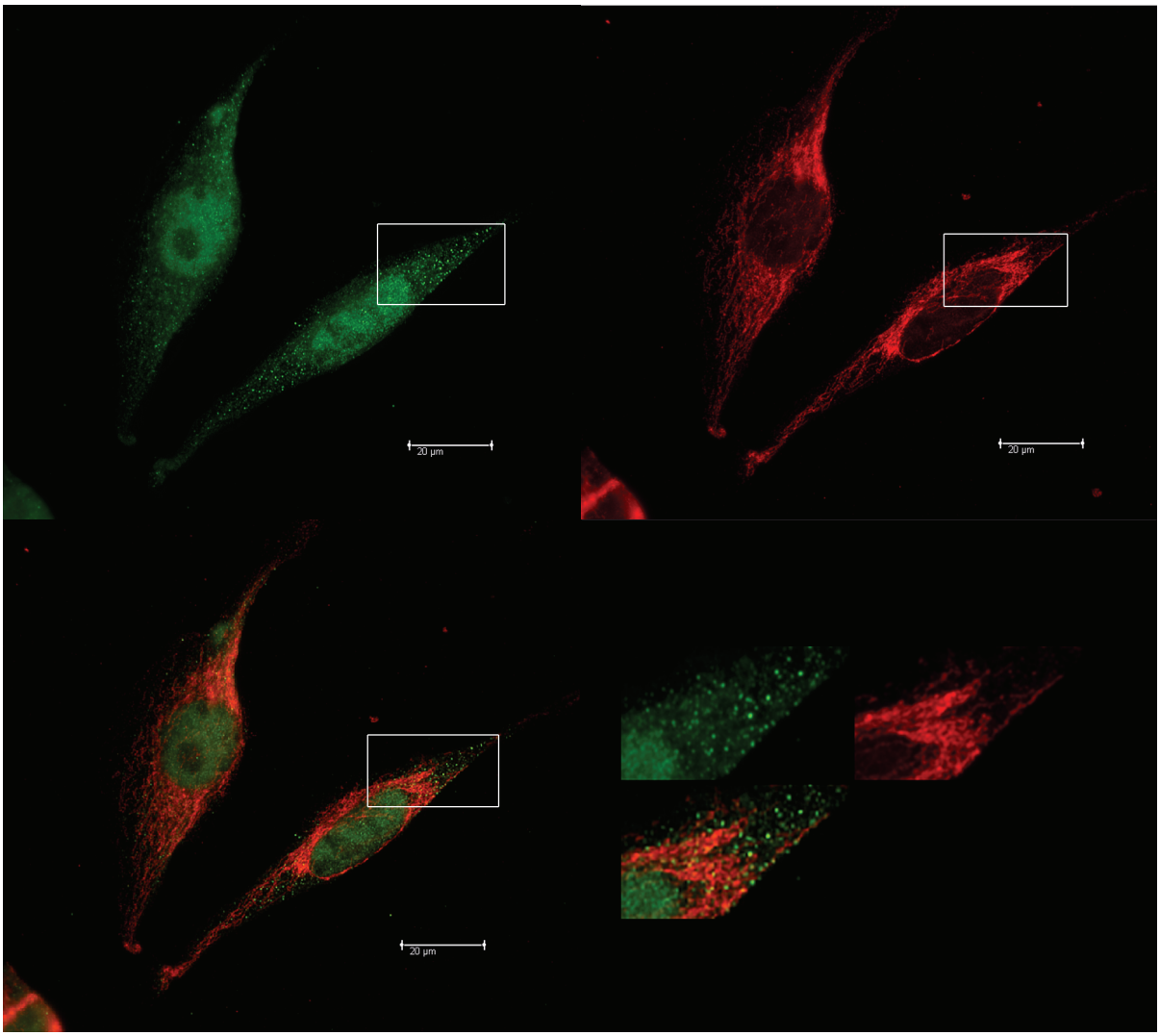


Fig. 3.22 Representative example of labelling after treatment with 2B12 (5 mg/ml, green channel) and Hsp-60 (1:100, red channel). Visualized by proper biotin-conjugated secondary antibodies and Avidin-FITC (1:600), Avidin-Texas Red (1:600), respectively. Green punctuate staining by 2B12 represents the localization of APP, solid red formations refer to mitochondria. The overlay of channels expresses the colocalization. No colocalization could be detected. Picture was taken with fluorescent microscope. Adequate controls were performed (data not shown).

4. DISCUSSION

Beta-amyloid (Aβ) plays a crucial role in the pathogenesis of Alzheimer's disease (AD). Aβ is produced by proteolytic cleavage of beta-amyloid precursor protein (APP). Therefore, the understanding of exact trafficking pathways and processing of APP in a cell is vital information for all prospective treatments of AD based on interaction with Aβ production.

The aim of this project was to confirm the results obtained by Pei San Ho (2007) concerning the internalisation of 2B12, and make a further research into the intracellular localization ant trafficking of 2B12. 2B12 is a monoclonal antibody developed by Thomas et al. (2006), raised against immunogenic peptide representing the 15 amino acid sequence spanning the β-secretase cleavage site of human APP. The characterisation and binding of 2B12 to APP in MOG cells was confirmed by ELISA and Western Blotting techniques. It was also shown that 2B12 is capable of reducing the levels of secreted Aβ (Thomas et al., 2006). It was proposed that 2B12 would enter the cells via natural internalisation (endocytosis) after binding to APP exposed on the cell surface. This hypothesis was based on previous observations made by Koo et al. (1996) and Yamazaki et al. (1996), who used labelled anti-APP monoclonal antibodies to examine the trafficking of APP. Importantly, they showed that binding of such antibody did not alter the natural trafficking of APP and that the antibodies remained attached to APP as a complex at least until degradation, therefore results obtained by this technique are relevant. The hypothesis was confirmed by Pei San Ho's (2007) experiments with live MOG cells, where she observed time-dependent internalisation of 2B12 via non-penetrated plasma membrane.

4.1 Labelling of fixed permeabilizated MOG cells by 2B12

Labelling was performed on cells fixed with 2% formaldehyde solution in 0.1% PBS. Cell membranes were permeabilizated with 0.1% Triton X-100. Triton was present during the whole process of ICC, including blocking, incubation with both primary and secondary antibodies and avidin-FITC. The presence of permeabilizating Triton leads to more apparent and distinctive labelling as reported by Pei San Ho (2007).

As expected, labelling of MOG cells by 2B12 led repeatedly to punctuate perinuclear staining (see **Fig. 3.15**), very similar to Pei San Ho's experiments with 2B12 and other's experiments with different antibodies binding to APP (e.g. Koo and Squazzo, 1994; Yamazaki et al., 1996). My results therefore confirm that 2B12 recognizes and detects APP in MOG cells.

4.2 Labelling of live MOG cells by 2B12 in a time course experiment

This experiment should have demonstrated the internalization (endocytosis) of 2B12 attached to APP molecules exposed on the cell surface. Although I exactly followed Pei San Ho's procedure (2007) and worked in the same laboratory, I did not come to the same results. The staining was often very weak, sometimes the amount of internalized APP did not increase with time, and there were significant differences among individual repetitions of the experiment (data not shown). The experiment was repeated five times (n = 5). The explanation for this discrepancy between mine and previous results is probably the decreased stability of 2B12, which might have not survived the conditions of time-course experiment (4 hours at 37 °C). On contrary, the antibody was stable enough for experiments with fixed cells (incubated for 1 hour at room temperature). The decline in stability of 2B12 aliquots used at the time of my stay in the laboratory was subsequently confirmed and measured by ELISA. Causes of the decline remain unknown.

The failure of this experiment led to constrictions of planned colocalization experiments.

4.3 Evaluating the primary antibodies – subcellular markers for usage with MOG cells

One of the most important aspects of my work was the choice of reliable primary antibodies, which would precisely visualize cell compartments concerning the trafficking of APP and which I was interested in. The fact that 2B12 is of mouse origin narrowed the number of available antibodies, because they were intended to be used together in colocalization experiments – all other mouse antibodies had to be omitted, because of the detection by secondary antibodies. It was necessary to test the antibodies – subcellular markers and optimize the ICC protocol (especially the dilutions) individually.

Anti – TGN-46 (Fig. 3.8), anti – Hsp-60 (Fig. 3.14) and anti-EEA-1 (Fig. 3.11) worked well and in accordance with manufacturer's datasheet, labelling selectively trans-Golgi network, mitochondria, and early endosomes, respectively. They also labelled expected bands in Western-blotting analysis, suggesting they were able to recognize targeted proteins in MOG cells. However, it might be beneficial to try anti-EEA-1 antibody from another manufacturer in order to obtain more accurate localization of early endosomes.

Anti-LAMP-1 had lots of non-specific staining in Western-Blotting analysis (Fig. 3.3). M6PR produced weak signal in ICC experiments (Fig. 3.13). It might be beneficial to try other manufacturer's antibodies for comparison.

4.4 Colocalization experiments

Colocalization experiments were performed to elucidate the distribution and trafficking pathway of 2B12 (APP) in MOG cells. After being synthesized, APP is modified in endoplasmic reticulum (ER) and Golgi apparatus (GA) and trafficked via constitutive secretory pathway (including trans-Golgi network – TGN) to the cell surface (Vetrievel, 2006). A portion of APP molecules are then internalized and trafficked via endocytic pathway (endosomes) either to end up for degradation in lysosomal compartment or to be recycled back to the cell surface (Koo et al., 1996) – see section **1.2. -** The role of amyloid precursor protein (APP) in AD - for details.

The concomitant secretion and internalization of APP makes the trafficking analysis complicated. Koo et al. (1996) and Yamazaki et al. (1996) used a more specific approach, using monoclonal antibodies to label APP molecules exposed on the cell surface. Therefore they were able to track the internalized molecules specifically. My efforts to use a similar technique for 2B12 were rendered impossible because of the lack of stability of this antibody – see above. Colocalization experiments had to be restricted to fixed cells only, limiting the value of achieved results.

Based on the information available in literature and briefly mentioned above, I expected to observe a colocalization of 2B12 signal (APP) with following antibodies' signals and compartments: anti-EEA-1 (early endosomes), anti-M6PR (late endosomes), anti-LAMP-1 (lysosomes), anti-Bcl-2 (endoplasmic reticulum), and anti-TGN-46 (trans-Golgi network). On contrary, no colocalization was expected for anti-Hsp-60 (mitochondria).

Despite expectations, **anti-TGN-46** signal did not significantly co localize with 2B12. Anti-TGN-46 produced a clear labelling pattern of solid sections of rather membranous type in the perinuclear area (Fig. 3.16A). No colocalization was observed within these sections. Although some colocalization signal could be seen in the form of dotted labelling in the cytoplasm in pictures taken by laser scanning confocal microscope (Fig. 3.16B), it seemed to be due to non-specific binding of anti-TGN-46. However, APP should be present in TGN as it's trafficked through it as a part of secretory pathway. The failure of detection of the colocalization might have several causes. The signal of APP could have been masked by very strong signal of anti-TGN-46 (to test this, I suggest performing further experiments with more diluted anti-TGN-46, or usage of different antibody labelling TGN-46 protein). The levels of APP present in TGN in MOG cells could have been too low to be visualized by 2B12 antibody (I suggest further testing with cell cultures secreting higher amounts of APP).

Colocalization was successfully confirmed for **anti-M6PR** (Fig. 3.21), **anti-LAMP-1** (Fig. 3.18) **and anti-Bcl-2** (Fig. 3.20) antibodies. For all of them, significant colocalization signal could have been traced in the pictures from confocal microscope. Number of repetitions for each experiment was sufficient to obtain reproducible and repeatable results (n = 3 at least). All necessary controls were performed to ensure that the effect of non-specific binding was not significant. Importantly, even though labelling pattern of 2B12 (APP) and relevant subcellular marker was often similar (discrete punctuate pattern in cytoplasm), they were not identical in any pair of 2B12 and any subcellular marker. That means the observed colocalization cannot be explained by possible overlay of excitation spectrums of FITC and Texas red dyes and the setup of microscope detectors.

Results for **anti-EEA-1** were unclear. The number of observed colocalization incidents was little (Fig. 3.19). With n = 3 no significant colocalization could have been confirmed despite of results suggesting that it is probably present. However, APP should be present in early endosomes as it gets there right in the process on endocytosis from the cell surface. Therefore experiments with live cells, tracking the APP molecules from the plasma membrane could help to solve the discrepancy. Testing with another antibody labelling early endosomes could be helpful as well.

No colocalization was observed with **anti-Hsp-60** antibody labelling mitochondria (Fig. 3.22), which is in concordance with the expectations. This proves the ability of used experimental method to achieve negative results.

Antibody	Expactation	Result
anti – TGN-46	expected	negative
anti – EEA-1	expected	unclear (further exp. required)
anti – M6PR	expected	confirmed
anti – Hsp-60	not expected	negative
anti – LAMP-1	expected	confirmed
anti – Bcl-2	expected	confirmed

Fig. 4.1 Summary of results of colocalization experiments for individual antibodies (subcellular markers)

4.5 Future experiments

Further research into this field is recommended. Results of colocalization experiments with anti-TGN-46 and anti-EEA-1 did not meet the expectations and need to be repeated and/or modified (different cell cultures, maybe transfected cells overproducing APP; different primary antibodies against the same proteins, or against other proteins labelling the same cell compartments). After the stability of 2B12 is strengthened, colocalization experiments with live cells should be performed as planned. In this type of experiment living MOG cells would be incubated with 2B12 for various periods of time, then fixed and exposed to primary antibodies labelling desired cell compartments. Only APP molecules present on the cell surface would be labelled and traced. Applying this strategy, we could obtain data for 2B12 localization in cell compartments after various periods of time after the internalization started. In future studies, transfected cells overexpressing APP could be used to investigate the trafficking of 2B12 (APP) in real-time. Another primary antibody recognizing human APP could be used simultaneously with 2B12 to obtain clearer results.

4.6 Conclusion

2B12 antibody developed by Thomas et al. (2006) represents a promising approach to AD therapy. Careful experimental examination of 2B12, its properties and mode of action is vital. Thomas et al. (2006) showed that 2B12 bound strongly and selectively to APP and was capable of decreasing the production of Aβ in human cell lines. They proposed that 2B12 would bind to APP molecules exposed on the cell surface and be internalized as a complex via natural endocytic pathway. This hypothesis was supported by Pei San Ho's (2007) project

focused on the internalization. My project examined the distribution and trafficking of 2B12 in MOG cells. Most of the results were in concordance with up-to-date knowledge of APP trafficking, which supports the hypothesis of the 2B12 / APP interaction, indicating that 2B12 is internalised together with APP. Moreover, the results suggest that the 2B12/APP complex is stable within the cells, which is crucial for the Aβ production lowering effect and for future potential use of 2B12 as a disease modifying drug in AD therapy.

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ABSTRACT

Intracellular trafficking of an anti-Amyloid Protein Precursor antibody

Alzheimer's disease is characterized by over-accumulation of beta-amyloid peptide (Aβ) in the brain. Aβ is produced by proteolytic cleavage of beta-amyloid precursor protein (APP) by β- and γ-secretases. Novel monoclonal antibody, 2B12, has been shown to bind to β-secretase cleavage site of APP, reducing the production of APP, presumably by preventing the cleavage by steric hindrance. 2B12 is hypothesized to bind to APP molecules exposed on the cell surface and to be internalized in the form of complex with APP via natural endocytic pathway. This hypothesis was confirmed by San Pei Ho's (2007), who followed the internalization of 2B12 in living astrocytoma MOG-G-UVW, cells in time-course experiment.

This project is focused on intracellular trafficking of 2B12 and its localization within specific cellular compartments. Experiments were performed with fixed astrocytoma MOG-G-UVW cells (constitutively expressing APP). Originally planned experiments with live cells could not be performed due to decreased stability of 2B12 (causes remain unknown). 2B12 was tested for colocalization with polyclonal affinity purified antibodies labelling subcellular markers (proteins) associated with compartments known to participate in APP trafficking (endoplasmic reticulum, trans-Golgi network, early and late endosomes, and lysosomes). Primary antibodies were visualized by correspondent biotinylated secondary antibodies and fluorophores conjugated to avidin. Pictures were taken by fluorescent and confocal laser scanning microscope.

Based on up-to-date information of APP trafficking, 2B12 was expected to be present in all compartments mentioned above. The results proved the localization in endoplasmic reticulum, lysosomes and late endosomes. Data for early endosomes were unclear. 2B12 localization in trans-Golgi network was not confirmed.

Most of the results are in agreement with presumed 2B12 localization based on localization of APP and therefore provide another piece of evidence supporting the hypothesis of 2B12 being trafficked together with APP. Results for early endosomes and trans-Golgi network have to be revised.

ABSTRAKT

Intracelulární sledování pohybu protilátky proti amyloidnímu prekurzorovému proteinu

Alzheimerova choroba je neuropatologické onemocnění, klinicky se projevující postupným zhoršováním funkcí centrální nervové soustavy, především pak paměti, kognitivních funkcí, změněným chováním (včetně paranoii) a zhoršováním jazykových funkcí. Alzheimerova choroba je nejčastější příčinou demence ve středním a vyšším věku. Příčiny a samotný proces rozvoje choroby nejsou doposud plně objasněny, nicméně jedním z rozhodujících faktorů se zdá být nahromadění β-amyloidního peptidu (Aβ) v určitých oblastech CNS. Aβ je ovšem přirozeným a fyziologickým metabolitem a lze ho izolovat z tělesných tekutin zdravých jedinců.

Aβ vzniká proteolytickým štěpením amyloidního prekurzorového proteinu (APP), což je fyziologický, evolučně vysoce konzervativní, membránový protein. APP může být štěpen v takzvané neamyloidogenní cestě α-sekretázou a následně γ-sekretázou za vzniku dobře rozpustných peptidů nepodílejících se na vzniků depozitů, nebo β-sekretázou a γ-sekretázou za vzniku Aβ s tendencí k agregaci (amyloidogenní cesta). Pohyb APP v buňce je velmi komplexní. Po jeho syntéze je posttranslačně modifikován a distribuován sekreční cestou k buněčnému povrchu, kde je exponován. Poměrně rychle je APP podroben endocytóze a následně směrován do lysosomů k degradaci a nebo recyklován zpět na buněčný povrch. K amyloidogennímu štěpení (produkci Aβ) dochází z velké části v průběhu těchto recyklačních kroků.

Nová monoklonální protilátka 2B12 byla připravena v roce 2006 imunizací myší syntetickým peptidem obsahujícím sekvenci aminokyselin odpovídající místu štěpení APP β-sekretázou. Bylo prokázáno, že 2B12 se váže na APP a snižuje sekreci Aβ v buněčných kulturách. Předpokládá se, že navázaná protilátka stericky inhibuje štěpení APP β- sekretázou. Tento přístup má zřetelné výhody oproti ostatním imunologickým metodám založeným na aktivní či pasivní imunizaci proti Aβ. Klinické testy látky tohoto typu byly zastaveny pro rozvinutí zánětlivé reakce v CNS zprostředkované Th1 lymfocyty u několika jedinců. U 2B12, která se významně neváže na samotný Aβ, je toto riziko nízké.

Dle vyslovené hypotézy se 2B12 váže na molekuly APP exponované na buněčném povrchu (plazmatické membráně) a takto vzniklý komplex je internalizován přirozenou

endocytózou. Tato hypotéza vychází z předchozích studií a byla potvrzena prací Pei San Ho (2007), která sledovala internalizaci 2B12 v buněčné kultuře astrocytomu MOG-G-UVW.

Cílem této práce bylo získat další důkazy podporující výše zmíněnou hypotézu pomocí studia lokalizace 2B12 v jednotlivých buněčných kompartmentech, které se dle dostupných informací účastní koloběhu APP v buňce (ranné a pozdní endosomy, lysosomy, endoplazmatické retikulum - ER, vezikuly trans-Golgiho aparátu - TGN). Potvrzení selektivní přítomnosti 2B12 v těchto kompartmentech by bylo dalším důkazem naznačujícím správnost této hypotézy.

Lokalizace 2B12 byla studována pomocí imunocytochemických metod na buňkách lidského astrocytomu MOG-G-UVW, konstitutivně produkujícího APP. Původním záměrem bylo inkubovat 2B12 s živými buňkami a jejich fixaci provádět až před aplikací druhé primární protilátky. Tento postup by zajistil selektivní označení jen těch molekul APP, které již byly exponovány na buněčném povrchu a umožnil by sledovat proces endocytózy 2B12 (lokalizaci v jednotlivých kompartmentech) v čase. Kvůli snížené stabilitě 2B12 (důvod neznámý) tento přístup nemohl být realizován. Fixované a permeabilizované buňky byly tedy nejprve inkubovány s 2B12 a poté s jednou z komerčně dostupných protilátek vážících se na proteiny typické pro jednotlivé buněčné kompartmenty (EEA1 – ranné endosomy, M6PR – pozdní endosomy, LAMP1 – lysosomy, Bcl-2 – ER, TGN46 – síť vezikul trans-Golgiho aparátu, Hsp60 - mitochondrie). Navázané primární protilátky byly poté zviditelněny použitím patřičných sekundárních protilátek značených biotinem a fluorescenčních barviv (fluorescein a texaská červeň) konjugovaných s avidinem (klasický postup využívající silné vazby mezi biotinem a avidinem). Použitím odlišných fluorescenčních barviv pro 2B12 a druhou ze dvojice primárních protilátek v rámci jednoho pokusu bylo možné detekovat lokalizaci obou těchto protilátek zároveň i jejich případnou kolokalizaci, která se projevila složením obou základních barev. Významná kolokalizace byla považována za důkaz přítomnosti 2B12 v daném buněčném kompartmentu. Výsledky byly vyhodnocovány pomocí fluorescenčního mikroskopu a laserového konfokálního mikroskopu. Všechny pokusy byly opakovány minimálně třikrát (n = 3) a byly prováděny příslušné negativní kontroly sloužící k posouzení vlivu případného nespecifického vázání protilátek na dosažené výsledky.

Přítomnost 2B12 byla očekávána ve všech výše zmíněných kompartmentech kromě mitochondrií (testování přítomnosti 2B12 v mitochondriích mělo za cíl ověřit schopnost použité metody dojít k negativnímu výsledku). Přítomnost 2B12 byla prokázána v pozdních endosomech (anti-M6PR protilátka, obr. 3.21), lysosomech (anti-LAMP1, obr. 3.18) a endoplazmatickém retikulu (anti-Bcl-2, obr. 3.20). Údaje o přítomnosti 2B12 v časných

endosomech byly proměnlivé a odhalená lokalizace byla malá (anti-EEA1, obr. 3.19). Oproti očekávání se přítomnost 2B12 nepodařila prokázat v TGN (anti-TGN46, obr. 3.16). Přítomnost 2B12 v mitochondriích (anti-Hsp 60, obr. 3.22) byla negativní. Příčiny nesrovnalostí mezi přepokládanými a dosaženými výsledky mohou být různé. Lze doporučit opakování daných pokusů s jinými primárními protilátkami (jiný výrobce), případně testování na transfekčních buňkách produkujících vyšší než běžné hladiny APP.

Většina výsledků této práce nepřímo potvrzuje schopnost 2B12 vázat se na molekuly APP a být s těmito internalizována. Výsledky rovněž svědčí o značné stabilitě komplexu 2B12 – APP, která je podmínkou případného farmakologického účinku v podobě snížení produkce Aβ. 2B12 tak nadále zůstává potenciálně perspektivní možností terapie Alzheimerovy nemoci.