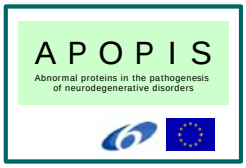

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A P O P I S

Abnormal proteins in the pathogenesis of neurodegenerative disorders

Instrument: Integrated Project

Thematic Priority: LIFESCIEHEALTH

Publishable Final Activity Report

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Project coordinator organisation name: VERUM - Foundation for Behaviour and Environment
Project website: http://www.verum-foundation.de/apopis

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Table of contents

1. PROJECT EXECUTION

1.1 Project objectives

The neurodegenerative diseases under investigation include dementing disorders like Alzheimer and Huntington disease, frontotemporal dementia (FTD), Lewy body dementia (LBD) and transmissible spongiform encephalopathies or prion diseases, as well as movement disorders like Parkinson disease, and motor neuron diseases like amyotrophic lateral sclerosis (ALS). Neither early diagnostic tools nor effective treatments exist at present for these disorders. A hallmark common to all of them is the deposition of abnormally folded protein aggregates in different brain regions. Yet, the events that trigger protein aggregation and the role these protein deposits play in disease progression are poorly understood. A small fraction of disease cases are associated with genetic mutations and the identification of disease-related genes with their subsequent characterization in cell culture and animal models has provided invaluable insights into the underlying pathological processes. However, the list of genes associated with neurodegenerative disorders is far from being complete and only little is known about the role of the encoded proteins, both in normal and pathologic conditions. The Consortium was established to provide a better understanding of the pathogenic mechanisms involved in neurodegeneration, to advance clinical detection and to develop strategies for the prevention and treatment of these disorders. It integrated 39 research groups from all over Europe to tackle the tasks in a multi-faceted approach, combining different disciplines to provide a more comprehensive view of the problem of protein aggregation and how it triggers neuro-degeneration.

1.2 Contractors involved

Name	**Short name**	**Country**	**Scientists in charge of the project**
VERUM - Stiftung für Verhalten und Umwelt	VERUM	Germany	Prof. Franz Adlkofer
Flanders Interuniversitary Institute for Biotechnology	VIB	Belgium	Prof. Bart De Strooper (Leuven) Prof. Christine van Broeckhoven (Antwerp)
Universität Zürich	UNIZH	Switzerland	Prof. Adriano Aguzzi, Prof. Christoph Hock, Prof. Roger M. Nitsch, Prof. Peter Sonderegger
Universität Basel	UNIBAS	Switzerland	Prof. Mathias Jucker
Eidgenössische Technische Hochschule Lausanne	EPFL	Switzerland	Prof. Patrick Aebischer
Eidgenössische Technische Hochschule Zürich	ETHZ	Switzerland	*left the contract in September 2004*
Albert-Ludwigs-Universität Freiburg	ALU.FR	Germany	Prof. Ralf Baumeister
Ludwig-Maximilians-Universität Muenchen	LMU.MUEN	Germany	Prof. Christian Haass
Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.	MPG	Germany	Prof. Rüdiger Klein (Martinsried) Prof. Kai Simons (Dresden)
Technische Universität Darmstadt	TUDarm	Germany	Prof. Boris Schmidt
Julius-Maximilians-Universität Würzburg	UNIWUE	Germany	Prof. Michael Sendtner
Max-Delbrück-Centrum für Molekulare Medizin	MDC	Germany	Prof. Erich Wanker
Parc Cientific de Barcelona	PCB	Spain	Dr. Marco Milán
European Molecular Biology Laboratory	EMBL	Germany	Dr. Luis Serrano
Institut National de la Santé et de la Recherche Medicale	INSERM	France	Prof. Alexis Brice

Université de Lille 2 - Droit et Santé	UNIV.LILLE	France	Prof. Luc Buée
Centre National de la Recherche Scientifique	CNRS	France	Dr. Frédéric Checler
National and Kapodistrian University of Athens	NKUA	Greece	Ass. Prof. Spiros Efthimiopoulos
The Hebrew University of Israel	HUJI	Israel	Prof. Hermona Soreq
Fondazione Cavalieri Ottolenghi	FCO.SI	Italy	Prof. Carlos Dotti
Università Vita-Salute San Raffaele	UNIHSR	Italy	Prof. Jacopo Meldolesi
CEINGE Biotecnologie Avanzate Scarl	CEINGE	Italy	Prof. Tommaso Russo
Università degli Studi di Genova	UNIGE	Italy	Prof. Claudio Russo
International Institute of Molecular and Cell Biology in Warsaw	IIMCB	Poland	Prof. Jacek Kuznicki
Universidade de Aveiro	UAVR	Portugal	Prof. Odete da Cruz e Silva
Uppsala Universitet	UU	Sweden	Prof. Lars Lannfelt
Lund Universitet	ULUND	Sweden	Prof. Anders Björklund
University College London	UCL	UK	Prof. Martin N. Rossor
University of Kent at Canterbury	UNIKENT	UK	Prof. Mick Tuite
King's College London	KCL	UK	Prof. Christopher Shaw
University of Cambridge	UCAM	UK	Dr. Maria Grazia Spillantini
EVOTEC Neurosciences GmbH	EVOTEC	Germany	Dr. John Pohlner
Cellzome AG	CELLZOME	Germany	Dr. Gerard Drewes
Philipps-Universität Marburg	UNIMAR	Germany	Prof. Wolfgang Oertel

1.3 Work performed

To foster the understanding of disease mechanisms APOPIS combined two workpackages (WP1 and WP2) on the basic biology of disease-related proteins with a clinical workpackage (WP3) on disease genetics and on novel imaging techniques and a workpackage on potential therapies (WP4). In WP1 the structural, genetic, biochemical, and cellular mechanisms involved in abnormal protein aggregation were investigated. WP2 dealt with the functional characterization of genes involved in Alzheimer and Parkinson disease and with the establishment of animal models to understand amyloid deposition. WP3 was directed to improve clinical detection. For this, two entirely different methodologies, genetics and neuroimaging, were applied in patients with suspected neurodegenerative diseases. WP4 focussed on the discovery and development of novel avenues for the treatment and eventual prevention of neurodegenerative diseases. Because these diseases are generally characterized by the presence of abnormal protein aggregates in different brain regions, prevention of aggregate formation was a common therapeutic objective.

1.4 End results

Substantial progress in our understanding of the various neurodegenerative conditions was achieved in the course of the project. This is reflected by a number of high-impact publications, many of them resulting from collaborations between partners. The main results obtained by the APOPIS Consortium are discussed in the following chapters:

1.4.1 Molecular mechanism of amyloid fibril formation and the associated cytotoxicity

A hallmark common to all human neurodegenerative diseases is the deposition of abnormally

folded protein aggregates in different brain regions. The pathogenic events that trigger protein aggregation and the underlying mechanism of toxicity remain, however, largely unknown. Moreover, although recent evidence suggests that soluble, prefibrillar aggregates – rather than the insoluble fibrils – are responsible for the neurotoxic effects, the identity of the toxic species is still a matter of debate.

We investigated in detail the molecular mechanisms that govern amyloid fibril formation and how this is linked to toxicity. This work provided important insight for the development of novel therapeutic strategies discussed later in this chapter.

The disease-associated proteins (like Abeta and Tau in Alzheimer disease (AD), alpha-synuclein in Parkinson disease (PD), huntingtin in Huntington disease (HD), PrP in prion diseases) do not share obvious sequence homology or folding patterns. However, X-ray fibre diffraction data indicate that when they aggregate, the amyloid fibrils they form share a characteristic cross-beta structure, suggesting that the key elements of the fibril formation process may be common to all proteins. We therefore speculated that by studying a highly simplified system – hexapeptides that are prone to polymerization into beta-sheets *in vitro* – we would be able to obtain molecular details of the general amyloid fibril formation process and derive rules that can be applied to any amyloid-forming protein. Thus, starting with a de novo designed hexapeptide that can form amyloid fibrils *in vitro*, we performed a saturation mutagenesis analysis, which allowed us to identify key residues and positions critical for amyloid formation. With this information we were able to derive a computer algorithm – the amyloid pattern – that can be used to identify amyloidogenic regions in any (disease-linked) protein. The amyloid pattern was applied to proteins associated with neurodegenerative disorders (Abeta, Tau and prion protein) to reveal their amyloidogenic regions. When tested *in vitro*, the identified hexapeptides retained the capacity to form fibrils *per se*, thus validating the approach. Based on these data we proposed that the force driving amyloid formation is localized in a short stretch of the protein. This so-called “amyloid stretch hypothesis” was validated by introducing a six-residue amyloidogenic stretch into a non-amyloidogenic protein, which was sufficient to convert it into an amyloid-prone molecule that was toxic to cells. This work set up the basis for a novel therapeutic strategy: the attempt to inhibit amyloid fibril formation by designing or by screening for molecules that specifically bind the fragments identified as amyloidogenic, as discussed in section 1.4.2.

To get insights into the pathogenic events that might trigger protein aggregation *in vivo*, we investigated how different physiochemical parameters (e.g. pH, temperature, ion strength, protein concentration) and lipids affect Abeta aggregation. We found that Abeta oligomerization is accelerated at acidic pH, both for the wild-type peptide as well as for a peptide carrying the Arctic mutation (AbetaArc), which is known to favour the formation of protofibrils (soluble prefibrillar intermediates in the fibrillization process). Moreover, AbetaArc fibrillization was more dependent on ionic strength than the wild-type peptide. Finally, we identified certain lipids (docosahexaenoic acid, DHA) that are capable of stabilizing Abeta protofibrils and might thus play a role in pathogenesis.

To identify which of the protein forms appearing during amyloid formation are responsible for cytotoxicity, we followed two approaches. Retinoic-acid-differentiated PC12 cells were challenged with either synthetic amyloidogenic hexapeptides or with AbetaArc. Both these experiments identified prefibrillar aggregates as responsible for cell impairment. As expected, the presence of the protofibril-stabilizing lipid DHA increased the toxicity of the Abeta preparations. Interestingly, toxic prefibrillar species from different hexapeptides displayed identical structure by electron microscopy, suggesting a common toxic pathway for amyloidoses. We next validated the results obtained in PC12 cells on primary neuronal cultures. Toxic aggregates obtained from full length Abeta1-42 or a smaller Abeta16-21 hexapeptide preferentially localized in synapses, leading to the re-organization of the underlying actin cytoskeleton. This process did not involve stereo-specific interactions between membrane and

toxic species suggesting that it is not receptor mediated. Notably, the degree of neurotoxicity of these Abeta species seemed to depend on the cell membrane composition.

In addition to the direct cytotoxic effects discussed above, protein aggregates may indirectly affect neuronal function by inducing astrocyte activation and the release of substances with potential neurotoxic effects. We investigated this possibility by administering Abeta peptide to primary cultures of rat astrocytes. This treatment induced phenotypical alterations that qualitatively resembled the changes observed upon cytokine treatment, namely COX-2 induction with subsequent release of PGE2, and secretion of interleukin 6. Even if these changes were weaker compared with those obtained with a mix of cytokines, they suggest that aggregated forms of Abeta are able to bring astrocytes to a state of activation similar to that occurring upon CNS inflammation. Interestingly, the Abeta effect on astrocyte activation was stronger if the peptide preparation was left 24 hours at 4 degrees, a treatment that favours the appearance of aggregates. More importantly, the astrocytic activation process was able to produce a reinforcement of the Ca^{2+}-dependent glutamate release that occurs upon acute stimulation of astrocytes. Altogether these data suggest that, when stimulated by Abeta, astrocytes can affect synaptic function by activating their release of neurotransmitters and neurotransmission modulators, therefore defining an additional toxic route for Abeta aggregates.

1.4.2 Development of inhibitors of aggregation or of the associated toxicity

Because of the toxicity associated with protein aggregates, prevention of aggregate formation represents an attractive therapeutic strategy to treat neurodegenerative disorders. The identification of aggregation inhibitors relays largely on the availability of suitable assay systems to screen for active compounds from large compound libraries. We developed cell-free, as well as yeast-, *C. elegans*- and *Drosophila*-based assays that are suitable for low-, medium- or high-throughput screenings. In these assays, protein aggregation and the effect of compounds on this process can be easily monitored. Moreover, in the small animal models (*C. elegans* and *Drosophila*) protein aggregation is linked to toxicity. Thus, it is possible to screen for compounds that affect the toxic phenotype and not only protein aggregation, giving an extra value to these systems. Finally, yeast, *C. elegans* and *Drosophila* are well-characterized model organisms for which a number of genetic tools are available. Consequently, these models are also suitable to screen for genetic modifiers of aggregation or of the associated toxicity; genes that when overexpressed or suppressed affect the outcome phenotype. Such modifier genes constitute candidate therapeutic targets.

Our cell-free assay is an *in vitro* automated filter-binding assay that is based on the finding that protein aggregates are resistant to SDS denaturation and are therefore selectively retained on a filter. The aggregates can then be quantified by immunoblot analysis using specific antibodies. As for the yeast assay system, the yeast strains express human disease-linked proteins and as a consequence of protein aggregation there is either a change in the colour of the colony or growth arrest (the aggregated protein losses its biological function and can no longer complement the deletion of the yeast gene). The *C. elegans* models also express one or more human protein resulting in protein aggregation and an associated toxic phenotype. Moreover, we set up a system in which worms are grown in 96-well plates in a liquid format. This system, which includes a pipetting robot and a worm sorter, is therefore suitable for high-throughput screenings (HTS). The *Drosophila* models rely on the expression of Abeta or Tau in the eye resulting in age-dependent neurodegeneration of retinal photoreceptor cells and thus in defects in the morphology of the eye. We also have available a *Drosophila* model of HD that expresses human huntingtin in photoreceptor cells in the eye.

We used the *in vitro* filter-binding assay based on mutant huntingtin to screen a chemical library of more than 180,000 chemical compounds and identified several inhibitors of huntingtin aggregation. Some of these inhibitors were also active in mammalian cells expressing mutant

huntingtin and in the *Drosophila* model of HD. We chose twelve of the most promising compounds to evaluate their bioavailability, toxicity and brain penetration in wild-type mice. Three candidate compounds showing the best pharmacological profiles were selected and tested in a mouse model of HD. The inhibitors tested did not improve the disease phenotype and did not reduce the amount of aggregates in the brains. We believe that this is due to only partial brain penetration of the inhibitor and, therefore, plan to synthesize chemical derivatives of hit compounds to achieve more favourable pharmacological profiles. In a separate effort, we screened approximately 5,000 natural compounds and identified (-)-epigallocatechin-3-gallate (EGCG) as a potent inhibitor of mutant huntingtin aggregation. EGCG is a polyphenol present in green tea that is known to cross the blood brain barrier. It not only blocked mutant huntingtin assembly in the filter binding assay but also suppressed mutant huntingtin-associated toxicity in yeast and *Drosophila* models of HD. Interestingly, some of the inhibitors of huntingtin aggregation also blocked Abeta aggregation *in vitro*, reduced Abeta neurotoxicity in primary cortical neurons and reduced Abeta-induced retina degeneration in the *Drosophila* AD model mentioned above. The identified group of compounds corresponds to a new class of molecules that might be useful for drug development. Similar screenings using the yeast and *C. elegans* models were carried out, and we were able to identify compounds with anti-aggregation and anti-neurodegeneration activities, respectively. Moreover, the yeast, *C. elegans* and *Drosophila* strains were used for genetic screenings and several genetic enhancers and suppressors of protein aggregation or neurodegeneration were identified. We are currently further characterizing the candidate chemical compounds and the genetic modifiers identified in these screenings. These compounds have the potential to become therapeutic agents to treat neurodegenerative diseases. The genetic modifiers, on the other hand, might reveal novel points for therapeutic intervention, enlarging the list of targets at which the disease process can be tackled.

In addition to these HTS, we followed a rational approach to 'design' anti-aggregation agents. Structure-based design of amyloid inhibitors has still to deal with the lack of structural information at atomic resolution. However, identification of the regions triggering amyloidogenesis by using the amyloid pattern algorithm offers the possibility to rationally attempt the inhibition of amyloid fibril formation by designing or by screening for molecules that specifically bind the fragments identified as amyloidogenic. We undertook an approach aiming at inhibiting amyloid formation using D-hexapeptides. Short synthetic D-peptides designed to target amyloid stretches should break the chirality of L-species impairing their growth and/or disassembling them. As a first target for inhibition we selected a well-characterized amyloid-forming hexapeptide. A D-hexapeptide library was then screened for their capacity to prevent beta-sheet formation (evaluated by circular dichroism) and amyloid generation (evaluated by electron microscopy). Based on these data we were able to extract a set of empirical rules to design D-hexapeptide inhibitors targeted to any amyloidogenic stretch. To validate the approach, we applied these rules to design D-peptide inhibitors to the amyloidogenic stretch in Abeta (16KLVFFA21). The 'designed' D-peptide could indeed prevent amyloid formation and amyloid-induced cytotoxicity in a dose-dependent manner. Moreover, it was very effective at disassembling preformed fibrils, correlating with an almost complete protection against amyloid-induced cell death. More importantly, the successful leads against the hexapeptide retained their efficacy against full-length Abeta. In a second approach we evaluated candidate organic molecules for their capacity to inhibit Abeta and Tau amyloid formation and to disassemble preformed fibrils. We were able to identify some derivatives of tetracycline as well as non-peptidic gamma-secretase inhibitors as promising anti-aggregation compounds. Interestingly, some of them also functioned to disassemble preformed fibrils.

1.4.3 Insights into the cell biology of beta- and gamma-secretases

We dedicated a large part of our efforts to investigate various aspects of beta- and gamma-

secretases. Knowledge in this area is crucial, since these enzymes are important targets for the treatment of AD. Very little is known on the physiological role of the beta-secretase BACE1 and its homologue BACE2. Due to their high homology it is likely that active site inhibitors for BACE1 will also affect BACE2 protease activity. To shed light on the *in vivo* functions of these two proteases and in an attempt to predict the *in vivo* consequences of the therapeutic inhibition of beta-secretase, we generated transgenic mice, in which either BACE1, BACE2 or both were inactivated. Surprisingly, our BACE1 mice displayed a complex phenotype that was not described in previous reports. A variable but significant number of BACE1 offspring died in the first weeks after birth. The surviving mice remained smaller than their littermate controls and presented a hyperactive behaviour. In contrast, BACE2 knock-out mice, that were first generated and described in this Consortium, displayed an overall healthy phenotype. The combined deficiency of BACE2 and BACE1 enhanced the BACE1-/- lethal phenotype. More detailed analysis of BACE1 deficient mice identified a defect in peripheral nerve myelination. We could show that BACE1 is expressed at high levels at time points when peripheral nerves become myelinated and that the absence of BACE1 resulted in hypomyelination of peripheral nerves and aberrant axonal segregation of small-diameter afferent fibres. A similar phenotype was observed in mice with mutations in neuregulin 1 (NRG1), an axonally expressed factor required for glial cell development and myelination. We then showed that BACE1 deficiency causes an accumulation of unprocessed NRG1 *in vivo* and demonstrated in cell culture experiments that NRG1 is a novel BACE1 substrate. Thus, BACE1 is required for myelination and correct bundling of axons by Schwann cells, probably through processing of NRG1. These novel data altogether challenge the general idea of BACE1 as a safe drug target and call for some caution when claiming that no side effects should be expected from blocking BACE1 activity. Previous reports suggested that the proteolytic activity of BACE1 towards APP is modulated by the membrane lipid composition. These experiments, however, relied mostly on APP and BACE1 overexpression, which can cause misdistribution of the proteins. We investigated the distribution of APP and BACE1 in membrane compartments at endogenous protein levels and in relevant systems, namely primary neuronal cultures and brain tissue. We demonstrated that a subset of BACE1 localizes to membrane microdomains rich in sphingolipids and cholesterol (rafts), whereas APP is excluded from these microdomains, therefore limiting the accessibility of BACE1 to its substrate APP. We also demonstrated that primary neurons in culture undergo progressive cholesterol loss of up to ~25% compared to young neurons. Loss of cholesterol is accompanied by disorganization of rafts, displacement of BACE1 to a non-raft compartment where it co-localized with APP, and increased amyloidogenic processing. Similar results were obtained by pharmacologically depleting comparable amounts of cholesterol from the membrane of young neurons in culture. This age-dependent cholesterol reduction observed in cultured primary neurons also occurs *in vivo* in mouse brain. To analyze the relevance of cholesterol loss for AD, we investigated APP processing in mice deficient in the cholesterol-synthesizing enzyme Seladin-1. The reduced cholesterol levels in these mice were associated with disorganized rafts, increased beta-cleavage of APP and higher Abeta levels. One important implication of this work is that pharmacological enhancement of Seladin-1 activity may be a novel Abeta-lowering approach for the treatment of AD.

The gamma-secretase complex consists of four core subunits – presenillin (PS), nicastrin, Aph1 and Pen2 – and is responsible for the processing of a number of type I membrane proteins, most notably APP and Notch. Two different genes encode Aph1A and Aph1B in human. A duplication of the Aph1B gene in rodents has given rise to a third gene, Aph1C. There are also two genes for PS (PSEN1 and PSEN2 encoding PS1 and PS2, respectively) both in human and mice. Therefore, at least four different gamma-complexes are possible in human and six in rodent. Nothing is known on whether all these complexes exist *in vivo* and whether they have different activities or substrate specificities. In co-immunoprecipitation experiments at

endogenous protein levels to avoid artefacts linked to overexpression, we found evidence for the existence of several independent gamma-secretase complexes (containing either Aph1A, 1B or 1C and either PS1 or PS2) that can coexist in the same cell type. To address the possibility that such complexes perform different functions, we have generated mice in which Aph1A, Aph1B or Aph1C genes were inactivated, as well as double and the triple knock-out mice. PS1, PS2 and double PS knock-out mice were already available. Whereas Aph1A-/- embryos are lethal, mice deficient in Aph1B, 1C or both (which can be considered as a model for total Aph1B loss in human) survive into adulthood. However, Aph1BC-/- deficiency causes a mild but significant reduction in APP processing in selective regions of the adult brain, and a specific behavioural deficit, which is characteristic of human neurological disorders like schizophrenia. We are therefore currently analyzing a possible deficit in the dopaminergic system in Aph1BC deficient mice. These data already indicate that at least some of the functions performed by Aph1B and 1C cannot be taken over by Aph1A, either because the gamma-complex they form possess different properties, or because their tissue distribution does not overlap. These studies also suggest that specifically targeting the Aph1B-containing gamma-complex (and sparing the Aph1A-containing complex) might be a more suitable AD therapy. We also carefully compared the phenotype of Aph1 deficient mice with that of mice deficient in other gamma-secretase components. Interestingly, Aph1A or PS1 loss in fibroblasts derived from knock-out animals caused an ~70% reduction in gamma-secretase activity, however, the phenotypical alterations in Aph1A-/- mice are more severe than PS1 deficiency and more similar to the phenotype observed in PS1/PS2 double knock-out embryos that are 100% deficient in gamma-secretase processing. These differences in the Aph1A and PS1 null phenotypes might be due to subtle differences in the activity of PS1-deficient versus Aph1A-deficient gamma-secretase complexes *in vivo* that were not readily seen in the fibroblast cell culture system. Alternatively, differences in tissue expression of the different isoforms of the gamma-secretase complex could explain the observed heterogeneity in function of the different gamma-secretase complexes.

In addition to the studies in mice, we were able to reconstitute successfully the complete functional gamma-secretase complex in yeast by co-expression of the four core subunits. We subsequently demonstrated that gamma-complex assembly occurs in the early secretory pathway, with Pen2 being the limiting factor responsible for stabilizing the PS-heterodimer after the PS endoproteolysis that occurs during complex maturation. We further identified Rer1, a putative ER-retention/retrieval protein, as responsible for ER retention of unassembled Pen2. Consistent with Pen2 being rate limiting for gamma-secretase complex assembly, we demonstrated that Rer1 overexpression stabilised Pen2 and resulted in enhanced complex formation. We have thus isolated the first gamma-secretase complex assembly factor.

Finally, we investigated in detail the exact subcellular localization where beta- and gamma-cleavage occur. Our data are consistent with endosomes being the major site for both processing events and, therefore, for Abeta generation. Moreover, we showed that a subset of the generated Abeta is then routed to vesicles of the multivesicular bodies, which upon fusion with the plasma membrane liberate Abeta-containing exosome vesicles extracellularly, thus defining a novel route for Abeta secretion. The physiological relevance of these findings is highlighted by the observation that exosomal proteins accumulate around amyloid plaques in AD patients, suggesting that exosome-associated Abeta could play a role in plaque formation.

1.4.4 Development of inhibitors of beta- and gamma-secretases

Whereas the work on inhibitors of aggregation and of aggregate-toxicity described in section 1.4.2 is relevant to most neurodegenerative diseases, a substantial part of our efforts focused on AD, more specifically, on the search of beta- and gamma-secretase inhibitors. In this case we target the more upstream event of Abeta generation rather than Abeta aggregation or toxicity. We performed a HTS to identify gamma-secretase inhibitors from a library of ~280,000

compounds. The ~500 candidate compounds obtained were subsequently clustered with respect to structural and molecular features, giving rise to 10 different groups or hit clusters. We then validated representative compounds from each cluster in *in vitro* cell-free gamma-assays and in a cellular Abeta secretion assay. A variety of compounds related to the hits in the individual clusters have either been purchased or synthesized with the goal of enlarging the hit population and to get hints on a possible structure-activity relationship. Upon evaluation of the structural parameters and the compounds´ physicochemical properties we concluded that none of the hits derived from this screening campaign was sufficiently promising to justify a full-blown Hit-to-Lead programme. In a separate approach we intended to obtain better gamma-secretase inhibitors by chemical modification of already described ones. Adding a membrane anchor to the known peptidomimetic gamma-secretase inhibitor DAPT resulted in novel inhibitors that were still active in *in vitro* reconstituted gamma-secretase assays; they were, however, inactive in cellular assays. We also synthesized derivatives of non-steroidal anti-inflammatory drugs (NSAIDs) that were recently identified as gamma-secretase modulators. They reduce the generation of the more amyloidogenic Abeta42 while increasing that of shorter, less amyloidogenic peptides, most notably Abeta38. Derivatisation of the carboxylic acid common to all NSAIDs did not improve the inhibitor's potency compared to their precursors. Indeed, most of the approximately 150 NSAID carboxylic acid derivatives tested had lost activity, indicating an important contribution of the carboxylic acid to target affinity.

As for beta-secretase, we synthesized and tested about 130 non-peptidic, cell-permeable candidates. Several of these compounds exhibited weak potency (IC50 = 10-150 µM) and therefore require further improvements. We also developed a first irreversible BACE inhibitor that offers a multitude of applications in the investigation of BACE1 because of its covalent attachment to the enzyme.

1.4.5 Novel AD animal models

For the study of any human disorder animal models are crucial, which reproduce (some) aspects of the human disease and are then suitable to test the efficacy of novel therapeutic interventions. The animal models we generated carry one or more human disease-associated genes and typically reproduce the characteristic protein aggregation and neurodegeneration observed in human patients. The value of these animal models is not restricted to their use in therapeutic development. A detailed anatomical and behavioural analysis has provided novel insights into disease mechanisms and helped us to better understand the human disease. In section 1.4.2 we have already discussed the small animal models (*C. elegans* and *Drosophila*); this section is dedicated to mouse models, which are more relevant to the human condition.

One of our mouse models expresses human APP carrying both the Swedish and the Arctic mutation (APPArcSwe mice). Analysis of these animals showed an early accumulation of intracellular Abeta aggregates by ~3 month of age not seen in APPswe mice (carrying only the Swedish mutation). Extracellular amyloid plaques developed at around 5-6 month of age and were markedly accelerated in APPArcSwe double mutant mice. Because the Arctic mutation favours protofibril formation, these mice constitute a relevant model to test therapeutic strategies targeting protofibrils (see section 1.4.10).

We also generated mouse models exhibiting an extremely early onset of plaque deposition. Thus, double transgenic mice overexpressing human mutant APPswe and PS1L166P (APPPS1 mice) present cerebral amyloidosis and associated pathology as early as 6-8 weeks of age, which progresses thereafter. The early and robust onset of amyloid lesions makes these animals an invaluable model, and, as a consequence, these mice have already been distributed worldwide. Similar models were generated that express APPswe in combination with either mutant human PS1M146V or PS2N141I. Yet, none of these animals present the second hallmark of AD, namely tau pathology. We thus generated a triple transgenic line (MAD4) that

co-expresses APPswe, PS1M146V and mutant Tau (TauP301L). In these animals the plaque phenotype is enhanced by the tau transgene. Plaque formation starts before 5 months of age in the frontal cortex, spreading to other brain regions with increasing age, and is accompanied by reactive astrogliosis and cerebral amyloid angiopathy (CAA). More importantly, MAD4 animals develop progressive neurofibrillary tangles, thus more closely resembling the human condition. They constitute an excellent model to test how specific therapies affect plaques, tangles or both.

CAA occurs in AD as well as in normal aging and is normally characterized by deposition of amyloid in the walls of blood vessels, which weakens them and can lead to microhaemorrhages, thereby contributing to cognitive decline and dementia. The availability of mouse models of CAA is critical to test AD vaccination therapies, since CAA-related microhemorrhages have been suspected to be one of the side effects of Abeta immunotherapy. Inherited forms of CAA are associated with autosomal dominant mutations in a number of genes including APP, cystatin-C (CysC), transthyretin, gelsolin and BRI2. The APP Dutch E693Q mutation causes Hereditary Cerebral Hemorrhage with Amyloidosis (HCHWA) Dutch type, characterized by Abeta deposits in blood vessels but very few parenchymal amyloid plaques. Point mutations in CysC cause HCHWA-Icelandic type, whereas mutations in BRI2 gene are responsible for familial British and Danish dementias. We generated a number of mouse lines expressing some of these mutant proteins. Transgenic mice carrying the human APPDutch transgene exhibited extensive CAA, haemorrhages and neuroinflammation. The Abeta40-to-42 ratio was significantly higher in these mice than in APPwt mice or AD human brain. Genetically shifting the AbetaDutch40-to-42 ratio toward AbetaDutch42 by crossing APPDutch mice with transgenic mice producing mutated PS1 redistributed the amyloid pathology from the vasculature to the parenchyma. This suggests that different Abeta species can drive amyloid pathology in different cerebral compartments and has important implications for current anti-amyloid therapeutic strategies. This HCHWA-Dutch mouse model was the first described to develop robust CAA in the absence of parenchymal amyloid. A drawback of this model is the late onset of vascular amyloid deposition, which occurs only at approximately 22 months of age. To accelerate the onset of CAA, APPDutch mice have been crossed with BACE1 and APP23 (APPswe) transgenic mice. APPDutch/BACE1 mice revealed more vascular amyloid deposits compared to APPDutch mice, but also exhibited parenchymal amyloid. APPDutch/APP23 mice showed more vascular amyloid and, interestingly, less parenchymal amyloid compared to APP23 mice. Cerebral amyloid deposits in these double-transgenic mice mainly contained Abeta40, which in turn consisted of both, mutant and wild-type Abeta, resembling the amyloid of patients affected by HCHWA-Dutch. Mice transgenic for mutated CysC, intended as a model for HCHWA-Icelandic type, failed to show any amyloid deposition with aging. Interestingly however, when crossed with mice overexpressing APP (APP23 or APPPS1 mice) the CysC transgene caused a reduction in Abeta burden. We could show that CysC can indeed bind Abeta *in vitro*, thereby inhibiting fibril formation.

1.4.6 Advances in Parkinsonian syndromes

A significant part of our efforts was dedicated to Parkinson disease, the second most common human neurodegenerative disease after Alzheimer disease. PD is a movement disorder that results primarily from the death of dopaminergic (DA) neurons in the substantia nigra, which innervate the striatum. Accompanying the loss of DA neurons is the accumulation of Lewy bodies, intracytoplasmic proteinaceous inclusions that contain alpha-synuclein. Like for most of the neurodegenerative disorders, no effective therapy is available. The most prevalent therapy is administration of levodopa, a precursor of dopamine that can cross the blood-brain-barrier and that benefits virtually all patients. However, efficacy tends to decrease as the disease progresses and chronic treatment results in adverse side effects (motor fluctuations, dyskinesias and neuropsychiatric problems). Several alternative therapies are currently being explored, such as neuroprotective approaches. Trophic factors represent one class of neuroprotective compounds

with the potential to stimulate regeneration in the damaged nigrostriatal system. To investigate which trophic factors are more relevant for survival of DA neurons, we generated mice in which expression of genes for neurotrophic factor receptors was specifically ablated in midbrain DA neurons. We could show that deletion of the Ret, but not TrkB, receptor causes ~20% loss of DA neurons in the substantia nigra, while striatum innervation was reduced by ~40%. Therefore, glial cell-derived neurotrophic factor (GDNF) that signals through the Ret receptor seems to be required for survival of DA neurons in the substantia nigra as well as for maintenance of target innervations. These data supports the use of GDNF as a treatment for PD.

Although most patients suffering from PD have a sporadic disease triggered by unknown environmental factors, several genetic causes have been identified in recent years, including mutations in alpha-synuclein (SNCA), parkin, PINK1, dardarin (LRRK2) and DJ-1. The function of these genes in the normal brain and the mechanism by which mutations trigger disease, remain largely unknown. Understanding the molecular mechanisms by which PD-linked mutations cause DA neuron degeneration will help us develop better therapies. One strategy that we used to understand the function of PD-associated genes both in health and disease was to generate various animal models (*C. elegans*, zebrafish, mouse) in which these genes were either specifically ablated or overexpressed. *C. elegans* has the advantage of possessing only eight dopaminergic neurons that can easily be monitored. Moreover, the genes involved in dopamine synthesis, transport, receptors, etc are evolutionarily conserved. We generated *C. elegans* strains that are deficient in each of the PD-related genes or that overexpress human alpha-synuclein. To investigate the functional connection between PD-related genes, the different strains were crossed creating multiple mutants in various combinations. The resulting strains were extensively characterized by testing movement patterns, brood size, morphological abnormalities as well as sensitivity to oxidative and ER stress upon treatment with pharmacological agents. Worms overexpressing mutated human alpha-synuclein presented with no phenotype despite the presence of aggregates. Wild-type alpha-synuclein, but not the mutated variant associated with PD, could protect worms from paralysis caused by the dopaminergic toxin 6-hydroxydopa (6-OHDA). Although the precise physiological function of DJ-1 remains obscure, accumulating evidence suggests that it functions as a redox-sensitive molecular chaperone that can protect against the deleterious effects of oxidative stress. Two DJ-1 homologues were identified in *C. elegans* and their deletion indeed rendered the animals more sensitive to oxidative stress. We also identified and characterized pdr-1, the *C. elegans* homolog of parkin that encodes an E3-ubiquitin-protein ligase, and showed that PDR-1 protein physically associates and cooperates with a conserved degradation machinery to mediate ubiquitin conjugation. Like for DJ-1, the deletion of pdr-1 only caused a mild phenotype, rendering the worms more sensitive to ER stress. Strikingly, in contrast to pdr-1 loss-of-function an in-frame deletion variant with altered solubility blocked the cellular degradation/detoxification machinery making worms highly vulnerable to protein folding stress. This *C. elegans* model thus recapitulates Parkin insolubility and aggregation similar to several parkin mutations in humans. Expression of mutant human alpha-synuclein in this pdr-1 mutant background resulted in severe developmental defects and lethality. This *C. elegans* double mutant strain was used in the screens mentioned in section 1.4.2 to identify chemical compounds and modifier genes capable of blocking neurotoxicity.

We also generated and characterized novel mouse PD models. We had available in our Consortium transgenic mice that either overexpress mutant alpha-synuclein or lack parkin. The alpha-synuclein transgenic animals have no overt motor abnormality despite the presence of alpha-synuclein aggregates. Similarly, parkin knock-out mice present only subtle behavioural and biochemical changes that reproduce some of the presymptomatic aspects of PD, though in the absence of neuronal degeneration. It is thought that loss of Parkin function can lead to the abnormal accumulation of Parkin substrates. Alpha-synuclein being one of the proposed Parkin substrates, we decided to address the possible functional relation between these two proteins by

crossing parkin deficient and alpha-synuclein overexpressing mice, as was done before with *C. elegans*. These mice did not show age-dependent changes in spontaneous locomotor activity and motor coordination indicative of possible deleterious synergistic effects of absence of Parkin and excess of alpha-synuclein, nor did they display dopaminergic neuron loss or alterations in striatal dopamine metabolism up to 17 months of age. However, detailed neuropathological analyses of these animals suggest that Parkin may directly or indirectly affect the ubiquitylation status of phosphorylated alpha-synuclein and have an impact on its subcellular localization in an undetermined way that is currently under investigation.

An additional strategy that we exploited to understand the function of specific genes linked to disease was to identify the binding partners of the encoded proteins. Knowing in detail what the disease-linked protein does in neurons, with which partners it has to interact to carry out its specific function and how mutations affect these processes offer different steps where specific compounds could act to prevent or correct the disease. We thus performed high-throughput screens to identify binding partners of each of the PD-related proteins. We then combined the protein-interaction data with the data we obtained in the genetic screens (section 1.4.2). This allowed us to generate a map of regulatory networks of proteins potentially involved in the disease mechanism (the "Parkinson Interactome"), which is continuously updated based on the results from new screens. The Parkinson Interactome constitute an important tool to understand and even predict how changes in the activity of a certain component of one pathway (by genetic mutation or by pharmacological intervention) can affect the function of other components of the same or of a linked pathways in the network. It is therefore an invaluable tool for therapeutic development.

1.4.7 Advances in motor neuron diseases

A special group of neurodegenerative disorders is characterized by the specific degeneration of motoneurons in the brain and spinal cord, which leads to a progressive weakness, atrophy of muscles and, eventually, death. These diseases include amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), progressive bulbar palsy, and primary lateral sclerosis. Motoneurons differ from other types of neurons by the length of their axons, which can reach >1 m in human adults. Structural and functional maintenance of these large cells are therefore heavily dependent on an efficient axonal transport. To better understand the function of the genes linked to motoneuron diseases in axon growth and maintenance as well as the mechanisms underlying the disease we have generated and characterized various mouse models. The progressive motor neuronopathy (pmn) mouse mutant, which resembles SMA by progressive degeneration of motoneurons in the early postnatal period, carries a mutation in the tubulin-specific chaperone E (Tbce) gene. The Tbce gene encodes a protein (cofactor E) that is essential for the formation of primary alpha-tubulin and beta-tubulin heterodimeric complexes. Motoneuron derived from pmn mutant mice and cultured *in vitro* presented with shorter axons, increased axonal diameter and axon swelling. Interestingly, the axonal defects could be rescued by ciliary neurotrophic factor (CNTF) but not by brain derived neurotrophic factor (BDNF) or GDNF, highlighting a potential of CNTF as a candidate therapeutic agent to treat motoneuron diseases. We investigated a second mouse model for SMA, the Smn mice that are heterozygote deficient for the survival motor neuron 1 (SMN1) gene. At the age of six months these mice develop a spinal motoneuron loss of ~40%. Notably, despite the significant motoneuron loss muscle power is not affected. We could demonstrate extensive axonal sprouting in these animals, which could be a compensatory mechanism allowing for normal muscle power despite significant motoneuron loss. Cultured SMN-deficient motoneurons presented shorter axons and decreased growth cone area. This is due to defects in the transport of the beta-actin mRNA in motor axons, for which SMN1 and its interacting partner hnRNP-R are required. The beta-actin cytoskeleton is known to play an important role for growth cone motility and axon growth and maintenance in motoneurons. We also utilized zebrafish as model organism to study the

function of these genes. Knocking down the expression of hnRNP-R in zebrafish resulted in specific defects of motor axon growth and in additional axon sprouting resembling spinal muscular atrophy in mouse models and in humans. Similarly, down-regulation of hnRNP-R in cultured motoneurons from wild-type mice correlated with a specific reduction of axon elongation.

Other beta-actin mRNA-binding proteins have been identified including members of the insulin-like growth factor 2 mRNA binding protein family (Igf2bp1-3). To identify novel factors with a possible function in axonal growth and maintenance, we performed protein interaction screens to identify Igf2bp1-binding partners. A number of novel components of the Igf2bp1 protein complex including mRNA binding proteins like the DEAH-box polypeptide DHX9 were identified. A possible role of these proteins in axonal function is currently under investigation.

1.4.8 Genetic basis of neurodegeneration

Most of the neurodegenerative conditions exist as sporadic and familiar forms. Whereas the cause of disease in sporadic cases is not clear, familiar cases are caused by mutations in specific genes. A significant amount of our efforts was dedicated to the identification of genes and gene mutations that either cause neurodegenerative disease or increase the risk of developing it.

In 10-43% of patients suffering from familial frontotemporal dementia (FTD) the disease is caused by mutations in the gene encoding the microtubule associated protein tau (MAPT) located in chromosome 17q21. This subtype of FTD is known as FTD with Parkinsonism (FTDP-17) and is neuropathologically characterized by tau-positive aggregates in the brain. However, autosomal dominant forms of FTD without MAPT mutations have been reported. One such form is FTD with tau-negative but ubiquitine-positive neuronal inclusions or FTDU-17, which is also linked to chromosome 17. In the APOPIS Consortium we succeeded in identifying the long-sought gene responsible for FTDU-17. This gene, progranulin (PGRN), lies in close proximity to MAPT, what has long impeded PGRN identification.

In the case of AD, known disease-causing genes are PSEN1, PSEN2 and APP, for which fully penetrant (causal) mutations have been described. Partially penetrant mutations that constitute risk factors for the more common late-onset form of the disease have been described in other genes, most notably in APOE. We identified a novel mutation in APP predicting a K724N substitution in the cytosolic domain. This is the most C-terminal APP mutation reported to date. Expression of APP K724N in cultured cells resulted in increased Abeta42 and decreased Abeta40 levels causing a near three-fold increase of the Abeta42/Abeta40 ratio. These data imply that in humans this novel APP mutation is a likely cause of disease. In the Consortium we further demonstrated that not only missense mutations in APP could cause AD, but also mutations or polymorphisms that increase APP expression. Thus, we were the first to show mutations in the APP promoter region that increase APP transcriptional activity as well as duplication of the APP locus as novel causes of autosomal AD. We also identified a novel locus at 7q36 linked to AD. Mutation analysis of coding exons of 29 candidate genes in this region spotted one linked synonymous mutation in exon 10 of the PAX transactivation domain interacting protein gene (PAXIP1). It remains to be determined whether PAXIP1 has a functional role in the expression of AD or whether another mutation at this locus explains the observed linkage. Moreover, we found two new candidate genes for sporadic, late-onset AD: the cholesterol hydroxylase gene (CH25H) and the ATP-binding cassette transporter A2 (ABCA2). The CH25H gene is highly expressed in specifically vulnerable brain regions of AD and we found an association of CH25H polymorphisms with different rates of brain Abeta deposition. Interestingly, the effect of ABCA2 on the genetic risk of sporadic AD seems to be population-dependent, suggesting specific interactions of the genetic background with environmental factors.

Parkinson disease, as mentioned in section 1.4.6, can be caused by autosomal dominant

mutations in SCNA, LRRK2, and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), or by autosomal recessive mutations in parkin, DJ-1 and Pink-1. SCNA (alpha-synuclein) mutations have been implicated in rare dominant forms of PD because of either missense mutations or gene triplications. We now showed that SNCA gene duplication is responsible for another subset of familial forms of PD. Interestingly, the phenotype in the 'duplicated' families was typical PD, in contrast to the ‘triplicated’ families that present with atypical physical signs indicative of neurodegeneration extending beyond the substantia nigra. Investigation of the SNCA promoter region revealed that the allele-length variability in a dinucleotide repeat sequence is associated with an increased risk of PD. The families with autosomal dominant parkinsonism that were negative for SNCA alterations were analyzed for mutations in the LRRK2 gene. The screening of LRRK2 exon 41 revealed that the frequency of the G2019S mutation was unexpectedly high in North-African families (~41%) as compared to European families (~2.9%). Haplotype analysis in the European and North African families suggested that there was a common founder for the G2019S mutation dating from the 13th century. The phenotype of G2019S carriers was similar to that of the idiopathic disease even in the homozygous patients, suggesting an absence of a dosage effect. To determine the mutational spectrum of the LRRK2 gene and to perform genotype-phenotype correlation studies, we sequenced the 51 exons of the LRRK2 gene in 234 index cases from families compatible with an autosomal dominant transmission and identified 46 rare variants including 7 potential new mutations and 28 frequent polymorphisms. Concerning forms of parkinsonism compatible with an autosomal recessive transmission, we could show that mutations in the DJ-1 gene are very rare. Regarding the Pink-1 gene, we directly sequenced it in 34 families linked to the Pink-1 locus and identified 10 pathogenic mutations including 8 novel ones. We also identified a novel promoter-exon1 deletion in the parkin gene that extended to exon 2 of the adjacent PACRG (Parkin co-regulated gene), both genes being transcriptionally co-regulated by means of a shared bidirectional promoter. The phenotypes of these patients were similar to those with parkin mutations. We also examined the contribution of MAPT to the genetic aetiology of PD. Our data implicate tau splicing in early-onset PD susceptibility. Finally, we aimed at identifying novel autosomal recessive genes accounting for PD. For this, we studied a sample of about 30 inbred individuals excluded for mutations in parkin, Pink-1, DJ-1 and LRRK2 genes. Our studies revealed a new locus in a 4 cM region (estimated to 4.6 Mb), and we are currently analyzing the genes in this region for its potential association with PD.

Among the motoneuron diseases, we investigated the genetic epidemiology of amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig’s disease. Thus far eight genetic loci (ALS1-8) have been linked to ALS, but the corresponding genes remain to be identified for four of them. We were able to map the gene defect associated with ALS6 to a locus in chromosome 16. Similarly, we successfully mapped the gene responsible for a form of ALS and FTD to a locus in chromosome 9. In both cases, we are fine mapping the regions to identify the responsible genes. We also investigated various candidate genes for their possible association to sporadic and familial ALS. Indeed, we found one haplotype across the intermediate filament alpha-internexin gene as significantly associated with susceptibility to ALS. Analysis of the Angiogenin gene also identified a sequence variant (Ile46Val) that is weakly associated with susceptibility to ALS.

1.4.9 Improving clinical detection

Most neurodegenerative diseases can only be diagnosed once symptoms are well established and therefore when substantial brain damage has already occurred. There is thus an urgent need for early diagnosis to be able to treat the diseases before brain damage is irreversible. One major tool that we used to detect disease-associated changes is brain imaging, which provides a non-invasive and reproducible method to analyse the structure and function of the brain in living patients. We also invested in the identification of candidate biomarkers in body fluids like blood

and cerebral spinal fluid (CSF), the presence of which indicate a particular disease state.

In order to identify early, pre-symptomatic changes we concentrated on young subjects predisposed to develop AD later in life because of a positive family history. In one approach we studied a family that carries the familial AD (FAD) mutation C410Y in the PSEN1 gene. The age of clinical manifestation of AD in this family is ~48 years. We examined by functional magnetic resonance imaging (fMRI) 2 mutation carriers 20 and 45 years old, as well as non-mutation carriers of the same family and non-related control individuals. We were able to detect an increase in memory-related brain activity in the 20-year-old mutation carrier, which probably reflects a compensatory effort to overcome preclinical neural dysfunction caused by first pathological changes. On the contrary, the middle-aged mutation carrier exhibited decreased memory-related brain activity, probably reflecting gross neural dysfunction in a more advanced stage of neuropathology. These data suggest that functional neuroimaging along with tasks that specifically challenge brain areas affected by AD pathology may reveal activity alterations decades before the clinical manifestation of disease.

In a second approach, we focused on young healthy individuals destined to develop AD on the basis of carrying mutations in APP or PSEN1 genes, and studied them by serial MRI scanning. Using semi-automated techniques, we detected significantly increased hippocampal and whole-brain atrophy rates in mutation carriers compared to controls, with differences increasing over time. Differences were evident 5.5 (in hippocampus) and 3.5 (in whole brain) years before clinical diagnosis. Interestingly, the patterns of tissue destruction were specific for PSEN1 and APP mutations, atrophy being more widespread in PSEN1 mutation despite comparable cognitive severity. These data thus highlight the value of longitudinal measures of atrophy rates to identify early changes in AD.

In a third approach, also evaluating autosomal dominant mutation carriers for AD we assessed whether metabolite abnormalities could be detected by magnetic resonance spectroscopy (MRS) prior to the onset of symptoms. We observed that mutation carriers indeed had significantly lower ratios of N-acetyl aspartate (NAA)/myo-inositol (MI) than control subjects, with both NAA and MI contributing to this effect. The lower NAA/MI ratio was detected up to 7-10 years before the expected age at onset and was lower as individuals approached this age. Therefore, metabolic changes can be detected by MRS in pre-symptomatic AD individuals and their magnitude is related to proximity of expected age at onset.

Finally, we analysed carriers of a single nucleotide polymorphism (SNP) in the cholesterol hydroxylase CYP46 gene associated with AD. CYP46 regulates the elimination of excess brain cholesterol and subjects with the homozygous CYP46 TT variant have significantly increased cholesterol levels in CSF and increased risk of AD. We examined a total of 116 young healthy subjects by voxel-based morphometry (VBM), a whole-brain unbiased objective technique suitable to characterise brain differences *in vivo* using structural MRI. We found consistently larger right temporal volumes in CT over TT carriers, highlighting the potential of VBM as a diagnostic tool. Interestingly, behaviourally measured memory performance did not show any significant differences between the two genetic variants. These data altogether highlight the value of brain imaging as a diagnostic tool suitable to detect early changes in the brain that occur even decades before the disease is manifested. The earlier the disease is detected the more effective the treatment can be expected to be. Brain imagining has also been used to discriminate between diseases with similar clinical manifestations. Thus, by using semi-automated measurement of regional atrophy rates we could differentiate AD from normal aging as well as AD from FTD with a relatively high degree of specificity. Similarly, we were able to identify longitudinal patterns of atrophy characteristic of progressive supranuclear palsy, which correlated well with regional pathology determined by post mortem histopathological analysis. These brain imaging tools should help clinicians make a more accurate diagnosis and therefore administer more adequate treatments.

We also explored brain imaging techniques that rely on the use of tracers. To study a possible correlation between amyloid plaque load and rates of cerebral atrophy, we used the radiolabelled amyloid-binding agent 11C-PIB for positron emission tomography (PET)-imaging and combined these data with serial volumetric MRI in a cohort of individuals with mild to moderate AD. These analyses revealed a positive correlation between rates of whole brain atrophy and whole brain and regional 11C-PIB uptake, corroborating the feasibility of this compound as a tracer for beta-amyloid PET-imaging *in vivo* and providing support for the central role of amyloid deposition in the pathogenesis of AD. In the PD field, we evaluated the recently developed 11C-ABP688 tracer that labels glutamatergic receptors in the mouse and monkey brain. Tracer pharmacokinetics and dynamics have been explored in a phase 1 study in healthy human volunteers. Brain uptake was adequate and tracer accumulation was according to the predicted distribution of the receptors, validating 11C-ABP688-PET imaging as a tool to evaluate PD patients.

In addition to brain imaging, we explored the potential of certain molecules that can be measured in body fluids as candidate biomarkers suitable for diagnosis. We had available in the Consortium blood samples from ~378 individuals at 3 age points (70, 77 and 82 years) obtained in the ULSAM longitudinal study that, since the 1970's, follows the health of men born in Uppsala between 1920 and 1924. We then measured Abeta40 and Abeta42 levels and examined a possible association between these Abeta levels and AD. We found that low plasma levels of Abeta40 at age 70 are associated with increased risk of incident AD, independent of ApoE genotype. Similarly, low plasma levels of Abeta42 were related to increased risk of AD, but the association was not statistically significant after adjustment for ApoE genotype. We also have available in the Consortium a CSF bank. We measured levels of total Tau in samples from AD and FTD patients and from control individuals. CSF Tau levels were highest in AD patients, followed by FTD patients and control subjects. The likelihood ratio for an individual with pathological elevated CSF Tau to have AD was 3.52 if compared to controls and 1.85 if compared to patients with FTD. Therefore, CSF total Tau levels appear to be a valid biomarker in the differential diagnosis of dementia. Also with the aim of identifying biomarkers in CSF, we used SELDI-TOF mass spectrometry to analyze and compare protein profiles from healthy control subjects, patients with AD and patients with other neurological diseases including other types of dementia. We found a diagnostic pattern that separates AD patients from healthy individuals and from other neurological diseases with high sensitivity and specificity. Furthermore, we found potential biomarker candidates such as Apolipoprotein AI, which was significantly reduced in patients with AD, and the unmodified form of Transthyretin (TTR) that was significantly increased. An N-terminally truncated form of TTR was reduced in all neurological conditions. Proteomic patterns in CSF might therefore constitute useful tools for the diagnosis of neurodegenerative diseases. We finally investigated the potential of Pin1 isovariants as a diagnostic marker in AD and other tauopathies. Pin1 is a peptidyl-prolyl isomerase that regulates APP and Tau phosphorylation and is itself a phosphoprotein. We demonstrated in 2-dimensional gel electrophoresis specific phosphorylation changes of Pin1 in human AD brains that were similar to those observed in a tau transgenic mouse model. Interestingly, these changes were age-dependent and correlated with the severity of tau pathology. Therefore, changes in Pin1 phosphorylation appear as valid novel biomarkers of the neurofibrillary degeneration process.

1.4.10 Development of therapies

For all the neurodegenerative conditions there is a frustrating lack of effective treatments. Available treatments can in the best of cases delay disease progression and temporarily ameliorate some of the symptoms. We already discussed, in sections 1.4.2 and 1.4.4, some significant advances that we achieved in the development of novel therapies (inhibitors of aggregation, of aggregate toxicity, beta- and gamma-secretase inhibitors). These are, however,

in most cases still far from clinical application. An important exception is the Abeta vaccination approach to treat AD. Abeta immunotherapy was shown to be beneficial in transgenic mouse models and members of this Consortium were the first to show beneficial effects also in AD patients in a phase II clinical trial from ELAN/Wyeth-Ayerst. To avoid/reduce the immunization-related meningoencephalitis observed in some of the patients (and which halted the clinical trial), we invested in the development of passive vaccination strategies. In one of the approaches, we isolated and immortalized memory B cells from vaccinated patients that showed strong antibody response and reduced cognitive decline in the phase II trial. One of the B cell clones produced an antibody, TAP-1, that specifically stained human amyloid-plaques on tissue sections of human AD brain. We further characterized this antibody in a number of assays and could show that it has a higher affinity for fibrillar than for monomeric forms of Abeta. Thus, the TAP-1 antibody might detect neo-epitopes generated by the pathologic aggregation of Abeta peptides. We then evaluated the therapeutic efficacy of this antibody in transgenic mouse models of AD. Preliminary analysis demonstrated that a small fraction of the antibody had crossed the blood brain barrier, and that there were no significant haemorrhages. In a separate approach, we developed conformation-specific monoclonal antibodies that bind specifically to Abeta protofibrils and were effective in reducing protofibrils-induced toxicity in cultured cells. We then tested these antibodies in the APPArcSwe mouse AD model. Our preliminary data suggest markedly lower Abeta protofibril levels and modestly lower levels of total insoluble Abeta in the brains of treated mice. Altogether these data, although preliminary, support passive Abeta vaccination as a promising treatment for AD patients.

2. DISSEMINATION AND USE

The results of research conducted within the APOPIS project, many of them deriving from collaborations between various partners, have been reported to the scientific community in about 200 mostly high-impact, peer-reviewed journals and at high-profile international conferences. At many occasions the public was informed about the increasing societal and economic burden of neurodegenerative diseases due to the growing human life expectancy all over the world. Public awareness was created for the fact that the consequences of this development can only be averted by successful research efforts resulting in the development of reliable preventive and therapeutic interventions. The exploitable results described below comprise only part of the findings obtained in the various laboratories within the course of the APOPIS project. Several contractors followed the advice of the Commission to withhold information on their results due to IPR reasons. Altogether, the APOPIS project contributes to achieving the Lisbon Agenda's goals of growth, competitiveness and employment not only by the scientific progress reached, but also by successfully combining the scattered European resources in our area of research which will certainly come to fruition only in the near future.

2.1 Mouse models with therapeutic value in Alzheimer disease research

Result description: Beta- and gamma-secretases are the two enzymes required to generate Abeta, the toxic entity in Alzheimer disease, and therefore their inhibition is considered a promising therapeutic strategy to treat or prevent the disease. We generated mouse models that lack either the beta-secretase (BACE1) or Aph1, one of the four core subunits of the gamma-secretase complex. Indeed, rodents have three Aph1 genes (Aph1A, Aph1B and Aph1C) and we generated single, double and triple knock outs. These models, among other purposes, can help us understand the physiological function of these proteins and predict the *in vivo* consequences of secretase therapeutic inhibition.

State of development: Scientific and/or technical knowledge (basic research)

IPR: Patent applied for but not yet granted; partnership/other contractual agreements

Collaboration sought or offered: Licence agreement, private-public partnership; further research or development support

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2.2 Raft domains in Alzheimer disease research

Result description: Our results from various experiments strongly support the notion that raft domains are involved in the beta-cleavage, A generation and plaque formation. A large body of evidence from recent years, including our own, suggests that the lipid composition of the membrane is crucial to determine the pathway for APP processing. Indeed, we and others have shown that beta- and gamma-secretases localize in these cholesterol-rich microdomains (8) (28) that therefore contain the complete A-generating machinery. Targeting raft domains that are specifically involved in the beta-cleavage could, therefore, be a potential therapeutic strategy to

combat Alzheimer's disease. In order to target the raft domains where beta-secretase localizes to, we are now testing compounds that a) preferentially localized to the raft domains and b) could be engineered to inhibit beta-secretase..

State of development: Scientific and/or technical knowledge (basic research) / We have acquired a palette of compounds from JADO TECHNOLOGIES that are engineered to be targeted to raft domains (raftophiles) and also to inhibit the beta-secretase, the first enzyme in the amyloidogenic processing pathway. These compounds will be tested first in a cell culture system. N2a neuroblastoma cells and/or HeLa cells stably expressing the wild type or the Swedish mutant APP (a better substrate for -secretase) will be used to study the potential of these compounds to inhibit beta-secretase activity and Abeta production. The primary aim is to develop or identify compounds that are targeted to raft domains and inhibit beta-secretase, thereby decreasing the generation of A peptides or disrupt the raft domains involved in Abeta generation.

IPR: Patent(s) applied for but not yet granted; partnership/other contractual agreement(s)

Collaboration sought or offered: Private-public partnership; financial support; further research or development support

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2.3 20S proteasome inhibitors

Result description: Small drug-like molecules inhibit 20 S proteasome activity. The tripeptide mimetics display enhanced solubility and selectivity over established compounds.

State of development: published in:
H. A. Braun, S. Umbreen, M. Groll, U. Kuckelkorn, I. Mlynarczuk, M. E. Wiegand, I. Drung, P. M. Kloetzel, B. Schmidt Tripeptide mimetics inhibit the 20S proteasome by covalent bonding to the active site threonines J. Biol Chem. 2005, 280 (31), 28394-28411.
I. Mlynarczuk-Bialy, H. Roeckmann, U. Kuckelkorn, B. Schmidt, S. Umbreen, J. Golab, A. Ludwig, C. Montag, L. Wiebusch, C Hagemeier, D. Schadendorf, P.-M. Kloetzel, U. Seifert. Combined Effect of Proteasome and Calpain Inhibition on Cisplatin-Resistant Human Melanoma Cells. Cancer Research 2006, 66(15), 7598-7605.

IPR: Patent(s) applied for but not yet granted; partnership/other contractual agreement(s); secret know-how

Collaboration sought or offered: Licence agreement

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2.4 Tau aggregation inhibitors

Result description: Small drug-like molecules inhibit tau protein aggregation

State of development: Prototype/demonstrator available for testing

IPR: Patent(s) applied for but not yet granted; secret know-how

Collaboration sought or offered: Licence agreement; venture capital/spin-off funding; available for consultancy

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2.5 D-peptide inhibitors of amyloid formation

Result description: The conversion of a soluble protein into ß-sheet-rich oligomeric structures and further fiber formation is a critical step in the pathogenesis of the group of human diseases known as amyloidoses. Drugs that interfere with this process may thus be able to prevent or reverse the effects of these diseases. Here, we present a general strategy to obtain D-peptides that specifically interact with protein amyloid stretches, thereby inhibiting amyloid formation and cell toxicity. This method is based on the combination of the results of screening a combinatorial D-peptide library for inhibitors of an amyloidogenic peptide with prior knowledge on the sequence determinants of amyloid fiber formation. D-peptides generated on these bases prevent amyloid formation and disassemble preformed fibrils of amyloid peptides and proteins. More importantly, they reverse amyloid-induced cytotoxicity of the Aß1-42 peptide in cell culture. This general approach opens new avenues for the rational generation of protein-specific D-peptides that can serve as leads for the development of antiamyloid drugs.

State of development: Scientific and/or technical knowledge (basic research): THe D-peptides have been tested in vitro for their properties to interfere with amyloid formation by different amyloidogenic hexapeptides. The D-peptide designed against the amyloid-beta peptide involved in Alzheimer's disease has proved to be effective against this peptide both in vitro and in cytotoxicity assays in cell culture. Currently these results are submitted to PNAS.

IPR: -

Collaboration sought or offered: Further research or development support: We would like to find partners to test the D-peptides in cell or animal models of Alzheimer's disease. We would be interested as well in stablishing contacts with pharmaceutical companies potentially interested in this strategy.

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2.6 Identification of gamma-secretase activity enhancer in mammalian cells and in Drosophila melanogaster

Result description: To identify modulators of gamma-secretase activity we utilized mammalian cells growing *in vitro*, expressing the human form of the Amyloid Precursor protein (APP). Gamma secretase activity was measured by ELISA. The identification of proteins capable of modifying gamma-activity was performed by siRNA technology. Proteins knocked down were those participating in the several aspects of membrane endocytic/exocytic dynamics. Out of several dozens tested, only one such proteins gave a mild (15%) but most reproducible reduction in Abeta production. Importantly, gamma activity reduction occurred in the absence of any major deleterious effect in the cells, as far as we could detect by simple morphological appearance or by the activation of apoptotic pathways. To define if the above in vitro results had an in vivo relevance, the same protein was suppressed in Drosophila melanogaster. Therefore Drosophila melanogaster expressing the truncated (beta cleavaged) form of APP were crossed with flies lacking the gene identified in the mammalian cells in vitro, and the effect on gamma activity measured by visual and biochemical means. This experiment revealed that the lack of this protein leads to reduced gamma secretase activity. As far as we have evaluated, the presence of the mutantion does not overtly affect Drosophila survival and behaviour. The possible importance of this molecule as future drug target will be tested in neuronal cells of the mammalian nervous system, both in vitro and in situ in animal models of Alzheimer's disease.

State of development: Scientific and/or technical knowledge (basic research)

IPR: Secret know-how

Collaboration sought or offered: none

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2.7 Novel, genetically-engineered strains of the yeast S. cerevisiae

Result description: Strains that allow for detection of both chemical entities and cellular factors (proteins) that facilitate or inhibit aggregation of Abeta42 or Prpcan. They can be used (potentially) in high throughput screening of compounds but this would require further development.

State of development: Scientific and/or technical knowledge (basic research)

IPR: No patent position; may need to obtain license from FoldRX, Boston, if need to fully exploit.

Collaboration sought: none

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2.8 SOD1 aggregation in yeast: a model suitable for high-throughput compound screening

Result description: Amyotrophic lateral sclerosis is a fatal illness due to the progressive loss of motor neurons. Mutations in only one gene SOD1, have been proven to be pathogenic through toxic misfolding and aggregation. We have stably transformed the yeast Saccharomyces cerevisiae ERG6- knock out strain to overexpress either wild type, G37R, G85R or G93A mutant SOD1. Detergent resistant protein aggregated only form from the mutant proteins. We have screened 600 of the NINDS licenced compound library in the G93A SOD1 strain and validated a significant (>35% decrease in G93ASOD1 aggregation) in 15 compounds. These are now being tested in several mammalian cell models.

State of development: Scientific and/or technical knowledge (basic research)

IPR: Patent(s) applied for but not yet granted; partnership/other contractual agreement(s)

Collaboration sought: Licence agreement; private-public partnership; financial support; further research or development support

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2.9 FP-CIT SPECT in rodents

Result description: This technique allows a three-dimensional, quantitative in vivo evaluation of the dopaminergic innervation to the striatum, thus presenting an important tool to assess rodent models of Parkinson's disease in vivo.

State of development: Scientific and/or technical knowledge (basic research): To monitor the dopaminergic innervation in the striatum, I-123-FP-CIT is injected i.v. and the specific striatal binding is determined quantitatively by SPECT using a small animal 10-pinhole collimator and a rotating double-headed gamma-camera (E.CAM, Siemens Medical Solutions, Illinois, USA) with a field of view of 533 x 387 mm. For the reconstruction of transversal slices, a multiplicative iteration algorithm has been developed especially designed for pinhole SPECT (HiSPECT, Scivis GmbH, Göttingen, Germany). The transverse slices are reoriented using a Hermes workstation (Hermes Medical Solutions, Stockholm, Sweden) and the “multi modality application” (Hermes Medical Solutions). ROI´s for the striata and the background are drawn on the reconstructed SPECT images. These ROI´s are saved as template for the evaluation of all SPECT images. For the analysis of striatal 123I-FP-CIT-binding, three consecutive transversal slices with the highest count rate in the striata are selected, and the template is positioned manually without changing the size and form of the ROI´s on the SPECT images. In each slice the striatum to background ratio is calculated. The average of the ratios for the left and right striatum, respectively, are used for the statistical evaluation. Our preliminary work has allowed demonstrating a highly significant linear correlation of the specific striatal FP-CIT binding with the DA concentration in untreated 6-OHDA mice to validate the technique.

IPR: Secret know-how

Collaboration sought: Financial support: In vivo drug testing for substances evaluating their putative affection of the nigrostriatal dopaminergic system.

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