

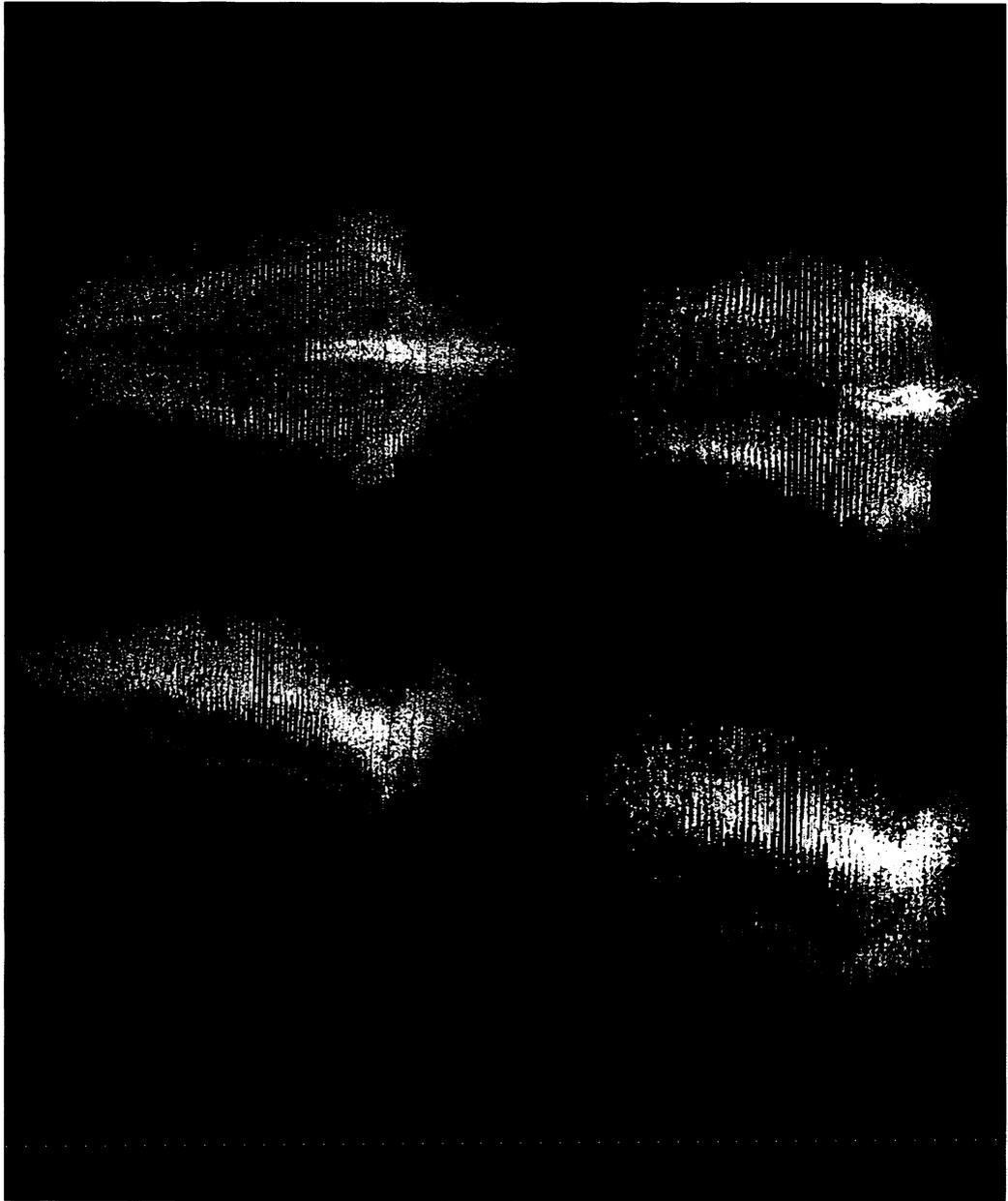
4.1.93

January-February 1993

4 Z 90.393-4

F65-19a

Dementia



S. Karger
Medical and Scientific
Publishers
Basel · Freiburg
Paris · London
New York · New Delhi
Bangkok · Singapore
Tokyo · Sydney

KARGER

1266 4

Dementia

Editor-in-Chief

V. Chan-Palay, New York, N.Y.

Editorial Board

L. Amaducci, Florence
M.J. Ball, Portland, Oreg.
K. Beyreuter, Heidelberg
D.M. Bowen, London
A. Brun, Lund
S.H. Ferris, New York, N.Y.
D.C. Gajdusek, Bethesda, Md.
C.G. Gottfries, Gothenburg
L. Gustafson, Lund
K. Hasegawa, Kawasaki
F.F. Hefti, Los Angeles, Calif.
A.S. Henderson, Canberra
S. Hirai, Gummaken
F. Javoy-Agid, Paris
A.D. Korczyn, Tel Aviv

C.L. Masters, Melbourne
R. Mayeux, New York, N.Y.
E. Melamed, Petah-Tiqva
D.L. Murphy, Bethesda, Md.
D.S. Olton, Baltimore, Md.
E.K. Perry, Newcastle upon Tyne
J.W. Pettegrew, Pittsburgh, Pa.
D. Price, Baltimore, Md.
J. Rogers, Sun City, Ariz.
A.D. Roses, Durham, N.C.
M. Roth, Cambridge
Y. Sakaki, Fukuokashi
H.M. Wisniewski,
Staten Island, N.Y.
M. Yoshida, Tochigi



KARGER



Contents Vol. 4, 1993

No. 1

Original Research Articles

Calbindin D-28k and Monoamine Oxidase A Immunoreactive Neurons in the Nucleus Basalis of Meynert in Senile Dementia of the Alzheimer Type and Parkinson's Disease
Chan-Palay, V.; Höchli, M.; Savaskan, E.; Hungerecker, G. 1

The Spatial Patterns of Plaques and Tangles in Alzheimer's Disease Do Not Support the 'Cascade Hypothesis'
Armstrong, R.A.; Myers, D.; Smith, C.U.M. 16

Brain Damage Caused by Ischemia: Pathophysiological and Pharmacological Aspects
Rami, A.; Kriegelstein, J. 21

Oral Tetrahydroaminoacridine Treatment of Alzheimer's Disease Evaluated Clinically and by Regional Cerebral Blood Flow and EEG
Minthon, L.; Gustafson, L.; Dalfelt, G.; Hagberg, B.; Nilsson, K.; Risberg, J.; Rosén, I.; Seiving, B.; Wendt, P.E. 32

Multichannel EEG Frequency Analysis and Somatosensory-Evoked Potentials in Patients with Different Types of Organic Dementia
Rosén, I.; Gustafson, L.; Risberg, J. 43

Increased Sweat Sodium Concentration in Patients with Alzheimer's Disease
Elmståhl, S.; Winge, L. 50

Psychometric Discrimination of Tetrahydroaminoacridine Responders in Alzheimer Patients
Alhainen, K.; Helkala, E.-L.; Riekkinen, P. 54

Letter to the Editor

Clinical Course and CSF Amyloid β Protein Precursor Having the Site of Application of the Protease Inhibitor (APPI) Levels in Patients with Dementia of the Alzheimer Type
Urakami, K.; Takahashi, K.; Okada, A.; Oshima, T.; Adachi, Y.; Nakamura, S.; Kitaguchi, N.; Tokushima, Y.; Yamamoto, S.; Tanaka, S. 59

No. 2

Original Research Articles

Distribution of Iron in the Basal Ganglia and Neocortex in Postmortem Tissue in Parkinson's Disease and Alzheimer's Disease
Griffiths, P.D.; Crossman, A.R. 61

Quantitative Assessment of the Synaptophysin Immuno-Reactivity of the Cortical Neuropil in Various Neurodegenerative Disorders with Dementia
Zhan, S.-S.; Beyreuther, K.; Schmitt, H.P. 66

Rare Neuropil Threads in Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia on Guam and in the Kii Peninsula of Japan
Wakayama, I.; Kihira, T.; Yoshida, S.; Garruto, R.M. 75

Reduced Phosphatidylinositol Kinase Activity in Alzheimer's Disease: Effects of Age and Onset
Jolles, J.; Bothmer, J.; Markerink, M.; Ravid, R. 81

Assessment of Depression in Alzheimer's Disease: Symptoms, Syndrome, and Computed Tomography Findings
Troisi, A.; Pasini, A.; Gori, G.; Sorbi, T.; Biagini, C.; Aulisi, A.; Baroni, A.; Ciani, N. 87

Pattern of Cerebral Metabolic Interactions in a Subject with Isolated Amnesia at Risk for Alzheimer's Disease: A Longitudinal Evaluation
Pietrini, P.; Azari, N.P.; Grady, C.L.; Salerno, J.A.; Gonzales-Aviles, A.; Heston, L.L.; Pettigrew, K.D.; Horwitz, B.; Haxby, J.V.; Schapiro, M.B. 94

Is Impaired Recall in Dementia of the Alzheimer Type a Consequence of a Contextual Retrieval Deficit?
Pollmann, S.; Haupt, M.; Romero, B.; Kurz, A. 102

Long-Term Effects of Bilateral Frontal Lobe Lesions from Neuropsychiatric and Neuroradiological Aspects
Hakola, H.P.A.; Puranen, M.; Repo, L.; Tiihonen, J. 109

Delirium in the Elderly: Relationship of Clinical Symptoms to Outcome
Wada, Y.; Yamaguchi, N. 113

Telephone-Assessed Mental State
Lanska, D.J.; Schmitt, F.A.; Stewart, J.M.; Howe, J.N. 117

Letter to the Editor

MRI Findings in an Individual at Risk for Familial Alzheimer's Disease
Ball, J.A.; Kennedy, A.M.; Roques, P.; Stevens, J.; Rossor, M.N. 120

.....
No. 3-4
.....

The 2nd International Conference on Frontal Lobe Degeneration of Non-Alzheimer Type

September 11-12, 1992, Lund, Sweden

Guest Editor: Arne Brun, Lund

Editorial: Dementia of Frontal Type Brun, A.	125
Frontal Lobe Degeneration of Non-Alzheimer Type Revisited Brun, A.	126
Overview of Dementia Lacking Distinctive Histology: Pathological Designation of a Progressive Dementia Knopman, D.S.	132
Presenile Dementia with Motor Neuron Disease Mitsuyama Y.	137
Clinical Picture of Frontal Lobe Degeneration of Non-Alzheimer Type Gustafson, L.	143
Progressive Frontal Dysfunction Benson, D.F.	149
The Clinical Pathological Correlates of Lobar Atrophy Neary, D.; Snowden, J.S.; Mann, D.M.A.	154
Spectrum of Frontal Lobe Dementia in a Swedish Family Passant, U.; Gustafson, L.; Brun, A.	160
Exclusion Mapping in Familial Non-Specific Dementia Brown, J.; Gydesen, S.; Sorensen, S.A.; Brun, A.; Duff, K.; Houlden, H.; Fidani, L.; Kullkarni, S.; Cummings, J.; Goate, A.; Rossor, M.; Hardy, J.	163
Neuropeptides in Cerebrospinal Fluid of Patients with Alzheimer's Disease and Dementia with Frontotemporal Lobe Degeneration Edvinsson, L.; Minthon, L.; Ekman, R.; Gustafson, L.	167
Preliminary Neurochemical Findings in Non-Alzheimer Dementia due to Lobar Atrophy Francis, P.T.; Holmes, C.; Webster, M.-T.; Stratmann, G.C.; Procter, A.W.; Bowen, D.M.	172
Prion Diseases in Humans and Their Relevance to Other Neurodegenerative Diseases Collinge, J.; Palmer, M.S.	178
Regional Cerebral Blood Flow in Frontal Lobe Dementia of Non-Alzheimer Type Risberg, J.; Passant, U.; Warkentin, S.; Gustafson, L.	186
Functional Activation of the Frontal Lobes. Regional Cerebral Blood Flow Findings in Normals and in Patients with Frontal Lobe Dementia Performing a Word Fluency Test Warkentin, S.; Passant, U.	188
Functional Imaging, the Frontal Lobes, and Dementia Friedland, R.P.; Koss, E.; Lerner, A.; Hedera, P.; Ellis, W.; Dronkers, N.; Ober, B.A.; Jagust, W.J.	192

Progressive Right Frontotemporal Degeneration: Clinical, Neuropsychological and SPECT Characteristics Miller, B.L.; Chang, L.; Mena, I.; Boone, K.; Lesser, I.M.	204
---	-----

Neuropsychological Findings in Frontal Lobe Dementia Elfgrén, C.; Passant, U.; Risberg, J.	214
---	-----

Assessment of Neuropsychological Dysfunction in Frontal Lobe Degeneration Stuss, D.T.	220
--	-----

Progressive Language Dysfunction and Lobar Atrophy Snowden, J.S.; Neary, D.	226
--	-----

Frontal Lobe Cognitive Functions in Aging: Methodologic Considerations Bauer, K.; Miller, B.L.; Lesser, I.M.	232
---	-----

.....
No. 5
.....

Original Research Articles

Galaninergic Innervation of the Cholinergic Vertical Limb of the Diagonal Band (Ch2) and Bed Nucleus of the Stria terminalis in Aging, Alzheimer's Disease and Down's Syndrome Mufson, E.J.; Cochran, E.; Benzing, W.; Kordower, J.H.	237
--	-----

The Ratio of Diffuse to Mature Beta/A4 Deposits in Alzheimer's Disease Varies in Cases with and without Pronounced Congophilic Angiopathy Armstrong, R.A.; Myers, D.; Smith, C.U.M.	251
--	-----

Phosphorylation of Tau by Cyclic-AMP-Dependent Protein Kinase Robertson, J.; Loviny, T.L.F.; Goedert, M.; Jakes, R.; Murray, K.J.; Anderton, B.H.; Hanger, D.P.	256
--	-----

Dendritic Atrophy and Remodeling of Amygdaloid Neurons in Alzheimer's Disease Scott, S.A.	264
--	-----

Induction of Memory and Cortical Cholinergic Neurochemical Recovery with Combine Fetal Transplantation and GM1 Treatments in Rats with Lesions of the NBM Santucci, A.C.; Gluck, R.; Kanof, P.D.; Haroutunian, V.	273
--	-----

Variation in Psychiatric and Behavioural Symptoms at Different Stages of Dementia: Data from Physicians' Examinations and Informants' Reports Forsell, Y.; Jorm, A.F.; Winblad, B.	282
---	-----

Influence of Feelings of Burden on the Caregiver's Perception of the Patient's Functional Status Mangone, C.A.; Sanguinetti, R.M.; Baumann, P.D.; Gonzalez, R.C.; Pereyra, S.; Bozzola, F.G.; Gorelick, P.B.; Sica, R.E.P.	287
---	-----

A Comparison of Cognitive Impairments in Dementia of the Alzheimer Type and Depression in the Elderly Geffen, G.; Bate, A.; Wright, M.; Rozenbils, U.; Geffen, L.	294
--	-----

.....
No. 6
.....

Original Research Articles

APP Expression in Primary Neuronal Cell Cultures from P6 Mice during in vitro Differentiation	301
Dichgans, M.; Mönning, U.; König, G.; Sandbrink, R.; Masters, C.L.; Beyreuther, K.	
Heparan Sulfate Expression Patterns in the Amyloid Deposits of Patients with Alzheimer's and Lewy Body Type Dementia	308
Van Gool, D.; David, G.; Lammens, M.; Baro, F.; Dom, R.	
Growth Hormone Secretion in Alzheimer's Disease: Studies with Growth Hormone-Releasing Hormone Alone and Combined with Pyridostigmine or Arginine	315
Ghigo, E.; Nicolosi, M.; Arvat, E.; Marcone, A.; Danelon, F.; Mucci, M.; Franceschi, M.; Smirne, S.; Camanni, F.	
Neuropsychological Heterogeneity in Mild Alzheimer's Disease	321
Binetti, G.; Magni, E.; Padovani, A.; Cappa, S.F.; Bianchetti, A.; Trabucchi, M.	

Epidemiology of Depressive Symptoms in Elderly Primary Care Attenders	327
Evans, S.; Katona, C.	
Brain Perfusion Imaging in Parkinson's Disease and Alzheimer's Disease Demonstrated by Three-Dimensional Surface Display with ¹²³ I-Iodoamphetamine	334
Tachibana, H.; Kawabata, K.; Tomino, Y.; Sugita, M.; Fukuchi, M.	
Age at Onset and SPECT Imaging in Alzheimer's Disease	342
Caffarra, P.; Scaglioni, A.; Malvezzi, L.; Previdi, P.; Spreafico, L.; Salmasso, D.	
Potential Biological Targets for Anti-Alzheimer Drugs	347
Allain, H.; Belliard, S.; de Certaines, J.; Bentué-Ferrer, D.; Bureau, M.; Lacroix, P.	
Author Index	353
Subject Index	355

.....
S. Karger
Medical and Scientific Publishers
Basel · Freiburg · Paris · London
New York · New Delhi · Bangkok
Singapore · Tokyo · Sydney

.....
Drug Dosage
The authors and the publisher have exerted every effort to ensure that drug selection and dosage set forth in this text are in accord with current recommendations and practice at the time of publication. However, in view of ongoing research, changes in government regulations, and the constant flow of information relating to drug therapy and drug reactions, the reader is urged to check the package insert for each drug for any change in indications and dosage and for added warnings and precautions. This is particularly important when the recommended agent is a new and/or infrequently employed drug.

.....
All rights reserved.
No part of this publication may be translated into other languages, reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying, recording, microcopying, or by any information storage and retrieval system, without permission in writing from the publisher or, in the case of photocopying, direct payment of a specified fee to the Copyright Clearance Center (see 'Information for Readers and Subscribers').

© Copyright 1993 by S. Karger AG,
P.O. Box, CH-4009 Basel (Switzerland)
Printed in Switzerland on acid-free paper by
Thür AG Offsetdruck, Pratteln

Martin Dichgans^a
Ursula Mönning^a
Gerhard König^a
Rupert Sandbrink^a
Colin L. Masters^b
Konrad Beyreuther^a

^a Center for Molecular Biology, University of Heidelberg, FRG;

^b Department of Pathology, University of Melbourne, Parkville, Vic., Australia

APP Expression in Primary Neuronal Cell Cultures from P6 Mice during *in vitro* Differentiation

Key Words

Alzheimer's disease
Amyloid protein precursor
Cerebellar neurons
Sialic acid

Abstract

Primary neuronal cell cultures from P6 mice were investigated in order to study amyloid protein precursor (APP) gene expression in differentiating neurons. Cerebellar granule cells which strongly express APP 695 allowed the identification of three distinct isoforms of neuronal APP 695. The high-molecular-weight form of APP 695 is sialylated. The expression pattern of neuronal APP 695 changes during *in vitro* differentiation. Sialylated forms become more abundant upon longer cultivation time. The secreted forms of sialylated, neuronal APP 695 are shown to comigrate with APP isolated from cerebrospinal fluid. We suggest that the different sialylation states of APP 695 may reflect the modulation of cell-cell and cell-substrate interactions during *in vitro* differentiation and regeneration.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by intracellular neurofibrillary tangles, extracellular plaques and cerebrovascular amyloid in the brains of affected individuals. The principal component of the deposits is the β A4 protein, which is derived from a larger amyloid protein precursor (APP) [1]. Several alternative splice forms of this membrane-spanning glycoprotein have been identified so far [2-7]. As shown by different authors, APP 695 is the predominantly expressed form in the developing central nervous system, whereas peripheral tissues mainly express the KPI-containing APPs [4, 6, 8-11]. Biochemical analyses have shown that APP is posttranslationally modified by N- and O-glycosylation as well as tyrosine sulfation and serine phosphory-

lation [12, 13]. Proteolytic cleavage within the amyloidogenic region leads to secretion of a large soluble NH₂-terminal APP fragment into the surrounding environment [12, 14, 15]. Such carboxyl-terminal truncated molecules are found in cerebrospinal fluid (CSF) and blood [12, 16, 17]. APPs circulating in blood are mainly derived from APP 751 and 770. They may be secreted by platelets and leukocytes and were shown to be identical with protease nexin 2 [18-21]. In contrast, soluble APP found in CSF mainly lacks the KPI insert, reflecting the predominant expression of APP 695 in the central nervous system.

It has been suggested by several authors that APP 695 plays an important role in neuronal differentiation and regeneration [22-24].

Cerebellar cells derived from early postnatal brain share many of the features characteristic for their counter-

parts in vivo such as cell migration, axonal sprouting, synapse formation and electric activity [25–28]. Thus they provide an ideal model for investigating APP biosynthesis during differentiation processes and formation of neuronal networks. Using this approach, we have studied the biogenesis of APP in developing neurons. Our findings show that APP is indeed strongly expressed by neuronal cells. Differentiation of these cells in vitro was associated with changes in posttranslational modification of amyloid protein precursor.

Materials and Methods

Cell Culture

The cell culture protocol employed was a modification of the methods described by Schnitzer et al. [29].

Cerebelli were obtained from postnatal Balb/c mice (P6). At this stage of development, granule cells constitute approximately 90% of all cerebellar neurons [30]. After removal of the meninges, cerebelli were incubated for 15 min in a solution of 10 mg/ml trypsin in Ca^{2+} - and Mg^{2+} -containing Hanks balanced salt solution (HBSS⁺). Cerebelli were rinsed three times in Ca^{2+} - and Mg^{2+} -free HBSS, followed by dissociation of the cells. This was done in 0.5 mg/ml DNase I in HBSS⁺ by trituration through the narrowed bore of a fire-polished Pasteur pipette. Cells were distributed to polylysine-coated (100 $\mu\text{g}/\text{ml}$) culture dishes containing 2 ml of Earl's Basal Medium (BME; Gibco) buffered with sodium bicarbonate (10 mM) and supplemented with 10% horse serum (Gibco). Neurons were plated at a density of about 5×10^4 cells/cm². Cultures were maintained in a humidified incubator at 37 °C in 5% CO₂.

Immunocytochemistry

Coverslips were removed from culture dishes, briefly rinsed with phosphate-buffered saline (PBS; pH 7.2) and fixed with freshly prepared 4% paraformaldehyde (in PBS; pH 7.2) at room temperature for 15 min. After permeabilization with 0.3% TX-100 in PBS for 5 min, nonspecific binding sites were blocked with 1% human serum albumin, 10% FCS in PBS for 30 min at room temperature (RT). Cells were then incubated with primary antibodies in PBS (0.3% TX-100, 1% human serum albumin) at 4 °C overnight, extensively washed with PBS and incubated with fluorescein- and rhodamine-conjugated secondary antibodies for 1 h at RT. Coverslips were washed for 45 min in PBS before being mounted upside down on a drop of aqueous mounting medium (Moviol including 2.5% DABCO). Cells were examined on a Zeiss axioplan microscope equipped with phase contrast and epifluorescence optics for rhodamine and fluorescein.

Antibodies were used at the following dilutions: polyclonal anti-Fd-APP [12], 1:400; monoclonal anti-GFAP (Serva), 1:50; monoclonal anti-neurofilament (Dakopatts), 1:50; fluorescein-conjugated goat anti-rabbit (Sigma) 1:100; rhodamine-conjugated goat anti-mouse (Jackson), 1:100.

Biosynthetic Labeling

After removal of the culture medium, cells were radioactively labeled with 120 μCi of [³⁵S]methionine (Amersham) in 1.5 ml of

methionine-free Dulbecco's modified Eagle's medium (DMEM) for 4 h. The conditioned medium was then cleared by centrifugation and stored at –20 °C. Cells were washed once with phosphate-buffered saline. For lysis, cells were resuspended in 0.2 ml of lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 2 mM phenylmethanesulfonylfluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1.6 mg/ml iodoacetamide] and incubated for 30 min on ice. Cell lysates were centrifuged at 10,000 g for 5 min, and the supernatants were stored at –20 °C until further use. The extraction pellet was discarded.

Immunoprecipitation

For immunoprecipitation of [³⁵S]methionine-labeled APP, 100 μl of cell lysate or 750 μl of conditioned medium were used. The cell lysate was diluted 1:1 with ice-cooled washing buffer B (20 mM Tris, pH 7.5, 500 mM NaCl, 0.5% Nonidet P-40, 2 mM PMSF). Lysate was preincubated for 2 h at room temperature with 10 μl of preimmune serum and 3 mg of protein A-Sepharose. Insoluble complexes were spun down and discarded. The supernatants were incubated for 1 h at room temperature with 5 μl of undiluted anti-CT or anti-FdAPP. The polyclonal anti-CT antiserum was raised against a synthetic peptide, which corresponds to the COOH-terminal 43 residues of APP. Polyclonal anti-FdAPP antiserum was raised against purified *Escherichia coli* FdAPP fusion protein consisting of APP 695 and the Fd fragment of the murine IgM heavy chain [12].

After addition of 2 mg of protein A-Sepharose, the mixture was incubated for 30 min at RT. Nonbound proteins were removed from Sepharose beads by sequential washing with buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 0.4% Nonidet P-40, 0.4% Triton X-100, 2 mM PMSF), washing buffer B and finally with TSA solution (20 mM Tris, pH 8.0, 150 mM NaCl). The immunoprecipitates were fractionated by SDS-PAGE (8%). Gels were soaked in enhancer solution, dried and exposed to Kodak X-OMAT AR film at –70 °C.

Secreted APP was precipitated under nondetergent conditions and analyzed by subsequent immunoblotting. Immunoblotting was performed as described by Mönning et al. [34].

Enzymatic Digestion

Prior to enzymatic digestion with neuraminidase from *Clostridium perfringens* (Sigma), immunoprecipitated proteins were denatured in 20 μl of 0.2% SDS, 2% MCE for 10 min at 100 °C followed by addition of 100 μl of sodium-acetate buffer (pH 6.0; 100 mM). Enzymatic digestion was performed with 10 mU of neuraminidase at 37 °C for 16 h. The proteins were dried and resuspended in Laemmli sample buffer. After boiling, labeled proteins were subjected to SDS-PAGE.

Results

Immunocytochemistry

For immunocytochemical localization of APP in primary neuronal cell culture, cerebellar cells from P6 mice were isolated and grown on poly-l-lysine-coated coverslips in the presence of 10% horse serum. Medium was not changed until cells were used for staining procedure, usually carried out after six days of cultivation. Staining

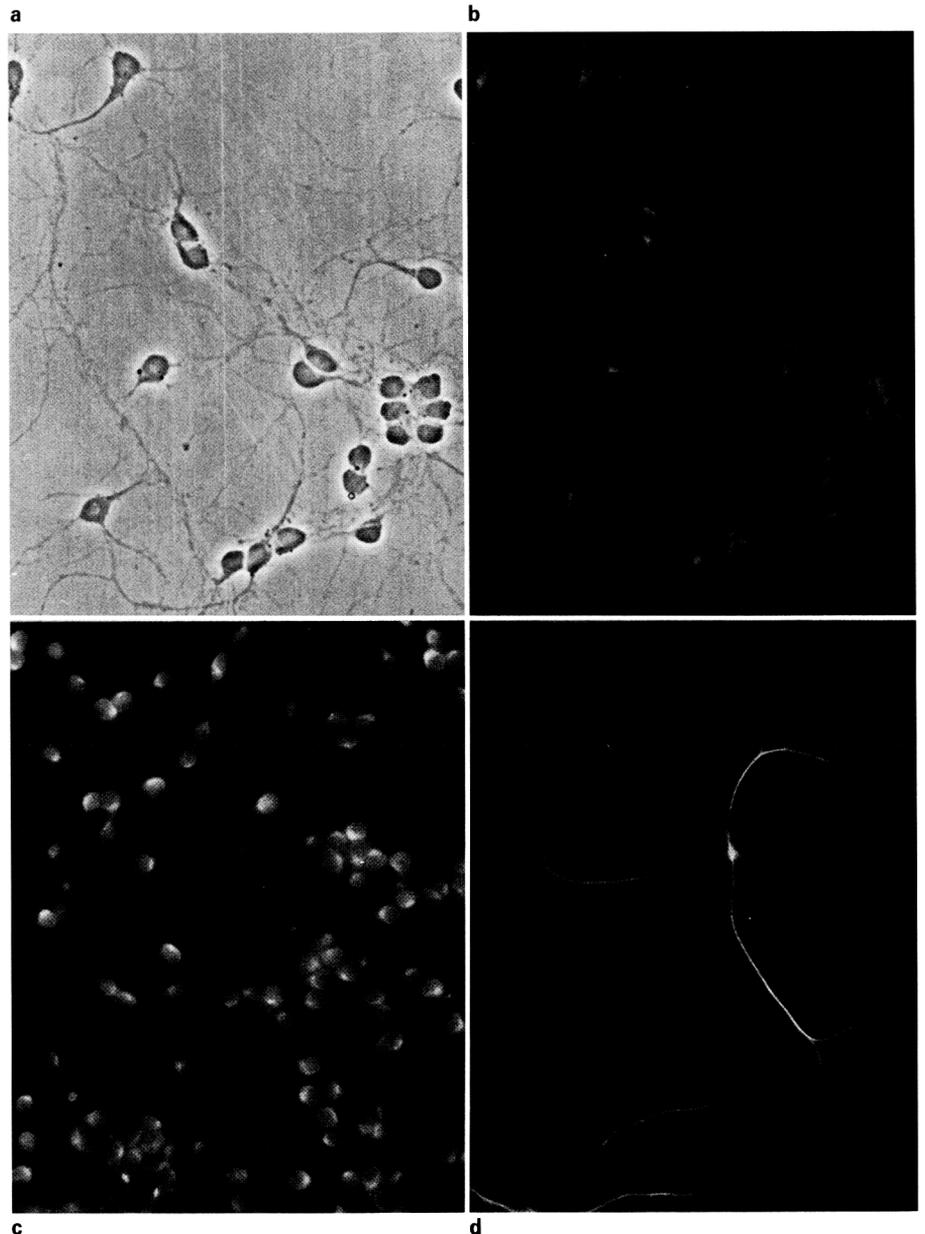


Fig. 1. Indirect immunofluorescence of primary cerebellar cell cultures. Cells were obtained from P6 mice and cultivated for 6 days. **a** Phase contrast. **b** Immunostaining with polyclonal anti-FdAPP serum. APP immunoreactivity is detected in granular cells. **c/d** Double staining with anti-Fd APP serum (**c**) and (**d**) monoclonal anti-GFAP (**d**). Neurons are the main source of APP-immunoreactivity (**c**). Astroglia, identified by GFAP-staining (**d**) show a weak APP staining.

with polyclonal anti-Fd-APP showed an intense labeling of granular cells which represented about 90% of cells in culture (fig. 1a, b). Within these neurons, strong APP reactivity could be detected close to the nucleus at a somewhat excentric position. Double staining with anti-GFAP (glial fibrillary acidic protein) revealed only weak labeling of astroglia (fig. 1c, d). Fibroblasts and oligodendroglia, as identified by their morphology, also showed low APP reactivity (results not shown). Thus, under our in vitro conditions granular cells were the main source of APP.

Metabolic Characterization

In order to analyze APP expression within these cells proteins were metabolically labeled with [³⁵S]-methionine and immunoprecipitated with anti-CT, an antiserum raised against the synthetic cytoplasmic part of APP. As shown in figure 2a, three distinct bands with a molecular weight of approximately 102, 112 and 120 kD could be detected in detergent extracts from cell pellets. They correspond to translation products of APP 695 mRNA as was confirmed by RNA analysis (results not shown). These findings are in accordance with results

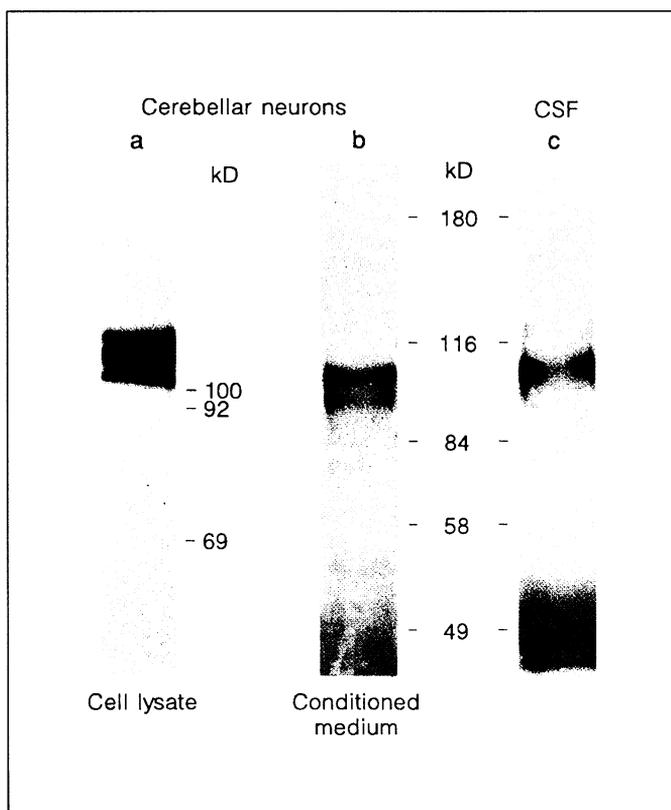


Fig. 2. Analysis of APP biosynthesis of cerebellar neurons. **a** Immunoprecipitation of APP from [³⁵S]methionine-labeled cell lysate of cerebellar neurons. Biosynthetic labeling was performed after 6 days of cultivation. The immunoprecipitates were analysed by autoradiography. Precipitation was done with the polyclonal anti-CT antiserum. **b** Identification of secretory APP from cerebellar neurons cultivated for 6 days. Conditioned medium of neurons was subjected to immunoprecipitation with anti-FdAPP serum. The immunoprecipitates were analyzed by immunoblotting. Detection of APP was performed with the monoclonal antibody 22C11. **c** Western Blot analysis of APP from cerebrospinal fluid. Detection of APP was done as described in **b**.

from other groups who were able to show, that APP 695 is the predominantly expressed form in neurons [3, 4, 6, 8, 9, 31].

For detection of secretory APP released by neuronal cells, conditioned medium was investigated. Since polyclonal anti-Fd APP did not precipitate soluble isoforms of mouse APP under high detergent concentrations necessary for radioactive immunoprecipitations followed by autoradiography, secretory APP was identified by Western blot analysis with monoclonal 22C11. Two immunoreactive bands with an apparent molecular mass of 95 and 105 kD were identified (fig. 2b). The upper band of secre-

tory APP was shown to comigrate with APP derived from cerebrospinal fluid (fig. 2c).

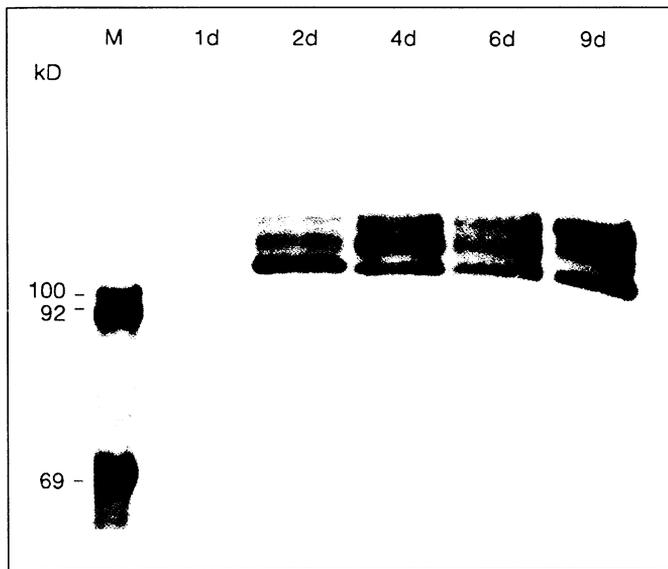
In order to assess APP biosynthesis during differentiation, cells were cultivated for different time periods (1, 2, 4, 6 and 9 days). At the end of each cultivation period, cells were metabolically labeled, immunoprecipitated with anti-CT and analyzed by SDS-PAGE. The autoradiography of the cell lysates is shown in figure 3a. The densitometric scanning analysis of the autoradiography is graphically demonstrated in figure 3b. At all time points investigated, cells in vitro expressed all three forms of APP 695. However, within prolonged cultivation time the ratio between these different forms changed. Whereas the lower band was dominating up to 48 h of cultivation, the higher molecular weight APP isoforms (112 kD and 120 kD) became more prominent upon longer cultivation time. At day 9 in culture, cells expressed large amounts of the high-molecular weight APP isoforms. The amount of secretory APP remained unchanged over the whole time investigated (results not shown).

Posttranslational Modifications

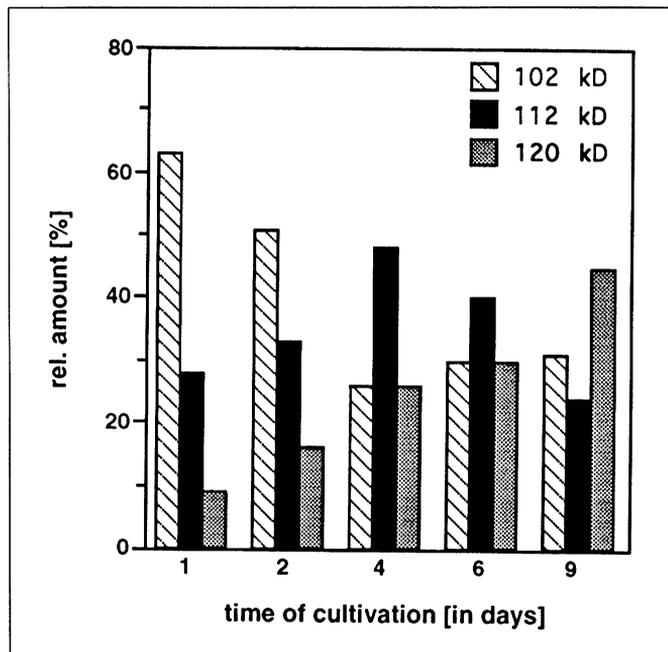
As could be shown by different authors, APP is post-translationally modified by N- and O-glycosylation as well as tyrosine sulfation and serine phosphorylation [12, 13]. APP has been proposed to be a cell adhesion molecule involved in cell-cell and cell-substrate interactions [31–34]. We have investigated whether neuronal APP is sialylated since sialic acid residues are known to play a key role in adhesion processes mediated by molecules like N-CAM (neuronal cell adhesion molecule) [35–37].

Immunoprecipitated APP was enzymatically digested with neuraminidase from *Clostridium perfringens* prior to separating proteins on SDS-PAGE. As shown in figure 4, the high molecular weight APP isoform (120 kD) disappeared after treatment with neuraminidase. Concomitant with the decrease of cell-associated 120-kD APP isoform there was an increase in the amount of cell-associated 112-kD APP. These experiments may indicate that the 112-kD APP isoform is the nonsialylated precursor of the high-molecular-weight APP isoform (120 kD). Sialylation of the 112-kD form gives rise to the apparent molecular weight shift of approximately 8 kD resulting in high-molecular-weight APP 695.

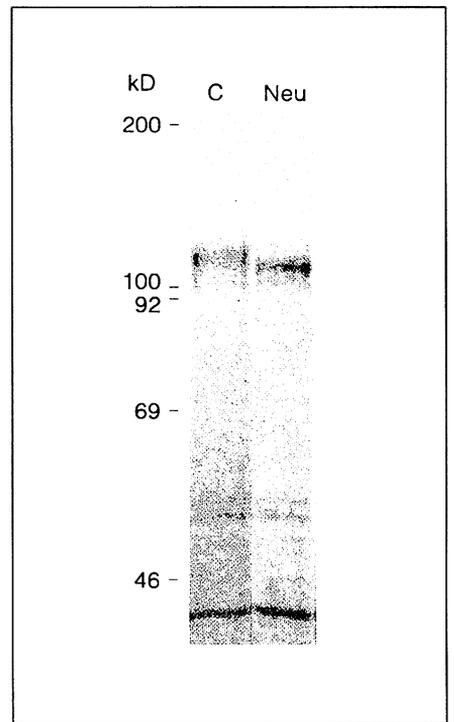
Cell associated, sialylated APP 695 was less prominent after 24 h of cultivation (8% of total APP) but contributes to about 45% of total APP upon 9 days of in vitro cultivation. This indicates that sialylation of neuronal APP 695 is regulated in a time-dependent way during in vitro differentiation.



3a



3b



4

Fig. 3. Expression of APP in cerebellar neurons during in vitro differentiation. **a** Cells were cultivated for different time periods. Biosynthetic labeling with [³⁵S]methionine was done at times indicated at the top of each lane. APP was isolated from cell pellets by immunoprecipitation with anti-CT antiserum. The immunoprecipitates were analyzed as in figure 2a. **b** Densitometric quantitation of the APP immunoprecipitation analysis (**a**) of cerebellar neurons cultivated for different time periods.

Fig. 4. Neuraminidase-treatment of cell-associated APP695 APP was immunoprecipitated from radiolabeled detergent extracts of cerebellar neurons with polyclonal anti-CT serum prior to enzymatic digestion with neuraminidase from *Clostridium perfringens* (Neu). Precipitates were analyzed by SDS-PAGE (8%) followed by autoradiography (C, non-digested APP).

Discussion

Primary neuronal cell culture from P6 mice was investigated to gain insight into APP expression during neuronal differentiation processes. Cerebellar neurons showed an intense labeling with anti-Fd APP, whereas astrocytes revealed only a weak APP immunoreactivity. This is in agreement with results from other groups, who have shown that neurons are the main source of cerebral APP production [10, 16, 31, 38, 39]. Large amounts of neuro-

nal APP are associated with the Golgi apparatus as revealed by light- and electron-microscopic immunocytochemical techniques [39, 40]. Our data support these results since neuronal APP immunoreactivity was intracellularly located proximal to the nucleus. Rapid turnover of transmembrane APP by cleavage within the β A4 sequence might explain the weak cell surface staining. This is evidenced by the fact that large amounts of secretory APP could be detected already on day 1 in vitro.

We have shown that APP 695 from cerebellar neurons is posttranslationally modified by sialylation. Sialic acid residues are known to have great influence on adhesion properties of cell surface molecules. The best studied example is N-CAM (neuronal cell adhesion molecule) which exists in a highly sialylated form as well as several isoforms containing less sialic acid [41, 42]. The sialylation of N-CAM is developmentally regulated [43]. Poly-sialylation of N-CAM is known to promote axonal branching and decrease cell adhesivity, whereas removal of polysialic acid increases axonal fasciculation [35–37].

APP was shown to be involved in cell-cell and cell-substrate interactions [31–34]. We therefore suggest that sialylation of APP 695 might be a mechanism by which neuronal cell adhesion properties are modulated. Three distinct forms of neuronal APP 695 can be identified by Western blot analysis. The 120-kD APP isoform was shown to be sialylated. During *in vitro* differentiation, changes in the expression pattern of neuronal APP 695 were observed. Sialylated APP was rarely detectable after cultivation for 24 h, but was increased up to 45% of total APP upon 9 days of cultivation. These changes could provide the basis for regulation of cell-cell and cell-substrate

interactions during *in vitro* differentiation of cerebellar neurons. The sialylation state seems to depend upon the state of differentiation of the neurons investigated. The presence of polysialylated cell associated APP may be a requirement for axonal branching and cell migration whereas a low sialylation state may be necessary for axonal fasciculation and cell adhesion.

We postulate that changes in the sialylation state of APP 695 play an important role in cellular interactions associated with neuronal cell migration and axonal guiding during early postnatal brain development. APP could thus belong to the group of cell surface molecules which are involved in the establishment of the neuronal network.

Acknowledgements

This work was supported by funds from the National Health and Medical Research Council, the Victorian Health Promotion Foundation, the Aluminium Development Corporation (to C.L.M.), the Cusanuswerk (to M.D.) and the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie (to K.B.)

References

- Kang J, Lemaire H-G, Unterbeck A, Salbaum JM, Masters CL, Grzeschik K-H, Multhaup G, Beyreuther K, Mueller-Hill B: The precursor of Alzheimer's disease amyloid β A4 protein resembles a cell-surface receptor. *Nature* 1987; 325:733–736.
- Ponte P, Gonzalez-De Whitt P, Schilling J, Miller J, Hsu D, Greenberg B, Davis K, Wallace W, Lieberburg I, Fuller F, Cordell B: A new A4 amyloid mRNA contains a domain homologous to serine protease inhibitors. *Nature* 1988;311:525–527.
- Tanzi RE, McClatchey AI, Lamperti ED, Villa-Komaroff L, Gusella GF, Neve RL: Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature* 1988;311:528–530.
- Kitaguchi N, Takahashi Y, Tokushima Y, Shiojiri S, Ito H: Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitor activity. *Nature* 1988;331:530–532.
- de Sauvage F, Octave JN: A novel mRNA of the A4 amyloid precursor gene coding for a possible secreted protein. *Science* 1989;245: 651–653.
- Golde TE, Estus S, Usiak M, Younkin LH, Younkin SG: Expression of β -amyloid protein precursor mRNA's: Recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR. *Neuron* 1990; 4:235–267.
- König G, Mönning U, Czech C, Prior R, Banati R, Schreiter-Gasser U, Bauer J, Masters CL, Beyreuther K: Identification and differential expression of novel alternative splice isoform and the β A4 amyloid precursor protein (APP) mRNA in leukocytes and brain microglial cells. *J Biol Chem* 1992;267:10804–10809.
- König G, Salbaum JM, Wiestler O, Lang W, Schmitt HP, Masters CL, Beyreuther K: Alternative splicing of the β A4 amyloid gene of Alzheimer's disease in cortex of control and Alzheimer's disease patients. *Mol Brain Res* 1991; 9:259–262.
- Koo EH, Sisodia SS, Cork LC, Unterbeck A, Bayne RM, Price DL: Differential expression of amyloid precursor protein mRNA's in cases of Alzheimer's disease and in aged nonhuman primates. *Neuron* 1990;2:97–104.
- LeBlanc AC, Chen HY, Autilio-Gambetti L, Gambetti P: Differential APP gene expression in rat cerebral cortex, meninges, and primary astroglial, microglial and neuronal cultures. *FEBS* 1991;292:171–178.
- Löffler J, Huber G: β -Amyloid precursor protein isoforms in various rat brain regions and during brain development. *J Neurochem* 1992; 59(4):1316–1324.
- Weidemann A, König G, Bunke D, Fischer P, Salbaum JM, Masters CL, Beyreuther K: Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* 1989;57:115–126.
- Suzuki T, Najrn AC, Gandy SE, Greengard P.: Phosphorylation of Alzheimer amyloid precursor by protein kinase C, *Neuroscience* 1992;48: 755–761.
- Sisodia SS, Koo EH, Beyreuther K, Unterbeck A, Price DL: Evidence that β -amyloid protein in Alzheimer's disease is not derived by normal processing. *Science* 1990;248:492–495.
- Esch FS, Keim PS, Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ: Cleavage of amyloid β peptide during constitutive processing of its precursor. *Science* 1990;248:1122–1124.
- Palmert MR, Podlisny MB, Witker DS, Oltersdorf T, Younkin LH, Selkoe DJ, Younkin SG: The β -amyloid protein precursor of Alzheimer disease has soluble derivatives found in human brain and in cerebrospinal fluid. *Proc Natl Acad Sci USA* 1989;86:6338–6342.

- 17 Podlisny MB, Mammen AL, Schlossmacher MG, Palmert MR, Younkin SG, Selkoe DJ: Detection of soluble forms of the β -amyloid precursor protein in human plasma. *Biochem Biophys Res Commun* 1990;167:1094–1101.
- 18 Smith RP, Darryl A, Higuchi GJ, Broze JR: Platelet coagulation factor XIa-inhibitor, a form of Alzheimer amyloid precursor protein. *Science* 1990;248:1126–1128.
- 19 Mönning U, König G, Prior R, Mechler H, Schreiter-Gasser U, Masters CL, Beyreuther K: Synthesis and secretion of Alzheimer amyloid β A4 precursor protein by stimulated human peripheral blood leukocytes. *FEBS* 1990;277:261–266.
- 20 Oltersdorf T, Fritz LC, Schenk DB, Lieberburg I, Johnson-Wood KL, Beattie EC, Ward PJ, Blacher RW, Dovey HF, Sinha S: The secreted form of the Alzheimer's amyloid precursor protein with the Kunitz domain is protease nexin-II. *Nature* 1989;341:144–147.
- 21 van Nostrand WE, Wagner SL, Suzuki M, Choi BH, Farrow JS, Geddes JW, Cotman CW, Cunningham DD: Protease nexin II, a potent antichymotrypsin, shows identity to amyloid β -protein precursor. *Nature* 1989;341:546–549.
- 22 König G, Masters CL, Beyreuther K: Retinoic acid induced differential neuroblastoma cells show increased expression of the β A4 amyloid gene of Alzheimer's disease and an altered splicing pattern. *FEBS Lett* 1990;269:305–310.
- 23 Scott JN, Parhad IM, Clark AW: β -Amyloid precursor protein gene is differentially expressed in axotomized sensory and motor systems. *Mol Brain Res* 1991;10:315–325.
- 24 Milward EA, Papadopoulos R, Fuller SJ, Moir RD, Small D, Beyreuther K, Masters CL: The amyloid protein precursor in Alzheimer's disease is a mediator of the effects of nerve growth factor on nerve outgrowth. *Neuron* 1992;9:129–137.
- 25 Rakic P: Neuron-Glia relationship during granular cell migration in developing cerebellar cortex: A Golgi and electron microscopic study in *Macacus rhesus*. *J Comp Neurol* 1971;141:283–312.
- 26 Trenkner E, Smith D, Segil N: Is cerebellar granule cell migration regulated by an internal clock? *J Neurosci* 1984;4:2850–2855.
- 27 Edmondson JC, Hatten ME: Glial-guided granule neuron migrating in vitro: A high resolution time-lapse video microscopic study. *J Neurosci* 1987;7:1928–1934.
- 28 Hockberger PE, Tseng H-Y, Connor JA: Development of rat cerebellar Purkinje cells: Electrophysiological properties following acute isolation and in long term culture. *J Neurosci* 1989;9:2258–2271.
- 29 Schnitzer J, Schachner M: Expression of Thy-1, H-2, and NS-4 cell surface antigens and tetanus toxin receptors in early postnatal and adult mouse cerebellum. *J Neuroimmunol* 1981;1:429–456.
- 30 Hatten ME: Neuronal regulation of astroglial morphology and proliferation in vivo. *J Cell Biol* 1985;100:384–396.
- 31 Shivers BD, Hilbich C, Multhaup G, Salbaum M, Beyreuther K, Seeburg P: Alzheimer's disease amyloidogenic glycoprotein: Expression pattern in rat brain suggests a role in cell contact. *EMBO J* 1988;7:1365–1370.
- 32 Schubert D, Saitoh T, Cole G: The regulatory role of amyloid β protein precursor secretion and its modulatory role in cell adhesion. *Neuron* 1989;3:689–694.
- 33 Breen KC, Bruce M, Anderton BH: Beta amyloid precursor protein mediates neuronal cell-cell and cell-surface adhesion. *J Neurosci Res* 1991;28:90–100.
- 34 Mönning U, König G, Banati RB, Mechler H, Czech C, Gehrman J, Schreiter-Gasser U, Masters CL, Beyreuther K: Alzheimer β A4-amyloid protein precursor in immunocompetent cells. *J Biol Chem* 1992;267:23950–23956.
- 35 Doherty P, Cohen J, Walsh FS: Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. *Neuron* 1990;5:209–219.
- 36 Hoffman S, Edelman GM: Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc Natl Acad Sci USA* 1983;80:5762–5766.
- 37 Landmesser L, Dahm L, Tang J, Rutishauser U: Polysialic acid as a regulator of intramuscular nerve branching during embryonic development. *Neuron* 1990;4:655–667.
- 38 Card JP, Meade RP, Davis LG: Immunocytochemical localization of the precursor protein for β -amyloid in the rat central nervous system. *Neuron* 1988;1:835–846.
- 39 Schubert M, Prior R, Weidemann A, Dirksen H, Multhaup G, Masters CL, Beyreuther K: Localization of Alzheimer β A4 amyloid precursor protein at central and peripheral synaptic sites. *Brain Res* 1991;563:184–194.
- 40 Palacios G, Palacios JM, Mengod G, Frey P: Beta amyloid precursor protein localization in the Golgi apparatus in neurons and oligodendrocytes: An immunocytochemical, structural and ultrastructural study in normal and axotomized neurons. *Mol Brain Res* 1992;15:195–206.
- 41 Hoffman S, Sorkin BC, White PC, Brackenbury R, Mailhammer R, Rutishauser U, Cunningham B, Edelman GM: Chemical characterization of neural cell adhesion molecule purified from embryonic brain membranes. *J Biol Chem* 1982;257:7720–7729.
- 42 Rothbard JB, Brackenbury R, Cunningham BA, Edelman GM: Difference in the carbohydrate structures of neural cell-adhesion molecules from adult and embryonic chicken brains. *J Biol Chem* 1982;257:11064–11069.
- 43 Edelman GM, Chuong C-M: Embryonic to adult conversion of neuronal cell adhesion molecules in normal and staggerer mice. *Proc Natl Acad Sci USA* 1982;79:7036–7040.