Geneeskundige Stichting Koningin Elisabeth

Fondation Médicale Reine Elisabeth

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F.M.R.E. - G.S.K.E.
3, avenue J.J. Crocq laan
Bruxelles 1020 Brussel
Tel.: +32.2.478.35.56
Fax: +32.2.478.24.13
E-mail: fmre.gske@skynet.be

Geneeskundige Stichting Koningin Elisabeth

2002

Inleiding
Verslag Activiteiten van de GSKE – FMRE

Het jaar 2002, was het eerste van de drie werkingsjaren voor de verschillende onderzoeksploegen die gesteund worden door de Stichting.

Ter gelegenheid van de 75ste verjaardag, heeft de Raad van Beheer een aanmoedigingssignaal willen geven door het totale bedrag van de kredieten te verhogen.

Dit bedrag, vastgelegd door de Raad van Beheer op advies van het Wetenschappelijk Comité bijgestaan door enkele internationale specialisten, is verdeeld over 20 universitaire ploegen die intens neurowetenschappelijk onderzoek verrichten in ons land.

Een bijzonder project heeft de aandacht getrokken van de beheerraad, vooral onze Erevoorzitter H.K.H. Prinses Astrid van België was zeer geinteresseerd. Het project handelt over "de toekomst van de patiënten in een Persisterende Vegetatieve Status".

Door een aantal bijzondere omstandigheden is over deze problematiek in ons land weinig gekend. Daarom heeft de beheerraad voorgesteld om de nationale enquête te steunen en de concrete voorstellen die eruit voortvloeien over te maken aan de bevoegde instanties. Dit werk werd uitgevoerd in samenwerking met het ministerie van Sociale Zaken, Volksgezondheid en Leefmilieu, die bijzonder geinteresseerd is in dit project. De resultaten zullen verspreid worden begin 2003.

Het is altijd een heerlijk moment om in het begin van het jaar het wetenschappelijk activiteitenrapport van de Stichting te kunnen voorstellen.

Een verslag waarop we zeer fier zijn gezien de kwaliteit van het uitgevoerde onderzoek in ons land dat gesteund wordt door de Stichting.

Het is eveneens een vorm van erkenning naar de beheerraad toe, voor zijn begrip, zijn steun en zijn keuze, om het thema van de neurowetenschappen te blijven behouden als het handelsmerk van de Geneeskundige Stichting Koningin Elisabeth.

Prof. Dr. Th. de Barsy

Brussel, januari 2003

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Introduction
Rapport d'Activités de la FMRE - GSKE

L'année 2002 a été la première année de mise en place des programmes de 3 ans pour les différentes équipes de recherche qui bénéficient d'un soutien de la Fondation.

A l'occasion du 75ième anniversaire, le Conseil d'Administration a voulu donner un encouragement supplémentaire en augmentant le montant total des crédits. Vingt équipes universitaires, témoins d'une activité scientifique intense dan le domaine des neuro-sciences dans notre pays, se partagent le montant décidé par le Conseil, sur l'avis du Comité Scientifique élargi aux experts internationaux.

Un projet particulier a retenu toute l'attention du Conseil d'Administration et, plus précisément, celle de notre présidente d'honneur, S.A.R. la Princesse Astrid du Belgique, à savoir "le devenir de patients présentant un Etat Végétatif Persistant".

Cette problématique très ponctuelle était mal connue dans notre pays et suite à une série de circonstances particulières, le Conseil a proposé de soutenir une enquête nationale et de présenter les propositions concrètes aux instances officielles. Ce travail a été fait en collaboration avec le Ministère des Affaires Sociales, de la Santé Publique et de l'Environnement qui a marqué un vif intérêt pour le projet. Les résultats seront largement diffusés au début de l'année 2003.

C'est toujours un moment très agréable de pouvoir, en début d'année, présenter le rapport de l'activité scientifique de la Fondation, source de fierté vu la qualité de la recherche effectuée dans notre pays et soutenue par la Fondation.

C'est également un moment de reconnaissance vis-à-vis du Conseil d'Administration pour sa compréhension, son soutien et son choix de continuer à maintenir le thème de neuro-sciences comme image de marque de la Fondation Médicale Reine Elisabeth.

Prof. Dr. Th. de Barsy

Bruxelles, janvier 2003

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Annual Report of the Research Group of

Prof. Dr. Ampe C.

Universiteit Gent
(U.Gent)

Prof. Dr. Christophe Ampe,

Department of Biochemistry,
Faculty of Medicine and Health Sciences,
Ghent University.
A. Baertsoenkaai 35
9000 Gent
Tel.: 09/331 3336
Fax: 09/331 3595
christophe.ampe@rug.ac.be

Researchers involved

Dr. Marleen Van Troys (Post Doctoral Fellow FWO)
Dr. Anja Lambrechts (Post Doctoral Fellow FWO)
Lic. Stefaan Rossenu (Ph D student, AAP-Ghent University) (part of the year)
Lic. Debby Polet (Ph D student, GSKE)
Lic. Shirley Leyman (Ph D student, IWT)
Lic. Sylvie Veniere (Ph D student, IWT) (part of the year)
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Mevr. Daisy Dewitte (Technician)

Collaborations with

Prof. Dr Ono Shoichiro (Emory University, Atlanta, USA)
Prof. Dr Reinhard Fässler (MPI, Martinsried, Germany)
Prof. Dr Frank Gertler (MIT, Cambrige, USA)

Role and regulation of actin binding proteins of the profilin, cofilin, β-thymosin and Ena/VASP families in actin filament dynamics during neuronal outgrowth.

Context and aim

Actin based cell motility is essential for cell migration. During development, neural cells extend processes that are guided to their destination by short and longe range repulsive or attractive guidance cues (Mueller, 1999). Formation of these processes is critically dependent on dynamic turn over of actin filaments and on transient formation of adhesive structures resembling focal contacts. Since the actin system is located in the periphery of the cell and in the filopodia of the growth cones, it is thought that the microfilament is the machinery that receives the transduced guidance information (reviewed by Suter and Forscher, 1998). An emerging picture from the last years is that the balance of actin polymerization/depolymerization is important (neither too little is good, nor too much). This balance is dictated by the activities of several actin binding proteins. The actin binding proteins studied here : **Ena/VASP-proteins, profilins, cofilins and thymosin β-members**, each modulate a different point of the actin polymerization cycle (Ampe and Vandekerckhove, 1999). This also enables cells to regulate distinct steps of the cycle differentially. It is also evident that the various actin binding proteins act in concert (Carlier 1998, see also below) necessitating to study combined effects of actin binding proteins. For instance, relevant to this project, we previously demonstrated that EVL and profilins, both actin binding proteins are also partner proteins. The simultaneous up-regulation of EVL and profilin IIa expression in brain in mouse embryos (Lanier et al., 1999, Lambrechts et al., 2000b) suggests an important role for the interaction of these proteins at this stage of neuronal development. EVL, an Ena/VASP-family member, nucleates actin polymerization in vitro (Lambrechts et al., 2000a) and profilins promote actin filament elongation if free polymerizing filament ends are available (if filaments are capped profilins reduce the length of filaments) (Pantaloni and Carlier, 1993; Lambrechts et al., 2000b). The combined action of Ena/VASP proteins and profilins on actin dynamics and on neurite formation is subject of our research, as well as the effects of cofilin and thymosin β family members. With the exception of the latter family all actin binding proteins are regulated by a variety of signal transduction mechanisms. For this project we focus on the regulation of 1) EVL by Protein kinase A and possibly by interaction with n-Src kinase and FE65, of 2) profilins and cofilins by phosphoinositides, and 3) of cofilin by LIM-kinases or other kinases.

The correct balance of actin polymerization/depolymerization appears critical for correct neuronal outgrowth. We chose actin binding proteins known or suspected from genetic or biochemical studied to be involved in neurite formation and that act at different steps of the actin polymerization cycle. Ena/VASP-proteins and profilins may work synergistically to promote filament formation. Profilins and cofilins are dynamizers of filament turn-over and profilins and mammalian β-thymosins work antagonistically. We investigate the role of these various key actin binding proteins in neurite extensions. Our long term goal is to understand the interplay (and the way it is regulated) of the actin binding proteins during neuronal outgrowth.

Research

The model systems. PC-12 cells (a rat pheochromocytoma cell line) extend neurites, observable with a light microscope, upon stimulation with nerve growth factor (NGF) and forskolin (FS)[1] . We are developing an assay system to measure number and length of neurites formed by a Tet-on PC12 cell line, upon doxycycline-induced expression of a given actin binding proteins. This is based on our initial observation that doxycycline-induced overexpression of thymosin β4, an actin binding protein known to inhibit actin polymerization, results in inhibition of outgrowth of neurites when these cells are NGF and forskolin stimulated (see Figure 1, Rossenu et al., unpublished). As we anticipate that effects will be dosage dependent we take care to monitor the expression levels of the heterologous proteins. We do this either by selecting cells with comparable enhanced Green Fluorescent Protein (eGFP)-expression when cells express actin binding proteins as eGFP-fusion proteins, or by monitoring expression levels by Western blotting when the GFP-moiety interferes with the activity of the protein (as is for instance the case with tagged profilins (Lambrechts et al., 2002). In addition we use NG108-15 cells, which display larger fan shaped growth cones, and primary murine hippocampal neurons. These are mainly used for studying *in vivo* interaction via co-immunoprecipitation and co-immunolocalization of the actin binding proteins (or their partners). Co-immunolocalizations of profilins are, however, momentarily hampered by the very special fixation conditions required for immunostaining of these proteins. We also initiated studies on *Caenorhabditis elegans* as a model organism. These have, so far, focused on the localization of proteins (see below) in order to select interesting proteins for subsequent molecular genetics approaches.

A thymosin β family member from C. elegans has altered actin binding properties. We completed biochemical characterization of the WT tetrathymosin β and of profilin isoforms from C. elegans. Tetrathymosin β belongs to the thymosin β-family (Van Troys et al., 1999) but, in contrast to its mammalian analogue, is not a pure actin sequestering agent. This is due to its four times repeated structure. Each repeat has actin binding capacity but the combination renders new properties to this protein. The protein is present early in embryogenesis and concentrated in the nerve ring later on (see figure 2a, Van Troys et al., in preparation, collaboration with Prof. S. Ono). The *Drosophila* homologue is involved in brain development (Boquet et al., 2000) and the knock-out of the *C. elegans* protein results in a lethal "dumpy " phenotype in early adults (Van Troys et al., in preparation). So far, neuronal staining for this protein in adult worms was experimentally negative, a phenomenon also observed for other neuronal proteins in this model organism. Therefore we will take a different approach and are currently isolating the promotor region of the tetraThymosin β gene to express GFP under the control of this promotor, enabling imaging of expression *in vivo*. In a later stage, mutants defective in a given actin binding activity, can be expressed from this construct and used to monitor rescue of the lethal phenotype.

C. elegans profilin 1 is a classical profilin and expressed in the nerve ring. Based on the genome sequence of *C. elegans* it was predicted that this organism has three profilin like proteins. The similarity between these proteins is rather low. During the past year we cloned

1 cAMP works synergistically with Nerve Growth Factor (NGF) on neurite extension in PC12 (Gunning et al., 1981).

and biochemically characterized these three isoforms and showed that they are *bona fide* profilins (Polet et al. in preparation). We raised antibodies against the three isoforms. One profilin isoform is specifically expressed in body wall muscle and one expresses after morphogenesis and in adults in the pharyngeal canal. Profilin 1 is expressed during entire embryogenesis[2]. Interestingly, it is also abundantly present in the nerve ring in embryos (see figure 2b, Polet et al., unpublished, collaboration with Prof. S. Ono). We faced the same problems for neuron staining in adults as described above for tetraThymosin β. Therefore the promotor region of profilin 1 was isolated. Cloning of potential partner proteins such as Unc 34 (the *C. elegans* homologue of Mena, see below) is ongoing.

Mutants of cofilin and EVL. Time was devoted to construct a number of cofilin and EVL mutants. Cofilin is regulated by PIP_2 and by LIM-kinase. There is a clear indication that LIM-kinase inhibits cofilin activity and this reduces neurite extension (Meberg and Bamburg, 2000). However the effect of PIP_2 was largely ignored in these studies although this phosphoinositide also inhibits cofilin activity. Into a PIP_2-gain of function mutant (Van Troys et al., 2000 and unpublished), mutations mimicking the phospho- and the dephospho-form of cofilin were introduced. Transfection of these constructs and selection of stable cell lines showed that in most cases only small levels of overexpression are tolerated. This may be due to a cell division or adhesion defect. Prior to introducing these mutants into our PC12-assay system this issue needs to be addressed, this is currently ongoing (Leyman et al., unpublished). EVL, like its family members Mena and VASP, contains an N-terminal EVH1-domain, a central proline rich region and a C-terminal EVH2 domain (Gertler et al., 1996). The proline rich region is involved in profilin, SH3-domain and WW-domain binding (Lambrechts et al., 2000a). The EVH2-domain contains a hypothetical actin monomer binding, an actin filament binding region and a dimerization domain. Various EVL EVH2-domain mutants (analogous Mena deletion mutants were obtained via collaboration) aimed at disrupting its actin binding functions were constructed. We now have alanine mutants in the EVH2-domain in each of these regions. The biochemical characterization of these mutants has started. Co-immunoprecipitation studies with GST-fusion constructs of the FE65 WW-domain and the SH3-nSrc domain showed that they may associate with EVL, Mena and VASP in NG108 and PC12 cells. Therefore a second set of mutants aimed at disrupting profilin and or SH3/WW-domain binding will be constructed. The goal here is to dissect the combined effect of EVL (or Mena) and profilin on actin dynamics, and to understand their interplay in neuronal outgrowth (see also research on profilin).

At least one of the functions of mammalian profilin I is required for proper neurite formation. Profilins display several activities: actin binding (promotion or inhibition of actin polymerization depending on the availability of free polymerizing ends), phosphoinositide binding and interaction with proline-rich sequences. Mammals have four profilin isoforms of which two: profilin I and IIa, are expressed in neuronal tissues (Lambrechts et al., 2000b). These two isoforms have similar actin binding properties but have complementary affinities for the other two interaction partners (Lambrechts et al., 1997; 2000b). Genetic evidence implicates a role for profilin I in neuronal outgrowth (Lanier et al., 1999) but isoform IIa is the most abundant form in neurons. Therefore we are addressing the role of both isoforms. We constructed various profilin mutants aimed at disrupting each of the three activities. However our results showed that the

2 RNAi of this profilin isoform is lethal (Fraser et al., 2000), of the other two forms it is not (Polet et al., unpublished).

situation is somewhat more complex because actin and PIP_2 on the one hand, and polyproline and PIP_2 on the other, have overlapping binding sites in profilin (Lambrechts et al., 2002). The profilin I mutants R74E (only defective in actin binding) and R136D (defective in PIP_2 and polyproline binding) have been biochemically characterized (Lambrechts et al., 2002). We introduced these or WT profilin I in the PC-12 Tet-on system described above. For work with the mutants care was taken to select stably transfected cell lines expressing similar levels of WT and mutants in order to be able to compare the effects of the mutants on neurite extension. We first probed the effect on NGF/FS stimulated neurite extension after induced expression of WT profilin at three different cellular profilin I concentrations (doxycycline itself has no effect on neurite extension in the parental cell line). Preliminary data indicate that the number of neurites per cell decreases with increasing WT profilin I expression and thus the population of cells shifts to more cells with no or fewer neurites. At first impression this is consistent with the observed in vitro sequestering activity of high profilin concentrations. However, in a next experiment in which we investigated the effect of mutant R74E, which lacks actin binding capability, we noticed that this mutant has a similar effect. By contrast, cells expressing mutant R136D appear to have more cells with more neurites compared to the NGF induced parental PC12-cell line. These results were unexpected but suggest that PIP_2-binding or polyproline interaction is involved in neurite outgrowth and may even be dominant over actin sequestering. Since mutant R136D has reduced affinity for both these ligands, we constructed, based on our experience with profilin IIA (Lambrechts et al., 2002) a profilin I mutant W3A only defective in binding polyproline. Selection of cell lines is now ongoing. These will allow us to probe more specifically the effect of disruption of the profilin-Mena or -EVL interaction. Similar experiments are planned for WT and mutant profilin IIa eventually combined with RNAi of profilin I to probe the effect of isoform IIa alone.

Figure 1: Thymosin β4 fused to enhancedGFP was stably transfected in Tet-on PC12-cells (Clontech). Cells were treated with (+) doxycycline (dox) or left untreated (-). In a control experiment only eGFP was induced to express. After 24 hours cells were treated with nerve growth factor and forskolin and allowed to form neurites during 48 hours. Cells were fixed and stained with phalloidine Texas red (lower panels), a specific probe for filamentous actin. Cells expressing thymosin β4-eGFP (or eGFP) are green (upper panels). Note that cells expressing thymosin β4-eGFP do not form neurites anymore but still form filopodia (lower middle).

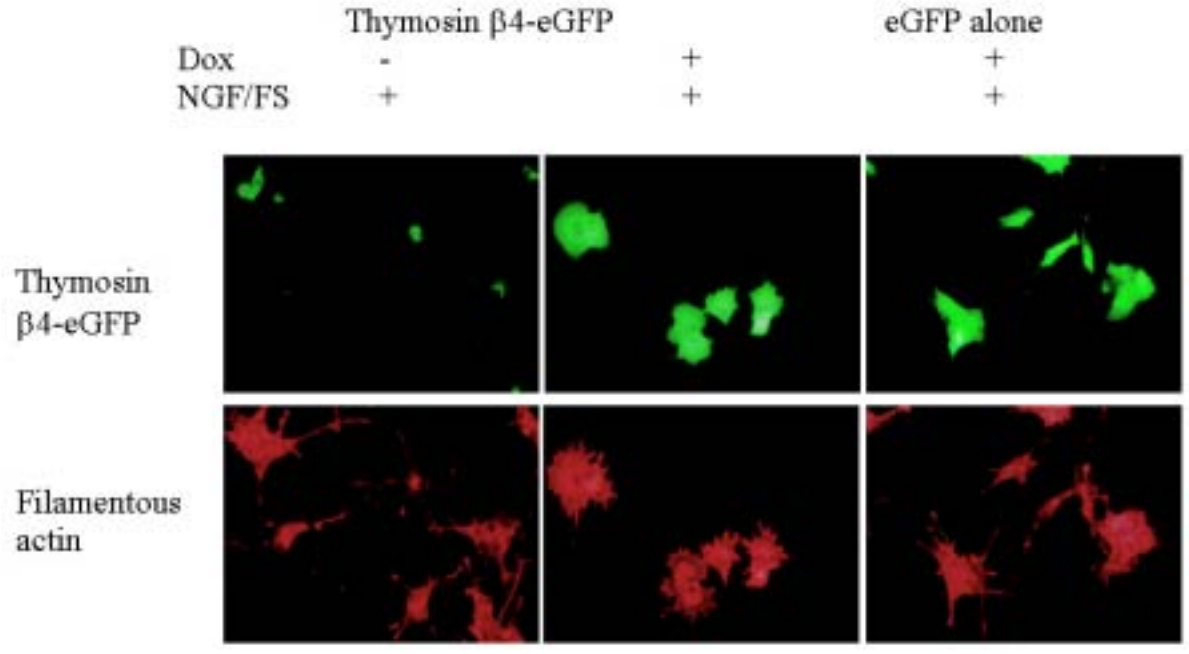
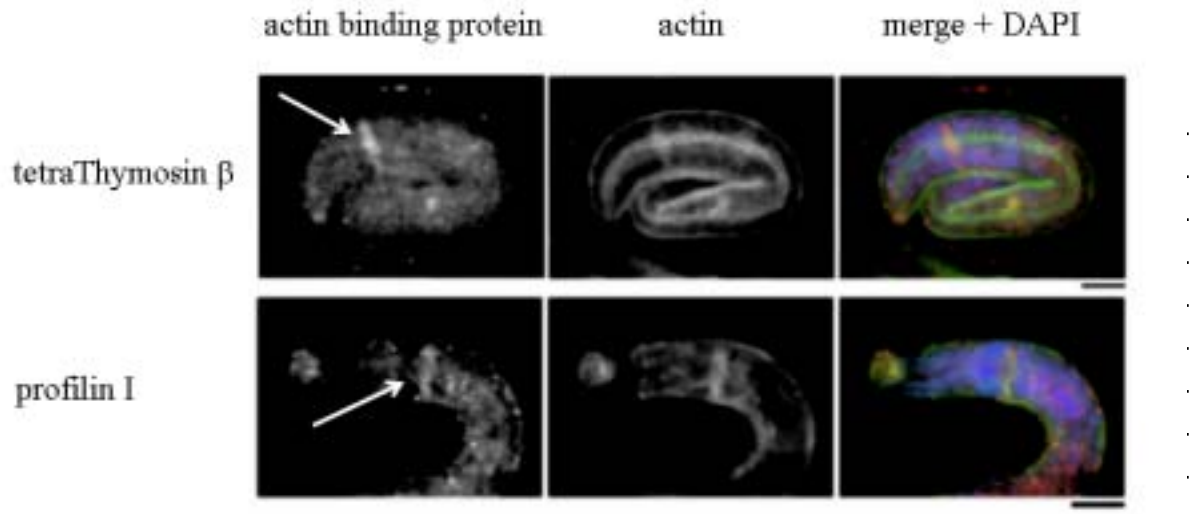


Figure 2: Larvae of C. elegans were stained for tetraThymosin β (upper left), profilin I (lower left) and actin (middle). The brightly stained area (arrow) is the circumpharyngeal nerve ring from where neurons start. In the merged picture is the actin binding protein in red, actin in green and DAPI (a marker for the nucleus) in blue.



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Annual Report of the Research Group of

Prof. Dr. Arckens L.

Katholieke Universiteit Leuven
(K.U.Leuven)

Prof. Dr. Lutgarde Arckens

K.U.Leuven
Faculteit Wetenschappen
Departement Biologie, Afdeling Dierkunde
Laboratorium voor Neuroëndocrinologie en Immunologische Biotechnologie
Naamsestraat 59
3000 Leuven
tel: +32 16 32 39 26

Members of the research group:
Stefan Clerens, Lieselotte Cnops, Sandy Jacobs, Ann Massie, Katrien Van Damme, Gert Van den Bergh, Estel Van der Gucht

In collaboration with
Erik Vandenbussche (1), Ulf T.Eysel (2)

(1) Laboratory of Neuropsychology, Medical School, K.U.Leuven
(2) Laboratory of Neurophysiology, Medical School, Ruhr Universität Bochum

INVESTIGATION OF THE MOLECULAR MECHANISMS UNDERLYING REORGANIZATION OF CORTICAL TOPOGRAPHY AFTER LIMITED SENSORY DEAFFERENTATION

Extracellular GABA concentrations in area 17 of cat visual cortex during topographic map reorganization following binocular central retinal lesioning.

γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system of mammals, plays an important role in cortical reorganization following sensory deprivation, by regulating the level of cortical inhibition and gating changes in receptive field size and synaptic efficacy. In cats it has been shown that two weeks after the induction of binocular retinal lesions, GABAergic inhibition, as determined by immunocytochemistry, is decreased in the deafferented region of area 17, whereas three months post-lesion, normal GABAergic control is restored within the cortical scotoma. In this study we used in vivo microdialysis to investigate the extracellular GABA concentrations one to two months post-lesion, in the sensory-deprived and remote, non-deprived region of area 17. Data were collected at those sample times and sites for which the extracellular glutamate concentrations had been determined in a previous investigation to elucidate the role of this excitatory neurotransmitter in cortical reorganization. As for glutamate, we observed significantly increased extracellular GABA concentrations in remote, non-deprived area 17, whereas in the deafferented area 17, extracellular GABA concentrations were comparable to those observed in normal control subjects. These data suggest that one to two months post-lesion the deafferented cortex behaves like normal visual cortex, in contrast to the remote, non-deprived cortex. Notwithstanding the increase in extracellular GABA concentration with 134%, the parallel increase in glutamate concentration with 269% could give rise to a net increase in excitability in remote area 17. We therefore suggest that LTP-like mechanisms, and thereby cortical reorganization, might still be facilitated, while possible excessive hyperexcitability is balanced by the moderately increased GABAergic control.

The critical period for visual cortex plasticity in cats: identification of age-dependent proteins using fluorescent 2D difference gel electrophoresis and mass spectrometry.

Although the mammalian brain remains capable to adapt to changes in the sensory input throughout the entire animals life, there is a marked difference in this capability between young and adult animals. Young cats within a critical period respond to these input changes by modifying their cortical connections, while in adult animals this cortical plasticity is greatly reduced. The molecular basis of this age-dependent difference in modifiability of the visual cortex between kittens and adult cats is, until now, not known in great detail.

In an attempt to unravel the proteins involved in this age-dependent cortical plasticity, we compared the protein expression levels of visual area 17 of 30-day old kittens and adult cats, using two-dimensional difference gel electrophoresis (2D-DIGE), combining a recently developed fluorescent pre-labeling technique for the quantitative analysis of proteins on two-dimensional electrophoresis gels, with mass spectrometry for protein identification. This let us to identify 32 proteins showing differential expression levels, of which 18 were more

abundantly expressed in kitten striate cortex and 14 were more abundant in adult cats.

Next to a number of metabolic enzymes, we isolated several proteins related to axon growth and growth cone guidance (collapsin response mediator proteins, CRMPs) and to the formation of new cytoskeletal filaments (cofilin, T-complex proteins 1 alpha and zeta) in kittens, probably making the rapid outgrowth of new connections possible after sensory changes. In adult cats, the expression level of glial fibrillary acidic protein (GFAP) was raised in comparison to kittens, an observation which has already been implicated in the termination of the critical period in kittens in earlier studies (Müller C.M., 1990, Glia 3,487-494).

J. Neurochemistry, Van den Bergh et al., in press.

Low-abundance age-dependent proteins in cat visual cortex: identification using high-performance liquid chromatography, 2D difference gel electrophoresis and mass spectrometry

Two-dimensional difference gel electrophoresis (2D-DIGE), in combination with mass spectrometry, is a highly effective method for the rapid and reproducible detection of differentially expressed proteins. This approach, however, has the unfortunate drawback that it preferentially displays rather abundantly expressed proteins. Nevertheless, comparison of the protein expression levels of the primary visual cortex of adult cats and 30-day-old kittens, resulted in the identification of several proteins related to postnatal brain development and possibly age-dependent plasticity as well. The goal of the present study was the selective enrichment and identification of low-abundance proteins within the same paradigm. Hereto, we performed a reversed-phase chromatography pre-fractionation of our tissue lysate to separate the proteins in four fractions based on their hydrophobicity prior to 2D-DIGE analysis. This approach not only confirmed the differential expression levels of a number of proteins from the previous study, but also identified 3 additional proteins preferentially expressed in kitten visual cortex and 5 additional proteins with higher expression levels in adult cat visual cortex. These spots were not visible on the total tissue lysate protein maps, thus representing proteins of lower abundance.

Characterization of development- and plasticity-related genes in cat visual cortex

As described above the 2D-DIGE - mass spectrometry approach identified fourty proteins with an age-dependent expression in cat primary visual cortex. Twenty-one proteins were found to be more abundant expressed in kitten visual cortex (P30), whereas in adult cats nineteen differentially expressed proteins were identified.

The aim of this study was to investigate the distribution and function of these proteins with an age-dependent expression that are possibly involved in developmental or even adult brain plasticity. We have focused our attention towards CRMPs (Collapsin Response Mediator Proteins), important for the growth cone collapse, synaptotagmin-1, a calcium-binding synaptic vesicle protein regulating neurotransmitter release at the synapse, and dynamin-1, a GTP-binding protein involved in endocytosis and vesicle recycling in nerve terminals. As a fast

screening method, in situ hybridization with cat-specific oligonucleotides was applied in order to determine the overall distribution of the mRNA's coding for CRMP-1to -5, synaptotagmin-1 and dynamin-1 throughout the brain, with special emphasis on the visual system. The first results showed that CRMP-1,-4,-5 and synaptotagmin-1 mRNA were more expressed in kitten visual cortex (P30) and that dynamin-1 and CRMP-2 mRNA levels were higher in adult, in agreement with the results obtained by 2D-DIGE. To quantify the protein expression differences between kitten (P30) and adult cat visual cortex or the age-dependent expression of proteins in kittens of different ages (P10, P30, P120), we chose a Western blotting approach with commercial antibodies. The specificity of the antibodies has been established in 2D western blots. In all instances the antibodies recognized the relevant CRMP protein spots. 1D western blot experiments will allow determining the expression levels of each protein in function of age and should therefore allow investigating the involvement of these proteins in brain plasticity. A high expression of a protein during the critical period could mean that the protein is important for the development of the neuronal network in this postnatal period in which the brain is highly susceptible to changes in visual stimuli. In a next step we will analyze the visual cortex of monocular and binocular deprived kittens to learn more about the function of developmentally regulated proteins. For proteins abundant in adult brain, we will investigate the visual cortex of isolated hemisphere cats and adult cats with small retinal lesions to elucidate a possible role in adult brain plasticity.

Molecular cloning and differential expression of the cat immediate early gene *c-fos*

Recently, the effect of binocular central retinal lesions on the expression of immediate early genes in the visual system of adult cats was demonstrated using in situ hybridization and immunocytochemistry (Arckens et al., 2000). The present study was undertaken to quantify cat *c-fos* mRNA expression differences in the cat primary visual cortex after sensory deafferentation. Prior to quantification, DNA fragments obtained using reverse transcription-polymerase chain reaction (RT-PCR) in combination with rapid amplification of complementary DNA ends (RACE) were cloned and sequenced. This provided us with the necessary sequence information to prepare cat-specific *c-fos* primers for the development of a new quantitative RT-PCR assay. We optimized a reverse transcription-competitive polymerase chain reaction (RT-cPCR) method with a heterologous DNA fragment (competitor) as external standard to quantify relative amounts of cat *c-fos* mRNA expression levels. Internal standardization was accomplished by quantifying, in a parallel RT-cPCR, a well-characterized housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This cat-specific RT-cPCR assay allowed us to measure *c-fos* mRNA expression levels in central and peripheral regions of primary visual cortex in normal and retinal lesion cats.

Molecular Brain Research, Van der Gucht et al., in press.

Differential expression of *c-fos* in subtypes of GABAergic cells following sensory stimulation in the cat primary visual cortex

Recent immunocytochemical stainings on cat visual cortex, visually stimulated for one hour, showed strongly induced Fos expression in cortical neurons. We initiated immunocytochemical double staining experiments with different cytochemical markers to unravel the neurochemical and morphological character of these activated neurons showing Fos induction after sensory stimulation. Double stainings between Fos and glutamic acid decarboxylase (GAD) demonstrated the presence of Fos in the nuclei of GABAergic neurons of the primary visual cortex. To further subdivide this Fos/GABAergic cell population we investigated whether Fos co-localized with parvalbumin, calbindin or calretinin. Co-localization of Fos with these calcium-binding proteins delineated distinct neuronal subclasses of Fos-immunoreactive neurons in supra- and infragranular layers of cat area 17. Quantitative analysis of the proportion of immunoreactive local-circuit neurons revealed that 35% of the GABAergic neurons showed Fos induction in supragranular layers, whereas in infragranular layers merely 10% of the GABAergic cells revealed Fos expression. Fos co-existed in about 24% of the calbindin-immunopositive cells within supra-and infragranular layers, but only a minority of the parvalbumin and the calretinin neuronal subgroups were immunopositive for Fos in the corresponding layers of area 17. These findings suggest that visual stimulation induces Fos expression in distinct subsets of inhibitory neurons in cat primary visual cortex.

European Journal of Neuroscience, Van der Gucht et al., 16:1620-1626, 2002

Neurochemical organization of the cat lateral geniculate nucleus complex and zonal parcellation of the ventral lateral geniculate nucleus: A Fos immunocytochemical study.

Using a cat specific Fos antibody, we examined the expression profile of the immediate early gene c-fos in thalamic structures receiving direct or indirect trans-synaptic retinal input within the cat lateral geniculate nucleus (LGN). Visual stimulation succeeding a period of darkness induced considerable Fos expression in the dorsal and ventral part of the LGN (dLGN and vLGN), the perigeniculate nucleus (PGN) and the nucleus reticularis thalami (RT). In particular, Fos immunoreactivity was present in the nuclei of geniculate cells in both A laminae and all C laminae of the dLGN. Interestingly, a characteristic Fos expression profile was apparent for the vLGN. Fos is therefore considered to be an efficient anatomical marker allowing the parcellation of the cat vLGN in a medial and lateral subdivision. Double immunocytochemical stainings between Fos and seven cellular markers, i.e. GAD (glutamic acid decarboxylase), three calcium-binding proteins (Calbindin, Parvalbumin and Calretinin), Enkephalin, Neuropeptide Y and Neurofilament protein (SMI-32), revealed the neurochemical character of distinct subpopulations of visually responsive neurons expressing Fos in different subcortical compartments of the LGN. This immunocytochemical study also revealed distinguished neurochemical reactivity for each of the cellular markers within the medial and lateral zone of the vLGN. These findings extend and confirm that the medial zone of the cat vLGN might be homologues to the rodent intergeniculate leaflet (IGL).

Distribution and Morphological Characterization of Phosphate-Activated Glutaminase-Immunoreactive Neurons in Cat Visual Cortex

Phosphate-activated glutaminase (PAG) is the major enzyme involved in the synthesis of the excitatory neurotransmitter glutamate in cortical neurons of the mammalian cerebral cortex. In this study, the distribution and morphology of glutamatergic neurons in cat visual cortex was monitored through immunocytochemical stainings for PAG. We first determined the specificity of the anti-rat brain PAG antibody, raised in rabbits, for cat brain PAG. We then examined the laminar expression profile and the phenotype of PAG-immunopositive neurons in area 17 and 18 of the cat visual cortex. Neuronal cell bodies with moderate to intense PAG immunoreactivity were distributed throughout cortical layers II to VI and near the border with the white matter of both visual areas. The largest and most intensely labelled cells were mainly restricted to cortical layers III and V. Careful examination of the typology of PAG-immunoreactive cells based on the size and shape of the cell body, together with the pattern of the immunoreactive dendritic processes, indicated that the vast majority of these cells were pyramidal neurons. However, PAG immunoreactivity was also observed in a paucity of non-pyramidal neurons in cortical layers IV and VI of both visual areas 17 and 18. We therefore witnessed PAG as a neurochemical marker allowing the identification of the cortical neurons that use the excitatory amino acid glutamate as their neurotransmitter in cat visual cortex.

Distribution of the AMPA2 glutamate receptor subunit in adult cat visual cortex

In this study, we revealed the distribution of the AMPA2 glutamate receptor subunit (AMPA2) in the visual cortical areas 17 and 18 of the adult cat by means of different techniques. *In situ* hybridization, using a cat-specific radio-active labeled oligo-nucleotide probe, showed that AMPA2-mRNA was expressed mainly in cortical layers II/III and V/VI with a lower expression in layer IV and practically no signal in layer I. Immunocytochemistry, using a polyclonal AMPA2-subunit specific antibody, showed almost exclusively immunoreactivity in the somata and dendrites of pyramidal neurons in cortical layers II/III and V/VI. Only a very faint signal was detected in layer IV. Neurons with little or no AMPA2 have AMPA receptors that are highly permeable to calcium. By determining the location of AMPA2, this study provides a clear examination of the distribution of Ca^{2+}-impermeable AMPA receptors in cat visual cortex. The functional implication of the absence of AMPA2 in cortical layer IV and thus the presence of Ca^{2+}-permeable AMPA receptors in this layer, is still speculative and has yet to be elucidated.

Brain Res., Van Damme et al., 960:1-8, 2003

Laminar and cellular distribution of three NMDA receptor subunits (NR1, NR2A and NR2B) in adult cat visual cortex

We examined the mRNA and protein distribution profile of three N-methyl-D-aspartate (NMDA) receptor subunits, NR1, NR2A and NR2B, in area 17 and 18 of adult cat visual cortex. *In situ* hybridization revealed NR1 and NR2B mRNA expression mainly in the supra- and infragranular layers, while for NR2A the highest mRNA levels were detected in cortical layers II/III, IV and VI. Immunocytochemistry with NR subunit-specific antibodies demonstrated a characteristic

laminar and cellular distribution pattern for each of the three NMDA receptor subunit proteins. Moreover, the laminar expression profile of all three NR subunit proteins coincides with their mRNA expression in the corresponding layers of visual areas 17 and 18. Immunoreactivity for the NR1 and NR2B subunits was found predominantly in the somata and dendrites of pyramidal neurons in the supra- and infragranular layers and in cell bodies and dendrites of small non-pyramidal neurons in layer IV. In contrast, NR2A immunoreactvity was restricted to the cell bodies of pyramidal neurons in cortical layers II/III and VI and to small non-pyramidal neurons mainly located in deep layer IV. Our study demonstrates that each NMDA receptor subunit, NR1, NR2A and NR2B, has a specific but widespread distribution in adult cat visual cortex, consistent with the important role for NMDA receptors in a variety of neuronal cortical functions.

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Annual Report of the Research Group of

Prof. Dr. Bellefroid E.

Université Libre de Bruxelles
(U.L.B.)

Eric Bellefroid

ULB-IBMM
Laboratoire d'Embryologie Moléculaire
Rue des Profs. Jeener et Brachet 12
6041 Gosselies
Belgium
Tel.: ++ 32 2 650 97 32
Fax: ++ 32 2 650 97 33
e-mail: ebellefr@ulb.ac.be

Notch signaling during vertebrate early neural development.

1 Characterization of MyT1 in Drosophila and mouse

Previously, we showed that the Xenopus Myt1 gene coding for a C2HC-type zinc finger protein is highly conserved during evolution. It is specifically expressed in neuronal precursor cells and is positively regulated by bHLH proneural factors such as X-NGNR-1 and negatively regulated by the Notch/Delta signal transduction pathway. We also showed that when overexpressed in embryos, X-MyT1 synergizes with proneural factors to promote neuronal differentiation, apparently by making cells insensitive to lateral inhibition (Bellefroid et al., 1996; Perron et al., 1999).

Recently, we found in the Drosophila genome a sequence that is predicted to encode a C2HC-type zinc finger protein with similarity to the vertebrate MyT1 genes. No corresponding EST sequence is available. A project has been initiated in order to verify that the predicted transcript and protein exist in Drosophila and to determine whether the dMyT1 protein has a function similar to that found in Xenopus. In collaboration with F. Agnes (Université Paris Sud), we have started to study the expression of the dMyT1 gene during embryonic development by whole-mount in situ hybridization. In stage 13 to 16 embryos, dMyT1 staining is found in the developing nervous system in post-mitotic neurons. It thus appears that the MyT1 expression profile observed in Drosophila is similar to that described in vertebrate. Further in situ hybridation experiments are ongoing in order to precise the d-MyT1 expression profile during neurogenesis. We are currently isolating by RT-PCR a dMyT1 cDNA to study the function and biochemical properties of the corresponding protein. In particular, the DNA-binding properties of the dMyT1 protein, its ability to function as a transcriptional activator and its role in neuronal differentiation will be investigated in both drosophila and Xenopus embryos.

A family of three genes encoding MyT1 type C2-H2 zinc finger transcription factors exist in the mouse genome. Two of them, NZF2/MyT1-l and NZF3 are, like in Xenopus, expressed in the entire nervous system in newly-postmitotic neurons, suggesting that they function as a panneural transcription factors associated with neuronal differentiation (Weiner et al., 1997). To study the function of these genes and demonstrate their requirement for neuronal differentiation, we decided to create mouse cell lines deficient for these genes. By screening a mouse BAC library, we isolated genomic clones containing NZF2 and NZF3 that are currently used to construct the targeting vectors.

2 Regulation by Notch of XNAP gene expression

We have shown that the Xenopus XNAP gene that encodes an evolutionarily conserved protein containing two repeated ankyrin motifs is strongly upregulated in response to Notch activation and that when overexpressed in embryos, it reduces the activation of Notch target genes and as a consequence increases the number of primary neurons that forms within the domains of the neural plate where neurogenesis normally occurs (Lamar et al., 2001; Lahaye et al., 2002). Based on these observations, and the finding by others that XNAP can directly interact and forms a ternary complex with Notch ICD and Su(H), we concluded that XNAP constitutes a novel component of the Notch pathway and that it may in a feed-back loop modulate its activity.

To determine whether the XNAP gene is a direct target of Notch and to study the mechanisms of its transcriptional activation, we isolated a mouse XNAP genomic clone and determined its transcription start site. We created a luciferase reporter construct containing 4kb of the XNAP gene upstream regions. This construct was transfected in 293T cells with or without an expression vector for Notch ICD. As positive and negative controls, we used a wild-type and Su(H) mutated HES1 promoter which is a well known effector of Notch. We could show that the XNAP reporter construct is strongly activated by Notch ICD and are currently testing shorter fragments to localize the sequences required for Notch ICD activation. The implication of the Su(H) transcription factor in the induction of XNAP will be tested by analysing the effect of overexpressing activated or dominant negative forms of Su(H) on the luciferase activity of the XNAP reporter construct and by measuring its expression in Su(H) deficient cell lines.

3 DNA binding and transcriptional properties of XHRT1

The Hairy-related transcription factor (HRT) genes encode a subfamily of basic helix-loop-helix (bHLH) transcription factors that show sequence similarity to the Hairy and Enhancer of split (H/E(spl)) genes. Compared to the other H/E(spl) proteins, these HRT proteins are characterized by an invariant glycine instead of a proline residue in the basic region and by the substitution of the WRPW carboxy-terminal motif by a YxxW motif, followed by the TE(I/V)GAF sequence (Davis and Turner, 2001). Together with the group of D. Christophe (ULB), we have recently isolated one member of this HRT gene family, XHRT1. This gene marks in early embryos floor plate and hypochord precursor cells and its expression in ectodermal cells is induced by Notch signaling (Pichon et al., 2002).

In collaboration with the group of D. Christophe, we analysed its DNA-binding and transcriptional activity. We found that, compared to HES1, XHRT1 binds in vitro to the different E-box motifs but with distinct affinities and identified the sequence "ggCACGTGcc" as the XHRT1 highest affinity binding site. In transiently transfected 3T3 cells, XHRT1 inhibits the expression of a luciferase reporter gene under the control of a promoter containing multimerized XHRT1 consensus binding sites and in embryos, inhibits the expression of chordin, a notochord marker. The C-terminal YRPW motif is not involved in this repression and cannot interact with the Groucho/TLE corepressor proteins. Deletion analysis of XHRT1 indicated that the repression of transcriptional activity as well as the inhibition of axial mesoderm formation require the Orange domain and the intermediate region between the Orange and YRPW C-terminal motif. In a yeast two hybrid screen, we identified a novel protein, OIP (Orange interacting protein), that associates with the orange domain of XHRT1. Overexpressed OIP protein is found distributed uniformely within the cell and coexpression of XHRT1 caused accumulation of OIP in the nucleus. The interaction with OIP appears functional as cotransfection of OIP increases XHRT1 transcriptional repression activity in a dose dependent manner. These results strongly suggest that OIP plays an important role in modulation of the XHRT1 activity.

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Annual Report of the Research Group of

Prof. Dr. De Schutter E.

Universitaire Instelling Antwerpen (U.I.A.)

Prof. Dr. E. De Schutter

In collaboration with:
S.-L. Shin and P. Fagerstedt

Theoretical Neurobiology,
Born-Bunge Foundation,
University of Antwerp
Universiteitsplein 1
B-2610 Antwerpen

Analysis of Purkinje cell firing: relationship between simple spikes and complex spikes

Introduction

Purkinje cells (PCs), the only output neuron in the cerebellar cortex, are innervated by two major afferent systems, the mossy fibers and the climbing fibers (Eccles et al. 1967; Ito 1984). Mossy fibers originate from a variety of brain stem and spinal nuclei and carry a broad range of signals, including proprioception from the periphery, efferent copy of the output of motor cortex areas, and inputs from sensory and motor association areas. The mossy fibers innervate granule cells whose axons synapse onto PCs, giving rise to simple spikes (SSs). Climbing fibers originate solely from the inferior olive, a nucleus that projects only to the cerebellum. Activation of climbing fiber synapses causes a large dendritic calcium spike in Purkinje cells called the complex spike (CS). Although they often also fire in close association with movement, they have been suggested to have an instructive or error-corrective role (Marr 1969; Albus 1971). Thus, these two input systems have no direct interaction in the cerebellar cortex, but they share postsynaptic targets, such as Purkinje, Golgi and basket cells (Hamori and Szentagothai 1966; Schulman and Bloom 1981; Ito 1984), which may permit functional interaction.

Such functional interactions may be represented in the correlation between SS and CS firing of individual PCs. For example, it has been reported that the pattern of SS firing is influenced by preceding CSs (Sato et al. 1992) and that the occurrence of CSs can be predicted by increased SS firing rates 150 ms before a CS (Miall et al. 1998). However, these previous studies concerned the pattern of SS firing over several hundreds milliseconds around a CS and did not permit a study of SS-CS interactions on a shorter time scale.

We found that the correlation between a CS occurrence and changes in SSs firing rate in a 100 ms time window surrounding the CS shows not only the three previously described SS firing patterns after a CS, pure pause, pause facilitation, or pause reduction (Sato et al. 1992), but also a slower SS firing before a CS.

Methods

Surgical preparation

Nineteen male Sprague-Dawley rats (300-400 g, Iffa Credo, Brussels, Belgium) were anesthetized with a mixture of ketamine HCl (75 mg/kg; Ketalar, Parke-Davis, Warner Lambert Manufacturing, Dublin, Ireland) and xylazine HCl (3.9 mg/kg; Rompun, Bayer, Leverkusen, Germany) in normal saline (0.9 % NaCl, Baxter, Lessine, Belgium) by intraperitoneal injection. A craniotomy exposing Crus I and II of left cerebellar hemisphere was performed (see (Vos et al. 1999), for detailed description). Supplemental doses (one-third initial dose) were given intramuscularly to maintain deep anesthesia as evidenced by the lack of a pinch withdrawal reflex and/or lack of whisking. All experimental methods were approved by the University of Antwerp and conformed to the guidelines of the European Commission.

Recordings

Single unit recordings were made in the cerebellar cortex with tungsten microelectrodes (impedance ~10 Mohm, FHC, Bowdoinham, ME). Signals were filtered and amplified (bandpass = 0.4 - 10 kHz; gain = 5000-10000) using a multichannel neuronal acquisition processor (Plexon Inc., Austin, TX). Spike waveforms were first discriminated with a real-time hardware-implemented combined time-voltage window discriminator (Nicolelis and Chapin, 1994). The identification of PCs, and the separation of SSs and CSs, were based on the presence of secondary and/or tertiary spikes in CSs (Fig. 1A). After experiments CSs and SSs were further separated with off-line spike sorting software (Plexon Inc., Austin, TX).

Data analysis

Offline analysis of PC firing patterns was carried out using Matlab 6.0 (The Mathworks, Inc., Natick, MA) and Microsoft Excel (Microsoft, Redmond, WA). PCs were classified into three types (PC type1, PC type2 or PC type3) based on the comparison of SS firing rate in a 50 ms window preceding the last SS before, and following the first SS after, each CS. CSs where other CSs occurred within the time window of SS firing before or after the CS were discarded from the analysis (Fig. 1B). When the mean firing rates of SSs before and after a CS were not significantly different ($p > 0.05$, Student's t-test), a PC was classified as PC type 1 (pure-pause PC). PCs showing significantly increased mean firing rates after a CS were classified as PC type 2 (pause-facilitation PC), while PCs where the mean firing rate after a CS was decreased were classified as PC type 3 (pause-reduction PC; c.f. Sato et al. 1992). In every PC, each CS was classified according to the difference in the number of SSs before and after the CS (Fig 2B). The classification was carried out in thresholds from 0 to 8. If the difference in the number of SSs before and after a CS was equal to or less than the threshold the CSs were classified as CS type 1. If larger, it was classified as CS type 2, and if smaller, as CS type 3.

The first three SS interspike intervals (ISIs) before and after a CS and the ISI between SS-CS and CS-SS in each recorded PC were compared with those from shuffled CS and SS trains. Because the duration of the spike discrimination window in experiments was set to 3 ms, all simulated ISIs shorter than 3ms were excluded from the analysis. To avoid breaking null hypothesis by the pauses after CSs, ISIs between CSs interrupted by SSs, were excluded for simulated SSs, while all ISIs between CSs were used in the simulation. The ISI histograms (ISIH) from recorded and simulated spike trains were compared by the Chi-square test. Significantly different bins, as determined by the analysis of residuals, were used for deciding if distributions from the recordings were shifted to the left (+1), to the right (-1), or not shifted (0), compared to the simulated spike trains (Fig. 3B).

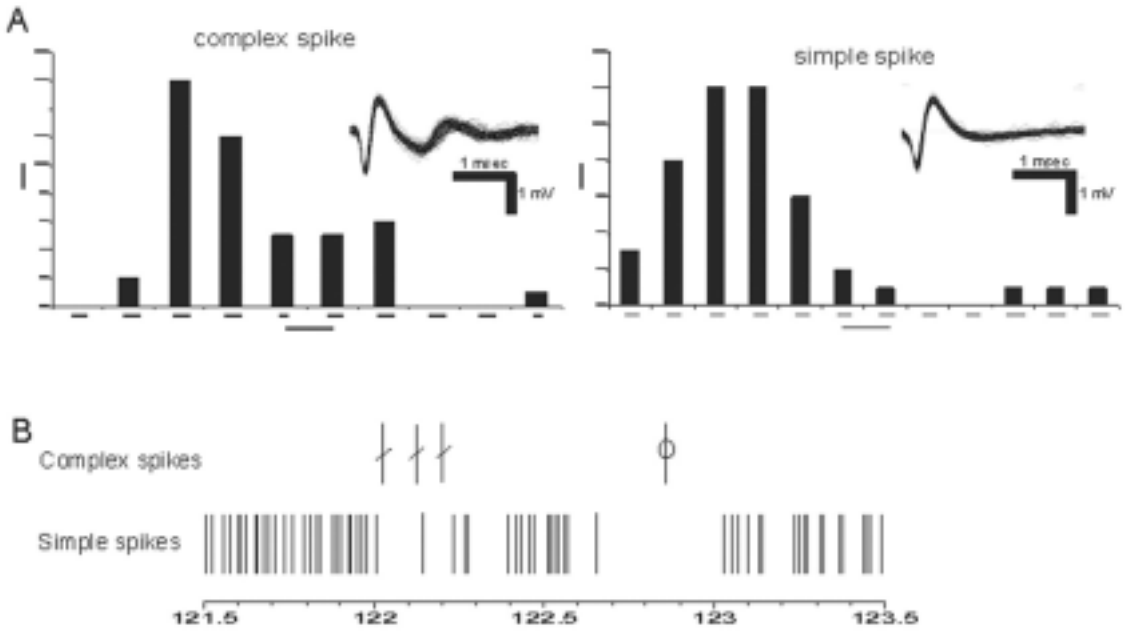
Results

Effect of complex spike on subsequent simple spike firing rate

Based on the presence of simple (SSs) and complex spikes (CSs) in extracellular single unit recordings, 47 cells in 19 experiments were identified as Purkinje cells (PCs). The mean firing rate of SSs was 34.4 ± 22.7 Hz (mean ± SD) and that of CSs was 0.8 ± 0.3 Hz (Fig. 1A). PCs

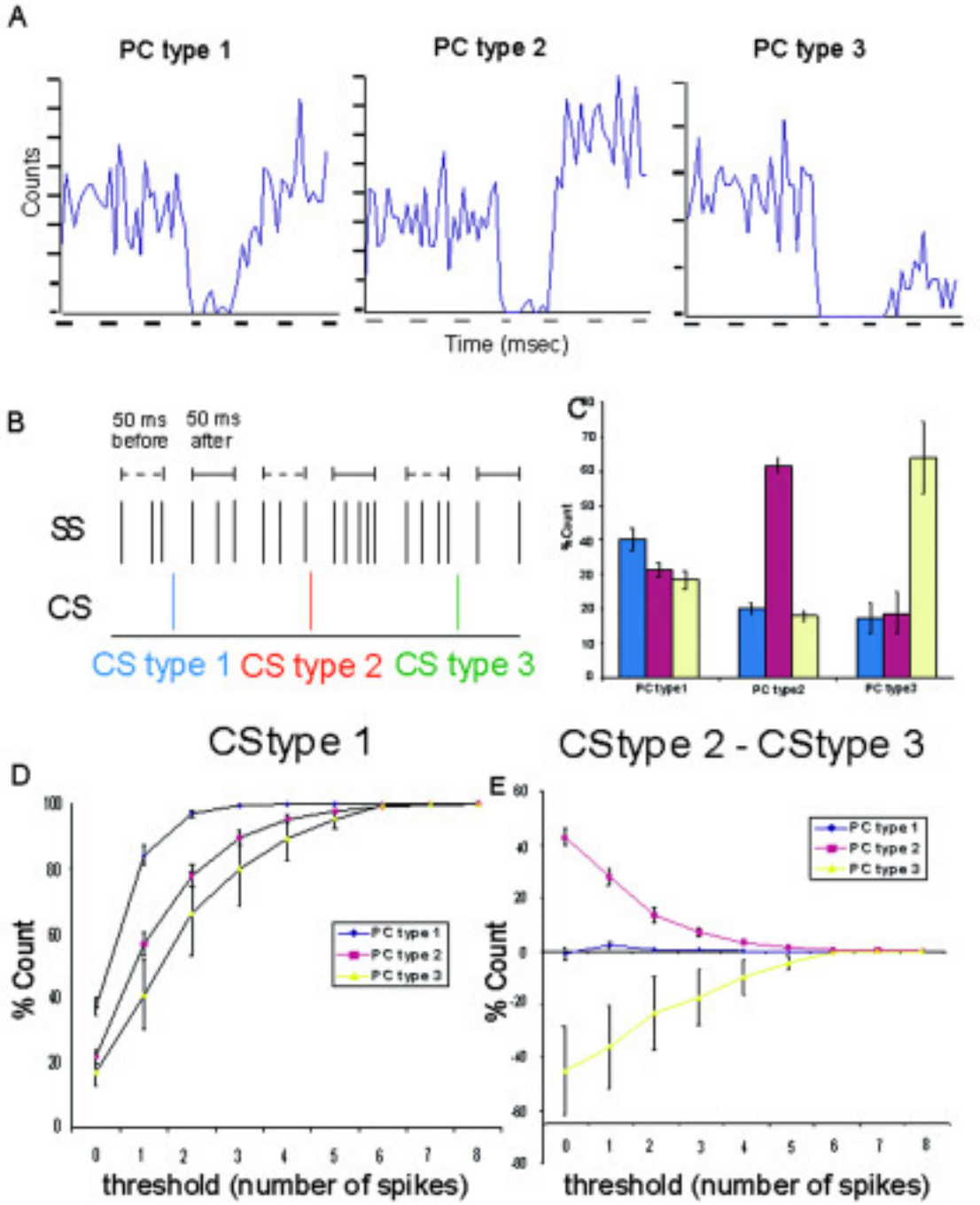
showed spontaneous firing of SSs interspersed with single or multiple CSs (Fig. 1B). Correlation analysis between SS firing rate before and after a CS made it possible to categorize PCs into three types termed PC type 1 (n = 9), PC type 2 (n = 34), and PC type 3 (n = 4) (Fig. 2A). The mean firing rate of SSs in PC type 2 (34.4 ± 19.4 Hz (mean ± SD)) was significantly faster than that of PC type 1 (20.0 ± 11.8 Hz (Student's t-test, $p > 0.05$)). PC type 3 (66.9 ± 36.9 Hz) was faster than PC type 2 and PC type 3, but this was not statistically significant.

Fig 1: Firing rates and firing pattern of simple (SS) and complex (CS) spikes. The firing rates of SSs ranged from 7.06 to 116.7 Hz (A, right) and those of CSs ranged from 0.37 to 1.85 Hz (A, left). The identification of SS and CS was based on the waveform (insets). SSs were interspersed with single or multiple CSs (B). The CSs used in the analysis are marked by a circle and those not used are marked by a slash. CSs were filtered out if multiple CSs appeared between SSs (the first two slashes) or if the ISI between CSs were less than 50 ms (the third slash).



The mean firing rate of CSs in all PC types did not differ significantly (0.8 ± 0.4 Hz in type 1 and 2, and 0.9 ± 0.4 Hz in type 3). The crosscorrelation between SSs that were not interrupted by any CS in 50 msec and a CS could be divided into three groups using the same classification of PCs as previous reported (Sato et al. 1992), but this does not necessarily means that each individual CS in each PC type will always relate to SS firing rate in a same way.

Fig 2: PC types and CS types. PCs were classified into PC type 1 (**A**, left) if there was no difference in firing rate of SSs before and after a CS, into PC type 2 (**A**, middle) if SSs became faster after a CS or into PC type 3 (**A**, right) if SSs became slower after a CS in 50 ms window preceding the last SS before and following the first SS after each CS. In all PC types, CS was classified into CS type 1 if there was no difference in the number of SSs before and after the CS, into CS type 2 if the number of SSs was increased after a CS, or into CS type 3 if decreased in 50 ms window preceding the last SS before and following the first SS after each CS (**B**) by setting different thresholds. When the threshold was 0, PC type 1, PC type 2 and PC type 3 had different composition of CS type 1, CS type 2 and CS type3 (**C**). As the threshold was increased, CS type 1 in PC type 1 reached the 100 % level faster than the other types, PC type 2 and PC type 3 which didn't show significant difference (**D**). But the difference between CS type 2 and CS type 3 was conserved throughout all thresholds (E).

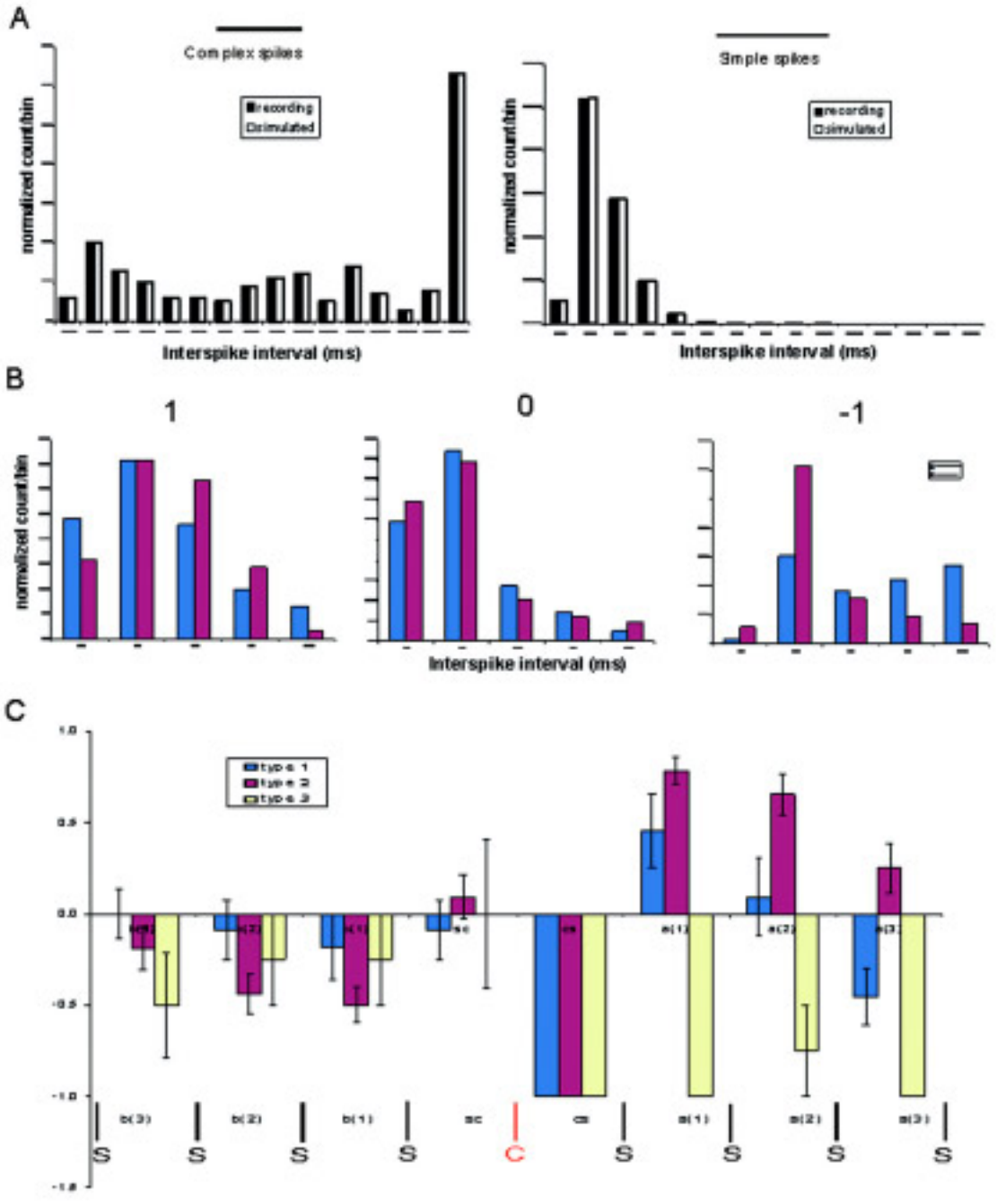


Thus, each CS from each of the PC types were classified into three types, CS type 1, CS type 2, or CS type3 (Fig. 2B). When the threshold was 0, PC type 1 had equivalent number of CSs in all CS types, PC type 2 included significantly more CS type 2, and PC type 3 included more CS type 3 (Fig 2C). As the threshold was increased (see Methods), CS type 1 in PC type 1 reached the 100 % level faster than CSs in the other PC types (Fig. 2D), while the difference between CS type2 and CS type 3 was conserved up to threshold 5 (Fig. 2E). Although in PC types there were all CS types, CS type 2 and CS type 3 in PC type 1 was weaker than those were in PC type 2 and PC type 3.

Interaction between the complex spike and simple spiking before and after

When the mean firing rates of SSs before and after a CS were compared for the classification of PCs, it was not possible to look at the exact pattern of SS around a CS. In a second study of the SS firing, three consecutive ISIs between SSs and pauses before and after a CS in each PC type were measured and compared to a simulated PC spike train constructed from the same recording to look for deviations from random behavior (see Methods). ISI distribution in total spike trains of reconstructed SSs and CSs were similar to that from original data (Fig. 3A). The distribution of three consecutive ISIs between SSs and the pause before and after a CS could show a shift either to left (+1), right (-1), or no shift (0) (Fig 3B). Pauses after a CS were significantly longer than that from simulation in all PC types, but pauses before a CS seemed to be a random. While the ISI after a CS shows the same characteristics for PC type 1, PC type 2 and PC type 3, the ISI before a CS were lengthened compared to the result in simulation (Fig. 3C).

Fig 3. Characteristic of interspike interval (ISI) distributions. Normalized ISI distributions of SSs (**A**, left) and CSs (**A**, right) from simulated data were the same as those from recorded ones. (**B**) Examples of classification. If each of the three consecutive ISIs between SSs and pauses before and after a CS was shifted to left, it was given value, +1 (**B**, left). If there was no difference in distribution (Chi-square test, p 0.05), it was given value, 0 (**B**, middle). If it was shifted to right, it was given value, -1 (**B**, right). The mean SD of the given values of each ISIs in each PC type is shown in **C**. Pauses after a CS were longer than predicted by simulation, and ISIs after a CS showed the characteristic of each PC types well. However, the pause before a CS was not different from prediction but ISIs before a CS were mainly shifted to right which represents slower firing than predicted by simulation.



Discussion

As reported previously (Sato et al. 1992) the SS firing after a CS in PCs showed three different patterns such as pure pause, pause followed by facilitation and pause followed by reduction. The analysis by Sato et al., however, was based on average firing behavior only. When analyzing individual CSs we found that for each type of PC an simple spiking after the pause can show any of the three patterns. In other words, the classification by Sato et al. describes a trend but does not predict SS patterns in single trials.

An unexpected finding of this study is that SS firing before a CS is slower, independent of the pattern after the CS. This could cause misclassification of PCs into PC type 2. In fact, 5 out of 34 PCs were classified as PC type 2 because of the slower firing before a CS even though SSs were not facilitated after a CS. This result is different from that of Miall et al (1998) who found an increased SS firing rate before a CS, but they looked at a different time window (150 ms before CS) compared to our data (on average 100 to 0 ms before CS). The mechanism causing slower SS firing shortly before a CS is unknown at present and requires further study.

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Annual Report of the Research Group of

Prof. Dr. Goffinet A.

Université Catholique de Louvain (U.C.L.)

Andre M. GOFFINET, MD, PhD

Unité de Génétique du Développement
Avenue E. Mounier, 73, Bte 7382
1200 Bruxelles
T: +32-(0)2 764 7386
F: +32-(0)2 764 7430
Email: Andre.Goffinet@gede.ucl.ac.be

Team at UCL:

Nina IGNATOVA (PhD student, FMRE), Yves JOSSIN (PhD student, FRIA), Fadel TISSIR (PhD), Lanrun GUI (Bursary, UCL), Chuan-En WANG (Bursary, UCL)

Collaborations at UCL:

F. Lemaigre (HORM), C. Sindic (Neurology), M. Vikkula (BCHM)

External collaborations:

I. Bar, O. De Backer, A. Kuvbachieva, C. Lambert de Rouvroit (FUNDP, Namur)
D. Blaas (Vienne), J. Herz (Dallas), G. Meyer (La Laguna), J. Nimpf (Vienne), JY Sire (Paris)

Genetic determinants of mouse brain development : The reelin signaling pathway.

1. Reelin and brain development.

1.1 Interactions between reelin and its receptors

In 1999, together with the group of J. Herz (Dallas), we showed that two receptors of the lipoprotein receptor family, namely the very low density lipoprotein receptor VLDLR and apolipoprotein E receptor type 2 (ApoER2) are reelin receptor. In order to define better the region of the Reelin protein that is implicated in receptor binding, parts of the reelin cDNA were constructed and expressed in HEK297 cells. After verification that the proteins are secreted normally and thus presumably well folded, the supernatant from transfected cells is used as a ligand to interact with the extracellular part of VLDLR and ApoER2 produced in fusion with the Fc fragment of human IgG in a pull-down assay. Using this assay, we showed that the N-terminal part of Reelin, up to and including domains one, two, three and four is incapable of interacting with the receptors. On the other hand, a robust pull-down signal is obtained with a construction containing reelin domains 3-6 or domains 3-8. The terminal part of reelin (domains 7 and 8) do not bind. When smaller constructs are used, no binding is detected suggesting low affinity binding to several sites. When reelin is added to target neurons, the Dab1 adaptor is phosphorylated on tyrosine (Y). In order to assess whether reelin binding to VLDLR and ApoER2 is sufficient to explain the actions of reelin or whether engagement of another, thus far unknown receptor is required, we compared the binding of reelin constructs with their ability to generate Dab1 phosphorylation when added to primary neuronal cultures. There is a full correlation between the ability to bind to receptors and to stimulate Dab1 phosphorylation, suggesting that receptor binding is sufficient to activate the Dab1 part of the reelin signalling network. These studies will be part of the PhD thesis of Y. Jossin. Some of these results are in press (Jossin et al., 2002) and the others have been submitted.

1.2. Reelin in human brain development.

In collaboration with G. Meyer (La Laguna), we undertook a study of expression of the Dab1 gene in the developing human brain. Both in situ hybridization and monoclonal antibodies against Dab1 were used. These monoclonal antiDab1 antibodies have been developed by C. Lambert and the production of new antibodies against VLDLR and ApoER2 are currently under way. These studies on human cortical development allowed us to define better the succession of events and to demonstrate that cortical plate cells express Dab1 as expected, but also that Dab1 (mRNA and protein) are quite strongly expressed in precursor cells in the ventricular zone. This has been confirmed by other laboratories in rodents and points to a role of the reelin pathway in the regulation of neural stem cells. Furthermore, Cajal-Retzius cells, the main producers of reelin were shown to express also Dab1, indicating the presence of an autocrine loop. These findings are in press (Meyer et al, In press).

1.3. Production of antibodies against VLDLR and ApoER2

The availability of good antibodies is requisite to cell biological studies of reelin an dits partners. Following the same strategy that allowed us to generate antireelin monoclonal antibodies, we decided to produce monoclonal antibodies against VLDLR and ApoER2 by immunising mutant mice in which the corresponding proteins are defective and that are consequently able to mount an immune response even against highly conserved proteins. Fusion proteins were synthetised in E.Coli using appropriate vector constructs are purified using Nickel affinity chromatography. They were then used as antigens for immunisation, and spleen fusion to hybridoma cells was used to generate monoclonal antibodies. Using this strategy, in collaboration with the laboratories of J. Nimpf and D. Blaas (Vienna) and with C. Lambert (Namur), several antibodies against both proteins have been generated. These reagents yield good results in western blot and immunoprecipitation and some seem to reveal a specific signal in immunohistochemistry. However, immunohistochemical results remain suboptimal and we have decided to pursue production of more monoclonal antireceptor antibodies. Among the antibodies generated, two (one against ApoER2 and one against VLDLR) are able to stimulate Dab1 phosphorylation when used in combination. The production and characterization of these antibodies is part of the thesis of N. Ignatova.

1.4. Studies of the Reelin protein in neurological diseases

Using antibody 142 that reacts with a reelin N-terminal epitope, and another antibody directed against the C-terminal epitope, we have analyzed reelin in the human cerebrospinal fluid in normal subjects at different ages, in a spectrum of neurological diseases and in three cases of schizophrenia. We were able to find the different fragments of reelin resulting from processing, but did not detect any significant modification of the reelin immunoreactivity in any of the disorders examined. This work, carried out by N. Ignatova in collaboration with Ch. Sindic, UCL, is submitted for publication.

2. Reelin and cortical evolution (Tissir et al., 2002d).

For several years, we have proposed that reelin may have played a role during cortical evolution and we have analyzed expression of reelin during cortical development in representatives of all amniote lineages in order to assess this idea further. This series of studies has been completed with the analysis of reelin expression during brain development in the Nile crocodile. This was carried out by F. Tissir in collaboration with J-Y Sire (Paris), G. Meyer and C. Lambert, with help from the "Ferme aux Crocodiles" (Pierrelate, France). We showed that the pattern of reelin expression is comparable to that in chick. In addition, we were able to demonstrate expression of the p73 gene in reelin-positive cells in the MZ. This finding suggests strongly that Cajal-Retzius are evolutionary homologous and is now the basis of a new project in the laboratory aiming at demonstrating this idea further.

3. The Dab1 gene (Bar et al., 2002).

In collaboration with I. Bar and C. Lambert, we completed an extensive study of the Dab1 gene in mouse and man. The Disabled-1 (Dab1) gene encodes a key regulator of Reelin signaling. The Dab1 protein docks to the intracellular part of Reelin receptors VLDLR and ApoER2, and becomes tyrosine phosphorylated following binding of Reelin to cortical neurons. In mice, mutations of Dab1 and Reelin generate identical phenotypes. In humans, Reelin mutations are associated with brain malformations and mental retardation; mutations in DAB1 have not been identified. We defined the organization of Dab1, which is similar in man and mouse. The Dab1 gene spreads over 1100 kb of genomic DNA and is composed of 14 exons encoding the major protein form, some alternative internal exons, and multiple 5' exons. Alternative polyadenylation and splicing events generate Dab1 isoforms. Several 5' untranslated regions (UTR) correspond to different promoters. Two 5'UTR, 1A and 1B, are predominantly used in the developing brain. 5'UTR 1B is composed of 10 small exons spread over 800 kb. With a genomic length of 1.1 Mbp for a coding region of 5.5 kb, Dab1 provides a rare example of genomic complexity, which will impede the identification of human mutations.

4. Potential effectors of the reelin pathway

Putative partners of reelin signalling were studied following two approaches. First, as initiated in Namur, we performed differential screening of mRNA from reeler, scrambler and normal mouse embryonic brain using differential display and representational difference analysis. Data are still being processed and an original technical modification of RDA has been published as part of the thesis of A. Kuvbachieva (Kuvbachieva & Goffinet, 2002). In addition, we used the candidate gene approach and selected some protocadherins of the Flamingo pathway in drosphila, because mutations in these genes affect dendritic deployment in flies. As a first approach, we investigated the expression during brain development of the three mouse orthologs of drosophila flamingo, named Celsr1, 2 and 3 (Tissir et al., 2002a). Based on the expression patterns, we selected for more study the Celsr3 gene, which is expressed in neurons in parallel to their maturation and is developmentally regulated. The studies of Celsr3 are now continuing in FUNDP. We also cloned and studied the expression of the gene Ankrd6, which is probably the ortholog of Diego, an adapter of the Flamingo signalling pathway in flies (Tissir et al, 2002b).

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Annual Report of the Research Group of

Prof. Dr. Leybaert L.

Universiteit Gent
(U.Gent)

Correspondence to:

Luc Leybaert, M.D., Ph.D.

Dept. Physiology and Pathophysiology
Ghent University
De Pintelaan 185 (Block B, Rm 306)
B-9000 Ghent
Belgium
Tel.: +32 9 240 33 66
Fax: +32 9 240 30 59
E-mail: Luc.Leybaert@rug.ac.be

Glio-vascular calcium signaling and neurovascular coupling in the brain

Framework.

Our research work over the past few years has been directed towards the mechanisms and role of cell-to-cell calcium signal communication in the brain. Neurons communicate by electric and synaptic signaling, while non-excitable brain cells, like glial cells, exchange signals that involve changes in cytoplasmic free calcium concentration. We have focused our investigations on the communication of calcium signals between astrocytes and brain vascular endothelial cells, i.e. the cells that form the blood-brain barrier. Because of the important role of calcium ions as an intracellular messenger, astrocyte-endothelial calcium signals might constitute a message that affects and modulates the molecular transports over the blood-brain barrier. Neuronal activity can trigger calcium signals in astrocytes, and the working hypothesis is that astrocyte-endothelial calcium signals regulate the passage of energy substrates, e.g. glucose, over the barrier. According to this hypothesis, neuron-astrocyte-endothelial calcium signals might thus act as a signal involved in adapting the entry of glucose into the brain to the local metabolic needs of the neurons. The concept of matching glucose entry to neuronal metabolic and electric activity is known as "neurovascular metabolic coupling". This kind of coupling, including the classical "neurovascular coupling" to the blood-flow, forms the basis of the signals monitored with modern functional brain imaging techniques (PET, SPECT and fMRI). A better understanding of the coupling mechanisms is fundamental to a better understanding of functional brain imaging.

Results.

Our investigations, performed on cultures and co-cultures of astrocytes and endothelial cells and making use of focal photolytic[1] release of calcium or IP_3, have demonstrated that the two cell types can exchange calcium signals by the diffusion of IP_3 through gap junction channels and by paracrine purinergic communication[2-5]. Paracrine purinergic communication involves the release of ATP that can be triggered by increasing IP_3 in the cells and that, based on work with connexin mimetic peptides appeared to be mediated by the opening of connexin hemichannels[6], which are halve gap junction channels not connected to neighboring cells. We have further explored this purinergic signaling pathway in a study where ATP release was triggered by IP_3 or zero extracellular calcium exposure and where various pharmacological agents such as gap junction blockers, connexin mimetic peptides, trivalent ions, fenamates and ion channel blockers were applied[7]. This works shows that the ATP release pathways triggered by IP_3 or zero extracellular calcium are very similar, that this release pathway also allows the passage of substances in a bi-directional way and that the passage of substances through this pathway is compatible with free diffusion. Work with connexin transfected cell lines furthermore showed that connexin mimetic peptides inhibit triggered ATP release by a specific action on the connexin subunit, adding strong evidence for the involvement of ATP release through connexin hemichannels.

In a second part of our work, we have investigated the role of cytokines on the pathways of calcium signal communication between blood-brain barrier endothelial cells. Cytokines are known to be involved in the opening of the blood-brain barrier in neuroinflammatory diseases. Our work with TNF-α has demonstrated that this cytokine is a potent blocker of the endothelial connexin-dependent ATP release pathway described higher[8]. This work thus clearly shows that TNF-α inhibits intercellular purinergic signaling by disrupting cellular ATP release. This inhibitory action will have important consequences on purinergic signaling and will silence purinergic signal communication between astrocytes and endothelial cells and also between endothelial cells and blood cells such as leucocytes and immune cells. ATP has a proinflammatory role on these blood cells and an interruption of ATP signaling might thus modulate the complex interactions of these cells with the blood vessels in inflammatory brain disease.

Future prospects.

Our research efforts have been devoted to the mechanisms and modulation of brain calcium signaling. The goal for future work is to acquire insight into the fundamental relation between neuronal electric activity, astrocytic calcium signaling and the transport of energy substrates (glucose, lactate) in the basic unit formed by the neuron-astrocyte-endothelium. This will be approached, in a first step, by investigating the influence of calcium signals on cellular glucose handling mechanisms in these three cell types, and in a second step, by integrating the influence of neuronal activity into this picture. Calcium is a fundamental signaling ion and glucose the most fundamental metabolic molecule; we have extensive knowledge on both but we know little on their interactions. The aim is to perform basic work on the influence of calcium signals on cellular glucose homeostasis. In a second step, this work will be extended to the in vitro brain slice preparation, to investigate the concerted effects of neuronal electric activation, astrocyte-endothelial calcium signaling and glucose dynamics, using fast confocal laser scanning microscope (CLSM) imaging in combination with electrophysiology. Imaging efforts will be directed towards a multi-probe approach combining fluorescent dyes that monitor membrane potential, cytoplasmic calcium and glucose. Effort will also be invested in developing original techniques for fast CLSM imaging, and in recent work we have acquired experience in setting up a video-rate CLSM based on resonant scanner technology[9].

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Annual Report of the Research Group of

Prof. Dr. Maloteaux J.M.

Université Catholique de Louvain (U.C.L.)

Prof. Dr. Jean-Marie Maloteaux

Laboratoire de Pharmacologie
Université Catholique de Louvain
UCL 54. 10
Avenue Hippocrate, 54 - 1200 Bruxelles
Tel.: +32.2.764.54.10 - Fax: +32.2.764.54.60

Collaborator:
Prof. Dr. J.N. Octave
Prof. Dr. E. Hermans

Biochemical basis of the neurotoxicity in neurodegenerative diseases : focus on glutamate transporters

Besides its metabolic role, the amino acid glutamate constitutes the principal excitatory amino acid in the central nervous system of mammalian species. Through its binding to a variety of ionotropic (ion channels) and metabotropic (G-protein coupled) receptors, glutamate ensures many critical nervous. In addition to its role in the transmission of excitation inputs, glutamate is involved in complex activities, including learning, memory and synaptic plasticity. It is now generally accepted that ionotropic receptors induce rapid responses and are involved in the transmission of glutaminergic responses. In contrast, metabotropic receptors are responsible for the modulatory action of glutamate, by controlling both the release of glutamate in presynaptic neurones and the response induced by ionotropic receptors. Finally, metabotropic glutamate receptors are also expressed in astrocytes. Besides their metabolic and physical support roles in the central nervous system, astrocytes take part to the control of glutamate transmission by ensuring a critical function in the clearance of the neurotransmitter from the synaptic cleft. In contrast to many neurotransmitters, glutamate is not degraded in the synaptic cleft, and its extracellular clearance strictly depends on the activity of specific glutamate transporters. Although neuronal cells express glutamate transporters (type EAAC1), glutamate uptake is essentially achieved by glial cells (mainly astrocytes) which express the two major glutamate transporters (types GLT-1 and GLAST). Recent studies revealed that the expression and activity of glutamate transporters are dynamically controlled and that such regulation could participate in the short and long term modulation of glutamate transmission. Such modulation may further result from alteration in the proliferation of glial cells (gliosis processes)..

One of the principal research theme of the neuropharmacology group in the laboratoire de Pharmacologie Expérimentale of the Université catholique de Louvain concerns the study of the mechanisms of the regulation of receptors and other pharmacological targets involved in nervous transmission (in particular cell membrane transporters). In this respect, the aim of this research funded by the Fondation Médicale Reine Elisabeth is to characterise the fundamental mechanisms involved in the regulation of glutamate transporters in diverse models reflecting the physiology of glial cells. As indicated below, the regulation of glutamate transport is evaluated in primary cultured astrocytes, in C6 glioma cells and in mesenchymal stem cells exposed to growth factors favouring their differentiation into glial-like cells.

Metabotropic glutamate receptor mediated regulation of glutamate uptake in primary culture of astrocytes.

There is considerable evidence that the activity of glial glutamate transporters can be dynamically regulated. However, little is known about the physiological stimuli that contribute to such process. Since astrocytes were shown to express group I metabotropic glutamate receptors (mGluR1 and mGluR5), the present study was aimed at evaluating their possible

involvement in the regulation of the glutamate transporters. We elaborated a model of primary culture of rat cortical astrocytes (~90% of positive GFAP cells) in which the functional expression of glutamate transporters and receptors was monitored by fluorescence imaging using the Na^+ and Ca^{2+} sensitive dyes (SBFI and Fura2), respectively. In optimal culture conditions, the vast majority of cells were found to express glutamate transporters (mainly GLAST) and at least 60 % responded to the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG). The modulation of glutamate transporter activity was evaluated by measuring [^{3}H]-aspartate uptake in cells previously stimulated with DHPG (50 µM). Our results show that exposure to this agonist significantly enhanced the transporter activity (up to 30 %). This effect was only observed after brief treatment (15 sec) and was inhibited by a highly selective mGluR5 antagonist, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP). In contrast, prolonged treatments (up to 48 h) had only modest effect on the activity of glial glutamate transporters. In conclusion, these results suggest that mGluR5 may contribute to the control of the glutamate transporters activity in astrocytes. Further studies are in progress to precise the physiological relevance and to elucidate the biochemical mechanism involved in such regulation.

Modulation of modulation of the neuronal/epithelial glutamate transporter by neurotensin in C6 transfected cells.

Protein kinase C was previoulsy shown to modulate directly both the expression and the activity of the neuronal glutamate EAAC1 transporter. However, only very few reports are available concerning the physiological signals that could initiate such regulation. The aim of this work was to study the possible regulation of the neuronal EAAC1 glutamate transporter by the neuropeptide neurotensin (NT) via its G protein coupled receptor. A close relationship has been described between glutamatergic and neurotensinergic systems. For instance, both in vivo and in vitro, NT was shown to enhance glutamate release in striatal neurons and cortical slices. In the present study, the effects of NT on the aspartate uptake were examined in rat C6 glioma cells stably transfected with the high affinity NT receptor cDNA. These cells express endogenously the neuronal EAAC1 glutamate transporter. Our results indicate an increase in aspartate uptake after acute NT treatment. The effect of NT was inhibited by cytochalasin D, an actin disrupting agent, and colchicine, an inhibitor of microtubules formation. Moreover, the phospholipase C inhibitor, U73122, blocks this effect indicating the implication of the cytoskeleton and the early NT receptor transduction pathways. All the other signaling pathway, including protein kinase C, inhibitors failed to prevent the effect of NT. This increase in aspartate uptake induced by NT was also shown to result from an increase in cell surface expression of EAAC1. This regulation may help to understand the probable fine and crucial interactions between synaptic G protein coupled receptors and EAAC1 in the same synaptic element.

Caracterisation of glutamate uptake in bone-marrow mesenchymal stem cells after in vitro differentiation.

Some years ago, researchers have raised the possibility to differentiate bone-marrow stem cells into cells that express neuronal and astroglial markers in vivo. This finding has opened new therapeutic perspectives in the field of neurodegenerative diseases. The aim of our research is to evaluate whether differentiation of these mesenchymal stem cells into astrocytes can be induced in vitro. Although such differentiation can be evaluated through the detection of astroglial markers, we also investigate the ability of the cells to take up glutamate. Indeed, a critical role of astrocytes in the central nervous system is the active transport of this excitatory amino acid. Bone-marrow mesenchymal stem cells were isolated from tibias and femurs from 8-week-old Lewis female rats. The ends of the bones were cut, and the marrow was extruded using a needle and syringe. Collected cells were plated on 2 x 75-cm^2 tissue culture flask in culture medium supplemented with 10% fœtal bovine serum. After 24hr, this medium was renewed and nonadherent cells were eliminated . The medium was replaced every 3-4 days as the cells were grown to confluency. The cells were lifted by incubation with trypsin/EDTA, and were maintained beyond passage 20. Immunocytochemical characterisation demonstrated that these cells are CD90 positive (bone-marrow marker) and CD45 negative (leucocyte common antigen). These results which have been confirmed by cell fluocytometry indicate that the culture consisted of purified bone marrow stem cells. When cultured during 10 days in a medium enriched with retinoïc acid, these cells tend to show a neurone-like morphology (retracted cytoplasm with extended processes). Immunochemical analysis (immunocytochemistry) revealed that the cells cultured in the presence of retinoïc acid are Nestin positive (Neuro-epithelial Stem Cell Intermediate Filaments). This result was confirmed by reverse transcription/polymerase chain reaction (RT-PCR). In contrast, when cells were grown during 10 days in a medium containing G5 (insulin, transferrin, selenite, biotin, hydrocortisone, fibroblast growth factor and epidermal growth factor), fibroblast growth factor (FGF2) (days 1-5) and ciliary neurotrophic factor (CNTF) (days 6-10), a modest induction of Nestin expression was detected, but a significant increase in the expression of the neuronal glutamate transporter (EAAC1) was observed (protein detected by immunocytochemistry and mRNA detected by RT-PCR). This effect was correlated with an increase in the sodium-dependent uptake of tritiated aspartate (substrate of glutamate transporter), which was inhibited by the glutamate transporter blocker L-trans-pyrrolidine-2,4-dicarboxylic acid (tPDC). Together these results indicate that manipulating the composition of mesenchymal stem cell culture medium contributes to their differentiation into cells showing some morphological and functional properties of neuro-astroglial cells. This study could indicate that the concept of plasticity of bone marrow stem cells initially demonstrated in vivo can also be observed in vitro. However, further studies are required to precisely define the astroglial nature of the cells that have been obtained in our experimental conditions.

Annual Report of the Research Group of

Prof. Dr. Maquet P.

Université de Liège
(U.Lg)

Centre de Recherches du Cyclotron

Université de Liège, B30, Sart Tilman
B-4000, Liège, Belgique
Tel.: +32.4.366.23.16 - Fax: +32.4.366.29.46
E-mail: pmaquet@ulg.ac.be

Introduction

As scheduled, the MR scan has been installed in the Cyclotron Research Centre (CRC) during September 2002. It is running since November. At present, we are getting familiar with the scanner and checking the image quality and stability.

In the same time, we are installing the material for the presentation of visual stimuli and the recording of subjects' responses. We are also working on the synchronization between the scanner and the stimulus presentation. The first fMRI acquisitions will probably take place in January 2003. Although any prediction remains difficult at this stage, it is likely that routine daytime fMRI acquisitions will begin during spring 2003.

Meanwhile, we are upgrading the computing resources available in the CRC. We recently acquired a server which would automatically archive the raw MR data and leave the data available for up to 7 days to the research fellows. Likewise, we are progressively increasing the computational capacity of the CRC for fMRI data analysis.

Part One. States of altered consciousness

Sleep studies

This research includes 2 main streams: (1) Generality of our previous results, (2) Functional significance of the brain reactivations during post-training sleep

Generality of previous results

For obvious reasons, the sleep studies performed in 2002 were acquired using positron emission tomography (PET). We previously showed that the waking experience influences regional brain activity and functional connectivity during subsequent sleep (Maquet, Laureys et al. 2000; Laureys, Peigneux et al. 2001). During REM sleep, several brain areas, activated during the execution of a probabilistic serial reaction time (SRT) task (an implicit learning task where the sequence of stimuli is generated by a probabilistic artificial grammar), are significantly more active in subjects previously trained on the task than in non-trained subjects. Recent data further show that cerebral reactivations during post-training REM sleep occur only if there is some material to be learned (Peigneux, Laureys et al. submitted). No reactivation is observed during REM sleep if subjects are submitted to a random sequence of items in the SRT task. Moreover, during REM sleep, subjects trained to the probabilistic sequence show significantly more activation in some occipital, parietal and premotor areas than subjects trained to the random sequence.

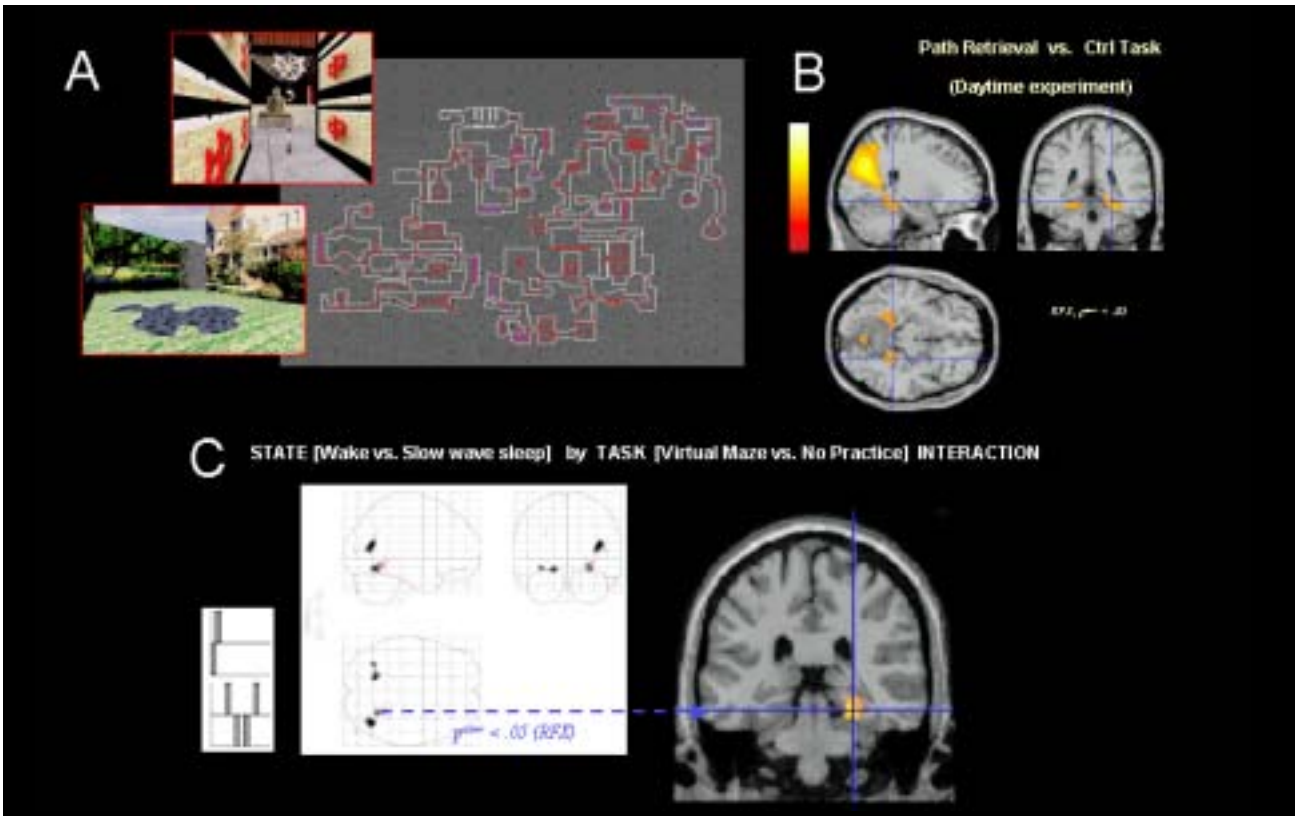
In order to show that the post-training sleep reactivation is task-dependent, we considered an explicit memory task whereby the subjects are asked to explore a large virtual reality maze, which looks like a large city with various districts. This spatial learning is allowed for 4 hours. The subjects are hourly tested on the knowledge they have acquired about the city. They are scanned during the immediate post-training night, both during sleep and wakefulness. Control populations include subjects scanned during sleep but not trained to the task and subjects awake, scanned while they are exploring the maze.

This task was chosen for 2 reasons. First, the functional anatomy of spatial learning in humans has been previously characterized using similar stimuli. This task is known to involve the parietal cortex and the hippocampal formation. Second, in rats, the reactivation of neuronal ensembles within the hippocampus has been described during sleep after spatial learning. We thus have both anatomical and functional prior knowledge about these experimental conditions.

According to our prediction, the preliminary results show that the activity in the right parahippocampal gyrus is both (a) increased during maze exploration (12 waking subjects) and (b) significantly different during post-training slow wave sleep in trained (N = 6) than untrained (N = 6) subjects (figure 1).

A manuscript describing these results is in preparation (Peigneux, Laureys et al. in preparation).

Figure 1. A. Virtual maze used in the experiment. Insets: examples of two maze locations. **B.** During wakefulness, path retrieval (as compared to rest) activates the parahippocampal regions as well as occipital and parietal areas. **C.** In the right parahippocampal gyrus, the activity changes significantly more in slow wave sleep (as compared to wakefulness) in trained subjects more than in control subjects.



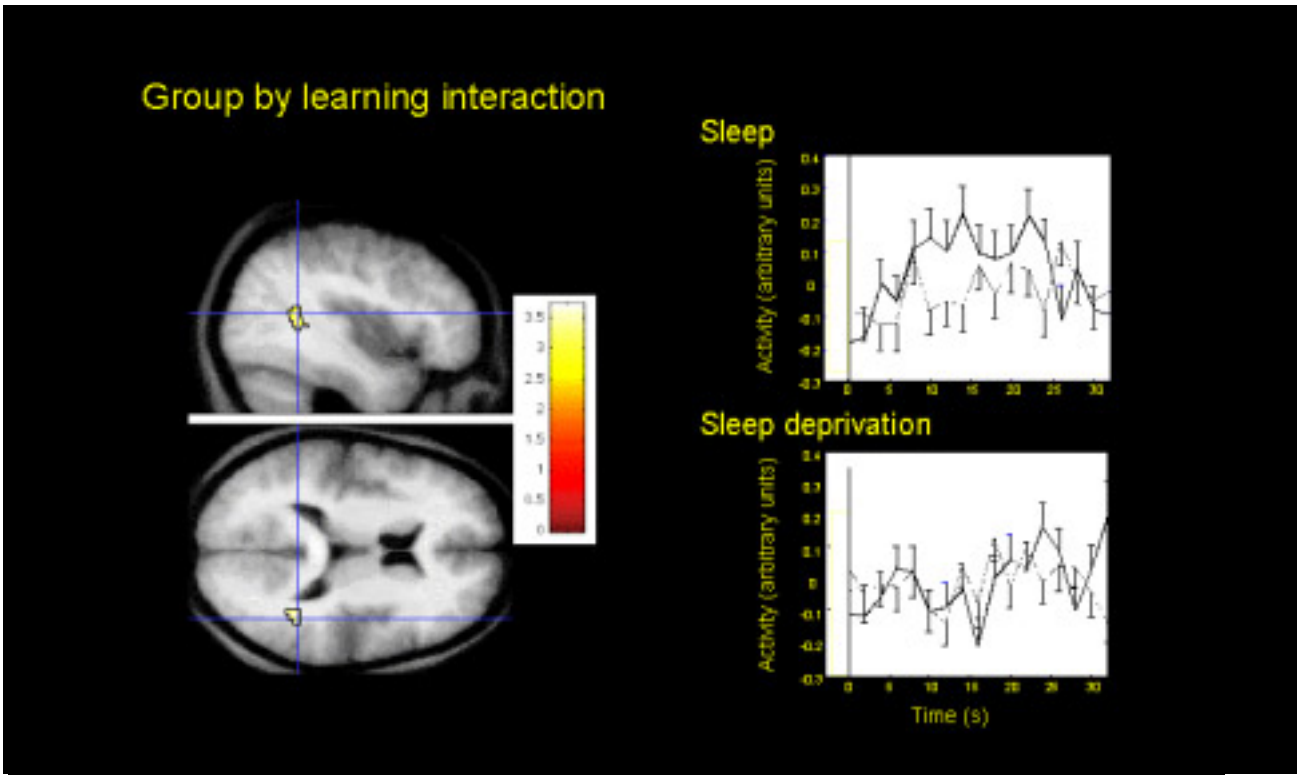
Functional significance of the brain reactivations during post-training sleep

The issue is to provide evidence that the reactivations we observed during post-training sleep were related to memory trace consolidation. To approach this issue, we have to develop different types of experimental designs (sleep deprivation, interference …). During the last year, we showed that sleep deprivation during the first post-training night hampers the

consolidation process and leaves the memory trace easily disrupted by subsequent material. We trained normal subjects on a pursuit task where the target trajectory was predictable only on the horizontal axis. Half of them were sleep-deprived on the first post-training night. Three days later, functional MRI revealed task-related increases in brain responses to the learned trajectory, as compared to a new trajectory. In the sleeping group as compared to the sleep deprived group, subjects' performance was improved, and their brain activity was greater in the superior temporal sulcus (STS, see figure 2). Increased functional connectivity was observed, in sleeping subjects (in contrast to the sleep deprived subjects) and for the learned trajectory (as compared to the new trajectory), between the STS and the cerebellum, and between the supplementary eye field and the frontal eye field. These differences indicate sleep-related plastic changes during motor skill learning, in areas involved in smooth pursuit eye movements.

This experiment was performed in the Wellcome Department of Imaging Neuroscience and is now published in the Journal of Neuroscience (Maquet, Schwartz et al. accepted). Similar experiments are now scheduled in Liège as soon as the MR scan will be available for scanning. One candidate task is the visual texture discrimination task because the perceptual learning it induces is known to be sensitive to sleep deprivation. Furthermore, we showed that this perceptual learning depends on the primary visual cortex (Schwartz, Maquet et al. 2002).

Figure 2. The superior temporal sulcus is significantly more active in the learned condition in sleeping subjects. Left panel. The statistical results, displayed at P < 0.001 are superimposed on the average normalized structural MR image of the group. Right panel. Peri-stimulus time courses of STS response (continuous line, responses to the learned trajectory; dotted line, for the new trajectory; top row: sleep group; bottom row: sleep deprivation group). Error bars represent standard error of the mean across subjects.



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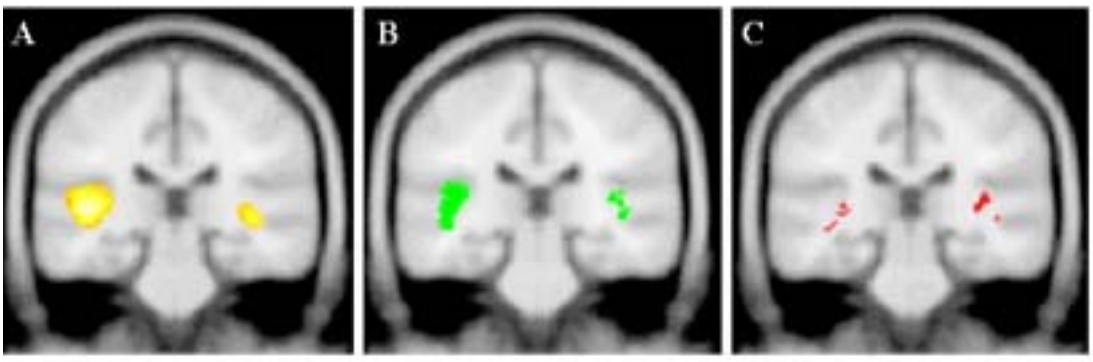
Studies on patients with altered states of consciousness (coma, vegetative state, minimally conscious state)

We continue our efforts to better understand sensory perception in severely brain injured patients using functional neuroimaging. So far, we have used PET imaging to assess auditory, noxious somatosensory and visual processing. The minimally conscious state (MCS) is a recently defined clinical condition. It consists in a severe alteration of consciousness, differing from vegetative state (VS) by the preservation of some inconsistent evidence of awareness. However, the behavioral signs of consciousness are often difficult to interpret and the frequency of misdiagnosis is high between these two conditions. By means of H_2O PET imaging, we investigated changes in regional cerebral blood flow induced by auditory click stimuli in five MCS and 15 VS patients, compared to 15 healthy controls. A psychophysiological interaction analysis also studied functional connectivity between the secondary auditory cortex and other brain areas in these patients. In both MCS patients and controls, auditory stimulation activated bilateral superior temporal gyrus (Brodmann areas - BA- 41, 42 and 22). In VS patients, the activation was restricted to BA 41 and 42 bilaterally (Figure 3). Moreover, MCS patients demonstrated significantly better functional connections than VS patients, between BA 42 and a set of cerebral areas including: posterior superior and middle temporal gyrus (BA 22, 21), posterior inferior frontal cortex (BA 44, 45) and prefrontal cortex (BA 9, 10, 46). These regions have recently been involved in higher order auditory processing and auditory attention. Although assumptions about the level of awareness of these patients are difficult to make, our findings suggest that the cerebral activity observed in MCS patients is more likely to lead to higher order integrative processes, thought to be necessary for the gain of a certain level of awareness (Paper in preparation).

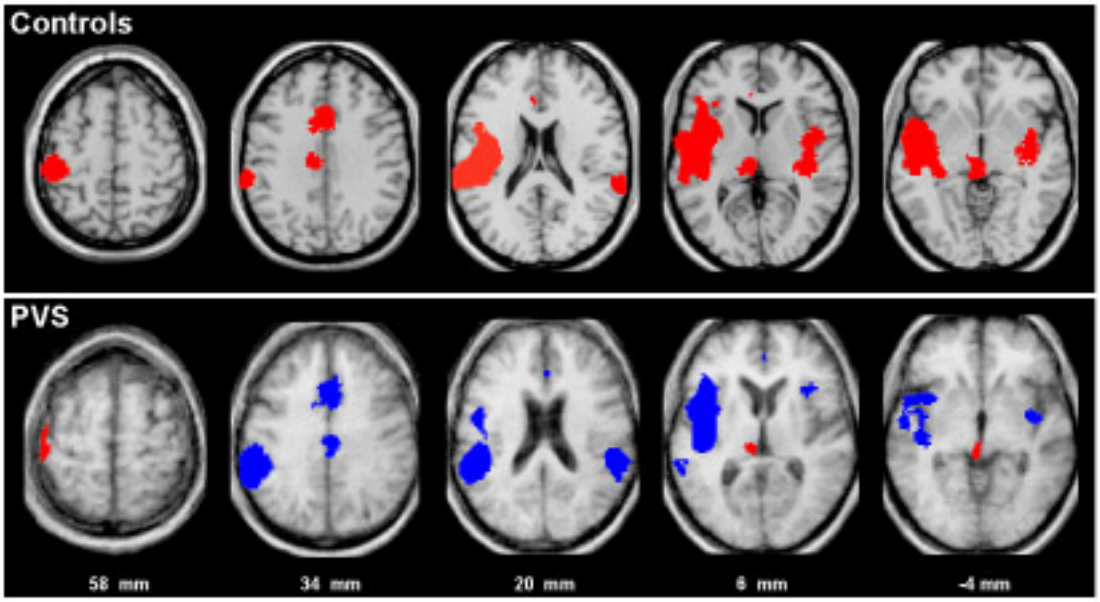
Figure 3. Brain areas showing an increase in regional cerebral blood flow during auditory stimulation in controls, shown in yellow. B and C. Areas of increase of rCBF during auditory stimulation that are common to controls and respectively MCS patients (B, shown in green) and VS patients (C, shown in red). Results are projected on a coronal section of a normalized brain MRI template, 28 mm posterior to the anterior commissural line. Results were thresholded at uncorrected p <0.001.



Our group was the first to study pain perception in persistent vegetative state patients (Laureys et al., 2002) Using PET, we measured changes in regional cerebral blood flow during high intensity electrical stimulation of the median nerve at the wrist compared to rest in fifteen non-sedated patients and in fifteen healthy controls. Evoked potentials were recorded

simultaneously. Brain glucose metabolism was also quantified in each patient. The stimuli were experienced as highly unpleasant to painful in controls. In patients, overall cerebral metabolism was 40% of normal values. Nevertheless, noxious somatosensory stimulation activated midbrain, contralateral thalamus and primary somatosensory cortex in each and every vegetative patient, even in the absence of detectable cortical evoked potentials. Secondary somatosensory, bilateral insular, posterior parietal and anterior cingulate cortices did not show activation in any patient (Figure 4). Moreover, in patients in a persistent vegetative state, as compared to controls, the activated primary somatosensory cortex was functionally disconnected from higher-order associative cortices. We then turned to pain perception in minimally conscious states. Here, our results show a preservation of activation in associative and limbic structures during noxious stimulation (Paper in preparation).

Figure 4 (Top) Brain regions, shown in red, that activated during noxious stimulation in controls [subtraction stimulation-rest]. (Bottom) Brain regions that activated during stimulation in PVS patients, shown in red [subtraction stimulation-rest] and regions that activated less in patients than in controls [interaction (stimulation versus rest) x (patient versus control)], shown in blue. Projected on transverse sections of a normalized brain MRI template in controls and on the mean MRI of the patients (distances are relative to the bicommisural plane).



Finally, we are studying visual and emotional processing in comatose, vegetative and minimally conscious patients in collaboration with the Erasme Hospital (ULB). We first have studied healthy volunteers and are currently including patients in both Liège and Brussels. Our future goal is to study residual brain function and plasticity in these patient populations by use of functional MRI.

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Part Two. Controlled processes and automatic processes, in physiological and pathological conditions.

Episodic memory

In last year report, we presented the results of an experiment on a major concept in episodic memory: information binding. In summary, we emphasized different frontal regions involved in retrieval of words, when colour was interfering or when it was bound to word. This experiment is now published (1).

Language

In last year report, we described an experiment contrasting high frequency non-words and words processing: a posterior part of the right superior temporal gyrus was activated to process non-words similar to words. The experiment is now published (2).

Executive functioning

Recently, Miyake et al. (2000) administered a set of executive tasks to a large group of young subjects in order to examine the dissociation between three often-postulated executive functions (shifting, updating, and inhibition). Confirmatory factor analysis indicated that these three executive functions were moderately correlated with one another, but were clearly separable. We have developed a research program in order to explore the unity and diversity of cerebral areas activated during executive tasks. For each executive function identified by Miyake et al., three tasks having distinct non-executive requirements have been administered.

Three different experimental tasks requiring information updating were contrasted to 3 adapted reference tasks that required no necessity to update information. Twelve subjects were selected and 144 scans were acquired. The result of a conjunction analysis revealed that the updating process relies on activation of anterior prefrontal, dorsolateral prefrontal, parietal and cerebellar areas (Figure 5).

Similarly, three different tasks were designed to explore shifting processes. The conjunction analysis revealed a common activation in right inferior parietal cortex and left precuneus (Figure 6).

Finally, three different tasks involving inhibition were explored: the Stroop task (the goal is to inhibit the automatic reading of a color name to name the colour in wich the word is written), the "stop signal" (the goal is to suppress the overlearned motor response to a stimulus) and the anti-saccade task (which requires to suppress an automatic saccade to a visual target). There was no common activation at our chosen level of significance ($p<0.001$). See Figure 7 for the antisaccade task.

Figure 5. Information updating (3 tasks): common activation in frontal, parietal and cerebellar areas

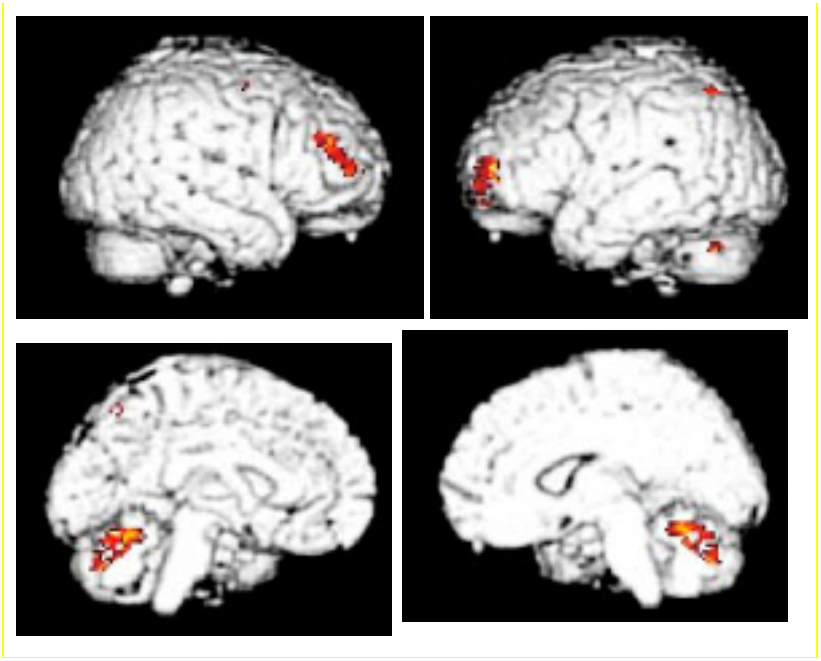


Figure 6. Shifting (3 tasks): common activation in parietal associative cortices

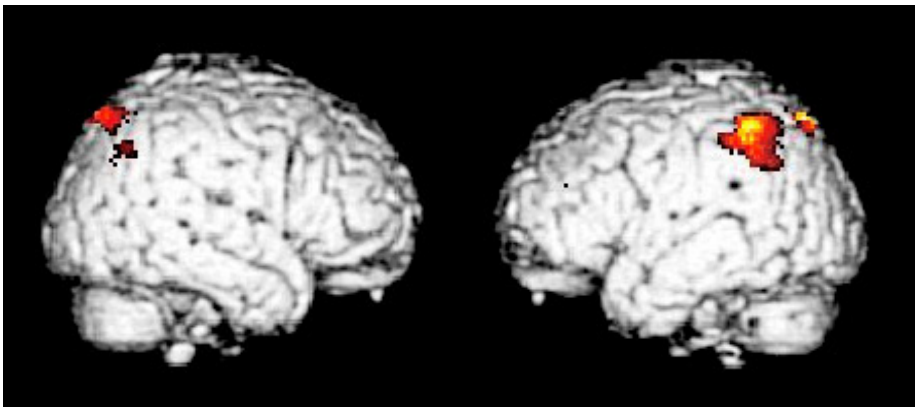
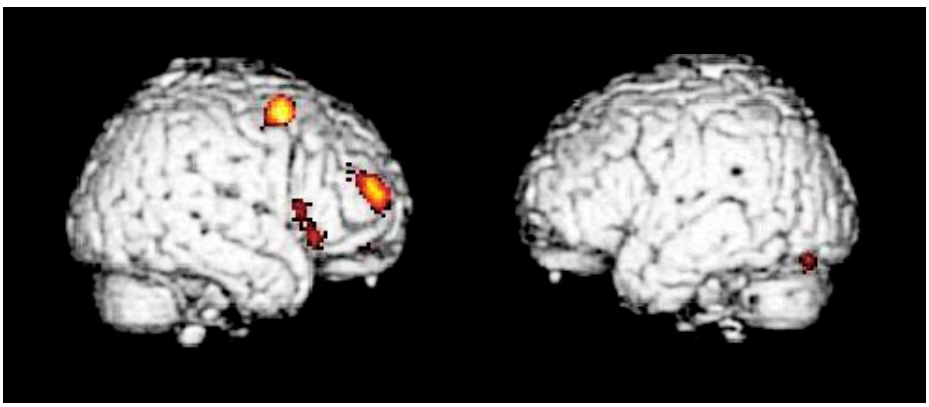


Figure 7. Inhibition: the example of anti-saccades task and frontal activation



The first analyses of this huge amount of data (1) confirm that the updating function depends on the activation of an integrated fronto-parietal network (2) demonstrate that shifting abilities are essentially dependant on parietal associative cortices and (3) confirm the idea reported in the literature that there are different inhibitory functions that depend on the recruitment of different brain regions. These results have been published in abstract form (10, 11)

Physiopathology of dementia

The evolution of brain metabolism in Alzheimer's disease (AD) and the significance of the decrease of activity have been reviewed (3). A new method has been published to increase the sensitivity of metabolic imaging to discriminate between AD patients and controls (4).

In another study, a series of inhibition tasks has been administered to patients with AD and to age-matched controls. Patients were divided in two populations: one group of patients had hypometabolism confined to posterior associative temporo-parietal cortices, while another group had a decrease of brain activity in both posterior and frontal cortices. The performances of AD patients were lower than those obtained in normal controls, but there was no difference between the two patient groups. So, frontal hypometabolism is not required to observe executive impairment in AD. We hypothesized that functional dysconnection between frontal and posterior cortices might be responsible for executive dysfunction in AD (5).

We already referred to the Stroop task, a classical inhibition tests where an automatic process (word reading) is opposed to a more controlled process (colour naming). In such a task, there are two kinds of items: in congruent trials, the word 'red' for example is written in red. In non-congruent trials, the word red is written in blue. The subject has always to name the colour. The Process dissociation Procedure developed by Jacoby (and presented in our project) has been applied to the Stroop task. According to the procedure, two conditions are administered to the subjects. In the inclusion condition, composed of congruent items, . controlled and automatic processes act together to facilitate the answer. In the exclusion condition, non-congruent items are presented and both processes have an opposite action on the response (this is the interference condition). Based on results obtained in the two conditions, it is possible to estimate the contribution of controlled and automatic processes. The Stroop task has been proposed to 20 patients with AD and to 20 age-matched controls. Results confirm that there is an interference effect and that both patients and controls are slower and make more errors in the non-congruent condition. However, the decrease of performance in the interference condition is more important for AD. Process dissociation shows that AD patients are less capable to control colour naming, and that their responses are more frequently based on the automatic word reading. So, this experiment is in agreement with the hypothesis of a decrease of controlled inhibition processes in AD, associated, in our mildly affected patients, with preserved automatic processes.

In another study, we demonstrated that, the controlled free recall of a list of words was associated to the metabolic activity measured in frontal cortex of AD patients. On the other hand, the more automatic, semantic cued retrieval of verbal information was related to

activity in the parahippocampal cortex. This is in agreement with theories that posit two different circuits for episodic and semantic verbal memory (7)

The influence of automatic processes was also explored in AD patients and controls that had to remember a task previously performed with and without actual manipulation of objects. Object manipulation remained effective to improve performance in AD (Lekeu et al, accepted). Similarly, specific techniques using automatic learning such as object manipulation allowed AD patients to learn how to use a mobile phone (8).

Emotion

The neural correlates of two hypothesized emotional processing modes - i.e. schematic and propositional modes - were investigated with positron emission tomography (9). Nineteen subjects performed an emotional mental imagery task while mentally repeating sentences linked to the meaning of the imagery script. In the schematic conditions, participants repeated metaphoric sentences, whereas in the propositional conditions, the sentences were explicit questions about specific emotional appraisals of the imagery scenario. Five types of emotional scripts were proposed to the subjects (happiness, anger, affection, sadness and a neutral scenario). The results supported the hypothesized distinction between schematic and propositional emotional processing modes. Specifically, schematic mode was associated with increased activity in the ventromedial prefrontal cortex (figure 8) whereas propositional mode was associated with activation of the anterolateral prefrontal cortex (figure 9). In addition, interaction analyses showed that schematic versus propositional processing of happiness (compared to the neutral scenario) was associated with increased activity in the ventral striatum whereas "schematic anger" was tentatively associated with activation of the ventral pallidum.

Figure 8. Ventromedial prefrontal activation during "hot" (schematic) processing of emotion

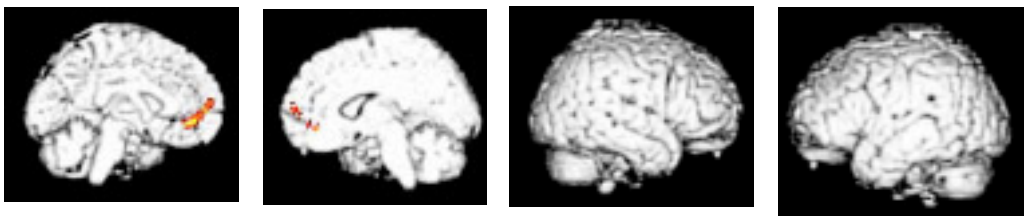
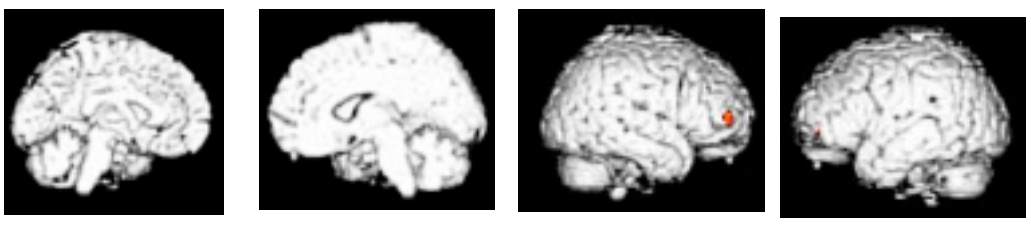


Figure 9. Lateral prefrontal activation during controlled processing of emotion



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Abstracts

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Annual Report of the Research Group of

Prof. Dr. Moonen G.

Université de Liège
(U.Lg)

Centre de Recherches en Neurobiologie
cellulaire et moléculaire
Université de Liège

Faculté de Médecine

Laboratoire du PROFESSEUR G. MOONEN

Institut Léon Fredericq - 17 place Delcour - B-4020 Liège - Belgique

Principal investigator: G. MOONEN

Senior Co-investigators:
P. LEPRINCE
B. MALGRANGE
J.-M. RIGO
B. ROGISTER
S.BELACHEW

Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro

Bernard Rogister, Sabine Wislet-Gendebien, and Pierre Leprince.

Neuregulin 1 (Nrg1) isoforms have been shown to influence the emergence and growth of oligodendrocytes, the CNS myelin-forming cells. We have demonstrated that embryonic striatal NP synthesize NRG-1 transcripts and proteins, as well as ErbB2 and ErbB4 -but not ErbB3 receptors. Striatal neuroepithelial precursors (NP) coexpress ErbB2 or ErbB4 with Nrg1 and predominantly synthesize a transmembrane Type III isoform called SMDF/CRD-NRG. To examine the biological effect of Nrg1, we added soluble ErbB3 (sErbB3) to growing neurospheres. This inhibitor decreased NP mitosis and increased their apoptosis, resulting in a significant reduction in neurosphere size and number. When NP were induced to migrate and differentiate by adhesion of neurospheres to the substratum, the level of type III NRG-1 isoforms detected by RT/PCR and Western blot decreased in the outgrowth in parallel with a decrease in Nrg1 fluorescence intensity in differentiating astrocytes, neurons and oligodendrocytes. Pretreatment of growing neurospheres with sErbB3 induced a three fold increase in the proportion of oligodendrocytes generated from migrating NP after neurosphere adhesion. This effect was not observed with an unrelated soluble receptor. Addition of sErbB3 after adhesion did not change the proportion of oligodendrocytes in the neurosphere outgrowth but enhanced their expression of galactocerebroside and myelin basic protein. We propose that both Type III Nrg1 signaling and released soluble ErbB receptor may modulate oligodendrocyte development from NP (Calaora et al., 2001).

We have expressed as a recombinant protein the intracellular domain of SMDF/CRD-NRG and we have purified it. Then we raised a rabbit polyclonal antiserum against this intracellular part of SMDF/CRD-NRG (SMDF-IC). Using purified IgG, we observed in immunocytofluorescent studies that SMDF-IC is present at plasmic membrane level in proliferating NP and in nuclei of differentiating neurons and oligodendrocytes but not in astroglial nuclei. Indeed, SMDF-IC exhibits a strong nucleus localization signal. We made the hypothesis that there is a cleavage of SMDF/CRD-NRG and the SMDF-IC is then transferred to nucleus. This nuclear translocation is followed by a oligodendroglial or neuronal differentiation. It has been recently demonstrated that SMDF/CRD-NRG is cleaved at the plasmic membrane level (Frenzel and Falls, 2001). So it is important to look for the protein(s) interacting with SMDF-IC and which could be translocated into the nucleus with SMDF-IC and there behave(s) like a transcription factor. We have started the co-immunoprecipitation studies using our polyclonal anti-SMDF-IC.

More recently, we have demonstrated that NRG-2 but not NRG-3 is expressed in proliferating neurospheres. No NRG-2 expression is observed in NP after 5 days of differentiation. Moreover, the expressed NRG-2 isoform is not related to an already described protein (Busfield et al., 1997; Higashiyama et al., 1997). NP NRG-2 isoform is characterized by an Ig domain, then an EGF domain followed by an α and a β domain, before the transmembrane and the cytoplasmic domain. Moreover exon recombination is responsible for a sliding of the open reading frame in the β exon, leading to an a new amino acid sequence in the protein isoform. This association of both α and β domain was not already described for a NRG-2 isoform. This $\alpha\beta$ region has a

important function because it is the target of an eventual shedding enzyme. We have cloned this NP-NRG-2 isoform and we are expressing it in order to get the recombinant protein and antibodies.

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Regulation of proliferation of oligodendrocyte precursors : role of glycine, serotonin and inhibitors of cyclin-dependent kinases.

Belachew, S., Nguyen, L., Malgrange, B., Rogister, B. and Moonen G.

We have recently demonstrated that oligodendrocyte progenitor cells (OPCs) as well as central nervous system (CNS) stem cells do express neurotransmitter receptors (Belachew et al., 1998a, 1998b, 2000a; Nguyen et al., 2001; Nguyen et al., 2002). In particular, OPCs have been shown to express type 3 serotonin ($5HT_3R$) and glycine (GlyR) ionotropic receptors. In vitro, serotonin enhance the rate of OPC differentiation induced by mitogen (i.e. platelet-derived growth facto, PDGF) withdrawal (Rocher et al., 2002). 5-HT-dependent enhancement of OP cell differentiation is purely mediated by $5\text{-}HT_3R$ activation and occurs until the pre-myelinating $GalC^+$ stage (Rocher et al., 2002). We also provided evidence that serotonin does not significantly interact with OP cell cycle progression both in the presence and in the absence of mitogen (Rocher et al., 2002). Future experiments are however needed to address the following issues: i) the possible role of metabotropic 5-HT receptors in oligodendroglial differentiation, ii) the role of serotonin and 5-HT3Rs in other experimental paradigms of oligodendroglial differentiation in vitro and notably T3-induced lineage progression, iii) the intracellular pathway(s) linking 5-HT3R activation to yet broadly unknown molecular cascades involved in the regulation of OP cell differentiation.

Cultured OPCs also express the ionotropic glycine receptor (GlyR) and glycine transporters 1 and 2 (GLYT1, GLYT2), the activation of which induces membrane depolarization followed by the opening of voltage-gated calcium channels (VGCC) and thus influx of intracellular calcium (Belachew et al., 1998b, 2000a). Considering this latter effect, we hypothetized that glycine

could alter events such as oligodendroglial proliferation and differentiation that are likely dependent upon intracellular calcium concentration. OPCs proliferation was quantified by a bromodeoxyuridine incorporation assay and differentiation was assessed by measuring the relative proportions of A2B5, O4 and galactocerebroside immunophenotypes during in vitro oligodendrocyte maturation.Without interfering with the in vitro survival of oligodendroglial cells, we showed that glycine induced a dose-dependent mitogenic effect on A2B5-positive OPCs that is suppressed by nifedipine, a L-type VGCC blocker (Belachew et al., 2000b). Although not completely inhibiting this process at any stage, glycine also significantly decreased the rate of oligodendrocyte differentiation (Belachew et al., 2000b). This work thus supports our hypothesis that glycine can regulate oligodendrocyte development by a mechanism involving a modulation of intracellular calcium homeostasis. Glycine released by neurons might consequently serve as a physiological signal between neurons and OPCs during oligodendrogliogenesis, possibly together with other neurotransmitters. Therefore, pharmacological manipulations of such receptors might provide new pathways for remyelination strategies.

Proliferation of OPCs is a crucial process controlling myelination in the central nervous system. Previous studies have demonstrated a correlation between neurotransmitter receptor activation, OPC proliferation rate and cyclin E/cyclin-dependent kinase-2 (cdk2) activity (Ghiani et al., 1999a, 1999b; Ghiani et Gallo, 2001). To establish a causal link between cyclin E/cdk2 activity and OPC proliferation, we selectively modulated cdk2 activity in vitro by transfection of cultured OP cells. Dominant-negative (van den Heuvel et al., 1993) (Dn)-cdk2 overexpression inhibited mitogen-induced OP cell proliferation, whereas wild-type (wt)-cdk2 prevented cell cycle arrest caused by anti-mitotic signals. Dn-cdk2- or wt-cdk2-mediated regulation of G_1/S transition, per se, did not influence initiation of OP differentiation (Belachew et al., 2002a). To study the function of cyclin E/cdk2 in OP cells during development in vivo, we analyzed cdk2 and cyclin E expression in cells acutely isolated from transgenic mice expressing the green fluorescent protein (GFP) under the control of the 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNP) gene promoter (Belachew et al., 2001, 2002; Yuan et al., 2002). Both cyclin E/cdk2 protein levels and activity were decreased in GFP^+ oligodendrocyte lineage cells between postnatal day 4 and 30 (Belachew et al., 2002a). Immunostaining of $NG2^+/GFP^+$ OP cells in brain tissue sections showed a 90% decrease in overall cell proliferation and cdk2 expression between perinatal and adult cells (Belachew et al., 2002a). However, cdk2 expression within the proliferating (i.e. expressing proliferating cell nuclear antigen) OP cell population was maintained throughout development (Belachew et al., 2002a). Our data indicate that: i) cyclin E/cdk2 activity plays a pivotal function in OP cell cycle decisions occurring at G_1/S checkpoint; ii) initiation of OP differentiation is independent of cyclinE/cdk2 checkpoint, and iii) intrinsic differences in cyclin E/cdk2 expression and activity may underlie the slowly proliferative state that characterizes so-called "quiescent" adult OP cells in vivo.

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Phenotypic plasticity of radial glial cells *in vitro* and *in vivo*.
Leprince, P. and Moonen, G.

Understanding the molecular mechanisms that control the bidirectional transition between radial glia and astrocytes is a prerequisite for attempting to manipulate the phenotype of astrocytes in the adult brain for experimental or therapeutic neuroblasts grafting purposes. Much information on that transdifferentiation process could be obtained by analyzing and comparing the proteomes of radial glia and astrocytes and of the various phenotypes that can be generated in vitro under the above-mentioned conditions of culture.

One prerequisite for those studies is to obtain as pure cultures as possible as starting material and the following approaches were designed for that purpose. The first involves the growth of cerebellar glial cells obtained from E16 embryos in a synthetic medium designed for glial cultures (G-5 supplement, (Bottenstein, 1985)). This medium strongly induces in these cells a phenotype of glial progenitors with a very high proliferation rate, expression of Radialine and Nestine by nearly all cells, very rare expression of GFAP and poor survival of differentiating neurons. Complementary to this, the continuous growth of the same cells in medium supplemented with 10 % Horse serum strongly favours the astroglial phenotype of highly stellate cells expressing high levels of GFAP and no Radialine or Nestine.

The second approach involves the FACS-sorting of cell suspensions made from cultures of cerebellar glia of GFEA mice (Nolte et al., 2001) . These mice express the fluorescent EGFP protein under the control of a GFAP promoter and have all the cells in their astroglial lineage totally green. Preliminary sorting of those cells is now possible using the new instrument available at the pathology department of the ULg and conditions are now tested to maintain and expand the selected cell population. Indeed one drawback of the sorting of these cells is the necessity to trypsinize and vigorously dissociate them, which is detrimental to their survival.

We have used the technique of isoelectric focusing on immobilized pH gradients (IPG) to separate the proteins extracted from some cultures of radial glia and astrocytes. Total protein extracts, including both soluble and insoluble proteins, have been prepared by using an extraction buffer that contains strong chaotropes and reducing agents while remaining compatible with the conditions of isoelectric focusing. A few protein maps have already been obtained and have been stained with a new fluorescent dye used for analytical purpose (Rabilloud et al., 2001). Gel images have been acquired with the laser-scanner Typhoon 9200 from Amersham and differences in spots locations and intensities occurring between cell phenotypes are detected using the ImageMaster 2-D program from Amersham. Relevant protein spots that show differential expression between cell phenotypes and culture conditions will be obtained in preparative gels that will be loaded with higher quantities of starting material. Those protein spots will be subjected to sequencing by mass spectrometry in collaboration with Prof E. De Pauw, laboratory of mass spectrometry, University of Liège

We have started investigations on the identity of neuron-derived activities that modulates the expression of *Radialin* by cerebellar glial cells. One such activity that is specifically released

by cerebellar granule neurons in vitro but is not produced by other neurons or by non-neuronal cells suppresses the expression of Radialin. Radial glia phenotype can by reinduced, both *in vitro* and *in vivo*, from GFAP-positive adult cortical astrocytes in response to a diffusible glial differentiation signal released by embryonic forebrain: the radializing factor RF60 (Hunter and Hatten, 1995). RF60 is a protein of about 60 kDa produced by embryonic neurons. Its expression is down-regulated perinatally when the radial glial population decreases (Hunter and Hatten, 1995). As only an effect on the morphology of glial cells had been documented for this activity, we set to show that embryonic neurons are also able to reinduce the expression of Radialin by cerebellar glial cells that have been cultured in medium without serum. Indeed such an activity is found in medium conditioned by embryonic neurons but not by postnatal glia. The next step will be the identification of this activity and for that purpose we have established a collaboration with Prof. Sharon Juliano, dept of Anatomy and cell biology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA. In her laboratory a preliminary characterization of this activity has been done (Gierdalski and Juliano, 2002) and we will take responsibility for identifying by 2D-electrophoresis the relevant proteins in sample that she will send to us.

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Cell biology for prevention and treatment of sensorineural deafness

Malgrange, B.[1], Lefebvre, P.P.[1], Belachew, S.[1], Rigo, J.M.[1], Nguyen, L.[1], Thiry, M.[2] and Moonen, G.[1]

[1]Centre de Neurobiologie Cellulaire et Moléculaire, Université de Liège, Belgique
[2]Service de Biologie Cellulaire, Université de Liège, Belgique

Development of the organ of Corti, the auditory sense organ of mammals, involves the differentiation of sensory hair cells (HC) (inner and outer) and non sensory supporting cells (SC) including Deiters' cells, pillar cells, inner phalangeal cells, tectal cells and Hensen's cells. Each of these cell types has a distinct morphology that contributes to the complex structural and functional properties of the organ of Corti. In mammals, embryonic HCs and SCs proliferation within the sensory epithelia peaks during late embryogenesis (e.g. between embryonic days 13 (E13) and 15 (E15) in the mouse) (Ruben, 1967) and HC production never occurs at later stages in normal conditions. To obtain a production of new (supernumerary) HCs in a neonatal sensory epithelium without induction of proliferation, immature cells must be present and retain the potentiality to undergo straightforward differentiation into HCs. We showed that acutely dissociated cells from the newborn rat organ of Corti, developed into so-called otospheres consisting of 98% nestin (+) cells when plated on a non-adherent substratum in the presence of either Epidermal Growth Factor (EGF) or Fibroblast Growth Factor (FGF2). Within cultured otospheres, nestin (+) cells were shown to express EGFR and FGFR2 and rapidly give rise to newly formed myosin VIIA (+) HCs and $p27^{KIP1}$ (+) SCs. Myosin VIIA (+) HCs had incorporated bromodeoxyuridine (BrdU) demonstrating that they were generated by a mitotic process. Ultrastructural studies confirmed that HCs had differentiated within the otosphere, as defined by the presence of both cuticular plates and stereocilia. This work raises the hypothesis that nestin (+) cells might be a source of newly generated HCs and SCs in the injured postnatal organ of Corti (Malgrange et al., 2002a).

In parallel, we showed that when fetal rat organ of Corti explants are cultured, supernumerary outer hair cells (OHCs) and supernumerary Deiters' cells are produced, without any additional cell proliferation. Analysis of semi- and ultrathin sections revealed that supernumerary OHCs are produced at the distal edge of the organ of Corti. Quantitative analysis of cell types present in the organ of Corti demonstrates that, when the number of OHCs increases: 1) the total number of cells remains constant; 2) the number of Deiters' cells increases; 3) the number of tectal cells decreases and of Hensen's cells decrease. Using specific HC markers, i.e. jagged2 (Jag2) and Math1, we show that in addition to existing OHCs, supernumerary OHCs, tectal cells and Hensen's cells expressed these markers in E19 organ of Corti explants after 5 days *in vitro*. The results of this study suggest that Hensen's cells retain the capacity to differentiate into either tectal cells, which differentiate into OHCs, or into undertectal cells which differentiate into Deiters'cells (Malgrange et al., 2002b).

The identification of the genes involved in HC development could provide some leads to understand regeneration. Actually, several genes are known to be implied in inner ear morphogenesis and HC development and differentiation including genes coding for membrane-bound signalling proteins, various transcription factors, cyclin-dependant inhibitors and

secreted factors, and these genes may also be involved in HC regeneration. In embryonic day 19 organs of Corti, we showed that roscovitine, a chemical inhibitor of cyclin-dependent kinases, significantly increased the number of HCs and corresponding SCs by triggering differentiation of precursor cells without interacting with cell proliferation. Immunohistochemical analysis indicated that roscovitine specific intracellular targets, CDK1, 2, 5, 7 and ERK1/2 were expressed in the organ of Corti and especially in Hensen's cells. Affinity chromatography studies showed a tight correlation between the protein levels of CDK1, 2 and 5 and the rate of roscovitine-induced supernumerary cells in the organ of Corti. In addition, we demonstrated that basal kinase activity was both higher and more roscovitine sensitive to pharmacological inhibition at the specific developmental stages that allow the emergence of supernumerary cells. The effects of roscovitine required the Math1 transcription factor, as shown by the lack of effect of roscovitine on organs of Corti derived from Math1-null mice. These results suggest that CDKs are involved in the normal development of the organ of Corti and that, at least in E19 embryos, inhibition of CDKs is sufficient to trigger the differentiation of HCs and SCs, presumably from the Hensen's cells progenitors.

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Neurotransmitters as developmental signals

Nguyen, L., Rogister, B., Moonen, G. and Rigo, J.M.

Gamma-aminobutyric acid (GABA) and its type A receptor ($GABA_AR$) are present in the immature central nervous system (CNS) and may function as growth-regulatory signals during the development of embryonic neural precursor cells. In the present study, based on their isopycnic properties in a buoyant density gradient, we developed an isolation procedure that allowed us to purify proliferative neural precursor cells from early postnatal rat striatum, which expressed the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). These postnatal striatal PSA-NCAM+ cells were shown to proliferate in the presence of epidermal growth factor (EGF) and formed spheres that generated preferentially neurons in vitro. We demonstrated that PSA-NCAM+ neuronal precursors from postnatal striatum expressed $GABA_AR$ subunits in vitro and in situ. GABA elicited chloride currents in PSA-NCAM+ cells by activation of functional $GABA_AR$ that displayed a typical pharmacological profile. $GABA_AR$ activation in PSA-NCAM+ cells triggered a complex intracellular signaling combining a tonic inhibition of the mitogen-activated protein kinase cascade and an increase of intracellular calcium concentration by

opening of voltage-gated calcium channels. We observed that the activation of $GABA_AR$ in PSA-NCAM+ neuronal precursors from postnatal striatum inhibited cell cycle progression both in neurospheres and in organotypic slices. Furthermore, postnatal PSA-NCAM+ striatal cells synthesized and released GABA, thus creating an autocrine/paracrine mechanism that controls their proliferation. We showed that EGF modulated this autocrine/paracrine loop by decreasing GABA production in PSA-NCAM+ cells. This demonstration of GABA synthesis and GABAAR function in striatal PSA-NCAM+ cells may shed a new light in the understanding of key extrinsic cues that regulate the developmental potential of postnatal neuronal precursors in the CNS.

In this work, we have assessed the presence of ionotropic glycine receptors (GlyRs) in neurogenic progenitors purified from the newborn rat striatum and expressing the polysialylated form of the neural cell adhesion molecule (PSA-NCAM). Using immunocytochemical analysis, we report that GlyRs are expressed in neurogenic Tuj1+ PSA-NCAM+ progenitors from the striatum, both in vitro and in situ. To ascertain whether these GlyRs were functional in vitro, whole-cell patch-clamp recordings of neurogenic PSA-NCAM+ progenitors demonstrated that glycine triggered inward strychnine-sensitive currents in 64 % of the cells with an EC50 value of 86.1 ± 1.2 μM. Moreover, those glycine-evoked currents were completely and reversibly inhibited in a concentration-dependent manner by the GlyR antagonist strychnine with an IC50 of 1.2 ± 1.8 μM. Altogether, the present data show that functional strychnine-sensitive GlyRs are expressed in neurogenic PSA-NCAM(+) progenitor cells isolated from the newborn rat striatum.

β-CCB induces apoptosis of cultured cerebellar granule neurons via the mitochondrial pathway

Hans, G., Malgrange, B., Belachew, S., Moonen, G. and Rigo, J.M.

In addition to their role in neurotransmission, inhibitory and excitatory neurotransmitters have been involved in other processes such as neuronal survival and death. We had previously shown that β-carbolines, which are inverse agonists of the $GABA_A$ receptor, induce the apoptotic cell death of cultured cerebellar granule neurons. However, the cellular events leading to this neuronal death remained uninvestigated.

During the last year, we were able to show that β-CCB-induced CGN apoptosis depends on caspase 9 and caspase 3 activation suggesting a mitochondrial implication. Furthermore, caspase activation is preceded by cytochrome c release into the cytosol and is accompanied by a nuclear translocation of the AIF. However, these changes do not depend of a mitochondrial membrane depolarisation as demonstrated by JC-1 confocal microscopy and FACS. Further investigations are required to address the question of how mitochondrial pro-apoptotic factors are release and to assess an eventual link between the effect of β-CCB at the $GABA_A$ receptor and its ability to trigger apoptosis in neuronal cultures.

Differentiation of mesenchymal stem cells in neural cells - cellular and molecular influences

Sabine Wislet-Gendebien, Pierre Leprince, Gustave Moonen and Bernard Rogister

Recently, several observations are in favor that adult somatic stem cells are not tissue-specific but exhibit a phenotypic plasticity. By example, when mesenchymal stem cells isolated from hematopoietic bone marrow are injected into the intra-cerebral ventricles of newborn rats, these cells differentiate into neurones and astrocytes (Kopen et coll., 1999). These mesenchymal stem cells are able to differentiate into neurons in an other in vivo model and it was demonstrated that these newly-formed neurons are included into neuronal pathway and thus could be activated as a normal neuron (Brazelton et coll., 2000; Mezey et coll., 2000). More recently, mesenchymal stem cells could be used in grafting experiments in order to repair myelin after a demylination (Sasaki et coll., 2001).

We have established the culture method in order to isolate and cultivate mesenchymal stem cells from adult rat and mice. We have characterized those cells in an immunological but also on a functional point of view and we demonstrate that those mesenchymal stem cells could differentiate into adipocytes, osteocytes and chondrocytes. In particular culture conditions they become able to express the nestin, an intermediate filament protein which is highly expressed in immature cells of the nervous system. We demonstrated that lysophosphatidic acid and thrombin could repress this expression. Moreover the nestin expression is only observed in mesenchymal stem cell cultures which are old enough : before ten passages in culture, no nestin could be seen in favourable culture conditions. It means that mesenchymal stem cells need to mature or to transform in vitro, in order to acquire the ability to express nestin. This nestin expression property is a hallmark of other modifications : if mesenchymal stem cells are able to and allowed to expressed nestin, they can form clusters in non-adherent culture conditions, just like neural stem cells do.

When nestin(+) but not nestin(-) mesenchymal stem cells are co-cultivated with neural stem cells in suspension, they rapidly form heterogenous spheres. If those spheres are plated on adherent surface for 5 days, one can observe a cell differentiation and 40 % of original mesenchymal stem cells express two astroglia-specific markers : GFAP and GLAST. We demonstrated using two procedures that those GFAP(+) cells are from stromal origin and are not a consequence of a cell fusion between a mesenchymal stem cell and a neural stem cell. Finally, nestin(-) mesenchymal stem cells will not express GFAP when they are co-cultivated with neural stem cells and a direct cell-to-cell contact is necessary to observe a GFAP expression by nestin(+) mesenchymal stem cells.

We have started the molecular characterization of the signal which stimulate the astroglial differentiation of mesenchymal stem cells.

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Annual Report of the Research Group of

Prof. Dr. Orban G.

Katholieke Universiteit Leuven
(K.U.Leuven)

Prof. Dr. G. Orban

K.U. LEUVEN - Faculteit der Geneeskunde
Labo voor Neuro- en Psychofysiologie
Campus Gasthuisberg
Herestraat 49
3000 Leuven
Tel.: +32.16.34.57.40
Fax: +32.16.34.59.93

Selectivity of monkey V4 neurons for kinetic patterns.

Since some time we have established that high in the ventral pathway (in IT cortex) single neurons are selective for 2D shapes defined by motion, as well as for 2D shapes defined by luminance (Sáry et al. Science 1993). The kinetic shapes were defined by direction differences and remained static. Much effort has gone since then in finding out at which earlier level selectivity for kinetic boundaries -figures or gratings- first emerges. We established that MT/V5 does not contribute much to this process (Marcar et al. J. Neurophysiology, 1995, Lauwers et al., J. Comp . Neurology 2000), nor V1 (Marcar et al. J Neurophysiology, 2000). A small percentage of V2 neurons are tuned for kinetic grating orientation but tuning is weak and latency long, suggesting that these could be the reflection of a feedback signal, rather than the emergence of a selectivity. Since human imaging had indicated that a region (KO), which may correspond to V4, processes kinetic patterns (Orban et al. PNAS 1995, Dupont et al. Cerebral Cortex 1997, Van Oostende et al. Cerebral Cortex 1997) and in monkey V4 is activated by kinetic patterns (Nelissen et al. Soc. Neurosci. Abstr. 2000, Fize et al., Soc Neurosci Abstr. 2001), we started to study selectivity of V4 neurons for kinetic patterns.

Up to now we have recorded from one monkey and only from dorsal V4 until now (for technical reasons), but the plan is to compare dorsal and ventral V4. The methods are standard, but much effort has gone in the sampling strategy. Neurons are searched for with luminance and motion defined patterns in strict alternation. All neurons, after exploration of the receptive field and optimization of size and position of stimuli are tested with kinetic and luminance gratings. In the first test, eight orientations of a luminance defined gratings and of parallel and orthogonal kinetic gratings are tested as well as eight axis of motion of transparent motion and 16 direction of simple motion in which all pixels move in the same direction. All stimuli are textured random patterns in which either direction of motion or luminance is manipulated. In the parallel and orthogonal kinetic gratings motion is parallel or orthogonal to the kinetic boundaries respectively. Once a cell has been tested with the first test or as soon as the first test reveals no selectivity, neurons are tested with a kinetic shape test. This test includes eight different simple 2D shapes (the same as originally used by Sary et al. , and also used in the fMRI of monkeys) defined either by luminance (two directions of contrast) or by motion direction (two different axis of motion).

So far we have recorded from 156 V4 neurons which were responsive to stimuli of the first test. Of these 68 (44%) were tuned for orientation of the luminance grating (tested by ANOVA). Of these 11 (16%) were also selective for kinetic orientation. The preferred orientation and tuning was similar for the two types of pattern, but on average the response of these 11 neurons was stronger for luminance defined than for kinetic gratings. In addition 88 V4 neurons were tested for shape selectivity and 14 were found selective. Of these 9 (64%) were also selective for kinetic shapes. Selectivity was similar for the types of shapes but again responses were stronger for the luminance defined than the kinetic shapes.

In addition to comparing selectivity for the two types of patterns, we also compiled the average response of all neurons to the different patterns (averaged over all orientations or directions of motion). This tests the idea that fMRI might represent only the average spike activity of the neurons. Spontaneous activity (n=156) equals 2 spikes /sec; response to kinetic gratings 9.3 and 9.2 spikes/sec for parallel and orthogonal respectively, 8 and 9.4 spikes/sec for transparent and simple motion and 11.8 spikes/sec for luminance defined gratings. In the fMRI V4 responds more to kinetic gratings than to simple motion.

Plans for next year: increase the V4dorsal sample and include V4 ventral neurons. We will also test other speeds, spatial frequencies and sizes in parallel with the fMRI studies (which we are currently analyzing). We will also test other types of boundaries.

Annual Report of the Research Group of

Prof. Dr. Pandolfo M.

Université Libre de Bruxelles
(U.L.B.)

Principal Investigator: Massimo Pandolfo, MD

Université Libre de Bruxelles
Clinique universitaires de Bruxelles
Hôpital Erasme
Route de Lennik 808
B-1070 Bruxelles
Tel.: +32.2.555.31.11

Pathogenesis of cellular dysfunction and death in frataxin deficiency

Activities during the first year of the grant have been focused on reorganizing the laboratory after moving from the Centre Hospitalier de l'Université de Montréal, on recovering and testing materials and cell lines shipped from Montréal, on establishing a new colony of mice, and on developing a new mouse model for Friedreich Ataxia.

Two graduate students and a postdoc have been recruited for the project. One of the graduate students is paid on FMRE funds.

Progress concerning each of the specific aims of the project is detailed below.

Specific aims 1 and 2

These aims include the analysis of oxidative stress and mitochondrial dysfunction in tissue samples from Friedreich ataxia patients and in cellular and animal models of the disease.

Most of the work done in 2001 has been preliminary and aimed to provide the necessary reagents.

In particular, fibroblasts from Friedreich Ataxia patients and stably transfected mouse embryonic carcinoma P19 cells shipped from Montréal have been put in culture and characterized for frataxin levels by Western blood analysis. All the relevant cell lines have been recovered and are now grown in the Brussels laboratory.

Concerning mouse models, knock-in mice carrying a $(GAA)_{230}$ triplet repeat in the first intron of the frataxin gene and frataxin knock-out mice have been shipped from Montréal. Colonies have been established at the Institute of Developmental Biology of ULB in Gosselies and the first knock-in/knock-out mice have been generated through the appropriate crossings. These animals are deficient in frataxin (20 % of well-type levels) but do not express a behavioral phenotype. Tissue samples have been obtained from these mice at different stages of development, pre- and postnatal. Formalin-fixed paraffin-embedded sections are ready for immuno-histochemical and cytochemical staining.

A study on the expression of mitochondrial ferritin in heart samples from Friedreich Ataxia patients has been carried out. The underlying hypothesis was that this recently discovered form of ferritin might be induced in the patient's mitochondria as an antioxidant defense against the iron accumulation occurring in these organelles. However, rather surprisingly, preliminary results do not show any mitochondrial ferritin induction in Friedreich's heart, contrary to what has been observed in sideroblastic anemia. This finding is of interest because it suggests that iron in Friedreich's mitochondria is not stored in a redox-inactive form as it would be within mitochondrial ferritin, so it may cause oxidative stress to a much higher degree than the iron stored in mitochondria of sideroblasts. This is in agreement with the fact that sideroblasts mitochondria seem to preserve normal function and structure, contrary to mitochondria in Friedreich's heart.

Specific aim 3

Using the reagents that we have recovered, we have now undertaken a collaboration with Professor Cocozza of the University of Naples for a study on the activation of the stress kinases pathways (MKK4, JNK, ERK, p38) in frataxin-deficient P19 cells during neuronal differentiation.

This study is underway and preliminary results should be available shortly.

Specific aim 4

We have analyzed the gene expression in frataxin deficient mice (knock-in / knock-out mice with 20 % of wild type frataxin levels) that not show symptoms of visible pathology, so as to avoid detecting changes due to cell loss or inflammation.

A custom 10K element cDNA microarray was probed with cDNA from three brain regions dissected from four frataxin knock-out / knock-in mice and from littermate controls.

Several analyses were performed including comparison of gene expression in males and females and identification of genes that were altered in common in cervical spinal cord and brainstem. Each of these analyses identified different but overlapping set of genes, which suggests that there is a significant neuroprotective compensatory response to the 80 % reduction in frataxin. Genes with significant alterations included those within functional categories of iron homeostasis regulation and oxidative stress response, in addition to signal transduction, metabolic, and the neuronal development pathway. These results need to be confirmed using more animals and independent methods such as RT-PCR.

Annual Report of the Research Group of

Prof. Dr. Parmentier M.

Université Libre de Bruxelles
(U.L.B.)

Promoter: Marc Parmentier,

IRIBHN, ULB Erasme,
808 route de Lennik,
1070 Brussels

Co-promoters:
Jean-Marie Boeynaems,
Catherine Ledent

Local collaborators:
Sébastien Ballet, Jérôme Becker, Jean-Marie Boeynaems, Didier Communi, Laila El Asmar, Patrick Laurent, Catherine Ledent, Frédéric Libert, Arielle Maho, Isabelle Migeotte, Jean-François Mirjolet, Audrey Sutherland, Jean-Yves Springael, Gilbert Vassart, Valérie Wittamer.

1. Overview.

The activities of the group are centered onto G protein-coupled receptors, which represent the largest family among membrane receptors. They all share a common structural organization with seven transmembrane segments, and a common way of modulating cell function through a family of heterotrimeric G proteins. About 160 G protein-coupled receptor types and subtypes have been functionally characterized to date in mammalian species, and about 120 orphan receptors are presently available in the literature or the databases. Orphan receptors potentially constitute elements of unknown communication pathways in various systems. The general aim of this program is to identify novel receptors playing a role in brain physiology. The characterization of the function of known receptors or recently characterized receptors, by using, among other approaches, knockout models, has also been pursued.

2. Cloning and expression of orphan neuropeptide receptors

With the aim of identifying the natural ligands of orphan receptors, we have established over the past years a collection of cell lines expressing genes encoding putative neuropeptide receptors collected in the databases and the human genome sequencing program. Altogether, about ninety orphan receptors have now been cloned, most of them of human origin. The coding region is inserted in a bicistronic expression vector that directs the production of a transcript encoding both the receptor and the aminoglycoside 3' phosphotransferase (G418 resistance), through an internal ribosome entry site (IRES). This expression vector has been widely used in the laboratory and allows to obtain high expression levels and long term stability of the cell lines. The recombinant plasmids were expressed in CHO cells adapted to functional screening. After selection with neomycin, individual clones were isolated and tested for receptor expression by northern blotting.

The screening is based on the coexpression of a receptor, mitochondrial apoaequorin and a transduction protein ($G_{\alpha 16}$) allowing to couple most receptors to the activation of phospholipase Cβ and calcium mobilization. In this system, the cells are incubated with coelenterazine, the aequorin co-factor, for reconstitution of the active form of the enzyme. When the cells are exposed to an agonist of the receptor, intracellular calcium release results in the activation of aequorin, that oxidizes coelenterazine and yields apoaequorin, coelenteramide, CO_2 and light. Light emission is recorded in a microplate luminometer and integrated over a period of 20 to 30 seconds. This system turned out to display a high signal to noise ratio (> 50), a high throughput, and excellent reproducibility and signal stability over time. This assay is now used routinely for both the screening of biological activities in complex mixtures and the pharmacological characterization of receptors.

As some receptors display constitutive activity, or other unknown characteristics that result in the counterselection of cell lines expressing them, we have introduced over the past year an inducible expression system, based on the tet repressor, that allows to keep cell lines without receptor expression, and to boost the expression upon addition of tetracyclin analogs. This system, that has required the adaptation of the cell lines and vectors used, has allowed to express efficiently receptors that were not functional in the systems used previously, as a

consequence of poor expression or deletions in the coding sequence.

The candidate peptidergic receptors have been tested for their functional response to a variety of known peptides, fractions of porcine brain extracts and other natural sources of potential agonists, as well as libraries of random peptides. Acidic or methanolic extracts of porcine brain and other tissues were fractionated by HPLC before testing. Several biological activities have resulted from this screening, and these activities are being described below.

3. Characterization of ligands for the orphan GPR7 and GPR8 receptors

GPR7 and GPR8 are two structurally related orphan G protein-coupled receptors, presenting high similarities with opioid and somatostatin receptors. Two peptides, L8 and L8C, derived from a larger precursor, were recently described as natural ligands for GPR8 (Mori et al., Patent Application WO 01/98494A1). L8 is a 23-amino acid peptide, whereas L8C is the same peptide with a C terminus extension of 7 amino acids, running through a dibasic motif of proteolytic processing. Using as a query the amino acid sequence of the L8 peptide, we have identified in DNA databases a human gene predicted to encode related peptides and its mouse ortholog. By analogy with L8 and L8C, two peptides, named L7 and L7C could result from the processing of a 125-amino acid human precursor through the alternative usage of a dibasic amino acid motif. The activity of these four peptides was investigated on GPR7 and GPR8. In binding assays, L7, L7C, L8, and L8C were found to bind with low nanomolar affinities to the GPR7 and GPR8 receptors expressed in Chinese hamster ovary (CHO)-K1 cells. They inhibited forskolin-stimulated cAMP accumulation through a pertussis toxin-sensitive mechanism. The tissue distribution of prepro-L7 (ppL7) and prepro-L8 (ppL8) was investigated by reverse transcription-PCR. Abundant ppL7 transcripts were found throughout the brain as well as in spinal cord, spleen, testis, and placenta; ppL8 transcripts displayed a more restricted distribution in brain, with high levels in substantia nigra, but were more abundant in peripheral tissues. The ppL7 and ppL8 genes therefore encode the precursors of a class of peptide ligands, active on two receptor subtypes, GPR7 and GPR8. The distinct tissue distribution of the receptor and peptide precursors suggest that each ligand and receptor has partially overlapping but also specific roles in this signaling system (Brezillon et al. 2003).

4. Ongoing characterization of additional receptors

Human chemokine receptor (HCR) is a putative chemokine receptor sharing high similarity with CCR1, CCR2, CCR3 and CCR5. We generated monoclonal antibodies directed at human HCR, and studied its distribution in human leukocyte populations and cell lines, and its regulation following maturation or activation of these populations. In peripheral blood leukocytes, HCR is expressed on CD4+ and CD8+ T lymphocytes, including most memory and part of naive cells, but is absent from B cells. HCR is present on monocytes and macrophages. Monocyte-derived dendritic cells harbored HCR, and expression was enhanced following stimulation by lipopolysaccharides, poly (I:C), IFN-gamma or CD40L. Neutrophils strongly expressed HCR. This large distribution across leukocyte populations, and the up-regulation during DC maturation, represent a new profile among chemokine receptors (Migeotte et al. 2002). Expression of this

orphan receptor has also been demonstrated by immunohistochemistry in neuroglial cells (unpublished).

In parallel, we have pursued the characterization of the natural ligand of the orphan receptor ChemR23, that was previously isolated from human ascitic fluid and identified by mass spectrometry. The human recombinant protein was shown to promote chemotaxis of dendritic cells and macrophages. This work is in the process of being fulfilled and should be published shortly (Wittamer et al. in preparation). The orphan receptors GPR41 and GPR43 have been identified as responding to short chain fatty acids. The pharmacology and function of these receptors is being worked out presently (Le Poul et al. unpublished).

5. Insect neuropeptide receptors

We have, in collaboration with the laboratory of A. De Loof (KUL) applied some of the procedures used for the characterization of human receptors, to insect receptors studied in that laboratory. The bioluminescent Ca^{2+}-sensitive reporter protein, aequorin, was employed to develop an insect cell-based functional assay system for monitoring receptor-mediated changes of intracellular Ca^{2+}-concentrations. Drosophila Schneider 2 (S2) cells were genetically engineered to stably express both apoaequorin and the insect tachykinin-related peptide receptor, STKR. Lom-TK III, an STKR agonist, was shown to elicit concentration-dependent bioluminescent responses in these S2-STKR-Aeq cells. This aequorin-based method was also utilized to study receptor antagonists (Torfs et al. 2002c).

The activity of a series of synthetic tachykinin-like peptide analogs was studied by means of microscopic calcium imaging on recombinant neurokinin receptor expressing cell lines. A C-terminal pentapeptide (FTGMRa) is sufficient for activation of the stomoxytachykinin receptor (STKR) expressed in Schneider 2 cells. Replacement of amino acid residues at the position of the conserved phenylalanine (F) or arginine (R) residues by alanine (A) results in inactive peptides (when tested at 1 μM), whereas A-replacements at other positions do not abolish the biological activity of the resulting insectatachykinin-like analogs. Calcium imaging was also employed to compare the activity of C-terminally substituted tachykinin analogs on three different neurokinin receptors. The results indicate that the major pharmacological and evolutionary difference between tachykinin-related agonists for insect (STKR) and human (NK1 and NK2) receptors resides in the C-terminal amino acid residues (R versus M). A single C-terminal amino acid change can turn an STKR-agonist into an NK-agonist and vice versa (Torfs et al. 2002b). Structure-function analysis of insect and human neurokinin-like peptides and cognate receptors suggested that the differential Arg-Met preference appears to be a major coevolutionary change between insect and human peptide-receptor couples (Torfs et al. 2002a).

6. Further characterization of the NPFF1 and NPFF2 receptors

We have previously characterized an orphan GPCR, previously designated OR143, as a functional high affinity receptor for Neuropeptide FF (NPFF) and related peptides. This receptor was reported as the $NPFF_2$ receptor. In collaboration with C. Mollereau (Toulouse), we have further investigated the differential pharmacology of the $NPFF_1$ and $NPFF_2$ receptors, which belong to an opioid-modulatory system including also two peptide precursors (pro-NPFF(A) and pro-NPFF(B)). The pharmacological and functional profiles of human $NPFF_1$ and $NPFF_2$ receptors expressed in Chinese hamster ovary (CHO) cells were compared by determining the affinity of several peptides derived from both NPFF precursors and by measuring their abilities to inhibit forskolin-induced cAMP accumulation. Each NPFF receptor recognizes peptides from both precursors with nanomolar affinities, however, with a slight preference of pro-NPFF(A) peptides for $NPFF_2$ receptors and of pro-NPFF(B) peptides for $NPFF_1$ receptors. BIBP3226 and BIBO3304, two selective neuropeptide Y Y1 receptor antagonists, display relative high affinities for NPFF receptors and exhibit antagonist properties towards $hNPFF_1$ receptors. The structural determinants responsible for binding of these molecules to NPFF receptors were investigated and led to the synthesis of $hNPFF_1$ receptor antagonists with affinities from 40 to 80 nM. Our results demonstrate differences in pharmacological characteristics between $NPFF_1$ and $NPFF_2$ receptors and the feasibility of subtype-selective antagonists (Mollereau et al. 2002).

7. Purinergic receptors.

Purinergic receptors constitute a subfamily of G protein-coupled receptors that contains 5 functional receptors (P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11). Three of these receptors have been described in our laboratory (P2Y4, P2Y6 and P2Y11).

Dendritic cell (DC) pulsing with antigen-encoded mRNA results in the loading of both major histocompatibility complex class I and II antigen presentation pathways and the delivery of an activation signal, leading to the induction of a potent primary immune response. We have characterized two signaling pathways utilized by extracellular mRNA to activate DC, and a novel ligand, poly(A), mediates signaling through a receptor that can be inhibited by pertussis toxin and suramin and can be desensitized by ATP and ADP, suggesting a P2Y type nucleotide receptor (Ni et al. 2002).

ATP has been reported to inhibit or stimulate lymphoid cell proliferation, depending on the origin of the cells. Agents that increase cAMP, such as PGE2, inhibit human $CD4^+$ T cell activation. We demonstrated that several ATP derivatives increase cAMP in both freshly purified and activated human peripheral blood $CD4^+$ T cells. The rank order of potency of the various nucleotides was: ATPγS = BzATP > ATP > 2-methylthio-ATP >> dATP, 2-propylthio-β, γ-dichloromethylene-D-ATP, UDP, UTP. This effect did not involve the activation of A_2Rs by adenosine or the synthesis of prostaglandins. Taken together, our results suggest that extracellular adenine nucleotides inhibit $CD4^+$ T cell activation via an increase in cAMP mediated by an unidentified P2YR (Duhant et al . 2002). We have also studied the functional consequences of adenine nucleotides action on human monocyte-derived dendritic cells (DC), we have compared the effects of an ATP analog active on the P2Y11 receptor, on the responses

to three DC stimuli, TNF-alpha, LPS, sCD40L. Our study supports the concept that, like prostaglandin E2 and other agents increasing cyclic AMP, adenine nucleotides favor either a Th2 response or tolerance (Wilkin et al. 2002).

8. Structure-function of CCR5 and chemokines

We have continued to analyze the structure-function relation of CCR5. It was shown previously that the N-terminal extracellular domain of the receptor is essential for its coreceptor activity, but that the second and other extracellular loops also contribute to the complex interaction with the env protein. In binding and functional assays, the second extracellular loop of CCR5 was found to provide specificity for the MIP-1α, MIP-1β and RANTES ligands. It was also found the N-terminus contributed to the binding of chemokines and HIV Env proteins.

CC-chemokine receptor 5 (CCR5) is the principal coreceptor for macrophage-tropic strains of human immunodeficiency virus type 1 (HIV-1). We have generated a set of anti-CCR5 monoclonal antibodies and characterized them in terms of epitope recognition, competition with chemokine binding, receptor activation and trafficking, and coreceptor activity. MC-1 and MC-6 inhibited RANTES, MIP-1β, and Env binding, whereas MC-5 inhibited MIP-1β and Env but not RANTES binding. MC-6 induced signaling in different functional assays, suggesting that this monoclonal antibody stabilizes an active conformation of CCR5. Flow cytometry and real-time confocal microscopy showed that MC-1 promoted strong CCR5 endocytosis. MC-1 but not its monovalent isoforms induced an increase in the transfer of energy between CCR5 molecules. Also, its monovalent isoforms bound efficiently, but did not internalize the receptor. In contrast, MC-4 did not prevent RANTES binding or subsequent signaling, but inhibited its ability to promote CCR5 internalization. These results suggest the existence of multiple active conformations of CCR5 and indicate that CCR5 oligomers are involved in an internalization process that is distinct from that induced by the receptor's agonists (Blanpain et al. 2002). CCR5 receptor oligomerization was further studied by bioluminescence resonance energy transfer (BRET) in cells expressing physiological levels of receptors. A strong energy transfer could be observed, in the absence of ligands, in whole cells and in both endoplasmic reticulum and plasma membrane subfractions, supporting the hypothesis of a constitutive oligomerization that occurs early after biosynthesis. No change in BRET was observed upon agonist binding, indicating that the extent of oligomerization is unrelated to the activation state of the receptor. In contrast, a robust increase of BRET, induced by a monoclonal antibody known to promote receptor clustering, suggests that microaggregation of preformed receptor homo-oligomers can occur. Taken together, our data indicate that constitutive receptor homo-oligomerization has a biologically relevant significance and might be involved in the process of receptor biosynthesis (Issafras et al. 2002). These observations can likely be broadened to many members of the GPCR family.

Using site-directed mutagenesis and molecular modeling in a combined approach, we demonstrated that a cluster of aromatic residues at the extracellular border of transmembrane helices 2 and 3 are involved in chemokine-induced activation. These aromatic residues are involved in interhelical interactions that are key for the conformation of the helices and govern

the functional response to chemokines in a ligand-specific manner. We therefore suggest that transmembrane helices 2 and 3 contain important structural elements for the activation mechanism of chemokine receptors, and possibly other related receptors as well (Govaerts et al. 2003). We have also compared the binding and functional properties of chemokine variants onto wild-type CCR5 and CCR5 point mutants. Altogether, our results suggest that the core domains of MIP-1α and RANTES bind distinct residues in CCR5 extracellular domains, while the N-terminus of chemokines mediates receptor activation by interacting with the transmembrane helix bundle (Blanpain et al. 2003). Finally, additional mutagenesis studies on MIP-1β have shown that basic residues Arg18, Lys19, and Arg22 of the chemokine are essential for its CCR5 binding properties, without a primary effect on CCR5 activation (Bondue et al. 2002).

9. Further characterization of a mouse knock-out model for the A_{2a} adenosine receptor.
Adenosine is released from metabolically active cells or generated extracellularly. It is a potent biological mediator modulating the activity of numerous cell types, including neurons, platelets, neutrophils and mast cells, and smooth muscle cells in bronchi and vasculature. Most of these effects contribute to the protection of cells and tissues during stress conditions such as ischaemia. We had previously generated a knockout model for the A_{2a} receptor, which is abundant in basal ganglia, vasculature and platelets, and is considered as a major target for caffeine. In these mice, caffeine was turned into a depressant of exploratory activity. Knockout animals also scored higher in anxiety tests, were more aggressive, and had increased blood pressure and heart rate. Platelet aggregation was increased (Ledent et al. Nature 388: 674-678, 1997).

Additional experiments were made in collaboration with various groups around the world, in order to delineate further the role of adenosine receptors in various aspects of physiology. To clarify the relative roles of A_2 adenosine receptor subtypes in the regulation of coronary flow and myocardial contractility, coronary vascular and functional responses to adenosine and its analogs were examined in isolated wild-type and A_{2A} receptor knockout mouse hearts. It was concluded that A_{2B} adenosine receptor activation increases coronary flow and developed pressure in isolated murine hearts (Morrisson et al. 2002). Another study indicated that the presence of adenosine A_3 receptors may either inhibit or negatively modulate coronary flow mediated by other adenosine receptor subtypes (Taludker et al. 2002).

A large body of evidence indicates important interactions between the adenosine and opioid systems in regulating pain at both the spinal and supraspinal leve. To investigate whether there are any compensatory alterations in opioid systems in A_{2A} knock-out animals, we have performed quantitative autoradiographic mapping of mu, delta, kappa opioid, and ORL1 receptors in the brains and spinal cords of wild-type and homozygous mice. In addition, mu-, delta-, and kappa-mediated antinociception using the tail immersion test was tested in wild-type and homozygous A_{2A} receptor knock-out mice. A significant reduction in [^{3}H]deltorphin-I binding to delta receptors and a significant increase in [^{3}H]CI-977 binding to kappa receptors was detected in the spinal cords but not in the brains of the knock-out mice. Mu and ORL1 receptor expression were not altered significantly. Moreover, a significant reduction in delta-

mediated antinociception and a significant increase in kappa-mediated antinociception were detected in mutant mice, whereas mu-mediated antinociception was unaffected. Comparison of basal nociceptive latencies showed a significant hypoalgesia in knock-out mice when tested at 55°C but not at 52°C. The results suggest a functional interaction between the spinal delta and kappa opioid and the peripheral adenosine system in the control of pain pathways (Bailey et al. 2002).

We have shown previously that the severity of handling-induced convulsions during ethanol withdrawal was reduced in A_{2A} receptor knock-out ($A_{2A}R^{-/-}$) mice. We have further characterized the role of adenosine A_{2A} receptors in ethanol consumption and neurobiological responses to this drug of abuse. $A_{2A}R^{-/-}$ mice showed increased consumption of solutions containing ethanol compared with wild-type ($A_{2A}R^{+/+}$) control mice. This slightly higher ethanol consumption was also related to increased ethanol preference. In contrast, $A_{2A}R^{-/-}$ mice showed normal consumption of solutions containing either sucrose or quinine. Relative to $A_{2A}R^{+/+}$ mice, $A_{2A}R^{-/-}$ mice were found to be less sensitive to the sedative effect of ethanol, as measured by more rapid recovery from ethanol-induced loss of righting reflex, and to the hypothermic effects of ethanol, although plasma ethanol levels did not differ significantly between the two genotypes. The selective adenosine A_{2A} receptor antagonist ZM 241385 significantly attenuated ethanol-induced hypothermia in CD1 mice. To assess whether ethanol administration would induce differential tolerance in $A_{2A}R^{-/-}$ and wild-type mice, we administered ethanol over 4 consecutive days and found no difference in the development of tolerance; however, female $A_{2A}R^{-/-}$ mice showed a lower tolerance-acquisition rate. These data suggest that activating the A_{2A} receptors may play a role in suppressing alcohol-drinking behavior and is associated with the sensitivity to the intoxicating effects of acute ethanol administration (Naasila et al. 2002).

10. Characterization of a mouse knock-out model for the central cannabinoid receptor CB1.

We had previously generated a knockout model for the CB_1 receptor, the central receptor for the active compounds of Cannabis, including Δ^9-tetrahydrocannabinol (Δ^9-THC), and for the endogenous cannabinoid anandamide. The effects of cannabinoids on body temperature, nociceptive threshold, locomotor activity and blood pressure were not observed for mutant mice, demonstrating that the main pharmacological responses to Δ^9-THC are indeed mediated by the CB_1 receptor. Since an interaction between the opioid and cannabinoid systems had been proposed, morphine-induced antinociception and hypothermia, as well as its reinforcing properties and the development of tolerance and physical dependence had been investigated as well. The antinociceptive effects of morphine and the development of tolerance to morphine were not modified. However, the reinforcing effects of the drug and the morphine withdrawal syndrome were affected (Ledent et al. Science 285 : 401-404, 1999).

This model was further tested in collaboration with a number of groups. To establish the role of the CB1 cannabinoid receptor in several emotional-related behavioural responses, including aggressiveness, anxiety, depression and learning models, using CB1 knockout mice. We evaluated the spontaneous responses of CB1 knockout mice and wild-type controls under different behavioural paradigms, including the light/dark box, the chronic unpredictable mild stress, the resident-intruder test and the active avoidance paradigm. Our findings showed that CB1 knockout mice presented an increase in the aggressive response measured in the resident-intruder test and an anxiogenic-like response in the light/dark box. Furthermore, a higher sensitivity to exhibit depressive-like responses in the chronic unpredictable mild stress procedure was observed in CB1 knockout mice, suggesting an increased susceptibility to develop an anhedonic state in these animals. Finally, CB1 knockout mice showed a significant increase in the conditioned responses produced in the active avoidance model, suggesting an improvement of learning and memory processes. These findings demonstrate that endogenous cannabinoids through the activation of CB1 receptors are implicated in the control of emotional behaviour and participate in the physiological processes of learning and memory (Martin et al. 2002). We have also evaluated the possible role of CB1 cannabinoid receptor in responses induced by acute and repeated nicotine administration. Our results demonstrate that some acute effects and motivational responses elicited by nicotine can be modulated by the endogenous cannabinoid system and support the existence of a physiological interaction between these two systems (Castane et al. 2002).

A study That used a number of specific agonists and antagonists of cannabinoid receptors suggested that there is a non-CB1 non-CB2 receptor present in the brain of CB1-/- mice (Monory et al. 2002). Other data suggest that the novel receptor might play a role in anxiety (Haller et al. 2002).

Activation of cannabinoid receptors causes inhibition of spasticity, in a mouse model of multiple sclerosis, and of persistent pain, in the rat formalin test. The endocannabinoid anandamide inhibits spasticity and persistent pain. It not only binds to cannabinoid receptors but is also a full agonist at vanilloid receptors of type 1 (VR1). We found that vanilloid VR1 receptor agonists (capsaicin and SDZ-249-665) exhibit a small, albeit significant, inhibition of spasticity that can be attenuated by the vanilloid VR1 receptor antagonist, capsazepine. Arvanil, a structural "hybrid" between capsaicin and anandamide, was a potent inhibitor of spasticity at doses where capsaicin and cannabinoid CB1 receptor agonists were ineffective. The anti-spastic effect of arvanil was unchanged in cannabinoid CB1 receptor gene-deficient mice or in wildtype mice in the presence of both cannabinoid and vanilloid receptor antagonists. Likewise, arvanil exhibited a potent analgesic effect in the formalin test, which was not reversed by cannabinoid and vanilloid receptor antagonists. These findings suggest that activation by arvanil of sites of action different from cannabinoid CB1/CB2 receptors and vanilloid VR1 receptors leads to anti-spastic/analgesic effects (Brooks et al. 2002).

Inhibition of prostaglandins synthesis does not completely explain non-steroidal anti-inflammatory drug-induced spinal antinociception. Among other mediators, endocannabinoids are involved in pain modulation. We have suggested that at the spinal level, indomethacin

induces a shift of arachidonic acid metabolism towards endocannabinoids synthesis secondary to cyclooxygenase inhibition. In addition, it lowers NO levels with subsequent higher levels of endocannabinoids (Guhring et al. 2002).

Anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) are the most active endocannabinoids at brain (CB1) cannabinoid receptors. CB1 knockout mice were compared with wildtype littermates for their ability to degrade AEA through an AEA membrane transporter (AMT) and an AEA hydrolase (fatty acid amide hydrolase, FAAH). AMT and FAAH activity were found to increase with age in KO, but not WT, mice and to be higher in the hippocampus than in the cortex of all animals. These results suggest that invalidation of the CB1 receptor gene is associated with age-dependent adaptive changes of endocannabinoid metabolism which appear to correlate with the waning of the anxiety-like behaviour exhibited by young CB1 KO mice (Maccarrone et al. 2002).

11. Knock out of orphan receptors.

We have continued the breeding and the analysis of the phenotype of the mouse knock out models generated for the orphan receptors JP05/GIR, ACCA, PRPR/GPR10 and GPR-NGA. Some of these data will be finalized and published shortly

12. Development of microarrays

We have initiated the construction of human and mouse microarrays that will be used in the study of signaling pathways of receptors in cell lines as well as in comparing phenotypes of knock out and control lice. The validation steps of the construction, hybridization and analysis of these arrays have been fulfilled, and actual experiments will be carried out in the near future on several models.

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Annual Report of the Research Group of

Dr. Ris L.

Université de Mons Hainaut
(U.M.H.)

Dr. L. Ris

University of Mons-Hainaut
Laboratory of Neurosciences
Place du Parc, 20
B-7000 Mons
Tel.: +32.65.37.35.70
Fax: +32.65.37.35.73

During last year, our laboratory tackled three projects. The first was vestibular compensation; the second concerned perturbations of long-term potentiation (LTP) in transgenic mice related to Alzheimer's disease; and the third studied was related to the mechanisms of late-LTP.

I. VESTIBULAR COMPENSATION

Background

At rest, in the absence of any movement of the head, the neurons of the vestibular nucleus are spontaneously active. After a unilateral labyrinthectomy, this basal activity which is very important for our static equilibrium, ceases. However, in the guinea pig, it recovers completely in a few days. We have investigated the potential mechanisms underlying this recovery, a dramatic example of brain plasticity.

Experiments on brainstem slices

We investigated the properties of the vestibular neurons in brainstem slices, using intracellular microelectrodes. Responses to currents injected intracellularly in vestibular neurons from control animals were compared to those obtained in vestibular neurons from animals labyrinthectomized one week earlier. Using this technique, first we have demonstrated that the performances of the spike generator, which transforms synaptic currents into a pattern of action potentials, is not changed by labyrinthectomy (NeuroReport, 2002). Then, we investigated the pacemaker currents in the vestibular neurons. We have found that one of them, the low-threshold calcium current, was increased after labyrinthectomy. Using antibodies against the 3 species of low-threshold calcium channels (α1G, α1H and α1I), we have shown that the observed increase in calcium current was not related to an increase synthesis of these channels (this work is submitted for publication). It shows that adult neurons deprived of one of their major synaptic inputs undergo modifications in their electroreceptors, a form of plasticity which has been hitherto underestimated.

Experiments on neuronal culture

In this approach, we intended to isolate in a co-culture the neurons of the vestibular ganglion and the neurons of the vestibular nucleus, in order to be able to study phenomena similar to vestibular compensation on a more simple preparation. Up to now, we have succeeded to obtain such a co-culture living for 30 days. In a first step, we studied the electrophysiological maturation of the vestibular neurons in culture in the absence of the vestibular afferent fibers. After an initial regression due to the plating procedure, the neurons matured. However, the degree of maturation achieved after 30 days was less than observed in neurons from adult animals and similar to that shown by neurons from newborn rats. (Neuroscience Letters, in press).

II. SYNAPTIC PLASTICITY IN ALZHEIMER'S DISEASE

For a few years, we are collaborating with the laboratory of Prof. VAN LEUVEN (KUL) which has generated different strains of transgenic mice in relation with Alzheimer's disease. On one hand, it is well known that patients suffering from Alzheimer's disease are afflicted with severe memory deficits. On the other hand, there is now general agreement among the scientific community that memories are underlied by changes in synaptic strength. We therefore decided to study the long-term potentiation (LTP) of the synaptic strength induced by high frequency stimulation of the presynaptic fibers in hippocampal slices of transgenic mice related to Alzheimer's disease. In 1999, we discovered that LTP was impaired in mice carrying a mutation of the amyloid precursor protein (APP) (Moechars et al., The Journal of Biological Chemistry, 1999, 274, 6483-6492). This year we found that a neuronal deficiency of presenilin 1 corrects the hippocampal LTP-defect of APP [V717] transgenic mice (Journal of Neuroscience, 2002).

III. MEMORY AND LONG-TERM POTENTIATION

We are currently interested in the mechanisms of the LTP which can be induced in hippocampal slices. We usually induced LTP in the synapses between Schaffer's collaterals and CA1 hippocampal neurons by high frequency stimulation of the Schaffer's collaterals. In this case, the major triggering event is an entry of Ca^{++} in the CA1 neurons through the NMDA receptors. The experiment carried on this year was based on the following assumptions: (1) a Ca^{++} entry is necessary to induce LTP. (2) Ca^{++} is stored intracellularly in the endoplasmic reticulum. When those stores decline, a signal is generated which induces the opening of particular channels in the plasma membrane (store-operated channels). This mechanism of replenishment of the Ca^{++} store is called "Capacitative Calcium Entry". (3) In fibroblasts lacking presenilin, CCE is increased. This year, we have demonstrated that a chemical induction of CCE in the neurons of hippocampal slices triggered a LTP. This means that presenilin 1 is involved in the mechanism of replenishment of Ca^{++} stores in intact neurons embedded in intact neuronal networks. This work has been submitted to the European Journal of Neuroscience (Ris et al., submitted).

PUBLICATIONS 2002

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THESIS

- On December 17 (2002), Laurence RIS defended a thesis entitled " Modifications de l'excitabilité intrinsèque des neurones du noyau vestibulaire induites par une labyrinthectomie ipsilatérale " to obtain the degree of " Agrégé de l'Enseignement Supérieur ".

Annual Report of the Research Group of

Prof. Dr. Schiffmann S.N.

Université Libre de Bruxelles
(U.L.B.)

Promoter:

Prof. Dr. Serge N. Schiffmann

Laboratory of neurophysiology,
Department of neuroscience
Faculty of medicine
808 route de Lennik - CP601
B-1070 Bruxelles

Senior collaborators:
Jean-Marie Vanderwinden
Alban de Kerckhove
David Gall

I. Physiology and physiopathology of the basal ganglia system

We studied the modulation of the corticostriatal glutamatergic transmission by adenosine in the accumbens nucleus on brain slices from wild-type and A_{2A} receptor-deficient mice ($A_{2A}r^{-/-}$) (7). We showed that the A_{2A} receptor is not involved in the regulation of the basal excitatory synaptic transmission in the accumbens. Long term potentiation (LTP) of the AMPA receptor-mediated synaptic transmission could be elicited in both wild-type and $A_{2A}r^{-/-}$ mice. However, LTP appeared to be quantitatively modulated by the A_{2A} receptor pathway since the level of potentiation was reduced in mutant mice (7). This observation was confirmed by a decrease in the LTP level in wild-type striatal slices treated with a specific A_{2A} receptor antagonist and the involvement of the cAMP cascade has been suggested since an inhibitor of PKA similarly reduced the LTP level in wild-type slices. The A_{2A} receptor is therefore implicated in the induction of cortico-accumbal LTP, an effect that could be related to its involvement in long-term behavioral sensitization to repeated dopaminergic treatment. We have arguments supporting that A_{2A} receptor-mediated effects are related to post-synaptic mechanisms and our working hypothesis is that the induction of LTP would be different in striatopallidal neurons bearing A_{2A} receptor and in striatonigral neurons that do not. To test this hypothesis, we developped the single-cell RT-PCR approach in order to identify the recorded neurons and to classify them in the two main subpopulations. We are now able to detect mRNAs encoding D_1, D_2, D_3, D_4, D_5 receptors, A_1, A_{2A}, A_{2B}, A_3 receptors, GAD65, GAD67, substance P and enkephalin in single neurons. Our preliminary results combining single cell PCR and recording of synaptic plasticity by patch clamp suggest that striatopallidal or striatonigral neurons behave differentially when an identical protocol of induction of synaptic plasticity was used.

In line with our previous studies, we studied the interactions between A_{2A} and dopamine D_2 receptor in controlling the neuronal excitability of striatal neurons. The objective is to clearly dissect out the effects related to receptors heterodimerization from those due to activation or inhibition of intracellular cascades. Different strategies were started including the recording of D_2 receptor-activated K^+ channel in whole cell configuration, recording of the same K^+ channel in outside-out configuration and recording of the D_2 receptor-mediated modulation of L-type Ca^{2+} currents in whole cell together with the blockade of any putative effect of A_{2A} receptor via an intracellular cascade with the $G_{s\alpha}$-selective G protein antagonist NF449. We also previously developed the analysis of the sodium current recorded in conditions of PKA activation using a minimal molecular model of the channel gating mechanism to fit experimental data. The validity of this model and the accuracy of its predictions was further evaluated in a series of experiments dealing with the modulation of sodium currents by serotonin (5).

We have pursued the construction of transgenic mice allowing the study of the specific roles of striatopallidal or striatonigral neurons. We have now obtained clones of ES cells expressing the floxed GAD67 gene and will proceed to the steps of incorporation of this construct in the genome of wild type mice. We have also obtained mice strains expressing the CRE recombinase under the control of the A_{2A} receptor promotor inserted in a BAC (bacterial artificial chromosome). These lines are currently crossed with a reporter strain (Rosa26) in order to determine whether they selectively expressed CRE in striatopallidal neurons.

Striatopallidal and striatonigral neurons are differentially affected in pathologies of the basal ganglia system through mechanisms remaining unknown. We have developed an experimental model of Huntington's disease in rat or mouse by using subchronic injection of 3-nitropropionic acid (3NP) and demonstrated the high reproducibility of this model in terms of lesion. Using this model, we have now determined the time-course of neurochemical changes occurring within both the striatum and the cerebral cortex (2). We found that the occurrence of striatal lesions was accompanied by: (i) a strong transcriptional alteration within the degenerative lateral striatum, (ii) receptor upregulations within the preserved medial striatum and (iii) transcriptional increases within the unaltered cerebral cortex. These phenomena were preceded by transcriptional modifications the day before lesion formation. Of great interest, we found that the density of A_{2A} receptor binding sites, known to be located on the striato-pallidal neurons, was downregulated at the time of symptoms worsening and strongly upregulated within the spared striatum after the lesion occurrence. This study thus highlights the differential modulations of striatal and cortical signalling produced by 3NP in this phenotypic model of Huntington's disease.

The high reproductibility of this 3NP model allows studies of neuroprotection. We have characterized the neuroprotective effect of adenosine amine congener (ADAC), a specific A_1 receptor agonist known to be devoid of any of the side effects usually impairing the clinical use of such compounds. We have observed that an acute, treatment with ADAC (100 μg/kg/d) not only strongly reduces the size of the striatal lesion (-40 %) and the remaining ongoing striatal degeneration (-30 %), but also prevents the development of severe dystonia of hindlimbs (3). Electrophysiological recording on cortico-striatal brain slices demonstrated that ADAC strongly decreases the post-synaptic field excitatory potential amplitude by 70 % whereas it has no protective effect up to 1μM against the 3NP-induced neuronal death in primary striatal cultures. This suggests that ADAC protective effects may be mediated pre-synaptically by the modulation of the energetic impairment-induced striatal excitotoxicity. Altogether, our results indicate that A_1 receptor agonists deserve further experimental evaluation in animal models of Huntington's disease (3).

II. Involvement of the regulation of calcium homeosatsis by calcium binding proteins in the cerebellar physiology

We have pursued the study of the cerebellar physiology of different strains of mice deficient in calcium binding proteins such as calretinin, calbindin and parvalbumin, through an approach combining genesis of new transgenic mice, in vitro electrophysiology, in vivo electrophysiology and behavioural analysis (8).

In this line, we have used patch clamp recording techniques in acute slice preparation to investigate the effect of a null mutation of the calretinin gene on the intrinsic electroresponsiveness of cerebellar granule cells at a mature developmental stage (11). Calretinin deficient granule cells exhibit faster action potentials and generate repetitive spike discharge showing an enhanced frequency increase with injected currents. These alterations disappear when 0.15 mM of the exogenous fast calcium buffer BAPTA is infused in the cytosol

to restore the calcium buffering capacity. A mathematical model has been proposed demonstrating that the observed alterations of the granule cell excitability can be explained by a decreased cytosolic calcium buffering capacity due to the absence of calretinin. This result suggests that calcium binding proteins modulate intrinsic neuronal excitability and may therefore play a role in the process of information transfer (11).

The absence of calretinin in cerebellar granule cells constitute a main hypothesis consistent with the perturbations that we previously demonstrated in $Cr^{-/-}$ mice. To investigate this hypothesis, we specifically rescued the expression of calretinin in the cerebellar granule cells of $Cr^{-/-}$ mice. The calretinin expression was targeted to cerebellar granule cells by using a fragment of the gene coding for the $GABA_A$ $\alpha 6$ subunit encompassing the promoter and the exons 1 to 8. This part of the gene has been previously shown to allow restricted transgene expression in cerebellar granule cells. We obtained several lines of transgenic $Cr^{-/-}$ mice exhibiting a selective and restricted re-expression of calretinin in granule cells as demonstrated by in situ hybridization, RT-PCR and imunohistochemistry. A first series of preliminary experiments in these strains of mice suggested that the rescue of calretinin in granule cells dose-dependently restores a normal firing behavior of Purkinje cells recorded in alert mice.

III. Molecular characterization of the gastrointestinal pacemaker mechanism.

In line with our previous studies, the distribution of the interstitial cells of Cajal (ICC), identified by their immunoreactivity for the receptor tyrosine kinase KIT (KIT+ ICC), was characterized in several human disorders of the gastrointestinal (GI) transit. The pathology of the cardia in Allgrove's (3A) syndrome (6) and the pathology of a case of chronic intestinal pseudoobstruction (4) have been reported. Other diseases and a quantitative pathological approach are currently being considered.

The distribution of the intermediate filament nestin in the postnatal human GI tract has been clarified. Nestin immunoreactivity is present in some, but not all, ICC and in enteric glial cells. Fibroblast-like cells (FLC), enteric neurons and smooth muscle cells are nestin negative (9).

The distribution of the Calcium-activated K^+ channel SK3 has been investigated in man and mouse. This channel could play an important role in the control of excitability of the smooth musculature. Recent literature provided conflicting results about the distribution of SK3 immunoreactivity (SK3-ir) in the GI tract, claimed to be present either on KIT-ir ICC or on enteric nerves and smooth muscle cells. We have established that SK3-ir is actually exclusively present on CD34-ir FLC but not on KIT-ir ICC. Furthermore, in human disorders (infantile hypertrophic pyloric stenosis and Hirschsprung's disease) where we have previously demonstrated a lack of KIT-ir and in a model of ICC deficient mice (W^{lacZ}/W^{v}), the distribution of SK3-ir cells, as well as of CD34-ir FLC, was unaffected. By semi quantitative PCR, the level of expression of SK3 mRNA in the intestine was similar in ICC deficient (W^{lacZ}/W^{v}) mice and healthy littermates (10). Our observations suggest for the first time the possible involvement of KIT negative, SK3-ir FLC in the control of excitability of the GI smooth musculature. This

represents a novative concept in the field of GI motility that we currently investigate by means of a transgenic mouse model allowing conditional expression of SK3.

The search for genes involved in the pacemaker function of ICC has been initiated in the laboratory. Substractive PCR experiments have been performed in order to identify genes expressed in the normal muse jejunum but absent in the jejunum of ICC deficient (W^{lacZ}/W^{v}) mice. This represents an innovative approach in the field of ICC. Candidate genes picked up in 2 independent experiments are currently evaluated by quantitative PCR, Northern blotting and in situ hybridization.

In collaboration with Janssen Research Foundation (Beerse, Belgium), transgenic mice have been produced which express a " enhanced green fluorescent protein " (EGFP) under the control of the c-kit gene promoter. Tests are currently under progress to confirm that the various KIT-expressing cell types (including the ICC in the GI tract) actually express the EGFP. This approach - that has been awarded a patent - may open numerous opportunities for the study of ICC development and function in vivo et in vitro.

IV. CCR5 chemokine receptor trafficking

In collaboration with Dr Cedric Blanpain and Prof. Marc Parmentier (ULB), we studied the receptor internalisation and trafficking kinetics in living cells expressing a recombinant CCR5 chemokine receptor tagged with EGFP (enhanced green fluorescent protein) and various of its mutants, by means of time-lapse confocal microscopy and FRAP (fluorescence recovery after photobleaching) (1).

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Annual Report of the Research Group of

Prof. Dr. Van Broeckhoven C.

Universitaire Instelling Antwerpen (U.I.A.)

Laboratorium voor Moleculaire Genetica

Born-Bunge Stichting (BBS)
Universitaire instelling Antwerpen
Departement Biochemie/Biomedische Wetenschappen
Universiteitsplein 1
B-2610 Antwerpen

Principal Investigator
Prof. Dr. Christine Van Broeckhoven, Ph.D., D.Sc.

Co-Investigator
Prof. Dr. Peter De Jonghe, MD, PhD
Prof. Dr. Vincent Timmerman, PhD

Personnel paid by GSKE
Technician
Tim De Pooter (01/07/2002)

Personnel involved in the project
Scientific personnel

Nadia Ammar (01/01/2002 - 31/12/2002), Dominique Audenaert, Katrien Coen (01/10/2002), Liesbet Deprez (01/10/2002), Ines Dierick (01/10/2002), Berten Ceulemans, Lieve Claes, Marc Cruts, Joy Irobi, Samir Kumar-Singh, Bart Dermaut (01/01/2002 - 31/12/2002), Albena Jordanova (01/11/2002 - 31/10/2002), Eva Nelis, Rosa Rademakers, Jessie Theuns, Kristien Verhoeven, Nathalie Verpoorten, Monika Zabielski (21/06/2002)

Technicians

Samira Bel Kacem, Ellen Corsmit, Els De Vriendt, An Jacobs, Ann Löfgren, Karin Peeters (75%), Sally Serneels, Iris Smouts (25%), Marleen Van den Broeck, Veerle Van Gerwen, Krist'l Vennekens

ALZHEIMER'S DISEASE

Alzheimer disease (AD) is the most frequent form of senile dementia, a progressive degenerative disorder of the central nervous system leading to memory and cognitive dysfunction due to neurodegeneration particularly in the hippocampus and the cerebral cortex. The major risk factor for AD is ageing; however, several studies have provided substantial evidence that genetic factors also play an important role in AD pathophysiology. To date, 3 genes have been identified that, when mutated, cause presenile AD (onset age < 65 years): the amyloid Aß precursor protein gene (APP) and the presenilin 1 and 2 genes (PSEN1, PSEN2) (see "Alzheimer Disease Mutation Database": http://molgen-www.uia.ac.be/ADMutations). Multiple studies have shown that allele ε4 of the apolipoprotein E gene (APOE-ε4) is a risk factor for both, presenile and senile (onset age > 65 years) AD. With recent advances in molecular genetic understandings of AD, the borders between different clinical phenotypes of dementia are blurring with mixed phenotypes making up a continuous spectrum from pure forms of AD to pure forms of other dementias, of which the most important is frontotemporal dementia (FTD). Recent studies have shown that 10-43% of all familial FTD cases are associated with mutations in the gene encoding the microtubule associated protein tau (MAPT). Because of the substantial clinical overlap and phenotypic heterogeneity in these autosomal dominant dementias, careful neuropathological brain examination is crucial in establishing a definite diagnosis. For AD, both Aß plaques and tau-positive neurofibrillary tangles are a prerequisite for the neuropathological diagnosis of AD. FTD can be pathologically classified into three different categories: FTD with neuronal and glial tau depositions, FTD with ubiquitin-positive inclusions and FTD with neuronal loss and spongiosis but without intracellular inclusions. Clinically there are no features that can reliably distinguish between these three groups.

Although insights in molecular genetics of dementias have advanced rapidly during the last years, a substantial number of dementia cases, including autosomal dominant cases, cannot be explained by mutations in the known dementia genes. Also, the normal and aberrant functions of the known dementia genes are poorly understood and no adequate model organisms for this devastating disorder were generated. Therefore, the objectives of this project are to identify novel early-onset dementia genes using genome scans in informative dementia families and to characterize the contribution of these dementia genes in population-based samples. In addition, we aim at better understanding the pathomechanisms of dementia by studying the in vitro effect of newly identified dementia genes on Aß processing and other relevant processes and by constructing good model organisms for AD and other dementias. Finally, also by studying pathological genotype-phenotype correlations we aim at developing new insights in the biochemistry and pathophysiology of AD and related dementias.

Positional cloning of novel dementia genes

Introduction

Multiple families exist that segregate dementia in a manner compatible with autosomal dominant inheritance and in which no mutation was identified in the known dementia genes. This demonstrates that additional, as yet unknown genes exist that, when mutated, cause

autosomal dominant dementia. Our aim is to identify novel causative genes using genome scans in informative dementia families. To this end, we have available DNA samples of 10 autosomal dominant AD families derived from a population-based study of EOAD in the 4 northern provinces of The Netherlands and metropolitan Rotterdam. In 8 families (Table 1), no mutation was identified in the known AD genes APP, PSEN1 and PSEN2.

Table 1 Characteristics of the autosomal dominant AD families derived from the population-based Rotterdam study. The 2 most informative families used in the genome scans are presented in bold.

Family	# Generations	Mean onset age ± SD	# Individuals	# Patients
1027	4	63 ± 6	20	3
1034	4	61 ± 6	55	5
1083	**4**	**62 ± 7**	**84**	**13**
1094	4	70 ± 10	27	7
1104	4	52 ± 7	55	9
1125	3	58 ± 9	11	5
1242	4	72 ± 8	50	4
1270	**4**	**68 ± 8**	**91**	**15**

Genome scan in dementia family 1083

We performed a genome-wide scan in the most informative Dutch dementia family: family 1083 (Rademakers et al., 2002). In family 1083, a four-generation family segregating autosomal dominant dementia, of which the proband was diagnosed with AD according to the NINCDS-ADRDA criteria, the clinical diagnosis was unequivocal in some patients and no autopsy was available. Prior to the genome scan, an extensive clinical follow-up was performed in combination with an update of the pedigree. Reliable clinical information was available for 12 of the 16 affected family members. The mean age at onset in these 12 patients was 64.2 years (range 53 to 79 years). The clinical presentation of dementia was highly suggestive for frontotemporal dementia (FTD) in 10 patients, whereas AD and/or vascular dementia could not be excluded in 2 patients. Brain autopsy in one patient showed atrophy of frontal lobes with severely dilated ventricles. No neuritic or diffuse plaques, ballooned cells, Pick bodies or Lewy bodies were seen. Staining with anti-ubiquitin antibody showed small numbers of intracytoplasmic neuronal inclusions and a moderate number of ubiquitin-positive neurites in the second layer of the frontal and temporal cortex. Phosphorylation-dependent and independent antibodies against tau protein did not stain these inclusions.

DNA was available for 40 individuals: 8 patients, 3 spouses and 29 at risk individuals. In the genome scan, two-point LOD score analyses identified only one marker, D17S1868 at 17q11-q21, with a conclusive LOD score of 3.48 at $\theta = 0$. To define the minimal candidate region, 22 additional chromosome 17 microsatellite markers were analyzed for linkage. The highest LOD score of 4.69 was obtained with D17S951 in the absence of recombinants (Rademakers et al., 2002). Multipoint LOD-score analysis between D17S1787 and D17S958 provided a maximum LOD score of 5.51 at D17S951 ($\theta = 0$) in the interval D17S1789-D17S951-D17S1860. By haplotype reconstruction we were able to identify a risk haplotype that was present in all patients

including 2 stroke patients. Most patients shared the complete risk haplotype spanning the 32 cM region; however, meiotic recombinants in 4 patients defined a candidate region of 4.8 cM between D17S1787 and D17S958.

The candidate region on chromosome 17 contains MAPT in which more than 20 different mutations have been found that lead to familial FTD. We performed an extensive mutation analysis by sequencing all 15 coding exons of MAPT in 7 patients segregating the risk haplotype and 1 unrelated control individual (Rademakers et al., 2002). Because many MAPT mutations affect alternative splicing of exon 10, at least 60 bp of intronic sequence flanking exon 10 was analyzed. Also exon 0 and 1 kb sequence upstream of exon 0, including the 5' untranslated region (5'UTR) and the core- and proximal promoter, as well as intron 13 and 150 bp of the 3'UTR were sequenced. No disease-related mutations were identified.

Family 1083 represents the fifth dementia pedigree without demonstrable MAPT mutations and the third such pedigree with conclusive linkage to chromosome 17q21. With a maximum multipoint LOD score of 5.51 at D17S951 ($\theta = 0$), this is the highest linkage reported for a dementia family linked to this chromosomal region. The 4.8 cM region between D17S1787 and D17S958 completely falls within all regions previously published and significantly reduces the 17q21 locus for dementia without MAPT mutations. Although we cannot exclude the possibility of a mutation in an as yet unanalyzed region of MAPT, the apparent absence of MAPT mutations combined with the lack of tau neuropathology suggests that another defective gene at 17q21 might be responsible for FTD with tau-negative and ubiquitin-positive inclusions (Rademakers et al., 2002).

Other functional candidate genes from within the linked region that we analyzed are saitohin (STH), the gene encoding the glial fibrillary acidic protein (GFAP) and PSEN homologue number 2 (PSH2). Sequence analysis of STH in patients of family 1083 filed to identify a disease-ralated mutation. Mutations in exons 1 to 9 including the alternatively used exon 7A were excluded in patients of family 1083. Sequence analysis of the single exon of PSH2 demonstrated that mutations in PSH2 do not explain the dementia in family 1083.

Family ascertainment

We have a collection of around 350 DNA samples of demented patients that were sent for genetic screening to our diagnostic unit. In addition, we have DNA samples of about 800 demented cases from a Belgian prospective study. From these collections, patients with an early onset of neurodegenerative dementia with positive family history were screened for the 5 major causal dementia genes (APP, PSEN1, PSEN2, PRNP, MAPT). From those patients in whom we did not detect a mutation in these genes, we have currently selected 28 cases that have a family history suggestive for autosomal dominant transmission. Of these patients, families are being contacted to participate in a research program aiming at the identification of the genes responsible for dementia in these families.

Association studies of AD genes

We have large population-based and hospital-based case/control samples of both, presenile and senile AD cases, some of which are derived from genetic isolates. These samples are being used for extended molecular genetic analyses of functional candidate genes for AD and dementia in general.

APOE in AD and FTD

The -491A/T polymorphism in the promoter of the apolipoprotein E gene (APOE) was reported to be associated with AD. We studied the effect of this -491A/T promoter polymorphism on plasma apoE levels and risk for AD in the Dutch population-based case-control study. We found a modest but statistically significant effect of the -491A/T polymorphism on plasma apoE levels independent of the APOE genotype (Roks et al., 2002). The lowest plasma levels were measured for the AA genotype, highest levels for the TT genotype, and intermediate levels for the heterozygotes. There was a small effect of the -491AA genotype on AD risk that disappeared after adjusting for APOE genotypes. Our data suggest that the -491A/T polymorphism has an APOE genotype-independent effect on plasma apoE levels but no APOE-independent effect on AD risk.

Although the apolipoprotein E4 (apoE4) allele has consistently been associated with AD and other types of dementia in many studies, its association with FTD is controversial. Recently it has been shown that semantic dementia, the temporal variant of FTD, may be associated with higher frequencies of the apoE4 allele. We have studied apoE in our sporadic FTD patient population and have assessed whether patients with predominance of temporal atrophy have higher frequencies of the apoE4 allele. Patients were ascertained through a clinico-epidemiological survey of patients with FTD in The Netherlands. Predominant temporal atrophy was semiquantitatively assessed on CT and/or MRI. Nine of 31 patients with temporal atrophy fulfilled the criteria for semantic dementia, 4 patients showed severe problems in language comprehension, although the diagnosis of semantic dementia could not be definitely established due to incomplete or inconclusive neuropsychological testing while the remaining 18 patients showed mainly decreased spontaneous speech and word finding difficulties. APOE genotype and allele frequencies were calculated for each group and compared with non-demented controls. The FTD patients had the APOE4/E4 genotype frequency of 6.0%, compared with 2.3% of non-demented controls ($p=0.04$). This genotype was present in 9.7% of patients with the temporal variant of FTD ($p=0.01$) compared with non-demented controls, compared with only 4.5% in patients with frontotemporal atrophy ($p=0.5$). The frequency of the apoE4 allele in all FTD patients was 21.9%, compared with 15.3% in the non-demented controls ($p=0.02$). In patients with temporal atrophy the apoE4 allele frequency was as high as 29.0% ($p=0.004$), whereas in the patients with frontotemporal atrophy this was only 18.7% ($p=0.3$). Our results show that the apoE4 allele frequency is increased in patients with the temporal variant of FTD compared with non-demented controls.

Nicastrin haplotype in AD

Mutation analysis of the nicastrin gene (NCSTN) in presenile AD patients revealed 14 single nucleotide polymorphisms (SNPs), among which 1 missense mutation (N417Y). In an in vitro assay, N417Y did not show increased Aß secretion suggesting that this mutation is unlikely to be pathogenic. SNP haplotype estimation in presenile AD indicated that 1 haplotype (hapB) showed suggestive association with familial presenile AD (p=0.10), but not with senile AD. In the APOE ε4 negative, familial EOAD subgroup the overall haplotype distribution was significantly different from controls (p=0.01, OR = 4.1, 95% C.I. 1.2-13.3) due to a highly increased frequency of hapB (Dermaut et al., 2002). These results further emphasize the importance of γ-secretase dysfunction in the etiology of familial presenile AD.

In vitro studies of mutations in Aß

In APP, 16 different mutations are identified that lead to AD or a related disorder (http://molgen-www.uia.ac.be/ADMutations/). Four mutations (Ala692Gly or Flemish APP, Glu693Gln or Dutch APP, Glu693Gly or Arctic APP and Asp694Asn or Iowa APP) are different from the others, since they are located within the sequence encoding the Aß fragment and result in altered Aß peptides. Mutations in the Aß sequence of APP present with variable disease phenotypes. While patients with the Dutch APP mutation have predominantly hemorrhagic strokes, Flemish APP patients develop both strokes and AD. To determine whether these diverse clinical and pathological presentations are due to mutant Aß or APP, we studied the effect of the Flemish, Dutch, and wild-type Aß/APP on phosphorylation of specific tau epitopes observed in AD (Kumar-Singh et al., 2002b). No effect was observed in differentiated SH-SY5Y cells either stably expressing APP or treated with synthetic Aß12-42. However, we did observe a paradoxical temporal difference in the neurotoxic potential of mutant and wild-type Aß. While long 24-h incubation at physiological levels of Aß showed a higher amount of apoptosis for Dutch Aß, a short 2-h incubation showed elevated apoptosis for Flemish and wild-type Aß. The altered aggregating properties of Aß, with Dutch Aß aggregating faster and Flemish Aß slower than wild type, elucidated a discrete two-phase Aß neurotoxicity. We propose here that, at least in vitro, Aß might be neurotoxic in an initial phase due to its soluble oligomeric or other early toxic Aß intermediate(s), which is perhaps distinct from the late neurotoxicity incurred by aggregated larger assemblies of Aß.

Pathological studies of mutations in Aß

AD is characterized by deposition of Aß in diffuse and senile plaques, and variably in vessels. Mutations in the Aß-encoding region of APP are frequently associated with very severe forms of vascular Aß deposition, sometimes also accompanied by AD pathology. We earlier described a Flemish APP (A692G) mutation causing a form of early-onset AD with a prominent cerebral amyloid angiopathy and unusually large senile plaque cores. The pathogenic basis of Flemish AD is unknown. By image and mass spectrometric Aß analyses, we demonstrated that in contrast to other familial AD cases with predominant brain $Aß_{42}$, Flemish AD patients predominantly deposit $Aß_{40}$ (Kumar-Singh et al., 2002a). On serial histological section analysis we further showed that the neuritic senile plaques in APP692 brains were centered on vessels.

Of a total of 2400 senile plaque cores studied from various brain regions from three patients, 68% enclosed a vessel, whereas the remainder were associated with vascular walls. These observations were confirmed by electron microscopy coupled with examination of serial semi-thin plastic sections, as well as three-dimensional observations by confocal microscopy. Diffuse plaques did not associate with vessels, or with neuritic or inflammatory pathology. Together with earlier in vitro data on APP692, our analyses suggest that the altered biological properties of the Flemish APP and Abeta facilitate progressive Abeta deposition in vascular walls that in addition to causing strokes, initiates formation of dense-core senile plaques in the Flemish variant of AD.

PERIPHERAL NEUROPATHIES

We aim to identify novel loci and genes in which mutations result in known or currently unknown forms of inherited peripheral neuropathies (IPN). Genetic linkage studies and positional cloning efforts have mapped more than 30 loci and identified 18 genes for distinct inherited peripheral neuropathies. Correlation between genotypes and phenotypes are made using clinical, neurophysiological and neuropathological data. The timely publication of large data sets from the Human Genome Project provides new opportunities for molecular genetic laboratories. It is our priority to make optimal use of these opportunities and to force breakthroughs in all our research topics by mapping and identifying the genes involved. The identification of these genes is the first step towards a better understanding of fundamental biological processes operating in myelination, axon-glia interaction, structure of the axonal cytoskeleton and axonal transport. Here we provide the most relevant data on our mutation screening in known and novel genes for IPN. These mutations are updated in our mutation database (Inherited Peripheral Neuropathies Mutation Database at http://molgen-www.uia.ac.be/CMTMutations/).

In 2002, we wrote a review paper on clinical and genetic aspects of hereditary neuropathy with liability to pressure palsies (HNPP) caused by a 1.4 Mb deletion on chromosome 17p11.2 (Pou-Serradell et al., 2002). In addition we characterised a smaller HNPP deletion patient (van de Wetering et al., 2002). We focussed our mutation analysis on the known genes causing recessive Charcot-Marie-Tooth (CMT) neuropathy. Mutations in periaxin (*PRX*) on chromosome 19q13 were recently reported in autosomal recessive DSS and CMT4F. PRX is a novel cytoskelet-associated protein with two PDZ-domains, and two alternative splice-variants, L- and S-periaxin. The PDZ domains are involved in membrane-protein interactions that stabilise the myelin sheaths in the peripheral nerve. We have screened DSS and CMT patients with a severe phenotype and found a nonsense (C715X) and frameshift (R82fsX96) mutation in a Turkish and Belgian family (Takashima et al., 2002). This finding was possible thanks to a collaboration with our colleagues in Houston, USA. For another recessive type of CMT, we screened the myotubularin related protein 2 (*MTMR2*) gene in a consanguineous Turkish CMT family compatible with linkage to 11q22, and now reported a homozygous missense mutation (R283W) associated with recessive CMT4B1 with myelin outfoldings (Nelis et al., 2002a). Recently, mutations in the ganglioside differentiation associated protein 1 (*GDAP1*) were shown to be responsible for autosomal

recessive demyelinating CMT (CMT4A) as well as for autosomal recessive axonal CMT with vocal cord paresis (ARCMT2C). We screened the coding region of *GDAP1* for the presence of mutations in 7 autosomal recessive CMT families in which the patients were homozygous for markers of the CMT4A locus at chromosome 8q21.1. We detected a nonsense mutation in exon 5 (S194X), a 1bp-deletion in exon 6 (G261fsX284) and a missense mutation in exon 6 (R282C). Mutations in *GDAP1* result in an early onset, severe clinical phenotype. The range of nerve conduction velocity (NCV) is variable. Some patients have normal or near normal NCVs suggesting an axonal neuropathy while others have severely slowed NCVs compatible with demyelination. The peripheral nerve biopsy findings are equally variable and show features of both demyelination and axonal degeneration (Nelis et al., 2002b).

A single large American family with autosomal dominant juvenile amyotrophic lateral sclerosis (ALS4) linked to 9q34 has been reported in the literature. The diagnosis of ALS4 was based on the combination of signs of lower motor neuron pathology with mild pyramidal tracts signs. Atypical features are: normal life expectancy, absence of bulbar involvement and predominantly distal weakness and atrophy. We studied 3 families with distal hereditary motor neuropathy (HMN) and pyramidal tract signs, and found linkage to the ALS4 locus on 9q34. The clinical features in our families are strikingly similar to the ALS4 phenotype in the American family. We therefore suggested that ALS4 and distal HMN with pyramidal tract signs could be the same disorder (De Jonghe et al., 2002). In 1996, we mapped the locus for distal hereditary motor neuropathy type II (distal HMN II) on chromosome 12q24 in an extended Belgian family. We constructed a clone contig and partial transcript map of the 12q24 region, and localised several genes and ESTs. We now reported the exclusion of 21 candidate genes (Irobi et al., 2002).

EGR2/Krox-20, a Cys_2-His_2 zinc finger transcription factor, plays an essential role in the regulation of myelination in the peripheral nervous system. Dominant and recessive mutations in EGR2 are associated with CMT1, DSS and CH. One recessive mutation (I268N) is known to affect the inhibitory domain that binds the NAB transcriptional co-repressors, NAB1 and NAB2. This mutation abolishes the interaction of EGR2 with the NAB co-repressors and thereby increases transcriptional activity. Therefore, we hypothesised that mutations in the EGR2 interacting domains or NAB conserved domains 1 (NCD1) of NAB1 and NAB2 might be associated with the pathogenesis of inherited peripheral neuropathies in currently unexplained cases. However, we did not find disease-causing mutations within the NCD1 domain. Additionally, we performed a mutation analysis of the complete coding region on genomic DNA to rule out both NAB genes completely. Again, we did not find any disease-causing mutation. Most probably both genes, NAB1 and NAB2, are not involved in the pathogenesis of Charcot-Marie-Tooth disorders (Venken et al., 2002).

Recently mutations in the SPTLC1 subunit of serine palmitoyltransferase have been shown to cause the common form of dominant hereditary sensory neuropathy (HSN1). Serine palmitoyltransferase (SPT) is a heterodimeric molecule made up of two subunits, SPTLC1 and SPTLC2. In collaboration with our Australian colleagues, 12 index patients from families with presumed genetic sensory neuropathies were screened for SPTLC2 mutations. No mutations were found suggesting that SPTLC2 mutations are not a common cause for genetic sensory neuropathies (Dawkins et al., 2002).

Mutations in MPZ, the major protein of the myelin sheath in peripheral nerves, cause the inherited peripheral neuropathies Charcot-Marie-Tooth disease type 1B (CMT1B), Dejerine-Sottas syndrome (DSS) and congenital hypomyelination (CH). In 1994, we reported a de novo insertional mutation (c.662_663GC) in a DSS patient. This insertion results in a frame shift mutation (A221fs) altering the C-terminal amino acid sequence of MPZ. In collaboration with German colleagues, we tried to clarify the molecular disease mechanisms in this sporadic DSS patient. We constructed wild type MPZ and the c.662_663GC mutant expression cassettes by site-directed mutagenesis and transfected the constructs into S2 insect cells. The mutant MPZ showed complete loss of adhesion (Ekici et al., 2002).

EPILEPSIES

Epilepsy is a common complex neurological disorder caused by interplay between hereditary and environmental factors that affects over 3% of the population at some time during life. Systematic genetic linkage analyses of pedigrees with a mendelian inheritance with reduced disease penetrance have fully demonstrated the contribution of genetic factors. A genetic contribution has been suggested for about 40% of epilepsy patients. In recent years, progress has been made in understanding the genetic etiology of epilepsies. Molecular genetic studies have mapped the disease locus for about 20 epilepsy syndromes. So far, mutations in 10 genes have been identified in pure epilepsy syndromes. Ion channel mutations have been reported in several families with distinct epilepsy phenotypes: ADNFLE (autosomal dominant nocturnal frontal lobe epilepsy) is associated with mutations in *CHRNA4* and *CHRNB2*, while mutations in *KCNQ2* and *KCNQ3* lead to BFNC (benign familial neonatal convulsions). In GEFS+ (generalized epilepsy with febrile seizures plus) mutations in *SCN1B*, *SCN1A*, *SCN2A* and *GABRG2* have been reported. More recently, *de novo* mutations in *SCN1A* in patients with SMEI (severe myoclonic epilepsy of infancy or Dravet syndrome) were described. Also, mutations in *LGI1* were shown to cause partial epilepsy with auditory features and mutations in *GABRA1* are associated with JME (juvenile myoclonic epilepsy).

Sampling of epilepsy families

Patients and families with different epilepsy syndromes are collected through the department of Neurology (P. De Jonghe) and Child Neurology (B. Ceulemans) of the University Hospital Antwerp (UZA). A network for referral for genetic studies is being constructed with the help of neurologists and child neurologists working in other university hospitals. We have already collected DNA samples of patients with distinct epilepsy syndromes, including some large pedigrees. These pedigrees are analysed for intra- and interfamilial clinical variability. At the moment, we collected DNA samples of 241 unrelated epilepsy patients, 18 multiplex families (5 or more patients) and 61 nuclear families (2 to 4 patients), representing 16 different epilepsy syndromes. In addition to DNA extractions, cell lines are made by EBV transformation of lymphocytes. The project was approved by the Commission of Medical Ethics of the University of Antwerp (file 99/023). Every participating person signed an informed consent.

Mutation analysis of known genes and genotype-phenotype correlations

First, we analysed the sodium channel *SCN1A* for 7 isolated patients with *severe myoclonic epilepsy of infancy* (SMEI). We found heterozygous dominant mutations that were absent from the parents and controls, demonstrating that these mutations are *de novo* mutations (Claes et al. Am J Hum Genet 68:1327-1332, 2001). The majority of these mutations were nonsense or frameshift mutations, except for 1 missense mutation. Probably these mutations cause haploinsufficiency of the sodium channel. We analysed an additional 9 SMEI patients (Claes et al. Hum. Mutation, revised version under review) and observed mostly *de novo* missense mutations located in important regions of the channel. These findings demonstrate for the first time that *de novo* mutations can cause epilepsy and also demonstrate that different mutations in the same gene (*SCN1A*) can cause different epilepsy phenotypes (SMEI versus GEFS+).

Further we analysed the genes *SCN1B, SCN2B, GABRG2, KCNQ2, KCNQ3* and *LGI1* in specific samples consisting of patients with several phenotypes: febrile seizures (FS), febrile seizures plus (FS+), GEFS+, absence epilepsy, JME, ADNFLE, partial epilepsy syndromes, BFNC, Lennox-Gastaut and Ohtahara.

In a sample of 74 patients with GEFS+, FS en FS+ we identified a mutation in *SCN1B* in one patient. The patient belongs to a family with FS+ and very early onset absence epilepsy and the mutation segregates with the disease (Audenaert et al. Neurology, under review). The mutation results in alternative splicing using an exonic cryptic acceptor site resulting in an in-frame deletion of 5 critical amino acids in the extracellular Ig-like region. The mutation probably causes the subunit to lose its function. This mutation is the second mutation reported in SCN1B worldwide, and the first deletion mutation in this gene.

No mutations were detected in the mutation analysis of *SCN2A, SCN2B* and *GABRG2*. The potassium channels *KCNQ2* en *KCNQ3* were analysed in 22 patients with BFNC, Lennox-Gastaut, Ohtahara and epilepsy syndromes at young age. In *KCNQ2*, a mutation resulting in the deletion of 1 amino acid was identified (data not published). No mutations were found in *KCNQ3*. Currently, *LGI1* is being analysed in 48 patients with partial epilepsy syndromes. No mutations have been identified yet.

Identification of novel genetic factors for epilepsy

Several multiplex epilepsy families were selected for parametric linkage studies. Simulation studies were performed to estimate the power of these families. We selected 7 families that had a maximum simulated LOD score of 2 or more. A genome wide scan using a 10 cM dense mapping panel was performed in these families. Significant linkage (LOD score > 3) was found in 4 families. A novel and unique locus was identified in each family. Currently, we are refining the candidate regions by analysing additional markers and we are screening positional and functional candidate genes. Also, 1 family showed significant linkage in a region that overlaps with the FEB4 locus on chromosome 5q. Mutations in the gene VLGR1 (very large G protein-coupled receptor 1) have been identified for FEB4, but only a small portion of patients with FS or GEFS+ carries a disease-causing mutation in VLGR1. Currently, we are analysing VLGR1 in the 5q-linked family.

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ACTIVITIES 2002

THESES

Ph.D. Theses

- J. Irobi 'Molecular genetic analysis of distal hereditary motor neuropathy type II (distal HMN II)', 'Moleculair genetische analyse van distale hereditaire motorische neuropathie type II (distale HMN II)
  Promotors: C. Van Broeckhoven & V. Timmerman, April 12th, 20020
- B. Dermaut 'Molecular genetic analysis of causal mutations en genetic risk factors in dementia', 'Moleculair genetische analyse van causale mutaties en genetische risicofactoren in dementie'
  Promotor: C. Van Broeckhoven, Co-promotor: M. Cruts, December 2nd, 2002

Licentiate Theses

- K. Coen 'Moleculair-genetisch onderzoek van erfelijke ulcero-mutlerende neuropathieën'
  Promotors: K. Verhoeven, V. Timmerman & P. De Jonghe, Begeleider: V. Van Gerwen
- L Deprez 'Moleculair genetisch onderzoek van de intermediaire vorm van de ziekte van Charcot-Marie-Tooth en de hereditaire sensorische en autonome neuropathie type IV'
  Promotors: E. Nelis, V. Timmerman & P. De Jonghe, Begeleider: A. Jacobs
- I. Dierick ' Moleculaire genetica van distale hereditaire motorische neuropathieën (distale HMN)'
  Promotors: V. Timmerman & P. De Jonghe, Co-promotor: J. Irobi, Begeleider: E. De Vriendt
- E. Hermans 'Moleculaire genetica van erfelijke epilepsiesyndromen'
  Promotor: P. De Jonghe, Co-promotor: L. Claes
- M. Pellis 'Moleculaire genetische analyse van kandidaatsgenen voor de ziekte van Alzheimer'
  Promotor: M. Cruts
  Co-promotor R. Rademakers
- L. Tambuyzer 'Moleculair genetische analyse van CDK5 in het geheugen'
  Promotor: M. Cruts, Co-promotor: B. Dermaut, Begeleider: M. Van den Broeck
- S. Vingerhoedt 'Transgene APPT714I muizen voor Alzheimer dementie'
  Promotors: S. Kumar-Singh & C. Van Broeckhoven, Begeleider: K. Vennekens

MD Theses

- K. Smets 'Idiopathische epilepsieën: genetische en fenotypische aspecten'
  Promotor: P. De Jonghe, Co-promotor: B. Ceulemans

Graduation Reports

- T. De Pooter 'Invloed van h et amyloid procursor proteïne op de ontwikkeling van de ziekte van Alzheimer: Studie van de promotorregio en SNP-map'
  Stagebegeleider: J. Theuns

PRIZES, HONORS AND AWARDS

Prizes and Honors

- B. Dermaut Co-recipient of the 'Pharmacia Corporate Prize', 'Molecular genetic research of causal mutations and genetic risk factors in dementia', Antwerpen, Belgium, September 30, 2002

Awards

- M. Cruts: Travel Fellowship of the Alzheimer' Association to attend the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- B. Dermaut. Travel Fellowship of the Fund for Scientific Research - Flanders (FWO-F) to attend the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- B. Dermaut: Travel Fellowship of the Alzheimer' Association to attend the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- S. Kumar-Singh: Travel Fellowship of the Alzheimer' Association to attend the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- E. Nelis: Travel Award of the National Fund for Scientific Research to attend the 34th European Human Genetics Conference 2002, Strasbourg, France, 25-28 May 2002
- R. Rademakers: Travel Fellowship of the Fund for Scientific Research - Flanders (FWO-F) to attend the 34th European Human GeneticsConference 2002, Strasbourg, France, May 25-28, 2002
- R. Rademakers: Travel Fellowship of the Alzheimer' Association to attend the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- J. Theuns: Stipend to attend the 3rd Forum of European Neuroscience, Paris, France, July 13-17, 2002
- J. Theuns. Travel Fellowship of the Alzheimer's Association to attend the 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- V. Timmerman: Travel Fellowship of the Fund for Scientific Research - Flanders (FWO-V) to attend the 52nd Annual Meeting of the American Society of Human Genetics, Baltimore, MD, USA, October 15-19, 2002

PRESENTATIONS AT MEETINGS

Invited Lectures

- M. Cruts: Molecular Genetics of Alzheimer disease. 16th Reunion of the study group of Aging and Dementia. Tomar, Portugal, June 14-15, 2002
- P. De Jonghe: Molecular investigation of sensory motor inherited neuropathies. Xth International Congress on Neuromuscular Diseases. Vancouver, Canada, July 7-12, 2002
- P. De Jonghe: Febrile seizures and specific infancy syndromes. 5th European Congress on Epileptology, Madrid, Spain, 6-10, 2002
- P. De Jonghe: Genetics of epilepsy. Lectures in Neurology, ULB, Brussels, Belgium, November 12, 2002
- P. De Jonghe: New insight in CMT diseases. Sientific Symposium Paediatric Neurology 'Neuromuscular Diseases in Childhood: an update'. Leuven, Belgium, November 29, 2002
- P. De Jonghe: Severe myoclonic epilepsy of infancy. Symposium on myoclonic epilepsies of infancy, childhood, adolescense and adulthood. Seattle, USA, December 5-6, 2002
- P. De Jonghe: Molecular genetics of distal hereditary motor neuropathies. Oxford Department of Clinical Neurology Lecture 2002. Oxford, UK, December 13, 2002
- B. Dermaut: Molecular genetics of dementia: a spectrum of disorders. Research seminar. Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, TX, USA, January 15, 2002
- S. Kumar-Singh: Vascular problems in Alzheimer's disease. The 15th ECNP Congress. Madrid, Spain, October 5-9, 2002
- V. Timmerman: European Molecular Genetics Quality Network, External Quality Assessment for Charcot-Marie-Tooth, Erlangen-Nürnberg, Germany, January 25-27, 2002, assessor
- V. Timmerman: Molecular genetics of the intermediate type of Charcot-Marie-Tooth disease. The 47th Annual Meeting of the Deutsche Gesellschaft für Neuropathologie und Neuroanatomie (Joint Meeting of the Belgian, Dutch and German Societies of Neuropathology). Aachen, Germany, October 9 -12, 2002
- C. Van Broeckhoven: Recente Ontwikkelingen in de Neurogenetica. Neurostaf AZ Middelheim. Antwerpen, Belgium, January 21, 2002
- C. Van Broeckhoven: Effects of genetic testing for major neurodegenerative brain diseases on modern medicine. Public Forum - HGM2002 'Genomic Biotechnology and Bio-economy in the 21st Century'. Shanghai, China, April 13, 2002
- C. Van Broeckhoven: Alzheimer's Disease. European Human Genetic Conference 2002. Strasbourg, France, May 25-29, 2002
- C. Van Broeckhoven: Genetics of early-onset Alzheimer's disease in a population - based sample. 8V International Conference on Alzheimer's Disease and Related Disorders. Stockholm, Sweden, July 20-25, 2002

Oral Presentations

- N. Ammar: GDAP1 mutations are associated with demyelinating and axonal AR-CMT. The 47th Annual Meeting of the Deutsche Gesellschaft für Neuropathologie und Neuroanatomie (Joint Meeting of the Belgian, Dutch and German Societies of Neuropathology), Aachen, Germany October 9 -12, 2002
- L. Claes: De novo mutations in the sodium channel gene SCN1A cause severe myoclonic epilepsy of infancy. VIB-Seminar, Blankenberge, Belgium, March 7-8 (2002)
- S. Claes: A genome-wide scan in extended families with bipolar disorder from an isolated region in North Sweden reveals candidate loci on chromosomes 2q and 9q. Xth World Congress on Psychiatric Genetics. Brussels, Belgium, October 9-13, 2002

- B. Dermaut: Nicastrin modifies risk for familial early-onset Alzheimer's disease in a Dutch population-based sample. 8th International Conference on Alzheimer's Disease and Related Disorders, Stockholm, Sweden, July 20-25, 2002
- J. Irobi: Distal Hereditary Motor Neuropathies type II: the hunt for the gene. UZA Research Club, March 13, 2002
- R. Rademakers: Reducing the candidate region for chromosome 17Q21 linked frontotemporal dementia. VIB-Seminar, Blankenberge, Belgium, March 7-8 (2002)
- J. Theuns: Presenilin 1 expression altered by promoter variations associated with increased risk for Alzheimer's disease. 3rd Forum of European Neuroscience, Paris, France, July 13-17 2002
- N. Verpoorten: Purification of motor and sensory neurons for differential gene expression studies. The Society for Neuroscience 2002, Annual Meeting in Orlando, Florida, USA, November 2-7, 2002

PUBLICATIONS FOR LAY-ORGANISATIONS

CMT Belgium:

- Joy Irobi: Waarom kiest een Nigeriaanse doctoraatsstudente om vier jaar lang onderzoek uit te voeren in het laboratorium Moleculaire Genetica aan de Universiteit Antwerpen? Februari 2002
- Eva Nelis: Mutaties in het GDAP1 en het LMNA gen veroorzaken recessieve CMT, mei 2002
- Vincent Timmerman: Tien jaar onderzoek naar de CMT1A duplicatie op chromosoom 17, december 2002

Annual Report of the Research Group of

Prof. Dr. Van Camp G.

Universitaire Instelling Antwerpen (U.I.A.)

Prof. Van Camp G.

Department of Medical Genetics,
University of Antwerp
Universiteitsplein 1
2610 Antwerpen
Tel.: +32.3.820.25.70
Fax: +32.3.820.25.66

Functional analysis of a gene for progressive hearing impairment

Introduction

This project concerns the functional characterization of *DFNA5*, a gene for a non-syndromic, autosomal dominant, progressive, sensorineural type of hereditary hearing impairment. The hearing loss starts at the high frequencies at an age between 5 and 15. Only at an older age the lower frequencies become affected. The *DFNA5* mutation responsible for this hearing impairment is a complex intronic deletion/insertion that, on the mRNA level, leads to exon 8 skipping and results in a frameshift and premature protein truncation (Van Laer et al., 1998). The exact expression pattern, the subcellular localization and the putative physiological function of DFNA5 remain undeciphered.

Research progress during the past year

1. The elucidation of the DFNA5 expression pattern by immunohistochemistry and in-situ hybridization

An antiserum was raised against a mixture of 2 peptides followed by purification of the antiserum using affinity chromatography with the specific peptides ("double XP" programme, Eurogentec). The thus generated antibodies did not recognize DFNA5 using Western blotting. Only aspecific bands were detected. As a consequence, this antiserum is useless.

Therefore, we have decided to return to a previously generated antiserum. The latter antiserum, raised against the complete recombinant DFNA5 protein, is applicable for Western blot after purification using affinity chromatography followed by a preadsorption step with total homogenate of brain derived from homozygous DFNA5 knockout mice. As the resulting antiserum still wasn't specific enough for immunochemistry, we are currently trying to adjust the preadsorption procedure in order to obtain an antiserum that can be applied for immunohistochemistry. Simultaneously, we are working on the optimization of the parameters for in-situ hybridization experiments in the mouse inner ear.

2. The elucidation of the subcellular localization of DFNA5

A DFNA5-GFP fusion protein was transfected into HEK293T and in COS-1 cells. The subcellular localization of DFNA5-GFP was cytoplasmic. As the HEK293T and the COS-1 cell lines might not express the necessary partners, which consequently might lead to an incorrect cytoplasmic localization, 4 additional cell lines will be transfected with DFNA5-GFP: an estrogen receptor-positive and an estrogen receptor-negative breast cancer cell line (Thompson et al., 1998; Van Laer et al., 1998), and an etoposide-resistent and an etoposide-sensitive melanoma cell line (Lage et al., 2001).

3. Characterization of a DFNA5 knockout mouse

To replace the genetic background of the DFNA5 mouse, which was generated in the hearing impaired 129 mouse strain, matings between DFNA5 heterozygous mice and C57Bl/6J (showing age-related hearing loss) and CBA/Ca (a good hearing reference strain) inbred mice are being

set up during 10 generations. After 10 generations 99.9 % of the genetic material will belong to the strain with which the DFNA5 mouse is being crossed. At this moment, the first homozygous mice of the 10th generation in the C57Bl/6J genetic background are being born, and heterozygous mice of the 9th generation in the CBA/Ca genetic background are available.

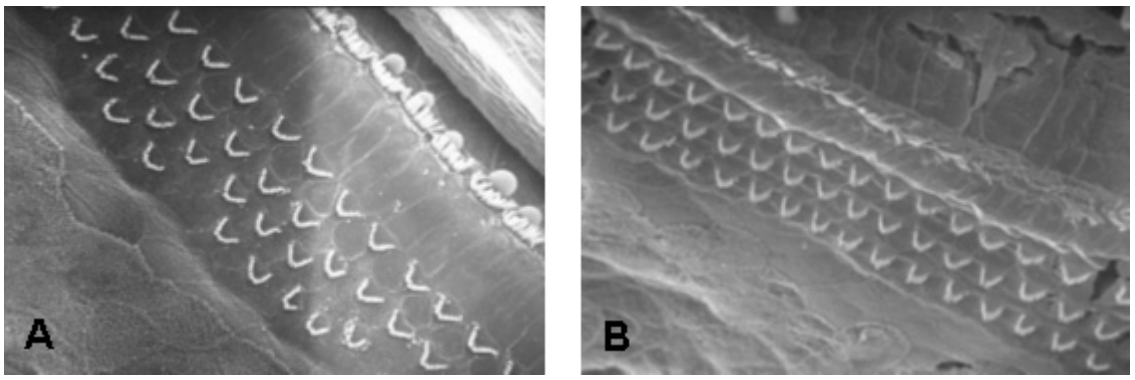
The first experiments to phenotype the DFNA5 mouse were performed using homozygous mice from the 6th generation for both genetic backgrounds (98.5 %). Using RT-PCR on brain mRNA we could demonstrate the presence of mutant *DFNA5* transcript in heterozygous and homozygous DFNA5 mice. However, we could demonstrate the complete absence of DFNA5 protein using Western blotting on brain tissue derived from homozygous animals, indicating that the DFNA5 mouse is a knockout.

Frequency-specific BERA (Brain Evoked Response Audiometry) tests were performed for mice with the C57Bl/6J genetic background aged between 4 and 5 months and between 7 and 8 months, and for mice with the CBA genetic background aged between 3 and 4 months and between 7 and 8 months. No significant differences were measured between DFNA5 knockout mice and their wild-type littermates for either age or either genetic background. The mice with the CBA genetic background will be retested when they are older than 1 year.

In addition, we could not detect a significant difference in vestibular function between DFNA5 knockout mice and their wild-type littermates using a series of simple tests (swimming test, elevated platform test, reaching response, negative geotaxis, circling behaviour, air righting reflex and contact righting reflex). These tests however are neither specific nor sensitive. Therefore, we intend to measure the vestibulo-ocular reflex and the optokinetical reflex in collaboration with Dr. van Alphen and Dr. De Zeeuw of the Erasmus University (Rotterdam).

Electronmicroscopical examinations revealed significant differences between DFNA5 knockout mice and their wild-type littermates at the level of the outer hair cells of the apical turn ($p=0.005$). Outer hair cells normally occur in 3 carefully arranged rows. DFNA5 knockout mice however, show stretches of 4th row outer hair cells and a disturbance of the pattern (Figure 1). Additional experiments in both genetic backgrounds are necessary to confirm and further characterize this phenomenon.

Figure 1: Electronmicroscopical picture of the organ of Corti of (A) a DFNA5 knockout mouse and (B) a wild-type littermate.



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Annual Report of the Research Group of

Prof. Dr. Vandenberghe R.

Katholieke Universiteit Leuven
(K.U.Leuven)

Prof. Dr. Vandenberghe Rik

Universitair Ziekenhuis Gasthuisberg
Herestraat 49
3000 - Leuven
Laboratorium voor Experimentele Neurologie

Tel.: +32.16.34.42.80
Fax: +32.16.34.42.85
E-mail: rik.vandenberghe@uz.kuleuven.ac.be

The functional neuroanatomy of naming, reading and knowing in the intact brain and in Alzheimer's disease

In the first year of this project we have initiated a new research programme to define the functional neuroanatomy of semantic processing and semantic decline, through the combination of behavioral studies, fMRI and PET studies in cognitively intact subjects, in patients with incipient or early Alzheimer's disease, and in patients with primary progressive aphasia. We are studying directly in vivo the substrate for acquired cognitive dysfunction at different levels: Behavioral performances, brain structure (atrophy), brain activity patterns as well as neurotransmitter systems. We are testing a model of functional specificity of different components of the semantic network for word and pictures. The protocol has been approved by the Ethics Committee, University Hospital Gasthuisberg.

1. Studies in normal volunteers:

Left anterior temporal pole is involved in processing the meaning of words and pictures. It is also strongly activated when subjects read sentences instead of lists of unconnected words. This response to sentences is surprising given the fact that deficits in sentence processing due to agrammatism are commonly associated with lesions of the inferior frontal gyrus, rather than temporal cortex. Using PET we have recently demonstrated that this response of temporal cortex to sentences is related to the propositional content of the sentences and not to grammatical processing per se.

In the past we have studied semantic processing of words and pictures using PET and a modified version of the "Pyramids and Palm Trees". We have recently adapted this experiment for epoch-based fMRI. This required a number of major modifications. In the anterior temporal pole fMRI images suffer signal loss due to susceptibility artifacts induced by the nearby air cavities. We adapted the MRI acquisition protocol to minimize these artefacts by using a 8-channel parallel head coil together with parallel imaging techniques in a sagittal plane. Preliminary fMRI findings revealed anterior and lateral temporal activations significant at a corrected $P<0.05$ for single subjects. In the next months we are planning a series of 10 elderly controls.

We are developing a paradigm to examine which ventral occipitotemporal areas are specifically activated by processing of words or pictures and by processing form or meaning. We will use event-related fMRI and tachistoscopic presentation of words and pictures in left or right visual field. We will vary the stimulus duration and the task. Methodologically, this study will build on the experience that we acquired during the past year in a series of event-related experiments using tachistoscopic presentation of grating stimuli in left or right visual field for the study of visuospatial attention.

2. Studies in patients

We will apply the same semantic task as described in 1b. The first patients, including 1 patient with early-onset Alzheimer's disease, 1 patient with primary progressive aphasia and 2 subjects with mild cognitive impairment will be scanned this month.

A high resolution structural MRI will be used for voxel-based morphometry

Each patient will also undergo a PET study of N-[^{11}C]methylpiperidin-4-yl propionate, a marker for acetylcholinesterase in vivo. The production of this marker as well as the mathematical kinetic models have been optimized during the past year. We recently scanned the first young control and two patients and are planning 15 more subjects during the next months.

Conclusion: During the first year we have implemented the fMRI and PET methodology that is necessary for this project. For fMRI this required the development of a new acquisition method to minimize temporal susceptibility artifacts and the implementation of event-related methodology. For PET this required the capability to produce a new marker for acetylcholinesterase and model its kinetics. The second year will be devoted to the acquisition of data in patients and elderly controls.

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Annual Report of the Research Group of

Dr. Vanderhaeghen P.

Université Libre de Bruxelles (U.L.B.)

Dr. Pierre Vanderhaeghen

Institute of Interdisciplinary Research (IRIBHN)
ULB, Campus Erasme
808, Route de Lennik
B-1070 Brussels
Tel.: +32.2.555.41.86
Fax: +32.2.555.46.55

MOLECULAR AND CELLULAR MECHANISMS OF DEVELOPMENT OF NEURONAL CONNECTIVITY IN THE CEREBRAL CORTEX.

INTRODUCTION.

The cerebral cortex contains the highest centers for the processing and integration of information in our brain. It is organized in several areas, each of which is specialized in particular functional modalities, such as the somatosensory areas for touch perception, or motor areas for motor control. In correlation with its complex functions, the neocortex is characterized by a high diversity of neuronal connections, each cortical neuron displaying a specific pattern of connectivity. Despite their potential importance in human pathology (in particular for mental disorders and epilepsy), the molecular mechanisms of the generation of distinct functional areas and specific neuronal connections in the cortex remain largely unknown (1-3).

Recently we have started the characterization of the ephrin/Eph family of axon guidance molecules (4) during the development of connectivity of a major cortical network : the connections between the thalamus (which serves as the main relay to transmit input to the cortex) and the cortex, or thalamo-cortical connections. We showed that members of the ephrin/Eph gene family display multiple gradients of expression in the developing thalamus and cortex, and that in ephrin-A5 mutant mice the cortical somatosensory map is distorted, although its topography is still preserved (5,6).

The identification of molecular factors like the ephrins capable of (re)specifying cortical connectivity has important implications for our understanding of normal and pathological brain development, but also in the long run for the rational design of neural regeneration therapies.

However many questions remain concerning the roles of ephrins in the cortex, their cellular modes of action and the nature of upstream mechanisms responsible for the patterning of the neocortex.

To address these issues we have focused this year on two distinct and complementary approaches :

(1) *In vivo study of the role of ephrin genes in cortical development using mouse transgenics.*

(2) *In vitro study of the cellular effects of ephrins and other signaling pathways in cortical development using an organotypic culture system.*

RESULTS.

In vivo study of the role of ephrins in the developing cortex : combining loss and gain of function approaches.

The analysis of ephrin-A5 mutant mice was only partially informative because of the compensation by other members of the ephrin/Eph family, and because of the pattern of expression of ephrin-A5 in non-cortical regions of the brain (5,6). In order to test for the effects of a more complete loss of ephrin function in the developing cortex, we have generated

compound mutants for ephrin-A5 and for its receptors EphA4 and EphA7, expressed in complementary gradients in the thalamus.

Retrograde axon tracing analysis of these compound mutant mice first revealed a disruption of somatosensory thalamocortical topography, providing in vivo evidence that EphA4 and EphA7, together with their ligand ephrin-A5, act as thalamic axon guidance receptors that control topographic specificity *within* the somatosensory area.

Surprisingly we also found aberrant projections *between* individual thalamic nuclei and cortical areas, in particular between the motor and somatosensory systems: in these mutants, thalamic nuclei from the anterior thalamus that normally only project to frontal motor areas start to project more caudally to the somatosensory areas. These results constitute the first direct evidence for the involvement of axon guidance factors in the generation of area-specific thalamocortical projections, and indicate that the same system of mapping label coordinates is used for the generation of topographic order of thalamocortical projections *between* and *within* individual cortical areas, suggesting an economical model of development of cortical connectivity (7).

Our data also point to a previously overlooked mechanism of generation of target-specificity in the developing brain. Indeed, using expression pattern analysis of ephrin/Eph genes in the early forebrain and anterograde axon tracing of mutant embryonic brains, we were able to show that ephrins were in fact controlling the initiation of target-specificity of cortical innervation through the early topographic sorting of thalamocortical axons in an intermediate target, the ventral telencephalon, which was further demonstrated by in vitro analyses (see section 2). Given the unique nature of the thalamocortical defects revealed in these mutants (an aberrant innervation of sensory areas by the motor system), we are now planning to analyze the functional consequences of these defects using behavioural and physiological techniques.

In many aspects of developmental genetics, gain of function analyses constitute a useful complementary approach to loss of function. Therefore we have started to generate mice that display a *gain* of function of ephrin-A5, specifically in the developing cortex, using the cre/lox system transgenic technology, that allows to modify a locus in a temporally and spatially controlled way (8).

Our previous studies have indeed revealed an interesting opportunity : several Eph receptor genes (in particular EphA7) are also expressed in gradients in the developing cortex, but their gradients of expression are inverted when compared to ephrin-A5 (our unpublished datas). Ectopic gradients of ephrin-A5 can thus be obtained by placing the ephrin-A5 coding sequence under the control of the regulatory sequences of the EphA7 gene.

This has now been achieved using a bacterial artificial chromosome (BAC) containing all the regulatory sequences of the EphA7 gene (previously isolated in the lab) where the ephrin-A5 coding sequence (flanked by a conditional lox/GFPstop/lox cassette) has been knocked in the first coding exon of the EphA7 gene by homologous recombination in E coli, followed by generation of the corresponding transgenic mice (9). Analysis of this line using in situ

hybridization and immunohistochemistry for GFP has enabled to show that the transgene faithfully reproduces the endogenous graded expression patterns of the EphA7 gene in the cortex, thalamus, and midbrain (V. Depaepe, and P. Vanderhaeghen, unpublished data)

This line has been crossed with several cre-expressing lines available in the lab (the Emx1-cre line, allowing recombination in the early developing dorsal telencephalon, the NEX-cre line allowing recombination in the later developing cortex). Mice resulting from these crosses will thus display ectopic gradients of ephrin-A5 specifically in the early and late developing cortex.

These various lines are now being analyzed with our previously used neuroanatomical techniques (quantitative histochemistry and axon tracing experiments (5,7)) to look for inversions and disruptions of various features of cortical sensory maps, focusing on the motor, somatosensory and visual systems. The combined analysis of these gain of function mutants should enable us to determine how the distribution of cortical ephrins can influence the functional organization of cortical maps.

In vitro study of ephrins and other factors controlling the development of cortical connectivity

Our in vivo studies on ephrin/Eph mutants has enabled to show that ephrins were required for the generation of area-specific patterns of thalamocortical projections (7 ; see section1). To determine more directly by which mechanism endogenous ephrins control the topographic guidance of thalamic axons, we have developed a new organotypic guidance assay (7,10). In this assay, explants from early embryonic thalamus are isolated from GFP-expressing mice and co-cultured with a whole-mount preparation of telencephalic vesicle, including the ventral and dorsal telencephalon. The growth preference of the thalamocortical axons can subsequently be quantified after 3 days in vitro using optical density measure of the GFP fluorescent signal.

This organotypic assay faithfully recapitulates several important aspects of the topography of thalamocortical outgrowth observed *in vivo* : in particular, axons from the rostral thalamus show a strong preference for the rostral part of the ventral telencephalon and avoid its caudal part, while caudal thalamic axons tend to invade more caudal territories of the ventral telencephalon (10). The addition of soluble Eph receptor fusion proteins (such as EphA3-Fc) to the culture medium represents a powerful way to inhibit the function of all endogenous ephrinA ligands (4). This resulted in the loss of growth preference and in some cases a caudalization of the outgrowth of rostral axons in the ventral telencephalon. To confirm that ephrin-A5 constituted at least a part of this ephrin-mediated guidance we next performed the assay using telencephalon isolated from ephrin-A5 -/- embryos. Again this resulted in a caudal shift of axon outgrowth, although of a less severe extent than with addition of ephrin/Eph antagonists, suggesting that other as yet unidentified ephrins are acting in concert with ephrin-A5 in the ventral telencephalon.

Taken together these in vitro results show that endogenous ephrin-A ligands distributed in the ventral telencephalon are required for the initiation of the topography of thalamocortical projections. This is fully consistent with the phenotypes described above in the ephrin/Eph

mutant mice: in the absence of appropriate ephrin/Eph signaling, thalamic axons from the rostral thalamus growing from the presumptive motor thalamic nuclei, start to invade more caudal territories in the ventral telencephalon, resulting in an aberrant shift of their projections to more caudal cortical areas such as the somatosensory cortex (7,10).

The same in vitro system has also been used to identify other signaling pathways potentially involved in the development of thalamocortical topography. Indeed, in collaboration with the groups of Drs F. Polleux (INSERM, Bron, France) and F. Guillemot (IGBMC, Strasbourg, France), we were able to show that the transcription factor Neurogenin-2, which is expressed in the developing thalamus in a graded pattern similar to Eph receptors, is also involved in the response of thalamocortical axons to guidance cues in the early telencephalon (10). Current work now focuses on the characterization of the potential molecular links between Neurogenin-2 and Eph genes in the developing forebrain, by studying the expression patterns of Eph genes in Neurogenin mutants and of Neurogenin genes in Eph mutants.

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Publications resulting from the FMRE Research Programme in 2002:

1 Dufour A, Seibt J, Passante L, Depaepe V, Ciossek T, Frisen J, Kullander K, Flanagan J, Polleux F, and Vanderhaeghen P. Area-Specificity and Topography of Thalamocortical Projections Controlled by Ephrin/Eph genes. Submitted.

2 Seibt J, Schuurmans C, Dehay C, Vanderhaeghen P, Guillemot F, and Polleux F. Neurogenin2 specifies the connectivity of thalamic neurons by controlling axon responsiveness to intermediate target cues. Submitted.

Annual Report of the Research Group of

Prof. Dr. Vanduffel W.

Katholieke Universiteit Leuven
(K.U.Leuven)

Prof. Dr. Wim Vanduffel

Lab. Neuro- en psychofysiologie
Fac. Geneeskunde, K.U. LEUVEN
Herestraat 49
B-3000 Leuven

Collaborators:
D. Fize, K. denys, K. Nelissen, H. Peuskens,
S. Sunaert, J. Todd, G. Orban

Functional Magnetic Resonance Imaging (fMRI) in awake behaving monkeys: establishing the link between human imaging and monkey electrophysiology.

During the past couple of years we developed and improved functional magnetic resonance imaging in awake and behaving monkeys. This year we made significant progress for two of the four general aims of the current GSKE-project: 1) clarifying potential homologies between human and non-human primates; 2) extensive mapping of the (visual) cortex within individual monkeys.

In line with the first aim, we conducted several fMRI experiments in parallel in humans and monkeys. In general we found, that early visual areas (V1, V2, V3, V4, MT and FST) and ventral stream areas (TEO, TE) were functionally very similar in the two primate species (at least for the tests that were conducted, see below). In posterior parietal cortex, however, we found several examples of pronounced functional differences between human and the monkeys. This suggest that human posterior parietal cortex underwent more pronounced evolutionary changes compared to early visual cortex and ventral stream areas.

Extracting the Third Dimension from Motion: Differences in Human and Monkey intraparietal Cortex. (Science, 2002)

In a first study, we compared the neuronal substrate involved in the processing of 3-dimensional-structure-from-motion (3D-SFM) *in awake* human and non-human primates. The two species share a network of early and middle level visual areas (V2, V3, V4, MT/V5, and FST) with similar sensitivity for 3D-SFM (see Fig. 1). These areas in monkeys and humans could be identified independently using the combination of retinotopic mapping (see below) and motion-sensitivity tests (see Vanduffel et al, 2001). At least for V4 and MT/V5, and their presumptive human homologues, multiple control experiments demonstrated the specificity of this activation: To control for differences in second order stimulus features such as spatial variations in speed, line length and orientation changes over time, we presented slowly rotating 3D displays, as well as in plane rotating, expanding and contracting 2D displays. In most 3D-SFM sensitive brain regions in monkey and human as described above, the two main 3D stimuli (original and slow) yielded statistically higher activity levels compared to the three 2D control stimuli. Pronounced functional differences between the two species were observed in V3A and the intraparietal cortex, which are unlikely to reflect attentional factors (see purple dashed outline in Fig. 1). Indeed it could be argued that the 3D moving stimuli are more interesting to observers compared to their 2D counterparts, or that humans covertly attend more to the 3D stimuli than monkeys. Functional interspecies differences might thus be biased towards regions strongly modulated by attention, such as the intraparietal cortex. To control for possible differences in attention, 3 human subjects were scanned while they performed a 1-back task, equalizing and drawing attention towards the 3D and 2D moving stimuli. Furthermore, one monkey and two human subjects were scanned using a demanding high acuity fixation task (see also Vanduffel et al., 2001), equalizing but drawing attention away from the stimuli presented in the background. Both these positive and negative attentional control tests yielded very similar activity patterns in the two species as obtained during the

standard 3D-SFM tests. These results indicate that the aforementioned functional interspecies differences observed under passive viewing conditions, are unlikely to reflect differences in attention between humans and monkeys.

The widespread human intraparietal 3D-SFM sensitivity might indicate the existence of an additional motion pathway in the human, projecting from V3A to the intraparietal sulcus. Although differences between human and monkey parietal cortex have been suggested, direct functional comparisons could not be conducted. The sole direct comparisons published to date reported only similarities between human and monkey prefrontal cortex. The present results suggest that under evolutionary pressure parietal, but not earlier regions adapted to implement human-specific abilities such as excellent motion-dependent 3D vision for manipulating fine tools (supporting online text). This adaptation could involve displacement of homologous regions. Alternatively, new posterior parietal areas could have been evolved prior the enormous frontal enlargement in recent hominid development. The latter mechanism has been suggested for areas 39 and 40. Finally, the present results call for follow-up studies investigating whether similar functional dissimilarities in the intraparietal sulcus exist for the processing of other 3D cues such as disparity (supporting online text), and to what extent the intraparietal species-differences in 3D-SFM processing are related to behavioral performance.

In a second experiment we directly compared the activation of the object-related 'lateral occipital' complex in humans and monkeys

Retinotopic mapping in awake monkeys suggests a different organization for dorsal and ventral V4. (submitted)

Typically, visual cortical areas in the macaque can be identified based upon differences in anatomical connections, cyto- and myeloarchitecture, functional properties and retinotopic organization. Using a combination of these four criteria, monkey visual cortex has been tentatively parcellated into more than 30 different visual areas (Felleman&Van Essen). However, although borders of early visual areas are well-established, this has proven to be more complex in extrastriate cortex. In fact, area MT/V5 is the only higher Tier area beyond V2 from which there is general agreement about its location and borders. Indeed, alternative division schemes have been proposed for area V4, as well as the complete extent of the inferotemporal cortex, and the posterior parietal cortex.

To resolve most of the discrepancies between the existing maps, it would be necessary to perform large-scale invasive experiments in single subjects. With the currently available technology, however, it would be unrealistic to achieve this by electrophysiological recordings. The development of non-invasive functional imaging techniques during the past decade has led to the paradoxical situation that, although 10 years ago virtually nothing was known about the retinotopic organization of human visual cortex, currently it has been mapped in a more systematic way compared to that in non-human primates. Actually, using fMRI it became possible to obtain detailed retinotopic maps and to identify areal boundaries from a considerable portion of the visual cortex of single human subjects. We exploited these

advantages of whole-brain in-vivo imaging techniques and applied them to awake monkeys.

In this study, we used static wedge-stimuli confined to the respective portion of the visual field. In an attempt to drive inasmuch areas as possible, each epoch contained randomly alternating colored and achromatic checkerboards, as well as moving dots or lines. Areal boundaries were based upon the representations of the vertical and horizontal meridian, and the upper and lower visual field. Finally, a small central stimulus (1.5 degrees radius), in combination with static concentric annuli covering different eccentricities, were presented in order to define eccentricity lines in the early visual areas. This also allowed us to calculate magnification factors for striate cortex.

The observed retinotopic organization of the early visual areas was completely consistent with the classical view (see Fig. 2): a vertical meridian marks the transition from V1 to V2, and a horizontal meridian specifies the border between V2 to V3. More anteriorly, V3 shared the second representation of a vertical meridian with V4. In addition, we could localize consistently the anterior border of ventral V4 (as a representation of the HM). Contrary to V4v, we could not determine the anterior border of V4d based upon retinotopic criteria. Areas V3A and MT showed a straightforward retinotopic pattern: both areas have a lower and upper visual field representation split by a horizontal meridian. Dorsally as well as ventrally, MT is bordered by a representation of a vertical meridian. A prominent foveal visual field representation was present in areas PO/V6, LIP and TEO. In all hemispheres, we found a representation of the upper visual field in the posterior part of the intraparietal sulcus. Furthermore, the eccentricity maps provided evidence for an asymmetric organization of ventral and dorsal V4: contrary to all other early visual areas including V4v, the eccentricity lines ran almost parallel to the areal boundaries in V4d. These results suggest a different functional organization in dorsal and ventral V4, similar as has been observed in humans.

Monkey equivalent of the human lateral occipital cortex. (in preparation)

In 1995, Malach et al. reported fMRI evidence for object-related activation in the lateral-posterior aspect of the human occipital lobe by comparing objects to uniform texture patterns. The activated region abutted the posterior aspect of the motion-sensitive area MT/V5. This 'lateral occipital' or LO region included a parietal and a temporal branch. This LO is a 'complex' of multiple areas in 'Lateral Occipital' cortex, which share a greater fMRI response to images of objects, compared to non-object controls. Results in 'LO' are consistent with earlier electrophysiology in comparable regions of the macaque. Follow-up fMRI-studies confirmed that parts of LO are involved in object recognition (Kourtzi and Kanwisher, 2001; Bar et al., 2001; Grill-Spector et al., 2000; James et al., 2000; Lerner et al., 2002). The latter studies localized regions that seem to generalize across lower-order visual cues including size, shape and perhaps viewpoint (Grill-Spector et al., 1999; Vuilleumier et al., 2002) as one might expect in a 'object-selective' computation, although results in the human studies remain controversial.

In a third experiment, we wished to verify to which monkey areas the human 'LO' complex might correspond to. To test this, we presented exactly the same stimuli as being used in the Kourtzi/Kanwisher study to 4 fixating monkeys and 11 human subjects. We compared fMRI activity related to viewing images of objects and line drawings of objects versus their scrambled counterparts, a classical test to define the 'LO' in humans (see Fig. 3).

To account for differences in attention between the stimuli (and between the species), we included the same control test as performed in the 3D-SFM experiment: the subjects (2 humans and 2 monkeys) performed a high acuity fixation task (see above) while the stimuli of interest were projected in the background.

In line with all previous results, we find in the 11 human subjects higher activity related to intact images compared to scrambled images in 'LOS', the occipital part of the intraparietal sulcus and the fusiform gyrus (see Fig. 3). In monkeys, this contrast revealed differential MR signals in extrastriate visual areas V3, V4, and almost the complete inferotemporal cortex (from TEO to TE). Furthermore, almost he complete extent of the lateral bank of the intraparietal sulcus and portions of prefrontal cortex were more activated during the presentation of the intact versus the scrambled images of objects. Thus in monkeys and by inference in also in humans, the LO complex consists of multiple extrastriate visual areas. In the images of objects are very powerful stimuli that activate the almost the complete extent of extrastriate cortex.

Furthermore, whereas the occipito-temporal part of the 'LO complex' is very similar in humans and monkeys, there is a much larger involvement of monkey intraparietal and frontal cortex for the processing of 2D images of objects (compared to equivalent regions in humans). Surprisingly, this interspecies difference for 2D shape processing is opposite to that observed for 3D structure-from-motion (see above). This double dissociation for the intraparietal cortex (human > monkey for 3D-SFM, and monkey > human for 2D shape), indicates that this region has significantly different functional properties in the two primate species.

Altogether, these results demonstrate that early visual areas and the occipito-ventral but not occipito-parietal cortex is very similar in the two species. This might imply that the dorsal 'where' pathway evolved differently in humans compared to monkeys while the ventral 'what' pathway underwent much less evolutionary changes. Furthermore, these results also indicate that one has to be extremely careful by comparing human fMRI data with electrophysiological data from the monkey. While anatomically regions might 'seem' to be equivalent (e.g. 'intraparietal' cortex), this does not necessarily imply that this holds true for its functions. To reveal conclusive evidence about potential 'homologies', it is mandatory to perform large-scale functional comparative studies in the two species.

Fig. 1: Comparison of 3D-SFM sensitivity in the monkey and human. Both panels represent the statistical parametric map (t-scores) for the subtraction (3D-2D) overlaying the flattened right hemisphere of monkey M4 and the 1 human subject (P < 0.05, corrected for multiple comparisons). The purple outline indicates V3A and the intraparietal sulcus: areas where pronounced inter-species differences were observed.

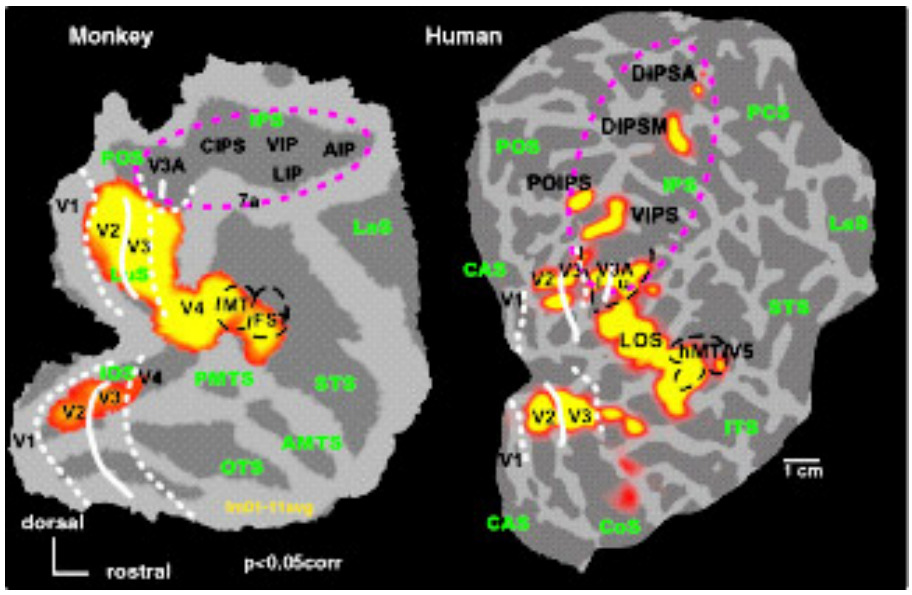


Fig. 2: T-score map of horizontal (red-yellow color-code) and vertical (blue color-code) meridian representations upon the flattened cortical reconstructions of the left hemispheres of M1 and M5 and right hemispheres of M3 and M4. White solid and dashed lines represent the horizontal and vertical meridians, respectively.

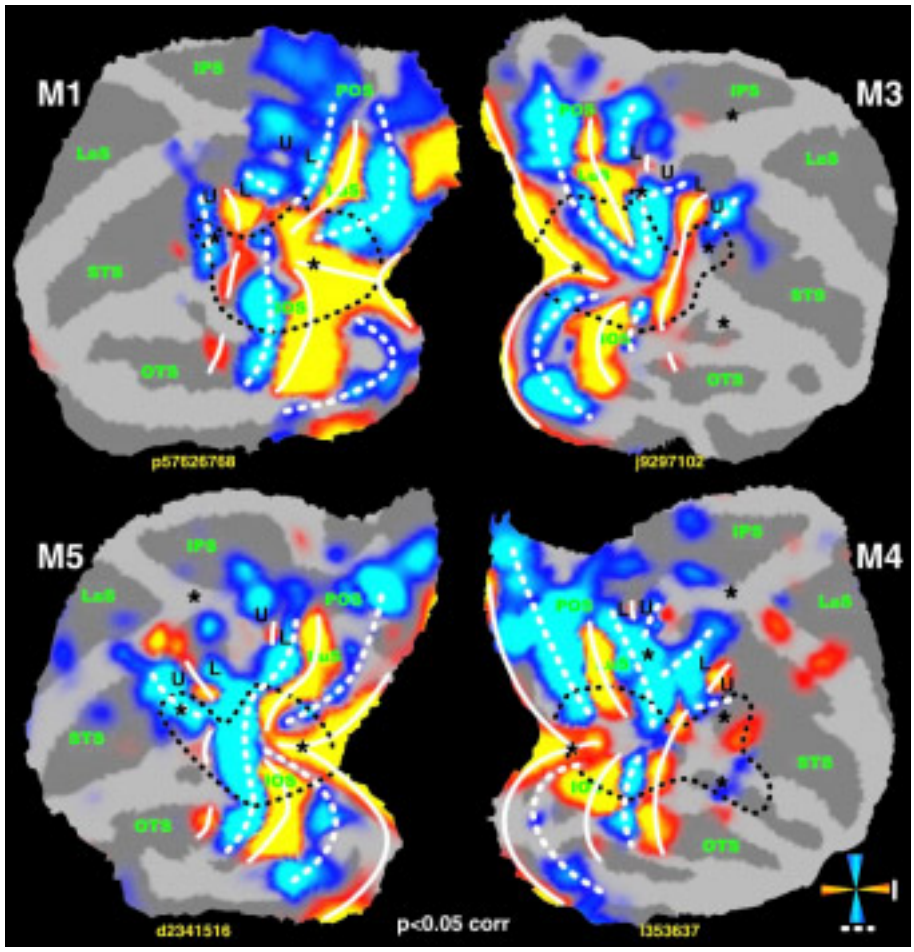
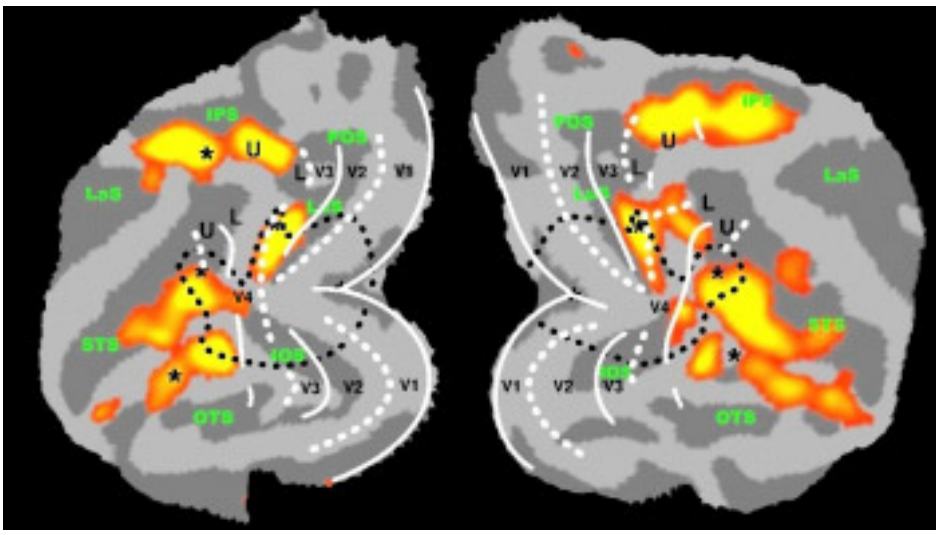
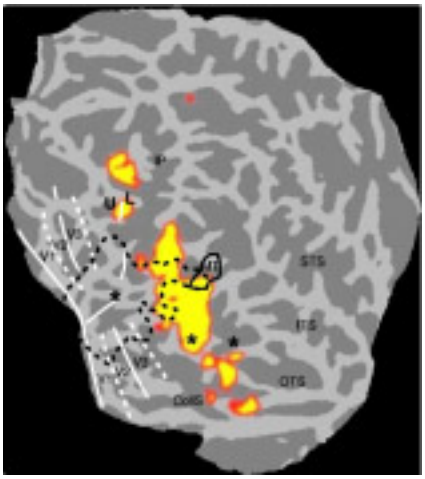


Fig. 3: Flatmaps of the right and left hemisphere of the group analysis (4 monkeys) with retinotopical borders (white and dashed white lines), foveal representation (dashed black lines and asterix)





T-score map for the contrast normal versus scrambled images ($p < 0.05$)

Same comparison (normal versus scrambled images) for 1 human subject.
Note the pronounced activations in the IPS for the monkey. Only posterior parietal cortex is activated in the human.

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Prof. Dr. Vogels R.

Katholieke Universiteit Leuven
(K.U.Leuven)

Prof. Dr. Rufin Vogels

Faculteit Geneeskunde
Campus Gasthuisberg
Labo Neuro- en Psychofysiologie
Herestraat 49
3000 Leuven
Tel.: +32.16.34.58.39
Fax: +32.16.34.59.93

Shape representations in macaque inferior temporal cortex

Macaque inferior temporal (IT) cortex consists of a number of visual areas that are supposed to be involved in object recognition and categorization. Single cell studies in these areas showed responses selective for object attributes such as shape, color, and texture. However, it is not yet clear which and how stimulus dimensions are coded in IT and to what degree object recognition performance correlates with the stimulus selectivity in IT. The solution of these questions will be imperative for understanding object recognition and categorization in non-human and human primates.

In the present studies, we have addressed these issues by (1) studying the coding of cues that signal 3D shape, and (2) correlating recognition performance and IT single cell responses by a manipulation of stimulus familiarity. The single cell recording studies are carried out in awake rhesus monkeys.

1. Coding of 3D shape cues.

a. 3D shape of single curved surfaces

Although objects are three-dimensional, little is known about how the brain codes 3D-shape. Our previous work in the macaque has shown that inferior temporal (IT) neurons code for 3D-shape. In these studies, 3D shape was manipulated by means of the binocular disparity cue, which is a reliable depth cue. It was found that IT neurons, mostly from the lower bank of the Superior Temporal Sulcus (TEs), are selective for the disparity-defined curvature, signaling the difference between a convex and a concave 3D shape. The 3D shape preference is preserved at different positions-in-depth, indicating that these TEs neurons respond to spatial variations in disparity. Indeed, some TEs neurons code for first-order disparities (disparity gradients), while others for second-order disparities (disparity curvature) and the responses can depend on curvature direction (e.g. horizontal versus vertical cylinders).

It is well established that other cues besides binocular disparity can signal depth structure, e.g. motion, texture, shading and other figural depth cues, and thus the question arises whether IT neurons also code depth from these other cues. So far, we have determined whether IT neurons also code for 3D shape-from-texture. In a first series of experiments, we compared the responses to texture patterns that produce a percept of a convex or a concave surface, i.e. a single curved surface. However, selective responses to these texture patterns can be merely due to a selectivity for 2D-textures changes, and it is known that IT neurons can be tuned to 2D-textures. To assess whether the texture selectivity is related to 3D shape instead of 2D texture, we determined the interaction of the texture with the binocular disparity cue. Thus, we examined whether the disparity-defined 3D shape selectivity of IT neurons was affected by the presence of a texture curvature cue, that signaled either the same or opposite curvature as the 2nd order disparity cue, or just a flat surface. The effect of the texture cue was determined at different reliabilities of the disparity cue, by manipulating stereo coherence. The latter is defined by the proportion of pixels that signal the correct disparity, i.e. the one corresponding to the curved surface. In addition, we assessed behaviorally in humans and in monkeys the effect of the same texture patterns on 3D-shape judgements.

The human psychophysical study clearly showed consistent effects of the texture cue on the 3D-shape judgements and the size of this effect depended, as predicted, on the degree of stereo coherence. When the stereo coherence is high, i.e. with a reliable stereo cue, the perceived 3D-shape was determined predominantly by the stereo cue. However, at low stereo coherences, i.e. an unreliable stereo cue, the percept was dominated by the texture cue. These results indicated that (1) the texture patterns that we employed produce a robust percept of 3D shape (convex versus concave) in man, and, (2) the texture cue affected strongly the perceived 3D shape when the stereo cue is weak.

In a subsequent single cell study in 3 macaque monkeys, we determined whether the 3D-shape selectivity of disparity selective TEs neurons is affected by the texture cue. First, we established in preliminary tests that the neurons showed a higher-order disparity selectivity, i.e. respond to disparity-defined 3D shape and not merely to position-in-depth (zero order disparity). Second, we measured their responses to the "flat", convex and concave texture patterns, the same stimuli that were employed in the psychophysics. The binocular disparity cue could signal either a convex or a concave curvature, and this at 5 different levels of stereo-coherence (range: 0-80%). The results showed that (1), as expected, the selectivity (difference between responses to concave and convex stimuli) decreases with decreasing levels of stereo-coherence for the flat texture pattern, (2) the responses at high (>40%) stereo coherences are dominated by the disparity cue, (3) the responses at <40% stereo coherences depend on the texture cue, (4) the 3D-shape preference in the texture-only condition (0% stereo coherence) correlated significantly (r = .3) with the 3D-shape preference when only the disparity cue was present (flat texture, 80% stereo coherence), showing that the two depth cues tend to converge at the single cell level, and (5) the 3D-shape selectivity was significantly greater when the two cues signaled the same curvature (e.g. both convex) than when they signaled the opposite curvature (e.g. texture cue signaling concave and disparity cue signaling convex).

In the above study, effects of the texture cue at the single cell level were, although reliable, of a lesser magnitude than expected from the human psychophysical results. This (quantitative) discrepancy between TEs single neuron selectivity and human psychophysics for the same manipulation of the same stimuli can be due to (1) a brain region different from TEs supports the behaviorally observed interaction of the disparity and texture cues, and/or (2) the texture depth cue is processed more weakly by monkeys than by humans. The latter was tested by having monkeys categorize the same stimuli as being convex or concave. This psychophysical study was conducted on 2 of the 3 monkeys of which we obtained single cell data. First, the monkeys were extensively trained in the categorization of disparity-defined 3D shapes (convex vs. concave) using random dot patterns. Second, we reduced the degree of stereo coherence and the frequency of reward for correct responses. Third, we tested their categorization of the flat, convex and concave texture patterns used in the single cell study. In the latter tests, reward was unrelated to the response of the monkey so that we could measure their "spontaneous" categorization of the texture patterns as being convex or concave. These tests were run at different levels of stereo coherence and with congruent or conflicting disparity and texture depth cues. The results showed that (1), as in humans, performance was higher when the stereo and texture cues were congruent than when they conflicted, indicating, an effect of the texture cue on 3D shape perception, but,

(2) this effect of the texture cue was weaker than in humans but similar to the one observed at the single cell level. Thus these results of the monkey psychophysical study are in line with the single cell study indicating a significant, albeit weak, processing of the texture depth cue.

b. 3D orientation of planes

Very recently, neurons in the macaque parietal cortex have been shown to be selective for the orientation of disparity and texture gradients (planes tilted in depth), and their preferred 3D orientation defined by either texture or disparity correlates (Tsutsui et al., Science, 2002). The degree of convergence of the texture and disparity cues that these authors found was much larger than that we observed for curved surfaces in IT. It is possible that this apparent discrepancy reflects a regional difference (parietal vs. IT cortices) in the coding of 3D shape, a difference between the coding of first (planes) and second (curved) -order surfaces, or reflects the higher saliency of the texture depth cue for planes compared to the curved surfaces. To sort this out, we recorded the responses of single TEs neurons in monkeys for planes at 4 orientations in depth and this when the 3D orientation was defined either by disparity or by texture. We observed a high correlation between the preferred 3D orientation for the disparity and texture defined planes ($r = .8$), even higher than that observed in parietal cortex and larger than that found for the curved surfaces in our first study. These results demonstrate that in IT there is convergence of different depth cues at the single cell level. It is likely that the weaker texture coding found for the curved surfaces is due to the weaker saliency of the texture cue in the latter case, which is consistent with the monkey psychophysical study.

Further work will address the coding of another monocular depth cue: shading.

2. Correlating object recognition performance and IT single cell responses.

Relating object recognition performance and IT single neuron selectivity requires that one quantifies each. In order to quantify object recognition performance one has to degrade the image or use small image differences. We have opted for degrading the image of an object by using backward masking, a procedure that is widely used in human psychophysics to study object recognition. It has been shown that human subjects can recognize objects at shorter masked exposures after several days of training. This training effect is largely specific to the trained objects, allowing a comparison of behavioral and neural responses to untrained and trained objects. In fact, using fMRI, Grill-Spector et al. (NatureNeuroscience, 2000) have shown increased activation of object-related areas (LO) for trained compared to untrained masked images, suggesting that, at least in humans, ventral stream areas underlie the training-induced changes in masked recognition.

We have trained two monkeys in a temporal same-different task in which the animal has to decide whether or not two successively presented stimuli, seperated by a delay of 500 msec, are different. The latter task allows flexible introduction of novel images. The first stimulus was followed by a mask. The exposure time of the first stimulus, and thus the stimulus-mask onset asynchrony (SOA) was manipulated. As stimuli, we used several sets of 10 grayscale images of common objects and abstract patterns.

The first monkey showed excellent performance at short SOAs and, as expected from the human psychophysical studies, a higher performance for the images that were extensively trained as short SOAs compared to novel images. Single cell recordings in this animal, while he was performing the behavioral task, showed significantly stronger responses to the trained than to untrained images, when presented at short SOA (12.5 msec). At a long SOA, responses to trained images tended to be smaller for the highly familiar trained than for the much less familiar untrained images, in agreement with the previously observed reduction of neural responses to familiar images. In addition, the average stimulus selectivity was larger for the trained than for the untrained images. Thus, in this monkey there was a practice-induced correlation of the behavioral recognition performance and IT selectivity.

The second monkey was a much slower learner than the first one and even after extensive training his performance at short SOAs was worse than that of the first monkey. Although training at short SOAs improved his performance, this training effect was equal for trained and untrained images. Single cell recordings in this animal showed little effect of training at short SOAs, but a familiarity effect at long SOAs with less selectivity for the less familiar compared to the highly familiar stimuli. However, further analysis showed that his performance level for different sets of untrained images correlated significantly with the single cell selectivity for these images. Thus, also this animal showed a correlation between behavioral recognition performance and single cell selectivity, but in this case it was not practice-induced.

Currently we are extending this study in two ways. First, we will determine whether active stimulus discrimination during training at short SOAs is necessary to obtain the practice-induced effect. This will be tested by measuring the single cell responses to highly and less familiar stimuli when these stimuli are irrelevant for correct task performance. The images will be familiarized while the monkey is performing a same-different task using luminance changes of the fixation spot as discriminanda and in which the images are irrelevant for solving the task. Second, we noted that in both animals responses to the masks were absent. This disagrees with the results of our previous study in passively fixating monkeys in which about 50% of the neurons showed mask responses (Kovacs et al., PNAS, 1995). Whether the absence of mask responses in the present study is related to stimulus differences between the two studies or reflects the extensive training will be examined by recording in a naïve, fixating monkey, eventually followed by training

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Fondation Médicale Reine Elisabeth
Geneeskundige Stichting Koningin Elisabeth

F.M.R.E. – G.S.K.E.

Mailing address:

The director:

Fondation Médicale Reine Elisabeth
Geneeskundige Stichting Koningin Elisabeth
Prof. Dr. Th. de Barsy, Director
3, avenue J.J. Crocq laan
1020 Bruxelles - Brussel
Belgium
Tel. & Fax: +32.2.478.35.56
(and also Tel. +32.10.43.02.11 - Fax: +32.10.41.19.72
Centre Neurologique W. Lennox, B-1340 Ottignies)

and

The Secretary:

Fondation Médicale Reine Elisabeth (F.M.R.E.)
Geneeskundige Stichting Koningin Elisabeth (G.S.K.E.)
Mr. Erik Dhondt
Tel.: +32.2.478.35.56
Fax: +32.2.478.24.13
E-mail: fmre.gske@skynet.be